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HEPATITIS A VIRUS (HAV) IN FOODS: STUDIES ON RAPID DETECTION, SURVIVAL AND INACTIVATION OF THE VIRUS AND ITS TRANSFER

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Department of Biochemistry, Microbiology and Immunology, Faculty of Medicine

Sabah P. Bidawid

ABSTRACT

Various outbreaks of hepatitis A have been associated with the consumption of foods contaminated with hepatitis A virus (HAV). In this study, experiments were designed to develop rapid molecular methods to detect HAV in produce, to investigate its heat inactivation in dairy products and gamma irradiation resistance in produce, to assess its survival under modified atmosphere packaging (MAP) and to determine the amount of virus transferred from human hands to foods. The results demonstrated the following:

1. Immunomagnetic beads-PCR (IM-PCR), positively-charged 1MDS filters (F), or a combination of both (F-IM-PCR) methods were used to capture, concentrate and rapidly detect HAV in experimentally-contaminated lettuce and strawberries. Although the detection limit (10 PFU) of the F-IM-PCR was slightly less sensitive than that by the IM-PCR (0.5 PFU), the F-IM-PCR method has the potential to be of a greater sensitivity when detecting the virus in larger analytical units of foods (> 50 mL) than the IM-PCR which is restricted to ≤ 20 mL volumes.

2. Studies on the thermal resistance of HAV in three types of dairy products containing increased amounts of fat content (skim milk, homogenized milk; 3.5% MFG, and table cream; 18% MFG) indicated that increased fat content seems to protect HAV and render it more heat-resistant as compared to those of lesser fat content. A list of time and temperature combinations required to achieve a 1 to 5 $\log_{10}$ reduction in HAV titre is presented.

3. Exposure of HAV to gamma irradiation (^{60}Co) doses ranging between 1 and 10 kGy showed that D_{10} values of 2.73 and 2.97 kGy were needed to achieve a 1-$\log_{10}$ reduction in HAV inoculated onto lettuce and strawberries, respectively.

4. Various MAP environments did not influence HAV survival on lettuce stored at 4°C. At room temperature, however, an atmosphere consisting of 70% CO_2 : 30% N_2 slightly ($p=0.06$) enhanced virus survival as compared to other storage conditions.

5. Approximately 10% of HAV inoculated onto the fingerpads of human volunteers was transferred to lettuce by touching. Treatment of the fingerpads with water, topical agents, or alcohol significantly ($p<0.05$) reduced virus transfer from 10% down to $\leq 0.64\%$.

Dedicated to my Family

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LIST OF ABBREVIATIONS

BE: Beef extract

BSA: Bovine serum albumin

CAP: Controlled atmosphere packaging

CTAB: Cetyltrimethyl ammonium bromide

DMSO: Dimethyl-sulfoxide

EBSS: Earle's Balanced salt solution

EDTA: (Ethylenedinitrilo) tetraacetic acid disodium salt

FBS: Fetal bovine serum

FRhK-4 : Fetal rhesus kidney cell line

HACCP: Hazard analysis critical control point

HAV : Hepatitis A Virus

HM-175: A tissue culture adapted HAV first isolated from a patient in a hospital in Melbourne, Australia; the "H" signifies hepatitis, "M" is the electron microscopist and "175" is the laboratory accession number.

HEPES: (N-2-hydroxyethyl piperazine-N-2-ethanesulfonic acid)

MAb: Monoclonal antibody

MAP Modified atmosphere packaging

MEM: Minimum essential medium

mL: Millilitre

MPC-6: Magnetic particle concentrator

PBS: Phosphate-buffered saline

PCR:	Polymerase chain reaction
PFU:	Plaque forming unit.
PV:	Poliovirus
RT-PCT:	Reverse transcriptase-polymerase chain reaction
μL:	Microlitre

1. ABSTRACT

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Hepatitis A virus (HAV), the causative agent of hepatitis A, is the sole member of the genus *Hepatovirus* in the family Picornaviridae. It is a small, non-enveloped, spherical particle of cubic symmetry containing a single-stranded RNA genome, and is known for its thermostability, acid-resistance and long-term survival in various environments. HAV transmission occurs by either person-to-person contact through the fecal-oral route, or via the consumption of contaminated food and water.

Of all 52 notifiable infectious diseases in the US, hepatitis A ranked fifth among the top ten most frequently reported ones, and sixth among the top 10 foodborne pathogens in the US in 1995 (CDC, 1995,1997). Approximately 50% of clinically apparent acute viral hepatitis in the US is attributable to hepatitis A (Alter et al., 1997). Hepatitis A is also included in the listing of Severe Hazards in Appendix V of the US Food and Drug Administration, and is the only reportable foodborne viral disease in North America (Cliver, 1997). Hepatitis A virus continues to cause outbreaks in various settings resulting in considerable morbidity and economic loss. Almost 10% of hepatitis A infections are attributable to contaminated foods.

Most of the published research has focused on the detection, survival and inactivation of HAV in clinical, environmental and shellfish samples. Very little, if any, research has addressed these aspects in other types of foods such as fruits, vegetables and dairy products, despite the fact that many of these foods have been incriminated in various outbreaks. Furthermore, although foodhandlers have been shown to contribute significantly to food contamination, no information is available with respect to the

amount of virus transfer from contaminated fingerpads of foodhandlers to foods.

This study was designed to address the following (a) develop rapid methodology to rapidly concentrate and detect HAV in fruits and vegetables, b) investigate the inactivation of HAV in fruits and vegetables by gamma irradiation, c) investigate the heat inactivation of HAV in different types of dairy products, d) assess the survival of HAV on lettuce stored under various modified atmospheres, and e) conduct studies on the rate of transfer of HAV from contaminated fingerpads of adult volunteers to lettuce.

Immunomagnetic beads-PCR (IM-PCR), positively-charged 1MDS filters (F), or a combination of both (F-IM-PCR) methods were used to capture, concentrate and rapidly detect hepatitis A virus (HAV) in experimentally-contaminated samples of lettuce and strawberries. Direct reverse transcriptase-polymerase chain reaction (RT-PCR) amplification of the collected HAV-beads complex demonstrated a detection limit of as low as 0.5 plaque forming units (PFU) of the virus (in 1 mL wash solution) from the produce, which was several hundred-fold more sensitive than that demonstrated by conventional RT-PCR. In separate trials, virus-containing wash solutions from the produce were passed through 1MDS filters, and the captured virus was eluted with 10 mL volume of 1% beef extract (BE), pH 9.5. Of the 62% filter-captured HAV, an average of 34.8 ± 2.66 % was eluted by 10 mL of 1% BE. Direct PCR amplification of 2 μL from the 10 mL eluates did not produce a positive signal. Using the F-IM-PCR methodology, as little as 10 PFU present on each piece of the lettuce or strawberry was detectable. This was nearly 20 times less sensitive than the detection limit of 0.5 PFU exhibited by the IM-PCR, which could be due to virus loss during the multi-step capture,

recovery and concentration process. However, due to the large volumes (10 or 50 mL) used in the F-IM-PCR, the sensitivity of detection could be much greater than that of the IM-PCR, which is restricted to volumes of ≤20 mL. The F-IM-PCR method potentially provides for a greater sensitivity of detection than the IM-PCR, since low levels of virus could be detected from large volumes of sample (≥ 50 mL), as compared to the maximum of ≤ 20 mL that can be used in the IM-PCR method. Although positively-charged filters captured a greater amount of virus than both the IM-PCR and F-IM-PCR methods, PCR amplification signals from BE eluates were inconsistent, and thus could not be reliably used to detect HAV from produce.

The effects of gamma irradiation (^{60}Co) on HAV was investigated by exposing inoculated lettuce and strawberries to radiation doses ranging between 1 and 10 kGy. Following exposure, the level of HAV inactivation was assessed by determining the titre of the residual virus by the plaque assay. Doses of 2.72 and 2.97 kGy were needed to achieve a 1-log reduction (D_{10} value) in the population of HAV on lettuce and strawberries, respectively. At present, gamma irradiation doses of up to 1 kGy are allowed in the US for the purposes of pest disinfestation and sprout inhibition. Based on our data, this would result in approximately a 0.20 $\log_{10}$ reduction in HAV on produce. Thus gamma irradiation by itself is probably not sufficient to inactivate HAV on produce to levels that render these foods safe for consumption. Therefore, either higher doses are needed or other treatments such as the use of ozone or high intensity UV light could be used in combination with gamma irradiation to achieve greater levels of HAV inactivation on lettuce and strawberries.

Experiments were also performed to determine the thermal resistance of hepatitis A virus (HAV) in three types of dairy products containing increased amounts of fat content (skim milk, homogenized milk; 3.5% MFG, and table cream; 18% MFG). HAV-inoculated dairy products were introduced into custom-made U-shaped microcapillary tubes, which in turn were simultaneously immersed in a waterbath, using custom-made floating boats and a carrying platform. Following exposure to the desired time and temperature combinations, the contents of each of the tubes was retrieved and was tested by the plaque assay to determine the reduction in virus titre. Our data indicated that <0.5 min at 85°C was sufficient to cause a 5-log reduction in HAV titre in all three dairy products, whereas at 80°C, ≤0.68 min (for skim and homogenized milk) and 1.24 min (for cream) were needed to cause a similar log reduction. Using a non-linear 2-phase negative exponential model (two compartment model) to analyse the data, it was found that at temperatures of 65, 67, and 69°C, significantly ($p<0.05$) higher exposure times were needed to achieve a 1-log reduction in virus titre in cream (ranging between 1.11 to 7.34 min) than for skim and homogenized milk (0.79 to 2.68 min). At 71°C, a significantly ($p< 0.05$) higher exposure time of 0.52 min (for cream) as compared to ≤0.18 (for skim and homogenized milk) was required to cause a 1-log reduction in virus titre. A similar trend of inactivation was observed at 73 and 75°C where significantly ($p< 0.05$) higher exposure times of 0.29 to 0.36 min for cream were needed to cause a 1-log reduction in HAV titre as compared to ≤0.17 min for skim and homogenized milk. These results lead to the conclusion that an increase in the fat content played a protective role, and further contributed to the heat stability of the virus

in such products.

Experiments were designed to study the effect of different modified atmospheres on the survival of hepatitis A virus (HAV) on lettuce stored at room temperature (RT) and 4°C. HAV was inoculated onto pieces of lettuce and allowed to dry. The inoculated lettuce pieces were then incubated at RT and 4°C for 12 days at ambient air and under various modified atmospheres (CO_2:N_2; 30:70, 50:50, 70:30 and 100% CO_2) inside plastic bags of low O_2 permeability. Incubation for 12 days at 4°C showed that the lowest HAV survival rate (47.54%) was on lettuce stored in a petri-dish, whereas the greatest survival rates at 4°C (83.61 and 71.64%) were on lettuce stored under higher CO_2 levels of 70 and 100%, respectively. Statistical analysis of the 4°C data indicated that HAV titres decreased for all packages, but without a significant difference between the package types. At RT, however, a significantly ($p \leq 0.0001$) lower survival rate of HAV was observed (0.01%) for lettuce stored in a petri-dish. Incubation under various MA environments at RT showed that the greatest survival rate (42.8%) was seen on lettuce stored under 70% CO_2: 30% N_2, which was almost half the survival rate (83.61%) observed under the same condition at 4°C. Overall much lower survival rates of ≤8.6% were observed on lettuce stored under other MAP environments at RT. Statistical analysis for the lettuce data (RT) indicated a highly significant ($p \leq 0.0002$) decrease in HAV titre with increasing storage time and between package types, except for lettuce stored under 70% CO_2:30% N_2, where the decrease in virus titre was not significant ($p = 0.066$). These data indicate that MAP does not enhance HAV survival when present on the surface of produce incubated at 4°C. The slight improvement in

virus survival seen in the presence of high CO_2 levels at RT may be attributed to the inhibition of enzymatic activities in the lettuce that cause pre-mature spoilage of the product, and thus may have reduced exposure of the virus to potential toxic by-products.

Hepatitis A virus is an important pathogen which has been responsible for many foodborne outbreaks. HAV-excreting foodhandlers, especially those with poor hygienic practices, can contaminate the foods they handle. Consumption of such foods without further processing has been known to result in cases of infectious hepatitis. Since quantitative data on virus transfer during the contact of hands with foods is not available, we investigated the transfer of HAV from artificially-contaminated fingerpads of adult volunteers to pieces of fresh lettuce. Touching the lettuce with artificially-contaminated fingerpads for 10 seconds at a pressure of 0.2 to 0.4 kg/cm^2 resulted in the transfer of $9.2 \pm 0.86\%$ of the infectious virus. The pre-treatments tested to interrupt virus transfer from contaminated fingerpads included: 1) a hard water rinse and towel-drying, 2) application of a domestic or commercial topical agent followed by water rinse and towel-drying, and 3) exposure to a hand gel with 62% ethanol or 75% aqueous ethanol solution, but without any water rinse or towel drying. Treatment of the fingerpads with either of the above agents prior to touching the lettuce showed that the amount of infectious virus transferred to lettuce was significantly ($p < 0.05$) reduced from 9.2% to between 0.30 and 0.64% (depending on the topical agent used), which is up to a 30-fold reduction in virus transfer. Surprisingly, no virus transfer to lettuce was detected when the fingerpads were rinsed with water alone prior to touching the lettuce. However, further experimentation with the water rinse using smaller volumes of water (1 mL

instead of 15 mL) showed a 0.31 ± 0.07 % rate of virus transfer to lettuce. This variability in virus transfer rates following the water rinse may indicate that the volume itself, at least in part, influences virus removal from the fingerpads. This study provides novel information with respect to the rate of virus transfer to foods, as well as a model to investigate the transfer of viral and other foodborne pathogens from contaminated hands to foods, and approaches to reducing the amount of virus transferred from the contaminated fingerpads to foods.

The findings in these separate, yet interrelated, studies should help to i) better understand and assess the role of foodhandlers in contributing to virus spread to foods; ii) provide a rapid method to detect the virus in produce, iii) provide data on the inactivation kinetics of HAV in irradiated produce and heat-treated dairy products, as well as its survival under modified atmosphere packaging environments.

2. GENERAL INTRODUCTION

2. GENERAL INTRODUCTION

Infection with hepatitis A virus (HAV) leads to the disease hepatitis A which is a common form of acute viral hepatitis in many parts of the world, and is responsible for significant world-wide morbidity and occasional mortality (Prevot et al., 1993; Koff, 1998).

Transmission of the virus occurs either by person-to-person contact or through the ingestion of fecally-contaminated foods and water. Various epidemiological studies have conclusively shown that many hepatitis A outbreaks have been associated with the consumption of various types of contaminated foods, such as fecally-contaminated and improperly-cooked shellfish (Halliday et al., 1991; Desenclos et al., 1991), as well as salads, fruits, sandwiches and dairy products (Cliver, 1985; Anon., 1997). Although a number of these outbreaks have been attributed to the contamination of foods by infected foodhandlers, the actual transfer of the virus and the amount that can be transferred remain to be demonstrated.

Incrimination of foods as vehicles of transmission for HAV has been based on an epidemiological association rather than the actual detection of the virus in suspect foods. This is due to a number of factors such as i) the unavailability of the implicated food by the time symptoms of hepatitis A appear in patients; ii) the fact that HAV may be present in only low numbers in foods (e.g., 0.2 to 224 infectious units/100g shellfish) compared to $> 10^6$ infectious units/g of fecal material (Dienstag et al., 1975; Williams and Fout, 1992; Yotsuyanagi et al., 1993); and iii) the inability of field strains of HAV isolates to grow in cell culture (Bradley et al., 1984; Melnick, 1990). Traditional methods for the

isolation and concentration of viruses in general are cumbersome, time-consuming, and require multi-step manipulations which may result in virus loss or inactivation (Lopez-Sabater et al., 1997). The emergence of the RT-PCR, as well as the development of rapid methods such as magnetic beads and positively-charged filters to capture and concentrate viruses, have provided powerful tools to isolate and detect HAV. These methods, however, have been applied mostly to the detection of HAV in fecal, environmental and shellfish samples, but not in produce which have been associated with many foodborne outbreaks (Cliver, 1985; Dalton et al, 1996; Anon., 1997).

Numerous studies have shown that HAV survives well in the environment and is fairly resistant to inactivation by various chemical and physical agents (Sobsey et al., 1988; Mbithi et al., 1991, 1992; Nasser, 1994). Heating at high temperatures, e.g., 85°C for 1.5 min, (Millard et al., 1987), as well as the use of gamma irradiation were found to be effective in inactivating the virus in biological samples and shellfish (Sullivan et al., 1971, 1973; Thomas et al., 1981,1982; Elliott et al., 1982; White et al., 1990; Mallett et al., 1991). However, studies on the inactivation of HAV in other types of foods such as dairy products, fruits and vegetables, have been lacking despite the fact that some of the recent foodborne hepatitis A outbreaks have been associated with these foods (Dalton et al., 1996; Anon, 1997).

Due to an increase in the volume of foods traded internationally, as well as an increase in consumer demand for ready-to-eat fresh-cut fruits and vegetables, there is a higher potential for food contamination with various pathogens, including HAV. Some of these foods are transported in large containers sealed under various modified

atmospheres to maintain freshness and prolong shelf-life by inhibiting the growth of contaminating spoilage bacteria (Dodds, 1995; Zagory, 1995). Although many studies have been carried out with respect to the effect of modified-atmosphere packaging (MAP) on bacteria, no studies have been reported on the effect of MAP on the survival of HAV.

In this thesis, experiments were designed to a) investigate the use of magnetic beads and positively-charged filters, either separately or in conjunction with RT-PCR to capture, concentrate and rapidly detect HAV in experimentally-contaminated lettuce and strawberries, b) determine time and temperature combinations required to inactivate HAV in dairy products of differing fat content, c) determine D_{10} values (doses) of gamma irradiation needed to inactivate HAV in lettuce and strawberries, d) investigate the survival of HAV stored on lettuce under differing modified atmospheric environments, and e) study the rate of virus transfer from contaminated fingerpads of adult volunteers to lettuce.

3. REVIEW OF THE GENERAL LITERATURE

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Hepatitis A Virus

Classification. Following its first identification by Feinstone et al. (1973), HAV was designated as enterovirus 72 based on initial observations of similarities in size, acid-resistance, virion proteins and single-stranded RNA genome of positive polarity with other entero- and cardioviruses (Matthews, 1982; Melnick,1982; Gust et al.,1983a). However, several studies have demonstrated that HAV possesses several characteristics which distinguish it from other classical enteroviruses (Hollinger and Ticehurst, 1996). These differences include i) only one serotype (Lemon and Binn, 1983) with one immunodominant neutralization site (Stapleton and Lemon, 1987; Ping et al., 1988); ii) stability at pH 1.0 (Scholz et al., 1989); iii) lack of cross-reaction with monoclonal antibodies specific to enteroviruses (Yousef et al., 1987); iv) resistance to chemical and physical factors that can inactivate other picornaviruses (Lemon, 1985; Parry and Mortimer, 1984; Siegl and Eggers, 1982; Siegl et al., 1984); v) difficulty to adapt to growth in cell cultures and its relatively slow replication usually without cytopathic effects (Provost and Hilleman, 1979; Purcell et al., 1984; Ticehurst et al., 1989; Siegl et al., 1991; Siegl, 1992); and vi) dissimilar nucleotide, amino acid sequences and protein sizes (Najarian et al., 1985; Cohen et al., 1987; Hyypia et al., 1987; Palmenberg, 1987, 1989; Ticehurst et al., 1988). Therefore, based on the above characteristics, Melnick (1990) proposed that HAV be placed in the genus *Heparnavirus* in the family Picornaviridae. The International Committee for the Taxonomy of Viruses has now placed HAV as the sole member of the genus *Hepatovirus* in the family Picornaviridae

(Minor, 1991).

Structure. The virions of HAV are small (27-32 nm in diameter), non-enveloped icosahedral particles with 32 capsomers, which are indistinguishable from those of other picornaviruses (Cook et al., 1976; Feinstone et al., 1973; Siegl, 1982). The proteinaceous capsid consists of three major polypeptides (VP1: 30-33k, VP2: 24-26k, VP3: 21-23k) which are similar in size to those of other picornaviruses (Cooper et al., 1978). A fourth, minor polypeptide (VP4: 7-14) has also been identified, and was found to be similar to that present in other picornaviruses. Mapping with monoclonal antibodies suggests the presence of multiple epitopes overlapping as an immunodominant neutralization site, although a second independent site may also exist (Stapleton and Lemon, 1987; Ping and Lemon, 1992). The capsid surrounds a 7.5 kb positive-sense single-stranded RNA genome whose organization resembles that of other picornaviruses. The highly conserved genome (Ticehurst et al., 1988; Lemon et al., 1992) consists of three distinct parts; an untranslated 5'-end which is characterized by the presence of a small protein (Vpg), a large open reading frame which is translated into a single polyprotein, and a short untranslated 3'-end which contains a poly-A tail (Siegl et al., 1981). The open reading frame encodes all of the viral proteins, with regions designated as P1 for capsid proteins and P2 and P3 for non-structural proteins (Rueckert and Wimmer, 1984).

Biophysical characteristics. Various populations of HAV with different buoyant densities in CsCl ranging between 1.20 and 1.48 g/c^3, as well as sedimentation coefficients (SC) in sucrose gradients ranging between 50S-230S have been identified. Generally, density gradient measurements indicate the presence of three types of HAV

particles with densities of 1.20-1.31, 1.32-1.34 and 1.40-1.48 g/c^3 (Siegl et al., 1981; Bradley et al., 1975,1984; Siegl, 1984: Lemon et al., 1985; Coulepis et al., 1982,1987). The least dense procapsid particles (Bishop and Anderson, 1993), which are abundant in feces during the early stages of infection, represent empty, premature or defective particles lacking RNA (Bradley et al., 1977; Tratschin et al., 1981). These procapsid particles have also been identified in cell culture (Ruchti et al., 1991). The more typical mature and complete intact HAV virions are those with densities of 1.32-1.34 g/c^3 (Bradley et al., 1978; Siegl, 1984). The heaviest particles (1.40-1.48 g/c^3) detected from feces have a more open conformation of the viral capsid which allows for the binding or penetration of cesium chloride, resulting in a heavier form of the particles (Lemon, 1985; Coulepis et al., 1987; Hollinger and Ticehurst, 1996). Similar groups of multiple buoyant density picornaviruses have also been described (Yamaguchi-Koll et al., 1975; Wiegers et al., 1977; Cooper et al., 1978).

Analysis of HAV by rate-zonal ultracentrifugation in sucrose gradients indicates sedimentation coefficients ranging from 50S to 230S (Bradley et al., 1978; Siegl and Frosner, 1978), which is consistent with the three alternative particle types (Coulepis et al., 1987). Mature and intact virions were found to have a sedimentation coefficient of 156 to 160S, whereas empty particles sedimented at 50 to 90S. Multiple sedimentation rates have also been reported in other enteroviruses (Wiegers et al., 1977). Super dense poliovirus particles were found to sediment at 160 and 220S in neutral gradients, although these particles exhibited multiple sedimentation rates ranging between 35 and 220S in non-neutral gradients (Coulepis et al., 1987).

HAV strains. Although many strains of HAV have been isolated from different regions of the world, they all belong to one serotype (Rakela et al., 1976; Lemon and Binn, 1983; Lemon et al., 1990; Jansen et al., 1990; Robertson et al., 1991). A number of these strains have been well characterized, including CR326 (Costa Rica, 1960), MS-1 (New York, 1964), HM-175 (Australia, 1976), GBM (Germany, 1976) and PA21 (Panama, 1980). Differentiation among HAV strains has been based on nucleotide sequence analysis (Tsarev et al., 1991; Robertson et al., 1992; Graff et al., 1994), hybridization patterns (Lemon et al., 1987), T1-oligonucleotide mapping (Weitz and Siegl, 1985), as well as different growth characteristics (Siegl et al., 1984). Seven genotypes have been designated based on nucleotide sequence analysis of a variable 168-base region in the open reading frame at the junction of 1D and 2A genes of 152 HAV strains collected from different global geographical regions (Robertson et al., 1992). These genotypes demonstrated differences of 15 to 25% in the nucleotide sequence. Genotype I includes most of the human HAV strains such as HM-175, MS-1, MBB, CR 326 (82 of 104 human strains studied), while genotype II (which shows an intermediate sequence diversity from genotype I) contains a single isolate, CF-53 (Jansen et al., 1990). Strains that demonstrated a 20% difference in nucleotide sequence from that of genotype I were grouped into genotype III which includes the PA 21 simian isolate from Panama (Lemon et al., 1982), as well as most of the remaining human strains (Jansen et al., 1990; Robertson et al., 1991). Genotype VII, a single strain (SLF 88) isolated from Sierra Leone in 1988, differs from genotype II by about 17% in sequence. Both genotypes II and VII show a disparity in 20-25% in nucleotide sequence from other

HAV strains (Robertson et al., 1992). Genotypes IV, V, and VI are all isolates from the Old World monkey species. These strains tend to differ genetically as much between themselves as they do from the human isolates (Robertson et al., 1992). The epidemiological or pathological implications of different HAV genotypes is unknown, although clusters within genotypes were found to predominate in certain geographical regions of the world. Genotyping, however, might be useful in determining if a particular outbreak was caused by a domestically circulating strain or due to the importation of a strain predominating in a particular region. Consequently, control measures could be initiated based on such findings.

Cell culture. Wild-type HAV strains do not or are very difficult to adapt and grow in vitro in cell cultures. Even after several weeks of blind passages that may result in successful growth, HAV usually results in a persistent and protracted infection which does not produce cytopathic effects despite accumulation of viral antigen (Bradley et al., 1984; Melnick, 1990). The first successful propagation of HAV strain CR-326 in a fetal rhesus monkey kidney cell line (FRhK-6) was reported by Provost and Hilleman (1979). Since then, several other strains have been successfully grown in a number of cell cultures. This was evident by the successful growth of HAV in FRhK-4 cell line (Flehmig, 1980), human diploid embryo fibroblasts (Gauss-Muller et al., 1981), Vero cells (Locarnini et al., 1981), primary African green monkey kidney cells (AGMK; Daemer et al., 1981), human hepatoma tissue (PLC/PRF/5; Frosner et al., 1979a) and continuous human diploid lung cells (MRC5). Growth of clinical isolates in FRhK-4 cells was found to be enhanced by 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole

(Widell et al., 1988). Since HAV does not usually produce discernible cytopathic effects, virus multiplication and the presence of virus antigens is usually detected by using methods such as immunoelectron microscopy, direct immunofluorescence, radioimmunoassay, and enzyme-linked immunosorbent assays (Feinstone et al., 1973; Purcell et al., 1976; Mathiesen et al.,1977; Duermeyer et al., 1979; Asher et al., 1987), or by hybridization to detect HAV RNA (Jansen et al., 1985, 1990; Ticehurst et al., 1987). Both traditional plaque assays and the radioimmunofocus assay-RIFA- (Lemon et al., 1983) have been used to quantify infectivity titres of HAV.

HAV replicates poorly in cells and results in a persistent infection where most of the HAV progeny (> 80%) remains cell-associated and the mechanism of virus spread to adjacent cells remains unknown (Wheeler et al., 1986). Even well adapted strains of HAV require several days or weeks to reach a maximal concentration of propagated virus, with typical yields ranging between 10^6 to 10^9 $TCID_{50}$ (tissue culture infectious dose)/mL following mechanical lysis of the cells. The degree to which HAV is released from cells is variable and depends on a combination of HAV strain, cell type and temperature (Binn et al., 1984; Siegl et al., 1984). In some cell cultures, most of the HAV remains cell-associated (Frosner et al., 1979a), whereas in others a substantial release of virus into the culture medium has been observed (Flehmig, 1980), without noticeable cytopathic effects. Lemon and Binn (1985) indicated that the type of cell membrane may play a role in virus assembly and release from infected cells, based on the observation that HAV particles are lipid-associated..

The mechanism by which HAV enters susceptible cells remains unknown. In a

recent study by Kaplan et al. (1996), three protective MAbs were found that were directed against an epitope expressed on the cell surface of the susceptible African green monkey kidney (AGMK) cell culture. Using one of the MAbs and an AGMK cell cDNA library cloned into a shuttle vector transfected into Hela cells, Kaplan et al. (1996) identified an attachment receptor for HAV, which they termed HAVcr-1. Nucleotide sequencing of HAVcr-1 cDNA revealed a 2093 bp sequence with a poly (A) tail at its 3' end, which encoded a putative polypeptide of 451 amino acids with the typical features of a class 1 integral membrane glycoprotein. This novel molecule was closely related to the mucin-like cell surface receptor family, and contained a cysteine-rich region with homology to the variable domains of membranes of the immunoglobulin super family. HAVcr-1 is an attachment or binding receptor for HAV, but it is still unknown if it is the functional receptor responsible for the internalization of the virus into the host cell.

Several trials with different substances have demonstrated that pre-treatment of cell culture with trypsin or the addition of serum inhibits HAV attachment to cells (Zajac et al., 1991), whereas calcium appears to promote cell attachment (Stapleton et al., 1991; Zajac et al., 1991). Although virus penetration of susceptible cells could occur rapidly following attachment, inefficient uncoating of the virus seems to account, at least partly, for its slow replication inside the host cell. Various investigators have attributed poor viral replication to a number of factors summarized by Hollinger and Ticehurst (1996) as follows: a) inefficient uncoating (Bishop and Anderson, 1993); b) asynchronous replication in certain cells (Cho and Ehrenfeld, 1991); c) inability to shut off host cell macromolecular synthesis (Glass et al., 1993); d) inefficient post-

translational processing (Borovec and Anderson, 1993); e) a limited pool of available viral RNA for translation or replication, f) host-mediated HAV down-regulation (Nuesch et al., 1993); and g) defective-interfering particles which interfere with HAV replication (Garelick et al., 1988).

The development of a cytopathic effect (CPE) by adapted strains depends on the interaction between particular HAV strains and cells at certain passage levels. In general, a CPE develops anywhere from 2 to 14 days after inoculation (Anderson et al., 1987; Cromeans et al., 1988, 1989; Borovec and Anderson, 1993). Studies on the replication of the cytopathic mutant HM-175A demonstrated that CPE developed 2 to 3 days after inoculation yielding 20 infectious units of HAV per cell, equivalent to approximately $10^{8.7}$ PFU/mL (Anderson et al., 1988 ; Borovec and Anderson, 1993).

Stability and Survival of HAV

Physicochemical stability of HAV. Numerous studies on the effects of chemical and physical agents on HAV have demonstrated that this virus, unlike other picornaviruses, is fairly resistant. Although entero- and cardioviruses have been shown to be stable at pH 3.0 (Newman et al., 1973; Siegl et al., 1984; Scholz et al., 1989), their infectivity is rapidly lost at lower pH levels (Farber and Dong, 1946). HAV, on the other hand, remains infectious at pH 1.0 for 5 h at room temperature (Coulepis et al., 1987; Scholz et al., 1989). Highly purified HAV was even more stable retaining infectivity for more than 8 h at pH 1.0, suggesting the presence of specific genes conferring acid-stability rather than the protection of the virus by cellular debris (Lemon and Binn, 1985; Scholz et al., 1989).

HAV was also found to be resistant to several chemical agents that effectively inhibited the growth of certain other picornaviruses. Some of these agents included arildone and disoxaril, which are a group of antiviral agents which inhibit entero- and rhinoviruses by preventing capsid uncoating (Rossmann and Rueckert, 1987). Similarly, HAV was resistant to the effect of 2 mM guanidine, whereas poliovirus type 1 (Mahoney) was inhibited even at a concentration of 1 mM (Siegl and Eggers, 1982). HAV is also resistant to Freon (dichlorodifluoromethane), Genetron or Arklone (trichlorotrifluoroethane), which allows for the incorporation of these agents in certain procedures to concentrate the virus, as well as to 20% ether and chloroform (Havens, 1945; Provost et al., 1975; Locarnini et al., 1978; Siegl, 1984). HAV retained infectivity following its exposure to 1.5 mg/L free chlorine (Peterson et al., 1983), 30 mg/L potassium permanganate and chloramine for 15 min, 0.1% w/v formaldehyde for 60 min, 2% gluteraldehyde for 1 min, 1.1 W (UV) for 1 min, 12 kGy gamma irradiation, 70% ethanol for 12 h (Siegl et al., 1984), ozone (Herbold et al., 1989; Vaughn et al., 1990), as well as to many other disinfectants and chemical agents (Mbithi et al., 1990, 1993; Thraenhart, 1991). Despite its resistance to a wide range of physical and chemical agents, HAV has been shown to be sensitive to certain agents such as the anitiviral amantadine and ribavirin compounds which inhibit uncoating (Superti et al., 1987, 1989), as well as β-propiolactone and high concentrations of potassium permanganate, iodine, chlorine, and formaldehyde (Hollinger and Ticehurst, 1996).

Several studies have also demonstrated the resistance of HAV to heat (Table 1). Siegl et al. (1984) found that, unlike other picornaviruses that cannot survive exposure to

Table 1. Heat-resistance and inactivation of hepatitis A virus in various substances.

Suspending substances	Temp. (°C)	Exposure time	Inactivation partial	Inactivation complete[a]	Reference
Cockles	85-90	1-1.5 min		+[b]	Millard et al. (1987)
Milk	62.8	30 min	+		Parry and Mortimer (1984)
	71.6	15 sec		+	"
Feces	56	30 min	+		Havens (1945); Ward et al. (1958)
Serum	56	30 min		+	Ward et al. (1958)
	98	1 min		+	Krugman et al.(1970)
Tissue-	85	1 min	+		Scheid et al. (1982)
culture	60	2h	+		Flehmig et al. (1985)
	70	30 min		+	"
Phosphate-	56	6-12 h	+		Thraenhart (1991)
buffered-		24h	+		"
saline		60 6-12 h	+		"
	70	4 min		+	"
	75	30 sec		+	"
	80	5 sec		+	"
	85	<5 sec		+	"

[a] "Complete" refers to no viral infectivity detected.
[b] Indicates the outcome of the heat treatment.

56°C for 10 min at pH 7, the temperature at which 50% of HAV particles disintegrate and release their RNA was 61°C as compared to 43°C for poliovirus. As is the case for other enteroviruses, the addition of cations such as 1M $MgCl_2$ (which is known to stabilize enteroviruses) to HAV suspensions increased its heat resistance. This was demonstrated when, in the presence of 1M $MgCl_2$, the 50% HAV inactivation temperature was shifted from 61 to 81°C, with the generation of RNA-free capsids that retained antigenicity (Siegl et al., 1984).

Various means have been used to inactivate HAV in foods to render them safe for human consumption, with heat being one of the most effective. Heat, however, can only be applied to certain types of foods that can withstand high temperatures, e.g., certain dairy products. For example, pasteurization has been applied widely and successfully for inactivating pathogenic bacteria in milk. One study, however, has found routine milk pasteurization to be ineffective against HAV (Parry and Mortimer, 1984).

Heat also has its limitations when applied to shellfish, as at the temperature (85°C for 1.5 min) needed to inactivate HAV (Millard et al., 1987), both the texture and the palatability of the product are affected. This is also true when attempting to inactivate the virus in other products such as fresh fruits (e.g., strawberries) and vegetables (e.g., lettuce) which cannot withstand temperatures needed for virus inactivation. Therefore, further studies are required to determine appropriate time and temperature combinations that would be applicable to inactivate HAV in dairy products.

Ionizing gamma irradiation has been successfully used to destroy bacterial and fungal contaminants in a variety of foods (Dubey and Thayer, 1994; Farkas, 1998).

Gamma irradiation studies have demonstrated that, due to their small size and genetic make-up, much higher doses are required to inactivate viruses than those required to inactive bacteria or fungi (Monk et al., 1995; Farkas, 1989; Patterson, 1993). Nevertheless, this technology could be very useful, either as a stand-alone approach or in conjunction with other methods, to inactivate HAV in produce. Currently, in different parts of the world, many foods , including strawberries, are being irradiated at various doses. Most studies have addressed the effects of gamma irradiation on HAV in biological samples (Sullivan et al., 1971, 1973; Thomas et al., 1981,1982; Elliott et al., 1982; White et al., 1990), but there is little information on the effects of gamma irradiation on HAV in foods other than shellfish (Mallette et al, 1991) (Table 2).

The survival of HAV in clinical and environmental samples has been well investigated (Table 3). The virus remained stable and retained infectivity for marmosets even when present in dried feces which had been stored for one month at 25°C and relative humidity of 42% (McCaustland et al., 1982). HAV has also been shown to remain infectious for several days in fresh water, seawater, wastewater, soil, marine sediment, live oysters, cookies (Sobsey et al., 1988), as well as on hands, environmental and inanimate surfaces (Mbithi et al., 1991, 1992).

Modes of Transmission

Transmission. Virus transmission is mainly by the fecal-oral route. This occurs either by person-to-person contact or by ingesting contaminated foods and water (Cliver, 1995; Dalton et al., 1996; Anon, 1997). Parenteral administration of blood or blood as other means of virus transmission (Giles et al., 1964; Aach et al., 1968).

Table 2. Inactivation of some viruses by ^{60}Co gamma irradiation.

Virus	Medium	D_{10} values (kGy)[a]	Reference
Hepatitis A virus	Oysters & clams	2.0[b]	Mallett et al. (1991)
Rotavirus SA11	Oysters & clams	2.4	"
Poliovirus	Oysters & clams	3.1	"
"	Eagles' medium[c]	4.9	Sullivan et al. (1971)
Coxsackievirus B-2	Ground beef	6.8	Sullivan et al. (1973)
"	Eagles' medium	4.4	Sullivan et al. (1971)
Newcastle disease virus	Egg fluid	5.5	Thomas et al. (1981)
"	Eagles' medium	5.2	Sullivan et al. (1971)
Echovirus	Eagles' medium	5.2	"
Adenovirus	Eagles' medium	4.9	"
Reovirus I	Eagles' medium	4.2	"
Simian virus 40	Eagles' medium	4.5	"
F-coliphage	Clams	13.5	Harewood et al. (1994)

[a] D_{10} is the dose, in kGy, required to reduce the virus population by 1 $\log_{10}$ (=90% inactivation).

[b] The listed values reflect the maximum irradiation dose, irrespective of the temperature of the medium.

[c] Eagles' minimum essential medium plus 2% serum.

Table 3. Survival of hepatitis A virus in various menstruum.

Sample	Temp. (°C)	Exposure time	Stability[a] /survival	Reference
Phosphate-	-20	months	+[b]	Siegl et al. (1984)
buffered-	-70	months	+	"
saline	4	16wk	+	"
	RT[c]	1-4wk	+	"
	25	56-84d	+	Sobsey et al. (1988)
	32	1-2 wk	+	Siegl et al. (1984)
	37	1-2 wk	+	"
Feces	25	30d	+	McCaustland et al. (1982); Sobsey et al. (1988)
	4	> 16wk	+	Siegl et al. (1984)
Inanimate surfaces	RT	hours	+	"
Hands	RT	hours	+	Mbithi et al. (1992)
Cream cookies	21	14-30d	+	Sobsey et al. (1988)
	49	7-14d	+	"
Environmental[d]	25	56-84d	+	"
Oysters	12-24	≥5d	+	"
Lettuce	22.5	≥12d	+	Bidawid et al. (1999)
	4	≥12d	+	(submitted for publication)
Milk (3.5% fat)	4	≥27d	+	Bidawid et al. (1999) (unpublished data)

[a] Loss in virus titre (≥50%) might have occurred at the mentioned conditions.

[b] Indicates virus survival or stability at corresponding conditions.

[c] Room temperature.

[d] Water and soil.

products (Azimi et al., 1986; Seeberg et al., 1981) and aerosols have also been suggested

Person-to-Person contact: Poor hygienic practices, as well as inadequate sanitary conditions play an important role in the spread of HAV from infected individuals to healthy ones. Many hepatitis A outbreaks have been documented in different settings and groups such as military camps, religious and social communities, day-care centres, nurseries, as well as among homosexuals, illicit-drug abusers and through blood product transfusions (Gerritzen, 1992; Mannucci, 1992; Peerlinck and Vermylen, 1993; Johnson et al., 1995; MMWR, 1996). The increased frequency of hepatitis A among homosexual men is evident by the higher prevalence of anti-HAV antibodies in these individuals when compared to the general population (Corey and Holmes, 1980; Christensen et al., 1982; Coutinho et al., 1983; Keeffe, 1986). Furthermore, there seems to be an increase in the incidence of hepatitis A among these groups as was documented by a CDC report which indicated that the rate of hepatitis A in Toronto's homosexual community had more than quadrupeled in one year, climbing from 13.3 cases per 100,000 in 1990 to 56.3 cases per 100,000 in 1991 (CDC, 1992). In this particular setting, the male:female ratio associated with this incidence was estimated at 1.3:1 in 1990 compared to 12:1 in 1991.

A number of factors have been described as contributing to the increase in the prevalence of hepatitis A among the homosexual community, such as multiple sexual partners, duration of homosexuality, oral-anal sexual practices, as well as the presence of other venereal diseases (CDC, 1992).

Illicit drug use has contributed to an increase in the number of hepatitis A cases,

whereby combined with poor hygienic practices, drug abusers become infected with the virus as a result of using contaminated needles or through the ingestion of fecally-contaminated illicit drugs (Widell et al., 1982; Akriviadis and Redeker, 1989).

Several reports have also documented transfusion-associated HAV among hemophiliacs, thalassemics and hemodialysis patients. Furthermore, transfusion-associated hepatitis A in intensive-care units may infect health care personnel and result in outbreaks (Hollinger et al., 1983; Noble et al., 1984; Azimi et al., 1986). Infection with the virus occurs as a result of transfusion of contaminated blood which has been collected during the short viremic stage, before the onset of illness (Hollinger et al., 1975).

Transmission through contaminated foods: Although foodborne hepatitis A outbreaks account for < 10% of hepatitis A cases, the implications of such outbreaks are enormous (Dalton et al., 1996). Foods become contaminated either at the site of harvest e.g., shellfish from contaminated waterbeds, or during food processing at various stages of food processing e.g., foodhandlers (Cliver, 1985). Many foodborne hepatitis A outbreaks have been documented (Table 4) including the large outbreak in Shanghai, China, in 1988 in which more than 300,000 individuals were infected due to the consumption of contaminated clams, with secondary cases occurring through person-to-person contact (Halliday et al., 1991). More recent outbreaks were reported in Denver, Colorado, where more than 5000 individuals were exposed to the virus as a result of consuming gourmet foods prepared by an infected foodhandler, as well as an outbreak in Michigan, in which more than 200 school children were infected due to the consumption

Table 4. Foodborne outbreaks of hepatitis A.

Year	Location	No. of cases	Implicated food	Original source of contamination	Reference
1980	Oklahoma, US	8	Cheese	Foodhandler	Cliver (1985)
1983	Aberdeen, UK	24	Raspberries	Food pickers[a] ?	Reid & Robinson (1987)
1983	Texas, US	121	Lettuce/tomato	Foodhandler	Cliver (1985)
1986	Florida, US	103	Green salad	Foodhandlers	Lowry et al. (1989)
1988	Shanghai, China	292,301	Cockles	Water[a]	Halliday et al. (1991)
1988	Florida, US	61	Oysters	Water[a]	Desenclos et al., 1991
1988	Kentucky, US	202	Lettuce	Irrigation water[a]?	Rosenblum et al. (1990)
1989	Village, UK	>50	Bread	Foodhandler	Warburton et al. (1991)
1990	Missouri, US	110	Salad, lettuce	Foodhandler	MMWR (1993)
1990	California, US	6,408	Cucumber/onion	Foodhandler	Anon. (1992)

Table 4 con'd

1990	Georgia, US	28	Strawberries	Food picker[a]	Niu et al. (1992)
1991	Wisconsin, US	230	Sandwiches	Foodhandler	MMWR (1993)
1992	Denver, US	43[b]	Gourmet food	Foodhandler	Dalton et al. (1996)
1994	New York, US	79	Baked goods	Foodhandler	Weltman et al. (1996)
1996	Quebec, Canada	7	Breakfast/food	Foodhandler	Anon. (1998)
1997	Michigan, US	213	Strawberries	Unknown	Anon. (1997) Hutin et al. (1999)

[a] Contamination at point of harvest.

[b] 5000 to 15,000 individuals might have been exposed to the virus.

of HAV-contaminated imported frozen strawberries used in a school lunch program (Anon, 1997; Hutin et al., 1999).

Chemical treatment of sewage has been shown to be ineffective against HAV (Bowen and McCarthy., 1983; Bloch et al., 1990; Gerba & Rose, 1990). As a consequence, sewage contamination of drinking water can also result in outbreaks of hepatitis A (Lippy and Waltrip, 1984).

The large numbers of individuals affected by foodborne hepatitis A outbreaks illustrate the considerable impact of these outbreaks in terms of cost for surveillance, diagnosis, vaccination, hospitalization, recovery and absenteeism, as well as reprocessing or destroying large quantities of suspect foods. Since food establishments can be frequented by individuals or groups from many different geographical locations, the potential for spread of the virus to far beyond the local area is greater than may occur in settings such as hospital nurseries, day-care centre or communal settings.

Infected foodhandlers have been implicated as the index case in a number of foodborne hepatitis A outbreaks (Levy et al., 1975; Denes et al., 1977; Cliver, 1985; Dalton et al., 1996; Anon, 1996). Since HAV continues to be shed in the feces during the asymptomatic incubation period, foodhandlers with poor hygienic practices may contaminate foods that are either consumed raw, partially cooked, or fully cooked prior to handling and serving (Rakela et al., 1976; Hooper et al., 1977; Rosenblum et al., 1991). This is particularly true in the case of fresh and ready-to-eat foods such as fruits and vegetables which are consumed raw or require only minimal cooking. Once contaminated, these foods become vehicles for virus transmission to patrons (Cliver,

1985). It has been well recognized that the availability of proper sanitary conditions, as well as the practice of adequate hygienic standards such as thorough hand washing with water and soap , are the most important measures in controlling the spread of HAV from infected individuals to foods. Although many such cases of foodhandler-associated outbreaks have been reported, no studies have been conducted to investigate the actual amount of HAV that can be transferred from contaminated hands to foods.

Infection and Disease

Infection, pathogenesis and clinical diagnosis of HAV. Infection with HAV leading to acute viral hepatitis follows the ingestion of fecally-contaminated material containing the virus. Four clinical phases of the disease have been described by Koff (1993): a) an incubation period ranging between 10-50 days with an average of 28 days, b) preicteric phase in which the infection is either subclinical (without apparent symptoms) or anicteric (appearance of symptoms but without jaundice), c) icteric phase which is usually characterized by jaundice, dark urine, and pale yellowish stool, and d) a convalescent period which could take a few weeks or longer.

During the incubation period, the patient remains asymptomatic and virus transmissibility is at its greatest. Not all infected individuals develop symptoms, i.e., more than 50% of adults infected with HAV develop symptoms, while approximately 85% of children under 5 years of age remain asymptomatic (Gingrich et al., 1983; Chin et al., 1991; CDC, 1992). Symptoms of HAV infection include fever, anorexia, fatigue, malaise, myalgia, nausea, vomiting, diarrhea, as well as right upper abdominal pain. Hepatocellular damage of the liver results in increased levels of the alanine amino-

transferase (ALT) enzyme. Symptoms may last 1-2 weeks followed by a jaundiced stage which is usually accompanied by dark urine and pale feces. During the jaundice phase, the virus continues to be shed in the feces of the patient for up to 2 weeks. Occasionally, more extensive necrosis of the liver occurs resulting in severe damage to the hepatic functions and leading to a fatal stage of fulminant hepatitis, which is a rare event. The case-fatality rate in the U.S. due to hepatitis A is approximately 0.4% (0.1% for children ≤14 years old, 0.3% for patients 15-39 years of age); the highest case-fatality rate (1.75 to 2.1%) is among persons >40 years of age (Hollinger and Ticehurst, 1996; CDC, 1999). Among individuals with fulminant hepatitis, the mortality can be >50% despite modern medical interventions such as liver transplantation (CDC, 1999). Approximately 50% of the clinically apparent cases of acute viral hepatitis in the USA has been attributed to hepatitis A (Koff, 1998).

In most cases, however, acute hepatitis A is self-limiting and resolves itself within a few weeks, and does not develop into a chronic carrier state (Lemon, 1985; Sjogren et al., 1987). The age of the individual seems to be the major determining factor as to whether infection becomes inapparent or subclinical, anicteric or icteric (Forbes and Williams, 1990; Tilzey and Banatvala, 1991).

Since HAV infects cells but does not cause cell disintegration when exiting the cell, suggests that the pathology of the liver may not be due to the virus itself, but rather to immune mechanisms that promote cell-mediated lysis by natural killer cells and cytotoxic T cells (Siegl and Weitz., 1993). Auto-antibodies to liver membrane antigens can be induced during HAV infection which might trigger auto-immune chronic active

hepatitis in susceptible subjects (Weidmann et al., 1984; Jansen et al., 1984; Vento et al., 1991). Severe glomerulonephritis, evanescent skin rash, transient arthralgia, arthritis and cutaneous-vasculitis have also been shown to accompany HAV infection (Morita et al., 1981; Dan and Yaniv, 1990; Karayiannis et al., 1990).

Although hepatitis A is usually not as severe as hepatitis B or C, relatively recent findings suggest otherwise. Although rare, cholestatic hepatitis (deep jaundice, acholic stools, pruritis) with massive acute hemolysis that may persist for 2 to 8 months has been observed (Gordon et al., 1984; Lyons et al., 1990; Schiff, 1992). Furthermore, several reports have documented a relapsing form of HAV infection (Karayiannis et al., 1990; Glikson et al., 1992). McDonald et al. (1989) observed a prolonged (1034 days) rise in anti HAV IgM antibodies with liver enzymes remaining elevated for 4 years. Relapsing hepatitis A infection usually occurs in 3 to 20% of patients after a short period of approximately 4 to 15 weeks (Sjogren et al., 1987; Glikson et al., 1992). Other complications associated with hepatitis A infection include Guillain-Barré syndrome and other neurological complications (Tabor, 1987).

During the incubation period and prior to the onset of clinical symptoms, HAV can be detected in the blood and stool. In the brief viremic phase, the virus is present in relatively low titres in the blood (10^4 particles/mL) as compared to approximately 10^9 particles/g in the feces (Dienstag et al., 1975; Yotsuyanagi et al., 1993). HAV is excreted in the feces of infected individuals 3 to 10 days prior to the onset of clinical symptoms and for two weeks after the appearance of jaundice (Flehmig et al., 1977; Coulepis et al., 1980); communicability is very high during this period (Denes et al.,

1977). The virus can usually be detected in these clinical samples by a variety of methods such as immune electron microscopy (IEM), enzyme immunoassay (EIA) or radioimmunoassay (RIA), hybridization assays, or RT-PCR (Feinstone et al., 1973; Sjogren et al., 1987; Jansen et al., 1990, 1991; Robertson et al., 1991, 1992; Niu et al., 1992; Seeling et al., 1992). Detection of anti-HAV IgM and/or IgG indicates either an acute recent infection or a previous infection, respectively. Recovery from hepatitis A usually results in immunity for many years (Skinhoj et al., 1977).

Prevention and Control

Prophylaxis and vaccination. Passive immunization with human immune globulin containing IgG anti-HAV antibodies has been used as a pre-exposure, as well as a post-exposure, prophylactic measure against clinical disease. Guidelines for the immune globulin prophylaxis against hepatitis A published by the U.S. Public Health Service Advisory Committee (MMWR, 1981; MMWR, 1996) suggest the use of immunoglobulin prior to or within two weeks after the onset of illness, following which its effectiveness is greatly reduced. Post-exposure prophylaxis has been recommended for a) household and sexual contacts of hepatitis A cases; b) outbreaks in day-care centres, residential facilities, or the military; c) potential common-source outbreaks, e.g., a foodhandler with acute hepatitis A if exposure is recognized before appearance of cases; e) health-care personnel who might be at risk; f) travellers to high-risk areas; g) handlers of recently imported chimpanzees; and h) the staff of some residential facilities where the disease is endemic.

Good hygienic practices and the prevalence of acceptable sanitary facilities have

always been recognized for their importance in controlling the spread of HAV (Gust and Feinstone, 1990; Melnick, 1990). However, as sanitation improves, the likelihood of infection with HAV is delayed to adulthood, at which time infection is more pronounced and the likelihood of symptoms appearing is considerably higher. Therefore, vaccination has been pursued as an alternative to immunoglobulin therapy. Currently, two inactivated HAV vaccines have been approved in the U.S. Both the Havrix® (1992-SmithKline Beecham) and Vaqta™ (1996-Merck) are whole-virus preparations, prepared by growing attenuated HAV strains (HM-175 for Havrix®; CR 326F for Vaqta™) in MRC-5 cells, followed by inactivation with formaldehyde. The vaccine is given as a primary and a booster dose at 6-12 month intervals, and has been found to be highly immunogenic resulting in efficacies of protection against clinical hepatitis A of 94 and 100% for Havrix® and Vaqta, respectively (Sjogren et al., 1987; Werzberger et al., 1992; Innis et al., 1994). Both vaccines may be stored for two years at 4°C without any adverse effects on immunogenicity, and are considered to be safe in humans, with only minor side effects (André et al., 1992). The vaccines provide protection for at least 10 years (Koff, 1998). They are recommended for persons travelling or working in countries with high or intermediate rate of hepatitis A, children in day-care centres and in communities that have high rate of hepatitis, military personnel, foodhandlers, homosexual men, illegal-drug users, persons who have an occupational risk for infection, and persons who have chronic liver disease (Hollinger and Ticehurst, 1996; MMWR, 1996)

Antiviral agents. HAV replication can be inhibited by a number of antiviral

agents. Amantadine and ribavirin were found to inhibit virus uncoating in the endosomes or lysosomes of infected cells, whereas 2-deoxy-D-glucose seems to interfere with the late stages of the HAV replication cycle which are energy-dependent (Widell et al., 1986; Superti et al., 1987, 1989; Passagot et al., 1988). Atropine, protamine and zinc were found to inhibit HAV replication (Anderson et al., 1987; Biziagos et al., 1990), as was also shown with sulphated polysaccharides such as iota-, lambda-, and kappa-carrageenans which inhibited HAV replication in human hepatoma cells (Girond et al., 1991). Other compounds such as dextran sulfate, 6,4'-dichloro-flavan can inhibit viral antigen synthesis (Superti et al., 1989; Mastromarino et al., 1991).

Epidemiology of HAV

Hepatitis A is prevalent globally and is one of the most common causes of infectious jaundice in many regions of the world (Hollinger and Ticehurst, 1996). The epidemiology of hepatitis A is greatly influenced by the level of sanitary conditions, hygienic practices, as well as socioeconomic background. Since HAV transmission occurs mainly through the fecal-oral route, close to 100% of the population in developing regions of the world becomes infected and develop immunity within the first decade of life, as a result of overcrowding and nonexistent or inadequate sanitary conditions (Szmuness et al., 1977; Frosner et al., 1979; Gust et al., 1979, Tassopoulos et al., 1986; Coulepis et al., 1987; Tsega et al., 1990). By the time they reach adulthood, most of the population has been naturally infected with the virus resulting in a lasting immunity, and therefore clinical cases of hepatitis A are uncommon in developing countries. Due to improvements in sanitary conditions, water supply, and better hygienic

awareness, the endemicity of hepatitis A in developing countries appears to be shifting towards a more epidemic pattern similar to that usually observed in developed countries (Shaw et al., 1989, 1990; Chin et al., 1991; Innis et al., 1991; Midthun et al., 1991; Hadler, 1991). The continuing decline in endemicity, however, is also causing an increase in the size of the susceptible population (Midthun et al., 1991), which in turn require a serious consideration of vaccination against the virus (WHO, 1988). In developed modern societies, on the other hand, the greater emphasis which has been placed on sanitary conditions and personal hygienic practices has limited both the spread of and exposure of individuals to HAV. Consequently, exposure of children to HAV is limited, resulting in a mostly susceptible adult population (Szmuness et al., 1976; Gust et al., 1978; Iwarson et al., 1978; Schenzle et al., 1979).

Globally, hepatitis A seems to follow a cyclical epidemic pattern with a peak occurring almost every 10 years, with a higher number of cases appearing in the fall and winter months (Polakoff, 1990; Shaw et al., 1990; Ross et al., 1991). It is estimated that the annual world-wide incidence of the disease exceeds 1.4 million cases, at a health cost of between 1.5 and 3 billion dollars. Due to improved sanitary and hygienic measures in the developed regions of the world, this phenomenon of cyclical peaks appears to be disappearing and is being replaced by smaller and less pronounced peaks that seem to occur at 4-year intervals (Ng et al., 1989; Melnick, 1990).

Hepatitis A accounted for approximately 50% of all viral hepatitis reported in the US in 1993, representing 27,797 reported cases (estimated total cases = 83,391) in which around 20% more males were affected than females (Hadler, et al., 1991; Koff, 1998;

CDC, 1999). Surveillance of hepatitis A cases in 1991 in the U.S., Canada and Australia demonstrated that there was an increase over previous years (1985 to 1990), particularly among homosexual and bisexual males (CDC, 1992). In addition, a 5-fold increase was reported in British Columbia and a 4-fold increase was seen in both Montreal and Toronto (Ng et al., 1989). Similar trends of an increase in hepatitis A cases over the same 5-year period, was also reported in the United Kingdom and other developed countries (Polakoff, 1990; Kani et al., 1991; Tilzey and Banatvala, 1991).

Outbreaks of hepatitis A in day-care centres in the U.S. account for 9 to12 % of all cases (Vernon et al., 1982; Margolis and Shapiro, 1992). Among the many factors that influence the spread of the virus in such settings are the size of the institution, the number and age of children, the number of non-toilet trained children, hours of operation, the number of supervisors, as well as the awareness of and compliance with acceptable hygienic standards by the staff (Hadler and McFarland, 1986). Since in most situations the index case remains asymptomatic, the virus continues to spread, infecting others until clinical symptoms start to appear (usually in an adult) (Hadler et al., 1980; Vernon et al., 1982; WHO, 1987). Day-care centres often act as a source of the virus that can infect families and the community at large (Pohjanpelto and Ponka, 1985; Hadler and McFarland, 1986; WHO, 1987).

The frequency of hepatitis A outbreaks in schools is usually much lower than that observed in day-care centres. Furthermore, outbreaks of the disease tend to cluster in either girls or boys, which may be attributed to closer contact between pupils of the same sex and the segregation of toilet facilities (Skidmore et al., 1982; Reid and Carter, 1986;

Naus et al., 1989).

Hospital-acquired (nosocomial) outbreaks of hepatitis A among patients, as well as health care personnel have been reported (Bryne, 1966; Coulepis et al., 1980; Goodman et al., 1982; klein et al., 1984; Drusin et al., 1987; Rosenblum et al., 1991). Pediatric wards are particularly important with regards to outbreaks of hepatitis A in hospital settings. Infected children continue to shed the virus in large amounts in their stools for up to 6 months, despite their asymptomatic condition (Rosenblum et al., 1991). When such children are admitted to a hospital for an unrelated illness, nurses and other staff members may not take the extra precautionary measures that are appropriate for HAV. As a result, nurses may become infected, and in turn spread the infection unknowingly to other children (Goodman et al., 1982; Klein et al., 1984; Rosenblum et al., 1991).

In contrast to person-to-person mode of virus spread, sudden bursts of explosive epidemics of hepatitis A outbreaks within communities or confined populations usually result from fecal contamination of a single-source, such as drinking water, food, or milk (Mosley, 1959; Taylor et al, 1966; Mosley, 1967; Tihen and Mailloux, 1974; Melnick et al., 1976; Mathiesen et al., 1977; Melnick, 1982; Gust et al., 1983b; Metcalf et al., 1984). This is particularly true in the industrialized countries where contaminated foods serve as vehicles for virus transmission (Lowry et al., 1989; Block et al., 1990; Warburton et al., 1991).

As mentioned previously, various types of foods, including water, have been incriminated as being the source of hepatitis A outbreaks, most prominent amongst which is shellfish (Portnoy et al., 1975; Cliver, 1985; Halliday et al., 1991). During the

process of feeding, bivalve mollusks can filter up to 38 litres (US 10 gallons) of water per hour over a short period of time, during which HAV can be concentrated approximately 100-fold and can persist for about 7 days (Enriques et al., 1992). For this reason, many outbreaks of hepatitis A have been associated with the consumption of raw or improperly cooked shellfish (Metcalf et al., 1980; Richards, 1985; Desenclos et al., 1991; Halliday et al., 1991; Le Guyader et al., 1993; Straub et al., 1994).

Drinking water may become contaminated with HAV-containing fecal material either during storage or distribution, and has been shown to cause large community-based outbreaks of hepatitis A (Mosley, 1965; Lippy and Waltrip 1984). Leaks in HAV-containing sewage effluent can also contaminate shellfish beds, which result in the contamination of shellfish being cultivated in these waters. An outbreak of hepatitis A was also reported in a public swimming pool (Mahony et al., 1992).

Concentration and Detection of HAV

No one cell line is currently recommended for HAV propagation (Jothikumar et al., 1998) due to the inability or difficulty of wild-type HAV to grow in cell culture. As a result, detection of HAV in clinical, environmental and food samples has relied heavily on one or more of the following methods: (i) immune electron microscopy (Feinstone et al, 1973); (ii) immunofluorescence (Mathiesen et al., 1977); (iii) enzyme-linked immunosorbent assay- ELISA- (Coulepis et al., 1985); (iv) radioimmunoassay- RIA- (Coulepis et al., 1985); (v) radioimmunofocus assay-RIFA- (Lemon, et al., 1983); (vi) nucleic acid hybridization (Jansen et al., 1985; Jiang et al., 1987; Ticehurst et al., 1987; Shieh et al., 1991; Zhou et al., 1991; Romalde et al., 1994; Romalde, 1996) and

vii) reverse transcriptase-polymerase chain reaction- RT-PCR (Rotbart, 1990; Atmar et al., 1993; Goswami et al., 1993; Graff et al., 1993; Tsai et al., 1993; Deng et al., 1994; Le Guyader et al., 1994; Jaykus et al., 1995,1996; Cromeans et al., 1997; Dix and Jaykus, 1998; Jothikumar et al., 1998).

The RT-PCR is becoming the method of choice for the rapid, specific and sensitive detection of HAV from various samples. However, clinical, environmental and food samples have been shown to contain a number of organic and inorganic substances such as humic acid, fulvic acid, tannic acid, proteins, glycoproteins, polysaccharides, and metals that inhibit or interfere with the PCR reaction (Shieh et al., 1995; Kreader, 1996; Ijzerman et al., 1997). Therefore, methods have been and continue to be developed to capture and concentrate the virus and separate it from inhibitors present in clinical, environmental or food (shellfish) materials. Among such methods is the use of microporous filters (positively- or negatively-charged) and magnetic beads to capture the virus from large sample volumes, followed by reconcentrating the virus by eluting it in a small volume of eluent or by re-suspending the beads in a small (few μL) volume.

Concentration, isolation and detection of HAV. HAV can be isolated from feces, sewage effluents, environmental samples such as river water, as well as shellfish, and other types of foods. The amount of virus present in the above mentioned sources can vary greatly from very large numbers e.g., $> 10^6$ virions /g feces of infected individual to around 0.2 to 2 x 10^2 virions/ 100 g shellfish (Williams and Fout, 1992). The amount of virus present in environmental waters can vary greatly, although it has been suggested that the numbers are very low (Tsai et al., 1993; Schwab et al., 1995)

requiring their concentration from large volumes (gallons) of water (APHA, 1997). The amount of enteric viruses observed in surface waters in selected European and US waters ranged from 0.1 to 620 PFU/L (Gerba and Rose, 1990). Although the amount of virus present in other types of foods such as fruits and vegetables is not known, only a few virus particles/g may be present. Therefore, it is more feasible to detect the virus directly from feces than it is to detect it directly from environmental or food samples. To detect HAV from environmental and food samples, it is necessary to first capture and concentrate the virus from large quantities of environmental or food samples down into a smaller volume from which the virus can be isolated, purified and then detected by an appropriate method (Tsai et al., 1993).

Traditional methods used to concentrate viruses include, but are not limited to: 1) precipitation by polyethylene glycol and ammonium sulfate (Philipson et al., 1960; Trepanier et al, 1981; Payment and Trudel, 1985; Lewis and Metcalf, 1988; Zhou et al., 1991; Jaykus et al., 1993; Traore et al., 1998), 2) hydroxyextraction-dialysis with polyethylene glycol (Cliver, 1967; Shuval et al., 1967; Sobsey, 1976; Ramia and Sattar, 1980), 3) adsorption-precipitation by aluminum hydroxide (Wallis and Melnick, 1967a,b; Payment et al., 1976; Dobberkau et al., 1981), 4) molecular filtration (Trudel and Payment, 1980), 5) adsorption-elution-precipitation (Sobsey et al., 1978; Howard, 1980; Lewis and Metcalf, 1988; Zhou et al., 1991; Jothikumar and Cliver, 1997; APHA, 1997), 6) organic flocculation (Katzenelson et al., 1976), and 7) column chromatography (Elkana et al., 1983; Schwab et al., 1995).

In the past few years a number of more rapid, less cumbersome, and more PCR-

compatible procedures have been developed to concentrate and isolate HAV from environmental and food samples. Among these methods are i) ultrafiltration by Centriprep-100 and Centricon-100, to concentrate HAV from large volumes of sewage and ocean water down to approximately 100 µL suitable for direct RT-PCR (Tsai et al., 1993); ii) the use of the cationic detergent CTAB (cetyltrimethyl ammonium bromide) to precipitate nucleic acids and viruses from oyster extracts (Zhou et al., 1991; Jaykus et al., 1995); iii) Pro-Cipitate precipitation (Schwab et al., 1995 ; Dix and Jaykus, 1998); and iv) antigen-capture using antibody-coated solid polystyrene surfaces or magnetic beads (Jansen et al., 1990; Robertson et al., 1992; Graff et al., 1993; Morace et al., 1993; Prevot et al., 1993; Yotsuyangi et al., 1993; Deng et al., 1994; Monceyron and Grinde, 1994; Grinde et al., 1995; Lopez-Sabater et al., 1997; Jothikumar et al., 1998).

Since the scope of the present study focusses on the capture and concentration of HAV in foods by positively-charged filters and magnetic beads, only an in-depth description of these latter methods will be presented in this thesis.

Electropositive filters are composed of a fiber glass surface-modified resin which efficiently adsorbs the negatively-charged virus without the need to condition the environmental sample (e.g., water) by pH adjustment or addition of cations such as $MgCl_2$ and $AlCl_3$ (Wallis and Melnick, 1967a,b; Wallis et al., 1972; Farrah et al., 1976) to optimize virus concentration (Sobsey and Jones; 1979; Sobsey and Glass, 1980; Borrego et al., 1991; Ma et al., 1994). Positively-charged filters are made by soaking them for 2 h at room temperature in solutions such as polyethyleneimine Nalco 7111, chitosan, polymyxin B, and benzyl-dimethyl-hexadecylammonium chloride (Borrego et

al., 1991). Different types of electronegative (e.g., Filterite) and electropositive (e.g., MK, Virosorb 1 MDS) filters have been studied for their efficiency in capturing and recovering HAV (Sobsey and Glass, 1980; Melnick et al., 1984; Sobsey et al., 1985; Borrego et al., 1 991; Ma et al., 1994).

Virosorb 1MDS (Cuno, Inc., Meriden, Conn.) is a positively-charged filter widely used to concentrate viruses from large quantities of environmental and other types of waters. Efficient virus elution from filters has been achieved using different solutions such as glycine-NaOH, (pH 9.5-11.5) and proteinaceous solutions such as beef extract of pH 9 to 9.5 (Gerba et al., 1978; Sobsey and Jones, 1979; Sobsey and Glass, 1980; Goyal et al., 1980; Hejkal et al., 1982; Stetler et al., 1992; Ma et al., 1994; APHA, 1997), and urea-arginine-phosphate buffer, pH 9.0 (Jothikumar et al., 1990, 1995; Jothikimar and Cliver, 1997). Despite the variation in HAV recovery rates, proteinaceous eluents such as beef extract often result in good recoveries of virus from large quantities of water. However, due to its competition with the virus for the positive charges in the filters, beef extract does not allow re-adsorption to a second, smaller adsorbing filter to further concentrate the virus to smaller volumes. To reconcentrate the virus, organic flocculation of the beef extract can be done by lowering the pH to 3.5. The virus-adsorbed flocs can then be precipitated by centrifugation and the virus recovered by solubilizing the flocs in a small volume of phosphate buffer solution (Katzenelson et al., 1976; Shields and Farrah, 1986).

However, despite good recoveries, the detection of HAV directly from beef extract eluates has not been successful since beef extract has been shown to interfere with or

inhibit the PCR due to its rich proteinaceous nature (Schwab et al., 1995, 1996). Therefore, antigen-capture methods used to separate the virus from the inhibitory effects of clinical, environmental or food materials, have been used by other investigators to separate the virus from the inhibitory effect of the beef extract. The antigen-capture methodology relies on capturing the virus from the sample by antibodies which are adsorbed to or coated onto solid-phase polystyrene tubes or magnetic bead particles, respectively. The captured virus is then separated from the sample either by discarding the sample from the tube leaving the virus adsorbed to the tube-coated antibody, or by magnetically separating the bead-adsorbed virus from the sample and resuspending the beads in a small fraction of solution compatible with the PCR (Jansen et al., 1990; Robertson et al., 1992; Graff et al., 1993; Deng et al., 1994; Monceyron and Grinde, 1994; Grinde et al., 1995; Lopez-Sabater et al., 1997; Jothikumar et al., 1998). The efficiency of virus capture by antibody-coated magnetic beads and by positively-charged filters, as well as the sensitivity of detection of the captured virus by PCR will be discussed in detail in subsequent sections in this thesis.

4. OBJECTIVES

4. OBJECTIVES

Hepatitis A virus continues to be endemic in developing countries. Outbreaks of the disease also continue to occur despite improvements in sanitary conditions and educational programs on the importance of hygiene, both in the home and in food establishments (CDC, 1992, 1994). Major foodborne outbreaks of hepatitis A (Halliday et al., 1991; Dalton et al., 1996; Anon, 1997), have further re-enforced the need to learn more about the transfer of HAV from infected foodhandler to foods, its survival on produce, its inactivation in certain types of foods such as produce and dairy products, as well as to develop methods to rapidly concentrate and detect the virus from produce by molecular techniques.

This study was, therefore, designed to achieve the following objectives:

(1) To determine the required time and temperature combinations necessary to inactivate HAV in dairy products of different fat content.

(2) To determine the D_{10} values (dose) of ionizing gamma irradiation required to inactivate HAV in lettuce and strawberries.

(3) To determine the survival of HAV on lettuce stored under modified atmosphere packaging.

(4) To study the transfer of HAV from experimentally-contaminated fingerpads to lettuce, before and after treatment of the fingerpads with water and topical agents.

(5) To develop methods to rapidly capture, isolate and concentrate HAV from experimentally-inoculated lettuce and strawberries for rapid detection by RT-PCR.

5. GENERAL MATERIALS AND METHODS

5. GENERAL MATERIALS AND METHODS

This section describes the materials and methods that are common to either all or most of the experimental work presented in this thesis. Materials and methods relating to specific sections will be detailed where appropriate.

Cells. A seed culture of FRhK-4 (fetal rhesus monkey kidney) cells was kindly provided to us by Dr. M.D. Sobsey of the University of North Carolina, Chapel Hill, North Carolina. The cells were cultivated and maintained as described by Sobsey et al. (1985). Unless otherwise stated, all reagents for the growth medium were purchased from Canadian Life Technologies- Gibco BRL Products, Burlington, Ont. The medium was prepared by mixing the reagents listed in Table 5. Following thorough mixing, the solution was filter-sterilized through a 0.22 μm pore size low protein-binding bottle-top filter (Xymotech, Toronto) into 500 mL or 1 L volumes, and stored at 4°C. The cell monolayers were maintained in the same medium, but with only 2% FBS.

Preparation of the cells for storage. FRhK-4 cell suspensions for storage in liquid nitrogen were prepared by seeding each 175 cm^2 cell culture flask (VWR/Corning, Montreal, Que.) with about 10^5 cells suspended in 40 mL of growth medium. The flasks were then incubated at 37°C for 4 days. The cells were harvested as follows: the spent medium was discarded and the monolayer was washed twice with Dulbecco's phosphate-buffered saline (PBS). This was followed by the addition of 4 mL of a commercial 1x solution of trypsin-EDTA (0.05% of 1:250 Trypsin, 0.53 mM EDTA). The flasks were manually rotated to completely cover the cell monolayer with the mixture, and incubated at 37°C for 10-15 min. The cells were dislodged with occasional

Table 5. The growth medium for FRhK-4 cells.

Reagent	Volume (mL)
Eagle's minimum essential medium (10X stock)	400
Double distilled deionized water	2960
Fetal bovine serum (FBS)	400
7.5% Sodium bicarbonate (BDH, Toronto, Ont.)	60
1.5 M HEPES (N-2-hydroxyethyl piperazine-N-2-ethanesulfonic acid)	40
200 mM (100X) Glutamine	40
10 mM (100X) Non-essential amino acids	40
200 µg/mL (in saline) Kanamycin	40
50 mg/mL Gentamycin	4
250 µg/mL Amphotericin B (Fungizone)	16
TOTAL	4000 mL

manual rotation, followed by the addition of 6 mL of the growth medium, the cells were further dispersed by repeated pipetting and transferred to a 50 mL centrifuge tube (Diamed, Mississauga, Ont.). A few microlitre volume of the cell suspension was used for cell counting in a hemocytometer (VWR). The remaining cell suspension was centrifuged at 800 x g for 10 min, and then washed twice with fresh PBS at the same speed. The packed cells were resuspended to a final concentration of 1.0×10^7 cells/mL in a freezing solution consisting of the growth medium supplemented with 20% FBS (instead of 10%) and 10% dimethyl-sulfoxide (DMSO), which were pre-sterilized either by autoclaving or by filtration through a 0.22 μm pore size filter (Millipore Inc., Toronto, Ont.). Aliquots (1 mL) of the cell suspensions were dispensed into 1.8 mL plastic cryovials (Nunc/Canadian Life Technologies, Burlington, Ont.). The vials were placed in a Cryomed programmable freezing controller (Cryomed, Mich.) and were subjected to freezing to -90°C at the rate of 1°C/min. The frozen vials were then transferred immediately into a liquid nitrogen tank (-196°C). One week later, a vial was removed and the cells were cultured to ensure the success of the freezing process.

For day-to-day use, FRhK-4 cells were cultured by seeding about 10^5 cells in each 75 cm^2 flask (VWR/Corning) using 20 mL of growth medium. When larger quantity of the cells were needed, 10^6 cells were seeded in each of 175 cm^2 flasks using 35 to 40 mL of growth medium. To evenly distribute the cells, the tightly-capped flasks were rocked gently before incubation on a flat surface at 37°C incubator. The cells were examined daily, with the cultures being split when the monolayers were complete (usually 4-5 days). A split ratio of 1:4 of a complete monolayer gave four 75 cm^2 flasks in 4 days.

When preparing cell monolayers in 12-well plates (Fisher Scientific/Costar, Ottawa, Ont.) for plaque assays, cells were trypsinized, harvested, counted in a hemocytometer, and resuspended in growth medium to give 2.0×10^4 cells/mL. One mL quantities of the cell suspension were then dispensed into each well of a 12-well plate and were incubated overnight at 37°C in a 5% CO_2 atmosphere (Fisher Scientific).

Viruses. A laboratory adapted HM-175 strain of HAV (Minor, 1991) was received from Dr. Sobsey. Stock virus was prepared as described by Mbithi et al. (1991, 1992). FRhK-4 monolayers were infected at a multiplicity of infection of 0.01. The virus was allowed to adsorb for 90 min at 37°C and the infected cultures were then kept in maintenance medium until 75 to 80% (5-7 days of incubation) of each monolayer showed virus cytopathology. They were then frozen (-20°C) and thawed three times, and the culture fluid centrifuged for 10 min at 1000 x g. The virus-containing supernatant was then dispensed in 0.5 or 1 mL quantities in 2 mL cryogenic vials, and stored at -80°C. The stock virus titre was 2×10^7 PFU/mL.

The Sabin strain of poliovirus (PV) type 1 was obtained through the courtesy of Mr. D. Mcleod of Health Canada (Ottawa, Ont.). FRhK-4 cells were used for propagating this virus as well. The procedures for the preparation and preservation of the virus pools were the same as described above for HAV.

Plaque Assays. Assays to determine the plaque forming units (PFU) of both viruses were carried out in overnight-grown FRhK-4 monolayers using 12-well plastic plates (Fisher Scientific) as described by Sattar et al. (1989) and Mbithi et al. (1991). Three wells were used for each virus dilution tested. Each well received 0.1 mL of the

inoculum, and the virus was allowed to adsorb for 90 min at 37°C. The overlay medium consisted of two separate components, namely supplemented 2x MEM and 2x agarose. To prepare 15 mL of the 2x supplemented MEM, the following were added: 3 mL of 10x MEM, 6.11 mL deionized water, 0.6 mL FBS (to give 4% concentration), 0.3 mL each of non-essential amino acids, glutamine and HEPES, 0.45 mL of sodium bicarbonate, 0.03 mL of gentamycin, 3.52 mL of kanamycin, 0.24 mL of amphotericine B (fungizone), and 0.15 g magnesium chloride. Following thorough mixing, the solution was filter-sterilized through a 0.22 μm pore size low protein binding filter, and was incubated in a waterbath at 42°C. The 2x agarose (Sigma Type II) was prepared by melting down 1.5 g agarose in 100 mL double distilled deionized water by heating in a microwave oven, followed by autoclaving at 121°C for 10 min, and the preparation was then placed in a 42°C waterbath. The final complete overlay medium for the plaque assay was prepared by combining equal volumes of the supplemented MEM solution and the 2x agarose, and then dispensing them in 2 mL quantities onto the infected monolayer in each well of the 12-well microtitre plate. The overlay was allowed to solidify (approximately 30 min) and the plates incubated in a humidified 5% CO_2 incubator at 37 °C for 8 days for HAV (unless otherwise stated) and 2 days for PV1. For counting the plaques, the cell monolayers were first fixed overnight by the addition of 2 mL (per well) of a 3.7% solution of formaldehyde (BDH, Toronto, Ont.) in normal saline. The agar overlay was then removed and the cells were stained for 10 min with a 0.1% aqueous solution of crystal violet (Fisher Scientific). The stained monolayers were washed in running tap water and allowed to dry at room temperature. Alternatively, the

stain was aspirated from the wells by vacuum. In all assays, positive and negative controls were included. The limit of virus detection using this technique was 1 to 3 PFU for both HAV and PV1.

Preparation of lettuce and strawberries. Romain lettuce and strawberries were purchased locally. Individual lettuce leaves were cut into intact rectangular pieces of approximately 6-cm x 7-cm (unless otherwise stated), washed with a mild detergent (Ivory™ - Procter and Gamble, Toronto, Ont.) , thoroughly rinsed in water for 1-2 min, and allowed to dry for approximately 20-30 min in a laminar flow hood. Intact, large-sized individual strawberries were also washed and dried in the same manner. Each side (front and back) of the lettuce pieces was then exposed to UV light for 1 min, whereas the uneven elliptical shape of the strawberries limited UV exposure to the two side surfaces. This process was carried out to reduce and/or inactivate contaminating microorganisms that might have interfered with the plaque assay.

Virus inoculation and recovery from lettuce and strawberries. Each individual irradiated piece of lettuce or strawberry was transferred into a clean and UV-irradiated weighing boat (unless otherwise stated) using a sterile pair of forceps. Portions (10 μL) of HAV of a known titre was spread evenly over a demarcated area of approximately 2-cm x 1-cm along the length of the lettuce stem, and over approximately a 1-cm diameter area on each strawberry. The inoculated virus was recovered either immediately (time zero), or after drying in a laminar flow hood for 20-30 min, or following stated periods of incubation. Virus recovery from the lettuce or strawberries was done by washing it off the produce by repeated (>25 times) pipetting of the

demarcated (inoculated) area with 1 mL PBS through the fine end of a sterile 1 mL-capacity tip fitted onto a 1000P Gilson pipettor. During pipetting, the boat was tilted slightly forward to allow for the wash solution to collect at the lower corner of the boat. At the end of the washing, the solution, as well as any visible droplets remaining on the lettuce/strawberry, was recollected from the boat using the same tip. Serial dilutions were then prepared from the wash solution, and plaque assayed as described above. The use of the weighing boats in the wash process provided a very smooth and non-stick surface which allowed the retrieval of almost the full 1 mL (approximately 970-980 μL) of wash solution. To establish a baseline for virus recovery from lettuce, 10 μL of HAV was spread evenly over a demarcated area of approximately 2 cm x 1 cm along the length of the lettuce stem or on the strawberry and was allowed to dry in a laminar flow hood for 20-30 min. This was followed by recovering the virus as described.

6. RAPID CONCENTRATION AND DETECTION OF HEPATITIS A VIRUS (HAV) IN LETTUCE AND STRAWBERRIES

6. RAPID CONCENTRATION AND DETECTION OF HEPATITIS A VIRUS (HAV) IN LETTUCE AND STRAWBERRIES.

INTRODUCTION

Infection with hepatitis A virus (HAV) results in the disease hepatitis A, which is one of the most common forms of acute viral hepatitis in much of the world (Koff, 1998). Based on its genetic, biophysical and biochemical characteristics, HAV has been removed from the genus *Enterovirus* and placed into its own genus *Hepatovirus* in the family *Picornaviridae* (Minor, 1991). It is a 27 nm diameter, non-enveloped virus, with four capsid proteins (VP1, VP2, VP3, VP4) encompassing a highly conserved positive-polarity single-stranded RNA genome. Although many strains have been isolated worldwide, they all belong to a single serotype (Hollinger and Ticehurst, 1990). New clinical isolates of HAV may not successfully grow in cell culture, or could take weeks of passaging to establish infection, which may or may not produce a noticeable cytopathic effect (CPE). Provost and Hilleman (1979) successfully cultivated and adapted HAV to grow in cell lines, and thus opened the door to study this laboratory-adapted virus in detail.

Transmission of HAV occurs mostly through the fecal-oral route, by person-to-person contact, or through the ingestion of fecally contaminated foods (Cliver, 1985 ; Cliver et al., 1992; Anon, 1993). Many foods have been implicated, including shellfish, sandwiches, dairy products, and fruits and vegetables. Recent foodborne outbreaks of hepatitis A such as the one in Denver, Colorado in 1992 (Dalton et al., 1996) due to the consumption of contaminated gourmet foods prepared by an infected foodhandler, and in

Michigan (Anon, 1997) due to consuming contaminated strawberries, have emphasized the need to develop rapid methods to detect the virus in foods such as produce.

Traditional methods to capture, concentrate and detect HAV in environmental samples and foods are lengthy, cumbersome, suffer from low sensitivity and the possibility of virus inactivation or loss during the various steps (De Leon and Sobsey, 1991; Romalde, 1996; Lopez-Sabater, et al., 1997). Although RT-PCR has recently emerged as one of the most rapid and sensitive molecular techniques to detect viruses at very low levels, it suffers some drawbacks. These include inhibition of the reverse transcriptase-polymerase chain reaction (RT-PCR) by various substances (Ijzerman et al. 1997; Richards, 1999), and that a positive result does not indicate the presence of a live and/or infectious virus (Harris and Griffiths, 1992; Cook and Myint, 1995; Richards, 1999). Although HAV and other enteric viruses may be present in large numbers in stool ($\geq 10^6$ infectious units/g), they are usually present in low numbers in foods, e.g., 0.2 to 224 infectious particles / 100 g shellfish meat (Williams and Fout, 1992). Therefore, various methods have been developed to capture, concentrate, isolate and identify these viruses from foods. Since most viruses carry a net negative surface charge and have an isoelectric point below pH 7.0 (Brinton and Lauffer, 1959; Mandel, 1971; Kessick and Wagner, 1978), adsorption-elution methods using microporous positively-charged filters have been used to capture viruses from drinking and environmental waters (Sobsey and Jones, 1979; Melnick et al., 1984; Sobsey et al., 1985; Borrego et al., 1991; Ma et al., 1994; Schwab et al., 1995, 1996; Jothikumar and Cliver, 1997) Magnetic bead particles coated with specific antibodies have also been used by a number of investigators to

capture, separate and concentrate many types of microorganisms, including enteric viruses, with their subsequent detection by RT-PCR (Safarik et al., 1995). However, these applications have been limited to the capture and detection of the virus from clinical (Jansen et al., 1985,1990; Robertson et al., 1992; Muir, et al.,1993), environmental (Graff et al., 1993; Prevot, et al., 1993; Deng et al., 1994; Schwab, et al., 1996; Jothikumar, et al., 1998), meat (Bennet et al., 1995; Colman et al., 1995) and shellfish samples (Zhou , et al., 1991; Jaykus, et al., 1993, 1996; Le Guyader, et al., 1994; Atmar et al., 1995).

In this study, we adapted the magnetic bead and positively-charged filter methods, either separately or combined, to capture and concentrate HAV from lettuce and strawberries. This was followed by its rapid detection by a one-tube RT-PCR method.

MATERIALS AND METHODS

Cells and Viruses. A seed culture of FRhK-4 cells and HAV (strain HM-175) and a seed of the Sabin vaccine strain of poliovirus (PV1) were used in this study (please see General Materials and Methods).

Inoculation of Fruits and Vegetables. Locally purchased Romain lettuce and strawberries, were selected as representative of a vegetable and fruit, respectively. Individual lettuce leaves were cut into pieces of ca. 6 cm x 7 cm, washed, dried and inoculated with 10 μL of a known plaque forming unit-PFU- (10^3 for the magnetic bead experiments, or 10^4 to 10^5 for the 1MDS positive filters) of HAV as described in General Materials and Methods. The inoculated lettuce pieces were placed inside sterile weighing boats, whereas the strawberries were put inside sterile glass beakers. The boats and the beakers were then covered with UV-sterilized Parafilm™ and incubated at 4°C overnight.

Virus Recovery. Using a flat-end sterile pair of forceps, each inoculated strawberry was transferred into a clean and UV-irradiated weighing boat similar to that containing the pieces of lettuce. The inoculated virus on the lettuce or strawberries was recovered by washing as described in General Materials and Methods. The virus-recovery solution was then used to investigate the capture, concentration, detection and quantitation of the virus as described below.

Plaque Assay. Please see General Materials and Methods.

RNA Extraction. The QIAamp viral RNA extraction kit (Qiagen, Mississauga, Ont.), which is based on the guanidinium thiocyanate RNA extraction procedure, was used to extract RNA from the HAV stock culture. Briefly, HAV particles present in 140μL cell-culture supernatant placed in a 1.5 mL microfuge tube were lysed by thorough mixing with 560 μL prepared buffer AVL (high molarity chaotropic salt agent) containing carrier RNA. After a 10 min incubation at room temperature, 560 μL of cold absolute ethanol was added and throughly mixed with the above mixture. Of this, 630 μL was transferred to the QIAamp spin column and centrifuged at 6000 x g for 1 min to get rid of unwanted contaminants while the RNA remains bound to the spin column membrane. The RNA-bound column was then washed twice (500 μL each) with buffer AW to further remove contaminants and traces of ethanol. The bound RNA was then eluted from the column membrane by the addition of 50 μL of preheated (80°C) RNase-free water and centrifugation at 6000 x g for 1 min. The extracted viral RNA was serially diluted and subjected to RT-PCR to determine the sensitivity of detection and the amount of RNA to be used as a positive control in the RT-PCR.

RT-PCR and Hybridization. The Titan™ One tube RT-PCR kit (Boehringer Mannheim, Laval, Quebec) was used according to the manufacturer's instructions. Briefly, the following mixture was prepared in a 0.2 mL microfuge tube:10 μL 5x RT-PCR buffer with Mg^{2+}, 1 μL of 10 mM of each of dATP, dCTP, dGTP and dTTP, 1 μL of 20 mM each of upstream and downstream primers, 2.5 μL of 100 mM DTT-solution, 1.5 μL of RNAse Inhibitor (40U/μL- Pharmacia, Laval), 1 μL enzyme mixture (Expand™ ; Taq and Pwo DNA polymerase) and 27 μL DEPC-treated distilled water. To the above 48 μL mixture, 2 μL of either extracted RNA template or HAV-containing cell culture supernatant (which had been subjected to 95°C for 5 min) was added. The same HAV-specific primers (upstream 5'-GTTTTGCTCCTCTTTACCATGCTATG-3' and downstream 5'-GGAAATGTCTCAGGTACTTTCT TTG-3'; producing a 247 bp amplicon) and thermocycling conditions as previously described by Deng et al. (1994) were used. The tubes were incubated in a Perkin-Elmer Cetus thermocycler model 9600 (Perkin-Elmer, Montreal, Quebec) programmed to run the following programs consecutively: 42°C for 30 min, 99°C for 5 min, 5°C for 5 min, and then 35 cycles of denaturation at 95°C for 1 min and annealing and extension at 60°C for 1 min, with an additional 7 min extension at 60°C. PCR amplicons were resolved on a 2% agarose gel, visualized by ethidium bromide staining, and photographed. The PCR bands were then transferred onto a positively-charged nylon membrane (Boehringer Mannheim) for hybridization. A specific internal oligoprobe (5'- TCA ACA ACA GTT TCT ACA GA - 3') located between nucleotides 2232 and 2251 (Deng et al., 1994) was labelled with digoxigenin-ddUTP according to the instructions in the DIG oligonucleotide 3'-end

labelling kit (Boehringer Mannheim) as follows: 5 μL (100 pmol) oligoprobe, 4 μL each of tailing buffer and $CoCl_2$, 1 μL DIG-ddUTP solution and 1 μL (~50 units) terminal transferases enzyme were mixed in a microfuge tube. Double distilled water (DDW) was added to make up to a final volume of 20 μL and incubate at 37°C for 15 min, then place on ice. The reaction was stoped by adding 2 μL of glycogen-EDTA solution, pH 8, and the labelled oligonucleotide was precipitated by the addition of 2.5 μL 4M LiCl and 75 μL of pre-chilled absolute ethanol. After 2h incubation at -20°C, the pellet was centrifuged at 12000 x g, washed with 50 μL cold 70% ethanol, dried under vacuum and dissolved in 20 μL of sterile DDW. Labelled oligonucleotide was stored at - 20°C. Membranes were pre-hybridized at 42°C overnight and then hybridized with the Dig-labelled probe at the same temperature in a hybridization oven (Robins, Model 400, VWR, Montreal, Quebec). The hybridization signal was obtained by immunological color detection according to the instructions in the DIG Nucleic Acid Detection kit (Boehringer Mannheim).

Sensitivity of detection of RT-PCR. Quantities of 2 μL containing 128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.12, 0.06, 0.03, and 0.015 PFU of HAV were amplified by PCR as described. The highest virus dilution which demonstrated a positive amplification band, confirmed by the Dig-labelled internal oligoprobe, was considered as the limit of detection.

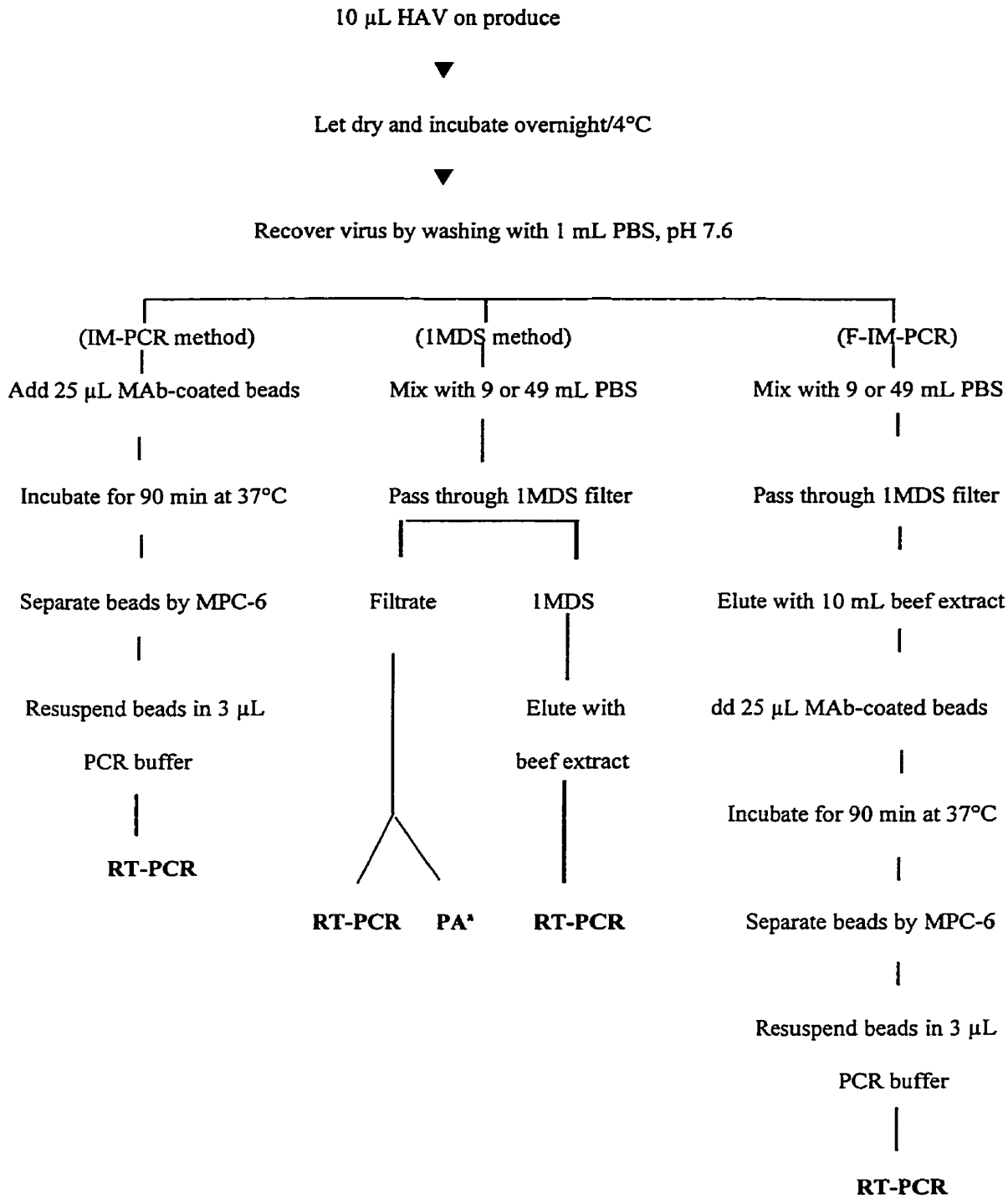
Antigen-Capture by Magnetic Beads (IM-PCR Method). Magnetic Dynabeads M-280 (Dynal, Great Neck, NY) were coated with purified specific anti-HAV K3-2F2 monoclonal antibodies (MAb) (Serum Commonwealth Labs.,Victoria, Australia) according to the manufacturer's instructions. Briefly; a portion of beads (see below) was incubated overnight at 4°C with a pre-determined amount of MAb, on a gently rotating roller to

maintain the beads in suspension. The beads were then collected at the side of the tube by using a Dynal magnetic particle concentrator (MPC-6), the supernatant discarded, and the beads washed four times with 1 mL phosphate-buffered saline (PBS), pH 7.2, containing 0.1% bovine serum albumin (BSA) [PBS-BSA]. The MAb-coated beads were then resuspended in 1% BSA (Sigma Chemicals, Oakville, Ont.) and were incubated for 30 min at room temperature on a roller. The beads were then separated, washed four times, resuspended in PBS-BSA, stored at 4°C and used within 2-3 weeks.

Optimal MAb to beads ratio was determined by cross-titration whereby 1000 μg of beads (100 μL) was coated with serial dilutions containing 7.5, 10, 12.5, 15, 17.5, and 20 μg MAb. Portions (25 μL) of the coated beads were then incubated with HAV dilutions containing 0.1, 0.5, 1, 10^1, 10^2 , 10^3, 10^4, and 10^5 PFU/mL, for 90 min at 37°C, on a gently rotating roller. The beads (with the captured virus) were then collected, washed and resuspended in either 1 mL PBS (for plaque assay) or in 3μl PCR buffer from the Titan™ Kit for the RT-PCR assay (see Fig.1).

To investigate non-specific binding of HAV to the beads, 25 μL portions of uncoated beads (10 mg/mL) were incubated with 1 mL solutions containing 10^1, 10^2, 10^3, 10^4 or 10^5 PFU of HAV. The beads were magnetically separated to the side of the tube and the supernatant decanted into a clean tube. The beads were washed four times each with 500 μL PBS-BSA and resuspended in 3 μL PBS, while the wash solutions (2 mL in total) were added to the 1 mL decanted supernatant. The beads were then run through an RT-PCR reaction, whereas the combined wash and solution suspension was examined by the plaque assay. The same procedure was applied to determine the percentage of virus

Figure 1: Schematic illustration of the immunomagnetic-polymerase chain reaction (IM-PCR), the 1MDS filtration (F), and the combined filtration-immunomagnetic (F-IM-PCR) methods used to concentrate and detect hepatitis A virus (HAV) from produce. Ten μL portions of HAV of a known titre were inoculated onto lettuce or strawberries, let dry and incubated overnight at 4°C. The virus was recovered in 1 mL PBS and processed as illustrated.



[a] Plaque assay.

specifically captured by the antibody-coated beads, except that 25 μL of MAb-coated beads was incubated with 1 mL containing 10^3 PFU of HAV.

The sensitivity of detection of the IM-PCR was determined by incubating 25 μL portions of MAb-coated beads with 1 mL quantities of HAV dilutions containing 80, 40, 20, 10, 5, 2.5, 1, 0.5, 0.25, 0.1, and 0.05 PFU, and the beads were then amplified by the RT-PCR.

In applying the IM-PCR technique to capture and detect HAV from lettuce and strawberries, the demarcated areas on the produce were inoculated with 10 μL portions containing 80 , 40, 20, 10, 5, 2.5, 1, 0.5, 0.25, 0.1or 0.05 PFU, allowed to air-dry, and were then recovered with 1 mL PBS as described above. MAb-coated beads were then incubated with the 1 mL of recovery solution (PBS, pH 7.6), following which the beads were separated and assayed by the Titan™ one-tube RT-PCR. To determine if the presence of other viruses could affect the IM-PCR, 10 μL of HAV (10^3 PFU) and 10 μL of PV1 (10^5 PFU) were inoculated onto the same demarcated areas on the produce, and were then recovered, captured and detected by the IM-PCR as described above.

Antigen-Capture by Positively-charged 1MDS Filters. Positively-charged, 47 mm diameter, 0.2 μm pore size, 1MDS filters (Cuno Inc., Meriden, CT) were used to capture HAV from inoculated lettuce and strawberries. The filters were sterilized by autoclaving and housed in a sterile Nalgene filtration unit (VWR). Experiments were designed to investigate the following: 1) the efficiency of virus recovery rates from filters, as well as PCR compatibility, using 1 and 10 mL volumes of 1 and 3% beef extract (BE) eluents; 2) investigate the capture and concentration of HAV from BE eluates by magnetic

beads; and 3) examine HAV recovery and detection from lettuce and strawberries using a combined filtration and bead procedure. In initial experiments, 10 mL volumes of PBS seeded with 10^5 PFU of HAV were filtered through 1MDS, and the filtrates were collected and tested by both RT-PCR and the plaque assay. The filter-adsorbed virus was eluted from the filters by overlaying them with 1 or 10 mL of 1 or 3% BE which, after a few minutes of holding time, was passed twice through the 1MDS filter by a drop-by-drop process (Borrego et al., 1991). A cordless pipettor was used to apply suction through the pressure relief valve at the filter holder to drain off as much as possible of the retained eluate from the filter. When 1 mL of BE was used, a washing step was added whereby, using sterile forceps, the filter was transferred into sterile 50 mL capacity centrifuge tubes, and was washed with the same collected eluate, by either repeated pipetting (> 25 times), or by vigorous shaking using a mechanical Wrist Action shaker (Burrell, Pittsburgh, PA) for 10 to 15 min. Using forceps, the filters were moved to the centre of the tube and were made to adhere or stick to the inside wall by pressing the filters against them. In order to drain off as much as possible of the retained eluent from the filters, the tubes were then centrifuged at 800 x g for 15 min. These virus-containing eluates were then collected and assayed by both the PCR and plaque procedures. The same process of virus recovery from filters was repeated using 1 and 10 mL volumes of 3% BE. Since virus recovery was the lowest when using the 1 mL eluates, whereas comparable recoveries were obtained with 10 mL of either 1 or 3% BE (results section), further experiments were carried out using 10 mL of 1% BE to elute the virus from the 1MDS filters.

In a separate experiment used to simulate the use of larger volumes of wash

solution to recover the virus from the produce, 1 mL containing 10^5 PFU of HAV was thoroughly mixed with 49 mL of PBS, and the entire solution (50 mL) was filtered through the 1MDS. The filtrates, as well as the virus eluted from the filters by 10 mL of 1% BE, were assessed by the plaque assay (Fig.1).

To further concentrate the eluted virus, as well as to remove the inhibitory effect of BE on the RT-PCR, a combined filtration and magnetic beads procedure was investigated whereby anti-HAV MAb-coated beads were added to the virus-containing 10 mL of BE eluates. The beads were then collected, resuspended in 3 μL PBS, and tested by the PCR as described earlier (see IM-PCR). In this procedure, two sets (each consisting of duplicates) each of lettuce and strawberries were inoculated with 10 μL of HAV dilutions in PBS containing 1000, 100, 50, 25, 10, 1 or 0.1 PFU, and allowed to dry. The virus was then recovered with 1 mL PBS from each of the samples, mixed with either 9 or 49 mL of PBS and passed through the 1MDS filters. The filtrates were saved, and HAV was eluted from the filters with 10 mL of BE. One set of the eluates was processed by the RT-PCR directly, whereas to the second set, MAb-coated beads were added to capture the virus, which was then subjected to RT-PCR, thus combining both the filtration and immunocapture methods (F-IM-PCR; Fig.1).

RESULTS AND DISCUSSION

Virus Recovery. Plaque assays demonstrated that with our elution method an average of 84 and 81% of HAV could be recovered from the lettuce and strawberries, respectively. These rates were used as the baseline virus titre in subsequent experiments. The weighing boats provided a very smooth and non-stick surface which allowed the

retrieval of approximately 970-980 μL of the 1 mL of wash solution .

RT-PCR. As low as 100 pg of HAV RNA produced a sharp amplification signal in the Titan™ single tube RT-PCR. Therefore, this amount was used as a positive control in RT-PCR reactions. Comparable positive PCR signals were obtained from the amplification of both the extracted RNA or from intact viruses whose RNA was released by heat (95°C / 5 min) at the start of the RT-PCR reaction (Fig. 2a). The identity of the 247 bp band obtained was confirmed by southern hybridization using the Dig-labeled oligo probe (Fig. 2b). Therefore, in our experiments, 2 μL from a virus stock solution was used directly in the RT-PCR reaction as a positive control. As low as 0.5 PFU was detected by the RT-PCR, although the sensitivity of detection varied between 0.5 and 2 PFU (Fig. 3). Other investigators have also reported detection sensitivities ranging from as little as 0.05 PFU to 10 PFU in a reaction sample (Atmar et al., 1993; Tsai et al., 1993; Monceyrone and Grinde, 1994; Puig et al, 1994; Grinde et al., 1995). It appears that this variation is inherent in the RT-PCR itself rather than the Titan™ one-tube RT-PCR system which, in our experience, demonstrated a greater sensitivity and reproducibility than the RT-PCR assays which incorporated only the Taq DNA polymerase. This increased sensitivity could be attributed to the higher fidelity of the enzymes used in the Titan™ RT-PCR system (i.e., Expand™ which includes both Taq and Pwo DNA polymerases) that allow for better proof-reading of the template and minimize the effect of any interference by secondary RNA structures (Frey and Suppmann, 1995; Kubler and Frey, 1995).

Figure 2: (a) Amplification by the Titan™ one-tube RT-PCR system of extracted RNA from hepatitis A virus (HAV) and from intact whole HAV. Lanes 1 and 2: extracted RNA; Lane M, DNA Marker ladder 123 bp; Lanes 3 and 4: 4×10^4 PFU of HAV. (b) Southern hybridization with Dig-labeled oligoprobe.

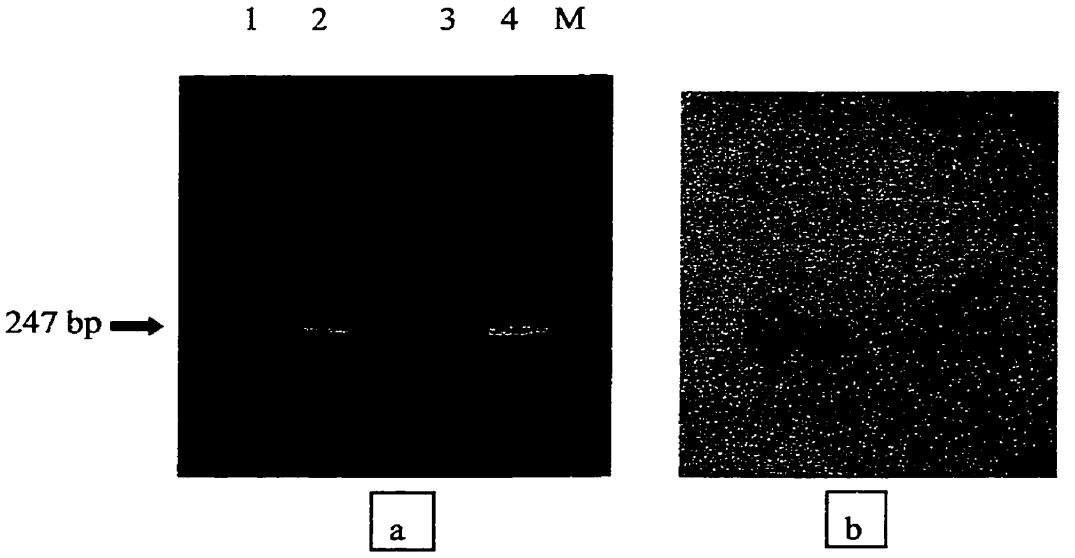
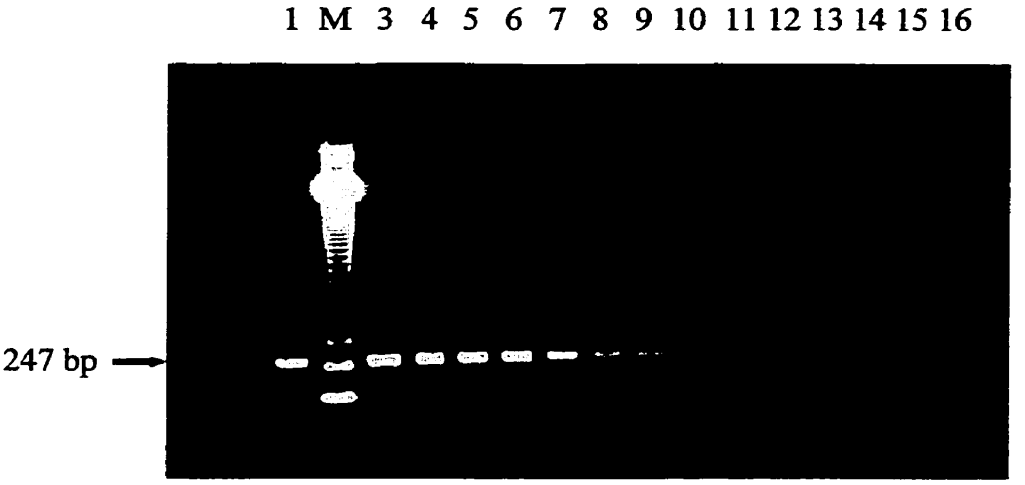

1 2 3 4 M
247 bp
a
b

Figure 3: Sensitivity of detection of hepatitis A virus (HAV) by the Titan™ one-tube RT-PCR system. Lane1, HAV positive control; Lane M, DNA Marker ladder 123bp; Lanes 3 through 16, HAV at total concentrations of 128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.12, 0.06, 0.03, 0.015 PFU, respectively.


1 M 3 4 5 6 7 8 9 10 11 12 13 14 15 16
247 bp

Virus Capture and Detection by Magnetic Beads and RT-PCR (IM-PCR).

a) Effect of magnetic beads on FRhK-4 cells and PCR performance: FRhK-4 cell monolayers inoculated with magnetic beads (2 mg) and observed for 14 days did not show any noticeable toxic or cytopathic effects. The presence of 3 μL of beads (300 μg) in the RT-PCR reaction mix, although occasionally producing a weaker band intensity, did not inhibit the reaction nor affect the sensitivity of detection. Monceyron and Grinde (1994) also observed a decrease in band intensity when similar type of beads were present in the PCR reaction, and therefore, with-held the beads upon transferring the cDNA to the PCR mix. However, Hedrum et al. (1992) using the same type of beads, did not notice any differences, and thus retained the beads in the PCR reaction mix. Therefore, in our study, the bead-virus complex was transferred to the single-tube RT-PCR reaction tube, where the entire amplification process was completed.

b) Optimization of the ratio of MAb to magnetic beads: Cross-titration studies demonstrated that both 10 and 12.5 μg of MAb per 1000 μg (100 μL) beads produced similar PCR band intensities (Table 6). In our experiments, however, we chose to coat the beads with 12.5 μg of MAb (2.5 μL purified MAb per 100 μL beads), to ensure that sufficient antibody was present to capture the virus, as well as to reduce non-specific binding by coating more of the bead surface area with the specific antibody. The use of 12.5 μg of MAb falls within the previously suggested range of 10 to 20 μg MAb per mg of beads (Luk and Lindberg, 1991; Monceyrone and Grinde, 1994; Grinde et al., 1995).

Table 6. Cross-titration to determine the optimal ratio of anti-hepatitis A virus (HAV) monoclonal antibody (MAb) and magnetic beads .

HAV-PFU/mL	MAb concentration (μg) 7.5	10	12.5	15	17.5	20	Magnetic bead concentration
0.1	-[a]	-	-	-	-	-	1000 μg
0.5	±[b]	+[c]	+	+	+	+	
1	+	+	+	+	+	+	
101	+	+	+	++[d]	++	++	
102	+	+	++	++	++	++	
103	+	++	++	++	++	++	
104	++	++	++	++	++	++	
105	++	++	++	++	++	++	

1000 μg portions of beads were coated with variable concentrations of MAb. Portions (25 μL) of the coated beads were then incubated with various HAV concentrations (PFU; plaque forming units) and assayed by PCR.

[a] No visible PCR amplification band.

[b] An occasional positive band by PCR.

[c] Faint positive band by PCR.

[d] Strong positive band by PCR.

c) Non-specific binding of HAV to uncoated beads: In the presence of uncoated beads, PCR amplification bands were not visible at HAV concentrations of 10^1 and 10^2 PFU, a faint band was occasionally visible at 10^3 PFU, and slightly stronger signals were observed at 10^4 and 10^5 PFU. Accurate determination of the rate of non-specific binding was not feasible due to an inconsistency in plaque assay results which could be due to i) a variation in the initial number of virus particles which adsorbed to the beads; ii) variation in the number of virus particles removed from the beads by the washing process; iii) the volume of washing solution; and iv) the vigorousness of the wash process itself. Overall, a range of 0.6 to 2.6% (average 1.5 ± 0.58 %) non-specific virus adsorption was obtained by plaque assays (Table 7). Pre-treatment of the beads with 1% BSA prior to incubation with HAV solutions, as well as vigorous washing of the beads after incubation, reduced the non-specific adsorption to approximately 0.93 ± 0.86 % and resulted in only the occasional faint band seen at 10^3 PFU. Moncyrone and Grinde (1994) also observed that HAV bound non-specifically to magnetic beads, but they did not indicate the actual percentage of non-specific binding.

d) Specific binding of HAV to MAb-coated beads: When HAV-MAb-bead suspensions were inoculated onto cell monolayers, an inconsistency in plaque counts, as well as in the size and discreteness of plaque zones was observed, making it difficult to accurately determine the rate of specific binding directly from the bead-HAV complex. However, plaque assay data from the supernatant was more consistent and showed that approximately 43 to 71% (average 58.4 ± 4.99 %) of the virus remained unadsorbed in the supernatant, indicating that the amount of virus captured by the

Table 7. Specific and non-specific binding of hepatitis A virus (HAV) by magnetic beads.

		Percent (± SE) specific binding of virus to MAb-coated beads			Percent (± SE) non-specific binding of HAV to uncoated beads		
HAV PFU/ mL (PBS)	Trial	Plaque assay [a] Beads	Plaque assay [a] Supernatant	Beads RT-PCR	Plaque assay [a] Beads	Plaque assay [a] Supernatant	Beads RT-PCR
103	1	29	71	+[c]	0.6	99.4	-[d]
	2	48	52	+	1.4	98.6	-
	3	57	43	+	2.6	97.4	±[e]
	4	34	66	+	ND[f]	ND	ND
	5	40	60	+	ND	ND	ND
Average		41.6 ± 4.99[b]	58.4 ±4.99		1.5 ± 0.58[b]	98.46 ±0.58	

[a] The percentage of specific binding of HAV to beads was derived by subtracting the amount (%) of the unbound HAV in the supernatant from 100%, which was the amount of input virus.

[b] The standard error of the mean (SE) for the specific and non-specific binding by the beads is similar to the SE for the supernatant, since the (%) values reflect the difference from 100%.

[c] Positive PCR amplification signal.

[d] No PCR amplification signal.

[e] An occasional faint PCR amplification signal.

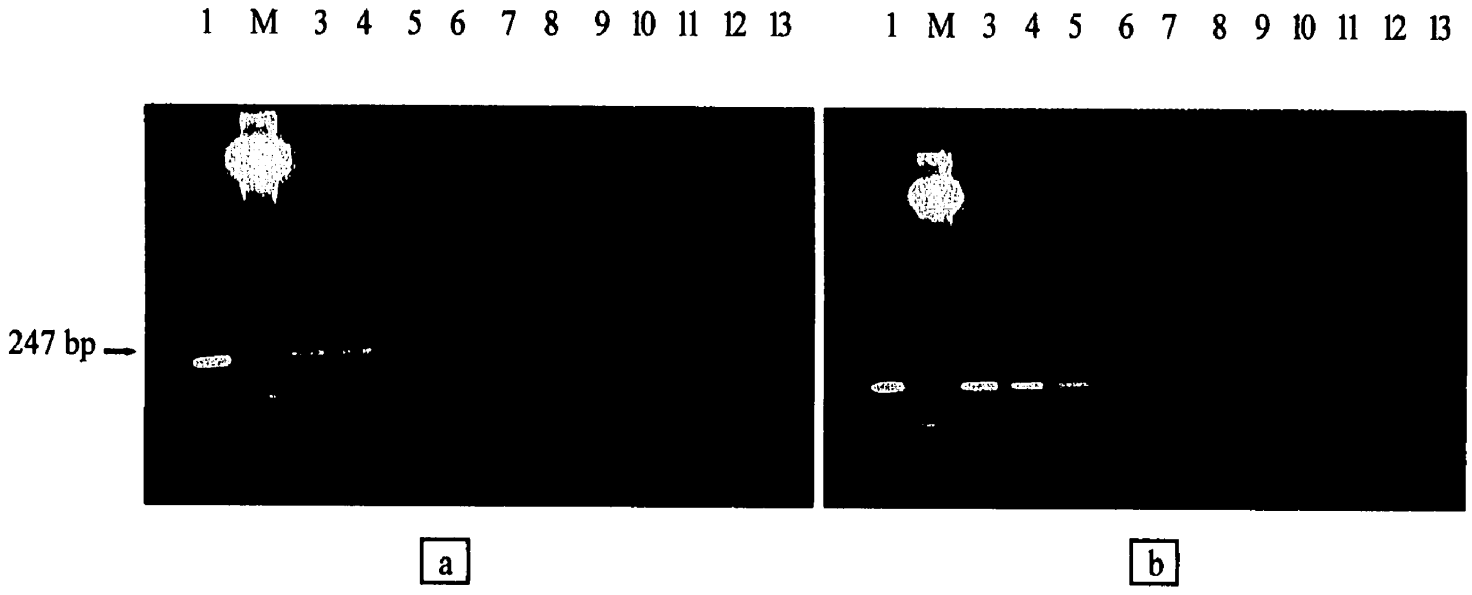
[f] Not done.

MAb-coated beads ranged between 29 and 57% (average 41.6 ± 4.99 %; Table 7). Based upon an average rate of 1% non-specific adsorption, it was estimated that an average of 40.6% of the virus bound specifically to the beads. This indicates that specific binding of HAV to MAb-coated beads was around 42 times greater than the nonspecific binding of the virus to the uncoated beads, which is lower than the estimated value of a 100-fold difference between the specific and non-specific HAV binding as was suggested by Monceyron and Grinde (1994). Vigorous washing (vortexing) of the beads for four times with PBS-BSA did not produce a noticeable reduction in the specific binding of the virus to the MAb-coated beads, confirming previous observations by other investigators (Monceyron and Grinde, 1994; Jothikumar and Cliver, 1997).

e) IM-PCR sensitivity of detection: PCR bands obtained from test samples in which MAb-coated beads were incubated with various concentrations of HAV in PBS, demonstrated a detection limit ranging between 0.5 and 10 PFU (Fig. 4a). Although similar to the detection limit of the conventional RT-PCR, the IM-PCR was > 100 times more sensitive since it detected the virus in a 1 mL volume, as compared to the same amount of virus present in the 2 μL used in the conventional RT-PCR reaction.

f) Detection limits of IM-PCR for produce: Although as low as a total of 2.5 PFU of HAV was detectable from lettuce and strawberries, the sensitivity of detection frequently varied between 5 and 10 PFU. Overall, this was approximately 5 to 10-fold less sensitive than the minimum of 0.5 PFU detected by the IM-PCR from PBS (Fig. 4b). The

Figure 4: (a) Sensitivity of detection of hepatitis A virus (HAV) from phosphate-buffered saline (PBS) by the immunomagnetic-PCR (IM-PCR). Lane1, HAV positive control; Lane M, DNA Marker ladder 123 bp; Lanes 3 to 13, contain HAV at total concentrations of 80, 40, 20, 10, 5, 2.5, 1, 0.5, 0.25, 0.1, 0.05 PFU, respectively. (b) Sensitivity of detection of hepatitis A virus (HAV) from lettuce or strawberries by the immunomagnetic-PCR (IM-PCR). Lane 1, HAV positive control; Lane M, DNA Marker ladder 123 bp; Lanes 3 to 13 contain HAV at inoculated concentrations of 80, 40, 20, 10, 5, 2.5, 1, 0.5, 0.25, 0.1, 0.05 PFU, respectively.


1 M 3 4 5 6 7 8 9 10 11 12 13
1 M 3 4 5 6 7 8 9 10 11 12 13
247 bp
a
b

lower sensitivity may be due to a number of factors such as the loss of virus during recovery by washing from produce (average recovery of 82%) and variation in virus recovery from lettuce/ strawberries due to inoculation with minute viral quantities. Taking these factors into consideration, as well as the 41.6% immunocapture efficiency by the beads, it is suspected that only half or less (1.6 and 3.3) of the 5 and 10 PFU, respectively, were actually captured and detected. Deng et al. (1994) demonstrated that the ratio of HAV particles to infectious units was 79:1, which, when applied to our IM-PCR findings would indicate that a detection limit of 5 to 10 PFU corresponds to approximately 395 and 790 virus particles, respectively. Considering an average rate of virus recovery from the produce to be 82% (of which 42% would be captured by the beads) would indicate that 126 to 252 virus particles were required to be present on the produce to result in visible detection by the IM-PCR, which is at least 2 to 30-fold higher than the 6 to 60 (Prevot et al., 1993) and four (Deng et al., 1994) virus particles detected in spiked environmental samples.

Data reported by other investigators reflect a variation in the detection limits obtained by the immunocapture-PCR method. Prevot et al. (1993) demonstrated a detection limit of 1 PFU/ mL HAV from water, whereas Grinde et al. (1995) routinely detected 10 PFU/mL of rotavirus from seawater. Detection limits of 0.047 PFU from 80 μL of HAV- seeded fecal samples by immunocapture-PCR (Jansen et al., 1990), and 0.04 PFU from 1 mL environmental samples when using a pre-concentration step followed by an immunocapture-PCR (Jothikumar et al., 1998) have also been reported. These variations demonstrate that, despite its greater sensitivity, the immunocapture-

PCR method can be influenced by a number of factors such as the type of virus, types of immunoaffinity solid phase used (i.e., polypropylene surfaces, different types of beads), the types of immunoaffinity capture system used (i.e., beads coated with antibody, or streptavidin beads coated with biotinylated antibodies or oligoprobes), and the type and source of sample (clinical, environmental water or sewage, shellfish, etc.).

Virus Capture and Detection by1MDS Positive Filters and RT-PCR (F-IM-PCR).

The use of 10 mL of 3% BE resulted in up to a 47% (average 36.2 ● 4.03 %) recovery of adsorbed virus from the 1MDS filter, while 38% (average 27.5 ● 8.4 %) was present in the filtrate, indicating that ca 15% of the virus remained on the filter after elution. In contrast, elution with 1 mL of 3% BE resulted in a maximum recovery of 32% (average 21 ± 4.78 %) while ca 30% of the virus remained adsorbed to the filter (Table 8). In both instances, the average efficiency of virus capture by the filter was approximately 62%. When 10 mL of 1% BE was used, up to 41% (average 34.8 ± 2.66 %) of HAV was recovered, compared to 28% (average 19.7 ± 3.42 %) with 1 mL of 1% BE (Table 8). This indicated that approximately 15 and 21 % of the virus remained adsorbed to the filter when 10 mL of 3 and 1% BE, respectively, were used. Therefore, the 1 mL volumes of both 1 and 3% BE resulted in the lowest virus recovery rates from the filters.

The percentage of virus recovery from positive filters reported by other investigators have demonstrated a broad range in recovery rates of from 2 to ≥90%. These differences could be attributed to many factors, such as the type of virus being eluted, the type and thickness of filters used, different types and concentrations of eluents, the pH of the

Table 8. Rates of hepatitis A virus (HAV) recovery from 1MDS filters using different volumes and concentrations of beef extract (BE).

Recovery rates[a]					
Trial	1% BE[a]		3% BE[a]		1% BE[b]
	1 mL eluate	10 mL eluate	1 mL eluate	10 mL eluate	50 mL eluate
1	17[c]	32	10	47	24
2	28	41	32	28	39
3	12	37	17	33	35
4	22	29	25	37	27
Average	19.7 ± .4 %[d]	34.8 ± 2.66 %	21.0 ± 4.78 %	36.2 ± 4.03 %	31.3 ± 3.47 %

[a] Virus recovery rate when 10 mL virus -containing PBS was filtered through 1MDS, and then recovered with 1 or 10 mL of 1 or 3% BE, pH 9.5.

[b] Virus recovery rate when 50 mL virus -containing PBS was filtered through 1MDS, and then recovered with 10 mL of 1% BE, pH 9.5.

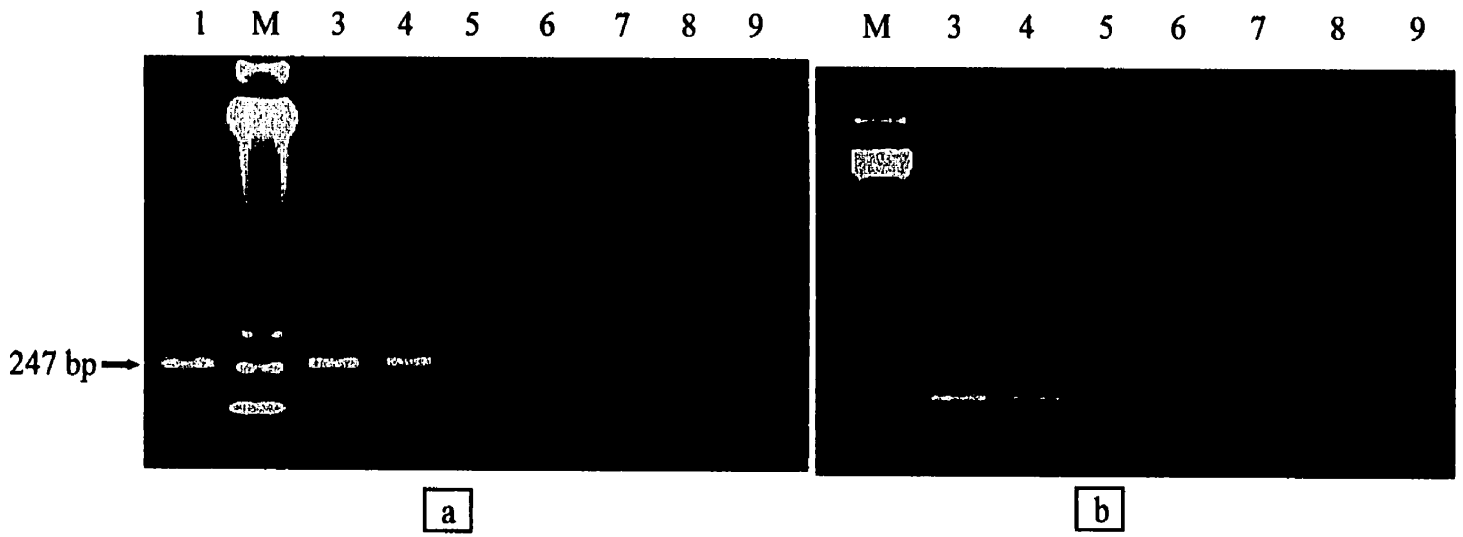
[c] Percent recovery rate.

[d] Standard error of the mean.

sample, the type of sample, as well as additional steps that might have been incorporated in the concentration procedure. Overall, the viral recovery rates observed in our study were comparable to those reported by other investigators who used BE to recover virus from 1MDS filters (Sobsey and Glass, 1980; Sobsey et al., 1985; Borrego et al., 1991; Sobsey, 1993; Schwab et al., 1995). Direct PCR amplification of 2 μL volumes from the 10 mL eluates of both the 1 and 3% BE failed, in most instances, to produce a clear amplification band, whereas a positive band was occasionally seen when using 1 mL of 1% BE, and to a lesser extent a faint band was obtained from the 3% BE. These data reflect similar findings by other investigators with respect to the inhibitory effect of BE on the PCR reaction. Since 1% BE gave comparable recovery to 3% BE, and was less inhibitory to the PCR, further experiments were performed using 10 mL of 1% BE (results not shown).

Since direct RT-PCR detection was not feasible from the 10 mL virus-containing eluates, MAb-coated beads were used to capture and concentrate the virus from the eluate, as well as to separate it from the inhibitory effects of BE. Therefore, the beads were incubated with the eluates, separated, and then amplified by RT-PCR, which resulted in a positive amplification signal. The F-IM-PCR procedure was then further evaluated for its ability to capture, concentrate and detect different concentrations of HAV recovered from inoculated lettuce and strawberries. Our data indicated that around 10 to 50 PFU needed to be present on the lettuce and/or the strawberries in order to produce a positive PCR signal (Fig. 5). Trials with filtering 50 mL of virus-seeded PBS (1 mL virus plus 49 mL PBS) through the 1MDS filter, and virus elution with 10 mL of 1% BE, resulted in comparable efficiencies of virus recovery from the filter (average 31.3 ± 3.47 %), as compared to those obtained when

Figure 5: Sensitivity of detection of hepatitis A virus (HAV) from lettuce and strawberries by the combined filtration-immunomagnetic-PCR (F-IM-PCR). For lettuce (a): Lane 1, HAV positive control; Lane M, DNA Marker ladder 123 bp; Lanes 3 to 9 contain HAV at concentrations of 1000, 100, 50, 25, 10, 1, 0.1 PFU, respectively. For strawberries (b): Lane M, DNA Marker ladder 123 bp; Lanes 3 to 9, contain HAV at concentrations of 1000, 100, 50, 25, 10, 1, 0.1 PFU, respectively.


1 M 3 4 5 6 7 8 9
M 3 4 5 6 7 8 9
247 bp
a
b

10 mL volumes of PBS were used (Table 8). Therefore, comparable rates of virus capture by and elution from the 1MDS filters would be expected if larger volumes (e.g., > 50 mL) were used as washing solutions to recover the virus from larger amounts of produce.

CONCLUSIONS

HAV experimentally inoculated onto lettuce and strawberries was recovered by a washing procedure, resulting in 84 and 81% recovery rates, respectively. Three methods, namely immunomagnetic beads, positively-charged filters, and a combination of both, were used to capture and concentrate the recovered virus, with its subsequent rapid detection by a one-tube RT-PCR system. Although very sensitive, the conventional RT-PCR is limited to a sample volume of only a few microlitres. The IM-PCR, however, successfully detected the same amount of virus in a 1 mL volume, and thus was ≥100-fold more sensitive than the conventional RT-PCR. The IM-PCR method, however, is restricted to maximum volumes of ≤20 mL. Although positively-charged filters captured > 60% of the virus, of which up to 47% was recoverable by different concentrations of BE, direct RT-PCR from the eluates were at best inconsistent in demonstrating a positive PCR signal. The sensitivity of the combined F-IM-PCR method was ≥10 PFU, as compared to 0.5 PFU for the IM-PCR method. However, the potential of the F-IM-PCR method for detecting low levels of virus in a larger sample volume (≥50 mL), makes this procedure of potentially much greater sensitivity than the IM-PCR which is limited to a maximum sample volume of ≤20 mL.

7. HEAT INACTIVATION OF HEPATITIS A VIRUS (HAV) IN DAIRY PRODUCTS

7. HEAT INACTIVATION OF HEPATITIS A VIRUS (HAV) IN DAIRY PRODUCTS

INTRODUCTION

Hepatitis A virus (HAV), the causative agent of hepatitis A, is a member of the Picornaviridae family. Because of its unique genetic and physical characteristics, HAV has now been assigned to a distinct genus, hepatovirus (Minor, 1991). It is a 27 nm icosahedral, non-enveloped virus, containing a 7.5 kb positive-stranded RNA genome, which is enclosed by a capsid protein shell consisting of VP1, VP2, VP3, and VP4 glycoproteins. It has only recently been successfully propagated in cell culture (Flehmig, 1980; Provost and Hilleman, 1979). Although similar to other enteroviruses in morphology and composition, HAV exhibits greater resistance to inactivation by various chemical and physical agents, including heat (Gust et al., 1983b; Flehmig et al., 1985; Sattar et al., 1989; Hollinger and Ticehurst, 1990). Outbreaks of foodborne hepatitis have been well documented epidemiologically with a wide variety of foods, such as shellfish, salads, fruits and dairy products being implicated (Raska et al., 1966; Cliver, 1983; Anon, 1994). Most investigations have focused on the use of heat as a method of inactivating HAV in shellfish. Koff and Sear (1967) indicated that it took 4 to 6 min for the internal temperature to reach 100°C in steamed clams, and Millard et al. (1987) suggested that an internal temperature of 85-90°C for 1 min would completely inactivate HAV in cockles. Kaplan and Melnick (1952) demonstrated that protein-rich foods such as ice cream, cream, and milk can exert a protective effect on poliovirus when exposed to heat. Investigating the effect of heat on the inactivation of HAV in various solutions,

including milk, Parry and Mortimer (1984) indicated that HAV suspended in protein rich media was more resistant to heat and that routine pasteurization was not sufficient to fully inactivate the virus in milk. This study, however, was not conclusive, nor did it provide details on the type of milk used, the experimental procedure, or an indication of the required temperatures for HAV inactivation in dairy products.

Raska et al. (1966) described a hepatitis A epidemic in which 439 individuals became ill due to the consumption of milk, which was contaminated before processing. Further investigation found that the water supply used on the dairy farm was contaminated with raw sewage. As well, a breakdown of the pasteurizer most likely contributed to the survival of HAV. In addition, a recent situation arose where a frontline packaging employee at a major cheese manufacturer in western Canada was diagnosed as being positive for HAV. Since it was not feasible to determine if the cheese lot handled by that employee became contaminated, the cheese had to be re-worked to inactivate any HAV present. However, due to the lack of data with respect to appropriate temperatures required to heat-inactivate the virus in dairy products, the cheese had to be reprocessed at high temperatures (85°C for 1 min) at a considerable expense. This incident, as well as the recent resurgence of foodborne hepatitis A outbreaks, strongly reinforces the need for more relevant data on the inactivation of HAV in foods such as dairy products.

In the present study, we investigated the heat resistance of HAV in dairy products of different fat content, with the objective to determine the appropriate time-temperature combinations required to achieve various levels of HAV inactivation.

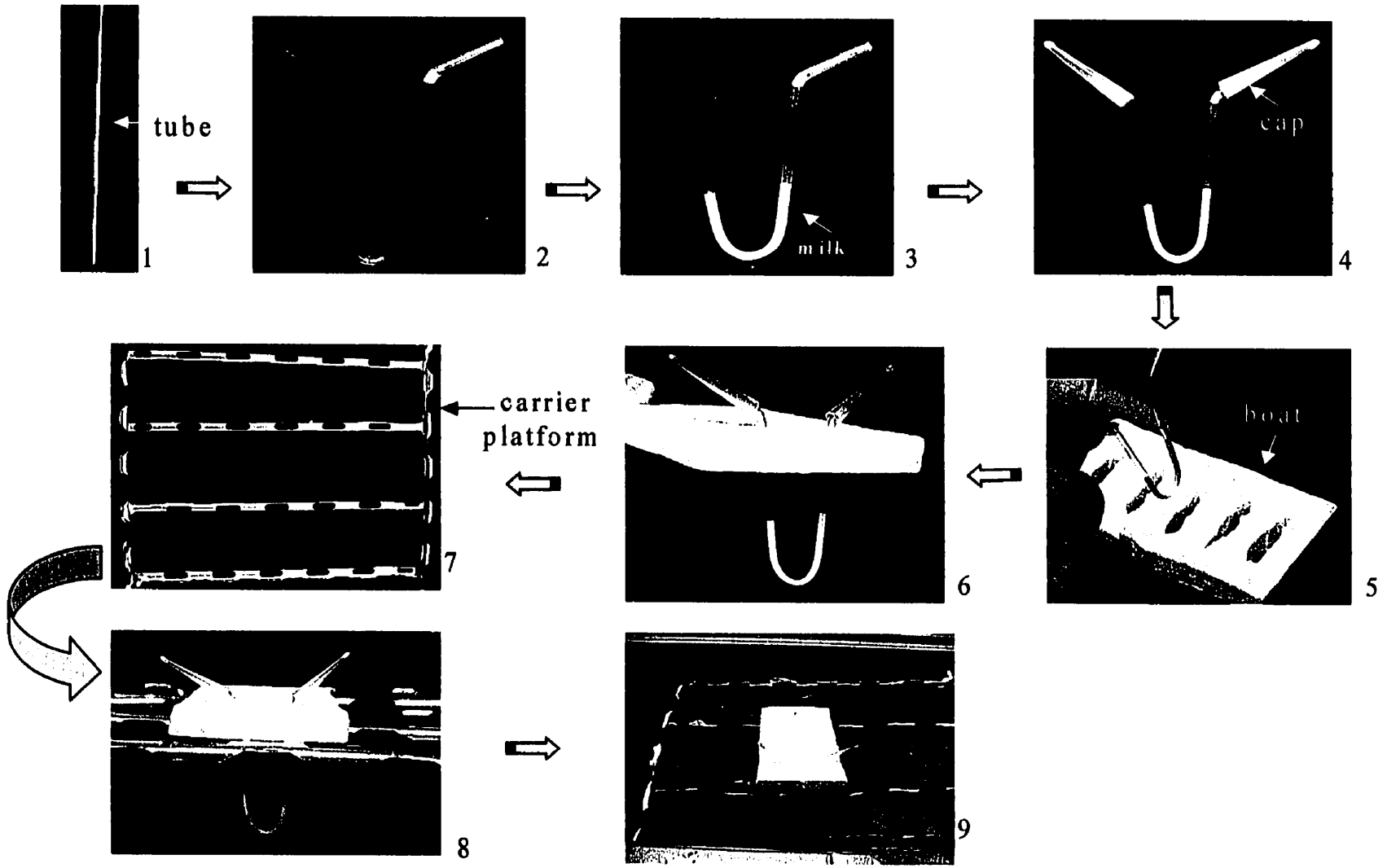
MATERIALS AND METHODS

Dairy products. Three different types of dairy products were chosen for this study; skim milk (0% fat), homogenized milk (3.5% fat), and table cream (18% fat). Each of the dairy products was sterilized by steaming in an autoclave at 121°C for 20 min to destroy any microbial cells present. The dairy products were seeded with HAV at a final concentration of ca 2 x 10^6 PFU/ mL (plaque forming units).

Parameters of heat inactivation. Each of the virus-seeded dairy products was subjected to temperatures of 65, 67, 69, 71,73, 75, 80 and 85°C. At each temperature (except 80 and 85°C), the exposure times were 0.5, 1, 2, 4, 6, 8, 10, 12, 14, and 16 min. For 80°C, exposure times were 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, and 6 min, whereas for 85°C, exposure times were the same as for 80°C, but were not extended beyond 4 min. All heat inactivation tests were conducted using a digitized circulating waterbath (Lauda, Model RMS 20, Baxter; Mississauga, Ont.).

Test procedure. Straight 200 µL capacity disposable microcapillary tubes (Fisher Scientific) were modified by heat to form a U-shaped tube (U-tubes) with two side arms extending outwards, at approximately a 45° angle (Fig. 6). Micropipet tips (200-250 µL capacity) used in Gilson^R pipettors were cut in half, and the half with the wide end, i.e., the end which fits onto the pipettor, was discarded. The narrow fine end of the other half of the tip was sealed off by heat. Both of the U-tubes and the sealed-end tips were sterilized in separate containers by autoclaving at 121°C for 20 min, and allowed to thoroughly dry in a hot air oven. An aliquot (100 µL) of virus-seeded dairy

Figure 6: Methodology for heat-inactivation of hepatitis A virus (HAV) in dairy products. (1) Straight 200 μL capillary tube; (2) The capillary tube was bent by heat to a U-shaped form; (3) HAV-seeded dairy product (100 μL) was introduced into U-shaped microcapillary tubes; (4) The tubes were then capped; (5-6) inserted in a Styrofoam^{R} boat; (7-8) overlayed on a carrier metal platform; and then (9) placed in a waterbath at the desired temperature. The tubes were then removed at the specific times of exposure, the contents retrieved and plaque-assayed to determine the residual virus titre.


tube
1
2
milk
3
cap
4
boat
5
6
carrier
platform
7
8
9

product was introduced into the U-tube through a marked opening in one of the two side arms, followed by capping both openings securely with the sterile sealed-end tips. This was done to prevent possible aerosol contamination of the dairy product, and its leakage from the tube as a result of air pressure build up inside the tube during exposure to heat in the waterbath. For each temperature and time, triplicate U-tubes were tested. To ensure uniformity and consistency of exposure to heat, all virus-seeded U-tubes were immersed instantaneously in the waterbath by using custom-designed floating Styrofoam^{R} boats anchored on a specially-designed aluminum carrier platform (Fig. 1). Briefly, Styrofoam^{R} pieces (boats) measuring 4 cm x 9 cm x 1 cm thick were cut, and slots were made in each boat to accommodate six U-tubes, with the body of each tube protruding through the slot while being held in place by the two side arms (Fig. 1). The aluminum carrier consisted of a 20 x 30 cm rectangular aluminum frame with two aluminum rods welded to its inside, creating three internal space compartments of equal sizes (Fig. 1). This system allowed for the simultaneous testing of up to 60 U-tubes.

Timing was started when the thermocouple inserted into one of the control U-tubes (containing dairy product without virus) indicated that the desired internal temperature in the dairy product had been reached. At the end of each time interval (0.5-16 min), one boat was removed and placed immediately in ice water. The sealing tips at both side arms were removed, and the contents of each of three U-tubes (of the six) was pumped out with a pipettor into 900 μL of PBS in a 1.5 mL microcentrifuge tube, and then serially diluted. A portion (100 μl) of each of the dilutions was then inoculated onto a FRhK-4 cell monolayer in wells in a 12-well cluster plate (Costar, Cambridge,

Mass.) . Each dilution was tested in triplicate wells. The contents of each of the other three U-tubes was directly inoculated into the respective monolayer-containing wells. Positive controls consisted of virus-seeded dairy product without heat treatment, whereas negative controls consisted of uninoculated, unheated product.

Retrieval of the dairy product from the U-tube. Following heat inactivation, the yellow tips were removed from the side arms, and discarded. A sterile micropipet tip, secured on a Gilson^{R} pipettor, was inserted into the marked mouth (opening) of the side arm through which the dairy product was initially introduced. The dairy product was then pumped out through the opening on the other side arm, directly into a microcentrifuge tube. This procedure reduced the possibility of contaminating the heat-exposed dairy product with any virus particles that might have stuck to the side arm through which the product was initially introduced. Prior to heat experimentation, a plaque assay was done to assess the virus titre in the dairy product following retrieval from the U-tube, and to determine if any virus had attached to the side arm through which it was introduced. Repeated trials indicated that the virus titre in the retrieved (output) product was similar to the initial titre.

Statistical Analysis. A 2-phase negative exponential model, extensively used in pharmaco- kinetics where it is referred to as a two compartment model (Gibaldi and Perrier, 1975), was used to analyse the heat inactivation data. The formula used was:

$HAVtitre = 2 \times 10^{7}\left(\propto \times 10^{-\frac{t}{k_1}} + (1-\propto) \times 10^{-\frac{t}{k_2}}\right)$, where $\propto$ denotes the proportion of one of the subpopulations of microorganisms, t denotes the length (min) of heat exposure, and

k_1 and k_2 denote the respective kill rates. Two other models; logistic and the Gompertz (Zwietering et al., 1990) were also used for comparative analysis. Data was analysed using the statistical analysis software package S-PLUS (StatSci, MathSoft, Inc., Seattle, Washington).

RESULTS

For all experiments, the average number of PFU obtained from triplicate wells was used to determine the $\log_{10}$ reduction in virus titre following exposure to heat at various time intervals.

Dairy products. To prevent contamination of the cell culture in the plaque assays, initial attempts were made to filter the dairy products (skim and homogenized milk, and table cream) through a 0.22 μm filter to remove any microbial contaminants that might be present in these products before they were seeded with virus. Due to constant clogging of the filters, this was easily achieved with skim, but not with homogenized milk and table cream. Therefore, these products were sterilized by steaming in an autoclave at 121°C for 20 min, without any noticeable adverse effects.

Effect of dairy products on cell monolayers. None of the dairy products inoculated onto the FRhK-4 cell monolayers in the wells showed any toxic effects on the cell monolayers. Furthermore, plaque counting was not hindered by any obvious opaqueness from the dairy products. Table cream (undiluted) occasionally condensed in certain areas of the well resulting in some noticeable turbidity, but this was confined to the overlay media and did not affect plaque counting. Intermittent gentle shaking of the 12-well plate aided in dispersing and maintaining an even distribution of the cream

over the entire area of the well. In addition, none of the dairy products affected either the overlay removal or crystal violet staining.

Heat inactivation. In assessing the various data obtained from heat inactivation of HAV, three statistical models were used, i.e., the 2-phase negative exponential, the logistic and the Gompertz models. As indicated by the r^2 values, in almost all cases (except for skim milk at 75°C) the 2-phase negative exponential model gave higher r^2 values than either the logistic or Gompertz model, and therefore better predictive values (Table 9). Thus, the 2-phase negative model was used throughout in analysing our data. The patterns of inactivation of HAV in the three dairy products as a function of time and temperature are illustrated in Figs. 7a-c.

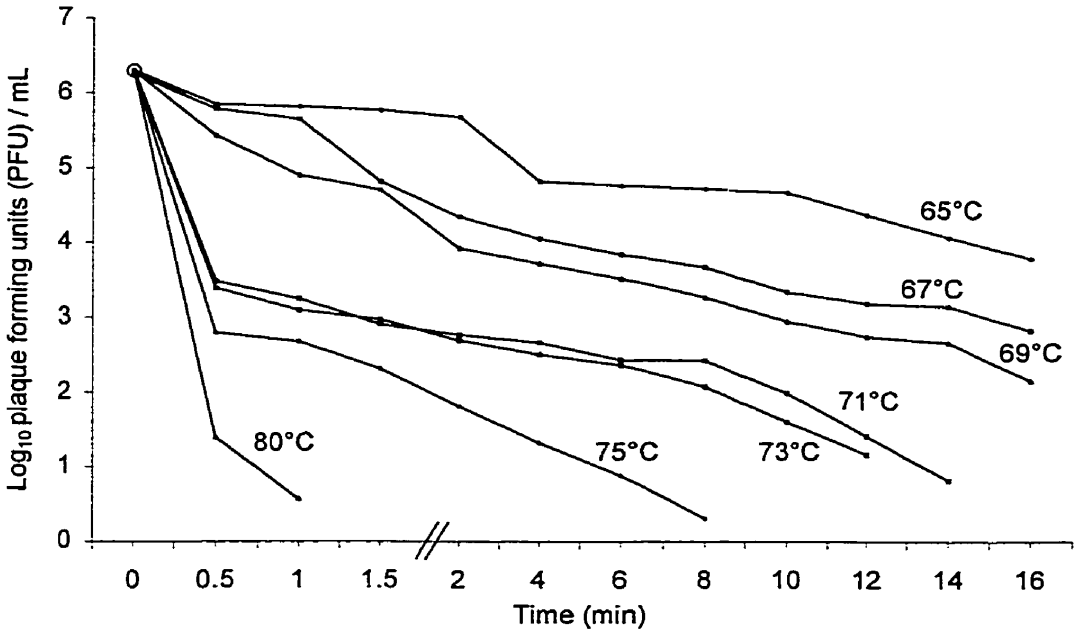
At temperatures of 65, 67, and 69°C, exposure times of 2.68, 1.00 and 0.73 min (skim), 6.20, 1.19 and 0.79 min (homogenized), and 7.34, 2.82 and 1.11 min (cream) were required, respectively, to achieve a 1-$\log_{10}$ reduction in virus titre. For a 4-log reduction at the above temperatures, exposures times of 33.81, 21.69 and 15.56 min (skim), 32.60, 24.99 and 19.00 min (homogenized), and 36.85, 29.59 and 21.88 min (cream) were required, respectively (Table 10). At 71°C, however, exposure times (min) of 0.16 (skim), 0.18 (homogenized), and 0.52 (cream) were sufficient to reduce HAV by 1-log, whereas a 4-log reduction required 6.55, 8.31 and 12.67 min for skim, homogenized, and cream, respectively (Table 10). To achieve a 1-log reduction in titre at 73 and 75°C, exposure times of 0.16 and 0.13 min (skim), 0.17 and 0.15 min (homogenized), and 0.36 and 0.29 min (cream), respectively, were required. Further analysis of the data obtained at 73 and 75°C indicated that 5.27 and 0.89 min (skim),

Table 9. Proportion of variance (r^2) values for three models fitted to reductions of HAV in skim, homogenized milk and table cream.

Milk type	Temperature (°C)	Models		
		2-phase	logistic	Gompertz
Skim	65	0.97	0.95	0.94
	67	0.99	0.97	0.97
	69	0.99	0.98	0.98
	71	0.99	0.97	0.97
	73	0.99	0.98	0.98
	75	0.98	0.99	0.99
Homogenized	65	0.98	0.95	0.94
	67	0.99	0.98	0.98
	69	0.98	0.98	0.98
	71	0.99	0.98	0.98
	73	0.99	0.98	0.98
	75	0.99	0.99	0.99
Cream	65	0.98	0.95	0.93
	67	0.99	0.98	0.97
	69	0.99	0.97	0.97
	71	0.98	0.97	0.97
	73	0.99	0.95	0.95
	75	0.99	0.95	0.95

Figure 7: Pattern of hepatitis A virus inactivation in a) skim milk; b) homogenized milk and c) table cream, as a function of time and temperature. Portions (100 μL) HAV-inoculated dairy products were introduced into U-shaped capillary tubes, and exposed to different temperature and time combinations. The dairy products were then retrieved and plaque-assayed to determine the inactivation parameters of HAV.

(a): Heat inactivation of hepatitis A virus (HAV) in skim milk



(b): Heat inactivation of hepatitis A virus (HAV) in homogenized milk

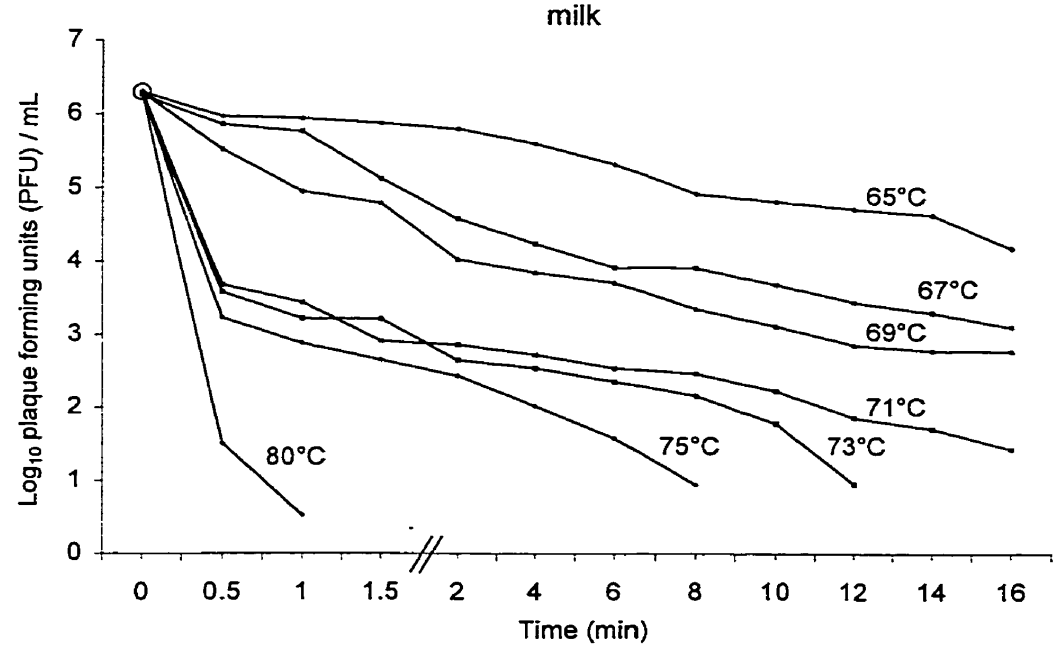


Fig. 7 cont'd

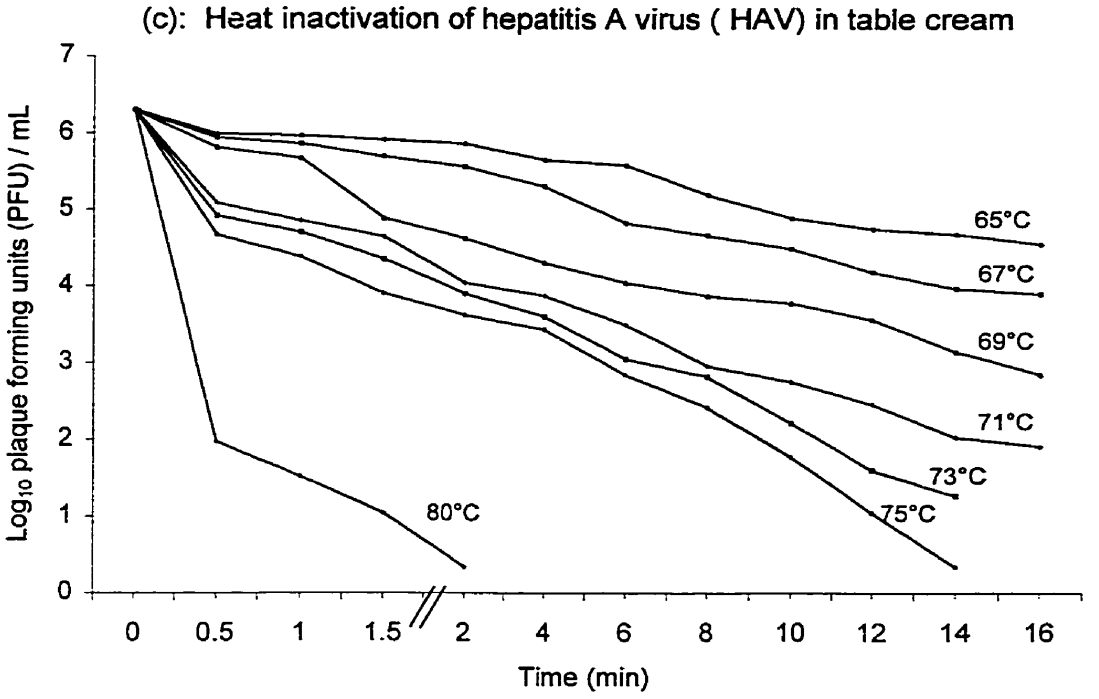

(c): Heat inactivation of hepatitis A virus (HAV) in table cream
Log10 plaque forming units (PFU) / mL
0
1
2
3
4
5
6
7
0
0.5
1
1.5
2
4
6
8
10
12
14
16
Time (min)
65°C
67°C
69°C
71°C
73°C
75°C
80°C

Table 10. Heating time required for a 1 to 5 log reduction of HAV in skim milk, homogenized milk and table cream.

		Skim milk		Homogenized milk		Cream	
Temperature	Log_{10}	Time to reduction	Standard error	Time to reduction	Standard error	Time to reduction	Standard error
C°	reduction	(min)		(min)		(min)	
65	1	2.68[a]	0.51	6.20[b]	0.44	7.34[b]	0.34
	2	11.33[a]	2.17	15.00[ab]	0.89	17.18[b]	0.80
	3	22.57	4.47	23.80	1.37	27.02	1.25
	4	33.81	6.78	32.60	1.84	36.85	1.71
	5	45.06	9.10	41.40	2.32	46.69	2.16
67	1	1.00[a]	0.08	1.19[a]	0.09	2.82[b]	0.28
	2	2.35[a]	0.18	3.13[b]	0.24	11.22[c]	0.90
	3	10.98[a]	1.35	13.60[a]	1.64	20.40[b]	1.60
	4	21.69	2.91	24.99	3.22	29.59	2.31
	5	32.40	4.46	36.39	4.80	38.77	3.02
69	1	0.73[a]	0.06	0.79[a]	0.06	1.11[b]	0.10
	2	1.62[a]	0.12	1.74[a]	0.14	3.83[b]	0.34
	3	7.23[a]	0.65	8.57[a]	1.07	12.82[b]	1.34
	4	15.56	1.53	19.00	2.64	21.88	2.37
	5	23.89	2.42	29.45	4.21	30.94	3.40
71	1	0.16[a]	0.01	0.18[a]	0.01	0.52[b]	0.08
	2	0.32[a]	0.03	0.37[a]	0.02	1.63[b]	0.18
	3	0.56[a]	0.04	0.63[a]	0.04	7.09[b]	0.53

Table 10 cont'd.

	4	6.55[a]	0.43	8.31[b]	0.58	12.67[c]	0.95
	5	13.64[a]	0.93	17.49[b]	1.26	18.24[b]	1.36
73	1	0.16[a]	0.01	0.17[a]	0.01	0.36[b]	0.05
	2	0.32[a]	0.02	0.35[a]	0.02	1.38[b]	0.11
	3	0.52[a]	0.03	0.59[a]	0.04	5.38[b]	0.27
	4	5.72[a]	0.35	5.95[a]	0.43	9.39[b]	0.46
	5	12.81	0.83	12.58	0.96	13.40	0.65
75	1	0.13[a]	0.02	0.15[a]	0.01	0.29[b]	0.04
	2	0.27[a]	0.04	0.30[a]	0.02	0.71[b]	0.07
	3	0.41[a]	0.06	0.48[a]	0.03	4.16[b]	0.19
	4	0.89[a]	0.12	2.80[b]	0.21	8.21[c]	0.36
	5	5.45[a]	1.09	7.11[a]	0.59	12.27[b]	0.54
80	1	0.10	NA	0.10	NA	0.12	NA
	2	0.20	NA	0.21	NA	0.23	NA
	3	0.30	NA	0.31	NA	0.35	NA
	4	0.41	NA	0.41	NA	0.46	NA
	5	0.59	NA	0.68	NA	1.24	NA
85	>5	<0.5	NA	<0.5	NA	<0.5	NA

[a-c] Times to *n* log reductions (when comparing each of the three dairy products at the same temperature and for the same log (reduction) are not significantly different ($p>0.05$) if they share the same superscript. Different superscripts indicate significant ($p<0.05$) differences. If there are no superscripts for a given temperature and log reduction, then there are no significant differences between the milk types.

[d] Data for 85°C is not listed because complete viral inactivation occurred in ≤0.5 min.

5.95 and 2.80 min (homogenized), and 9.39 and 8.21 min (cream) were sufficient to achieve a 4-log reduction in HAV titre (Table 10). For all dairy products exposed to 80°C, ≤0.46 min was required to cause a 4-log reduction in titre, whereas 0.59 min (skim), 0.68 min (homogenized) and 1.24 min (cream) were needed to cause a 5-log reduction. At 85°C, a 5-log reduction in titre in all dairy products was obtained in < 0.5 min (Table 10).

DISCUSSION

When compared to other viruses, HAV is quite resistant to inactivation by various chemical and physical methods (Mbithi et al., 1990, 1993; Thraenhart, 1991). Heat, however, can be an effective means of inactivating HAV. In studies done in other labs, complete inactivation (> 6 $\log_{10}$ PFU/mL) of HAV in PBS was observed in 5 sec at 85°C, 30 sec at 75°C and 30 min at 62.8°C (Millard et al., 1987), while only partial inactivation (infectivity was reduced by > 10^4) of tissue-culture propagated HAV was demonstrated when the virus was exposed to 85°C for 1 min (Scheid et al., 1982). HAV, however, exhibited more heat stability and was only partially inactivated when suspended in protein-rich solutions. This was demonstrated by the appearance of the disease in human volunteers who were fed both virus-containing fecal material or serum heated at 56°C for 30 min (Havens, 1945; Ward et al., 1958). In addition, heating virus-containing milk at 62.8°C for 30 min and 71.6°C for 15 sec resulted in only partial inactivation of HAV as indicated by a 3- and 2-log reduction in virus titre, respectively (Parry and Mortimer, 1984). Complete inactivation of HAV in serum pool was only achieved at 98°C for 1 min (Krugman et al., 1970).

In our study, the effect of heat on the inactivation of HAV in three different dairy products was investigated to determine the time and temperature combinations required for HAV inactivation, and to examine the effect of different levels of fat on the heat stability of the virus. The heat inactivation studies covered a wide range of temperatures, and included those generally used for pasteurization, as well as those used to inactivate HAV in shellfish (i.e., 80°C). To ensure even and simultaneous exposure to temperature, an apparatus was devised using custom-made devices and capillary tubes to accommodate the test samples. The use of narrow diameter thin-walled capillary tubes allowed for an even and quick distribution of heat, with the desired internal temperature being reached within 3-5 sec. This also allowed for cooling down (in ice water) of the dairy products within a few seconds. The design of the carrier platform allowed for the simultaneous exposure of all capillary tubes at any one particular test temperature over the entire incubation period. Thus, this system significantly reduced possible variation with respect to temperature and exposure period. The use of floating boats also contributed to the ease of handling many test samples at the same time.

Our inactivation data indicated two distinct patterns. The first was observed at the higher temperatures of 80 and 85°C, where a linear sharp decline in virus titre was observed. The second pattern was seen at temperatures ranging between 65 and 75°C, where a non-linear and slower mode of inactivation was demonstrated over a much broader time. HAV survival kinetics at these lower temperatures exhibited an initial phase of rapid virus inactivation within the first 1 min, followed by a plateau phase of slower virus inactivation. A possible explanation for the occurrence of these two

distinct patterns is that exposure to heat within the first minute might have resulted in inactivation of freely suspended viral particles, along with the virus particles present on the outside of the viral aggregates. This may have left the remaining population of viruses in an aggregated form, which would have required longer times of exposure to achieve the same level of inactivation. In general, the longer times needed to achieve the same log reduction of HAV in cream as compared to milk could have been due to the high fat content (18%) in the product protecting the virus, thus rendering it more heat resistant.

Three different statistical models were compared in the assessment of the heat inactivation data. Although the 2-phase model was used since it described the data well, there are also good empirical reasons for choosing this model. It has generally been found that, with heat treatment, the inactivation of microbial pathogens (particularly bacteria) follows a negative exponential decrease. If there are two subpopulations of microorganisms each having different heat sensitivities, then the two-phase exponential model should more accurately describe the overall reduction in the levels of microorganisms. Other models that have been applied in the literature to describe non-linear inactivation kinetics are the logistic and the Gompertz models (Zwietering et al., 1990). These latter models, however, are more suitable for use when the data are sigmoidal in shape, rather than the distinct 2-phased data which was observed in our study. An illustration of this was the higher r^2 values obtained with the 2-phase model in almost all cases (except for skim milk at 75°C) as compared to the other two models (Table 9). Inactivation of HAV in both skim and homogenized milks

followed a similar pattern of inactivation, whereas HAV in table cream required a greater amount of heat to reach inactivation levels comparable to those obtained in skim and homogenized milks (Figs. 7a-c). This could be explained by the fact that both skim and homogenized milks are more similar in their fat content (0 and 3.5% fat), whereas table cream at the higher fat content of 18% might have protected the virus. Extrapolation of our data suggests that the current minimum standards for high temperature short time (HTST) pasteurization in the USA (71.7°C /15 sec) would result in approximately a 1.58 and 1.43 log reduction in HAV titre in skim and homogenized milk, respectively, whereas for cream (HTST 74.7°C /15 sec), a 0.87 log reduction would be achieved. The data generated in this study provides information that can also be used by dairy manufacturers for the re-processing of contaminated products.

8. INACTIVATION OF HAV IN FRUITS AND VEGETABLES BY GAMMA IRRADIATION

8. INACTIVATION OF HEPATITIS A VIRUS (HAV) IN FRUITS AND VEGETABLES BY GAMMA IRRADIATION.

INTRODUCTION

With the increasing demand for fresh fruits and vegetables, as well as market globalization, an increase in foodborne illness due to produce is a real possibility as a result of contamination of foods with pathogenic microorganisms. One such pathogen, hepatitis A virus (HAV), has been implicated in a variety of food-related outbreaks. Recent foodborne outbreaks of hepatitis A (Dalton et al., 1996; Anon, 1997) underline the need to investigate proper means to inactivate this virus in foods such as produce. Although heat is used with considerable success in inactivating most microbial pathogens, it may not be applicable to foods such as fruits and vegetables which are mostly consumed raw or after minimal processing. In addition, chemicals such as chlorine which are commonly used in the produce industry are, for the most part, ineffective against viruses (Hollinger and Ticehurst, 1990; Mbithi et al, 1990). Gamma irradiation offers a safe alternative as a food decontamination method (WHO, 1981, 1994; Diehl, 1992; Patterson, 1993; Monk et al., 1995; Farkas, 1998). Gamma ionizing radiation has been the focus of numerous investigations with respect to its use and effectiveness in the inactivation of bacteria (Clavero et al., 1994; Monk et al., 1994, Corry et al., 1995; Monk et al, 1995; ; Collins et al., 1996; Byun et al., 1998; Farkas, 1998) and parasites (Sivinski, 1985; Dubey and Thayer, 1994) in foods. Currently, greater than 40 different types of foods in more than 32 countries are subjected to gamma irradiation for sprout inhibition, pest quarantine control, as well as shelf-life

extension (Olszyna-Marzys, 1992; Farkas, 1998). The widespread application of gamma irradiation used for such purposes is, however, limited due to a number of factors which include the effect of gamma rays on the texture, appearance, and sensory palatability of food products, as well as consumer concerns (Diehl, 1992; Patterson, 1993; Monk et al., 1995; Farkas, 1998). Although there have been a number of research studies directed at examining the effects of gamma irradiation against bacterial and/or fungal contaminants, only a limited number of them have addressed the use of gamma irradiation to inactivate viruses in various biological fluids and food products (Sullivan et al., 1971, 1973; Thomas et al., 1981,1982; Elliott et al., 1982; White et al., 1990; Mallett et al., 1991). Because of their small size and genetic make-up (particularly single stranded RNA), viruses have been shown to be more resistant to inactivation by ionizing radiation than bacteria, parasites, or fungi (Farkas, 1989; Monk et al., 1995; Patterson, 1993). The objective of this study was to determine the D-values required to inactivate HAV in fruits (strawberries) and vegetables (lettuce).

MATERIALS AND METHODS

Irradiation source. A Gamma Cell 220 irradiator, using a cobalt (^{60}Co) source (Nordion International Inc., Kanata, Ontario, Canada) and delivering 0.4 Mrad (4 kGy) per hour was used in this study.

Preparation of lettuce and strawberry samples. Romain lettuce and strawberries were purchased locally. Individual lettuce leafs were cut into intact rectangular pieces of approximately 3.5 cm x 8.5 cm, washed and UV-sterilized as described in General Materials and Methods. Each piece of lettuce was placed inside a sterile 50 mL

centrifuge tube, whereas each strawberry was placed inside a sterile 50 mL beaker. A portion (10 µL) of a known plaque forming unit (PFU) concentration of HAV was spread evenly over a demarcated area of approximately 2 cm x 1 cm along the length of the lettuce stem, and over approximately a 1-cm diameter area on each strawberry, and allowed to dry in a laminar flow hood for 20-30 min. The centrifuge tubes were then re-capped, the beakers covered with UV-sterilized parafilm, and both incubated overnight at 4°C. The lettuce-containing centrifuge tubes were positioned vertically in a 2-L capacity glass beaker, which was then placed inside the housing section of the gamma cell. For the strawberries, the small beakers containing the berries were placed inside the 2-L beaker. Uninoculated lettuce and strawberries, serving as irradiated controls, were also included with the inoculated samples during irradiation. Both positive (HAV-inoculated) and negative (uninoculated) unirradiated controls were also used. Exposure of the inoculated and uninoculated lettuce and strawberries to gamma irradiation was done at ambient temperature. After total average doses of 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 kGy, samples (lettuce or strawberries) were removed, stored at 4°C (< 3h), and then processed for virus recovery and plaque assay.

Statistical Analysis. A linear to the log scale and a logistic model were used to determine D_{10} values. For the linear model, the following formula was used:

$$\text{HAV titre} = 10^{\left(b - \frac{r}{d}\right)}$$

, where b was the $\log_{10}$ initial load (= 6.003 ± 0.042), d the increase in radiation that leads to a 1-$\log_{10}$ reduction in HAV load (= 2.72 ● 0.05), and

r the level of radiation in kGy. The formula used for the logistic model was:

$$\text{HAV titre} = \frac{10^b}{1 + Ar^k}$$, where b is the $\log_{10}$ initial load (= 5.68 ± 0.05), $A = 10^{-a}$, where a =2.46 ± 0.19, k =2.36 ± 0.09 and r is the level of radiation in kGy and (Gerwen and Zwietering, 1998). Analysis of variance by the log linear and the logistic models was performed using the Statistical Analysis System S-PLUS (StatSci, MathSoft, Inc., Seattle, Washington).

RESULTS

Virus recovery. Please refer to General Materials and Methods for details. Plaque assays demonstrated that an average of 87 ±6.8 and 83 ± 7.2% of the virus was recovered from the un-irradiated lettuce and strawberry controls, respectively. This was used as the baseline virus titre when determining the PFU count following produce exposure to gamma irradiation.

Effect of gamma irradiation on the inactivation of HAV in lettuce and strawberries. Plaque assays of HAV following exposure to various doses of gamma irradiation indicated that there was a decrease in virus titre (PFU) as the irradiation dose was increased (Figs. 8 and 9). Table 11 illustrates the gradual log reduction in HAV as a result of exposure to gamma irradiation doses ranging between 1 and 10 kGy. Doses of 2.72 and 2.97 kGy were required to reduce HAV populations by 1 log (90% kill) on lettuce and strawberries, respectively (Figs. 8 and 9). There was no noticeable deterioration in the texture or appearance of either the lettuce or the strawberries, even at the highest dose of 10 kGy.

Figure 8. Inactivation of hepatitis A virus in lettuce using gamma irradiation. HAV-inoculated lettuce pieces were exposed to irradiation doses ranging between 1 to 10 kGy; the residual virus was then recovered and plaque-assayed. Both the log-linear and logistic models were used to analyse the data.

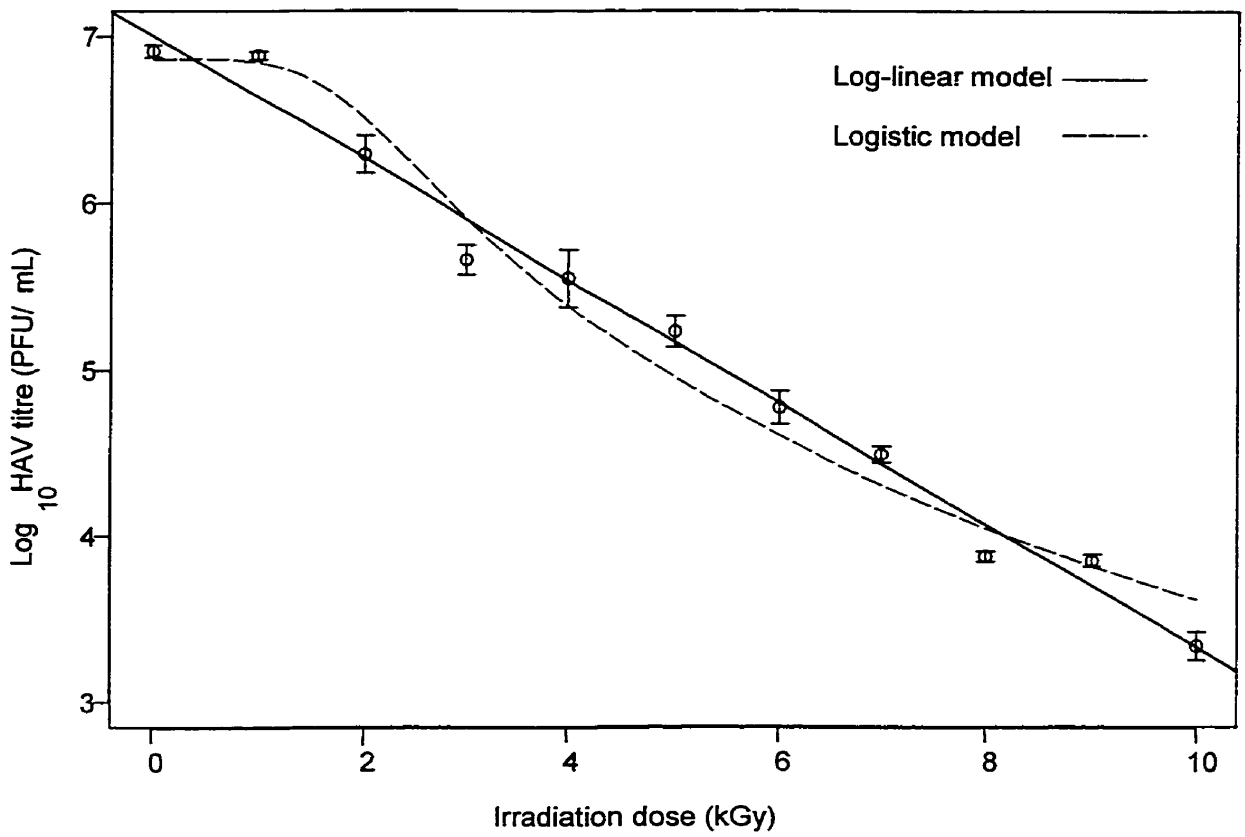

Log-linear model
Logistic model
Log 10 HAV titre (PFU/ mL)
7
6
5
4
3
0
2
4
6
8
10
Irradiation dose (kGy)

Figure 9. Inactivation of hepatitis A virus on strawberries using gamma irradiation. HAV-inoculated strawberries were exposed to irradiation doses ranging between 1 to10 kGy; the residual virus was then recovered and plaque-assayed. Both the log-linear and logistic models were used to analyse the data.

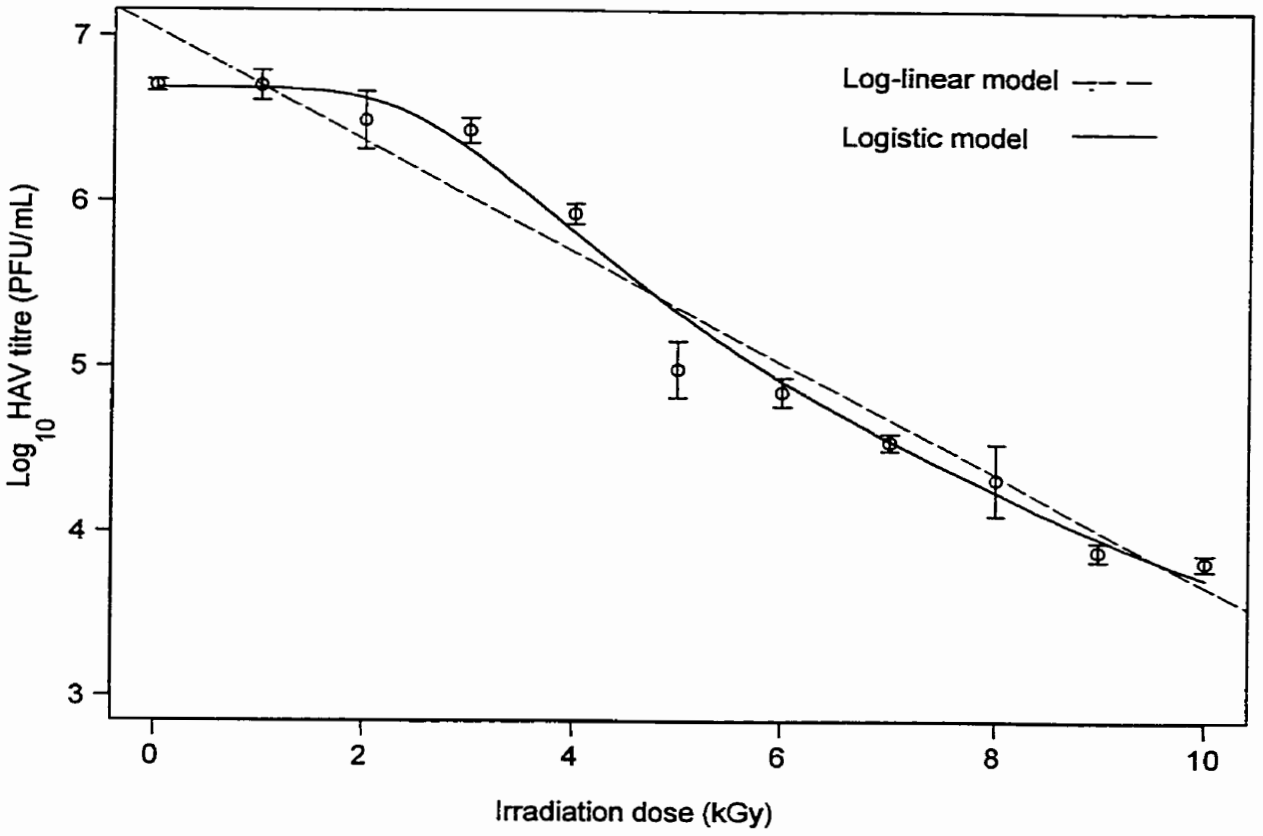

Log-linear model
Logistic model
Log10 HAV titre (PFU/mL)
7
6
5
4
3
0
2
4
6
8
10
Irradiation dose (kGy)

Table 11. The effect of various gamma irradiation doses on the survival of hepatitis A virus in lettuce and strawberries.

Irradiation dose (kGy)	Log_{10} HAV titre (PFU/mL)	
	Lettuce	Strawberries
1	6.90 ± 0.00[a]	6.60 ± 0.02
2	6.20 ± 0.10	6.41 ± 0.06
3	5.60 ± 0.00	6.41 ± 0.06
4	5.50 ± 0.10	5.89 ± 0.06
5	5.20 ± 0.10	5.00 ± 0.00
6	4.77 ± 0.67	4.81 ± 0.07
7	4.47 ± 0.01	4.50 ± 0.01
8	3.87 ± 0.02	4.30 ± 0.01
9	3.85 ± 0.07	3.88 ± 0.17
10	3.36 ± 0.06	3.83 ± 0.02

Inoculated lettuce and strawberries were exposed to 1 to 10 kGy doses of gamma irradiation. Samples were removed at 1kGy intervals, the virus recovered and plaque-assayed for residual titre.

[a] Log_{10} HAV titre ±SE.

DISCUSSION

Gamma irradiation is re-emerging as an alternative technology for pathogen inactivation in foods, especially for produce. With today's increasing demand for high-quality ready-to-eat foods including fresh-cut fruits and vegetables, irradiation, possibly in combination with other processes, may provide a suitable means of enhancing product safety.

Currently, standards exist for the maximum allowable gamma irradiation dose that can be used for different foods. These WHO standards which are based on nutritional, toxicological and microbiological criteria (WHO, 1981; 1994), indicate that irradiation doses of $\leq$10 kGy for any purpose are considered safe. A number of studies have determined D values for the inactivation of different viruses, mostly in cell culture fluid, biological, and environmental samples. However, only a few studies have examined the use of gamma irradiation on viruses in or on foods. This has included work on poliovirus in fish fillets (Heidelbaugh and Giron, 1969), coxsackievirus B in ground beef (Sullivan et al., 1973), and rotavirus and HAV in clams and oysters (Mallett et al., 1991). Based on WHO recommendations (1994) and what is currently acceptable for use, irradiation doses ranging from 1 to 10 kGy were used in our study. Strawberries were selected to represent a fruit type which has been implicated in recent outbreaks, and lettuce was selected as a representative of a vegetable product which is widely consumed in salads and sandwiches, and has been epidemiologically associated with various hepatitis A outbreaks (Anon, 1993; Cliver, 1985).

Statistical analysis indicated that the log-linear model provide a better fit than the logistic model for lettuce, whereas the logistic model was found to provide a better fit than the log linear model for the strawberries.

The effectiveness of gamma irradiation is dependent on various factors such as the size of the virus, the suspension medium and/or type of food product, and the temperature of exposure (Patterson, 1993; Farkas, 1998). Comparison of the data with those of other investigators showed that our D_{10} values were similar to those of Mallett et al. (1991), who reported values of 2.0 kGy for HAV and 2.4 kGy for rotaviruses in both clams and oysters. Our D_{10} values for HAV were lower than the D_{10} value of 3.1 kGy found for poliovirus in clams and oysters (Mallett et al.,1991), and 7 kGy for coxsackievirus B-2 in ground beef (Sullivan et al., 1973). The reason for the greater resistance to gamma irradiation exhibited by polio- and coxsackieviruses is not clear, since these viruses are similar structurally and genomically to HAV. However, it is possible that this increased resistance might have been due to the type of food material (e.g., shellfish, ground beef) in which these viruses were present This is supported by previous findings that the D_{10} values for polioviruses II and III ranged between 4.1 and 5.4 kGy when these viruses were suspended in maintenance medium + 2% serum, compared to a D_{10} value of 1.1 kGy for poliovirus III when suspended in distilled water (Sullivan et al., 1971).

Currently in the US, doses of up to 1 kGy are permitted on produce for purposes of pest disinfestation and sprout inhibition (Nordion International Inc., Kanata, Ont., Canada-personal communication). Based on our data, this would result in approximately

a 0.02 $\log_{10}$ reduction in HAV titre on lettuce and strawberries. Thus, although gamma irradiation may be effective in reducing bacterial spoilage organisms, irradiation at the currently allowable doses would result in less than a 1-log reduction of HAV in such produce. Thus, either higher doses of gamma irradiation or the use of a combination of gamma irradiation plus other hurdles, such as high-intensity UV light or ozone treatment (Byun et al., 1998), would provide for a greater level of HAV inactivation in these products.

9. SURVIVAL OF HAV ON LETTUCE STORED UNDER MODIFIED ATMOSPHERE PACKAGING (MAP) ENVIRONMENTS

9. SURVIVAL OF HAV ON LETTUCE STORED UNDER MODIFIED ATMOSPHERE PACKAGING (MAP) ENVIRONMENTS.

INTRODUCTION

The past decade or two has seen a major increase in worldwide trade, as well as in consumer demand for a wide variety of fresh and minimally-processed foods that are free of pesticides and chemical preservatives such as salt and nitrite. Technological advances have led to the development of a number of packaging techniques that have been shown to contribute significantly to the extension of the shelf-life of various types of foods. Vacuum packaging, controlled atmosphere packaging and modified atmosphere packaging are the most commonly used techniques used by the food industry to prolong the shelf-life of foods.

In vacuum packaging, the food is placed in a plastic package of low O_2 permeability, the air evacuated and the package sealed. As the food product and contaminating microorganisms consume the trapped residual O_2, elevated levels of CO_2 are produced inside the package. In controlled atmosphere packaging, food products in bulk, such as apples and pears, are stored in enclosed warehouses to maintain a certain atmosphere around the product as a result of the natural respiratory activity of the fruit, which reduces O_2 and increases CO_2 sufficiently to slow ripening and slow deterioration. Modified atmosphere packaging (MAP) is a process which involves the packaging of food so that the atmosphere is different from the normal composition of air (78.08% N_2,20.96% O_2, 0.03% CO_2, variable traces of water and traces of inert gases). Modified

atmospheres can be established by flushing with gas mixtures of desired composition, or by passively allowing air-packed products to generate a modified atmosphere inside the package as a result of natural respiration. The resulting modified atmospheres extend the shelf life of foods by inhibiting chemical, enzymatic and microbial spoilage.

Controlled atmosphere packaging (CAP) of fresh fruits and vegetables was named one of the 10 most significant innovations in food science between 1939 and 1989 by the Institute of Food Technologists (Anon., 1989). CAP relies on the use of reduced levels of O_2 while maintaining the food under a controlled environment of increased CO_2 or selective mixtures of gases to limit respiration and ethylene production, thereby delaying and/or inhibiting chemical, enzymatic and microbial spoilage.

More than 4% of all fresh vegetables in the USA, 75% of California strawberries and up to $40 million (US) worth of fresh-cut vegetables for retail are distributed under MAP conditions. Overall, MAP technology represents up to $4 billion in sales of fresh produce (Anon., 1991), and is expected to reach $8 billion by the year 2000.

Three gases are generally used in MAP: nitrogen (N_2), oxygen (O_2), carbon dioxide (CO_2) or various mixtures of the three. The choice of gas mixture used depends on the type of product and its expected shelf life (Goodburn and Halligan, 1988) . MAP exerts a dual effect in its role of prolonging the shelf-life of food products such as fruits and vegetables. The first of MAP's effects is on the physiological nature of the food. The basic mechanism by which MAP operates is that elevated CO_2 and/or low O_2 reduce ethylene production, which directly affects both the chlorophyll and carotenoid components of the food. Ethylene is a colorless, odourless and tasteless gas which is

considered a plant hormone. It induces rapid ripening and overripening in many fruits, and premature yellowing in many vegetables. Decreased ethylene production reduces the chlorophyllase enzyme, thus reducing the breakdown of chlorophyll and subsequently delaying food degradation (Weichmann, 1986; Martens and Baardseth, 1987). Carotenoids are fat-soluble pigments which impart color to fruits and vegetables. They vary in their stability but, due to their unsaturated nature, are generally susceptible to oxidation. Ethylene is known to accelerate the biosynthesis of carotenoids, therefore the reduction in ethylene production which occurs in MAP also reduces the biosynthesis of carotinoids, thereby delaying the appearance of color to fruits and vegetables (Zagory, 1995).

In addition to its physiological role, MAP also prolongs the shelf-life of food by inhibiting the growth of contaminating spoilage microorganisms such as bacteria and fungi. CO_2 selectively inhibits the growth of gram-negative bacteria (Enfors and Molin, 1981) such as *Pseudomonas* spp., which typically grow rapidly and produce the off-flavours and odours associated with food spoilage. Most of the CO_2 dissolves in the food material or enters the bacterial cell. CO_2 is thought to inhibit bacterial growth by lowering the intracellular pH which slows down or inhibits the activity of many intracellular enzymes. Other bacteria, such as the lactic acid bacteria, are not usually affected by high CO_2 levels. Thus, they tend to predominate in MAP foods and help to prolong shelf life by producing compounds which are inhibitory towards spoilage bacteria (Dodds, 1995).

Different types of packaging films have been used to maintain the modified atmospheres required for various products. These films consist of plastic polymers that provide protection, strength, sealability, clarity and a printable surface. They vary in thickness, as well as permeability to O_2 and CO_2 gases, thus restricting the movement of these gases through the bag and allowing for the establishment of a modified atmosphere. The most commonly used films for MAP of fresh produce are made of low density polyethylene (LDPE) or polypropylene (PP) which is characterized by a high gas, but low water vapour permeability. High permeability films allow greater escape of gases such as CO_2 from the package, whereas low permeability ones restrict the movement of gases. The type of film or plastic bag used is dependent on the type of food product and its gas requirements and respiration characteristics (Brody, 1995; Zagory, 1995). The largest use of MAP or vacuum packaging for produce is for fresh-cut lettuce, which accounts for about 15% of the total California iceberg lettuce. Almost 750 million pounds of lettuce are MA packaged annually in the USA, as well as over 150 million packages of other freshly prepared vegetables (Brody, 1995).

The ability of elevated CO_2 and reduced O_2 to inhibit microbiological spoilage of fresh-cut produce is one of the most compelling reasons for the use of MAP. However, one of the major concerns associated with the use of MAP is that of product safety. The desired suppression of spoilage microorganisms may create opportunities for slower growing and potentially pathogenic bacteria (Genigeorgis, 1985; Hintlian and Hotchkiss, 1987; Farber, 1991). Therefore, there is concern that the delay of product spoilage by MAP allows many contaminating pathogens an extended period of growth, which may

render the product potentially hazardous before it is overtly spoiled (Dodds, 1995). Fresh, whole produce has not traditionally been associated with foodborne disease. However, produce can become contaminated with a variety of pathogenic microorganisms either during processing, handling by an infected foodhandler, or when irrigated with and harvested from fecally-contaminated soils. This is especially important when dealing with psychrotrophic pathogenic microorganisms such as *Listeria monocytogenes*, non-proteolytic *Clostridium botulinum*, *Aeromonas hydrophila* and *Yersinia enterocolitica*, all of which can grow at refrigeration temperatures. In addition, *Salmonella*, *Staphylococcus aureus* and *Bacillus cereus* can grow at elevated refrigeration temperatures of between 5 to 12°C (Genigeorgis, 1985; Hintlian and Hotchkiss, 1987).

Although MAP inhibits the growth of bacterial and fungal spoilage micro-organisms, its effect on the survival of enteric viruses, including HAV, has not been investigated and remains unknown. Increase in demand for fresh-cut produce is associated with an increase in trade, mass production and handling of these foods. This provides a greater potential for the contamination of foods with HAV, which has been associated with foodborne outbreaks Therefore, in this study, experiments were designed to investigate the effect of different modified atmospheres on the survival of HAV on lettuce stored at 4°C and room temperature.

MATERIALS AND METHODS

Inoculation and recovery of HAV from lettuce. Please refer to General Materials and Methods for details. Each piece of lettuce was placed in a clean and UV-

treated weighing boat, and 10 μL of virus inoculum was spread over the demarcated area on the lettuce, allowed to dry and recovered as described in previous sections.

Storage of inoculated lettuce under normal and modified conditions. Once the virus inoculum on the lettuce had dried, the weighing boats accommodating the inoculated pieces of lettuce were processed and stored as follows:

1. Boats containing HAV-inoculated and un-inoculated lettuce pieces were placed inside large plastic petri-dishes (150 x 15 mm). Approximately 5 mm V-shape cuts were made at the corners of each boat to allow for air and humidity exchange after the petri-dish lid was placed on top of the boat. Moisture was provided by placing a moistened piece of cloth inside the petri-dish. These experiments were designed to determine the survival of HAV on lettuce stored in open air.

2. Another batch of boats containing HAV-inoculated and un-inoculated lettuce pieces was inserted into10 cm x 21 cm Cryovac barrier plastic bags of low O_2 permeability (3-6 cc/100 in^2-day-atm, 4.4°C and 0% RH). The plastic bags were then processed as follows:

   1) one set of bags was heat-sealed using an impulse MP-12 sealer (Chiswick, Mississauga, Ont.). This represented lettuce stored in normal atmospheric air (21% O_2, 78% N_2, 0.03% CO_2).

   2) one set of bags was placed in a Multivac heat-seal packaging machine model A 300/16 (Knud Simonsen Industries Ltd., Rexdale, Ont.) connected to a proportional gas mixer. The gas mixtures delivered to the plastic bags consisted of the following percentages of CO_2:N_2; 30:70, 50:50, 70:30; as well as 100% CO_2 (Please see Appendix A).

3. HAV was seeded into 20 mL of PBS, pH 7.2, at a concentration of 2 x 10^5 PFU/mL PBS. The PBS was divided into two 10 mL portions. One portion was incubated at 4°C, whereas the second was incubated at room temperature. This experiment was designed to determine the survival of HAV in PBS, as compared to survival on lettuce.

Sample incubation and processing. Inoculated and un-inoculated lettuce samples were divided into two batches with one incubated at 4°C and the second at room temperature. The experimental design of this study is illustrated in Table 12.

Samples were removed on days 1, 3, 6, 9 and 12 to determine virus survival by the plaque assay. On each of these sampling days, samples were removed (in triplicate) and were processed. Prior to opening the plastic bags to recover the virus from the lettuce, the air inside the bags was analysed for its gas content. For gas analysis: a 1-cm diameter sticky Nickels Adhesive (MOCON-Modern Control Inc., Minneapolis, MN) was firmly pressed onto each plastic bag. The needle of a Pressure-Lok® Series A-2 syringe (Chromatographic Specialty, Brockville, Ont.) was then inserted through the sticky button, and a 0.1 mL air sample was withdrawn. The air sample was analysed with a Varian 3300 Gas Chromatography (GC) instrument (Varian Canada Inc., Mississauga, Ont.). The results of the analysis were recorded and plotted automatically by a Varian 4270 Integrator (Varian Canada). The plastic bags were then cut-open inside a laminar flow hood, and the boats containing the lettuce were retrieved. The lettuce was inspected visually to assess any changes in the color and texture of the lettuce. The virus was then recovered from all pieces of lettuce, including those that were incubated in petri-dishes. Virus recovery was done as was described in General Materials and Methods.

Table 12. Experimental design for the survival of hepatitis A virus (HAV) on lettuce stored under modified atmosphere packaging (MAP) environments at room temperature and 4°C.

	Petri-dish[a] (open air)	Sealed air[b] in a bag (O_2:N_2:CO_2)	Modified gaseous composition[c]				HAV in PBS[e]
			30 : 70 CO_2: N_2	50 : 50 CO_2: N_2	70 : 30 CO_2: N_2	100% CO_2	
DAY 1[d] : RT	3[d]	3	33	33	33	33	Tubes[e]
4°C	3	3					
DAY 3 : RT	33	33	33	33	33	33	
4°C							
DAY 6 : RT	33	33	33	33	33	33	
4°C							
DAY 9 : RT	33	33	33	33	33	33	
4°C							
DAY 12 : RT	33	33	33	33	33	33	
4°C							

[a] Weighing boats containing HAV-inoculated and un-inoculated lettuce (3 pieces of each) were placed inside petri-dishes containing moistened cloth (for humidity). The corners of the boats were cut into a v-shape to allow for a flow of air in and out of the boats.

[b] Boats containing inoculated and un-inoculated lettuce were place in a plastic bags and heat sealed.

[c] Boats containing inoculated and un-inoculated lettuce were place in a plastic bags, the atmospheric air was vacuumed out and replaced by different gaseous mixtures (as indicated above), and the bags were heat-sealed automatically.

[d] Lettuce samples were incubated at both room temperature (RT) and 4°C. Samples were removed on days 1,3,6,9 and 12, and virus recovered and plaque-assayed for virus titre. Samples (inoculated and un-inoculated controls) were tested in triplicate.

[e] Tubes containing phosphate-buffered saline (PBS) seeded with a known virus titre, were incubated at RT and 4°C for 12 days, and samples werewithdrawn on the same days of lettuce sampling and plaque-assayed to determine virus survival in PBS.

Serial decimal dilutions were made from the recovered virus solution, and were plaque assayed to determine the virus titre, as was described in General Materials and Methods.

Statistical analysis. The ANOVA model used was as follows:

$\log_{10}$ HAV titre$_{ij} = \mu + P_i + \beta d + \gamma_i d + \varepsilon_{ij}$. Where μ is the common intercept, P_i is the intercept for package type i when adjusted for the common intercept (i.e., $P_i + \mu$), β is the overall slope, d is days of incubation, γ_i is the slope for package type i adjusted for the common slope, and ε_{ij} is the error for sample j in package type i. For the $_{ij}^{th}$ sample, the $\log_{10}$ HAV titre is the mean of triplicate test samples.

RESULTS

Virus recovery from lettuce averaged 78.21 ± 14.97 %. This was considered as the baseline level to determine virus survival on lettuce under various conditions. Visual inspection of the lettuce incubated at 4°C showed that on day 6 some pieces of lettuce showed minimal browning, mainly in those incubated in the petri-dish and in bags sealed in ambient air. Some browning was also observed in pieces of lettuce incubated under 100% CO_2. For lettuce incubated at room temperature, a similar pattern of browning of lettuce to that incubated at 4°C was observed, but was more prominent and started to appear on day 3. This pattern continued to day 12 by which time, more than half of the area of the lettuce leaves in the petri dish were brown. When comparing packages incubated at room temperature, less browning was observed in lettuce stored under sealed air and under 100% CO_2, as compared to other packages, but was relatively more prominent than the observed browning in lettuce stored under sealed air and under 100%

CO_2 at 4°C. Some brown spots also began to show on lettuce stored under 70:30% (CO_2:N_2) by day 9. Gas chromatography analysis indicated that in most sealed bags, the ratio of gas mixtures flushed in the bags remained essentially similar to its initial composition. Only a marginal increase in CO_2 levels (< 10%) was observed in some of the bags.

Plaque assay determinations demonstrated that on day 12 at 4°C incubation, 80.33 ± 17.35 % of HAV in PBS was still viable, whereas HAV on lettuce stored under other conditions (i.e., petri-dish, air-bag and high CO_2 environments at 4°C) showed survival rates ranging between 47.54 and 71.64%, with an overall average of 64% (Table 13 and Figs. 10 and 11). Incubation at room temperature resulted in a 31.93 ± 12.95 % survival rate of HAV in PBS on day 12, compared to survival rates ranging between 0.01 and 42.79% (overall average of 11.62%) under all other air and MAP conditions. Survival rates of HAV on days 1, 3, 6, 9 and 12 are listed in Table 13.

The results of the statistical analysis of the data by ANOVA can be seen in Table 14. At 4°C, the decrease in HAV titre was not significantly different ($p > 0.05$) between the package types. When HAV was incubated at room temperature, a more prominent loss in virus titre was observed by day 12. A 31% survival rate of HAV in PBS was seen, as compared to 80.3% at 4°C. A greater loss in virus titre was observed when HAV-inoculated lettuce was stored in a petri-dish at room temperature (0.01% survival) as compared to 4°C (47.5 % survival). During storage at room temperature, there was a highly significant ($p \leq 0.0001$) decrease in HAV titre in all packages, except at

Table 13: Survival of hepatitis A virus on lettuce incubated at room temperature and 4°C under various normal and modified atmosphere packaging (MAP) environments.

Atmosphere	Day[a]	Survival (%) at 4°C	Survival (%) at room temperature
Virus recovery[b] (from lettuce) Baseline=100%	0	78.21 (± 17.21)[c]	
Dish[d]	1	102.46 (± 27.83)	39.44 (± 12.06)
	3	70.49 (± 14.29)	9.59 (± 3.22)
	6	50.66 (± 15.06)	6.23 (± 1.71)
	9	54.67 (± 24.40)	0.50 (± 0.17)
	12	47.54 (± 15.18)	0.01 (± 0.00)
Air[e]	1	107.38 (± 20.29)	62.71 (± 22.31)
	3	82.79 (± 15.57)	48.53 (± 16.33)
	6	58.20 (± 14.57)	14.51 (± 4.27)
	9	70.74 (± 27.06)	7.57 (± 3.72)
	12	61.72 (± 27.23)	6.15 (± 4.07)
30:70[f] (CO_2:N_2)	1	89.35 (± 15.96)	76.23 (± 19.88)
	3	78.69 (± 13.69)	72.87 (± 22.72)
	6	60.66 (± 14.01)	10.00 (± 3.15)
	9	68.28 (± 23.79)	12.45 (± 7.47)
	12	62.87 (± 21.74)	5.29 (± 1.28)
50:50[f] (CO_2:N_2)	1	118.45 (± 23.78)	75.41 (± 16.150
	3	90.17 (± 16.64)	59.02 (± 14.41)
	6	73.77 (± 15.36)	56.48 (± 22.39)
	9	73.53 (± 27.29)	25.41 (± 10.66)
	12	59.84 (± 15.70)	6.89 (± 2.99)

70:30[f] (CO_2:N_2)	1	104.92 (± 22.01)	79.51 (± 10.27)
	3	86.07 (± 17.79)	45.25 (± 13.35)
	6	90.17 (± 26.73)	48.61 (± 13.56)
	9	88.53 (± 22.54)	41.15 (± 9.84)
	12	83.61 (± 23.63)	42.79 (± 23.29)
100% CO_2[f]	1	91.81 (± 13.63)	36.48 (± 17.39)
	3	89.35 (± 11.48)	14.51 (± 4.27)
	6	85.25 (± 21.83)	15.16 (± 5.33)
	9	101.40 (± 36.31)	9.59 (± 2.21)
	12	71.64 (± 24.43)	8.60 (± 3.82)
PBS[g]	1	111.07 (±21.09)	116.40 (± 20.20)
	3	112.30 (±14.91)	90.99 (± 14.42)
	6	102.46 (± 15.70)	53.77 (± 24.27)
	9	98.36 (± 18.62)	46.40 (± 23.96)
	12	80.33 (± 17.35)	31.93 (± 12.95)

[a] All inoculated and uninoculated samples were incubated at 4°C and room temperature for 12 days. Samples were removed on day 1, 3, 6, 9 and 12. Residual virus was recovered and plaque-assayed to determine survival.

[b] Ten µL of HAV was deposited onto lettuce, allowed to dry and then recovered. The percent recovery rate was determined as follows: $= \frac{\text{Titre of recovered virus}}{\text{Titre of virus at time zero}} \text{ X } 100$. This was considered as the baseline (100%) for virus recovery.

[c] Percent (%) virus survival (± standard error).

[d] Inoculated lettuce was placed inside a petri-dish which was loosely covered to allow for air-flow to the lettuce.

[e] Inoculated lettuce was heat-sealed inside a plastic bag containing normal atmospheric air.

[f] Inoculated lettuce was heat-sealed inside plastic bags flushed with various gas mixtures (CO_2:N_2; 30:70, 50:50, 70:30 and 100% CO_2).

[g] HAV was seeded in PBS stored at 4°C and room temperature.

Figure 10: Survival of hepatitis A virus on lettuce stored under air and various modified atmospheres at room temperature. Inoculated and uninoculated lettuce pieces were incubated at room temperature for 12 days. Samples were removed on days 1, 3, 6, 9 and 12, the residual virus recovered and plaque-assayed for titre.

[a] Input virus titre at time zero.

[b] 10 μL of HAV deposited onto lettuce, allowed to dry and then recovered. The recovery rate (%) was determined as follows:

$$= \frac{\text{titre of virus recovered after drying}}{\text{Input virus titre at time zero}} \times 100$$

. This was considered as the baseline (100%) for virus recovery.

[c] Inoculated and uninoculated lettuce pieces were placed inside a loosely-covered petri-dish to allow for air flow to the lettuce.

[d] Inoculated lettuce sealed inside plastic bags with normal atmospheric air.

[e] Inoculated lettuce sealed inside plastic bags with CO_2:N_2 atmospheres of 30:70, 50:50, 70:30 or 100% CO_2.

[f] HAV in PBS stored at room temperature.

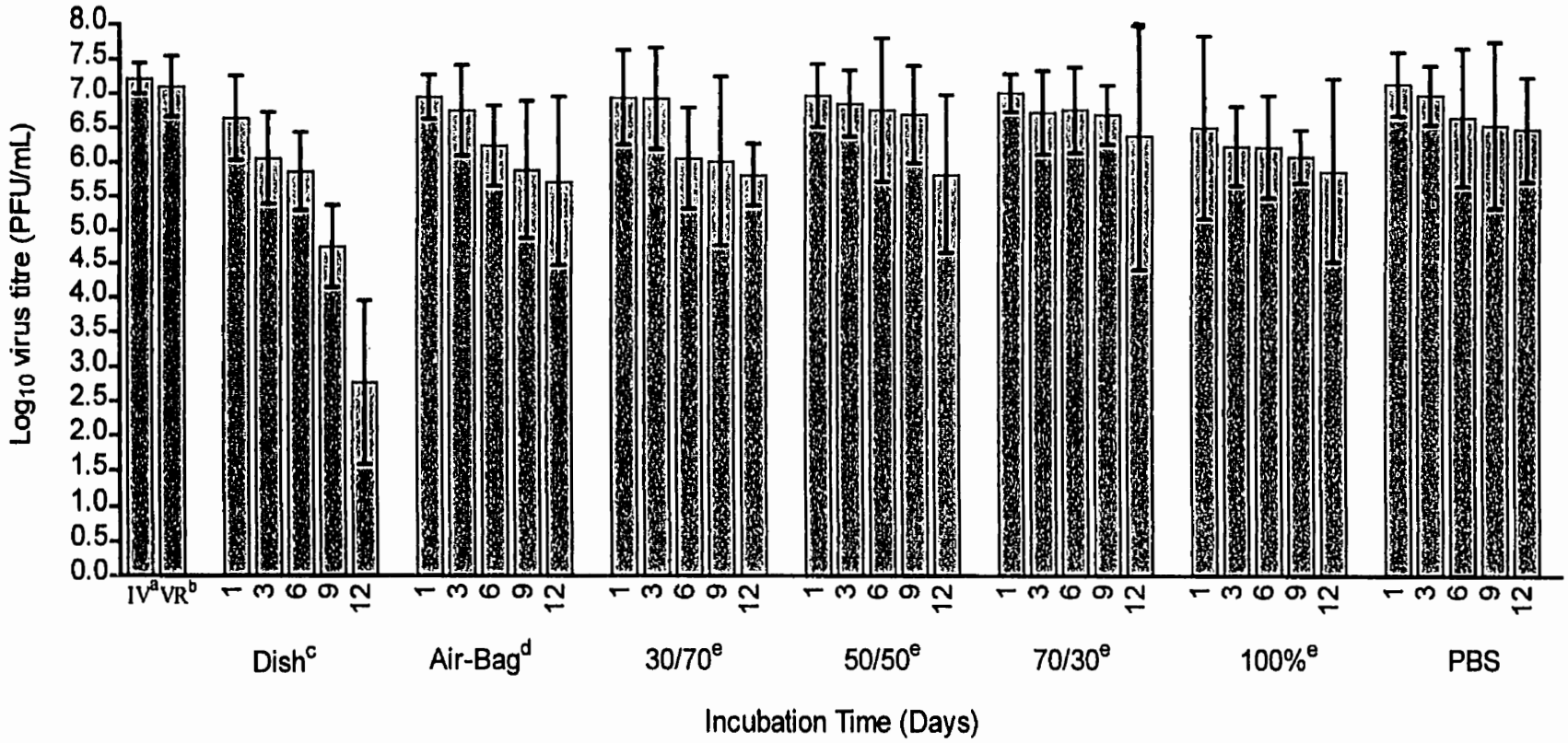

Log10 virus titre (PFU/mL)
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
IV[a]VR[b]
1 3 6 9 12
Dish[c]
Air-Bag[d]
30/70[e]
50/50[e]
70/30[e]
100%[e]
PBS
Incubation Time (Days)

Figure 11: Survival of hepatitis A virus on lettuce stored under air and various modified atmospheres at 4°C. Inoculated and uninoculated lettuce pieces were incubated at 4°C for 12 days. Samples were removed on days 1, 3, 6, 9 and 12, the residual virus recovered and plaque-assayed for titre.

[a] Input virus titre at time zero.

[b] 10 µL of HAV deposited onto lettuce, allowed to dry and then recovered. The recovery rate (%) was determined as follows:

$$= \frac{\text{titre of virus recovered after drying}}{\text{Input virus titre at time zero}} \times 100$$

.This was considered as the baseline (100%) for virus recovery.

[c] Inoculated and uninoculated lettuce pieces were placed inside a loosely-covered petri-dish to allow for air flow to the lettuce.

[d] Inoculated lettuce sealed inside plastic bags with normal atmospheric air.

[e] Inoculated lettuce sealed inside plastic bags with CO_2:N_2 atmospheres of 30:70, 50:50, 70:30 or 100% CO_2.

[f] HAV in PBS stored at 4°C.

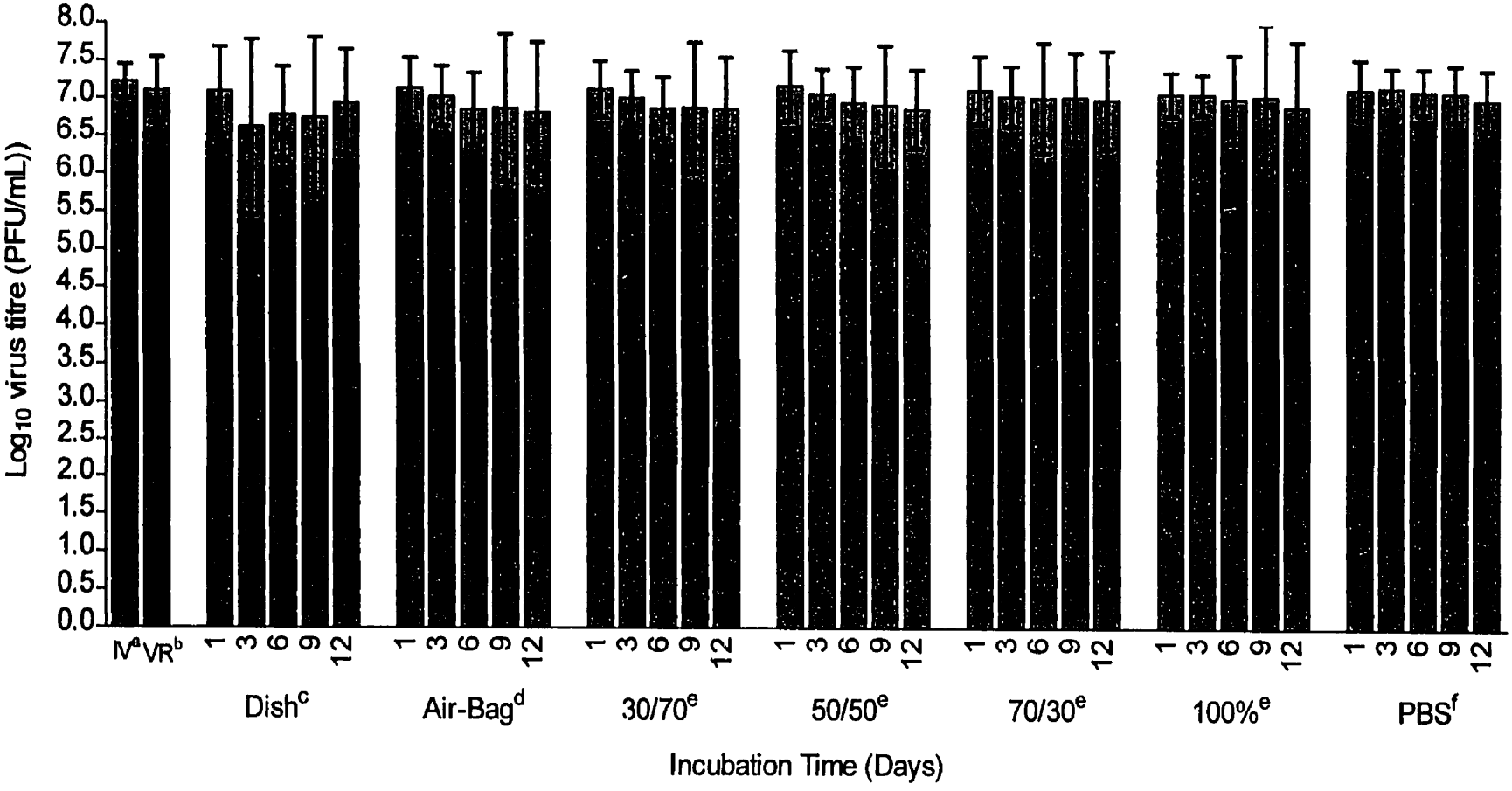

8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Log10 virus titre (PFU/mL))
IVa VRb
1 3 6 9 12
Dishc
Air-Bagd
30/70e
50/50e
70/30e
100%e
PBSf
Incubation Time (Days)

Table 14. Results of statistical analysis of the data by ANOVA.

Temperature	Medium	Slope $\log_{10}$ HAV titre/day	p (slope=0)	Standard error of slopes
4°C[a]	Dish	-0.031	0.03	0.014
	Air	-0.028	0.05	
	30:70	-0.018	0.2	
	50:50	-0.027	0.058	
	70:30	-0.009	0.54	
	100%CO_2	-0.014	0.33	
	PBS	-0.013	0.36	
Room temperature	Dish[b]	-0.328	≤0.0001	0.024
	Air	-0.12	≤0.0001	
	30:70	-0.113	≤0.0001	
	50:50	-0.102	≤0.0001	
	70:30	-0.045	0.066	
	100%CO_2	-0.05	0.042	
	PBS	-0.062	0.012	

[a] No slopes were found to be significantly different using Tukey's multiple range test (Miller, 1966).

[b] The slopes for the open dish was significantly different from all other slopes, but all other slopes were not significantly different from each other using Tukey's multiple range test.

70 % CO_2 : 30% N2, with increasing storage time, and that the slopes differed ($p \leq$ 0.0002) between package types.

DISCUSSION

Although there have been many studies examining the survival of both spoilage and pathogenic bacteria in various MAP environments, little work has been done examining the effect of MAP on the survival of foodborne viruses. Since HAV is one of the most common viral food contaminants, we designed experiments to determine its survival on lettuce, when incubated at 4°C and room temperature under normal air and different MAP conditions for up to 12 days.

The earlier appearance and more prominent browning of lettuce incubated at room temperature than at 4°C is attributed to the effect of the incubation temperature. Exposure to CO_2 levels above a tolerance limit can cause physiological damage to produce (Zagory and Kader, 1988). Therefore, the high CO_2 levels might have also caused some injury to the lettuce and contributed to the increased browning under 100% CO_2 at both temperatures. Overall, the organoleptic quality of lettuce appeared to be much better when stored under MAP conditions than when incubated in air.

Despite the fact that lettuce is a living tissue that continues to respire by consuming O_2 and releasing CO_2, there was very little change in the overall gaseous composition inside the bags. This may be attributed to a number of factors such as the small surface area of the lettuce relative to the bag, the lack of O_2 or high CO_2 in the gas mixtures which might have resulted in a more rapid death of the lettuce tissue, as well as the low permeability of the plastic bag which essentially restricted the movement of

gases. The use of low permeability bags was necessary to maintain the gas ratios as constant as possible.

Our data demonstrated that by the 12th-day of incubation, ≥50% of HAV on lettuce stored at 4°C was still viable. The lowest survival rate (47.5%) was seen with lettuce stored in a petri-dish, whereas the highest rates of virus survival at 4°C i.e., 83.6 and 71.6%, occurred for lettuce stored under 70% CO_2:30% N_2 and 100 % CO_2, respectively. The latter two survival rates were similar to those (80.3%) for HAV in stored PBS, at 4°C.

Incubation of HAV-inoculated lettuce under various MAP conditions at room temperature demonstrated that the greatest HAV survival rate (42.8%) was on lettuce incubated under an atmosphere of 70% CO_2: 30% N_2, which was almost half the survival rate (83.6%) observed under the same conditions at 4°C. Much lower survival rates, ranging between 5.3 and 8.6%, were observed under other MAP conditions at room temperature. HAV titres decreased significantly ($p \leq 0.0001$) for almost all package types, with HAV titres having decreased more rapidly for the open dish than for all other package types at room temperature.

An increase in CO_2 levels at room temperature seems to have slightly improved virus survival, as compared to other modified-atmospheres. Overall, however, virus survival rates were significantly ($p<0.001$) lower under all conditions at room temperature in comparison to survival at 4°C. Since the lower rate of virus survival (47.5%) in petri-dishes at 4°C was not significantly different from lettuce stored in other atmospheres at 4°C, it is concluded that MAP at 4°C did not significantly enhance the

survival of HAV. However, MAP environments containing a high CO_2 level (70%) may have contributed to some improvement in virus survival at room temperature. Unlike bacteria, viruses are essentially inert particles that do not carry out enzymatic or metabolic activities on their own, and, after entry into the cell, are entirely dependent on the metabolic activities of the host cell for their replication.

Since HAV was inoculated onto the lettuce surface, the virus would be in an inert state which might not be affected by high CO_2, even if it enters the virus. In comparison, CO_2 inside a bacterial cell can result in a decrease in the intracellular pH, which can affect crucial metabolic activities. The reason for the slight overall improvement observed in virus survival on lettuce stored in an atmosphere containing 70 and 100% CO_2 at room temperature is not known, although it might be possible that inhibition of ethylene production by high CO_2 levels could have contributed to better virus survival while on the surface of the produce. Since CO_2 inhibits bacterial growth, it might be possible that it could have an inhibitory effect on virus replication when the virus is inside a host cell. These possibilities, however, require further investigation.

In conclusion, our study has demonstrated that only at room temperature was there significantly better virus survival in a high CO_2 environment (i.e., $\geq$70%) as compared to all other storage or atmospheric conditions. Further studies of virus survival in MAP environments with a wider variety of foods and with other foodborne viruses may be warranted.

10. CONTAMINATION OF FOODS BY FOOD HANDLERS: EXPERIMENTS ON HEPATITIS A VIRUS TRANSFER AND ITS INTERRUPTION

10. CONTAMINATION OF FOODS BY FOOD HANDLERS: EXPERIMENTS ON HEPATITIS A VIRUS TRANSFER AND ITS INTERRUPTION.

INTRODUCTION

Hepatitis A is a common form of acute viral hepatitis in many parts of the world. It is responsible for significant world-wide morbidity and occasional mortality (Prevot et al., 1993; Koff, 1998). Outbreaks of hepatitis A occur periodically throughout the world with fecally contaminated food and water being the main vehicles (Atmar et al., 1993). Although less than 10% of the cases of hepatitis A in the US are associated with foodborne outbreaks (CDC, 1994), substantial costs are incurred by both society and the food industry as a result (Dalton et al., 1996). Foods implicated in these outbreaks include shellfish (Richards, 1985; Wang et al., 1989; Halliday et al., 1991; Desenclos et al., 1991; Enriquez, 1992; Le Guyader et al., 1994), sandwiches, dairy products, baked products, salads, fruits and vegetables (Feinstone, 1996; Cliver, 1997). Examples of such outbreaks include the one in Denver, Colorado, in 1992 in which more than 5000 individuals were exposed to HAV due to the consumption of a variety of gourmet foods prepared by an infected foodhandler (Dalton et al., 1996). A recent outbreak in Michigan, which resulted in more than 200 cases of infectious hepatitis in school children, occurred due to the consumption of imported contaminated strawberries (Anon., 1997; Hutin et al., 1999). In nearly 50% of the cases of hepatitis A, the mode and vehicle(s) of virus spread remain unidentified (Hadler and Margolis, 1989).

A number of reports suggest that in many cases infected food handlers may play

an important role in food contamination (Cliver, 1985; Sobsey et al., 1988; Dalton et al., 1996). However, our understanding of the potential for hands to transfer viruses such as HAV to foods is limited and this, in turn, hampers the institution of proper measures of hand hygiene to reduce the risk of food contamination.

This study was designed to develop an experimental procedure to investigate the amount of HAV that can be transferred from artificially contaminated hands of adult volunteers to lettuce, both with and without prior treatment of hands with water, with a topical agent followed by rinsing with water and drying or with alcohol-based agents.

MATERIALS AND METHODS

Topical agents, water and drying. Standard hard water as well as two different types of topical agents were tested for their potential to interrupt the transfer of HAV. One of these products was a topical non-medicated (P1) Ivory soap (Proctor & Gamble Co., Toronto, Ont), whereas the second (P2) was a commercially used medicated (anti-bacterial). An alcohol-based gel hand rub containing 62% alcohol and an aqueous 75% alcohol solution were also included in this study. Hard water used in the washing process was prepared by mixing (all sterile solutions) 1 mL of reagent A (6.792 g $MgCl_2.6H_2O$ + 9.80 g $CaCl_2.2H_2O$ per 100 mL deionized water), 2 mL of reagent B (5.60 g $NaHCO_3$ per 100 mL deionized water), and 497 mL of deionized water (AOAC, 1995). Autoclaved square pieces (approximately 7 cm x 7 cm) of paper towel were used for fingerpad drying.

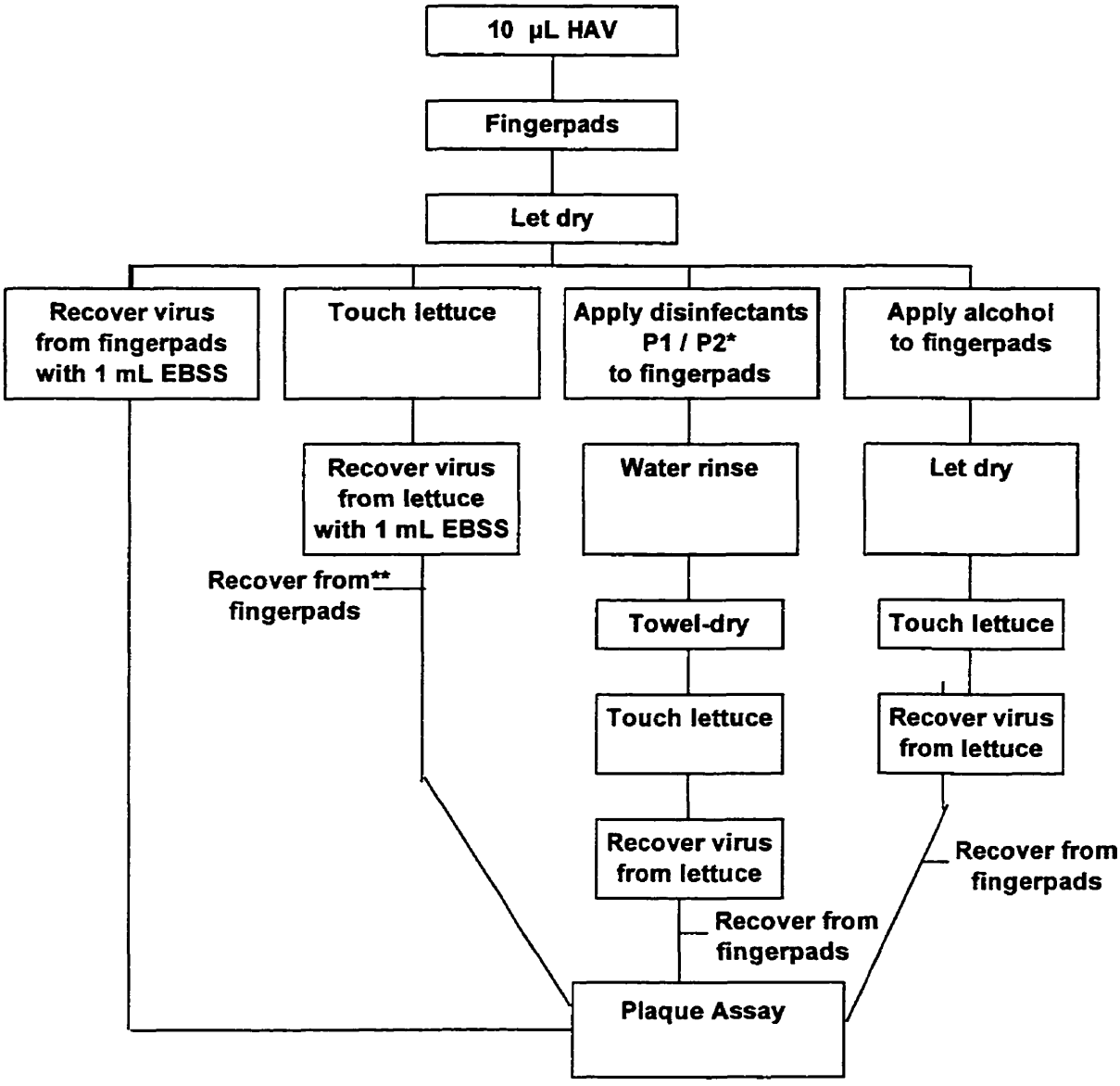
Inoculation and recovery of HAV from lettuce. Please see General Material and Methods.

Volunteers. A total of 11 males and females, ranging in age from 24 to 45 years, participated in this study. A known concentration of HAV was deposited on demarcated areas on the fingerpads during the experimental procedure. At the end of the experiments, the contaminated fingerpads were decontaminated by pressing for 4 min on a piece of paper towel soaked in a 10% solution of domestic bleach (Dutch Chemicals, Weston, Ont). The hands were then washed thoroughly with liquid soap and running tap water, and dried with a paper towel (Mbithi et al., 1993).

Protocol for virus transfer from hands to lettuce. The procedure used to assess virus survival on hands as described by Ansari et al., (1988, 1991) was adapted and modified to incorporate the transfer of the virus to lettuce (Please see Fig.12 and Appendix B). Before each experiment, the HAV stock was diluted to a final concentration of about 1.3×10^7 PFU/mL containing 5% FBS to simulate an organic (soil) load. For in-put virus control, 10 µL of this viral suspension (approximately 1.3×10^5 PFU) was immediately transferred into each of three microcentrifuge tubes containing 990 µL of Earle's Balanced Salt Solution (EBSS), using a 10 µL Eppendorf pipettor (Mandel, Toronto, Ont.). Serial $\log_{10}$ dilutions were made for the plaque assay.

To demarcate the areas for virus deposition, each fingerpad was pressed tightly against the mouth (8 mm inside diameter) of an empty sterile 1.8 mL-capacity plastic vial (Sarstedt Inc., St. Laurent, Quebec). A portion (10 µL) of the same virus suspension was placed at the centre of the demarcated area on each fingerpad. In each experiment, the actual amount of virus in the inoculum on the fingerpads (zero-minute control) was determined by immediately recovering the deposited virus separately from

Figure 12: Schematic illustration of the procedure used to determine the rates of hepatitis A virus (HAV) transfer from contaminated fingerpads of human volunteers to lettuce.



* P1/P2 : Topical disinfectants.

** The amount of virus remaining on the fingerpads was determined following the touching of the lettuce.

four fingerpads (two fingerpads of the left hand and two of the right hand) as follows: the contaminated area was placed over the mouth of a plastic vial identical to that used for fingerpad demarcation, but containing 990 μL of EBSS. With the fingerpad tightly pressed against the mouth, the vial was inverted (up-side-down) and held in place for 10 seconds, allowing full contact between the EBSS and the inoculated demarcated area. This was followed by 20 full inversions. The tube was inverted (up-side-down) for a second time and held for 10 seconds, followed by a second round of 20 more full inversions. The surface of the fingerpad was then slowly and tightly scraped on the inside rim of the vial to recover as much of the fluid as possible. Serial 10-fold dilutions of the eluates were made for the plaque assay.

To establish a baseline for the recovery of the virus from the fingerpads after drying, 10μL of virus suspension was deposited onto demarcated areas on four fingerpads (two from each hand) and was allowed to air-dry, keeping track of the time needed for the inoculum to dry on each of the fingerpads. Virus deposition on the fingerpads was staggered at a 3-min interval between fingerpads. This was followed by virus recovery from fingerpads as described above, using an identical plastic vial containing 1 mL of EBSS. Serial $\log_{10}$ dilutions were made for the plaque assay.

To determine the effect of water on virus removal from the fingerpads following drying of the inoculum, the inoculated area on the fingerpad was washed with water by placing the mouth (identical to the diameter of the Sarstedt vial) of a 25 cm^2 tissue culture flask (Canadian Life Technologies, Burlington, Ontario) containing 15 mL (or a 1.8 mL-capacity plastic vial containing 1 mL) of hard water and inverting the flask (or

the vial) back and forth 5 times. To towel-dry the fingerpad, a pre-sterilized piece of paper towel was placed on a weighing balance, and the fingerpad was then pressed slightly on the towel until the indicator on the balance (Sartorius- VWR, Mississauga, Ont.) showed a pressure ranging between 0.2 and 0.4 kg per cm^2, which was maintained for 10 seconds (Garner and Favero, 1985; Ansari et al., 1989). This was followed by virus recovery, as described earlier. The rate of virus transfer to lettuce following water treatment was determined by the same procedure, except that following towel-drying, the fingerpad was re-pressed for 10 seconds (0.2-0.4 kg/ cm^2 pressure) onto a demarcated area on a piece of lettuce placed in a weighing boat resting on the balance. The virus was then recovered from both the lettuce and the fingerpads, and plaque-assayed, as described previously.

The following experiment was done to determine the effect of applying a viscous topical agent [P1]or [P2] to the fingerpads. After air-drying of the deposited 10 μL virus, the topical agent was applied by inverting the vial containing 1 mL of the test product [P1 or P2] up-side-down onto the fingerpad while maintaining contact for 20 seconds, instead of the 10 second contact time used for liquid agents. The vial was then removed without scraping the fingerpad. The fingerpad was washed with hard water, towel-dried and the remaining virus on the fingerpad was recovered as described earlier. Serial 10-fold dilutions of the virus were then made for the plaque assay. To determine the extent of virus transfer to lettuce following the treatment with disinfectants (P1 and P2) and water, the same procedure was repeated except that following towel-drying, the fingerpad was re-pressed (0.2 and 0.4 kg/ cm^2 pressure) for 10 seconds onto a

demarcated area on a piece of lettuce. The virus was then recovered from both the lettuce and the fingerpads and plaque-assayed as described previously.

The same process was repeated to investigate the effect of a 62% alcohol-based gel and a 75% aqueous (liquid) alcohol on the inactivation and/or removal of HAV from fingerpads, except that following the 20-second contact (10 seconds for the liquid 75% alcohol), the fingerpads were allowed to air-dry rather than towel-dry. The remaining virus was recovered and assayed in the same fashion. Similarly, the extent of virus transfer to lettuce following alcohol treatment was determined as described for the P1 and P2 treatments.

Statistical analysis. For each experiment, four fingers (two on each hand) were used, and the experiment was repeated with at least two different volunteers. Since the plaque assay for each finger was done in triplicate, a total of at least 24 sets of data were obtained and averaged. The baseline virus recovery was determined by calculating the percentage of the virus titre recovered from the fingerpads or the lettuce as compared to the titre of virus deposited onto these surfaces. The rate of virus recovery from the fingerpads following treatment or following touching the lettuce was determined by calculating the percentage of the virus titre obtained from the fingerpads or the lettuce to that of the baseline. The extent of virus transfer to the lettuce was derived by calculating the percentage of the virus titre recovered from the lettuce as compared to that of the baseline recovery for lettuce. The results were statistically analyzed by using the Student's *t-test* (Bailey, 1995).

RESULTS

The extent of virus recovery from fingers and lettuce, as well as the rates of transfer of HAV from artificially contaminated fingerpads of human volunteers to lettuce can be seen in Table 15. Recovery rates of the dried virus inoculum from fingerpads and lettuce were 70.05 ± 3.49 and 75.38 ± 1.63 %, respectively. These were considered as the baseline (100%) for all other virus recovery and transfer data obtained in this study. When inoculated fingerpads were washed with water alone and towel-dried, 3.69 ± 0.65 % of the virus was recovered from the fingerpads , whereas treatment with P1 and P2 formulations (topical agents) followed by water and towel-drying resulted in 6.53 ±1.24 and 4.14 ± 0.82 % recovery rates, respectively. Exposing the dried virus inoculum on the fingerpads to a 62% alcohol-based rubbing gel resulted in a virus recovery of 64.3 ± 3.98 % compared to 24.11 ± 2.85 % recovery from fingerpads exposed to 75% prepared liquid alcohol.

The amount of the virus recovered from lettuce, which was touched by virus-contaminated fingerpads was 7.02 ± 0.65 %. Since the baseline virus recovery rate from lettuce was approximately 76 %, this would indicate that around 9.24 ± 0.86 % of the virus was transferred from the fingerpads to the lettuce by touching, without using any prior treatment of the fingerpads.

Approximately 53.41 ± 4.92 % of the virus was recovered from the fingerpads following transfer to lettuce. When the soiled fingerpads were first washed with water, towel-dried, and then pressed onto lettuce, the virus recovery rates were 0 and 6.16 ± 0.69 % from the lettuce and fingerpads, respectively.

Table 15. Recovery of hepatitis A virus (HAV) from contaminated fingerpads of human volunteers and rates of transfer to lettuce upon contact with fingerpads.

Test[a]	Virus recovery (%) from lettuce ± SE	Virus recovery (%) from fingers ± SE
a. Input Virus (1.29 x 10^5 PFU/10 µL)		
b. 10-Finger-R[b] *(time zero for fingerpads)*		77.50 ± 6.9
c. 10-Lettuce-R (*time zero for lettuce*)	88.50 ± 3.71	
d. 10-Finger-Dry-R[b] *(Baseline= 100% for Fingers)*[c]		70.50 ± 3.49
e. 10-Lettuce-Dry-R[b] *(Baseline= 100% for Lettuce)*[c]	75.83 ± 1.63	
f.. 10-Finger-Dry-Water-Towel-R		3.69 ± 0.65
g. 10-Finger-Dry⇒Lettuce-R	7.02 ± 0.65 (= 9.24 ± 0.86 % TR)[d]	53.41 ± 4.92
h. 10–Finger-Dry-Water-Towel⇒Lettuce-R[e] 10–Finger-Dry-Water-Towel⇒Lettuce-R[f]	0 0.23 ± 0.05 (=0.31 ± 0.07 % TR)	6.16± 0.69 5.94 ± 0.85
i. 10-Finger-Dry-P1-Water-Towel-R[b]		6.53± 1.24
j. 10-Finger-Dry-P1-Water-Towel⇒Lettuce-R	0.30 ± 0.06 (= 0.39 ± 0.08 % TR)	2.05 ± 0.4

Table 15 cont'd

k.	10-Finger-Dry-P2-Water-Towel-R[b]		4.14± 0.82
l..	10-Finger-Dry-P2-Water-Towel⇒Lettuce-R	0.26 ± 0.05 (= 0.34 ± 0.7 % TR)	5.17± 0.77
m..	10-Finger-Dry-62% Alcohol (gel)-Dry-R[b] *(Baseline=100% for Alcohol-gel)*[c]		64.32± 3.98
n.	10-Finger-Dry-62% Alc (gel)-Dry⇒Lettuce-R	0.49 ± 0.07 (= 0.64 ± 0.09 % TR)	45.73± 4.95
p.	10-Finger-Dry-75% aqueous Alc -Dry-R[b] *(Baseline=100% for 75% Alcohol)*		24.11± 2.85
q.	10-Finger-Dry-75% aqueous Alc-Dry⇒Lettuce-R	0.35 ± 0.06 (= 0.46 ± 0.08 % TR)	18.80 ± 3.52

[a] A list of the different experiments (a to q) and the steps of each experiment (separated by a dash) are listed.

[b] 10μL of HAV deposited on fingers or lettuce; R: Virus recovery from fingers or lettuce; ⇒ : indicates touching lettuce; Dry: Virus air-drying on fingers or lettuce; water: rinse; Towel: paper-towel-drying; P1, P2: Topical agents; Alc-gel: 62% Alcohol-based gel; 75% Alc (aqueous alcohol); SE: Standard error of the Mean.

[c] The baseline was calculated as follows: $\text{Baseline} = \frac{\text{Titre of recovered virus after drying}}{\text{Titre of virus at time zero}} \times 100$.

[d] The TR (Transfer rate) was calculated as follows: $\text{TR} = \frac{\text{Titre of recovered virus}}{\text{Baseline titre}} \times 100$.

[e] 15 mL of water was used to recover HAV from fingerpads..

[f] 1 mL of water was used to recover HAV from fingerpads.

When 1 mL volumes of water (instead of 15 mL) were used as a water rinse, virus recovery rates were 0.23 ± 0.05 and 5.94 ± 0.85 %, from lettuce and fingerpads, respectively. Treating contaminated fingerpads with either the P1 or P2 formulations followed by a water rinse and paper-towel drying prior to touching lettuce, resulted in recovery rates from lettuce of approximately 0.30 ±0.06 % (ca 0.39 ± 0.08 % transfer rate), whereas 2.05 ± 0.4 and 5.17 ± 0.77 % of the virus was recovered from P1- and P2-treated fingers, respectively, following touching the lettuce.

A virus recovery rate of 0.49 ± 0.07 % (equivalent to 0.64 ± 0.09 % transfer rate) was obtained from lettuce which was touched by contaminated fingerpads, and which had been subjected to treatment with a 62% alcohol-based gel and then allowed to dry. The virus recovery rates from the alcohol-gel-treated fingerpads following touching lettuce was 45.73 ± 4.95 %. In comparison, when the same process was repeated using 75% liquid alcohol instead of the gel, virus recovery rates of 0.35 ± 0.06 % (0.46 ± 0.08% transfer rate) and 18.8 ± 3.52 % were obtained from the lettuce and fingerpads, respectively.

DISCUSSION

The objective of these experiments was to determine the extent (percentage) of transfer of HAV from artificially inoculated fingerpads of human volunteers to lettuce through contact, before and after treatment of the fingerpads with different topical agents and water (Fig. 12).

Although the exact same steps, reagents, and techniques were used for all volunteers, variations among the different test subjects in some aspects of the testing procedure were commonly observed. This was evident by the wide span of virus drying time (6 to 14 min) on the fingerpads of different volunteers, different rates of virus recovery, as well as the differences in the amount of virus remaining on the fingerpads or transferred to lettuce following the various procedural steps. Most likely, the type of skin texture, its moistness, dryness, thickness, as well as other factors contributed to the observed variations.

The recovery rate of HAV from lettuce and fingerpads was comparable, i.e., 75.83 ± 1.63 and 70.50 ±3.49 %, respectively. The slightly ($p > 0.05$) lower recovery from the fingerpads may be due to the more complex skin texture and surface variation in the fingerpads as compared to lettuce. This complexity in skin texture may have contributed to the 2-fold higher standard error in virus recovery from the fingerpads as compared to that from lettuce.

An important outcome to consider in the present study is the finding that the highest level of virus transfer from contaminated fingerpads to lettuce (approximately 9.24%) was exhibited by touching the lettuce with the soiled fingerpads without any prior treatment of the fingerpads. Hollinger and Ticehurst (1996) indicated that the amount of HAV excreted in feces of infected individuals could be in the range of 10^6 to 10^9 virus particles per gram. However, the actual minimal infectious dose of HAV required to

cause human infection remains unknown, although one infectious unit might be sufficient to cause infection. Similarly, the amount of fecal material that might be present on human hands which become soiled due to unhygienic practices is unknown and could vary widely between different individuals. Nevertheless, taking into consideration the amount of virus present in feces, even a small amount of fecal material (e.g., 0.001 g) could easily contain 10^3 to 10^6 viruses. Assuming a ratio of infectious virus to virus particles in the range of 1:79 (Deng et al., 1994) , at least 13 to 13,000 infectious units could be present in 0.001 g. Using a worst case scenario of a 9.24% transfer rate of HAV from fingerpads to lettuce, indicates that at least 1 to 1300 infectious HAV units could be transferred to lettuce by the process of touching, which most likely would be sufficient to initiate infection in susceptible individuals.

Treatment of the fingerpads with either water and towel-drying, or with a topical agent (P1 or P2) followed by a water rinse and towel-drying reduced the amount of virus remaining on the fingerpads, and consequently resulted in a noticeable reduction in the rate of transfer of the virus from the fingerpads to the lettuce. This was demonstrated by virus recovery rates from the fingerpads of 5.94, 2.05 and 5.17% following water, P1 and P2, treatments, respectively. Similarly, lower rates of virus transfer to lettuce of 0 to 0.31, 0.39 and 0.34%, respectively, were obtained following the same treatments. Applying this finding to the above described scenario of fecally-soiled hands implies that the above three treatments would reduce or diminish the probability of contaminating

the lettuce by about 25 to 100-fold, and thus reduce the possibility of virus spread and subsequent infection, particularly if only a few virus infectious units were initially present on the fingerpads prior to treatment. Our data indicate that rinsing with water alone or using either topical agents (P1 and P2) plus a water rinse, resulted in similar ($p > 0.05$) rates of virus transfer to the lettuce. Treatment with water alone or with P2 along with a water rinse resulted in similar efficiencies ($p > 0.05$) of virus removal from the fingerpads. However, treatment with P1 followed by a water rinse resulted in a significantly ($p < 0.001$) greater recovery of the virus from the fingerpads, as compared to either water or P2 plus water treatments. It is not clear why the water rinse alone was as efficient in virus removal from the fingerpads as was the combined P2 and water rinse. One possible explanation could be that the viscous nature of the topical agent might have caused some de-aggregation of the virus on the fingerpads, thus increasing its counts in the plaque assay, or that the mere contact (without lathering) of the topical agent resulted in a thin residue which shielded the virus from the water rinse. The reason for the higher rate of virus removal from the fingerpads with P2 as compared to P1 is also unclear, although the medicated nature of this topical agent might have contributed to possible virus inactivation on the fingerpads.

Treatment of soiled fingerpads with a 62% alcohol-based gel was surprisingly inefficient in inactivating HAV, i.e., 64.3% of the inoculated virus was recovered from the fingerpads following this treatment. Furthermore, the rate of virus transfer to lettuce

was only 0.64 %, which was significantly higher ($p < 0.05$) than the transfer rates of 0.39 and 0.34%, respectively following treatment with the other topical agents P1 and P2. Following alcohol-gel treatment and touching the lettuce, 45.73% of the virus was recovered from the fingerpads, which was considerably higher ($p < 0.001$) than the amount obtained following all , except for the 62% alcohol-based gel. It is possible that mere contact of the gel with the virus inoculum on the fingerpads for 20 seconds, might not have been sufficient as compared to its application as a rubbing compound. This may be due to the viscous nature of the gel which might have either shielded the virus, de-aggregated it or fixed it to the fingerpads. When a 75% aqueous alcohol preparation was used, the transfer rate of HAV to lettuce was reduced to 0.46%, which was not significantly ($p > 0.05$) different from the rate of virus transfer to lettuce following treatment with the 62% alcohol-based gel. Thus, either alcohol treatment reduced the probability of virus transfer from fingerpads to the lettuce to levels comparable to the 0.3 to 0.39% transfer rate demonstrated by the water and topical agent treatments. Nevertheless, the amount of virus recovered from the fingerpads (18.80 %) following treatment with the 75% alcohol was significantly ($p < 0.001$) lower, as compared to the 45.73% recovery rate following treatment with the 62% gel. This indicates that the liquid form of alcohol, albeit at the slightly higher concentration, was clearly more efficient ($p < 0.001$) in inactivating HAV on the fingerpads than the 62% gel.

In conclusion, this study demonstrates that hand washing with water, topical agent

and alcohol-based solutions can significantly reduce the probability of virus transfer from contaminated fingerpads to produce during food handling. Consequently, the risk of virus spread and infection through foods is significantly ($p < 0.001$) reduced. None of the treatments completely removed or inactivated HAV on the fingerpads, and thus a proper, more thorough lathering with topical disinfectants and rinsing with water would be necessary to reduce the number of remaining virus on the fingerpads. These results also suggest that even after proper hand washing, care should be exercised by foodhandlers particularly when handling foods that are to be consumed raw or undercooked. The use of gloves should be encouraged, particularly when handling foods such as fresh-cut produce that require no processing prior to serving to consumers.

11. GENERAL DISCUSSION

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Hepatitis A is more prevalent and common in developing countries than in developed industrialized societies, which have managed to exert some control over its prevalence as a result of placing greater emphasis on improving sanitary conditions and hygienic standards. However, with today's global economy in which massive amounts of foods cross international borders on a daily basis, and in which there is an increasing amount of daily travel which sees millions of people crossing borders, the potential for more frequent occurrence of hepatitis A is very likely. This is well illustrated by the recent outbreak of hepatitis A in Michigan (Anon., 1997) due to the consumption of imported contaminated frozen strawberries, and two outbreaks in Finland, a very low endemic area for hepatitis A, due to the consumption of imported contaminated salad food items (Pebody et al., 1998). Similarly, a relatively recent report by CDC suggested that approximately 24 million American travellers are at risk of being exposed to HAV annually due to their travels to destination where hepatitis A is endemic. The total economic cost for one hepatitis A foodborne outbreak which occurred in Denver, Colorado, in 1992 (43 cases reported) was > US$ 800,000, which is an indication of the enormous economic burden associated with these outbreaks.

Although a number of studies have reported using chemical agents, heat and irradiation to inactivate HAV, most of these studies have been directed at HAV on environmental surfaces, as well as biological and shellfish samples. Very little work has

been done on the inactivation of HAV in other types of foods (i.e., fruits, vegetables, etc.), many of which have been incriminated in the spread of the virus and in common source outbreaks. Therefore, there is a lack of knowledge with respect to the inactivation of HAV in such foods, as well as no current information concerning the amount of virus transferred from foodhandlers to foods. Furthermore, many studies have addressed the survival rate of HAV in various settings but none, to the best of our knowledge, have addressed its survival under different modified atmospheres, which are widely used in the food industry to inhibit the growth of spoilage microorganisms and prolong the shelf life of foods.

The inability or difficulty in growing wild-type HAV in cell cultures has hampered efforts to study clinical, environmental or food isolates in greater detail. Therefore, research has been limited to the use of laboratory adapted strains of the virus, the first of which was reported by Provost and Hilleman (1979). The rapid development of sensitive molecular techniques to detect low levels of microorganisms, has made it possible to use techniques such as the RT-PCR to detect HAV in biological samples. However, factors such as the low numbers of virus and the presence of substances that inhibit the performance of the RT-PCR, have made it necessary to develop supplementary techniques to concentrate and isolate the virus from foods prior to its detection by the RT-PCR.

Therefore, the present study was designed to address all of the above issues and to

fill these gaps of knowledge. The use of the plaque assay for HAV provided a quantitative measure of determining the rate of virus inactivation by heat or gamma irradiation, as well as the rate of virus survival under MAP conditions and transfer from contaminated fingerpads of volunteers to lettuce. The use of the RT-PCR system in conjunction with concentration methods such as magnetic bead particles and positively-charged filters provided the opportunity to circumvent the effects of inhibitory substances in foods, as well as to rapidly concentrate and detect HAV in fruits and vegetables.

We devised a novel system to investigate the heat inactivation of HAV in dairy products. This system relied on the use of specifically-shaped microcapillary tubes (which accommodated the HAV-seeded milk samples) which were inserted into custom-designed floating boats resting on a metal platform. This allowed the simultaneous exposure of all samples to the waterbath temperature, once the boats were placed in the waterbath.

The heat inactivation results of HAV in dairy products demonstrated that dairy products containing a higher fat content somehow protected the virus and rendered it more heat-resistant. This was shown when a significantly ($p<0.05$) longer exposure time of the dairy product to a particular temperature was required to achieve the same amount of reduction in virus titre when HAV was present in table cream as compared to skim or homogenized milk. When examining inactivation data from temperatures close to those

routinely used in milk pasteurization, our results indicated that pasteurization, although efficient in inactivating bacterial pathogens, is not sufficient to inactivate HAV in dairy products. This study also provides a comprehensive list of time and temperature combinations required to achieve a 1 to 5-$\log_{10}$ reduction in HAV titre in three different dairy products.

Today, greater than 40 different types of foods in more than 32 countries are subjected to gamma irradiation to destroy bacterial and fungal food-contaminants and to prolong product shelf life (WHO, 1994; Byun et al., 1998; Farkas, 1998). Research on the effects of gamma irradiation on viruses have for the most part been limited to inactivation studies in biological fluids and laboratory media, and to a lesser extent in shellfish (Sullivan et al., 1971, 1973; Thomas et al., 1982; Mallett et al., 1991). In our study, we exposed HAV-inoculated lettuce and strawberries to gamma irradiation doses ranging between 1 and 10 kGy. Statistical analysis of the data using both the linear and logistic models led to D_{10} values of 2.73 and 2.97 kGy for lettuce and strawberries, respectively. These results showed that at the maximum level currently allowed to be used for produce, gamma irradiation by itself is not sufficient enough to inactivate HAV in fruits and vegetables. Therefore, the use of gamma irradiation in conjunction with other technologies such as heat, ozone treatment or UV light, should be considered to provide a realistic alternative to the use of harsh and toxic chemicals.

This study also provides the first report on the survival of HAV on lettuce stored

under various MAP environments. The findings that various MAP conditions did not have a significant ($p>0.05$) effect on HAV survival on lettuce stored at 4°C indicates that these environments do not affect the survival of the virus on the surface of the produce. As compared to other atmospheres, the reason for the better ($p=0.06$) survival of HAV on lettuce stored under 70% CO_2:30% N_2 at room temperature is not clear, although it could be due to the effect of CO_2 on the physiological integrity of the lettuce tissue rather than on the virus itself. This finding at room temperature is, however, not as pertinent as that at 4°C, since most produce is usually stored at refrigeration temperatures. Further studies to address the effect of MAP on the survival of foodborne viruses at different incubation temperatures may be warranted.

The rate of transfer of HAV from contaminated fingerpads of volunteers to lettuce through the process of lettuce handling (touching) was investigated in this study. The finding that approximately 10% of the virus was transferred from the fingerpads to the lettuce, provides valuable information with respect to virus spread from infected foodhandlers to foods. The significant ($p<0.05$) reduction in virus transfer (from 10 to $\leq 0.64\%$) following various treatments with water, topical agents and alcohol strongly demonstrates the importance of hand washing and adherence at all times to proper hygienic standards. The findings further indicate that even though virus transfer was significantly reduced following the various treatments, some virus still remained on the fingerpads, which might still constitute a risk. This study did not include aspects such as

the effect of friction and greater pressure on the amount of virus transfer to foods, the effect of lathering of topical agents on virus removal from fingerpads, or the effect of different volumes of wash solutions on virus removal from fingerpads. The method employed in this study, which was a modification of that described by Ansari et al. (1988), may need re-visiting to incorporate some of the observations and findings in the present study. Nevertheless, the present findings provide for a much greater understanding of virus transfer and the role of foodhandlers in virus transfer. This protocol also provides a starting point for further research into this very important area of virus epidemiology.

The combined use of antibody-coated magnetic beads and positively-charged 1MDS filters provided a means to concentrate the virus from large volumes of wash (virus recovery) solution. This allowed for easy detection of the virus by RT-PCR. Starting from large volumes of solution, this method could detect as low as 10 PFU inoculated onto lettuce or strawberries, demonstrating that it has the potential for greater sensitivity than either the RT-PCR alone or when used in combination with magnetic beads. This technique, however, suffers from some drawbacks such as the low levels of (~ 34%) virus elution by BE from the 1MDS filters and the 40% virus specific binding by the MAb-coated beads. These factors that caused virus loss most likely contributed to the lower sensitivity of this method as compared to others when eluting the virus in 10 mL volumes. Improving the rate of virus capture and elution from filters,

as well as capture by immunobeads will undoubtedly improve the sensitivity of this method. Future studies could be directed at investigating the potential use of volumes of > 50 mL (e.g., 200-250 mL) as a wash solution to recover the virus from a large volume of food.

Hepatitis A is currently ranked as sixth among the top ten foodborne diseases in the US, and continues to re-emerge unexpectedly causing large outbreaks associated with the consumption of various types of foods. Although foodborne outbreaks account for approximately 10% of hepatitis A cases, nevertheless, with the changing life style, massive globalization and trade, and greater demands for fresh ready-to-eat foods, one can predict that there will be a substantial increase in the number of HAV food-related outbreaks in the future. Thus, further research should focus on control measures that will reduce the risk of acquiring foodborne hepatitis A, especially in food products that receive minimal processing. These control measures will have to include the design and implementation of on-farm and food-service HACCP (hazard analysis critical control point) food safety programs.

In summary, it is hoped that the findings in this study will provide for a better understanding of the transfer of HAV to foods, as well as its inactivation, survival and detection in foods such as dairy products, fruits and vegetables.

12. CONCLUDING REMARKS

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The experimental work for this study was designed to address the five main objectives listed on page 45. The results outlined and discussed show that all of these objectives have been met and the following conclusions can be drawn from this study:

(1) The fat content of dairy products seems to protect HAV and render it more heat-resistant as compared to those of lesser fat content. As an example, significantly ($p<0.05$) longer exposure time was needed to achieve the same level of HAV inactivation in cream than in skim and homogenized milk, at the same temperature. Routine pasteurization most likely is not sufficient to inactivate HAV. A list of time and temperature combinations required to achieve a 1 to 5 $\log_{10}$ reduction in HAV titre is presented in Table 10.

(2) D_{10} values of 2.73 and 2.97 kGy were needed to achieve a 1-$\log_{10}$ reduction in HAV inoculated onto lettuce and strawberries, respectively.

(3) Various modified atmosphere packaging (MAP) environments did not influence HAV survival on lettuce stored at 4°C. At room temperature, however, an atmosphere consisting of 70% CO_2 : 30% N_2 slightly ($p=0.06$) enhanced virus survival as compared to other storage conditions.

(4) Approximately 10% of HAV inoculated onto the fingerpads of human volunteers was transferred to lettuce by touching. Treatment of the fingerpads with water, topical agents, or alcohol significantly ($p<0.05$) reduced virus transfer from 10% down to $\leq 0.64\%$.

(5) Of the three methods used to capture, concentrate and detect HAV in lettuce and strawberries, the combined F-IM-PCR could detect as low as 10 PFU from produce, which was slightly less sensitive than the other two methods (IM-PCR and 1MDS) used when dealing with small volumes (≤ 10 mL) of virus recovery solution. However, this method has the potential to be even more sensitive than the other two methods when detecting the virus in ≥50 mL of solution, i.e., from large analytical units of food.

13. FUTURE RESEARCH

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The results obtained from the various aspects of this study provide for potential future research projects that are either directly or indirectly related to this study.

1. Investigate the use of large volumes (200-250 mL) of PBS to recover HAV from a number of lettuce leaves, and then applying the F-IM-PCR to detect the recovered virus.
2. Conduct further studies on the transfer of HAV from contaminated fingerpads of volunteers to produce, incorporating some modifications in the methodology. Aspects to address would include, but not be limited to, the role of increased friction and pressure (between the fingerpads and the lettuce) in influencing the rate of virus transfer, and the effect of lathering and rubbing on virus removal from the fingerpads.
3. Comparison of the rate of HAV survival on different fruits and vegetables (i.e., lettuce, strawberries) under a variety of different conditions.
4. Genotyping of a collection of HAV strains isolated in Canada to determine their genotype using the scheme of Robertson et al. (1992), as well as to determine which of the genotypes are prevalent in Canada.
5. Investigate the mechanism of HAV attachment to foods, and factors that influence the attachment and removal of the virus from different foods.
6. Conduct studies on the resistance of HAV to commercially-available sanitizers.
7. Conduct a health risk assessment of HAV on produce.
8. The use of gene-chip technology to detect HAV in foods.

14. REFERENCES

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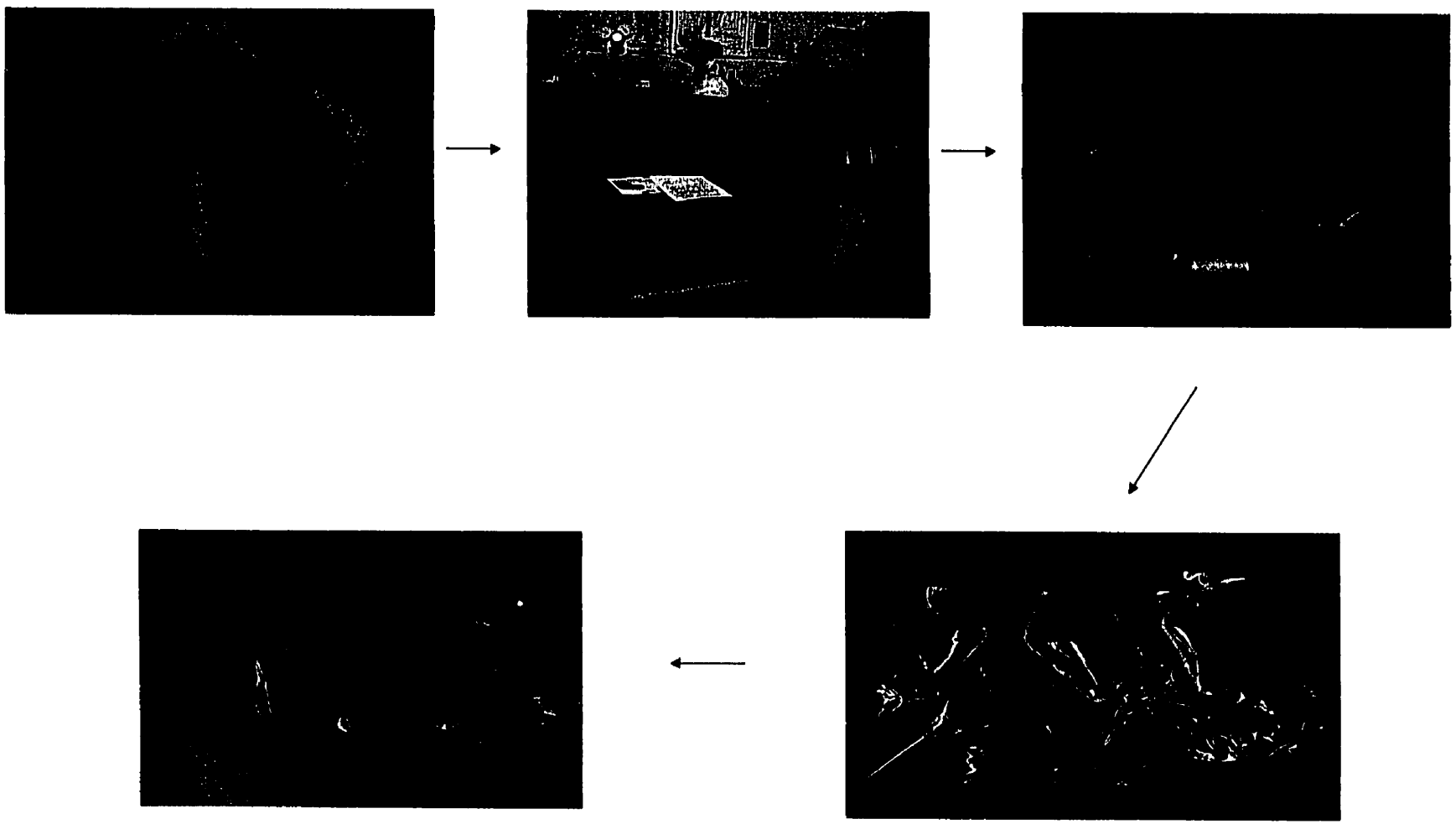
15. APPENDICES

APPENDIX A:

Methodology for the preparation of lettuce for storage under modified atmosphere packaging (MAP)

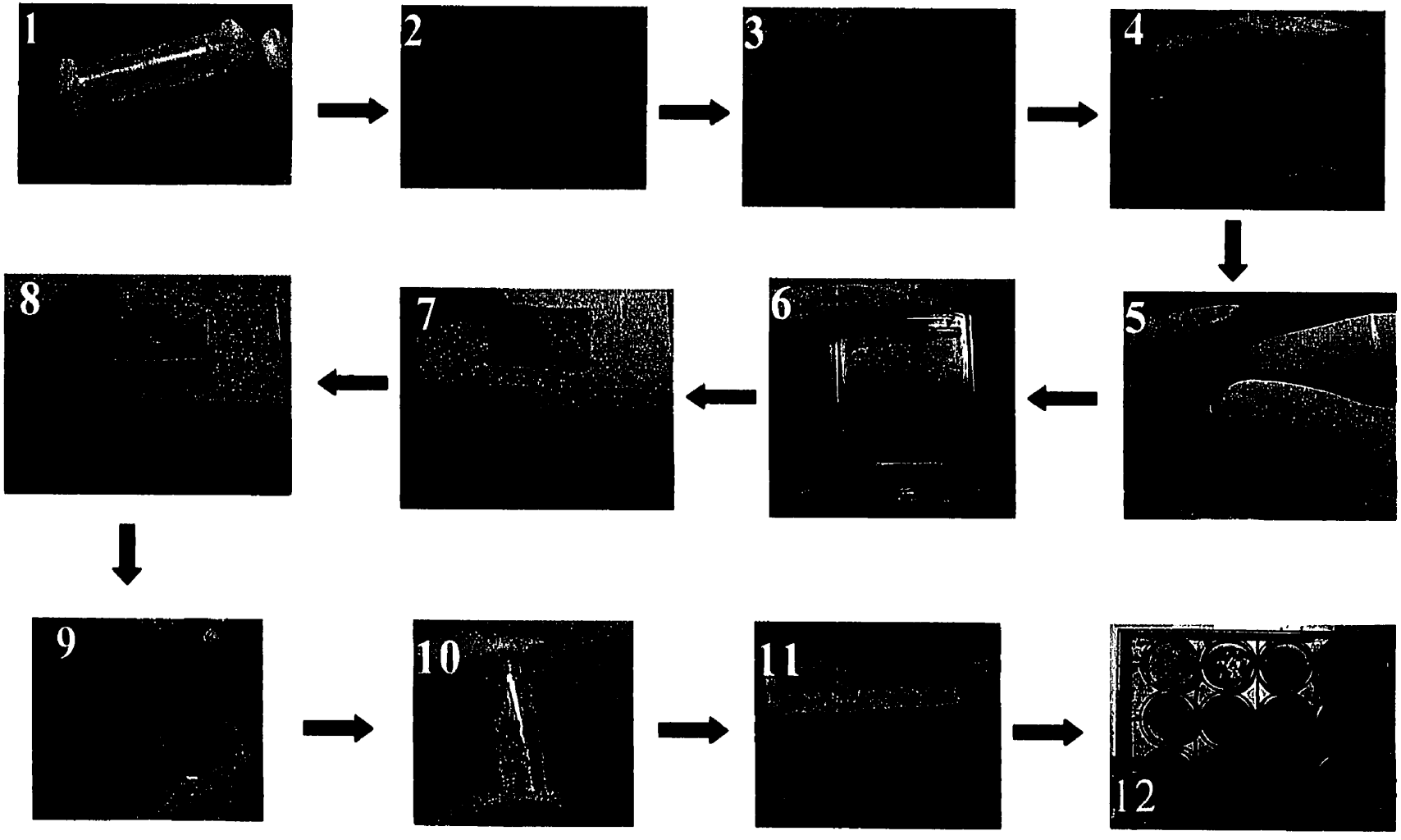
Individual lettuce leaves were cut into intact rectangular pieces of approximately 6-cm x 7-cm, washed with a mild detergent, thoroughly rinsed in water and allowed to dry for approximately 20-30 min in a laminar flow hood. Each side (front and back) of the lettuce pieces was then exposed to UV light for 1 min to reduce and/or inactivate contaminating microorganisms that might have interfered with the plaque assay. Lettuce pieces were then placed face-up in weighing boats, inoculated with 10 μL of hepatitis A virus (HAV) and allowed to air-dry for approximately 20 min. The boats were placed inside plastic bags. The plastic bags were then placed in a Multivac heat-seal packaging machine which automatically evacuates the air from inside the plastic bags, replaces it with pre-determined gas mixtures (N_2 and/or CO_2) and then heat-sealed the bags. The bags were incubated up to 12 days at both room temperature and 4°C. Bags were removed on days 1,3,6,9 and 12 and their gas content was measured by gas chromatography. The bags were then opened, virus recovered from the lettuce and its titre evaluated by plaque assay.



APPENDIX B:

Methodology for studies on the transfer of hepatitis A virus (HAV) from artificially-contaminated fingerpads of human volunteers to lettuce

1 & 2 : Demarcation of fingerpad(s) with a sterile cryogenic vial.
3: Inoculation of fingerpads with 10 µL of hepatitis A virus (HAV). The virus inoculums was allowed to air dry.
4 & 5 : Virus recovery from fingerpads in 1 mL Earle's Balanced Salt Solution (EBSS).
6: Washing off the virus from the fingerpads with 15 mL of hard water.
7: Drying of fingerpads by pressing on a sterile paper towel.
8: Virus transfer from fingerpads to lettuce by touching.
9: Virus retrieval from lettuce by repeated washing with 1 mL PBS.
10: Recovery of residual virus from fingerpads (following transfer to lettuce) in 1 mL PBS, by repeated washing.
11: Serially-diluted virus (recovered from lettuce and/or fingerpads) was plaque-assayed to determine the amount of virus transfer.
12. Plaque counting to determine virus titre.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APPENDIX C:

Publications and presentations

Publications:

1. Bidawid, S.*, Farber, J.M. and Sattar, S.A. 1999. Heat-inactivation of hepatitis A virus (HAV) in dairy foods. J. Food Prot. (Accepted).

2. Bidawid, S.*, Farber, J.M. and Sattar, S.A. 1999. Gamma irradiation of hepatitis A virus in fruits and vegetables. Int. J. food Microbiology (Accepted).

3. Bidawid, S.*, Farber, J.M. and Sattar, S.A. 1999. Rapid detection of hepatitis A virus in fruits and vegetables. J. Virol. Methods (Accepted).

4. Bidawid, S.*, Farber, J.M. and Sattar, S.A. 1999. Contamination of foods by foodhandlers: Experiments on hepatitis A virus transfer and its interruption. Appl. Environ. Microbiol. (Accepted).

5. Bidawid, S.*, Farber, J.M. and Sattar, S.A. 1999. Survival of hepatitis A virus on lettuce stored under modified atmosphere packaging. Food Microbiol. (Submitted).

6. Sattar, S.A. and Bidawid, S. (1999). Environmental considerations in preventing the foodborne spread of hepatitis A. In: *Foodborne Disease Handbook*. Hui, J., Gorham, R., Murrell, K.D. and Sattar, S.A. (eds.). Marcel Dekker, Inc., New York, NY. (In Press).

7. Sattar, S.A., Tetro, J., Bidawid, S. and Farber, J.M. 1999. Foodborne spread of hepatitis A: recent studies on virus survival, transfer and inactivation. Can. J. Infect. Dis. (Accepted).

Presentations:

1. Bidawid, S.*, Farber, J.M. and Sattar, S.A. (October, 1997). Heat-inactivation of hepatitis A virus (HAV) in dairy foods. Annual Meeting of the Canadian Association for Clinical Microbiology and Infectious Diseases, held in St. John's, NFLD.

2. Bidawid, S.*, Farber, J.M. and Sattar, S.A. (October, 1997). Rapid detection of hepatitis a virus in fruits and vegetables. Annual Meeting of the Canadian Association for Clinical Microbiology and Infectious Diseases, held in St. John's, NFLD.

3. Bidawid, S.*, Farber, J.M. and Sattar, S.A. (November, 1998). Gamma irradiation of hepatitis A virus in fruits and vegetables. Annual Meeting of the Canadian Association for Clinical Microbiology and Infectious Diseases, held in Toronto, Ont.

4. Bidawid, S.*, Farber, J.M. and Sattar, S.A. (November, 1998). Survival of hepatitis A virus on lettuce stored under various modified atmosphere packaging. Annual Meeting of the Canadian Association for Clinical Microbiology and Infectious Diseases, held in Toronto, Ont.

5. Bidawid, S.*, Farber, J.M. and Sattar, S.A. (November, 1999). Studies on hepatitis A virus transfer from contaminated fingerpads of volunteers to food. and its interruption. Annual Meeting of the Canadian Association for Clinical Microbiology and Infectious Diseases, held in Edmonton, Alta.

6. Sattar*, S.A., Bidawid, S. and Farber, J.M. (August 1998). Detection and thermal inactivation of hepatitis A virus in foods. Annual Meeting of the International Assoiation of Milk, Food and Environment Sanitarians, held in Nashville, Tennessee.