A STUDY OF THE EPIDEMIOLOGY OF THEILERIOSIS ON SMALLHOLDER DAIRY FARMS IN KIAMBU DISTRICT, KENYA

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ABSTRACT

A STUDY OF THE EPIDEMIOLOGY OF THEILERIOSIS
ON SMALLHOLDER DAIRY FARMS IN KIAMBU DISTRICT, KENYA

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University of Guelph, 1998

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This thesis describes a one year longitudinal study of East Coast fever (ECF)/*Theileria parva* infection on 90 smallholder dairy farms, randomly selected from dairy co-operative societies in Kiambu District, Central Highlands, Kenya. A total of 535 animals were examined monthly, providing 370.3 animal years of observation, 82.3 years contributed by calves less than one year of age.

Serological samples were assessed for *T. parva*-specific antibodies by a standardised and quantified ELISA, allowing identification of primary and secondary infections from serological profiles. In total, 54.5% of all samples tested positive. The overall rate of secondary infections in mature cattle of 18.4% was significantly lower ($p<0.001$) than the rate of seroconversion (primary infections) of 49.8% observed in calves.

While infection pressure was higher for calves, ECF disease risk was not. Although the crude mortality rate was higher ($p<0.001$) in calves (30.4%) than in mature cattle (6.3%), the ECF-specific morbidity and mortality rates were not different (7.3% *versus* 5.6% and 1.2% *versus* 1.7%, respectively). These relatively low ECF risks were associated with lower intensity of tick control practices than previously reported, particularly for calves.

Risks of *T. parva* infection/ECF were homogeneous across agro-ecological zone (AEZ) with the most important differences by grazing management system. Generalised

linear multi-level mixed models of measures of antibody activity and dichotomous (positive/negative) status both demonstrated significantly different age-profiles by grazing management system, but not by AEZ. Cattle kept under semi-/full-pasture grazing management both tested positive earlier and had higher levels of antibody activity than those housed in zero-grazing units. Strong farm clustering was observed for all outcome measures, but variability was greatest within semi-/full-pasture grazing systems.

Continued intensification of the smallholder dairy sector is expected to further depress the low level of challenge experienced by zero-grazing units such that disease control efforts are likely to be based on a risk-aversion rather than disease-reduction strategy. However, the more heterogeneous risk in grazing systems on highland margins demonstrates that these farms are likely to experience the greatest benefits of ECF control programmes.

DEDICATION

In memory of Robert Daniel O'Callaghan.

I miss you Dad.

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CHAPTER 1

INTRODUCTION

1.1 The Study

The study described in this thesis was concerned with better understanding the epidemiology of East Coast fever and *Theileria parva* infection. The objectives were designed from two perspectives; i) furthering the understanding of how productivity can be improved in a specific region and production system and ii) improving the general understanding of *T. parva* transmission dynamics in a defined system.

1.2 The Production System

Demand for animal products in developing countries is expected to increase substantially from the very low levels of consumption, relative to the developed world (Delgado, Crosson and Courbois, 1997). The predicted increase is due to a combination of rapid population growth, urbanization and rising incomes (Winrock, 1992; De Haan, Steinfeld and Blackburn, 1997). The International Food Policy Research Institute estimates that by the year 2010 per capita milk consumption in the developing world will have increased by 20% from 1995 levels (IFPRI, 1995). In the case of sub-Saharan Africa, demand for milk has continually exceeded local supply. Based on the trend in total regional dairy production over the previous three decades, Mbogoh (1984a, 1984b) predicted that the deficit for milk would exceed 10 million *tonnes* of whole-milk equivalents by the year 2000.

This situation has arisen despite substantial potential for dairy development noted in many of the countries of sub-Saharan Africa (ILCA, 1979, 1981; Walshe *et al.*, 1991). The current consensus is that dairy development, research and extension efforts are best targeted to the smallholder sector (Walshe, 1987; KARI, 1990; Sansoucy, 1995). The reasons most commonly cited for this rationale include the potential for improvement in productivity, the predominance of smallholdings in the most suitable dairy production zones, the large social benefits from supporting small-scale agriculture, and the sustainability of small-scale farming systems (Brumby and Scholtens, 1986; Ehui *et al.*, 1998).

Kenya is one of the few countries that has managed to successfully support smallholder dairy development (Brumby and Gryseels, 1985; Walshe *et al.*, 1991) and estimates of the contribution of smallholder dairy farmers to the total milk production vary from 60-90% (Mbogoh, 1984a, 1984b; Goldson and Ndeda, 1985; Kenya, 1986; Ministry of Livestock Development, 1989). Smallholder dairy farming in Kenya is practised as a component of subsistence agriculture and smallholders also grow food and cash crops. Farms tend to be located in the highland and peri-urban areas and are generally small, varying in size from two to five acres, with farmers keeping between one and ten milking cows. However, individual cow productivity remains low (Omore, McDermott and Arimi, 1994) and most of the gain in production in the smallholder sector has been a result of an expansion of the land resources and livestock committed to dairy production (Walshe *et al.*, 1991). Over 90% of the dairy farms in Kenya are owned by smallholder farmers who keep approximately 80% of the total estimated dairy herd in Kenya of three million cattle (Ministry of Livestock Development, 1989; Ministry of Agriculture, Livestock Development

and Marketing, 1996). These farmers are subject to a number of pressures, including rapid population growth, limited land resources and cultural traditions with strong attachments to land ownership, which have necessitated a shift to more intensive dairy production methods (Rendel and Nestel, 1983; Simpson, 1984; Von Massow, 1989; Walshe *et al.*, 1991). In consequence, between 65-80% of dairy cattle on smallholder farms are stall-fed for the greater part of the year (Goldsen and Ndeda, 1985; Gitau *et al.*, 1994c).

It is clear that the relatively easy gains in production made to-date cannot continue (Christiansen, 1989). Further development of the dairy industry is conditional on identification of opportunities for increased productivity and the limitations which prevent capitalising on them. In the case of smallholders, the major constraints have been identified as diseases, poor management, inadequate nutrition and lack of farm inputs (ILRAD, 1984; Goldson and Ndeda, 1985; Van Schaik *et al.*, 1996). Among those diseases which afflict smallholder dairy systems the most important are the parasitic tick-borne diseases (TBD) which result in substantial production losses (Mukhebi, Perry and Kruska, 1992; Winrock, 1992), in particular East Coast fever (ECF) caused by infection with *Theileria parva* (ILRAD, 1984).

1.3 Epidemiology of Theileria parva

Recent developments in the understanding of TBD epidemiology have focussed attention on the theory of endemic stability (Perry *et al.*, 1992) whereby early infection during a period of neonatal disease resistance results in the development of immunity in the cattle population in the absence of overt clinical disease (Norval, Perry and Young, 1992).

Under this scenario, the impact of disease is naturally limited and costly control efforts can be minimised. O'Callaghan *et al.* (1998) provided the theoretical underpinnings for endemic stability and confirmed that the pattern of disease in endemically unstable areas is expected to be non-linear across vector challenge (Perry and Young, 1995), suggesting that management practices and environmental factors are likely to have significant effect on the development of endemic stability. The recognition of the potential for endemic stability and the inability to find a single method to otherwise successfully control TBD has lead to the call for integrated strategies for disease control (Young, Groocock and Kariuki, 1988; Perry and Young, 1995). An improved understanding of the factors affecting endemic stability and the inter-relationships of management practices and disease dynamics in areas of potential endemic instability requires further elaboration. In the case of *T. parva*, the continued elaboration of the bovine immune response (Morrison and McKeever, 1998) and the discovery and development of novel vaccines (McKeever and Morrison, 1998) continue to raise questions regarding how these might best be integrated into the most efficient and sustainable combination of control measures. The answers will not be universal but will almost certainly be specific to different ecosystems, climatic zones, farming systems, breeds and types of cattle at risk. It is therefore vital that appropriately designed and structured epidemiological studies are conducted to derive information on the distribution and patterns of important tick-borne diseases which continue to limit the development of livestock production sectors such as smallholder dairying in the high potential highland areas of Kenya.

Kiambu District, located in the Central Province of Kenya, is considered

representative of the highland ecosystem in which ECF occurs and provides a range of grazing and dairy management practices for comparison. The smallholder dairy production system in Kiambu represents the probable future trends for other highland areas; increased intensification and better input and output markets. It was therefore a natural choice for a study of the impact and potential determinants of the epidemiology of ECF.

1.4 Objectives

In this context, a longitudinal prospective study was designed and executed, the specific objectives of which were developed as follows:

1. To determine the distribution of potential risk factors, at both farm and animal levels, hypothesized to be associated with the occurrence of ECF and to characterise the demographics of smallholder dairy production.
2. To evaluate the impact of ECF as assessed by measures of the rates of observed morbidity and mortality and to investigate the distribution of these measures across the risk factors identified above.
3. To investigate the rates of *T. parva* infection, as distinguished from the development of clinical disease, with a view to better understanding the dynamics of transmission and its inter-relationship with management and environmental factors.
4. To characterise the current epidemiological state of ECF with respect to endemic stability/instability for combinations of determinants identified above.
5. To utilise the results of the study to define appropriate target populations for ECF control strategies, both now and in the future.

The dynamic state of the smallholder dairy sector, with changing management and disease control practices, means that the current study will provide a baseline assessment of the epidemiology of ECF. It is hoped that, in combination with future studies of ECF, the inter-relationship between the transmission dynamics of *T. parva* and the design and impact of current and potential disease control strategies may be better elucidated.

CHAPTER 2

BACKGROUND AND REVIEW OF LITERATURE

2.1 Theileriosis/East Coast Fever

Norval, Perry and Young (1992) provide a thorough and comprehensive review of all aspects of the epidemiology of theileriosis in Africa. This chapter highlights salient features of the literature pertinent to the subject of this thesis.

Theileriosis refers to infections caused by several species of protozoan parasites of the genus *Theileria*. In sub-Saharan eastern, central and southern Africa the most important species is *Theileria parva*, the causative agent of the clinical syndrome in cattle known as East Coast fever (ECF). *Theileria parva* is transmitted transtadially by Ixodid ticks, the principal vector species being *Rhipicephalus appendiculatus*, commonly known as the African brown ear tick (Lawrence *et al.*, 1994).

Economic losses attributable to theileriosis include i) direct production losses through morbidity and mortality (Minjauw *et al.*, 1998a, 1998b) and reduced productivity (Rumberia *et al.*, 1993; Pegram *et al.*, 1996; Minjauw *et al.*, 1998c), ii) indirect production losses, where disease is perceived as a constraint to the ability to improve livestock production and genetic potential (Callow, 1983) and iii) costs incurred for disease control, research, training and extension services (Mukhebi *et al.*, 1992). Miller, Pino and McKelvey (1977) estimated an annual ECF mortality figure of approximately one million cattle in Kenya, Tanzania and Uganda, while Young *et al.* (1988) estimated that Kenya alone spent approximately US$ 10

million of foreign reserves in 1987 through the importation of acaricides and theileriacidal drugs and the provision of dipping and curative services.

2.2 Theileria parva *Life Cycle*

The life cycle of *T. parva* in cattle and in the ixodid tick *R. appendiculatus* is complex but well elucidated. *Theileria* sporozoites are injected with the saliva of the feeding tick and rapidly enter lymphoid cells of the host through a sequential process of attachment and internalisation (Shaw, 1996, 1997). Each sporozoite then grows into a schizont and the host lymphocyte becomes transformed (Ole-Moi Yoi, 1989), a process which can be reversed by theileriacidal drug treatment (Pinder *et al.*, 1981). Lymphoblastogenesis and clonal expansion of infected cells, including concurrent schizont division, occurs at a rapid rate giving rise to a 10-fold increase in infected cells every three days or less (Irvin, Ocama and Spooner, 1982). The proliferation and concomitant destruction of overgrown and infiltrated lymphoid tissue gives rise to the main pathogenic effects of ECF (Jarrett, Crighton and Pirie, 1969; Radley *et al.*, 1974). *Theileria parva* schizonts begin developing into merozoites 12-14 days after infection, thereby destroying the host cell and releasing the merozoites which in turn penetrate erythrocytes and develop into piroplasms.

Feeding ticks ingest and lyse infected erythrocytes resulting in the release of piroplasms in the tick gut. There, further development into the sexual stages known as micro- and macro-gametes takes place (Mehlhorn and Schein, 1984). Syngamy of gametes produces a zygote which enters the gut epithelium, begins to grow and, at a variable time after tick repletion, transforms to a kinete (Mehlhorn, Schein and Warneke, 1978). The

kinete itself grows and develops into a motile stage, a process which appears to be synchronized and perhaps controlled by moulting of the tick (Young and Leitch, 1980). Post-moulting, granular *e* cells in the tick salivary glands are available for infection and are believed to become infected by chance as kinetes migrate (Fawcett, Büscher and Doxsey, 1982a; Fawcett, Doxsey and Büscher, 1982b; Fawcett, Young and Leitch, 1985). On entry into the *e* cell, the kinete rounds up into a sporont. In general, tick feeding begins the process of sporogony (Fawcett *et al.*, 1982b, 1985), although this has been observed in a proportion of infected acini without feeding (Young, Leitch and Mutugi, 1984). Sporonts increase in size to sporoblasts and at a variable time, around 3-4 days after commencing feeding, sporozoites are ready for emission from the salivary gland acinus. Approximately 50,000 *T. parva* sporozoites are found per infected acinar cell in the adult tick (Fawcett *et al.*, 1985).

2.3 Rhipicephalus appendiculatus - *Life Cycle, Distribution, Dynamics & Infection*

All ixodid ticks have four stages of development: egg, larva, nymph and adult. *Rhipicephalus appendiculatus* is an obligate three host tick. That is, both the larva-to-nymph and nymph-to-adult moults occur off the host and unfed larvae, nymphs and adults must all find separate hosts (Norval *et al.*, 1992). *Rhipicephalus appendiculatus* is a relatively sedentary species of tick with very limited potential for migration under its own locomotion (Rechav, 1979). Rather, all stages of the tick have been observed to ascend vegetation and wait to attach to passing hosts (Short and Norval, 1981). Daily diurnal rhythms of detachment, observed in engorged larvae, nymphs and adults (Minshull, 1982), usually ensure that ticks end up in appropriate microhabitats to enable them to moult and survive

(Short *et al.*, 1989a, 1989b). In consequence, the host density, activity and coverage of the habitat as well as the longevity of the questing stages are crucial to the establishment and maintenance of the tick population (Sutherst, Wharton and Utech, 1978). In addition, as a three-host tick, *R. appendiculatus* is particularly susceptible to unfavourable environmental conditions and it is important that microclimatic conditions such as temperature, humidity and vegetation cover be suitable if the tick population is to survive. Thus, open grazing management systems where cattle move freely on pasture are much more likely to favour completion of the *R. appendiculatus* life-cycle than zero-grazing units, where, although cattle density is high, the environment is universally unsuitable for ticks.

Rhipicephalus appendiculatus is distributed throughout eastern, central and southern Africa. However, the distribution is not continuous, but rather is dependent on favourable climate and vegetation and the presence of suitable hosts (Norval *et al.*, 1992). Lessard *et al.* (1990) provide a review of the use of geographical information systems in assessing the distribution of the vectors of *T. parva* and Perry *et al.* (1990a) demonstrated a very close association between measures of ecological suitability, determined from a climate matching model (Sutherst and Maywald, 1985), and the recorded tick distribution in Kenya. Broadly speaking, *R. appendiculatus* is present in the Lake Victoria Basin in Western Kenya, the Central Highlands and the Coastal Lowlands.

In areas characterised by seasonal differences in temperature, rainfall, humidity and day-length, a distinct seasonality is observed in the activity and abundance of *R. appendiculatus*. In particular, unfed adult ticks remain inactive, in a state of behavioural diapause, when unsuitable conditions prevail (Short *et al.*, 1989a, Pegram and Banda, 1990).

This may be so pronounced that it results in ticks passing through only one generation per year (Short and Norval, 1981; Rechav, 1982; Pegram *et al.*, 1986). However, most of these observations have been made in southern Africa. In the equatorial region of eastern Africa, where day-length is virtually constant throughout the year and a prolonged dry season is absent, diapause does not occur (Branagan, 1973 a, 1973b, 1978) and larvae, nymphs and adults are usually present simultaneously on hosts throughout the year (Smith, 1969; Kaiser, Sutherst and Bourne, 1982; Matthysse and Colbo, 1987; Kaiser *et al.*, 1988). This is the situation in the Central Kenyan Highlands, where two rainy seasons occur annually. However, in other climatically extreme areas of Kenya, such as the Coastal Lowlands, seasonality has been observed (Newson, 1978; Newson and Punyua, 1978).

The simultaneous presence of all stages of *R. appendiculatus* has profound implications to the epidemiology of ECF in that transmission of *T. parva* can occur from both carrier cattle and from those undergoing clinical disease (Norval *et al.*, 1991). In areas in which a pronounced diapause occurs, emerging adult ticks induce acute ECF in the population of cattle which have remained susceptible since the previous season, but subsequent larval and nymphal stages then feed predominantly on surviving carriers. Since there is no transovarial transfer of infection in *R. appendiculatus*, virtually all infections in post-moulting nymph and adult ticks have been derived from carrier hosts. While *T. parva* infection in vector ticks can be transmitted transtadially through either the larva-to-nymph or nymph-to-adult moulting process, both the source of infection and the instar affect the prevalence and distribution of intensity of infection in subsequent ticks. Ochanda *et al.* (1996) demonstrated that infections derived from ticks feeding on acutely infected hosts

resulted in a high prevalence of infections in the subsequent instar and that these infected ticks were distinguished by large numbers of infected salivary gland acini. In contrast, ticks feeding on carrier cattle were infected less often and with lower intensity infections. This pattern was consistent between nymphs and adult ticks (infected as larvae and nymphs respectively), but with overall greater prevalence and intensity in adults *versus* nymphs.

2.4 The Host - Infection, Immunity and Dynamics

Although Young *et al.* (1983a) demonstrated that sporozoites from one infected adult tick salivary gland cell are sufficient to infect and kill Friesian cattle, the severity of *T. parva* infections has been observed to be dependent on the dose of sporozoites inoculated (Jarret *et al.*, 1969; Radley *et al.*, 1974; Dolan *et al.*, 1984; Purnell *et al.*, 1973; Morrison *et al.*, 1981), the virulence and pathogenicity of the stock of *T. parva* (Barnett and Brocklesby, 1966; Norval *et al.*, 1992) and the breed/type of the host (Young, 1981; Moll *et al.*, 1986). Historically, indigenous Zebu breeds have been considered more resistant than exotic Taurine breeds (Guilbride and Opwaka, 1969) which were restricted from "African" areas (i.e. out with areas occupied by white settlers) in Kenya for many years (Swynnerton, 1986). However, this perception is based predominantly on anecdotal information (Norval *et al.*, 1992).

From the earliest outbreaks of theileriosis in animals moved from disease free to endemic areas, scientists recognized that the animals which survived the epidemic were subsequently immune to rechallenge. The use of these "salted" animals in endemic areas became accepted practice (Lawrence, 1992). Elucidating the bovine immune response to *T.*

parva infection has been, and continues to be, the focus of considerable research efforts, with reviews of progress appearing regularly in the recent literature (Morrison *et al.*, 1989; McKeever and Morrison, 1990; Morrison, Taracha and McKeever, 1995a; Morrison and McKeever, 1998). It is now accepted that protection against ECF is a result of cell-mediated immunity, specifically that class I major histocompatibility complex (MHC) restricted CD8(+) cytotoxic T-lymphocytes (CTL) act in conjunction with CD4(+) helper T cells to recognize and kill lymphocytes containing *T. parva* schizonts (Taracha, Awino and McKeever, 1997). Further, antigenic diversity of the parasite and "competition" between parasite epitopes for induction of CTL responses are likely to account for the differences in parasite strain cross-protective properties (Morrison, 1996a, 1996b). This accounts for disease breakthroughs by antigenically different strains of ECF observed under heterologous challenge. With respect to humoral immunity, anti-schizont antibody has not been demonstrated to play a role in the protection against disease (Muhammed, Lauerman and Johnson, 1975; Emery, 1981), although the observation by Musoke *et al.* (1992) that serum from repeatedly challenged animals neutralized the infectivity of sporozoites for cattle, provided the basis for the development of a novel subunit vaccine (McKeever and Morrison, 1998).

Although the Cape buffalo (*Cyncercus caffer*) is generally considered the definitive host of *T. parva* (Lawrence, 1992), it is clear that both parasite and vector populations can be maintained in the bovine host in the absence of alternative hosts (Norval *et al.*, 1992). This phenomenon may be explained by the finding of a long-term *T. parva* carrier state in cattle (Young *et al.*, 1986; Kariuki *et al.*, 1995) and the very high prevalence of such carriers

in cattle populations in the field in Kenya (Young *et al.*, 1986; Kariuki, 1990; Watt, Kiara and Sporagano, 1998). The theory behind this empirical observation was explored in a mathematical model of *T. parva* transmission developed by Medley, Perry and Young (1993). It was demonstrated that there was a non-linear relationship between level of vector challenge and proportion of hosts infected, such that at all but the lowest rates of tick attachment, the majority of cattle were previously infected carriers.

2.5 Epidemiological States of Theileriosis - Endemic Stability

Over years of empirical observation and field experience in the epidemiology of tick-borne diseases, researchers developed the important concept of endemic stability (Perry *et al.*, 1992). Endemic stability was considered to exist where the incidence rate and mortality from clinical disease were low and restricted to younger age groups but where antibody prevalence and relative level of tick challenge were concomitantly high (Norval *et al.*, 1992; Perry *et al.*, 1992; Perry and Young, 1995). In the case of *T. parva*, endemic stability was associated with the early infection of calves of resistant breeds under continuous *R. appendiculatus* challenge (Yeoman, 1966; Moll, Lohding and Young, 1984; Moll *et al.*, 1986; Norval *et al.*, 1992). Although specific to another tick-borne disease (Heartwater - *Cowdria ruminantium* infection), recent theoretical modelling work by O'Callaghan *et al.* (1998) confirms that endemic stability is indeed due principally to the protection of calves against disease by either innate or maternally-derived factors. Perhaps more importantly, however, the hypothetical patterns of disease incidence, case-fatality proportion and antibody prevalence (as a surrogate measure of prior infection), described by Perry and Young (1995)

across a range of epidemiological states, are shown to be due to a non-linear relationship between the severity of disease at the population level and the level of vector challenge, such that, those areas defined to be endemically unstable may be characterised by low or high incidence of disease (Mukhebi *et al.*, 1998). An understanding of the transmission dynamics of *T. parva* and of the resulting relationships between the epidemiological states of theileriosis is vital to designing and assessing disease control strategies (O'Callaghan *et al.*, 1998).

2.6 Diagnosis of East Coast Fever / Theileria parva *Infection*

Throughout most of Africa, the diagnosis of ECF continues to be predominantly based on the presence of what are considered to be characteristic clinical signs, including pyrexia, lymphadenopathy, pulmonary oedema, subcutaneous oedema, petechiation of mucus membranes, diarrhoea, lachrymation and corneal opacity (Norval *et al.*, 1992). Lack of ancillary supporting facilities for laboratory diagnosis and absence of reliable field diagnostic tests means that the majority of passive reports of ECF are based on clinical diagnosis alone. Even the relatively simple procedures of lymph node biopsy smears or thin blood films are rarely utilised and in any case may only serve to demonstrate that the animal is a previously infected carrier, while the ability to differentiate *Theileria* species on microscopic morphology alone is highly dependent on the skill of the operator (FAO, 1984).

The most widely used serum antibody assay for *T. parva* has been the indirect fluorescent antibody (IFA) test using crude schizont antigen (Burridge and Kimber, 1972; Goddeeris *et al.*, 1982). Unfortunately, the assay is relatively slow and labour intensive,

requires a subjective assessment of the degree of fluorescence and exhibits cross-reactivity with other *Theileria* species (Burridge and Kimber, 1972; Burridge *et al.*, 1974a; Burridge, Brown and Kimber, 1974b; Williamson, Lesan and Awich, 1990).

A recently developed enzyme-linked immunosorbent assay (ELISA) using a recombinant polymorphic immunodominant molecule specific to *T. parva* (Toye *et al.*, 1991, 1996) has demonstrated a sensitivity in excess of 99% and a specificity of between 94% and 98% in experimental and field sera (Katende *et al.*, 1998).

Other molecular diagnostic tools for the detection of parasite DNA and RNA in both vectors and hosts have been developed, including DNA probes (Allsopp *et al.*, 1993; Shayan *et al.*, 1998) and polymerase chain reactions (PCR) (Bishop *et al.*, 1992; Bishop, Sohanpal and Morzario, 1993; Watt *et al.*, 1997, 1998).

2.7 Vector Control

The principal method of controlling East Coast fever in Kenya has, and continues to be, the application of acaricides to cattle by plunge dipping or spraying (back-pack or spray-race). These methods attempt to i) reduce vector:host contact, thereby reducing the opportunity for transmission of the parasite, and ii) to directly kill the tick and prevent successful completion of the tick life-cycle, thereby reducing the vector population (Norval *et al.*, 1992). More recently, acaricides have also been applied mixed in a petroleum jelly-base, in systemic spot-on or pour-on preparations, and in slow-release devices such as impregnated ear and tail-tags (Young *et al.*, 1985a, Young, DeCastro and Kiza-Aura, 1985b; Rechav, 1987).

The history of dipping in Kenya has been reviewed by Ngulo (1975) and Keating (1983). In general, it has failed to control the spread and intensity of ECF and is never likely to achieve its original goal of vector eradication (Young *et al.*, 1988). In keeping with other parts of Africa and elsewhere, improper or ineffective application of acaricides resulted in severe problems with acaricide resistance in ticks in Kenya (Crampton and Gichanga, 1979; Chema, 1984), such that the Veterinary Department eventually assumed control over which products could be used (Kenya, 1976). Nevertheless, escalating costs have eroded the government's ability to finance and maintain its policy of subsidised and monitored tick control, a fact which has been reflected in a deteriorating infrastructure and a shift among smallholder dairy farmers to alternate control methods (Gitau *et al.*, 1994c, 1997).

Alternative methods of controlling the vector such as quarantine of animals, pasture spelling, restrictions of livestock movement, rotational burning of pastures and planting of tick-killing or repelling plants have all been utilised in the past with varying results. In addition, newer technologies, including selection for tick-resistance in hosts, development of tick vaccines, use of pheromones in tick-decoys and manipulation of hybrid sterility between closely related tick-species are under investigation (Norval *et al.*, 1992).

In the case of smallholder dairy farms, the shift toward intensive management with cattle confined to zero-grazing units, where the only possibility for vector contact is through the introduction of tick-infested fodder, has helped to reduce the incidence of tick-borne diseases in general (Maloo *et al.*, 1994; Gitau, 1998). However this is likely to result in the maintenance of a susceptible population with a concomitant shift of primary infection to older age groups (O'Callaghan *et al.*, 1998).

2.8 ECF Chemotherapy

Neitz (1953) demonstrated that early and prolonged treatment with chlortetracycline prevented the development of ECF following infection with *T. parva* and Brocklesby and Bailey (1962) utilised concurrent application of *T. parva* infected ticks and oral tetracycline as a technique for immunisation. These results formed the basis for the "infection and treatment" method of immunisation, however, certain stocks of *T. parva* broke through the protective chemoprophylaxis such that it was necessary to establish an immunizing dose which would not overcome the oxytetracycline regimen (Mutugi *et al.*, 1988). Spooner (1990) subsequently demonstrated that oxytetracycline acts by slowing down the division of schizonts and their host cells and thus exerts only a limited suppressive effect in the early stages of infection.

It was not until the 1980's that three therapeutic agents were developed and registered for the treatment of theileriosis, namely parvaquone (Clexon®) and buparvaquone (Butalex®) (Wellcome Pharmaceutical, United Kingdom), and halofuginone (Terit®, Hoechst Pharmaceutical, Germany). Successful field trials of parvaquone (Chema *et al.*, 1986) and halofuginone (Chema *et al.*, 1987) were conducted in Kenya. However, Dolan (1986a, 1986b) also demonstrated a high prevalence of carriage of *T. parva* in cattle treated with these drugs. While all three compounds are efficacious in the treatment of ECF, this depends on an early diagnosis and administration of a full therapeutic dose (McHardy, 1989). Unfortunately, the prohibitively high cost of these drugs has resulted in their limited use by smallholder farmers.

2.9 Immunisation against ECF

In a logical extension to the observations made concerning the acquisition of solid immunity in recovered animals post-natural infection, scientists began to experiment with methods of immunisation. Early efforts, involving transfer of a suspension of splenic and lymph node cells from *T. parva*-infected cattle (Theiler, 1911a, 1911b), resulted in a considerable proportion of lethal infections, while some animals did not acquire immunity. Although Neitz (1953) and Brocklesby and Bailey (1962) demonstrated the protective effect of tetracycline administration, it was not until a method of producing stabilites of sporozoites was developed (Cunningham *et al.*, 1973) which allowed cattle to be infected with a predetermined dose, that the "infection and treatment" method of immunisation using chemoprophylaxis became feasible (Brown *et al.*, 1977; Radley, 1978). Subsequently, parvaquone (Dolan *et al.*, 1984; Dolan, 1986b; Young *et al.*, 1990) and buparvaquone (McHardy and Wekesa, 1985; McHardy, 1989; Mutugi *et al.*, 1988, 1991; Young *et al.*, 1990) were also evaluated in infection and treatment immunisation and a variety of field studies were conducted in Kenya (Dolan, 1985; Morzaria *et al.*, 1988; Young *et al.*, 1990; Young *et al.*, 1992). However, there are several problems with the "infection and treatment" method of immunisation including breakthrough of heterologous strains, severe clinical reactions on immunisation, the need to ensure a cold chain for delivery of product, the potential for vaccine contamination spreading other organisms, and the potential for long-term carriers created by vaccination to introduce new strains of *T. parva* directly and through sexual recombination with endemic strains (Morzaria, 1996).

In consequence, efforts have continued to develop new sub-unit vaccines based on

important antigens of *T. parva*, as determined from studies of the bovine immunological response (Morrison, Taracha and McKeever, 1995b; Musoke *et al.*, 1996; McKeever and Morrison, 1998). This research has identified two candidate antigens, the p-67 surface antigen found on the *T. parva* sporozoite (Musoke *et al.*, 1992; Musoke, Nene and Morzaria, 1993; Nene *et al.*, 1992, 1996) and the unique polymorphic immunodominant molecule (PIM) found on both sporozoites and intracellular schizonts of *T. parva* (Toye *et al.*, 1991, 1996) and recently exploited as a diagnostic antigen (Katende *et al.*, 1998).

2.10 Integrated Strategies for Controlling Tick-borne Diseases

The failure to find a single strategy to successfully control ECF and other tick-borne diseases has prompted the call for integration of the available technologies to provide an economically viable and sustainable system, robust to breakdowns of individual control methods (Young *et al.*, 1988; Perry and Young, 1995). In the context of sub-Saharan Africa, such integrated control strategies are likely to be complex and highly variable as they must take into account specific ecosystems and climatic zones, co-distributions of different tick-borne diseases and their vectors, the diversity of farming systems and land-use patterns, the distribution of different breeds and types of cattle at risk and the presence of alternate hosts for both vectors and parasites (Norval *et al.*, 1992). Decisions regarding which combinations of methods and levels of intensity of control will minimise the risk of disease outbreaks and maximize the economic returns are unlikely to be straightforward, given the likely complexity of their interactions. While it is possible to devise field trials or case studies for particular combinations of factors (Dolan, 1985; Young, 1985; Tatchel *et al.*, 1986; Young

et al., 1986; Kaiser *et al.*, 1988), the use of predictive models of the dynamics of disease transmission (Medley *et al.*, 1993; O'Callaghan *et al.*, 1998) which integrate vector (Floyd, Maywald and Sutherst, 1987a, 1987b) and host population dynamics are likely to prove essential tools in the study of epidemiological patterns of tick-borne diseases and thus help to determine optimum control combinations in the large variety of disease circumstances encountered.

2.11 The Epidemiology and Impact of ECF in Kenya

Much has been made of the importance and severity of ECF in sub-Saharan Africa and estimates of its economic impact, although varying widely, are inevitably substantial. De Haan and Bekure (1991) suggested that approximately half of all cattle deaths in sub-Saharan Africa were the result of diseases transmitted by external parasites, most importantly ticks. Miller *et al.* (1977) estimated a regional ECF mortality of one million cattle annually in Kenya, Tanzania and Uganda while Mukhebi *et al.* (1992) followed this with an estimate of annual mortality due to theileriosis across eastern, central and southern Africa of 1.1 million cattle, equivalent to a cost of US$ 168 million per year. In general these broad brush estimates are distinguished by being based on passive surveillance data recorded at regional or country levels, and hence tend to make the implicit assumption that the distribution of ECF is homogeneous across production systems and agro-ecological zones. However, a variety of surveys and more focussed studies on the epidemiology of ECF conducted in Kenya using both active and passive sampling have demonstrated that the distribution is not homogeneous.

The largest of these was a national cross-sectional serological survey in which approximately 18,000 cattle were sampled using a purposive selection technique. Twenty clusters, containing approximately 25 animals each, drawn from 4 - 5 herds chosen in close proximity, were sampled in each of 36 districts and samples tested using the indirect haemagglutination (IHA) test (FAO, 1975). The study showed variation in the prevalence of antibodies by district, with highest prevalence rates observed in the Lake Victoria Basin in Western Kenya (61%-100%) and intermediate rates in the Central Highlands (21-60%). Although the approximate age of the animals was recorded (as "aged adult", "young adult", "immature" and "calf") no attempt was made to provide a statistical representation of all age classes, making interpretation of the observed trend for increasing seroprevalence with age difficult. Nevertheless, the study provided a general picture of the relative distribution of the infection in the country at the time, although potential differences within any one district were obscured.

In a retrospective study of all ECF cases recorded by the Kenyan Veterinary Department over the years 1969-1987, Kariuki (1990) showed an increase in the annual number of cases from 1981 onwards. Kyule (1989) focussed his attention on the period of 1976-1986 and demonstrated an overall higher, but stable incidence of ECF in government designated "Tick Control Project" districts, but of an annually increasing incidence in non-project districts. Unfortunately, both studies were based on data derived from passive surveillance such that it is impossible to assess how much the potential for differential rates of misclassification bias, as disease diagnosis and available chemotherapeutic agents improved over time, may account for the trends observed. This effect is further compounded

in the case of Kyule (1989) where estimates of incidence were derived from reported cattle population numbers. Similar concerns occur with interpretation of the results of the study of Mulei and Rege (1989), where cases of ECF reported to the Ambulatory Clinic of the University of Nairobi's Department of Clinical Studies and assumed cattle populations were used to demonstrate a changing annual incidence of ECF in Kiambu District.

More focussed cross-sectional serological surveys have also been conducted on a smaller scale in Kenya. Young *et al.*(1986) and Morzaria, Musoke and Latif (1988) conducted studies in endemic areas of high challenge and demonstrated near 100% seroprevalence in mature animals and a high degree of passive transfer to newborn calves. In a step forward in study design, Deem *et al.* (1993) undertook a cross-sectional serological survey in the Coastal Lowland region but where selection was stratified by agro-ecological zone. This study was able to demonstrate differences in antibody prevalence rates to *T. parva* across agro-ecological zones which consequently led to speculation on how control efforts might best be focussed. In another cross-sectional serological survey, using a two-stage random selection procedure stratified on sublocations (which are the smallest administrative subdivisions in Kenya) and dairy co-operative societies/cattle diptank registers, Gitau *et al.* (1997) made a similar observation of differing seroprevalence to *T. parva* by agro-ecological zone in smallholder dairy farms of the Central Highlands and were also able to show simultaneous differences between grazing management systems.

Although prospective studies offer the advantage of being able to provide additional and more accurate information on incidence rates of morbidity and mortality, the logistics and costs associated with longitudinal monitoring usually constrain the size and geographic

representation of these studies. Nevertheless, the studies of Moll *et al.* (1984, 1986), which involved intensive monitoring of a relatively small cohort of calves in an endemic area of high challenge, provided the basis for quantifying the transmission dynamics of *T. parva* (Medley *et al.*, 1993). More recently, prospective studies of the epidemiology of ECF have focussed on the high potential smallholder dairy farming systems in both Coastal Lowland (Maloo *et al*, 1994) and Central Highland (Gitau, 1998) regions of Kenya. These studies have demonstrated that production losses due to ECF are highly variable across combinations of such factors as agro-ecological zone and grazing management system. Perhaps more importantly, however, they have provided a valid and robust picture of the epidemiology of ECF in an important production sector, which will ultimately allow more efficient and appropriate targeting of tick-borne disease control efforts.

CHAPTER 3

STUDY DESIGN AND DESCRIPTIVE DEMOGRAPHICS

3.1 Introduction

This study was conducted as a subsection of the larger integrated Smallholder Dairy Development Research Project (Gitau *et al.*, 1994c). The overall objective of the larger project was to obtain an accurate assessment of health and production constraints to the smallholder dairy industry in Kenya.

In this context, the assertions that cattle diseases are one of the major constraints to increased smallholder dairy production (ILRAD, 1983; Perry *et al.*, 1984; Goldson and Ndeda, 1985) and that the tick-borne parasitic diseases theileriosis, babesiosis and anaplasmosis, are the most important infectious diseases of cattle in Kenya (ILCA, 1981; ILRAD, 1983; Brumby and Sholtens, 1986; Young *et al.*, 1988), necessitated their incorporation in further investigation, with particular reference to determining their importance relative to other potential constraints. In this chapter, the design of the longitudinal observational study as it applies to monitoring of East Coast fever is described.

In Kenya, the growth of large urban centres and increased population pressures in fertile rural areas provided an increased demand (and price) for dairy products while concomitantly requiring further intensification of smallholder agriculture, particularly in peri-urban areas (Lahloukassi, Rey and Faye, 1994). Large scale farmers were able to transport, process and market milk through a central body, the Kenya Cooperative

Creameries (KCC). However, the traditional smallholders, who practised dairying as a component of a subsistence level mixed farming system, were able to increase milk production to match demand, but their access to expanding markets was limited by their geographical location and capital costs of adequate transportation. In response to demand, cooperative societies of smallholder farmers were formed according to existing administrative boundaries to provide a link in the milk collection and transportation system. Under the system in place during the period of the study, farmers continued to produce milk to meet personal and local demands but were then able to deliver excess milk daily to a conveniently located collection station where the milk was weighed, recorded, pooled with that of other local producers and transported to the local dairy society, usually found in an adjacent small urban centre. Here, unprocessed milk was available for direct sale by the society. Excess was temporarily stored and transported daily to the nearest KCC processing plant. Farmers "sold" their excess milk directly to the dairy society and were charged a small administrative fee per litre. During the period of the study, strict rules were in place governing the processing and sale of milk and milk-based value added products, which restricted the dairy societies to selling excess directly to the KCC. There was also a hierarchy of pricing and both the farmers and the societies received higher prices for locally purchased milk than for their excess, such that the majority of the milk (58%) produced by smallholders was sold locally (Ombui *et al.*, 1995). More recent liberalization of government policies are likely to result in an increased role in the processing and production of milk and milk products for the dairy societies themselves.

3.2 Methods and Materials for Longitudinal Study

3.2.1 Study Population

The population of interest consisted of smallholder dairy farmers of the central Kenyan highlands. Smallholder dairying in this area was considered to be experiencing significant constraint due to the presence of theileriosis. Kiambu District, a triangular shaped area of 2448 km^2 (193,500 ha, of which approximately 75% is considered suitable for agriculture), situated just north and west of the capital city Nairobi, was chosen as a representative fertile highland area of high dairy potential (Figure 3.1). The vast majority of farmers in Kiambu district are smallholders, occupying an area of 94 820 ha and practising mixed agriculture including dairying, livestock production and food and cash crops. With an estimated total population of just over one million in 1992 (based on a 1989 census figure of 914,412 persons and an approximate annual growth rate of 3.3%), Kiambu district had a population density of over 400 persons/km^2 (Central Bureau of Statistics, 1989). There were fifteen active dairy societies in Kiambu district and approximately 75-80% of the total milk marketed was produced by smallholder farmers owning fewer than ten milking cows (Ministry of Livestock Development, 1989). In 1991, 30 604 781 kg of milk was received by dairy societies in Kiambu district, of which 16 603 388 kg was sold to the KCC and 13 844 907 kg sold locally, earning farmers a total of Kshs. 120,079,967 (Ministry of Livestock Development, 1992).

Geographically, the area is spread over altitudes between 1200 metres (to the east and south bordering Nairobi) and 2000 metres above sea level (to the west bordering the Great Rift Valley) and incorporates various agro-ecological zones (Figure 3.2). Two distinct rainy

seasons occur annually. The long rains cover mid March to June while the short rains occur from mid October to December. Higher altitude zones are characterized by cooler temperatures, higher rainfall, larger farms, and a wider variety of crops than lower altitude zones. Annual average rainfall varies between 600 and 2500 mm (German Agricultural Team, 1982). Total average rainfall over the one year period of the study was 970 mm.

3.2.2 Sampling Frame/Selection of Study Farms

Since both dairy cooperative society and farm differences were of interest, study farms were selected by means of a two-stage stratified random sampling process. From a list of the fifteen dairy societies operating in the Kiambu District (Table 3.1), six societies were randomly selected (one dairy society, Sigona, was excluded from the sampling frame because of its very small membership). Owing to the fact that dairy societies were originally formed along existing divisional administrative boundaries there is little geographical overlap between societies, such that, selection by society provided a crude geographical stratification as well. The six selected dairy societies included Chania, Kiambaa, Kikuyu, Lari, Limuru and Nderi (Figures 3.1 and 3.2). Managers of the selected societies were approached with letters of support provided by the Kiambu District Veterinary Officer (Ministry of Livestock Development) and the district representative of the Ministry of Cooperative Development.

Each dairy society was visited personally and the manager was asked to provide a list of all active members of that society. Active membership was defined as a member who was presently producing milk and consequently had an active account on the society's payroll.

Previous members who were not presently providing milk to the society were to be excluded. One society selected, Lari Dairy Farmers' Cooperative Society, could not differentiate active producers from those retired or not presently producing and so provided a list of all previous members. Dairy society co-operation was exemplary with 100% participation.

Individual farms were randomly selected in the second stage of sampling. From each of the six dairy society members lists, fifteen farms were randomly selected. A contingency list of five farms per society was additionally selected to serve as replacement farms in cases of non-response from any of the originally sampled farms. All randomization procedures were performed using a table of random numbers. Letters of introduction, briefly describing the study and requesting participation, were distributed to the first fifteen farmers selected from each of the six dairy societies. Subsequent to the delivery of these letters, the farms were visited. If the farmer was active and willing to participate, the farm was enrolled in the study; if not, a replacement farm was selected in order from the five contingency farms. The societies selected, the number of farmers and collection routes, the amount of milk received daily and the proportion sold locally are presented in Table 3.1.

The overall sample size of ninety farms was based on logistical, more so than statistical, considerations. Ninety farms was considered to be the practical maximum that could be visited during a month of field-visits. The potential power of this sample design could not be estimated *a priori*, since little information concerning prevalence and no information on the distribution of management practices existed.

3.2.3 Data Collection

Each of the 90 farms was visited once every month for 12 months. The first visit was conducted in July 1991 and the last in June 1992. Farm visits were conducted on a regular rotating pattern from Monday to Thursday of each week such that, after the initial visits, a fixed inter-visit interval of approximately 30 days (1 month) was maintained for individual farms.

Two four-wheel drive vehicles were required to transport the two teams of personnel and equipment to the farms. The teams were composed of the author, five other veterinarians and one or two technicians. Farm location and introductory visits were conducted in conjunction with an official from the local dairy society or the divisional animal production officer from the Ministry of Livestock Development.

During the first visit a comprehensive initial farm-survey questionnaire was administered by one of the veterinarians conversant in Kikuyu (the local language). Questions covered a broad spectrum of topics including farm management, disease control and prevention practices, health history, nutrition, housing, and farm demographics. With respect to tick-borne diseases, questions were designed to include those characteristics considered to a) have a plausible biological association with the level of exposure of the vector and host and consequently subsequent parasite challenge; b) be associated with potential iatrogenic means of infection; or c) affect the level of immunity to tick-borne diseases. At the farm level, these included such factors as grazing system, method and frequency of tick control and any differences by age of animal or season of year, type of housing, actual or potential contact of dairy animals with other livestock or wildlife, use and

type of bedding, disinfection of housing areas, separation of calves from adults, timing and delivery of colostrum and age and method of dehorning. The complete Initial Farm Survey is included as Appendix 1.1.

In addition, an individual-animal questionnaire was administered for each bovid on the farm, collecting data on individual animal variables such as age, breed, sex, disease history, disease prevention practices administered to individual animals, production levels, reproductive performance, and the findings of a complete physical examination. Counts of 'standard ticks' according to the method of Norval *et al.* (1992) were conducted on each animal at every visit. The milk production of each lactating cow on the day prior to the visit was recorded. As part of the physical examination, all calves were weighed at every visit (up to a size corresponding to approximately 80 kg body weight) using an Avery AKL 150 suspension scale (Avery Kenya Ltd. Factory Street, Nairobi, Kenya). A calf was defined to be any bovid less than 12 months of age. For each animal, serum was separated from blood collected in plain vacutainers and whole blood was collected in EDTA coated vacutainers (Becton Dickson and Company, Rutherford, New Jersey). Samples were chilled for transport.

At each of the subsequent monthly visits, a shorter follow-up adult or calf survey was administered, in which health and production events (disease diagnosis and treatment, calving, oestrus, calf weights, etc) and management events (timing of last treatment for ticks) occurring in the past month were recorded for every bovid which had been present on the farm at the previous visit. With respect to animals initially classified as calves, once they reached 12 months of age these animals were considered to be adults and follow-up adult

surveys were prepared at all subsequent observations. To improve diagnosis and recording of disease events between monthly visits, farmers were reimbursed chemotherapeutic costs and veterinary consultation fees when they provided written records of disease diagnosis and treatment details by the local veterinary staff. An initial adult or calf questionnaire (as appropriate) was administered for all new cattle that joined the farm through birth, purchase or as gifts. A follow-up adult or calf survey form was recorded for any animal withdrawn from the farm through death, slaughter or sale, detailing the reason and date of withdrawal. If the farmer had not recorded the exact date of withdrawal, it was estimated as closely as possible by relating it to the other events over the month since the previous visit. This principle was applied to other events as well (such as estimation of birth dates on the initial survey). Dates had to be estimated in this way for approximately 10% of farmers. Initial and follow-up adult and calf questionnaires are included as Appendices 1.2 through 1.5.

During the final monthly visit, an economics questionnaire covering both the farming operation and household income and expenditures was interviewer-administered. A detailed account of each farm commodity sector (e.g. dairy, poultry, coffee, maize) listing all inputs and their costs and outputs and their prices were tabulated.

All questionnaires were structured to maximize the number of closed form (categorical) questions to ease administration, minimize variation and improve precision. Variation because of interviewer bias was minimized by the use of one person to conduct all initial interviews. Data on the type of management practice (for example, type of animal housing) were verified by physical inspection of the farm at the time of the interview.

3.2.4 Laboratory Analysis

Complete blood counts [red (RBC) and white (WBC) blood cell count, mean corpuscular volume (MCV), haemoglobin concentration (HB) and calculated microhaematocrit (MCT)] were performed on EDTA samples using a Model ZM Coulter-Counter (Coulter Electronics, France). Packed cell volume (PCV) was estimated using a Clay Adams microhaematocrit centrifuge and reader (Becton Dickson and Company, Rutherford, New Jersey). Total Protein (TP) determination was made using a clinical refractometer. Thin film smears were made fresh and stained daily with giemsa stain for use in white blood cell differential counts and parasite screening examinations.

Sera were screened for antibodies specifically directed against *T. parva* by a recently developed enzyme-linked immunosorbent assay (ELISA) but where the unique schizont antigen protein utilised (Toye *et al.*, 1991, 1996) was chromatographically separated and purified from cell-culture schizont lysate rather than by means of later developed recombinant technologies (Katende *et al.*, 1998).

3.2.5 Data Storage and Handling

Laboratory results, farm management and individual animal data were entered and stored in separate database files in Dbase (DBASE IV Plus, Ashton Tate, Torrance, California, USA). Data files were then screened for proper coding, missing and out of range results and any errors in data entry were corrected.

3.2.6 Data Analysis - Descriptive Statistics

Descriptive statistics were calculated using SAS statistical software (SAS® System for Windows™, Version 6.12, SAS Institute Inc., Cary, North Carolina, USA).

3.3 Results

Detailed methods and results are contained in specific chapters. Results are reported herein with respect to response rate, demography and data availability.

3.3.1 Response Rate

Voluntary enrollment rate among eligible farms was 98% (88/90). Of the 90 farms originally randomly selected from dairy co-operative society membership lists, 8 (5 from Lari Society) could not participate due to a variety of reasons including recent sale or death of animals, retirement and movement out of the district, while two actually refused to participate. All 10 of these farms were replaced from contingency lists such that the target number of 90 farms post initial visit was achieved.

A total of 10 farmers actively withdrew their participation over the course of the study, for a voluntary withdrawal rate of 11% (10/90) after one year. Thirty-one animals (26 adults, 5 calves) were undergoing observation on these farms at the time of exclusion. Although the rate of withdrawal followed no discernable pattern, the majority of the farmers that actively withdrew (8/10) cited "excessive" blood sampling as the predominant reason. In addition, there were two passive exclusions when one farmer sold his only cow and a lone adult cow present on another farm died. Neither farmer replaced the animal lost, resulting

in an attrition rate of 2% (2/90). Thus, the overall farm participation rate was calculated as 87% (78/90) with the loss to observation due to farmer withdrawal of 31 animals (Table 3.2).

3.3.2 Farm Demographics

Demographic data collected at the level of the farm are summarized in Tables 3.3 and 3.4. Detailed presentation by geographical stratification based on dairy cooperative society membership is presented in Appendix 2 with appropriate measures of association.

Both the mean farm size and the mean number of dairy animals present on the farm were relatively low at 4.6 acres and 4.4 cattle (3.6 adults, 0.8 calves) respectively. Significant differences were noted using multi-comparison methods between dairy cooperative societies (refer to Appendix 2). Further, the median farm size of three acres and median number of three dairy animals indicate a right skewed distribution toward smaller farms with fewer cattle. The smallest farm consisted of 0.25 acres. At the same time, these farms were supporting a mean family size of 6.5 members with a maximum of 15. Number of family members was significantly different according to dairy cooperative society (Fisher's Exact Test $p<0.01$).

The mean number of years spent dairy farming was 14.8 years (median of 14.5 years) with no significant differences between dairy cooperative societies. Four farmers (4.4%) had been dairy farming for less than one year. Some 3.3% (3/90) of farmers indicated that dairying was the sole on-farm practice while the vast majority of farmers (94.4% = 85/90) grew subsistence crops such as maize (the dietary staple) and other vegetables. Most farmers (82.2% = 74/90) indicated they raised other livestock including sheep (46.7% = 42/90), goats

(16.7% = 15/90), chickens (55.6% = 50/90), swine (4.4% = 4/90) and donkeys (13.3% = 12/90). The main cash-crop grown by farmers was coffee (15.6% = 14/90), while one farmer also grew tea and two marketed pyrethrum. Off farm employment was relatively rare, with 6.7% (5/90) of farm owners employed off of the farm in a part-time capacity and 17.8% (16/90) employed in a full-time non-farm-related job.

3.3.3 Distribution of Farm Factors

The distribution of possible determinants of tick-borne diseases measured at the farm level are summarized in Tables 3.5, 3.6 and 3.7. Detailed information presented by geographical stratification on dairy cooperative society is again presented in Appendix 2 with appropriate measures of association.

The majority of farmers (92.2% = 83/90) practised some form of tick control. This was nearly evenly split between acaricide dipping (41.1% = 37/90) and spraying by means of a hand pump/pressure operated spraying unit (48.9% = 44/90). The method of tick control was associated with dairy cooperative society (Fisher's Exact Test $p<0.006$). Only 2 farmers indicated that they used hand applied (i.e. paint brush/sponge) acaricide solutions and both did so on an irregular basis according to tick burden. Seven farmers (7.8%) practised no tick control, while 5 of the remaining 83 farms which utilized acaricide (6.0%) reported that they did not treat calves. Of the 78 farmers who did treat their calves for ticks, the mean age at first treatment was 4.8 months with a median age of 4 months and a maximum of 24 months. There were no significant differences in age at first treatment between dairy cooperative societies. Eighty-two out of 83 (98.8%) farmers who practised tick control stated they were

attempting to control theileriosis, anaplasmosis and babesiosis, while 36 of these same 83 farmers (43.4%) suggested they were also attempting to control cowdriosis.

The most intensive tick control regimen of one acaricide treatment per week was also the most popular, reportedly being practised by 62.2% (56/90) of farmers, while 21.1% (19/90) reportedly treated every second week; 3.3% (3/90) on a monthly basis and the remainder less frequently (5/90 = 5.6%) or not at all (7/90 = 7.8%). No farmers reported ever treating more frequently than once per week and frequency of tick control was not significantly associated with dairy cooperative society (Fisher's Test of Association $p<0.388$).

With respect to grazing practice, 47.8% (43/90) of farmers operated a full zero grazing system while 18.9% (17/90) practised semi-zero grazing and the remaining 33.3% (30/90) grazed their animals on pastures. Grazing system was significantly associated with dairy cooperative society (Chi-Square Test of Association $p<0.001$).

Less than half (41.1% = 37/90) of farmers reported that no permanent housing or shelter was available to the animals. When available, the physical type of housing for adult animals was quite varied. Broadly summarized, the type of housing was approximately equally divided between that with dirt flooring and that with concrete flooring and that enclosed with walls *versus* an open shelter. Again, presence/type of housing was significantly associated ($p<0.001$) with dairy cooperative society based on the Chi-Square Test of Association. While the majority of farmers (64.4% = 58/90) reported not using bedding for adults, the remainder were split between cut grass (15.6% = 14/90) and wood shavings (20.0% = 18/90), a practice also associated with dairy cooperative society

($p<0.001$).

No farmers (0/90) indicated that their animals ever came into contact with wildlife species, while 18.9% (17/90) indicated contact between their dairy animals and other livestock on the farm with the potential of mechanically transmitting ticks. Eighty-three of 90 (92.2%) farmers practised some form of dehorning of their cattle. Of these 83, one farmer indicated that he used a completely bloodless method (elastic bands). Similarly, most farmers indicated that they dehorned during a period when the horns had become large and significantly vascularized; 46.7% (42/90) stated that they dehorned animals at 5 months of age or older.

With respect to separation of calf and dam and method of delivery of first colostrum there was a nearly equal division with 47.8% (43/90) of farmers separating animals immediately postpartum or within 4 hours. This was also reflected by the proportion of those that allowed free-choice suckling (52.2% = 47/90) and those who pail (44.4% = 40/90) or bottle (3.3% = 3/90) fed. Regardless of method, 96.7% (87/90) of farmers indicated that the delivery of first colostrum was within 6 hours of birth and 61.1% (55/90) stated that it was prior to two hours post-partum.

Calf housing was more common than adult housing with 6.7% (6/90) of farms having no calf housing facilities. The remainder were housed either in groups or as individuals on dirt or concrete. Four farmers housed their calves in their own dwelling, while three farmers had individual calf pens with raised wooden floors. Similarly, more farmers (95.5% = 86/90) provided bedding for their calves; this was divided between cut grass (52.2% = 47/90) and wood shavings (43.3% = 39/90) as per the adults. As for mature cattle, the presence/type of

housing of calves and the use/type of bedding of calves were both significantly associated with dairy cooperative society ($p<0.001$ and $p=0.006$ respectively). Calf housing was cleaned relatively frequently with 6.7% (6/90) of farms cleaning daily and 46.7% (42/90) cleaning at least twice per week; 26.7% (24/90) of farmers indicated that they cleaned out the calf housing area monthly or less frequently.

3.3.4 Longitudinal Data Available

A total of 535 animals were identified during the one year period of observation. Initial adult surveys were obtained for 334 animals. Of these, 315 were present on the 90 sampled farms at the time of the initial visit, while 19 additional adult animals joined 11 farms over the study period as they were purchased or received as gifts. With respect to calves, 72 initial surveys were obtained for calves present on 42 farms at the time of the initial visit, while a further 129 initial surveys were prepared during the remaining rounds of study visits for calves born on 66 farms. A total of 201 initial calf surveys were completed for calves on 77 farms. On 13 farms (14.4%), no calf was observed. Table 3.8 provides the distribution of initial and follow-up adult and calf surveys prepared during the 12 monthly rounds of farm visits.

Of the 334 animals initially identified as adults, 211 of the 315 (67%) present at the time of the initial farm visit were followed for the complete 12 month period. One-hundred and six were lost to follow-up over the course of the study and 17 of the 19 which were enrolled after the initial farm visit, were present at the final farm visit. Similarly, for the 201 animals initially categorized as calves, 57% (41/72) of those present on initial inspection

were still present at final observation, while 96 calves which were born after the initial farm visit were also present at the completion of the study, although 15 of these calves were initially observed at the final farm visit. Of the 64 calves lost to follow-up, 31 of these were calves present at the initial inspection and 6 of these losses occurred after the animals had reach 12 months of age and had subsequently been reclassified as adults. Of the remaining 33 calf losses which were enrolled after the initial farm visit, 10 were calves lost before an initial examination could be conducted.

Thus a total of 170 animals were lost to follow-up and 283 animals were observed for only a portion of the study period. Table 3.9 provides the distribution of number of observations recorded for all animals enrolled in the study. Table 3.10 indicates the disposition of the 10 calves lost to follow-up prior to an initial examination and Table 3.11 indicates the disposition of those 160 animals lost after an initial survey had been prepared for each.

3.3.5 Calculation of Period of Observation

Although specific dates were recorded, wherever possible, for additions and losses of animals, the design of the study and the nature of timing of farm management practices (both with repetitive and relatively static monthly events), suggested that the procedure for calculation of total period of animal observation from longitudinal survey records could be simplified for further analysis. Thus, the following rationale was adopted: Since the inter-visit interval for individual farms was almost universally fixed and equivalent at 30 days, follow-up surveys completed for adults and calves which were actually examined at both

visits, were assumed to contribute 1 animal month of observation. Further, owing to the fact that initial animal surveys gathered retrospective individual animal information on a variety of areas such as health and application of disease prevention practices, they were also considered to cover a period at least equivalent to 1 animal month. It was anticipated that the retrospective accuracy for this period would be high, avoiding the potential for recall bias. Finally, the timing of animal additions (88% of which were from births of calves) and losses/withdrawals was assumed to be random, occurring across the monthly inter-visit interval with a mean period of observation equivalent to 15 days. Thus animals for which an initial animal survey was recorded after the initial farm visit, and for animals where follow-up surveys which indicated a loss to follow-up, each contributed an additional one-half animal month of observation. However, with respect to the 10 calves which were born and subsequently lost to follow-up before an initial examination could be conducted, these animals contributed no additional period of observation.

Under these assumptions, the collected data provided a total of 4444 animal months (370.33 animal years) of observation composed of 3456.5 months contributed by adult animals and 987.5 months from calves. Further details of the calculated contributions by source and within each age group are presented in Table 3.12.

Table 3.1: Fifteen smallholder Dairy Cooperative Societies operating in Kiambu District in July, 1991 and their corresponding demographic indices.

Dairy Cooperative Society	Approximate Number of Active Members	Number of Collection Centres	Approximate Daily Milk Intake (kg)	Approximate Percentage of Intake Sold Locally
Limuru[1]	4000	9	20000	67
Githunguri	4000	10	16500	25
Kiriita	2000	10	9000	34
Ndumberi	1600	7	11000	81
Kiganjo	1200	15	6000	70
Chania[1]	1100	43	3500	14
Gatamaiyu	900	5	2000	25
Nderi[1]	750	6	3000	80
Kabete	600	10	3000	80
Kiambaa[1]	550	14	2300	75
Kikuyu[1]	550	7	2086	90
Kamaihia	470	13	8000	12
Kinale	460	3	1200	33
Lari[1]	200	1	900	100
Sigona	35	1	640	80

[1] - Dairy Cooperative Society randomly selected for participation in study

Table 3.2: Disposition of 12 of 90 enrolled farms which were not participating in a longitudinal study conducted in Kiambu District from July 1991 - June 1992 at the time of the final round of farm visits. The round of observation (visit number) at which they announced their withdrawal or were reported lost from the study and the numbers of adults and calves present on the farm at the time of withdrawal/loss to follow-up are indicated.

Farm	Dairy Cooperative Society	Round of Withdrawal	# Adults Present	# Calves Present	Reason For Exclusion
1	Chania	3	2	0	Voluntary withdrawal
2	Chania	7	1	0	Voluntary withdrawal
3	Kiambaa	4	1	1	Voluntary withdrawal
4	Kikuyu	3	7	1	Voluntary withdrawal
5	Lari	6	2	1	Voluntary withdrawal
6	Lari	6	1	0	Voluntary withdrawal
7	Lari	9	3	1	Voluntary withdrawal
8	Lari	11	4	0	Voluntary withdrawal
9	Limuru	6	2	0	Voluntary withdrawal
10	Nderi	9	3	1	Voluntary withdrawal
11	Limuru	4	1	0	Sold only cow
12	Chania	10	1	0	Only cow died
	Totals		(26+2)[1]	5	33 Animals

[1] - With respect to disposition of individual animals, 26 of these 28 adults were considered to have been excluded due to voluntary farm withdrawal while the remaining two animals were classified as lost to follow-up due to sale and death respectively.

Table 3.3: Distribution of agro-ecological zone classification for 90 smallholder dairy farms enrolled in a longitudinal study conducted in Kiambu District from July 1991 - June 1992.

Agro-Ecological Zone[1]	Number of Farms (n=90)	Percentage
UH 1	6	6.7
UH 2	15	16.7
LH 1	2	2.2
LH 2	23	25.6
LH 3	13	14.4
UM 1	2	2.2
UM 2	22	24.4
UM 3	7	7.8
Total	90	100.0

[1]See text for description and Figure 3.2 for distribution. Where "UM" refers to Upper Midland zones, "LH" refers to Lower Highland zones and "UH" refers to Upper Highland zones.

Table 3.4: Demographic statistics for farm-level continuous variables of 90 smallholder dairy farms enrolled in a longitudinal study conducted in Kiambu District from July 1991 - June 1992.

	Mean	Median	Min - Max	SD
Farm size (acres)	4.6	3	0.25 - 25	4.7
Number of dairy cattle	4.4	3	1 - 24	4.1
Number of years dairy farming	14.8	14.5	0[1] - 34	9.2
Number of family members	6.5	3	1 - 15	3.2
Age (months) at first tick control treatment for treated calves	4.8	4	1 - 24	3.0

[1] - "0" recorded for those practising dairy farming less than one full year

Table 3.5: Demographic statistics for grazing management and tick control practices reported on initial survey of 90 smallholder dairy farms enrolled in a longitudinal study conducted in Kiambu District from July 1991 - June 1992.

	Number of Farms (n=90)	Percentage
Grazing System		
Full/Pasture grazing	30	33.3
Semi-zero grazing	17	18.9
Zero-grazing	43	47.8
Dairy Cattle Contact Other Livestock With Tick-Transmitting Potential	17	18.9
Tick Control Method		
Dipping	37	41.1
Spraying	44	48.9
Hand application	2	2.2
None practised	7	7.8
Tick Control Frequency	(n=83)[1]	
≤1 week	56	67.5*
>1 week to ≤2 weeks	19	22.9*
> 2 weeks	8	9.6*
Frequency altered by season	9	10.8*
Only adults treated for ticks	5	6.0*

[1] - based on n=83 farms reporting tick control being practised

Table 3.6: Demographic statistics for adult and calf housing management reported on initial survey of 90 smallholder dairy farms enrolled in a longitudinal study conducted in Kiambu District from July 1991 - June 1992.

	Number of Farms (n=90)	Percentage
Housing of Mature Animals		
No housing available	37	41.1
Enclosed housing + Dirt floor	8	8.9
Enclosed housing + Concrete floor	19	21.1
Open housing + Dirt floor	7	7.8
Open housing + Concrete floor	19	21.1
Bedding of Mature Animals		
No bedding provided	58	64.4
Grass bedding	14	15.6
Wood shavings	18	20.0
Calf Housing		
Housed in owner's house	4	4.4
Free or tethered outdoors	6	6.7
Grouped + Concrete floor	11	12.2
Grouped + Dirt floor	41	45.6
Individually + Concrete floor	6	6.7
Individually + Dirt floor	19	21.1
Individually in raised pens	3	3.3
Calf Bedding		
No bedding	4	4.4
Grass bedding	47	52.2
Wood shavings	39	43.3

Table 3.7: Demographic statistics for calf feeding and rearing management reported on initial survey of 90 smallholder dairy farms enrolled in a longitudinal study conducted in Kiambu District from July 1991 - June 1992.

	Number of Farms (n=90)	Percentage
Calf/Dam Separation		
Calves Immediately Separated From Dams	37	41.1
Calves with Dams <4 Hours Post-Partum	6	6.7
Calves with Dams 4-24 Hours Post-Partum	42	46.7
Calves with Dams 24-72 Hours Post-Partum	3	3.3
Calves with Dams >72 Hours Post-Partum	2	2.2
Colostrum Feeding Method		
Colostrum Free Choice	47	52.2
Colostrum by Nursing Bottle	3	3.3
Colostrum by Pail Feeding	40	44.4
Timing of First Colostral Feed		
Colostrum <2 Hours Post-Partum	55	61.1
Colostrum 2-6 Hours Post-Partum	32	35.6
Colostrum 6-12 Hours Post-Partum	2	2.2
Colostrum 12-24 Hours Post-Partum	1	1.1
Reported Calf Tick-Borne Disease Death Within 12 Months	3	3.3

Table 3.8: Number and distribution of initial and follow-up adult and calf surveys recorded during a longitudinal study conducted in Kiambu District from July 1991 - June 1992, presented by round of observation (visit number) at which they were obtained.

Round of Observation	Initial Adult Surveys	Follow-up Adult Surveys	Initial Calf Surveys	Follow-up Calf Surveys
1	315	-	72	-
2	4	315	12	72
3	0	307	8	76
4	3	302	14	77
5	4	295	15	82
6	3	291	10	83
7	2	287	12	81
8	1	282	8	81
9	0	285	9	78
10	2	277	12	78
11	0	277	14	85
12	0	270	15	87
Totals	334	3188	201	880

Table 3.9: Frequency distribution of total number of observations per animal obtained during a longitudinal study conducted in Kiambu District from July 1991 - June 1992, and subdivided on the basis of the classification of the animal with respect to age (0-12 months of age = calf, > 12 months = adult) at the time of initial observation.

Number of Observations	Initial Adults	Initial Calves
"0"	-	10[1]
1	0	15[2]
2	15	23
3	11	14
4	13	17
5	13	13
6	15	16
7	16	12
8	6	10
9	14	13
10	3	9
11	13	7
12	215[3]	42[4]
Totals	334	201

[1] - 10 Calves lost to follow-up prior to initial examination.
[2] - 15 Calves born between round 11 and round 12 visit, i.e. with initial calf survey only at Round 12.
[3] - 4 of 215 Adults lost to follow-up between round 11 and round 12 visit, i.e. where round 12 follow-up adult survey form is a withdrawal form.
[4] - 1 of 42 Calves lost to follow-up between round 11 and round 12 visit, i.e. where round 12 follow-up calf survey form is a withdrawal form.

Table 3.10: Disposition of 10 calves lost to follow-up prior to initial examination during a longitudinal study conducted in Kiambu District from July 1991 - June 1992.

Reason for Loss		Number
Slaughtered for consumption		3
Given away as gift		1
Died	Stillborn	1
	Dystocia	1
	Trampled by dam	1
	Atresia coli	1
	Sudden/Unexplained	2
Total		10

Table 3.11: Disposition of 160 animals with an initial observation/examination and which were subsequently lost to follow-up during a longitudinal study conducted in Kiambu District from July 1991 - June 1992. Losses are subdivided on the basis of the classification of the animal with respect to age (0-12 months of age = calf, > 12 months = adult) at the time of loss to follow-up.

Reason for Loss to Follow-up		Adults	Calves	Total
Sold/Given as gift		55 (52+3)[1]	23	78
Death		18 (17 +1)[1]	19	37
Farm withdrawal		26 (25 +1)[1]	5	31
Consumed for meat		3 (2 +1)[1]	1	4
Excluded	Owner Change	4	0	4
	Dangerous Bull	3	0	3
Unspecified		3	0	3
Total		112 (106+6)[1]	48	160

[1] - 6 animals which were lost to follow-up as adults were initially examined while still categorized as calves

Table 3.12: Calculation of period of animal observation from surveys recorded during a longitudinal study conducted in Kiambu District from July 1991 - June 1992, subdivided on the basis of the classification of the animal with respect to age (0-12 months of age = calf, > 12 months = adult) at the time of each observation/survey:

a) Adult Animals

Source/Survey	Number	Period of Animal Observation Contributed	
		Months	Years
Initial observation at round 1 visit	315	315	26.25
Initial observation at other round	19	9.5	0.792
Follow-up observation	3076	3076	256.333
Loss to follow-up observation	112	56	4.667
Total		3456.5	288.04[1]

b) Calves:

Source/Survey	Number	Period of Animal Observation Contributed	
		Months	Years
Initial observation at round 1 visit	72	72	6
Initial observation = loss to follow-up	10	0	0
Initial observation at other round	119	59.5	4.958
Follow-up observation	832	832	69.33
Loss to follow-up observation	48	24	2
Total		987.5	82.29[1]

[1]-Total period of observation = 4444 months or 370.33 years

Figure 3.1: Map of Kiambu District, Kenya showing broad agro-ecological classifications and locations of 6 dairy cooperative societies participating in a longitudinal study conducted from July 1991 - June 1992.

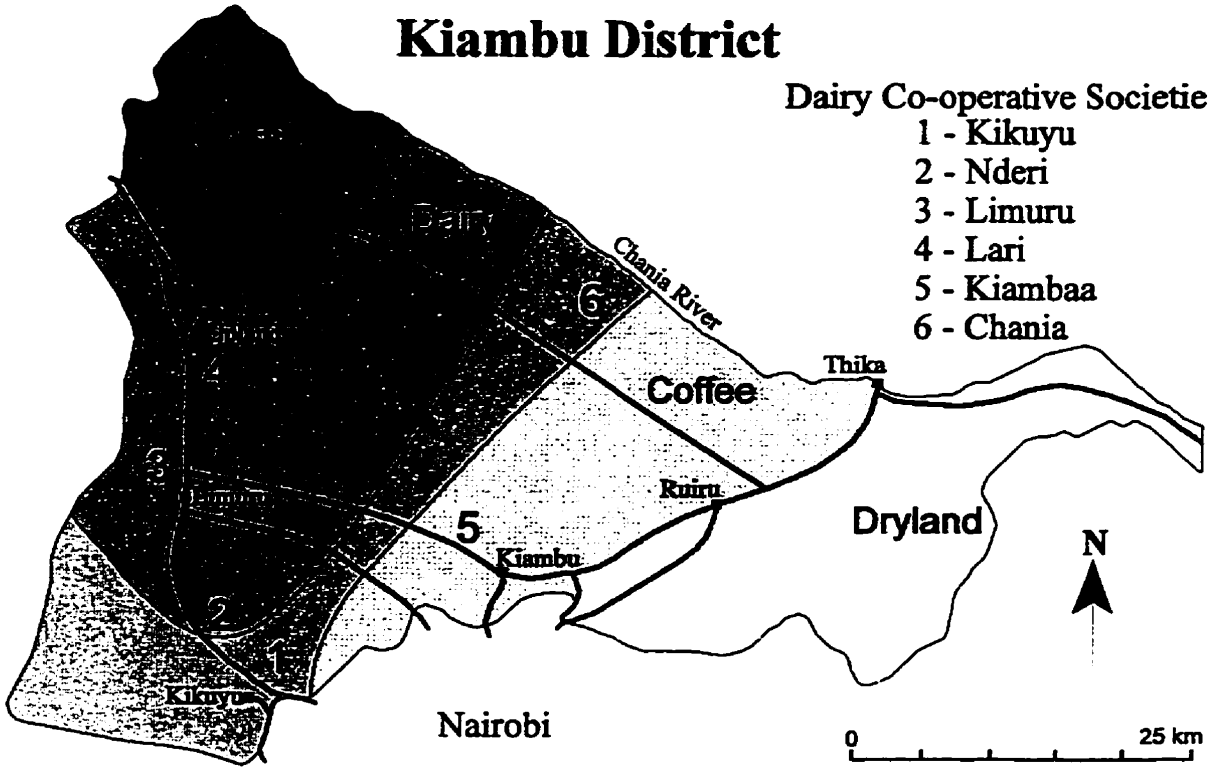
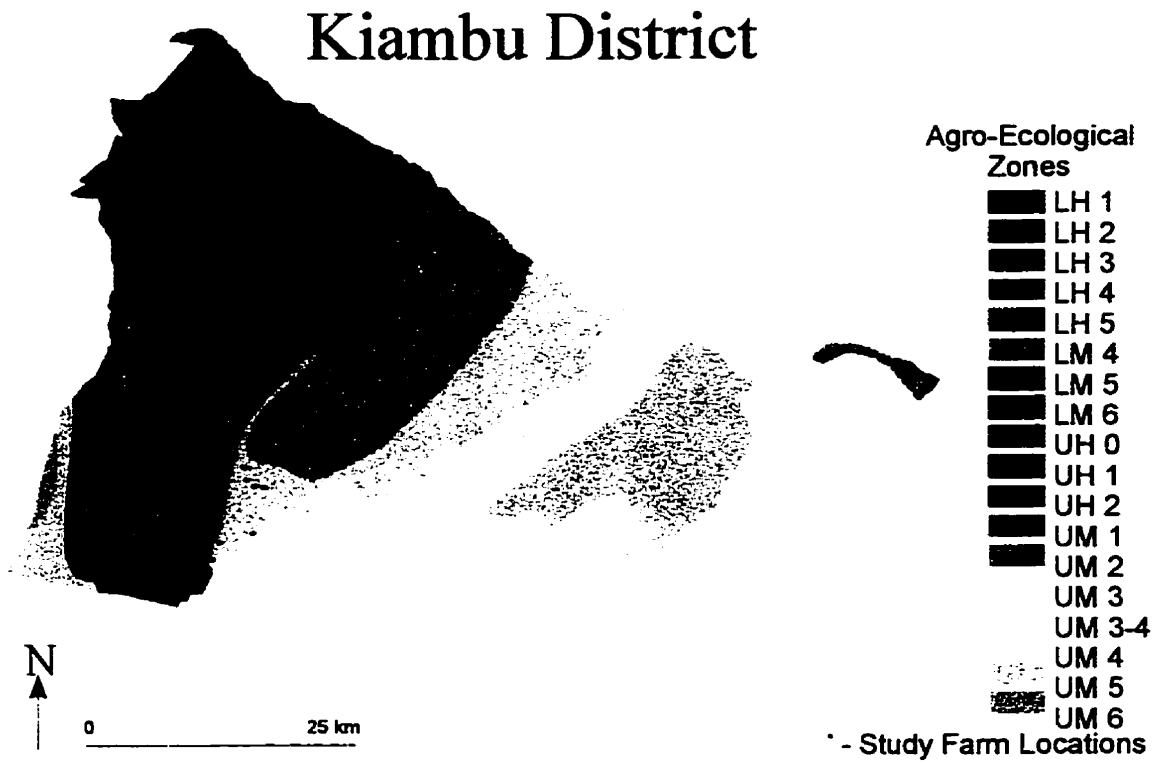


Figure 3.2: Map of Kiambu District, Kenya showing discrete agro-ecological zones and locations of 90 smallholder dairy farms participating in a longitudinal study conducted from July 1991 - June 1992.



CHAPTER 4

SEROLOGICAL ANALYSES

4.1 Introduction

Decisions on how to interpret serological test results and contend with missing data occur in all studies which record serological responses. Often it appears that these decisions are determined informally and the basis for them are rarely discussed. Nevertheless, these decisions may have an important impact on the interpretation of the study results.

Predominantly, results of serological assays have been interpreted as dichotomous "positive" or "negative" outcomes, and much attention is often focussed on the determination of an appropriate test cut-off level (Wright *et al.*, 1993). However, for longitudinal studies, a key feature is how to standardise and quantify results across time so that temporal patterns in antibody titres or profiles of serological test results can be compared to determine seroconversion or other events of interest. Further, in the analysis of longitudinal studies, most statistical software, while allowing for right or left censoring of observations, requires otherwise complete data for each subject over time. Thus, for animals with single missing values, the missing observation either needs to be estimated or all data on that animal are lost.

In this chapter the sources and impact of errors in the interpretation of serological data from basic principles will be presented. Methods for standardising and quantifying serological results over time, which were applied to the *T. parva* schizont antibody ELISA

test results, will be presented and their influence on the interpretation of results explored.

4.2 Available Data

A total of 4433 sampling opportunities arose over the period of the study, where "sampling opportunity" is defined as an observation at which the study animals were available for examination and hence excludes withdrawal or loss to follow-up observations. For this study, these figures are composed of 334 initial and 3076 follow-up adult and 191 initial and 832 follow-up calf observations (see Table 3.12).

At the time of the monthly physical examination, a blood sample was collected from each animal for subsequent serological analysis. Where possible, a total of approximately 10 - 15 ml of whole blood was collected in two plain vacutainers (Becton Dickson and Company, Rutherford, New Jersey, USA). Each vacutainer was individually labelled with a unique code comprising the dairy co-operative society number, farm identification number, animal name and date of collection. Samples were chilled immediately after collection and transported to the laboratory daily.

On arrival in the laboratory, the vacutainers were allowed to stand at room temperature until maximum clot retraction had occurred. Final separation was achieved by centrifugation. Separated serum was then pipetted into between 2 - 4 cryopreservation vials per animal, each aliquot containing approximately 1 - 2 ml of serum. Cryo-preservation vials were labelled with the same unique code as the original sample from which the serum was derived. Aliquots of serum were then frozen and stored at -20°C.

Serum were stored and screened by month of collection, a single aliquot from each

animal sampled during a given month of farm visits being removed from storage, thawed and stored at 4°C immediately prior to screening.

4.3 Potential Sources of Errors and Their Detection

With respect to the collection, coding and handling of serological samples prior to and during testing and the subsequent handling and analysis of test results, two types of errors may occur; errors of omission - where a result is missing and untraceable, and what may be termed errors of translocation - where a value is recorded, but it is erroneous.

4.3.1 Errors of Omission

Errors of omission may be the result of failure to collect a sample, the physical loss or destruction of a sample, or as the result of a unidirectional error of translocation at the point of labelling of the original sample or sub-sample such that the result generated was recorded elsewhere. Fortunately, when they occur, errors of omission are easily recognised as missing observations.

A total of 4433 serum sampling opportunities arose during the study. In total, there were 29 recorded animal examinations (0.65% = 29/4433) for which no corresponding serum sample was screened initially or found on retrospective investigation. Table 4.1 presents the number and distribution of present and missing serum samples by age-class of the animals and sample collection opportunity. Of these 29 "missing" samples, nine (all calves) involved the initial examination, two cases (both adult) coincided with the final sampling opportunity, while 18 other missing serum samples (14 adults/4 calves) had both a previous and a

subsequent monthly screening test result. The implications for estimating missing values with respect to the temporal relationship between missing samples and tested samples for the same animal will be discussed subsequently.

With regard to initial examinations of calves, in 9 of 191 cases (4.71%) farmers objected to blood collection from very young stock, usually calves only hours or days post-partum. However, for all of these calves, routine sampling was conducted at all subsequent examinations. In the 16 cases of missing results for adult animals, one case was a result of a farmer who consented to physical examination of his sole animal, but objected to collection of a blood sample. This farmer voluntarily withdrew from the study at the subsequent farm visit. The other 15 adult missing value cases and the 4 calf missing value cases which occurred during follow-up visits all had blood samples reportedly collected but not screened serologically. The apparent loss of these samples can be explained principally due to breakage of vacutainers during transport or centrifugation. Unfortunately, no formal record was made of these losses during the study. However, although both vacutainers and freezing tubes were labelled with a unique date and sorting code combination, the potential for "loss" of longitudinally collected samples due to mislabeling (see section *4.3.2 Errors of Translocation*) must also be acknowledged.

4.3.2 Errors of Translocation

In the context of this study, errors of translocation may occur at three distinct steps in the process of sample collection, processing and analysis. First, at the point of animal identification. The error may involve a failure to accurately identify an animal, or

alternatively to erroneously mislabel the collected sample. Second, at the time of sample processing, where separated serum was transferred from the original vacutainer to freezing tube, a mislabeling or transfer error may occur. Third, during the actual serological analysis and subsequent data entry, errors in transferring information on freezing tubes to laboratory templates or typographical data entry errors were possible.

Unlike errors of omission, by their nature errors of translocation are often difficult to detect. Whilst every effort was made to minimize the potential for mislabeling errors, it was impossible to document such occurrences directly. Instead, it was necessary to retrospectively examine longitudinal patterns of serological screening test results in an attempt to identify anomalous individual values which are deemed to be "outliers" (inconsistent with the trend observed). For example, where an appropriately standardised and quantified assay has been utilised, a negative test result in the midst of 11 strong positive outcomes (Figure 4.1 A), or *vice versa* (Figure 4.1 B), would strongly suggest that the lone outlying result occurred either due to mislabeling or data-entry error. While the data-entry errors were easily corrected when identified, this was not true for anomalies which were attributed to mislabeling of samples. A total of 14 such aberrant unexplained screening results were identified (14/4404 = 0.32%).

4.4 Strategies for Minimizing Errors and Their Impact

Efforts to minimise the potential impact of errors on subsequent analyses were both pro-active in terms of prevention of errors and reactive, in the form of data correction.

4.4.1 Prevention of Errors

A set of cross-referencing and checking procedures was established to minimise the opportunities for both errors of omission and translocation.

Interviewers on each monthly farm visit were accompanied by a list of all bovids present on the farm at the previous visit. This form provided both the unique farm identification number and the individual animal's identification (name or number), gender, physical description, reproductive status/stage of gestation, and in the case of calves, their previous weight. Pairs of veterinarians, working from copies of this list, labelled vacutainers with a unique code comprising the dairy co-operative society number, farm identification number, animal identification and date of collection using indelible ink markers at the time of sample collection. Where two vacutainers of blood were collected for one animal, these were secured together for transport to the laboratory. This protocol was intended to ensure, particularly in the early stages of the study before examiners became familiar with individual livestock, that errors in animal identification and failure to collect samples from all animals present were minimized. Further, on two farms where farm labourers were unable to consistently identify individual animals by name, numbered ear-tags were inserted and the animals identified accordingly.

The collection of annotated copies of farm-visit lists, documenting animal removals and additions noted during the farm visit, was made available to laboratory technicians at the time of serum separation to ensure that smudged or partially illegible labels could be interpreted and correctly reproduced on the freezing tubes. Unfortunately, the potential for transfer of serum from the original vacutainers to incorrectly labelled freezing tubes was

entirely dependent on technician competence and vigilance.

Finally, to guarantee that all samples available from a given round of farm visits were actually screened, microtitre plate templates were created from copies of farm-visit lists at the time of thawing and organization of samples for serological screening.

4.4.2 Correction of Errors

To maintain continuity of longitudinal data, it was deemed necessary to impute all missing serological results. The alternative, leaving the values as missing, would result in the omission of entire sets of longitudinal observations for 29 individual animals (29/535 = 5.4%) from subsequent statistical analyses. While a number of imputation methods, including simple deterministic approaches, regression predictions, hot deck procedures, etc. are available, owing to the very small numbers of observations involved and the nature of antibody dynamics, a simple method was adopted. Missing samples which occurred at an initial or final sampling opportunity were allocated the value of the subsequent or previous screening result, while missing samples occurring within the period of animal observation were assigned a value equal to the mean of the previous and subsequent screening results. Although such a crude algorithm for generating missing values may result in both a reduction in overall variance and a bias toward the mean, the fact that the imputations were performed for so small a proportion of observations (0.65% = 29/4433) suggests that any such effect would be virtually negligible. This is particularly true when we realize that, in the absence of seroconversion or an anamnestic humoral immune response, the dynamics of antibody decay ensure that adjacent observations, even at monthly intervals, result in serial test results

that are strongly correlated (see section *7.6.1 Variance Components*). In addition, this method of imputation was very unlikely to either create or obscure instances of seroconversions or sero-events (see section *5.4.2 Definitions of Seroconversion and "Sero-Event"*).

The impact of the 14 aberrant "outlier" observations, considered to have resulted from mislabeling errors, was considered to be minimal in that their very small frequency (0.32% = 14/4404) would result in only trivial differences in the observed variation. In addition, unless occurring in the final month of an individual's observation, a single isolated error of translocation was unlikely to affect the interpretation of the longitudinal serological profile (see section *5.4.2.2 Sero-Event Classification Criteria*) and then only if the error resulted in a distinct increase in OD value. As outliers (either high or low), were only identifiable on the basis of their inconsistency with the overall longitudinal profile, their rate of occurrence, although considered low, cannot be estimated. Thus, rather than attempt to adjust or correct only the apparent errors of translocation, suspected outliers were left unchanged and included in subsequent analyses, to acknowledge this relatively small source of error as a source of test variation.

4.5 Enzyme-Linked Immunosorbent Assay (ELISA)

A simple, indirect, antibody-capture enzyme-linked immunosorbent assay (ELISA) was utilized to screen all serum samples for *T. parva*-specific antibodies (Katende *et al.*, 1998).

Specific target antigen consisted of *Theileria parva* schizont antigen p85, referred to

as the polymorphic immunodominant molecule (PIM) (Toye *et al.*, 1991, 1996). At the time of sample analysis, the 85 kilodalton protein was chromatographically extracted and purified from cell-culture schizont lysate (Katende, personal communication). Subsequently, this antigen was produced by means of recombinant technology, when it was confirmed to be superior to other *T. parva* antigens in its ability to detect antibodies in the sera of experimentally infected cattle, providing an overall test sensitivity of >99% with a concomitant specificity of between 94% and 98%, based on a range of sera from experimentally infected cattle and field sera from endemic and *T. parva*-free areas as applied in an optimized and standardised ELISA protocol (Katende *et al.*, 1998).

Specific antibodies in bovine test sera were detected using a trivalent conjugate. This comprised three individual anti-bovine immunoglobulin conjugates (Igs) prepared by conjugation of horse radish peroxidase R 3.0 (HRP, Serva) to goat anti-bovine Ig, according to the method described previously by Katende *et al.* (1990).

4.5.1 Assay Protocol

Nunc Polysorb 96 MicroWell plates (Nalge Nunc International, Roskilde, Denmark) were utilized in all ELISA assays. Plates were coated at a concentration of 100 ng/well by the addition of 100 µl/well of PIM antigen solution diluted to a concentration of 1.0 µg/ml in 0.01 M Dulbecco's phosphate buffered saline (DPBS), pH 7.4, and incubated either overnight at 4°C, or for one hour at room temperature.

Excess antigen solution was then discarded and the uncoated binding sites on the walls of the MicroWells were blocked by the addition of 300 µl/well of a 1% casein solution

in DPBS followed by incubation for 2 hours at 37°C. Blocking solution was then removed and the plates washed a minimum of 5 times with 0.15 M sodium chloride (normal saline) containing 0.1% Tween 20. Plates were then sealed and stored at -20°C for future use. Shortly prior to use, plates were removed from storage and allowed to return to room temperature.

Test and control sera were diluted to a final working dilution of 1:50 by the addition of 5 µl of serum to 245 µl of a 5% skimmed milk solution prepared in DPBS with 0.1% Tween 20. Serum dilutions were mixed thoroughly and 100 µl transferred to the wells of an antigen-coated MicroWell plate in two replicates (i.e. 100 µl/well). In addition, a pair of conjugate control wells (i.e. containing only 5% skimmed milk solution) were also present on each plate (Figure 4.2). Antibody:antigen binding was facilitated by intermittent gentle agitation of each plate on a micro-agitator (Heidolph, France), approximately every 15 minutes during the course of a 1 hour, room temperature incubation.

Unbound antibodies were removed from the plates by extensive washing which involved a series of 10 rinses with normal saline and 0.1% Tween 20 followed by a 15 minute soaking interval. The washing protocol was performed twice for each plate before the addition of 100 µl/well of a trivalent anti-bovine immunoglobulin horseradish peroxidase (HRP) conjugate diluted to 1:3000 in DPBS containing 0.1% Tween 20 and 2.5% skimmed milk (Katende *et al.*, 1990). Plates were again incubated for one hour at room temperature under a protocol of intermittent agitation and then washed as previously described.

A colourimetric reaction was initiated by the addition of 100µl/well of sodium citrate buffer, pH 4.0, containing 1% hydrogen peroxide as substrate and 40 mM 2,2'-azino-*bis* 3-

ethylbenz-thiazoline-6-sulphuric acid diammonium salt (ABTS) as chromogen. After the addition of the substrate:chromogen solution, plates were incubated in the dark and were subjected to periodic gentle agitation, as previously described, to ensure maximum enzymatic exposure and hence optimal colour development. Beginning at approximately 10 - 15 minutes post substrate:chromogen addition, the light absorbance, in units of optical density (OD) at a wavelength of 414 nm, was determined using a Titertek Multiscan MCC340 spectrophotometer (Titertek Instruments Inc., Huntsville, Alabama, USA). Subsequently, multiple OD readings per plate were made over time of colour development at intervals of between approximately 10 to 15 minutes until the OD reading for the strongest positives observed on each set of plates reached a plateau.

4.5.2 Control and Standardisation Reference Sera

Control sera consisted of aliquots of pooled sera from one positive reactor (C9) and three negative animals (BJ202, BJ212 and BJ28). Aliquots were maintained at -80°C and were thawed to room temperature immediately prior to use. Animal C9 was originally infected twice by *T. parva* (Muguga stock); being initially immunized by infection and treatment and subsequently challenged by application of ticks infected with the same strain. Serum collected from C9 with an antibody titre greater than 1:5000, as determined by the indirect fluorescent antibody test (IFAT) (Goodeeris *et al.*, 1982), was considered to be the laboratory standard reference serum for anti-*T. parva* antibodies. Animals BJ202 and BJ212 were uninfected control animals, while BJ28 had been experimentally infected with *Babesia bigemina* organisms only. All animals were otherwise maintained in tick-free isolation.

In addition to two replicate wells for the conjugate control, each 96 MicroWell plate contained duplicate samples of all 4 control sera. Figure 4.2 provides an example template indicating the position of samples on each plate.

4.6 Assay Standardisation

For the purposes of this study, the primary interest in enzyme immunoassays is assessing the presence and possibly quantifying the level of specific antibody generated by an immunological response in a given subject. However, in order for the test results to be meaningfully interpreted the assay itself should be not only highly sensitive and specific, both immunologically and epidemiologically, but also repeatable. An assay must be optimized for validity and reliability and standardized for consistency within and between laboratories (Wright *et al.*, 1993). Wherever possible, potential sources of variation should be minimized within the assay protocol, accounted for by subsequent adjustment methods, or at the very least detected by a set of quality-control criteria, so that all results produced by a single assay can be directly comparable. It is particularly important in the case of large-scale longitudinal serological studies where the large numbers of samples which must be screened will require testing to be carried out over prolonged periods and consequently under a potential variety of laboratory conditions. In addition, for such studies, maintaining the uniformity of the quantification of the assay is vital since the goal may be to evaluate, not only dichotomous test assessments of positive/negative or time to seroconversion, but also to compare and contrast amounts of antigen-specific antibodies or to examine patterns of antibody levels over time.

Although the enzyme immunoassay protocol described by Katende *et al.* (1998) for the recombinant antigen ELISA is essentially identical (with only minor modifications) to that utilized and described herein, at the time of screening of the samples this assay had been optimised for the detection of *T. parva* specific antibodies, but standardization efforts had concentrated solely on minimizing intra-plate variation. Correction for inter-plate variation was by subtraction of conjugate control OD's of individual plates. Inter-plate variation was assessed on a daily basis by direct comparison of OD values of control sera and, over a broader time-scale, by examining repeatability of the dichotomous assessment (i.e. positive/negative) of test samples from known positive and negative reactors. No attempt was made to obtain quantitative data from the assay.

To obtain the maximum information from this assay in the analysis of longitudinally collected serum samples, it was necessary to interpret on a quantitative, rather than a dichotomous, scale. There are a variety of methods of expressing the results of serological activity amplification assays in at least a semi-quantitative manner (Wright, Kelly and Gall, 1987; Wright *et al.*, 1993). These methods all require the generation of some type of standardised reference criteria (e.g. a standard reference or conversion curve) and therefore rely on the repeatability of individual test outcomes (Tijssen, 1985). Thus it was essential to ensure that inter-plate variation was minimized for meaningful comparisons to be made across plates over the range of sampling times.

Broadly speaking, inter-plate variation is a consequence of any and all factors which affect the performance of an ELISA and which may vary from plate-to-plate, either spatially or temporally. At its lowest resolution, this may be inflated by inter-operator, inter-run or

even inter-laboratory variation. However, in the context of this study, where all serological analyses were conducted by the same operator working within the same laboratory and using the same equipment and reagents, inter-plate variation may be principally attributed to those factors which alter over longer time-scales, i.e. days to months and may be beyond the immediate control of the operator. Examples include environmental conditions such as temperature and humidity, quality of deionised water supply, subtle differences in osmolality or pH of batches of reagents, either as prepared in the laboratory or purchased from commercial sources, reduction in efficacy of stored reagents, variation in performance of dispensing equipment, etc.

The goal of minimising the effect of these factors on test outcomes was accomplished through an adaptation, from first principles, of a timing protocol/targeting method similar to that described by Wright *et al.* (1987), followed by the generation of an assay-specific quantification standard curve and its further use in a post-assay adjustment technique.

4.6.1 Establishment of Standardising Protocol

Wright *et al.*'s (1987) method of reducing the inter-plate variation involved the use of a flexible reading-time post addition of substrate:chromogen such that the OD units for all serum samples on a given microtitre plate are determined at the same time that a standard positive control reference sample on that plate has reached a specified, pre-determined "target" value. The implicit assumption being that all factors affecting the rate of colourimetric development of the targeting control affected the rate of colourimetric development of all samples present on the same microtitre plate similarly and proportionally.

As demonstrated below, this assumption is valid if the chosen positive control OD target value is reached while it and all accompanying samples are still in the approximately linear phase of their colourimetric development.

To fully appreciate the rationale behind such a standardization method, an understanding of the mechanism and kinetics of enzyme activity is necessary.

4.6.1.1 Principles and Quantification of Enzyme Kinetics

The enormous capacity for enzymes to catalyse a substrate to product transformation is a function of their high specificity for the substrate and their ability to lower the activation energy barrier, i.e. the level at which a substrate molecule has achieved sufficient energy to proceed through unstable, but higher energy, transition intermediates to a lower energy-content final product. For example, in the case of the enzyme catalyst horse radish peroxidase (hydrogen-peroxide oxidoreductase) acting at room temperature in the decomposition of hydrogen peroxide, the activation energy is lowered from 70 kJ/mol to 7 kJ/mol, resulting in an acceleration of the reaction rate (k) by a factor in the region of 8.8×10^{10} (Tijssen, 1985).

For a simple single-substrate enzyme, the reaction of enzyme (E) and substrate (S) to form an intermediate complex (ES) in the liberation of product (P) and hence free enzyme, can be represented by the equation:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

where the k_1, k_{-1} and k_2 represent the corresponding reaction rate constants at each step. Given that, under a fixed set of conditions, the rate at which an individual enzyme molecule is capable of converting substrate does not change, the initial overall conversion rate (v_0) is therefore dependent on the relative amounts of substrate and enzyme present initially. More specifically, the rate constants are the constants of proportionality under the *Law of Mass Action*, which states that the rate of a reaction is proportional to the product of the concentrations of the reactants. This rate achieves a maximum (V_{Max}) at enzyme saturation, i.e. when virtually every enzyme molecule is present in a substrate complex (ES). At this point, the speed of release of product (P) is determined by k_2, the rate constant of the limiting step in the reaction. The relationship can be expressed as:

$$V_{Max} = k_2 \cdot [ES]$$

In the situation of continued excess concentration of substrate (i.e. where it can be assumed that every freed enzyme molecule is rebound with a substrate molecule into a substrate complex) this can be alternately expressed as:

$$V_{Max} = k_{cat} \cdot [E]_0$$

where k_{cat} is termed the "catalytic constant" (k_2) and $[E]_0$ is the concentration of enzyme initially present. For low amounts of enzyme V_{Max} will be correspondingly low and is therefore effectively a constant over the duration of the period of incubation as substrate will be in excess of enzyme throughout. This is reflected by a near linear accumulation of product over time. However, when sufficient enzyme is present to catalyse significant

amounts of substrate, not only will V_{Max} be higher, but it will be achieved over a shorter period of time. This is particularly likely to be true if lower initial levels of substrate are utilized in the system.

Michaelis and Menten (1913) demonstrated that the actual enzyme:substrate complex reaction speed is so fast that, beyond the first instants of the reaction, the complex is more or less in equilibrium at all times, i.e. their so-called *pseudo-steady state hypothesis* (Murray, 1989). Under this assumption, the overall initial conversion rate (v_0) is expressed by the equation:

$$v_0 = \frac{V_{Max} \cdot [S]_0}{[S]_0 + K_m} = \frac{k_{cat} \cdot [E]_0 \cdot [S]_0}{[S]_0 + K_m}$$

where K_m is the Michaelis constant which can be expressed as:

$$K_m = \frac{k_{-1} + k_2}{k_1}.$$

For example, when the initial concentration of substrate ($[S]_0$) = K_m, the enzyme reaction begins at half of its maximum possible rate. Further, it is clear that the conversion rate of substrate will vary with time of reaction and, using the Michaelis-Menten solution, can be described by the differential equation:

$$\frac{d[S]}{dt} = -\frac{V_{Max} \cdot [S]_t}{[S]_t + K_m} = -\frac{k_{cat} \cdot [E]_0 \cdot [S]_t}{[S]_t + K_m}$$

where $[S]_t$ = the concentration of substrate left at time t.

Solving for time (t), with initial conditions of enzyme concentration ($[E]_0$) and substrate concentration ($[S]_0$) yields the following equation:

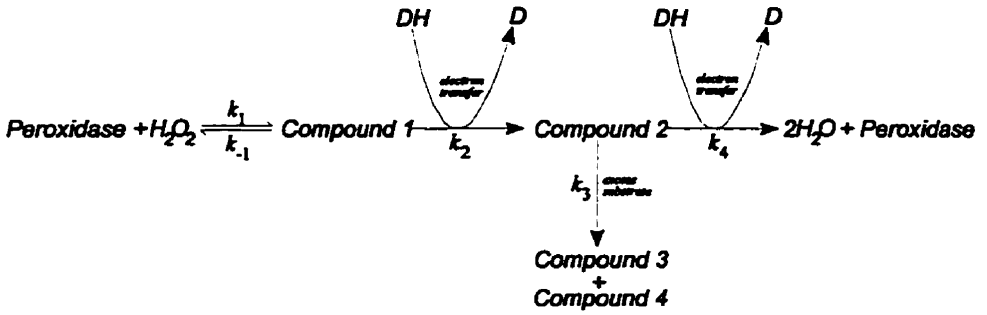
$$t = \frac{K_m \cdot \ln[S]_0 + [S]_0 - K_m \cdot \ln[S]_t + [S]_t}{k_{cat} \cdot [E]_0}$$

Although this equation does not have an analytical solution, it may be applied in a numerical analysis using appropriate software. Maple V Release 3 software (Waterloo Maple Software, University of Waterloo, Waterloo, Ontario, Canada) was used to yield plots of concentration of substrate ($[S]_t$), or the proportion of substrate converted to product ($([S]_0 - [S]_t)/[S]_0$) *versus* incubation time (t = time post addition of substrate). Such plots inevitably yield a characteristic hyperbolic reaction curve. Figure 4.3 contrasts this Michealis-Menten relationship to a constant conversion rate for two relative enzyme concentrations and demonstrates the dual principles that a) the approximation of linearity of product formation is adequate over the initial stages of the enzyme reaction and b) for a fixed set of conditions, the length of the incubation period over which this linear approximation is maintained is inversely proportional to the initial enzyme concentration.

Peroxidases act by transferring hydrogen from a hydrogen donor (DH) to the peroxide molecule according to the equation:

$$H_2O_2 + 2DH \xrightarrow{POase} 2H_20 + 2D$$

More specifically, horseradish peroxidase is divalently oxidized by peroxide to form Compound 1 (C1), which is reduced back to the initial state by two successive univalent interactions with hydrogen donors (DH) as detailed below:



Where, Compound 2 (C2) is the one-electron oxidized, intermediate form and Compounds 3 and 4 are inactivated forms of the enzyme complex which occur in the situation of hydrogen peroxide excess.

The system is therefore effectively a multi-substrate reaction since ABTS (40 mM 2,2'-azino-*bis* 3-ethylbenz-thiazoline-6-sulphuric acid diammonium salt) is acting as both a hydrogen donor and a chromogen in that the pre-oxidation form (DH) is relatively colourless with a maximum absorbance at 340 nm, while the oxidized product (D) is strongly coloured (green) with a maximum absorbance at 414 nm.

Although the enzyme kinetics of horseradish peroxidase are necessarily more complex than those described for a simple single-substrate system, it is possible to simplify them (see Appendix 3) to yield a set of dynamic equations expressed in terms of fixed reaction rate constants (k_x's) and three initial conditions; i) enzyme concentration ($[E]_0$), ii) initial "substrate" concentration ($[H_2O_2]_0$) and iii) initial concentration of chromogen/hydrogen donor ($[DH]_0$). While it is not possible to provide a closed-form

analytical solution for this set of equations, a numerical analysis was again performed utilising Maple V Release 3 (Waterloo Maple Software, University of Waterloo, Waterloo, Ontario, Canada). Figure 4.4 presents plots of A) proportion of oxidised, colourimetric product (D) produced and B) proportion of substrate ($S = H_2O_2$) consumed, respectively, *versus* incubation time for a fixed enzyme concentration and set of theoretical reaction rate constants. Each plot graphically contrasts the effects of relative amounts of initial substrate versus initial chromogen for three scenarios; i) equivalent amount of each; $[DH]_0=[S]_0$, ii) chromogen in excess; $10*[DH]_0=[S]_0$ and iii) substrate in excess; $[DH]_0=10*[S]_0$, such that, when the initial substrate concentration ($[S]_0$) is equivalent to, or in excess of the initial chromogen concentration ($[DH]_0$), the initial chromogen concentration becomes the limiting factor with the curve of proportion of product produced being equivalent and virtually identical to that generated by the simple single-substrate enzyme system (Figure 4.3). In contrast, only in the situation of low initial concentration, relative to chromogen, does the initial substrate concentration become the limiting factor, altering the curve of product production with respect to the maximum achievable colourimetric development.

Fortunately, since the concentration of hydrogen peroxide (2.2 mM) used in the ELISA protocol was much greater than the concentration of ABTS (0.2 mM), the Michaelis-Menten approximation of a simple single-substrate enzyme system can be used in place of the more complex hydrogen peroxide/ABTS system to explore the relationship between variation in environmental conditions and variation in rate of production of colourimetric product.

4.6.2 Contrasting a Fixed-time Reading Frame versus Targeting Protocol

While V_{Max} varies linearly with the amount of enzyme present, which in turn is proportional to the amount of bound antibody, K_m is independent of the enzyme concentration. Rather, K_m and k_{cat} are constant for a given set of environmental and assay conditions (pH, buffer composition, temperature, etc.).

As previously stated, the ELISA protocol was optimized such that, although conducted at room temperature, all incubation periods were of sufficient length (1 hour under intermittent agitation) to ensure binding of antigen-specific antibodies and subsequently anti-bovine conjugate reached equilibrium. If we, therefore, make the reasonable assumption that changes in environmental conditions do not significantly affect the amount of enzyme present in the assay for a given sample, but they alter both the K_m and k_{cat} similarly and proportionally, then Figure 4.5 illustrates the potential for higher variation in resulting sample test outcomes for a fixed-time reading frame *versus* reading at a control reference target OD as follows.

Using the implicit solution of the Michaelis-Menten equation defined previously as an approximation to the hydrogen peroxidase system, Figures 4.5 A and B provide identical plots of time by substrate conversion for a reference control ("Target") and a test sample ("Sample") under two sets of hypothetical environmental conditions, where the reference control is a 'strong positive reactor', equivalent to stating that a greater concentration of enzyme is present in the target than the sample, $[E]_{Target} > [E]_{Sample}$. In this example, Target-2 and Sample-2 possess the same concentration of enzyme as Target-1 and Sample-1,

respectively, but their corresponding K_m and k_{cat} are reduced to one-half the Target-1/Sample-1 values. Although the reduction in proportionality constants is necessarily extreme for demonstration purposes, we nevertheless could imagine this situation to correspond to such factors as a lower ambient room temperature for screening tests conducted in a colder season of the year. Figure 4.5 A indicates the corresponding proportions of substrate converted to product for Target-1 (*T1*), Sample-1 (*S1*), Target-2 (*T2*) and Sample-2 (*S2*) when all outcomes are assessed at a fixed time post addition of substrate (t^*). Under this approach, not only is there considerable variation in the observed sample outcome values, S1 >> S2, but also between the actual target outcomes as well, T1 >> T2. In contrast, in Figure 4.5 B, sample outcomes ($S1^*$ and $S2^*$) are assessed when both Target-1 and Target-2 outcomes are equivalent to a single target value, T^* (equivalent to *T1* in Figure 4.3A), but each occurring at different incubation times, *t1* and *t2* respectively (noting that $t2>t1=t^*$). Clearly, by definition all variation in target outcomes has been removed, however, the magnitude of the absolute difference between sample outcomes has also been substantially reduced.

Although not identical in value, it is clear from this example that the use of a targeting protocol can significantly reduce the variation between sample outcome measures (*S1:S2 versus S1*:S2**) under situations of varying of proportionality constants. The remaining slight absolute difference in value between the sample outcomes ($S1^\bullet > S2^*$) is a function of the non-linear relationship present in the system. This non-linearity has further implication in terms of the choice of an appropriate outcome targeting value.

4.6.3 Choice of Outcome Targeting Value

To minimize the variation in standardized outcome measures, it is necessary to reduce the effective impact of the non-linear dynamics of the enzyme:substrate reaction. This can perhaps most easily be accomplished by selection of a targeting value on the earlier, near-linear phase of the hyperbolic reaction curve.

Figures 4.6 A and B assume the same theoretical enzyme:substrate system and hypothetical test conditions as per Figure 4.5. However, while Figure 4.6 A also utilizes the same targeting value, T^*, as per Figure 4.5 B (and hence the figures themselves are identical), Figure 4.6 B demonstrates the effect on standardization of sample outcomes when a higher targeting value is utilized. Essentially the relationship is such that, when the concentration of enzyme in the target is greater than that in the sample ($[E]_{Target} > [E]_{Sample}$), then the farther from the approximately linear portion of the substrate conversion curve the targeting value is chosen (T', where $T'>T^*$) the greater will be the subsequent variability in standardized sample outcomes ($S1'>S2'$ *versus* $S1^*>S2^*$). In a corollary to this relationship, Figure 4.7 illustrates that this principle can also apply when the concentration of enzyme in the sample is greater than that in the target ($[E]_{Sample} > [E]_{Target}$) but holds true only over a limited range of increasing target values. In fact, under this scenario, the relationship between sample variability and chosen target value is quadratic as, at the extreme target values, the sample outcomes converge toward complete substrate conversion. Notably, however, the magnitude of the difference between the samples is reversed (S1'<S2' in Figure 4.7 *versus* S1*<S2* in Figure 4.6).

Clearly it would be desirable to establish an immunoassay to work in conditions of very high initial substrate concentration, $[S]_0$, such that $[S]_0+K_m \approx [S]_0$, and thus $v_0 \approx V_{Max}=k_{cat}\cdot[E]_0$, which could be maintained for more prolonged periods and result in an effectively linear production of product over the entire incubation period. Unfortunately, as indicated previously, the very high concentrations of substrate which can be required for complete saturation of enzyme (e.g. $[S]_0=100\cdot K_m$) can also result in direct enzyme inhibition by the substrate, as is the case with the hydrogen peroxide/horse radish peroxidase system (Tijssen, 1985). This becomes particularly problematic when a single initial concentration of substrate is to be applied to a potentially wide range of unknown concentrations of enzyme (amounts of antibody), as occurs in most enzyme-immunoassays. In such a situation, the limiting factor becomes the desire not to overly inhibit low-positive reactions such that they become false negatives, and hence a certain degree of non-linearity among strong-positive reactions must be accepted.

Having demonstrated that the longer the incubation time or greater the enzyme concentration, the greater the departure from the assumption of linearity of substrate transformation and hence the larger the sample variation, it might seem inherently logical to select a targeting value which would be achieved relatively quickly post-addition of substrate. However, while this would ensure minimal variation by enzyme concentrations and hence direct comparability of outcome measures, it could also compromise the ability to discriminate quantitatively between measures.

4.6.3.1 Maximization of Quantitative Discrimination

The ability to discriminate between quantitative outcome measures of enzyme amplification assays is a function of measurement scale and resolution. Alternatively, the problem can expressed as one of attempting to maximize the range of assay outcome measures across the potential range of enzyme concentrations while simultaneously minimizing the variation between repeated measures within the same enzyme concentration. The application of these principles may again be best illustrated graphically.

Figure 4.8 describes a second theoretical enzyme:substrate system and includes a series of plots of proportion of substrate consumed over time of reaction, generated from the Michaelis-Menten relationship, in which each line corresponds to a linear reduction by 5% of initial enzyme concentration ($[E]_0$), but where K_m and k_{cat} are held constant in each case. Assuming that the assay response error relationship is fixed (i.e. precision of measurment of OD is not variable) and that the range of potential enzyme concentrations are representative of a population of serologically positive samples, then it is clear that the maximum variation in outcome measures, and hence the maximum absolute difference in outcome measure for any two initial enzyme concentrations, occurs at an incubation time just prior to the point where the outcome measures for the highest enzyme concentrations (strongest positive reactors) cease diverging and begin to converge. This time is represented on the figure as the *"Point of Maximum Resolution"*, and is clearly beyond the linear phase of substrate conversion for higher enzyme concentrations. It is also evident that beyond this point, the higher enzyme concentrations continue to converge toward maximum substrate conversion while the lower enzyme concentrations, although continuing to diverge with respect to one

another, by virtue of their continued conversion of substrate at, albeit lower but approximately linear rates, they too converge with respect to higher enzyme concentrations. The overall effect is one of an initial increase in resolution across enzyme concentration, up to the *"Point of Maximum Resolution"*, followed by a decrease in resolution which progressively effects higher to lower enzyme concentrations over incubation time. This phenomenon occurs as a result of the fact that the substrate conversion process proceeds at different non-linear rates for each enzyme concentration.

It was therefore necessary to strike a balance between minimizing sample variation (by means of a low/linear target value) and maximizing quantitative resolution. Figure 4.8 demonstrates that this compromise could be accomplished through the choice of a targeting value at or near the maximum extent of the linear phase of substrate conversion for the greatest enzyme concentration.

4.6.4 Adopted Targeting Protocol

The implementation of the targeting method first involved the simultaneous screening of an entire set of monthly serum samples (approximately 400 samples on 10 MicroWell plates). As previously described, multiple readings at approximately 10 to 15 minute intervals over the time post-addition of substrate:chromogen were made for each of the plates. For those samples with the fastest developing OD readings and the reference positive control serum (C9), the mean of each set of paired serum samples was plotted over incubation time. From these plots, the point of maximum colourimetric development was identified for the strongest positive samples and the approximate corresponding OD value

for the reference positive control determined at the same incubation time. Notably, the strongest positive samples demonstrated significantly higher and more rapid colourimetric development than the reference positive control serum (C9), such that, their point of maximum colour development (approximately 1.2 OD units) was achieved while the reference control samples had attained only approximately one-half that value (≈ 0.500 OD units). This value of the positive reference control was adopted as the test target value.

The protocol of multiple timed readings post-addition of substrate (see section *4.5.1 Assay Protocol*) was implemented for all subsequent MicroWell plates of samples. Thus, for each plate, a set of 4 - 6 timed readings were generated, covering an incubation period of between 25 and 90 minutes duration. The results of the mean OD value for replicates of the positive reference control were compared and the reading at which this value was closest to the test target value but where the results for strong positive samples present on the plate were also between the end point of the linear phase and the point of maximum resolution of colourimetric development (see Figure 4.8) was selected. Mean OD values for all replicates of samples present on the plate were recorded from this reading as raw unadjusted data. A total of 103 MicroWell plates of samples were assayed using the targeting protocol. Table 4.2 provides the distribution of selected targeting times for these plates.

Serum samples were prepared in batches on 8 to 10 MicroWell plates per assay, each plate requiring approximately 90 seconds to be read by the spectrophotometer. Unfortunately, the resulting 10 to 15 minute interval between subsequent readings of an individual plate precluded a highly focussed or predictive targeting (e.g. where target-time was predicted based on a previous reading), such that the "best" reading usually under or

over-shot the target value. This situation was further compounded when it was discovered that two different aliquots of the strong positive control differed in target value, a second aliquot yielding a target value of approximately 0.350 OD units when the first aliquot had reached 0.500 units. Figure 4.9 presents the frequency distribution of mean OD values at target incubation time for the 45 plates targeted with aliquot 1 (A) and 58 plates targeted with aliquot 2 (B) of the reference positive control (C9). Similarly, Figure 4.10 presents the frequency distribution of mean OD values at target incubation for samples from negative control animals BJ202 (A), BJ212 (B) and BJ28 (C) which were present on each of 103 plates. On each figure a continuous normal distribution of equivalent mean and standard deviation is superimposed which fits the distribution reasonably well, suggesting that at least some of the variation observed is due to what was effectively a random starting point for the timed readings.

Figure 4.11 illustrates the relationship between the targeted value of the reference positive control and the corresponding test result for each of the negative controls, and supports the above assertion by demonstrating the consistent positive correlation between target-time OD values on the same plate.

4.7 Quality Control

Although the use of a targeting protocol is effective in minimizing inter-plate variation, it has little or no effect on intra-plate variation. Where reference positive and negative controls are being used in a targeting and refinement protocol, it is important for inter-plate standardisation and quantification that intra-plate variation be simultaneously

minimised.

It is possible to estimate the degree of intra-plate variation without resorting to the use of a relatively large number of sample replicates per plate, dramatically increasing the cost and reducing the speed and efficiency of the assay. With the exception of edge effects, sources of intra-plate variation are those which may differ from well-to-well and are usually associated with poor operator technique or equipment error, e.g. pipetting or dilution errors, etc. Thus, careful and consistent laboratory technique combined with strict rejection criteria applied to sets of duplicate samples can be an effective method of quality control.

All samples, including conjugate and positive and negative controls were applied to each 96 MicroWell plate in duplicate (Figure 4.2). For each paired sample, the mean, standard deviation and coefficient of variation were calculated. Figure 4.11 A presents the frequency distribution of level of standard deviation observed between the final raw test results for the 4422 pairs of replicates (9 of 4404 samples had 4 replicates) processed, demonstrating that 90.88% (4019/4422) exhibited standard deviations of 0.015 OD units or less, rising to 98.6% (4362/4422) for values of 0.030 OD units or less (equivalent to a difference between replicates of 0.030 and 0.060 OD units, respectively). Further, for any pair of replicates, when the coefficient of variation exceeded 15% and the standard deviation exceeded 0.025 OD units, the results were rejected and the sample repeated. This combination of rejection criteria was chosen to reflect the greater importance of correctly discriminating OD results at or around the negative cut-off, particularly in consideration of subsequent seroconversion classification criteria (see section *5.4.2.1 Seroconversion Classification Criteria*). In contrast, slightly higher standard deviations were tolerated for

strongly positive samples with a concomitant lower coefficient of variation and *vice versa* for very low negative samples. In total, 62 samples (1.40% = 62/4422) were rejected and repeated. Figure 4.11 B presents a scatter plot of the standard deviation *versus* coefficient of variation for the 4422 pairs of final raw test results, clearly indicating the absence of observations above the quality control criteria and demonstrating the predominance of low standard deviation/low coefficient of variation combinations.

4.8 Establishment of Standard Titration Quantification Curve

To quantify the assay-specific relationship between the serological test result, expressed in OD units at a single dilution, and the antibody activity (which is a function of the quantity and affinity of the antibody for the test antigen) present in the sample, as assessed by traditional titration methods, a standard titration curve was generated as follows.

Beginning at the initial working dilution of 1/50, 158 different sera from animals whose raw targeted test values covered the range of positive OD values (>0.125 OD units) were serially diluted in a two-fold fashion on MicroWell plates to end dilutions of 1/6400 for weak positive samples and to 1/204800 for strong positive reactors. Each plate contained positive and negative control sera and was assayed using the ELISA test and targeting protocol described. Raw OD test results for each individual were then plotted against the natural log transformation of the denominator of each dilution and a curve of the equation

$$OD = \frac{a}{[\ln(dilution)]^{b}+c}$$

was fitted to the data by a non-linear optimisation process (Corel® Quattro® Pro 7, Corel

Corporation, Ottawa, Ontario, Canada). For each animal the best-fit parameter estimates for *a*, *b* and *c* were utilised in the inverse equation

$$\ln(dilution) = \left(\frac{a}{cut\text{-}off} - c\right)^{\frac{1}{b}}$$

to determine more precisely the corresponding "end-dilution", i.e. beyond which the sample would test below the negative cut-off (= 0.125 OD units). Two samples, where the original raw OD values at the working dilution of 1/50 were very close to the cut-off level (0.130 & 0.135), both yielded results of 0.118 OD units on titration and were thus excluded from the optimisation process.

Thus a total of 156 end-dilutions were available. From each of these, the natural log of 50 was subtracted; the difference between the two values being equivalent to the additional factor by which the original sample needed to be diluted to achieve a negative test result and hence a reflection of the extra amount of antibody present in the sample. In turn, these values were plotted against the initial test result in OD units obtained at the working dilution of 1/50 (Figure 4.13). Next, to quantify the mean relationship between the two measures, a sigmoid curve of the equation

$$Test\ OD = \frac{Max * Min * e^{[\ln(enddilution) - \ln(50)] * r}}{Max - Min + Min * e^{[\ln(enddilution) - \ln(50)] * r}}$$

where:

Max = Maximum OD obtained at ELISA saturation

Min = Minimum possible OD (= negative cut-off of 0.125 OD units)

r = coefficient of slope between *Max* and *Min*

was fitted to the data using least squares non-linear regression (Proc NLIN, SAS® For Windows ™, Version 6.12, SAS Institute Inc., Cary, North Carolina, USA). Specifically, the multivariate secant method of false position (DUD method of Ralston and Jennrich, 1978) was employed to derive estimates for the parameters r and *Max*, *Min* having been constrained to the negative cut-off threshold (0.125). Parameter estimates, their asymptotic standard errors and correlation are presented for this equation in Table 4.3 and the corresponding best-fit curve is superimposed on the scatter plot of raw data in Figure 4.13. Overall model fit was assessed by examination of residuals. Figure 4.14 presents scatter plots of standardised residual (A), predicted value (B) and leverage (C) *versus* observed OD reading and of standardised residual *versus* leverage (D).

Two important features of the relationship between the serological test result and the natural log of the titre are notable from these plots. First, owing to the choice of targeting value, over the greater portion of the interval of observed OD values (i.e. from approximately 0.200 through 0.900 OD units), the relationship is effectively straight (Figure 4.13). Only at the extreme titre values does the ELISA test approach its colourimetric saturation level. Second, variation increases across this same interval but subsequently decreases as saturation is approached (Figure 4.14 A&B). As expected, leverage increases substantially with increasing titre (Figure 4.14 C). This is principally explained by the saturation effect and the strong correlation observed between the r and *Max* estimates. Fortunately, no inordinately large residuals are noted for observations exhibiting higher leverage values (Figure 4.14 D).

4.9 Post-Assay Adjustment of Optical Density Values

Despite the apparent success of the targeting protocol, as demonstrated by the shape and fit of the standard titration curve, the distribution of control OD values at target time (Figures 4.9 and 4.10) indicates that the method employed was only partially effective and that sufficient inter-plate variation existed post-targeting to warrant a further refinement of raw OD values. The positive correlations observed between the same control results and the consistency in relative magnitude (Figure 4.11) suggested a method of post-assay adjustment of raw targeted results based on the departure of control sample values from their mean at-target value as follows.

4.9.1 Calculation of Post-Targeting Control Optical Density Ratios

As noted, every MicroWell plate contained paired replicates for each of the 3 negative control sera (BJ202, BJ212 and BJ28) and the positive target sera (C9; aliquot 1 or 2) such that an overall mean OD value for each negative control was calculated from raw "at-target values" recorded on the 103 plates assayed (Figure 4.10 A-C, respectively). In the case of the positive target, separate means were calculated at target OD values on the 45 plates which contained aliquot 1 (Figure 4.9 A) and on the 58 plates which contained aliquot 2 (Figure 4.9 B).

For each plate, 4 "control-OD ratios" were created by dividing the mean OD value of each pair of control replicates on the plate by the overall mean at-target value of that control sera/aliquot. Clearly, the control OD ratio for the reference/target positive sample estimates the degree of OD development of a given plate relative to the overall mean target

value achieved. That is, a ratio >1 suggests the target OD for the plate over-shot the mean value, while those <1 indicate the target OD had yet to be achieved at the time of reading. Figure 4.11 demonstrates the positive correlation between negative and positive control OD values at target time and establishes the consistency of effect of over or under targeting of a given plate. In consequence, inter-plate variation was further reduced by a post-targeting plate-adjustment step whereby all raw sample OD values on a given plate were altered by a factor equivalent to the relative degree of over or under development of that plate.

4.9.2 Calculation of Post-Targeting Plate-Adjustment Factor

A specific adjustment factor was calculated for each plate as follows. First, from each set of four control OD ratios, the median of the three negative-control ratios was selected, along with the positive-target-OD ratio. Next, the coefficient of variation (relative to the corresponding mean of all 103 plate ratios, where the mean of positive ratios = 1 and the mean of median negative ratios = 0.97) was calculated for each. Finally, the plate adjustment factor was calculated as the inverse of the sum of the ratios, where each ratio was weighted by the coefficient of variation of the opposite ratio, and where the positive control ratio received twice the weight of the median negative control ratio. The calculation is given by the following equation

$$AF = \frac{(2 * CVMedNeg + CVPos)}{(2 * CVMedNeg * PosRatio) + (CVPos * MedNegRatio)}$$

Positive target and negative control samples were not measured without error. The impact of such errors on the process of post-target adjustment was minimised by combining

the target positive and the median negative control OD ratios in a method of calculation weighted in favour of the ratio closest to its overall mean. The increased weighting given to the positive control OD ratio was a reflection of its relative importance as in the original targeting protocol. In general, the process of post-targeting adjustment was undertaken as a refinement or fine-tuning exercise only and, where possible, efforts were made to minimise the impact of errors which biased away from mean at-target values. Figure 4.15 presents the frequency distribution of 103 plate adjustment factors, showing that the maximum degree of post-target adjustment was of the order of ±25%, while for the majority of plates (53.3% = 55/103) it was less than ±10%.

4.9.3 Sample Correction by Plate-Adjustment Factor

Although the post-assay adjustment factor was plate-specific, in consideration of the non-linearity of colourimetric development, within each plate its method of application was conditional on the raw test result of the sample. Samples which it was certain would be at or below the linear phase of their OD development even after adjustment (<0.500 OD units), were corrected by multiplication of their mean OD value by the plate adjustment factor. For samples with mean raw OD values ≥ 0.500 OD units, a three step process of adjustment was employed. First, using the equation describing the standard quantification curve (Table 4.3) the sample mean OD value was transformed to the equivalent ln(dilution) value. Second, this value was corrected by multiplication by the plate adjustment factor. Third, the corrected ln(dilution) value was back-transformed to yield an adjusted sample OD value. The effect of these transformations was to provide a decreasing degree of adjustment as OD values

approach the maximum or saturation level. In fact, the shape of the standard quantification curve means there is little difference between transformation and direct multiplication for mean OD values less than approximately 0.900 OD units, while for values greater than this, the effect of larger adjustment factors is dampened out. This is illustrated in Figure 4.16 which presents a scatter plot of mean raw test result *versus* the post-adjustment value in OD units for all 4404 samples.

4.10 Distribution of Final OD values

Although the post-targeting adjustment process altered some raw test results by a factor of as large as 1.25 or as small as 0.8 (Figure 4.15), because adjustment factors were relatively symmetrically distributed around the mean, the overall effect on the distribution of results as a whole was minimal. This is demonstrated in Table 4.4 which contrasts descriptive statistics calculated for raw test results *versus* those for adjusted values. Values for mean, standard deviation, median, minimum and maximum are nearly identical for the two distributions. Similarly, the number of observations declared negative ($\leq$0.125 OD units) is reduced by only 14 (2029 to 2015) by the process of post-targeting adjustment. Figure 4.17 presents the frequency distribution of final post-adjustment test optical density values for the 4404 samples analysed and the 29 missing values imputed from post-adjustment results. The distribution of adjusted test values is markedly skewed toward higher values with a mean value of 0.231 OD units but a median of only 0.139 OD units. The standard deviation was 0.235 OD units and values varied from 0 through 1.126 OD units. In total, 54.5% (2418/4433) of adjusted values tested positive (>0.125 OD units),

while 13.6% (603/4433) yielded test results greater than the approximate mean of the target control (i.e. >0.500 OD units).

Table 4.1: Number and distribution of serum samples collected in a longitudinal study conducted in Kiambu District from July 1991 - June 1992 and screened by ELISA, presented by age class (0-12 months of age = calf, >12 months = adult) and blood collection/sampling opportunity.

Age Class	Examination	Serum Sample Screened		Total
		Yes	No	
Adult	Initial	334	0	334
	Follow-up	3060	16[1]	3076
Calf	Initial	182	9[2]	191
	Follow-up	828	4[3]	832
	Totals	4404	29[4]	4433

[1] - Of these 16 sampling opportunities, two occurred at the point of final examination of the animal. For one of these, the farmer denied the request to collect blood but otherwise consented to the animal being examined. However, this farmer withdrew from the study at the subsequent farm visit. The remaining 14 missing serum samples have both a previous and subsequent monthly test result.

[2] - Four of these nine calves were examined on the initial farm visit. The remaining five animals were first examined as newborns, four at less than 1 week of age. In all cases, the farmers specifically requested that blood samples not be collect from very young stock. However, none of the seven farmers making this request withdrew from the study.

[3] - All of these missing serum samples have both a previous and subsequent monthly test result.

[4] - In no case has any animal more than one missing serum sample over its period of observation.

Table 4.2: Frequency distribution of time of development (time post-addition of substrate) at reading for 103 MicroWell plates containing serum samples collected in a longitudinal study conducted in Kiambu District from July 1991 - June 1992 and analysed by an enzyme-linked immunosorbent assay in a targeting protocol.

Target Time (minutes)	Frequency (# plates)
25	1
30	11
34	2
35	2
37	2
40	7
43	1
45	9
48	4
50	1
52	2
55	9
60	48
70	1
75	2
90	1
Total	103

Table 4.3 Estimates of mean and asymptotic standard errors for parameters contained in the non-linear model of format

$$Test\ OD = \frac{Max * Min * e^{[\ln(enddilution) - \ln(50)] * r}}{Max - Min + Min * e^{[\ln(enddilution) - \ln(50)] * r}}$$

(where *Max* = Maximum OD obtained at ELISA saturation, *Min* = constrained to negative cut-off = 0.125 and *r* = coefficient of slope between *Max* and *Min*), describing the relationship between sample test optical density unit at 1/50 working dilution, as assayed using targeted ELISA protocol, and natural log transformation of dilution factor required for sample to achieve the negative threshold (=0.125 OD units), for 156 serum samples collected in a longitudinal study conducted in Kiambu District from July 1991 - June 1992 and titrated to extinction.

Parameter	Estimate	Asymptotic Standard Error
r	0.822	0.0194
Max	1.127	0.0275
Correlation (*r*:*Max*)	-0.833	

Table 4.4: Comparison of descriptive statistics of raw targeted test results and post-adjustment values of 4404 samples analysed and the 29 imputed values imputed for serum samples collected in a longitudinal study conducted in Kiambu District from July 1991 - June 1992 and analysed by means of a enzyme-linked immunosorbent assay in a targeting protocol.

Statistic	Raw Targeted Results	Adjusted Values
n	4433	4433
Mean (OD Units)	0.231	0.228
Standard Deviation (OD Units)	0.236	0.233
Median (OD Units)	0.139	0.137
Min - Max (OD Units)	0 - 1.126	0 - 1.151
Number Negative[1]	2029	2015
Percentage Positive[1]	54.2	54.5

[1] - negative refers to OD values ≤0.125 OD units

Figure 4.1: Plots of monthly serological screening results for two animals in a longitudinal study conducted in Kiambu District from July 1991 - June 1992, demonstrating the identification of mislabeled samples/data entry errors (■) from "acceptable" (●) results for (**A**) an otherwise consistently strong positive reactor and (**B**) an otherwise consistently negative reactor.

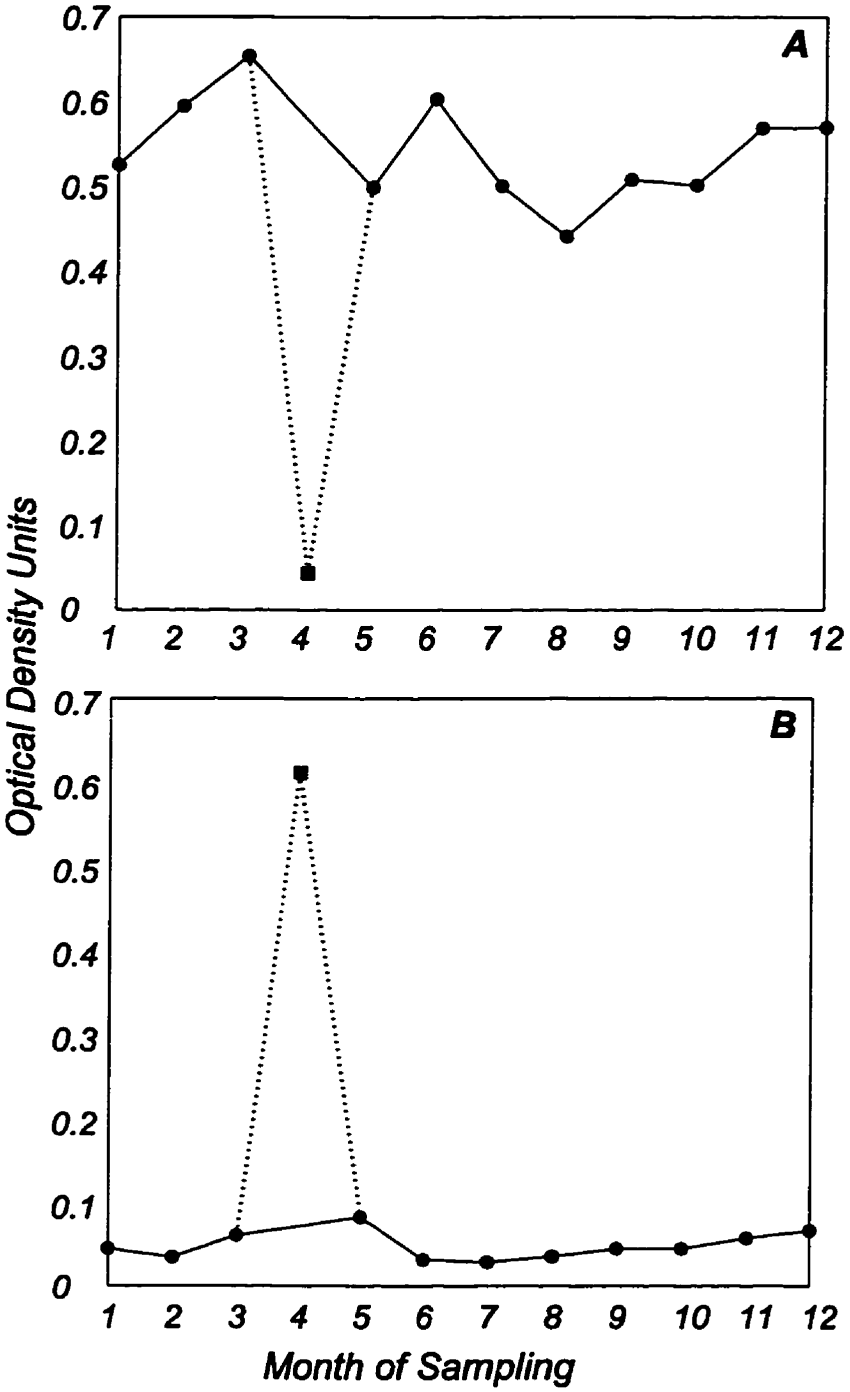


Figure 4.2: Example template of typical 96 MicroWell microtitre plate showing positioning of control and test serum samples collected in a longitudinal study conducted in Kiambu District from July 1991 - June 1992 and analysed by an enzyme-linked immunosorbent assay in a targeting protocol.

	1	2	3	4	5	6	7	8	9	10	11	12
1	Conj.	Neg. 3	4	8	12	16	20	24	28	32	36	40
2	Conj.	Neg. 3	4	8	12	16	20	24	28	32	36	40
3	Pos.	1	5	9	13	17	21	25	29	33	37	41
4	Pos.	1	5	9	13	17	21	25	29	33	37	41
5	Neg. 1	2	6	10	14	18	22	26	30	34	38	42
6	Neg. 1	2	6	10	14	18	22	26	30	34	38	42
7	Neg. 2	3	7	11	15	19	23	27	31	35	39	43
8	Neg. 2	3	7	11	15	19	23	27	31	35	39	43

Figure 4.3: Comparison of characteristic Michaelis-Menten hyperbolic curve of substrate conversion *versus* linear approximation for production of product for "high" (E_0) and "low" ($0.2*E_0$) initial enzyme concentrations.

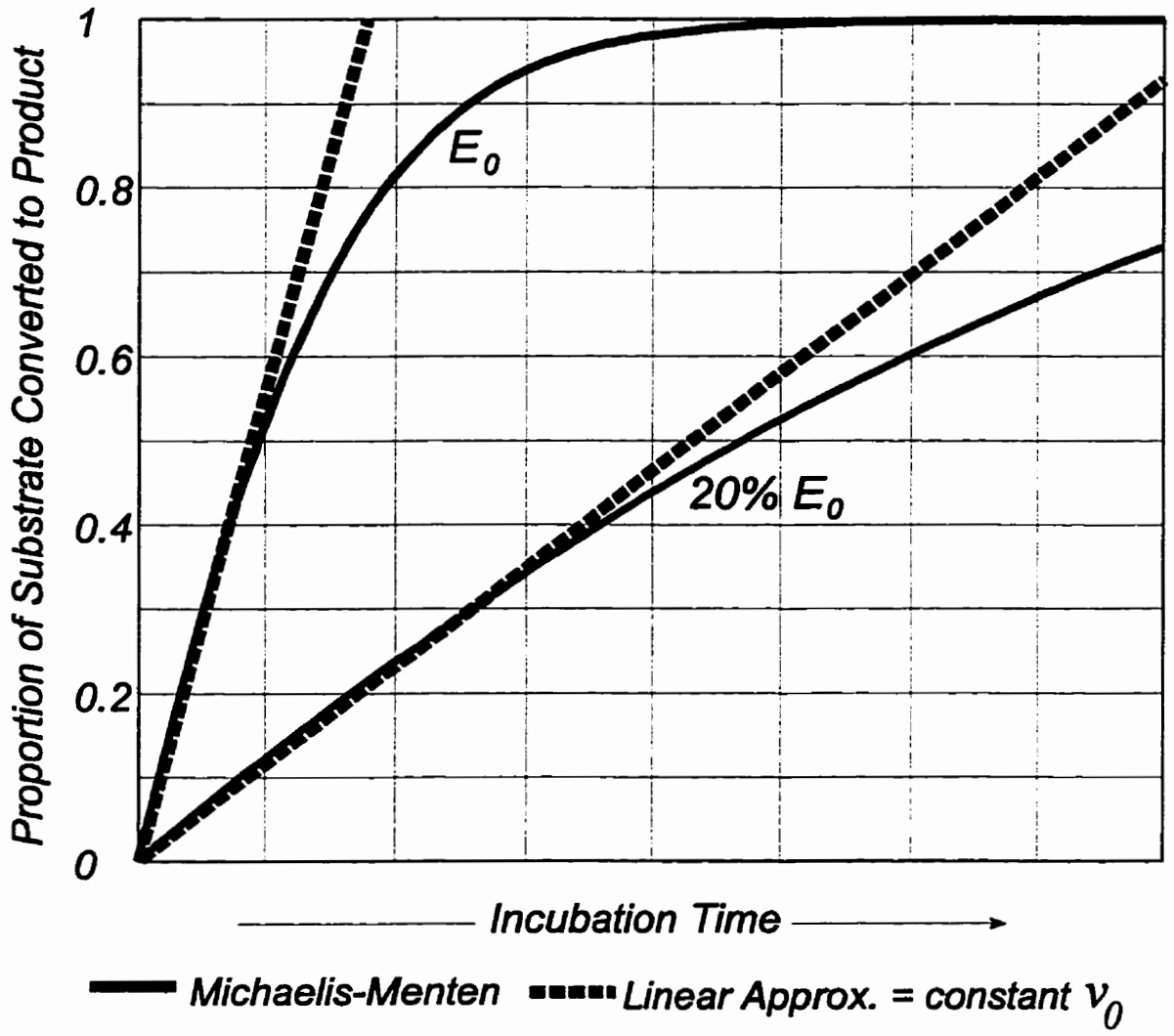


Figure 4.4: Comparison of proportion of colourimetric product produced (**A**) and proportion of substrate (H_2O_2) consumed (**B**) for a given horse-radish peroxidase enzyme concentration but under three scenarios of relative amounts of initial substrate ($[S]_0$) *versus* initial chromogen ($[DH]_0$); i) equivalent amounts of each - $[DH]_0=[S]_0$, ii) chromogen in excess - $10*[DH]_0=[S]_0$ and iii) substrate in excess - $[DH]_0=10*[S]_0$.

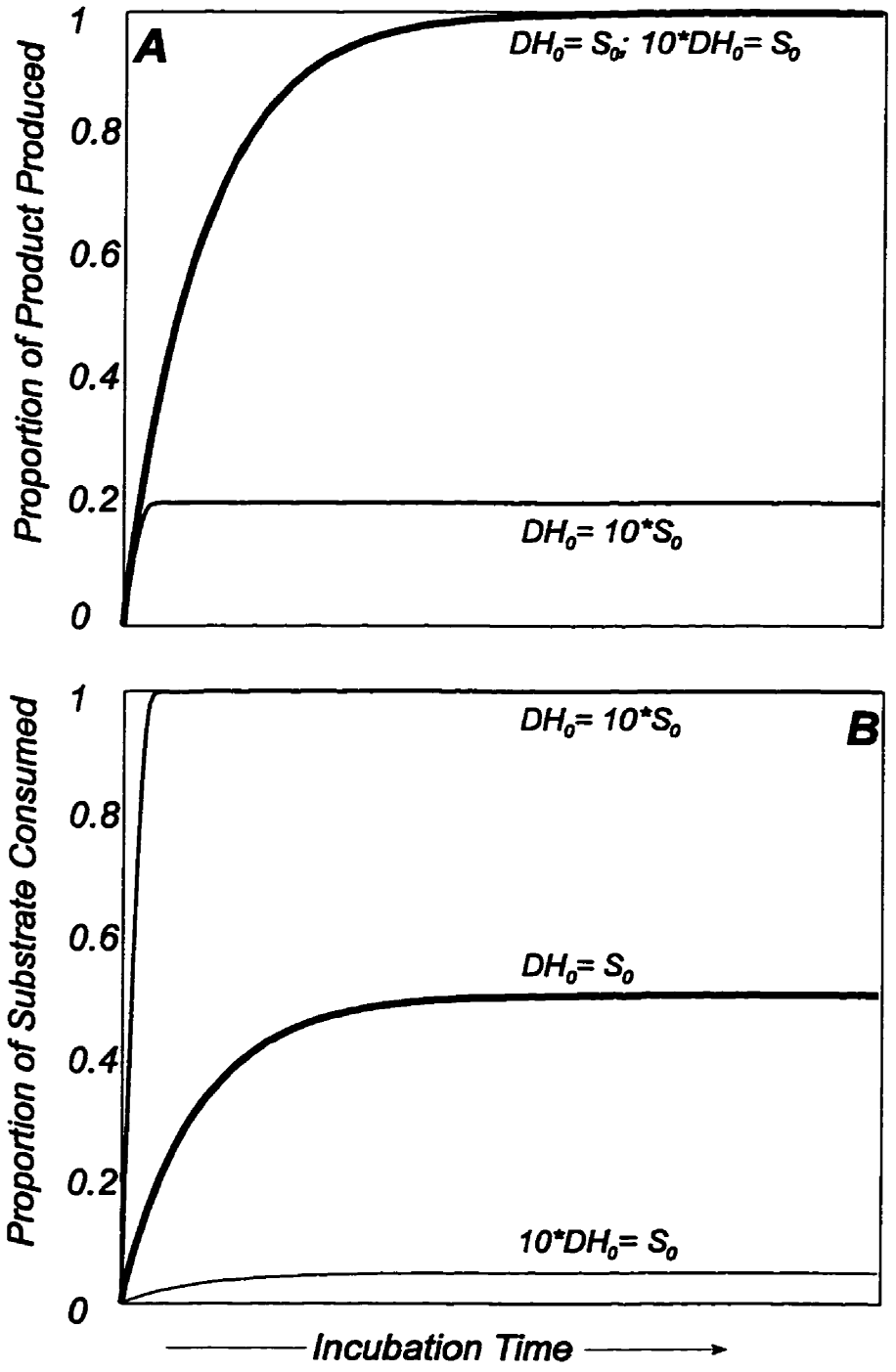


Figure 4.5: Comparison of proportion of substrate converted to product in a sample (S) and a target (T) by a theoretical simple enzyme:substrate system operating under two different sets of environmental conditions (1 & 2) where the time at measurement (t) is determined using a fixed-time reading frame (**A**) *versus* a targeting protocol (**B**), demonstrating the reduced variation in both sample and target values for **B**.

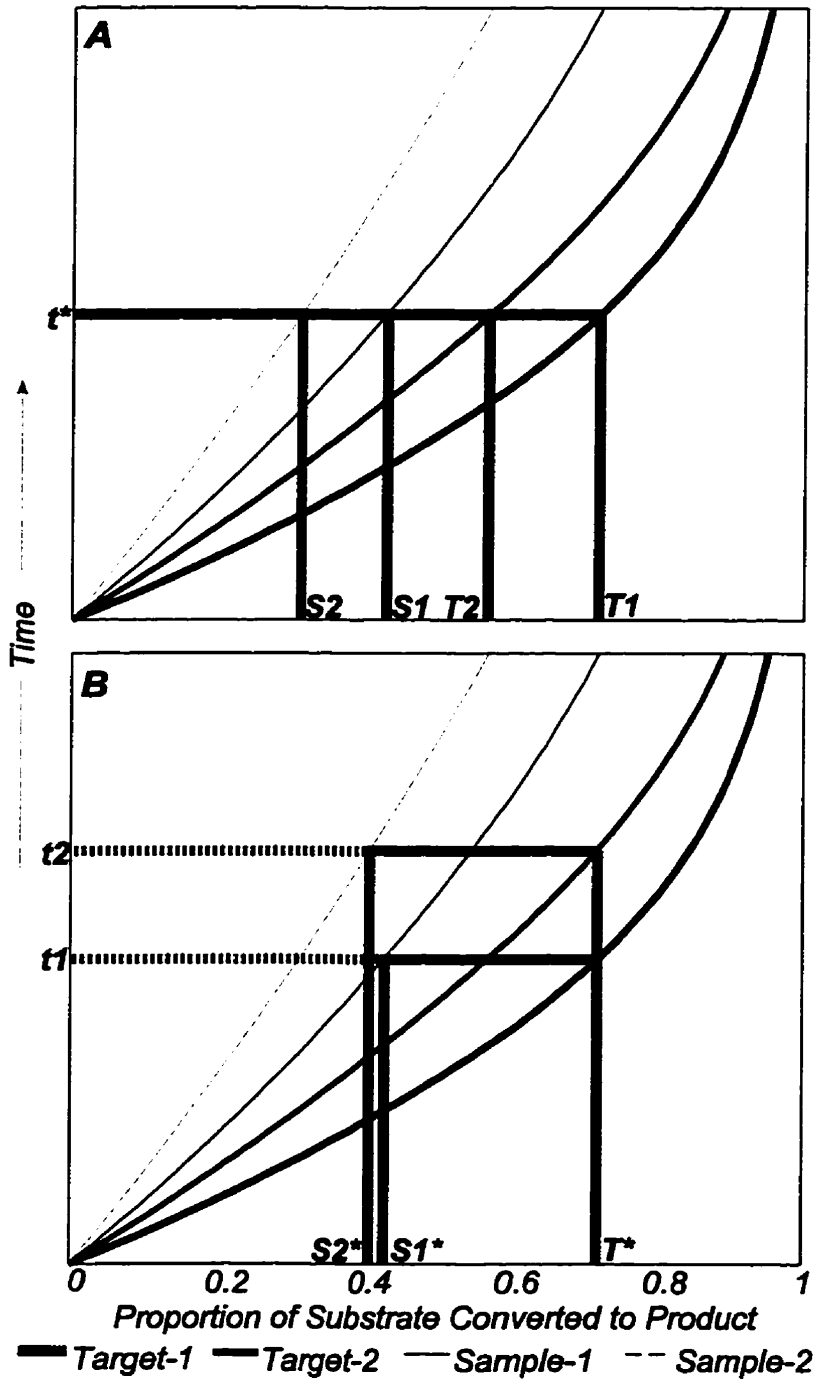


Figure 4.6: Comparison of proportion of substrate converted to product in a sample (S) by a theoretical simple enzyme:substrate system operating under two different sets of environmental conditions (1 & 2) where the time at measurement (t) of sample is determined using a targeting protocol where $[E]_{Target} > [E]_{Sample}$ and where the target value lies on the linear portion of the development curve (**A**) and beyond the linear period of development (**B**), demonstrating the reduced variation in sample values for **A**.

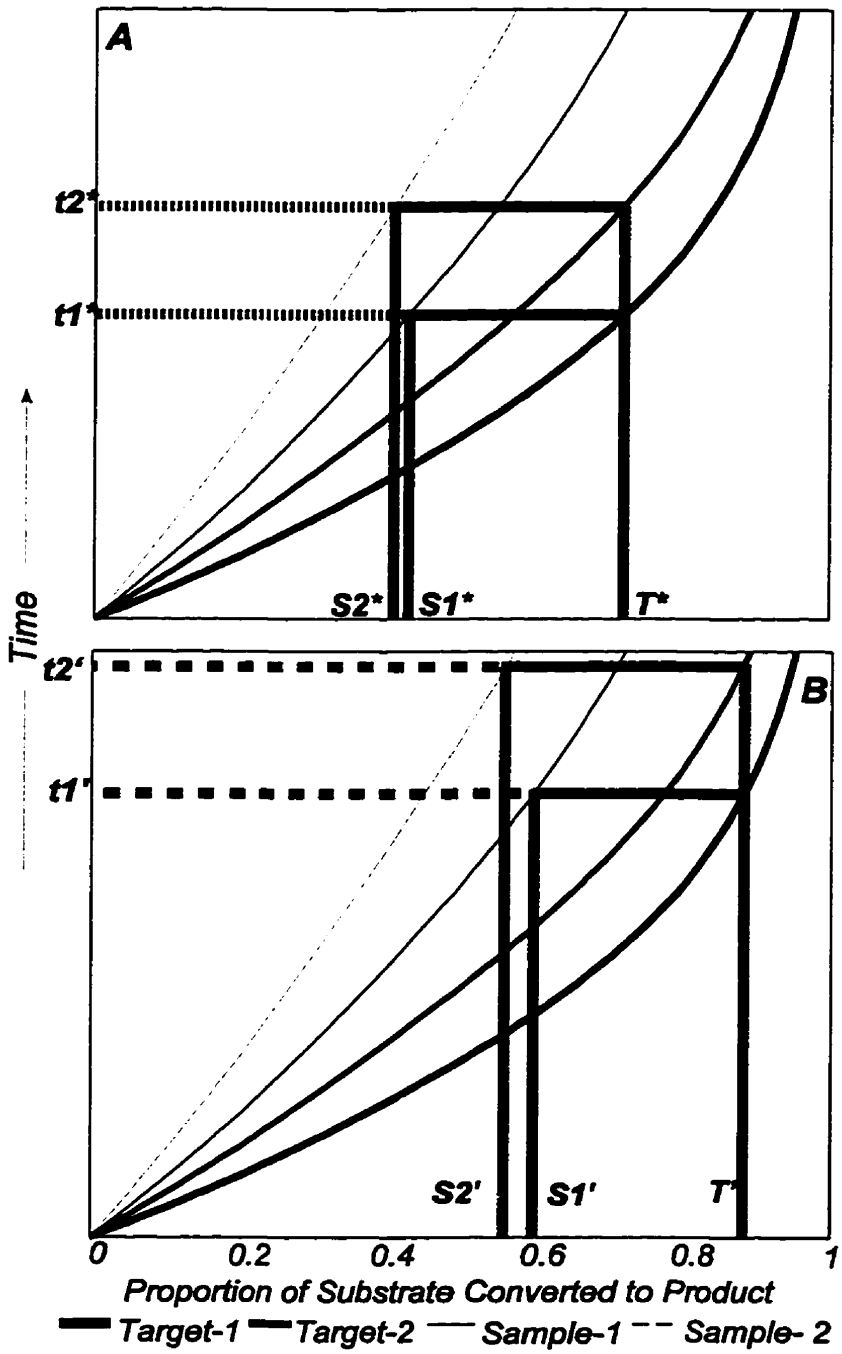


Figure 4.7: Comparison of proportion of substrate converted to product in a sample (S) by a theoretical simple enzyme:substrate system operating under two different sets of environmental conditions (1 & 2) where the time at measurement of sample (t) is determined using a targeting protocol where the target value lies at a "high" (**A**) *versus* "low" (**B**) value on the linear portion of the development curve but where $[E]_{Sample} > [E]_{Target}$ demonstrating the quadratic increase and decrease of sample variance with increasing target value.

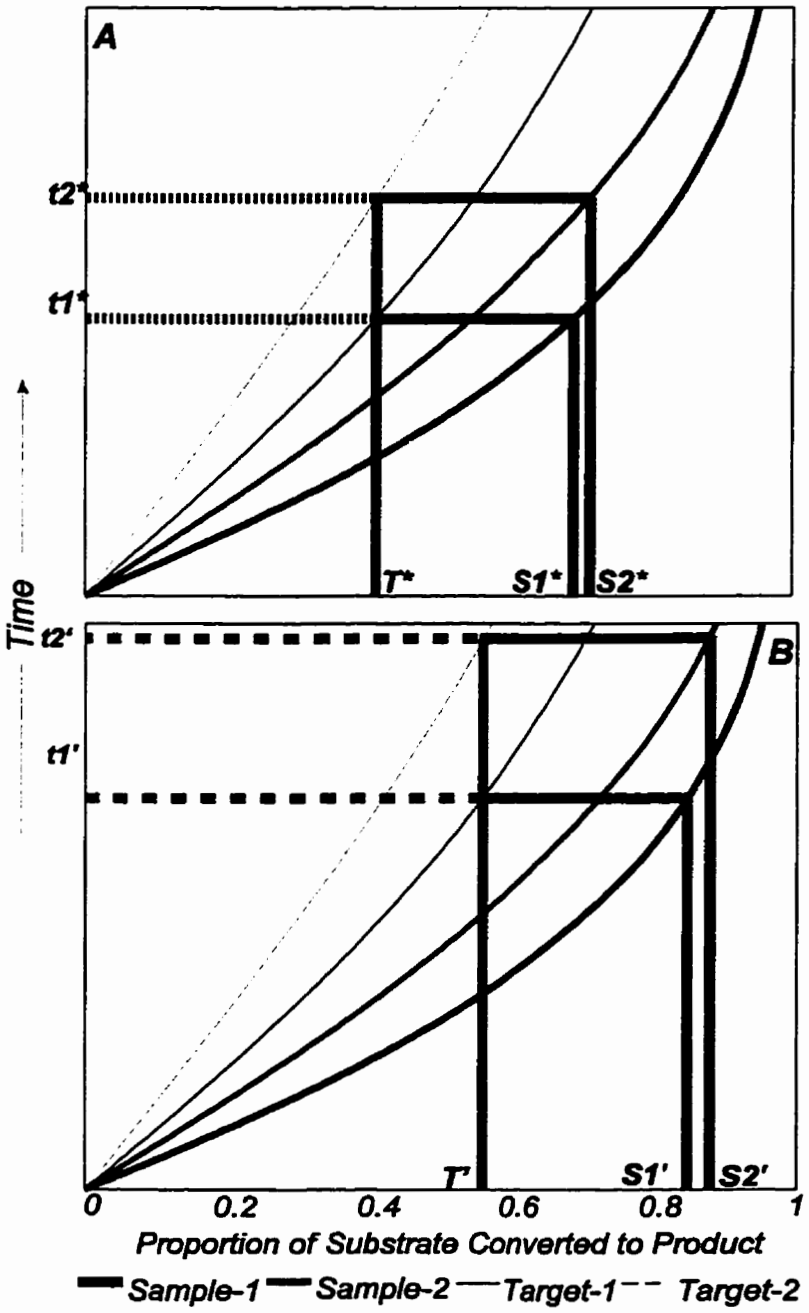


Figure 4.8: Contrasting proportion of substrate converted to product over time for a range of initial enzyme concentrations operating at fixed and equivalent environmental conditions in a theoretical simple enzyme:substrate system, illustrating the concept of point of maximum resolution *versus* targeting value at near end of linear phase of substrate conversion curves.

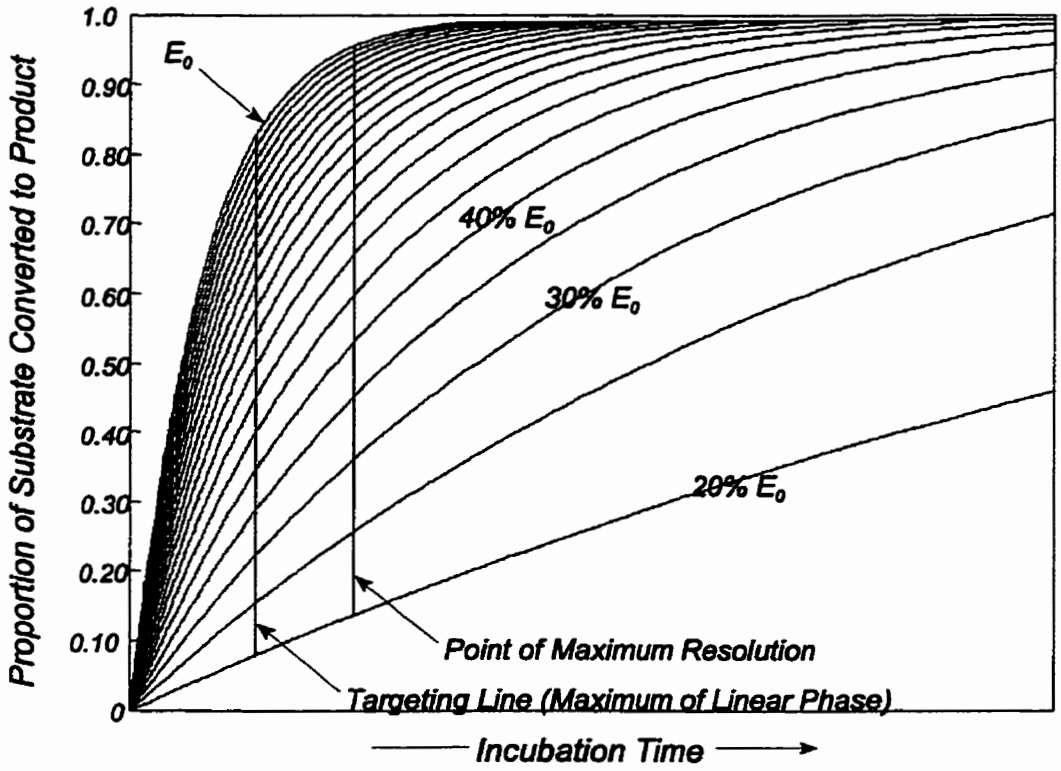


Figure 4.9: Frequency distribution of 103 mean test values at target incubation time for aliquot 1 (**A**) and aliquot 2 (**B**) of reference positive control sample (C9) utilised as a target in an enzyme-linked immunosorbent assay analysis of serum samples collected in a longitudinal study conducted in Kiambu District from July 1991 - June 1992.

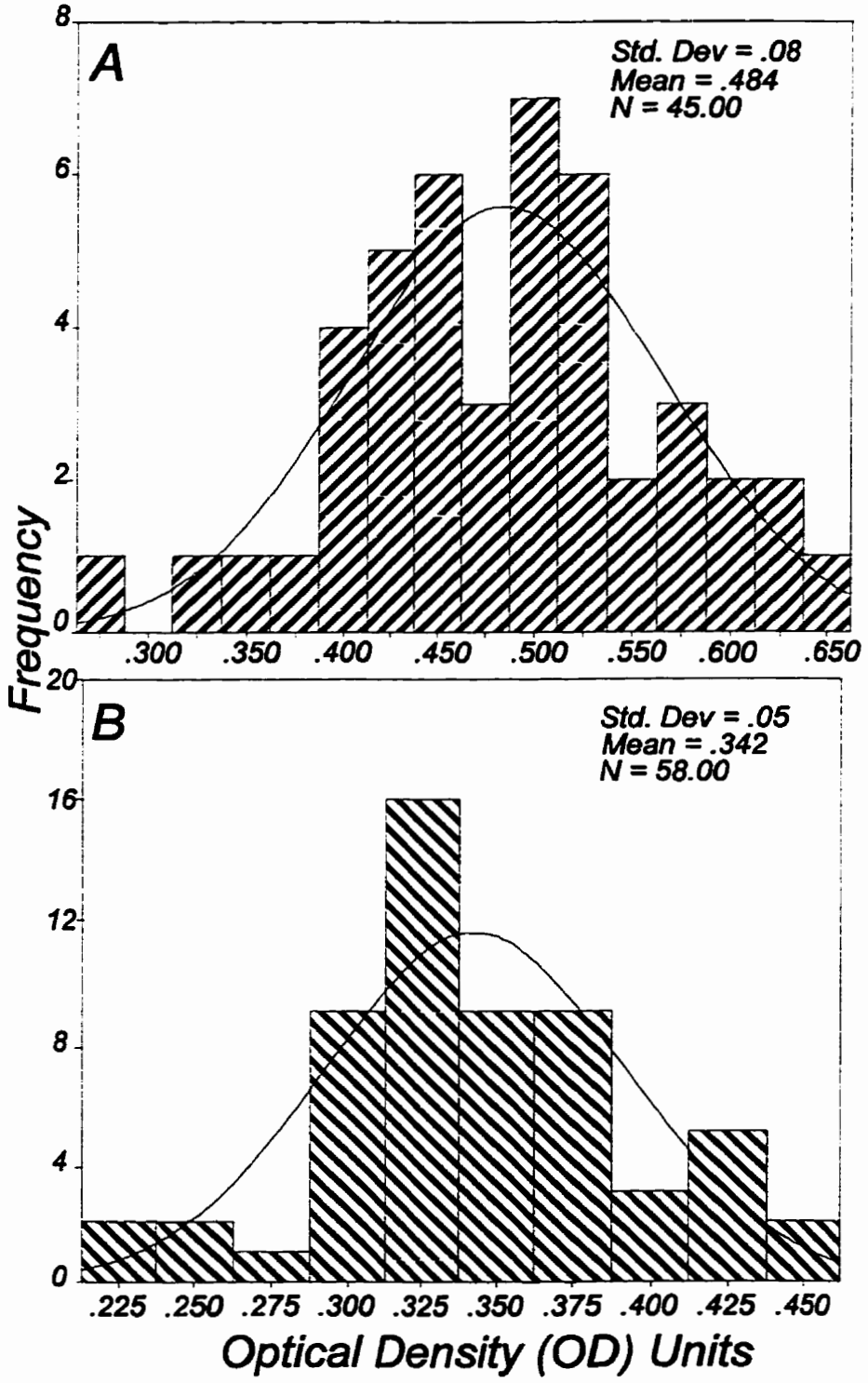


Figure 4.10: Frequency distributions of 103 mean test values at target incubation time for BJ202 (**A**), BJ212 (**B**) and BJ28 (**C**); utilised as reference negative control samples in an enzyme-linked immunosorbent assay analysis of serum samples collected in a longitudinal study conducted in Kiambu District from July 1991 - June 1992.

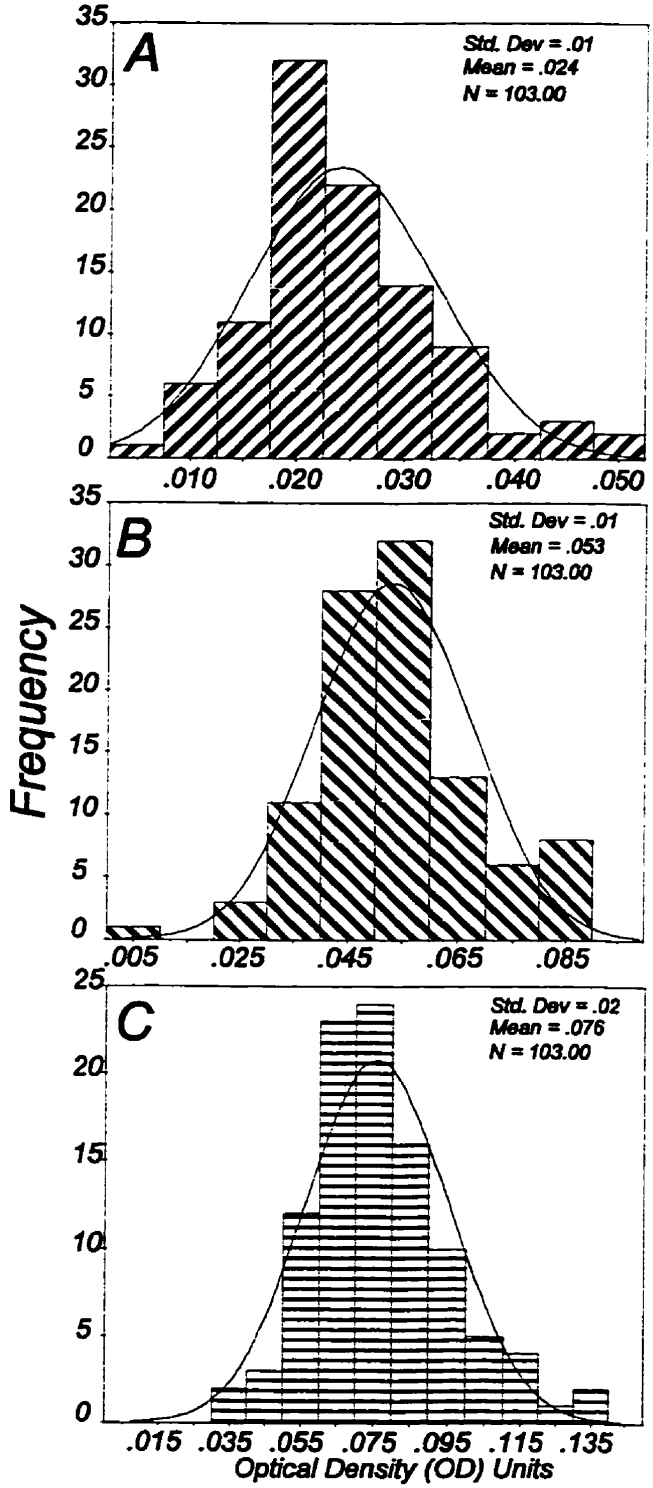


Figure 4.11: Scatter plots of mean test values for reference negative control samples (BJ202, BJ212 and BJ28) *versus* corresponding mean reference positive control sample (C9) value at target time on 103 plates utilised in an enzyme-linked immunosorbent assay analysis of serum samples collected in a longitudinal study conducted in Kiambu District from July 1991 - June 1992.

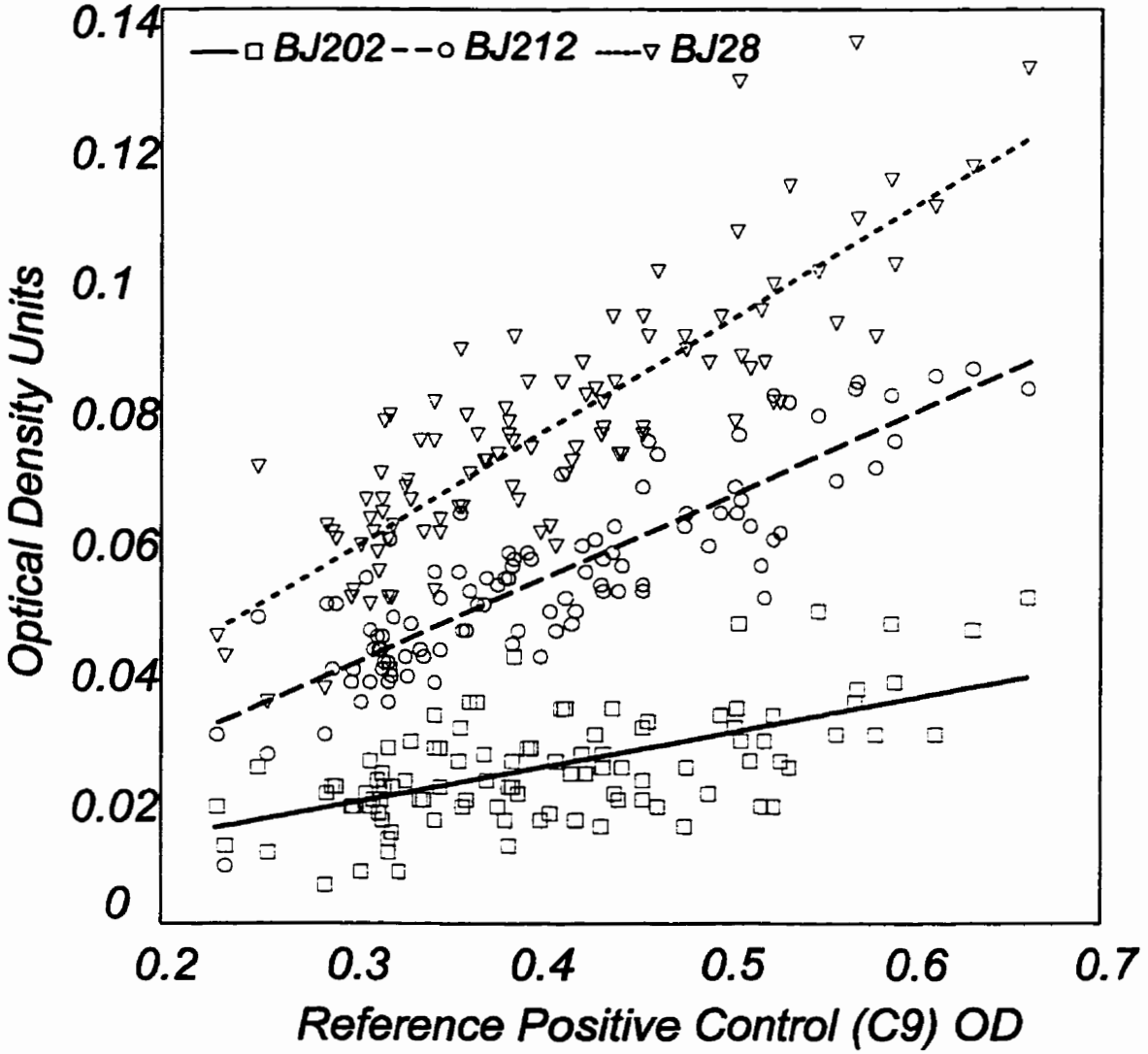


Figure 4.12: Frequency distribution (A) and scatter plot *versus* coefficient of variation (B) for standard deviations observed between 4422 serum samples collected in a longitudinal study conducted in Kiambu District from July 1991 - June 1992 and tested as pairs of replicates in an enzyme-linked immunosorbent assay analysis using a targeting protocol.

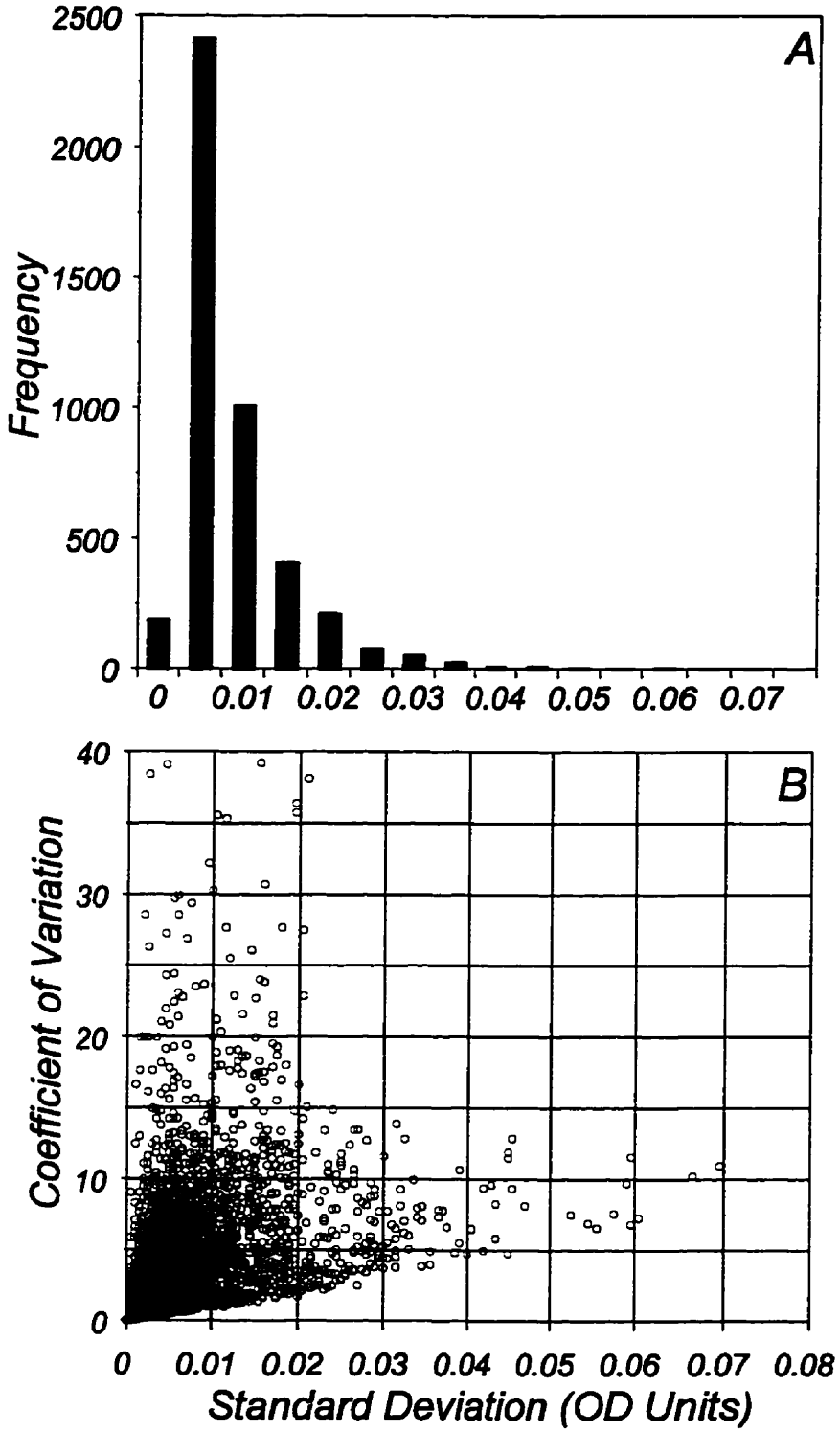


Figure 4.13: Scatter plot of mean optical density value at target time and at 1/50 working dilution *versus* natural log transformation of [end dilution (at negative cut-off)/working dilution] for 156 serum samples collected in a longitudinal study conducted in Kiambu District from July 1991 - June 1992 and titrated to extinction as paired replicates in an enzyme-linked immunosorbent assay analysis using a targeting protocol. Superimposed on plot is non-linear best fit standard quantification curve of equation

$$Test\ OD = \frac{Max * Min * e^{[\ln(enddilution) - \ln(50)] * r}}{Max - Min + Min * e^{[\ln(enddilution) - \ln(50)] * r}}$$

where *Max* = 1.126 OD units, *r* = -0.822 and *Min* = 0.125 OD units.

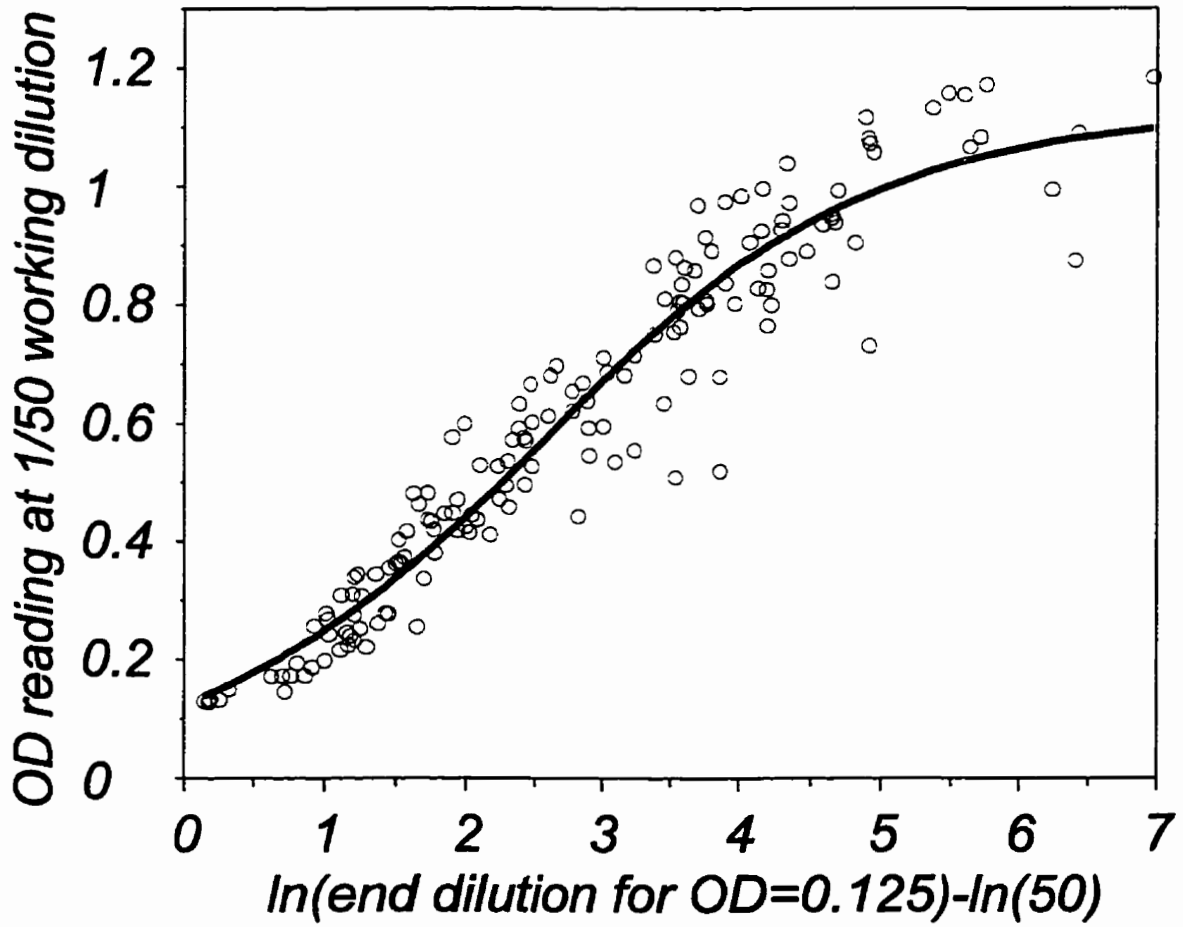


Figure 4.14: Scatter plots of standardised residual (A), predicted value (B) and leverage (C) *versus* observed OD reading at 1/50 working dilution and of standardised residual *versus* leverage (D) for standard quantification curve (Figure 4.13) based on non-linear least squares fit of 156 serum samples collected in a longitudinal study conducted in Kiambu District from July 1991 - June 1992 and titrated to extinction as paired replicates in an enzyme-linked immunosorbent assay analysis.

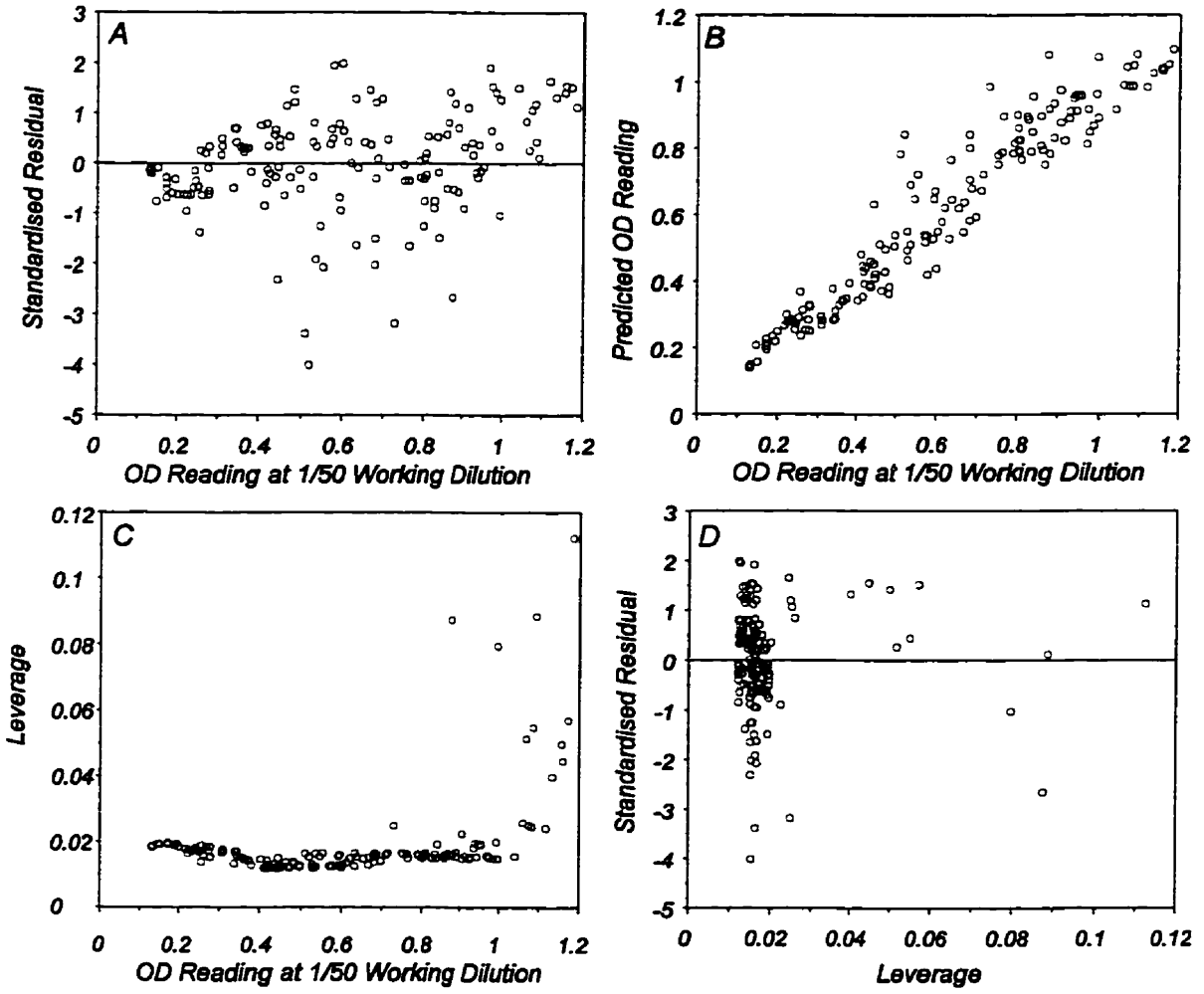


Figure 4.15: Frequency distribution of 103 plate adjustment factors, derived from positive target and negative control samples present on MicroWell plates in an enzyme-linked immunosorbent assay, and utilised in a post-targeting refinement of raw test results for serum samples collected in a longitudinal study conducted in Kiambu District from July 1991 - June 1992.

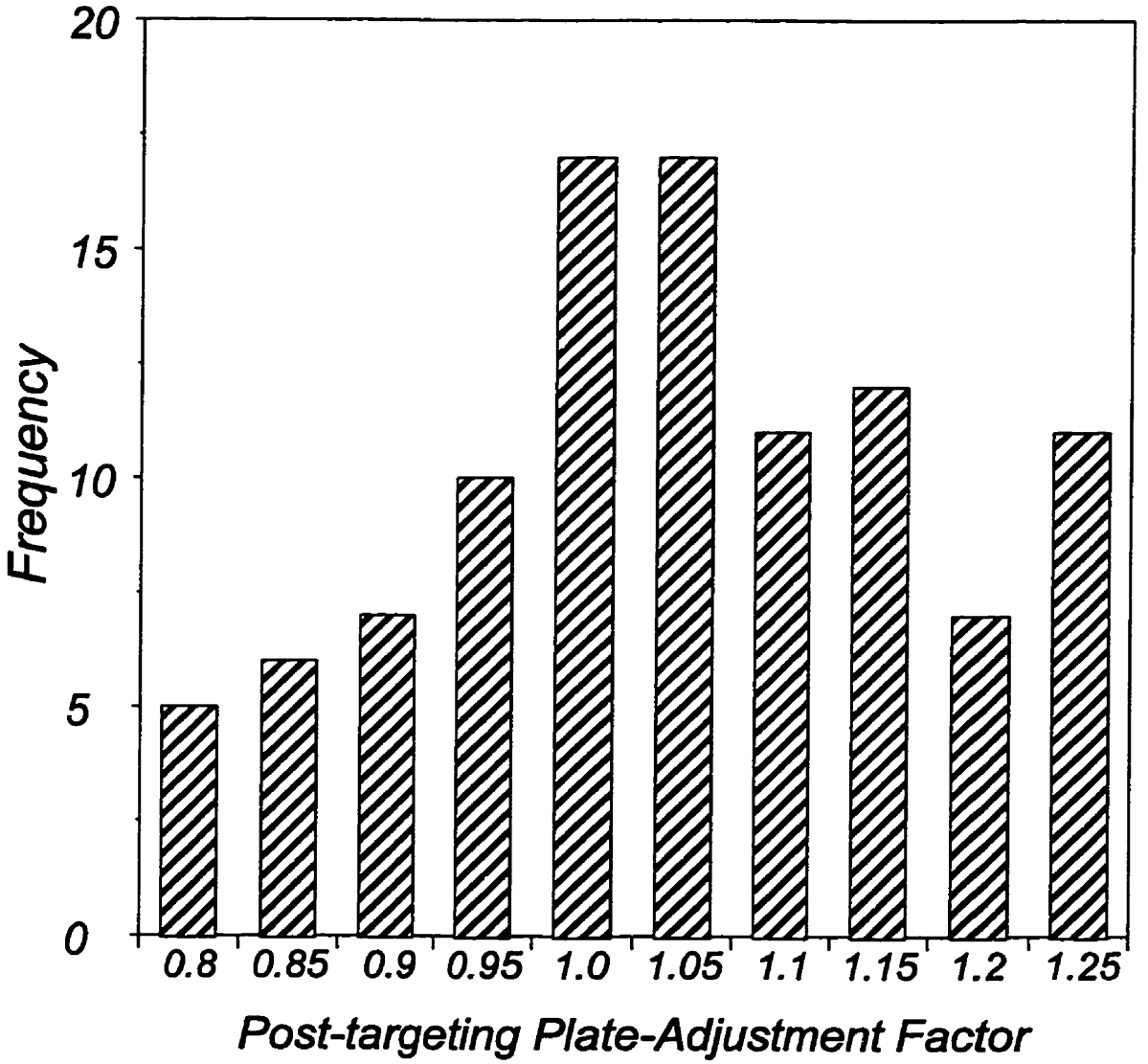


Figure 4.16: Scatter plot of raw at-target test result *versus* post-targeting adjusted value for 4404 serum samples collected in a longitudinal study conducted in Kiambu District from July 1991 - June 1992 and analysed as paired replicates in an enzyme-linked immunosorbent assay analysis using a targeting protocol.

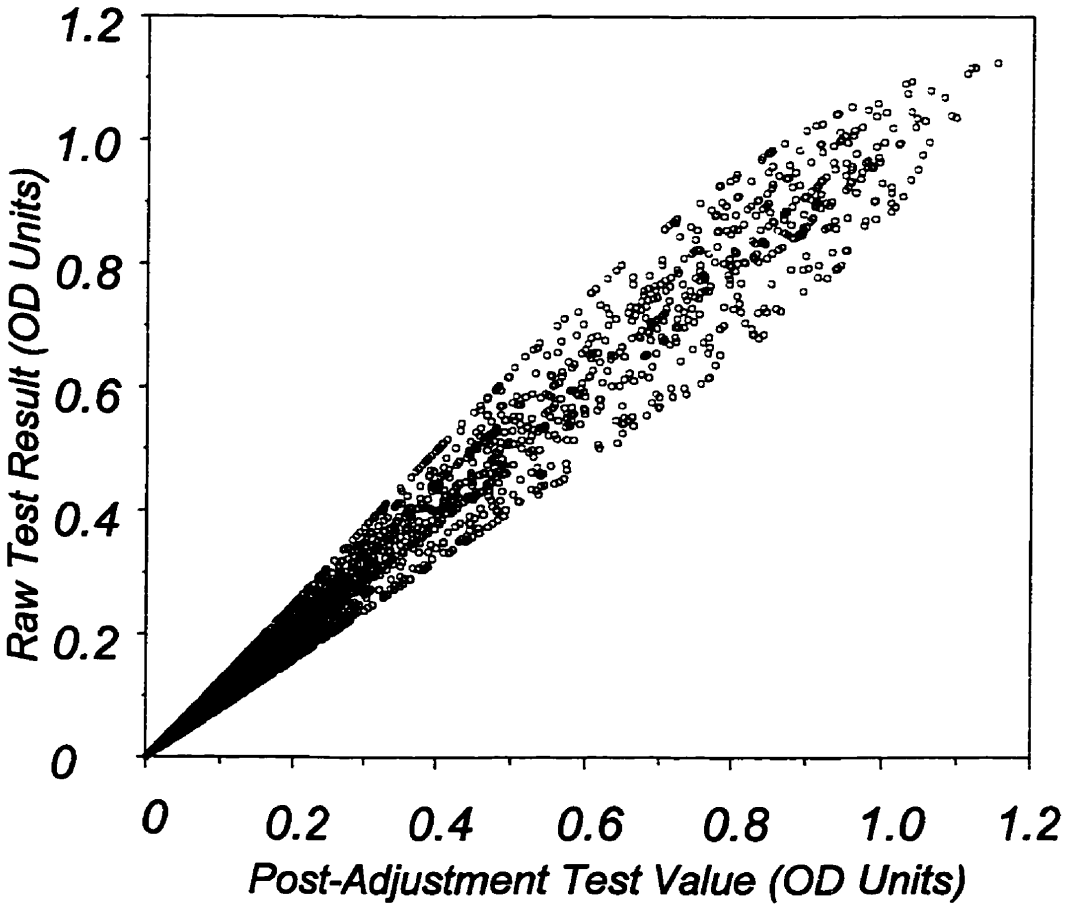
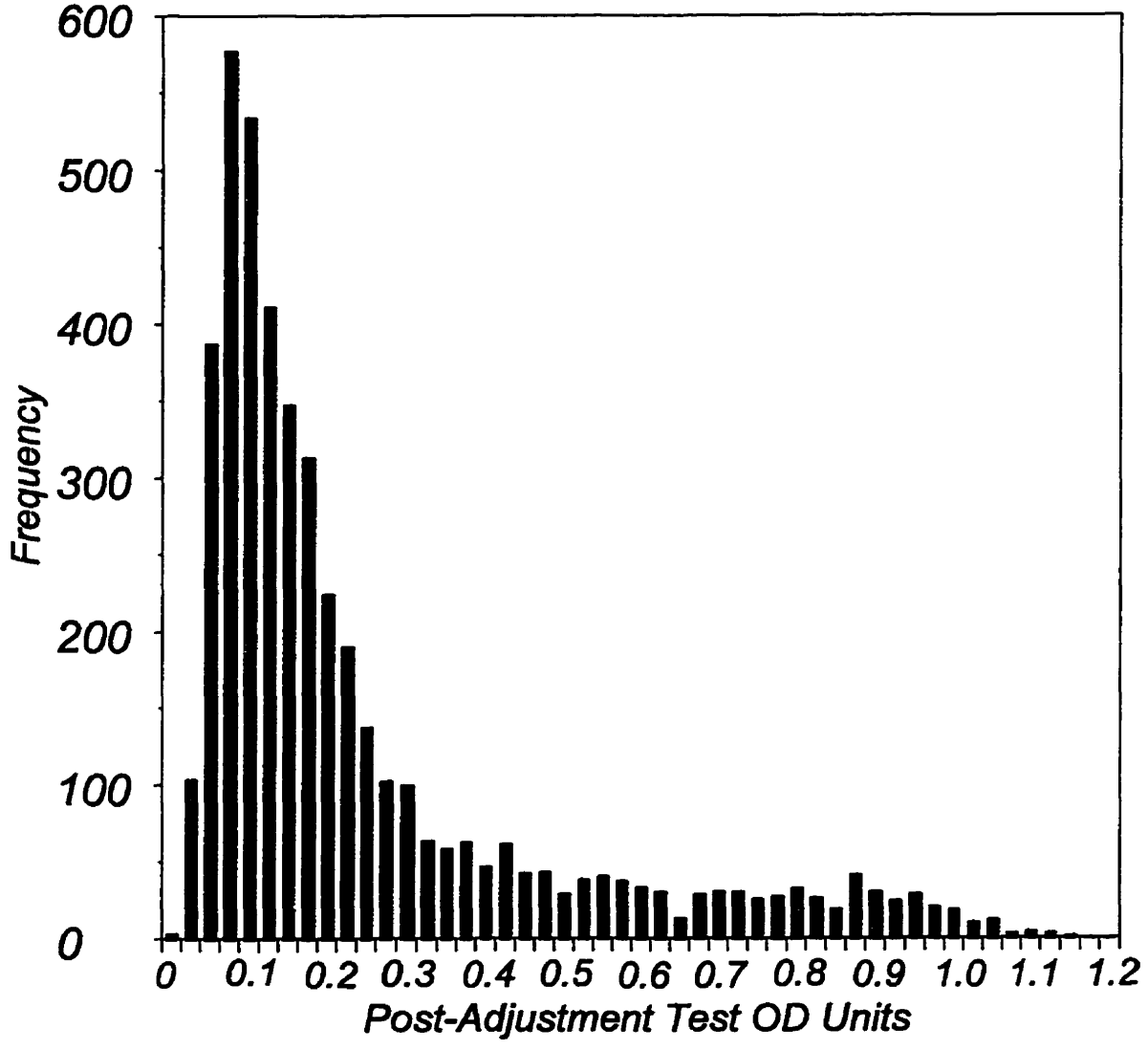


Figure 4.17: Frequency distribution of post-targeting adjusted test value for 4404 serum samples collected in a longitudinal study conducted in Kiambu District from July 1991 - June 1992 and analysed as paired replicates in an enzyme-linked immunosorbent assay analysis using a targeting protocol.



CHAPTER 5

INCIDENCE OF EAST COAST FEVER MORTALITY, MORBIDITY AND *THEILERIA PARVA* SEROLOGICAL EVENTS

5.1 Introduction

Although a more detailed understanding of the occurrence of both infection with *Theileria parva* and clinical East Coast fever, as required by epidemiologists, can be satisfied by appropriately designed prospective studies, the measures of incidence they generate present a statistical conundrum. Specifically, the rates at which events occur are derived variables, based on a ratio of the number of counts observed over a measured time interval. In consequence, they are potentially inaccurate in that they appear to present continuously distributed data but, depending on the potential distributions of numerator and denominator, some values can not be obtained, while several combinations yield the same ratio. Perhaps more importantly, there are seldom clear distributional assumptions on which statistical assessment can be based, while the indiscriminate use of normal approximation methods may lead to spurious statistical inference (Sokal and Rohlf, 1981). Nevertheless, in the case of tick-borne disease (TBD), rates of infection and disease are inherently understandable and provide both biologically meaningful and economically important measures (Mukhebi *et al.*, 1992; Norval *et al.*, 1992).

This chapter documents the definitions and occurrence of ECF mortality and morbidity and of *T. parva* infection as assessed by serological measures. Incidence rates are

presented as density functions, i.e. per animal year of observation. Approximate 95% confidence intervals on rates are calculated under the assumption that the numerator of the rates could be considered a Poisson variable (Kahn, 1983; Rothman and Greenland, 1998) and that they are estimates of a single randomly selected group. Thus, at low values (e.g. mortality) the confidence limits are non-symmetrical, as constrained by 0, but become more symmetrical as the number of events increases (e.g. sero-events).

The purpose of the presentation is to focus on the patterns and magnitudes of rates, overall and as distributed across potential risk factors. As such, statistical analysis herein is limited, but the justification, methods and results of a more comprehensive and appropriate statistical assessment of the data are presented in detail in Chapter 7. Although significance tests, in which two rates were contrasted, were conducted based on the relative risk (ratio of incidence densities where the denominators were cattle years of risk, as calculated using Epi Info 6, Version 6.04b, Centres for Disease Control and Prevention, Atlanta, Georgia, USA), no adjustments for multiple comparisons have been made to correct experimentwise error rates. Thus statistical inferences are made with caution.

5.2 Mortality

As detailed in chapters two and three, on each follow-up farm visit interviewers were accompanied by a list of all cattle present on the farm at the previous visit, including individual animal identification features and, in the case of pregnant cows, the stage of gestation and projected calving date. Thus, the "loss to follow-up" of individual animals, including newborn calves which had not actually been examined post-partum, was quickly,

consistently and accurately identified, and the reason ascertained subsequently from the farmer.

In the case of losses reported as mortalities, it was originally intended that a full post-mortem examination be conducted on each bovine which died during the study period. The initial study design included the provision for this service on farmer notification of a death, to be conducted either by the study participants themselves, or through the ambulatory care service of the Faculty of Veterinary Medicine, University of Nairobi. Unfortunately, despite offering this service on a 24-hour call-out/no-fee basis, in only one case was such a notification received, while on a second occasion, study participants conducted a post-mortem examination on an animal which had died immediately prior to their arrival. The reason for this set of circumstances appears to have been two-fold.

First may have been the difficulty in contacting the Faculty of Veterinary Medicine. Almost no farmers possessed telephones, requiring that messages be delivered in person or passed through the daily milk pick-up service to their dairy co-operative society or another telephone source. A further exacerbating factor was the relative unreliability of the telephone system. For prolonged periods of the study, the exchange which serviced the campus was non-functional. Second may have been owing to farmer reticence. It is not unusual for animals which die to be subsequently butchered and consumed, particularly if there is no overt clinical illness or if the "death" was effectively emergency slaughter (e.g. in response to trauma or dystocia). In this context, the removal of a carcass for post-mortem examination or the delay in awaiting inspection may have been seen to represent non-reimbursable and hence unacceptable losses to the farmer.

In response to this situation, when a death occurred without a subsequent post-mortem examination being conducted, the recorded cause of death was derived from the following hierarchy of rules, as appropriate (see *5.3.1 Diagnosis of East Coast Fever*):

1) Putative diagnosis made by study veterinarians for animals they observed to be clinically ill prior to death.

2) Putative diagnosis (as recorded on treatment reimbursal records) made by a local veterinarian called to treat a clinically ill animal prior to death.

3) Tentative diagnosis made by study veterinarians based on farmers account of history and accompanying clinical signs.

4) Peracute deaths, i.e. in the absence of any clinical illness or attributable cause, were recorded as "Unknown" or "Sudden" deaths.

Thus, while there is a relatively high degree of confidence that all mortalities were identified, in some cases, the putative cause of death may be less certain. More specifically, with respect to mortalities attributed to ECF, deaths were further classified as suspected or, when initial diagnosis was accompanied by supporting serological or post-mortem findings, as confirmed.

5.2.1 Total Mortality

A total of 43 deaths were recorded from the 535 animals which contributed a total of 370.3 animal years of observation during the year-long study, yielding a crude mortality proportion of 8.0% (= 43/535) and a crude overall mortality rate of 11.6% (11.6 deaths per 100 animal years of observation = 43/370.3). Hereafter, the term rate refers to an incidence

density measure based on animal years at risk while cumulative incidence rates will be referred to as proportions. On the basis of the age category of the animal at the time of death these mortalities were further subdivided into 18 "adult" cattle (i.e. > 1 year of age), and 25 "calves" (≤ 1 year of age). Table 5.1 provides information concerning the dairy co-operative society, agro-ecological zone, farm of origin, grazing management system, timing, age at death and putative cause of death for the 18 adult cattle mortalities. Tables 5.2 and 5.3 provide similar details for the 19 mortalities in calves which were observed at least once prior to their death and the 6 calves which died prior to their initial examination, respectively.

The overall crude mortality rate of 6.3% calculated for adult cattle (18/288.0) was significantly lower ($p<0.001$) than the rate of 30.4% observed for calves (25/82.3). Alternatively, as proportions, 5.1% (17/334) of all animals initially observed as adults and 12.9% of all animals initially observed as calves (26/201) were recorded to have died during the study period.

Table 5.4 provides periods of animal observation utilised as denominators in subsequent tables of incidence rates, as subdivided on the basis of i) agro-ecological zone, ii) grazing management system of the farm of origin and iii) age classification group.

5.2.2 Mortality in Adult Cattle

Of the 18 deaths of adult cattle, five (27.8%) were tentatively attributed to East Coast fever (Table 5.1), yielding a putative overall ECF mortality rate of 1.7% (5/288.0) in adult cattle (Table 5.5). Two of these five suspected ECF deaths were further confirmed, one on

the basis of post-mortem examination and the second by virtue of an immediately pre-mortem *T. parva*-specific serological increase in an animal diagnosed with clinical ECF. Three deaths (3/288.0 = rate of 1.0%) were peracute in nature and undiagnosed. Three deaths were associated with the peri-parturient "downer-cow" syndrome, one of which was distinguished by a severe and prolonged dystocia, although the calf survived. Three deaths were noted to be of gastro-intestinal origin, namely bloat, grain overload and rumen impaction, while two additional deaths were attributed to pneumonia. A further cow died of toxic mastitis and another animal was slaughtered after fracturing its femur.

Deaths of adult cattle were reported from all dairy co-operative societies and in all agro-ecological zones. Age at time of death varied from 1 to 10 years, with a mean age of 4.8 years and a median age of 5 years. One farm (Farm #14, Kikuyu Dairy Co-operative Society) reported more than one adult mortality, although, of the three adult deaths which occurred on this farm, one was attributed to suspected ECF. Twelve deaths (66.7%) occurred on farms practising zero-grazing management from which 42.7% of the total adult observations were made.

Estimates of cause-specific mortality rates for adult cattle are presented in Table 5.5, by agro-ecological zone and, with reference to East Coast fever, also by grazing management system. All five suspected and both confirmed adult ECF deaths occurred on farms practising zero-grazing, yielding putative and confirmed adult ECF mortality rates on zero-grazing farms of 4.1% and 1.6% respectively. There were no adult ECF deaths reported on farms practising semi- or full-pasture grazing. Three of the five suspected ECF deaths occurred on zero-grazing farms in the upper midland (UM) agro-ecological zone and two

(both of the confirmed ECF deaths) occurred on farms in the lower highland (LH) zone, yielding a putative and a confirmed adult ECF mortality rate of 4.7% and 3.4% for zero-grazing farms in each zone respectively. Two of the three undiagnosed, sudden deaths occurred in the LH zone (rate of 1.8%), while the remaining death was reported on a farm in the upper highland (UH) zone (rate of 1.3%). When suspected ECF deaths and undiagnosed/sudden deaths were combined, their total incidence rate was 2.7% (8/288.0) in adult cattle, summarised from rates of 3.0%, 3.5% and 1.3% in the UM, LH and UH agro-ecological zones respectively. This pattern was similarly reflected in total adult mortality rates of 7.1%, 7.1% and 4.0% observed across the same zones. No significant differences in mortality rates were detected by agro-ecological zone, based on approximate 95% confidence intervals.

5.2.3 Mortality in Calves

Of the 25 deaths of calves, 19 occurred in animals older than 1 month, i.e. which had been examined at least once previously (Table 5.2), while 6 occurred in newborns prior to initial examination (Table 5.3). Estimates of cause-specific mortality rates (CSMR) for calves are presented in Table 5.6, by agro-ecological zone and, with reference to ECF, also by grazing management system. One calf (from a semi-/full-pasture grazing farm in the lower highland agro-ecological zone) was reported to have died from suspected ECF, yielding a putative overall ECF mortality rate of 1.2% (1/82.3) in calves. This rate was not significantly different from the putative ECF rate observed in mature cattle (p=0.74). In contrast, eight deaths (CSMR 9.7% = 8/82.3) were attributed to diarrhoea-induced

dehydration, while seven (CSMR 8.5% = 7/82.3) were peracute/undiagnosed. Also, four deaths (CSMR 4.9% = 4/82.3) were the result of poisoning, two of these as a result of the ingestion of acaricide concentrate and one from overdose of a topical acaricide spray.

Although deaths of calves were reported from five of six dairy co-operative societies (no calf deaths were reported in Limuru Dairy Co-operative Society) and in all agro-ecological zones, there was an apparent pattern of higher mortality rates in "lower" (UM & LH) zones. In fact, one calf death, a dystocia-related stillbirth, was reported from Lari Dairy Co-operative Society in the upper highland (UH) zone. Overall crude calf mortality rates of 36.9%, 39.6% and 5.0% were calculated for UM, LH and UH zones respectively, however, the approximate 95% confidence interval for the crude mortality rate in the UH zone overlapped with those for the UM and LH zones. Further, cause-specific mortality rates of diarrhoea/dehydration, unknown/sudden deaths and poisoning (which in total account for 76% of all calf deaths = 19/25), were virtually identical between UM *versus* LH agro-ecological zones at 13.4% *versus* 12.2%, 10.1% *versus* 12.2% and 6.7% *versus* 6.1% respectively (Table 5.6).

Fifteen of the 25 calf deaths (60.0%) occurred on farms practising zero-grazing management. Age at time of death varied from 0.5 months (i.e. death prior to initial examination) to 8 months, with an mean age of 3.5 months and a median age of 3 months.

Although five farms reported more than one calf death (Kikuyu Dairy Co-operative Society/Farm #'s 9, 13 and 14 & Nderi Dairy Co-operative Society/Farm #'s 1 and 7), it is notable that the sole farm which reported more than one adult mortality (Farm #14, Kikuyu Dairy Co-operative Society) also reported the most calf mortalities at four; one

unknown/sudden death and three from diarrhoea/dehydration.

5.2.4 Overall East Coast Fever Mortalities and Undiagnosed/Sudden Deaths

Table 5.7 presents summary (i.e. irrespective of age) estimates of cause-specific mortality rates for ECF and undiagnosed/sudden deaths. The overall mortality rate attributed to ECF was relatively low at 1.6%, with an approximate 95% confidence interval of 0.7% - 3.5%. This was composed of a mortality rate of 3.1% on zero-grazing farms *versus* 0.5% on farms practising semi- or full-pasture grazing, although these rates were not significantly different (p=0.09). Further, within zero-grazing farms, there was no significant (p=0.71) difference in ECF mortality rates between those located in the upper midland (UM) agro-ecological zones (3.5%) and the lower highland (LH) zones (2.7%). The overall rate for ECF mortalities which could be confirmed falls to 0.5% (1.3% in zero-grazing farms). In contrast, the overall rate of peracute, unexplained deaths was higher at 2.7%. However, it is of interest to note that the pattern of relatively higher rates in the lower agro-ecological zones observed for putative ECF was maintained, with rates of unexplained deaths in the UM and LH agro-ecological zones of 2.3% and 4.1 % respectively, contrasting with a rate of 1.0% in the UH zone, although again all approximate 95% confidence intervals overlapped. Assuming a worst case scenario, where all unexplained deaths were actually due to undiagnosed ECF, yields an overall cause-specific mortality rate of 4.3%, composed of rates of 4.7%, 6.2% and 1.0% from the UM, LH and UH agro-ecological zones respectively.

5.3 Morbidity

The difficulties farmers experienced in contacting the Faculty of Veterinary Medicine adversely impacted the ability of study members to derive accurate and confirmed diagnoses for clinical illness. As per the aforementioned post-mortem service, the initial study design envisioned the provision of a free ambulatory care service to farmers on notification of animal illness. In this manner, clinical examination, collection of laboratory samples and administration of appropriate chemotherapeutic agents would be conducted by study veterinarians. Unfortunately, as noted, this system failed to function adequately. To minimize the uncertainty of disease diagnoses made days to weeks after a clinical episode, a strategy whereby farmers enlisted the services of their local veterinarian in the diagnosis and treatment of disease on an emergency basis and were later reimbursed for full professional and therapeutic costs on submission of a written account by the attending veterinarian was adopted. Unfortunately, although local veterinary practitioners had access to a wide range of appropriate therapeutic agents, no laboratory or ancillary diagnostic support was available to them. In general, this process was well utilised by farmers and generated considerable good-will towards the study, although in some cases farmers were either unable or chose not to seek veterinary aid but rather administered therapy themselves or simply exercised benign neglect.

In the context of this study, morbidity may be simply defined as the discernable evidence of ill-health. Cause-specific morbidity rates were, therefore, calculated based on episodes where clinical signs were attributed to a particular disease or process.

5.3.1 Diagnostic Categories of East Coast Fever

In the majority of cases, the diagnosis of ECF was made on the basis of what were considered to be characteristic clinical signs, either at the mercy of the diagnostic acumen of the local veterinary practitioners, or the farmers themselves. Since there are no consistent pathognomonic signs of ECF there was considerable scope for error or misdiagnosis. Fortunately, although no ancillary diagnostic tests, such as direct parasite detection, were available at the time of clinical illness, animals were being simultaneously sampled longitudinally for serological screening. It was thus possible to contrast temporally a serological profile of *T. parva*-specific antibodies (Katende *et al.*, 1998) generated for each animal with the reported occurrence of clinical disease, to assess the consistency of a putative diagnosis of ECF. Therefore, with respect to the determination of ECF-specific morbidity rates, three ECF-diagnostic categories were created: 1) Reported/Suspected, 2) Confirmed Serologically and 3) Missed Diagnosis.

5.3.1.1 ECF Reported/Suspected

All cases of clinical illness where a putative diagnosis of ECF had been made by either the study veterinarians, a local veterinary practitioner, or the farmer, were classified as "Reported/Suspected ECF".

5.3.1.2 ECF Reported and Confirmed Serologically

All reported/suspected cases of ECF where the animal's serological profile of *T. parva*-specific antibodies demonstrated an increase in level temporally related to the

occurrence of clinical disease and consistent in pattern and magnitude with either a primary seroconversion or a secondary anamnestic immune response, as appropriate (refer to section *5.4 Serological Profiles*), were considered to be cases of ECF which were "Confirmed Serologically".

5.3.1.3 Missed ECF Diagnosis

Serological profiles of *T. parva*-specific antibodies were also contrasted with the occurrence of incidents of clinical disease where a diagnosis other than ECF had been made by either the study veterinarians, the local veterinary practitioner, or the farmer. This process was repeated for episodes of non-specific and/or mild illness noted by the farmer or as an incidental finding on physical examination, but where no diagnosis had been sought or established. Where the pattern of the antibody level exhibited an increase of sufficient magnitude and appropriate timing relative to the incident, suggestive of either a primary seroconversion or a secondary anamnestic immune response, as appropriate, the episode was classified as a "Missed Diagnosis" of ECF.

5.3.2 Incidence of East Coast Fever

Incidence rates of ECF morbidity were derived for four combinations of ECF diagnostic categories. Each combination of cases and fatalities and the rationale for its consideration are given below:

Category 1: Includes all suspected non-fatal cases and deaths, but excludes missed cases. This category yields rate estimates based on clinical evaluation only. It most

closely approximates what might be expected from a passive disease reporting system where ancillary laboratory diagnosis was not available. Sources of error include both incorrect and missed diagnoses of ECF.

Category 2: Includes suspected non-fatal cases and deaths which were subsequently confirmed, but excludes unconfirmed non-fatal cases and deaths and missed cases. This is the most conservative set of criteria in that it incorporates a two-step process with a serial interpretation whereby an initial diagnosis based on clinical signs must be supported by supplementary assessment. Bias is unidirectional toward the null since incorrect diagnosis of ECF as an inflationary source of error is eliminated. Hence this category may be considered to yield minimum estimates for rates.

Category 3: Includes suspected non-fatal cases and deaths which were subsequently confirmed and all missed cases, but excludes unconfirmed non-fatal cases and unconfirmed deaths. Both errors which bias toward and away from the null have been excluded from this category. The inclusion of mild and sub-clinical cases which were noted as a result of ancillary screening suggests omniscience and thus this combination is considered to yield the most accurate reflection of the true rates.

Category 4: Includes all suspected non-fatal cases and deaths and all missed cases. This least conservative combination will yield the highest estimate of rates since only errors which bias toward the null have been eliminated. Estimates may be considered a maximum of the likely interval.

5.3.3 East Coast Fever Morbidity in Adult Cattle

Twelve adult animals were suspected to have suffered from non-fatal ECF over the course of the longitudinal study. Of these, one animal (Chania Dairy Co-operative Society, Farm #1) was reported to have experienced two distinct episodes of ECF three months apart, although neither could be confirmed on the basis of serology. However, in two other cases (Kiambaa Dairy Co-operative Society, Farm #12 - Figure 5.1C & Limuru Dairy Co-operative Society, Farm #12 - Figure 5.1E) animals which were reported to have suffered episodes of clinical ECF, which were confirmed serologically, were also noted to have experienced a missed occurrence of ECF; 5 months prior and 6 months subsequently, respectively. Table 5.8 provides information regarding the dairy co-operative society, agro-ecological zone, farm of origin, grazing management system, timing, age, initial serological status and correlation with serological profile (Figure 5.1) for the 13 suspected episodes of ECF in adult cattle. Suspected cases of ECF occurred in five of six dairy co-operative societies (no cases were reported from Nderi Dairy Co-operative Society) and in all three agro-ecological zones. Age of suspected ECF cases in adult animals varied from 1 to 6.5 years, with a mean age of 3.5 years and a median age of 3 years. However, of the 13 suspected cases, 5 (38.5%) were subsequently confirmed on the basis of serology. Figure 5.1 illustrates the longitudinal serological profiles of these 5 animals (Table 5.8).

In contrast, Table 5.9 provides details for 9 missed cases of ECF detected in adult cattle, 7 of these occurring in animals in which an independent diagnosis of ECF was not made. The longitudinal serological profiles of these 7 animals are presented in Figure 5.2. In 78% (7/9) of missed cases in adult cattle, the predominant presenting complaint was

respiratory in origin, either coughing or increased respiratory rate and sounds ● dyspnea. However, none of the animals experienced marked respiratory distress, 6 of the 7 were apyrexic ($\leq$ 39.0^{0}C) at the time of examination and 2 (one of which was pyrexic) were considered to be sufficiently ill to warrant further treatment. When it was administered, treatment consisted of injection with oxytetracycline, which proved consistently successful. Missed cases of ECF occurred in three of six dairy co-operative societies (Kiambaa, Kikuyu and Limuru Dairy Co-operative Societies) but were drawn from all three agro-ecological zones. Age of missed ECF cases in adult animals varied from 1.5 to 7.5 years, with a mean age equivalent to the median age of 4 years.

Thus, in adult cattle there were a total of 8 suspected but unconfirmed, 5 suspected and confirmed and 9 missed clinical episodes along with 3 suspected and 2 confirmed fatalities due to ECF. Table 5.10 presents the incidence of ECF morbidity in adult cattle by agro-ecological zone and grazing management system for each of the 4 aforementioned combinations of ECF diagnostic criteria. The overall ECF morbidity rate, based on suspected non-fatal cases and deaths which were confirmed combined with missed diagnoses (Category 3), was 5.6% (16/288.0; 95% CI 3.4% - 9.0%), . Within this same category, there was no difference between zero-grazing and semi-/full-pasture grazing farms with rates of 5.7% and 5.4%, respectively (p=0.97). Similarly, rates in upper midland (UM), lower highland (LH) and upper highland (UH) agro-ecological zones were broadly similar at 4.0%, 6.2% and 6.6%, respectively, with largely overlapping approximate 95% confidence intervals. However, within the UM zone, the rates for zero-grazing *versus* semi-/full-pasture grazing were 1.6% *versus* 8.4% respectively, in contrast to corresponding rates of 10.1%

versus 1.9% in the LH zone, although neither difference was statistically significant (p=0.13 for each contrast). The addition of suspected but unconfirmed non-fatal ECF cases and fatalities (Category 4) raised the overall incidence rate to 9.4% (27/288.0); composed of 10.6% in zero-grazing farms *versus* 8.5% in semi-/full-pasture grazing farms and 11.1%, 7.9% and 9.2% in UM, LH and UH agro-ecological zones respectively. However, within the UM and LH zones, the rates for zero-grazing *versus* semi-full-pasture grazing farms were more similar at 11.0% *versus* 11.3% and 10.1% *versus* 5.6%, respectively. There were no significant differences between rates by either agro-ecological zone or grazing management system.

5.3.4 East Coast Fever Morbidity in Calves

A total of 4 calves were suspected to have suffered from non-fatal East Coast fever. One of these calves (Kiambaa Dairy Co-operative Society, Farm #12 - Figure 5.3D) was noted to have suffered a missed ECF diagnosis 6 months prior to the suspected, but ultimately unconfirmed, clinical episode. Details for the 4 suspected episodes of ECF in calves are presented in Table 5.11 and Figure 5.3. Suspected cases of ECF occurred in three dairy co-operative societies (Chania, Kiambaa and Nderi Dairy Co-operative Societies) and in two agro-ecological zones (UM and LH zones). Age of suspected ECF cases in calves varied from 2 to 10 months, with a mean age of 7.2 months and a median age of 8.5 months. However, 2 (50%) of the suspected non-fatal cases in calves were subsequently confirmed on the basis of serology. Figure 5.3 A & B present the serological profiles for these 2 animals.

Table 5.12 contains details for a further 4 missed ECF cases detected in calves, 3 of these occurring in animals in which an independent clinical diagnosis of ECF was not made. The longitudinal serological profiles of these 4 calves are illustrated in Figure 5.3 C - F. Similar to the pattern observed in adult cattle, the dominant clinical presentation of all missed ECF diagnoses in calves was with respiratory signs, although unlike adult cattle, 75% (3/4) were also pyrexic (> 39.0^{0}C). Two cases were misdiagnosed as calf-pneumonia but were successfully treated by injection of oxytetracycline. Although one of the remaining two calves was mildly pyrexic, neither was considered sufficiently ill to warrant treatment. Missed cases of ECF were noted from four of six dairy co-operative societies (no missed cases were noted from Nderi or Lari Dairy Co-operative Societies) and in two agro-ecological zones (UM and LH zones). Age of missed ECF cases in calves varied from 2 to 10.5 months, with a mean age equivalent to the median age of 6.2 months.

In calves there were a total of 2 suspected but unconfirmed, 2 suspected and confirmed and 4 missed clinical episodes along with 1 death due to suspected ECF. Table 5.13 presents the incidence rates of East Coast fever morbidity among calves by agro-ecological zone and grazing management system for each of the 4 combinations of ECF diagnostic criteria. The overall ECF morbidity rate, based on suspected non-fatal cases and deaths which were confirmed combined with missed diagnoses (Category 3), was 7.3% (6/82.3; 95% CI 3.3% - 15.9%). From this same category, the overall rates for zero-grazing and semi-/full-pasture grazing farms were 5.5% and 8.7% respectively (p=0.88). Rates in upper midland (UM) and lower highland (LH) agro-ecological zones were 6.7% and 12.2% respectively. Although no confirmed, unconfirmed or missed cases of ECF were observed

in calves in the upper highland (UH) zone or from zero-grazing farms in the upper midland (UM) zone, all approximate 95% confidence intervals demonstrated varying degrees of overlap. Thus while rates for zero-grazing *versus* semi-/full-pasture grazing were broadly similar in the LH zone at 14.1% and 10.8%, respectively, the rate for semi-/full-pasture grazing farms in the UM zone was higher at 25.7%, but the difference was not significant (p=0.07). The overall incidence rate for all non-fatal ECF cases, deaths and missed ECF diagnoses in calves (Category 4) was 10.9% (10.9/100 calf years of observation); composed of 5.5% in zero-grazing farms and 15.2% in semi-/full-pasture grazing farms (p=0.34) and 13.4% and 15.3% in UM and LH agro-ecological zones (p=0.85), respectively. However, within the UM zone, the rate for semi-full-pasture grazing farms at 51.3% was significantly higher than the rate of 0% observed on zero-grazing farms (p=0.005), while the rates for zero-grazing *versus* semi-full/grazing farms in the LH zone were higher, but similar at 14.1% *versus* 16.2%, respectively (p=0.88).

5.3.5 Total ECF Morbidity Incidence

Table 5.14 presents estimates of cause-specific morbidity rates for ECF by agro-ecological zone and grazing management system for each of the four combinations of ECF diagnostic criteria, summarised across age groups. With respect to ECF morbidity, defined as suspected non-fatal cases and deaths which were confirmed and then combined with missed diagnoses (Category 3), the overall rate across age groups was 5.9%; 7.3% in calves and 5.6% in adults (p=0.92). Further within this diagnostic category, the overall summary rates for zero-grazing *versus* semi-/full-pasture grazing farms were nearly identical at 5.6%

versus 6.2%, respectively, while broadly similar rates of 4.7%, 7.5% and 5.2% were observed for UM, LH and UH agro-ecological zones, respectively. Nevertheless, within the UM zone the rates for zero-grazing *versus* semi-/full-pasture grazing farms were sharply different at 1.2% *versus* 11.6, respectively (p=0.02). However, the direction of this difference was reversed within the LH zone with corresponding rates of 10.8% *versus* 4.2% (p=0.14), the latter being similar to the rate of 5.2% observed for semi-/full-pasture grazing farms in the UH zone. Overall minimal (Category 2) and maximal (Category 4) morbidity incidence rates of 2.4% and 9.7%, respectively, were identical or nearly identical to corresponding rates of 2.4% and 9.4% observed in adults and 2.4% and 10.9% observed in calves.

Despite the relative similarities in rates observed between age groups, grazing management systems and agro-ecological zones, a distinct clustering of morbidity outcomes by farm of origin was noted. Table 5.15 demonstrates that, of a total of 36 ECF diagnostic category 4 events (all non-fatal ECF cases, deaths and missed ECF diagnoses) recorded, 58.3% of these (21/36) occurred on 6 farms (6/90 = 6.7%), 7 (= 19.4%) episodes being reported in 5 animals from a single farm (Kiambaa Dairy Co-operative Society, Farm #12).

5.4 Serological Profiles

Serology has long been utilised to investigate the immune status of individuals with respect to detecting antibodies formed in response to exposure to pathogens or more generally to antigens. Typically, the results of such serological tests have been dichotomised into a positive/negative outcome, based on a pre-determined threshold antibody level, and

reported as prevalence data. Occasionally, pre- and post-exposure or acute and convalescent samples have been serially diluted to extinction and the semi-quantitative titres (largest dilution for which a positive test result is still obtained) compared to provide ancillary support to clinical diagnoses. However, longitudinal observations and serological profiles may themselves be utilised to derive information on the incidence of immunological changes in response to infection, particularly where infection may result in sub-acute or non-specific clinical signs. Such incidence may be measured with respect to detecting a change in health status, e.g. negative animals which subsequently test positive, or through identification of relatively sudden and significant increases in the amount of antigen-specific antibodies in animals already considered to be positive.

5.4.1 Rationale for Interpretation

The rationale for interpretation of longitudinal antibody profiles is based on the mechanisms and dynamics of the immune system.

5.4.1.1 Primary versus Secondary Immune Responses

For the purposes of this study, we may consider that the role of the immune system is to protect an individual against pathogenic viruses, microorganisms and parasites. An immune response is composed of two main phases; recognition of antigen followed by an effector phase. The response is therefore characterised by two important features, namely specificity and memory. The system is capable of distinguishing between foreign antigen and self-molecules and of retaining memory of an initial antigen encounter such that a

subsequent encounter results in an enhanced or modified response to that antigen (Roitt, 1997).

The production of antibodies is part of the effector arm of the humoral immune response. B-lymphocytes are stimulated to produce antigen-specific antibody when endogenously synthesized cell-surface immunoglobulin receptors (of the same specificity as the antibody) recognise antigen, either independently or more usually in cooperation with a sub-set of T-lymphocytes. However, each lymphocyte only recognises one antigen specifically and thus the ability to respond to the enormous variety of potential antigens requires a large number (approximately 10^{12}) of different lymphocyte antigen-receptor carriers. It ensues that there must be only a relatively small number of such clones initially which can recognize any particular specificity. It is therefore essential that clones of responding cells be expanded in the initial phases of a primary immune response to provide sufficient effector cells to counter infection. Activated B-cells proliferate and irreversibly differentiate into plasma cells which are almost wholly devoted to the production of secreted antibody. However, not all stimulated B-cells completely mature, such that clonal expansion also produces an expanded population of partially differentiated but quiescent "memory cells" with the same antigen-specific recognition capacity as the original clones. Plasma cells and indeed secretory antibody itself have relatively short life spans, however, the memory B cell population is extremely long-lived, perhaps lasting for the lifetime of an individual even in the absence of further antigen exposure, likely being maintained by a continuous process of recruitment and selection (Male *et al.*, 1991). Consequently, if an individual encounters the antigen again, the secondary (anamnestic) immune response is both

more rapid and more effective than the primary response. In addition, the secondary humoral response is characterised by a switch in class of immunoglobulin, from IgM to IgG, and an increase in binding affinity of the antibodies being synthesised; so called affinity maturation.

In comparison, the primary immune response is characterised by a lag-phase and an intermediate level of lower affinity antigen-specific antibody, while subsequent responses to the same antigen are faster and yield higher levels of greater affinity antibody, but always of the same specificity. The relative patterns of IgG and IgM immunoglobulin production on primary and secondary immune responses are illustrated in Figure 5.4.

5.4.1.2 Passive Transfer of Maternal Antibodies

In addition to endogenous production in response to antigen exposure, antibodies can also be passively absorbed by neonates via trans-placental transfer, or in the case of cattle, solely from maternal colostrum. Hence, newborn calves who are completely naive with respect to antigen exposure may still exhibit positive tests for antigen-specific antibody. The level of circulating antibodies in such calves is dependent on the amount present in the dams's colostrum, the success of passive transfer and time since gut closure (which occurs within hours of birth). The rate of degradation of maternally-acquired anti-schizont antibodies is relatively high such that, in the absence of antigen exposure, an exponential decline in antibody level is observed which falls below the detectable threshold within a few months (Burridge and Kimber, 1973a; Gitau, 1998; Mining *et al.*, 1998).

5.4.2 Definitions of Seroconversion and "Sero-Event"

Although the above description is an oversimplification of the various structures and mechanisms of the immune system, it is sufficient to illustrate the basis on which longitudinal serological profiles were assessed for evidence of primary (seroconversion) or secondary/anamnestic immune responses (sero-event) to *T. parva*. To formulate a set of objective classification criteria, further essential assumptions were made as follows:

- The ELISA test is immunologically specific, detecting only *T. parva*-specific antibodies and is epidemiologically sensitive and specific with respect to the chosen cut-off level of 0.125 OD units (Katende *et al.*, 1998).
- Calves are born completely naive to *T. parva*. There is no trans-placental or intra-uterine infection and thus newborn calves which test positive on initial post-partum examination do so as a result of passive transfer of maternal antibodies. Experimentally, primary infection produces detectable schizont-specific antibodies 2-4 weeks after infection (Burridge and Kimber, 1973a).
- In the absence of rechallenge, antibodies to *T. parva*, generated in a primary immune response (seroconversion), decline to an undetectable level (<1/40 titre on indirect fluorescent antibody test) by 6 months post-infection (Burridge and Kimber, 1973b).
- Based on experimental (Burridge and Kimber, 1973a; Mining *et al.*, 1998) and field (Gitau, 1998) studies where maternally derived antibodies were observed to have declined to negative levels between the third and sixth month of life, respectively, the absolute maximum duration of detectable

maternally-derived antibodies is assumed to be 8 months.

- By definition, a primary immune response can only occur in an immunologically naive animal. Since only newborn and neonatal calves can be confirmed not to have been exposed to *T. parva*, all other animals were assumed to have been previously infected and thus were assessed for evidence of a secondary immune response only.

Classification criteria were divided into those which defined seroconversion (primary immune response) and those which constituted presumptive evidence of a secondary immune response (sero-event).

5.4.2.1 Seroconversion Classification Criteria

A) Calves born sero-negative

Calves that tested negative on their first post-partum sample must either have been born to a sero-negative dam or they experienced a failure of passive transfer. Clearly, if such a calf remained sero-negative throughout its period of observation then seroconversion did not occur and the calf was considered to remain fully naive. If the calf subsequently tested positive and remained positive at an antibody level > 0.150 OD units (i.e. marginally greater than the test cut-off of 0.125 OD units to acknowledge test variation) for at least two consecutive tests thereafter, or if the positive test occurred in the final month of observation, then the animal was declared to have seroconverted. Examples of serological profiles of five calves which initially tested negative but were subsequently observed to have seroconverted under this criterion and in the absence of clinical signs are presented in Figure 5.5 A, B, F,

G & H. Subsequent to such a seroconversion, sero-event classification criteria (see section *5.4.2.2 Sero-Event Classification Criteria*) were applied in assessing whether an anamnestic immune response occurred later. However, if only a single positive test result was recorded before the calf once again tested negative, or below the 0.150 OD threshold, then regardless of the magnitude of the positive test, it was considered to be a false positive result and the calf remained classified as fully naive and at risk of seroconversion, should those criteria be later met.

B) Calves born sero-positive

By definition, calves which tested positive at initial post-partum examination were also naive, the test result as a consequence of passive transfer of colostral antibodies. Thus, these calves were expected to exhibit a declining antibody level over subsequent tests such that, in the absence of seroconversion, this level would become negative (Burridge and Kimber, 1973b). Once these calves experienced a negative test then the seroconversion criteria for calves born sero-negative were applied. Figure 5.6 A-G and I-L illustrate serological profiles for 11 calves which demonstrate evidence of both decay of passively acquired maternal antibodies to negative levels and subsequent seroconversion. The maximum period of decay in these calves before reaching a negative result was four months. Thus, a calf which initially tested positive but whose serum antibodies subsequently declined to undetectable levels and remained negative thereafter, did not seroconvert. If, however, the antibody level of a calf did not decline but subsequently began to rise at any point after an initial positive test result (whether the decline reached a negative level or not) then seroconversion was considered to have occurred. Figure 5.6 H provides an example of such

a calf. Alternatively, if the decline in antibody level was observed to plateau at a positive level (> 0.150 OD) or was considered to be prolonged beyond the maximum duration of maternally-derived antibody (> 8 months) then a seroconversion was recorded at the point of the plateau.

C) Positive and Negative Calves < 1 year of Age on Initial Observation

For calves which were not observed from birth (e.g. identified at first farm visit, or subsequently purchased), the age of the calf at initial observation was considered in the interpretation of its serological profile. Calves ≤ 7 months of age were classified according to the seroconversion rules for newborn calves as determined by initial test result. Figure 5.5 C, D & E illustrate seroconversions observed in 3 such calves which tested negative on initial examination. Calves > 7 months of age were assessed under the more stringent sero-event criteria regardless of the initial test result, since it was theoretically possible for them to have experienced seroconversion and a decline to negative levels. By definition, calves > 7 months of age with positive test results on initial examination had seroconverted previously and must thereafter be assessed under sero-event criteria.

5.4.2.2 Sero-Event Classification Criteria

An anamnestic humoral immune response must be evidenced by a rapid increase in amount of antigen-specific antibody, of relatively large magnitude compared to that observed in a primary response, which is sustained, or followed by a decline in level consistent with the natural metabolism of circulating antibodies (Roitt, 1997). By this definition, patterns of increase in ELISA test results observed for those animals reported to have suffered a

concomitant episode of ECF (suspected cases which were subsequently confirmed - Figure 5.1), or which coincided with more mild clinical signs (missed ECF diagnoses - Figure 5.2) were determined to be consistent with a secondary immune response, as illustrated in Figure 5.4. In most of these cases a dramatic increase in optical density units, often greater than 0.300 OD units, was detected over two consecutive tests (i.e. 1 month apart) which temporally flanked the clinical episodes, followed by a variable period of slow decline. However, the minimum increase noted in these instances was of magnitude 0.140 OD units, while in some cases the increase was observed to extend over three consecutive tests. Such increases were observed in animals with initial antibody levels up to and beyond that of the strong positive control samples (i.e. > 0.500 OD units).

A subsequent examination of the serological profiles generated for all other animals revealed many similar patterns, strongly suggestive of anamnestic immune responses, but occurring in otherwise healthy and apparently unaffected animals. Figure 5.7 A-X presents several examples of these profiles. The majority of individual profiles were suggestive of a single anamnestic immune response having occurred during the period of observation, however, in a few cases two distinct sero-events were discernible. For example, Figure 5.7 W & X both demonstrate evidence of a marked sero-increase having occurred between the sixth and seventh rounds of sampling and again between the eleventh and twelfth rounds. To estimate the incidence of such sero-events in animals (other than neonatal calves subject to seroconversion criteria as defined above), a sero-event was only documented where the profile of the increase was deemed to have met all the properties of an anamnestic immune response as follows:

A) Magnitude of Increase - Since it was not possible to undertake titration of all samples, any increase in antibody levels must be assessed with respect to measures of optical density units. Figures 4.13 and 4.14 demonstrate two key features. First, even after test standardisation, there is variation in the relationship between antibody titre (established by serial dilution to extinction), and optical density units at the single working dilution. This variation increases with test result since the OD reading approaches a maxima while titre theoretically does not. Hence, although a particular OD value may be maintained concomitant with a declining antibody titre, any increase in value is synonymous with either test variation or a true increase in the amount of antibody. Fortunately, the majority of variation is observed between animals (with different titres), rather than for repeated samples within the same animal. Second, the best-fit curve for the data is approximately straight line over the interval (0.125 through approximately 0.900 OD units) in which the majority (Figure 4.13) of the positive test results were observed. Specifically, over this portion of the curve an increase of 0.200 OD units corresponds to an increase of approximately one natural log in titre (2.7 times increase in amount of antibody). Similarly, a doubling of the end dilution ($ln[2] = 0.7$) corresponds to an increase of approximately 0.125 OD units in the test result obtained. It was therefore deemed sufficient to assume a simple straight line relationship between antibody titre and test OD measurement and utilise a single magnitude of increase criterion of at least 0.150 OD units (more than a doubling of antibody titre) across the entire interval of possible OD values. Although a change of 0.150 OD units is consistent with a greater change in titre for higher OD values, given the increased variation observed at these levels, this methodology constitutes an overall more conservative assessment.

B) Rapidity of Increase - With a sampling interval of 1 month, it is not possible to be certain when antibody levels peaked during a period of sero-increase. Thus, as observed in the clinical cases, the minimum magnitude of rise in OD level was calculated over one or two subsequent rounds as necessary.

C) Persistence of Increase - Owing to the dynamics of antibody production and degradation, it is essential that, for any such sero-increase to be considered legitimate, it must show evidence of maintenance. This is partially dealt with by the fact that the initial rise is allowed to occur over up to two subsequent test results. Under these circumstances, if the highest individual test value is considered the peak (which may occur at either the second or third subsequent test) then the next sample may be i) on a plateau with the peak antibody tests, ii) slightly above due to variation in test results or iii) below as antibody levels have already begun to decline. In consequence, for the sero-event definition criteria, the value of the third or fourth test (corresponding to a one and two month duration of increase, respectively) must arbitrarily yield an OD value at least equal to 50% of the total increase observed. For example, for the minimum criterion of an OD increase of 0.150 OD units over one test interval, (from first to second test), in order for this to be considered an event, the value of the third test must be higher than that of the first test by 0.075 OD units. This essential difference would rise to 0.250 OD units where the initial increase was of magnitude 0.500 OD units, etc. Any event not satisfying this criterion is considered to have resulted from an erroneous test value. Further, as per calf-seroconversion rules, if the maintenance of rise can not be so established due to lack of subsequent observations (e.g. rise occurred over final month of the study, or immediately prior to loss from observation), then a rise of

sufficient magnitude alone will be considered sufficient evidence of a sero-increase event.

5.4.2.3 Refinement of Sero-Event Classification Criteria

As a method of assessing the validity of the chosen necessary level of sero-increase (i.e. >0.150 OD units ≈ doubling of titre), the criteria were made progressively more stringent. That is, the number of sero-events were recalculated as the minimum essential magnitude was sequentially increased to 0.165, 0.180 and 0.200 OD units, with all other criteria remaining unchanged. However, in acknowledgment of the fact that newborn calves were the only animals which could be absolutely categorized as naive, the sero-conversion criteria were considered to reflect the certainty of classification and alternatives were not considered.

5.4.3 Incidence of Seroconversions and Sero-Events in Calves

Forty-one animals, composed of 38 calves (≤ 1 year of age) and 3 adults (> 1 year of age at time of seroconversion but observed negative prior to 7 months of age) drawn from all dairy co-operative societies and in all agro-ecological zones, demonstrated seroconversion to *T. parva*, 37 of these (90.2%; 34 calves and all 3 adults) in the absence of any recognised clinical signs. There were 3 additional cases of sero-events in calves, all of which coincided with the appearance of clinical signs and all of which exhibited a sero-increase > 0.200 OD units in magnitude.

The rates of seroconversion and seroconversion + sero-events observed in calves are presented in Table 5.16 by agro-ecological zone and grazing management system. The

overall seroconversion rate was 49.8% (95% CI 36.7% - 67.6%), composed of rates of 41.4% and 56.5% on zero-grazing and semi-/full-pasture grazing farms, and 46.9%, 61.2% and 35.4% in UM, LH and UH agro-ecological zones, respectively. No significant differences were detected between any of these rates, however, the pattern of higher incidence on semi-/full-pasture grazing farms is more pronounced when comparisons are made within agro-ecological zone. That is, the rate for semi-/full-pasture grazing farms is more than double the rate for zero-grazing farms within the UM zone at 77.0% *versus* 36.3% (p=0.15). A similar pattern is observed within the LH zone, but the absolute difference is smaller with rates of 70.4% and 49.3%, respectively (p=0.50). Excluding the UH zone and summarizing across UM and LH zones yields rates of seroconversion of 41.4% (15/36.3 years of observation) *versus* 69.5% (19/27.3 calf years of observation) for zero-grazing *versus* semi-/full-pasture grazing farms (p=0.13). However, although there is an apparent, but non-significant, trend for differences in the rates of seroconversion by grazing management system in the UM and LH zones, the mean age at time of seroconversion apparently differs more between these two zones rather than by grazing management system within a zone.

Table 5.17 presents the mean age, its standard deviation, the median age and the observed minimum and maximum in ages at time of seroconversion, by grazing management system and agro-ecological zone, for the 41 animals which seroconverted. While no statistically significant differences were detected for any age-to-seroconversion contrasts, it is of interest to note that the mean age at time of seroconversion observed for zero-grazing and semi-/full-pasture grazing farms in the UM zone are virtually identical with values of

0.39 years and 0.38 years respectively. This in contrast to corresponding means of 0.55 years and 0.61 years noted within the LH zone and of 0.56 years for semi-/full-pasture grazing farms in the UH zone. Further, although the overall mean age at time of seroconversion in the UM zone (0.38 years) is apparently lower than the overall mean in the LH zone (0.59 years), the corresponding summary values for median age at seroconversion of 0.39 years and 0.42 years are very similar, indicative of the skewed distribution of higher ages observed in the LH zone, where two animals were noted to have seroconverted at 17 and 20 months of age.

It may be argued that survival analysis of time-to-seroconversion is a superior method of assessing the force of infection in a cohort of calves. However, within the current study, the variable timing of calf entry, the high degree of right censoring due to mortality and sale and the large variation in age of calves at initial farm visit resulted in a relatively small total period of calf observation, such that a more formal analysis restricted to naive calves only was considered likely to be uninformative.

5.4.4 Incidence of Sero-Events in Adult Cattle

A total of 78 sero-events with a magnitude of increase in *T. parva*-specific antibodies in excess of 0.150 ELISA OD units were observed in adult cattle, 83.3% (65/78) of these in the absence of a concomitant clinical episode. Animals which experienced detectable sero-events were observed in all agro-ecological zones and from all dairy co-operative societies. Increasing the acceptable criteria for minimal magnitude of sero-increase from 0.150 through 0.165 and 0.180 to 0.200 OD units resulted in a corresponding reduction of total defined

sero-events to 68, 58 and 53 respectively. The 32.0% (25/78) reduction in total sero-events from 0.150 OD to 0.200 OD units, was composed of 24 sub-clinical events and one clinical episode of ECF. Although this reduction may seem substantial, it was distributed equally across agro-ecological zone and grazing management classifications, such that the overall pattern of incidence of sero-events was maintained. These relationships are demonstrated in Table 5.18 which presents the incidence rates of sero-events observed in adult cattle by minimum essential magnitude of serological increase, agro-ecological zone and grazing management system. Considering only serological increases of magnitude 0.200 OD units or greater, the overall incidence of sero-events was 18.4% (53/288.0; 95% CI 14.1 - 24.1), significantly lower ($p<0.001$) than the rate of seroconversions observed in calves. The rate in mature cattle was composed of average rates of 12.2% and 23.0% on zero-grazing and semi-/full-pasture grazing farms and 16.2%, 16.1% and 25.1% in UM, LH and UH agro-ecological zones, respectively. Although the approximate 95% confidence intervals for these summary rates were observed to overlap, as with seroconversions, the characteristic higher rates in semi-/full-pasture grazing farms is most pronounced in the UM agro-ecological zone, where a rate of 6.7% was calculated on zero-grazing farms in contrast to a rate of 33.8% on semi-/full-pasture grazing farms ($p=0.02$). Further, although the direction of the difference in rates appears to be slightly reversed in the LH zone, with corresponding values of 18.5% *versus* 13.3%, the difference is not statistically significant ($p=0.46$) and it is important to note that two animals which were observed to have seroconverted as adults (> 1 year of age) were from semi-/full-pasture grazing farms in the LH zone. In consequence, despite the large and statistically significant differences observed between the rates of seroconversions in calves

and the sero-event rates in mature cattle, the overall patterns of incidence may be best investigated by summarizing across age group, that is combining seroconversions and sero-events.

5.4.5 Total Incidence of Seroconversions and Sero-Events

Total incidence rates of seroconversion and sero-events in all animals, irrespective of age, are presented in Table 5.19 by agro-ecological zone and grazing management system, for increasingly conservative criteria of definition of sero-events. Once again, the relative reduction in incidence rates with increasing magnitude of sero-increase is virtually uniform across agro-ecological and grazing management subclassifications, such that the overall pattern observed is consistent. The total incidence rate under the most conservative sero-event classification (> 0.200 OD units) was 26.2% (97/370.3; 95% CI 21.5% - 31.9%), that is, an average of one such event approximately every 4 animal-years. Summary rates of 24.1%, 25.3% and 27.2% for UM, LH and UH agro-ecological zones, were nearly equivalent, while the total rate of 31.3% observed in semi-/full-pasture grazing farms was significantly higher than the corresponding rate of 19.5% from farms practising zero-grazing management (p=0.03). Once again, however, this difference in the overall summary rates was principally attributable to pronounced differences within the UM zone. The rate of seroconversion and sero-events observed on semi-/full-pasture grazing farms located in the UM zone (43.9%) was over three times the rate observed on zero-grazing farms (14.0%) in the same zone (p=0.002). In contrast, the rates by grazing management system in the LH zone, 29.0% and 25.8% respectively, were more similar to each other and to the rate

observed on semi-/full-pasture grazing farms in the UH zone (27.2%).

Table 5.1: Deaths of "adult" (i.e. > 1 year of age) cattle recorded over 288 animal-years of observation in a longitudinal study conducted in Kiambu District from July 1991 - June 1992.

Dairy Co-operative Society	AEZ[1]	Farm	Grazing System[2]	Visit	Age (years)	Diagnosis/Cause of Death
Chania	UM2	6	Zero	11	10	Suspected ECF
Kiambaa	UM2	1	Zero	10	6	Lumpy Skin Disease + Rumen Impaction
Kiambaa	UM2	7	Zero	4	4	Pneumonia
Kiambaa	UM2	8	Zero	2	6	Suspected ECF
Kikuyu	LH2	1	Graze	9	6.5	Post-calving Downer, Hypocalcemia
Kikuyu	LH2	4	Graze	2	1.5	Unknown - Sudden Death[3]
Kikuyu	LH2	5	Zero	6	3.5	Confirmed ECF (sero-increase)[4]
Kikuyu	LH3	8	Zero	6	3.5	Confirmed ECF (post-mortem)[4]
Kikuyu	LH3	10	Zero	4	4	Grain Overload
Kikuyu	UM3	14	Zero	12	6	Suspected ECF
Kikuyu	UM3	14	Zero	4	6	Toxic Mastitis
Kikuyu	UM3	14	Zero	5	7.5	Bloat
Lari	UH2	1	Graze	11	3.5	Unknown - Sudden Death
Lari	UH1	8	Graze	5	2.5	Pneumonia
Limuru	UH2	5	Graze	12	1.5	Trauma - Fractured Femur
Limuru	LH2	7	Zero	12	7	Severe Dystocia - Downer Cow
Nderi	LH2	1	Graze	11	7	Post-calving Downer, Hypocalcemia
Nderi	LH2	15	Zero	2	1	Unknown - Sudden Death

[1] - Agro-ecological Zones (AEZ) where "UM" refers to the upper midland zone, "LH" to the lower highland zone and "UH" to the upper highland zone.
[2] - Grazing management where "Zero" refers to a zero-grazing system and "Graze" documents animals kept on pasture.
[3] - "Unknown - Sudden Death" refers to peracute death, in the absence of clinical signs, and not subject to post-mortem examination.
[4] - Confirmation of death due to East Coast fever (ECF) was either by means of post-mortem examination or evidence of a serological response in an animal which subsequently died.

Table 5.2: Deaths of calves recorded over 83 calf-years of observation in a longitudinal study conducted in Kiambu District from July 1991 - June 1992, where death occurred after initial examination but before the animal reached 1 year of age.

Dairy Co-operative Society	AEZ[1]	Farm	Grazing System[2]	Visit	Age (months)	Diagnosis/Cause of Death
Kiambaa	UM2	4	Zero	11	6	Unknown - Sudden Death[3]
Kiambaa	UM3	12	Graze	3	2.5	Unknown - Sudden Death
Kikuyu	LH2	5	Zero	5	4.5	Salt Poisoning
Kikuyu	LH3	8	Zero	7	1.5	Diarrhoea/Dehydration
Kikuyu	UM3	9	Semi	8	6.5	Poisoning (Consumed)
Kikuyu	UM3	9	Semi	8	6.5	Poisoning (Consumed)
Kikuyu	LH3	10	Zero	2	6	GIT Obstruction/Bloat
Kikuyu	LH3	11	Zero	6	4	Poisoning (Acaricide Spray)
Kikuyu	UM3	13	Zero	12	3	Diarrhoea/Dehydration
Kikuyu	UM3	14	Zero	4	4.5	Unknown - Sudden Death
Kikuyu	UM3	14	Zero	5	8	Diarrhoea/Dehydration
Kikuyu	UM3	14	Zero	11	1.5	Diarrhoea/Dehydration
Kikuyu	UM3	14	Zero	11	1.5	Diarrhoea/Dehydration
Kikuyu	LH2	15	Semi	8	5.5	Suspected ECF
Nderi	LH2	1	Graze	3	2	Diarrhoea/Dehydration
Nderi	LH2	1	Graze	10	6	Diarrhoea/Dehydration
Nderi	LH2	1	Graze	11	6	Unknown - Sudden Death
Nderi	LH2	3	Graze	4	2	Diarrhoea±Pneumonia
Nderi	LH3	9	Zero	12	6	Unknown - Sudden Death

[1] - Agro-ecological Zones (AEZ) where "UM" refers to the upper midland zone, "LH" to the lower highland zone and "UH" to the upper highland zone.
[2] - Grazing management where "Zero" refers to a zero-grazing system and "Semi" or "Graze" documents animals kept on pasture.
[3] - "Unknown - Sudden Death" refers to peracute death, in the absence of clinical signs, and not subject to post-mortem examination.

Table 5.3: Deaths of calves born to dams enrolled in a longitudinal study conducted in Kiambu District from July 1991 - June 1992, where death occurred prior to initial examination and sampling, i.e. < 1 month of age.

Dairy Co-operative Society	AEZ[1]	Farm	Grazing System[2]	Visit	Diagnosis/Cause of Death
Chania	LH1	1	Graze	6	Unknown - Sudden Death[3]
Chania	UM2	12	Zero	5	Atresia Coli
Kikuyu	UM3	13	Zero	5	Trampled by Dam
Lari	UH1	10	Graze	10	Dystocia - Still Birth
Nderi	LH2	7	Zero	11	Dystocia
Nderi	LH2	7	Zero	11	Unknown - Sudden Death

[1] - Agro-ecological Zones (AEZ) where "UM" refers to the upper midland zone, "LH" to the lower highland zone and "UH" to the upper highland zone.
[2] - Grazing management where "Zero" refers to a zero-grazing system and "Graze" documents animals kept on pasture.
[3] - "Unknown - Sudden Death" refers to peracute death, in the absence of clinical signs, and not subject to post-mortem examination.

Table 5.4: Calculation of period of animal observation from surveys recorded in a longitudinal study conducted in Kiambu District from July 1991 - June 1992; subdivided on the basis of i) the agro-ecological zone (AEZ) and ii) grazing management system of the farm of origin and iii) the classification of the animal with respect to age (0-12 months of age = calf, > 12 months = adult) *at the time of each observation/survey*:

AEZ[1]	Grazing System[2]	Period of Observation Contributed by					
		Adults		Calves		TOTAL	
		Months	Years	Months	Years	Months	Years
UH	Semi/Grazing	909	75.8	237.5	19.8	1146.5	95.5
LH	Zero-Grazing	714	59.5	170.5	14.21	884.5	73.7
	Semi/Grazing	646	53.8	221.5	18.5	867.5	72.3
UM	Zero-Grazing	761.5	63.5	264.5	22.0	1026	85.5
	Semi/Grazing	426	35.5	93.5	7.79	519.5	43.3
TOTAL		3456.5	288.0	987.5	82.3	4444	370.3

[1] - Agro-ecological Zones (AEZ) where "UM" refers to the upper midland zone, "LH" to the lower highland zone and "UH" to the upper highland zone.
[2] - Grazing management where "Zero-Grazing" refers to a zero-grazing system and "Semi/Grazing" documents animals kept on pasture.

Table 5.5: Cause-specific mortality rates of "adult" (i.e. > 1 year of age) cattle recorded in a longitudinal study conducted in Kiambu District from July 1991 - June 1992 and expressed as number of deaths per 100 "adult" animal years of observation. Crude numbers from which rates are determined are presented beside the rate in square brackets while approximate 95% confidence intervals are presented below each rate.

Diagnosis/Cause of Death	Agro-Ecological Zones[1]			Total Mortality Rate
	UM (1,2,3)	LH (1,2,3)	UH (1,2)	
East Coast Fever (suspected)	3.0 [3] (1.0-8.9)	1.8 [2] (0.5-6.4)	0 (0-5.1)	1.7 [5] (0.7-4.1)
Zero-grazing	4.7 [3] (1.6-13.4)	3.4 [2] (0.9-12.3)	-	4.1 [5] (1.7-9.5)
Semi/Grazing	0 (0-10.8)	0 (0-7.1)	0 (0-5.1)	0 (0-2.3)
East Coast Fever (confirmed)	0 (0-3.4)	1.8 [2] (0.5-6.4)	0 (0-5.1)	0.7 [2] (0.2-2.5)
Zero-grazing[2]	0 (0-6.1)	3.4 [2] (0.9-12.3)	-	1.6 [2] (0.4-5.9)
Semi/Grazing[2]	0 (0-10.8)	0 (0-3.4)	0 (0-5.1)	0 (0-2.3)
Unknown - Sudden Death	0 (0-3.4)	1.8 [2] (0.5-6.4)	1.3 [1] (0.2-7.5)	1.0 [3] (0.4-3.1)
Respiratory: (Pneumonia)	1.0 [1] (0.2-5.7)	0 (0-3.4)	1.3 [1] (0.2-7.5)	0.7 [2] (0.2-2.5)
GIT: (Rumen Impaction, Bloat, Grain Overload)	2.0 [2] (0.6-7.4)	0.9 [1] (0.2-5.0)	0 (0-5.1)	1.0 [3] (0.4-3.1)
Periparturient: (Dystocia, Hypocalcemia, Downer)	0 (0-3.4)	2.6 [3] (0.9-7.8)	0 (0-5.1)	1.0 [3] (0.4-3.1)
Mastitis	1.0 [1] (0.2-5.7)	0 (0-3.4)	0 (0-5.1)	0.4 [1] (0.1-2.0)
Trauma	0 (0-3.4)	0 (0-3.4)	1.3 [1] (0.2-7.5)	0.4 [1] (0.1-2.0)
TOTAL	7.1 [7] (3.4-14.6)	7.1 [8] (3.6-13.9)	4.0 [3] (1.3-11.6)	6.3 [18] (4.0-9.9)

[1] - Agro-ecological Zones (AEZ) where "UM" refers to the upper midland zone, "LH" to the lower highland zone and "UH" to the upper highland zone.

[2] - Grazing management where "Zero" refers to a zero-grazing system and "Semi/Grazing" documents animals kept on pasture.

Table 5.6: Cause-specific mortality rates of calves (i.e. ≤ 1 year of age) recorded in a longitudinal study conducted in Kiambu District from July 1991 - June 1992 and expressed as number of deaths per 100 "calf" years of observation. Crude numbers from which rates are determined are presented beside the rate in square brackets while approximate 95% confidence intervals are presented below each rate.

Diagnosis/Cause of Death	Agro-Ecological Zones[1]			Total Mortality Rate
	UM (1,2,3)	LH (1,2,3)	UH (1,2)	
East Coast Fever (suspected)	0 (0-12.9)	3.1 [1] (0.5-17.3)	0 (0-19.4)	1.2 [1] (0.2-6.9)
Zero-grazing[2]	0 (0-17.4)	0 (0-27.0)	-	0 (0-10.6)
Semi/Grazing[2]	0 (0-49.3)	5.4 [1] (1.0-30.7)	0 (0-19.4)	2.2 [1] (0.4-12.3)
Diarrhoea/Dehydration	13.4 [4] (5.2-34.5)	12.2 [4] (4.8-31.5)	0 (0-19.4)	9.7 [8] (4.9-12.3)
Unknown - Sudden Death	10.1 [3] (3.4-29.6)	12.2 [4] (4.8-31.5)	0 (0-19.4)	8.5 [7] (4.1-17.6)
Poisoning (Consumed or Applied)	6.7 [2] (1.8-24.4)	6.1 [2] (1.7-22.3)	0 (0-19.4)	4.9 [4] (1.9-12.5)
Dystocia-related	0 (0-12.9)	3.1 [1] (0.5-17.3)	5.0 [1] (0.9-28.6)	2.4 [2] (0.7-8.9)
GIT Obstruction/Bloat	0 (0-12.9)	3.1 [1] (0.5-17.3)	0 (0-19.4)	1.2 [1] (0.2-6.9)
Congenital Anomaly (Atresia Coli)	3.4 [1] (0.6-19.0)	0 (0-11.8)	0 (0-19.4)	1.2 [1] (0.2-6.9)
Trauma (Trampled)	3.4 [1] (0.6-19.0)	0 (0-11.8)	0 (0-19.4)	1.2 [1] (0.2-6.9)
TOTAL	36.9 [11] (20.6-66.0)	39.6 [13] (23.3-68.1)	5.0 [1] (0.9-28.6)	30.4 [25] (20.6-44.8)

[1] - Agro-ecological Zones (AEZ) where "UM" refers to the upper midland zone, "LH" to the lower highland zone and "UH" to the upper highland zone.
[2] - Grazing management where "Zero" refers to a zero-grazing system and "Semi/Grazing" documents animals kept on pasture.

Table 5.7: Cause-specific mortality rates due to East Coast Fever (ECF) and unknown/undiagnosed deaths recorded in a longitudinal study conducted in Kiambu District from July 1991 - June 1992 and expressed as number of deaths per 100 animal years of observation. Crude numbers from which rates are determined are presented beside the rate in square brackets while approximate 95% confidence intervals are presented below each rate.

Diagnosis/Cause of Death	Agro-Ecological Zones[1]			Total Mortality Rate
	UM (1,2,3)	LH (1,2,3)	UH (1,2)	
East Coast Fever (suspected)	2.3 [3] (0.8-6.8)	2.0 [3] (0.7-6.0)	0 (0-4.0)	1.6 [6] (0.7-3.5)
Zero-grazing[2]	3.5 [3] (1.2-10.3)	2.7 [2] (0.7-9.9)	-	3.1 [5] (1.3-7.4)
Semi/Grazing[2]	0 (0-8.9)	1.4 [1] (0.2-7.8)	0 (0-4.0)	0.5 [1] (0.1-2.7)
East Coast Fever (confirmed)	0 (0-3.0)	1.4 [2] (0.4-5.0)	0 (0-4.0)	0.5 [2] (0.1-2.0)
Zero-grazing	0 (0-4.5)	2.7 [2] (0.7-9.9)	-	1.3 [2] (0.3-4.6)
Semi/Grazing	0 (0-8.9)	0 (0-5.3)	0 (0-4.0)	0 (0-1.8)
Unknown - Sudden Death[3]	2.3 [3] (0.8-6.8)	4.1 [6] (1.9-9.0)	1.0 [1] (0.2-5.9)	2.7 [10] (1.5-5.0)
TOTAL[4]	4.7 [6] (2.1-10.2)	6.2 [9] (3.2-11.7)	1.0 [1] (0.2-5.9)	4.3 [16] (2.7-7.0)

[1] - Agro-ecological Zones (AEZ) where "UM" refers to the upper midland zone, "LH" to the lower highland zone and "UH" to the upper highland zone.
[2] - Grazing management where "Zero" refers to a zero-grazing system and "Semi/Grazing" documents animals kept on pasture.
[3] - "Unknown - Sudden Death" refers to peracute death, in the absence of clinical signs and not subject to post-mortem examination.
[4] - "Total" deaths are composed of all suspected ECF mortalities and all undiagnosed, peracute deaths.

Table 5.8: Cases of non-fatal East Coast Fever (ECF) in "adult" (i.e. > 1 year of age) cattle recorded over 288 animal-years of observation in a longitudinal study conducted in Kiambu District from July 1991 - June 1992.

Diary Co-operative Society	AEZ[1]	Farm	Grazing System[2]	Visit	Age (years)	Initial Serological Status	Serological Profile (Corresponding Figure)
Chania	LH1	1	Graze	7, 10[3]	1.5	Moderate Positive	No serological increases[3]
Chania	LH1	3	Semi	8	6.5	Low Positive	Serological increase noted (Fig 5.1A)
Kiambaa	UM2	4	Zero	5	3.5	Low Positive	Serological increase noted (Fig 5.1B)
Kiambaa	UM3	12	Graze	9 (4)[4]	1.5	Moderate Positive[4]	Serological increase noted (Fig 5.1C)[4]
Kiambaa	UM3	12	Graze	12	2.5	Moderate Positive	No serological increase
Kiambaa	UM3	13	Zero	12	6	Low Positive	No serological increase
Kikuyu	LH3	8	Zero	12	1	Negative	Serological increase noted (Fig 5.1D)
Kikuyu	UM3	13	Zero	9	5.5	Negative	No serological increase
Kikuyu	UM3	14	Zero	11	5	Low Positive	No serological increase
Lari	UH1	7	Graze	7	2	Negative	No serological increase
Lari	UH2	11	Graze	9	5.5	Negative	No serological increase
Limuru	UH2	12	Graze	4 (10)[5]	2	Moderate Positive[5]	Serological increase(s) noted (Fig 5.1E)[5]

[1] - Agro-ecological Zones (AEZ) where "UM" refers to the upper midland zone, "LH" to the lower highland zone and "UH" to the upper highland zone.

[2] - Grazing management where "Zero" refers to a zero-grazing system and "Semi/Grazing" documents animals kept on pasture.

[3] - Episodes of clinical ECF reported on both occasions but neither confirmed by serology

[4] - Clinical ECF reported at time of 9th round of farm visits and confirmed by serology, however, also previous serological and clinical evidence suggestive of undiagnosed ECF at round 4 visit (Table 5.9)

[5] - Clinical ECF reported at time of 4th round of farm visits and confirmed by serology, however, also subsequent serological and clinical evidence suggestive of undiagnosed ECF at round 10 visit (Table 5.9).

Table 5.9: Incidents of non-specific and/or mild clinical illness in "adult" (i.e. > 1 year of age) cattle recorded over 83 calf-years of observation in a longitudinal study conducted in Kiambu District from July 1991 - June 1992 which were subsequently correlated with serological evidence of *Theileria parva* infection; suggestive of undiagnosed ECF.

Diary Co-operative Society	AEZ[1]	Farm	Grazing System[2]	Visit	Age (years)	Dx / Signs / Tx	Serological Profile (Corresponding Figure)[3]
Kiambaa	UM3	12	Graze	4	1.5	Cough, Apyrexial, No Tx	Seroincrease: low - strong positive (Fig 5.2A)
Kiambaa	UM3	12	Graze	4	1.5	Dull, Prescapular lymph node, Oxytet.	Seroincrease: moderate - strong positive (Fig 5.1C)
Kikuyu	LH3	8	Zero	5	7.5	Recumbent, Hypocalcemia, Calcium	Seroincrease: negative - moderate positive (Fig 5.2B)
Kikuyu	LH3	10	Zero	9	5.5	Cough, Apyrexial, No Tx	Seroincrease: low - very strong positive (Fig 5.2C)
Kikuyu	UM3	14	Zero	5	2.5	Pyrexia, Dyspnea, Oxytet.	Seroincrease: negative - low positive (Fig 5.2D)
Limuru	UH2	1	Graze	4	4	Cough, Apyrexial, NoTx	Seroincrease: low - moderate positive (Fig 5.2E)
Limuru	UH2	12	Graze	5	4.5	Cough, Rales, Apyrexial, Oxytet.	Seroincrease: moderate - very strong positive (Fig 5.2F)
Limuru	UH2	12	Graze	10	2	Dyspnea, Apyrexial, No Tx	Seroincrease: strong - very strong positive (Fig 5.1E)
Limuru	LH3	14	Zero	8	7	Cough, Apyrexial	Seroincrease: low - moderate positive (Fig 5.2G)

[1] - Agro-ecological Zones (AEZ) where "UM" refers to the upper midland zone, "LH" to the lower highland zone and "UH" to the upper highland zone.
[2] - Grazing management where "Zero" refers to a zero-grazing system and "Semi/Grazing" documents animals kept on pasture.
[3] - Corresponding serological profiles are presented in referenced figure

Table 5.10: Morbidity incidence rates of East Coast Fever in "adult" (i.e. > 1 year of age) cattle recorded in a longitudinal study conducted in Kiambu District from July 1991 - June 1992 and expressed as number of cases per 100 "adult" years of observation. Crude numbers from which rates are determined are presented beside the rate in square brackets and include mortalities according to corresponding classification criteria while approximate 95% confidence intervals are presented below each rate.

Classification of ECF Diagnosis	Agro-Ecological Zones[1]			Total Morbidity Rate
	UM (1,2,3)	LH (1,2,3)	UH (1,2)	
1) Suspected non-fatal ECF cases and deaths, whether confirmed or unconfirmed.	9.1 [9] (4.8-17.3)	5.3 [6] (2.4-11.6)	4.0 [3] (1.3-11.6)	6.2 [18] (4.0-9.9)
Zero-grazing[2]	11.0 [7] (5.3-22.8)	5.0 [3] (1.7-14.8)	-	8.1 [10] (4.4-14.0)
Semi/Grazing[2]	5.6 [2] (1.5-20.5)	5.6 [3] (1.9-16.4)	4.0 [3] (1.3-11.6)	4.9 [8] (2.5-9.6)
2) Suspected non-fatal cases and deaths which were confirmed by serology or post-mortem.	2.0 [2] (0.6-7.4)	3.5 [4] (1.4-9.1)	1.3 [1] (0.2-7.5)	2.4 [7] (1.2-5.0)
Zero-grazing	1.6 [1] (0.3-8.9)	5.0 [3] (1.7-14.8)	-	3.2 [4] (1.3-8.4)
Semi/Grazing	2.8 [1] (0.5-16.0)	1.9 [1] (0.3-10.5)	1.3 [1] (0.2-7.5)	1.8 [3] (0.6-5.3)
3) Suspected non-fatal cases and deaths which were confirmed by serology or post-mortem and missed cases as demonstrated by longitudinal serology.	4.0 [4] (1.6-10.4)	6.2 [7] (3.0-12.8)	6.6 [5] (2.8-15.5)	5.6 [16] (3.4-9.0)
Zero-grazing	1.6 [1] (0.3-8.9)	10.1 [6] (4.6-22.0)	-	5.7 [7] (2.8-11.8)
Semi/Grazing	8.4 [3] (2.9-24.8)	1.9 [1] (0.3-10.5)	6.6 [5] (2.8-15.5)	5.4 [9] (2.9-10.4)
4) Suspected non-fatal ECF cases and deaths, whether confirmed or unconfirmed and missed cases as demonstrated by longitudinal serology.	11.1 [11] (6.2-19.9)	7.9 [9] (4.2-15.1)	9.2 [7] (4.5-19.1)	9.4 [27] (6.4-13.6)
Zero-grazing	11.0 [7] (5.3-22.8)	10.1 [6] (4.6-22.0)	-	10.6 [13] (6.2-18.1)
Semi/Grazing	11.3 [4] (4.4-29.0)	5.6 [3] (1.9-16.4)	9.2 [7] (4.5-19.1)	8.5 [14] (5.1-14.2)

[1] - Agro-ecological Zones (AEZ) where "UM" refers to the upper midland zone, "LH" to the lower highland zone and "UH" to the upper highland zone.

[2] - Grazing management where "Zero" refers to a zero-grazing system and "Semi/Grazing" documents animals kept on pasture.

Table 5.11: Cases of non-fatal East Coast Fever (ECF) in calves (i.e. ≤1 year of age) recorded over 288 animal-years of observation in a longitudinal study conducted in Kiambu District from July 1991 - June 1992.

Diary Co-operative Society	AEZ[1]	Farm	Grazing System[2]	Visit	Age (months)	Initial Serological Status	Serological Profile (Corresponding Figure)[3]
Chania	LH1	3	Semi	6	9	Low Positive	Serological increase noted (Fig 5.3A)
Kiambaa	UM3	12	Graze	8(2)[4]	8	Strong Positive[2]	Serological decline noted[4]
Kiambaa	UM3	12	Graze	12	2	Low Positive	Declining Maternal Ab
Nderi	LH3	9	Zero	4	10	Low Positive	Serological increase noted (Fig 5.3B)

[1] - Agro-ecological Zones (AEZ) where "UM" refers to the upper midland zone, "LH" to the lower highland zone and "UH" to the upper highland zone.
[2] - Grazing management where "Zero" refers to a zero-grazing system and "Semi/Grazing" documents animals kept on pasture.
[3] - Corresponding serological profiles are presented in referenced figure.
[4] - Clinical ECF reported at time of 8th round of farm visits but unconfirmed by serology, however, previous serological and clinical evidence of undiagnosed ECF at round 2 visit (Table 5.12, Figure 5.3D).

Table 5.12: Incidents of non-specific and/or mild clinical illness in calves (i.e. ≤1 year of age) recorded over 83 calf-years of observation in a longitudinal study conducted in Kiambu District from July 1991 - June 1992 which were subsequently correlated with serological evidence of *Theileria parva* infection; suggestive of undiagnosed ECF.

Diary Co-operative Society	AEZ[1]	Farm	Grazing System[2]	Visit	Age (months)	Diagnosis / Clinical Signs / Treatment	Serological Profile (Corresponding Figure)[3]
Chania	LH1	1	Graze	3	5	Pneumonia, Weak, Pyrexial, Bilateral rales, Oxytet.	Seroincrease: negative - strong positive (Fig 5.3C)
Kiambaa	UM3	12	Graze	2	2	Cough, Apyrexial, NoTx	Seroincrease: low - moderate positive (Fig 5.3D)
Kikuyu	UM3	9	Semi	2	10.5	Pyrexial, Elevated respiratory rate, No Tx.	Seroincrease: moderate - strong positive (Fig 5.3E)
Limuru	LH2	7	Zero	2	7.5	Pneumonia, Pyrexial, Elevated respiratory rate, Oxytet.	Seroincrease: moderate - strong positive (Fig 5.3F)

[1] - Agro-ecological Zones (AEZ) where "UM" refers to the upper midland zone, "LH" to the lower highland zone and "UH" to the upper highland zone.
[2] - Grazing management where "Zero" refers to a zero-grazing system and "Semi/Grazing" documents animals kept on pasture.
[3] - Corresponding serological profiles are presented in referenced figure

Table 5.13: Morbidity incidence rates of East Coast Fever in calves (i.e. ≤ 1 year of age) recorded in a longitudinal study conducted in Kiambu District from July 1991 - June 1992 and expressed as number of cases per 100 "calf" years of observation. Crude numbers from which rates are determined are presented beside the rate in square brackets and include mortalities according to corresponding classification criteria while approximate 95% confidence intervals are presented below each rate.

Classification of ECF Diagnosis	Agro-Ecological Zones[1]			Total Morbidity Rate
	UM (1,2,3)	LH (1,2,3)	UH (1,2)	
1) Suspected non-fatal ECF cases and deaths, whether confirmed or unconfirmed.	6.7 [2] (1.8-24.4)	9.2 [3] (3.1-27.0)	0 (0-19.4)	6.1 [5] (2.6-19.2)
Zero-grazing[2]	0 (0-17.4)	7.0 [1] (1.2-39.9)	-	2.8 [1] (0.5-15.6)
Semi/Grazing[2]	25.7 [2] (7.0-93.6)	10.8 [2] (3.0-39.5)	0 (0-19.4)	8.7 [4] (3.4-22.3)
2) Suspected non-fatal cases and deaths which were confirmed by serology or post-mortem.	0 (0-12.9)	6.1 [2] (1.7-22.3)	0 (0-19.4)	2.4 [2] (0.7-8.9)
Zero-grazing	0 (0-17.4)	7.0 [1] (1.2-39.9)	-	2.8 [1] (0.5-15.6)
Semi/Grazing	0 (0-49.3)	5.4 [1] (1.0-30.7)	0 (0-19.4)	2.2 [1] (0.4-12.3)
3) Suspected non-fatal cases and deaths which were confirmed by serology or post-mortem and missed cases as demonstrated by longitudinal serology.	6.7 [2] (1.8-24.4)	12.2 [4] (4.8-31.5)	0 (0-19.4)	7.3 [6] (3.3-15.9)
Zero-grazing	0 (0-17.4)	14.1 [2] (3.9-51.3)	-	5.5 [2] (1.5-20.1)
Semi/Grazing	25.7 [2] (7.0-93.6)	10.8 [2] (3.0-39.5)	0 (0-19.4)	8.7 [4] (3.4-22.3)
4) Suspected non-fatal ECF cases and deaths, whether confirmed or unconfirmed and missed cases as demonstrated by longitudinal serology.	13.4 [4] (5.2-34.5)	15.3 [5] (6.5-35.8)	0 (0-19.4)	10.9 [9] (5.8-20.8)
Zero-grazing	0 (0-17.4)	14.1 [2] (3.9-51.3)	-	5.5 [2] (1.5-20.1)
Semi/Grazing	51.3 [4] (19.9-132)	16.2 [3] (5.5-47.8)	0 (0-19.4)	15.2 [7] (7.4-31.4)

[1] - Agro-ecological Zones (AEZ) where "UM" refers to the upper midland zone, "LH" to the lower highland zone and "UH" to the upper highland zone.

[2] - Grazing management where "Zero" refers to a zero-grazing system and "Semi/Grazing" documents animals kept on pasture.

Table 5.14: Total morbidity incidence rates of East Coast Fever in cattle (irrespective of age) recorded in a longitudinal study conducted in Kiambu District from July 1991 - June 1992 and expressed as number of cases per 100 animal years of observation. Crude numbers from which rates are determined are presented beside the rate in square brackets and include mortalities according to corresponding classification criteria while approximate 95% confidence intervals are presented below each rate.

Classification of ECF Diagnosis	Agro-Ecological Zones[1]			Total Morbidity Rate
	UM (1,2,3)	LH (1,2,3)	UH (1,2)	
1) Suspected non-fatal ECF cases and deaths, whether confirmed or unconfirmed.	8.5 [11] (4.8-15.3)	6.2 [9] (3.2-11.7)	3.1 [3] (1.1-9.2)	6.2 [23] (4.1-9.3)
Zero-grazing[2]	2.3 [2] (0.6-8.5)	8.1 [6] (3.7-17.8)	-	5.0 [8] (2.5-9.4)
Semi/Grazing[2]	9.2 [4] (3.6-23.8)	6.9 [5] (3.0-16.2)	3.1 [3] (1.1-9.2)	7.1 [15] (4.3-11.7)
2) Suspected non-fatal cases and deaths which were confirmed by serology or post-mortem.	1.6 [2] (0.4-5.7)	4.1 [6] (1.9-9.0)	1.0 [1] (0.2-5.9)	2.4 [9] (1.3-4.6)
Zero-grazing	1.2 [1] (0.2-6.6)	5.4 [4] (2.1-14.0)	-	3.1 [5] (1.3-7.4)
Semi/Grazing	2.3 [1] (0.4-13.1)	2.8 [2] (0.8-10.1)	1.0 [1] (0.2-5.9)	1.9 [4] (0.7-4.9)
3) Suspected non-fatal cases and deaths which were confirmed by serology or post-mortem and missed cases as demonstrated by longitudinal serology.	4.7 [6] (2.1-10.2)	7.5 [11] (4.2-13.5)	5.2 [5] (2.2-12.3)	5.9 [22] (3.9-9.0)
Zero-grazing	1.2 [1] (0.2-6.6)	10.9 [8] (5.5-21.5)	-	5.7 [9] (3.0-10.7)
Semi/Grazing	11.6 [5] (4.9-27.0)	4.2 [3] (1.3-12.2)	5.2 [5] (2.2-12.3)	6.2 [13] (3.6-10.5)
4) Suspected non-fatal ECF cases and deaths, whether confirmed or unconfirmed and missed cases as demonstrated by longitudinal serology.	11.6 [15] (7.1-19.2)	9.6 [14] (5.7-16.1)	7.3 [7] (3.6-15.1)	9.7 [36] (7.0-13.5)
Zero-grazing	8.2 [7] (4.0-17.0)	10.8 [8] (5.5-21.5)	-	9.4 [15] (5.7-15.5)
Semi/Grazing	18.5 [8] (9.4-36.5)	8.3 [6] (3.8-18.1)	7.3 [7] (3.6-15.1)	10.0 [21] (6.5-15.2)

[1] - Agro-ecological Zones (AEZ) where "UM" refers to the upper midland zone, "LH" to the lower highland zone and "UH" to the upper highland zone.

[2] - Grazing management where "Zero" refers to a zero-grazing system and "Semi/Grazing" documents animals kept on pasture.

Table 5.15: Six farms on which more than one mortality and/or episode of clinical illness was attributed to East Coast Fever, as recorded in a longitudinal study conducted in Kiambu District from July 1991 - June 1992.

Dairy Co-operative Society	AEZ[1]	Farm	Grazing System[2]	Visit	Age Group	Category of ECF diagnosis
Chania	LH1	1	Graze	7*	Adult*	Unconfirmed Case
				10*	Adult*	Unconfirmed Case
				3	Calf	Misdiagnosed Case
Chania	LH1	3	Semi	8	Adult	Confirmed Case
				6	Calf	Confirmed Case
Kiambaa	UM3	12	Graze	4**	Adult**	Misdiagnosed Case
				9**	Adult**	Confirmed Case
				12	Adult	Unconfirmed Case
				4	Adult	Misdiagnosed Case
				2***	Calf***	Misdiagnosed Case
				8***	Calf***	Unconfirmed Case
				12	Calf	Unconfirmed Case
Kikuyu	LH3	8	Zero	6	Adult	Confirmed Death
				12	Adult	Confirmed Case
				5	Adult	Misdiagnosed Case
Kikuyu	UM3	13	Zero	12	Adult	Unconfirmed Death
				9	Adult	Unconfirmed Case
				5	Adult	Misdiagnosed Case
Limuru	UH2	12	Graze	4****	Adult****	Confirmed Case
				10****	Adult****	Misdiagnosed Case
				5	Adult	Misdiagnosed Case

[1] - Agro-ecological Zones (AEZ) where "UM" refers to the upper midland zone, "LH" to the lower highland zone and "UH" to the upper highland zone.
[2] - Grazing management where "Zero" refers to a zero-grazing system and "Semi/Grazing" documents animals kept on pasture.
*to **** - Denotes multiple events recorded in a single animal.

Table 5.16: Incidence rates of seroconversions[1] and sero-events in calves (i.e. ≤ 1 year of age) expressed as number of events per 100 "calf" years of observation[1] as observed in a longitudinal study conducted in Kiambu District from July 1991 - June 1992. Crude numbers of events from which rates are determined are presented beside the rate in square brackets as number of events without clinical signs + number of events with concomitant clinical signs, either diagnosed as ECF or undiagnosed while approximate 95% confidence intervals are presented below each rate.

Criteria of Definition of Sero-Event	Agro-Ecological Zones[2]			Total Incidence Rate
	UM (1,2,3)	LH (1,2,3)	UH (1,2)	
Seroconversion	46.9 [13+1] (28.0-78.8)	61.2 [17[1]+3] (39.6-94.6)	35.4 [7[1]] (17.1-73.0)	49.8 [37[1]+4] (36.7-67.6)
Zero-grazing[3]	36.3 [8] (18.4-71.7)	49.3 [5[1]+2] (23.9-102)	-	41.4 [13[1]+2] (25.1-68.3)
Semi/Grazing[3]	77.0 [5+1] (35.3-168)	70.4 [12[1]+1] (41.2-121)	35.4 [7[1]] (17.1-73.0)	56.5 [24[1]+2] (38.5-82.7)
Seroconversion + Sero-Events[4]	50.3 [13+2] (30.5-83.0)	67.3 [17[1]+5] (44.5-102)	35.4 [7[1]] (17.1-73.0)	53.5 [37[1]+7] (39.8-71.8)
Zero-grazing	36.3 [8] (18.4-71.7)	56.3 [5[1]+3] (28.5-111)	-	44.1 [13[1]+3] (27.2-71.7)
Semi/Grazing	89.8 [5+2] (43.6-185)	75.8 [12[1]+2] (45.1-127)	35.4 [7[1]] (17.1-73.0)	60.8 [24[1]+4] (42.1-87.9)

[1] - Four subclinical seroconversions were recorded in animals >1 year of age which had initially been observed as calves. Two of these were on Semi/Grazing farms in the LH zone (at 17 and 20 months of age), one on a Zero-grazing farm in LH zone (at 13 months of age) and one in the UH zone (at 14 months of age). Denominators for incidence rate calculations incorporate the additional periods of observation accordingly.

[2] - Agro-ecological Zones (AEZ) where "UM" refers to the upper midland zone, "LH" to the lower highland zone and "UH" to the upper highland zone.

[3] - Grazing management where "Zero" refers to a zero-grazing system and "Semi/Grazing" documents animals kept on pasture.

[4] - All sero-events in calves not defined as seroconversions corresponded to serological increases >0.200 Standardised OD Units

Table 5.17: Age in years at time of seroconversion for at-risk animals observed to have seroconverted in a longitudinal study conducted in Kiambu District from July 1991 - June 1992. Seroconversions include those events without clinical signs and those events with concomitant clinical signs, either diagnosed as ECF or undiagnosed.

Age at Sero-conversion (years)	Agro-Ecological Zones[1]							Summary		
	UM (1,2,3)			LH (1,2,3)			UH (1,2)			
	Zero[2]	Graze[2]	Total	Zero	Graze	Total	Graze/ Total	Zero	Graze	Total
Mean	0.39	0.38	0.38	0.55	0.61	0.59	0.56	0.47	0.54	0.51
Standard Error	0.071	0.051	0.044	0.11	0.13	0.09	0.13	0.065	0.074	0.053
n	8	6	14	7	13	20	7	15	26	41
Min - Max	0.13-0.83	0.17-0.54	0.13-0.83	0.21-1.08	0.23-1.67	0.21-1.67	0.08-1.17	0.13-1.08	0.080-1.67	0.08-1.67
Median	0.35	0.41	0.39	0.58	0.4	0.42	0.46	0.4	0.42	0.4

[1] - Agro-ecological Zones (AEZ) where "UM" refers to the upper midland zone, "LH" to the lower highland zone and "UH" to the upper highland zone.
[2] - Grazing management where "Zero" refers to a zero-grazing system and "Graze" documents animals kept on pasture.

Table 5.18: Incidence rates of sero-events in "adult" (i.e. > 1 year of age) cattle expressed as number of events per 100 "adult" years of observation as observed in a longitudinal study conducted in Kiambu District from July 1991 - June 1992. Crude numbers of events from which rates are determined are presented beside the rate in square brackets as number of events without clinical signs + number of events with concomitant clinical signs, either diagnosed as ECF or undiagnosed while approximate 95% confidence intervals are presented below each rate.

Criteria of Definition of Sero-Event	Agroecological Zones[1]			Total Incidence Rate
	UM (1,2,3)	LH (1,2,3)	UH (1,2)	
Serological Increase >0.150 OD Units	24.3 [20+4] (16.3-36.1)	24.1 [23+4] (16.4-34.7)	35.6 [22+5] (24.5-51.9)	27.1 [65+13] (21.7-33.8)
Zero-grazing[2]	11.8 [6+1] (5.3-22.8)	28.6 [14+3] (17.8-45.8)	-	19.5 [20+4] (13.1-29.0)
Semi/Grazing[2]	47.9 [14+3] (29.9-76.7)	18.6 [9+1] (10.1-34.2)	35.6 [22+5] (24.5-51.9)	32.7 [45+9] (25.1-42.7)
Serological Increase >0.165 OD Units	21.2 [17+4] (13.9-32.4)	19.6 [18+4] (12.8-29.4)	33.0 [20+5] (22.4-48.7)	23.6 [55+13] (18.6-29.9)
Zero-grazing	10.1 [5+1] (4.3-20.6)	21.8 [10+3] (12.8-37.4)	-	15.5 [15+4] (9.9-24.1)
Semi/Grazing	42.3 [12+3] (25.6-69.7)	16.7 [8+1] (8.8-31.8)	33.0 [20+5] (22.4-48.7)	29.7 [40+9] (22.5-39.2)
Serological Increase >0.180 OD Units	18.2 [14+4] (11.5-28.8)	17.8 [17+3] (11.4-27.3)	26.4 [15+5] (17.1-40.8)	20.1 [46+12] (15.6-26.0)
Zero-grazing	8.4 [4+1] (3.4-18.4)	18.5 [9+2] (10.3-33.1)	-	13.0 [13+3] (8.0-21.2)
Semi/Grazing	36.6 [10+3] (21.4-62.7)	16.7 [8+1] (8.8-31.8)	26.4 [15+5] (17.1-40.8)	25.4 [33+9] (18.8-34.4)
Serological Increase >0.200 OD Units	16.2 [12+4] (10.0-26.3)	16.1 [15+3] (10.0-25.1)	25.1 [14+5] (16.1-39.2)	18.4 [41+12] (14.1-24.1)
Zero-grazing	6.7 [3+1] (2.5-16.2)	18.5 [9+2] (10.3-33.1)	-	12.2 [12+3] (7.4-20.1)
Semi/Grazing	33.8 [9+3] (19.3-59.1)	13.0 [6+1] (6.3-26.8)	25.1 [14+5] (16.1-39.2)	23.0 [29+9] (16.8-31.6)

[1] - Agro-ecological Zones (AEZ) where "UM" refers to the upper midland zone, "LH" to the lower highland zone and "UH" to the upper highland zone.

[2] - Grazing management where "Zero" refers to a zero-grazing system and "Semi/Grazing" documents animals kept on pasture.

Table 5.19: Total incidence rates of seroconversion and sero-events in all animals (i.e. irrespective of age) expressed as number of events per 100 animal years of observation as observed in a longitudinal study conducted in Kiambu District from July 1991 - June 1992. Crude numbers of events from which rates are determined are presented beside the rate in square brackets as number of events without clinical signs + number of events with concomitant clinical signs, either diagnosed as ECF or undiagnosed while approximate 95% confidence intervals are presented below each rate.

Criteria of Definition of Sero-Event	Agro-Ecological Zones[1]			Total Incidence Rate
	UM (1,2,3)	LH (1,2,3)	UH (1,2)	
Seroconversions + Serological Increases >0.150 OD Units	30.3 [33+6] (22.2-41.4)	33.6 [40+9] (25.4-44.4)	35.6 [29+5] (25.5-49.7)	32.9 [102+20] (27.6-39.3)
Zero-grazing[2]	17.5 [14+1] (10.6-28.9)	33.9 [19+6] (23.0-50.1)	-	25.1 [33+7] (18.5-34.2)
Semi/Grazing[2]	55.4 [19+5] (37.3-82.5)	33.2 [21+3] (22.3-49.4)	35.6 [29+5] (25.5-49.7)	38.8 [69+13] (31.3-48.2)
Seroconversions + Serological Increases >0.165 OD Units	28.0 [30+6] (20.2-38.7)	30.1 [35+9] (22.5-40.5)	33.5 [27+5] (23.7-47.3)	30.2 [92+20] (25.1-36.4)
Zero-grazing	16.4 [13+1] (9.8-27.5)	28.5 [15+6] (18.6-43.6)	-	22.0 [28+7] (15.8-30.6)
Semi/Grazing	50.8 [17+5] (33.6-76.9)	31.8 [20+3] (21.2-47.7)	33.5 [27+5] (23.7-47.3)	36.5 [64+13] (29.2-45.6)
Seroconversions + Serological Increases >0.180 OD Units	25.6 [27+6] (18.2-36.0)	28.8 [34+8] (21.3-38.9)	28.3 [22+5] (19.4-41.1)	27.5 [83+19] (22.7-33.4)
Zero-grazing	15.2 [12+1] (8.9-26.0)	25.8 [14+5] (16.5-40.3)	-	20.1 [26+6] (14.2-28.4)
Semi/Grazing	46.2 [15+5] (29.9-71.4)	31.8 [20+3] (21.2-47.7)	28.3 [22+5] (19.4-41.1)	33.2 [57+13] (26.2-41.9)
Seroconversions + Serological Increases >0.200 OD Units	24.1 [25+6] (17.0-34.2)	25.3 [32+5] (18.4-34.9)	27.2 [21+5] (18.6-39.9)	26.2 [78+19] (21.5-31.9)
Zero-grazing	14.0 [11+1] (8.0-24.5)	25.8 [14+5] (16.5-40.3)	-	19.5 [25+6] (13.7-27.6)
Semi/Grazing	43.9 [14+5] (28.1-68.6)	29.1 [18+3] (19.0-44.4)	27.2 [21+5] (18.6-39.9)	31.3 [53+13] (24.6-39.8)

[1] - Agro-ecological Zones (AEZ) where "UM" refers to the upper midland zone, "LH" to the lower highland zone and "UH" to the upper highland zone.

[2] - Grazing management where "Zero" refers to a zero-grazing system and "Semi/Grazing" documents animals kept on pasture.

Figure 5.1: Serological profiles of "adult" (i.e. > 1 year of age) cattle reported to have suffered a non-fatal episode of East Cost Fever during a longitudinal study conducted in Kiambu District from July 1991 - June 1992 and where clinical diagnosis (see Table 5.8) is supported by serological pattern. Solid line denotes test cut-off.

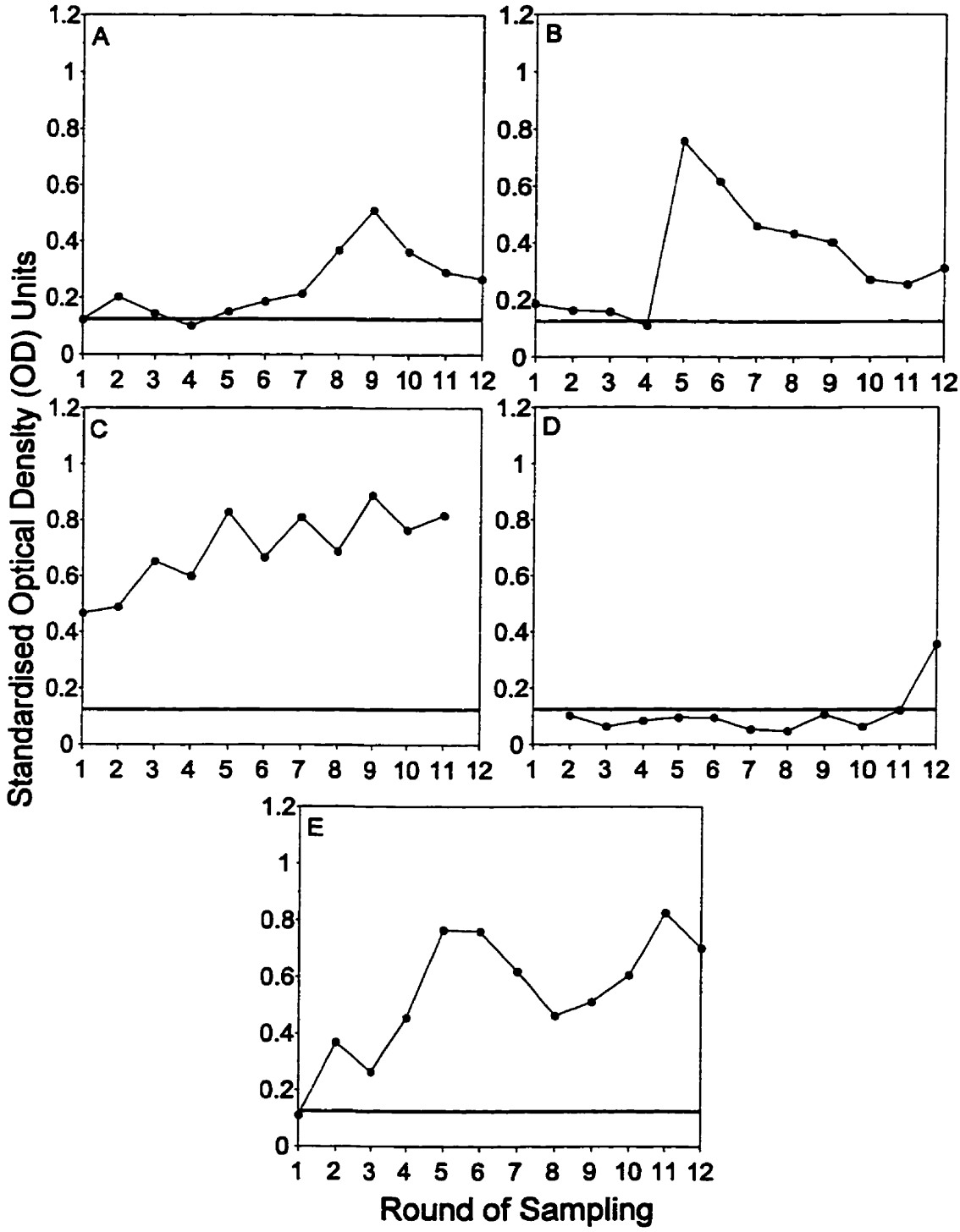


Figure 5.2: Serological profiles of "adult" (i.e. > 1 year of age) cattle which experienced non-specific and/or mild clinical illness during a longitudinal study conducted in Kiambu District from July 1991 - June 1992 (see Table 5.9) where illness was subsequently correlated with serological evidence of *Theileria parva* infection; suggestive of undiagnosed ECF. Solid line denotes test cut off.

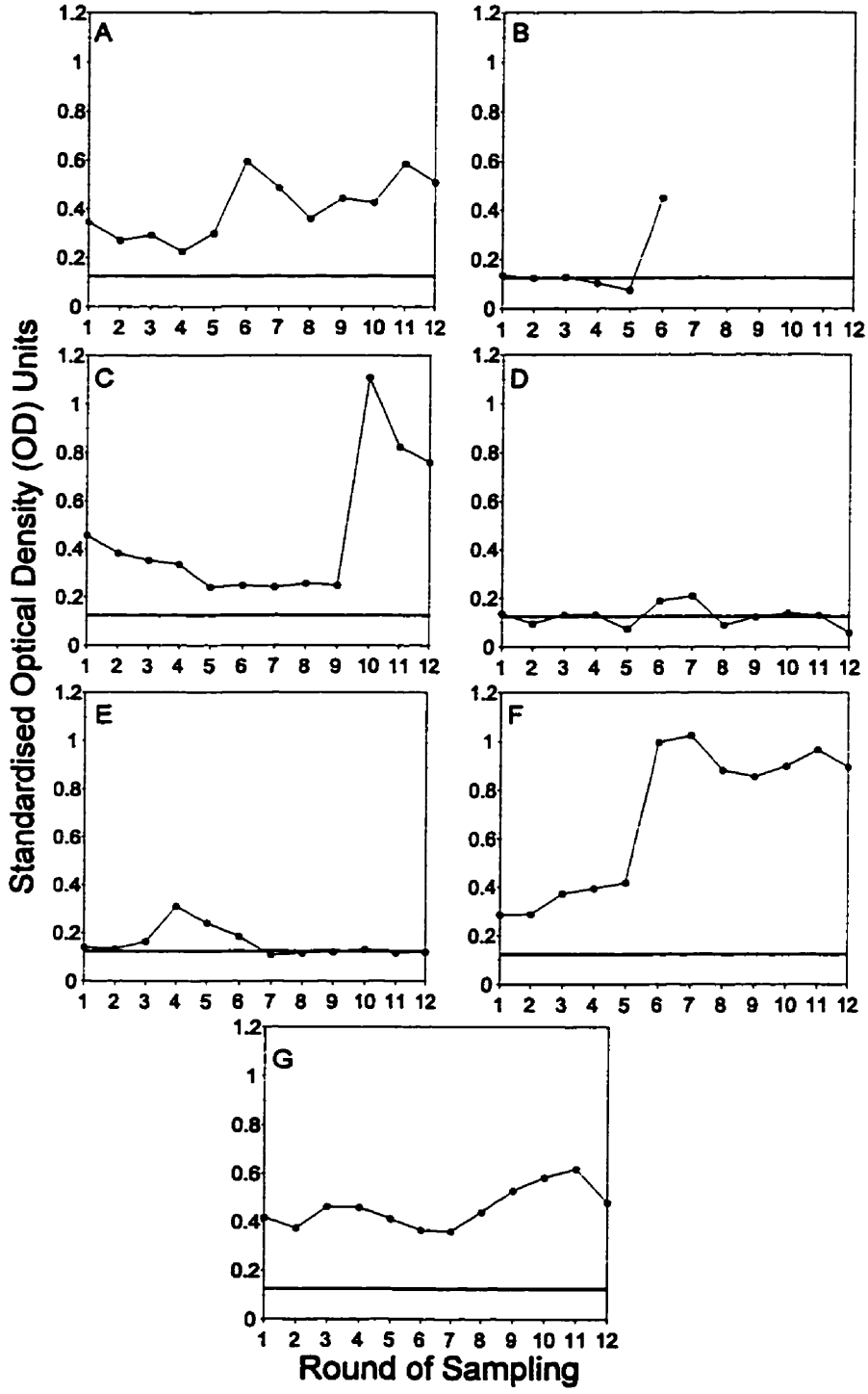


Figure 5.3: Serological profiles of calves (i.e. ≤ 1 year of age) reported to have suffered either a non-fatal episode of East Cost Fever (A,B - see Table 5.11) or experienced non-specific and/or mild clinical illness (C,D,E,F - see Table 5.12) during a longitudinal study conducted in Kiambu District from July 1991 - June 1992 where presumptive diagnosis of ECF/occurrence of illness was subsequently correlated with serological evidence of *Theileria parva* infection. Solid line denotes test cut-off.

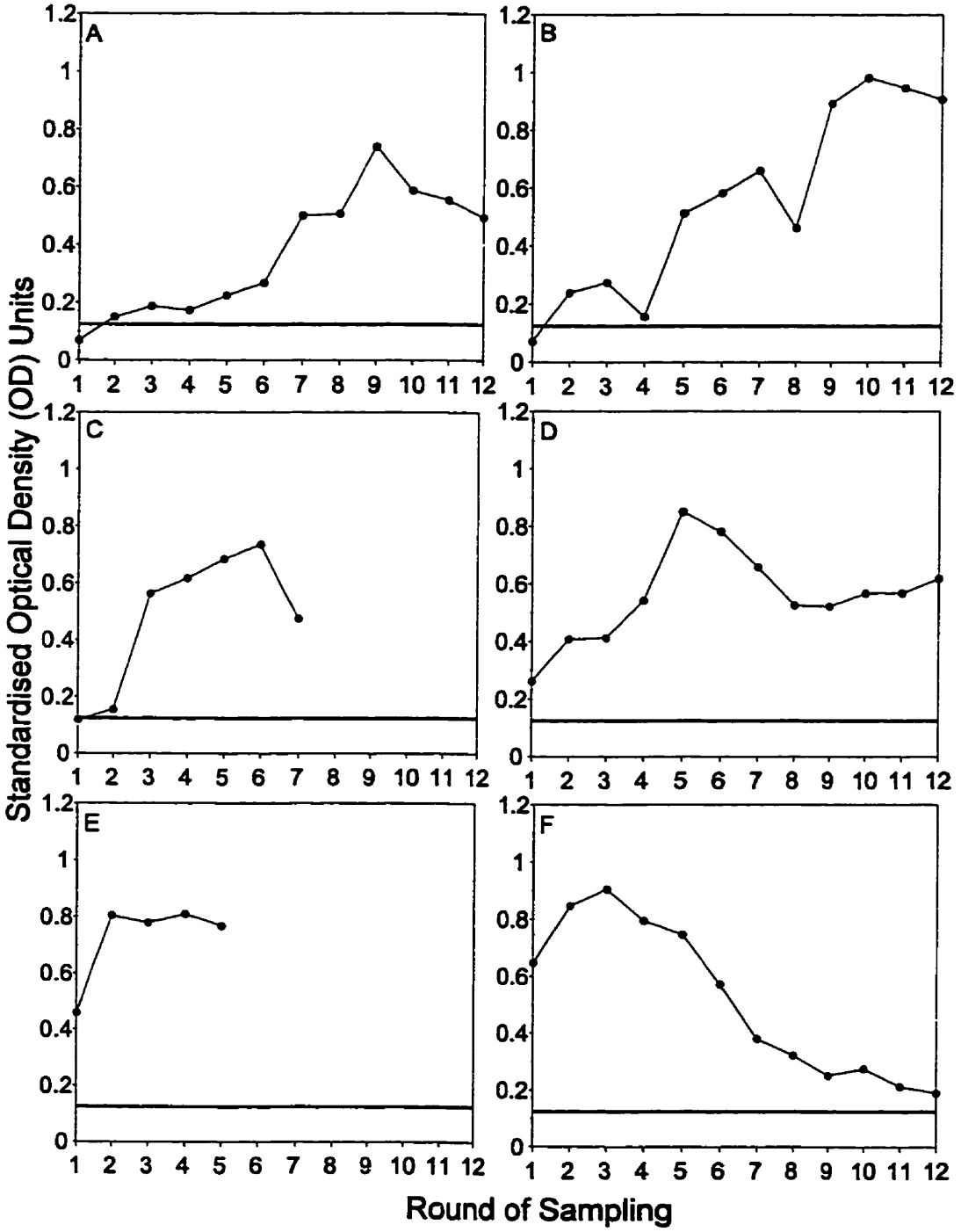


Figure 5.4: Primary and secondary antibody responses to antigen challenge (after Figure 1.3 Male, Champion, Cooke & Owen, 1991). Primary antigen exposure is followed by a lag period, after which IgM appears first followed by IgG. Secondary challenge results in a more rapid and enhanced response, dominated by the greater production of higher affinity IgG. In both cases, antibody response declines after reaching a peak.

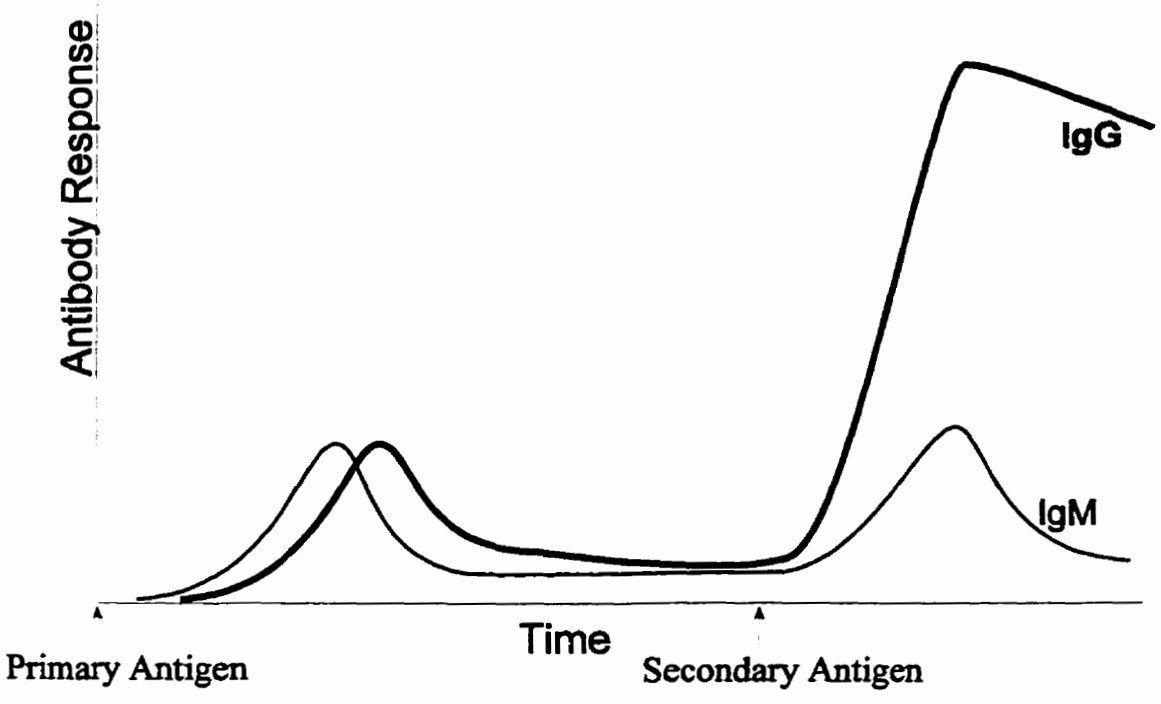


Figure 5.5: Examples of serological profiles of calves observed during a longitudinal study conducted in Kiambu District from July 1991 - June 1992 (where occasion of first post-partum sampling is denoted by * or age at first sampling is provided) which demonstrate evidence of seroconversion; suggestive of sub-clinical *Theileria parva* infection, either in the absence of maternally acquired antibodies or post-decay. Solid line denotes test cut-off.

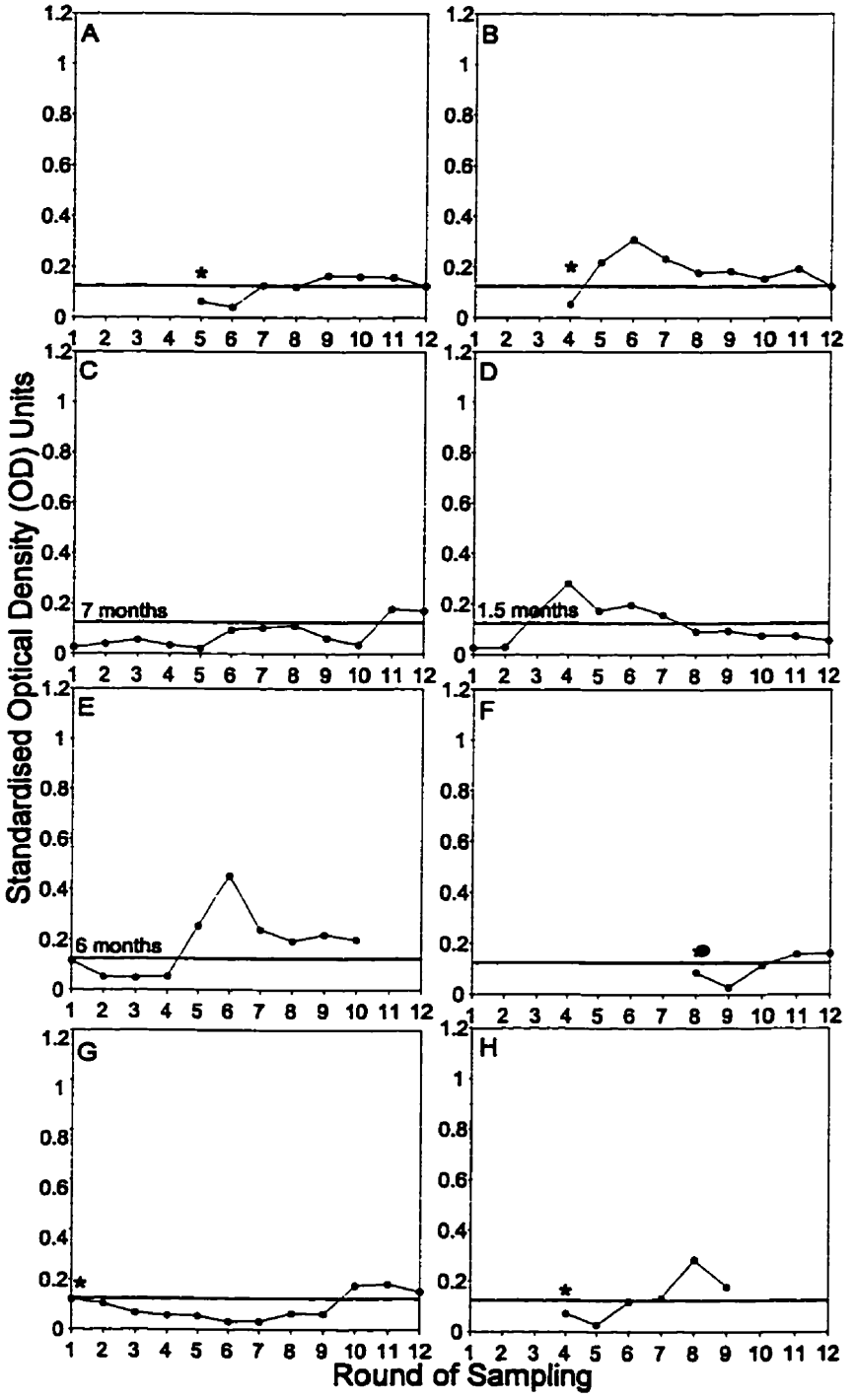


Figure 5.6: Examples of serological profiles of calves born during a longitudinal study conducted in Kiambu District from July 1991 - June 1992 (where occasion of first post-partum sampling is denoted by *) which demonstrate evidence both of decay of passively acquired maternal antibodies and subsequent seroconversion; suggestive of sub-clinical *Theileria parva* infection. Solid line denotes test cut-off.

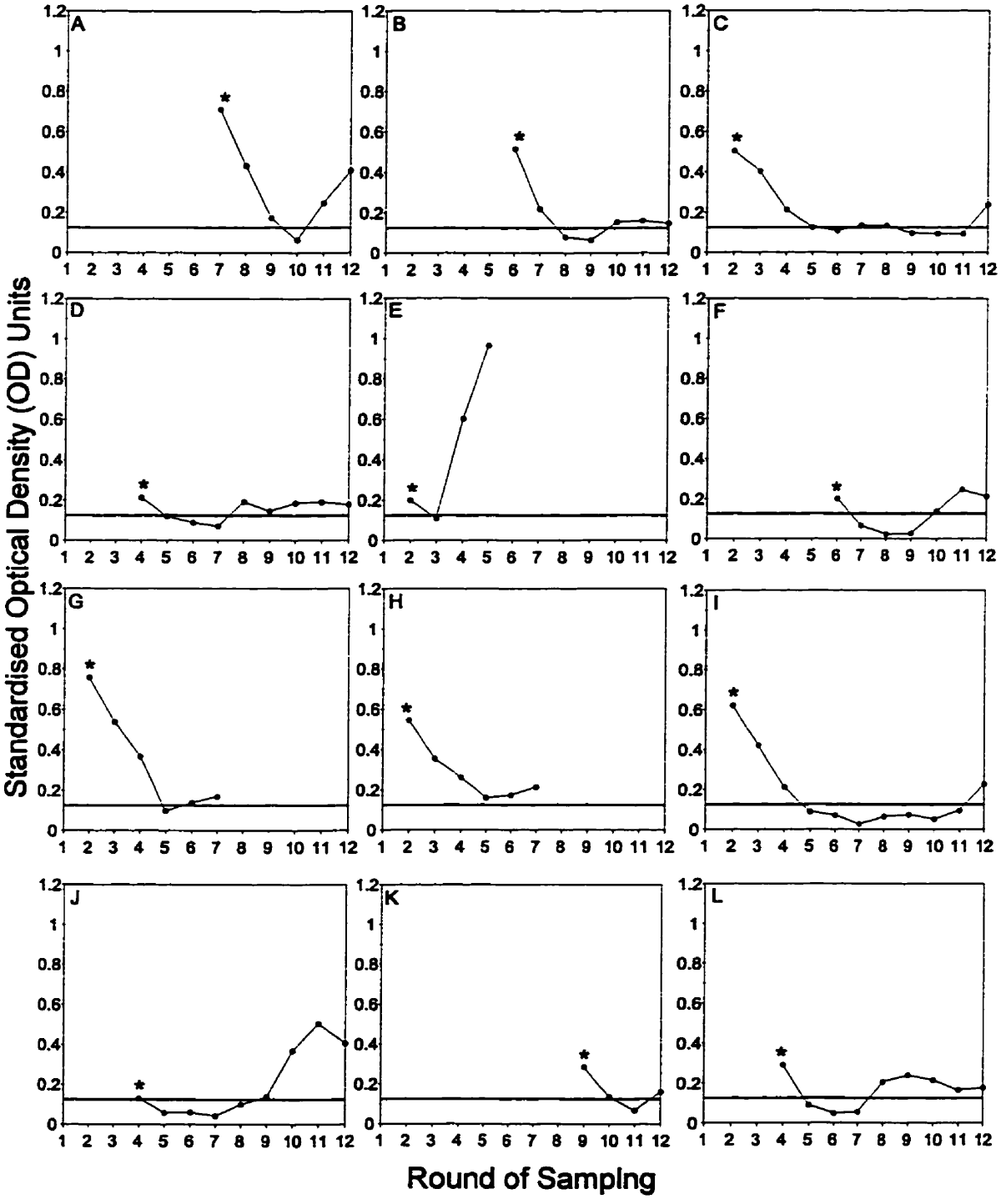


Figure 5.7: Examples of serological profiles of cattle observed during a longitudinal study conducted in Kiambu District from July 1991 - June 1992 (where age at first sampling is provided) which demonstrate patterns suggestive of anamnestic responses to sub-clinical *Theileria parva* infection; defined to be "sero-events". Solid line denotes test cut-off.

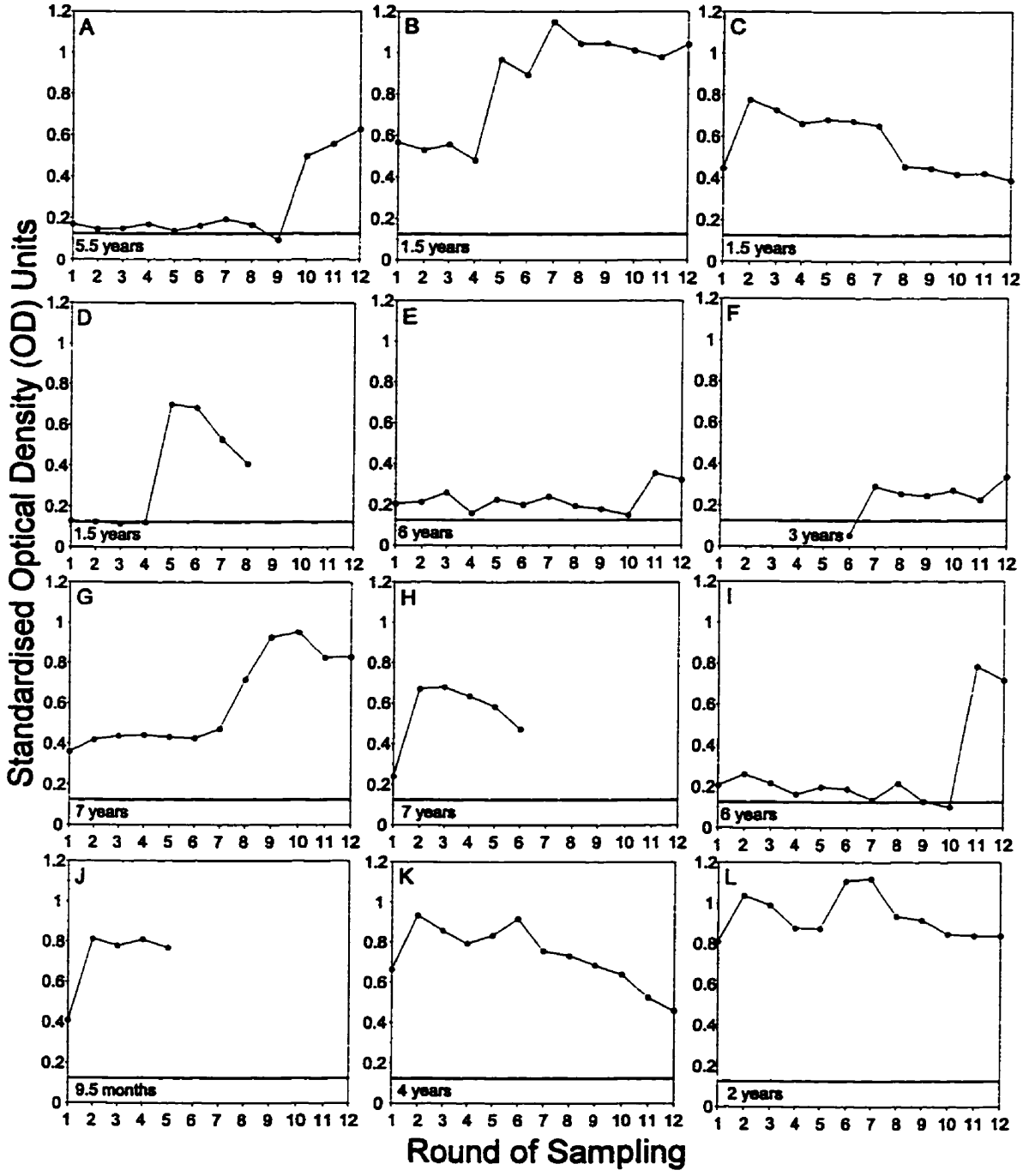
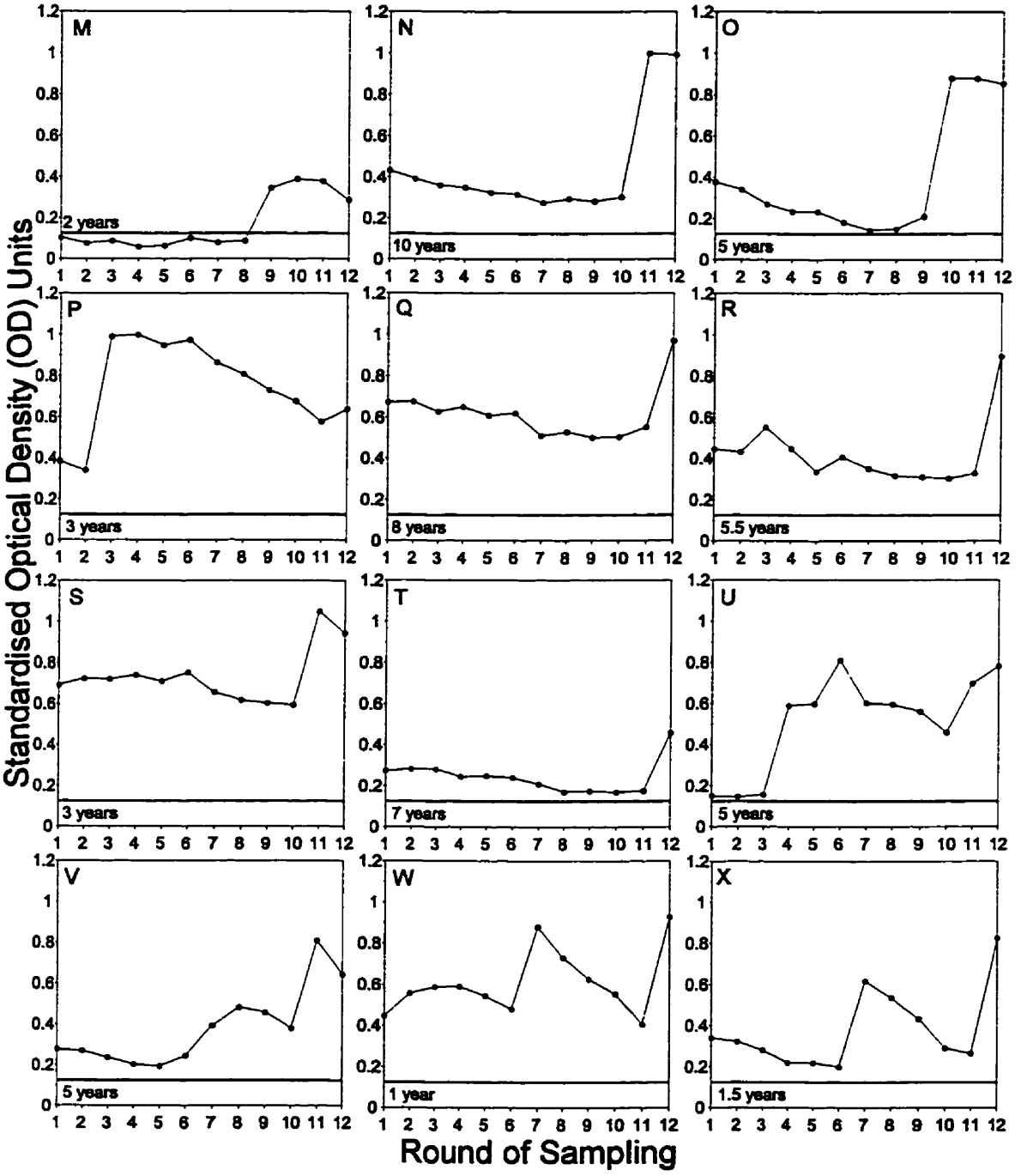


Figure 5.7: (continued) Further examples of serological profiles of cattle observed during a longitudinal study conducted in Kiambu District from July 1991 - June 1992 (where age at first sampling is provided) which demonstrate patterns suggestive of anamnestic responses to sub-clinical *Theileria parva* infection; defined to be "sero-events". Solid line denotes test cut-off.



CHAPTER 6

TICK CONTROL METHODS AND FREQUENCY OF ACARICIDE APPLICATION

6.1 Introduction

Vector control has been the predominant method of controlling the diseases caused by *T. parva* infection since the finding in 1903 that the organism was naturally transmitted transtadially by the ixodid tick *R. appendiculatus* (Lounsbury, 1904).

In Kenya (then the East African Protectorate), following the first outbreak of ECF in the fertile "White Highlands" (which included Kiambu District), the Kenya Veterinary Department sequentially adopted measures first introduced in South Africa for the purpose of eradicating ECF (Diesel, 1948). Between 1904 to 1911 eradication efforts focussed on quarantine of infected premises and restriction of cattle movements (Stordy, 1908), however, they were largely ineffective (Brandt, 1913; Walker, 1974). In 1912 settlers borrowed an additional tactic from South Africa and began plunge dipping cattle in acaricide solution to rid them of ticks.

The history of dipping in Kenya has been reviewed by Ngulo (1975) and Keating (1983). Despite a delay in construction caused by the First World War, by 1920, 206 plunge dip tanks were in operation in the country (Anon., 1921) and a Cattle Cleansing Ordinance was announced. However, progress in implementation was slow since the government was not able to advance adequate funds and the cost of dip tank construction was prohibitive to small farmers. It was not until the Ordinance was officially adopted in 1937 as the Cattle

Cleansing Act that it became an offense for any settler to fail to dip cattle regularly in the correct concentration of acaricide solution. In 1944, dipping was extended from the "White Highland" district to bordering African Reserves. However, despite costs being split equally between the government and local councils, it was not until after independence in 1963 that a massive programme of dip construction began on a large scale (Ngulo, 1975), such that, by 1980 over 4923 dip tanks were in operation (Keating, 1983). Although problems of tick resistance to acaricide solutions were noted through the 1950's and 60's (Kane, 1976), by 1975 the true extent of the problem became apparent after several country-wide surveys revealed that *R. appendiculatus* was resistant to several acaricides then in use (Crampton and Gichanga, 1979; Chema, 1984). In 1976 the Veterinary Department assumed control over which products could be used (Kenya, 1976) and also took over control of dipping management in 20 districts (including Kiambu), thereafter designated as "Project Tick Control Districts" (Kenya, 1977), with the aim of saving the Kenyan dairy industry from ECF (Chema, 1981). Within these districts, the Department subsidized dipping services by maintaining dip tanks, providing acaricide and employing dip tank attendants. Dip tank attendants were held legally responsible for ensuring regular and adequate dipping of all cattle in correct concentrations of acaricide solutions, as determined by monthly samples submitted for testing (Kenya, 1986; Kyule, 1989). By 1986 there were 14 veterinarians and 15 livestock officers supervising 1268 attendants managing over 4000 dip tanks in the Project Districts (Kenya, 1986). In addition, for each dip tank, a committee composed of elected representatives from local stock-owners was formed to liaise with the Veterinary Department. However, a combination of factors including increasing acaricide resistance,

rapidly increasing costs of new acaricides purchased using foreign currency reserves, the high cost of dip tank construction and maintenance, the need for a year-round sustainable supply of large volumes of water, the large amount of acaricide necessary for the initial charging of a tank and the requirement for an adequate number of cattle to use a tank to make its operation economically viable, conspired to erode the government's ability to finance and maintain its policy of subsidised tick control. In consequence, farmers were forced to assume a progressively greater degree of responsibility for other tick control on their animals. In turn, this has manifest itself as a shift towards methods of tick control, such as the use of hand-held or back-pack pressure sprayers in the application of acaricide, which, although less economical on the large scale and potentially less efficacious than plunge dipping (Norval *et al.*, 1992), are more sustainable in the context of small-scale dairy production.

The objective of this chapter is twofold. First, to assess what tick control methods were in use by small scale dairy farmers of Kiambu district and to explore the demographic patterns of method and frequency of acaricide application, with particular respect to ecoclimatic suitability for ticks (as assessed by agro-ecological zone) and level of potential tick exposure of cattle (via contrasting grazing management systems). Second, to investigate whether the incidence of ECF or the incidence of *T. parva*-specific seroconversions and sero-events differed with pattern of tick control practised.

6.2 Reported Tick Control Methods and Frequency of Application

At the time of the initial farm visit, a comprehensive farm-survey questionnaire was

administered to the farm owner/manager by a study veterinarian conversant in Kikuyu (the local language). With regard to tick control and related management practices, 16 open and closed formed questions sought to establish whether or not tick control was practised on the farm and if so, the method and frequency of acaricide application, any seasonal or age-related differences in these practices and the age of calves at first application (see Questions 24 - 39 of Initial Farm Survey, Appendix 1.1). Summary descriptive statistics generated from responses to these questions are presented in Table 3.5 (see also section *3.3.2.2 Distribution of Farm Factors*). In addition, a more detailed statistical analysis of i) the distribution of responses relative to dairy co-operative society membership, agro-ecological zone of origin and grazing management system practised and ii) the co-distribution of responses was undertaken. Owing to the relatively strong collinearites between the demographic covariates (for example see Appendix 2), all comparisons were conducted on a univariate basis. However, for categorical variables which contrasted types of tick control procedures but which included a "no tick control" level, two separate assessments were made, one including and the second excluding the "no tick control" observations. Thus, where dual p-values appear in the text separated by a slash (e.g. p=0.14/0.76), the second value reports the level of significance observed for the same pair of covariates, but where observations for the 7 farms which reportedly did not practice tick control have been excluded. Statistical analyses were conducted using SAS statistical software (SAS® System for Windows™, Version 6.12, SAS Institute Inc., Cary, North Carolina, USA). Significance of observed associations between categorical responses was assessed by a two-tailed Fisher's Exact Test using the algorithm of Mehta and Patel (1983). Covariates measured on a continuous scale were

transformed by natural log transformation to more closely approximate normal distributions, where necessary, with an overall test of association based on an unbalanced one-way analysis of variance. Multiple comparison of means was undertaken by the Tukey-Kramer studentised range test, which minimises the maximum experimentwise error rate and produces results that, while still conservative, are usually closer to the intended significance level of α=0.05 than other methods (Dunnett, 1980; Sokal and Rohlf, 1981).

6.2.1 Distribution and Demographic Associations of Responses

The reported method of acaricide application was associated with both dairy co-operative society membership ($p<0.001/<0.001$) and agro-ecological zone of origin ($p<0.001/<0.001$), but not with grazing management system ($p=0.14/0.76$). Details of distribution of reported method of application by demographic covariates are presented in Table 6.1. Approximately two-thirds of farms in the upper highland (UH) and lower highland (LH) zones (66.7% = 14/21 and 63.2% = 24/38, respectively) reported using the method of back-pack spraying of acaricide *versus* plunge dipping of cattle, which was reportedly practised by the remaining 33.3% (7/21) of farms in the UH zone and by 23.7% (9/38) of farms in the LH zone. This pattern was completely reversed in the upper midland (UM) zone where 67.7% (21/31) of farms reported plunge dipping, with 19.4% (6/31) indicating they used back-pack spraying. Interestingly, of the 83 farms which reported practising some tick control, the proportions of plunge dipping *versus* spraying were identical on zero-grazing farms (48.6% = 18/37) and not completely dissimilar on semi-/full-pasture grazing farms at 39.6% (19/46) and 56.5% (26/46), respectively. However, of the

7 farms which reported no tick control, 6 also practised a zero-grazing system.

In contrast to the reported method of application, the reported frequency of application of acaricide was associated with grazing management system (p=0.011/0.032) but not with either dairy co-operative society membership (p<0.17/<0.18) or agro-ecological zone of origin (p=0.070/0.071). Although within both zero-grazing and semi-/full-pasture grazing systems the overall trend for a higher proportion of farms reporting weekly (53.5% = 23/43 and 70.2% = 33/47, respectively) *versus* fortnightly (16.3% = 7/43 and 25.5% = 12/47, respectively) acaricide application was preserved, there was an apparent shift toward less intensive control frequencies on zero-grazing farms where 16.3% (7/43) reported monthly or less frequent application compared to only 2.1% (1/47) of semi-/full-pasture grazing farms. A similar, but non-significant pattern of shift toward lower frequency of application was observed for farms in the LH zone. Full details of distribution of reported frequency of acaricide application are reported in Table 6.2.

There was a strong collinearity between the reported method and reported frequency of acaricide application (p<0.001) for the 83 farms which controlled ticks; documented in Table 6.3. Virtually all of the farms (94.6% = 35/37) which reported plunge dipping also indicated that they did so on a weekly basis. In contrast, there was a greater distribution in reported frequency of application for those farms which reported using the back-pack spraying method, with 45.5% (20/44) reporting weekly, 40.9% (18/44) reporting fortnightly and 13.6% (6/44) reporting monthly or less frequent applications. In consequence, comparisons by reported frequency of application are likely only to be valid within the spraying method, and similarly, contrasts between stated method of application are

effectively made within weekly reported frequency.

Few farms (5/90 = 5.6%) reported applying acaricide to mature animals only, with no significant differences by dairy co-operative society membership (p=0.241/0.321) or agro-ecological zone of origin (p=0.263/0.363). However, although 4 of these 5 farms also practised zero-grazing, when farms which reported no tick control were excluded from consideration, the association between age group treated for ticks and grazing management system lost statistical significance, hence p=0.025/0.17 (Table 6.4).

A total of 78 farms reported applying acaricide to "calves". Table 6.5 presents the mean, standard error, median, minimum and maximum for age at first acaricide application and assesses associations by dairy co-operative society membership (p=0.16), agro-ecological zone of origin (p=0.005) and grazing management system (p=0.34). Although the range of reported ages (23 months; minimum = 1 to maximum = 24) is relatively large, it is of interest to note that the mean age very closely approximates the median age in virtually all categories, suggesting a symmetrical distribution. The only significant (α = 0.05) difference in mean age at first acaricide application was noted as a higher mean age (6.4 months) on farms in the UM zone *versus* the UH (4.0 months) and LH (4.2 months) zones. However, Table 6.6 also demonstrates an overall significant association between age at first acaricide application and stated method of tick control (p<0.001), predominantly due to farms reporting plunge dipping having a higher (α = 0.05) mean age (6.1 months) than those utilising back-pack spraying (3.9 months), such that the higher age in the UM zone may be a consequence of the higher proportion of farms in this zone undertaking plunge dipping of livestock (Table 6.1).

Other factors thought to possibly be related to the reported method and frequency of application of acaricide included farm size and number of dairy animals present. Tables 6.7 and 6.8 present the distribution of each by method and frequency of application and age group subjected to tick control. Neither covariate was statistically significantly associated with any reported tick control practice.

6.3 Observed Tick Control Methods and Frequency of Application

Although the method and frequency of acaricide application was reported by farmers at the time of the initial farm visit, given the potential importance of these factors in the epidemiology of tick-borne diseases, it was deemed essential to provide a method of validating the stated intensity of control efforts. Consequently, a system of monitoring was implemented at the animal level in conjunction with longitudinal sampling. Specifically, two questions regarding the monthly history of tick control as applied to an individual were incorporated into the follow-up adult and follow-up calf questionnaires asked at each monthly farm visit (see Appendix 1.4, Appendix 1.5). However, the format of the initial question, which concerned frequency of application, was altered to elicit information on the time between successive acaricide applications, the inter-application interval. This was done in an attempt to prevent farmers simply reasserting potentially erroneous statements regarding the number or frequency of acaricide applications. The two closed formed questions appeared as follows:

```
When was this animal last treated for ticks?
      0) do not treat for ticks
      1) < 3 days
      2) 3 - 7 days
      3) 8 days - 2 weeks
      4) 2 - 3 weeks
      5) 3 weeks - 1 month
      6) > 1 month

If this animal was treated for ticks, was it dipped or sprayed?
      1) sprayed
      2) dipped
```

The results of the first question were compared to the stated intensity of tick control using the following interpolation. For animals reportedly subject to weekly acaricide treatment, the maximum inter-application interval could be 7 days, larger intervals would be inconsistent with a weekly reported frequency. Similarly, the maximum inter-application interval under a fortnightly treatment regimen would be 2 weeks. For all other intervals, the maximum frequency of tick control would be once per month.

6.3.1 Tick Control Inter-Application Intervals

Table 6.9 presents the distribution of 4433 inter-application intervals recorded during the monthly farm visits cross-classified by the frequency of application of tick control reported at the initial farm visit. Of 2507 observations made on animals reportedly subject to weekly application of acaricide, 52.2% (1310/2507) recorded inter-application intervals consistent with the stated frequency of tick control (i.e. "< 3 days" or "3 - 7 days"), 9.7% (242/2507) were consistent with a maximum frequency of fortnightly treatment ("8 days - 2 weeks"), 14.5% with monthly or less frequent treatment ("2 - 3 weeks", "3 weeks - 1 month", "> 1 month") and 23.6% (591/2507) had not been treated. Similarly, for the 1152

observations made on animals reported to be under a system of fortnightly acaricide application, 63.8% (735/1152) recorded inter-application intervals consistent with the stated frequency of tick control ("< 3 days" , "3 - 7 days", "8 days - 2 weeks"), 18.6% (214/1152) were consistent with monthly or less frequent treatment ("2 - 3 weeks", "3 weeks - 1 month", "> 1 month") and 17.6% (203/1152) had not been treated.

Tables 6.10 and 6.11 present the same information as above but where observations have been divided into 1033 observations of calves (≤ 1 year of age) and 3400 observations of adults (> 1 year of age), respectively, demonstrating that 48.7% (636/1307) of observations of "not treated" or treated "> 1 month" previously, were recorded in calves. On farms reporting weekly acaricide application, however, a percentage of 60.9% (371/609) of observations recording "not yet treated" is not necessarily inconsistent, given a mean age at first treatment of 5.2 months (Table 6.6) and a population biased toward newborn/younger animals by a high mortality rate (30.4/100 calf years - Table 5.6). Similarly, on farms which reported fortnightly dipping (mean age at first acaricide application = 4.1 months - Table 6.6), 47.9% (124/259) of calf observations recorded no tick treatment having been applied.

While it is possible to rationalise the distribution of inter-application intervals observed in calves, the situation is not so straightforward in adult animals, where 40.1% (761/1898), 30.2% (270/893) and 20.9% (57/273) of observations made on animals reported to be under systems of weekly, fortnightly and monthly or less frequent acaricide application, respectively, recorded inter-application intervals which were inconsistent with the stated frequency of tick control (Table 6.11). It is also of interest to note that 6.3% (21/336) of observations made on adult animals from farms that reported that tick control was not

practised, indicated that animals on 4 of 7 of these farms had been treated by back-pack sprayer application of acaricide at least once during the longitudinal study (Table 6.12).

6.3.2 Farm Reclassification

On the basis of the clear inconsistencies observed between the longitudinal records of inter-application intervals and the stated frequency of acaricide application, farms were reclassified to more accurately reflect the observed intensity of tick control efforts. The primary assumption of the rationale for reclassification was that inter-application interval information was of superior quality than the information recorded on the initial farm visit and hence more likely to accurately reflect the true intensity of tick control efforts.

6.3.2.1 Reclassified Method of Acaricide Application

The uncertainty regarding tick control practices was not just restricted to the frequency of acaricide application. Although all 44 farms which reported using back-pack sprayers at the initial farm survey did so subsequently, 6 of 37 farms (16.2%) which initially reported plunge dipping of livestock actually sprayed their animals. Table 6.12 compares the reported method of application to the reclassified method of application based on longitudinal observations.

6.3.2.2 Reclassified Frequency of Application of Tick Control

Farms which reported practising tick control were reclassified based on the proportion of inter-application intervals recorded for adult animals which were consistent

with the reported treatment frequency. Two threshold proportions, a "conservative" level of 66.7% (two-thirds) and a "liberal" level of 33.3% (one-third), were employed in an iterative reclassification process as follows. When the farm proportion of consistent adult inter-application intervals was equal to or above the threshold value, the farm classification remained unchanged, however, when the proportion was observed to be below the threshold value, the farm was reclassified to the next frequency of application category (e.g from weekly to fortnightly), the proportion of consistent intervals recalculated and the process repeated until no further reduction in frequency of application category was warranted under that threshold.

Four farms that reported that no tick control was practised but were subsequently recorded to have treated their animals once over the course of the study, were reclassified in the monthly or less frequent acaricide application category. Two other farms, which initially reported that acaricide was applied tri-annually and quarterly but where shorter inter-application intervals were reported each month, were reclassified in the fortnightly or weekly category according to the proportion of consistent adult inter-application intervals by the liberal and conservative criteria.

Table 6.13 presents the frequency of application of tick control reported on initial farm visit *versus* the maximum potential farm frequency of application as derived using liberal and conservative reclassification criteria. Under the conservative criteria, 6 of 56 farms (10.7%) which reported weekly acaricide application were reduced in classification to the fortnightly category while 27 (48.2%) were further reduced to the monthly or less frequent category. This compares to 15 (26.8%) and 13 (23.2%), respectively, when the

liberal threshold was applied. Similarly, 8 of 18 farms (42.1%) which originally reported fortnightly acaricide application, were reduced to the monthly category by the conservative process while 1 (5.3%) of these farms was so reclassified using the liberal classification criteria. Of the 2 farms which actually applied acaricide more frequently than stated, both were reclassified as fortnightly under the conservative method, while one was actually raised to the weekly category by the liberal approach.

6.3.3 Distribution and Demographic Associations of Reclassified Farms

Having reclassified 11.1% (10/90) of farms for the method of tick control practised and 52.2% (47/90 - conservative) or 38.9% (35/90 - liberal) of farms with respect to the intensity of acaricide application, it was essential that the distribution and demographic association of the various tick-control covariates be reevaluated.

Table 6.14 presents the distribution of the observed method of tick control for each of three demographic covariates. Contrasting it to Table 6.1 (method of control reported initially), we note that the associations with dairy co-operative society membership and agro-ecological zone of origin remain, but that the UH and LH zones are more similar in percentage of farms practising plunge dipping (23.8% = 5/21 and 18.4% = 7/38, respectively) and back-pack spraying (76.2% = 16/21 and 71.0% = 27/38, respectively). This again contrasts with the UM zone where, although the percentage of farms utilising back-pack spraying has increased from 19.4% (6/31) to 35.5% (11/31), plunge dipping remains the dominant method of acaricide application, being practised by 61.3% (19/31) of farms.

The demographic distributions of farm frequency of acaricide application, as derived

under liberal and conservative reclassification criteria, are presented in Tables 6.15 and 6.16, respectively. Comparing the results of applying the liberal threshold (Table 6.15) to the distribution observed for the reported frequency of application (Table 6.2), we note that, although there is a shift toward lower frequency categories, this is relatively uniformly distributed such that there is still no association by dairy co-operative society membership or agro-ecological zone of origin. However, there has been a more pronounced effect with respect to grazing management system, demonstrated by increased statistical significance of association ($p<0.001$ *versus* $p=0.01$). This is owing to a differential degree of reclassification between the two systems having exaggerated the differences at the extremes of the treatment frequency scale. For example, under the liberal criteria, 11.6% (5/43) of zero-grazing farms *versus* 51.1% (24/47) of semi-/full-pasture grazing farms were considered to be applying acaricide weekly compared to 46.5% (20/43) *versus* 8.5% (4/47), respectively, doing so a maximum of once per month. This differential shift is even more pronounced under the conservative reclassification criteria (Table 6.16) with 1 (2.3%) zero-grazing farm *versus* 22 (46.8%) semi-/full-pasture grazing farms considered to be applying acaricide weekly compared to 32 (74.4%) *versus* 13 (27.7%) farms, respectively, doing so monthly or less frequently. Further, the more profound differential reclassification of zero-grazing *versus* semi-/full-pasture grazing farms under the conservative method is also observed as a significant association by agro-ecological zone of origin ($p<0.001$), since higher frequencies of application observed in the UH zone are likely due to the fact that 95.3% (20/21) of farms in this zone practise semi-/full-pasturing grazing.

An additional benefit of farm reclassification was the reduction in collinearity

between observed method and observed frequency of acaricide application. Table 6.17 assesses this association for both liberal and conservative classification criteria, demonstrating that, although still significantly associated ($p=0.031$ and $p=0.034$, respectively), there is a relatively more homogenous distribution of frequencies of application by plunge dipping and back-pack spraying than observed in Table 5.6 for reported method and intensity, i.e. the covariates are less collinear such that it may be possible to simultaneously assess them in subsequent multiple variable models (see section *6.5 Derivation of Time-varying Frequency of Application Covariates*).

Table 6.18 confirms that age of calves at first acaricide application remains associated with the observed method of acaricide application ($p<0.001$) being significantly ($\alpha = 0.05$) higher on farms practising plunge dipping, but not associated with frequency of application under either the liberal ($p=0.84$) or conservative ($p=0.13$) classifications. However, in contrast to the lack of association by reported method or reported frequency observed for farm size (Table 6.7) and number of dairy animals (Table 6.8), Tables 6.19 and 6.20 demonstrate significant associations with conservative frequency of acaricide application for farm size ($p=0.005$) and number of dairy animals ($p=0.022$), respectively. Farms which were conservatively classified to be practising monthly or less frequent acaricide application under the conservative method, were noted to be smaller (2.9 acres) than those in the fortnightly (6.8 acres) or weekly (6.5 acres) categories and to have had fewer dairy animals (3.4) than either fortnightly (5.4) or weekly (5.6) farms ($\alpha = 0.05$).

6.4 East Coast Fever Morbidity and Sero-Events

In addition to contrasting changes in the distribution by various demographic covariates, the incidence of clinical ECF and serological patterns suggestive of anamnestic immune response were compared for combinations of reported and observed method and frequency of tick control covariates. Incidence rates, approximate 95% confidence intervals and statistical assessment of singular contrasts were calculated according to the methods described in Chapter 5.

6.4.1 Determination of Periods of Risk

To compare incidence rates between reported *versus* observed method and frequency of acaricide application, it was necessary to calculate periods of risk for each combination of covariate categories. These were derived from the number of observations of adults and calves in each category using the rationale presented in section *3.3.4 Calculation of Period of Observation*. Table 6.21 presents the periods of risk in months calculated for combinations of reported method and frequency, while Tables 6.22 and 6.23 do so for observed method and frequency as defined under liberal and conservative reclassification criteria, respectively.

Unfortunately, for several combinations of tick control categories, the total period at risk was very small. This was particularly true for combinations which included hand application of acaricide (Total = 90.5 months) and for farms which were classified as conducting no tick control under both liberal and conservative classifications (124 months). Also, in the case of calves, relatively low values were also observed for several

combinations. Extreme caution must be exercised in the interpretation of rates which are based on small at risk periods, and the uncertainty of these estimates is reflected by the very wide confidence intervals.

6.4.2 ECF Morbidity Incidence

ECF morbidity rates were calculated using numbers of cases derived from the Category 4 definition (see section *5.3.2 Incidence of East Coast Fever)*, i.e. including all reported/suspected non-fatal cases and deaths attributed to ECF, regardless of whether they were confirmed subsequently, and all missed cases as detected by increase in *T. parva*-specific antibodies concomitant with mild/subclinical disease.

Morbidity incidence rates for combinations of reported method and frequency of tick control are presented in Table 6.24 and for combinations of observed method and frequency, as defined by liberal and conservative methods, in Tables 6.25 and 6.26, respectively.

Given the degree of uncertainty associated with those rates with wide confidence intervals, it is difficult to make definitive statements concerning patterns or trends within combinations of method and frequency of acaricide application. However, an examination of the summary rates for all animals suggests a pattern consistent across reported and observed methods and frequencies of acaricide application, with incidence of morbidity i) highest on farms practising fortnightly tick control and lower where maximum frequency of acaricide application is either weekly or monthly and ii) approximately equivalent between plunge dipping *versus* back-pack spraying. For example, for observed method of tick control, rates of ECF-specific morbidity for all ages were estimated to be 8.8% on farms

practising plunge dipping *versus* 10.1% where back-pack spraying of acaricides was utilised (p=0.72; Tables 6.25 and 6.26). Under the conservative classification criteria (Table 6.26), rates of 8.8%, 18.6% and 4.9% were also observed for weekly, fortnightly and monthly frequency of acaricide application categories, respectively. Further, the approximate 95% confidence intervals for fortnightly and monthly categories did not overlap. However, within weekly and monthly categories, these summary rates were composed of plunge dipping rates of 10.7% and 7.7% respectively (p=0.39), with no cases observed in the fortnightly category. Thus, the higher overall rate for the fortnightly category, was principally due to observations made on farms practising back-pack spraying (20.9%) such that, within this method of control, farms practising monthly (3.8%; p=0.009) and weekly application (6.9%; p=0.04) exhibited significantly lower rates.

6.4.3 Incidence of Seroconversion and Sero-Events

Crude numbers of seroconversions and sero-events were derived from longitudinal serological profiles based on definitions detailed previously (see section *5.4.2 Definitions of Seroconversion and "Sero-Event"*) and where sero-increases of > 0.200 OD units were observed in all sero-events.

Seroconversion and sero-event incidence rates for combinations of reported method and frequency of tick control (Table 6.27) and for combinations of observed method and frequency, as defined by liberal (Table 6.28) and conservative (Table 6.29) methods, are presented, summarised for all cattle and also separately for adult cattle and calves. Examination of the summary rates for all animals suggests that relative relationship between

rates is also consistent across reported and observed methods and frequencies of acaricide application. However, while the pattern of incidence of seroconversions and sero-events agrees with that for ECF morbidity, i.e. highest on farms practising fortnightly tick control and lower where maximum frequency of acaricide application is either weekly or monthly, the summary rates of seroconversions and sero-events are higher on farms practising back-pack spraying *versus* plunge dipping. For the reported method of acaricide application (Table 6.27), the rate on farms conducting back-pack spraying was 32.8% *versus* 17.5% for plunge dipping farms (p=0.008). These rates altered slightly to 28.1% and 20.2%, respectively, (p=0.16) based on the observed method of tick control (Tables 6.28 and 6.29).

With respect to frequency of acaricide application, rates of 27.9%, 38.2% and 18.2% were observed for weekly, fortnightly and monthly frequency of acaricide application categories, respectively under the conservative reclassification criteria (Table 6.29). Within weekly and monthly categories, these summary rates were composed of plunge dipping rates of 23.0% and 18.0% (p=0.59) and back-pack spraying rates of 32.7% and 18.2%, respectively. While the rate for the fortnightly/dipping combination (10.4%) was also lower than for the corresponding fortnightly/spraying category (38.0%), this was based on relatively few observations (7.8 animal years of observation - Table 6.23) and hence exhibited a very wide confidence interval. Thus, for farms which practised back-pack spraying there was no difference in the monthly *versus* fortnightly rates (p=0.61) but the rate on farms which were considered to be applying acaricide monthly or less frequently was lower than both of these rates (p=0.004; p<0.001, respectively). Interestingly the rates of seroconversions/sero-events on farms which either reported or were confirmed not to practice

tick control were the lowest observed, at 11.0% and 9.7% respectively, although these rates were again based on very few observations.

6.5 Derivation of Time-varying Frequency of Application Covariates

Although the reclassified frequency of acaricide application is likely to more accurately reflect the patterns of tick control, since the covariates are estimates of the maximum intensity of tick control being practised by a farm, they are by default static over time. However, even under the conservative reclassification criteria up to 33.3% of inter-application intervals may not be consistent with the maximum farm frequency of tick control. Thus these farm-level covariates do not account for potential sources of variation such as seasonal changes in tick control, or varying intensity of acaricide application by different groups of animals on the farm. To more fully assess any temporal relationships between longitudinal serological measures and method and intensity of acaricide application, it was necessary to account for the variation observed between and within animals on a farm. This was accomplished through the creation of a time-varying covariate and its application in subsequent statistical models (see section *7.3 Independent Covariates*).

For each set of liberal and conservative farm classifications, a time-varying variable recording the number of monthly applications of acaricide was derived from the longitudinal inter-application intervals for each animal as follows. Where a monthly inter-application interval was consistent with the reclassified farm frequency (e.g. interval "3-7 days" for weekly category), then the variable was allocated the maximum possible number of monthly acaricide applications as per the reclassified frequency (weekly = 4 applications). If the

inter-application interval was inconsistent with the reclassified farm frequency, then the variable was assigned the maximum number of monthly treatments consistent with the observed inter-application interval (e.g. interval "2 - 3 weeks" for weekly category = 1 application). In all cases, when the farmer indicated that an animal had not been treated since the previous visit, or was not yet being treated for ticks, a value of 0 was allocated. This system creates a variable with values 0, 1, 2 and 4, which is recorded monthly, is potentially time-variant but animal specific and is restricted in distribution only by the most probable frequency of acaricide application, as assessed by the overall inter-application interval pattern observed in adult animals on the farm. A potential weakness of this method may be its tendency to underestimate the variability of the true tick control frequency since it assumes that the frequency of acaricide application is at a maximum over the period of the study and thus acknowledges only reductions in the intensity of control efforts. Nevertheless, given the apparent inability of farmers to correctly report tick control management practices and in the absence of a more active farm-monitoring system, the time-variant covariate created almost certainly provides the most realistic representation possible of the actual frequency of tick control being practised, in the absence of detailed records of the dates of acaricide application.

Table 6.30 presents the distribution of number of monthly applications of acaricide allocated to the time-varying covariate under liberal and conservative reclassification criteria, across frequency of application of tick control reported on the initial farm visit. Under the liberal reclassification criteria, 51.8% (1299/2507) of observations on farms reporting weekly acaricide application and 32.3% (372/1152) of observations on farms

reporting fortnightly tick control yielded values for number of applications of acaricide in the previous month which differed from the stated intensity of tick control. Corresponding values of 54.6% (1369/2507) and 42.7% (492/1152) were derived under the conservative reclassification method. However, despite these relatively large departures from the reported frequency of tick control, Table 6.31 contrasts the number of monthly applications of tick control derived for the time-variant covariate by liberal *versus* conservative criteria and demonstrates that, although 30.0% (27/90) of farms were classified differently under the two thresholds, only 7.9% (348/4433) of the actual monthly values differed. Consequently, although the two reclassification methods utilised markedly different thresholds, the difference between them, with respect to their use in statistical models, may not be large.

Table 6.1: Initial Visit - Distribution (with respect to dairy cooperative society membership, agro-ecological zone classification and grazing management system) of method of application of tick control practised, as reported on initial farm visit in a longitudinal study conducted in Kiambu District from July 1991 - June 1992.

Demographic Covariates	N	Reported Method of Application of Tick Control								Fisher's Exact Test of Association[1]
		Plunge Dipping		Spraying		Hand Applied		None		
		Freq	%	Freq	%	Freq	%	Freq	%	
Dairy Cooperative Society										
Chania	15	12	80.0	3	20.0	0	0	0	0	
Kiambaa	15	9	60.0	3	20.0	0	0	3	20.0	
Kikuyu	15	7	46.7	7	46.7	0	0	1	6.7	p <0.001/<0.001
Lari	15	5	33.3	10	66.7	0	0	0	0	
Limuru	15	3	20.0	10	66.7	1	6.7	1	6.7	
Nderi	15	1	6.7	11	73.3	1	6.7	2	13.3	
Agro-Ecological Zones[2]										
UH (1,2)	21	7	33.3	14	66.7	0	0	0	0	p <0.001/<0.001
LH (1,2,3)	38	9	23.7	24	63.2	2	5.3	3	7.9	
UM (1,2,3)	31	21	67.7	6	19.4	0	0	4	12.9	
Grazing System[3]										
Zero-grazing	43	18	41.9	18	41.9	1	2.1	6	14.0	p = 0.144/0.755
Semi/Grazing	47	19	40.4	26	55.3	1	2.1	1	2.1	
Total	90	37	41.1	44	48.9	2	2.2	7	7.8	

[1] - Two-tailed Fisher's Exact Test calculated using network algorithm of Mehta & Patel (1983). First p-value is for data set including farms which reported no tick control, second value assesses association when these farms are excluded.

[2] - Agro-ecological Zones (AEZ) where "UM" refers to the upper midland zone, "LH" to the lower highland zone and "UH" to the upper highland zone.

[3] - Grazing management where "Zero-grazing" refers to animals housed within a zero-grazing unit and "Semi/Grazing" documents animals with access to/kept on pasture.

Table 6.2: Initial Visit - Distribution (with respect to dairy cooperative society membership, agro-ecological zone classification and grazing management system) of frequency of tick control practised, as reported on initial farm visit in a longitudinal study conducted in Kiambu District from July 1991 - June 1992.

Demographic Covariates	N	Reported Frequency of Application of Tick Control								Fisher's Exact Test of Association[2]
		Weekly		Fortnightly		≤ Monthly[1]		None		
		Freq	%	Freq	%	Freq[1]	%	Freq	%	
Dairy Cooperative Society										
Chania	15	11	73.3	4	26.7	0	0	0	0	
Kiambaa	15	11	73.3	1	6.7	0	0	3	20.0	
Kikuyu	15	11	73.3	2	13.3	1	6.7	1	6.7	p = 0.17/0.18
Lari	15	9	60.0	5	33.3	1	6.7	0	0	
Limuru	15	6	40.0	4	26.7	4	26.7	1	6.7	
Nderi	15	8	53.3	3	20.0	2	13.3	2	13.3	
Agro-Ecological Zones[3]										
UH (1,2)	21	14	66.7	6	28.6	1	4.8	0	0	p = 0.070/0.071
LH (1,2,3)	38	20	53.6	8	21.0	7	18.4	3	7.9	
UM (1,2,3)	31	22	71.0	5	16.1	0	0	4	12.9	
Grazing System[4]										
Zero-grazing	43	23	53.5	7	16.3	7	16.3	6	14.0	p = 0.011/0.032
Semi/Grazing	47	33	70.2	12	25.5	1	2.1	1	2.1	
Total	90	56	62.2	19	21.1	8	8.9	7	7.8	

[1] - "≤ Monthly" refers to tick control practised monthly or less frequently.
[2] - Two-tailed Fisher's Exact Test calculated using network algorithm of Mehta & Patel (1983). First p-value is for data set including farms which reported no tick control, second value assesses association when these farms are excluded.
[3] - Agro-ecological Zones (AEZ) where "UM" refers to the upper midland zone, "LH" to the lower highland zone and "UH" to the upper highland zone.
[4] - Grazing management where "Zero-grazing" refers to animals housed within a zero-grazing unit and "Semi/Grazing" documents animals with access to/kept on pasture.

Table 6.3: Initial Visit - Association between method of tick control and frequency of application for 83 farms which practised tick control, as reported on initial farm visit in a longitudinal study conducted in Kiambu District from July 1991 - June 1992.

Reported Method of Application of Tick Control		Reported Frequency of Application of Tick Control			Totals
		Weekly	Fortnightly	≤ Monthly[1]	
Plunge Dipping	Frequency	35	1	1	37
	%	42.2	1.2	1.2	44.6
	Row %	94.6	2.7	2.7	
	Column %	62.5	5.3	12.5	
Spraying	Frequency	20	18	6	44
	%	24.1	21.7	7.2	53.0
	Row %	45.5	40.9	13.6	
	Column %	35.7	94.7	75.0	
Hand Applied	Frequency	1	0	1	2
	%	1.2	0	1.2	2.4
	Row %	50.0	0	50.0	
	Column %	1.8	0	12.5	
Totals		56	19	8	83
		67.5	22.9	9.6	100

Test of association: $p<0.001$ - Two-tailed Fisher's Exact Test calculated using network algorithm of Mehta & Patel (1983).

[1] - "≤ Monthly" refers to tick control practised monthly or less frequently.

Table 6.4: Initial Visit - Distribution (with respect to dairy cooperative society membership, agro-ecological zone classification and grazing management system) of age groups treated with tick control, as reported on initial farm visit in a longitudinal study conducted in Kiambu District from July 1991 - June 1992.

Demographic Covariates	n	Age Groups Reportedly Treated With Tick Control						Fisher's Exact Test of Association[1]
		All Animals		Mature Cattle Only		None		
		Freq	%	Freq	%	Freq	%	
Dairy Cooperative Society								
Chania	15	13	86.7	2	13.3	0	0	
Kiambaa	15	11	73.3	1	6.7	3	20.0	
Kikuyu	15	12	80.0	2	13.3	1	6.7	p = 0.241/0.321
Lari	15	15	100.0	0	0	0	0	
Limuru	15	14	93.3	0	0	1	6.7	
Nderi	15	13	86.7	0	0	2	13.3	
Agro-Ecological Zones[2]								
UH (1,2)	21	21	100.0	0	0	0	0	p = 0.263/0.363
LH (1,2,3)	38	33	86.8	2	5.3	3	7.9	
UM (1,2,3)	31	24	77.4	3	9.7	4	12.9	
Grazing System[3]								
Zero-grazing	43	33	76.7	4	9.3	6	14.0	p = 0.025/0.167
Semi/Grazing	47	45	95.7	1	2.1	1	2.1	
Total	90	78	86.7	5	5.6	7	7.8	

[1] - Two-tailed Fisher's Exact Test calculated using network algorithm of Mehta & Patel (1983). First p-value is for data set including farms which reported no tick control, second value assesses association when these farms are excluded.

[2] - Agro-ecological Zones (AEZ) where "UM" refers to the upper midland zone, "LH" to the lower highland zone and "UH" to the upper highland zone.

[3] - Grazing management where "Zero-grazing" refers to animals housed in a zero-grazing unit and "Semi/Grazing" documents animals with access to/kept on pasture.

Table 6.5: Initial Visit - Distribution (with respect to dairy cooperative society membership, agro-ecological zone classification and grazing management system) of calf age at first application of tick treatment for farms which treated calves, as reported on initial farm visit in a longitudinal study conducted in Kiambu District from July 1991 - June 1992.

Demographic Covariates	n	Age of Calves at First Application of Tick Control (Months)				Test of Association/ Multiple Comparison[1]
		Mean	Standard Error	Median	Min - Max	
Dairy Cooperative Society						p = 0.16
Chania	13	6.0	0.8	6.0	2 - 12	A
Kiambaa	11	6.6	1.8	5.0	2.5 - 24	A
Kikuyu	12	4.2	0.5	4.0	1 - 6	A
Lari	15	4.2	0.5	4.0	1 - 8	A
Limuru	14	4.0	0.4	3.2	2 - 6	A
Nderi	13	4.4	0.4	4.0	3 - 8	A
Agro-ecological Zones[2]						p = 0.005
UH	21	4.0	0.4	4.0	1 - 8	A
LH	33	4.2	0.3	4.0	1 - 8	A
UM	24	6.4	0.9	6.0	2.5 - 24	B
Grazing Management[3]						p = 0.34
Zero-grazing	33	5.0	0.4	5.0	1 - 12	A
Semi/Grazing	45	3.4	0.5	4.0	1 - 24	A
Total	78	4.8	0.3	4.0	1 - 24	

[1] - Overall test of association is based on unbalanced one-way analysis of variance of transformed dependent variable; ln(Calf Age at First Treatment). The Tukey-Kramer studentised range test (which minimises the maximum experimentwise error rate) was performed for multiple comparison of means of the transformed dependent variable; ln(Calf Age at First Treatment). Categories with the same letter are not considered to differ significantly from one another.

[2] - Agro-ecological Zones (AEZ) where "UM" refers to the upper midland zone, "LH" to the lower highland zone and "UH" to the upper highland zone.

[3] - Grazing management where "Zero-grazing" refers to animals housed within a zero-grazing unit and "Semi/Grazing" documents animals with access to/kept on pasture.

Table 6.6: Initial Visit - Distribution of calf age at first application of tick treatment by method of tick control and frequency of application on farms which treated calves, as reported on initial farm visit in a longitudinal study conducted in Kiambu District from July 1991 - June 1992.

Tick Control Practices	n	Age of Calves at First Application of Tick Control (Months)				Test of Association/ Multiple Comparison[1]
		Mean	Standard Error	Median	Min - Max	
Reported Frequency of Application of Tick Control						p = 0.61
Weekly	52	5.2	0.5	4.8	1 - 24	A
Fortnightly	19	4.1	0.3	4.0	2 - 6	A
≤ Monthly[2]	7	4.3	0.5	4.0	3 - 6	A
Reported Method of Application of Tick Control						p = <0.001
Plunge Dipping	32	6.1	0.7	6.0	2 - 24	A
Spraying	44	3.9	0.2	4.0	1 - 8	B
Hand Application	2	4.0	1.0	4.0	3 - 5	A/B
Total	78	4.8	0.3	4.0	1 - 24	

[1] - Overall test of association is based on unbalanced one-way analysis of variance of transformed dependent variable; ln(Calf Age at First Treatment). The Tukey-Kramer studentised range test (which minimises the maximum experimentwise error rate) was performed for multiple comparison of means of the transformed dependent variable; ln(Calf Age at First Treatment). Categories with the same letter are not considered to differ significantly from one another.

[2] - "≤ Monthly" refers to tick control practised monthly or less frequently.

Table 6.7: Initial Visit - Distribution of farm size by method of tick control, frequency of application and age group treated, as reported on initial farm visit in a longitudinal study conducted in Kiambu District from July 1991 - June 1992.

Tick Control Practices	n	Farm Size (Acres)				Test of Association/ Multiple Comparison[1]
		Mean	Standard Error	Median	Min - Max	
Reported Frequency of Application of Tick Control						p = 0.12/0.31
Weekly	56	4.7	0.7	3.0	0.25 - 25	A
Fortnightly	19	5.7	1.0	4.0	0.5 - 15	A
≤ Monthly[2]	8	4.2	1.1	3.0	1.25 - 11	A
None	7	1.8	0.4	2.0	0.5 - 3.5	A
Reported Method of Application of Tick Control						p = 0.26/0.73
Plunge Dipping	37	5.0	1.0	3.0	0.25 - 25	A
Spraying	44	4.9	0.6	4.0	0.25 - 15	A
Hand Application	2	2.1	0.9	2.1	1.25 - 3	A
None	7	1.8	0.4	2.0	0.5 - 3.5	A
Age Groups Reportedly Treated with Tick Control						p = 0.13/0.39
All Animals	78	5.0	0.6	3.8	0.25 - 25	A
Mature Cattle Only	5	2.5	0.8	2.5	1 - 5.5	A
None	7	1.8	0.4	2.0	0.5 - 3.5	A
Total	90	4.6	0.5	3.0	0.25-25	

[1] - Overall test of association is based on unbalanced one-way analysis of variance of transformed dependent variable; ln(Farm Size). The first p-value corresponds to the data set including farms which reported no tick control while the second value assesses the relationship when these farms are excluded. The Tukey-Kramer studentised range test (which minimises the maximum experimentwise error rate) was performed for multiple comparison of means of the transformed dependent variable; ln(Farm Size). Categories with the same letter are not considered to differ significantly from one another.

[2] - "≤ Monthly" refers to tick control practised monthly or less frequently.

Table 6.8: Initial Visit - Distribution of number of dairy animals present on farm by method of tick control, frequency of application and age group treated, as reported on initial farm visit in a longitudinal study conducted in Kiambu District from July 1991 - June 1992.

Tick Control Practices	n	Number of Dairy Animals Initially Present on Farm				Test of Association/ Multiple Comparison[1]
		Mean	Standard Error	Median	Min - Max	
Reported Frequency of Application of Tick Control						p = 0.82/0.62
Weekly	56	4.0	0.5	3.0	1 - 20	A
Fortnightly	19	5.5	1.3	4.0	1 - 24	A
≤ Monthly[2]	8	3.6	0.5	4.0	1 - 5	A
None	7	5.5	2.1	4.0	1 - 17	A
Reported Method of Application of Tick Control						p = 0.73/0.51
Plunge Dipping	37	3.7	0.5	3.0	1 - 15	A
Spraying	44	4.8	0.7	4.0	1 - 24	A
Hand Application	2	3.5	1.5	3.5	2 - 5	A
None	7	5.5	2.1	4.0	1 - 17	A
Age Groups Reportedly Treated With Tick Control						p = 0.47/0.21
All Animals	78	4.4	0.5	3.0	1 - 24	A
Mature Cattle Only	5	2.4	0.5	2.0	1 - 4	A
None	7	5.5	2.1	4.0	1 - 17	A
Total	90	4.4	0.4	3.0	1-24	

[1] - Overall test of association is based on unbalanced one-way analysis of variance of transformed dependent variable; ln(Initial Number of Dairy Animals). The first p-value corresponds to the data set including farms which reported no tick control while the second value assesses the relationship when these farms are excluded. The Tukey-Kramer studentised range test (which minimises the maximum experimentwise error rate) was performed for multiple comparison of means of the transformed dependent variable; ln(Initial Number of Dairy Animals). Categories with the same letter are not considered to differ significantly from one another.

[2] - "≤ Monthly" refers to tick control practised monthly or less frequently.

Table 6.9: Longitudinal Study - Comparison of frequency of application of tick control reported on initial farm visit and inter-application intervals observed for all animals during a longitudinal study conducted in Kiambu District from July 1991 - June 1992.

Inter-Tick Control Application Interval Recorded During Longitudinal Observation		Reported Frequency of Application of Tick Control				Totals
		Weekly	Fortnighly	≤ Monthly[1]	None	
		n=2507	n=1152	n=333	n=441	n=4433
		56.6%	26.0%	7.5%	9.9%	100.0%
< 3 days	Frequency	361	78	25	0	464
	%	14.4	6.8	7.5	0	10.5
3 - 7 days	Frequency	949	436	66	1	1452
	%	37.8	37.8	19.8	0.2	32.8
8 days - 2 weeks	Frequency	242	221	20	0	483
	%	9.7	19.2	6.0	0	10.9
2 - 3 weeks	Frequency	79	48	22	0	149
	%	3.1	4.2	6.6	0	3.3
3 weeks - 1 month	Frequency	24	42	10	0	76
	%	1.0	3.6	3.0	0	1.7
> 1 month[2]	Frequency	261	124	97	20	502
	%	10.4	10.8	29.1	4.6	11.3
not treated[3]	Frequency	591	203	93	420	1307
	%	23.6	17.6	28.0	95.2	29.5

[1] - "≤ Monthly" refers to tick control practised monthly or less frequently.
[2] - "> 1 month" documents animals treated with tick control in the past, but at least prior to the preceding visit.
[3] - "not treated" recorded for calves not yet treated for ticks and adult animals not being treated.

Table 6.10: Longitudinal Study - Comparison of frequency of application of tick control reported on initial farm visit and inter-application intervals observed for calves (i.e. ≤ 1 year of age) during a longitudinal study conducted in Kiambu District from July 1991 - June 1992.

Inter-Tick Control Application Interval Recorded During Longitudinal Observation		Reported Frequency of Application of Tick Control				Totals
		Weekly	Fortnightly	≤ Monthly[1]	None	
		n=609	n=259	n=60	n=105	n=1033
		58.9%	25.1%	5.8%	10.2%	100.0%
< 3 days	Frequency	41	11	3	0	55
	%	6.7	4.2	5.0	0	5.3
3 - 7 days	Frequency	132	71	11	0	214
	%	21.7	27.4	18.4	0	20.7
8 days - 2 weeks	Frequency	29	30	2	0	61
	%	4.8	11.6	3.3	0	5.9
2 - 3 weeks	Frequency	19	5	2	0	26
	%	3.1	1.9	3.3	0	2.5
3 weeks - 1 month	Frequency	4	9	1	0	14
	%	0.7	3.5	1.7	0	1.4
> 1 month[2]	Frequency	13	9	5	0	27
	%	2.1	3.5	8.3	0	2.6
not treated[3]	Frequency	371	124	36	105	636
	%	60.9	47.9	60.0	100	61.6

[1] - "≤ Monthly" refers to tick control practised monthly or less frequently.
[2] - "> 1 month" documents animals treated with tick control in the past, but at least prior to the preceding visit.
[3] - "not treated" recorded for calves not yet treated for ticks.

Table 6.11: Longitudinal Study - Comparison of frequency of application of tick control reported on initial farm visit and inter-application intervals observed for "adult" (i.e. > 1 year of age) cattle during a longitudinal study conducted in Kiambu District from July 1991 - June 1992 where inconsistent combinations are in bold typed.

Inter-Tick Control Application Interval Recorded During Longitudinal Observation		Reported Frequency of Application of Tick Control				Totals
		Weekly	Fortnightly	≤ Monthly[1]	None	
		n=1898	n=893	n=273	n=336	n=3400
		55.8%	26.3%	8.0%	9.9%	
< 3 days	Frequency	320	67	22	0	409
	%	16.9	7.5	8.1	0	12.0
3 - 7 days	Frequency	817	365	55	**1**	1238
	%	43.0	40.9	20.1	**0.3**	36.4
8 days - 2 weeks	Frequency	**213**	191	18	0	422
	%	**11.2**	21.4	6.6	0	12.4
2 - 3 weeks	Frequency	**60**	**43**	20	0	123
	%	**3.2**	**4.8**	7.3	0	3.6
3 weeks - 1 month	Frequency	**20**	**33**	9	0	62
	%	**1.0**	**3.7**	3.3	0	1.8
> 1 month[2]	Frequency	**248**	**115**	92	**20**	475
	%	**13.1**	**12.9**	33.7	**6.0**	14.0
not treated[3]	Frequency	**220**	**79**	**57**	315	671
	%	**11.6**	**8.8**	**20.9**	93.8	19.8

[1] - "≤ Monthly" refers to tick control practised monthly or less frequently.
[2] - "> 1 month" documents animals treated with tick control in the past, but at least prior to the preceding visit.
[3] - "not treated" recorded for adult animals not being treated.

Table 6.12: Longitudinal Study - Comparison of method of application of tick control reported on initial farm visit and method of application of tick control observed during a longitudinal study conducted in Kiambu District from July 1991 - June 1992.

Observed Method of Application of Tick Control Recorded During Longitudinal Observation		Reported Method of Application of Tick Control				Totals
		Plunge Dipping	Spraying	Hand Applied	None	
		n=37	n=44	n=2	n=7	n=90
Plunge Dipping	Frequency	31	0	0	0	31
	%	83.8	0	0	0	34.5
Spraying	Frequency	6	44	0	4	54
	%	16.2	100	0	57.1	60.0
Hand Applied	Frequency	0	0	2	0	2
	%	0	0	100	0	2.2
None	Frequency	0	0	0	3	3
	%	0	0	0	42.9	3.3

Table 6.13: Longitudinal Study - Comparison of frequency of application of tick control reported on initial farm visit and reclassified maximum potential farm frequency of application as derived, using liberal and conservative criteria, from inter-application intervals observed during a longitudinal study conducted in Kiambu District from July 1991 - June 1992.

Reclassification of Farm Frequency of Application of Tick Control[3]		Reported Frequency of Application of Tick Control				Totals
		Weekly	Fortnighly	≤ Monthly[1]	None	
		n=56	n=19	n=8	n=7	n=90
Liberal Classification Criteria[3]						
Weekly	Frequency	28	0	1	0	29
	%	50.0	0	12.5	0	32.2
Fortnightly	Frequency	15	18	1	0	34
	%	26.8	94.7	12.5	0	37.8
≤ Monthly[1]	Frequency	13	1	6	4	24
	%	23.2	5.3	75.0	57.1	26.7
None[2]	Frequency	0	0	0	3	3
	%	0	0	0	42.9	3.3
Conservative Classification Criteria[3]						
Weekly	Frequency	23	0	0	0	23
	%	41.1	0	0	0	25.6
Fortnightly	Frequency	6	11	2	0	19
	%	10.7	57.9	25.0	0	21.1
≤ Monthly[1]	Frequency	27	8	6	4	45
	%	48.2	42.1	75.0	57.1	50.0
None[2]	Frequency	0	0	0	3	3
	%	0	0	0	42.9	3.3

[1] - "≤ Monthly" refers to tick control practised monthly or less frequently. Three of seven farms which reportedly did not practise tick control treated animals once during the longitudinal study, while one such farm treated their animals twice.

[2] - "None" now documents farms which reportedly did not treat for ticks and on which an animal was never observed to have been treated.

[3] - Farm reclassification by an iterative process of reduction in frequency of application category until 33.3% (= liberal criteria) or 66.7% (= conservative criteria) of observed inter-application intervals were consistent.

Table 6.14: Longitudinal Study - Distribution (with respect to dairy cooperative society membership, agro-ecological zone classification and grazing management system) of method of tick control practised as observed during a longitudinal study conducted in Kiambu District from July 1991 - June 1992.

Demographic Covariates	n	Observed Method of Tick Control								Fisher's Exact Test of Association[1]
		Plunge Dipping		Spraying		Hand Applied		None		
		Freq	%	Freq	%	Freq	%	Freq	%	
Dairy Cooperative Society										
Chania	15	12	80.0	3	20.0	0	0	0	0	
Kiambaa	15	7	46.7	8	53.3	0	0	0	0	
Kikuyu	15	6	40.0	8	53.3	0	0	1	0	p <0.001/<0.001
Lari	15	3	20.0	12	80.0	0	0	0	0	
Limuru	15	3	20.0	10	66.7	1	6.7	1	6.7	
Nderi	15	0	0	13	86.7	1	6.7	1	6.7	
Agro-Ecological Zones[2]										
UH (1,2)	21	5	23.8	16	76.2	0	0	0	0	p <0.001/<0.001
LH (1,2,3)	38	7	18.4	27	71.0	2	5.3	2	5.3	
UM (1,2,3)	31	19	61.3	11	35.5	0	0	1	3.2	
Grazing System[3]										
Zero-grazing	43	15	34.9	24	55.8	1	2.3	3	7.0	p = 0.323/0.824
Semi/Grazing	47	16	34.1	30	63.8	1	2.1	0	0	
Total	90	31	34.5	54	60.0	2	2.2	3	3.3	

[1] - Two-tailed Fisher's Exact Test calculated using network algorithm of Mehta & Patel (1983). First p-value is for data set including farms which did not practise tick control, second value assesses association when these farms are excluded.

[2] - Agro-ecological Zones (AEZ) where "UM" refers to the upper midland zone, "LH" to the lower highland zone and "UH" to the upper highland zone.

[3] - Grazing management where "Zero-grazing" refers to animals housed within a zero-grazing unit and "Semi/Grazing" documents animals with access to/kept on pasture.

Table 6.15: Longitudinal Study - Distribution (with respect to dairy cooperative society membership, agro-ecological zone classification and grazing management system) of maximum potential farm frequency of tick control practised, as derived from inter-application intervals observed during a longitudinal study conducted in Kiambu District from July 1991 - June 1992 and reclassified under liberal criteria.

Demographic Covariates	n	Maximum Potential Farm Frequency of Application of Tick Control under Liberal Reclassification Criteria[5]								Fisher's Exact Test of Association[2]
		Weekly		Fortnightly		≤ Monthly[1]		None		
		Freq	%	Freq	%	Freq	%	Freq	%	
Dairy Cooperative Society										
Chania	15	3	20.0	10	66.7	2	13.3	0	0	
Kiambaa	15	6	40.0	3	20.0	6	40.0	0	0	
Kikuyu	15	4	26.7	6	40.0	4	26.7	1	6.7	p = 0.18
Lari	15	8	53.3	6	40.0	1	6.7	0	0	
Limuru	15	5	33.3	5	33.3	4	26.7	1	6.7	
Nderi	15	3	20.0	4	26.7	7	46.7	1	0	
Agro-Ecological Zones[3]										
UH (1,2)	21	11	52.4	8	38.1	2	9.5	0	0	p = 0.17
LH (1,2,3)	38	9	23.7	13	34.2	14	36.8	2	5.3	
UM (1,2,3)	31	9	29.0	13	41.9	8	25.8	1	3.3	
Grazing System[4]										
Zero-grazing	43	5	11.6	15	34.9	20	46.5	3	7.0	p <0.001
Semi/Grazing	47	24	51.1	19	40.4	4	8.5	0	0	
Total	90	29	32.2	34	37.8	24	26.7	3	3.3	

[1] - "≤ Monthly" refers to tick control practised monthly or less frequently.
[2] - Two-tailed Fisher's Exact Test calculated using network algorithm of Mehta & Patel (1983).
[3] - Agro-ecological Zones (AEZ) where "UM" refers to the upper midland zone, "LH" to the lower highland zone and "UH" to the upper highland zone.
[4] - Grazing management where "Zero-grazing" refers to animals housed within a zero-grazing unit and "Semi/Grazing" documents animals with access to/kept on pasture.
[5] - Farm reclassification by an iterative process of reduction in frequency of application category until 33.3% of observed inter-application intervals were consistent.

Table 6.16: Longitudinal Study - Distribution (with respect to dairy cooperative society membership, agro-ecological zone classification and grazing management system) of maximum potential farm frequency of tick control practised, as derived from inter-application intervals observed during a longitudinal study conducted in Kiambu District from July 1991 - June 1992 and reclassified under conservative criteria.

Demographic Covariates	n	Maximum Potential Farm Frequency of Application of Tick Control under Conservative Reclassification Criteria[5]								Fisher's Exact Test of Association[2]
		Weekly		Fortnightly		≤ Monthly[1]		None		
		Freq	%	Freq	%	Freq	%	Freq	%	
Dairy Cooperative Society										
Chania	15	2	13.3	3	20.0	10	66.7	0	0	
Kiambaa	15	5	33.3	2	13.3	8	53.3	0	0	
Kikuyu	15	3	20.0	3	20.0	8	53.3	1	6.7	p = 0.028
Lari	15	7	46.7	7	46.7	1	6.7	0	0	
Limuru	15	4	26.7	3	20.0	7	46.7	1	6.7	
Nderi	15	2	13.3	1	6.7	11	73.3	1	6.7	
Agro-Ecological Zones[3]										
UH (1,2)	21	10	47.6	8	38.1	3	14.3	0	0	p <0.001
LH (1,2,3)	38	6	15.8	6	15.8	24	63.1	2	5.3	
UM (1,2,3)	31	7	22.6	5	16.1	18	58.1	1	3.2	
Grazing System[4]										
Zero-grazing	43	1	2.3	7	16.3	32	74.4	3	7.0	p <0.001
Semi/Grazing	47	22	46.8	12	25.5	13	27.7	0	0	
Total	90	23	25.6	19	21.1	45	50.0	3	3.3	

[1] - "≤ Monthly" refers to tick control practised monthly or less frequently.
[2] - Two-tailed Fisher's Exact Test calculated using network algorithm of Mehta & Patel (1983).
[3] - Agro-ecological Zones (AEZ) where "UM" refers to the upper midland zone, "LH" to the lower highland zone and "UH" to the upper highland zone.
[4] - Grazing management where "Zero-grazing" refers to animals housed within a zero-grazing unit and "Semi/Grazing" documents animals with access to/kept on pasture.
[5] - Farm reclassification by an iterative process of reduction in frequency of application category until 66.7% of observed inter-application intervals were consistent.

Table 6.17: Longitudinal Study - Association between observed method of application of tick control and maximum potential farm frequency of application of tick control for farms which practised tick control, as derived from inter-application intervals observed during a longitudinal study conducted in Kiambu District from July 1991 - June 1992 and reclassified under liberal and conservative criteria.

Maximum Potential Farm Frequency of Application of Tick Control By Reclassification Criteria[2]		Observed Method of Application of Tick Control			Totals
		Plunge Dipping	Spraying	Hand Applied	
		n=31	n=54	n=2	n=87
Liberal Classification Criteria[2]					
Weekly	Frequency	15	12	2	29
	%	48.4	22.2	100	33.3
Fortnightly	Frequency	9	25	0	34
	%	29.0	46.3	0	39.1
≤ Monthly[1]	Frequency	7	17	0	24
	%	22.6	31.5	0	27.6
Conservative Classification Criteria[2]					
Weekly	Frequency	12	10	1	23
	%	38.7	18.5	50.0	26.5
Fortnightly	Frequency	3	15	1	19
	%	9.7	27.8	50.0	21.8
≤ Monthly[1]	Frequency	16	29	0	45
	%	51.6	53.7	0	51.7

Tests of association: p=0.031 (liberal criteria)/p=0.034 (conservative criteria) - Two-tailed Fisher's Exact Test calculated using network algorithm of Mehta & Patel (1983).

[1] - "≤ Monthly" refers to tick control practised monthly or less frequently.
[2] - Farm reclassification by an iterative process of reduction in frequency of application category until 33.3% (= liberal criteria) or 66.7% (= conservative criteria) of observed inter-application intervals were consistent.

Table 6.18: Longitudinal Study - Distribution of calf age at first application of tick treatment, as initially reported on farms treating calves, by observed method of tick control and maximum potential farm frequency of application of tick control, as derived from inter-application intervals observed during a longitudinal study conducted in Kiambu District from July 1991 - June 1992 and reclassified under liberal and conservative criteria.

Observed Tick Control Practices	N	Age of Calves at First Application of Tick Control (Months)				Test of Association/ Multiple Comparison[1]
		Mean	Standard Error	Median	Min - Max	
Liberal Maximum Potential Farm Frequency of Application of Tick Control[3]						p = 0.84
Weekly	29	4.6	0.4	4.0	1 - 12	A
Fortnightly	31	5.2	0.7	4.0	1 - 24	A
≤ Monthly[2]	18	4.7	0.3	5.0	3 - 6.5	A
Conservative Maximum Potential Farm Frequency of Application of Tick Control[3]						p = 0.13
Weekly	23	4.0	0.3	4.0	1 - 8	A
Fortnightly	19	4.6	0.5	4.0	2 - 12	A
≤ Monthly[2]	36	5.5	0.6	6.0	1 - 24	A
Observed Method of Application of Tick Control						p <0.001
Plunge Dipping	26	6.5	0.83	6.0	2 - 24	A
Spraying	50	4.0	0.2	4.0	1 - 8	B
Hand Applied	2	4.0	1.0	4.0	3 - 5	A/B
Total	78	4.8	0.3	4.0	1 - 24	

[1] - Overall test of association is based on unbalanced one-way analysis of variance of transformed dependent variable; ln(Calf Age at First Treatment). The Tukey-Kramer studentised range test (which minimises the maximum experimentwise error rate) was performed for multiple comparison of means of the transformed dependent variable; ln(Calf Age at First Treatment). Categories with the same letter are not considered to differ significantly from one another.

[2] - "≤ Monthly" refers to tick control practised monthly or less frequently.

[3] - Farm reclassification by an iterative process of reduction in frequency of application category until 33.3% (= liberal criteria) or 66.7% (= conservative criteria) of observed inter-application intervals were consistent.

Table 6.19: Longitudinal Study - Distribution of farm size by observed method of tick control and maximum potential farm frequency of application of tick control, as derived from inter-application intervals observed during a longitudinal study conducted in Kiambu District from July 1991 - June 1992 and reclassified under liberal and conservative criteria.

Observed Tick Control Practices	N	Farm Size (Acres)				Test of Association/ Multiple Comparison[1]
		Mean	Standard Error	Median	Min - Max	
Liberal Maximum Potential Farm Frequency of Application of Tick Control[3]						p = 0.16
Weekly	29	5.8	1.0	5.0	0.25 - 24	A
Fortnightly	34	4.9	0.9	3.0	0.5 - 25	A
≤ Monthly[2]	24	3.1	0.6	2.0	0.25 - 11	A
None	3	2.3	0.9	3.0	0.5 - 3.5	A
Conservative Maximum Potential Farm Frequency of Application of Tick Control[3]						p = 0.005
Weekly	23	6.5	1.1	5.0	0.25 - 24	A
Fortnightly	19	6.8	1.4	5.0	1 - 25	A
≤ Monthly[2]	45	2.9	0.4	2.0	0.25 - 11	B
None	3	2.3	0.9	3.0	0.5 - 3.5	A/B
Observed Method of Application of Tick Control						p = 0.81
Plunge Dipping	31	4.6	0.9	3.0	0.25 - 24	A
Spraying	54	4.9	0.7	3.4	0.25 - 25	A
Hand Applied	2	2.1	0.9	2.1	1.25 - 3	A
None	3	2.3	0.9	3.0	0.5 - 3.5	A
Total	90	4.6	0.5	3.0	0.25-25	

[1] - Overall test of association is based on unbalanced one-way analysis of variance of transformed dependent variable; ln(Farm Size). The Tukey-Kramer studentised range test (which minimises the maximum experimentwise error rate) was performed for multiple comparison of means of the transformed dependent variable; ln(Farm Size). Categories with the same letter are not considered to differ significantly from one another.

[2] - "≤ Monthly" refers to tick control practised monthly or less frequently.

[3] - Farm reclassification by an iterative process of reduction in frequency of application category until 33.3% (= liberal criteria) or 66.7% (= conservative criteria) of observed inter-application intervals were consistent.

Table 6.20: Longitudinal Study - Distribution of number of dairy animals initially present on farm by observed method of tick control and maximum potential farm frequency of application of tick control, as derived from inter-application intervals observed during a longitudinal study conducted in Kiambu District from July 1991 - June 1992 and reclassified under liberal and conservative criteria.

Observed Tick Control Practices	N	Number of Dairy Animals Initially Present on Farm				Test of Association/ Multiple Comparison[1]
		Mean	Standard Error	Median	Min - Max	
Liberal Maximum Potential Farm Frequency of Application of Tick Control[3]						p = 0.27
Weekly	29	5.2	0.8	4.0	1 - 20	A
Fortnightly	34	4.2	0.8	3.0	1 - 24	A
≤ Monthly[2]	24	3.8	0.7	3.0	1 - 17	A
None	3	3.3	1.2	4.0	1 - 5	A
Conservative Maximum Potential Farm Frequency of Application of Tick Control[3]						p = 0.022
Weekly	23	5.6	1.0	4.0	1 - 20	A
Fortnightly	19	5.4	1.2	4.0	2 - 24	A/B
≤ Monthly[2]	45	3.4	0.5	3.0	1 - 17	B
None	3	3.3	1.2	4.0	1 - 5	A/B
Observed Method of Application of Tick Control						p = 0.89
Plunge Dipping	31	3.9	0.6	3.0	1 - 15	A
Spraying	54	4.7	0.6	3.5	1 - 24	A
Hand Applied	2	3.5	1.5	3.5	2 - 5	A
None	3	3.3	1.2	4.0	1 - 5	A
Total	90	4.4	0.4	3.0	1 - 24	

[1] - Overall test of association is based on unbalanced one-way analysis of variance of transformed dependent variable; ln(Initial Number of Dairy Animals). The Tukey-Kramer studentised range test (which minimises the maximum experimentwise error rate) was performed for multiple comparison of means of the transformed dependent variable; ln(Initial Number of Dairy Animals). Categories with the same letter are not considered to differ significantly from one another.

[2] - "≤ Monthly" refers to tick control practised monthly or less frequently.

[3] - Farm reclassification by an iterative process of reduction in frequency of application category until 33.3% (= liberal criteria) or 66.7% (= conservative criteria) of observed inter-application intervals were consistent.

Table 6.21: Longitudinal Study - Calculation of period of animal observation from surveys recorded in a longitudinal study conducted in Kiambu District from July 1991 - June 1992; subdivided on the basis of i) the reported method of application and ii) the reported frequency of application of tick control by iii) the classification of the animal with respect to age (0-12 months of age = calf, > 12 months = adult) *at the time of each observation/survey*:

Reported Method of Application of Tick Control	Reported Frequency of Application of Tick Control				Total
	Weekly	Fortnightly	≤ Monthly	None	
		ADULT CATTLE			
Plunge Dipping	1189.5	20.5	22	-	1232
Spraying	727	885.5	198.5	-	1811
Hand Applied	24	0	52.5	-	76.5
None	-	-	-	337	337
Total	1940.5	906	273	337	3456.5
		CALVES			
Plunge Dipping	337.5	4	2	-	343.5
Spraying	239.5	247	42	-	528.5
Hand Applied	0	0	14	-	14
None	-	-	-	101.5	101.5
Total	577	251	58	101.5	987.5
		ALL ANIMALS			
Plunge Dipping	1527	24.5	24	-	1575.5
Spraying	966.5	1132.5	240.5	-	2339.5
Hand Applied	24	0	66.5	-	90.5
None	-	-	-	438.5	438.5
Total	2517.5	1157	331	438.5	4444

Table 6.22: Longitudinal Study - Calculation of period of animal observation from surveys recorded in a longitudinal study conducted in Kiambu District from July 1991 - June 1992; subdivided on the basis of i) the observed method of application and ii) the maximum potential frequency of application of tick control (as derived from inter-application intervals and reclassified under liberal criteria) by iii) the classification of the animal with respect to age (0-12 months of age = calf, > 12 months = adult) *at the time of each observation/survey*:

Observed Method of Application of Tick Control	Derived Monthly Frequency of Application of Tick Control under Liberal Reclassification Criteria[1]				Total
	Weekly	Fortnightly	≤ Monthly	None	
		ADULT CATTLE			
Plunge Dipping	722	193	150	-	1065
Spraying	544	1025	645.5	-	2214.5
Hand Applied	76.5	0	0	-	76.5
None	-	-	-	100.5	100.5
Total	1342.5	1218	795.5	100.5	3456.5
		CALVES			
Plunge Dipping	177	60	64.5	-	301.5
Spraying	201	299.5	148	-	648.5
Hand Applied	14	0	0	-	14
None	-	-	-	23.5	23.5
Total	392	359.5	212.5	23.5	987.5
		ALL ANIMALS			
Plunge Dipping	899	253	214.5	-	1366.5
Spraying	745	1324.5	793.5	-	2863
Hand Applied	90.5	0	0	-	90.5
None	-	-	-	124	124
Total	1734.5	1577.5	1008	124	4444

[1] - Farm reclassification by an iterative process of reduction in frequency of application category until 33.3% of observed inter-application intervals were consistent.

Table 6.23: Longitudinal Study - Calculation of period of animal observation from surveys recorded in a longitudinal study conducted in Kiambu District from July 1991 - June 1992; subdivided on the basis of i) the observed method of application and ii) the maximum potential frequency of application of tick control (as derived from inter-application intervals and reclassified under conservative criteria) by iii) the classification of the animal with respect to age (0-12 months of age = calf, > 12 months = adult) *at the time of each observation/survey*:

Observed Method of Application of Tick Control	Derived Monthly Frequency of Application of Tick Control under Conservative Reclassification Criteria[1]				Total
	Weekly	Fortnightly	≤ Monthly	None	
		ADULT CATTLE			
Plunge Dipping	629	93	343	-	1065
Spraying	506.5	707	1001	-	2214.5
Hand Applied	24	52.5	0	-	76.5
None	-	-	-	100.5	100.5
Total	1159.5	852.5	1344	100.5	3456.5
		CALVES			
Plunge Dipping	155	22	124.5	-	301.5
Spraying	190.5	210	248	-	648.5
Hand Applied	0	14	0	-	14
None	-	-	-	23.5	23.5
Total	345.5	246	372.5	23.5	987.5
		ALL ANIMALS			
Plunge Dipping	784	115	467.5	-	1366.5
Spraying	697	917	1249	-	2863
Hand Applied	24	66.5	0	-	90.5
None	-	-	-	124	124
Total	1505	1098.5	1716.5	124	4444

[1] - Farm reclassification by an iterative process of reduction in frequency of application category until 66.7% of observed inter-application intervals were consistent.

Table 6.24: Longitudinal Study - Morbidity incidence rates of East Coast Fever in cattle by reported method and frequency of application of tick control recorded in Kiambu District from July 1991 - June 1992 and expressed as number of cases per 100 years of observation by age-classification of animals. Crude numbers from which rates are determined are presented beside the rates in square brackets and include mortalities while approximate 95% confidence intervals are presented below the rates.

Reported Method of Application of Tick Control	Reported Frequency of Application of Tick Control				Total
	Weekly	Fortnightly	≤ Monthly	None	
		ADULT CATTLE			
Plunge Dipping	6.1 [6] (2.8-13.2)	58.5 [1] (10.3-331)	54.6 [1] (9.6-309)	-	7.8 [8] (3.9-15.4)
Spraying	6.6 [4] (2.6-17.0)	16.3 [12] (9.3-28.3)	6.0 [1] (1.1-34.2)	-	11.3 [17] (7.0-18.0)
Hand Applied	0 (0-192)	-	0 (0-87.8)	-	0 (0-60.2)
None	-	-	-	7.1 [2] (2.0-26.0)	7.1 [2] (2.0-26.0)
Total	6.2 [10] (3.4-11.4)	15.9 [13] (10.1-29.5)	8.8 [2] (2.4-32.1)	7.1 [2] (2.0-26.0)	9.4 [27] (6.4-13.6)
		CALVES			
Plunge Dipping	10.7 [3] (3.6-31.4)	0 (0-1150)	0 (0-2300)	-	10.5 [3] (3.6-30.8)
Spraying	0 (0-192)	24.3 [5] (10.4-56.9)	0 (0-110)	-	11.4 [5] (4.8-26.6)
Hand Applied	-	-	85.7 [1] (15.1-486)	-	85.7 [1] (15.1-486)
None	-	-	-	0 (0-45.4)	0 (0-45.4)
Total	6.2 [3] (2.1-18.3)	23.9 [5] (10.2-56.0)	20.7 [1] (3.6-117)	0 (0-45.4)	10.9 [9] (5.8-20.8)
		ALL ANIMALS			
Plunge Dipping	7.1 [9] (3.7-13.4)	49.0 [1] (8.6-277)	50.0 [1] (8.8-283)	-	8.4 [11] (4.7-15.0)
Spraying	5.0 [4] (1.9-12.8)	18.0 [17] (11.2-28.8)	5.0 [1] (0.9-28.3)	-	11.3 [22] (7.5-17.1)
Hand Applied	0 (0-192)	-	18.0 [1] (3.2-102)	-	13.3 [1] (2.3-75.1)
None	-	-	-	5.5 [2] (1.5-20.0)	5.5 [2] (1.5-20.0)
Total	6.2 [13] (3.6-10.6)	18.7 [18] (11.8-29.5)	10.9 [3] (3.7-32.0)	5.5 [2] (1.5-20.0)	9.7 [36] (7.0-13.5)

Table 6.25: Longitudinal Study - Morbidity incidence rates of East Coast Fever in cattle by observed method and frequency of application of tick control (as reclassified under liberal criteria) recorded in Kiambu District from July 1991 - June 1992 and expressed as number of cases per 100 years of observation by age-classification of animals. Crude numbers from which rates are determined are presented beside the rates in square brackets and include mortalities while approximate 95% confidence intervals are presented below the rates.

Observed Method of Application of Tick Control	Derived Monthly Frequency of Application of Tick Control under Liberal Reclassification Criteria[1]				Total
	Weekly	Fortnightly	≤ Monthly	None	
		ADULT CATTLE			
Plunge Dipping	6.7 [4] (2.6-17.1)	6.2 [1] (1.1-35.2)	16.0 [2] (4.4-58.3)	-	7.9 [7] (3.8-16.3)
Spraying	8.8 [4] (3.4-22.7)	15.2 [13] (8.9-26.0)	3.7 [2] (1.0-13.6)	-	10.3 [19] (6.6-16.1)
Hand Applied	0 (0-60.2)	-	-	-	0 (0-60.2)
None	-	-	-	11.9 [1] (2.1-67.6)	11.9 [1] (2.1-67.6)
Total	7.2 [8] (3.6-14.1)	13.8 [14] (8.2-23.2)	6.0 [4] (2.3-15.5)	11.9 [1] (2.1-67.6)	9.4 [27] (6.4-13.6)
		CALVES			
Plunge Dipping	20.3 [3] (6.9-59.8)	0 (0-76.8)	0 (0-71.5)	-	11.0 [3] (4.1-35.1)
Spraying	0 (0-22.9)	20.0 [5] (8.6-46.9)	0 (0-31.1)	-	9.2 [5] (4.0-21.7)
Hand Applied	85.7 [1] (15.1-486)	-	-	-	85.7 [1] (15.1-486)
None	-	-	-	0 (0-196)	0 (0-196)
Total	12.2 [4] (4.8-31.4)	16.7 [5] (7.1-39.1)	0 (0-21.7)	0 (0-196)	10.9 [9] (5.8-20.8)
		ALL ANIMALS			
Plunge Dipping	9.3 [7] (4.5-19.3)	4.7 [1] (0.8-26.9)	11.2 [2] (3.1-40.8)	-	8.8 [10] (4.8-16.2)
Spraying	6.4 [4] (2.5-16.6)	16.3 [18] (10.3-25.8)	3.0 [2] (0.8-11.0)	-	10.1 [24] (6.8-15.0)
Hand Applied	13.3 [1] (2.3-75.1)	-	-	-	13.3 [1] (2.3-75.1)
None	-	-	-	9.7 [1] (1.7-54.8)	9.7 [1] (1.7-54.8)
Total	8.3 [12] (4.7-14.5)	14.4 [19] (9.3-22.6)	4.8 [4] (1.9-12.2)	9.7 [1] (1.7-54.8)	9.7 [36] (7.0-13.5)

[1] - Farm reclassification by an iterative process of reduction in frequency of application category until 33.3% of observed inter-application intervals were consistent.

Table 6.26: Longitudinal Study - Morbidity incidence rates of East Coast Fever in cattle by observed method and frequency of application of tick control (as reclassified under conservative criteria) recorded in Kiambu District from July 1991 - June 1992 and expressed as number of cases per 100 years of observation by age-classification of animals. Crude numbers from which rates are determined are presented beside the rates in square brackets and include mortalities while approximate 95% confidence intervals are presented below the rates.

Observed Method of Application of Tick Control	Derived Monthly Frequency of Application of Tick Control under Conservative Reclassification Criteria[1]				Total
	Weekly	Fortnightly	≤ Monthly	None	
ADULT CATTLE					
Plunge Dipping	7.6 [4] (3.0-19.6)	0 (0-49.6)	10.5 [3] (3.6-30.9)	-	7.9 [7] (3.8-16.3)
Spraying	9.5 [4] (3.7-24.4)	20.4 [12] (11.7-35.6)	3.6 [3] (1.2-10.6)	-	10.3 [19] (6.6-16.1)
Hand Applied	0 (0-192)	0 (0-87.8)	-	-	0 (0-60.2)
None	-	-	-	11.9 [1] (2.1-67.6)	11.9 [1] (2.1-67.6)
Total	8.3 [8] (4.2-16.3)	16.9 [12] (9.7-29.5)	5.4 [6] (2.5-11.7)	11.9 [1] (2.1-67.6)	9.4 [27] (6.4-13.6)
CALVES					
Plunge Dipping	23.2 [3] (7.9-68.3)	0 (0-209)	0 (0-37.0)	-	11.0 [3] (4.1-35.1)
Spraying	0	22.9 [4] (8.9-58.8)	4.8 [1] (0.9-27.4)	-	9.2 [5] (4.0-21.7)
Hand Applied	-	85.7 [1] (15.1-486)	-	-	85.7 [1] (15.1-486)
None	-	-	-	0 (0-196)	0 (0-196)
Total	10.4 [3] (3.5-30.6)	24.4 [5] (10.4-57.1)	3.2 [1] (0.6-18.2)	0 (0-196)	10.9 [9] (5.8-20.8)
ALL ANIMALS					
Plunge Dipping	10.7 [7] (5.2-22.1)	0 (0-40.1)	7.7 [3] (2.6-22.6)	-	8.8 [10] (4.8-16.2)
Spraying	6.9 [4] (2.7-17.7)	20.9 [16] (12.9-34.0)	3.8 [4] (1.5-9.9)	-	10.1 [24] (6.8-15.0)
Hand Applied	0 (0-192)	18.0 [1] (3.2-102)	-	-	13.3 [1] (2.3-75.1)
None	-	-	-	9.7 [1] (1.7-54.8)	9.7 [1] (1.7-54.8)
Total	8.8 [11] (4.9-15.7)	18.6 [17] (11.6-29.7)	4.9 [7] (2.4-10.1)	9.7 [1] (1.7-54.8)	9.7 [36] (7.0-13.5)

[1] - Farm reclassification by an iterative process of reduction in frequency of application category until 66.7% of observed inter-application intervals were consistent.

Table 6.27: Longitudinal Study - Incidence rates of seroconversion and sero-events by reported method and frequency of application of tick control recorded in Kiambu District from July 1991 - June 1992 and expressed as number of cases per 100 years of observation by age-classification of animals. Crude numbers of events from which rates are determined are presented beside the rates in square brackets as number of events without clinical signs + number of events with concomitant clinical signs while approximate 95% confidence intervals are presented below the rates.

Reported Method of Application of Tick Control	Reported Frequency of Application of Tick Control				Total
	Weekly	Fortnightly	≤ Monthly	None	
TOTAL: Seroconversion + Serological Increase >0.200 OD Units					
Plunge Dipping	16.5 [16+5] (10.8-25.2)	0 (0-188)	100 [1+1] (27.4-365)	-	17.5 [17+6] (11.7-26.3)
Spraying	29.8 [20+4] (20.0-44.3)	37.9 [27+8] (26.7-51.6)	25.0 [5] (10.7-58.4)	-	32.8 [52+12] (25.7-41.2)
Hand Applied	50.0 [1] (8.8-283)	-	90.2 [4+1] (38.5-211)	-	79.6 [5+1] (36.5-174)
None	-	-	-	11.0 [4] (4.3-28.1)	11.0 [4] (4.3-28.1)
Total	21.9 [37+9] (16.4-29.2)	36.0 [27+8] (26.1-50.5)	43.5 [10+2] (24.9-76.0)	11.0 [4] (4.3-28.1)	26.2 [78+19] (21.5-31.9)
ADULT CATTLE: Serological Increase >0.200 OD Units					
Plunge Dipping	8.1 [5+3] (4.1-15.9)	0 (0-225)	109 [1+1] (29.9-398)	-	9.7 [6+4] (5.3-17.9)
Spraying	19.8 [8+4] (11.3-34.6)	28.5 [17+4] (18.6-43.5)	18.1 [3] (6.2-53.3)	-	23.8 [28+8] (17.2-33.0)
Hand Applied	50.0 [1] (8.8-283)	-	68.6 [3] (23.3-202)	-	62.8 [4] (24.4-161)
None	-	-	-	10.7 [3] (3.6-31.4)	10.7 [3] (3.6-31.4)
Total	13.0 [14+7] (8.5-19.9)	27.8 [17+4] (18.2-42.5)	35.2 [7+1] (17.8-69.4)	10.7 [3] (3.6-31.4)	18.4 [41+12] (14.1-24.1)
CALVES: Seroconversion + Seroevents					
Plunge Dipping	46.2 [11+2] (27.0-79.1)	0 (0-1150)	0 (0-2300)	-	45.4 [11+2] (26.5-77.7)
Spraying	60.1 [12] (34.4-105)	68.0 [10+4] (40.5-114)	57.1 [2] (15.7-208)	-	63.6 [24+4] (44.0-91.9)
Hand Applied	-	-	171 [1+1] (47.0-625)	-	171 [1+1] (47.0-625)
None	-	-	-	11.8 [1] (2.1-67.0)	11.8 [1] (2.1-67.0)
Total	52.0 [23+2] (35.2-76.8)	66.9 [10+4] (40.0-112)	82.8 [3+1] (32.2-213)	11.8 [1] (2.1-67.0)	53.5 [37+7] (39.8-71.8)

Table 6.28: Longitudinal Study - Incidence rates of seroconversion and sero-events by observed method and frequency of application of tick control (as reclassified under liberal criteria) recorded in Kiambu District from July 1991 - June 1992 and expressed as number of cases per 100 years of observation by age-classification of animals. Crude numbers of events from which rates are determined are presented beside the rates in square brackets as number of events without clinical signs + number of events with concomitant clinical signs while approximate 95% confidence intervals are presented below the rates.

Observed Method of Application of Tick Control	Derived Monthly Frequency of Application of Tick Control under Liberal Reclassification Criteria[1]				Total
	Weekly	Fortnightly	≤ Monthly	None	
TOTAL: Seroconversion + Serological Increase >0.200 OD Units					
Plunge Dipping	21.4 [12+4] (13.1-34.7)	9.5 [2] (2.6-34.6)	28.0 [3+2] (11.9-65.5)	-	20.2 [17+6] (13.5-30.3)
Spraying	30.6 [15+4] (19.6-47.8)	35.3 [31+8] (25.8-48.3)	13.6 [9] (7.2-25.9)	-	28.1 [55+12] (25.8-40.3)
Hand Applied	79.6 [5+1] (36.5-174)	-	-	-	79.6 [5+1] (36.5-174)
None	-	-	-	9.7 [1] (1.7-54.8)	9.7 [1] (1.7-54.8)
Total	28.4 [32+9] (20.9-38.5)	34.9 [33+8] (23.0-42.3)	16.7 [12+2] (9.9-28.0)	9.7 [1] (1.7-54.8)	26.2 [78+19] (21.5-31.9)
ADULT CATTLE: Serological Increase >0.200 OD Units					
Plunge Dipping	10.0 [4+2] (4.6-21.8)	6.2 [1] (1.1-35.2)	24.0 [1+2] (8.2-70.6)	-	11.3 [6+4] (6.1-20.7)
Spraying	19.8 [5+4] (10.4-37.7)	26.9 [19+4] (17.9-40.4)	11.2 [6] (5.1-24.3)	-	20.6 [30+8] (15.0-28.3)
Hand Applied	62.8 [4] (24.4-161)	-	-		62.8 [4] (24.4-161)
None	-	-	-	11.9 [1] (2.1-67.6)	11.9 [1] (2.1-67.6)
Total	17.0 [13+6] (10.9-26.5)	23.6 [20+4] (15.9-35.2)	13.6 [7+2] (7.1-25.8)	11.9 [1] (2.1-67.6)	18.4 [41+12] (14.1-24.1)
CALVES: Seroconversion + Seroevents					
Plunge Dipping	67.8 [8+2] (36.8-125)	20.0 [1] (3.5-113)	37.2 [2] (10.2-136)	-	51.7 [11+2] (30.2-88.5)
Spraying	59.7 [10] (32.4-110)	64.1 [12+4] (39.5-104)	24.3 [3] (8.3-71.5)	-	53.7 [25+4] (37.4-77.1)
Hand Applied	171 [1+1] (47.0-625)	-	-	-	171 [1+1] (47.0-625)
None	-	-	-	0 (0-196)	0 (0-196)
Total	67.4 [19+3] (44.5-102)	56.8 [13+4] (35.4-90.9)	28.2 [5] (12.1-66.1)	0 (0-196)	53.5 [37+7] (39.8-71.8)

[1] - Farm reclassification by an iterative process of reduction in frequency of application category until 33.3% of observed inter-application intervals were consistent.

Table 6.29: Longitudinal Study - Incidence rates of seroconversion and sero-events by observed method and frequency of application of tick control (as reclassified under conservative criteria) recorded in Kiambu District from July 1991 - June 1992 and expressed as number of cases per 100 years of observation by age-classification of animals. Crude numbers of events from which rates are determined are presented below the rate in square brackets as number of events without clinical signs + number of events with concomitant clinical signs while approximate 95% confidence intervals are presented below the rates.

Observed Method of Application of Tick Control	Derived Monthly Frequency of Application of Tick Control under Conservative Reclassification Criteria[1]				Total
	Weekly	Fortnightly	≤Monthly	None	
TOTAL: Seroconversion + Serological Increase >0.200 OD Units					
Plunge Dipping	23.0 [11+4] (13.9-37.9)	10.4 [1] (1.8-59.1)	18.0 [5+2] (8.7-37.1)	-	20.2 [17+6] (13.5-30.3)
Spraying	32.7 [15+4] (20.9-51.1)	38.0 [22+7] (26.9-54.5)	18.2 [18+1] (11.7-28.5)	-	28.1 [55+12] (25.8-40.3)
Hand Applied	50.0 [1] (8.8-283)	90.2 [4+1] (38.5-211)	-	-	79.6 [5+1] (36.5-174)
None	-	-	-	9.7 [1] (1.7-54.8)	9.7 [1] (1.7-54.8)
Total	27.9 [27+8] (20.1-38.8)	38.2 [27+8] (27.5-53.2)	18.2 [23+3] (12.4-26.6)	9.7 [1] (1.7-54.8)	26.2 [78+19] (21.5-31.9)
ADULT CATTLE: Serological Increase >0.200 OD Units					
Plunge Dipping	11.4 [4+2] (5.2-25.0)	0 (0-49.6)	14.0 [2+2] (5.4-36.0)	-	11.3 [6+4] (6.1-20.7)
Spraying	21.3 [5+4] (11.2-40.5)	30.6 [14+4] (19.3-48.3)	13.2 [11] (7.4-23.6)	-	20.6 [30+9] (15.0-28.3)
Hand Applied	50.0 [1] (8.8-283)	68.6 [3] (23.3-202)	-	-	62.8 [4] (24.4-161)
None	-	-	-	11.9 [1] (2.1-67.6)	11.9 [1] (2.1-67.6)
Total	16.6 [10+6] (10.2-26.9)	29.6 [17+4] (19.3-45.2)	13.4 [13+2] (8.1-22.1)	11.9 [1] (2.1-67.6)	18.4 [41+12] (14.1-24.1)
CALVES: Seroconversion + Seroevents					
Plunge Dipping	69.7 [7+2] (36.7-132)	54.6 [1] (9.6-309)	28.9 [3] (9.8-85.0)	-	51.7 [11+2] (30.2-88.5)
Spraying	63.0 [10] (31.2-116)	62.9 [8+3] (35.1-112)	38.7 [7+1] (19.6-76.4)	-	53.7 [25+4] (37.4-77.1)
Hand Applied	-	171 [1+1] (47.0-625)	-	-	171 [1+1] (47.0-625)
None	-	-	-	0 (0-196)	0 (0-196)
Total	66.0 [17+2] (42.2-103)	68.3 [10+4] (40.7-115)	35.4 [10+1] (19.8-63.5)	0 (0-196)	53.5 [37+7] (39.8-71.8)

[1] - Farm reclassification by an iterative process of reduction in frequency of application category until 66.7% of observed inter-application intervals were consistent.

Figure 6.30: Longitudinal Study - Comparison of frequency of application of tick control reported on initial farm visit and number of monthly applications of tick control for "adult" cattle and calves as derived using liberal or conservative farm classifications and inter-application intervals observed in a longitudinal study conducted in Kiambu District from July 1991 - June 1992.

Derived Monthly Frequency of Application of Tick Control[3]		Reported Frequency of Application of Tick Control				Totals[2]
		Weekly	Fortnighly	≤ Monthly[1]	None	
Liberal Classification Criteria[3]						
4 (Weekly)	Adult Obs.	**1045**	0	*27*	*0*	1072
	Calf Obs.	**163**	0	*4*	*0*	167
2 (Fortnightly)	Adult Obs.	*320*	**663**	*23*	*0*	1006
	Calf Obs.	*53*	**117**	*7*	*0*	177
≤ 1 (≤ Monthly[1])	Adult Obs.	*313*	*151*	**166**	*21*	651
	Calf Obs.	*22*	*18*	**13**	*0*	53
0 (None)	Adult Obs.	*220*	*79*	*57*	**315**	671
	Calf Obs.	*371*	*124*	*36*	**105**	636
Conservative Classification Criteria[3]						
4 (Weekly)	Adult Obs.	**980**	*0*	*0*	*0*	980
	Calf Obs.	**158**	*0*	*0*	*0*	158
2 (Fortnightly)	Adult Obs.	*247*	**556**	*50*	*0*	853
	Calf Obs.	*49*	**104**	*11*	*0*	164
≤ 1 (≤ Monthly[1])	Adult Obs.	*451*	*258*	**166**	*21*	896
	Calf Obs.	*31*	*31*	**13**	*0*	75
0 (None)	Adult Obs.	*220*	*79*	*57*	**315**	671
	Calf Obs.	*371*	*124*	*36*	**105**	636
Totals[2]	Adult Obs.	1898	893	273	336	3400
	Calf Obs.	609	259	60	105	1033

[1] - "≤ Monthly" refers to tick control practised monthly or less frequently.
[2] - "Total" refers to the total number of initial and follow-up surveys recorded at which an animal was present and hence a tick application interval reported.
[3] - Farm reclassification by an iterative process of reduction in frequency of application category until 33.3% (= liberal criteria) or 66.7% (= conservative criteria) of observed inter-application intervals were consistent.

Figure 6.31: Longitudinal Study - Comparison of number of monthly applications of tick control for "adult" cattle and calves as derived using liberal and conservative farm classifications and inter-application intervals observed in a longitudinal study conducted in Kiambu District from July 1991 - June 1992.

Derived Monthly Frequency of Application of Tick Control under Liberal Reclassification Criteria[3]		Derived Monthly Frequency of Application of Tick Control under Conservative Reclassification Criteria[3]				Totals[2]
		4 (Weekly)	2 (Fortnightly)	≤ 1 (≤ Monthly)	0 (None)	
4 (Weekly)	Farms	**23**	*6*	*0*	*0*	29
	Adult Obs.	**980**	*92*	-	-	1072
	Calf Obs.	**158**	*9*	-	-	167
2 (Fortnightly)	Farms	*0*	**13**	*21*	*0*	34
	Adult Obs.	-	**761**	*245*	-	1006
	Calf Obs.	-	**155**	*22*	-	177
≤ 1 (≤ Monthly)	Farms	*0*	*0*	**24**	*0*	24
	Adult Obs.	-	-	**651**	-	651
	Calf Obs.	-	-	**53**	-	53
0 (None)	Farms	*0*	*0*	*0*	**3**	3
	Adult Obs.	-	-	-	**671**	671
	Calf Obs	-	-	-	**636**	636
Totals[2]	Farms	23	19	45	3	90
	Adult Obs.	980	853	896	671	3400
	Calf Obs.	158	164	75	636	1033

[1] - "≤ Monthly" refers to tick control practised monthly or less frequently.
[2] - "Total" refers to the number of initial and follow-up surveys recorded at which an animal was present and hence a tick application interval reported.
[3] - Farm reclassification by an iterative process of reduction in frequency of application category until 33.3% (= liberal criteria) or 66.7% (= conservative criteria) of observed inter-application intervals were consistent.

CHAPTER 7

MULTI-LEVEL MODELS OF SEROLOGICAL DATA

7.1 Introduction

The objective of this chapter is to explore the relationships between serological data and a variety of farm, animal and observation (i.e. time) level variables derived from a hierarchically structured population through the development of multi-level generalised linear mixed statistical models. Serological data include both measures of the amount of *T. parva*-specific antibody and a dichotomous assessment of test results as positive or negative. Age-profile relationships of these serological measures as dependent variables by combinations of hypothesized risk factors are assessed as a surrogate method of investigating potential differences in the level of infection challenge.

It is the rule rather than the exception that data from observational studies are derived from a hierarchically structured population. The existence of different levels of aggregation is neither accidental nor can it be ignored. At the simplest level, in the veterinary context, animals are grouped within farms and subject to "farm-effects". Animals within a group are not independent of other group members whom they both influence and are influenced by. This is particularly true in the case of infectious diseases where the per-susceptible risk of infection is dependent on the prevalence of infectious and immune individuals, the so-called "herd effect" (Anderson and May, 1991). The consequences of ignoring such clustering of observations may be to overlook the importance of the effects of aggregation and to render

invalid statistical inference from traditional techniques based on the assumptions of independence. Although researchers have long recognised this issue, only recently have developments in "multilevel modelling" made available powerful statistical techniques for analysing data with multiple levels of aggregation.

7.2 Dependent Variables

Longitudinal serological data yielded two main outcomes of interest:

1) Post-targeting adjusted optical density (OD) values, as a surrogate measure of the amount of *T. parva*-specific antibody present.

2) Serological status, based on dichotomisation of the above into negative and positive results, i.e. values below or above the negative cut-off (0.125 OD units), respectively.

Models with the adjusted OD values as the dependent variable attempted to estimate the relationship between independent variables and the amount of antibody present, while those using a binary transformed outcome sought to assess risk factors associated with an increased likelihood of possessing antibodies at a level beyond a given threshold, the negative cut-off. Although there was a degree of correlation between the two outcomes, each provided a different emphasis across the scale of measurement resolution. For example, a relatively small, inconsequential increase in OD units, but across the negative threshold value (e.g. 0.100 OD units to 0.150 OD units), results in a change in test classification from negative to positive, while a much larger, and hence more important change, occurring above the threshold (e.g. 0.200 OD units to 0.900 OD units) would have no effect on an already positive classification. Thus, the two models need not necessarily have yielded the same set

of risk factors or statistically significant covariates.

Estimates from generalised linear multilevel mixed models of continuous variables are based on the assumptions of multivariate normality and homoscedasticity (McCullagh and Nelder, 1989). Although the presence of a skewed or kurtotic outcome variable is not necessarily synonymous with a non-normal model error distribution, it suggests that a normalising transformation of the data may be indicated by subsequent model evaluation. Figure 7.1A presents a histogram of adjusted OD units on which a normal distribution of equivalent mean and standard deviation has been superimposed, demonstrating both a pronounced skewness to the right (skewness coefficient = 1.74) and severe leptokurtosis (coefficient of kurtosis = 2.25). Figure 7.1B shows the normalising effect of taking the natural log transformation of the adjusted OD values where the post-transformation frequency distribution is virtually free of skewness (0.014) and kurtosis (0.145), suggesting this would be an appropriate transformation in the event of failure of model assumptions (see section *7.6 Models of Adjusted Optical Density Units*). However, the effect of such a transformation on model interpretation and emphasis also needs to be considered.

Figure 7.2 displays the relationship between adjusted and transformed OD values over the interval of observations. For non-transformed adjusted OD values, 45.5% are below the negative cut-off level of 0.125 (Table 7.1) but they cover 11.1% of the observed OD interval (0.125/1.126). In contrast, for the natural log transformed OD values, "negative" observations span 68.7% (4.829/7.028) of the post-transformation interval of values (Table 7.1). With this transformation, the uninterpretable differences in "negative antibody levels" exert an undue influence on model parameter estimation. In effect, the model for the

transformed variable will be reduced in its ability to assess covariate relationships across the interval of positive test results and would become more similar to the model of discrete outcomes in its interpretation.

In the case of multilevel models, even if the normality assumption fails to hold, the parameter estimates are consistent but not fully efficient, although the standard error estimates and corresponding confidence intervals will not generally be consistent (Goldstein, 1995). Thus, while models based on the natural log transformed OD values may provide a superior framework through which to make statements of parameter significance, the interpretation of estimates from models of untransformed adjusted OD values is more intuitive and meaningful.

7.3 Independent Variables

Continuous and categorical variables created from observations made at the farm, animal and observation level and thought to have a plausible biological association with the level of exposure of the vector and host or to affect the level of *T. parva*-specific antibodies in a host, were assessed for significance in statistical models of serological outcomes. Examples include agro-ecological zone of origin and grazing management system at the farm level, gender and breed at the animal level, and age and time at the observation level. In some cases, variables could be assessed at different levels and for different combinations. For example, frequency of application of tick control was assessed at the farm-level for recorded *vs* reclassified management categories (see section *6.3.2 Farm Reclassification*) and at the observation-level as a time-varying covariate (see section *6.5 Derivation of Time-*

varying Frequency of Application Covariates). Although the number of standard ticks (Norval *et al.*, 1992) and the presence of smaller ticks were measured at the observation level and assessed directly through the use of a variety of lag-effect/rolling average combinations, these covariates were also assessed at the animal and farm level by summary statistics and cumulative measures. Table 7.2 provides a non-exhaustive list of the main independent variables investigated.

7.4 Model Building and Parameter Estimation

All models were fitted using MLwiN software and its predecessor, MLn software (Multilevel Models Project, Institute of Education, University of London, London, UK).

Owing to the clear hierarchical structure of the data, with longitudinal observations made on animals housed within farms, three corresponding levels of random effects were incorporated within each model to account for effects of clustering (McDermott, Schukken and Shoukri, 1994; McDermott *et al.*, 1997). Models were first developed with single random-effect parameter estimates at each level, reflecting a simple variance structure and later, where possible, more complex variance structures were modelled as a function of explanatory variables (Goldstein, 1995). All model building and refinement was undertaken manually. Choice of independent variables to be included in models as either fixed or random effects was based first on biological plausibility of association and subsequently on statistical significance. No specific algorithms or selection procedures were utilised. Beginning with a simple three-level variance-components-only model that incorporated a grand-mean intercept, model development proceeded by the addition and testing of fixed-

effect parameters as described in section *6.3* (*Independent Variables*) followed by combinations of random-effect variables.

7.4.1 Continuous Dependent Variables

Adjusted OD values and natural log transformed OD values were each modelled directly as a continuous dependent variable. Model parameters were first estimated using an iterative process of fixed- and random-effect (residual) estimation beginning with ordinary-least-squares estimates and proceeding until convergence was achieved (i.e. the estimates for all parameters did not change). This process is known as Iterative Generalised Least Squares (IGLS) and yields maximum-likelihood estimates when the residuals have normal distributions (Goldstein, 1986). However, since this process takes no account of the sampling variation of the fixed parameters, the maximum-likelihood estimates produced for the random parameters are biased, particularly when sample size is small. Thus, model estimates were refined by an adapted process known as Restricted Iterative Generalised Least Squares (RIGLS) or Restricted Maximum Likelihood (REML) which yield unbiased estimates under the assumption of multivariate normality (Goldstein, 1989). Details of the computational methods employed in MLwiN RIGLS estimation are supplied by Goldstein and Rasbash (1992).

Individual fixed-effect terms and groups of dummy variables for categorical variables were tested for significance in nested models by a likelihood-ratio test based on change in deviance (McCullagh and Nelder, 1989). Given the nature of the outcome of interest and the longitudinal structure of repeated observations, i.e. as measurement occasions within each

animal, the effective aim of each model was to fit the pattern of antibody level or presence over age/time (maternal antibody decline, development of new infections and re-infections) for each animal (Goldstein, 1979). Particular attention was paid to variables demonstrated to be associated with prevalence of antibody to *T. parva*, such as agro-ecological zone (Deem *et al.*, 1993; Gitau *et al.*, 1997) and grazing management system (Moll *et al.*, 1986; Maloo *et al.*, 1994; Gitau, 1998). Specifically, the potential for these variables to affect the shape and position of the antibody curve was accounted for by their incorporation in the models, first as simple fixed effects (different intercept but parallel slopes) and then as interaction terms with age (different slopes). In addition, a similar set of age covariates was included in all models to acknowledge the difference in dynamics of antibodies acquired by maternal transfer to newborns (Burridge and Kimber, 1973a; Gitau, 1998; Mining *et al.*, 1998). Finally, complex variation was modelled as differences in farm-level variance by grazing management system and within the animal level as variable slopes over time in the study, based on the seroconversion and sero-event classification of the animal (see section *5.4.2 Definitions of Seroconversion and "Sero-Event"*).

Although the preliminary set of models, developed as described above, accounted for clustering of animals within farms, they made the implicit assumption that level-1 residuals (i.e. departures of the longitudinal measures made within the same animal from the animals underlying antibody curve) were independent. In light of the long persistence of antibodies relative to the sampling interval (Burridge and Kimber, 1973b), there is likely to be autocorrelation between the residuals. To account for this fact, a correlation component (Yang, Rasbash and Goldstein, 1998) was added to the models whereby the covariance

between level-1 residuals was modelled as a negative exponential function of the difference in continuous time between measurements such that, with increasing time difference the covariance tends toward a fixed value, usually assumed to be zero (Goldstein, Healy and Rasbash, 1994). The covariance between level-1 residuals was estimated in its simplest form by:

$$cov(e_t, e_{t-s}) = \sigma_e^2 * e^{-\alpha s}$$

where:

σ_e^2 is the error variance

s is the time difference between measurements

α is the covariance parameter

This correlation-adjustment procedure added the single parameter α to the model, allowing an assessment of its significance by change in deviance. Further, since all intervals between observations in the data set were fixed and equivalent, the level-1 correlation matrix is described by a first-order autoregressive (AR-1) series such that the correlation between adjacent observations (i.e. separated by a single unit of time) was estimated by $e^{-\alpha}$ = the correlation coefficient ρ. The value of this coefficient gives an indication of the appropriateness of an assumption of independence of longitudinal antibody measures.

7.4.2 Discrete Dependent Variable

Binary (negative/positive) classifications were modelled as proportions using the logit link function under the assumption of a binomial distribution of responses. Model

parameters were first estimated using RIGLS in a marginal quasi-likelihood (MQL) method with a first-order approximation of the Taylor-series expansion and level-1 variance constrained to the binomial assumption (Goldstein, 1991; Breslow and Clayton, 1993). While this combination of methods is simpler, computationally faster and more likely to reach convergence than other methods of estimation (Goldstein *et al.*, 1998), it tends to underestimate fixed and random effect parameters when there are few level-1 units per level-2 unit or where higher level variances are large (Goldstein and Rasbash, 1996). Subsequently, each model was also estimated using RIGLS in a predicate (or penalised) quasi-likelihood (PQL) process with a second-order approximation of the Taylor-series expansion and with level-1 variance constrained to the binomial assumption and also estimating an extra-binomial variance parameter (Goldstein, 1995). Further, with binary (0,1) data the likelihood-ratio test statistic is considered unreliable (Goldstein, 1995) and so approximate (Wald) chi-square tests were employed for hypothesis testing of linear combinations of fixed and random effect parameters, thus assessing significance to model fit (Goldstein *et al.*, 1998). Unlike the method of Generalized Estimating Equations (GEE) of Liang and Zeger (1986), MLwiN was unable to incorporate a correlation component for models of discrete outcomes.

In general, quasi-likelihood iterative estimation methods experience a variable degree of bias towards the null for both random- and fixed-effect parameter estimates (Goldstein, 1995). Second-order PQL estimates are considered the least biased of the quasi-likelihood methods (Goldstein and Rasbash, 1996), but in some cases failed to converge (Goldstein *et al.*, 1998). Thus, although considered to still be in the developmental stage (Goldstein *et al.*,

1998), a full-Bayesian approach to fitting the discrete models was undertaken using Metropolis-Hastings sampling in a Markov Chain Monte Carlo (MCMC) process (Gilks *et al.*, 1993; Gilks, Richardson and Spiegelhalter, 1996). In general, MCMC estimation takes account of the uncertainty associated with the estimates of the random parameters and hence, unlike other methods, does not overestimate the model precision (Goldstein, 1995). Specifically, the MLwiN process utilised the adaptive method of specifying the proposal distribution with acceptance rate of 50% and a tolerance of 10%. Diffuse priors were specified and a burn-in of 500 iterations was followed by a monitoring period of 500,000 iterations for the variance components only model and 1,000,000 iterations for other models, each with a thinning factor of 25 (i.e. every 25th value stored). Significance of MCMC parameter estimates was assessed by 95% confidence intervals derived from kernel-density traces of the posterior distributions (Goldstein *et al.*, 1998). Although a parametric bootstrap estimation method (Efron and Tibshirani, 1993), which can also reduce the uncertainty and prove efficient in correcting the downward bias of PQL parameter estimates, was available in MLwiN, owing to the failure of some models to converge under PQL methods, it was not employed.

7.5 Checking Model Assumptions

For each multilevel model, adequacy of assumptions was assessed by examination of residuals. Standardised residuals were calculated for random-effect parameters at each level by dividing their raw residuals by the appropriate standard errors. These "diagnostic" residuals were plotted against their equivalent normal scores and against the fixed-

component predicted value. Efficient techniques for influence analysis are not yet available for multilevel models (Goldstein, 1995).

7.6 Models of Adjusted Optical Density Units

7.6.1 Variance Components

Table 7.3A presents parameter estimates from the three-level variance-components-only model of adjusted OD values showing that, when the autocorrelation of observation-level errors is ignored, the farm-level variance accounts for 48.0% (0.028/0.0583) of the total observed variation with 37.8% observed at the animal level and 14.2% at the observation level. However, when an AR-1 correlation matrix was fitted to longitudinal observations (Table 7.3B), the strong clustering by farm persisted with 49.1% (0.028/0.057) of variation occurring between farms, but the contribution of between-animal variation fell to 24.6% (0.014/0.057) while the proportion of total variation occurring between longitudinal observations rose to 26.3%. This correlation component (i.e. estimation of the parameter α) was highly significant to overall model fit, resulting in a change in model deviance of 1620.81 for 1 degree-of-freedom ($p<<0.001$) and yielding an estimate of the correlation coefficient ($\hat{\rho}$) of 0.76.

7.6.2 Model Screening

Eight model formulations by combination of i) level of model specification, ii) presence of AR-1 autocorrelation structure and iii) estimation of animal-level random effects, were developed for adjusted OD values. Table 7.4 compares the overall fit of these models

by contrasting the changes in deviance and degrees-of-freedom of each relative to the variance-components-only model (Table 7.3A). In all cases, models which incorporated a correlation component were markedly superior to the equivalent models which did not (all $p<<0.001$), indicating that the addition of fixed- and random-effect parameters was unable to fully account for the correlation between longitudinal serological measures. Further, consistently better fit was demonstrated by models in which random slopes over time in the study for animal-level antibody curves were differentiated on the basis of whether or not an animal had been defined to have undergone seroconversion or to have experienced a sero-event, rather than by the grazing management system it was subject to. This is a reflection of the fact that the definition of sero-events was based on the presence of an increasing antibody titre and the corresponding shape and pattern of the serological profile (see section *5.4.2.2 Sero-Event Classification Criteria*) while sero-events occurred under both zero-grazing and semi-/full-pasture grazing systems. Finally, it was possible to reduce each fully specified, "maximal model" by removing between 11 and 18 fixed-effect parameters without significantly affecting model fit (all $p>0.05$).

7.6.3 Fully Specified Model

Table 7.5 presents the random- and fixed-effect parameter estimates for the three-level fully specified maximal model of adjusted OD units when an AR-1 autocorrelation structure was incorporated and when animal-level random effects differed by sero-event status. For the addition of 37 parameters, the fully specified model exhibits a change in deviance from the variance-components-only model of 2618.89 ($p<<0.001$), while the level-

1 correlation coefficient was halved ($\hat{\rho} = 0.38$).

Longitudinal serological profiles of 72 newborns which experienced passive transfer of maternal antibodies were modelled by the inclusion of a discrete intercept (Newpas) and linear (AgeNpas), quadratic (Age^2Npas), cubic (Age^3Npas) and quartic (Age^4Npas) age-interaction terms as fixed effects. Since all newborn animals are by definition left justified with respect to age, modelling their profiles over age was equivalent to modelling over time in the study. In addition to the fixed effects listed above (which estimate the mean antibody curve over age/time), the intercept, linear and quadratic covariates were also treated as random variables at the animal level. That is, for each newborn with passive transfer a separate antibody curve was fitted such that the variances in the departures (residuals) of intercept (σ^2_{Newpas}), linear ($\sigma^2_{AgeNpas}$) and quadratic ($\sigma^2_{Age^2Npas}$) components of each curve from the mean curve described by the fixed effect variables were estimated under the assumption of multivariate normality. However, histograms and normal probability plots of standardised residuals for each of Newpas (intercept), AgeNpas (linear) and Age^2Npas (quadratic) random effects presented in Figure 7.3 suggest at least moderate kurtosis with one large value for linear and quadratic random effects. The covariances between intercept and linear terms ($\sigma_{Newpas, AgeNpas}$) and between linear and quadratic terms ($\sigma_{AgeNpas, Age^2Npas}$) were also estimated and corresponding scatter plots of standardised residuals provided in Figure 7.4 A & B, respectively, demonstrate the negative correlations observed in both cases. That is, the higher the intercept OD value (initial observation in newborns), the more pronounced is the linear component of the decline. Further, these estimates allowed the animal-level variance for newborns with passive transfer to be described as a quartic function of age according to

the following equation:

$$Total\ \sigma^2_{age_i} = \sigma^2_{Newpas} + 2*\sigma_{Newpas,AgeNpas}*age_i + \sigma^2_{AgeNpas}*age^2_i + 2*\sigma_{AgeNpas,Age^2Npas}*age^3_i + \sigma^2_{Age^2Npas}*age^4_i$$

The mean relationship between adjusted OD values and age, for newborns with passive transfer of maternal antibodies, is illustrated in Figure 7.5A along with the corresponding age variance relationship. Overall the mean level of adjusted OD values declines precipitously over the first 3 months of observation to below the negative cut-off level. Thereafter, the decline becomes slower until approximately one year of age when it begins to rise dramatically. Concomitant to the initial decline in mean, the total variation also begins to decline but rises slightly through the intermediate interval of ages (2 - 6 months) before falling sharply again until 12 months after which it rises in parallel with the mean. The two factors which have the greatest impact in generating these patterns may be best described with reference to the set of individual curves derived from residuals for each calf, as plotted in Figure 7.5B (note that curves incorporate farm-level residuals). First, while the greater proportion of calves exhibit declining maternal antibodies across the age interval, at least two demonstrated pronounced increases at young ages (one of these was responsible for the large standardised residuals observed in Figure 7.3) and the curves for two others began to rise noticeably only near the final observations. Second, there appeared to be a differential rate of right censoring with age as calves which are not lost to follow-up were predominantly those exhibiting low adjusted OD values, at least through 1 year of age.

The serological profiles of the remaining 453 animals were modelled by a combination of time- and age-effect covariates. Although age and time effects are still

correlated in animals other than newborns, in the circumstances of a longitudinal study of 1 year duration made on animals varying in age up to 13 years, the time and age covariates may be considered to be evaluating relationships on different temporal scales.

With respect to time effects in these animals, separate linear and quadratic terms were estimated as fixed effects for the 71 animals which were defined to have either seroconverted or experienced a sero-event (of 0.150 OD unit increase = ETime and $ETime^2$ respectively) and for those 382 which had not (NTime and $NTime^2$ respectively), where time referred to the time since enrolment in the study and was also measured in years. Further, these parameters and their corresponding intercept terms (Event, NoEvent) were included as random variables at the animal level, such that, for each group variances and covariances were estimated with interpretation analogous to that described above for newborns with passive transfer.

For animals not defined to have experienced a sero-event, all random-effect parameters other than the intercept covariate converged to zero estimates, indicating that the overall fixed effect of a reduction in mean adjusted OD values over time was consistent across all the non-event group, save for variation in the value of the initial (= intercept) observation (Figure 7.6). That is, animals not considered to have experienced a sero-event tended to exhibit declining antibody levels. In contrast, the sero-event group was characterised by a mean increase in adjusted OD values over time post-initial observation and considerably more variation in the linear and quadratic components of the individual antibody curves (Figure 7.7). This is perhaps unsurprising since second-order polynomial curves were being used to approximate what may be more accurately described as a step-

function of sero-increase, the timing and magnitude of which was variable. Nevertheless, the contrast between the random-effect estimates for the event and non-event groups indicates that serological profiles which were consistent with primary or anamnestic immune responses (see section *5.4.1 Rationale for Interpretation*) were responsible for the vast majority of the variation observed between animals.

In the fully specified maximal model separate intercepts and linear and quadratic age-interaction terms were fitted as fixed effects for each combination of grazing management system and agro-ecological zone. For example the relationship between adjusted OD value and age for animals housed on zero-grazing units in the lower highland (LH) zone was described by the variables LHZero (intercept; age = 0), LHZAge (linear age effect) and $LHZAge^2$ (quadratic age effect), where age was measured in years. Figure 7.8 presents the mean-age relationships with adjusted OD values, superimposed on individual curves for sero-event and non-sero-event animals, over the one year study period, observed in each combination of grazing management system and agro-ecological zone of origin. Corresponding parameter estimates in Table 7.5 indicate that, within each of the UM and LH zones, the age:OD relationship on semi-/full pasture grazing farms was distinguished from that on zero-grazing farms by a larger intercept and linear component and more negative quadratic component, suggesting higher OD levels in early years but interestingly, lower levels in older animals. In addition, decomposing farm-level variance by grazing management system showed that the variation in adjusted OD values observed between semi-/full-pasture grazing farms (0.032) was more than ten times the variation observed among zero-grazing farms (0.0029), suggesting a stronger farm clustering effect. However,

no significant differences were detected between agro-ecological zones after the effect of grazing management system and age had been accounted for and the fully specified maximal model was subsequently reduced.

7.6.4 Reduced Model

Fixed- and random-effect estimates for the most parsimonious reduction of the fully specified maximal model described above are presented in Table 7.6. The reduced model retained the variance/covariance structure of the fully specified model (with the exception of the removal of those random-effect variables which converged to zero variance estimates) and provided a near identical estimate of the level-1 correlation coefficient ($\hat{\rho} = 0.39$), but was altered by the reduction of fixed-effect variables concerned with assessing the serological profiles across age. Through a sequential process of reparamaterisation and significance testing of independent variables, all differences by agro-ecological zone were removed ($\chi^2_9 = 15.10$; p=0.09). Next, although linear and quadratic age-interaction terms were retained by grazing management system, a single overall intercept (*versus* separate intercepts by grazing system) was fitted for the 453 animals other than newborns with passive transfer of maternal antibodies ($\chi^2_1 = 3.29$; p=0.07). Finally a non-significant quadratic age-effect for zero-grazing farms ($\chi^2_1 = 1.81$; p=0.18) was deleted. The total change in deviance between the full and reduced model was 18.86 for 11 degrees of freedom (p=0.06).

Although the estimate for farm-level variance on zero-grazing farms nearly doubled to 0.0054 in the reduced model, it was still significantly less than the slightly increased

estimate of 0.036 for semi-/full-pasture grazing farms ($\chi^2_1 = 13.82$; $p<0.001$). Further the mean fixed-effect difference between the age profile for zero-grazing farms (ZAge covariate = linear effect only) and that for semi-/full-pasture grazing farms (GAge + $GAge^2$ = linear and quadratic components) was also statistically significant ($\chi^2_1 = 11.70$; $p<0.001$). The difference in age:OD relationships by grazing management system may be best illustrated graphically. Figure 7.9 presents scatter plots of adjusted OD units by age for (B) the 206 animals (other than newborns with passive transfer) on farms practising zero-grazing and for (C) the 247 animals on semi-/full-pasture grazing farms. The patterns demonstrate a greater number of observations with higher OD values at younger ages on semi-/full-pasture grazing farms. This observation is further reinforced by plots of curves for sero-event and non-sero-event animals by grazing management system, derived from the reduced model of adjusted OD units and presented in Figure 7.10. Semi-/full-pasture grazing farms have greater numbers of both sero-event and nonsero-event observations of higher OD values at earlier ages than zero-grazing farms. The overall mean age:OD relationships which derive from these trends are depicted in Figure 7.11, illustrating that the increase in OD values with age is faster and more pronounced on semi-/full-pasture grazing farms, but appears to be more sustained on zero-grazing farms across the interval of ages observed. However, the validity of stating there is a decline in mean OD values at higher ages on semi-/full-pasture grazing farms must be considered in light of the relatively small number of observations made at these ages and the appropriateness of fitting a quadratic function to the data. Nevertheless, there are clear and "statistically significant" differences in age profiles between grazing management systems. However, a histogram (Figure 7.12A) and normal probability plot

(Figure 7.12B) of level-1 standardised residuals from the reduced model, demonstrated marked kurtosis (coefficient of kurtosis = 17.85) but little skewness (skewness coefficient =0.146), suggesting that the assumption of normality, and hence the validity of statistical inference, was suspect. In response to this finding, the entire model development process detailed above was repeated with the natural log transformed OD values as the dependent variable. Plots of standardised residuals from these models indicated that the assumption of multivariate normality was more closely approximated, yet for each permutation the *ln*(OD) model demonstrated the same patterns and trends of significant and non-significance of fixed- and random-effect variables as the corresponding adjusted OD value model. Since the interpretation and inference of the adjusted OD models was more straightforward (see section *7.2 Dependent Variables*), preference was given to the presentation of these results.

7.7 Models of Dichotomised Negative/Positive Results

7.7.1 Variance Components

Table 7.7 presents parameter estimates from three-level (farm, animal and observation) variance components only models in which a logistic transformation of binary (positive/negative) serological status was modelled as the dependent variable using predictive quasi-likelihood (PQL) estimation (with and without a level-1 extra-binomial variance parameter) and in a Markov Chain Monte Carlo (MCMC) Bayesian estimation process which assumed level-1 binomial errors. In all three cases the farm-level variance estimate was only approximately 60% of the estimated between-animal variance. This relative relationship between farm and animal variability was the inverse of that observed

in variance component models of adjusted OD values, suggesting that, although there was clustering by farm (i.e. animals within a given farm more or less likely to test positive), the clustering of dichotomous responses within individuals was more pronounced. This effect may be largely attributed to the dynamics of the immune system. Animals testing negative tend to remain so until challenged whereupon they either seroconvert (if naive) or experience an anamnestic response (sero-event), remaining positive for a variable period thereafter, which is largely dependent on whether or not or when they are re-exposed. However, even after this animal-level clustering was accounted for, when the level-1 variance was not constrained to the binomial assumption (Table 7.7 B), considerable underdispersion (extra-binomial variance parameter estimate = 0.62) was exhibited, further inflating the estimate of animal-level variance. Such marked underdispersion is also a reflection of the non-independence of the longitudinal measures.

Parameter estimates derived from the MCMC estimation were higher than those obtained under PQL methods. When the level-1 variance was constrained to the binomial assumption, the farm- and animal-level variance estimates obtained by PQL were virtually half (54.5% and 53.4% respectively) those obtained under MCMC, while the intercept estimate achieved 70% of the MCMC value (Table 7.7C). This suggests that, despite the use of the RIGLS process in a second-order PQL estimation, considerable bias toward the null remained. When the observed probability is very large or small or there are a many level-2 units where the responses are all 0's or 1's, convergence may not be possible under PQL methods and even if it is achieved, the estimates are usually not unbiased (Goldstein, 1995).

7.7.2 Model Screening

Problems of non-convergence of PQL estimates persisted for models of increased size and complexity, particularly when an extra-binomial variance parameter was estimated. As a result, complex variance structures could not be evaluated and model development was restricted to assessing fixed-effect variables. Variables available were identical to those offered to adjusted OD value models, however, in light of the inability to derive estimates for more fully-specified maximal models, model building could only proceed through a forward selection process. Two categories of models are presented, first those in which age-interaction terms were assessed and second, those in which sero-event status-time in the study interaction terms were added to these models.

7.7.3 Age-Profile Models

Tables 7.8 and 7.9 present parameter estimates for three-level age-profile models of binary serological status estimated under PQL and MCMC methods respectively. In each model, separate intercept terms (age = 0 years) are estimated for newborns with passive transfer of maternal antibodies (Newpas) and for all other animals (ConsOther). Further, as per models of adjusted OD values, linear, quadratic, cubic and quartic newborn-age interaction parameter estimates were all "significant", based on Wald approximations of chi-square, while different age-profiles were fitted for animals housed under zero-grazing *versus* semi-/full-pasture grazing by the incorporation of linear (ZAge; zero-grazing - age) and linear (GAge; semi-/full-pasture grazing - age) and quadratic (GAge2; semi-/full-pasture grazing - age^2) terms respectively.

The age-seroprevalence profiles generated from the fixed effect estimates for newborns with passive transfer of maternal antibodies are presented for both PQL and MCMC models in Figure 7.13. Broadly speaking, the profiles are in agreement, showing a markedly declining proportion of sero-positive calves over the first 3 months, followed by an increasing proportion to approximately 7 months of age and then a decline to near zero values. The pattern is consistent with that observed in the continuous adjusted OD model; the initial rapid loss of maternally acquired antibodies followed by increasing numbers of seroconversions but with a differential rate of loss to follow-up subsequently biasing toward negative/non-seroconverted animals.

As per variance component models, the MCMC estimate for each fixed and random parameter exhibited greater departure from the null and marginally larger standard deviation than the biased PQL estimate, although this was again most pronounced for random variables. All MCMC estimates demonstrated symmetry about the mean (mean ≈ median) and were significant based on 95% confidence intervals derived from lower (2.5%) and upper (97.5%) percentiles of the kernel density trace of the posterior distribution (Goldstein *et al.*, 1998). The inclusion of fixed-effect variables resulted in a decrease in the estimate of animal-level variance, but a marginal increase in the variance between farms, from the corresponding variance components only models.

7.7.4 Age and Time Models

Age-profile only models were extended by the addition of sero-event status-time interaction terms for animals other than newborns with passive transfer, where time was

recorded as the time since initial observation. Tables 7.10 and 7.11 present parameter estimates for three-level age/time models of binary serological status estimated under PQL and MCMC methods respectively. PQL models estimating a binomial underdispersion parameter failed to converge entirely while those with level-1 variance constrained to the binomial assumption also failed to converge under the strict tolerance criteria (parameters do not change by more than 0.01) but settled into oscillation between two very similar sets of parameter values, before iterations were manually halted. Unsurprisingly, all MCMC estimates exhibited greater departure from the null than PQL estimates, but all fixed effects were determined to be significant by Wald test under the PQL method and by 95% confidence intervals of posterior parameter distributions under MCMC estimation.

The addition of time covariates restored farm- and animal-level variance estimates to near the initial variance-components-only values. Figure 7.14 presents histograms and normal probability plots of standardised residuals by farm, animal and observation level for the PQL model with level-1 variance constrained to the binomial assumption, demonstrating that the assumption of normality of errors is adequate at both the farm and the animal levels. However, the marked kurtosis present at the observation level, with the presence of several large residuals, indicates the presence of strong underdispersion, due largely to the non-independence of longitudinal errors.

As addressed previously, the effect of simultaneously incorporating the effects of age and time in a model is to allow for differential dynamics over the two temporal scales. For example, the negative linear (Ntime) and positive quadratic ($NTime^2$) time-effect covariates for animals which were not considered to have experienced seroconversion or a sero-event

indicate that a declining antibody level observed in the absence of rechallenge results in an increasing (but non-linear) proportion of sero-negatives, over a 1 year period of longitudinal observation. However, the positive linear (ETime) and negative quadratic ($ETime^2$) time-effect estimates for sero-event animals and the positive linear age-effect covariates suggest that the frequency of the sero-events (challenge/rechallenge) is sufficiently high to sustain a high proportion of sero-positives across all ages.

Little difference was observed for the linear, quadratic, cubic and quartic age parameter estimates (and hence the age-seroprevalence profiles, presented in Figure 7.13) of newborns with passive transfer of maternal antibodies. However, under both PQL and MCMC estimation, the addition of time effects by sero-event status resulted in larger positive linear parameter estimates by grazing management system and, for semi-full-pasture grazing farms, only a marginally more negative quadratic effect. The effect of these differences in age/grazing management interaction terms on the seroprevalence profiles is illustrated in Figure 7.15. The mean age-profiles for each grazing management system derived from age-effect only model MCMC estimates (A) and from age and time-effect model MCMC estimates (B) are presented. From both models, there is a clear trend for an increase in the proportion of sero-positives with increasing age with the initial rate of increase being higher on semi-/full-pasture grazing farms than on zero-grazing farms. Based on the age-effect only model, by approximately 3 years of age a mean of 80% of animals were estimated to be sero-positive on semi-/full-pasture grazing farms, while it required approximately 5 years on zero-grazing farms to achieve this same level. However, when the time-effects were accounted for, the mean estimate of 80% sero-positive is achieved earlier

in both grazing management systems, at approximately 2 and 3 years respectively, although the difference between the two has been reduced. Also, the pronounced decline in the proportion of sero-positives at higher ages on semi-/full-pasture grazing farms, as suggested by the age-effect only model, has been sharply reduced with the age/time-effect model estimates. However, the question again arises as to whether it is legitimate to extrapolate across higher ages given a quadratic fit and limited observations.

Figure 7.16 displays the crude age-seroprevalence profiles (derived from longitudinal observations for 453 animals other than newborns with passive transfer of maternal antibodies) by grazing management system, confirming that the greater prevalence observed on semi-/full-pasture grazing farms does occur in the 1 - 3 year age group and that thereafter, although continuing to rise in parallel, the profiles for the two grazing systems are virtually indistinguishable. More specifically, the apparent difference between the two age-seroprevalence relationships for older age groups, suggesting that older animals on semi-/full-pasture grazing farms are less likely to test positive, is almost entirely based on the differences observed in those few animals greater than 8 years of age.

Table 7.1: Comparison of descriptive statistics of post-adjustment test results and natural log transformed values of 4404 samples analysed and the 29 imputed values imputed for serum samples collected in a longitudinal study conducted in Kiambu District from July 1991 - June 1992 and analysed by an enzyme-linked immunosorbent assay in a targeting protocol.

Statistic	Adjusted Results (OD Units)	Transformed Values (*ln(OD)*)
N	4433	4433
Mean	0.231	-1.896
Standard Deviation	0.236	0.942
Median	0.139	-1.966
Min - Max	0 - 1.126	-6.908 - 0.120
Negative Cut-off	0.125	-2.079
Number Negative[1]	2015	-
Percentage Positive[1]	54.5	-

[1] - negative refers to OD values ≤ 0.125 OD units

Table 7.2: Continuous and categorical farm, animal and observation-level variables derived from observations made in a longitudinal study conducted in Kiambu District from July 1991 - June 1992 and assessed in hierarchical generalised linear mixed multilevel models of serological responses.

FARM-LEVEL	
Agro-Ecological Zone of Origin	categorical
Upper Midland (1-3)	
Lower Highland (1-3)	
Upper Highland (1&2)	
Grazing Management System	categorical
Zero-Grazing Unit	
Semi-/Full-Pasture Gazing	
Dairy Animals in Zero-Grazing Units Contact Other Livestock on Farm (y/n)[1]	categorical
Method of Tick Control Practised[2]	categorical
Plunge Dipping	
Back-Pack Spraying	
Hand Applied	
No Tick Control	
Frequency of Application of Acaricide[2]	categorical
Weekly	
Fortnightly	
Monthly or Less Frequently	
No Tick Control	
Only Mature Animals Treated for Ticks (y/n)	categorical
Age of Calves at First Tick Treatment	continuous
Separation of Calf and Dam	categorical
Separated within 4 hours of birth	
Separated within 24 hours of birth	
Colostrum delivered within 2 hours of birth (y/n)	categorical
Calf Death From Tick-Borne Disease in Past Year (y/n)	categorical
Dairy Farming Experience in Years	continuous
Farm Size in Acres	continuous
Number of Dairy Animals on Farm	continuous
Farm Average Standard[3] Tick Count	continuous
Farm Proportion of Observations of Small Ticks	continuous
Farm Proportion of Observations of Standard Ticks	continuous
Farm Proportion of Observations of Standard and/or Small Ticks	continuous

Table 7.2 (continued): Continuous and categorical farm, animal and observation-level variables derived from observations made in a longitudinal study conducted in Kiambu District from July 1991 - June 1992 and assessed in hierarchical generalised linear mixed multilevel models of serological responses.

ANIMAL-LEVEL	
Gender (Male/Female)	categorical
Breed ("exotic"/crossbred)	categorical
Newborns with passive transfer of maternal antibodies (y/n)	categorical
Mean Count of Standard[3] Ticks	continuous
Proportion of Animal Observations of Small Ticks[4]	continuous
Proportion of Animal Observations of Standard Ticks	continuous
Proportion of Animal Observations of Standard and/or Small Ticks	continuous
> 10% of Animal Observations of Small Ticks[4] (y/n)	categorical
OBSERVATION-LEVEL	
Age of animal in years	continuous
Time of observation in years	continuous
Number of Acaricide Applications[5]	
Weekly	continuous & categorical
Fortnightly	
Monthly or Less Frequently	
No Tick Control	
Count of Standard[3] Ticks	continuous
Presence of Small Ticks (y/n)	categorical

[1] - (y/n) denotes binary categorical variables, i.e. satisfied by yes or no responses.
[2] - Farm method and frequency of application of acaricide variables assessed for each of a) reported and b) reclassified by an iterative process of reduction in stated frequency of application category until 33.3% (= liberal criteria) or 66.7% (= conservative criteria) of observed inter-application intervals were consistent.
[3] - Standard ticks assessed according to the method of Norval *et al.* (1992).
[4] - Covariate screened as continuous and refined to dichotomous categorical variable
[5] - Two time-varying covariates generated from reclassified farm frequency of application category (see 3 above) and observed inter-application intervals and screened as continuous and categorical variables.

Table 7.3: Parameter estimates from three-level variance-components-only models, without (A) and with (B) an auto-regressive (AR-1) correlation component, where adjusted optical density (OD) units, derived from observations made in longitudinal study conducted in Kiambu District from July 1991 - June 1992, were modelled as dependent variable.

A)

Level	Parameter	Description	Estimate	SE
Random Effects				
3	σ^2_k	$Farm_k$ - level Variance	0.028	0.0051
2	σ^2_{jk}	$Animal_{jk}$ - level Variance	0.022	0.0016
1	$\sigma^2_{e\,ijk}$	$Observation_{ijk}$ - Error Variance	0.0083	0.00019
Fixed Effects				
	$Cons_{ijk}$	$Intercept_{ijk}$	0.250	0.0196

B)

Level	Parameter	Description	Estimate	SE
Random Effects				
3	σ^2_k	$Farm_k$ - level Variance	0.028	0.0051
2	σ^2_{jk}	$Animal_{jk}$ - level Variance	0.014	0.0017
1	$\sigma^2_{e\,ijk}$	Obs_{ijk} - Error Variance	0.015	0.0010
	α	AR-1 autocorrelation where: $\sigma e_t e_{t-s} = \sigma^2_e * e^{-\alpha s}$	0.28	0.0078
Fixed Effects				
	$Cons_{ijk}$	$Intercept_{ijk}$	0.255	0.0196
Model Deviance (reduction in -2*log likelihood from non-correlation model) $\chi^2 = 1620.81$, df = 1				

Table 7.4: Changes in model deviance (calculated as reduction in -2*log likelihood) and degrees of freedom from variance-components-only model (Table 7.3A) for eight model permutations by combination of (i) model specification, (ii) presence of AR-1 correlation component and (iii) estimation of random time-effects, where adjusted optical density (OD) units, derived from observations made in a longitudinal study conducted in Kiambu District from July 1991 - June 1992, were modelled as the dependent variable.

Model Specification	Level 1 Auto-Correlation	Animal-Level Random Slopes over Time by:	Δ Deviance (from variance components model)	Δ Degrees of Freedom (from variance components model)	p for Max *vs* Reduced Model	Table
Fully Specified Maximal Model	AR-1	Sero-event Definition	2618.89	41(37)[1]	0.064	6.5
		Grazing System	2222.23	41	0.18	-
	None	Sero-event Definition	2383.53	40(38)[1]	0.10	-
		Grazing System	1872.27	40	0.12	-
Most Parsimonious Reduced Model	AR-1	Sero-event Definition	2600.03	26	-	6.6
		Grazing System	2198.97	23	-	-
	None	Sero-event Definition	2366.26	27	-	-
		Grazing System	1853.25	27	-	-

[1]- values in brackets differ by the number of random effect parameter estimates in fully specified maximal model which converged on zero-variance estimates.

Table 7.5: Parameter estimates for random- and fixed-effects from three-level, fully-specified maximal model incorporating AR-1 correlation component and separate random time-effects by sero-event definition, where optical density (OD) units, derived from observations made in a longitudinal study conducted in Kiambu District from July 1991 - June 1992, were modelled as the dependent variable.

Random Effects					
Level	Parameter	Description	Estimate	SE	Corr
$Farm_k$	$\sigma^2_{Zerograzing\ k}$	Variance of zero-grazing farms.	0.0029	0.0013	1
	$\sigma^2_{Grazing\ k}$	Variance of grazing farms.	0.032	0.0075	1
$Animal_{jk}$	$\sigma^2_{Newpas\ jk}$	Variance and covariance terms of newborns with passive transfer of maternal antibodies, for intercept (Newpas) and linear (AgeNpas) and quadratic (Age^2Npas) effects of age in years.	0.053	0.010	1
	$\sigma_{Newpas\ jk,\ AgeNpas\ jk}$		-0.087	0.018	-0.45
	$\sigma^2_{AgeNpas\ jk}$		0.71	0.14	1
	$\sigma_{AgeNpas\ jk,\ Age^2Npas\ jk}$		-0.52	0.14	-0.89
	$\sigma^2_{Age^2Npas\ jk}$		0.47	0.15	1
	$\sigma^2_{Event\ jk}$	Variance and covariance terms of animals which experienced sero-events for intercept (Event) and linear (ETime) and quadratic ($ETime^2$) effects of time in years.	0.041	0.0085	1
	$\sigma_{Event\ jk,\ ETime\ jk}$		-0.039	0.0099	-0.26
	$\sigma^2_{ETime\ jk}$		0.56	0.11	1
	$\sigma_{ETime\ jk,\ ETime^2\ jk}$		-0.57	0.12	-0.93
	$\sigma^2_{ETime^2\ jk}$		0.67	0.14	1
	$\sigma^2_{NoEvent\ jk}$	Variance and covariance terms of animals which did not experience seroevents for intercept (NoEvent) and linear (NTime) and quadratic ($NTime^2$) effects of time in years.	0.011	0.0010	1
	$\sigma_{NoEvent\ jk,\ NTime\ jk}$		0[1]	0[1]	
	$\sigma^2_{NTime\ jk}$		0[1]	0[1]	1
	$\sigma_{NTime\ jk,\ NTime^2\ jk}$		0[1]	0[1]	
	$\sigma^2_{NTime^2\ jk}$		0[1]	0[1]	1
Obs_{ijk}	$\sigma^2_{e\ ijk}$	Error Variance	0.0052	0.00017	
	α	AR-1 autocorrelation where: $\sigma e_t e_{t-s} = \sigma^2_e * e^{-\alpha s}$	0.96	0.037	

[1]- random effect parameter estimates which converged on zero-variance estimates.

Table 7.5 (continued): Parameter estimates for random- and fixed-effects from three-level, fully-specified maximal model incorporating AR-1 correlation component and separate random time-effects by sero-event definition.

Fixed Effects			
Parameter	Description	Estimate	SE
Newpas	Intercept (Newpas) and linear (AgeNpas), quadratic (Age^2Npas), cubic (Age^3Npas) and quartic (Age^4Npas) age-effect terms for newborns with passive transfer of maternal antibodies, where age is measured in years.	0.484	0.0350
AgeNpas		-2.828	0.236
Age^2Npas		7.560	0.920
Age^3Npas		-8.823	1.413
Age^4Npas		3.660	0.702
UMZero	Intercept (UMZero) and linear (UMZAge) and quadratic ($UMZAge^2$) age-effect terms for zero-grazing animals within the Upper Midland agro-ecological zone, where age is measured in years.	0.090	0.0247
UMZAge		0.0342	0.00934
$UMZAge^2$		-0.00142	0.000856
UMGraze	Intercept (UMGraze) and linear (UMGAge) and quadratic ($UMGAge^2$) age-effect terms for grazing animals within the Upper Midland agro-ecological zone, where age is measured in years.	0.227	0.0620
UMGAge		0.0382	0.0223
$UMGAge^2$		-0.00193	0.00212
LHZero	Intercept (LHZero) and linear (LHZAge) and quadratic ($LHZAge^2$) age-effect terms for zero-grazing animals within the Lower Highland agro-ecological zone, where age is measured in years.	0.116	0.0283
LHZAge		0.0375	0.0117
$LHZAge^2$		-0.000817	0.00112
LHGraze	Intercept (LHGraze) and linear (LHGAge) and quadratic ($LHGAge^2$) age-effect terms for grazing animals within the Lower Highland agro-ecological zone, where age is measured in years.	0.165	0.0453
LHGAge		0.0498	0.0122
$LHGAge^2$		-0.00407	0.00121
UHGraze	Intercept (UHGraze) and linear (UHGAge) and quadratic ($UHGAge^2$) age-effect terms for grazing animals within the Upper Highland agro-ecological zone, where age is measured in years.	0.131	0.0397
UHGAge		0.0493	0.0115
$UHGAge^2$		-0.00398	0.00119
ETime	Linear (ETime) and quadratic (Etime2) time-effect terms for sero-event animals, where time is the period of longitudinal of observation in years.	0.176	0.0955
$ETime^2$		-0.0376	0.108
NTime	Linear (NTime) and quadratic (Ntime2) time-effect terms for non-sero-event animals, where time is the period of longitudinal of observation in years.	-0.114	0.0225
$NTime^2$		0.0393	0.0207
Hand	Farms utilizing hand-applied tick control.	0.224	0.0738
Small Ticks	Animals where small ticks were recorded for greater than 10% of longitudinal observations.	0.0769	0.0211
Model Deviance (reduction in -2*log likelihood from variance components model)χ^2=2618.89, df=41(37)			

Table 7.6: Parameter estimates for random- and fixed-effects from three-level, most parsimonious reduced model incorporating AR-1 correlation component and separate random time-effects by sero-event definition, where optical density (OD) units, derived from observations made in a longitudinal study conducted in Kiambu District from July 1991 - June 1992, were modelled as the dependent variable.

Random Effects					
Level	Parameter	Description	Estimate	SE	Corr
$Farm_k$	$\sigma^2_{Zerograzing\ k}$	Variance of zero-grazing farms.	0.0054	0.0019	1
	$\sigma^2_{Grazing\ k}$	Variance of grazing farms.	0.036	0.0081	1
$Animal_{jk}$	$\sigma^2_{Newpas\ jk}$	Variance and covariance terms of newborns with passive transfer of maternal antibodies, for intercept (Newpas) and linear (AgeNpas) and quadratic (Age^2Npas) effects of age in years.	0.054	0.010	1
	$\sigma_{Newpas\ jk,\ AgeNpas\ jk}$		-0.081	0.017	-0.41
	$\sigma^2_{AgeNpas\ jk}$		0.74	0.15	1
	$\sigma_{AgeNpas\ jk,\ Age^2Npas\ jk}$		-0.57	0.15	-0.91
	$\sigma^2_{Age^2Npas\ jk}$		0.52	0.16	1
	$\sigma^2_{Event\ jk}$	Variance and covariance terms of animals which experienced seroevents for intercept (Event) and linear (ETime) and quadratic ($ETime^2$) effects of time in years.	0.042	0.0087	1
	$\sigma_{Event\ jk,\ ETime\ jk}$		-0.039	0.010	-0.26
	$\sigma^2_{ETime\ jk}$		0.55	0.11	1
	$\sigma_{ETime\ jk,\ ETime^2\ jk}$		-0.56	0.12	-0.93
	$\sigma^2_{ETime^2\ jk}$		0.67	0.14	1
	$\sigma^2_{NoEvent\ jk}$	Variance of animals which did not experience seroevents for intercept (NoEvent).	0.011	0.00099	1
Obs_{ijk}	$\sigma^2_{e\ ijk}$	Error Variance	0.0052	0.00018	
	α	AR-1 autocorrelation where: $\sigma e_t e_{t-s} = \sigma^2_e * e^{-\alpha s}$	0.95	0.037	

Table 7.6 (continued): Parameter estimates for random- and fixed-effects from three-level, most parsimonious reduced model incorporating AR-1 correlation component and separate random time-effects by sero-event definition, where optical density (OD) units, derived from observations made in a longitudinal study conducted in Kiambu District from July 1991 - June 1992, were modelled as the dependent variable.

Fixed Effects			
Parameter	Description	Estimate	SE
Newpas	Intercept (Newpas) and linear (AgeNpas), quadratic (Age^{2}Npas), cubic (Age^{3}Npas) and quartic (Age^{4}Npas) age-effect terms for newborns with passive transfer of maternal antibodies, where age is measured in years.	0.466	0.0344
AgeNpas		-2.816	0.238
Age^{2}Npas		7.509	0.921
Age^{3}Npas		-8.714	1.411
Age^{4}Npas		3.609	0.700
ConsOther	Intercept for all other animals	0.136	0.0163
ZAge	Linear age-effect term for zero-grazing animals, age in years.	0.0225	0.00257
GAge	Linear age-effect term for grazing animals, age in years.	0.0515	0.00731
GAge^2	Quadratic age-effect term for grazing animals, age in years.	-0.00392	0.000754
ETime	Linear (ETime) and quadratic (Etime2) time-effect terms for sero-event animals, where time is the period of longitudinal observation in years.	0.179	0.0950
ETime^2		-0.0401	0.108
NTime	Linear (NTime) and quadratic (Ntime2) time-effect terms for non-sero-event animals, where time is the period of longitudinal observation in years.	-0.111	0.0224
NTime^2		0.0376	0.0207
Hand	Farms utilizing hand-applied tick control.	0.243	0.0854
Small Ticks	Animals where small ticks were recorded for greater than 10% of longitudinal observations.	0.0707	0.0204
Model Deviance (reduction in -2*log likelihood from variance components model) $\chi^2 = 2600.03$, df=26			

Table 7.7: Parameter estimates from three-level variance-components-only models where a logistic transformation of binary outcome (positive/negative=optical density units >/≤0.125, derived from observations made in a longitudinal study conducted in Kiambu District from July 1991 - June 1992) was modelled as the dependent variable i) using a penalised quasi-likelihood (PQL) second order iterative estimation procedure, (A) with level-1 variance constrained to the binomial assumption and (B) estimating an extra-binomial variance parameter at level 1, and ii) in a Bayesian process using (C) a Metropolis-Hastings sampling method in a Markov Chain Monte Carlo (MCMC) iterative estimation procedure utilising 500,000 iterations.

A)

Parameter	Description	Estimate	SE
Random Effects			
σ^2_k	$Farm_k$ - level Variance	3.54	0.91
σ^2_{jk}	$Animal_{jk}$ - level Variance	5.44	0.61
σ^2_1	$Observation_{ijk}$ - Binomial Variance	1	-
Fixed Effects			
$Cons_{ijk}$	$Intercept_{ijk}$	0.608	0.269

B)

Parameter	Description	Estimate	SE
Random Effects			
σ^2_k	$Farm_k$ - level Variance	3.81	0.99
σ^2_{jk}	$Animal_{jk}$ - level Variance	6.25	0.67
σ^2_1	Obs_{ijk} - Extra-binomial Variance	0.62	0.014
Fixed Effects			
$Cons_{ijk}$	$Intercept_{ijk}$	0.632	0.281

C)

Parameter	Description	Mean	SD	Mode	2.5%	97.5%
Random Effects						
σ^2_k	$Farm_k$ - level Variance	6.50	1.74	6.04	3.73	10.52
σ^2_{jk}	$Animal_{jk}$ - level Variance	10.18	1.36	9.96	7.82	13.15
σ^2_1	$Observation_{ijk}$ - Binomial Variance	1	-			
Fixed Effects						
$Cons_{ijk}$	$Intercept_{ijk}$	0.875	0.342	0.872	0.211	1.547

Table 7.8: Parameter estimates for random- and fixed-effects from three-level, most parsimonious reduced models incorporating fixed age-effects only, where a logistic transformation of binary outcome (positive/negative=optical density units >/≤ 0.125, derived from observations made in a longitudinal study conducted in Kiambu District from July 1991 - June 1992) was modelled as the dependent variable using a penalised quasi-likelihood (PQL) second order iterative estimation procedure with level-1 variance constrained to the binomial assumption and estimating an extra-binomial variance parameter at level 1.

Parameter	Description	Binomial		Extra-binomial[1]	
		Est.	SE	Est.	SE
Random Effects					
σ^2_k	$Farm_k$ - level Variance	4.12	1.01	4.42[1]	1.07
σ^2_{jk}	$Animal_{jk}$ - level Variance	5.00	0.60	5.68	0.64
σ^2_1	$Observation_{ijk}$ - Binomial Variance	1	-	0.64[1]	0.015
Fixed Effects					
Newpas	Intercept (Newpas) and linear (AgeNpas), quadratic (Age^{2}Npas), cubic (Age^{3}Npas) and quartic (Age^{4}Npas) age-effect terms for newborns with passive transfer of maternal antibodies, where age is measured in years.	9.12	1.62	9.31	1.39
AgeNpas		-99.99	17.61	-102.50	14.9
Age^{2}Npas		323.20	61.75	333.40	52.06
Age^{3}Npas		-413.90	84.50	-429.30	71.12
Age^{4}Npas		177.50	38.77	184.60	32.54
ConsOther	Intercept for all other animals	-1.05	0.38	-0.808[1]	0.38
ZAge	Linear age-effect term for zero-grazing animals, where age is measured in years.	0.46	0.075	0.40[1]	0.075
GAge	Linear age-effect term for grazing animals, where age is measured in years.	0.98	0.19	0.97[1]	0.18
GAge^2	Quadratic age-effect term for grazing animals, where age is measured in years.	-0.085	0.020	-0.090[1]	0.020

[1] - Denotes parameter estimates which did not meet convergence criteria (parameter tolerance = 0.01).

Table 7.9: Parameter estimates for random- and fixed-effects from three-level, most parsimonious reduced model incorporating fixed age-effects only where a logistic transformation of binary outcome (positive/negative=optical density units >/≤ 0.125, derived from observations made in a longitudinal study conducted in Kiambu District from July 1991 - June 1992) was modelled as the dependent variable in a Bayesian process using a Metropolis-Hastings sampling method in a Markov Chain Monte Carlo (MCMC) iterative estimation procedure in which level-1 variance was constrained to the binomial assumption. Parameter estimates are derived from 1,000,000 iterations.

Parameter	Description	Mean	SD	Mode	2.5%	97.5%
Random Effects						
σ^2_k	$Farm_k$ - level Variance	6.91	1.82	6.47	4.00	11.14
σ^2_{jk}	$Animal_{jk}$ - level Variance	8.14	1.13	7.93	6.19	10.59
σ^2_1	$Observation_{ijk}$ - Binomial Variance	1	-			
Fixed Effects						
Newpas	Intercept (Newpas) and linear (AgeNpas), quadratic (Age^2Npas), cubic (Age^3Npas) and quartic (Age^4Npas) age-effect terms for newborns with passive transfer of maternal antibodies, where age is measured in years.	10.817	1.761	10.471	7.663	14.564
AgeNpas		-118.34	19.44	-114.25	-159.78	-83.25
Age^2Npas		375.99	69.45	356.93	248.54	532.46
Age^3Npas		-474.98	96.14	-445.89	-694.14	-300.56
Age^4Npas		201.83	44.52	189.03	122.37	304.15
ConsOther	Intercept for all other animals	-1.091	0.443	-1.103	-1.940	-0.202
Zage	Linear age-effect term for zero-grazing animals, where age is measured in years.	0.523	0.078	0.524	0.369	0.676
Gage	Linear age-effect term for grazing animals, where age is measured in years.	1.136	0.208	1.139	0.725	1.541
$Gage^2$	Quadratic age-effect term for grazing animals, where age is measured in years.	-0.098	0.022	-0.098	-0.143	-0.054

Table 7.10: Parameter estimates[1] for random and fixed effects from three-level, most parsimonious reduced models incorporating fixed age and time effects where a logistic transformation of binary outcome (positive/negative=optical density units >/≤ 0.125, derived from observations made in a longitudinal study conducted in Kiambu District from July 1991 - June 1992) was modelled as the dependent variable using a penalised quasi-likelihood (PQL) second order iterative estimation procedure with level-1 variance constrained to the binomial assumption.

Parameter	Description	Binomial[1]	
		Est.	SE
Random Effects			
σ^2_k	$Farm_k$ - level Variance	3.04	0.79
σ^2_{jk}	$Animal_{jk}$ - level Variance	5.66	0.71
σ^2_1	$Observation_{ijk}$ - Binomial Variance	1[1]	-
Fixed Effects			
Newpas	Intercept (Newpas) and linear (AgeNpas), quadratic (Age^2Npas), cubic (Age^3Npas) and quartic (Age^4Npas) age-effect terms for newborns with passive transfer of maternal antibodies, where age is measured in years.	9.266	1.644
AgeNpas		-100.2	17.85
Age^2Npas		322.60	62.50
Age^3Npas		-412.20	85.45
Age^4Npas		176.6	39.19
ConsOther	Intercept for all other animals	-1.146	0.394
ZAge	Linear age-effect term for zero-grazing animals, where age is measured in years.	0.721	0.086
GAge	Linear age-effect term for grazing animals, where age is measured in years.	1.45	0.201
$GAge^2$	Quadratic age-effect term for grazing animals, where age is measured in years.	-0.108	0.0212
ETime	Linear (ETime) and quadratic (Etime2) time-effect terms for sero-event animals, where time is the period of longitudinal of observation in years.	5.041	1.729
$ETime^2$		-3.841	1.707
NTime	Linear (NTime) and quadratic (Ntime2) time-effect terms for non-sero-event animals, where time is the period of longitudinal of observation in years.	-4.781	0.882
$NTime^2$		1.917	0.804

[1]-Model parameter estimates did not meet convergence criteria (parameter tolerance = 0.01) but experienced small oscillations. The equivalent model in which an extra-binomial variance parameter at level 1 was estimated experienced significant instability and failed to converge entirely.

Table 7.11: Parameter estimates for random- and fixed-effects from three-level, most parsimonious reduced model incorporating fixed age and time effects where a logistic transformation of binary outcome (positive/negative=optical density units >/≤ 0.125, derived from observations made in a longitudinal study conducted in Kiambu District from July 1991 - June 1992) was modelled as the dependent variable using a Metropolis-Hastings sampling method in a Markov Chain Monte Carlo (MCMC) iterative estimation procedure in which level-1 variance was constrained to the binomial assumption. Parameter estimates are derived from 1,000,000 iterations.

Parameter	Description	Mean	SD	Mode	2.5%	97.5%
Random Effects						
σ^2_k	$Farm_k$ - level Variance	5.38	1.61	4.98	2.82	9.14
σ^2_{jk}	$Animal_{jk}$ - level Variance	9.80	1.36	9.56	7.43	12.78
σ^2_i	$Observation_{ijk}$ - Binomial Variance	1	-			
Fixed Effects						
Newpas	Intercept (Newpas) and linear (AgeNpas), quadratic (Age^2Npas), cubic (Age^3Npas) and quartic (Age^4Npas) age-effect terms for newborns with passive transfer of maternal antibodies, where age is measured in years.	11.332	1.625	10.837	8.575	14.768
AgeNpas		-123.27	17.56	-113.81	-159.66	-96.80
Age^2Npas		393.96	62.27	350.49	304.09	533.58
Age^3Npas		-500.29	86.32	-443.13	-700.61	-375.47
Age^4Npas		213.59	40.15	190.68	153.44	306.27
ConsOther	Intercept for all other animals	-1.254	0.459	-1.249	-2.157	-0.351
ZAge	Linear age-effect term for zero-grazing animals, where age is measured in years.	0.851	0.095	0.846	0.671	1.043
Gage	Linear age-effect term for grazing animals, where age is measured in years.	1.651	0.237	1.638	1.198	2.117
$Gage^2$	Quadratic age-effect term for grazing animals, where age is measured in years.	-0.121	0.025	-0.121	-0.170	-0.073
Etime	Linear (ETime) and quadratic (Etime2) time-effect terms for sero-event animals, where time is the period of longitudinal of observation in years.	5.013	1.769	4.960	1.584	8.548
$ETime^2$		-4.039	1.727	-4.028	-7.481	-0.678
Ntime	Linear (NTime) and quadratic (Ntime2) time-effect terms for non-sero-event animals, where time is the period of longitudinal of observation in years.	-5.246	0.925	-5.270	-7.043	-3.421
$NTime^2$		2.097	0.845	2.105	0.422	3.727

Figure 7.1: Frequency distributions of (A) adjusted optical density units (OD) and (B) natural log transformed optical density units (*ln* OD), derived from observations made in a longitudinal study conducted in Kiambu District from July 1991 - June 1992. Predicted equivalent normal distributions (based on mean and standard deviations) are superimposed as bold lines.

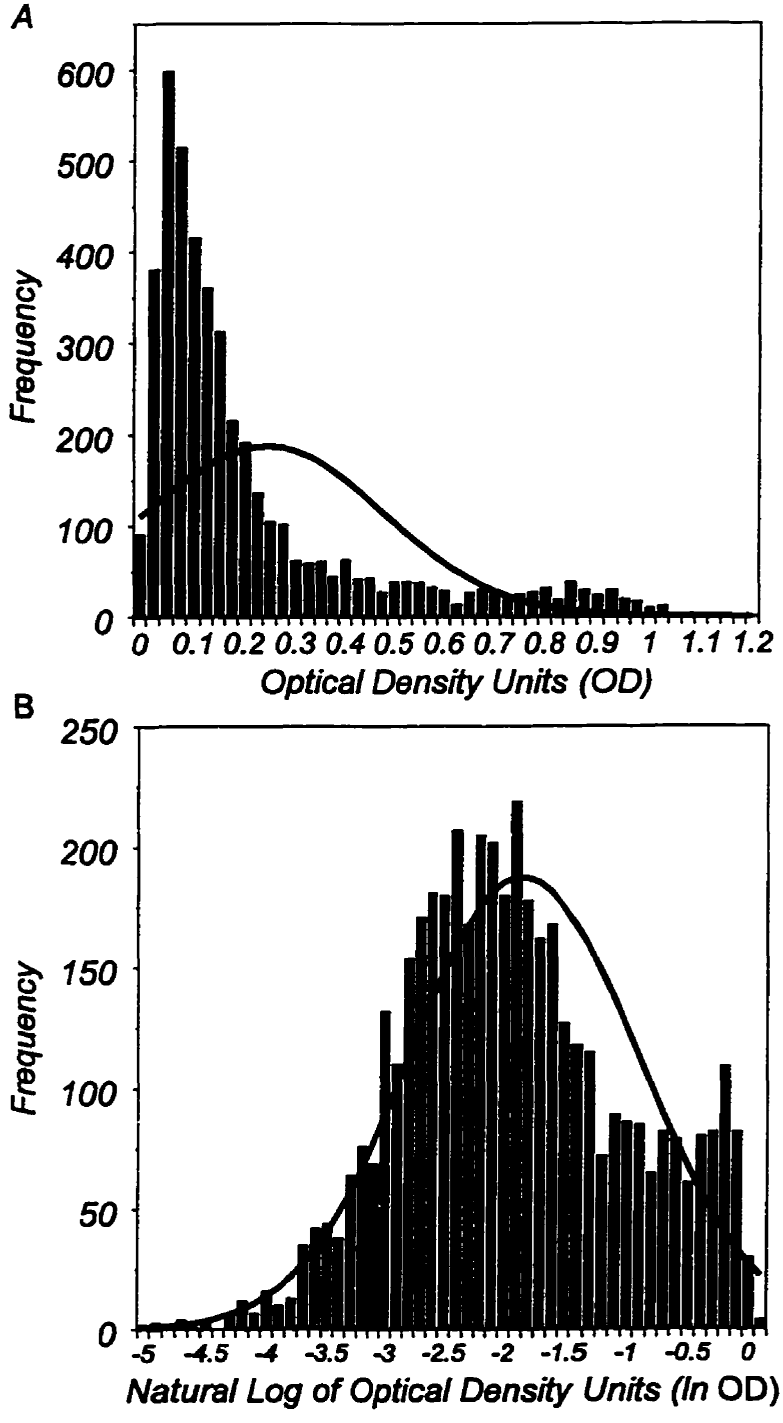


Figure 7.2: Plot demonstrating the relationship between adjusted optical density unit (OD) value and natural log transformed optical density unit (*ln* OD) value, derived from observations made in a longitudinal study conducted in Kiambu District from July 1991 - June 1992, over interval of observed OD values.

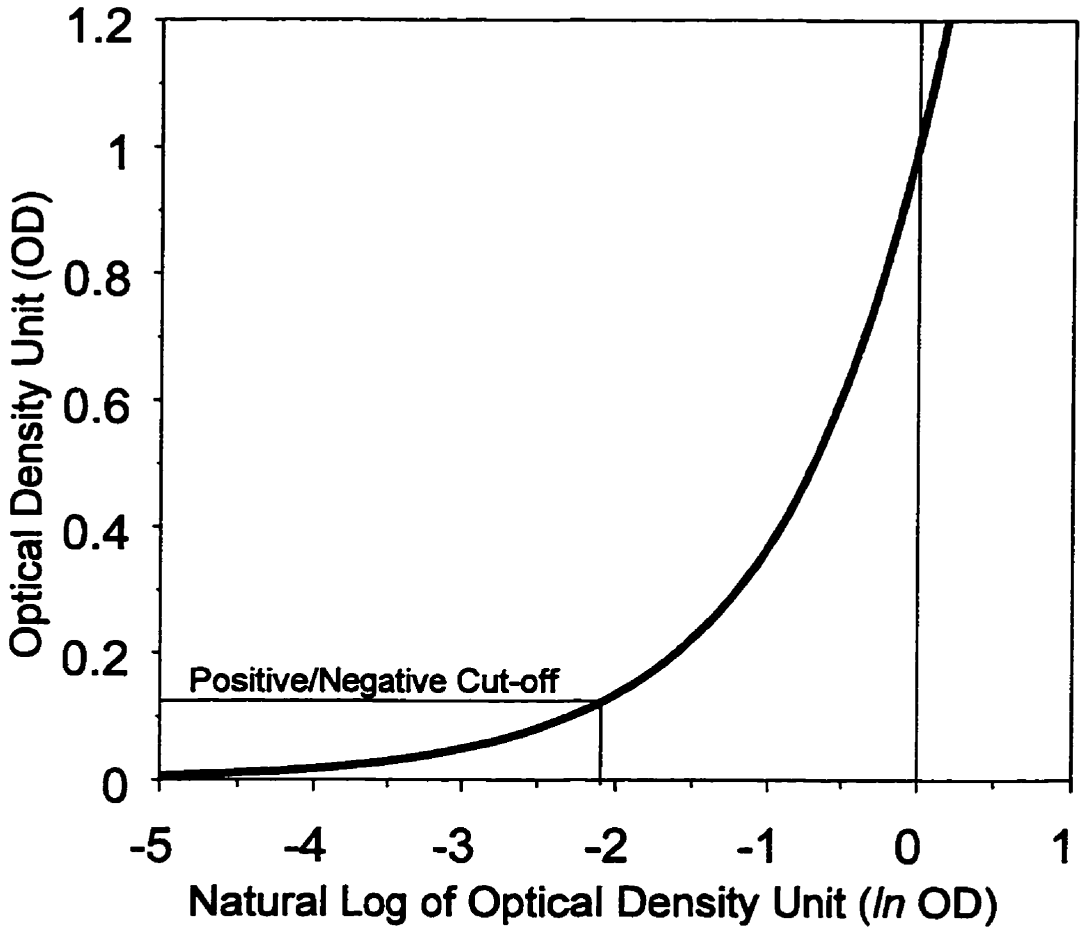


Figure 7.3: Histograms and normal probability plots of level-2 standardised residuals for newborns with passive transfer of maternal antibodies from three-level, fully specified maximal model incorporating AR-1 correlation component and separate random time-effects by sero-event definition, where adjusted optical density (OD) units, derived from observations made in a longitudinal study conducted in Kiambu District from July 1991 - June 1992, were modelled as the dependent variable (Table 7.5). Standard normal distribution is superimposed on histograms as bold lines.

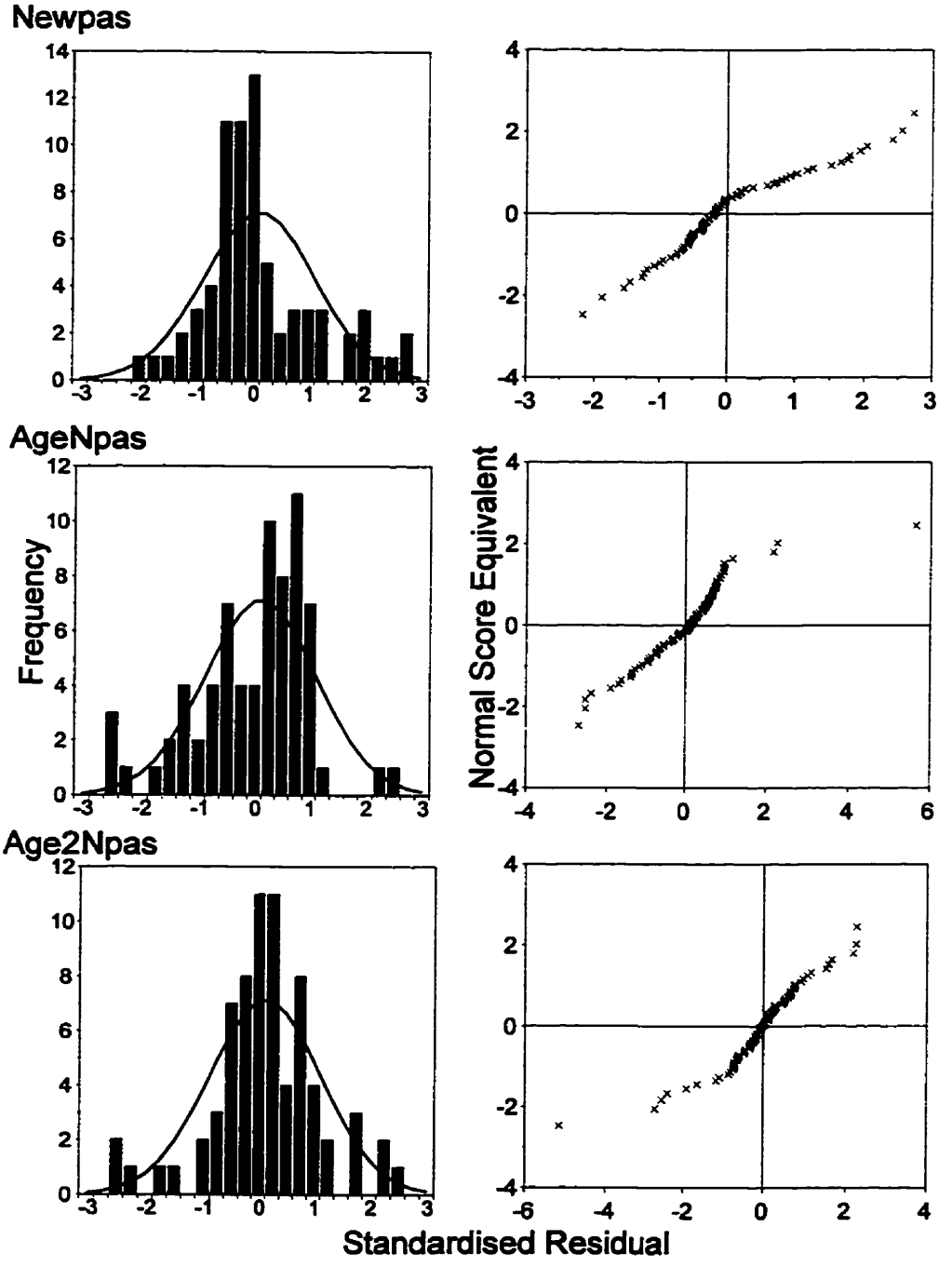


Figure 7.4: Plots of level-2 standardised residuals for newborns with passive transfer of maternal antibodies demonstrating correlations between (A) intercept (Newpas) and linear age-effect (AgeNpas) terms and (B) linear and quadratic (Age^2Npas) age-effect terms, from three-level, fully specified maximal model incorporating AR-1 correlation component and separate random time-effects by sero-event definition, where adjusted Optical Density (OD) units, derived from observations made in a longitudinal study conducted in KiambuDistrictfrom July 1991 - June 1992, were modelled as the dependent variable (Table 7.5).

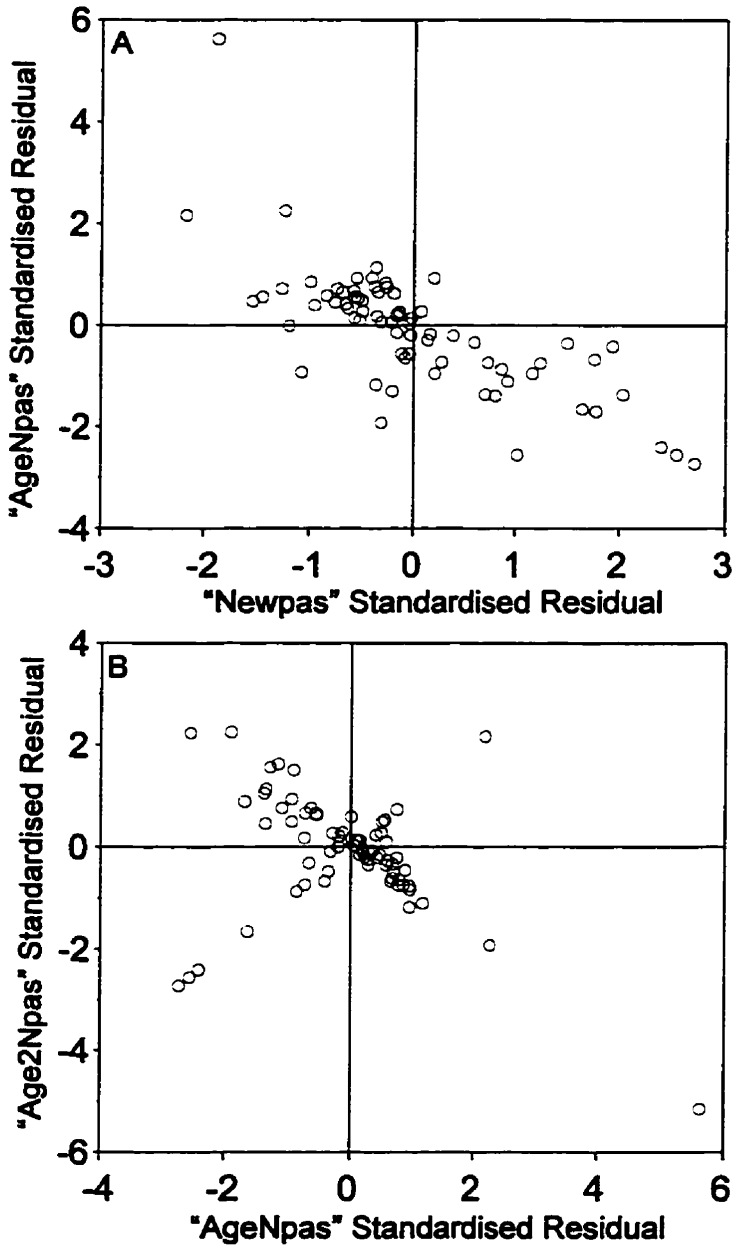


Figure 7.5: Plots of relationship between mean OD and total variance by age (A) and individual lines of OD by age (B) for newborns with passive transfer of maternal antibodies, from three-level, fully specified maximal model incorporating AR-1 correlation component and separate random time-effects by sero-event definition, where adjusted optical density (OD) units, derived from observations made in a longitudinal study conducted in Kiambu District from July 1991 - June 1992, were modelled as the dependent variable (Table 7.5).

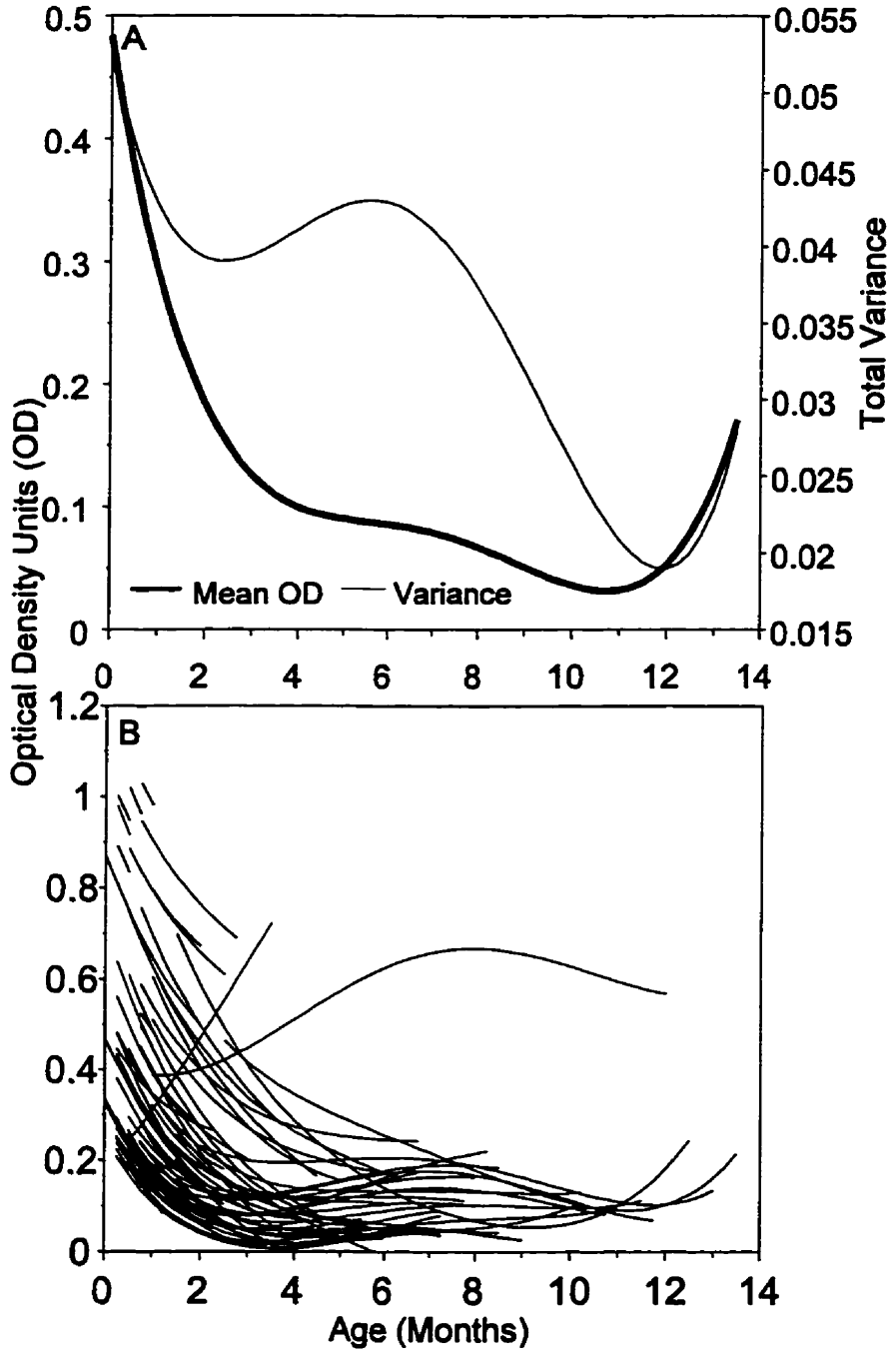


Figure 7.6: Plots of relationship between mean OD and total variance by time (independent of age effects) (A) and individual lines of OD by age (B) for animals (other than newborns with passive transfer of maternal antibodies) which did not experience sero-events, from three-level, fully specified maximal model incorporating AR-1 correlation component and separate random time-effects by sero-event definition, where optical density (OD) units, derived from observations made in a longitudinal study conducted in Kiambu District from July 1991 - June 1992, were modelled as the dependent variable (Table 7.5).

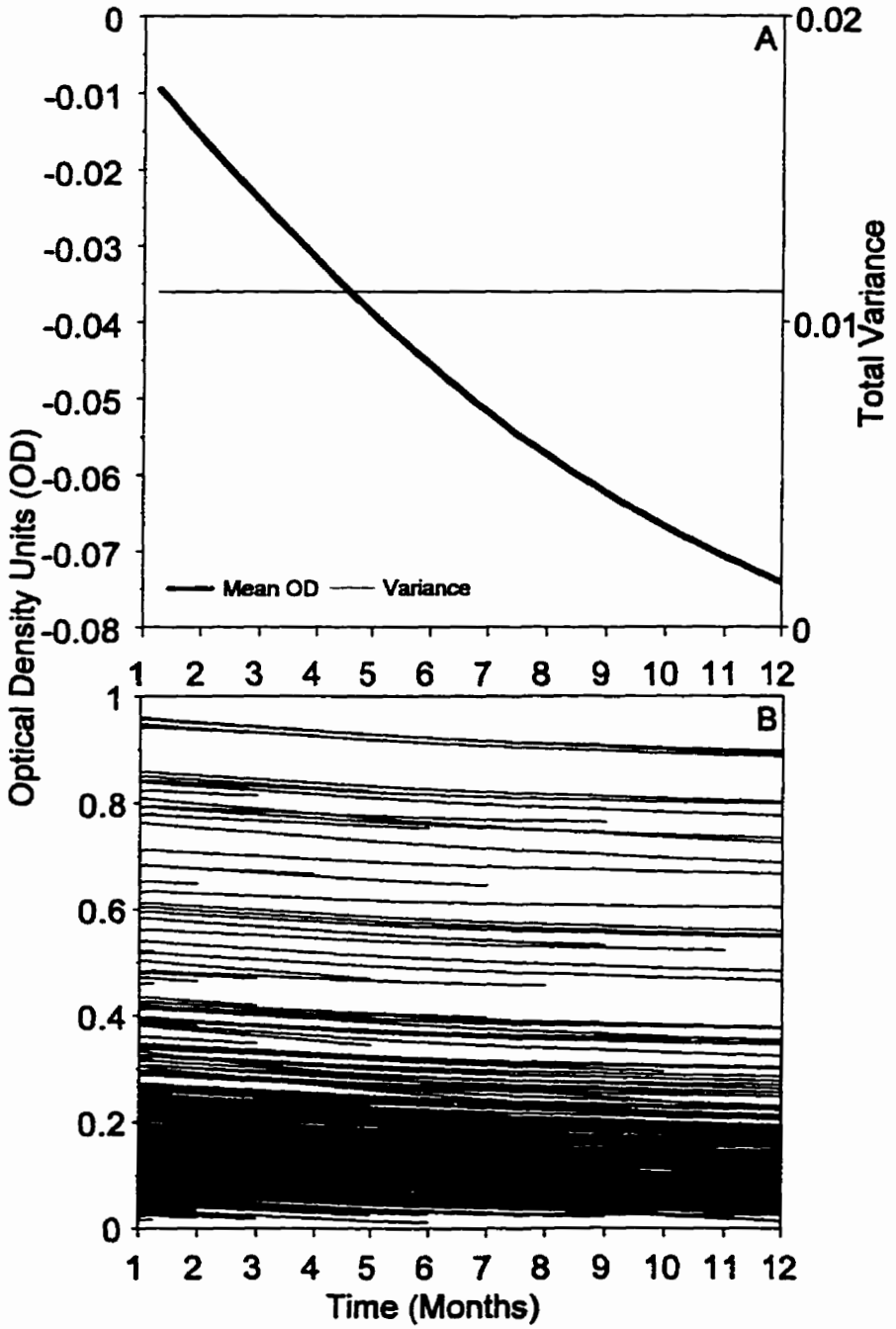


Figure 7.7: Plots of relationship between mean OD and total variance by time (independent of age effects) (A) and individual lines of OD by age (B) for animals (other than newborns with passive transfer of maternal antibodies) which experienced sero-events, from three-level, fully specified maximal model incorporating AR-1 correlation component and separate random time-effects by sero-event definition, where optical density (OD) units, derived from observations made in a longitudinal study conducted in Kiambu District from July 1991 - June 1992, were modelled as dependent variable (Table 7.5).

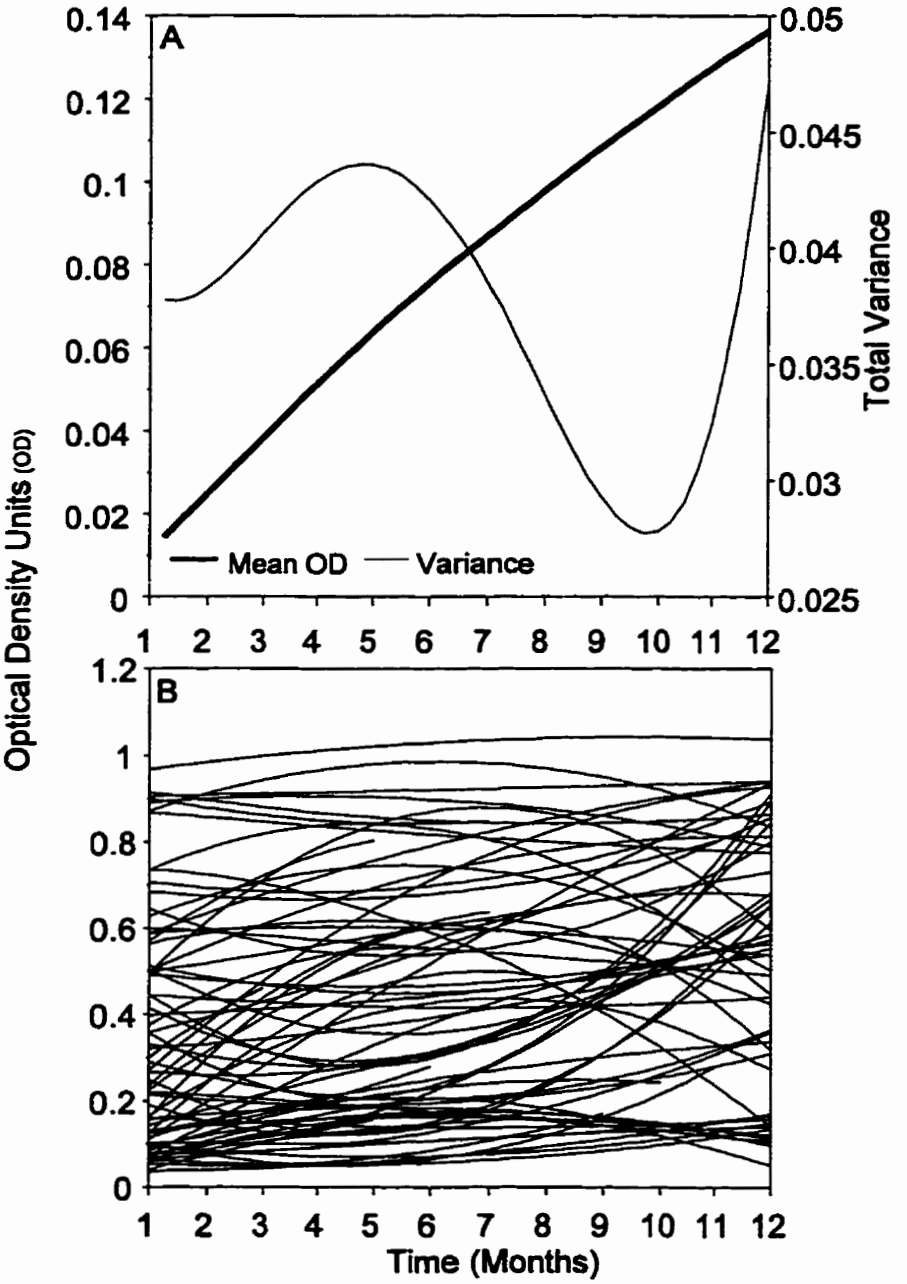


Figure 7.8: Plots of individual lines over age/time and overall mean-age relationship of optical density units (OD) for 453 animals other than newborns with passive transfer of maternal antibodies, by agro-ecological zone (UM, LH and UH) and grazing system (Zero-grazing, Grazing), from three-level, fully specified maximal model incorporating AR-1 correlation component and separate random time-effects by sero-event definition, where optical density (OD) units, derived from observations made in a longitudinal study conducted in Kiambu District from July 1991 - June 1992, were modelled as the dependent variable (Table 7.5).

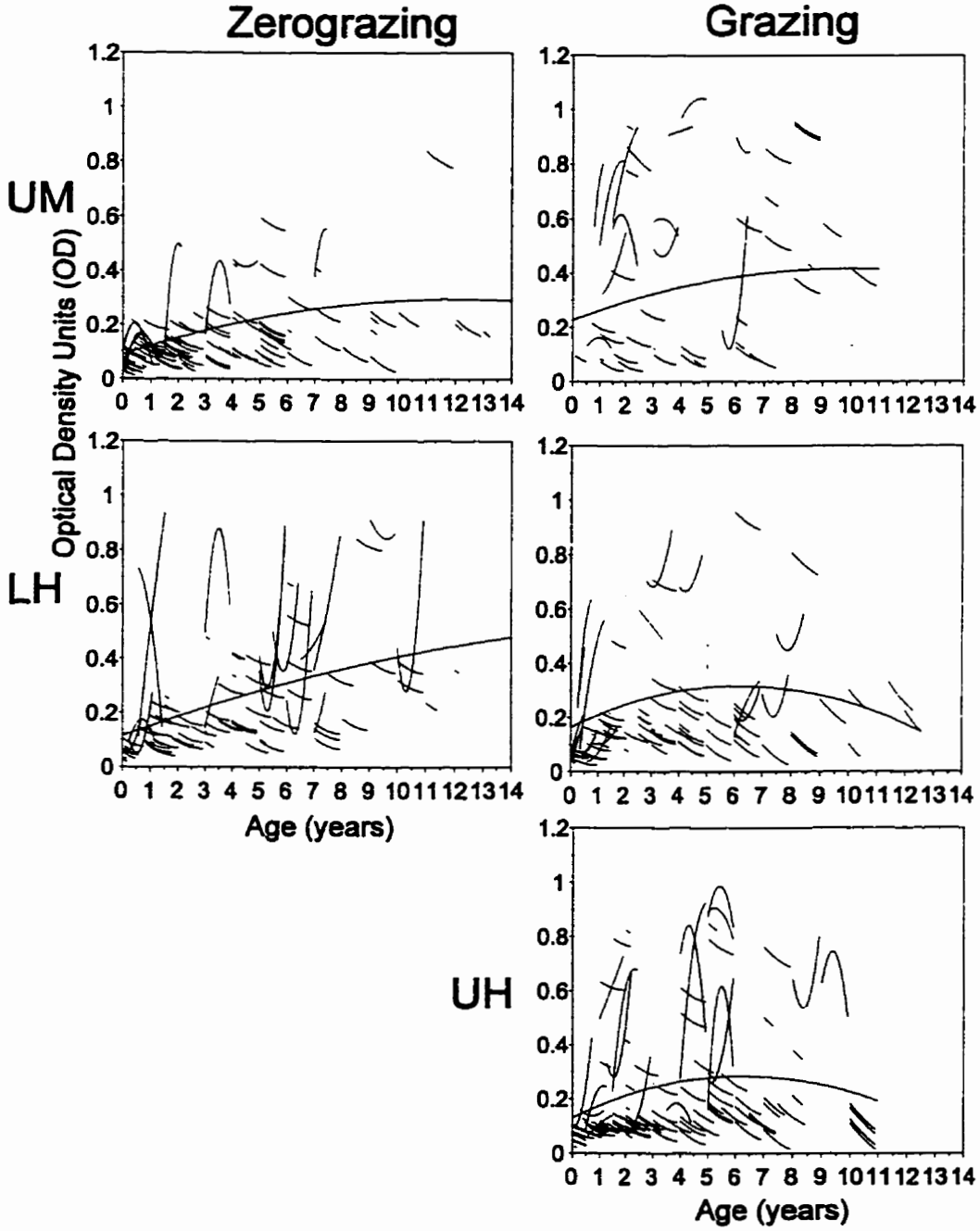


Figure 7.9: Plots of adjusted optical density (OD) units (derived from observations made in a longitudinal study conducted in Kiambu District from July 1991 - June 1992) by age for: (A) 72 newborns with passive transfer of maternal antibodies, (B) 206 other animals present on farms practising Zero-grazing and (C) 247 other animals present on farms practising semi- or full- grazing.

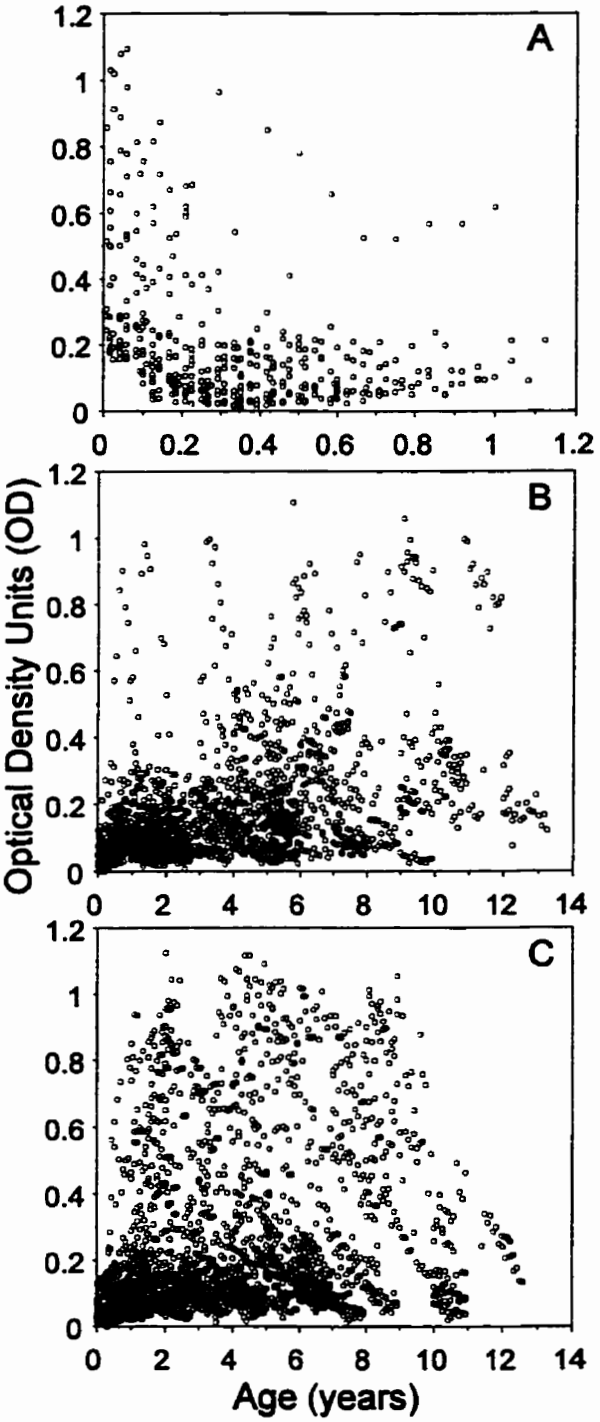


Figure 7.10: Plots of individual lines of optical density units (OD) over age/time for 453 animals, other than newborns with passive transfer of maternal antibodies, present on farms practising semi-/full-grazing, by sero-event definition, from three-level, most parsimonious reduced model incorporating AR-1 correlation component and separate random time-effects by sero-event definition, where optical density (OD) units, derived from observations made in a longitudinal study conducted in Kiambu District from July 1991 - June 1992, were modelled as the dependent variable (Table 7.6).

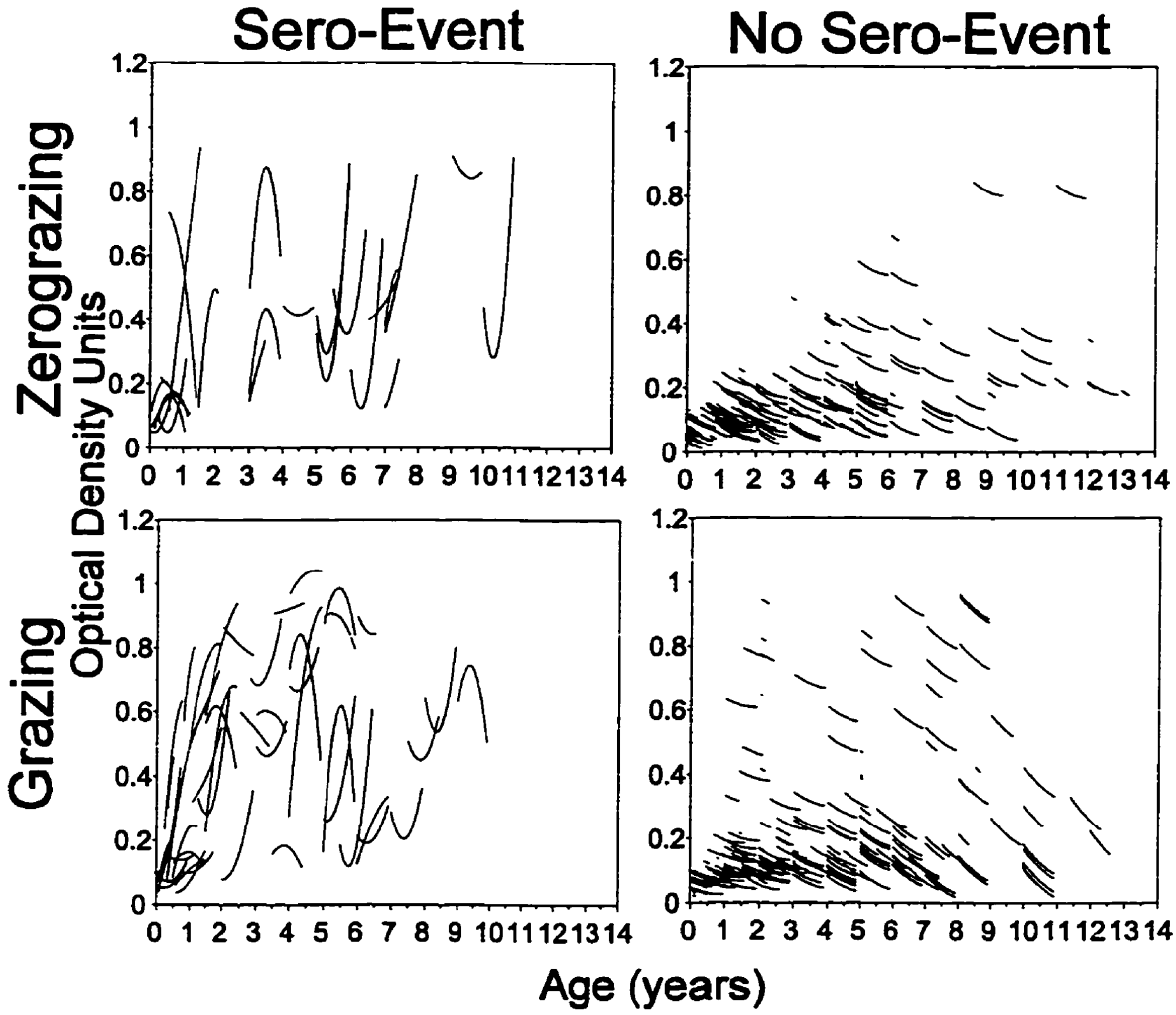


Figure 7.11: Comparison of plots of overall mean-age relationship of optical density units (OD) for 453 animals other than newborns with passive transfer of maternal antibodies, by grazing system (Zero-grazing, Grazing), from three-level, most parsimonious reduced model incorporating AR-1 correlation component and separate random time-effects by sero-event definition, where optical density (OD) units, derived from observations made in a longitudinal study conducted in Kiambu District from July 1991 - June 1992, were modelled as the dependent variable (Table 7.6).

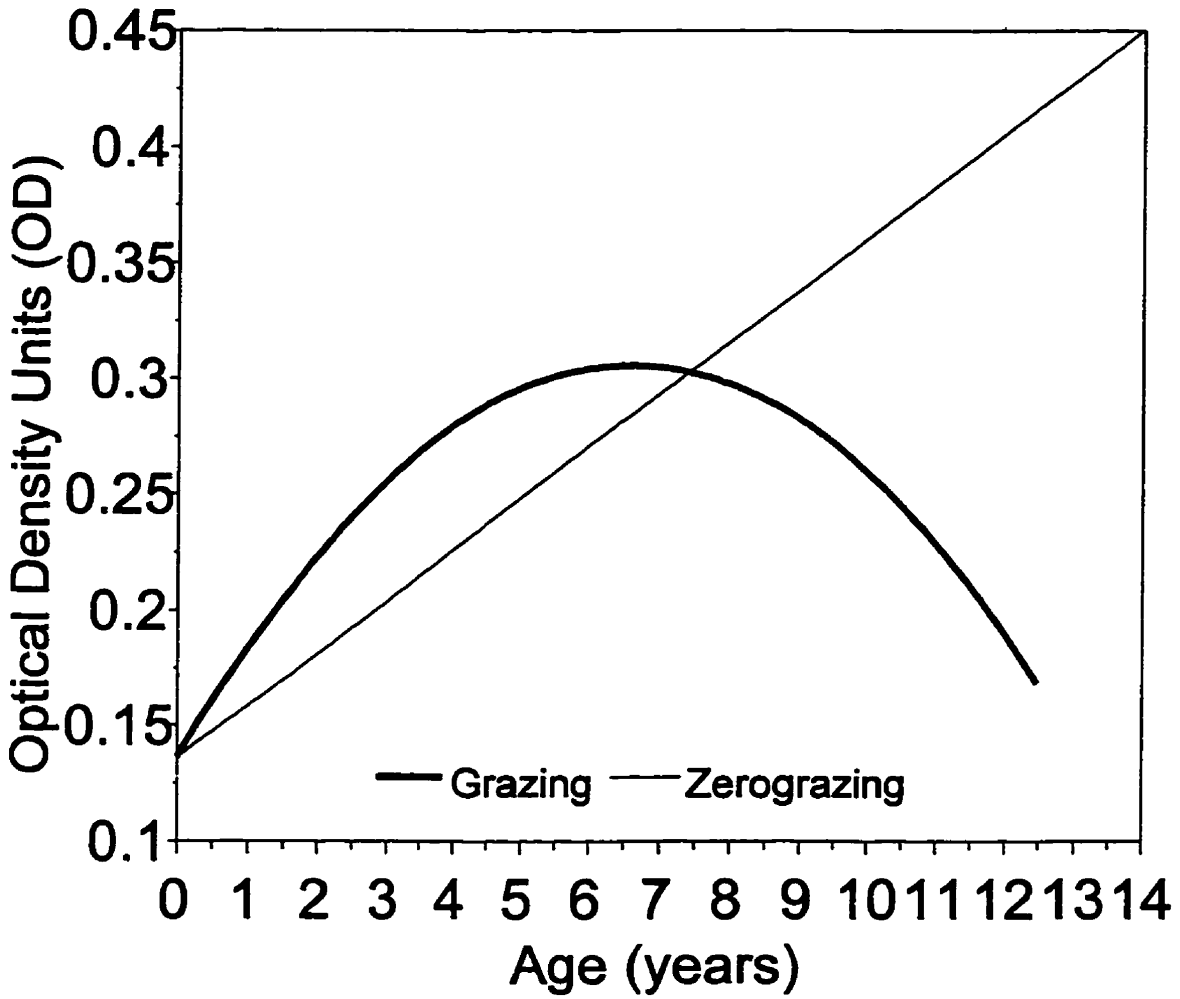


Figure 7.12: Histogram (A) and normal probability plot (B) of level-1 standardised residuals from three-level, reduced model incorporating AR-1 correlation component and separate random time-effects by sero-event definition, where optical density (OD) units, derived from observations made in a longitudinal study conducted in Kiambu District from July 1991 - June 1992, were modelled as the dependent variable (Table 7.6). Standard normal distribution is superimposed on histogram as bold line.

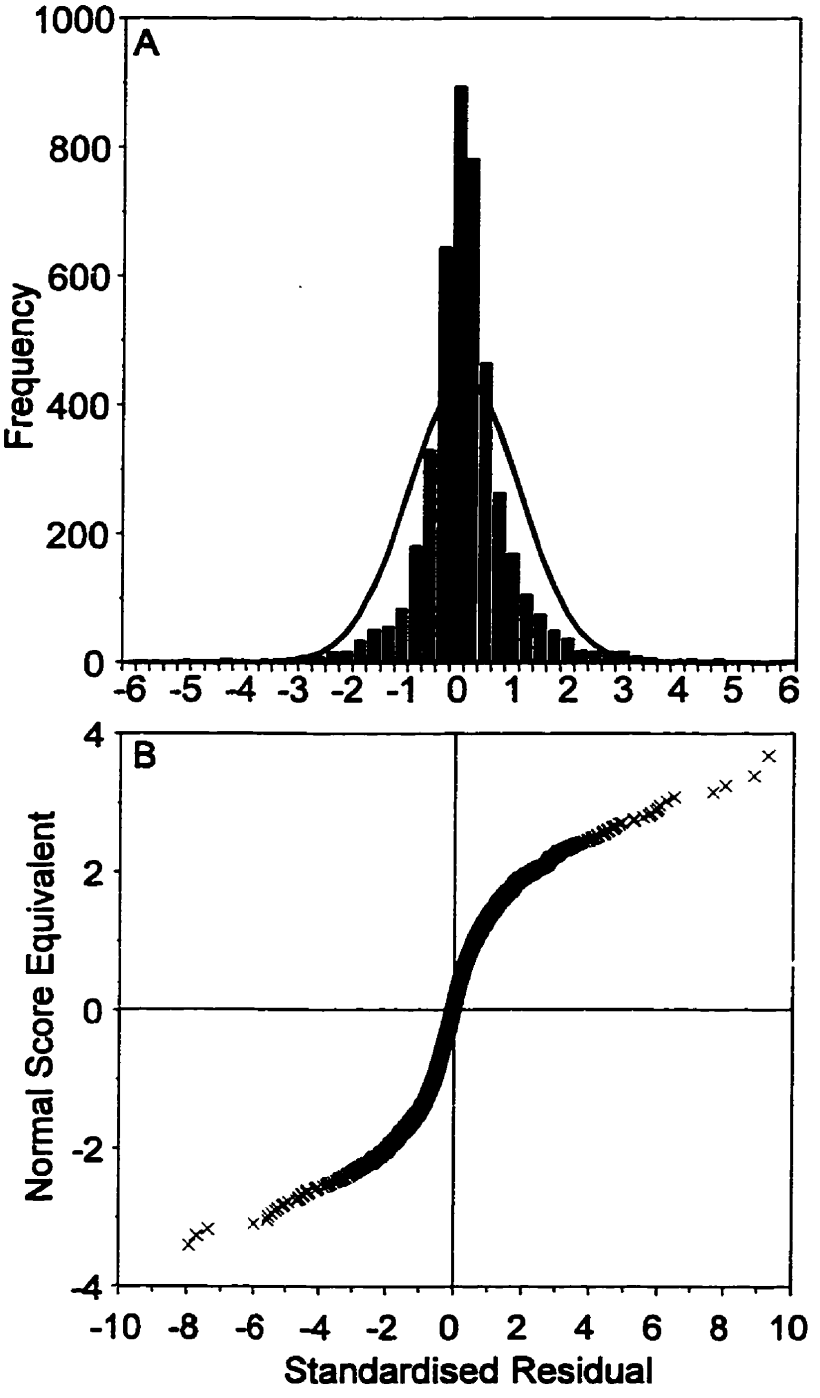


Figure 7.13: Age-seroprevalence profiles for newborns with passive transfer of maternal antibodies as estimated from three-level age-effect only models where a logistic transformation of binary outcome (positive/negative=optical density units >/≤ 0.125, derived from observations made in a longitudinal study conducted in Kiambu District from July 1991 - June 1992) was modelled as the dependent variable using i) a penalised quasi-likelihood (PQL) second order iterative estimation procedure with level-1 variance constrained to the binomial assumption and ii) a Metropolis-Hastings sampling method in a Markov Chain Monte Carlo (MCMC) iterative estimation procedure in which level-1 variance was constrained to the binomial assumption and parameter estimates are derived from 1,000,000 iterations.

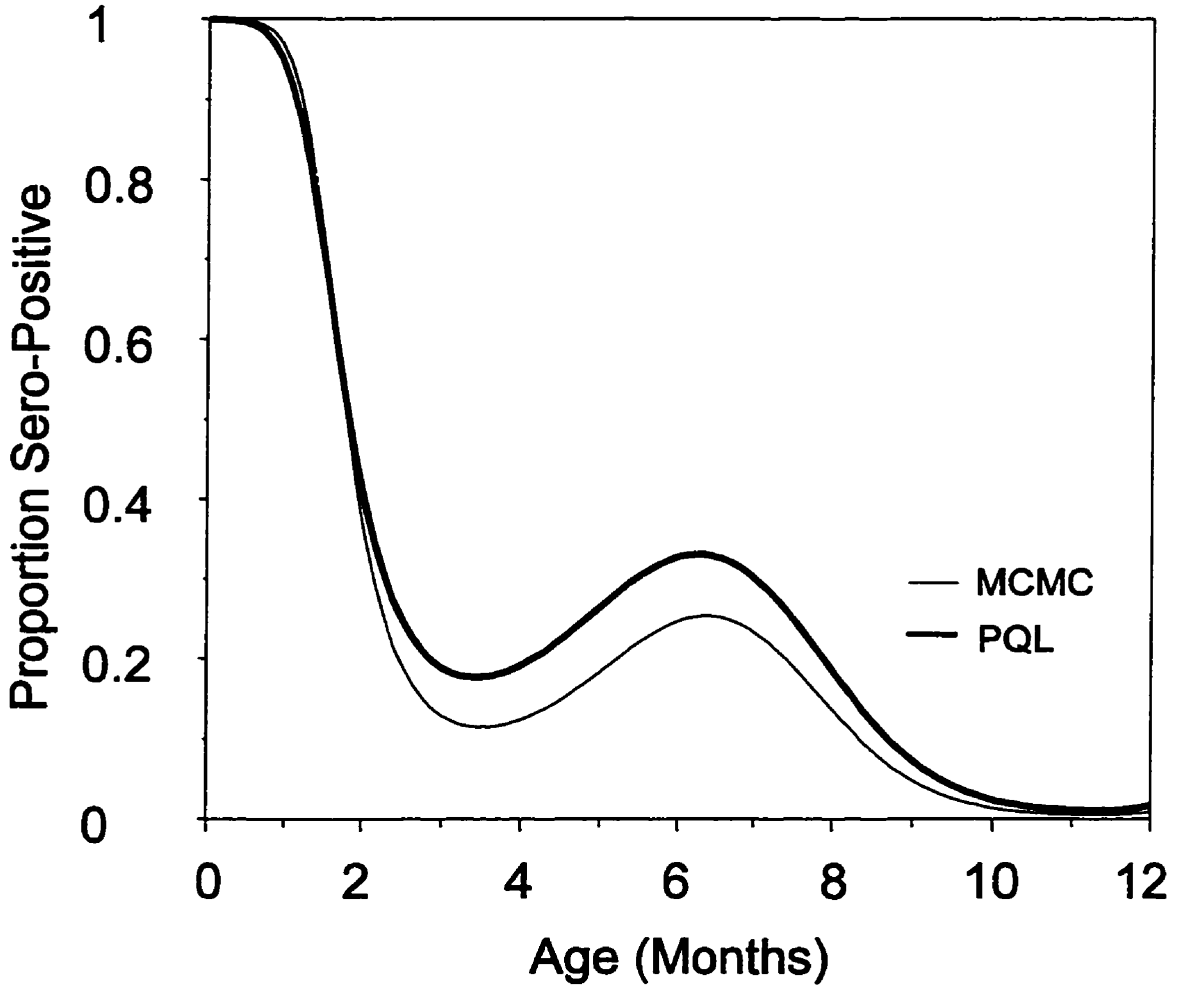


Figure 7.14: Histograms and normal probability plots of standardised residuals by level from three-level, most parsimonious reduced models incorporating fixed age and time effects where a logistic transformation of binary outcome (positive/negative=optical density units >/≤0.125, derived from observations made in a longitudinal study conducted in Kiambu District from July 1991 - June 1992) was modelled as the dependent variable using a penalised quasi-likelihood (PQL) second order iterative estimation procedure and where the level-1 variance was constrained to the binomial assumption. Standard normal distributions are superimposed on histograms as bold lines.

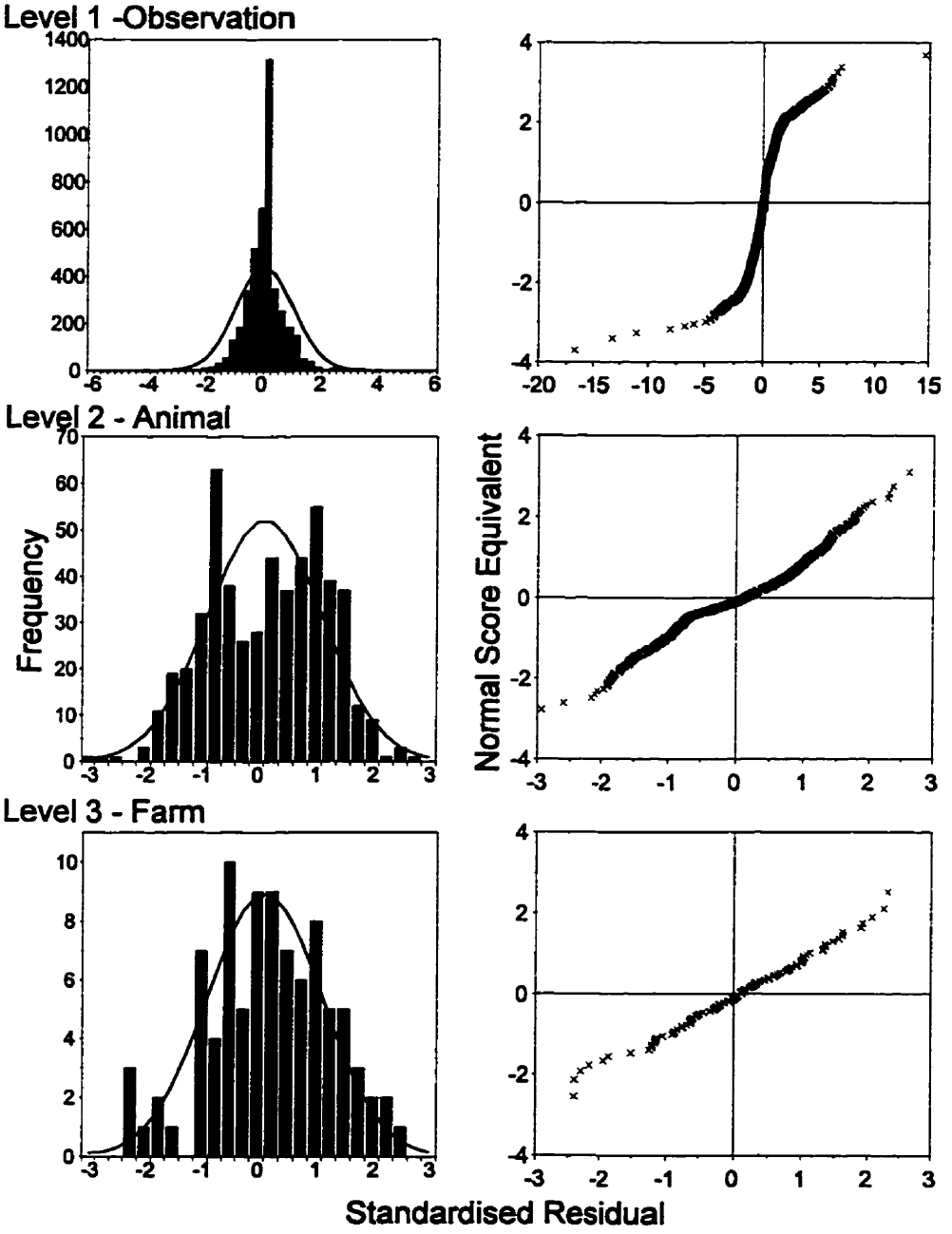


Figure 7.15: Age-seroprevalence relationship for 453 animals other than newborns with passive transfer of maternal antibodies by semi-/full-pasture grazing and zero-grazing farms as estimated from three-level reduced (A) age-effect only and (B) age and time-effect models where a logistic transformation of binary outcome (positive/negative=adjusted optical density units >/≤0.125, derived from observations made in a longitudinal study conducted in Kiambu District from July 1991 - June 1992) was modelled as the dependent variable using a Metropolis-Hastings sampling method in a Markov Chain Monte Carlo (MCMC) estimation procedure in which level-1 variance was constrained to the binomial assumption and parameter estimates are derived from 1,000,000 iterations.

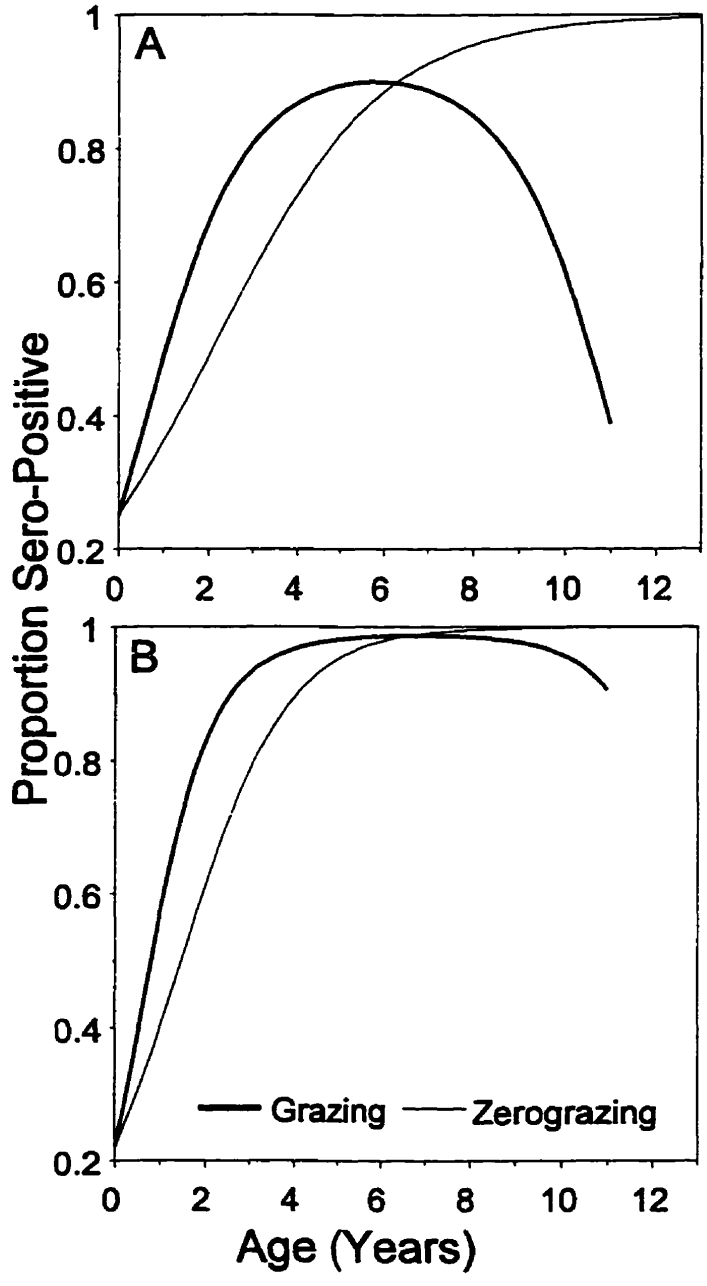
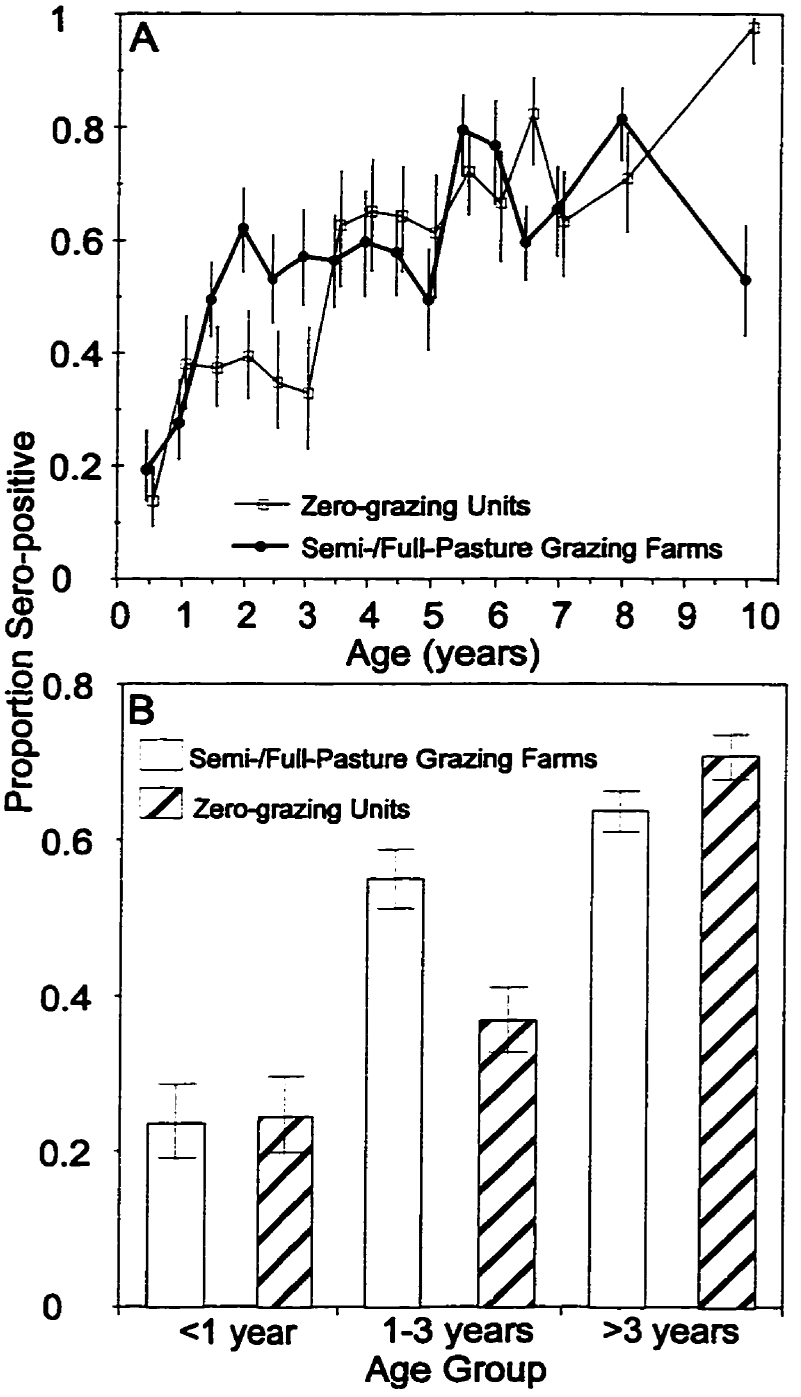


Figure 7.16: Crude age-seroprevalence proportions for 453 animals other than newborns with passive transfer of maternal antibodies by semi-/full-pasture grazing and zero-grazing farms derived using binary outcome (positive/negative=adjusted optical density units >/≤0.125, derived from observations made in a longitudinal study conducted in Kiambu District from July 1991 - June 1992) and calculated across age (A) and for 3 separate ageclasses (B).



CHAPTER 8

DISCUSSION

8.1 Study Design

This study utilised a stratified random sampling procedure followed by a period of active monitoring to derive estimates of the incidence of clinical ECF and *T. parva* infection on smallholder dairy farms in Kiambu District, Kenya. Although not without its weaknesses, a study design incorporating a system of active monitoring in combination with random sampling was deemed most appropriate to the overall Smallholder Dairy Development Project objective of obtaining an accurate assessment of health and productivity constraints to the smallholder dairy industry of the Central Kenyan Highlands (Gitau *et al.*, 1994c). Although more common, passive reporting systems and data generated from purposive sampling, or even cross-sectional studies, potentially suffer from both unreliability and bias. For example, unbiased estimates of mean calving intervals of 633 days (Odima *et al.*, 1994) and mean milk yields of 5.8 kg/day (Omore *et al.*, 1994; Omore, 1996), generated by parallel studies within the project framework, demonstrated markedly inferior production than shown by indices (460-480 days and 8.3 kg/day respectively) estimated by the National Dairy Development Programme (NDDP) for the same population (Van der Valk, 1987, 1988, 1992). These differences are most likely to have arisen owing to the NDDP method of convenience sampling and selective exclusion of farms with poorer performance. Similarly, a retrospective study of ECF incidence in Kiambu District (Mulei and Rege, 1989) used

cases of ECF observed by the Ambulatory Service of the University of Nairobi's Faculty of Veterinary Medicine and an estimated at-risk population, making the assumption that all cases of ECF were reported. The results of the current study shows that this assumption is untenable, as 70% (9/13) of clinical cases which exhibited concomitant serological evidence of infection were not diagnosed as ECF. Additionally, farmers often ignored animals perceived to be of low value (e.g. male dairy calves - Gitau *et al.*, 1994b) or self-treated clinical cases.

Cross-sectional studies can yield point estimates of *T. parva* seroprevalence and possibly risk-factor associations (when these are recorded). Relevant examples include purposive sampling studies conducted in the Lake Victoria Basin (Morzaria *et al.*, 1988) and country wide (FAO, 1975), and stratified random sampling by agro-ecological zone, but restricted to calves, in Kilifi District (Deem *et al.*, 1993). However, this design provides no or poor quality estimates of ECF incidence and mortality and hence may be of limited value in assessing the epidemiological state.

The current observational study, which utilised random sampling to select collaborating farms, was able to provide unbiased information on the distribution of management practices hypothesised to be risk factors. Further, the ECF morbidity and mortality estimates derived from longitudinal observations are more likely to be representative of the state of nature in the population of interest, namely smallholder dairy farms in Kiambu. Unfortunately, the ability to subsequently assess differences across the potential risk factors was dependent on their individual and concurrent distributions. Homogeneity of risk factors was not generally a problem in this study, with the possible

exception of breed of cattle, as virtually all animals were *Bos taurus* or had a vague and uncertain lineage which may have involved *Bos indicus* cross-breeding at least several generations previously. However, strong multi-collinearites were observed between several management practices and demographic covariates. As specific examples, i) all farms in the upper highland agro-ecological zone (UH) practised semi-/full-pasture grazing, while approximately two-fifths did so in each of the lower (LH) and upper (UH) highland zones, ii) acaricide application by plunge dipping was practised by two-thirds of farms in the upper midland (UM) zone but by approximately one-third of farms in either LH or UH zones, and iii) three-quarters of all zero-grazing farms applied acaricides monthly or less frequently (under conservative reclassification criteria) compared to only about one-quarter of semi-/full-pasture grazing farms. In consequence the ability to evaluate multiple contrasts was limited.

Studies with a greater component of experimental design sacrifice a measure of representativeness to ensure (or promote) orthogonality of contrasts. For example, other longitudinal studies of ECF on smallholder dairy farms, conducted in the Coastal Lowlands (Maloo *et al.*, 1994) and Central Highlands (Gitau *et al.*, 1997; Gitau, 1998) of Kenya, have attempted to avoid problems of homogeneity and collinearity of covariates by undertaking an initial cross-sectional survey followed by stratification on known or suspected risk factors in the longitudinal component. Maloo *et al.* (1994) stratified on agro-ecological zone, grazing management system, herd and cattle type and conducted a near census (80% smallholder dairy coverage) of Kaloleni Division before subsequently purposively selecting 30 free- (equivalent to semi/full-pasture grazing) and 30 zero-grazing smallholder dairy

herds, but within one zone. Thus, while they were able to confirm higher incidence of clinical ECF in all age groups on free-grazing farms, they could only make this inference within a single zone. Gitau *et al.* (1997) stratified and randomly sampled by agro-ecological zone and sublocation and then restricted their consideration to newborn female calves, purposively selecting three zones on the basis of antibody prevalence to *T. parva* and recruiting willing participants (with calves) within each zone. This study also experienced the problem of collinearity between agro-ecological zone of origin and grazing management system practised, but multiple variable analysis was nevertheless able to simultaneously demonstrate significant associations with both variables and ECF-specific morbidity rates in calves and also with survival functions for time to seroconversion (Gitau, 1998). However, unlike the current study or that of Maloo *et al.* (1994), inference could not be drawn concerning the degree and importance of *T. parva* transmission and challenge among adult animals.

An alternative, but much less efficient, method of dealing with multi-collinearites (in the absence of complete homogeneity of risk factors) is to simply increase sample size until sufficient power is achieved to simultaneously evaluate the desired contrasts. However, the study sample size of 90 farms was originally chosen based on logistical constraints. This was thought to be the maximum number of farms which could be visited while maintaining a sufficiently short interval to i) minimise recall bias, ii) accurately document incidents of disease and fatalities (Gitau *et al.*, 1994b), iii) record reproduction (Odima *et al.*, 1994), milk production (Omore, 1996), calf growth (Gitau *et al.*, 1994a) and iv) collect longitudinal serological data (O'Callaghan *et al.*, 1994). Another method to increase the apparent sample

size is to take more measurements per animal. Nonetheless, by their nature, longitudinal measures of antibody levels, daily milk production, calf growth, etc. are not independent, such that there is a law of diminishing returns in the amount of actual data generated as the degree of correlation increases with shorter intervals of observation. With the exception of the first round of sampling, when the administration of the comprehensive farm-survey required that several hours be devoted to each farm, the chosen minimum inter-visit interval of 30 days was maintained throughout the year-long study for a total of 334 adult animals and 201 calves on 90 farms. This compares favourably to monitoring levels achieved in other longitudinal studies of tick-borne diseases in Kenya. Maloo *et al.* (1994) also utilised a monthly sampling interval but did so for 110 animals on 60 farms. Gitau (1998) undertook fortnightly sampling and observation on 188 farms, using a cohort of 225 calves. Moll *et al.* (1986) undertook even more intensive weekly serological monitoring, but of a single cohort of 31 calves in a community of free-ranging indigenous cattle in the endemically stable Trans-Mara District of Kenya.

In retrospect, it is apparent that efforts to maintain the intensity of observation for the given sample size were sometimes at the expense of the type and quality of data collected. A specific weakness of this study was the lack of attention paid to documenting the presence and numbers (by instar), and determining infection status, in field samples of *R. appendiculatus*. In mitigation, full body, or even half-body tick counts are very time consuming and labour intensive, a situation further exacerbated when overlapping tick distributions require additional expertise in species differentiation. In this study, counts of "Standard Ticks", made according to the method of Norval *et al.* (1992) and documenting

only those adult ticks attached and engorging, without differentiating by species, were intended to be a crude measure of the overall level of tick challenge and efficacy of acaricide control. The presence of immature stages, and newly attached adults, irrespective of species, was also simply recorded in a "yes/no" fashion. Although both of these measures could be accommodated within the longitudinal sampling framework, they yielded few data on the role of the vector population. In contrast, under fortnightly observation, Gitau (1998) identified *R. appendiculatus* ticks by instar, gender and degree of engorgement, although he too restricted his analysis to simple presence/absence data for each combination and did not report collecting ticks from the field or determining their infection status. However, it has since been suggested that nymphal transmission of infection may be more important than first realized, both with respect to the proportion of infections and the dose of sposozoites administered (Medley, personal communication). This observation is based on the meticulous work by Ochanda *et al.* (1996) on the distribution of prevalence and intensity of infection in adult and nymphal *R. appendiculatus* by host stage of infection, incorporated in an adaptation of the original transmission dynamic model of *T. parva* (Medley *et al.*, 1993). In light of the above, it is important that future studies of ECF, and indeed tick-borne diseases in general, pay greater attention to the infection within the vector population and the role this plays in epidemiology of disease in the host.

Finally, with a voluntary enrollment rate among eligible farms of 98% and homogeneously distributed withdrawal and attrition rates of 11% and 2 % respectively after one year, non-response and loss-to-follow-up biases (Martin, Meek and Willeberg, 1987) were considered to be minimal in this study.

8.2 Serological Analyses - Test Standardisation and Quantification

Cross-sectional studies of ECF (FAO, 1975; Morzaria *et al.*, 1988; Deem *et al.*, 1993; Gitau *et al.*, 1997) have generally been concerned with documenting the sero-prevalence to *T. parva* as a measure of past exposure and have relied on diagnostic tests correctly classifying animals as positive or negative. Initial problems of cross-reactivity (Burridge *et al.*, 1974a, 1974b) and immunological sensitivity (Burridge and Kimber, 1973b) observed in the *T. parva* schizont indirect fluorescent antibody (IFA) test (Burridge and Kimber, 1972; Goddeeris *et al.*, 1982) have been solved by the development of an epidemiologically sensitive and specific enzyme-linked immunosorbent assay (ELISA), based on the polymorphic immunodominant molecule (PIM - Toye *et al.*, 1991, 1996). Although the antigen available at the time of serological analysis was the chromatographically separated and purified equivalent of the recombinant protein, developed and utilised later, the sensitivity and specificity of the assay are likely to be high, reflected by the 99% specificity and 94-98% sensitivity subsequently reported for the recombinant antigen (Katende *et al.*, 1998). However, beyond the potential for more accurate determination of age-specific serological profiles in the estimation of force of infection (Medley *et al.*, 1993), the usefulness of cross-sectional studies in understanding the dynamics and sequellae of infection has not been dramatically improved. Even the application of this recombinant antigen ELISA test in determining the time to seroconversion of naive calves in a longitudinal study (Gitau, 1998) provided accurate information on the initial acquisition of infection and not on the rate of occurrence or implications of repeated challenge.

By longitudinal monitoring of *T. parva* schizont-specific antibodies, the current study

sought to document cases of both initial seroconversion and subsequent anamnestic immune responses on rechallenge, to relate these events to the incidence of clinical disease and to thereby derive a better understanding of the population infection dynamics in the epidemiology of ECF. The interpretation of serological profiles was predicated on being able to repeatably and accurately determine the amount of antibody in a given sample. At the time of analysis of samples, efforts to standardise the ELISA test had focussed on minimising intra-plate variation and maximising test sensitivity and specificity. While it was theoretically possible to utilise the assay at that stage of development to derive estimates of the amount of antibody by titrating all serum samples to extinction, a conservative estimate is that this would have increased the total workload by a factor of 10 - 20, requiring in excess of a year to complete all 4404 samples. In consequence, considerable efforts were made, first, to standardise the protocol such that individual single-dilution values were comparable and second, to establish a standard titration-curve through which changes in single-dilution values could be explicitly quantified. The process of assay standardisation was based on the use of a targeting protocol (Wright *et al.*, 1987) and was derived through an application of the chemical properties and enzyme kinetics of the ELISA system (Tijssen, 1985). Its application resulted in arrays of longitudinal adjusted optical density (OD) values for study animals that could be interpreted and analysed with confidence.

8.3 Diagnosis of East Coast Fever

The hierarchical system of disease diagnosis which was ultimately employed in the current study was derived in response to logistical constraints, but was less rigorous than the

minimum field definition proposed prior to the development of serological assays (Yeoman, 1966). In the absence of ancillary diagnostic techniques, such as direct detection of schizonts in lymph node biopsies/thin film blood smears or of piroplasms in red blood cells 5-8 days subsequently, putative diagnosis of ECF was based exclusively on clinical signs. Clearly, this was less than ideal, but in animals which survived the episode of clinical disease it was possible to determine whether or not the serological profile was consistent with a diagnosis of ECF. The results from this work draw attention to the fact that the characteristic clinical signs attributed to ECF (Norval *et al.* , 1992) are not pathognomonic nor were they consistently present in even the majority of cases detected. Indeed for both adults and calves in the current study, more ECF was missed than was correctly diagnosed. Of the 13 reported incidents of ECF in mature cattle, 5 (38.5%) exhibited serological evidence suggestive of *T. parva* infection, while the serological profiles of 9 other adults demonstrated that the non-specific clinical signs observed were best attributed to a missed ECF diagnosis. Similarly, of the 4 reported cases of ECF in calves, 2 (50%) were confirmed subsequently, but a further 4 clinical incidents were later identified as mild ECF. These findings are consistent with those of Gitau (1998), where, despite more intensive longitudinal monitoring, approximately one-quarter of the non-fatal illnesses in calves attributed to ECF (7/26), failed to exhibit evidence of seroconversion. In the current study, the most notable clinical feature of the missed ECF diagnoses was the near uniform presence of mild respiratory signs such as cough, increased respiratory rate and sounds and dyspnea, observed in some combination in 85% (11/13) of cases. The complexity in establishing a case definition for ECF is illustrated by the wide range of classification of reactions to *T. parva* infection derived by a consensus

of expert opinion (Anon, 1989). The findings of this study serve to further demonstrate the unreliability of passive reports of ECF and also suggest that greater attention needs to be paid to accurately diagnosing ECF in the field. In addition, the value of interpreting longitudinal serological profiles is highlighted.

8.4 Measures of ECF in Calves and Mature Cattle

Crude calf mortality in the current study was high with nearly one-third of calves dying by one-year of age, although mortality attributed to ECF was very low at a rate of 1.2%, based on a single death reported on a semi-/full-pasture grazing farm. This ECF fatality constituted only 4.0% of all calf deaths, but yielded a case-fatality proportion of 14%, considering all cases of ECF in calves confirmed by serology. This contrasted with the results reported in the longitudinal study of Gitau (1998) in the adjacent Murang'a District of Kenya, where an overall ECF-specific mortality rate of 4.5% was estimated with 60% of all deaths attributed to ECF and a serologically-confirmed case-fatality proportion of approximately one-third. However, two-thirds of the calf deaths recorded in that study were from the sole agro-ecological zone not represented in the current study (upper midland zone 4), such that, when only the two zones in common (upper midland 1 and 2) are considered, the mortality rate estimate falls to 2.6%, although this still represents half of all calf deaths and the estimate of case-fatality proportion remains unchanged. Put simply, had one more calf death in the current study been attributable to ECF the comparable rates between the two studies would have been virtually identical.

With respect to the pattern of ECF-specific morbidity rates observed in calves, the

overall ECF morbidity rate of 7.3% (based on clinical signs and serological confirmation) observed in the current study agreed very closely with the rate of 6.5% observed by Gitau (1998) in agro-ecological zones common to the two studies. Further, the pattern of a higher rate in semi-/full-pasture grazing farms reported in that study was also observed between zero-grazing and semi-/full-pasture grazing farms in the upper midland (UM) zone of this study, but was even more extreme with no ECF cases reported in calves on zero-grazing farms. However, in the lower highland (LH) zone, the rates on zero-grazing and semi-/full-pasture grazing were virtually identical such that the interpretation of an overall higher rate on semi-/full-pasture grazing farms can not be made. Reasons for this variability are not readily apparent.

The rate of mortality attributed to ECF among mature animals (1.7%) was very close to that observed in calves, however, ECF now accounted for nearly one-third of all deaths in mature animals while the case-fatality proportion was also higher at 30%. It is also noteworthy that all 5 of these deaths occurred on zero-grazing farms, yielding a rate of 4.1%.

The overall ECF-specific morbidity rate of 5.6% observed in adult cattle was marginally less than the rate observed in calves. While the pattern of a higher rate of ECF morbidity on semi-/full-pasture grazing farms than zero-grazing farms in the upper midland zone was also consistent with that for calves, in the lower highland zone the direction of this pattern was again apparently reversed, although the difference in mature animals was of greater magnitude than calves. On the basis of the consistency of this relationship, it is worth suggesting that the effect of semi-/full-pasture grazing may be magnified within the upper midland agro-ecological zone.

Undoubtedly, the most pronounced and potentially important feature of the patterns of ECF morbidity and mortality observed in both calves and adults is the very strong farm clustering. Of a total of 36 suspected or confirmed, fatal or non-fatal cases of ECF reported over the period of the longitudinal study, 58.3% of these (21/36) occurred on 6.7% of all farms (6/90). This tendency could not be attributed to greater numbers of animals present on these farms, since the five farms which each reported two or three cases of ECF housed 3, 5, 5, 5 and 10 animals. On the sixth farm, a grazing farm in the lowest agro-ecological zone (UM), 7 ECF cases were recorded among 18 animals, suggesting that this combination of AEZ/grazing system may be unstable for ECF. East Coast fever impact may vary from farm-to-farm either because of clustering of infection or perhaps also because of variation in farm-level control (see section *8.7 Multilevel Modelling of Serological Profiles*).

8.5 Primary and Secondary Immune Responses: Seroconversions and Sero-events

The use of longitudinal arrays of standardised and quantified measures of *T. parva*-specific antibody to document and define rates of primary and secondary immune responses as surrogate measures of challenge represents one of the more novel aspects of this study. Time to seroconversion (Gitau, 1998; Moll *et al.*, 1984, 1986), age/seroprevalence profiles (Medley *et al.*, 1993) and analysis of longitudinal measures of binary (positive/negative) serological status (Maloo *et al.*, 1994; O'Callaghan *et al.*, 1994) have all been undertaken for *T. parva* in Kenya. However, this study documents the first efforts to quantify the rate and effect of anamnestic immune responses to draw inference regarding the degree and importance of *T. parva* transmission and challenge among previously infected/carrier

animals (Young, Leitch and Newson, 1981; Young *et al.*, 1986; Kariuki *et al.*, 1995). Recent theoretical work on the transmission dynamics of tick-borne diseases in general (Medley *et al.*, 1993; O'Callaghan *et al.*, 1998) has drawn attention to the importance of infections of low prevalence and intensity in ticks, as derived from feeding on long-term carrier animals, in the epidemiology of disease. However, in each case the implications for reinfection/rechallenge of carrier animals have been discussed but otherwise ignored for lack of empirical data.

The concept of seroconversion of immunologically naive animals has long been utilised to define primary infection, but certain aspects of the immune response to *T. parva* suggest that it is possible to detect anamnestic humoral immune responses on secondary infections. Specifically, although the humoral response is thought to have little role in the protective mechanism, anti-schizont antibody levels rise in response to infection but rapidly decline in the absence of repeated challenge and there is considerable antigenic variation among strains of the parasite.

Burridge and Kimber (1973b) demonstrated that the duration of the serological response to primary *T. parva* infection is not lifelong in the absence of repeated challenge, although animals that recover spontaneously from infection are solidly protected against developing clinical disease under homologous challenge for up to three and a half years thereafter (Burridge *et al.*, 1972). Further, studies using transfer of immune serum (Muhammed *et al.*, 1975) and generation of high schizont-specific titres by immunisation with either heat-killed schizont infected cells or semi-purified schizont antigens (Emery, 1981) did not demonstrate any protection against challenge. The continued development of

cellular and molecular techniques has allowed researchers to confirm and explore the original hypothesis that the protective mechanism of immunity to ECF was primarily cell-mediated and directed at the schizont-infected cell (Eugui and Emery, 1981). However, there are numerous strains of *T. parva* of varying pathogenicity and virulence, immunity to many of which is not necessarily cross-protective (Norval *et al.*, 1992). In addition, there is considerable heterogeneity in field-derived stocks and antigenic diversity in infections of individual animals is also well established (Conrad *et al.*, 1989). It has also been demonstrated that sexual recombination can occur between heterologous strains of *T. parva* in the gut of engorged larval or nymphal ticks (Gauer *et al.*, 1995) such that the antigenic composition of the parasite may differ after it passes through the tick. Thus, a combination of some degree of heterologous challenge and cross-protection is likely to have accounted for the appearance of concomitant clinical signs of ECF in some post-exposure sero-events.

Therefore, the justification for the sero-event definition criteria utilised in this study is that they were derived from basic immunological principles, founded on features of the bovine immune response to *T. parva*, and verified by patterns of antibody levels actually observed in clinical cases of ECF in cattle with serological evidence of prior infection. Similar subclinical sero-increases were observed in an ECF infection and treatment immunisation project in the central province of Zambia (DiGiulo, personal communication) which were noted to coincide with the end of a period of diapause of *R. appendiculatus* (Pegram and Banda, 1990) and were thus correlated with rechallenge of *T. parva*.

Seroconversion rates were consistently higher in calves from farms which practised semi-/full-pasture grazing than on zero-grazing farms, although the difference in rates was

most pronounced within the UM agro-ecological zone. The seroconversion rate in calves from the UH zone was the lowest rate observed for semi-/full-pasture grazing. The overall rate of seroconversion and sero-events observed on farms in the UM and LH zones suggested a level of challenge equivalent to infection every 16 animal-months on semi-/full-pasture grazing farms and every 27 animal-months on zero-grazing farms, while the corresponding level on UH zone semi-/full-pasture grazing farms was of infection every 34 animal-months. The pattern of sero-event rates in mature cattle was similar to that in calves, although the maximum (based on sero-increase > 0.150 OD units) rates were uniformly lower. The rate of sero-events detected in mature cattle was consistent with successful feeding by an infected tick approximately every 5 animal-years on zero-grazing farms and every 3 animal-years on semi-/full-pasture grazing farms. Maloo *et al.* (1994) reported nearly identical patterns, but of age-specific ECF incidence, with rates on free-grazing farms approximately twice the rates observed on zero-grazing farms and with rates in younger stock approximately twice the rates observed in older stock within each grazing management system. There are a variety of explanations for why the rates of primary infection in calves were nearly twice the rates of secondary challenge detected in adults. As discussed in detail below (see *8.6 Tick Control - Method and Frequency of Acaricide Application*), there was clear evidence that the intensity of vector control on calves was much lower and in some cases virtually non-existent for the greater part of their early life. It is also possible that the rate of sero-events underestimated the level of rechallenge in mature cattle for several reasons, both assay-related and biologically-based.

The criteria for definition of sero-events was predicated on the ability to detect an

increase in antibody level consistent with a humoral immune response. Although considerable and successful efforts were made to standardise and quantify the ELISA test, variation in single sample OD values increased with increasing titre such that it is possible that this variation may have obscured a true sero-increase in some cases. Further, the interval of possible single OD values approached a maximum level with increasing antibody titre. This was due to saturation of the substrate:chromogen system such that it was impossible to detect sero-increases in animals with initial OD test values near the maximum. More specifically, although an animal which maintained a uniformly high OD value over the duration of the test may have experienced at least one sero-event to sustain these antibody levels, this could not be documented explicitly. This was the rationale for modelling antibody levels rather than seroconversions and sero-events.

Finally, it is also possible that reinfection with *T. parva* did not always result in an anamnestic humoral immune response. This is discussed in greater detail subsequently (see section *8.7 Multilevel Modelling of Serological Profiles*).

8.6 Tick Control - Method and Frequency of Acaricide Application

One of the most remarkable results from this study was the finding that a large proportion of farmers did not actually practise either the method or intensity of tick control they initially reported. The extent of the differences, documented through longitudinal observation, necessitated that farms be reclassified to derive more accurate assessments of the true impact of control efforts. Although the use of liberal and conservative reclassification criteria reflects the degree of uncertainty associated with this process, there

can be little argument with the observation that farmers were simply not applying acaricide as frequently as they said they did, as more than two-thirds of the longitudinal observations on mature cattle were inconsistent with the stated intensity of tick control. That the difference in intensity was most pronounced for those farms which indicated they undertook weekly acaricide application suggests that farmers were strongly influenced by the historical legislation (e.g. Cattle Cleansing Act of 1937) which mandated tick control at this level. In addition, the fact that Kiambu was one of the Project Tick Control Districts (Kenya, 1977) where the government assumed responsibility for and heavily subsidised dipping services from 1976 (Kenya, 1976) through the late 1980's (Kyule, 1989) is likely to have further exacerbated farmers reluctance to admit to reduced acaricide use. However, even before farm reclassification was undertaken, approximately one-third of farmers admitted to having reduced the frequency of acaricide application to levels below that previously mandated, while after reclassification this proportion was nearer to three-quarters. It is possible that farmers actually feared prosecution by reporting minimal tick control efforts. This may also have been a contributing factor in the situation of those farmers which reported plunge dipping of livestock but subsequently performed back-pack spraying, although this could equally be explained by the continued collapse of the plunge dipping infrastructure (Kenya, 1986). The latter view is supported by the observation that approximately one-half of farms had switched to back-pack spraying and that the use of plunge dipping was strongly associated with region, being restricted to areas where dip tanks were still in operation. In at least one area, a co-operative group of local farmers had assumed responsibility for dip maintenance and operation.

It is of particular interest to note that the observation of disagreement between cross-sectional and longitudinal study results is not restricted to smallholder dairy farmers in Kenya. Chamboko *et al.* (1998) reported a similar situation with respect to a cross-sectional and subsequent longitudinal study on smallholder beef farmers in Zimbabwe and considerable insight can be gained from his experiences. There a long-standing, highly subsidised plunge-dipping service provided by the Department of Veterinary Services (Perry *et al.*, 1990b) began implementing an annual fee in July 1995 as part of a cost-recovery policy (Department of Veterinary Services, 1995). In the cross-sectional study conducted between April and June 1995, 217 randomly selected smallholder farmers in the lowveld reported practising plunge dipping with a median frequency of 44 immersions per annum (maximum = 48, minimum = 18) and 34% indicated they used supplementary control practices such as tick grease or home-made engine-oil mixtures. However, in the subsequent year-long study of 8 of these farms which began in November 1995 (i.e. after the introduction of the dipping charge), the actual median number of annual immersions (as documented from dip-tank records) was 9, with a minimum of 8 and a maximum of 12, while 88% (7/8) farms reported the use of supplementary tick control practices. The consistency of these observations, from different countries and across production systems, has wide-ranging implications for the interpretation and analysis of self-reported farm management data derived from cross-sectional studies. The potential to miss important associations or make invalid or biased statistical inference, even when data have been collected using formal random sampling procedures, reflects the value of conducting longitudinal studies. This justification is particularly important in consideration of the higher costs of longitudinal

studies, financially as well as in terms of time and labour commitments.

Whether Zimbabwean smallholders actually reduced the intensity of their tick-control practices in response to a relatively small levy, or whether they, like their Kenyan counterparts, simply exaggerated the intensity of their control efforts, is open for speculation. However, new and expensive acaricides do represent an increasing proportion of the costs of tick-borne disease control programmes (Mukhebi *et al.*, 1992; Mukhebi *et al.*, 1998) and the reduction in the intensity of tick control from historical levels to those observed in the current study, where control costs are being born by the farmer, was likely to have been at least partially dictated by the economics of small scale subsistence agriculture. Nevertheless, an hypothesized economic motivation for reducing tick control must be considered in the context of the relatively high value of an individual dairy cow, such that smallholder dairy farmers are unlikely to reduce tick control efforts below a threshold where the risk of death from tick-borne disease outweighs control costs. Under such logic, the intensity of tick control may serve as a useful surrogate measure of the impact of tick-borne diseases.

In this study, depending on the criteria used, between one-third and one-half of farms were reclassified as practising a maximum of monthly application of acaricide, a level of tick control which is unlikely to prevent the attachment and successful feeding of ticks and may therefore best be described as strategic.

This point also raises the issue of whether tick control is actually a dependent variable for the presence and number of ticks. That is, do farmers alter their control efforts in response to the perceived threat to tick-borne disease, such that more intensive tick control is synonymous with greater rather than lesser tick challenge? Although Gitau (1998)

dichotomised across tick control intensities, he detected a positive association between any degree of tick-control being practised and *T. parva* infection, when controlling for other covariates and hypothesized this to be the case. Broadly speaking the patterns noted in this study also agree with this hypothesis. The rates of sero-events and ECF-specific morbidity in mature cattle tended to follow each other, with greatest incidence observed at intermediate intensity of acaricide application (fortnightly - from reclassified farms) and with both measures consistently lower on farms reclassified as monthly or less frequent application and lowest on farms which practised no control over the entire period of observation. The fact that these rates were lower on farms which apparently did conduct intensive (weekly) tick control may be a reflection of their relative success in reducing the opportunity for ticks to attach and feed sufficiently to successfully transmit infection. However, although they did not explicitly report the intensity of application, Maloo *et al.* (1994) observed incidence rates of clinical ECF in the order of 3 to 5 times those observed in this study and attributed this principally to ineffective use of acaricide.

For an alternative explanation, we can turn to the non-linear relationship between incidence of disease and level of tick challenge observed for several tick-borne diseases (Norval *et al.*, 1992; Perry *et al.*, 1992), where incidences of primary infection and of clinical disease are highest at intermediate levels of tick challenge and lower with both increasing and decreasing intensities of tick control. These empirical observations based on field experiences led to the development of the theory of endemic stability (Perry and Young, 1995). Although this concept is explored in greater detail later in this chapter, recent theoretical work in the field of transmission dynamic modelling (O'Callaghan *et al.*, 1998)

indicates that it is principally due to the interplay between the protection of young calves against disease by either innate or maternally-derived factors and the mean age at first infection, as determined by tick challenge. However, this explanation makes the implicit assumption that a spectrum of epidemiological states exists within the smallholder dairy industry in the Kiambu District, conditional on the intensity of tick control being practised. In light of the findings of this study and those of Gitau (1998) in the adjacent Murang'a District, these statements may form a reasonable hypothesis for farms with semi-/full-pasture grazing systems but not necessarily for zero-grazing units. This is explored in greater detail later in this chapter (see section *8.8 ECF Transmission Dynamics and Epidemiological State in Kiambu*).

Farmers appeared to be less circumspect when reporting the intensity of tick control as applied to calves. Reported age at first application varied between 1 and 24 months, but a mean of approximately 5 months was broadly consistent with the observed inter-application intervals. When the reported age was compared between observed methods of tick control, farms which practised plunge dipping began treating calves at a significantly older age than those which utilised back-pack sprayers. This is unsurprising since back-pack spraying is conducted on the farm and requires little additional effort to include calves while plunge dipping is a more physically stressful process and is generally restricted to animals of sufficient size and rigour. It is clear that, regardless of the reported or reclassified farm frequency of acaricide application, calves were universally subjected to much lower levels of tick control. Whether this was a conscious decision by farmers to maximize the potential for infection in a period of neonatal disease resistance (Norval *et al.*, 1992; Perry and Young,

1995; O'Callaghan *et al.*, 1998), or whether it is simply a reflection of the lower inherent value smallholders placed on calves as replacement stock (Gitau *et al.*, 1994b), the overall effect may be beneficial, or at the very least does not appear to be deleterious, with the incidence of seroconversion in calves nearly triple the sero-event rate in mature animals while ECF-specific morbidity rates remained virtually identical between the two groups. More specifically, the incidence rate observed in calves suggests an overall inter-challenge interval of two years, while for mature cattle, the observed incidence of sero-events is more consistent with rechallenge in the order of once every 5 years.

The differences in incidence measures observed by method of acaricide application are more difficult to interpret, particularly in light of the strong collinearites with grazing management system and agro-ecological zone and with intensity of tick control, which remained even after farm reclassification. The traditional explanation for a higher incidence on farms which undertook back-pack spraying *versus* plunge dipping would likely focus on the relative efficiency of the two methods. Where properly maintained and charged by a trained attendant, plunge dipping results in complete wetting of the animal with an effective acaricidal solution. Back-pack spraying depends on the individual operator correctly diluting the stock solution, seldom achieves complete wetting and is thus associated with poorer results (Norval *et al.*, 1992). It is also worth noting that one calf died from acaricide poisoning as a result of back-pack spraying, suggesting that farmers may have been inappropriately diluting the acaricide concentrate solutions.

8.7 Multilevel Modelling of Serological Profiles

The decision to utilise generalised linear mixed multilevel models to analyse serological data was made for several reasons. Foremost among these was that the study design and sampling method yielded data from a distinctly hierarchically structured population. Repeated measures were made longitudinally within animals, which themselves were grouped within farms, such that, at neither the observation nor animal level could the traditional statistical assumption of independence be made (Sokal and Rohlf, 1981). The consequences of ignoring correlated responses is to underestimate error and parameter variance estimates such that the potential for making spurious statistical inference by erroneously rejecting the null hypothesis is increased (Goldstein, 1995). Correlation between longitudinal observations made within the same animal is largely a function of the dynamics of the humoral immune system, with slow decay and long persistence relative to the interval between observations (Burridge and Kimber, 1973b; Roitt, 1997) as was reflected by the strong correlation coefficients observed in subsequent analyses. Clustering of like responses within aggregations of animals (beyond that which can be explained by association with like management practices or risk factors, the "farm effect") has long been noted and a variety of post-hoc methods have been discussed for accounting for it (McDermott, Schukken and Shoukri, 1994). It is unsurprising that overall estimates of very strong farm effects were derived in this study since clustering is likely to be particularly important when investigating the epidemiology of infectious diseases, where the per-susceptible risk of infection is a function of the prevalence of infectious individuals and their "contact" with susceptibles (Anderson and May, 1991). Fortunately, the recent development of statistical methods

which utilise the increase in computing capacity has provided powerful tools to conduct appropriate analyses of multilevel data. (Goldstein, 1995; McDermott, 1995; McDermott *et al.*, 1997).

The capacity of these tools to detect not only fixed effect/mean relationships but to simultaneously model complex variation at all levels of aggregation was an additional factor in their selection (Goldstein *et al.*, 1998). Veterinary epidemiologists are becoming increasingly aware of the importance of assessing and providing biological interpretations of patterns of variability, rather than simply treating it as nuisance in the analysis of fixed effect risk factors (McDermott *et al.*, 1997).

Post-targeting adjusted optical density (OD) values were modelled as a measure of the amount of *T.-parva*-specific antibody present at a given time. The limitation of being able to detect sero-events only in animals with low to moderate antibody levels combined with the observation that antibody levels generally declined post-exposure, suggested that it would be appropriate to model the antibody levels themselves as a surrogate measure of the level of challenge an animal was subjected to. Although models which utilised a natural log transformation of the adjusted OD values more closely approximated the assumptions of normality, the loss of ability to resolve associations with higher antibody levels *within* the interval of positive values more than outweighed this advantage. The fact that serological profiles were derived over a one-year period of observation but that total risk of exposure was cumulative with respect to age of the animal, meant that both temporal scales had to be accommodated within the models. The overall mean OD relationship with age was assessed by fitting polynomial curves for a variety of age:fixed-effect interactions, while the temporal

variations observed across the study period was simultaneously accounted for.

Several interesting features emerged from the models developed. Although the strong overall farm clustering observed is consistent with what might be expected from an infectious disease, it is of particular interest to note that when this was apportioned by grazing management system, semi-/full-pasture grazing farms accounted for virtually all of it. By virtue of their combination of extremely restricted movement of cattle and lack of suitable microhabitats for moulting, the route of ticks, presumably brought into zero-grazing units on forage, is almost certainly one-way. That is, cattle in zero-grazing units may become infected from ticks which reach them, but they are unable to contribute infection back into the population of ticks, or at the very least do so at a much reduced level. In consequence the epidemiology of a tick-borne disease on zero-grazing farms is likely to more closely approximate that of a non-infectious disease, and the variability simply reflects the differences in rate of introduction of ticks which derived their infection elsewhere. However, because of their smaller cattle numbers, the ability to detect clustering of sero-positives and cases will be more limited. In contrast, the strong clustering observed on semi-/full-pasture grazing systems is more consistent with a vector-borne infection. Since animals on these farms are not only at risk of infection but act as sources of infection for others, the variability, due to the distribution of management/environmental factors within a system with a high host/vector exposure potential, is further magnified.

The difference between the zero-grazing and semi-/full-pasture grazing farms was also reflected by the difference in fixed-effect estimates for age-interaction terms. Although mean adjusted OD values rose with age for both grazing systems, the rate of increase across

younger ages was more rapid on semi/full-pasture grazing farms, but appeared to be more sustained across all age-groups on zero-grazing farms. The difference in levels of antibody across early ages was almost certainly a result of higher levels of tick challenge on semi-/full-pasture grazing farms than on zero-grazing units, as reflected by the greater incidence of seroconversion and sero-events observed on semi-/full-pasture grazing farms. These results are consistent with those observed in other longitudinal studies of ECF (Maloo *et al.*, 1994; Gitau, 1998). However, the number of animals in the current study decreased with age, such that the validity of extrapolating across higher ages is questionable. This is particularly true since the estimated relationships were constrained by the assumption of linear or quadratic fit. Nevertheless, a highly speculative explanation for the observed relationship can be derived from observations of the immune response to *T. parva*.

There is little doubt that cell-mediated immunity directed against the schizont infected lymphocyte provides the protective mechanism against ECF (Eugui and Emery, 1981) and that the humoral immune response may, more or less, be considered extraneous (Muhammed *et al.*, 1975). Under this scenario, repeated challenge may result in an increasing efficiency of anamnestic cell-mediated immunity to such an extent that the suppression of lympho-proliferation actually deprives the humoral immune system of antigen exposure essential for its own anamnestic response. Alternatively, repeated heterologous challenge may provide continued stimuli to the humoral immune system until the antigenic diversity is exhausted. In either case, the non-linearity observed with age on semi-/full-pasture grazing farms would be consistent with a higher rate of rechallenge achieving maximum stimulation of the cell-mediated immune response while a lower rate on zero-

grazing farms precludes this degree of stimulation being achieved within the life of the animal. In a variation of this explanation, it is possible that repeated exposure to sporozoites results in a sufficiently high anamnestic humoral immune response such that lymphocyte infection does not occur and hence schizont antigen does not appear. Although the latter explanation may be less speculative, since it is the basis for development of a novel *T. parva* sporozoite subunit vaccine (Morrison, Taracha and McKeever, 1995b; McKeever and Morrison, 1998), it is likely that the two mechanisms may operate synergistically.

In contrast to the observed differences by grazing management system, once these had been account for, there were no statistically significant differences between agro-ecological zones. At first this appears to be in direct contrast to the findings of the majority of cross-sectional and longitudinal serological surveys of *T. parva* (Deem *et al.*, 1993; Maloo *et al.*, 1994; Gitau *et al.*, 1997; Gitau ,1998). The explanation for this is likely to reside in the range and heterogeneity of zones surveyed within each study. Both Deem *et al.* (1993) and Maloo *et al.* (1994) conducted sero-surveys in Coast Province of Kenya in three of the four "coastal lowland" agro-ecological zones present, CL3, CL4 and CL5, and reported no significant differences between the wetter CL3 and CL4 zones. Gitau *et al.* (1997) conducted a cross-sectional survey in 5 zones in the lower highland and upper midland categories (LH1, UM1, UM2, UM3 and UM4) before purposively selecting 3 zones (UM1, UM2 and UM4) for follow-up studies (Gitau, 1998) and noted in each study that the UM4 zone was significantly different from the other zones, but observed no differences within them. Thus the results of the current study and that of Gitau *et al.* (1997) and Gitau (1998) are actually in agreement in that 4 of the zones in which Gitau *et al.* (1997) reported no

significant differences in sero-prevalence (LH1, UM1, UM2 and UM3) also demonstrated no-significant differences in the current study, while the only zone in which significant differences were detected by Gitau *et al.* (1997) was not sampled in the current study. The additional upper and lower highland zones observed in this study were also found not to be statistically significantly different, suggesting that the majority of the agro-ecological zones in which smallholder dairying is conducted in Kiambu District are relatively homogenous with respect to the suitability for *R. appendiculatus* and hence the epidemiology of ECF.

The inclusion of separate sets of random-effect estimates for study time by sero-event status served to demonstrate that the vast majority of the variation observed between the longitudinal serological profiles could be attributed to differences among those animals which were considered to have seroconverted or experienced sero-events. Although the rationale may be somewhat circuitous (given that the definition of sero-event required an increase in antibody levels) it is nevertheless reassuring to note that the trend among the 382 animals not considered to have been challenged/rechallenged was virtually homogenous and of a decline in adjusted OD units. This observation lends support to the validity of the sero-event criteria and to the process of modelling antibody levels as a surrogate measure of challenge. In contrast, the use of second order polynomial curves, to approximate a step-function of sero-increase of variable timing and magnitude, likely served to inflate the variation observed among the 71 seroconversion/sero-event animals.

The theory behind the development of models of serological status was based on the concept of the age-seroprevalence profile. In the case of directly transmitted microparasitic infections which induce lifelong humoral immunity in those who recover, the proportion of

seropositives in the population rises steadily with age as a consequence of the acquisition of infection (Anderson and May, 1991). Further, the rate of rise in seropositivity with age is a direct measure of the age-specific force of infection (i.e. the per capita rate at which susceptibles acquire infection) and statistical methods employing maximum likelihood procedures have been developed to estimate these from age-stratified serological data (Grenfell and Anderson, 1985). Unfortunately, important problems arise if the duration of measurable antibody following infection is not lifelong. Specifically, the probability of falsely identifying an individual as sero-negative increases with age and the resulting bias affects the age-specific rates of infection. (Ades and Nokes, 1993). These models also make the assumption that the humoral immunity induced is completely protective, which has great impact on the population dynamics of transmission. In consequence, it was not possible to employ the statistical methods derived from non-linear transmission dynamics models of infection, and generalised linear models were extended to fit and assess different age-seroprevalence profiles.

The models of dichotomous serological status (positive/negative) as the dependent variable agreed closely with the models developed for adjusted OD values in significance of fixed effect variables and overall interpretation. The failure of some models to converge under penalised quasi-likelihood (PQL) iterative estimation is a well documented phenomenon (Goldstein *et al.*, 1998) and along with the inherent bias toward the null (Goldstein, 1995; Goldstein and Rasbash, 1996) was the principal reason for undertaking a full Bayesian estimation using Metropolis-Hastings sampling in a Markov Chain Monte Carlo (MCMC) process (Gilks *et al.*, 1996). Although the magnitude of unbiased MCMC

estimates were consistently larger than those derived under PQL, parameter estimate significance and model interpretation did not change. Further, regardless of whether the model accounted for the dynamics of antibody responses over the period of the study by the inclusion of sero-event status:time interaction terms, the different relationships between age and seroprevalence described by these models for zero-grazing and semi-/full-pasture grazing farms was consistent, confirming that animals on semi-/full-pasture grazing farms were more likely to test positive at earlier ages than animals on zero-grazing farms, but that the differences between the two systems disappeared with increasing age of the animal. This pattern is perhaps best illustrated by the plot of crude seroprevalence proportions by age, suggesting that, up to approximately 3-4 years of age, the two systems are experiencing very different forces of infection, which appear to be equivalent thereafter. However, it is important to remember that each age group is effectively a cohort of animals at risk through time, such that, the interpretation of the apparent increase in seroprevalence of zero-grazing animals at 4 years of age may not be a reflection of a higher current force of infection in this age group but rather may indicate a historical reduction in force of infection on zero-grazing farms occurring four years previously. The second explanation is more likely to hold true since the adoption of zero-grazing management in Kiambu has been a relatively recent but subsequently progressive process. In fact, we can safely assume that the proportion of "zero-grazing" animals which actually began life in a semi-/full-pasture grazing system increases with age. However, even more specifically, the evidence may be considered presumptive of a relatively sudden large-scale shift to zero-grazing units 4 years prior to the beginning of the current study. Unsurprisingly, this coincides with the end of the consolidation phase

of the Netherlands-funded National Dairy Development Programme (NDDP) which offered a "zero-grazing" assistance package whereby smallholder dairy farmers could apply for grants to construct zero-grazing units and improve fodder and recycling of manure (De Jong and Zwart, 1994). This finding highlights another potentially important failure of the current study in that it made the implicit assumption that grazing system was a static management practice when in fact it appears to have altered quite dramatically in Kiambu. In retrospect, it is clear that zero-grazing farmers should have been questioned more closely about when they switched to zero-grazing. Perhaps more importantly, it also demonstrates the risk of using linear static statistical models to approximate dynamic processes. What was a risk factor for infection in the past may not have a significant association with the outcome as measured today and *vice versa*. In short, if modelling of age-profiles had not been undertaken in the current model, but simple static risk factors assessed, the difference between grazing management systems may not have been detected. In contrast, we would expect a repeated study contrasting zero-grazing *versus* semi-/full-pasture grazing farms conducted in Kiambu District today to reveal an even more pronounced difference in the age-serological profiles through the loss of older animals. Finally, no differences were detected between age-profiles for the limited range of agro-ecological zones sampled.

No association was detected with either the amount of *T. parva* antibody or dichotomous serological status (positive/negative) and the majority of covariates concerned with assessing the numbers of engorging adult instars or the presence of unfed adults or nymphae, when controlling for other significant covariates in multiple variable models. The sole exception was the higher mean antibody levels observed on animals where small, unfed

ticks were detected on more than 10% of longitudinal observations. In light of the aforementioned weakness of the tick data in this study and the fact that several summary, rolling average and lag-effect combinations of variables were assessed for significance, it would be foolhardy to attach undue importance to this association on its own. However, it is interesting to note that similar results emerged from the longitudinal study conducted by Gitau (1998), who demonstrated significant and positive univariate associations between *T. parva* antibody titre and dichotomous presence/absence data for all classes of *R. appendiculatus* ticks. However, in multiple variable models, a negative relationship emerged for those which had engorged. Similarly, for multiple variable models of time to sero-conversion, a positive association with the presence of nymphal *R. appendiculatus* was detected, but a concurrent negative association if the nymphs had fed to engorgement. The conclusion was that the intensity of follow-up was insufficient to more accurately document the success of tick feeding. However, we may better understand these observations if we consider two factors. First, the suggestion that nymphal transmission of infection may be of greater importance than originally suspected. Second, that the presence of small, unfed, newly attached ticks is possibly more indicative of the level of challenge a farm is subject to, while the proportion which feed to repletion (i.e. engorged tick counts) reflects the success and intensity of intermittent tick control efforts. In support of the second factor, although the process of sporogony in the tick (sporoblast developing into sporozoite) is generally assumed to be initiated by and occur over the first three to four days of feeding (Young *et al.*, 1983a, 1983b) such that the transmission of sporozoites to the host occurs after several days (Purnell *et al.*, 1973; Young *et al.*, 1975) it has been demonstrated that it can

be completed relatively quickly, even in the absence of feeding (Young *et al.*, 1984; Ochanda *et al.*, 1989), resulting in a more rapid infection of the host.

In summary, models of adjusted OD values and binary serological status confirmed that, not only were animals on semi-/full-pasture grazing farms more likely to test positive at younger ages than animals on zero-grazing farms, but over the same interval of ages their antibody levels were also higher.

8.8 ECF Transmission Dynamics and Epidemiological State in Kiambu

We can broaden the discussion to consider the population ecology of the vector and the dynamics of the vector:host interaction in the context of smallholder dairy farms in Kiambu District. Kiambu District has at least a moderate ecoclimatic suitability index for *R. appendiculatus* (Lessard *et al.*, 1990) and lacks seasonality of tick activity. It has been long associated with the presence of ECF. The district is distinguished as an area of high and rapidly increasing human population density (Central Bureau of Statistics, 1989) where small-scale subsistence agriculture is widespread but under ever-increasing pressure to intensify. The concurrent impact of the cultural practice of farm division among sons is illustrated by the fact that farms were generally small with few animals (Ministry of Livestock Development, 1989) but were supporting large and extended families. In addition, the degree of intensification is reflected by the fact that nearly half the farms enrolled in the current study had already adopted zero-grazing systems, from which animals emerged only infrequently, on such occasions as artificial insemination. Further, no farms reported the presence of any wildlife species which could act as efficient hosts for either the vector or

parasite and, with the exception of sale of newborn calves, the number of imports and exports noted on study farms was minimal and approximately equivalent such that few farms increased the number of cattle kept.

The picture emerges of a relatively fixed and stable cattle population kept intensively at high density and in effective isolation amidst a burgeoning human community. Superimposed on this, we find a species of tick, the free-living stages of which are sedentary, not moving over long distances under their own locomotion (Rechav, 1979) but relying on host animals to act as dispersal agents. In the absence of alternate hosts and the presence of a primary host on which all stages of its life cycle may be completed (Norval *et al.*, 1992) it is reasonable to assume that the vector population is maintained exclusively by the cattle population. However, the daily rhythms of detachment observed in engorged larvae, nymphs and adults (Minshull, 1982), which usually ensure that ticks end up in appropriate microclimatic conditions to enable them to moult and survive (Short *et al.*, 1989a, 1989b), can have little effect in zero-grazing units. Specifically, only on cattle which have free access to suitable microhabitats can the vector complete its life cycle and zero-grazing units, by definition, must result in a one-way movement of ticks. The corollary to this is that not only is the tick population predominantly maintained by animals with access to pasture, but so too is the parasite population. How do the patterns of infection and disease observed in the current study relate to this scenario and how does this affect our understanding of the epidemiology of ECF? We might paraphrase this question as "Where do smallholder dairy farms in Kiambu fit among the epidemiological states of ECF?".

Norval *et al.* (1992) defined endemic stability to be the situation where virtually no

clinical disease occurs in the face of relatively high levels of vector challenge. Recent theoretical work modelling the transmission dynamics of tick-borne diseases (O'Callaghan *et al.*, 1998) supports their original contention that this was principally due to the vast majority of the population becoming infected and immune during a period of age-specific resistance, a deduction which was also supported by the results of the current study where higher incidence of challenge observed in calves did not equate with a markedly higher ECF morbidity or mortality. Perhaps more importantly, it also confirmed the non-linear relationship between disease incidence and level of tick challenge described by Perry *et al.* (1992) where maximum disease impact occurs at intermediate challenge but with incidence subsequently declining in tandem with decreasing challenge over levels below this point. This has led to a refinement in terminology to include "endemically unstable - high incidence" and "endemically unstable - low incidence" epidemiological states (Mukhebi *et al.*, 1998). Although this model was developed for Heartwater (*Cowdria ruminantium*) infection, it was derived from the original work on ECF (Medley *et al.*, 1993) and endemic stability has been reported in one guise or another for the spectrum of tick-borne diseases (Perry and Young, 1995).

Pertinent evidence which emerged from the current study is as follows. The cattle population was virtually entirely composed of Taurine breeds. Although overall ECF-specific morbidity rates were low to moderate and were broadly equivalent between age groups, there was clear evidence of clustering by farm and all fatalities in mature cattle occurred in zero-grazing systems. Nevertheless, the case-fatality proportion was also uniformly low with the majority of incidents of clinical ECF so uncharacteristically mild

they initially went undiagnosed. Tick control efforts were predominantly strategic but a few farms did practise intensive acaricide application. Rates of seroconversion were higher in calves than rates of sero-events in mature cattle, but overall the level of challenge was higher in semi-/full-pasture grazing systems than on zero-grazing units, yet again, strong clustering was present in these farms. However, even the highest rate of seroconversion and sero-events in calves on semi-/full-pasture grazing farms in the upper midland agro-ecological zone was only equivalent to an approximate per calf rate of challenge of one infected tick per year. This contrasted with a mean inter-challenge interval of 5 years for mature cattle in zero-grazing units.

The strong farm clustering observed among semi-/full-pasture grazing farms may be the key observation. With their homogeneously lower levels of challenge, and their dead-end role in parasite and vector population dynamics, zero-grazing units are best described under the existing classification system as being "endemically unstable" but with low force of infection and low disease incidence. The fact that the infectious ticks, to which these farms are subject, doubtless derived their infection from animals on pasture (and hence more likely to be previously infected carriers), means that the intensity of infection in these ticks will be lower as well (Ochanda *et al.*, 1996), further reducing the incidence and severity of disease observed. The probability that cattle on these farms will receive a large infective dose of *T. parva* sporozoites is low and in fact, as the proportion of farms practising zero-grazing increases, the tick population and hence level of challenge these farms are subject to will further decline and the proportion of cattle which remain naive to *T. parva* and hence fully susceptible to disease on primary infection both increases and shifts right with respect to age.

In contrast, the higher mean level of challenge and greater heterogeneity observed for semi-/full-pasture grazing farms suggests that at least some of these farms may be approaching an endemically stable state. The unfortunate deduction implicit in this statement is that some find themselves in an endemically unstable - high disease incidence situation, contributing disproportionately to the overall ECF morbidity estimates. It is possible that a further few, perhaps those actually undertaking intensive and effective tick control, or in areas of lower ecological suitability for the tick, experience the endemically unstable - low incidence state of zero-grazing units, but with a greater probability of subsequent higher challenge.

8.9 Implications for ECF Control and Future Research

Although the overall ECF mortality and morbidity rates were relatively low, the differences in the level and variability of challenge observed between the two categories of grazing management systems practised by smallholder dairy farmers in Kiambu have significant implications for future disease control efforts in this area. In particular, on how the novel ECF subunit vaccines currently under development may be applied effectively and efficiently.

Their relatively uniform and significantly higher rate of seroconversion suggests that calves are treated fundamentally differently than mature cattle, but not by grazing management system. Nevertheless, the rate of challenge appears to be insufficient to ensure that the vast majority of calves are infected during a period of neonatal resistance, as would be required in an endemically stable situation (Norval *et al.*, 1992). In the case of zero-grazing units, the homogeneously low level of challenge, to which mature cattle are

subsequently subject, means that, despite a likely increase in the mean age at first infection and a larger proportion of cattle remaining naive to *T. parva* longer, the rate of ECF remains low. However, since the low level of challenge appears to be a function of strict stall-feeding of cattle, rather than more active tick control efforts, and hence a relatively immutable situation with respect to zero-grazing units, this epidemiological state may have been inappropriately termed "endemically unstable/low incidence" (Perry and Young, 1995; Mukhebi *et al.*, 1998). Rather, with levels of challenge likely to decline even further in Kiambu District as population and other pressures result in a greater proportion of the dairy cattle population kept under intensive management, zero-grazing units may be more appropriately classified as "low and stable challenge/low incidence". In this circumstance, the use of intensive tick-control practices is almost certainly unnecessary, while the application of mass vaccination may be considered inefficient, and potentially should only be recommended to individual problem farms or as a risk-aversion strategy. However, whether or not vaccination would be economical for these farmers is not a question which can be answered within the scope of this study.

With respect to those smallholder dairy farms practising semi- or full-pasture grazing, the picture is very different. Although the level of challenge is higher, more importantly it is much more variable. This suggests that it is likely to be more dependent on the distribution of other factors such as the environmental suitability for the tick and the intensity and success of vector control efforts. Whether Taurine breeds of cattle can exist in a state of endemic stability is a question open to debate (Norval *et al.*, 1992), but there is little doubt that vaccination would be appropriately targeted to those farms experiencing a high

incidence of morbidity and mortality due to ECF ("endemically unstable- high incidence"). However, the non-linearity in the relationship between rate of challenge and disease incidence at the population level (Perry and Young, 1995) means that recommendations concerning the concomitant use of tick-control measures in integrated programmes of ECF/TBD control are not straightforward. For example, where the level of vector challenge is sufficiently high (e.g. lower, warmer agro-ecological zones), the use of vaccination and a *reduction* in vector control efforts may prove to be the most efficient combination, allowing farms to progress to the endemically stable state without experiencing epidemic losses (Mukhebi *et al.*, 1998). More to the point, as the endemically stable state is approached, there is likely to be a diminishing rate of return, with the potential of reaching a point at which the population immunity is sufficiently high that vaccination is no longer economically viable. However, if the attainable level of challenge is insufficient to reach or too heterogenous to sustain a state of endemic stability (e.g. upper, cooler agro-ecological zones), then it is possible that an *increase* in the intensity of vector control efforts could act synergistically with vaccination. In any case, in the absence of being able to ensure a stable and low level of challenge, vaccination would need to be continued. However, the relative economic returns for combinations of each factor require further elucidation.

The speculative nature of this important section of the discussion serves to highlight the need for further research to provide a better understanding of the non-linear dynamics of the epidemiology of tick-borne diseases. In particular, the development and use of predictive infection dynamic models to assess the impact of integrated control strategies involving vaccination is vital. These models, under development for individual tick-borne diseases

(Medley *et al.*, 1993, personal communication; O'Callaghan *et al.*, 1998), require a good understanding of the biology and natural history of infection and the mechanisms of vaccine-induced protection. This understanding relies on well designed experimental and observational studies, such as that described by this thesis, so that potentially important factors, such as the dose response, the effect of repeated challenge on carriers, the role of different instars, the vector population dynamics and their interaction with tick-borne pathogens can be elucidated. The author continues to conduct research in this field.

CHAPTER 9

CONCLUSIONS

- The results of this prospective observational study, which utilised active monitoring, proved to be superior both to retrospective studies, based on passive disease surveillance, and to cross-sectional studies. It provided more accurate and reliable estimates of the incidence of East Coast Fever and the rate of *T. parva* challenge. These are vital to understanding the transmission dynamics, determining the epidemiological state and estimating the actual and relative impact on animal health and productivity. Although the use of formal random sampling procedures ensured that the estimates were unbiased and representative of the population of interest, it resulted in significant multicollinearity of risk factors.
- A combination of an initial cross-sectional survey, based on formal random sampling, followed by a prospective study, with sampling stratified on previously identified risk factors, is likely to prove the most informative and flexible structure in investigating the epidemiology of tick-borne diseases. This combination should be considered the standard for those future field studies which are essential to improving the understanding of tick-borne diseases in different livestock production systems.
- Wherever possible, management practices or farm policies should be verified by independent observation. Although overall farmer cooperation in the current study

was exemplary, there was clear and considerable self-reporting bias with respect to the method and intensity of application of acaricide reported.

- The interpretation of longitudinal serological profiles, derived from an epidemiologically sensitive and specific assay which had been appropriately standardised and quantified, proved useful in detecting anamnestic humoral immune responses as a surrogate measure of the rate of repeated *T. parva* challenge.
- This technique demonstrated potentially important differences between the rates of primary seroconversions in calves and secondary responses in mature animals, showing that calves were experiencing a higher force of infection.
- It also provided a mechanism through which mild and subclinical cases of ECF could be detected and suspected cases confirmed, provided the justification for modelling antibody levels directly, and highlighted the need for additional research to better understand the biological and epidemiological implications of repeated challenge of previously infected/carrier animals, particularly with respect to their subsequent ability to infect ticks.
- The preponderance of subclinical or mild clinical cases of ECF and the uncharacteristically low case-fatality proportions in Taurine breeds may be a function of the dose response effect, attributable to low intensity carrier-derived infections or a greater role of nymphae in *T. parva* transmission. This aspect of the epidemiology of ECF needs further investigation.
- It is vital to a better understanding of the transmission dynamics of tick-borne diseases that such factors as the true tick attachment rate by instar (as opposed to rate

of challenge of infectious ticks) and the prevalence and distribution of intensity of infection in the different instars be accurately documented. In the context of tick-borne diseases, those studies which consider only the epidemiology of infection and disease in the host population or give only a rudimentary consideration to the ecology and dynamics of infection in the vector can at best provide half the picture and perhaps none of the answers. Unfortunately, tick dynamics may be occurring on a different temporal scale and future studies will need to give careful consideration to how best to combine observation of the host and vector populations.

- Overall ECF-specific morbidity and mortality rates were low, but there was strong farm clustering of these outcomes by grazing management system, most strikingly observed on grazing farms in the lower agro-ecological (UM) zone.
- The large differences in tick control practices and crude mortality rates observed between mature cattle and calves, in combination with their higher rates of *T. parva* infection, indicate that calves are managed fundamentally differently than mature cattle, irrespective of other farm policies.
- The use of newly developed statistical tools to conduct appropriate multilevel analyses of hierarchically structured data provided not only statistically valid fixed-effect contrasts, but also a means through which complex patterns of variation at all levels of aggregation could be assessed.
- In this study, the observation of significantly greater variability in antibody levels among semi-/full-pasture grazing farms than between zero-grazing units was of at least equivalent importance to the finding that they also experienced higher levels of

challenge across younger age groups. This suggested that ECF control efforts, including immunisation, would prove most efficient and beneficial if correspondingly targeted at problem farms rather than applied under a policy of blanket coverage. The targeting of immunisation and other control measures to farms and production systems is a critical question that requires further research.

- The burgeoning human population, escalating value of land and cultural tradition of farm subdivision on inheritance continue to increase the pressure on smallholder dairy farmers in Kiambu to adopt intensive management practices such as zero-grazing of cattle. Results of the current study show that these cattle are already subject to a more homogeneous and low level of challenge such that any further reduction in this level will likely result in both the maintenance of a relatively large pool of mature cattle, completely naive to *T. parva* and hence fully susceptible to developing ECF, and an increase in the mean age at first infection.
- In light of the relative stability of the above situation and the anticipated decreasing tick population, the endemic stability paradigm of Norval, Perry and Young (1992; Perry and Young, 1995) needs to be augmented with another dimension, namely the level and stability of challenge.
- In the face of an evolving smallholder dairy sector, the current study should be considered the baseline from which future investigations of the epidemiology of theileriosis on smallholder dairy farms in Kiambu are based.

CHAPTER 10

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Appendix 1.1 - Initial Farm Survey

Circle or fill in the appropriate response where indicated.

1. Date (day/month/year):____/____/_____

2. Dairy Society:

1) Chania
2) Kiambaa
3) Kikuyu
4) Lari
5) Limuru
6) Nderi

3. Farm ID/ Cooperative #:_____

4. P.O. Box # & Post Office:_____

5. How long have you been keeping cows?_____(years)

6. What services of the Dairy Society do you make use of?

0) none
1) private artificial insemination services
2) private clinical veterinary care
3) buying feeds on credit
4) loans
5) cash advances on milk production
6) other. Specify:_____

7. How many members of your family presently live on the farm, or, are dependent on farm income?

Number of family members:_____

8. In addition to dairy farming, do you have other farm-related activities? If so, indicate which?

0) no other farming activities, i.e. only dairy
1) raising vegetables for home consumption
2) raising livestock for home consumption
3) raising cash crops/horticultural products
4) raising livestock for sale
5) other. Specify:_____

9. Do you employ non-family members on your farm? If so, how many?

0) no non-family employees
1) ____ non-family employees

10. For what periods of the year do you employ non-family workers?

1) all year
2) harvest time
3) according to the need/work load
4) other. Specify:_____

11. If you raise other horticulture products or cash crops on your farm, what are they?

1) coffee
2) tea
3) horticulture products (vegetables)
4) other. Specify:_____

12. Do you purchase fertilizer for farm use? If so to what do you apply it?

0) no fertilizer purchased
1) cash crops/horticultural products
2) cattle forage
3) cattle grain
4) other. Specify:_____

13. If you raise livestock, other than dairy animals, what do these include, and give approximate numbers of each?

	number
1) beef cattle	_____
2) sheep	_____
3) goats	_____
4) chickens	_____
5) other. Specify:_____	

14. Do these "other livestock" come in contact with dairy animals through housing, grazing, corral, etc.?

0) no contact with dairy animals
1) come in contact with dairy animals

15. What area of land does your farm include?

_____(acres)

16. Of your total farm area, what proportion is involved in dairying (housing, corral, pasture and area of crops devoted to dairy forage/grain)?

_____(percentage)

17. Of your total farm area, what proportion do you own?

_____(percentage)

18. Do you rent any other land for agriculture?

0) No
1) Yes

19. If yes, of your total farm area, what proportion do you rent?

_____(percentage)

20. And, what is the yearly cost of renting this land?

_____(Kenya shillings per acre)

21. Is the farm owner employed or has another business outside the farm?

0) No
1) Part time (<35 hrs per week)
   Specify Occupation:_____
2) Full time (>35 hrs per week)
   Specify Occupation:_____

22. If the farm owner is employed outside the farm, how many hours per day does he/she spend working on the farm?

_____(hours per day)

23. In the absence of the farm owner, who is responsible for farm labour/management? Indicate relationship to owner.

1) wife
2) son
3) daughter
4) employee
5) other. Specify:_____

24. Do you control ticks on your dairy animals? If so, by what method?

0) no tick control
1) dipping
2) spraying
3) combination of spraying/dipping.
   Describe:_____
4) other. Specify:_____

25. What diseases do you hope to prevent by tick control? (circle all appropriate response(s))

1) Theileriosis ("Ngai"/East Coast Fever)
2) Anaplasmosis ("Ndigana"/Gall-sickness)
3) Babesiosis ("Guthuguma Thakame"/Red Water)
4) Cowdriosis (Heartwater)
5) other. Specify:_____

26. What animals do you treat for ticks?

1) all animals
2) adult cattle only
3) calves only
4) other. Specify:_____

27. At what age do you begin tick control for calves? (write in appropriate space)

_____(weeks)

_____(months)

28. What time of the year do you control for ticks?

1) all year
2) wet/cold season only
3) other. Specify:_____

29. Is your method of tick control different depending on the season, i.e. wet vs dry?

0) No
1) Yes. Describe:_____

30. Is your method of tick control different for adults and calves?

0) No
1) Yes. Describe:_____

31. When you control for ticks, how often do you do it?

1) more than twice per week
2) twice per week
3) once per week
4) once every two weeks
5) once every three weeks
6) once per month
7) less than once per month
8) when the tick burden is high
9) other. Specify:_____

32. Is your frequency of tick control different depending on the season, i.e. wet vs dry?

0) No
1) Yes. Describe:_____

33. Is your frequency of tick control different for the adults and the calves?

0) No
1) Yes. Describe:_____

34. If you practise tick control by dipping, which dip tank do you use and how far is it from your farm?

Dip tank name:_____

Distance from farm (circle appropriate response):

1) < 1 km
2) 1 - 2 km
3) 2 - 3 km
4) > 3 km

35. If you practise tick control by spraying, which product/acaricide do you use?

Product Name:_____

Concentration of product ("conc"):_____

Acaricide:_____

36. What total volume of spray do you prepare?

Total Volume (litres):_____

37. How much of the concentrated product do you use to make this volume of spray?

Volume of Concentrate (litres):_____

38. Approximately how many litres of the final mixture do you spray on EACH ADULT?

Volume per Adult (litres):_____

39. Approximately how many litres of the final mixture do you spray on EACH CALF?

Volume per Calf (litres):_____

40. Where do your animals spend the day?

1) grazing/open pasture
2) corral +/- shelter (semi-zero grazing)
3) housed indoors (total zero grazing)
4) other. Specify:_____

41. Where are your animals kept at night?

1) open pasture +/- shelter
2) housed indoors
3) other. Specify:_____

42. How do your animals get access to forage?

1) grazing/pasture
2) cut or purchased and transported to animals(zero grazing)
3) combination of the above. Explain:_____
_____

43. If your animals graze or are on pasture, how far from the night housing/shelter do they travel?

1) <0.5 km
2) 0.5 - 1 km
3) 1 - 2 km
4) 2 - 4 km
5) 4 - 6 km
6) > 6 km

44. If your animals graze or are on pasture, are they ever close to areas where "wild animals" are seen? If so, which wild animals?

0) no wild animals in area
1) buffalo
2) bush buck/"antelopes"
3) other. Specify:_____

45. If your animals DO NOT GRAZE, what type of forage do you harvest/transport to feed them? Indicate the approximate amount of each fed per day in kg.

kg

1) Napier grass ____
2) kikuyu grass ____
3) legumes. Specify:_____
4) other. Specify:_____

46. If your animals ARE grazing on pasture, what size area do they have access to?

_____(acres)

47. Is housing/shelter available to the animals?

0) No
1) Yes

48. If yes, is the type of housing/shelter?

1) closed (roof and walls), wooden
2) closed (roof and walls), concrete
3) open, covered (roof, no walls)
4) natural shelter. Describe:_____
5) other. Specify:_____

49. If yes, what is the flooring type?

1) dirt
2) concrete
3) wood
4) other. Specify:_____

50. If yes, how are the adult animals kept in housing?

1) free (no stalls)
2) free with stalls
3) tied (no stalls)
4) tied in stalls
5) other. Specify:_____

51. Does your housing/grazing routine differ between wet season and dry season?

0) No
1) Yes. Describe:_____

52. Is bedding used in the housing area?

0) No
1) Yes

53. If yes, what kind of bedding is used?

1) straw
2) grass
3) shavings
4) other. Specify:_____

54. Do you disinfect your housing area you clean it out?

0) No
1) Yes. Specify disinfectant used:_____

55. What do you do with the manure from the housing area/pens/corral?

1) used as fertilizer on crops
2) sold
3) used as fuel
4) stored but not used
5) other. Specify:_____

56. Do you feed grain/concentrate to your cattle?

0) No
1) Yes

57. If you feed grain/concentrate, to which animals do you offer it? Indicate the approximate amount fed each group per day in kg.

	kg
1) lactating cows, early in lactation	____
2) lactating cows, throughout lactation	____
3) dry cows	____
4) weaned heifers	____
5) weaned bulls	____
6) unweaned calves	____

58. What is the source of the grain/concentrate you feed?

1) home grown/formulated
2) purchased grain/home mixing
3) purchased commercial prepared/mixed feed
4) other. Specify:_____

59. Do you provide any other nutritional supplements? If so what type? (circle all appropriate responses)

0) no other supplements
1) salt lick
2) salt and mineral mix
3) molasses
4) vitamins
5) antibiotics
6) other. Specify:_____

60. If you feed supplements, to which animals do you offer which supplement?

	Supplement(s)
1) lactating cows, early in lactation	_____
2) lactating cows, throughout lactation	_____
3) dry cows	_____
4) weaned heifers	_____
5) weaned bulls	_____
6) unweaned calves	_____

61. Where do your cows have access to water?

1) water provided in housing
2) water available on pasture/grazing
3) water available in pen/corral
4) other. Specify:_____

62. If water is provided in the housing area, or in the pen/corral, what is its source?

1) stream
2) pond
3) private ground pump
4) community ground pump
5) collected rain water
6) other. Specify:_____

63. If water is provided in the housing area, how is it delivered?

1) individual automatic waterers
2) individual animal buckets
3) communal automatic waterer/trough
4) communal bucket/trough
5) other. Specify:_____

64. If water is provided MANUALLY to BUCKETS or TROUGH, how often is it offered?

1) ad libitum (free choice, kept full)
2) once per day
3) twice per day
4) three times per day
5) other. Specify:_____

65. If water is available while grazing/on pasture, what is its source?

1) stream
2) pond
3) private ground pump
4) community ground pump
5) collected rain water
6) other. Specify:_____

66. If water is available while grazing/on pasture, how far must animals travel to it?

1) < 0.5 km
2) 0.5 - 1 km
3) 1 - 2 km
4) 2 - 3 km
5) > 3 km

67. If water is available while grazing/on pasture, how often do animals have access to it?

1) ad libitum (free choice, always available)
2) once per day
3) twice per day
4) three times per day
5) other. Specify:_____

68. Does your method/source/availability of water depend on the season, i.e. wet vs dry? If so, explain.

0) same year round
1) seasonal. Describe - give details:_____
_____
_____

69. Do you give any vaccines to the adults?

0) No
1) Yes. Specify vaccine, to whom and when:

Vaccine (product)	Animals (cows/heifers/calves)	When (age/month)
_____	_____	_____
_____	_____	_____
_____	_____	_____

70. Do you "deworm" the dairy animals?

0) No
1) Yes

71. If yes, what animals do you deworm?

1) all animals
2) lactating cows
3) non-lactating cows
4) weaned heifers and bulls
5) calves
6) other. Specify:_____

72. If yes, what product(s) do you use?

Deworming product(s):_____

73. If yes, what is the method of administration of this product?

1) injectable
2) oral
3) pour-on
4) other. Specify:_____

74. If yes, how often do you deworm?

1) once per year in wet season
2) once per year in dry season
3) twice per year
4) three times per year
5) other. Specify:_____

75. If yes, does your frequency of deworming vary between the adults and the calves?

0) doesn't differ
1) different between calves and cows.
Describe:_____

76. Who raises most of the calves on your farm?

1) owner
2) owner's relative (e.g. wife/son).
Specify:_____
3) employee

77. How many calves were born alive on your farm in the past 12 months?

_____calves born alive

78. How many calves were born dead or aborted in the past 12 months?

_____calves born dead/aborted

79. Of the calves born in the last 12 months that became sick and died, how many fall into each of the following categories?

	Number
1) Abortions:	_____
2) Weak Calves	_____
3) Diarrhoea:	_____
4) Pneumonia:	_____
5) Tick Borne Diseases:	_____
6) Bloat:	_____
7) Poisoning:	_____
8) Accidents:	_____
9) Difficult Calving:	_____
10) Congenital Abnormalities: (Birth Defects)	_____
11) Internal Parasites:	_____
12) Other Causes. Specify:	_____
13) Unknown sickness	_____

80. Where do most of your cows calve?

1) indoor calving pen (box stall)
2) pasture
3) outdoor pen or corral
4) stanchion/stall (tied install)
5) other. Specify:_____

81. If an indoor calving pen is used, what kind of bedding is used?

1) no bedding - dirt floor
2) no bedding - cement floor
3) no bedding - other. Specify:
4) straw
5) shavings
7) other. Specify:_____

82. For what percent of calvings is someone present?

1) 0 - 25%
2) 25 - 50%
3) 50 - 75%
4) 75 - 100%
5) unknown

83. Do you give any vaccines to the calves?

0) No
1) Yes. Specify what and when:_____
_____

84. How soon after calving do you separate the calf and dam?

1) immediately
2) < 4 hours
3) 4 - 24 hours
4) 24 - 72 hours
5) > 72 hours

85. How do you house the calves after separation from the dam?

1) tied, individual pens
2) free, individual pens
3) tied with cows, no pens
4) group pens
5) outdoors. Specify:_____
6) other. Specify:_____

86. If calves are initially kept individually, at what age do you usually begin to group them?

1) < 2 weeks
2) 2 - 4 weeks
3) 4 - 6 weeks
4) 6 - 8 weeks
5) > 8 weeks

87. If calves are put into group pens, how many are usually put per pen?

1) 2 - 4
2) 4 - 6
3) >6

88. Are your calf pens constructed with a raised floor?

0) No
1) Yes

89. <u>If the pen is raised</u>, what is the floor made of?

1) plain wood
2) slatted wood
3) expanded metal
4) other. Specify:_____

90. <u>If the pen is ground level</u>, what is the flooring?

1) dirt
2) concrete
3) wood
4) other. Specify:_____

91. Is bedding used in the calf pens?

0) No
1) Yes

92. If yes, what kind of bedding is used?

1) straw
2) grass
3) shavings
4) other. Specify:_____

93. If yes, how often do you completely clean out your calf pens or calf area?

1) daily
2) every few days
3) weekly
4) every 2 weeks
5) monthly or less frequently

94. If calf pens are cleaned out at weekly or greater intervals, how often are the wet spots cleaned out?

0) not done
1) daily
2) every few days
3) weekly
4) every two weeks or less frequently

95. Do you disinfect your calf pens after you clean them out?

0) No
1) Yes. Specify disinfectant used:_____

96. How do your calves usually receive their first colostrum?

1) free choice suckle
2) free choice suckle with supplementation
3) assisted suckle
4) nursing bottle
5) bucket
6) other. Specify:_____

97. How soon after birth do most of your calves receive colostrum?

1) < 2 hours
2) 2 - 6 hours
3) 6 - 12 hours
4) 12 - 24 hours
5) unknown

98. During the first three days of life, what are most calves fed?

1) mother's milk or colostrum
2) pooled fresh milk
3) milk replacer
4) other. Specify:_____

99. If calves are initially fed their mother's fresh milk or colostrum, at what age is this stopped?

1) < 3 days
2) 3 - 5 days
3) 5 - 7 days
4) > 7 days

100. Between one week of age and weaning, what is the main liquid feed in the calf's diet?

1) mother's milk
2) pooled fresh milk
3) milk replacer
4) other. Specify:_____

101. At what age is pail feeding of milk or milk replacer started?

0) pail feeding not used
1) < 1 week
2) 1 - 2 weeks
3) 2 - 3 weeks
4) 3 - 4 weeks
5) > 4 weeks

102. Before being put on the pail, or, if pail feeding is not used, how are most of your calves fed?

1) nursing bottle
2) suck the cow
3) other. Specify:

103. Until the calves are a month old, how often are they usually fed in a day?

1) once
2) twice
3) three times
4) four or more times

104. Until they are a month old, what volume of liquid food is each calf usually offered per feeding?

1) one litre or less
2) greater than 1 l but less than 2
3) greater than 2 l but less than 3
4) greater than 3 l but less than 4
5) greater than 4 l

105. At what age do you usually begin to offer calves grain/concentrate?

0) not offered
1) < 2 weeks
2) 2 - 3 weeks
3) 3 - 4 weeks
4) 4 - 6 weeks
5) 6 weeks - 2 months
6) 2 months - 3 months
7) > 3 months

106. Do you usually begin to offer forage (grass) to calves before they are weaned? If so, at what age?

0) Not done
1) < 2 weeks
2) 2 - 4 weeks
3) 4 - 6 weeks
4) 6 weeks - 2 months
5) 2 months - 3 months
6) > 3 months

107. What kind of forage is usually offered?

1) pasture/grazing
2) Napier grass
3) legumes. Specify:_____
4) other. Specify:_____

108. Do you usually begin to offer calves water before they are weaned? If yes, at what age?

0) Not offered
1) < 2 weeks
2) 2 - 4 weeks
3) 4 - 6 weeks
4) 6 weeks - 2 months
5) 2 months - 3 months
6) > 3 months

109. How is the water offered?

1) pail
2) nursing bottle
2) as per adult cows
3) other. Specify:_____

110. How often is water offered?

1) once daily
2) twice daily
3) when the pail is empty
4) ad libitum (free choice)
5) other. Specify:_____

111. By what age is weaning usually completed?

1) < 2 months
2) 2 - 3months
3) 3 - 4 months
4) 4 - 5 months
5) > 5 months

112. Do you usually dehorn your calves before they are weaned? If yes, at what age?

0) Not dehorned before weaning
1) < 2 months
2) 2 - 3months
3) 3 - 4 months
4) 4 - 5 months
5) > 5 months

113. How are most of your calves dehorned?

1) caustic paste
2) gouger
3) burned/disbudding
4) other. Specify:_____

114. Do you clean the udder/teats before and/or after milking?

0) udder/teats not cleaned
1) cleaned before milking only
2) cleaned after milking only
3) cleaned both before and after milking

115. If the udder is cleaned, what do you use?

1) water alone
2) water + disinfectant. Specify:_____
3) dry towel/cloth

116. If the udder is cleaned with water +/- disinfectant, is it dried?

0) wet udder not dried
1) udder dried before milking
2) udder dried after milking

117. If the udder is dried, what is used?

1) newsprint
2) disposable paper towels
3) reusable towel
4) other. Specify:_____

118. Do you wash/clean your hands before milking?

0) no, do not wash hands
1) yes, wash hands

119. If so, what do you use?

1) water alone
2) water + soap/disinfectant.
Specify product:_____
3) dry towel
4) other. Specify:_____

120. If you wash your hands, do you dry them before milking?

0) hands not dried before milking
1) hands dried before milking

121. If you dry your hands before milking, what do you use?

1) newsprint
2) disposable paper towels
3) reusable towel
4) other. Specify:_____

122. How do you dry off(stop milking) your cows?

1) suddenly stop milking
2) gradually reduce milking

123. When you dry off a cow what procedures/treatments do you perform?

0) no treatments
1) infuse all quarters with antibiotic.
   Specify product:_____
2) infuse mastitic quarters only with antibiotic.
   Specify product:_____
3) other. Specify:_____

124. Who does the milking on your farm?

1) employee(s)
2) family member(s). Specify:_____
3) both employee(s) and family member(s).
   Specify:_____

125. How many cases of mastitis have you had in the past year?

_____ cases of mastitis

126. Did you have to replace a cow due to mastitis in the past year?

0) No
1) Yes. Specify how many:_____

127. Approximately how much do you have to spend per treatment of a case of mastitis (drugs + professional fees)?

_____ (Kenya shillings per treatment)

128. Has your milk been rejected at the dairy due to mastitis in the past 12 months?

0) No
1) Yes. Specify how many times:_____

Appendix 1.2: Initial Adult Survey/Examination Form

To be filled out for **EACH** dairy animal older than one year of age.

Circle or fill in the appropriate response.

1. Date (day/month/year):____/____/____

2. Investigator Administering Survey:_____

3. Dairy Society:

    1) Chania
    2) Kiambaa
    3) Kikuyu
    4) Lari
    5) Limuru
    6) Nderi

4. Farm ID/Cooperative #:_____

5. Cow ID/Name or Number:_____

6. Breed:

    1) Friesian/Holstein
    2) Guernsey
    3) Aryshire
    4) Jersey
    5) Zebu/Boran
    6) Australian Milking Zebu
    7) Sahiwal
    8) Cross Breed. Specify:_____

7. Dam ID/#:_____

8. Breed of Dam:

    1) Friesian/Holstein
    2) Guernsey
    3) Aryshire
    4) Jersey
    5) Zebu/Boran
    6) Australian Milking Zebu
    7) Sahiwal
    8) Cross Breed. Specify:_____

9. Sire ID/#:_____

10. Breed of Sire:

1) Friesian/Holstein
2) Guernsey
3) Aryshire
4) Jersey
5) Zebu/Boran
6) Australian Milking Zebu
7) Sahiwal
8) Cross Breed. Specify:_____

11. Date of Birth (d/m/y):____/____/____, or, if unavailable, age in years:

_____years old

12. Sex:

0) male
1) female

HISTORY:

13. Parity Number: How many live births has this cow had?

_____ live births

14. Date of last Calving (d/m/y):____/____/____

15. Did any of the following occur, within the last 12 months:

1) abortion (born prematurely)
2) stillbirth (born dead)
3) dystocia (difficult birth)
4) deformed calf
5) retained placenta
6) metritis/endometritis
7) mastitis
8) other. Specify:_____

16. How long after calving was the first heat observed:

0) no heat yet observed
1) < 7 days
2) 1 - 2 weeks
3) 3 - 4 weeks
4) > 4 weeks

17. How was this cow last Bred:

1) natural service (Bull)
2) artificial Insemination
3) both. Give details - _____

18. Date of Last Breeding/Insemination (d/m/y):____/____/____

19. Number of Services Since Last Calving: 1 2 3 4 5 6+

20. Present weight (kg) of Milk per day from this Cow:

_____kg/day

21. Of the milk produced by this cow how much (kg) is being consumed by each of the following?

	kg
1) calf/calves	____
2) family	____
3) sold locally	____
4) sold to dairy society	____
5) other. Specify:_____	

22. Has this animal received any vaccinations in the past year? If so, specify what was administered and date administered.

0) no vaccines
1) vaccinated.

	Product	Date(s)
Give details:	_____	_____
	_____	_____
	_____	_____

23. Has this animal been "dewormed" in the past year? If so, specify what was administered and date administered.

0) not dewormed
1) dewormed.

	Product	Date(s)
Give details:	_____	_____
	_____	_____
	_____	_____

24. Has any prophylactic (preventative) medication been administered to this animal in the past year? If so, specify what was administered and date administered.

0) no preventative medication
1) preventative medication administered

	Product	Date(s)
Give details:	_____	_____
	_____	_____
	_____	_____

25. How would you classify this animals appetite?

0) anorexic
1) poor (less than normal)
2) normal (average)
3) greater than average

26. What are you presently feeding this animal and how much:

	kg
1) forage	____
2) grain/concentrate	____
3) mineral supplement	____
4) legumes. Specify:	_____
5) other. Specify:	_____

27. When was this animal last treated for ticks?

0) do not treat for ticks
1) < 3 days
2) 3 - 7 days
3) 8 days - 2 weeks
4) 2 - 3 weeks
5) 3 weeks - 1 month
6) > 1 month

Has this animal ever been diagnosed as having, or treated for any of the following illnesses? If so give details (date, severity, response to therapy, cost of therapy (drugs + professional fees)).

28. Theileriosis ("Ngai"/East Coast Fever)

0) No
1) Yes. Give details:

Date:	Severity:	Response:
_____	_____	_____
_____	_____	_____
_____	_____	_____

29. Anaplasmosis ("Ndigana"/Gall-sickness)

0) No
1) Yes. Give details:

Date: _____ Severity: _____ Response: _____
_____ _____ _____
_____ _____ _____

30. Babesiosis ("Guthuguma Thakame"/Red Water)

0) No
1) Yes. Give details:

Date: _____ Severity: _____ Response: _____
_____ _____ _____
_____ _____ _____

31. Cowdriosis (Heartwater)

0) No
1) Yes. Give details:

Date: _____ Severity: _____ Response: _____
_____ _____ _____
_____ _____ _____

32. Other Diseases. Specify Disease:_____

0) No other illnesses
1) _____. Give details:

Date: _____ Severity: _____ Response: _____
_____ _____ _____
_____ _____ _____

CLINICAL EXAMINATION:

33. Clinician performing Clinical Examination:_____

GENERAL INSPECTION:

34. Body Condition Score:

1) too thin
2) average
3) too fat

35. Respiratory Rate:_____(breaths/minute)

36. Cough:

0) absent
1) infrequent/"normal"
2) frequent/harsh. Describe:_____

37. Dyspnea:

0) absent
1) mild
2) moderate
3) severe

38. Nasal Discharge:

0) absent
1) serous
2) mucoid
3) purulent
4) haemorrhagic
5) other. Describe:_____

39. Mentation:

0) alert/normal
1) dull/depressed
2) other. Specify:_____

40. Gait/lameness:

0) normal
1) abnormal/lame. Describe:_____

PHYSICAL EXAMINATION:

41. Rectal Temperature:_____°C or _____°F

Vagina/Labia:

42. Labia:

0) normal
1) abnormal. Describe:_____

43. Vaginal Discharge:

0) absent
1) serous/mucoid
2) purulent/haemorrhagic
3) frank blood/blood tinged

44. Vaginal Mucus Membranes:

1) pink/normal
2) pale-white
3) jaundiced
4) cyanotic
5) petechiated (haemorrhages)
6) injected
7) other lesions. Describe:_____

45. Rumen Motility:

0) absent/no motility
1) weak contractions
2) strong contractions

46. Number of contractions per minute:_____

Lung Auscultation/Percussion:

47. Left side:

1) normal sounds/rhythm, no areas of dullness
2) mildly increased lung sounds (M1)
3) moderately increased lung sounds (M2)
4) markedly increased lung sounds (M3)
5) Areas of dullness on percussion/absence of normal lung sounds

48. Abnormal sounds present/loudest on:

1) inspiration
2) expiration
3) both inspiration and expiration

49. Area of lung field affected (increased sounds or areas of dullness):

1) generalized over lung field
2) antero-ventrally
3) dorso-caudally

50. Character of increased/abnormal sounds:

1) wheezes
2) crackles
3) other. Describe:_____

51. Right side:

1) normal sounds/rhythm, no areas of dullness
2) mildly increased lung sounds (M1)
3) moderately increased lung sounds (M2)
4) markedly increased lung sounds (M3)
5) Areas of dullness on percussion/absence of normal lung sounds

52. Abnormal sounds present/loudest on:

1) inspiration
2) expiration
3) both inspiration and expiration

53. Area of lung field affected (increased sounds or areas of dullness:

1) generalized over lung field
2) antero-ventrally
3) dorso-caudally

54. Character of increased/abnormal sounds:

1) wheezes
2) crackles
3) other. Describe:_____

Cardiac Auscultation:

55. Heart rate:_____(beats per minute)

56. Rhythm:

0) normal
1) abnormal. Describe:_____

57. Adventitial (abnormal/extra heart sounds/murmurs):

0) absent (normal heart sounds only)
1) adventitial sounds present

58. Location/Character (more than one possible, circle all appropriate)

1) loudest on left side
2) loudest on right side
3) loudest at heart base
4) loudest at heart apex
5) loudest at systoly
6) loudest at diastoly
7) high pitched sound
8) low pitched sound

59. Peripheral Pulse:

0) normal character and rhythm (coincides with auscultation)
1) weak/thready/abnormal rate/rhythm (i.e. pulse deficit)
2) pounding/exaggerated

60. Jugular Pulse:

0) absent
1) present but mild
2) present and moderate
3) marked/dramatic jugular pulse

61. Ocular Examination (eye, orbit, conjunctiva, discharge, etc.):

0) eyes normal
1) abnormality detected. Describe:_____

Oral Examination:

62. Mucus Membranes:

1) pink/normal
2) pale-white
3) jaundiced
4) cyanotic
5) petechiated (haemorrhages)
6) injected
7) ulcerated/erosions. Describe:_____
8) vesicles present. Describe:_____
9) other. Describe:_____

63. Dentition/Tongue/Buccal Papillae:

0) normal
1) abnormal. Describe:_____

Mammary Examination:

64. Udder Appearance/Conformation/Suspensory Ligaments:

0) normal
1) abnormal. Describe:_____

65. Udder Palpation/ Associated Lymph Nodes:

0) normal
1) abnormal. Describe:_____

Teats (Appearance/Palpation/Strip cup/CMT score):

66. Left fore:
0) normal
1) abnormal. Describe:_____

67. CMT score (LF):

0) -
1) +
2) ++
3) +++
4) ++++

68. Left hind:
0) normal
1) abnormal. Describe:_____

69. CMT score (LH):

0) -
1) +
2) ++
3) +++
4) ++++

70. Right fore:
0) normal
1) abnormal. Describe:_____

71. CMT score (RF):

0) -
1) +
2) ++
3) +++
4) ++++

72. Right hind:

0) normal
1) abnormal. Describe:_____

71. CMT score (RH):

0) -
1) +
2) ++
3) +++
4) ++++

72. Supernumerary Teats:

0) absent
1) present. Describe:

Number:_____ Size(s/m/l):_____ Location:_____

73. Skin Condition/Examination:

0) no abnormal findings/good condition
1) abnormal/pathological condition. Describe:___
_____

74. Superficial Lymph Nodes:

0) normal
1) abnormal. Describe each:

Node: _____ Size (cm): _____
_____ _____

75. Standard Tick Count:_____(standard ticks/whole body)

Limbs/Joints/Hooves/Coronary Bands:

76. Left fore:
0) normal
1) abnormal. Describe:_____

77. Left hind:
0) normal
1) abnormal. Describe:_____

78. Right fore:
0) normal
1) abnormal. Describe:_____

79. Right hind:
0) normal
1) abnormal. Describe:_____

RECTAL EXAMINATION:

Uterus:

80. Uterine Palpation:

0) normal
1) abnormal. Describe:_____

81. Preg Status:

1) Open/<42 days
2) 6 weeks - 12 weeks
3) 3 - 5 months
4) 5 - 7 months
5) 7 - 9 months

Ovaries:

82. Right Ovary (Describe structures present and size):

0) normal (describe structures)
1) abnormal. Describe:_____

Structure: _____ Size (cm): _____
_____ _____

83. Left Ovary (Describe structures present and size):

0) normal (describe structures)
1) abnormal. Describe:_____

Structure: _____ Size (cm): _____
_____ _____

84. Lymph Nodes/Kidneys/etc:

0) normal
1) abnormal. Describe:_____

85. Rumen:

0) not palpable
1) firm/doughy
2) gasseous
3) fluid consistency
4) other. Describe:_____

SAMPLES COLLECTED:

Check when collected

ALL ANIMALS:

Blood: i) 1 plain (red-top) vacutainer _____
ii) 1 EDTA (purple-top) vacutainer _____

LACTATING COWS

Blood: iii) 1 Citrate (grey-top) vacutainer _____

Milk: iv) Individual milk samples by quarter _____

OTHER SAMPLES. SPECIFY:_____
_____
_____

NOTE: Animals with lymphadenopathy and/or clinical signs of illness, need to have lymphnode aspirations performed.

OTHER CLINICAL SIGNS/HISTORY/PHYSICAL EXAMINATION FINDINGS:_____
_____
_____

CONDITION AND/OR DIAGNOSIS (If illness discovered):_____
_____

TREATMENT ADMINISTERED/PROCEDURES PERFORMED:

PROCEDURE	THERAPEUTIC AGENT	AMOUNT	ROUTE
_____	_____	_____	_____
_____	_____	_____	_____

INSTRUCTIONS/RECOMMENDATIONS TO OWNER:_____
_____
_____

Appendix 1.3: Initial Calf Survey/Examination Form

To be filled out for **EACH** dairy animal less than or equal to one year of age.

Circle or fill in the appropriate response.

1. Date (day/month/year):____/____/____

2. Investigator Administering Survey:_____

3. Dairy Society:

    1) Chania
    2) Kiambaa
    3) Kikuyu
    4) Lari
    5) Limuru
    6) Nderi

4. Farm ID/Cooperative #:_____

5. Calf ID/Name or Number:_____

6. Breed:

    1) Friesian/Holstein
    2) Guernsey
    3) Aryshire
    4) Jersey
    5) Zebu/Boran
    6) Australian Milking Zebu
    7) Sahiwal
    8) Cross Breed. Specify:_____

7. Dam ID/#:_____

8. Breed of Dam:

    1) Friesian/Holstein
    2) Guernsey
    3) Aryshire
    4) Jersey
    5) Zebu/Boran
    6) Australian Milking Zebu
    7) Sahiwal
    8) Cross Breed. Specify:_____

9. Sire ID/#:_____

10. Breed of Sire:

1) Friesian/Holstein
2) Guernsey
3) Aryshire
4) Jersey
5) Zebu/Boran
6) Australian Milking Zebu
7) Sahiwal
8) Cross Breed. Specify:_____

11. Date of Birth (d/m/y):___/___/___, or, if unavailable, age in weeks or months:

_____weeks old

_____months old

12. Sex:

0) male
1) female

HISTORY:

13. Has this calf experienced any "sickness" since birth? If yes, what were the clinical signs/diagnosis?

0) no sickness
1) diarrhoea
2) pneumonia
3) other. Specify:_____

14. Has this calf received any vaccinations since birth? If so, specify what was administered and date administered.

0) no vaccines
1) vaccinated.

	Product	Date(s)
Give details:	_____	_____
	_____	_____

15. Has this calf been "dewormed" since birth? If so, specify what was administered and date administered.

0) not dewormed
1) dewormed.

	Product	Date(s)
Give details:	_____	_____
	_____	_____

16. Has any prophylactic (preventative) medication been administered to this calf since birth? If so, specify what was administered and date administered.

0) no preventative medication
1) preventative medication administered

	Product	Date(s)
Give details:	_____	_____
	_____	_____

17. How would you classify this animals appetite?

0) anorexic
1) poor (less than normal)
2) normal (average)
3) greater than average

18. What are you presently feeding this calf and how much (litres or kilograms)?

	litres	kg
1) colostrum	____	____
2) milk	____	____
3) milk replacer	____	____
4) forage	____	____
5) grain/concentrate	____	____
6) mineral supplement	____	____
7) legumes. Specify:_____	____	____
8) other. Specify:_____	____	____

19. When was this animal last treated for ticks?

0) do not treat for ticks
1) calf not yet treated for ticks
2) < 3 days
3) 3 - 7 days
4) 8 days - 2 weeks
5) 2 - 3 weeks
6) 3 weeks - 1 month
7) > 1 month

Has this animal ever been diagnosed as having, or treated for any of the following illnesses? If so give details (date, severity, response to therapy, cost of therapy (drugs + professional fees)).

20. Theileriosis ("Ngai"/East Coast Fever)

0) No
1) Yes. Give details:

Date: _____ Severity: _____ Response: _____
_____ _____ _____

21. Anaplasmosis ("Ndigana"/Gall-sickness)

0) No
1) Yes. Give details:

Date: _____ Severity: _____ Response: _____
_____ _____ _____

22. Babesiosis ("Guthuguma Thakame"/Red Water)

0) No
1) Yes. Give details:

Date: _____ Severity: _____ Response: _____
_____ _____ _____

23. Cowdriosis (Heartwater)

0) No
1) Yes. Give details:

Date: _____ Severity: _____ Response: _____
_____ _____ _____

CLINICAL EXAMINATION:

24. Clinician performing Clinical Examination:_____

GENERAL INSPECTION:

25. Body Condition Score:

1) too thin
2) average
3) too fat

26. Respiratory Rate:_____(breaths/minute)

27. Cough:

0) absent
1) infrequent/"normal"
2) frequent/harsh. Describe:_____

28. Dyspnea:

0) absent
1) mild
2) moderate
3) severe

29. Nasal Discharge:

0) absent
1) serous
2) mucoid
3) purulent
4) haemorrhagic
5) other. Describe:_____

30. Mentation:

0) alert/normal
1) dull/depressed
2) other. Specify:_____

31. Gait/lameness:

0) normal
1) abnormal/lame. Describe:_____

PHYSICAL EXAMINATION:

32. Rectal Temperature:_____°C or _____°F

Vagina/Labia:

33. Labia:

0) normal
1) abnormal. Describe:_____

34. Vaginal Discharge:

0) absent
1) serous/mucoid
2) purulent/haemorrhagic
3) frank blood/blood tinged

35. Vaginal Mucous Membranes:

1) pink/normal
2) pale-white
3) jaundiced
4) cyanotic
5) petechiated (haemorrhages)
6) injected
7) other lesions. Describe:_____

36. Rumen Motility:

0) absent/no motility
1) weak contractions
2) strong contractions

37. Number of contractions per minute:_____

Lung Auscultation/Percussion:

38. Left side:

1) normal sounds/rhythm, no areas of dullness
2) mildly increased lung sounds (M1)
3) moderately increased lung sounds (M2)
4) markedly increased lung sounds (M3)
5) Areas of dullness on percussion/absence of normal lung sounds

39. Abnormal sounds present/loudest on:

1) inspiration
2) expiration
3) both inspiration and expiration

40. Area of lung field affected (increased sounds or areas of dullness):

1) generalized over lung field
2) antero-ventrally
3) dorso-caudally

41. Character of increased/abnormal sounds:

1) wheezes
2) crackles
3) other. Describe:_____

42. Right side:

1) normal sounds/rhythm, no areas of dullness
2) mildly increased lung sounds (M1)
3) moderately increased lung sounds (M2)
4) markedly increased lung sounds (M3)
5) Areas of dullness on percussion/absence of normal lung sounds

43. Abnormal sounds present/loudest on:

1) inspiration
2) expiration
3) both inspiration and expiration

44. Area of lung field affected (increased sounds or areas of dullness:

1) generalized over lung field
2) antero-ventrally
3) dorso-caudally

45. Character of increased/abnormal sounds:

1) wheezes
2) crackles
3) other. Describe:_____

Cardiac Auscultation:

46. Heart rate:_____(beats per minute)

47. Rhythm:

0) normal
1) abnormal. Describe:_____

48. Adventitial (abnormal/extra heart sounds/murmurs):

0) absent (normal heart sounds only)
1) adventitial sounds present

49. Location/Character (more than one possible, circle all appropriate)

1) loudest on left side
2) loudest on right side
3) loudest at heart base
4) loudest at heart apex
5) loudest at systole
6) loudest at diastole
7) high pitched sound
8) low pitched sound

51. Peripheral Pulse:

0) normal character and rhythm (coincides with auscultation)
1) weak/thready/abnormal rate/rhythm (i.e. pulse deficit)
2) pounding/exaggerated

52. Jugular Pulse:

0) absent
1) present but mild
2) present and moderate
3) marked/dramatic jugular pulse

53. Ocular Examination (eye, orbit, conjunctiva, discharge, etc.):

0) eyes normal
1) abnormality detected. Describe:_____

Oral Examination:

54. Mucous Membranes:

1) pink/normal
2) pale-white
3) jaundiced
4) cyanotic
5) petechiated (haemorrhages)
6) injected
7) ulcerated/erosions. Describe:_____
8) vesicles present. Describe:_____
9) other. Describe:_____

55. Dentition/Tongue/Buccal Papillae:

0) normal
1) abnormal. Describe:_____

Mammary Examination:

56. Udder and Teat Appearance/Conformation/Suspensory Ligaments:

0) normal
1) abnormal. Describe:_____

57. Udder Palpation/ Associated Lymph Nodes:

0) normal
1) abnormal. Describe:_____

58. Supernumerary Teats:

0) absent
1) present. Describe:

Number:_____ Size(s/m/l):_____ Location:_____

59. Skin Condition/Examination:

0) no abnormal findings/good condition
1) abnormal/pathological condition. Describe:
_____

60. Superficial Lymph Nodes:

0) normal
1) abnormal. Describe each:

Node: _____ Size (cm): _____
_____ _____

61. Standard Tick Count:_____(standard ticks/whole body)

Limbs/Joints/Hooves/Coronary Bands:

62. Left fore:
0) normal
1) abnormal. Describe:_____

63. Left hind:
0) normal
1) abnormal. Describe:_____

64. Right fore:
0) normal
1) abnormal. Describe:_____

65. Right hind:
0) normal
1) abnormal. Describe:_____

66. Weight of calf (to nearest one-half kg):_____(kg)

SAMPLES COLLECTED:

Check when collected

ALL CALVES:

Blood: I) 2 plain (red-top) vacutainers _____
ii) 1 EDTA (purple-top) vacutainer _____

Faecal Sample _____

OTHER SAMPLES. SPECIFY:_____
_____

NOTE: All animals with lymphadenopathy or clinical signs of illness need to have a lymph node aspiration performed.

OTHER CLINICAL SIGNS/HISTORY/PHYSICAL EXAMINATION FINDINGS:_____
_____

CONDITION AND/OR DIAGNOSIS (If illness discovered):_____
_____

TREATMENT ADMINISTERED/PROCEDURES PERFORMED:

PROCEDURE	THERAPEUTIC AGENT	AMOUNT	ROUTE

INSTRUCTIONS/RECOMMENDATIONS TO OWNER:_____
_____

Appendix 1.4: Follow-Up Adult Survey/Examination Form

To be filled out for **EACH** dairy animal older than one year of age.

1. Date (day/month/year):____/____/____

2. Investigator Conducting Examination:_____

3. Dairy Society: 1) Chania
   2) Kiambaa
   3) Kikuyu
   4) Lari
   5) Limuru
   6) Nderi

4. Farm ID/Cooperative #:_____

5. Cow ID/Name or Number:_____

HISTORY:

6. Did any of the following occur, since the last visit:

   0) normal calving/live calf
   1) abortion (born prematurely)
   2) stillbirth (born dead)
   3) dystocia (difficult birth)
   4) deformed calf
   5) retained placenta/metritis/endometritis
   6) mastitis
   7) other. Specify:_____
   8) None of the above

7. Has this animal been observed "on heat" since the last visit? If so, when? (NOTE: may be more than once):

   0) no heat yet observed
   1) heat observed (day/month/year):____/____/____
   and (day/month/year):____/____/____

8. If this cow was observed "on heat" since the last visit, was she bred and if so, how and on what day?:

   0) observed "on heat" but not bred
   1) natural service (Bull) same day as heat observed
   2) natural service (Bull) day after heat observed
   3) artificial insemination same day as heat observed
   4) artificial insemination day after heat observed
   5) both. Give details - _____

9. Present weight (kg) of Milk per day from this Cow:_____kg/day

10. Has this animal received any vaccinations, been dewormed, or received any prophylactic medication since the last visit? If so, specify what was administered and date administered.

0) no vaccines/deworming
1) vaccinated
2) dewormed
3) received prophylactic medication

Product Date(s)

Give details:_____ _____

11. What are you presently feeding this animal and how much:

	kg
1) forage	____
2) grain/concentrate	____
3) mineral supplement	____
4) legumes. Specify:_____	
5) other. Specify:_____	

12. When was this animal last treated for ticks?

0) do not treat for ticks
1) < 3 days
2) 3 - 7 days
3) 8 days - 2 weeks
4) 2 - 3 weeks
5) 3 weeks - 1 month
6) > 1 month

13. If this animal was treated for ticks, was it dipped or sprayed?

1) sprayed
2) dipped

14. Has this animal been ill/treated by a veterinarian since the last visit? If so give details (date, severity, response to therapy, cost of therapy (drugs + professional fees)).

0) No illnesses
1) _____. Give details:

Date:_____ Severity:_____ Response:_____

CLINICAL EXAMINATION:

15. Body Condition Score:

1) too thin
2) average
3) too fat

16. Rectal Temperature:_____°C or _____°F

17. Respiratory Rate:_____(breaths/minute)

18. Lung Auscultation/Cough/Dyspnea/Nasal Discharge:

0) normal
1) abnormal. Describe:_____

19. Vagina/Labia/Vaginal Discharge:

0) normal
1) abnormal. Describe:_____

20. Rumen Motility:

0) absent/no motility
1) number of contractions per minute:_____

21. Udder Appearance / Conformation / Suspensory Ligaments / Palpation / Associated Lymph Nodes:

0) normal
1) abnormal. Describe:_____

Teats (Appearance/Palpation/Strip cup/CMT score):

22. Left fore:
0) normal
1) abnormal. Describe:_____

23. CMT score (LF): (0,1,2 or 3)_____

24. Left hind:
0) normal
1) abnormal. Describe:_____

25. CMT score (LH): (0,1,2 or 3)_____

26. Right fore:
0) normal
1) abnormal. Describe:_____

27. CMT score (RF): (0,1,2 or 3)_____

28. Right hind:
0) normal
1) abnormal. Describe:_____

29. CMT score (RH): (0,1,2 or 3)_____

30. Skin Condition/Examination/Superficial Lymph Nodes:

0) no abnormal findings/good condition
1) abnormal/pathological condition.
Describe (give sizes of Lymph Nodes if enlarged):
_____

31. Standard Tick Count:_____(standard ticks/whole body)

32. Oral/Ocular Examination:

0) normal
1) abnormal. Describe:_____

33. Limbs/Joints/Hooves/Coronary Bands/Gait/Lameness:

0) normal
1) abnormal. Describe:_____

34. Uterine Palpation/Lymph Nodes/Kidneys/Rumen:

0) normal
1) abnormal. Describe:_____

35. Pregnancy Status:

1) Open/<42 days
2) 6 weeks - 12 weeks
3) 3 - 5 months
4) 5 - 7 months
5) 7 - 9 months

36. Right Ovary (Describe structures present and size):

0) normal (describe structures)
1) abnormal. Describe:_____

Structure:_____ Size (cm):_____

37. Left Ovary (Describe structures present and size):

0) normal (describe structures)
1) abnormal. Describe:_____

Structure:_____ Size (cm):_____

SAMPLES COLLECTED:

Check when collected

ALL ANIMALS:

Blood: I) 2 plain (red-top) vacutainers _____
ii) 1 EDTA (purple-top) vacutainer _____

LACTATING COWS

Milk: iii) Individual milk samples by quarter _____

OTHER SAMPLES. SPECIFY:_____

NOTE: Animals with lymphadenopathy and/or clinical signs of illness, need to have lymph node aspirations performed.

OTHER CLINICAL SIGNS/HISTORY/PHYSICAL EXAMINATION FINDINGS:_____

CONDITION AND/OR DIAGNOSIS (If illness discovered):_____

TREATMENT ADMINISTERED/PROCEDURES PERFORMED:

PROCEDURE	THERAPEUTIC AGENT	AMOUNT	ROUTE

INSTRUCTIONS/RECOMMENDATIONS TO OWNER:_____

Appendix 1.5: Follow-Up Calf Survey/Examination Form

To be filled out for **EACH** dairy animal less than one year of age.

1. Date (day/month/year):____/____/____

2. Investigator Conducting Examination:_____

3. Dairy Society:

1) Chania
2) Kiambaa
3) Kikuyu
4) Lari
5) Limuru
6) Nderi

4. Farm ID/Cooperative #:_____

5. Cow ID/Name or Number:_____

HISTORY:

6. Has this animal received any vaccinations, been dewormed, or received any prophylactic medication since the last visit? If so, specify what was administered and date administered.

0) no vaccines/deworming
1) vaccinated
2) dewormed
3) received prophylactic medication

	Product	Date(s)
Give details:	_____	_____

7. What are you presently feeding this calf and how much (litres or kilograms)?

	litres	kg
1) colostrum	____	____
2) milk	____	____
3) milk replacer	____	____
4) forage	____	____
5) grain/concentrate	____	____
6) mineral supplement	____	____
7) legumes. Specify:_____	____	____
8) other. Specify:_____	____	____

8. When was this animal last treated for ticks?

0) do not treat for ticks
1) < 3 days
2) 3 - 7 days
3) 8 days - 2 weeks
4) 2 - 3 weeks
5) 3 weeks - 1 month
6) > 1 month

9. If this animal was treated for ticks, was it dipped or sprayed?

1) sprayed
2) dipped

10. Has this animal been ill/treated by a veterinarian since the last visit? If so give details (date, severity, response to therapy, cost of therapy (drugs + professional fees)).

0) No illnesses
1) _____. Give details:

Date:_____ Severity:_____ Response:_____

CLINICAL EXAMINATION:

11. Body Condition Score:

1) too thin
2) average
3) too fat

12. Rectal Temperature:_____°C or _____°F

13. Respiratory Rate:_____(breaths/minute)

14. Lung Auscultation/Cough/Dyspnea/Nasal Discharge:

0) normal
1) abnormal. Describe:_____

15. Vagina/Labia/Vaginal Discharge:

0) normal
1) abnormal. Describe:_____

16. Rumen Motility:

0) absent/no motility
1) number of contractions per minute:_____

17. Udder Appearance / Conformation / Suspensory Ligaments / Palpation / Associated Lymph Nodes/Teats:

0) normal
1) abnormal. Describe:_____

18. Skin Condition/Examination/Superficial Lymph Nodes:

0) no abnormal findings/good condition
1) abnormal/pathological condition.
Describe (give sizes of Lymph Nodes if enlarged):
_____

19. Standard Tick Count:_____(standard ticks/whole body)

20. Limbs/Joints/Hooves/Coronary Bands/Gait/Lameness:

0) normal
1) abnormal. Describe:_____

21. Oral/Ocular Examination:

0) normal
1) abnormal. Describe:_____

22. Weight of calf (to nearest ½ kg):_____kg

SAMPLES COLLECTED:

Check when collected

ALL ANIMALS:

Blood: I) 2 plain (red-top) vacutainer _____
ii) 1 EDTA (purple-top) vacutainer _____

Faecal Sample _____

OTHER SAMPLES. SPECIFY:_____

NOTE: Animals with lymphadenopathy and/or clinical signs of illness, need to have lymph node aspirations performed.

OTHER CLINICAL SIGNS/HISTORY/PHYSICAL EXAMINATION FINDINGS:_____
_____

CONDITION AND/OR DIAGNOSIS (If illness discovered):_____
_____

TREATMENT ADMINISTERED/PROCEDURES PERFORMED:

PROCEDURE	THERAPEUTIC AGENT	AMOUNT	ROUTE

INSTRUCTIONS/RECOMMENDATIONS TO OWNER:_____

Appendix 2: Distribution of continuous and categorical farm-level variables of 90 smallholder dairy farms enrolled in a longitudinal study conducted in Kiambu District from July 1991 - June 1992, presented by geographical stratification on dairy cooperative society with appropriate measures of association.

Farm Size in Acres

Significant differences (α=0.05) between dairy cooperative societies indicated by multi-comparison method

	Mean	SD	Median	Min - Max	Tukey	
Overall	**4.64**	**4.71**	**3**	**0.25 - 25**		
Chania	3.31	3.81	2	0.5 - 15	B	
Kiambaa	3.22	3.74	1	0.25 - 12	B	
Kikuyu	4.92	6.24	2.5	0.25 - 24	B	
Lari	9.36	5.48	10	1.4 - 25		A
Limuru	4.17	2.49	4	0.5 - 10	B	A
Nderi	2.88	2.39	2	0.5 - 10	B	

Number of Dairy Cattle Present on Farm at Initial Sample

Significant differences (α=0.05) between dairy cooperative societies indicated by multi-comparison method

	Mean	SD	Median	Min - Max	Tukey
Overall	**4.37**	**4.10**	**3**	**1 - 24**	
Chania	2.40	1.35	2	1 - 6	A
Kiambaa	5.13	4.64	4	1 - 17	A
Kikuyu	5.73	6.09	4	1 - 24	A
Lari	4.40	2.64	4	1 - 10	A
Limuru	4.20	3.28	4	1 - 12	A
Nderi	4.33	4.67	3	1 - 20	A

Number of Full Years Spent Dairy Farming

No significant differences between dairy cooperative societies

	Mean	SD	Median	Min - Max	Tukey
Overall	**14.83**	**9.24**	**14.5**	**0 - 34**	
Chania	14.80	9.60	15	0 - 30	A
Kiambaa	17.60	9.79	20	0 - 31	A
Kikuyu	15.60	8.77	16	2 - 29	A
Lari	18.07	9.62	20	0 - 28	A
Limuru	10.80	9.16	10	0 - 34	A
Nderi	12.13	7.48	10	3 - 26	A

Number of Family Members on Farm or Dependent on Farm Income

Significant differences (α=0.05) between dairy cooperative societies indicated by multi-comparison method

	Mean	SD	Median	Min-Max	Tukey	
Overall	**6.52**	**3.22**	**6**	**1 - 15**		
Chania	5.40	3.74	5	1 - 15	B	A
Kiambaa	6.53	2.59	6	2 - 11		A
Kikuyu	4.87	2.39	5	1 - 10	B	
Lari	8.53	3.42	8	3 - 15		A
Limuru	7.60	2.29	7	5 - 12	B	A
Nderi	6.20	3.57	6	1 - 12	B	A

Age in Months at which Tick-Treatment of Calves Commences

No significant differences between dairy cooperative societies

	No. Farms	Mean	SD	Median	Min-Max	Tukey
Overall	**78**	**4.83**	**2.95**	**4**	**1 - 24**	
Chania	13	6.00	2.97	6	2 - 12	A
Kiambaa	11	6.55	5.96	5	2.5 - 24	A
Kikuyu	12	4.17	1.64	4	1 - 6	A
Lari	15	4.20	1.93	4	1 - 8	A
Limuru	14	4.04	1.45	3.25	2 - 6	A
Nderi	13	4.38	1.50	4	3 - 8	A

Agro-Ecological Zones - Chi-Square Test of Association = 178.34 d.f. = 35 p<0.001

	UH1		UH2		LH1		LH2		LH3		UM1		UM2		UM3	
	#	%	#	%	#	%	#	%	#	%	#	%	#	%	#	%
Overall	**6**	**6.7**	**15**	**16.7**	**2**	**2.2**	**23**	**25.6**	**13**	**14.4**	**2**	**2.2**	**22**	**24.4**	**7**	**7.8**
Chania	0	0	0	0	2	2.2	0	0	0	0	2	2.2	11	12.2	0	0
Kiambaa	0	0	0	0	0	0	0	0	0	0	0	0	11	12.2	4	4.4
Kikuyu	0	0	0	0	0	0	6	6.7	6	6.7	0	0	0	0	3	3.4
Lari	5	5.6	10	11.1	0	0	0	0	0	0	0	0	0	0	0	0
Limuru	1	1.1	5	5.6	0	0	8	8.9	1	1.1	0	0	0	0	0	0
Nderi	0	0	0	0	0	0	9	10.0	6	6.7	0	0	0	0	0	0

Grazing System - Chi-Square Test of Association = 42.75, d.f. = 10 p<0.001

	Zero		Semi-zero		Full/Pasture	
	Freq.	Percent	Freq.	Percent	Freq.	Percent
Overall	**43/90**	**47.8**	**17/90**	**18.9**	**30/90**	**33.3**
Chania	10/15	66.7	4/15	26.7	1/15	6.7
Kiambaa	7/15	46.7	5/15	33.3	3/15	20.0
Kikuyu	10/15	66.7	2/15	13.3	2/15	13.3
Lari	0/15	0	0/15	0	15/15	100
Limuru	6/15	40.0	3/15	20.0	6/15	40.0
Nderi	10/15	66.7	3/15	20.0	2/15	13.3

Tick Control Method - Chi-Square Test of Association = 32.34, d.f. = 15 p=0.006

	Dipping		Spraying		Hand Applied		None	
	Freq.	Percent	Freq.	Percent	Freq.	Percent	Freq.	Percent
Overall	**38/90**	**42.2**	**43/90**	**47.8**	**2/90**	**2.2**	**7/90**	**7.8**
Chania	12/15	80.0	3/15	20.0	0/15	0	0/15	0
Kiambaa	9/15	60.0	3/15	20.0	0/15	0	3/15	20.0
Kikuyu	8/15	53.3	6/15	40.0	0/15	0	1/15	6.7
Lari	5/15	33.3	10/15	66.7	0/15	0	0/15	0
Limuru	3/15	20.0	10/15	66.7	1/15	6.7	1/15	6.7
Nderi	1/15	6.7	11/15	73.3	1/15	6.7	2/15	13.3

Tick Control Frequency - Fisher's Exact Test p=0.234

	≤ 1 Week		> 1 Week to ≤ 2 Weeks		> 2 Weeks	
	Freq.	Percent	Freq.	Percent	Freq.	Percent
Overall	**56/83**	**67.5**	**19/83**	**22.9**	**8/83**	**9.6**
Chania	11/15	73.3	4/15	26.7	0/15	0
Kiambaa	11/12	91.7	1/12	8.3	0/12	0
Kikuyu	11/14	78.6	2/14	14.3	1/14	7.1
Lari	9/15	60.0	5/15	33.3	1/15	6.7
Limuru	6/14	42.9	4/14	28.6	4/14	28.6
Nderi	8/13	61.5	3/13	23.1	2/13	15.4

Availability and Type of Housing/Flooring for Mature Dairy Animals - Chi-Square Test of Association = 67.45, d.f. = 20 p<0.001

	No Housing		Enclosed Dirt Floor		Enclosed Concrete		Open Concrete		Open Dirt	
	Fq	%	Fq	%	Fq	%	Fq	%	Fq	%
Overall	**37/90**	**41.1**	**8/90**	**8.9**	**19/90**	**21.2**	**7/90**	**7.8**	**19/90**	**21.1**
Chania	3/15	20.0	1/15	6.7	1/15	6.7	0/15	0	10/15	66.7
Kiambaa	3/15	20.0	2/15	13.3	7/15	46.7	0/15	0	3/15	20.0
Kikuyu	2/15	13.3	4/15	26.7	6/15	40.0	2/15	13.3	1/15	6.7
Lari	15/15	100	0/15	0	0/15	0	0/15	0	0/15	0
Limuru	8/15	53.3	0/15	0	2/15	13.3	2/15	13.3	3/15	20.0
Nderi	6/15	40.0	1/15	6.7	3/15	20.0	3/15	20.0	2/15	13.3

Use and Type of Bedding for Mature Dairy Animals - Chi-Square = 52.32, d.f. = 10 p<0.001

	No Bedding Used		Grass Bedding		Wood Shavings	
	Freq.	Percent	Freq.	Percent	Freq.	Percent
Overall	**58/90**	**64.4**	**14/90**	**15.6**	**18/90**	**20.0**
Chania	6/15	40.0	9/15	60.0	0/15	0
Kiambaa	4/15	26.7	4/15	26.7	7/15	46.7
Kikuyu	8/15	53.3	1/15	6.7	6/15	40.0
Lari	15/15	100	0/15	0	0/15	0
Limuru	13/15	86.7	0/15	0	2/15	13.3
Nderi	12/15	80.0	0/15	0	3/15	20.0

Use and Type of Bedding for Calves - Fisher's Exact Test, p=0.006

	No Bedding Used		Grass Bedding		Wood Shavings	
	Freq.	Percent	Freq.	Percent	Freq.	Percent
Overall	**4/90**	**4.4**	**47/90**	**52.2**	**39/90**	**20.0**
Chania	1/15	6.7	14/15	93.3	0/15	0
Kiambaa	1/15	6.7	6/15	40.0	8/15	53.3
Kikuyu	1/15	6.7	5/15	33.3	9/15	60.0
Lari	0/15	0	7/15	46.7	8/15	53.3
Limuru	1/15	6.7	8/15	53.3	6/15	40.0
Nderi	0/15	0	7/15	46.7	8/15	53.3

Calf Housing/Flooring/Grouping Types - Chi-Square Test of Association = 60.69, d.f. = 30 p<0.001

	Outdoors		Owners House		Grouped Concrete		Grouped Dirt		Individual Concrete		Individual Dirt		Individual Raised	
	Fq	%	Fq	%	Fq	%	Fq	%	Fq	%	Fq	%	Fq	%
Overall	**6/90**	**6.7**	**4/90**	**4.4**	**11/90**	**12.2**	**41/90**	**45.6**	**6/90**	**6.7**	**19/90**	**19.0**	**3/90**	**3.3**
Chania	5/15	33.3	0/15	0	0/15	0	4/15	26.7	0/15	0	6/15	40.0	0/15	0
Kiambaa	0/15	0	0/15	0	2/15	13.3	8/15	53.3	1/15	6.7	3/15	20.0	1/15	6.7
Kikuyu	0/15	0	1/15	6.7	3/15	20.0	3/15	20.0	4/15	26.7	2/15	13.3	2/15	13.3
Lari	0/15	0	1/15	6.7	4/15	26.7	8/15	53.3	0/15	0	2/15	13.3	0/15	0
Limuru	1/15	6.7	0/15	0	0/15	0	11/15	73.3	0/15	0	3/15	20.0	0/15	0
Nderi	0/15	0	0/15	13.3	2/15	13.3	7/15	46.7	1/15	6.7	3/15	20.0	0/15	0

Timing of Calf/Dam Separation Post-Partum - Chi-Square = 21.77, d.f. = 20 p=0.353

	Immediately		<4 Hours		4-24 Hours		24-72 Hours		>72 Hours	
	Fq	%	Fq	%	Fq	%	Fq	%	Fq	%
Overall	**37/90**	**41.1**	**6/90**	**6.7**	**42/90**	**46.7**	**3/90**	**3.3**	**2/90**	**2.2**
Chania	7/15	46.7	1/15	6.7	6/15	40.0	1/15	6.7	0/15	0
Kiambaa	7/15	46.7	1/15	6.7	5/15	33.3	0/15	0	2/15	13.3
Kikuyu	6/15	40.0	0/15	0	8/15	53.3	1/15	6.7	0/15	0
Lari	9/15	60.0	0/15	0	6/15	40.0	0/15	0	0/15	0
Limuru	4/15	26.7	2/15	13.3	9/15	60.0	0/15	0	0/15	0
Nderi	4/15	26.7	2/15	13.3	8/15	53.3	1/15	6.7	0/15	0

Method of Delivery of Colostrum to Calves - Fisher's Exact Test, p=0.355

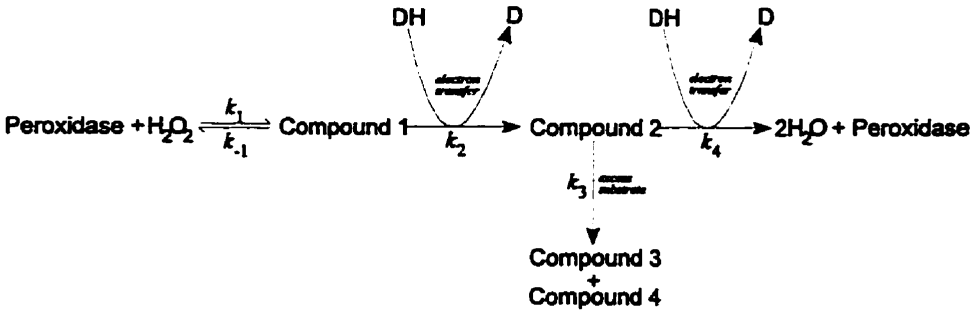
	Free Choice Suckle		Nursing Bottle		Pail or Bucket Feeding	
	Freq.	Percent	Freq.	Percent	Freq.	Percent
Overall	**47/90**	**52.2**	**3/90**	**3.3**	**40/90**	**44.4**
Chania	8/15	53.3	0/15	0	7/15	46.7
Kiambaa	5/15	33.3	1/15	6.7	9/15	60.0
Kikuyu	8/15	53.3	0/15	0	7/15	46.7
Lari	6/15	40.0	0/15	0	9/15	60.0
Limuru	10/15	66.7	1/15	6.7	4/15	26.7
Nderi	10/15	66.7	1/15	6.7	4/15	26.7

Timing of Delivery of First Colostrum Post-Partum - Fisher's Exact Test p=0.243

	<2 Hours		2-6 Hours		6-12 Hours		12-24 Hours	
	Freq.	Percent	Freq.	Percent	Freq.	Percent	Freq.	Percent
Overall	**55/90**	**61.1**	**32/90**	**35.6**	**2/90**	**2.2**	**1/90**	**1.1**
Chania	8/15	53.3	7/15	46.7	0/15	0	0/15	0
Kiambaa	6/15	40.0	8/15	53.3	1/15	6.7	0/15	0
Kikuyu	11/15	73.3	3/15	20.0	0/15	0	1/15	6.7
Lari	8/15	53.3	7/15	46.7	0/15	0	0/15	0
Limuru	11/15	73.3	4/15	26.7	0/15	0	0/15	0
Nderi	11/15	73.3	3/15	20.0	1/15	6.7	0/15	0

Appendix 3: Simplification of horseradish peroxidase enzyme kinetic quantification

Horseradish peroxidase is divalently oxidized by peroxide to form Compound 1 (C1), which is reduced back to the initial state by two successive univalent interactions with hydrogen donors (DH) as detailed below:



where, Compound 2 (C2) is the one-electron oxidized, intermediate form and Compounds 3 and 4 are inactivated forms of the enzyme complex which occur in the situation of hydrogen peroxide excess. Assuming $k_3 = 0$, we can generate the following set of equations describing the reaction:

$$E + H_2O_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} C_1$$

$$C_1 + DH \xrightarrow{k_2} C_2 + D$$

$$C_2 + DH \xrightarrow{k_4} E + 2\ H_2O + D$$

with the following sets of differential equations:

$$\frac{d[E]}{dt} = -k_1 \cdot [E] \cdot [H_2O_2] + k_{-1} \cdot [C_1] + k_4 \cdot [C_2] \cdot [DH]$$

$$\frac{d[H_2O_2]}{dt} = -k_1 \cdot [E] \cdot [H_2O_2] + k_{-1} \cdot [C_1]$$

$$\frac{d[C_1]}{dt} = k_1 \cdot [E] \cdot [H_2O_2] - k_{-1} \cdot [C_1] - k_2 \cdot [C_1] \cdot [DH]$$

$$\frac{d[DH]}{dt} = -k_2 \cdot [C_1] \cdot [DH] - k_4 \cdot [C_2] \cdot [DH]$$

$$\frac{d[C_2]}{dt} = k_2 \cdot [C_1] \cdot [DH] - k_4 \cdot [C_2] \cdot [DH]$$

$$\frac{d[D]}{dt} = k_2 \cdot [C_1] \cdot [DH] + k_4 \cdot [C_2] \cdot [DH]$$

$$\frac{d[H_2O]}{dt} = 2k_4 \cdot [C_2] \cdot [DH]$$

and the following initial conditions:

$$[E](0) = [E]_0$$

$$[H_2O_2](0) = [H_2O_2]_0$$

$$[C_1](0) = 0$$

$$[DH](0) = [DH]_0$$

$$[C_2](0) = 0$$

$$[D](0) = 0$$

$$[H_2O](0) = 0.$$

Noting that:

$$\frac{d[D]}{dt} + \frac{d[DH]}{dt} = 0$$

$$\therefore [D](t) + [DH](t) = Constant$$

$$\because [D](0) + [DH](0) = [DH]_0$$

$$\therefore [DH](t) = [DH]_0 - [D](t)$$

and similarly:

$$\frac{d[E]}{dt} + \frac{d[C_1]}{dt} + \frac{d[C_2]}{dt} = 0$$

$$\therefore [E](t) + [C_1](t) + [C_2](t) = Constant$$

$$\because [E](0) + [C_1](0) + [C_2](0) = [E]_0$$

$$\therefore [E](t) = [E]_0 - [C_1](t) - [C_2](t)$$

together these lead to the reduced set of differential equations:

$$\frac{d[H_2O_2]}{dt} = -k_1\cdot([E]_0 - [C_1] - [C_2])\cdot[H_2O_2] + k_{-1}\cdot[C_1]$$

$$\frac{d[C_1]}{dt} = k_1\cdot([E]_0 - [C_1] - [C_2])\cdot[H_2O_2] - k_{-1}\cdot[C_1] - k_2\cdot[C_1]\cdot([DH]_0 - [P])$$

$$\frac{d[C_2]}{dt} = (k_2\cdot[C_1] - k_4\cdot[C_2])\cdot([DH]_0 - [D])$$

$$\frac{d[D]}{dt} = (k_2\cdot[C_1] + k_4\cdot[C_2])\cdot([DH]_0 - [D]).$$

However, we also note that:

$$\frac{d[H_2O_2]}{dt} + \frac{d[C_1]}{dt} + \frac{1}{2}\cdot\left(\frac{d[C_2]}{dt} + \frac{d[D]}{dt}\right) = 0$$

$$\therefore [H_2O_2](t) + [C_1](t) + \frac{1}{2}\cdot[C_2](t) + \frac{1}{2}\cdot[D](t) = Constant$$

$$When\ t = 0\ \ Then\ [D](0) + [C_1](0) + [C_2](0) = 0$$

$$\therefore Constant = [H_2O_2]_0$$

$$\therefore [D](t) = 2\cdot([H_2O_2]_0 - [H_2O_2](t) - [C_1](t)) - [C_2](t)$$

allowing us to reduce the original system of 7 differential equations to the following 3:

$$\frac{d[H_2O_2]}{dt} = -k_1\cdot([E]_0 - [C_1] - [C_2])\cdot[H_2O_2] + k_{-1}\cdot[C_1]$$

$$\frac{d[C_1]}{dt} = k_1\cdot([E]_0 - [C_1] - [C_2])\cdot[H_2O_2] - k_{-1}\cdot[C_1]$$

$$- k_2\cdot[C_1]\cdot([DH]_0 - 2\cdot[H_2O_2]_0 + 2\cdot[H_2O_2] + 2\cdot[C_1] + [C_2])$$

$$\frac{d[C_2]}{dt} = (k_2\cdot[C_1] - k_4\cdot[C_2])\cdot([DH]_0 - 2\cdot[H_2O_2]_0 + 2\cdot[H_2O_2] + 2\cdot[C_1] + [C_2])$$

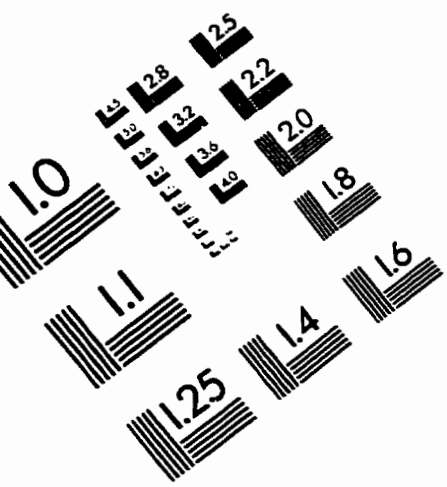
subject to:

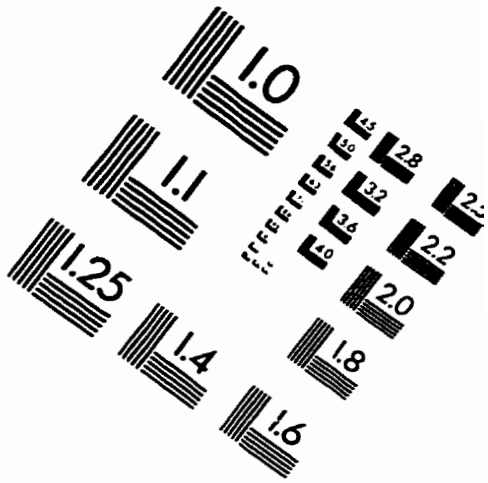
$$[H_2O_2](0) = [H_2O_2]_0$$

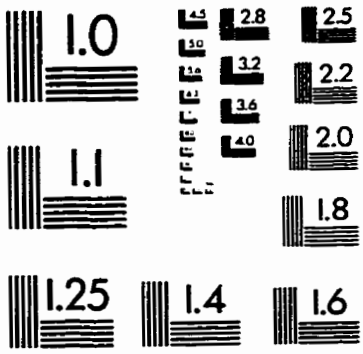
$$[C_1](0) = 0$$

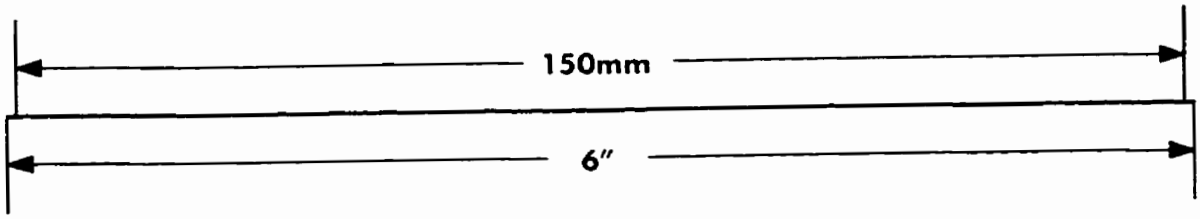
$$[C_2](0) = 0.$$

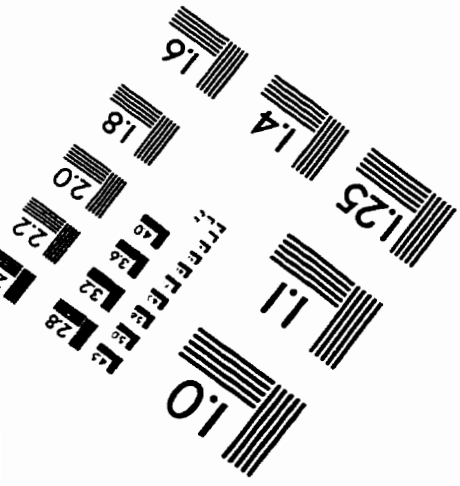
IMAGE EVALUATION
TEST TARGET (QA-3)

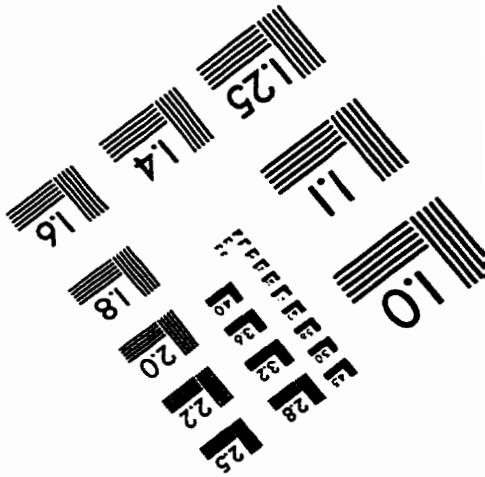












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