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STUDIES ON THE ROLE OF INANIMATE SURFACES AND HANDS IN THE SPREAD OF HEPATITIS A VIRUS AND THEIR CHEMICAL DISINFECTION

A Thesis Submitted to the School of Graduate Studies

University of Ottawa

In Partial Fulfillment of the Requirement for the Degree of Doctor of Philosophy

Department of Microbiology and Immunology, Faculty of Medicine

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UNIVERSITY OF OTTAWA

Ivinda ya tene, Mwana Munduume anengawa uta na maangi ni Ithe nikana akasyimie andu amake. Indi yu, uta na maangi ni mavuku na iandiki. Ngai ndethya umanthini wa uui!

In accordance with Akamba tradition, a son was bequeathed a bow and a quiver of arrows by his father for protection and as hunting aid. Bows and arrows have now been replaced by books and pens. God help me in my search for Truth and Knowledge.

Dedicated to my Parents

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LIST OF ABBREVIATIONS

AG : Alkaline glutaraldehyde

BCG : Bacillus Calmette-Guérin. An attenuated *Mycobacterium bovis* strain

CFU : Colony forming units

CR326 : A tissue culture adapted Hepatitis A virus strain first isolated from a Costa Rican patient

DPD : N,N-diethyl-*p*-phenylenediamine

EBSS : Earle's balanced salt solution

F : Variance ratio (S_1^2/S_2^2) also expressed as: between group variance/within group variance

FBS : Fetal bovine serum

FFE : Flexible fiberoptic endoscope

FP : Fingerpad

FRhK-4 : Fetal rhesus monkey kidney cell line

GI : Gastrointestinal endoscope

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, the "M" the electron microscopist and 175 is the laboratory accession number.

HCl : Hydrochloric acid

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

Ki : A constant representing rate of virus inactivation as $\log_{10}$ PFU over time

MEM : Minimum essential medium

$MgCl_2$: Magnesium chloride

mM : Milli-Molar

mL : Milli-Litre

NaOH : Sodium hydroxide

PBS : Phosphate buffered saline

PFU : Plaque forming units

ppm : Parts per million (milligram per litre)

PV : Poliovirus

RH : Relative humidity

TPB : Tryphosphate broth

µL : Microlitre

µg: Microgram

1. ABSTRACT

1. ABSTRACT

Hepatitis A continues to be a serious problem for human health. Apart from community-based cases, hepatitis A virus (HAV) causes outbreaks in hospitals, day-care centers and schools. The mechanisms of spread of the virus in such settings are poorly understood. This has, in turn, made it difficult to institute effective measures for its prevention and control. The present study was designed to (a) assess the vehicular role of human hands and environmental surfaces in the spread of HAV (b) evaluate the efficacy of chemical disinfectants and handwashing agents to eliminate HAV from inanimate and animate surfaces and (c) determine the efficacy of alkaline glutaraldehyde reuse against HAV and other microorganisms.

Each stainless steel disk (1 cm diameter) was contaminated with 10 µL (6.6×10^4 PFU) of fecally-suspended HAV (strain HM-175) and the disks held at either low (25±5%), medium (55±5%), high (80±5%) or ultra-high (95±5%) relative humidity (RH) at an air temperature of 5, 20 or 35°C. HAV survival was inversely proportional to the level of RH and temperature and the half-lives of the virus ranged from >7 days at the low RH and 5°C to about 2 hours at the ultra-high RH and 35°C. In parallel tests with fecally-suspended Sabin poliovirus (PV) type 1 (approximately 8.0×10^5 PFU/10 µL) at the low and ultra-high RH and 20°C, all PV infectivity was lost within 4 hours at the low RH, whereas at the ultra-high RH it remained detectable up to 12 hours. Overall, the effect of RH on virus survival was significant ($F=9.61$ $P<0.05$ for HAV; $F=5.99$, $P<0.05$). Similarly, the overall temperature effect on HAV survival was also significant ($F=34.24$, $P<0.05$). A significant effect on HAV survival due to RH-temperature interaction was also revealed ($F=18.30$ $P<0.05$). At 20°C, HAV survived much better than PV on non-porous environmental surfaces ($F=15.27$, $P<0.05$). Moreover, the ability of HAV to survive better at lower levels of RH is in direct contrast to the behavior of other picornaviruses. The ability of HAV to survive well may help explain the lack of a clear seasonal pattern in hepatitis A outbreaks in temperate regions.

HAV survival on hands was determined by depositing 10 µL (6.0×10^4 PFU) of

fecally-suspended virus on each fingerpad and eluting the inoculum after 0, 20, 60, 120, 180 and 240 minutes. Virus half-lives ranged from 5.5 to 7.7 hours and the rate of inactivation in $\log_{10}$ PFU/minute (*Ki*) was between 0.0015 and 0.0021; at 4 hours, 835 to 1,804 PFU could still be detected.

HAV inocula of 10 µL (approximately 1.0 X 10^4 PFU) placed on fingerpads or the metal disks were used to determine virus transfer to clean surfaces upon a 10-second contact at a pressure of nearly 0.2 kg/cm^2. When the inoculum was dried for 20 minutes, virus transfer from fingerpad-to-fingerpad, fingerpad-to-disk and disk-to-fingerpad ranged from 2,800 to 3,884 PFU, while 0-50 PFU could be transferred after 4 hours of drying. Elevating the contact pressure alone from 0.2 kg/cm^2 to 1.0 kg/cm^2 resulted in approximately a 3-fold increase in the amount of virus transferred. Incorporating friction (10 half-turns of the finger during a 10-second contact) with the low and high levels of pressure gave a 2-fold and 3-fold increase in virus PFU transferred, respectively. Pressure and friction were found to significantly affect HAV transfer ($F=33.98$; $p<0.05$) irrespective of the mode of transfer used. No statistically significant interaction was observed between mode of transfer and pressure or friction. The findings of this phase of the study suggest that human hands and inanimate surfaces may play an important role in the direct as well as indirect spread of HAV.

HAV disinfection was assessed on experimentally-contaminated metal disks. Ten uL of fecally-suspended HAV (approximately 1.9 X 10^5 PFU) was deposited at the center of each disk, dried for 20 minutes and then covered with 20 µL of the test product for 1 minute. Of the 23 formulations tested, only 2% alkaline glutaraldehyde, a quaternary ammonium formulation containing 23% HCl, and sodium hypochlorite (>5000 ppm of free chlorine) reduced the virus titer by >3 $\log_{10}$; phenolics, other quaternary ammonium-based formulations, iodine-based products, alcohols and 10% solutions of acetic, peracetic, citric and phosphoric acids were unable to do so. Exposure of the dried HAV inocula to 3% Virkon and sodium hypochlorite (with 800 ppm free chlorine) did not result in a >3 $\log_{10}$ reduction of the virus even after 40 minutes. These results show the relative resistance of HAV to a variety of commercial and

non-commercial disinfectants.

The ability of selected chemical disinfectants to interrupt HAV transfer from inanimate surfaces to hands was assessed. Ten µL of fecally-suspended HAV was dried on each metal disk for 1 hour under ambient conditions. The inoculum was then exposed to 20 µL of either tapwater or the product under test. Once the liquid had become visibly dry (about 40 minutes), virus transfer was attempted by pressing onto the disk a clean fingerpad for 10 seconds at a pressure of 1 kg/cm^2. The virus from the fingerpads was eluted and the eluates plaque assayed.

No virus was transferred from disks treated with a 3% solution of Virkon. The amount of virus transferred after tapwater (4.07±1.84%) treatment was significantly different from that with LpH (13.99±2.33%), a phenolic, and a sodium hypochlorite (13.37±6.10%) solution containing 800 ppm free chlorine. Amount of virus transferred after treatment with Unicide 128, quaternary ammonium-based product, was 7.94±2.32% and this was not found to be statistically significant (at the 95% confidence level) from the amounts transferred with sodium hypochlorite, LpH and tapwater.

For each of 3 volunteers used to evaluate efficacy of hygienic handwashing agents, 10 µL of fecally-suspended HAV (approximately 9.2X10^4 PFU) or PV (about 6.0X10^7 PFU) was placed on each fingerpad (FP) and the inoculum allowed to dry for 20 minutes. The contaminated area was then exposed to 1 mL of the handwashing agent under test for 10 seconds, rinsed in 15 mL of tapwater and dried with paper towel. The FP was treated with tryptose phosphate broth to elute any infectious virus remaining.

None of the eleven handwashing agents examined was able to reduce the infectivity titer of HAV and PV to an undetectable level. The least reduction in HAV titer was shown by an unmedicated soap (77.96±7.17%), while the highest level of reduction was given by Bacti-Stat Medicated Soap (92.04±4.02%). The lowest PV reduction was however shown by tapwater (85.22±2.91%) while the highest level of reduction was again by Bacti-Stat (98.39±1.98%). Significant differences were found between HAV and PV removal in all the agents except in Anti-Bacterial Triclosan Hand Soap and Scrub Stat IV. There was good

correspondence in the results of the fingerpad protocol and those obtained with the whole-hand washing test. These findings reinforce the need to assess the activity of such products against viruses prior to marketing.

In a separate set of experiments, the infectious virus remaining on the FP after exposure to a handwash, rinsing and drying was transferred to a stainless steel disk with a contact time of 10 seconds and a pressure of 1 kg/cm^2 without friction. Detectable amounts of both HAV and PV were transferred with all agents except an alcoholic solution of Savlon, 70% ethanol and Alcare.

Samples of 2% alkaline glutaraldehyde were collected over the 14-day reuse period from two manual and one automatic bath used for the disinfection of flexible bronchoscopes and gastrointestinal endoscopes at a neighboring hospital. The number of instruments put through each bath during the 14-day cycle was recorded. The disinfectant samples were tested for pH, glutaraldehyde concentration and protein content. Their germicidal activity was tested against a mixture of HAV, PV, *Pseudomonas aeruginosa* and *Mycobacterium gordonae* and separately against *Mycobacterium bovis* using a carrier test with 5% fetal bovine serum as the organic load and a contact time of 1 minute.

The initial pH of the disinfectant in all the baths was about 8.4. It remained between 8.2 and 8.3 in the two manual baths at the end of the cycle. The drop in the pH in the automatic bath was greater, the final value being 7.7. The starting glutaraldehyde concentration was about 2.25% and it fell to approximately 1% at the end of the 14-day reuse period in all the three baths. Protein concentration rose gradually in all the baths reaching a maximum of nearly 1074 µg/mL. The broad-spectrum germicidal activity of the disinfectant lasted only up to six days. This suggests a review of alkaline glutaraldehyde reuse in the disinfection of semi-critical instruments such as flexible fiberoptic endoscopes.

These findings should help in understanding the genesis of HAV outbreaks more clearly and in designing better measures for their prevention and control.

2. GENERAL INTRODUCTION

2. GENERAL INTRODUCTION

Hepatitis A continues to be an important problem of human health throughout the world (CDC, 1992; Polakoff, 1990). Epidemiological studies have conclusively shown that drinking fecally contaminated water and eating shell-fish, harvested from sewage-polluted waters and consumed without proper cooking, play a significant role in its spread (Halliday et al., 1991). However, in nearly 50% of the cases of hepatitis A the mechanisms and vehicle(s) of virus spread remain unidentified (Hadler and Margolis, 1989). Strong circumstantial evidence indicates that hands of health-care workers and food handlers can act as vehicles for HAV but the ability of the virus to remain viable on human hands remains undetermined. The virucidal efficacy of hand-washing agents is similarly unknown. The patterns of spread of hepatitis A in institutional settings, e.g. day-care centers, also suggest that environmental surfaces may play a role in the spread of the infection (Hadler et al., 1980) and the use of certain types of chemical disinfectants is recommended for the prevention and control of such spread (Thraenhart, 1991). So far, no published information has been available either on the ability of HAV to survive on environmental surfaces or on the comparative efficacy of chemicals in the their decontamination. Improperly decontaminated semi-critical instruments such as flexible fibreoptic endoscopes (FFE) could also spread hepatitis A (Hanson et al., 1989), but the HAV-inactivating capacity of disinfectants used in endoscopy is not known.

HAV was, therefore, the main focus of this laboratory-based study. The experiments were designed to determine the (a) influence of environmental factors on the capacity of HAV to survive on non-porous inanimate surfaces, (b) ability of HAV to remain viable on human hands, (c) sensitivity of HAV to commonly used environmental surface disinfectants, (d) extent of HAV elimination by hand-washing agents routinely used in health-care settings, (e) potential of disinfectants and hand-washing agents in interrupting the transfer of HAV between animate and inanimate surfaces and (f) efficacy of alkaline glutaraldehyde, under reuse conditions in an endoscopy unit, against HAV as well as poliovirus (PV) type 1 (Sabin), *Pseudomonas aeruginosa*, *Mycobacterium gordonae* (*M. gordonae*) and *Mycobacterium bovis* (*M. bovis*).

3. REVIEW OF THE GENERAL LITERATURE

3. REVIEW OF THE GENERAL LITERATURE

Hepatitis A Virus

Classification. Until recently, HAV was designated as Enterovirus 72 (Melnick, 1983) because of its size, acid resistance and single-stranded RNA genome of positive polarity. However, several studies in the past few years have revealed that HAV is different from classical enteroviruses in many respects (Bradley et al., 1984; Lemon, 1985; Parry and Mortimer, 1984; Siegl and Eggers, 1982; Siegl et al., 1984). In view of this evidence, Melnick (1990) proposed a genus *Heparnavirus* in the family Picornaviridae to accommodate it. The International Committee for the Taxonomy of Viruses has now placed HAV as the sole member of a new 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 capsomeres (Cook et al., 1976; Feinstone et al., 1973), The genome consists of linear, single-stranded, positive-sense RNA (Bradley et al., 1978; Gauss-Muller et al., 1984; Provost et al., 1975; Siegl et al., 1981). The genome organization is similar to that of other picornaviruses as it has a 5'-end (capped by a small protein, Vpg), a large open-reading frame responsible for a single polyprotein and a poly-A tail at the 3'-end (Siegl et al., 1981). The capsid is composed of three major polypeptides (VP1: 30-33k, VP2: 24-26k, VP3: 21-23k) similar in size to those of other picornaviruses (Cooper et al., 1978). The existence of a minor polypeptide (VP4: 7-14k), also identified in other picornaviruses, is suggested from molecular cloning and sequence data (Baroudy et al., 1985; Gust et al., 1983; Tratschin et al., 1981), although its presence has not been conclusively shown (Hollinger and Ticehurst, 1990). However, there are major differences in the amino acid sequences in the proteins of HAV and other picornaviruses (Palmenberg et al., 1984; Baroudy et al., 1985; Cohen et al., 1987).

Biophysical characteristics. Density gradient measurements indicate the presence of three types of virions with densities of 1.29-1.31, 1.33-1.34 and 1.40-1.48 g/cm^3 (Bradley et al., 1984; Coulepis et al., 1987). Particles with densities of 1.29-1.31 g/cm^3 (the less dense

particles) are empty, premature or defective virions and are abundant during early stages of infection (Bradley et al., 1977) while those showing densities of 1.33-1.34 g/cm^3 are mature and intact virions. The heavy particles (1.40-1.48 g/cm^3) have an open virion structure that binds or allows cesium chloride penetration (Coulepis et al., 1987; Lemon, 1985; Hollinger and Ticehurst, 1990). Similar groups of multiple buoyant density picornaviruses have also been described (Cooper et al., 1978; Wiegers et al., 1977; Yamaguchi et al., 1975).

HAV analysis by rate-zonal ultracentrifugation in sucrose indicates sedimentation coefficients ranging from 50S to 230S (Bradley et al., 1978; Siegl and Frosner, 1978). This finding is consistent with the belief that HAV has three alternative virion structures (Coulepis et al., 1987). Multiple sedimentation rates have also been reported in other enteroviruses (Wiegers et al., 1977) and super dense poliovirus particles sediment at 220 and 160 S in neutral gradients but the same particles may show multiple sedimentation rates ranging from 220 to 35 S in non-neutral gradients (Coulepis et al., 1987; Wiegers et al., 1977).

HAV strains and cell culture. There is only one serotype of HAV (Jansen et al., 1990; Lemon et al., 1990; Melnick, 1990; Robertson et al., 1991). However, seven groups of HAV strains are distinguishable on the basis of nucleotide sequence of selected capsid regions (Robertson et al., 1992). Genotype I includes the majority of HAV strains (82 of 104 human strains studied) while genotype II shows an intermediate sequence diversity from genotype I and is represented by a single isolate, CF-53 (France 1979; Jansen et al., 1990). Genotype III are strains that show 20% nucleotide sequence differences from genotype I and is comprised of a simian isolate from Panama (PA21, 1980; Lemon et al., 1982), a human isolate from Sweden (H-1221979; Jansen et al., 1990) and a Georgian (USA) isolate of 1976 (GA76; Robertson et al., 1991). Genotype VII, a single strain (SLF88) isolated from Sierra Leone in 1988, differs from genotype II by approximately 17% of base positions. Both genotypes II and VII show a disparity in 20-25% base positions 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 viruses

(Robertson et al., 1992).

The epidemiological or pathobiological relevance of HAV genotypes is unknown and more than 90% of HAV isolates examined have high sequence homology and belong to genotype I (Hollinger and Ticehurst, 1990; Robertson et al., 1992).

In cell culture, wild-type HAV produces a persistent, protracted, acytopathic infection (Bradley et al., 1984; Melnick, 1990). Ever since Provost and Hilleman (1979) reported the first *in vitro* propagation of HAV (strain CR-326), several other strains have been successfully grown in a number of cell culture systems. Flehmig (1980) managed to culture HAV in a fetal rhesus monkey kidney (FRhK-4) cell line. Human diploid embryo fibroblasts (Gauss-Muller et al., 1981), human primary liver carcinoma cells (PLC/PRF/5; Frosner et al., 1979), Vero cells (Locarnini et al., 1981) and African Green Monkey kidney cells (Daemer et al., 1981) have also been used to grow this virus.

The degree to which HAV is released from the cells is variable. In some cell culture systems, HAV appears to be tightly cell associated (Frosner et al., 1979) while in others there is substantial release of virus into the culture medium (Flehmig, 1980). HAV particles are also reported to be lipid-associated and non-neutralizable (Lemon and Binn, 1985). This means that cell membranes probably play a role in the assembly and release of HAV from infected cells (Lemon and Binn, 1985). The majority of HAV strains do not show cytopathology in most of the cell cultures listed above. Electron microscopy, immunofluorescence, radioimmunoassay and enzyme linked immunosorbent assay can be used to detect the presence of the virus in them (Asher et al., 1987; Duermeyer et al., 1979; Feinstone et al., 1973; Hollinger et al., 1975; Mathiesen et al., 1977).

Of the 20 human cell culture-adapted strains of HAV, nine can be distinguished from one another on the basis of growth characteristics (Binn et al., 1984; Bradley et al., 1984; Siegl et al., 1984), nucleotide sequences (Cohen et al., 1987), oligonucleotide maps (Weitz and Siegl, 1985) or hybridization patterns (Ticehurst et al., 1987; Lemon et al., 1987). Initial HAV characterization was performed with strain MS-1 (Boggs et al., 1970; Feinstone et al., 1973;

Krugman et al., 1967) but strains HM-175 and CR-326 are now the more widely studied (Cohen et al., 1987; Simmonds et al., 1985; Stapleton and Lemon, 1987). Mouse monoclonal antibodies produced against HM-175 are the reference reagents (McGregor et al., 1983; Ping et al., 1988). HM-175 and CR-326 are regarded as appropriate hepatitis A vaccine candidates due to their well characterized nature (Karron et al., 1988; Midthun et al., 1991).

Physicochemical stability. Even though enteroviruses are stable at pH 3.0 (Newman et al., 1973; Siegl et al., 1984), their infectivity is rapidly lost at pH below this (Faber and Dong, 1946; Loring and Schwerdt, 1944). HAV, on the other hand, appears to be stable for a few hours even at pH 1.0 (Coulepis et al., 1987; Scholz et al., 1989). Highly purified preparations of HAV also show this stability, suggesting the presence of a specific genetic marker for acid resistance rather than the protection of the virus by cellular debris (Lemon and Binn, 1985; Scholz et al., 1989).

The virus loses only 50% of its infectivity when heated in a liquid at 61°C for 10 minutes (Siegl et al., 1984). Complete HAV inactivation occurs at 87°C whereas poliovirus infectivity becomes undetectable in 10 minutes by heating at 67°C. As is true of enteroviruses, addition of 1 M $MgCl_2$ to HAV suspensions further enhances its heat resistance; the 50% virus inactivation temperature level is shifted from 61°C to 81°C (Siegl et al., 1984). HAV dried in feces and stored at 25°C and 42% relative humidity could retain its infectivity for marmosets even after one month (McCaustland et al., 1982). It can also remain infectious for several days in freshwater, seawater, wastewater, soil, marine sediment, live oysters, cookies and in feces dried on polystyrene surfaces (Sobsey et al., 1988).

Exposure of HAV suspensions to free chlorine levels of up to 1.5 mg/L could not render them non-infectious to marmosets (Peterson et al., 1983). HAV in tap water also shows a higher degree of resistance to ozone as compared to polioviruses (Herbold et al., 1989; Vaughn et al., 1990). Chloramine T (1 mg/L) and perchloracetic acid (300 mg/L) were ineffective against the virus after a 15-minute contact at 20°C (Hollinger and Ticehurst, 1990). Although HAV has been found to be sensitive to chemicals such as ß-propiolactone, potassium

permanganate and formaldehyde (Hollinger and Ticehurst, 1990), there is virtually no published information on the ability of the virus to withstand exposure to chemicals used in the decontamination of environmental surfaces, medical instruments and hands.

Outcome of HAV infection. Hepatitis A is an acute and self-limiting infection that does not develop into a chronic carrier state (Lemon, 1985; Sjogren et al., 1987). Clinically, hepatitis A is indistinguishable from other forms of hepatitis (Lemon et al., 1985). The infection may be inapparent but when clinical symptoms appear they may be with or without jaundice (Hollinger and Ticehurst, 1990). The ratio of icteric to anicteric cases has been estimated to range from 1:3.5 to 1:12 (Krugman et al., 1960; Routenberg et al., 1979).

The age of the individual seems to be the major determining factor as to whether the infection becomes an inapparent, anicteric or icteric case (Forbes and Williams, 1990; Hadler et al., 1980; Tilzey and Banatvala, 1991). More than 50% of infected adults develop clinical hepatitis (CDC, 1992; Chin et al., 1991) while nearly 85% of the cases in children younger than 10 years remain asymptomatic (CDC, 1992; Gingrich et al., 1983; Hollinger and Ticehurst, 1990). HAV infection rarely results in fulminant hepatic failure and the fatality rate is age-dependent so that it is 0.02%-0.03% for those under 55 years but as high as 1.5 % in those over 64 years (Tilzey and Banatvala, 1991). The overall mortality rate is 0.1% but it can be as high as 0.5% in icteric cases (Hollinger and Ticehurst, 1990).

Hepatic and extra-hepatic complications of hepatitis A. Unlike hepatitis B, for instance, hepatitis A is generally regarded as benign and not associated with hepatic or extra-hepatic complications (Gocke and Johnson, 1986). Recent findings suggest otherwise. Cholestatic hepatitis with massive acute hemolysis has now been reaffirmed (Lyons et al., 1990). Several reports have also documented a relapsing form of HAV infection (Glikson et al., 1992; Karayiannis et al., 1990). McDonald et al. (1989) have described a prolonged (over 1034 days) rise in anti-HAV IgM antibodies with liver enzymes remaining marginally elevated for 4 years and annual liver biopsies showing evidence of chronicity. This evidence concurs with that of Routenberg et al. (1979) and Cornu et al. (1983) who described chronic active

hepatitis A with necrosis although the patients reverted to normal within 12-15 months. Relapsing hepatitis A infection usually occurs after a short period (<3 weeks) and the prevalence among patients with acute hepatitis A is 3 to 20% (Glikson et al., 1992).

Among the reported extra-hepatic complications of hepatitis A are the Guillain-Barré syndrome and other neurologic complications (Tabor, 1987). Auto-antibodies to liver membrane antigens can be induced during HAV infection (Weidmann et al., 1984; Jensen et al., 1984) and this has been suggested as a trigger for auto-immune chronic active hepatitis in susceptible subjects (Vento et al., 1991). Severe glomerulonephritis, evanescent skin rash, transient arthralgia, arthritis and cutaneous-vasculitis have also been shown to accompany HAV infection (Dan et al., 1990; Morita et al., 1981; Karayiannis et al., 1990).

Epidemiology of hepatitis A

Global distribution. HAV infections occur endemically world-wide and in many temperate countries this occurrence depicts a cyclical epidemic peak of about 7-10 years (CDC, 1990; Polakoff, 1990; Ross et al., 1991; Shaw et al., 1990), though this phenomenon seems to be disappearing in most industrialized countries (Melnick, 1990) and smaller peaks of four year intervals are evident (Ng et al., 1989). The cyclical epidemic peaks of hepatitis A appear to represent the time needed to generate enough susceptible individuals to fuel an outbreak (Ross et al., 1991; Shaw et al., 1990). In developing countries, virtually all children are exposed to HAV by 15 years of age (Tassopoulos et al., 1986; Coulepis et al., 1987; Tsega et al., 1990) and therefore, the susceptible adult population is low and as such clinical HAV is uncommon.

In the U.S., there were 28,500 cases of hepatitis A for the year 1988 (CDC, 1990); this accounts for nearly 50% of all recorded cases of hepatitis in that year. From 1983 to 1989, the incidence of hepatitis A in that country increased from 9.2 to 14.5 cases per 100,000 (CDC, 1990), a frequency increase of 26% (Midthun et al., 1991). Figures from the first half of 1991 show the rates of hepatitis A in the U.S., Canada and Australia to be higher than in previous years (CDC, 1992); this increase was particularly prominent in homosexual/bisexual males. For

instance, Toronto reported 274 cases representing a four-fold increase over the same period in the previous year. A four-fold increase (20.70 per 100,000) was also reported for Montreal (CDC, 1992). Reports from British Columbia for 1988-1989 show a 48.9% increase (5-fold) in the cases of hepatitis A and this increase does not appear to be due to correcting, under-reporting or under-diagnosis in previous years (Ng et al, 1989). An increase in the number of recorded cases of this disease is also reported from the U.K. and other industrialized countries (Kani et al, 1991; Polakoff, 1990; Tilzey and Banatvala, 1991).

The endemicity of hepatitis A in developing countries appears to be changing towards a more epidemic pattern generally observed in industrialized countries (Chin et al., 1991; Innis et al., 1991; Midthun et al., 1991; Shaw et al., 1990; Shaw et al., 1989; Schwarz et al., 1991). This is most likely due to improvements in water supply and sanitation. Antibody surveys further substantiate the drop in the prevalence of the infection. For example, in Hong Kong 89.2% of individuals over 30 had antibodies against HAV as compared to 24% in those below this age (Chin et al., 1991). The continuing decline in the endemicity of hepatitis A in developing countries is increasing the size of the susceptible population (Midthun et al., 1991) and this points to the urgency for a suitable vaccine against this infection (WHO, 1988).

Community-based outbreaks. Community-based epidemics of hepatitis A are well documented, particularly in industrialized countries (CDC, 1990; Shaw et al., 1989). Such epidemics are usually large and often involve common source vehicles such as food or drinking water (Bloch et al., 1990; Lowry et al., 1989; Mosley, 1959; Taylor et al., 1966; Warburton et al., 1991).

Various types of shell-fish are particularly important as vehicles for HAV (Feingold, 1973; Halliday et al., 1991; Portnoy, 1975). During feeding, these bivalves, when cultivated in sewage-polluted areas, can concentrate HAV and other human pathogens to more than six times the levels found in the overlaying waters (Millard et al., 1987; O'Mahony et al., 1983). Recently, mussels (*Mytilus chilensis*) have been shown to concentrate HAV to levels nearly 100-fold higher than that in the surrounding waters (Enriquez et al., 1992). Eating of such

shell-fish raw or with improper cooking presents a high risk for contracting hepatitis A (Halliday et al., 1991).

Drinking water which is either improperly treated or fecally contaminated during distribution or storage may also result in large community-based outbreaks of hepatitis A. Lippy and Waltrip (1984) reported that 672 waterborne disease outbreaks occurred in the U.S. over a 35 year period (1946-1980), of which 68 were of hepatitis A. Drinking water has also been implicated in other outbreaks of the disease (Bloch et al., 1990; Bowen et al., 1983; Mosley, 1959; Taylor et al., 1966). Inadequate treatment and the higher resistance of HAV to levels of chlorine used in conventional methods of drinking water treatment are usually a factor in this regard (Bloch et al., 1990; Bowen et al., 1983).

Recently, a hepatitis A outbreak associated with swimming in a public pool has been reported (Mahony et al., 1992); free chlorine in the pool was found to be at the recommended level. Although outbreaks of hepatitis A are frequently associated with consumption of virus-contaminated food or water, these vehicles are incriminated in less than 7% of the reported cases of the disease in the U.S. (CDC, 1983; Lowry et al., 1989).

Outbreaks in institutional settings. Institutional outbreaks of hepatitis A are often related to eating establishments (Hooper et al., 1977; Levy et al., 1975; Lowry et al., 1989; Mishu et al., 1990), day-care centers (Hadler et al., 1980; Pohjanpelto and Ponka, 1985), institutions for the mentally retarded (Lehmann et al., 1978; Matthew et al., 1973), schools (Naus et al., 1989; Reid and Carter, 1986; Skidmore et al., 1982) and hospitals (Goodman et al., Klein et al., 1984; Rosenblum et al., 1991).

(a) Eating establishments. In eating establishments, the index case is commonly a food-handler (Denes et al, 1977; Levy et al., 1975) who may be asymptomatic or in the prodromal stage of the disease (Hooper et al., 1977). Fecal excretion of HAV is known to occur for several days before and after the on-set of the clinical phase (Rakela et al., 1976; Rosenblum et al., 1991). An HAV-excreting food-handler with poor personal hygiene may contaminate food that is consumed raw or cooked food prior to serving.

(b) Day-Care Centers. Outbreaks of hepatitis A in day-care centers are a major problem in many industrialized countries (Hadler and McFarland, 1986; Hadler et al., 1980; Pohjanpelto and Ponka, 1985; WHO, 1987). The index case in most such outbreaks is an asymptomatic child (Gingrich et al., 1983; Hadler et al., 1980), and the outbreak continues unnoticed till a clinical case occurs, usually in an adult (Hadler et al., 1980; Vernon et al., 1982; WHO, 1987). Day-care centers often act as sources of the virus for the infection in families and the community at large (Hadler and McFarland, 1986; Pohjanpelto and Ponka, 1985; WHO, 1987).

Hepatitis A in day-care centers in the U.S. accounts for 9-12% of the country-wide cases (Hadler and Margolis, 1989; Vernon et al., 1982). Among the important factors in the spread of the virus in day-care centers (Vernon et al., 1982) are the size of the institution, hours of its operation and the number of non-toilet trained children enrolled in it (Hadler and McFarland, 1986).

(c) Schools and institutions for the mentally-retarded. Almost 60% of the 20-year-old patients in institutions for the mentally-retarded have anti-HAV antibodies and by age 40 nearly all of these individuals show sero-conversion; comparable figures for the general community are 40% and 60% (Lehmann, et al, 1978). This may reflect the crowded and poor hygienic conditions in these institutions (Matthew et al., 1973). Unlike day-care centers, secondary cases of hepatitis A rarely occur in employees of institutions for the mentally-retarded (Matthew et al., 1973).

In developing countries, HAV outbreaks in schools rarely occur except among children of high economic class (Stroffolini et al., 1991). The frequency of hepatitis A outbreaks in normal schools in industrialized countries is also much lower than that observed for day-care centers and institutions for the mentally-retarded, but have been reported nonetheless (Naus et al., 1989; Reid and Carter, 1986; Skidmore et al., 1982). Secondary cases in teachers are infrequent (Naus et al., 1989; Reid and Carter, 1986) and this probably is due to a lower degree of physical contact with the pupils. But of significance is the observation that outbreaks

of the disease tend to cluster in either girls or boys (Naus et al., 1989; Reid and Carter, 1986); this may be due to closer contact between pupils of the same sex and the segregation of toilet facilities.

(d) Nosocomial outbreaks. Hospital-acquired hepatitis A is a frequent occurrence (Drusin et al., 1987; Goodman et al., 1982; Klein et al., 1984; Orenstein et al., 1981; Rosenblum et al., 1991) for patients as well as health-care personnel (Bryne, 1966). It has been suggested by many that the available figures of nosocomial cases of hepatitis A are underestimations (Coulepis et al., 1980; Mao et al., 1980; Rakela and Mosley, 1977).

Pediatric wards are particularly important with regards to outbreaks of hepatitis A in hospital settings. HAV-infected patients in such wards are often subclinical cases and recently infants have been shown to excrete relatively large amounts of the virus for 4 to 6 months (Rosenblum et al., 1991). This finding is an affirmation of the data of Tassopoulos et al., (1986) who found that HAV illness in children was more likely to have detectable fecal viral antigen (46%) than adults (14%) during the second week of infection. Pediatric patients may be admitted for diseases unrelated to viral hepatitis (Baptiste et al., 1987; Bryne, 1966; Edgar and Campbell, 1985; Orenstein et al., 1981; Seeberg et al., 1981). As a result, precautionary measures that are normally recommended for HAV cases may not be observed. Nurses in these institutions have been reported to spread HAV from one child to another (Klein et al., 1984) having initially contracted the infection from an asymptomatic child. Therefore, an important risk factor for infection in pediatric hospital is care of a primary infant-case by a nurse who in turn becomes a risk factor to other children in the unit (Rosenblum et al., 1991). Fecal incontinence in patients helps to spread the virus and also facilitates the contamination of the hospital environment (Bradley et al., 1976; Goodman et al, 1982).

Modes of HAV transmission

As an enteric virus, HAV is excreted in the feces of infected individuals and it spreads mainly by the fecal-oral route (Hollinger and Ticehurst, 1990) either directly or through certain vehicles. This mode of transmission is greatly facilitated by poor hygienic conditions. Presence

of HAV in oropharyngeal secretions and their role in the transmission of hepatitis A remain unresolved issues (Stapleton et al., 1991). Spread of hepatitis A also occurs through the parenteral administration of blood or blood products (Azimi et al., 1986; Seeberg et al., 1981).

Transmission through homosexual activity. Hepatitis A has been known to be a relatively common infection in homosexual men (Christenson et al., 1982; Corey and Holmes, 1980; Coutinho et al., 1983; Hoybye et al., 1980; Keeffe, 1986). Recent reports (CDC, 1992) from many parts of the world in fact show a further increase in its incidence in this high risk group; for example, the incidence rate for hepatitis A in Toronto's homosexual community climbed from 13.3 per 100,000 in 1990 to 56.3 per 100,000 in 1991 (CDC, 1992). The male:female ratio associated with this incidence was estimated at 1.3:1 for 1990 but 12:1 for the same period in 1991. The prevalence of anti-HAV antibodies in homosexual men is also higher than that observed in the general population (Corey and Holmes, 1980; Coutinho et al., 1983; Keeffe, 1986).

Multiple sexual partners, duration of homosexuality, presence of other venereal diseases and oral-anal sexual exposure were found to be significant factors in HAV outbreaks in homosexuals (CDC, 1992; Christenson et al., 1982; Corey and Holmes, 1980; Coutinho et al., 1983; Keeffe, 1986;). As many homosexuals work in food establishments and health-care settings, the continuing increase in the number of cases of hepatitis A in this group poses an increased risk to the health of the community in general (Christenson et al., 1982). There is therefore an urgent need to increase surveillance, launch public education campaigns and to develop better methods for preventing and controlling the spread of HAV in order to counteract this threat (CDC, 1992).

Transfusion-associated transmission. Hemophiliacs, thalassemics and hemodialysis patients have the same anti-HAV prevalence as the general population (Azimi et al., 1986; Hollinger et al., 1983; Noble et al., 1984; Seeberg et al., 1981; Szmuness et al., 1977). However, several reports have documented transfusion-associated HAV (Azimi et al., 1986; Giocola and Kasprisin, 1989; Hollinger et al., 1983; Seeberg et al., 1981). Viremia that occurs

during the late incubation period of HAV infection lasts no more than 3 weeks and precedes development of clinical symptoms (Boggs et al., 1970; France et al., 1946; Havens, 1946; Hollinger et al., 1975; Krugman et al., 1959). Thus, contaminated blood is not identified by serum aspartate aminotransferase or alanine aminotransferase screening (Giocola and Kasprisin, 1989). Transfusion-associated hepatitis A in intensive-care units may infect health-care personnel and precipitate institutional outbreaks (Azimi et al., 1986; Klein et al., 1984 Noble et al., 1984; Seeberg et al., 1981).

Transmission through drug abuse. Increasing numbers of cases of hepatitis A are being recorded in drug abusers who may become infected through the use of contaminated needles or ingestion of fecally-contaminated illicit drugs (Akriviadis and Redeker, 1989; CDC, 1988; Harkess et al., 1989; Widell et al., 1982). Poor personal hygiene appears to contribute to HAV transmission among drug addicts and the incidence of HAV in this group may be four times that of the general population (CDC, 1988). Illegal drugs are often smuggled in condoms concealed in the rectum (Sunddkvist et al., 1985) and the virus is perhaps ingested when an addict samples the drug.

Contamination of certain types of illicit drugs with HAV may also occur when night soil or waste-water is used in their cultivation (CDC,1988). Whereas we still do not fully understand the basis for the increased number of cases of hepatitis A in drug abusers, control measures directed against them do help interrupt outbreaks of this disease (Ng et al., 1989). Attempts to detect infectious HAV in incriminated samples of illegal drugs were unsuccessful (CDC, 1988).

Vehicles for HAV transmission

In developing countries, the relative importance of various vehicles of hepatitis A has not been clearly established. Wide-spread fecal contamination and lower sanitary and hygienic conditions suggest that the spread of the disease in such settings may occur simultaneously through several modes and vehicles. Furthermore, major water-borne outbreaks of hepatitis thought to be due to HAV have subsequently been shown to be due to hepatitis E virus, for

instance (Gust and Purcell, 1987; Khuroo, 1980; Ross et al., 1991). In many developing areas of the world, coprophagous insects such as house flies and cockroaches almost certainly play a role in the mechanical spread of HAV (Hollinger and Ticehurst, 1990; Melnick, 1953).

Even though in industrialized countries such as the U.S. food and drinking water are often associated with relatively large outbreaks of the disease (Denes et al., 1977; Hooper et al., 1977; Levy et al., 1975; Lowry et al., 1989; Mishu et al., 1990; Warburton et al., 1991), these vehicles are incriminated in only 7% of the total number of cases of hepatitis A reported (CDC, 1983; Lowry et al., 1989). The following will, therefore, focus on vehicles of HAV other than food and water.

Several reports have suggested the possibility that air could act as a vehicle for the spread of hepatitis A (Aach et al., 1968; Giles et al., 1964; Gould, 1946; Ipsen et al., 1950; Pickcles, 1930; Summerville and Clark, 1944). However, spraying or instillation of nasopharyngeal fluid from known cases of hepatitis A into the nose or throat of human volunteers produced equivocal results (Havens, 1946; Hollinger and Ticehurst, 1990; McCallum and Bradley, 1944; Neefe and Stokes, 1945). Aach et al. (1968), however, contend that airborne spread does not necessarily mean respiratory tract-to-respiratory tract transmission via air; virus-contaminated feces or other material may get aerosolized directly or dry and become re-suspended in air and inhaled. The virus may or may not multiply in the oropharynx before reaching the enteric tract. Whereas such a scenario for airborne transmission is theoretically sound, there is as yet no convincing epidemiological or experimental evidence that the virus can spread in this fashion. There are no published reports on the capacity of HAV to survive in air.

There is strong circumstantial evidence for the role of hands (Rosenblum et al., 1991) and environmental surfaces (Levy et al., 1975) as vehicles for hepatitis A and the available literature has been reviewed in Part 6 of this thesis.

Prevention and control of hepatitis A spread

Spread of viral diseases can be prevented or controlled through the application of the

following measures singly or in various combinations: vaccination, sanitation and public health, insect vector control, quarantine, screening of blood and blood products, immuno-prophylaxis, public education and disinfection and sterilization. Chemotherapy against viral pathogens is still in its developmental stages (Crance et al., 1990; Girond et al., 1991).

It is generally agreed that the reduction in the number of cases of hepatitis A in industrialized countries since the beginning of this century came about with the general improvements in sanitation and public health (Gust et al., 1988; Melnick, 1990). Properly administered immuno-prophylaxis has proven effective in preventing cases of hepatitis A in susceptible individuals visiting areas where the disease is endemic (CDC, 1990; Hoffman, 1992; Tilzey et al., 1992). Public education campaigns regularly warn people about the dangers of eating shellfish raw. But what is the current status of chemotherapy, vaccination and chemical disinfection in preventing and controlling the spread of hepatitis A?

Antiviral Agents. Recently, chemotherapeutic agents with anti-HAV activity have been reported (Biziagos et al., 1990; Crance et al., 1990; Girond et al., 1991; Mastromarino et al., 1991; Superti et al., 1989). Sulphated polysaccharides such as iota-, lambda-, and kappa-carrageenans all inhibit the replication of HAV in PLC/PRF/5 human hepatoma cells (Girond et al., 1991). Polyanions such as dextran sulfate and heparin are effective against HAV (Mastromarino et al., 1991) and 6,4'-dichloroflavan and 6,4'-dichloroisoflavan, though not virucidal, showed inhibitory effects on HAV antigen synthesis (Superti et al., 1989). Combinations of atropine and protamine were also shown to deter HAV replication though the steps affected remain undetermined (Biziagos et al., 1990). More research is, however, required to improve the selectivity index but, from these reports, the future of antivirals against HAV appears promising.

Vaccines. The presence of a single serotype of HAV makes the development and use of a vaccine very attractive (Jansen et al., 1990; Lemon et al., 1990; Robertson et al., 1991). A live attenuated vaccine is feasible but the risks associated with its use are very difficult to assess because there is no information on certain basic aspects of HAV and its pathogenesis

and pathology. For instance, how stable is the attenuation? What levels of liver enzymes are permissible with a live attenuated vaccine? What is the degree and length of fecal shedding? Would prolonged fecal shedding indicate HAV persistence and how does that relate to a continued provocation of a cellular response, the basis for pathogenesis of hepatitis?

Moreover, vaccine take, based on antibody production, is linked to hepatovirulence (Provost et al., 1986) and antibody response is considerably delayed following infection with live attenuated HAV raising questions on the fate of the virus (Siegl and Lemon, 1990). Surprisingly, the initial site of HAV replication in human hosts is not yet clearly defined (Stapleton et al, 1991; Zajac et al, 1991).

A formaldehyde-inactivated HAV vaccine has been tested in human volunteers in both the U.S. and the U.K. with considerable success in terms of clinical tolerance and sero-conversion (Hoffman, 1992; Tilzey et al., 1992); there was 100% sero-conversion in both children and adults after 2 doses. When compared to passive immunoglobulin administration this vaccine is 200 times more potent (Hoffman, 1992) and therefore highly immunogenic. Unfortunately, large scale production is extremely costly due to poor virus yields in cell cultures and this may limit its use in developing countries (Tilzey et al., 1992).

Attempts to produce a genetically-engineered HAV vaccine have not met with success because the viral protein produced is a weak antigen as it lacks the conformational integrity of its natural counterpart (Siegl and Lemon, 1990). This problem could perhaps be resolved but it may again make the vaccine uneconomical for use on a global basis.

Chemical disinfection and hygienic handwashing. Environmental decontamination and handwashing are practiced in an effort to prevent and control the spread of hepatitis A. However, little has been known about the efficacy of such measures in dealing with HAV. The available information in this regard will be reviewed in Parts 7 and 8 of this thesis.

4. OBJECTIVES

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In spite of our efforts to control the spread of hepatitis A, this disease continues to take its toll in both developing and industrialized countries (CDC, 1992; Giacola and Kasprisin, 1989; Hallidy et al, 1991; Klein et al, 1984; Ng et al, 1989; Rosenblum et al, 1991; Shaw et al, 1990; Tilzey and Banatvala, 1991; Warburton et al, 1991). In fact, in recent years the human health impact of this disease is on the rise (CDC, 1992; Tilzey and Banatvala, 1991). Changes in life-style in industrialized countries and 'development' *per se* in the Third World appear to be responsible for this increase (Gust et al, 1988; Innis et al., 1991). Effective chemotherapy and vaccination are not yet available for general use and the foregoing has high-lighted the gaps in our knowledge with regards to the epidemiology of hepatitis A in institutional settings. There is therefore a need; more than ever before, to better understand the mechanisms of spread of the infection so that informed approaches can be instituted against it.

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

(1) To assess the influence of air temperature and relative humidity on the capacity of HAV to survive on non-porous inanimate surfaces.

(2) To determine how well HAV could survive on human hands.

(3) To evaluate the role pressure and friction play in HAV transfer upon contact between animate and inanimate surfaces.

(4) To test, using a carrier test protocol, how well chemical disinfectants meant for use on environmental surfaces could inactivate HAV.

(5) To investigate, using hands of volunteers, the efficacy of HAV elimination by hand-washing agents used in health-care settings.

(6) To evaluate the comparative efficacy of alkaline glutaraldehyde reuse baths in the inactivation of HAV, poliovirus type 1 (Sabin), *M. gordonae*, *M. bovis* and *P. aeruginosa*.

5. GENERAL MATERIALS AND METHODS

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

Cells. A seed culture of FRhK-4 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). The growth medium was prepared as follows: Five hundred grams of powdered Eagle's minimum essential medium (Gibco, Grand Island, N.Y.) was reconstituted with 4,750 mL of double-distilled deionized water. Quantities of 475 mL were distributed into 500 mL glass bottles and autoclaved. The medium was allowed to cool before each bottle was first supplemented with 7.5 mL of a 7.5% stock solution of sodium bicarbonate (BDH, Toronto, Ontario), 5 mL of 1.5 M HEPES (N-2-hydroxyethyl piperazine-N-2-ethanesulfonic acid; Gibco) and 50 mL fetal bovine serum (FBS; Gibco). It then received 5 mL of 200 mM L-glutamine (Gibco) 5 mL of 10 mM non-essential amino acids (Gibco), 0.5 mL of 50 mg/mL gentamicin sulfate (Cidomycin; Roussel, Montreal) stock to a final concentration of 50 µg/mL and 5 mL of 10 mg/mL kanamycin sulfate stock (Gibco) to a concentration of 100 µg/mL. 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 490 cm^2 roller bottle (Costar, Cambridge, MA) with about 10^5 cells suspended in 60 mL of the growth medium. The roller bottles were then rotated at 37°C for eight days with a single medium change on day four. To harvest the cells, the spent medium was discarded and the monolayer washed three times with Dulbecco's phosphate buffered saline (PBS). It then received 2.5 mL of a commercial trypsin-EDTA mixture (Gibco) and the bottle was manually rotated to completely cover it with the mixture. The excess liquid was immediately discarded and the bottles incubated at 37°C for 10 minutes.

The cells were dislodged by gently tapping the bottle on the sides, 15 mL of the growth

medium was added and the cells further dispersed by repeated pipetting. The cell suspension was centrifuged in sterile 50 mL tubes (Costar, Cambridge, Mass.) at 1000 X *g* for 15 minutes and washed 3 times in fresh PBS at the same centrifugal speed. The packed cells were resuspended in 15 mL of supplemented MEM (no serum) containing additionally 15% FBS and 10% dimethyl-sulfoxide pre-sterilized by autoclaving. The cells in the suspension were counted in a hemocytometer and the density adjusted to 1.0×10^7 cells/mL. One mL quantities were aliquoted into 2 mL plastic cryovials (Product no. 72.694.006; Sarstedt, St. Laurent, Quebec). The vials were wrapped up in several layers of facial tissue and kept at 4°C for 6 hours before overnight storage at -20°C and a further 72 hours at -80°C. Finally, the vials were transferred to a liquid nitrogen tank (-196°C). A week later, one vial was removed and the cells cultured to ensure the success of the cell freezing process.

For day-to-day use, FRhK-4 cells were raised by seeding about 10^5 cells in each 75 cm^2 flask (Costar) using 20 mL of the growth medium discussed above. To evenly distribute the cells over the available area, the flasks, with the caps tightly closed, were rocked gently before incubation on a flat surface at 37°C in a walk-in incubator. The cells were examined every day and the cultures were split when the monolayers were complete (usually 3-5 days). A split ratio of 1:4 of a complete monolayer gave four 75 cm^2 flasks in 4 days while a single flask could give 3-4 12-well micro-culture plates (Costar) with complete monolayers in 3 days when 2 mL of cell suspension was seeded into each well.

Viruses. The HM-175 strain, the prototype of HAV (Minor, 1991), was also received from Dr. Sobsey. Stock virus was prepared by infecting FRhK-4 monolayers at a multiplicity of infection of 0.01. The virus was allowed to adsorb for 90 minutes at 37°C and the infected cultures were then kept in the maintenance medium till 75-80% (4-5 days of incubation) of each monolayer was affected by virus cytopathology. They were then frozen (-20°C) and thawed three times, and the culture fluid was centrifuged for 10 minutes at 1000 X *g*. The virus was concentrated 10-fold by polyethylene glycol hydroextraction as described by Ramia and Sattar (1979). Briefly, sterilized dialysis tubing (Spectrum, Los Angeles, CA) containing 100

mL of the clarified cell culture fluid was placed in a plastic tray and completely covered with polyethylene glycol (molecular weight 8,000; Matheson, Coleman and Bell, Norwood, Ohio). After overnight hydroextraction at 4°C, the residue in the dialysis tube was resuspended in 10 mL of Earle's balanced salt solution (EBSS). The concentrate was filtered through a 0.2 µm membrane filter (Nalge Co., Rochester, N.Y). Concentrated virus pools were aliquoted in 0.5 mL quantities and stored at -80°C.

The Sabin strain of poliovirus (PV) type 1 was obtained from the Laboratory Center for Disease Control (Ottawa, Ontario). FRhK-4 cells were used for propagating this virus as well. The procedures for the preparation, concentration and preservation of the virus pools were the same as described above for HAV.

Virus suspending medium. The fecal sample used in this study was obtained from a healthy 5-month old baby. It was prepared as a 10% (w/v) suspension in normal saline, clarified of gross particulate matter by a 10-minute centrifugation at 1000 X *g*, and passed through a 0.2 µm membrane filter (Nalge) to remove bacteria and fungi. The cytotoxicity of the filtrate and the presence of indigenous viruses in it were tested by inoculating 0.1 mL of it into each 3-day old FRhK-4 monolayer and incubating them at 37°C for at least two weeks under a liquid or agar overlay. There was no evidence of either cytoxicity, cytopathology or plaques.

Disk carriers. Stainless-steel disks (1 cm in diameter), punched out of locally purchased no. 4 finish polished sheets (0.75 mm thickness), were used as carriers to represent non-porous inanimate environmental surfaces.

Before use, the disks were soaked overnight in distilled deionized water containing a 10% solution of 7X (Flow Laboratories, McLean, VA) and then thoroughly rinsed under running distilled deionized water. The disks were then autoclave sterilized and dried overnight in an oven at 56-60°C.

Volunteers. Permission to place HAV and PV on the hands of adult volunteers was first obtained from our university's Ethics Committee. Any individual with cuts or abrasions on

the hands was automatically excluded from the study. Each volunteer was then thoroughly briefed on the experimental protocol and the risks involved before being asked to sign a consent form. The age of the participants ranged from 24 to 46 years.

At the end of the experiment, the volunteer was asked to gently press the experimentally-contaminated fingerpads on a piece of paper towel soaked in a 6% solution of sodium hypochlorite (Javex, Toronto, Ontario) for 3 minutes for their decontamination. The hands were then washed thoroughly with ordinary soap and running tap water and dried with a paper towel.

Plaque assays. Assays to determine the plaque forming units (PFU) of both the viruses were carried out in FRhK-4 monolayers using 12-well plastic plates (Costar). 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 minutes at 37°C. The overlay consisted of supplemented MEM, 2% FBS, 2 µg of amphotericin B (Fungizone; Gibco)/mL, 0.75% agarose (type II; Sigma, St. Louis, Mo.) and 26 mM magnesium chloride (BDH). The plates were sealed in laminated plastic bags (Dazey Corporation, Industrial Airport, KS) and incubated (8 days for HAV and 48 hours for PV) at 37°C. For counting plaques, the FRhK-4 monolayers were first fixed overnight in a 3.7% solution of formaldehyde (BDH) in normal saline. The agar overlay was removed and the cells stained for 3 minutes with a 1% aqueous solution of crystal violet (Fisher). The limit of virus detection in this technique was 3-4 PFU for both HAV and PV.

Statistical analyses. The zero-minute virus titer (PFU/mL) represents 91-100% of the in-put virus. The virus titer at each sampling time (X-value) is the amount of virus remaining expressed as a fraction of that eluted at zero minute. All observations were normalized to the zero minute value as 100%.

The normalized figures obtained at each RH and temperature level were analyzed graphically using a computer program (SigmaPlot Version 4.1; Jandel Scientific, Corte Madera, CA). At each temperature and RH level, both linear and log-linear plots of the normalized data against time were established.

To calculate the rates of virus inactivation and virus half-lives over 96 hours, first order linear-regression lines were generated and fitted to the log-linear plots (log_{10}) using the same program. The rate of virus inactivation (*Ki*) was obtained from the gradient of the regression lines as loss in virus infectivity titer in log_{10} PFU/hour. Similarly, the half-lives of the viruses were calculated from *Ki* as described by Segel (1976). The relationship between *Ki* and half-life is given by the quantity: *In* (N_O / N) = *Ki t*, where *In* = natural logarithm, N = amount of virus at the beginning of experimentation and N_O is the amount of virus remaining at time *t*. For t = ½ this quantity becomes: *In* 1/0.5 = *Ki* t½ which can also be written as *In*2 = *Ki* t½ but natural log 2 = 0.693 hence this can be rewritten as 0.693 = *Ki* t½ and therefore *Ki* = 0.693/t½ or t½ = 0.693/*Ki*. Where virus became undetectable before 96 hours, the *Ki* value was obtained over the duration of virus survival. The influence of RH and temperature on HAV survival was demonstrated by the half-lives, *Ki* values and plots of *Ki* against the RH or temperature.

To fully understand the effects of RH and temperature on the survival of HAV and PV, log_{10} PFU survival for each virus at each RH and temperature level was regressed against the time each virus survived (96 hours was the longest survival) using the least squares method in the SAS computer program (Statistical Analysis System, UNC, Chapel Hill, North Carolina) and the generated individual regression slopes analyzed, compared and tested in the same computer program for significant statistical differences. An overall effect on virus survival due to RH and due to temperature and the possible interaction between these two factors was also analyzed in the general linear model procedure in SAS.

HAV surviving on the fingerpads of each volunteer at each X-value was expressed as a fraction of HAV eluted at the zero minutes. These observations were then normalized to the zero-minute values as 100%. Such data was used to generate log-linear plots (log_{10}) in SigmaPlot. First-order linear regression lines were then fitted to the plots in the same program. HAV decay rates on fingerpads were obtained from the gradient of the fitted regression lines as loss of virus infectivity titer in log_{10} PFU per minute. The half-lives were calculated from

the decay rate constants (*Ki*), as discussed by Segel (1976).

HAV and PV $\log_{10}$ PFU survival or $\log_{10}$ PFU virus transferred was analyzed for each handwashing agent used in a 2-way analysis of variance (ANOVA) in SAS. *Post hoc* tests (Tukey) were carried out with the analysis.

6. HAV SURVIVAL ON INANIMATE AND ANIMATE SURFACES AND VIRUS TRANSFER ON CONTACT

6. HAV SURVIVAL ON INANIMATE AND ANIMATE SURFACES AND VIRUS TRANSFER ON CONTACT

INTRODUCTION

As has been mentioned before, food and drinking water are well-recognized as vehicles for hepatitis A. However, the exact vehicles for virus spread remain unidentified in nearly half of the reported cases of hepatitis A in the U.S. (Hadler and Margolis, 1989). The information summarized above argues against air as a vehicle for the direct spread of this infection. Does the spread of this virus occur by other means? If so, what might be the most likely candidate vehicles?

The available circumstantial evidence points to human hands and non-porous environmental surfaces; semi-critical medical instruments, such as flexible fiberoptic endoscopes, represent a form of non-porous inanimate surface and there is increasing concern with regards to their potential in the transmission of many types of human pathogens, including HAV (Hadler et al., 1980; Klein et al., 1984; Leger et al., 1975; Levy et al., 1975; Lowry et al., 1989; Mishu et al., 1990; Rosenblum et al., 1991; Warburton et al., 1991). The paucity of direct evidence for their role and relative significance as vehicles is due to the fact that epidemiological studies to generate such information are particularly difficult to design and conduct. Human hands and inanimate surfaces interact regularly in any domestic or institutional setting and contamination of one can often lead to the contamination of the other (Scott and Bloomfield, 1990). Therefore, the following summary will treat these two types of potential vehicles together.

Klein et al (1984) and Seeberg et al (1981) have described HAV outbreaks in pediatric wards that may have spread from the index cases via the contaminated hands of the health-care personnel. Hands of children and care-givers are believed to play an important role in the transmission of hepatitis A in day-care centers as well (Hadler and McFarland, 1986; Hadler et al., 1980). In many such institutions, personnel deployed to change diapers may also prepare and serve food (Hadler et al, 1980) and employees (including teachers) who care for ill children

seldom wash their hands between handling of children (Albert and Condie, 1981; Ekanem et al, 1983). Hepatitis A outbreaks associated with restaurants and cafeterias are often thought to result from the contamination of food by hands of employees excreting the virus (Denes et al., 1977; Hooper et al., 1977).

A large common source HAV outbreak involving sandwiches has been reported in which, by exclusion, the sandwich board was found to have been contaminated by the hands of the index case (Levy et al, 1975). HAV is said to have survived for several days on the sandwich board to contaminate a large number of sandwiches resulting in a community-based outbreak of hepatitis A. HAV-contaminated food trays in a cafeteria were believed to be responsible for an outbreak of hepatitis A (Denes et al., 1977).

Human pathogenic viruses can remain viable on an array of environmental surfaces for periods ranging from a few hours to more than several days (Ansari et al., 1991; Buckland and Tyrrell, 1962; Mahl and Sadler, 1975; Moe and Shirley, 1982). Further, viruses that are primarily transmitted through the fecal-oral route appear to spread more readily on contact between animate and inanimate surfaces (Moe and Shirley, 1975; Isaacs et al, 1989).

Hard surfaces as well as hands are often contaminated with enteropathogens particularly during outbreaks (Ekanem et al, 1983; Petersen and Bressler, 1986; Black et al, 1981; Scott et al, 1982; Schothurst et al, 1978; Mendes et al, 1978; Tebutt, 1986; Mackintosh and Hoffman, 1984).

There is experimental evidence to show that viruses do survive on both inanimate and animate surfaces to permit their transfer between them (Ansari et al., 1991; Gwaltney et al, 1978; Hendley et al, 1973; Halls et al, 1980).

In spite of the mounting evidence for the potential of human hands and non-porous inanimate surfaces as vehicles for hepatitis A, no published information has been available on the capacity of HAV to remain viable on them. Nor do we know anything about the extent of virus transfer between contaminated and uncontaminated surfaces and objects upon contact during routine activities.

MATERIALS AND METHODS

Protocol for virus survival on the metal disks. Before each experiment, the stock virus was diluted 10-fold in the fecal suspension. Ten µL of the fecally-suspended virus was placed, using a positive displacement pipette (Gilson Medical Instruments, Villiers-le-Bel, France), into each of two glass vials containing 990 µL EBSS; these acted as the in-put virus control. Each of two disks also received 10 µL of the virus; immediately after virus inoculation, each disk was placed into a glass vial with 990 µL of EBSS (zero min control). After sonication (Bransonic Ultrasonics Corp., Danbury, CT) for 10 minutes at room temperature, the eluates were recovered for plaque assay. With this procedure, 91-100% of the in-put infectious virus could be recovered from the disks.

For virus survival tests, one disk was placed in each well of a 24-well plastic plate (Costar), and 10 µL of fecally-suspended HAV or PV was placed in the center of each disk using a positive displacement pipette. The plates were then placed at the desired temperature and RH (see below). At each sampling time, three disks were removed and placed separately in vials containing 1 mL EBSS. Virus from the disks was eluted as described above. The eluates were stored at -80°C and all samples from a given experiment plaque assayed at the same time. Survival experiments were conducted over 96 hours and each experiment was repeated at least three times.

The survival of HAV at low (25±5%), medium (55±5%), high (80±5%) and ultra-high (95±5%) levels of RH, and air temperature of 5, 20 and 35°C was studied. The experiments on PV survival were conducted only at the low and ultra-high RH levels while keeping the air temperature at 20°C. The temperature of the incubator (Precision Scientific Co., Chicago, Ill.) used for holding the disks could be adjusted to the desired level. The ambient RH level ranged between 50 and 60%. The higher levels of RH were achieved and maintained by bubbling the air entering the incubator through sterile deionized water with one or more diffusing stones (Fisher Scientific, Ottawa, Ontario, Canada). The low level of RH was obtained by passing the air through a column of Drierite (Hammond, Xenia, OH). The air temperature and RH were

continuously monitored using a recording hygrothermograph (Cole-Parmer Instruments Co., Chicago, IL).

Protocol for virus survival on hands. Virus survival on the hands of volunteers was tested according to the procedure of Ansari et al. (1988). The hands were first washed in luke-warm running tap water for 30 seconds, disinfected by thoroughly rubbing them with 70% ethanol (BDH) followed by air drying. To demarcate the areas for virus deposition, each fingerpad was pressed hard over the mouth (8-mm inside diameter) of an empty plastic vial (no. 72.694.006; Sarstedt Inc., St. Laurent, Quebec).

Before each experiment, the stock virus was diluted 10-fold in the fecal suspension. Ten microliters of the fecally suspended virus was immediately placed, using a positive displacement pipette (Gilson Medical Instruments, Villiers-le-Bel, France), into each of two glass vials containing 990 µL of EBSS; these acted as the in-put virus control.

Ten microliters of the virus suspension (approximately 6.0 X 10^4 PFU) was also deposited at the center of the demarcated area on each fingerpad. In each experiment, the amount of virus in the inoculum on the fingerpads (0-minute control) was determined by immediately recovering the deposited virus separately from four fingerpads of the left hand as follows: the contaminated area was placed over the mouth of a vial identical to that used for fingerpad demarcation but containing 990 µL EBSS. The vial was inverted with the fingerpad still over it and held in position for 5 seconds. This was followed by 20 full inversions of the vial while still in place, then a further 5 seconds of soaking and 20 more full inversions. The surface of the fingerpad was then scraped on the inside rim of the vial to recover as much of the fluid as possible. This technique was found to recover approximately 70% of the in-put infectious virus. To determine HAV survival on hands, virus was randomly eluted from separate fingerpads 20, 60, 120, 180 and 240 minutes after their inoculation.

Five volunteers participated in the experiments on virus survival on hands and each experiment was performed 3 times on each volunteer.

Protocol for virus transfer experiments. The extent of infectious HAV transfer upon

fingerpad-fingerpad interaction and fingerpad-environmental surface contact was examined as follows: After letting the virus inoculum dry on the donor surface for the desired length of time, contact was made at the required pressure, with or without friction, with the recipient surface for 10 seconds. Virus was then eluted from the recipient as well as the donor surface. The two values thus obtained were added together to represent the total amount of infectious virus detectable on the donor before transfer and the amount of virus transferred was determined as a percentage of this total. The disk-to-fingerpad and the fingerpad-to-disk experiments were conducted on each of two volunteers, whereas the fingerpad-to-fingerpad mode involved Volunteer E as the donor and Volunteer A as the recipient.

To analyse the data obtained from HAV survival and transfer between the 3 models (fingerpad-fingerpad, fingerpad-disk, disk-fingerpad) over 4 hours using two volunteers, the data was normalized as above. Bar charts were then produced in a SigmaPlot computer program.

In order to assess the effects of pressure, friction and the transfer mode used on the amount of HAV transferred, the raw data was entered into the SAS program and a 3-way analysis of variance (ANOVA) performed. The possible interaction between the variables (pressure, friction and type of model) was also analysed. The Tukey test was performed *post hoc* in the same program. To compensate for differences in virus transfer due to time, an average of the 20 and 60 minutes HAV transfer was computed and used in the 3-way ANOVA.

The effect of a low (approximately 0.2 kg/cm^2) and a high (approximately 1.0 kg/cm^2) level of pressure was tested in the virus transfer experiments. The low level of pressure, with or without friction, was taken to represent ordinary touching of environmental surfaces. Animate-animate interaction through hand shake was represented by a pressure of 1.0 kg/cm^2 without friction while the same pressure with friction simulates opening of doors and other equivalent actions.

Depending on the type of experiment, a clean or HAV-contaminated disk was placed at the center of the pan of a scale with a digital read-out (Model No. 1206 MP; Sartorius,

Gottingen, Germany) and a virus donor or recipient fingerpad was pressed on it till the desired pressure was achieved and held there for 10 seconds; pressure was computed from balance reading and the area of the contaminated surface. For virus transfer from fingerpad to fingerpad, the recipient fingerpad was placed on the scale and contacted with the donor.

In an attempt to stardardize the friction to be applied during virus transfer, each volunteer was first thoroughly briefed on and given the opportunity to properly practice the following procedure: Once contact was made between the disk and the fingerpad and the desired pressure level was attained, the finger was rotated in half-circles 10 times over the 10-seconds period of contact; in the case of fingerpad-to-fingerpad transfer, the donor was required to apply friction.

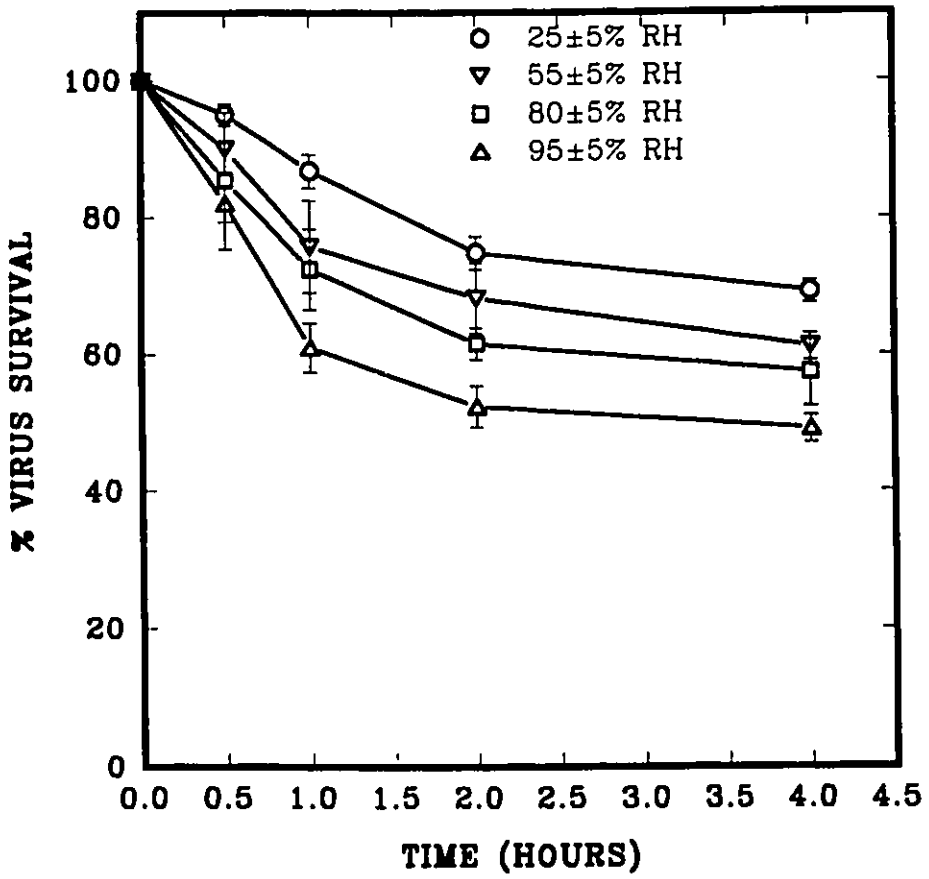
RESULTS

At 35°C and 95±5% RH, HAV became undetectable by 4 hours. As a result of this, comparison figures are given for all RH and temperature conditions at the 4 hours period. Furthermore, graphs of HAV inactivation have been expanded to show the first 4 hour more clearly although calculations of *Ki* and half-life values as well as multiple regression analysis using general linear models were applied on the whole data set.

There were significant overall effects on HAV survival due to RH ($F=9.61$, $P<0.05$), temperature ($F=34.24$, $P<0.05$) and RH-temperature interactions ($F=18.30$, $P<0.05$). PV survival was also significantly affected by RH ($F=5.99$, $P<0.05$). HAV survival at RH level of 25±5% (mean 50,057 PFU) was significantly different from 55±5% (mean 24,934 PFU), 80±5% (mean 21,137 PFU) and 95±5% (mean 20,847 PFU), with no significant differences between the latter three RH levels. PV survival was also significantly different at 25±5% (mean 78,954 PFU) than at 95±5% (mean 182,794 PFU). Similarly, HAV survival at 5°C (32,290 PFU) was significantly different from 20°C (mean 21,151 PFU) and 35°C (mean 19,290 PFU).

HAV survival at 5°C. At 5°C, HAV survival was inversely proportional to the level of RH and 70, 63, 58 and 50% of the in-put infectious virus remained viable at the end of 4 hours at the low, medium, high and ultra-high RH levels, respectively (Figure 1). As would be

Figure 1: Survival of hepatitis A virus on stainless steel disks held at an air temperature of 5°C and at RH levels of 25±5%, 55±5%, 80±5% and 95±5%.



Ten µL of fecally-suspended virus was placed at the centre of each disk and the disks held at the desired temperature and RH. The vertical error bars represent the standard deviation from the mean of six observations for each x value.

expected, the virus survived better at 5°C than at 20 or 35°C (Table 1).

HAV and PV survival at 20°C: As can be seen from Figure 2, the relationship between HAV survival and RH was the same as described for 5°C (Figure 1). The amounts of infectious in-put HAV that were detected at this temperature after 4 hours were approximately 52, 48, 34 and 28% in increasing order of RH. The actual numbers of PFU of HAV remaining at 96 hours were approximately 548, 415, 112 and 50 PFU at the low, medium, high and ultra-high RH levels, respectively.

Parallel experiments were also carried out at this air temperature and at the low and ultra-high RH levels to compare PV survival with that of HAV (Figure 2). At both the RH levels, the rate of loss of PV infectivity was faster than that of HAV. PV survival showed an exponential decay to about 28% in the first hour at the ultra-high RH and to about 12% during the same period at the low RH. Residual PV infectivity was approximately 13% for the ultra-high and <1.0% for the low RH at the end of 4 hours. PV infectivity was undetectable at 6 hours at 25±5% and after 12 hours at 95±5% RH.

The HAV decay rate at the high RH was >2.5 times higher than at the low RH and >3.5 times higher at the ultra-high RH level than at the low RH. The rate of decay of PV at the low RH was >106X faster than that of HAV under the same test conditions; even though PV survived better at the ultra-high RH (*Ki* 0.0984) than at the low RH (*Ki* 0.3928), its rate of decay was still >7.2 times faster than that of HAV at the higher humidity level. The half-life for HAV at 95±5% RH was 51 hours compared to 7 hours for PV at the same RH level and temperature (Table 1).

HAV survival at 35°C. At 35°C also, there was an increase in the rate of decay of HAV infectivity as the RH level was increased (Figure 3). At the ultra-high RH, virus infectivity became undetectable within 4 hours, whereas approximately 43, 38 and 20% of the in-put infectious virus remained detectable at the end of the same period for low, medium and

Table 1. Influence of air temperature and relative humidity on the half-lives([a]) and rates of inactivation (*Ki*) of fecally-suspended hepatitis A virus (HM-175) and poliovirus type 1 (Sabin) on non-porous inanimate surfaces

Virus	Temp.	Relative humidity			
		25±5%	55±5%	80±5%	95±5%
Hepatitis A	5°C	169[a] (0.0041)[b]	151 (0.0046)	123 (0.0056)	103 (0.0067)
	20°C	187 (0.0037)	128 (0.0054)	71 (0.0097)	51 (0.0136)
	35°C	65 (0.0106)	50 (0.0138)	21 (0.0329)	2 (0.0984)
Polio	20°C	2 (0.3928)	NT	NT	7 (0.0984)

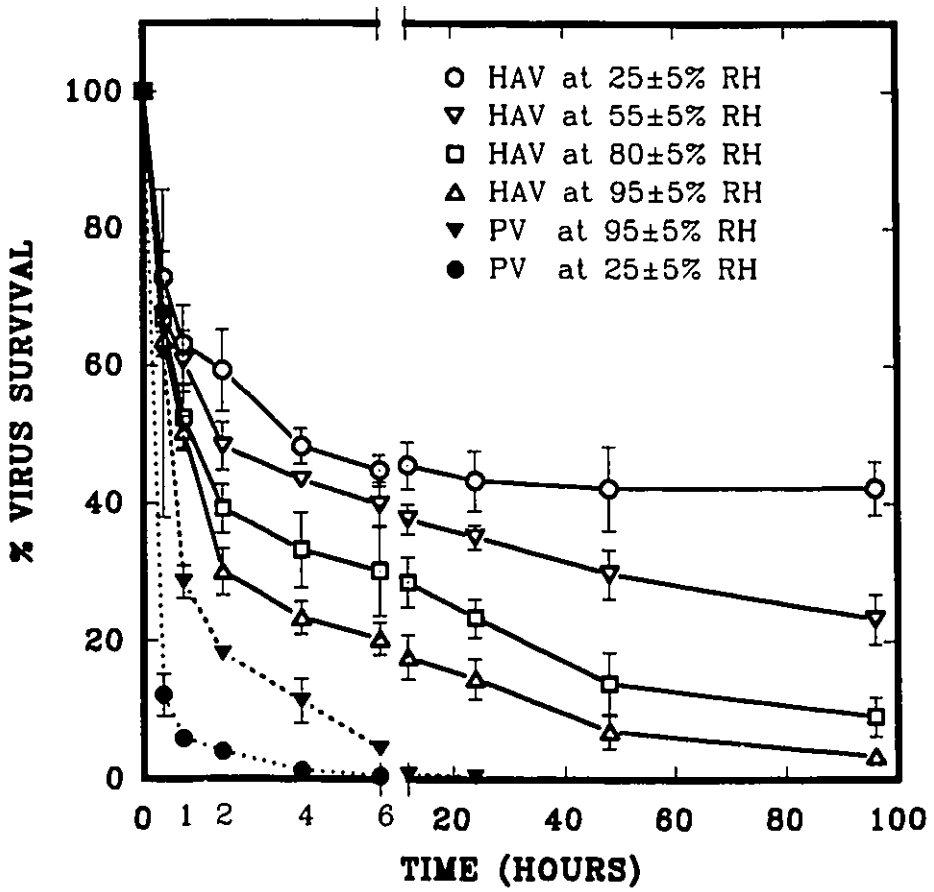
(Experimental procedure is as discussed on Figure 1).

[a] *Half-life* is expressed in hours.

[b] K_i, loss of virus infectivity, expressed as $\log_{10}$ PFU per hour.

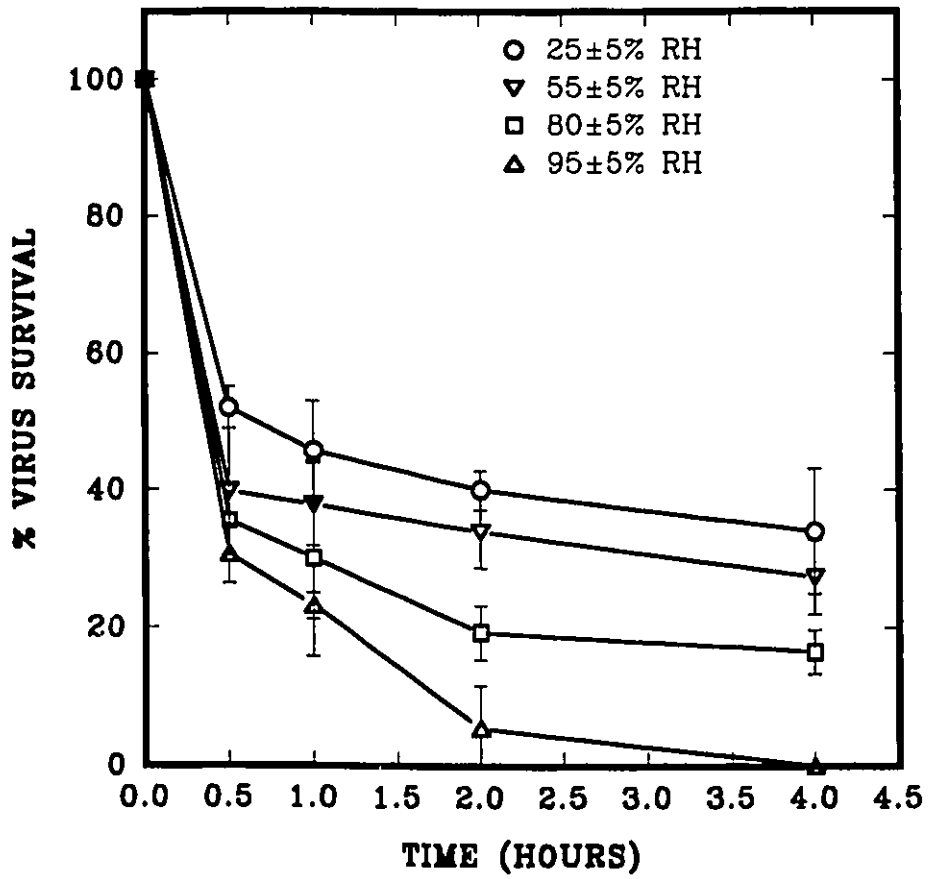
NT, Not tested.

Figure 2: Comparison of the survival of hepatitis A virus (HAV) and poliovirus (PV) type 1 (Sabin) on stainless steel disks held at an air temperature of 20°C and at RH levels of 25±5%, 55±5%, 80±5% and 95±5%.



Ten µL of fecally-suspended HAV or PV was placed at the centre of each disk and the disks held at the desired temperature and RH. The vertical error bars represent the standard deviation from the mean of six observations for each x value.

Figure 3: Survival of hepatitis A virus on stainless steel disks held at an air temperature of 35°C and at RH levels 25±5%, 55±5%, 80±5% and 95±5%.

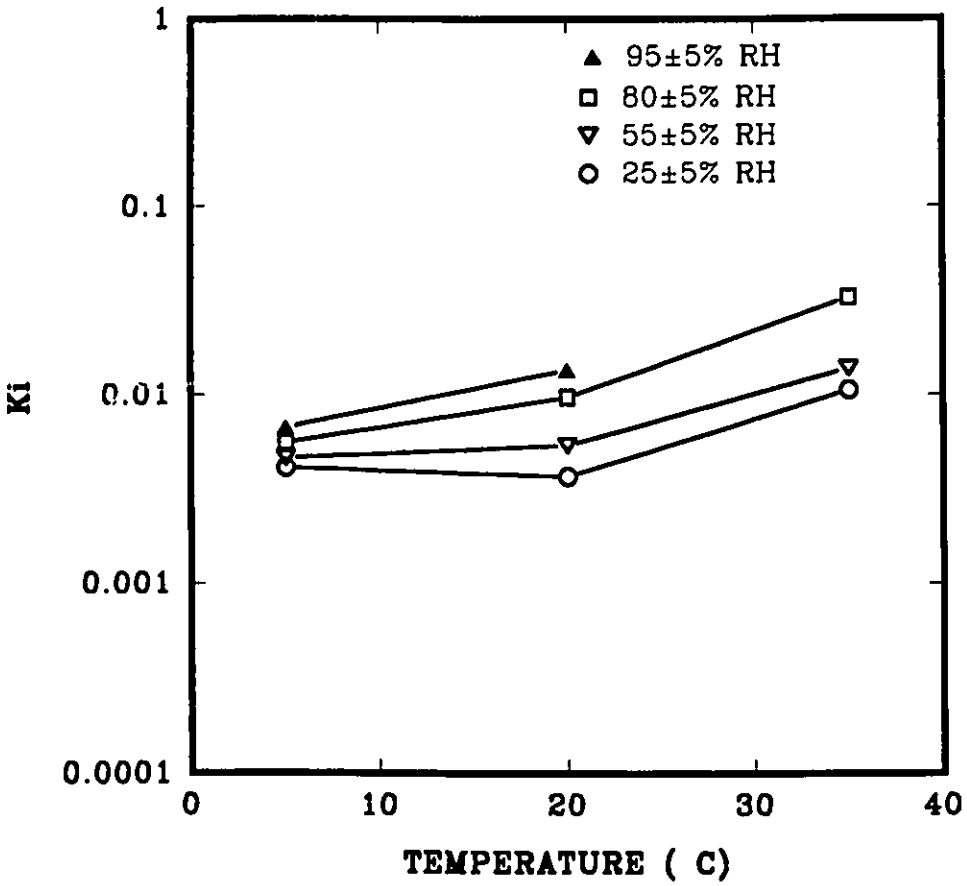


Ten µL of fecally-suspended virus was placed at the centre of each disk and the disks held at the temperature and RH. The vertical error bars represent the standard deviation from the mean of six observations for each x value.

high RH levels, respectively. The half-lives of the virus (Table 1) at 35°C were much shorter at all the RH levels tested than when this virus was held at the lower temperatures.

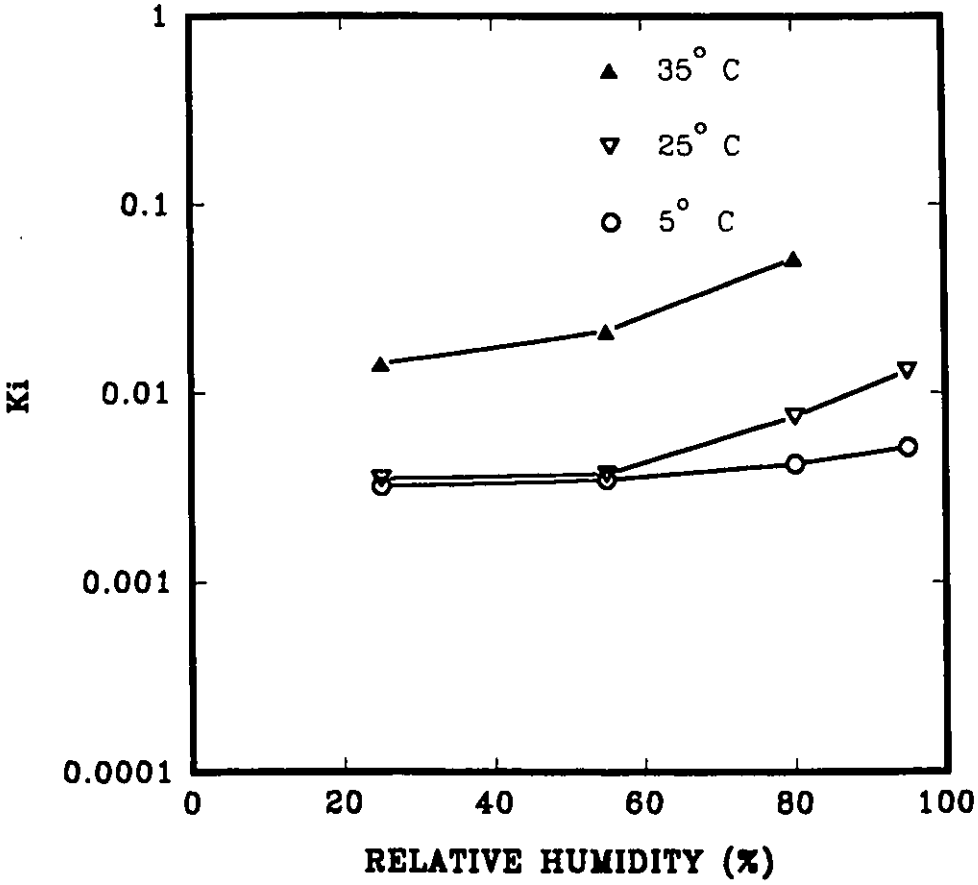
The influence of RH and temperature on HAV survival. The *Ki* values obtained at the various air temperatures and RH levels were plotted to ascertain the influence of RH on HAV survival (Figure 4). The differences in the effects of the higher levels of RH on HAV survival at 5°C were much less pronounced than those at 20 and 35°C. The difference in the *Ki* values for the low and medium RH levels at 5 and 20°C were minimal, but increased gradually as the RH was raised. The influence of air temperature on HAV inactivation was similar for 25±5% and 55±5%, but more obvious at the high and ultra-high RH (Figure 5).

Figure 4: Effect of relative humidity on the survival of hepatitis A virus on stainless steel disks held at air temperatures of 5°C, 20°C and 35°C.



Ten µL of fecally-suspended virus was placed at the centre of each disk and the disks held at the temperature and RH.

Figure 5: Effect of air temperature on the survival of hepatitis A virus on stainless steel disks held at relative humidity levels of 25±5%, 55±5%, 80±5% and 95±5%.

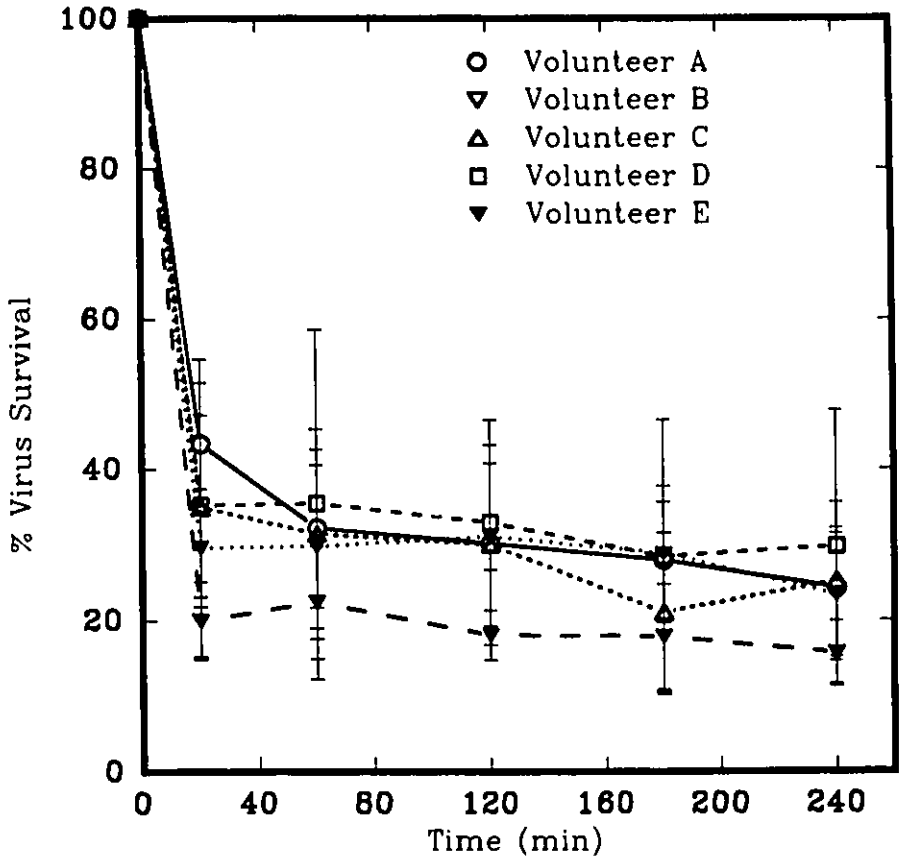


Ten µL of fecally-suspended virus was placed at the centre of each disk and the disks held at the temperature and RH.

Virus survival on fingerpads. The mean virus titer at zero minute on fingerpads in the HAV survival experiments was approximately $6.0X10^4$ (range $5.3X10^4$ to $7.2X10^4$) PFU in the 10 µl inoculum. The results of these experiments are presented in Figure 6. Nearly 68% of the input infectious virus became undetectable within the first 20 minutes after contamination of the fingerpads; this early loss in virus infectivity was particularly pronounced in Volunteer E. The rate of decay in virus infectivity then became essentially similar during the 20 to 240 minutes period for all the five volunteers. As can be seen from the data summarized in Table 2, the half-life of the virus was the shortest in Volunteer E (5.5 hours) and the longest in Volunteer D (>7.7 hours). The *Ki* values, expressed as the rate of loss in virus infectivity in $\log_{10}$ per minute, ranged from 0.0015 to 0.0021. However, the differences in virus half-lives and *Ki* values for the five volunteers were not found to be statistically significant. The amount of HAV detectable at the end of 4 hours ranged from 16 to 30% of the input dose; the actual PFU recovered at the end of 4 hours were 1,804 and 835 for volunteers D and E, respectively.

HAV transfer after 20 and 60 min of drying. The mean amount of virus at zero minutes on the fingerpads and disks was 3.1×10^4 and 2.9×10^4 PFU/mL, respectively. There was always virus transfer. A 3-way ANOVA revealed significant main effects due to mode of transfer, pressure and friction ($F=3.35$, $P<0.05$; $F=133.51$, $P<0.05$; and $F=81.29$, $P<0.05$, respectively). A *post hoc* analysis (Tukey tests) of the transfer mode main effect showed a significant difference in virus transfer between the fingerpad-fingerpad (Mean=634.08 PFU) and the disk-fingerpad (Mean=454.72). Similarly, there was a significant difference in the amount of virus transferred between the fingerpad-disk (Mean=607.21) and disk-fingerpad modes. The amount of virus transferred was not significantly different between the fingerpad-fingerpad and fingerpad-disk modes.

Figure 6: Hepatitis A virus survival on the hands of human volunteers.



Ten µL of fecally-suspended virus was placed on each fingerpad and virus eluted at the indicated times. Vertical error bars are standard deviations from the means of three observations at each x value.

Table 2. Hepatitis A virus survival expressed as virus half-life and as rate of virus inactivation *(Ki)* on fingerpads of 5 volunteers.

	Volunteer				
	A	B	C	D	E
Half-life in hours	6.10	7.20	6.10	7.70	5.50
Ki^a	0.0019	0.0016	0.0019	0.0015	0.0021
R-value[b]	0.7936	0.6471	0.7365	0.6700	0.6562

(Experimental procedure as discussed in Figure 6).

[a] Loss in $\log_{10}$ PFU per min. The difference between *Ki* values of the 5 volunteers was not significant ($[F_{0.05\ 0.5,\ 5,\ 15}] = 0.694$)

[b]Coefficient of determination (measure of closeness of fit of the scatter graphs to their regression)

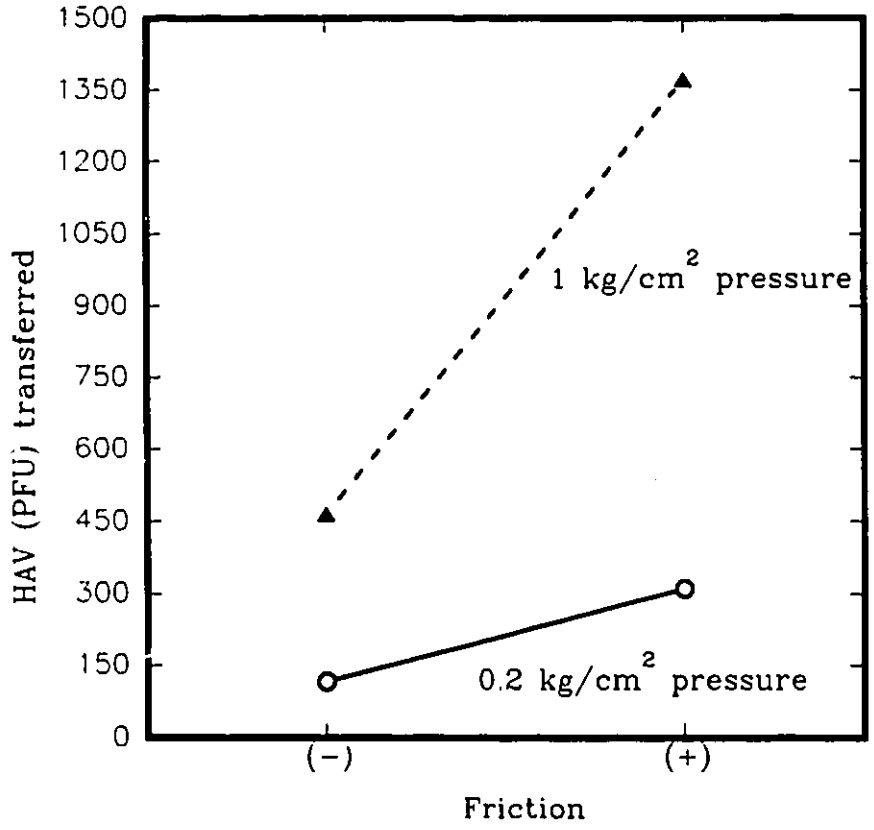
The increase in pressure from 0.2 kg/cm^2 to 1 kg/cm^2 resulted in a significant increase in the amount of virus transferred ($F=133.51$, $P<0.05$); the mean transfer rate went from 212.82 PFU at 0.2 kg/cm^2 to 917.85 PFU at 1 kg/cm^2. Similarly, the incorporation of friction resulted in a significant elevation in the amount of virus transferred ($F=81.29$, $P<0.05$). The mean HAV transfer rate with no friction was 290.27 PFU whereas, with friction, the mean value was 840.40 PFU. The combination of pressure and friction significantly affected the amount of HAV transferred ($F=33.98$, $P<0.05$). The 2-way effect of mixing pressure and friction is illustrated in Figure 7. The combination of friction and the higher pressure (1 kg/cm^2) resulted in a profound increase in HAV transfer (Mean=1,370.76). No statistically significant interactions were observed between mode of transfer and pressure and friction, mode of transfer and friction, or mode of transfer and pressure.

HAV transfer over a period of 4 hours. The ability of HAV to be transferred over a 4 hour period was assessed using 2 volunteers (A and E). The three modes of virus transfer were tested using only the low level of pressure without any friction. The mean amount of virus at zero minute in the 10 µL inoculum on fingerpads and disks in these experiments was $1.1X10^4$ ($1\text{-}1.2 \times 10^4$) PFU and 1.08×10^4 ($1.07\text{-}1.09 \times 10^4$ PFU), respectively.

Virus transfer from disks to fingerpads. As shown in Figure 8, nearly 33% of the in-put virus remained detectable on the disks at the end of 4 hours under ambient temperature (22±2°C) and relative humidity (45±5%). When a clean fingerpad was pressed against a disk with an inoculum dried for 20 minutes, about 26% (2,667 PFU) of the PFU could be transferred, whereas no detectable virus was transferred to fingerpads when the inoculum was left to dry on the disks for 4 hours.

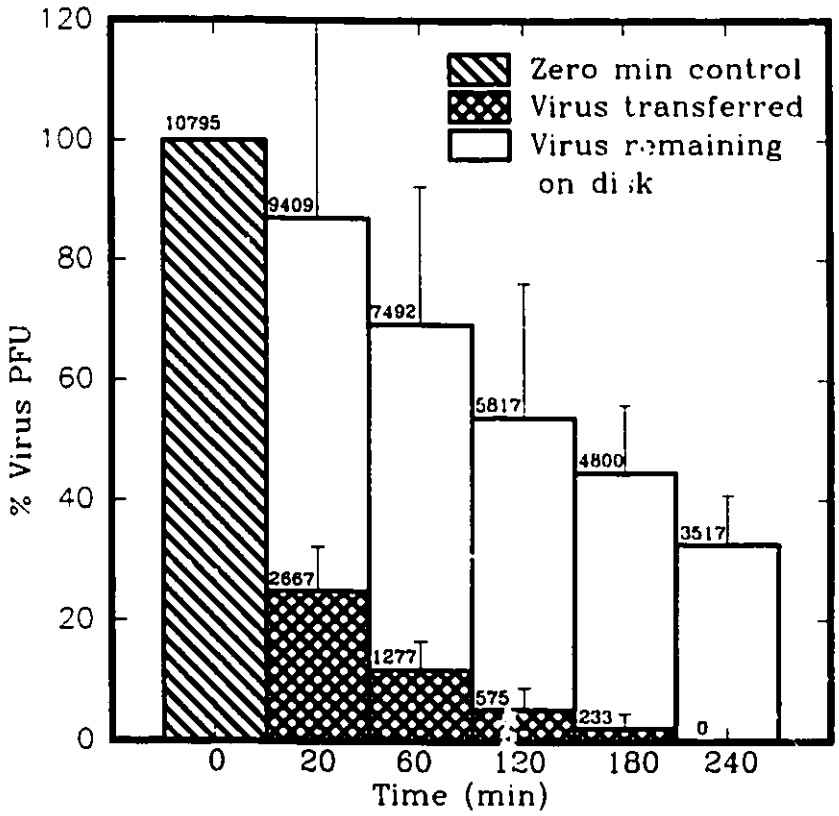
Virus transfer from fingerpads to disks. In this set of experiments, nearly 25% of the in-put virus PFU remained detectable on the fingerpads even after 4 hours (Figure 9). The amount of virus transferred from contaminated fingerpads to clean disks on contact was nearly 27% (3,484 PFU) of the virus remaining after 20 minutes of drying; when the inoculum was dried for 4 hours, 1.6% (50 PFU) of the surviving virus was transferred.

Figure 7: Influence of pressure and friction on the transfer of hepatitis A virus.



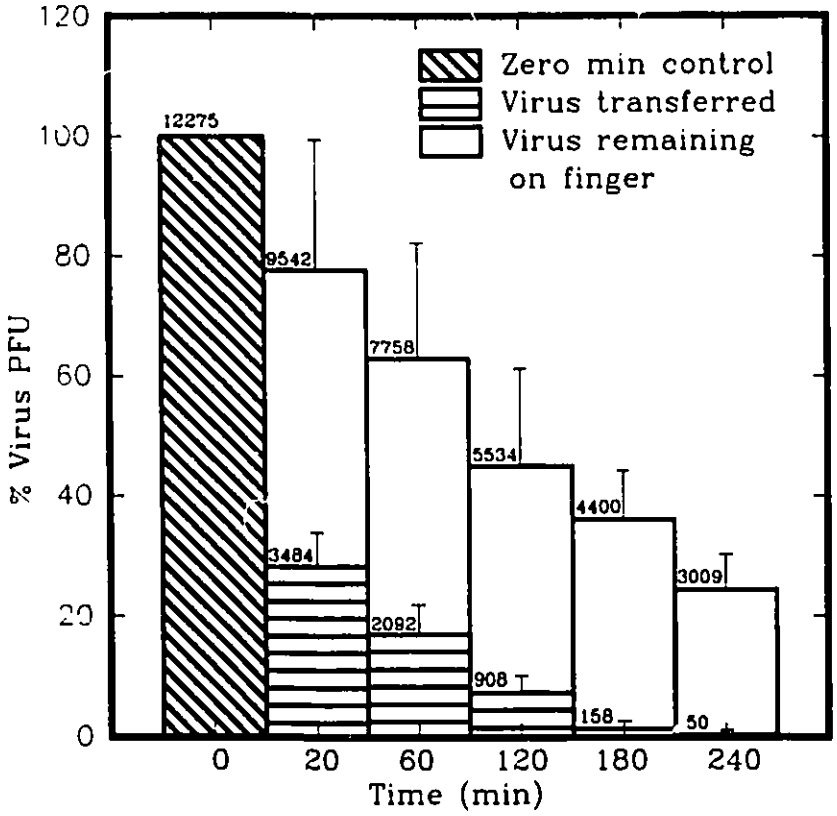
Ten µL of fecally-suspended virus was placed on each disk and at the indicated times virus transfer was attempted without friction.

Figure 8: Hepatitis A virus remaining on stainless steel disks and that transferred to fingerpads.



Ten µL of fecally-suspended virus was placed on each disk and at the indicated times virus transfer was attempted without friction. Vertical error bars represent standard deviations from the means of six observations. Numbers above the bars are the actual PFU detected.

Figure 9: Hepatitis A virus remaining on fingerpads and that transferred to stainless steel disks.



Ten µL of fecally-suspended virus was placed on each fingerpad and at the indicated times virus transfer was attempted without friction. Vertical error bars represent standard deviations from the means of six observations. Numbers above the bars are the actual PFU detected.

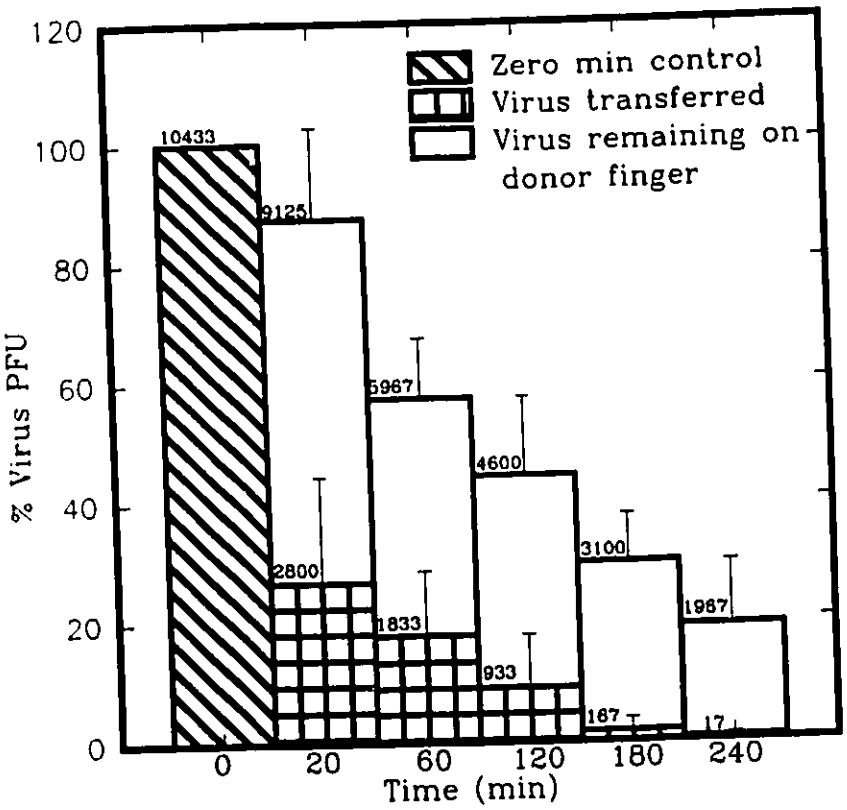
Virus transfer between fingerpads. About 10,433 PFU of HAV were deposited on the fingerpad of volunteer E but only 1967 (19%) remained at the end of 4 hours (Figure 10). The survival pattern was similar to that observed earlier (Figure 6). HAV transferred from the fingerpads of Volunteer E to the fingerpads of Volunteer A was 2,800 PFU after 20 minutes of drying (23% of the available PFU), but only 17 PFU (0.9%) at the end of 4 hours of drying.

DISCUSSION

It is generally believed that the survival of enveloped viruses on inanimate surfaces is favored when RH levels are below 50%; on the other hand, RH levels higher than 80% are considered to be more conducive to the survival of non-enveloped viruses (Buckland and Tyrrell, 1962; Sattar et al, 1986). The results of this study clearly show that the influence of RH on HAV survival on nonporous inanimate surfaces is very different from that of most other non-enveloped viruses tested (Buckland and Tyrrell, 1962; Sattar et al, 1986). A direct confirmation of this difference was obtained in this study by testing PV survival under the same experimental conditions. HAV survived better than PV irrespective of the air temperature and RH level tested. Here it is interesting to note that rotavirus, another non-enveloped virus, could also survive better on non-porous inanimate surfaces when the RH was at the low or medium level (Sattar et al, 1988).

Earlier it was shown that the infectivity of poliovirus and other enteric viruses decreases in parallel to the evaporation of water in the inocula (Ward & Ashley, 1977). The mechanism of inactivation by evaporation may be accelerated by exposure of viral particles to air at the liquid-air interface causing inactivation by release of viral genomes (Ward and Ashley, 1977). At the high and ultra-high RH levels used in this study, virus suspensions placed on the disks often remained visibly moist even after 4 hours. On the other hand, the inocula became visibly dry in 20 minutes or less when the RH was at the low or medium level. Yet, HAV survived better at the lower RH levels than at the high or ultra-high RH. Drying of the inocula alone, therefore, does not account for the observed difference in HAV survival.

Figure 10: Survival of hepatitis A virus on fingerpads of Volunteer E and the amounts transferred to Volunteer A.



Ten µL of fecally-suspended virus was placed on each donor fingerpad and the transfer attempted at the indicated times without friction. Vertical error bars are the standard deviations of three observations. Numbers above the bars are actual PFU detected.

The reasons for the higher stability of HAV at the lower RH levels remain to be elucidated, but a number of possibilities can be considered, singly or in combination, to explain the observed results. Association of HAV with cellular lipids is believed to protect it from neutralization by a variety of convalescent sera (Lemon and Binn, 1985). Such lipid association may confer on the virus a certain degree of resistance to environmental inactivation. In contrast to this, Scholz et al. (1989) have shown that the high acid resistance of HAV did not depend on the protection afforded by virus-associated cellular material. The presence of lipid material on a virus would tend to orient the virion at the air-water interface (Trouwborst et al., 1974) due to the hydrophobicity of the lipids; this could result in inactivation of the virus as explained by Ward and Ashley (1977). However, we believe that such a mechanism was not responsible for HAV inactivation seen in this study since the virus survived better in low RH levels where the air-water interface was more likely to exist than in an environment of air saturated with water as result of high RH levels (Figures 1-4).

The association of viruses with cellular membranes and lipids can often result in the presence of large clumps in the virus suspension. The preparation of virus used in this study was deliberately not purified to obtain monodisperse HAV in order to present as natural a preparation as possible. The presence of viral clumps, particularly if lipid associated, may further enhance virus survival, and lead to aberrations in virus survival curves (Berg et al., 1965). Single virions or clumps of HAV associated with lipid will tend to accumulate at the air-water interface. As the inoculum dries these virus clumps may tend to increase in size, offering further protection to the cell-associated viruses. At high ambient RH levels the hydrophobicity at the air-water interface will be decreased, and it is not known what effect this may have on the virus particles accumulated at the interface. Different sub-populations of HAV with different levels of sensitivity to RH may also be involved.

HAV may also have an inherently more stable molecular structure than many other enteroviruses including polioviruses. Sobsey et al. (1988) have evaluated HAV survival in a variety of environmental samples. In comparison with poliovirus, they found HAV to survive

much longer in waters and sediments. Furthermore, when dried onto polystyrene surfaces, HAV could still be detected after 1 month (Sobsey et al., 1988).

The half-lives of HAV indicated in Table 1 show a marked decline at 20°C and RH levels of 80±5% and 95±5% and at all RH levels at 35°C relative to values found at 5°C. Such decline would be consistent with enzymatic activity in the fecal suspending medium, and under near optimum conditions for enzyme activity HAV was not detectable after 4 hours. If enzymatic activity is indeed the cause of increased HAV inactivation it would be interesting to know the nature of the enzymes responsible.

As stated before, it is generally very difficult to determine if and to what extent environmental surfaces and fomites play a role in the spread of infectious agents. However, the ability of HAV to survive for long periods on the disks suggests that hard environmental surfaces as well as many types of fomites could act as potential vehicles for prolonged periods after HAV contamination, especially in settings such as hospital wards, day-care centers and restaurants (Cliver, 1983). Levy et al. (1975) have suggested that HAV contamination of a sandwich board by oropharyngeal secretions of an asymptomatic food handler resulted in an outbreak of hepatitis A in a restaurant.

Environmental surfaces may be particularly important in places which house infants and children (Scanlon and Leikkanen, 1973). It is known that children in day-care centers closely associate with surfaces and fomites. Children and infants may also excrete HAV for prolonged periods, sometimes up to 6 months (Rosenblum et al., 1991). The majority of young children also show no clinical symptoms after HAV infection and may serve as asymptomatic virus transmitters (Gingrich et al., 1983). The exchange of enteric organisms, such as HAV, between children and their environment may, therefore, be quite high. In this respect, the virus may silently circulate in the institution until susceptible adults become infected, often with secondary spread to the general community (Hadler et al., 1980).

Combinations of either of the two higher air temperatures with any of the 4 different RH levels selected in this study are considered representative of the environmental conditions

encountered in indoor settings, in temperate as well as tropical climates, at different times of the year. Refrigeration temperature is often around 5°C.

Although oropharyngeal secretions may contain HAV (Levy et al., 1975) and aerosols have been reported to initiate HAV outbreaks (Aach et al., 1968), the principal source of environmental contamination with this virus is the feces of infected individuals. Hence one of the possible mechanisms of HAV spread is through contact with environmental surfaces that are contaminated with HAV-containing fecal matter. In view of this, fecally-suspended HAV was used for all the survival experiments in our study. The fecal sample used here was diluted and filtered because the particulate matter in feces interfered with virus quantitation. In nature, however, HAV excreted in feces may either be adsorbed to or embedded in particles and this may afford greater protection to its infectivity.

The results presented here show that high levels of HAV remained infectious on both human hands and the disks; although the reasons for the large virus loss (68%) during the first 20 minutes requires further investigation. Because a fecal suspension of HAV was used to simulate natural conditions in which HAV contaminates both animate and inanimate surfaces, it is expected that the results may be an indication of what may occur in the field.

The period of experimentation shown here is within the limits for human hands to stay contaminated before a hand wash or interaction with other animate and inanimate environments. The metal disks used in this study were selected to represent environmental surfaces. The results of HAV survival on environmental surfaces show that stainless steel disks allow the virus to persist for several days; virus survival was similar on glass and plastic surfaces (Sattar et al., 1986; Ward et al., 1991).

Hepatitis A outbreaks are often associated with hospitals (Azimi et al., 1986; Gwaltney et al., 1978; Klein et al., 1984; Seeberg et al., 1981). The mode of transmission in these reports is unclear but the source is usually an asymptomatic child or adult. The observation that the spread of the infection is associated with nurses and other medical personnel suggests that human hands may play a vehicular role in virus transmission.

An assessment of HAV outbreaks in the community shows that diaper-changing personnel in many day-care centres also participate in food-handling activities (Ekanem et al., 1983; Hadler and McFarland, 1986); HAV may be acquired from an excretor child, the majority of whom are asymptomatic (Gingrich eta al., 1983). Although no such study has been done for HAV, the day-care environment has been found to be contaminated often with enteric organisms which share a similar mode of transmission (Ekanem et al., 1983; Keswick et al., 1983; Petersen and Bressler, 1986).

HAV on contaminated toys and other play items may persist for prolonged periods; toys are shared items in day-care centers and children are known to suck them frequently (Black et al., 1981; Ekanem et al., 1983). Such behavior tends to encourage virus transmission through the fecal-oral route among the children. The children also associate closely with the environment around them as well as with adults assigned to them in the day-care center (Black et al., 1981). Interaction between such a contaminated environment and the institutionalized population, especially children, may recycle the virus until suspectible hosts are infected, usually adults. Such a secondary spread of HAV often results in community-wide outbreaks (Hadler et al., 1980).

Restaurant-associated HAV outbreaks are frequently reported (Christenson et al., 1982; Leger et al., 1975; Levy et al., 1975; Mishu et al., 1990). In these instances, the index case is frequently a food-handler subclinically infected with HAV. Secondary spread of the disease is a prominent feature of the outbreaks (Christenson et al., 1982; Leger et al., 1975; Levy et al., 1975) and may occur over an extended period (Lowry et al., 1989) indicating a continuing source of infection. Refrigerated foodstuffs as well as foods stored at room temperature after handling are known to permit the survival of HAV for several days (Francis and Maynard, 1979) and together with the index case furnish a continued source of the virus. It has also been suggested that HAV which persisted on a sandwich board contaminated by hands moistened with oropharyngeal secretions from an anicteric food-handler was responsible for contaminating many sandwiches (Levy et al., 1975).

The potential of human hands to spread viral agents has serious implications in the food industry and eating establishments, and may be particularly important when foodstuffs do not require cooking after handling. Contamination of foodstuffs with HAV from a institutional food-handler can culminate in community-wide HAV outbreaks (CDC, 1990; Christenson et al., 1982; Hanrahan et al., 1984; Leger et al., 1975; Levy et al., 1975; Mishu et al., 1990).

Handwashing is probably the single most important measure for controlling and preventing the spread of infections (CDC, 1975; Isaacs et al., 1989; Rebrouck, 1986). However, the compliance of handwashing among medical personnel is frequently suboptimal (Albert and Condie, 1981; Fox et al., 1974) and is sometimes altogether ignored (Ekanem et al., 1983). Moreover, many commonly available surface disinfectants are ineffective against HAV (Table 3) and commonly available hand-washing agents are inefficient in removing or inactivating HAV or indeed PV (Tables 6, 8, and 9). Use of antiseptics has previously failed to contain nosocomial HAV (Azimi et al., 1986; Seeberg et al., 1981) thus permitting secondary spread of the virus in hospital wards.

The findings of this study clearly show that control and prevention of HAV outbreaks in settings such as day-care centers, hospitals, eating establishments and other similar facilities must place a greater emphasis on both human hands and environmental surfaces as virus vehicles.

Gwaltney et al. (1978) demonstrated that as much as 70% of the infectious rhinovirus on hands could be transferred to a recipient finger after a contact of only 10 seconds. Similarly, Hendley et al. (1973) have shown that dried rhinoviruses picked up from environmental surfaces may persist on human hands long enough to permit self-inoculation and that virus transmission may proceed by transfer of the virus from the hands of the infected person to an intermediary surface or directly to the fingers of a susceptible recipient. Respiratory syncytial virus has also been shown to survive in the environment, allowing its transfer to human hands (Hall et al, 1980) and studies on the transfer of human rotavirus between hands and inanimate surfaces also implicate hands and inanimate surfaces as potential rotavirus vehicles (Ansari et

al, 1988). All these studies suggest that human hands and environmental surfaces may be serve as routes of virus transmission.

Residual HAV on human hands at the end of 4 hours could also be transferred to other hands and may be enough to initiate infection upon self-inoculation. High levels of infectious HAV were exchanged between fingerpads and the disks over the 4 hour period. These results suggest that human hands and fomites are potential ways in which self-inoculation and communication of the virus can occur over an extended time. The significance of pressure and friction in determining the amount of HAV transferred in the 3 models examined is clearly shown. A literature survey reveals no previous quantitative studies where such interaction in the transfer of infectious virus has been documented.

Virus transfer between contaminated and clean surfaces is greatest when the inoculum is wet (Ansari et al., 1988; Gwaltney et al., 1978; Hall et al., 1980; Hendley et al., 1973) and is observed to decrease as the virus dries. Also, during drying some virus is usually inactivated. In the observed transfer of infectious virus at specific times the effects of drying on virus inactivation as well as the physical transfer of virus particles and their recovery are confounded. However, in order to assess the virus loss due to drying we used a reference point which was the indication that the virus inocula were visibly dry on the contaminated surfaces. An inoculum of 10 µL HAV in fecal suspension appeared dry after 18-20 minutes on the metal disks and 11-17 min on fingerpads. For this reason, 20 minutes was taken as the minimum time that HAV inocula would require to initially dry. The first reading was therefore taken at 20 minutes and indicates virus loss due to drying.

7. CHEMICAL DISINFECTION OF HEPATITIS A VIRUS-CONTAMINATED NON-POROUS INANIMATE SURFACES

7. CHEMICAL DISINFECTION OF HEPATITIS A VIRUS CONTAMINATED NON-POROUS INANIMATE SURFACES

INTRODUCTION

Chemical disinfection is often the first line of defence against infectious agents and various chemicals and formulations are regularly used in domestic and institutional settings to decontaminate inanimate surfaces and objects (Garner and Favero, 1985; Rutala, 1990; Springthorpe and Sattar, 1990). But these steps have not reduced the incidence of infections particularly in institutions that house children or critical care facilities. For instance, large HAV outbreaks continue to occur in both day-care centers and pediatric wards, especially neonatal intensive care units, where these agents are frequently used by trained and experienced personnel (Azimi et al., 1986; Hadler and McFarland, 1986; Hadler et al., 1980; Klein et al., 1984; Pohjanpelto and Ponka, 1985; Rosenblum et al., 1991; Seeberg et al., 1981; WHO, 1987).

It has now been established that children excrete HAV for prolonged periods compared to adults (Rosenblum et al., 1991; Tassopoulos et al., 1986). Lack of toilet training and use of diapers enhance the risk of environmental contamination with the virus (Bradley et al., 1976; Goodman et al., 1982; Vernon et al., 1982), especially in the event of an outbreak and asymptomatic illness among children (Gingrich et al., 1983). Day-care center environments have been found to be contaminated with enteric pathogens (Ekanem et al., 1981; Keswick et al., 1983; Petersen and Bressler, 1986) that are known to spread in a manner similar to that of HAV. Inanimate-animate interactions occur all the time and these two environments exchange microbes in the process. But the available chemical disinfectants have not been shown to be active against HAV even though these are recommended for surface disinfection in order to contain infections.

Recently, Ward et al. (1991) have shown that disinfection of experimentally-contaminated non-porous inanimate surfaces could successfully interrupt the spread of rotavirus spread to human volunteers. However, such interruption may not occur with HAV

due to its higher resistance to chemical disinfectants (Herbold et al., 1989; Siegl et al., 1984; Scholz et al., 1989).

MATERIALS AND METHODS

Disinfectants tested. The selection of the disinfectant formulations tested in this study was based on (a) manufacturer's recommended use for surface disinfection, (b) the type(s) and level(s) of active ingredients, (c) the results of previous tests with other viruses (Sattar et al., 1989; Lloyd-Evans et al., 1986) and (d) recent formulations introduced into the market with virucidal claims. Unless otherwise stated, tapwater was used for diluting a given product according to the directions on the label. A total of 23 chemicals were tested and are given in Table 3.

Free chlorine measurements. The sample cells (two glass bottles graduated to 25 mL; Hach Chemical Co., Ames, IA) were thoroughly cleaned with ordinary tap water and dried. To one of these was added ordinary tap water to the 25 mL mark and the sides of it dried with facial tissue before placing the cell in the cell holder on the meter (model Hach DR/2 Spectrophotometers; Hach). The chlorine meter scale for the DPD (N,N-diethyl-*p*-phenylenediamine) method was then slotted in the meter and the wavelength adjusted to 530 nm. Using the light control knob, the meter reading was adjusted to zero mg/L and the standardization procedure repeated two more times with the same water sample at intervals of 10 minutes until a zero reading was obtained every time.

Javex (Colgate-Palmolive Canada Inc., Toronto, Ontario) for domestic and institutional use comes as a 6% sodium hypochlorite solution containing approximately 60,000 mg/L free chlorine. The meter scale provided could only read about 2 mg/L free chlorine and therefore Javex was first diluted by four 10-fold steps in tapwater before a further 1:6 dilution to give approximately 1 mg/L free chlorine in a volume of 100 mL.

To 25 mL of diluted Javex in a clean dry cell was added all the contents of one DPD Free Chlorine Reagent Powder Pillow provided with the kit (Hach). The mixture was immediately mixed by swirling the cell several times.To read the free chlorine concentration,

Table 3: Formulations of chemical disinfectants tested and their efficacy against hepatitis A virus in a carrier test.

Disinfectant	Dilution tested	pH	Recommended for:	Log_{10} Reduction
Commercial Formulations				
(1) 2% alkaline glutaraldehyde	undiluted	7.5	Medical instruments and appliances	>4
(2) 6% sodium hypochlorite	1:68.8 (800 ppm free chlorine		General purpose disinfection	<1
	1:18 (3,000 ppm free chlorine)	10.9	General purpose disinfection	<1
	1:11 (5,000 ppm free chlorine)	11.2	Generalpurpose disinfection	>4
(3) 2.73% sodium chlorite & 15.10% organic acid	1:12	2.9	Medical instruments and environmental surfaces	<1
(4) 15.5% iodophor no. 1 & 6.5% phosphoric acid	1.214 (75 ppm titratable iodine)	2.8	Highly contaminated areas, isolation rooms and food contact surfaces	<1
(5) 9.1% iodophor no. 2 & 8.74% iodophor no. 3	1:173 (75 ppm titratable iodine)	5.2	Surfaces & Skin	<1
(6) 0.5% sodium *o*-benzyl-*p*-chlorophenol & 0.6% sodium lauryl sulfate	undiluted	12.7	Environmental surfaces	<1
(7) 0.1% *o*-phenyl-phenol, 70% ethyl alcohol, & 0.14% chlorhexidine gluconate	undiluted	2.4	Surfaces and instruments in medical, dental, and veterinary clinics	<1
(8) 0.4% QAC no. 1 and 23% HCl	undiluted	0.4	Toilet bowls, urinals, and enamel surfaces	>4
(9) 10.76% QAC no. 2, 10.76% QAC no. 3, & 16% isopropanol	1:500	6.3	General purpose surface and equipment cleaning	<1

Table 3 (cont'd.)

(10) 0.3% QAC no. 4, 45% iso-propyl alcohol, 3% triethylene glycol, & 2% propylene glycol	undiluted	7.6	Environmental surfaces	<1
(11) 2.7% QAC no. 5	undiluted	9.2	Environmental surfaces	<1
	1.33	8.9	Environmental surfaces	<1
(12) 2.7% QAC no. 5 & 70% ethanol	undiluted	10.1	Environmental surfaces	<1
(13) 7.3% *o*-phenylphenol and 7.4% p-tertiary amylphenol (LpH)	1:256	2.6		<1
(14) 3% Virkon[b]	3:100 (w/v)	2.3		<1
(15) Alkyl (C14, 50%; C12, 40%; C16, 10%) 1.355% di-methyl benzyl ammon. chloride & 1.005% dioctyl dimethyl ammonium chloride (Unicide 128)	1:128	3.0		<1
Noncommercial formulations				
(16) 10% chloramine-T	1:18 (3,000 ppm free chlorine)	7.7	Food equipment and environmental surfaces	<1
	1:11 (5,000 ppm free chlorine)	8.0	Food equipment and environmental surfaces	<1
(17) 3.125% iodophor no. 4	undiluted	1.8	Instruments and environmental surfaces	<1
(18) 70% ethanol	undiluted	5.2	Surface disinfection	<1
(19) 99.8% acetic acid	1:33	2.6	Environmental surfaces	<1
	1:10	2.2	Environmental surfaces	<1
(20) 35% peracetic acid	1:33	2.4	Isolators, medical and surgical equipment, and environmental surfaces	<1

Table 3 (cont'd.)

	1:10	2.2	Isolators, medical and surgical equipment, and environmental surfaces	<1
(21) 85% phosphoric acid	1:33	2.3	Environmental surfaces	<1
	1:10	1.8	Environmental surfaces	<1
(22) 10% citric acid	1:3	2.9	Environmental surfaces	<1
	1:2	2.6	Environmental surfaces	<1
(23) 30% hydrogen peroxide	1:10	6.8	Implants,ventilators, utensils, and thermoplastic equipment	<1
	1:5	6.6	Implants, ventilators, utensils, and thermoplastic equipment	<1

[a] QAC no. 1, *n*-alkyl (50% C-14, 40% C-12, 10% C-16) dimethyl benzyl ammonium chloride; QAC no. 2, *n*-alkyl (60% C-14, 30% C-16, 5% C-12, 5% C-18) dimethyl benzyl ammonium chloride; QAC no. 3, *n*-alkyl (68% C-12, 32% C-14) dimethyl ammonium chloride; QAC no. 4, *n*-alkyl (40% C-12, 50% C-14, 10% C-16) dimethyl benzyl ammonium chloride; QAC no. 5, *n*-alkyl(50% C-14, 40% C-12, 10% C-16) *n*-*n*-dimethyl *n*-benzyl ammonium chloride; iodophor no. 1, butoxypolypropoxypolyethoxy ethanol-iodine complex; iodophor no. 2 polyethoxypolypropoxypolyethoxy ethanol-iodine complex; iodophor no. 3, nonylphenoxypoly(ethyleneoxy) ethanol-iodine complex; iodine no. 4, trial formulation.

[b]**Virkon** = 50% triple salt of potassium monopersulphate, potassium hydrogen sulphate and potassium sulphate + 18% sodium hexanetaphate, 15% sodium dodecyl benzene and 10% malic acid, 5% sulfanic acid.

Ten µL fecally-suspended HAV was placed at the center of a stainless steel disk and dried for 20 or 60 minutes. The inocula was then flooded with 20 µL disinfectant and allowed to act for 1 minute or ten minutes before elution with 1 mL TPB.

the cell was placed in the cell holder on the meter as soon as the red color developed. This reading was multiplied with the two dilutions initially done to obtain the amount of free chlorine in undiluted Javex. The measurements were repeated at least four times and a mean figure established. The final figure was usually around 56,000 mg/L free chlorine.

Solutions with the various levels of free chlorine (800, 3,000 and 5,000 ppm) were obtained by dilution of a sample taken from the same Javex bottle.

Disinfectant Testing Procedure. A positive displacement pipette (Gilson, Medical Instruments, Villiers-le-Bel, France) was used to deposit 10 µL of the fecally-suspended HAV at the center of each disk placed in the wells of a 24-well plastic cell culture plate (Corning). The inoculum was allowed to dry by keeping the disks for 20 minutes in a laminar flow hood. Twenty µL of the product under test was then placed over the dried inoculum and allowed to remain in contact for 1 minute under ambient conditions. At the end of the contact time, each disk was placed in a glass vial containing 980 µL of tryptose phosphate broth (TPB; Difco, Detroit, MI). The contents of the vial were sonicated (Bransonic Ultrasonic Corp., Danbury, KS) for 10 minutes to achieve maximal elution of the virus from the disks. Control disks were treated in an identical manner except that instead of a disinfectant they received 20 µL of EBSS. In each experiment at least 3 disks were treated with the formulation under test and each experiment was repeated at least once.

In a kinetic study over a forty-minute period the activity of 3% Virkon (a recently introduced chemical disinfectant with virucidal activity claims; Pharmascience Inc., Montreal, Quebec), was compared to that of Javex with free chlorine at 800 ppm, the recommended level for surface disinfection in institutions (Rutala, 1990) using the same procedure. A formulation was regarded as effective against HAV if it could reduce the virus infectivity titer by at least 99.9% under the test conditions described.

Interruption of HAV Transfer. Ten µL of fecally-suspended HAV was placed at the center of each disk and the inoculum allowed to dry under ambient conditions. Twenty µL of the test disinfectant was then placed on the dried inoculum and the disinfectant was also

allowed to become visibly dry (about 45 minutes). Virus transfer was attempted by pressing onto the disk with a clean fingerpad of a volunteer with a pressure of 1 kg/cm^2 for 10 seconds. The amount of HAV transferred to the fingerpad was recovered as discussed in Part 6 and the eluents plaque assayed. Controls were set up in the same way and in parallel except that these were treated with dechlorinated tapwater in place of a chemical disinfectant.

Virus elution efficiency using radiolabelled viruses. Both HAV and PV were used to establish the efficiency of virus recovery from stainless steel disks carriers. FRhk-4 cells that had been growing at 37°C for three days were washed three times in PBS and infected with either HAV or PV at an MOI of 0.01 and the virus adsorbed for 45 minutes (PV) or 90 minutes (HAV) at the same temperature. After adsorption, the infected cells were maintained in regular maintenance medium for 3 hours (PV) or 30 hours (HAV). Twenty mL methionine-deficient maintenance medium (Fisher), without serum but containing 50 µCi/mL L-[^{35}S] methionine (Dupont Canada Inc., Markham, Ontario), was added after draining the regular medium. Incubation was resumed at 37°C until cytopathology was evident (approximately 18 hours for PV and 4 days for HAV). The viruses were harvested by freezing (-80°C) and thawing (room temperature) five times before centrifugation of the suspension at 3,000 X *g* for 15 minutes at 4°C.

Supernatant containing labeled virus was then dialysed against distilled water at 4°C overnight and sodium chloride added to final a concentration of 0.4 M before precipitation with 10% polyethylene glycol (PEG; molecular weight 8,000, Matheson, Coleman and Bell, Norwood, Ohio) overnight at 4°C with stirring. The PEG precipitate was obtained by centrifugation at 13,000 X *g* for 1 hour and resuspended in 50 mL of 50 mM tris-HCl buffer at pH 7.4. Virus containing the PEG pellet was then dissolved by vigorous shaking for 1 hour at room temperature and the virus extracted with an equal amount of freon followed by centrifugation at 5,000 X *g*. The freon layer and the interphase were further extracted with a further 50 mL 50 mM tris-HCl buffer. Virus extracts were then combined and reconcentrated with PEG as before and suspending the final PEG pellet with 50 mL tris-HCl buffer.

Magnesium chloride (BDH) at a final concentration of 2 mM and sarkosyl NL-97 (SLS; Sigma) to a final concentration of 1% were added to stabilize the virus and to discourage virus aggregation, respectively.

Six mL of this preparation was loaded onto a preformed discontinuous cesium chloride (Gibco) density gradient (1.1, 1.2, 1.3, 1.4, 1.5, 1.6 and 1.7 g/cm^3) in 0.05 M tris-HCl buffer at pH 7.4 containing 0.1% SLS 17 mL cellulose nitrate tubes (Beckman) overlayed with 30% and 20% sucrose (Gibco) also prepared in 0.05 tris-HCl buffer. The gradients were then centrifuged at 210,000 X g for 22.5 hours at 4°C before collecting 0.5 mL fractions from the bottom of each tube. Gradient fractions with high counts (10^8 for HAV and 10^9 for PV, counts per minute) were used to estimate virus recovery from the disks.

Ten µL of such gradient fraction was placed in the middle of a stainless steel disks and eluted immediately with 990 µL of either TPB or EBSS to establish recoverable amount of virus before drying. Other inocula were allowed to dry for 1 hour before addition of 20 µL of either alkaline glutaraldehyde, sodium hypochlorite (800 ppm free chlorine), dechlorinated tapwater, EBSS or TPB. The disks and their contents were then separately eluted with 980 µL EBSS. Each eluate (0.1 mL) was mixed with 2 mL scintillation fluid (Aquasol-2, New England Nuclear, Boston, MA) and counting in an LKB Liquid Scintillation counter (Model LKB 1214 Rackbeta, LKB, Turku, Finland).

Nearly all of the virus-incorporated radioactivity could be recovered from the wet as well as dried but untreated disks (Table 4). These results show high virus recovery rate in the carrier test protocol.

RESULTS

Activity of chemical disinfectants on HAV. The anti-HAV activity of the 23 chemical disinfectants examined is given in Table 3. Freshly activated 2% alkaline glutaraldehyde inactivated HAV to undetectable levels. On a comparative basis, sodium hypochlorite was found to be more effective against HAV than chloramine-T. At 5000 ppm free chlorine, sodium hypochlorite reduced virus titer to undetectable levels. However, lower

Table 4 : Efficiency of elution of radiolabelled hepatitis A virus and poliovirus (PV) type 1 (Sabin) from stainless steel disks using EBSS.

Treatment of virus inocula on disks	**% Radiolabelled virus recovered**	
	HAV	PV
Dechlorinated tapwater	97.83	100
Earles' balanced salt solution (EBSS)	97.83	98.82
Tryptose phosphate broth (TPB)	100	92.78
2% Alkaline glutaraldehyde	93.48	91.49
Javex (800 ppm free chlorine)	100	100

Ten µL of radiolabeled HAV or PV was placed at the center of a disk and dried under ambient conditions for 1 hour before treatment with 20 µL chemical disinfectant, TPB, EBSS or dechlorinated tapwater for 10 minutes. The disks were then placed in a vial of EBSS and sonicated for virus elution.

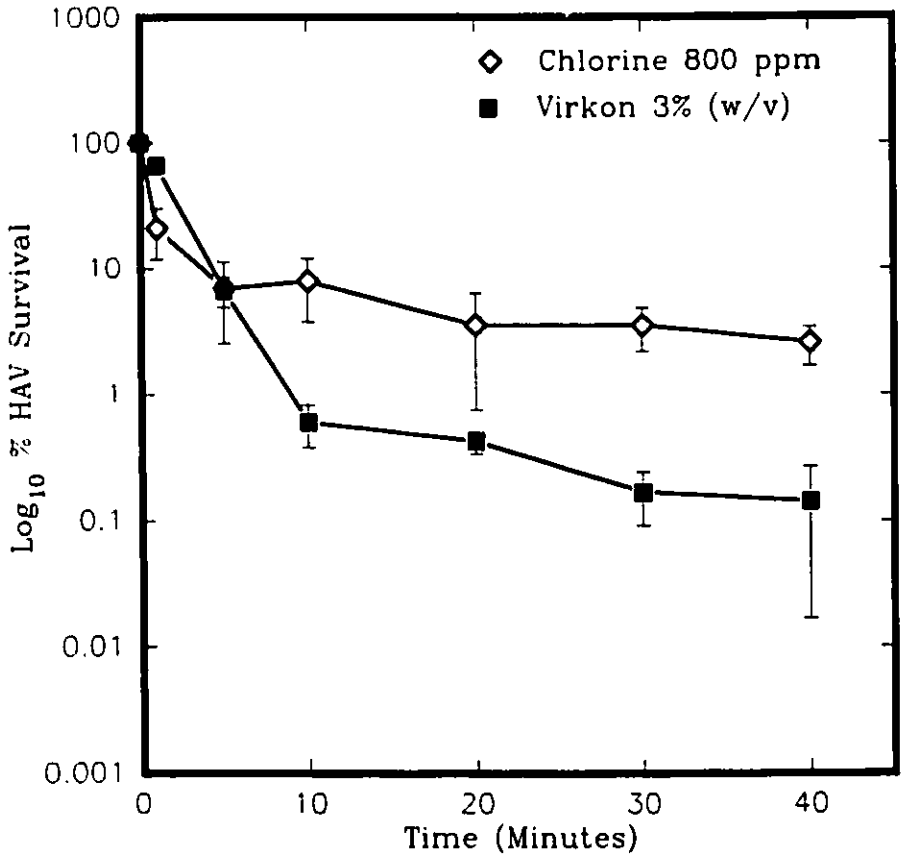
levels of free chlorine released from both of these products were found to be ineffective against HAV.

The three iodophor compounds were inactive against HAV either on their own or in combination with phosphoric acid. Similarly, quaternary ammonium compounds (QAC) as well as phenolic compounds were found to be ineffective against HAV. Combination of QAC with two different types of alcohol (isopropanol and ethanol) or triethylene and propylene glycol did not improve their activity against this virus. However, QAC number 1 (listed as no. 8 in Table 3), containing 23% hydrochloric acid, was able to reduce HAV titer to undetectable levels. Phenolic compounds incorporating sodium lauryl sulfate (a detergent) and chlorophenate or alcohol and chlorhexidine gluconate did not have enhanced activity against HAV. None of the acids was able to reduce virus titer by at least 3 $\log_{10}$, the criterion used for efficacy. Hydrogen peroxide at 3% and 6% was also ineffective against HAV. Virkon (3%) was similarly ineffective.

In the kinetic study (Figure 11), the activity of Virkon against HAV in the first one minute was the lowest (66.52±6.08%) compared to 21.02±9.22% detectable HAV on disks after Javex (800 ppm free chlorine) treatment. However, at 5 minutes, the amount of HAV on Virkon-treated disks was essentially similar to those of Javex.. At 20 minutes, HAV detectable on the disks was 3.56±2.81% and 0.42±0.09% for Javex and Virkon, respectively. Further reduction in virus titer occurred at 40 minutes and the surviving HAV survival at this point was 2.55±0.87% and 0.14±0.12% for Javex and Virkon, in that order.

Interruption of virus transfer through chemical disinfection. The amounts of HAV transferred from inocula treated with dechlorinated tap water ranged from 2.22 to 7.33% (mean of 4.07±1.84%) compared to virus transfer range of 6.85 to 18.90% (mean: 13.37±6.10%) and 11.76 to 16.22% (mean: 13.99±2.33%) given by sodium hypochlorite (800 ppm free chlorine) and LpH respectively (Table 5). Statistically significant differences were revealed between tap water and sodium hypochlorite and also between tap water and LpH. No significant differences were found between LpH and sodium hypochlorite.

Figure 11: Kinetics of hepatitis A virus inactivation by sodium hypochlorite (800 ppm free chlorine) and 3% Virkon.



Ten µL of fecally-suspended virus was placed on each disk and the inoculum allowed to dry for 60 minutes under ambient conditions. The dried inoculum was then covered with 20 µL of the product under test. The virus on the disks was eluted at the indicated times and plaque assayed. The vertical error bars are standard deviations from a mean of 3 observations.

Table 5: Interruption of hepatitis A virus transfer from stainless steel disks to fingerpads by chemical disinfectants.

Disinfectant	Mean % HAV transfer	Statistical significance@
Javex (800 ppm free chlorine)	13.37±6.10	***
Unicide 128 (dilution 1:128)¤	7.94±2.32	*
Tapwater (control)	4.07±1.84	
3% (w/v) Virkon	0.0	*
LpH (dilution 1:256)§	13.99±2.33	***

@Tukey's studentized range test for the variable "*HAV transfer*". Comparisons significantly different from tapwater, Javex and LpH at the 0.05 level are indicated by "***". Those comparisons that are indicated by "*" are not significantly different from tapwater.

¤ Alkyl (C14, 50%; C12, 40%; C16, 10%) 1.355% dimethyl benzyl ammonium chloride and 1.005% dioctyl dimethyl ammonium chloride.

§ 7.3% O-phenylphenol and 7.4% p-tertiary amylphenol.

Ten µL of fecally-suspended HAV was placed at the center of a stainless steel disk and dried for 60 minutes. The inocula were then flooded with 20 µL disinfectant and the fluid allowed to become visibly dry (about 45 minutes) before transfer to fingerpads was attempted.

Unicide 128-treated disks showed virus transfer ranging from 5.31 to 9.66% (mean: 7.94±2.32%) while disks treated with 3% Virkon gave no HAV transfer (Table 5). Compared to tapwater, HAV transferred from disks treated with Unicide 128 was not statistically significant and was also not significantly different from virus transfers given by sodium hypochlorite and LpH at the 95% confidence limit (Table 5). The differences in the means of sodium hypochlorite and LpH on the one hand and means of Unicide 128, tapwater and virkon on the other, were statistically significant ($F = 17.01$, $P<0.05$).

DISCUSSION

Out of the 23 disinfectants tested, only a freshly activated 2% alkaline glutaraldehyde solution, a QAC containing 23% hydrochloric acid and sodium hypochlorite that had at least 5,000 ppm free chlorine were active against HAV. The demonstrated effect of alkaline glutaraldehyde on HAV is consistent with the results obtained for other viruses on contaminated surfaces (Llyods-Evans et al., 1986; Sattar et al., 1989, Tyler et al., 1990) and suggests that 2% alkaline glutaraldehyde is effective against HAV even in the presence of an organic load. Alkaline glutaraldehyde is recommended for the disinfection of semi-critical medical instruments such as flexible fiberoptic endoscopes (FFE) which cannot be heat sterilized (Cole et al., 1990). However, the potency of alkaline glutaraldehyde under reuse conditions may be compromised due to its dilution, accumulation of organic matter, cumulative disinfection load and changing pH levels.

In this study, sodium hypochlorite was found to be more effective than chloramine T against HAV. Although sodium hypochlorite is a broad spectrum, inexpensive and fast-acting disinfectant, it is also known to be extremely sensitive to the presence of organic matter. Sattar et al. (1989) also found that at least 5,000 ppm of available chlorine was required for the disinfection of coxsackievirus B3 under similar test conditions. Similarly, inactivation of other non-enveloped enteric viruses required high levels of free chlorine (Lloyds-Evans et al., 1986). Free chlorine levels of 500 to 5,000 ppm are recommended for disinfection of environmental surfaces (CDC, 1982). The results of this study suggest that free chlorine concentrations in

excess of 5,000 ppm be used for effective disinfection of environmental surfaces contaminated with HAV and other non-enveloped viruses.

All the three iodine-based compounds tested for their efficiency to disinfect HAV on environmental surfaces were ineffective. Other studies have similarly found virucidal efficacy of iodophors to be limited (Lloyd-Evans et al., 1986; Sattar et al., 1989). Even in high concentrations and longer contact times, iodophors are ineffective against viruses (Wallbank et al., 1978).

QAC are widely used as disinfectants. In this study, 5 QAC-based formulations were examined and only one of these proved to be effective against HAV. This effective formulation contained 23% HCl and had a pH of 0.42, suggesting that its activity was due to the acid and not the QAC itself since other QAC with a higher concentration and/or in combination with other compounds such as alcohols were inactive against HAV. Ethanol or isopropanol alone, or isopropanol with triethylene and propylene glycol, when added to QAC did not improve the activity of QAC. The results obtained in this and previous studies (Lloyd-Evans et al., 1986; Sattar et al., 1989) suggest that QAC are unreliable virucidal disinfectants. Phenolic compounds tested in this study were unsatisfactory as HAV disinfectants. One of the phenol compounds contained ethanol and chlorhexidine gluconate but had no better HAV inactivating capacity than the other which contained chlorophenate and sodium lauryl sulfate. Previous studies have also shown phenolic compounds to have little or no virucidal effect on other picornaviruses (Rutala, 1989).

Isopropyl alcohol in combination with chlorhexidine gluconate has been reported to be effective against rotaviruses (Lloyd-Evans et al., 1986). In chimpanzee transmission experiments (Bond et al., 1983) 70% isopropyl alcohol and 80% ethanol are reported effective against hepatitis B virus within 10 and two minutes, respectively. This was not the case with HAV; alcoholic disinfectants on their own, or in combination with QAC, ethylene and propylene glycol, or chlorhexidine gluconate and phenol, or iodine and phosphoric acid were insufficient to inactivate this virus within one minute contact time. Different viruses require

different levels of alcohol for their effective disinfection and therefore the degree of alcohol dilution is limited (Prince et al., 1991). Other previous studies show activity of 70% alcohol against poliovirus to be variable while isopropyl alcohol was found to be ineffective after ten minutes of contact (Tyler et al., 1990).

All the four acids tested were unable to inactivate HAV on environmental surfaces even when the pH level for some of them was as low as 1.8. Only the acid containing QAC (pH <1.0), mentioned above, was effective. Enteroviruses as a group can remain viable at pH 3.0 (Newmann et al., 1973; Siegl et al., 1984), but HAV is even more acid resistant; recent studies by Scholz et al. (1989) have shown that purified HAV can survive exposure to pH 1.0 for several minutes. The capacity of HAV to bind cellular material to its surface (Lemon and Binn, 1985) may also play a role in protecting virus infectivity.

Peracetic acid tested in a previous study was found efficacious against rotaviruses even in as low concentrations as 0.1% pure acid (Lloyd-Evans et al., 1986). In this study, peracetic acid concentrations of up-to 3.5% (w/v) were unable to inactivate HAV. At both concentrations (3 and 6%), hydrogen peroxide was also found ineffective against HAV on surfaces, although its virucidal activity against rhinoviruses has been claimed (Mentel and Schmidt, 1973). Recent findings also show that, at both concentrations hydrogen peroxide is inactive against poliovirus, concurring with the results of this study (Tyler et al., 1990).

The implications of the amounts of HAV transferred to human hands from an environmental surface are unclear. No virus transfer studies have been done with virus inocula pre-treated with chemical disinfectants meant for environmental surface disinfection that can help in the interpretation of the results obtained in this study. However, HAV transferred range of 7.94±2.32 (3.5×10^3 to 4.0×10^3) for Unicide, to 13.99±2.33 (6.0×10^3 to 6.3×10^3 PFU) for LpH is large enough to cause an infection in susceptible host, since the dose required to initiate a viral infection for most viruses is usually small (Westwood and Sattar, 1976, Schiff et al., 1984). The results of these HAV transfer studies emphasize the fact that in addition to their inactivity against HAV, commonly available chemical disinfectants are also unable to interrupt

HAV spread through animate-inanimate contact.

8. *IN VIVO* EFFICACY OF HYGIENIC HANDWASHING AGENTS AGAINST HEPATITIS A VIRUS AND POLIOVIRUS

8. *IN VIVO* EFFICACY OF HYGIENIC HANDWASHING AGENTS AGAINST HEPATITIS A VIRUS AND POLIOVIRUS

INTRODUCTION

Regular and proper washing of hands by care-givers is universally recognized as crucial for infection control (Steere and Mallison, 1975). The limited number of studies conducted in clinical settings have demonstrated the importance of handwashing in controlling outbreaks of bacterial gastroenteritis (Black et al., 1981) and viral infections (Isaacs et al., 1989; Isaacs et al., 1991).

Although handwashing has been in use for sometime now, disease outbreaks continue to be reported which should have been contained or minimized by this measure (Black et al., 1981; Ekanem et al., 1983; Isaacs et al., 1989; Klein et al., 1984; Rosenblum et al., 1991; Seeberg et al., 1981). Even in neonatal intensive care units where strict measures of infection control are more likely to be practiced, large HAV outbreaks have been reported (Azimi et al., 1986; Giocola and Kasprisin, 1989; Klein et al., 1984; Noble et al., 1984; Rosenblum et al., 1991). The sustained incidence of HAV outbreaks in these settings suggests a breakdown of the traditional infection control measures (including handwashing using a handwashing agent) among the care-givers and this may in part account for the observed outbreaks (Rosenblum et al., 1991). Even though Rosenblum et al. (1991) do not report the degree of compliance with handwashing guidelines, other investigators (Albert and Condie, 1981; Doebbeling et al., 1992; Fox et al., 1974) have shown failure of medical and nursing personnel to comply with such guidelines.

There is a general conception that most microorganisms that care-givers pick up from patients remain on the skin only transiently and can be dislodged with bland soap and water (Ehrenkranz, 1992). But polioviruses have been shown to persist on human hands even after the most stringent methods of handwashing attempts (Eggers, 1989). Thus the persistence of HAV outbreaks in settings that are otherwise known for strict hand hygienic measures may reflect the non-removal of the virus from human skin by handwashing agents (Klein et al.,

1981; Rosenblum et al., 1991; Seeberg et al., 1981). HAV sensitivity to commonly used handwashing agents is yet to be established and handwashing control measures are yet to be validated for HAV. Similarly, the role of handwashing agents to interrupt HAV transfer is unknown.

MATERIALS AND METHODS

Hygienic handwashing agents tested. A total of 11 handwashing agents were tested. Seven of these preparations were received from the Infection Control Officer, Ottawa General Hospital, as handwashing preparations currently in-use at this hospital. The others were preparations recently acquired from the local market for use in our laboratory. The details of each of the 11 agents are shown in Table 6.

The fingerpad protocol. Participating volunteers were asked to wash their hands in luke-warm (about 40°C) running tapwater for 30 seconds and to apply 70% alcohol after drying them with ordinary paper towel (Kimberly-Clark Canada Inc., Mississauga, Ontario). To spread the alcohol, the hands were gently rubbed against each other and then air dried. The procedure for testing the potency of handwashing agents was as described previously (Ansari et al., 1989). Briefly, small areas of demarcation were made by pressing the fingerpads hard on the mouth (8-mm inside diameter) of an empty sterile plastic vial (no. 72.694.006; Sarstedt Inc., St. Laurent, Quebec, Canada). A virus inoculum of 10 µL of either HAV (approximately 9.2×10^4 PFU) or PV (approximately 6.0×10^7 PFU) suspended in 10% feces was placed at the center of the demarcated area on 3 fingerpads of one hand and then eluted immediately with 990 µL TPB

The five fingers on the other hand were similarly contaminated with HAV or PV as described for the input controls. After the virus dried, the inoculum from one fingerpad was eluted to determine the amount of virus surviving the initial drying process. Three fingerpads were used to assess the virus eliminating efficacy of each agent tested. For this purpose, the contaminated area of the fingerpad was placed over the mouth of a plastic vial containing 1 mL of the test agent. The vial was inverted and the test agent brought in contact with the

Table 6. Details of handwashing preparations tested.

Brand name	Active ingredients listed	In-use dilution	Manufacturer
1. Anti-bacterial Triclosan hand Soap	triclosan+0.5% 2,4,4'-trichloro-2'-hydroxy-diphenyl ether	none	G.H. Wood, Toronto, Ont.
2. Savlon	1.5% chlorhex. gluc., 15% cetrimide	1:30* in 70% ethanol	Ayerst, Montreal, Quebec.
3. Ethanol, Denatured D.A.G-2A	70% ethanol	8.3:10	BDH, Toronto, Ontario.
4. Septisol	0.75% hexachlorophene	1:2*	KVL, Cambridge, Ontario.
5. Scrub Stat IV	4% chlorhex. gluc. + 4% isopropanol	none	Huntington, Huntington, PA
6. Alcare	62% v/v or 53% w/w emolliented ethanol foam	none	Huntington, Huntington, PA
7. Aquaress	none	none	DEB Swarfega, Waterford, Ontario.
8. Bacti-Stat Soap	0.3% triclosan + isopropanol	none	Huntington, Bramalea, Ontario.
9. Bioprep Soap	0.1% chlorhex. gluc.+ didecyl dimethyl ammonium chloride + 5% isopropanol	none	
10. Dettol	4.8% 4-chloro-3,5-xylenol + 9.4% isopropanol	none	Reckitt-Colman, Lachine, Quebec.
11. Tapwater (40°C)	none	none	

*Manufacturer's recommended dilution

area for 10 seconds. The treated area was then rinsed in 15 mL of tapwater contained in a plastic tube; the tube, with the fingerpad pressing over its mouth, was subjected to three inversions in a total of three seconds. After the water rinse, the treated fingerpad was dried by pressing and holding it over a paper towel for 3 seconds. The virus remaining on the fingerpad was eluted as described for the input controls above.

Virus Transfer : The fifth finger was treated with the test handwashing agent, washed with 15 mL tapwater, dried with a paper towel and the remaining virus transferred to a stainless steel disk at a pressure of 1 kg/cm^2 without friction. To recover the virus transferred, the disk was placed in a vial containing 1 mL of TPB and the eluate plaque assayed.

Whole-hand protocol: The participating volunteer was not required to wash his/her hands prior to experimentation in order to reflect field practice where handwashing preparations are used without prewashing. As such, the results obtained would closely simulate what may occur in nature. These results could also be correlated with those obtained with the fingerpads (Ansari et al., 1989).

HAV and PV (equal amounts) were suspended in 10% fecal suspension to give approximately 1.3×10^5 PFU/mL and 1.1×10^8 PFU/mL, respectively. To estimate the amount of viruses recoverable from the hands after drying, a control was setup as follows. The virus inoculum (0.5 mL) was placed in the middle of the palm of one hand of the volunteer and the palms were rubbed together to contaminate them evenly. The palms were then allowed to air dry in a laminar flow hood for 20 minutes. With the help of an assistant, the viruses surviving drying were eluted by slowly pouring 20 mL of TPB over the hands while they were being rubbed together. The eluents were collected in a clean and sterile plastic bag about 30 cm in diameter and 60 cm deep; the volumes of eluent recovered ranged from 17 to 19.5 mL.

To test the handwashing preparations, 0.5 mL of it was placed at the center of one of the virus-contaminated palms and the preparation spread evenly over both hands by rubbing them together for 10 seconds. The assistant was then requested to slowly pour 500 mL lukewarm tapwater over the hands while they were rubbed together as in normal washing and

the hands dried with paper towel (Kimberly-Clark Canada Inc., Mississauga, Ontario). Viruses surviving this washing, rinsing and drying procedure were eluted from the hands using 20 mL TPB as described above. Two-fold dilutions were performed in TPB immediately to neutralize the effect of the handwashing preparation. All the eluents were filter sterilized before plaque assay.

RESULTS

An analysis of variance using a general linear model revealed no significant differences between the results of the 3 volunteers participating in this study. The means shown in Tables 7 and 8 are therefore means from nine experiments. The analysis also revealed significant differences between HAV and PV removal by the 11 eleven handwashing agents ($F= 102.14$, $P<0.05$) and also significant differences between the agents ($F = 17.10$, $P<0.05$).

Virus removal from fingerpads. Eleven commercial handwashing agents were compared for their ability to remove HAV from experimentally-contaminated fingerpads. Luke warm tapwater, used as a control, reduced the titer of the virus on the fingerpad by 79.74±4.80% compared to 77.96±7.17% HAV reduction observed for Aquaress (unmedicated ordinary soap), the mean differences being insignificant (Table 7). Bacti-Stat Medicated Soap, an agent containing 0.3% triclosan and isopropanol showed virus titer reduction of 92.04±4.02% compared to Antibacterial Triclosan Hand Soap that gave a virus titer reduction of 91.29±4.47% with no statistically significant differences between the two means.

Savlon, Scrub Stat IV and Biorep Antiseptic Lotion Hand Soap all contain different levels of chlorhexidine gluconate and alcohol (Table 6); the results observed for the latter 2 were comparable (Table 7). Savlon gave an HAV reduction of 90.91±5.08% while 70% ethanol , Alcare (containing 62% emolliented ethyl alcohol foam) and Dettol (containing 9.4% isopropyl alcohol) reduced HAV titer to 87.40±4.59%, 89.27±4.38%, 88.63±5.38% respectively. Septisol Antiseptic Liquid Soap showed an HAV reduction of 88.60±5.36% (Table 7). A *post hoc* analysis (Tukey tests) summarizes the differences between the agents

Table 7: Comparative efficacy of hygienic handwashing agents against hepatitis A virus (HAV) and poliovirus (PV) type 1 (Sabin) in the fingerpad protocol.

Handwashing agent	Mean % reduction		Tukey grouping	
	HAV	PV	HAV	PV
Anti-Bacterial Triclosan Hand Soap	91.29±4.47	94.80±3.76	A	A
Savlon	90.91±5.08	98.13±1.58	B	A
70% Ethanol	87.40±4.59	95.47±4.06	B	A
Septisol Antiseptic liquid Soap	88.60±5.36	94.37±4.34	B	A
Scrub Stat IV	89.57±6.70	91.45±3.49	A	A
Alcare	89.27±4.38	97.52±3.59	B	A
Aquaress	77.96±7.17	89.01±4.18	B	A
Bacti-Stat Medicated Soap	92.04±4.02	98.39±1.98	B	A
Bioprep Antiseptic Lotion Hand Soap	83.35±2.76	90.93±2.86	B	A
Dettol	88.63±5.38	93.77±4.14	B	A
Tapwater (40°C)	79.74±4.80	85.22±2.91	B	A

Ten µL of fecally-suspended virus was placed on each fingerpad and the inoculum dried for 20 Minute under ambient conditions. The contaminated area was then exposed to the agent under test, rinsed in tapwater and dried with a paper towel. The virus was eluted from the fingerpads and titrated.

examined (Table 7). Comparisons of the means by Tukey's studentized range test (using the general linear model procedure) showed all the agents to be significant different from tapwater except Aquaress (unmedicated soap; differences between the means = 5.03 to -12.70). All the agents were also significantly different from Aquaress (differences between the means = 7.59 to 12.26) at the 95% confidence limit.

In general, the amounts of PV removed from the fingerpads in each volunteer were more than the amounts of HAV removed by the same agent. For instance, Alcare reduced PV by 97.52±3.59% PV compared to 89.27±4.38% of HAV.

Alcohol-based products were generally more active on PV than they were on HAV (compare 95.47±4.06% PV reduction by 70% ethanol and 87.40±4.59% HAV reduction by the same product). Among the chlorhexidine containing substances, Savlon was better with a reduction of 98.13±1.58% PV (Table 7). Small differences were also noticed when fingerpads were treated with tapwater or unmedicated soap between PV and HAV with more mean % reduction occurring in PV antisepsis (compare 85.22±2.19% obtained with tapwater and 89.01±4.18% given by Aquaress against a reduction of 79.74±4.80% and 77.96±7.17% HAV reduction given by the same agents, respectively; Table 7.

Dettol, Septisol Antiseptic Liquid Soap and Bioprep Antiseptic Lotion Hand Soap were found to be more active on PV than they were on HAV (Table 7). Between the two triclosan-based products, Bacti-Stat Medicated Soap was better in virus removal than Anti-bacterial Triclosan Hand Soap (compare a reduction of 98.39±1.98% for the former and 94.80±3.76% for the latter).

Virus transfer to metal disks after handwashing. HAV transfer was the highest in fingerpads treated with tapwater alone (0.8055±0.1632%; Table 8). Virus treatment with Aquaress reduced the amount of HAV transfer to 0.3911±0.1522%. Among the other handwashing agents Antibacterial Triclosan Hand Soap had the highest amount of HAV transfer; 0.1456±0.0317%. Fingerpads treated with products containing high levels of alcohol (Savlon, Alcare and 70% ethanol; Table 6) had no HAV transfer (0%; Table 8). All other

Table 8: Interruption of hepatitis A virus (HAV) and poliovirus (PV) type 1 (Sabin) transfer from fingerpads to stainless steel disks by hygienic handwashing agents.

Handwashing agent	Mean % transfer		Tukey grouping	
	HAV	PV	HAV	PV
Anti-Bacterial Triclosan Hand Soap	0.1456±0.0317	0.0711±0.0220	A	B
Savlon	0.0	0.0		
70% Ethanol	0.0	0.0		
Septisol Antiseptic liquid Soap	0.0517±0.0232	0.0244±0.0142	A	B
Scrub Stat IV	0.0725±0.0183	0.0286±0.0177	A	B
Alcare	0.0	0.0		
Aquaress	0.3911±0.1522	0.1450±0.0781	A	B
Bacti-Stat Medicated Soap	0.0420±0.0175	0.0060±0.0055	A	B
Bioprep Antiseptic Lotion Hand Soap	0.0590±0.0191	0.0211±0.0093	A	B
Dettol	0.0350±0.0306	0.0250±0.0093	A	B
Tapwater (40°C)	0.8055±0.1632	0.4142±0.1831	A	B

Ten µL of fecally-suspended virus was placed on each fingerpad. The inoculum was allowed to dry for 20 minute under ambient conditions before exposure to test agent. The treated area was rinsed in tapwater and dried with a paper towel. The fingerpad was then contacted with a clean metal disk at a pressure of 1 kg/cm^2 without friction. The virus was eluted from the disk and titrated.

products showed a comparatively low HAV transfer with respect to the tapwater control (Table 8). Tukey tests revealed significant differences in amounts of HAV or PV transferred post treatment with each handwashing agent. Table 8 is a summary of this analysis.

There was no poliovirus transfer from the fingerpads treated with Savlon, 70% ethanol and Alcare (Table 8). Bacti-Stat Medicated Soap showed a mean virus transfer of 0.0060±0.0055%. As was the case with HAV, more PV was transferred by fingerpads treated with tapwater alone (0.4142±0.1831%). Aquaress had the second highest level of PV transfer (Table 8). Anti-bacterial Triclosan Hand Soap had a PV transfer of 0.0711±0.0220% while the other agents showed much lower virus transfer. Amounts of PV transferred across the handwashing agents was lower than the amounts of HAV transferred by the same volunteers (Tables 8).

Elimination of HAV using the whole-hand protocol. Table 9 shows results obtained with the whole-hand test using Volunteer 2. The percentage HAV reduction obtained by each agent was comparable to results realized in the fingerpad method with the same volunteer (Table 7). There were no statistically significant differences in HAV reduction in the fingerpad and those obtained using the whole-hand procedure.

As was the case with HAV, levels of PV reduction at the end of the handwashing event were comparable to those observed in the fingerpad procedure (Table 7 and 9). There were no statistically significant differences between the two methods.

DISCUSSION

Seven of the antiseptics tested (Table 6) are products currently in use at the Ottawa General Hospital. Savlon, ethanol and Dettol are also commonly used in other health-care settings. The efficacy of these agents against HAV and PV was similar in the three volunteers examined. Although this number is statistically small, the results suggest the reproducibility of the *in vivo* procedure used and concur with the findings with human rotavirus (Ansari et al., 1989). *In vivo* studies with either bacteria or viruses are few (Ansari et al., 1989; Doebbeling et al., 1992) and current testing of handwashing agents is based on *in vitro* suspension assays.

Table 9. Comparative efficacy of handwashing agents in the elimination of hepatitis A virus (HAV) and poliovirus (PV) type 1 (Sabin) using the whole-hand test protocol.

Handwashing agent	% Reduction (mean±SD)		Tukey grouping	
	HAV	Polio	HAV	polio
Triclosan Soap	88.98±1.73	96.63±2.86	B	A
Savlon	86.53±3.44	95.81±3.08	B	A
70% Ethanol	86.92±1.63	92.77±2.79	B	A
Septisol	89.20±0.81	92.03±6.82	A	A
Scrub Stat IV	81.15±1.15	89.27±1.41	B	A
Alcare	86.17±4.28	93.39±5.74	A	A
Aquaress	91.39±2.65	90.86±4.03	A	A
Bacti-Stat Soap	94.56±5.75	97.28±2.05	A	A
Bioprep Soap	81.44±1.59	88.99±2.59	B	A
Dettol	90.67±2.08	97.09±2.32	B	A
Tapwater (40°C)	81.57±4.55	85.06±3.73	A	A

Ten µL of fecally-suspended virus was placed on 3 precleaned and pre-demarcated FP and dried for 20 minute at ambient conditions. The first FP was treated with 1 mL handwashing agent for 10 seconds while the second and the third FP were treated with the agent water or water and then dried with paper towelling before eluting the remaining virus with TPB.

The results of this study have shown what may be the case in the field when handwashing agents are used to control the spread of HAV and PV infections, particularly in institutions.

Although the formulations tested in this study were better than tapwater and unmedicated soap in reducing the virus titer on hands, none of them showed a >3 $\log_{10}$ virus reduction at the end of the washing process. In fact, all showed HAV and PV transfer except for Savlon, 70% ethanol and Alcare (containing 62% alcohol) (Tables 8). Such residual virus on the hands may explain at least in part, the frequently reported HAV outbreaks in pediatric and intensive care units (Azimi et al., 1986; Giocola and Kasprisin, 1989; Klein et al., 1984; Noble et al., 1984; Rosenblum et al., 1991).

HAV and PV removal by the handwashing agents was comparable except that the amounts of PV removed were higher than those of HAV. This suggests that the capability of handwashing agents to remove virus from hands depends on the type of virus involved. Caution must, therefore, be exercised in extrapolating the results with one particular virus. Literature review also shows conflicting evidence on the efficacy of handwashing agents to control infections in institutions or in food handling establishments. Studies on diarrhoeal outbreaks in institutions have observed diarrhoea incidence to decline upon imposition of handwashing procedures (Black et al., 1981). Similarly, viral outbreaks in pediatric nurseries have also been contained by instituting stringent handwashing procedures with antiseptics (Isaacs et al., 1989; Isaacs et al., 1991). But transient bacteria have been shown to persist on hands after washing and only a few products are able to reduce their level significantly (Stiles and Sheena, 1987; Sheena and Stiles, 1982; Rotter and Koller, 1992). Moreover, some organisms have been shown to grow in antiseptics (Anderson et al., 1991).

Laboratory experiments show that handwashing with antiseptics and vigorous hand rubbing during this event is unable to remove poliovirus, leaving about 1% of the original inoculum (Eggers, 1989). The ineffectiveness shown by antiseptics against HAV and PV in this study suggest inadequacy of commonly available antiseptics to inactivate and remove viruses from human hands and affirms some of the above observations and indicates the potential for

viruses to spread horizontally even under conditions of high standards of hygiene.

Failure of antiseptics to inactivate and to remove the two viruses from human hands is confounded by lack of compliance with handwashing guidelines among health-care personnel (Albert and Condie, 1981). Even when practiced, the practice is often suboptimal (Fox et al., 1975) and in contrast to guidelines (AHA, 1974; Garner and Simmons, 1983; Steere and Mallison, 1975). However, the fact that HAV and PV could be transferred to an environmental surface after handwashing and drying with paper towel shows that careful handwashing *per se* is not enough to control the horizontal dissemination of these viruses.

Alcohol containing antiseptics show a good bacterial removal from the skin (Rotter and Koller, 1992). They were, however, ineffective against HAV and PV. Chlorhexidine gluconate containing products are widely used and are effective against bacteria (Ayliffe et al., 1975; Butz et al., 1990; Doebbeling et al., 1992; Sheena and Stiles, 1982), but are inadequate in removal of HAV and PV from hands even in combination with alcohols (Scrub Stat IV, Bioprep Antiseptic Lotion Hand Soap) or with cetrimide (Savlon). These results are in accord with those shown for human rotavirus (Ansari et al., 1989). The only PCMX-containing product (Dettol) examined in this study gave poor activity against both viruses and has also been shown to give similar results with bacteria (Sheena and Stiles, 1982) and rotaviruses (Ansari et al., 1989).

The triclosan containing handwashing agents were also ineffective against both viruses and their activity has been shown to be lower than that of chlorhexidine but higher than alcohol containing agents (Butz et al., 1990). The combination of triclosan and isopropanol alcohol (Bacti-Stat Medicated Soap) however, gave the highest virus reduction of the 11 agents examined suggesting its combination with isopropanol improves virus removal since this alcohol did not show the same degree of virus removal when combined with chlorhexidine or quaternary ammonium containing products (Tables 7 and 9).

This study has shown that handwashing agents do not remove HAV or PV from human hands. It is recognised the handwashing process among care-givers is suboptimal (Albert and

Condie, 1981; Doebbeling et al., 1992; Fox et al., 1974) and the frequent occurrence of HAV outbreaks in these institutions (Azimi et al., 1986; Giocola and Kasprisin, 1989; Klein et al., 1984; Noble et al., 1984; Rosenblum et al., 1991) may be accounted for by lack of compliance with handwashing guidelines, the inability of the agents to remove the virus from the hands, and also lack of interruption of virus transfer between animate and inanimate surfaces.

9. ALKALINE GLUTARALDEHYDE REUSE AND GERMICIDAL ACTIVITY AGAINST HEPATITIS A VIRUS AND OTHER MICROORGANISMS

9. ALKALINE GLUTARALDEHYDE REUSE AND GERMICIDAL ACTIVITY AGAINST HEPATITIS A VIRUS AND OTHER MICROORGANISMS

INTRODUCTION

Reusable medical devices that are damaged by heat require decontamination by chemicals (liquid or gas) between patients. In particular, flexible fiberoptic endoscopes (FFE), present a formidable challenge to the disinfection process because of their complex structure (Appendix B_4) and design which make most of their parts inaccessible to chemical disinfection (Axon, 1991; Babb and Bradley, 1991).

In the recent past the use of these instruments has proliferated enormously (Axon, 1991; van Gossum et al., 1989) and FFE-associated bacterial and viral infections are also being reported with increasing frequency (Alfa and Sitter, 1991; Alvarado et al., 1991; Fraser et al., 1992; Hanson et al., 1989; Karim et al., 1989). In a multi-state investigation (Kaczmarek et al., 1992) disinfection procedures for endoscopes were often found to be sub-optimal and nearly 24% of the samples obtained from internal channels of FFEs had bacterial contamination of at least 10^4 CFU.

During reuse, the germicidal activity of a chemical disinfectant can be affected by a number of factors. Dilution of the chemical may occur to levels below those that are recommended for its use. The concentration of alkaline glutaraldehyde (2-2.5%), a disinfectant most commonly used for the disinfection of semi-critical instruments, was shown to fall to less than 1% after 20 endoscopes were processes in a manual bath (Hanson et al., 1989). Higher dilutions of the disinfectant occur in automatic disinfectors and a fall from 2.4% to 0.5% after 26-30 cycles has been recorded (Leong et al., 1991). A recent Canadian study (Whyman et al., 1991) reported similar findings. This evidence raises concern for the efficacy of alkaline glutaraldehyde over its recommended reuse period. Most hospitals using alkaline glutaraldehyde for disinfection of FFE do not monitor its concentration during its period of reuse (Kaczmarek et al., 1992). Sterilog strips that are available for such monitoring are not

reliable (Power and Russell, 1988).

Apart from dilution, the pH level, organic load, aging and temperature at which alkaline glutaraldehyde is used are important for its germicidal activity (Gelinas and Goulet, 1983; Gorman et al., 1980). The number of instruments subjected to a chemical under reuse conditions also contributes to its loss of activity (Hanson et al., 1989; Leong et al., 1991; Whyman et al., 1991).

In this study, five different organisms were selected to monitor the germicidal activity of alkaline glutaraldehyde during its reuse in an endoscopy unit. The disinfectant samples were also tested for their pH, disinfectant concentration and protein content.

Poliovirus (PV) type 1 (Sabin) has been recommended as the prototype for determining the virucidal activity of chemical disinfectants (CGSB, 1991). However, the findings reported in Part 7 have shown HAV to be particularly resistant to a wide variety of disinfectants. We, therefore, decided to conduct a direct comparison of their resistance to alkaline glutarladehyde under reuse.

The selection of *P. aeruginosa* as one of the test organisms in this part of the study was based on the following considerations:

It is a non-fermentative, Gram-negative bacillus which is often found in soil and water (Palleroni, 1984). Its relevance to medicine is underscored by the fact that it can cause sev.re and often fatal disease in immuno-compromised patients. Those suffering from cystic fibrosis, cancer, diabetes or heart diseases are particularly at risk. For instance, 90% of cystic fibrosis patients show *P. aeruginosa* to be the primary pathogen (Marshall and Carroll, 1991). Because of its ability to acquire antibiotic resistance, its widespread distribution, ability to survive under a variety of conditions and a broad-spectrum host range, *P. aeruginosa* is the most common nosocomial pathogen in patients hospitalized for more than a week (Bodey et al., 1983; Boukadida et al., 1991). Moreover, among opportunistic infections with Gram-negative bacilli, those caused by *P. aeruginosa* are associated with particularly high mortality (Marshall and Carroll, 1991).

P. aeruginosa has also been cultured from both chemical disinfectants and antiseptics used in hospital settings (Anderson et al., 1991; Lee and Fiaklow, 1961). The disinfectant in these baths is often found to have heavy biofilms of this organism. This organism has been isolated from channels of endoscopes disinfected in automatic baths (Allen et al., 1987; Alvarado et al., 1991; Greene et al., 1974; Hanson et al., 1989).

Cases of tuberculosis are on the rise again in industrialized countries and infections due to atypical mycobacteria are also being reported with increasing frequency, particularly in association with AIDS and immuno-suppression (Dunmire and Breyer, 1991; Dunagan, 1990; Peters et al., 1989; Wolinsky, 1992; Zaman, 1991). This has further enhanced the urgency of instituting effective measures for the prevention and control of such infections.

Chemicals to be used for the disinfection of semi-critical instruments must be shown to be effective against mycobacteria (Favero and Bond, 1991; Rutala, 1990). The test protocols for the assessment of such activity recommend the use of *M. smegmatis* for the initial tests to be followed by confirmatory tests with *M. bovis* (AOAC, 1990). There is, however, considerable disagreement over the suitability of these two organisms as surrogates for *M. tuberculosis*. As a result, a number of other mycobacteria have been tested as potential substitutes. The notable species in this regard are: *M. terrae* (Gundermann, 1987; Sonntag et al., 1977), *M. avium* and *M. fortuitum* (Collins, 1986) and *M. chelonae* (Zaman, 1991). These species were considered unsuitable in this study due to various factors such as their potential pathogenicity to humans (Peters and Morice, 1991; Wolinsky, 1992) and variability in resistance to alkaline glutaraldehyde (Gundermann, 1987). Instead, we chose to include *M. gordonae* as a representative of mycobacteria here.

M. gordonae is considered as the least pathogenic of the Runyon Group II mycobacteria (Jarikre, 1991). It is found in water taps and soil (Wayne and Kubica, 1986). Although a saprophyte (Wolinsky, 1992), it is isolated from sputum and gastric lavage specimens (Wayne and Kubica, 1986) and more recently its association with infections in humans has been suggested (Barber et al., 1991; Jarikre, 1991; London et al., 1988; Tokars et

al., 1990).

MATERIALS AND METHODS

Viruses. Both HAV and PV stocks described below were cultivated in cells maintained in a medium containing no antibiotics to avoid any inhibition of the bacteria used in the mixture. The viruses used in this study were also from unconcentrated pools.

Organic Load. Whole fetal bovine serum used to culture FRhK-4 cells (Gibco) was obtained from one bottle, heat inactivated at 56°C for 30 minutes, cooled and aliquoted in 0.5 mL quantities that were then preserved at -20°C until needed.

Pseudomonas aeruginosa. ATCC strain #27853 of *P. aeruginosa* was received from the L.C.D.C. and was used throughout this study. Stock cultures were grown in trypticase soya broth (Difco) in 500 mL flasks, mounted onto a shaker (Lab-line Instruments; Melrose Park Ill), at 37°C for 48 hours. The cultures were washed three times in fresh broth before preservation. Day-to-day cultures were grown by inoculating a single drop from a stock culture into 12 mL of trypticase soya broth in a glass vial and incubated at 37°C, with the screw cap loose, for 48 hours. The cultures were vortexed once at 24 hours post incubation. *P. aeruginosa* colonies were enumerated by spreading 0.1 mL quantities onto a Bacto *Pseudomonas* Agar F (Difco). Colony counts were recorded after an incubation of 24 hours at 37°C and then on the third day of incubation. The count was expressed as colony forming units per mL (CFU/mL) according to the formula: CFU = colony count X dilution X 10, where the 10 is volume conversion of 0.1 mL inoculum to 1 mL.

Mycobacteria. The BCG vaccine strain of *M. bovis* (Wayne and Kubica, 1986), obtained from the L.C.D.C. as strain NT 416 (ATCC 35743), was used. It is widely used as the surrogate organism for disinfectant testing for tuberculocidal claims and is recommended for this purpose by the Association of Official Analytical Chemists (Beloian, 1990). A seed culture of *M. gordonae* (NRCT 1101) was also obtained from the L.C.D.C.

Stock cultures for both *M. gordonae* and *M. bovis* were grown as shake cultures in the same way as described for *P. aeruginosa* above, except that Bacto Middlebrook 7H9 broth

(Difco) containing 0.5% Tween-80 (Baker Chemical Co., Phillipsburg, N.J), supplemented with Bacto Middlebrook OADC enrichment (Difco), was used and growth allowed to occur for 21 days at 37°C, with vortex mixing every 2 days. Such cultures were separately washed three times in fresh broth before preservation. The day-to-day cultures were grown in 12 mL Bacto Middlebrook 7H9 broth containing 0.5% Tween-80 and supplemented as above, in screw capped glass vials held at 37°C. Bacterial enumeration was by spreading out 0.1 mL quantities onto Bacto Middlebrook 7H11 Agar containing 0.5% glycerol (BDH) and incubation at 37°C. Colonies were counted on day 14 for *M. gordonae* and on day 21 for *M. bovis*. A second and final count was made on the day 30. Gentamicin (Roussel), at a final concentration of 15 µg/mL, was added to 7H11 Agar when the medium was used for the detection of *M. gordonae* in the mixture while suppressing the growth of *P. aeruginosa*.

Preservation of bacteria. The washed bacterial cells were suspended in fresh broth containing 7% glucose (BDH) and 15% heat-inactivated FBS in 2 mL plastic vials that were brought to -20°C overnight before storing them to -80°C till required.

Glass cups as carriers. Glass cups (1 cm in diameter and height) were cut out of the bottom of Pyrex serum dilution tubes measuring about 1 cm in diameter and 7.5 cm in height. Before use, the glass cups were soaked in 10% nitric acid overnight at room temperature. They were then thoroughly rinsed under running tapwater and soaked in 7X cleaning solution (Fisher) for 24 hours. The detergent was removed by rinsing thoroughly under running deionized distilled water. The glass cups were allowed to dry at room temperature and then baked at 160°C for 2 hours in a glass beaker covered with aluminium foil. Such treatment of the cups before reuse was necessary to remove the organic material fixed to the glass by alkaline glutaraldehyde.

Disinfectant baths. The disinfection of endoscopes at the Ottawa General Hospital includes the following: pre-wash in warm tap water containing an enzymatic detergent (Metrizyme, Metrex Research Corp., Vancouver, B.C), a rinse in fresh warm tap water and then soaking in the disinfectant (without drying). Two manual and one automatic baths (EW-20,

Olympus, Tokyo, Japan) meant for the disinfection of gastrointestinal endoscopes and bronchoscopes at the Ottawa General Hospital were selected for this study. The manual baths were basins provided by Cidex (Johnson & Johnson Medical, Inc., Arlington, Texas). Each bath was carefully washed and dried before filling it with 4 litres of activated 2% alkaline glutaraldehyde. The automatic bath was washed and disinfected before adding 2 litres of fresh detergent into the detergent compartment and 15 litres of the disinfectant solution. The disinfectant was reused for 14 days in all the baths.

Collection of disinfectant samples. Approximately 15 mL of the disinfectant were collected daily from each bath except on days 1 and 2 (Saturday and Sunday) because the 14-day cycle at the hospital starts on a Friday. Samples from the automatic bath were taken promptly within 3 minutes of the start of the 20-minute disinfection phase. The solution in the manual baths was first thoroughly mixed before withdrawing the samples. For a control, a manual bath was set up with 4 L of freshly prepared alkaline glutaraldehyde solution and placed in the same room as the test baths. Disinfectant samples were collected from it over a 14-day period and tested for pH, glutaraldehyde concentration and protein in parallel with the test samples.

Microbial elution. A full-strength Bacto Middlebrook 7H9 broth containing 2% glycine was used as the eluent. A combination of this broth and glycine (7H9G) was found suitable to neutralize the disinfectant. Initial experiments were carried out by incubating a mixture of *M. gordonae*, *P. aeruginosa*, HAV and PV in 5 mL 7H9G for 4.5 hours in the laminar flow hood. On culturing, no significant titer reduction was found with any of the bacteria or virus when compared to a control comprised of the same organisms in 7H9 broth but without glycine. A parallel test with *M. bovis* gave similar results.

Preparation of mixed inocula. Twelve mL of a 21-day-old culture of *M. gordonae* was mixed with an equal amount of a 24-hour-old culture of *P. aeruginosa*. The mixture was then centrifuged at 3,000 X *g* for 60 minutes. The supernatant was discarded in a jar of 20% Javex. The bacterial pellet was resuspended in approximately 1.5 mL of a mixture of HAV and

PV. Each mL of the bacteria-virus mixture contained about 10^6 PFU of HAV, 10^7 PFU of PV, $10^{8.5}$ CFU of *P. aeruginosa* and $10^{6.5}$ CFU of *M. gordonae*. Since the virus pools contained 2% FBS, an additional amount of FBS was added to the mixture to bring the final serum concentration to 5%.

Germicidal test procedure. The bacteria-virus mixture was vortexed and 20 µL carefully placed at the bottom of each glass cup held in a well of a 96-well microtiter plate (Costar). The inocula were allowed to dry at ambient temperature and RH for 90 minutes in a laminar flow hood. Sixty µL of the disinfectant was placed on the dried inoculum using a 20 µL pipette (Gilson Medical Instruments, Villiers-le-Bel, France) and allowed to act for 10 minutes. Each glass cup was then dropped into a glass vial containing 1 mL 7H9G. The vial was gently vortexed for 1 minute before pipetting its contents up and down 5 times to ensure complete elution. Experiments with *M. bovis* were done in parallel and in the same way as for the mixed culture.

Removal of residual disinfectant. The removal of the disinfectant from the eluates was according to the procedure described by Blackwell and Chen (1970). The eluates were loaded onto pre-swollen (equilibrated) Sephadex LH-20 gels (Pharmacia, Fine chemicals, Uppsala, Sweden) in plastic cones (Amicon) placed in 50 mL plastic centrifuge tubes. The tubes were centrifuged at 2,000 X *g* for 5 minutes. When the microorganisms were suspended in the eluent (without disinfectant) and passed through the gel, the recovery rates were 77.43, 75.73, 92.30, 95.19, 76.94% for *M. gordonae, P. aeruginosa*, HAV, PV and *M. bovis*, respectively.

Enumeration of surviving microorganisms. A 0.1 mL amount of the gel filtrate was spread onto each bacteriological medium for the detection and enumeration of the bacteria in the mixture.

The remaining filtrate was passed through a 0.2 µm membrane filter (Nalgene) to remove the bacteria before plaque assay for the viruses. For the quantitation of PV, the membrane filtrate did not require any further manipulations. However, for HAV plaque assay,

the poliovirus in the mixture was first neutralized by incubating the mixture for one hour at 37°C, with an equal volume of diluted rabbit hyper-immune anti-poliovirus serum kindly provided by Dr. P. Payment of Institut Armand-Frappier, Montreal, Quebec. The undiluted serum had a 50% plaque reduction titer of over 1:30,000 and the final dilution used was pre-tested to neutralize nearly 10^6 PFU of PV.

Estimation of disinfectant pH. The pH of each disinfectant sample was determined using a Fisher Scientific (Model 220) pH meter.

Estimation of protein accumulation. Protein estimations in the disinfectant samples were made using a modification (Peterson, 1983) of the method of Lowry et al. (1951). The principal of the method is based on reduction of the phospho-molybdic-tungstic mixed acid chromagen (3 $H_2O.P_2O_5.13\ WO_3.5MoO_3.10\ H_2O$ and 3 $H_2O.P_2O_5.14\ WO_3.4MoO_3.10\ H_2O$) in the reagent of Folin and Ciocalteu (Peterson, 1983), the reduced form of which has an absorption maximum at 750 nm. The reducing agent in this case is the aromatic amino acid residues, tyrosine and tryptophan, and a slower reaction with copper chelates of the peptide chain or polar side chains, or both (Peterson, 1983) present in the sample.

The procedure was as follows: (a) the sample was diluted 100-fold in volumes of 1 mL using double distilled deionized water at room temperature, (b) 0.1 mL of 0.15% sodium deoxycholate (DOC; BDH) in deionized distilled water was added to the sample, mixed and allowed to stand at room temperature for 10 minutes, (c) 0.1 mL of 72% trichloroacetic acid (TCA; BDH) was added to this mixture and centrifuged at 3000 X *g* for 30 minutes, (d) the supernatant was decanted and the tubes left inverted over ordinary facial tissue paper for 1 hour before removing any other traces of supernatant with a Pasteur pipette, (e) 1 mL reagent A (prepared as in Appendix A2) was added to dissolve the precipitate allowing the reaction to proceed for 10 minutes at room temperature, (f) 0.5 mL of reagent B (prepared as in Appendix A3) was added and the reaction allowed to proceed for 30 minutes at room temperature (g) the absorbancy was read at 750 nm (visible light) using a Beckman Spectrophotometer Model DU-50 (Beckman Instruments, Inc., Fullerton, Calif), (h) controls of bovine serum albumin at 5, 10,

25, 50, 75 and 100 µg/mL were run in parallel and used to generate a standard curve. Final protein estimation was based on calculations according to the equation shown in Appendix A4 as well as readings obtained with the standard curve.

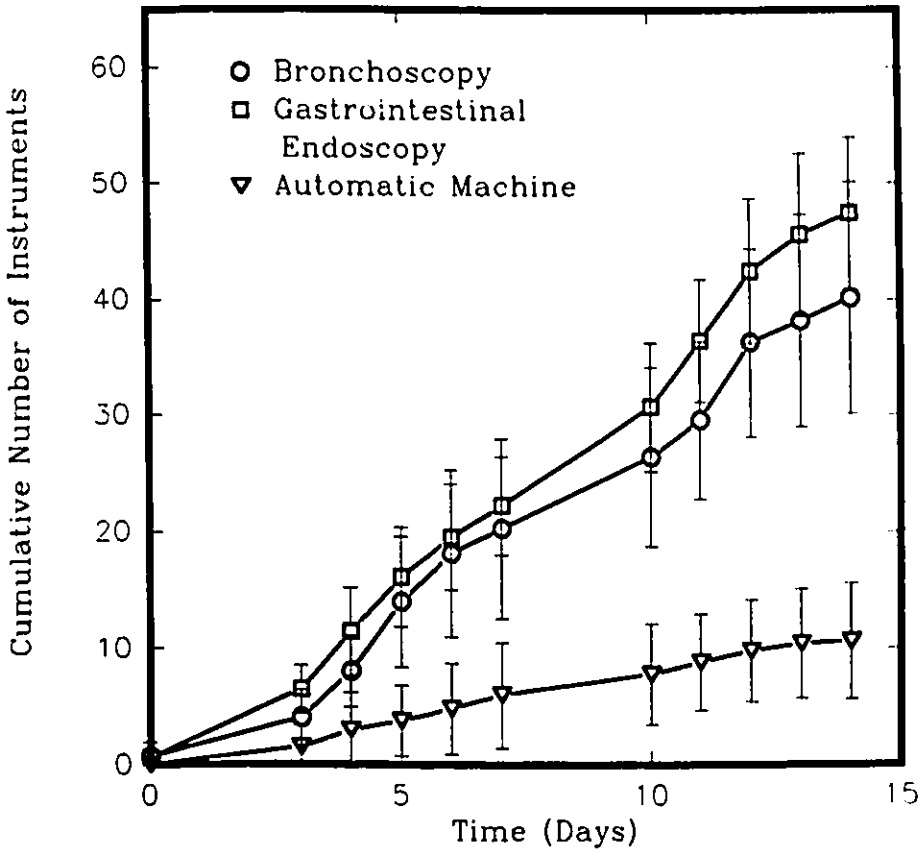
Estimation of disinfectant concentration. Whereas titremetric (Anderson, 1967) and spectrophotometric (Hadju and Hendriech, 1975; Power and Russell, 1988) methods are available for the estimation of alkaline glutaraldehyde under reuse conditions (Gormans and Russell, 1983), they all suffer from certain drawbacks. We chose to use the following method after extensive consultations with the manufacturer of glutaraldehyde-based products. It is based on the reaction of alkaline glutaraldehyde with hydroxylamine to produce hydrochloric acid (HCl) as one of the by-products (Appendix B_3) which is then titrated against 0.1 N NaOH.

(a) 5 mL of the disinfectant in 250 mL titration flask was diluted with 50 mL double distilled deionized water and the pH was then adjusted to 3, (b) 15 mL of 10% hydroxylamine hydrochloride (HNO_3.Cl) was added and the mixture stirred with a magnetic stirrer while titrating with NaOH, back to pH 3 (d) the glutaraldehyde concentration was calculated from the relationship: *% glutaraldehyde= V X N X 50 X 100/1000 X W. Where V= volume in mL of NaOH used; N = normality of NaOH; 50 = weight equivalent of glutaraldehyde; 1000 = conversion of mLs to litre; W = volume glutaraldehyde added.*

Cumulative loading of instruments. The Endoscopy Unit was requested to maintain an accurate record of the number of instruments processed daily through those reuse cycles of the baths that acted as the source of the disinfectant samples for this study. The cumulative number of instruments for the seven cycles for each one of the three types of baths is presented in Figure 12.

Rating of germicidal activity. A given sample of the disinfectant was considered as effective if it could reduce the infectivity titer of the test organism by at least 3 $\log_{10}$ under these test conditions. This arbitrary criterion of 3 $\log_{10}$ or greater reduction for the germicidal efficacy of chemical disinfectants is accepted by Canadian and U.S. regulatory agencies and is

Figure 12: Cumulative numbers of instruments in endoscope disinfection baths during a fourteen-day reuse period.



The number of instruments were recorded on a predesigned log sheet by a nurse for each bath separately.

included as a part of the standard virucidal test protocol of the Canadian General Standards Board (1991). When a sample met this criterion, it was given a rating of 100%. But if it failed, the percentage figure represents the actual loss it produced in the infectivity titer of the test organism. Since disinfectant samples from either seven (manual baths) or four (automatic bath) 14-day reuse cycles were tested, the percentage failure rate refers to the failed number of samples collected on a given day of the cycle out of the total.

RESULTS

pH levels. The mean pH values of the disinfectant control and the samples from the three types of baths are represented in Figure 13. The pH of the control sample was 8.48 at the outset and showed no significant change over the test period. At the beginning of the cycle, the pH of all the samples from the three baths was approximately 8.4. The samples from the automatic bath showed the most pronounced drop in their pH early in the reuse cycle and the reduction continued over the 14-day period; the pH value on the 14th day was 7.68±0.20. On the other hand, the pH of the samples from the bronchoscope bath remained fairly constant, with the 14th day value being 8.3±0.21. The samples from the GI bath showed a certain degree of fluctuation over the first 10 days of reuse, but remained essentially unaltered for the last 4 days, with the final, value being 8.16±0.15.

Glutaraldehyde concentration. As can be seen from Figure 14, the glutaraldehyde concentration in the control sample went from 2.25% at the beginning to a low of 1.8% on day 14. The mean of the initial concentration of the disinfectant in the samples from the three baths was between 2.23±0.12% to 2.27±0.11% with a range of 2.06% to 2.37%. Irrespective of the type of bath, there was a gradual decline in glutaraldehyde concentration over the period of the reuse and at the end of the 14th day, the levels recorded were 1.15±0.16%, 0.99±0.11% and 1.1±0.07% for the manual bronchoscope, the manual gastrointestinal endoscope and the automatic machine baths, respectively (Figure 14).

Protein concentration. Whereas no protein could be detected in any of the samples from the disinfectant control, the levels of protein gradually increased over the reuse period in

Figure 13: Alkaline glutaraldehyde pH levels in endoscope disinfection baths during a fourteen-day reuse period.

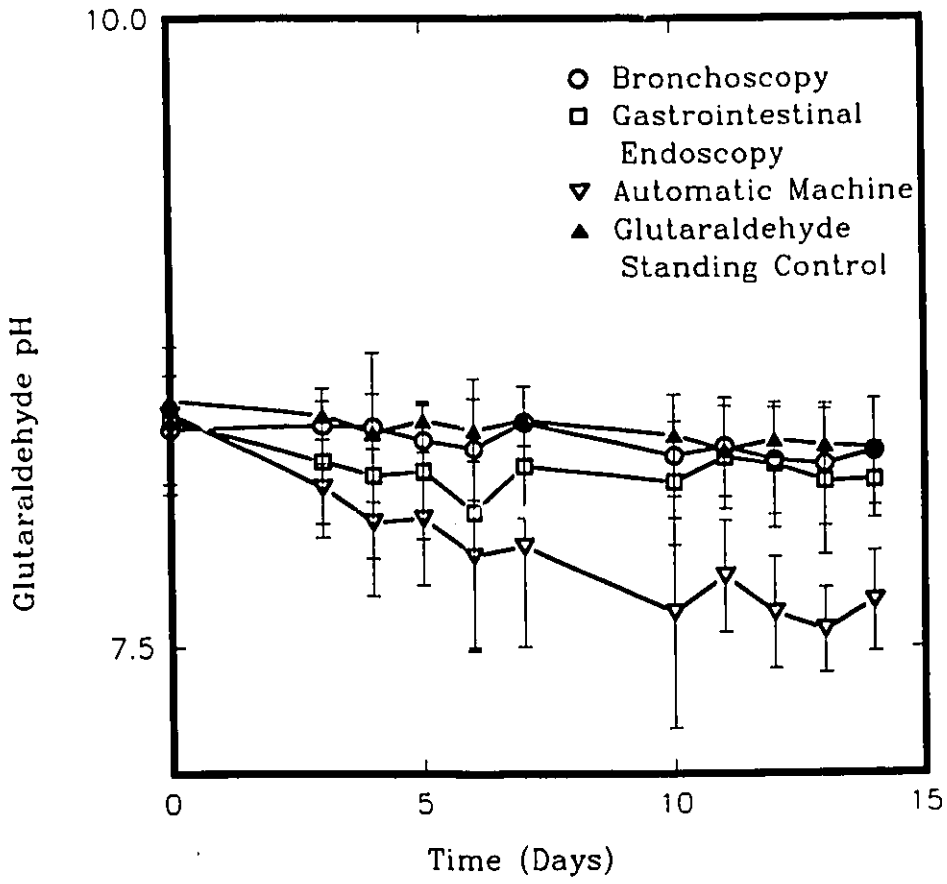
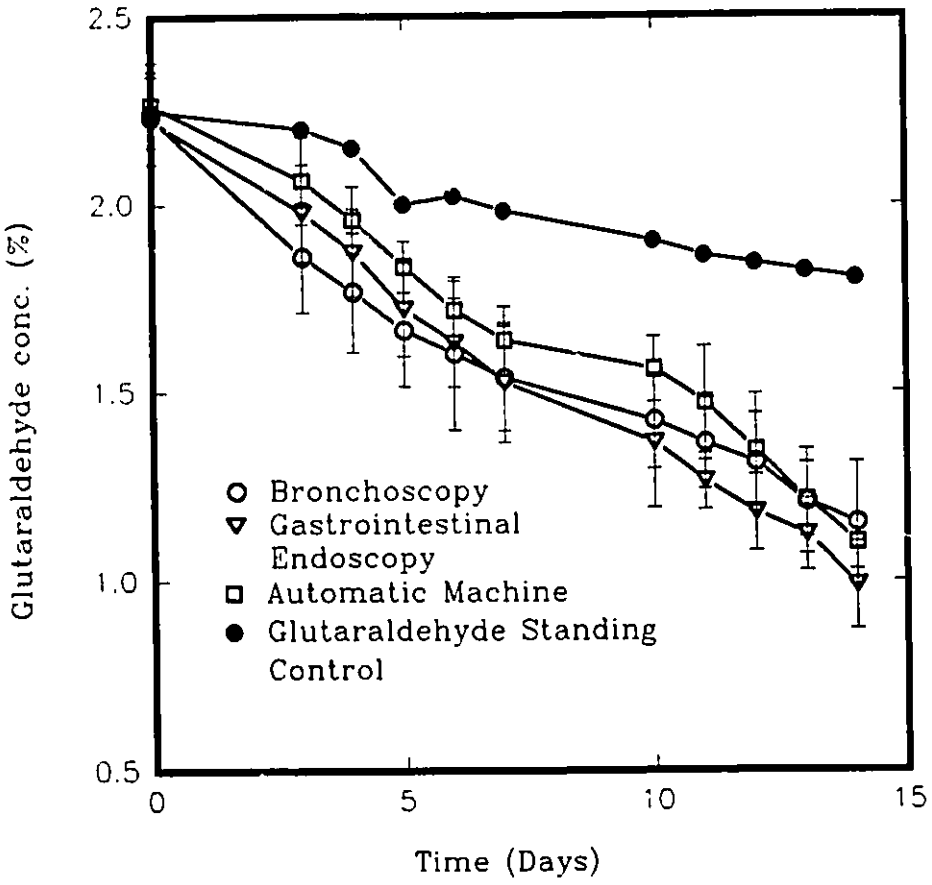


Figure 14: Alkaline glutaraldehyde concentration (%) in endoscope disinfection baths during a fourteen-days reuse period.



Vertical error bars represent standard deviations from the mean of seven observations.

the samples from the three baths (Figure 15). This increase was somewhat slow in the first five days with higher rate of accumulation during the remaining period of reuse. At the end of the 14th day the values for protein concentration in µg/mL were 1074.20±110.62, 1067.6±139.08 and 663.36±60.042 for the bronchoscope, gastrointestinal endoscope and the automatic baths, respectively (Figure 15).

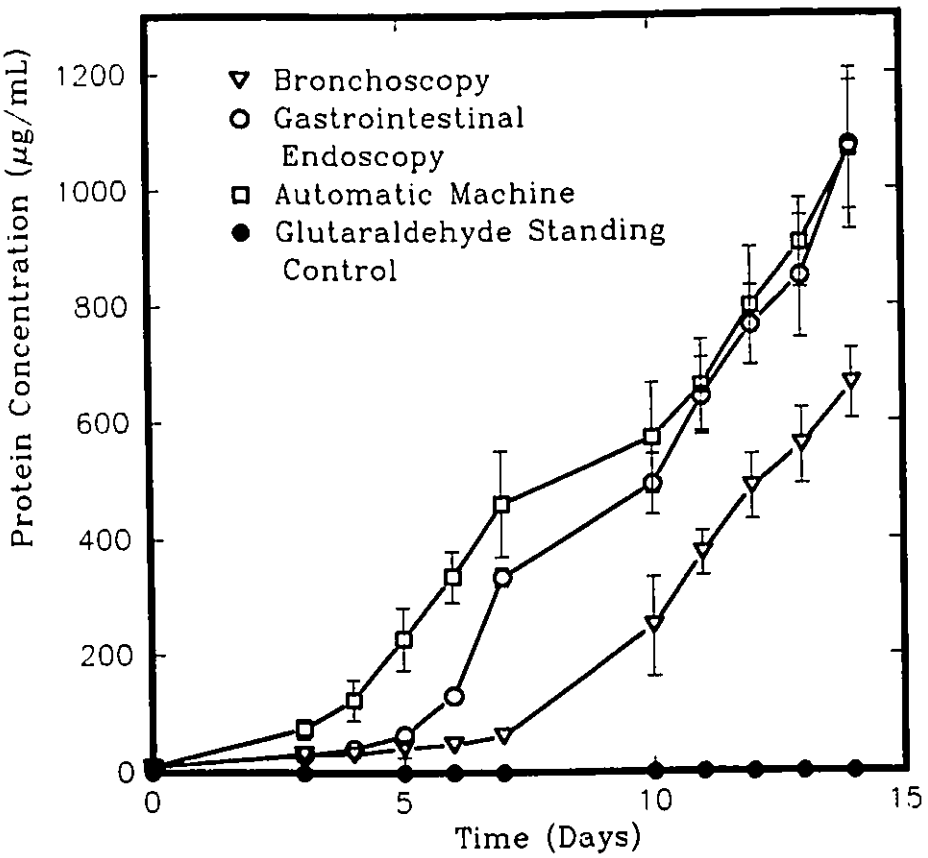
Germicidal activity. The samples from the control bath remained effective against all the test organisms even after 14 days. Those from the three test baths were able to reduce the infectivity titer of the five types of microorganisms to undetectable levels from day zero to day 6 of reuse. However, there were considerable variations in the disinfectant's broad-spectrum germicidal activity after this period of reuse. It is also worth noting here that once samples in a particular cycle began to fail against a test organism, the activity never showed an improvement over the rest of the cycle.

(a) Manual bronchoscope disinfection bath. All the disinfectant samples from this bath were able to inactivate *P. aeruginosa* even after 14 days of reuse. They also remained effective against *M. bovis* till the end of day 13 with one marginal failure on day 14 (Table 10). Failure of activity against *M. gordonae* first appeared on day 11 and became progressively worse over the remaining period of reuse; at the end of day 14, four of the seven (57.14%) samples did not meet the efficacy requirements (Table 10). The samples remained effective against HAV and PV till the end of day 10 and 11, respectively (Table 11). After this period the failure rate against both the viruses remained at 14.29%.

(b) Manual gastrointestinal endoscope disinfection bath. All the samples from this bath were also able to inactivate *P. aeruginosa*. The samples began to fail against *M. gordonae* starting at the end of day 7 (Table 12), with the final failure rate of 57.29 % (4/7). For *M. bovis*, only two of the samples showed a marginal failure at the end of day 14. The first failures against HAV and PV were recorded at the end of days 11 and 12, respectively (Table 13).

(c) Automatic bath. Samples from only 4 seven-day cycles could be collected from this

Figure 15: Protein accumulation in disinfection baths during a fourteen-day reuse period.



Vertical error bars represent standard deviations from the mean of seven observations

Table 10: Activity of alkaline glutaraldehyde, taken from a bronchoscope manual bath, against *M. gordonae* (M G) and *M. bovis* (M B).

	Day of testing													
	% Reduction in CFU													
	6		7		10		11		12		13		14	
Expt. no.	M G	M B	M G	M B	M G	M B	M G	M B	M G	M B	M G	M B	M G	M B
1	100	100	100	100	100	100	100	100	100	100	99.77	100	99.62	100
2	100	100	100	100	100	100	100	100	100	100	100	100	100	100
3	100	100	100	100	100	100	98.21	100	97.50	100	95.71	100	96.50	99.01
4	100	100	100	100	100	100	100	100	100	100	100	100	100	100
5	100	100	100	100	100	100	100	100	100	100	100	100	97.88	100
6	100	100	100	100	100	100	100	100	100	100	100	100	100	100
7	100	100	100	100	100	100	100	100	99.65	100	99.35	100	98.11	100
Failure rate (%)	0	0	0	0	0	0	14.29	0	28.58	0	42.86	0	57.14	14.29

Twenty µL mixed organisms or *M. bovis* were dried for 90 min in glass cups before addition of 60 µL the disinfectant for a contact time of 10 min. The disinfectant was then removed by gel filtration and further neutralized with 2% glycine in the eluent. The microorganisms were then eluted and titrated.

Table 11: Activity of alkaline glutaraldehyde, taken from a bronchoscope manual bath, against hepatitis A virus (HAV) and poliovirus (PV) type 1 (Sabin).

	Day of testing													
	% Reduction in PFU													
	6		7		10		11		12		13		14	
Expt. no.	HAV	PV	HAV	PV	HAV	PV	HAV	PV	HAV	PV	HAV	PV	HAV	PV
1	100	100	100	100	100	100	100	100	100	100	100	100	100	100
2	100	100	100	100	100	100	100	100	100	100	100	100	100	99.16
3	100	100	100	100	100	100	98.72	100	98.52	99.09	98.22	98.89	95.28	98.93
4	100	100	100	100	100	100	100	100	100	100	100	100	100	100
5	100	100	100	100	100	100	100	100	100	100	100	100	100	99.52
6	100	100	100	100	100	100	100	100	100	100	100	100	100	100
7	100	100	100	100	100	100	100	100	100	100	100	100	99.98	100
Failure rate (%)	0	0	0	0	0	0	14.29	0	14.29	14.29	14.29	14.29	14.29	14.29

Twenty µL mixed organisms were dried for 90 minutes in glass cups before addition of 60 µL the disinfectant and a contact time of 10 minutes. The disinfectant was then removed by gel filtration and further neutralized with 2% glycine in the eluent. The viruses were eluted and plaque assayed.

Table 12: Activity of alkaline glutaraldehyde, taken from a manual gastrointestinal endoscope bath, against *M. gordonae* (M G) and *M. bovis* (M B).

	Day of testing													
	% Reduction in CFU													
	6		7		10		11		12		13		14	
Expt. no.	M G	M B	M G	M B	M G	M B	M G	M B	M G	M B	M G	M B	M G	M B
1	100	100	100	100	100	100	99.60	100	98.80	100	98.10	100	96.64	100
2	100	100	99.82	100	99.30	100	99.10	100	97.30	100	96.80	100	96.20	98.96
3	100	100	100	100	100	100	100	100	100	100	100	100	100	100
4	100	100	100	100	100	100	100	100	100	100	100	100	100	100
5	100	100	100	100	100	100	99.40	100	98.80	100	97.90	100	96.60	100
6	100	100	100	100	100	100	100	100	100	100	100	100	100	100
7	100	100	99.81	100	99.70	100	99.10	100	98.20	100	96.70	100	96.14	98.69
Failure rate (%)	0	0	28.57	0	28.57	0	57.14	0	57.14	0	57.14	0	57.14	28.57

Twenty µL mixed organisms or *M. bovis* were dried for 90 minutes in glass cups before addition of 60 µL the disinfectant for a contact time of 10 minutes. The disinfectant was removed by gel filtration and further neutralized with 2% glycine in the eluent. The microorganisms were eluted and titrated.

Table 13: Activity of alkaline glutaraldehyde, taken from a manual gastrointestinal endoscope bath, against hepatitis A virus (HAV) and poliovirus (PV).

	Day of testing													
	% Reduction in PFU													
	6		7		10		11		12		13		14	
Expt. No.	HAV	PV	HAV	PV	HAV	PV	HAV	PV	HAV	PV	HAV	PV	HAV	PV
1	100	100	100	100	100	100	100	100	100	100	100	100	100	100
2	100	100	100	100	100	100	100	100	100	100	100	100	99.55	100
3	100	100	100	100	100	100	100	100	100	100	100	100	100	100
4	100	100	100	100	100	100	99.81	100	98.70	99.71	97.90	99.38	97.72	98.28
5	100	100	100	100	100	100	100	100	100	100	100	100	100	100
6	100	100	100	100	100	100	100	100	100	100	100	100	100	100
7	100	100	100	100	100	100	100	100	100	100	98.84	100	99.55	99.84
Failure rate (%)	0	0	0	0	0	0	14.29	0	14.29	14.29	28.58	14.29	42.29	28.58

Twenty µL mixed organisms were dried for 90 minutes in glass cups before addition of 60 µL the disinfectant for a contact time of 10 minutes. The disinfectant was then removed by gel filtration and further neutralized with 2% glycine in the eluent. The viruses were then eluted and plaque assayed.

bath. As was the case with the samples from the other two baths, all of the ones from this bath remained effective against *P. aeruginosa*. The first two failures against *M. gordonae* were noted at the end of day 10 (Table 14) and samples from only one of the four cycles remained effective against this bacterium at the end of day 14. Although the samples proved to be ineffective against *M. bovis* from the end of day 12, these failures were marginal. As can be seen from the results of the virucidal tests summarized in Table 15, the activity against PV remained undiminished till the end of day 12 whereas failures against HAV began to appear two days earlier.

A summary of the rate of alkaline glutaraldehyde failure with the respect of the microorganisms used and for each bath is shown on Table 16.

DISCUSSION

Although iodophors and alcohols are some times used to decontaminate semi-critical medical instruments between patients (Rutala et al., 1991; Gossum et al., 1989; Gorse et al., 1991), 2% alkaline glutaraldehyde is presently the most widely used disinfectant for this purpose (Babb and Bradley, 1991; Rutala et al., 1991). The activity of alkaline glutaraldehyde against microorganisms is well established (Tyler et al., 1990; Bailly et al., 1991) and the results given in Table 3 further substantiate this fact. There is, however, growing concern over the possible negative influence of disinfectant dilution, pH changes and protein accumulation in reuse baths on the germicidal activity of alkaline glutaraldehyde (Bailly et al., 1991; Cole et al., 1990; Gorman et al., 1980; Passagot et al., 1987). Furthermore, there are no officially recognized test protocols for pre-market evaluation of disinfectant products meant for reuse.

During the 14-day reuse period in this study, the concentration of the disinfectant was noted to fall steadily and in four instances the levels fell below the recommended 1%. The Sterilog Monitor failed to detect this drop in the disinfectant concentration. Power and Russel (1988) also found the Sterilog Monitor to be unreliable as an indicator of glutaraldehyde concentration.

At the end of the 14-day reuse period, the mean disinfectant level in the automatic bath

Table 14: Activity of alkaline glutaraldehyde, taken from an automatic bath, against *M. gordonae* (M G) and *M. bovis* (M B).

	Day of testing													
	% Reduction in CFU													
	6		7		10		11		12		13		14	
Expt. no.	M G	M B	M G	M B	M G	M B	M G	M B	M G	M B	M G	M B	M G	M B
1	100	100	100	100	100	100	100	100	100	100	100	100	100	100
2	100	100	100	100	99.76	100	99.63	100	99.43	100	98.98	99.10	97.38	99.70
3	100	100	100	100	99.79	100	99.59	100	99.55	99.88	97.48	99.42	97.64	99.27
4	100	100	100	100	100	100	100	100	99.81	100	98.80	100	98.11	100
Failure rate (%)	0	0	0	0	50	0	50	0	75	25	75	50	75	50

Twenty µL mixed organisms or *M. bovis* were dried for 90 minutes in glass cups before addition of 60 µL of the disinfectant and a contact time of 10 minutes. The disinfectant was then removed by gel filtration and further neutralized with 2% glycine in the eluent. The microorganisms were eluted and then titrated

Table 15: Activity of alkaline glutaraldehyde, taken from an automatic bath, against hepatitis A virus (HAV) and poliovirus (PV) type 1 (Sabin).

	Day of testing													
	% Reduction in PFU													
	6		7		10		11		12		13		14	
Expt. no.	HAV	PV	HAV	PV	HAV	PV	HAV	PV	HAV	PV	HAV	PV	HAV	PV
1	100	100	100	100	100	100	100	100	100	100	100	100	100	100
2	100	100	100	100	99.79	100	99.69	100	99.03	100	97.50	99.69	96.67	99.16
3	100	100	100	100	100	100	99.97	100	99.86	100	99.76	99.85	99.48	99.52
4	100	100	100	100	100	100	100	100	99.97	100	99.89	100	99.46	100
Failure rate (%)	0	0	0	0	25	0	50	0	75	0	75	50	75	50

Twenty µL mixed organisms were dried for 90 minutes in glass cups before addition of 60 µL the disinfectant for a contact time of 10 minutes. The disinfectant was then removed by gel filtration and further neutralized with 2% glycine in the eluent. Viruses were then eluted and plaque assayed.

Table 16 : Day of reuse at which samples of alkaline glutaraldehyde showed the first failure.

Test Organisms	Bronchoschope manual bath	Gastrointestinal endoscope manual bath	Automatic bath
M. gordonae	11	7	10
HAV	11	11	10
PV	12	12	13
*M. bovis**	14	14	12
P. aeruginosa	>14	>14	>14

* This organism was not tested as a part of the mixture.

was 1.1±0.07%. Others have shown the levels of the chemical to fall below 1% (Whyman et al., 1991) to as low as 0.27% (Leong et al., 1991) on the 4th day of reuse. The discrepancy between our results and those of the other groups may be due to the number of instruments that were washed and disinfected in the automatic bath; in our study, 4-16 instruments were put through the bath in a 14-day cycle whereas the number of instruments recorded for the same period was 60 and 52-60 by Whyman et al. (1991) and Leong et al. (1991), respectively. A mean alkaline glutaraldehyde concentration of just over 1.0% after the 4-16 machine cycles observed in this study emphasizes the fact that automatic baths severely dilute the product and the level of the disinfectant in these machines should be closely monitored with reliable laboratory tests.

In the manual baths, the relationship between the number of instruments disinfected and the dilution of the disinfectant was not as dramatic. After 30-54 instruments and on day 14, the level of AG in the bronchoscope bath was 1.15±0.16% whereas that in the gastrointestinal endoscope bath was 0.99±0.11%, with 41-59 instruments. Bageant et al (1981) found as much as 250 to 300 mL of rinse water trapped in automatic baths and this may contribute to disinfectant dilution. It has been suggested that the rubber and tubings in the automatic bath may also absorb AG (Power and Russell, 1989). A drop in disinfectant levels occurs, perhaps due to evaporation as well as polymerization, with the age of the solution and this is clearly indicated by the drop in the concentration of disinfectant in the standing control (Figure 14).

AG shows better germicidal activity around pH 8.0 (Bailly et al., 1988; Power and Russell, 1988). The drop in the pH of the disinfectant solution was most prominent in the automatic bath (Figure 13) and this may again be related to the higher dilutions of AG with the rinse water.

There was a substantial increase with reuse in the levels of protein in all the three baths (Figure 15). Higher levels of protein accumulation (0.02-0.1%) have been reported in the only other study to estimate protein accumulation during disinfectant reuse (Rehork and Azzaui, 1991). AG is considered to be more resistant to interference by organic matter than most other

disinfectants (Gelinas and Goulet, 1983; Robinson et al 1988) even though the germicidal action of AG may be due to its reaction with protein components of microorganisms (Gorman et al., 1980). As can be seen from Figures 14 and 15, there was a certain degree of inverse relationship between protein accumulation and the AG concentration.

All the five microorganisms examined were sensitive to the disinfectant prior to its reuse. The samples from the control bath also remained effective against all of them over the 14-day period of disinfectant aging. Failure of broad-spectrum germicidal activity of the product was first recorded at the end of day 7 when it was unable to reduce the titer of *M. gordonae* to the required level (Table 13). The next earliest set of failures was against HAV and these occurred at the end of day 10 (Table 16) and day 11 (Table 14). Therefore, a preliminary rating, in decreasing order of resistance to reused alkaline glutaraldehyde, of the four organisms in the mixture would be: *M. gordonae*, HAV, PV and *P. aeruginosa*. Even though *M. bovis* proved to be somewhat less resistance than *M. gordonae*, any direct comparison between them is not justified at this stage because *M. bovis* was not included in the mixture.

Protein accumulation and the number of instruments disinfected in the manual baths had increased significantly by day seven (Figure12 and 15) while the level of AG and pH had fallen markedly (Figure 13 and 14). At the same time the germicidal activity of AG commenced to show variable failure rates against the tested organisms (Table 11-16). Hence, there appears to be a correlation between these factors and germicidal activity. We are not aware of any other published reports where such a relationship has been demonstrated. Robinson et al. (1988) examined germicidal activity of glutaraldehyde-based products in dental clinics and found that both acid and alkaline solutions of glutaraldehyde retained their germicidal activity against *Salmonella choleraesuis, Staphylococcus aureus* and *P. aeruginosa* over 24 days of reuse; they used a suspension test to assess germicidal activity and the three species tested are all relatively sensitive vegetative bacteria. Our study used a more realistic carrier test and microorganisms of varying degrees of resistance to chemical disinfectants.

The studies by Isenberg et al. (1988) and Bageant et al. (1981) attempted to assess the effect of instrument- and organic-load on AG under simulated clinical use. Germicidal activity of AG against *M. bovis, M. smegmatis, Staph. aureus, P. aeruginosa* and *Candida albicans* remained over the entire reuse period of 14 days; the instruments used by them were not subjected to patient use prior to soaking in the AG bath. Our study, on the other hand, determined the germicidal activity of disinfectant samples collected from baths in an actual clinical setting.

The findings reported here suggest 2% alkaline glutaraldehyde may become ineffective against non-enveloped viruses and mycobacteria in much less than 14 days in reuse baths meant for the disinfection of semi-critical medical instruments such as fiberoptic endoscopes. Inadequate decontamination of such instruments between patients increases the risk of disease transmission. Particular attention must be paid to improving the basic design and functioning of the automatic bath because, in its present form, it appears to be incompatible with the recommended use of the disinfectant. During the course of this study, the bath tended to break down frequently and the personnel in the Endoscopy Unit often avoided its use.

It has been suggested that infections associated with endoscopic procedures (Leger et ai., 1980; Allen et al., 1987; Alvarado et al., 1991; Fraser et al., 1992) may have been due to failure of the disinfection procedure. The risk of spread of infections through improperly disinfected endoscopes is on the rise because of the continuing increase in the number of such procedures and the number of cases of infections due to mycobacteria, HAV and the human immuno-deficiency virus (HIV). In view of this, better protocols are urgently needed to assess the broad-spectrum germicidal activity of disinfectants to be reused in the decontamination of semi-critical instruments.

M. gordonae and HAV were the most resistant organisms in this study. Their sensitivtity to alkaline glutaraldehyde reflected sensitivity of the other organisms. It is not, however, known whether the sensitivity of *M. bovis* would be altered if it was tested in a mixed culture as the others. The colonies of this organism are white as opposed to those of *M.*

gordonae which are dark yellow and can be distinguished on this basis. However, the concentrations of gentamicin used here to inhibit *P. aeruginosa* were also inhibitory to *M. bovis* and lower concentration were inadequate to stop the growth of the former.

A search of antibiotic combinations need to be done to lower the concentrations while retaining efficacy against *P. aeruginosa* for an all mixed culture reuse test protocol. Nevertheless, a mixture of only *M. gordonae* and the virus HAV may satisfy the criterion for reuse test for alkaline glutaraldehyde in this glass carrier test. In this study (Table 11-16), the results obtained with these 2 organisms were predictive of the sensitivity of a broad spectrum of bacteria and viruses and such a test can be further evaluated on an inter-laboratory basis to constitute a standard germicidal test procedure for products meant for reuse.

10. GENERAL DISCUSSION

10. GENERAL DISCUSSION

The common denominator in the work presented here was HAV, with particular emphasis on approaches to preventing and controlling the spread of hepatitis A in institutional settings. Although studies on the structure and molecular biology (Robertson et al., 1992) of the virus were initiated essentially when the viral etiology of hepatitis A was established, the first *in vitro* culture of HAV was reported just over a decade ago (Provost and Hilleman, 1979). Therefore, the routine use of cell culture-adapted strains of the virus in research is a relatively recent development. However, it is worth noting here that no systematic studies have been carried out with such strains to understand better the genesis of hepatitis A outbreaks in in-door settings and means for their prevention and control. Therefore, this work was designed to fill this gap in our knowledge. Studies in the future should determine if wild-type HAV behaves in the same way as the cell culture-adapted strain used in this investigation. Work with the wild-type HAV may require the use of more expensive and elaborate techniques such as immuno-fluorescence microscopy, immuno-peroxidase and radio-immunofocus assay for the determination of virus infectivity (Nadala et al., 1990; Lemon et al., 1983).

The use of a plaque assay system for HAV made this investigation fully quantitative and the incorporation of PV in many aspects of this study provided a 'control' for the experimental protocols and a reference point to understand the behavior of HAV. In many tests, a mixture of the two viruses was used to make the results more directly comparable. The use of a mixture of the two viruses along with *P. aeruginosa* and *M. gordonae* in testing the germicidal efficacy of alkaline glutaraldehyde from reuse baths was an approach not reported before. It again permitted the simultaneous and direct comparison of the activity of the disinfectant against these four representative microorganisms.

The findings of this experimental study reinforce the potential of environmental surfaces and human hands as vehicles for HAV. This has a great deal of relevance not only for institutional settings and food establishments but also for ordinary house-holds. The fact that fecally-suspended HAV can survive for many days on non-porous inanimate surfaces and for

several hours on human hands, in conjunction with the relative resistance of the virus to chemical disinfection, requires a review of our measures for preventing and controlling outbreaks of hepatitis A through environmental control and hygienic handwashing. Our data may also help explain the continued occurrence of such outbreaks, especially in health-care settings where disinfection and handwashing are an integral part of infection control.

The factors underlying the presence or absence of seasonality in viral infections, especially in institutional settings in regions with a temperate climate, remain elusive (Nathanson, 1990); this is particularly true of how changes in seasons influence the resistance of the host to pathogens. Therefore, it would be simplistic to suggest that any one property of a given viral pathogen alone may be sufficient to explain the observed seasonal patterns, or lack thereof, associated with its infections. However, it would be fair to assume that the longer a virus can retain its infectivity outside the host the higher is the probability of it being acquired by other susceptible hosts in the vicinity.

Polio-, coxsackie-, echo- and rhinoviral infections are most frequent during late summer and early fall in the temperate regions of the world (Moore, 1982). But, poliovirus infections in Hawaii for example, with its relatively constant climate, occurred throughout the year with essentially the same frequency (Enright, 1954). All the entero- and rhinoviruses tested have been found to survive well in air and on environmental surfaces only at RH levels higher than 75% (Buckland and Tyrrell, 1962; Hemmes et al., 1960). In temperate regions, institutional outbreaks of rotaviral gastroenteritis occur almost exclusively during the winter months (Ansari et al., 1991). Rotaviruses have been found to retain their infectivity in air and on non-porous environmental surfaces at RH levels below 50% (Sattar et al., 1986; Sattar and Ijaz, 1987). A number of other human pathogenic viruses have been found to survive better at RH levels prevalent during periods when outbreaks due to them are more frequent (Buckland and Tyrrell, 1962; Harper, 1961; Hemmes et al, 1960; Sattar and Ijaz, 1987).

In contrast to the seasonality associated with viral infections listed above, institutional outbreaks of hepatitis A in temperate regions generally occur sporadically throughout the year

(Hadler and McFarland, 1986; Goodman et al, 1982; Mishu et al, 1990). Whenever seasonality has been noted in such outbreaks, the peak was in late autumn or during the winter months (Varughese, 1984; Hollinger and Ticehurst, 1990; Melnick, 1990). The data reported here clearly show that the ability of HAV to survive on non-porous environmental surfaces was less subject to differences in the levels of RH, particularly at 20°C. A direct comparison with the behavior of PV could be made because in these experiments a mixture of the two viruses was used (Figure 2). This pattern of HAV survival was unexpected and, as far as we are aware, information of this type has not been available in the published literature. It is, therefore, tempting to suggest from our findings that the lack of clear-cut seasonality with hepatitis A outbreaks may be, at least in part, due to the ability of HAV to survive well under a variety of RH levels.

In this study, we did not attempt to test the ability of HAV to survive on porous materials such as fabrics. Our earlier studies with a human rotavirus showed that recovery of infectious virus from experimentally-contaminated porous materials was highly variable (Sattar et al., 1986) and this made it difficult to design properly quantitative experiments; the variabilty may be due to a combination of factors such as the presence of biocides in various porous materials and irreversible binding of the virus to its fibers. In our opinion, these factors may also reduce the potential of porous materials to act as vehicles for HAV in natural settings.

Stainless steel disks were used throughout this study as representative non-porous inanimate surfaces because experiments with other human pathogenic viruses have shown that their behavior was the same on metal, glass or plastic surfaces (Sattar et al., 1986). The recovery of HAV and PV from the metal disks even after the drying of the inoculum and treatment with various chemical disinfectants was highly efficient. This was demonstrated conclusively using radio-labeled viruses. It should also be noted here that the same metal disks were used repeatedly in this study to simulate more closely the wear that occurs during the use of such surfaces in the field. Scanning electron microscopy of these disks revealed the details of their surface topography (Springthorpe and Sattar, 1990) and it is not difficult to see how

contaminants could be readily sequestered in the gaps and fissures invisible to the naked eye.

This study has also shown for the first time the ability of HAV to survive on human hands. This virus was able to retain its viability on the fingerpads of all the voluneers better than a human rotavirus (Ansari et al., 1988) as well as rhinovirus type 14 and parainfluenzavirus type 3 (Ansari et al., 1991). Whereas the exact reason for the large drop in HAV infectivity during the first 20 minutes (Figure 6) remains unknown at this time, there are several possible explanations for this phenomenon. A sub-population of the virus particles may be more sensitive to the initial drying of the inoculum, even though the inoculum may not become completely dry due to the moisture on the skin. The innate resistance mechanisms of the skin may have inactivated a proportion of the virus. There is growing evidence that epidermal keratinocytes have the capacity to elaborate specific pro-inflammatory cytokines, adhesion molecules and chemotatic factors responsible for antigen-independent cutaneous inflammation (Barker et al, 1991). Keratinocytes constitute 95% of the cell mass of human epidermis and are responsible for the biochemical and physical intergrity of the skin (Barker et al, 1991).

The presence of antibodies in sweat (Page and Remmington, 1967) and in the upper epidermis (Kaneko et al, 1982) may also have neutralized the virus. However, sweat samples obtained from two of our volunteers did not show measurable anti-HAV activity (results not shown).

Further work is needed to properly define the factors that govern the survival of HAV and other viruses on human skin. It would also be very useful to determine if the presence of anti-viral humoral antibodies and/or an active cell-mediated immune response influence virus survival on the skin. If the immune status is found to reduce virus survival on skin, this will be an indirect benefit of active vaccination. The vaccine, apart from the protecting the individual, could also reduce the potential of their hands as virus vehicles.

The ability of HAV to be transferred between contaminated and clean surfaces has important implications for infection control because it underscores the significance of regularly

decontaminating environmental surfaces in conjunction with proper and regular washing of hands. The types of surfaces and objects to be decontaminated would vary from setting to setting. Efforts are also needed to increase the awareness of this issue in care-givers and food-handlers in particular.

The relatively high resistance of HAV to a variety of environmental surface disinfectants and hygienic handwash agents raises the very practical issue of selecting the products to be used, especially when such a selection must take into consideration cost, toxicity, ease of application, storage and environmental safety, etc. None of the available commercial formulations may be considered entirely satisfactory in meeting all these requirements. This points to the need for developing more innovative formulations. At the same time, the availability of ineffective products on the market suggests that better test protocols are required to assess their germicidal efficacy before marketing. This is especially true of hygienic handwash agents because, at the present time, there are no requirements for pre-market testing of these products nor are there any proper and standardized protocols for conducting such evaluations.

Alkaline glutaraldehyde as a 2-2.5% solution is the most commonly used chemical for the disinfection of semi-critical instruments such as FFE (Axon, 1991). However, it is under attack these days because of the increasing concerns for its toxicity for humans (Macnab, 1991; Axon, 1991; Babb and Bradley, 1991). There are also reports which suggest that manufacturers' claims for the broad-spectrum germicidal efficacy of the product during the recommended period of reuse may be exaggerated (Whyman et al., 1991; Kaczmarek et al., 1992). The findings of our study further reinforce this concern.

The experimental design used by us represents a unique combination of field and laboratory-based components. The disinfectant samples were collected from the reuse baths in an actual endoscopy unit of a general hospital and the broad-spectrum germicidal activity of the collected samples was assessed using a modification of the standardized carrier test protocol (Canadian General Standards Board, 1991). Our protocol, using a mixture of test

microorganisms dried on a glass carrier, presented the disinfectant with a much more realistic challenge than that in previous studies (Bailly et al., 1991; Bageant et al., 1991; Cole et al., 1990; Robinson et al., 1988).

Although hepatitis A has a relatively low case fatality rate (Lemon, 1985; Ross et al., 1991), it is a debilitating disease and management of its cases produces a significant economic burden (Sherlock, 1989). In fact, its impact on human health appears to be increasing (CDC, 1992), particularly in association with drug addicts and homosexuals (CDC, 1992; Kani et al., 1991; Ng et al., 1989). Employment of such individuals in health-care professions and in food establishments increases the risk for community-wide outbreaks (Christenson et al., 1982). It is hoped that the findings of this study will be helpful in developing a better understanding of the vehicles of hepatitis A transmission and in instituting more appropriate measures for its prevention and control.

11. CONCLUDING REMARKS

11. CONCLUDING REMARKS

The experimental work for this study was designed to address the six main objectives listed on page 25. The results outlined and discussed show that all of these objectives have been successfully addressed and the following conclusions can be drawn based on the findings of this study:

(1) HAV suspended in feces can retain its infectivity for several days on non-porous inanimate surfaces and its capacity to survive was much better than that of poliovirus type 1 (Sabin). This observation reinforces the view that objects and environmental surfaces could play a role in the spread of hepatitis A. Under our experimental conditions, changes in levels of RH did not affect HAV survival on the metal disks as dramatically as that of the poliovirus. As was expected from previously available data, poliovirus survival was favored at 95±5% RH. HAV survival (22±2°C), on the other hand, was better at the lower levels of RH. This finding, reported for the first time in this study, was quite unexpected. This observation may be helpful in understanding the differences in the seasonality of hepatitis A and infections caused by entero- and rhinoviruses.

(2) Tests using 5 volunteers have established that HAV could survive on their hands for at least 5 hours. This is again the first time the viability of HAV on human skin was tested. The result clearly point to the potential of hands as vehicles for hepatitis A.

(3) Fecally-suspended HAV could be transferred readily from contaminated to clean surfaces upon contact. The amount of virus transferred increased when a higher level (1.0 kg/cm^2) of pressure was applied; application of friction during contact further enhanced virus transfer. As far as we are aware, these findings are also a 'first' for HAV. They also emphasize the need to consider hands and environmental surfaces together in any attempts to prevent and control the spread of hepatitis A.

(4) Our study using a carrier test protocol and fecally-suspended HAV represents the first comprehensive assessment of the resistance of this virus to chemical disinfectants meant for use on environmental surfaces. Only three of the 23 chemicals and formulations

tested were able to reduce the virus titer by >3 $\log_{10}$. This points to a need to review the present methods to assess the pre-market evaluation of disinfectant products and emphasizes the care required in the selection of disinfectants for use, particularly in day-care and health-care settings and food establishments.

(5) None of the hand-washing agents tested was able to reduce the HAV titer on hands by > 2 $\log_{10}$ either in the finger-pad protocol or the whole-hand test. Whereas it is not possible to comment on the implications of these results on the role of hand-washing in preventing and controlling the spread of hepatitis A, this study provides a basis for the development of an *in vivo* method to determine the relative efficacy of hand-washing itself as well as hygienic hand-wash agents in the elimination of HAV.

(6) The alkaline glutaraldehyde reuse study was conducted with samples collected from three different types of disinfectant baths in an endoscopy unit. The number of instruments processed through each bath was carefully recorded, and the disinfectant samples were analyzed for glutaraldehyde concentration, pH and protein content; their germicidal activity was determined in a carrier test using a mixture of four types of microorganisms as well as *M. bovis*. As far as we are aware, such an approach has not been reported in the published literature. The results show that the broad-spectrum germicidal efficacy of the product is compromised well before the 14-day reuse period recommended by the manufacturer. This situation must, therefore, be reassessed to reduce the possibility of endoscope-related spread of hepatitis A and other infections.

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APPENDICES

APPENDIX A_1
Radioimmunoassay Test Results for hepatitis A virus Antigen

Sample	Counts per minute	
	Trial 1	Trial 2
Clarified FRhk-4 Cell Medium From Control Cells	346	359
Clarified FRhk-4 Medium From Cells Infected with HM-175	1781	1659
Concentrated HM-175 Virsu Sample (10X)	9560	5588
Clarified FRhk-4 Medium From Cells Infected with Metcalf HAV Strain	1253	833
Concentrated Metcalf HAV Strain (10X)	4668	Not done
Clarified FRhk-4 Medium From Cells Infected with Rotavirus	Not done	358

These tests were conducted in the laboratory of Dr. R. K. Chaudhary at the Laboratory Center for Disease Control, Ottawa, Ontario.

APPENDIX B_1
Protein Estimation in Reused Samples of Alkaline Glutaraldehyde

Since protein concentration and absorbance depict a non-linear relationship, the amount of protein in reused alkaline glutaraldehyde samples was calculated according to Peterson (1983; Methods in Enzymology 91 : 95-119) and from the relationship:

$$A = P / (aP + b)$$

OR

$$A^{-1} = a + bP^{-1}$$

OR

$$A = aP^{b}$$

OR

$$P = (A / a)^{1/b}$$

Where A = Absorbance

P = Protein concentration

a and b are constants that can be given by the equation : $a = A^{-1} - bP^{-1}$ and

$b = (A_l^{-1} - A_h^{-1}) / (P_l^{-1} - P_h^{-1})$

Where $^{(-1)}$ is a reciprocal

A^{-1} and P^{-1} are the means of the reciprocal of the lowest and highest absorbance and Protein concentration of the standard. Subscript l and h indicate lowest and highest readings.

Hence the equation for the unknown is : $P = bA\ (1-aA)^{-1}$

These calculations can also be arrived at by plotting Log-Log plot of Absorbance and Protein concentration and hence : $1/b = \text{Log}\ (P_h / P_l) / \text{Log}\ (A_h / A_l)$

$1/a = \text{antilog}\ (b\ \text{Log}\ P_h - \text{Log}\ A_h)$ and where $1/a$ and $1/b$ are the intercept and the slope of the line

APPENDIX B_2

Reagents Used in Folin phenol Protein Quantitation Method

Based on the method of Lowry et al (1951) and the modification of Peterson (1983).

REAGENTS I : is a mixture of the following:

(i) 0.1% (W/V) copper sulphate ($CuSO_4.5H_2O$) in distilled water

(ii) 0.2% (W/V) sodium or potassium tartrate (both can be used in the same reagent) in distilled water

(i) and (ii) should be dissolved in the same reactor and in ca. half the final volume

(iii) 10% (W/V) Sodium carbonate (Na_2CO_3) in distilled water also in half the final volume

To the mixture of (i) and (ii) was added (iii) with stirring to give reagent (I). This reagent keeps indefinitely at 10°C.

REAGENT A : was obtained from mixing :

Two parts of 5% (W/V) sodium dodecyl sulphate (SDS; Sigma) in distilled water

One part of 0.8 M sodium hydroxide (BDH) in distilled water

One part of reagent (I) above

Reagent A keeps for 2-3 weeks at room temperature but can be stored at 4°C for long periods. SDS precipitates in low temperatures but redissolves on warming up to room temperature and the the solution should be discarded after formation of A dark precipitate.

REAGENT B : was prepared as follows :

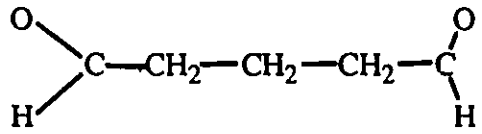
One part of 2 N Folin-Ciocalteu phenol reagent (Sigma)

Five parts of distilled deionized water

Reagent B is stable for months when stored in amber bottles at room temperature.

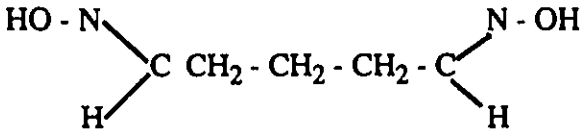
APPENDIX B_3

Assessment of Alkaline Glutaraldehyde Concentration (%)



+

$NH_2OH.HCl$



+

$2\ HCl\ +\ 2H_2O$

The resultant hydrochloric acid (HCl) was titrated against 0.1 Normal sodium hydroxide (NaOH)

Titrations of HCl (by product of the above reaction) have been claimed before (Anderson, 1967) although they lack universal acceptance and the literature is not in agreement as to their standard application (Gormans and Russell, 1983; Hadju and Hendriech, 1975; Power and Russell, 1988). However this type of titration is used by Johnson and Johnson Medical Inc. (Arlington, TX), the producers of cidex (2% alkaline glutaraldehyde) to evaluate the product concentration. The technique has not been used to evaluate cidex under reuse conditions before and this is the first time to do so.

APPENDIX B_4

A Diagram of Gastrointestinal Endoscope

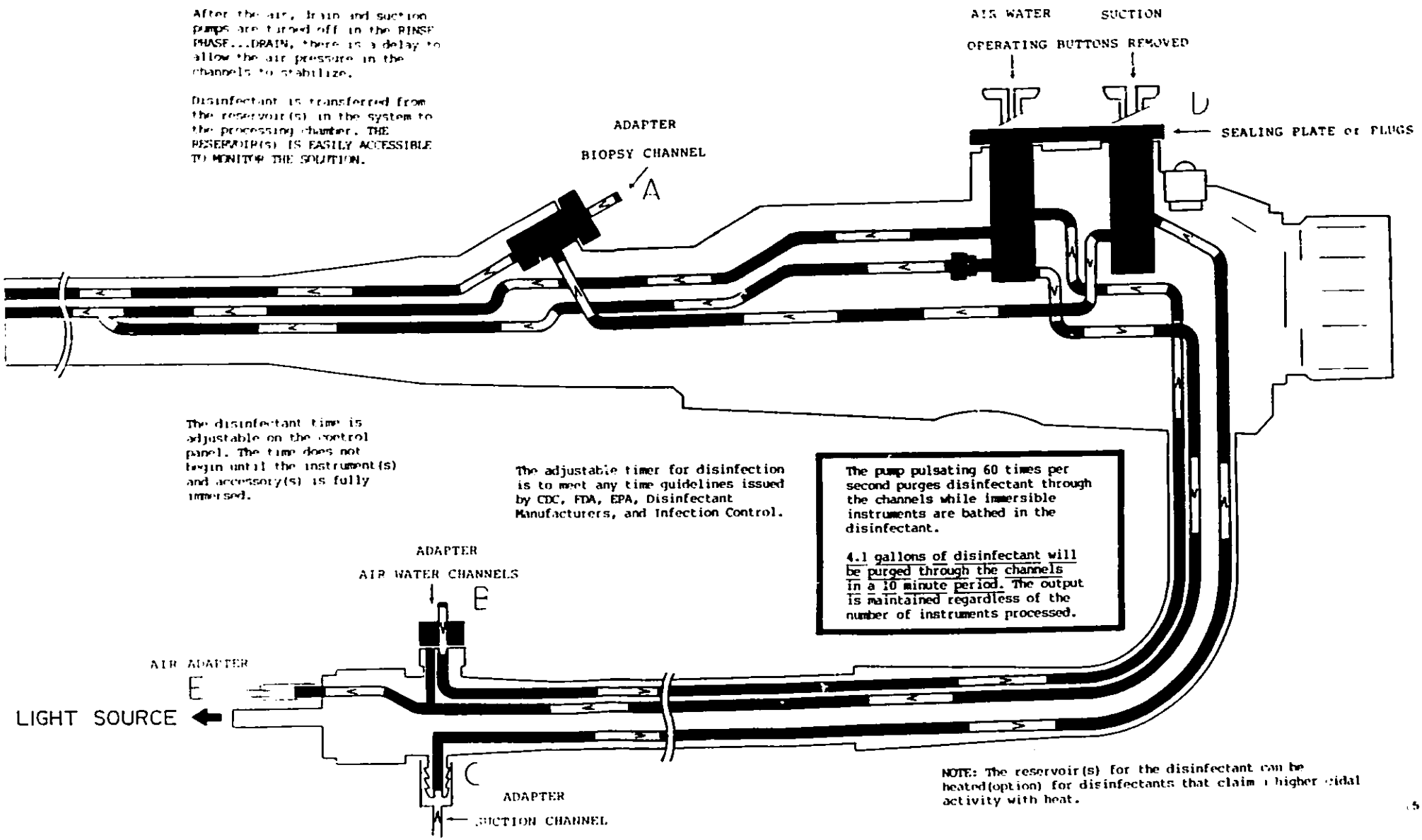
Shown on this diagram is the complex structure of an endoscope that present a special challange to disinfection.

GASTRO SCOPE

DISINFECTANT PHASE

After the air, brain and suction pumps are turned off in the RINSE PHASE...DRAIN, there is a delay to allow the air pressure in the channels to stabilize.

Disinfectant is transferred from the reservoir(s) in the system to the processing chamber. THE RESERVOIR(S) IS EASILY ACCESSIBLE TO MONITOR THE SOLUTION.



The disinfectant time is adjustable on the control panel. The time does not begin until the instrument(s) and accessory(s) is fully immersed.

The adjustable timer for disinfection is to meet any time guidelines issued by CDC, FDA, EPA, Disinfectant Manufacturers, and Infection Control.

The pump pulsating 60 times per second purges disinfectant through the channels while immersible instruments are bathed in the disinfectant.

4.1 gallons of disinfectant will be purged through the channels in a 10 minute period. The output is maintained regardless of the number of instruments processed.

NOTE: The reservoir(s) for the disinfectant can be heated(option) for disinfectants that claim higher cidal activity with heat.