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Effect of Relative Humidity and Air Temperature on Survival of Hepatitis A Virus on Environmental Surfaces

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Stainless steel disks (diameter, 1 cm) were contaminated with fecally suspended hepatitis A virus (HAV; strain HM-175) and held at low (25% ± 5%), medium (55% ± 5%), high (80% ± 5%), or ultrahigh (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 h at the ultrahigh RH and 35°C. In parallel tests with fecally suspended Sabin poliovirus (PV) type 1 at the low and ultrahigh RH, all PV activity was lost within 4 h at the low RH whereas at the ultrahigh RH it remained detectable up to 12 h. HAV could therefore survive much better than PV on nonporous environmental surfaces. Moreover, the ability of HAV to survive better at low levels of RH is in direct contrast to the behavior of other enteroviruses. These findings should help in understanding the genesis of HAV outbreaks more clearly and in designing better measures for their control and prevention.

In regions with a temperate climate, infections due to polio-, coxsackie-, and echoviruses generally reach a peak in summer and early fall (25). This suggests that relative humidity (RH) and air temperature may be important in the seasonality of enteroviral outbreaks (9). Whereas laboratory studies have shown that these viruses survive better in air (15) and on environmental surfaces (4, 30) when RH is higher than 80%, the role of air temperature in the seasonality of enteroviral infections is unclear.

Although hepatitis A virus (HAV) is presently classified as enterovirus type 72 (24), cases of hepatitis A in most places do not show any clear seasonal patterns (8); where seasonality is observed, the peaks are generally recorded in winter (16, 43). The virus is excreted in the feces of infected individuals, and consumption of fecally contaminated food or water is known to result in common-source outbreaks of the disease (8). The available epidemiological evidence suggests that except in rare cases (1), air does not play a significant role in the transmission of HAV (16).

Apart from the cases that occur in the general community, hepatitis A outbreaks are now a recognized problem in day-care centers (13), hospitals (11, 17), schools (26), institutions for the mentally handicapped (18, 21), and animal care facilities (38). In many of these outbreaks, the vehicles of virus spread remain obscure (12). Whereas hands may play an important role in virus transmission, fecally contaminated environmental surfaces (20) and fomites (7) have been incriminated in certain cases. The virus has also been found to survive well in dried feces (23) and on polystyrene surfaces held at 5 and 25°C (40). There is, however, no information on how well HAV survives on environmental surfaces and fomites in relation to a changing RH level. The present study was therefore designed to address this issue and to compare the persistence of HAV with that of poliovirus type 1 (Sabin), a prototypical enterovirus.

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MATERIALS AND METHODS

Cells. A seed culture of FRhK-4 cells was kindly provided to us by M. D. Sobsey (University of North Carolina, Chapel Hill). The cells were cultivated and maintained as described by Sobsey et al. (39). Briefly, Eagle minimum essential medium (GIBCO, Grand Island, N.Y.) with 10% fetal bovine serum (GIBCO), 2 mM glutamine (GIBCO), 0.1 mM nonessential amino acids (GIBCO), 50 μg of gentamicin sulfate (Cidomycin; Roussel, Montreal, Quebec, Canada) per ml, 100 μg of kanamycin (GIBCO) per ml, 0.015 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; GIBCO), and 0.113% sodium bicarbonate (BDH Chemicals, Toronto, Ontario, Canada) was used for growing the cells; they were maintained in the same medium but with only 2% fetal bovine serum.

Viruses. The HM-175 strain of HAV was also received from 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 min at 37°C, and the infected cultures were then kept in the maintenance medium until 75 to 80% (4 to 5 days of incubation) of each monolayer was affected by virus cytopathology. The cultures were frozen (−20°C) and thawed three times, and the culture fluid was centrifuged for 10 min at 1,000 × *g*. The virus was concentrated 10-fold by polyethylene glycol hydroextraction as described by Ramia and Sattar (29). Dialysis tubing (Spectrum Medical Industries Inc., Los Angeles, Calif.) 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 2 ml of Earle balanced salt solution. The concentrate was filtered through a 0.2-μm-pore-size membrane filter and divided into aliquots for storage at −70°C.

The Sabin strain of poliovirus type 1 (PV) was obtained from the Laboratory Center for Disease Control (Ottawa, Ontario, Canada). FRhK-4 cells were also used for working with this virus. The procedures for the preparation and concentration of the virus pools were the same as those 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% suspension in normal saline, clarified of gross particulate matter by 10 min of centrifugation at 1,000 × *g*, and passed through a 0.2-μm-pore-size membrane filter (Nalge Co., Rochester, N.Y.) to remove bacteria and fungi. The filtrate was noncytotoxic and free of indigenous viruses when tested as previously described (2).

Disks. Stainless steel disks (diameter, 1 cm), punched out of locally purchased, no.-4-finish polished sheets (thickness, 0.75 mm), were used as carriers to represent nonporous inanimate environmental surfaces. The method for their decontamination, cleaning, and sterilization before reuse has been described previously in detail (32).

Plaque assay. Assays to determine the PFU of both viruses were carried out with FRhK-4 monolayers in 12-well plastic plates (Costar, Cambridge, Mass.). Three wells were used for each virus dilution tested. Each well received 0.1 ml of the inoculum, and the virus was allowed to adsorb for 90 min at 37°C. The overlay consisted of Eagle minimum essential medium, 2% fetal bovine serum, 2 mM glutamine, 0.1 mM nonessential amino acids, 50 μg of gentamicin sulfate per ml, 50 μg of kanamycin sulfate per ml, 2 μg of amphotericin B (Fungizone; GIBCO) per ml, 0.015 M HEPES, 0.113% sodium bicarbonate, 0.75% agarose (type II; Sigma Chemical Co., St. Louis, Mo.), and 26 mM magnesium chloride (BDH Chemicals). The plates were sealed in laminated plastic bags (Dazey Corporation, Industrial Airport, Kans.) and incubated (8 days for HAV and 48 h for PV) at 37°C. The procedure for fixing and staining the monolayers prior to counting plaques has been described previously (32). The limit of virus detection in this technique was 3 to 4 PFU for both HAV and PV.

Experimental procedure. Before each experiment, the stock virus was diluted 10-fold in the fecal suspension. Ten microliters 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 of Earle balanced salt solution; these acted as the input virus controls. 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 Earle balanced salt solution; (0-min control). After sonication (Bransonic Ultrasonics Corp., Danbury, Conn.) for 10 min at room temperature, the eluates were recovered for plaque assay. With this procedure, 91 to 100% of the input 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 by 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 of Earle balanced salt solution. Virus from the disks was eluted as described above. The eluates were stored at −80°C, and all samples from a given experiment were plaque assayed at the same time. Survival experiments were conducted over 96 h, and each experiment was repeated at least three times.

The survival of HAV at low (25% ± 5%), medium (55% ± 5%), high (80% ± 5%), and ultrahigh (95% ± 5%) levels of RH and at air temperatures of 5, 20, and 35°C was studied. The experiments on PV survival were conducted only at the low and ultrahigh RH levels, and the air temperature was kept 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, Ohio). The air temperature and RH were continuously monitored by using a recording hygrothermograph (Cole-Parmer Instruments Co., Chicago, Ill.).

Data handling. The 0-min virus titer (in PFU per milliliter) represents 91 to 100% of the input virus. The virus titer at each sampling time (x value) is the amount of HAV remaining expressed as a fraction of that eluted at 0 min. All observations were normalized to the 0-min value, with the 0-min value as 100%.

The normalized figures obtained at each RH and temperature level were analyzed graphically in a computer program (SigmaPlot Version 4.0; Jandel Scientific, Corte Madera, Calif.). 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 h, first-order linear-regression lines were generated and fitted to the log-linear plots ($\log_{10}$) by the same program. The rate of virus inactivation (K_i) was obtained from the gradient of the regression lines as loss in virus infectivity titer (in $\log_{10}$ PFU per hour). Similarly, the half-lives of the viruses were calculated from K_i as described by Segel (35). When virus became undetectable before 96 h, the K_i value was obtained over the duration of virus survival. The influence of RH and temperature on HAV survival was demonstrated by the half-lives, K_i values, and plots of K_i against the RH or temperature.

RESULTS

At 35°C and 95% ± 5% RH, HAV became undetectable by 4 h. As a result of this, comparison figures are given for all RH and temperature conditions over the first 4 h. Furthermore, graphs of HAV inactivation have been expanded to show the first 4 h more clearly, although calculations of K_i and half-life were done for the whole set of data.

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 input infectious virus remained viable at the end of 4 h at the low, medium, high, and ultrahigh RH levels, respectively (Fig. 1). As would be 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 Fig. 2, the relationship between HAV survival and RH at 20°C was the same as that seen at 5°C (Fig. 1). The amounts of infectious input HAV that were detected at 20°C after 4 h were approximately 52, 48, 34, and 28%, in increasing order of RH. The actual numbers of PFU of HAV remaining at 96 h were approximately 548, 415, 112, and 50 PFU at the low, medium, high, and ultrahigh RH levels, respectively.

Parallel experiments were also carried out at 20°C at the low and ultrahigh RH levels to compare PV survival with that of HAV (Fig. 2). At both the RH levels, the rate of loss of PV infectivity was faster than that of HAV at any RH level. PV survival showed an exponential decay to about 28% in the first hour at the ultrahigh RH and to about 12% during the same period at the low RH. Residual PV infectivity was approximately 13% for the ultrahigh RH and <1.0% for the low RH at the end of 4 h. PV infectivity was

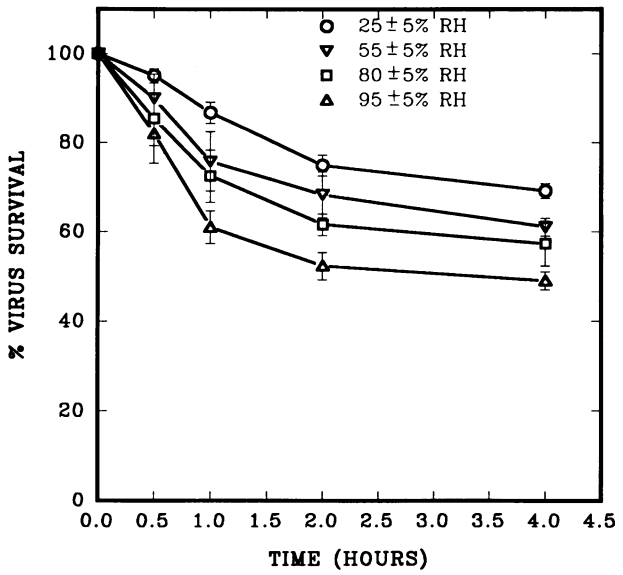

FIG. 1. Survival of HAV on stainless steel disks held at an air temperature of 5°C and at various RH levels. The vertical error bars shown represent the standard deviation from the mean of six observations for each x value.

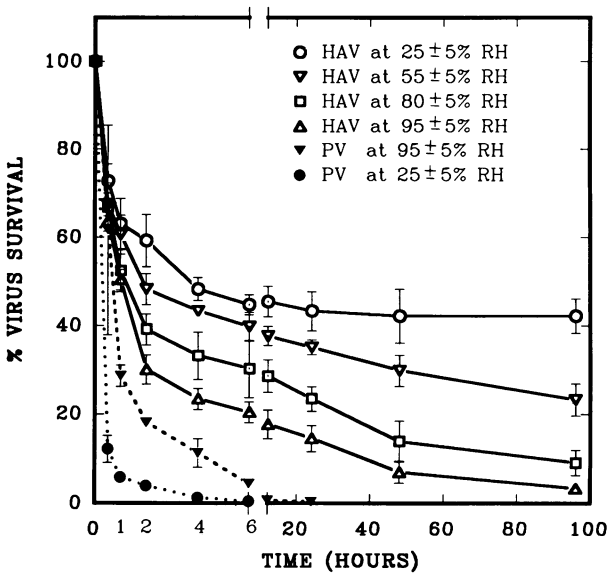

FIG. 2. Comparison of survival between HAV and PV on stainless steel disks held at an air temperature of 20°C and at various RH levels. The vertical error bars shown represent the standard deviation from the means of six observations for each x value.

undetectable after 6 h at 25% ± 5% RH and after 12 h at 95% ± 5% RH.

HAV decay rate was >2.5 times higher at the high RH than at the low RH and >3.5 times higher at ultrahigh RH than at the low RH. The rate of decay of PV at the low RH was >106 times faster than that of HAV under the same test conditions; even though PV survived better at the ultrahigh RH (K_i, 0.0984) than at the low RH (K_i, 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 h, compared with 7 h for PV at the same RH level and temperature (Table 1).

HAV survival at 35°C. At 35°C, there was also an increase in the rate of decay of HAV infectivity as the RH level was increased (Fig. 3). At the ultrahigh RH, virus infectivity became undetectable within 4 h, whereas approximately 43, 38, and 20% of the input infectious virus remained detectable at the end of the same period at low, medium, and 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 those seen when HAV was held at the lower temperatures.

Influence of RH and temperature on HAV survival. The K_i values obtained at the various air temperatures and RH levels were plotted to ascertain the influence of RH on HAV survival. As shown in Fig. 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 differences in the K_i values for the low and medium RH levels at 5 and 20°C were minimal, but they increased gradually as the RH was raised. The influence of air temperature on HAV inactivation was similar at 25% ± 5% and 55% ± 5% RH but was more obvious at the high and ultrahigh RH (Fig. 5).

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 nonenveloped viruses (4, 30). The results of this study clearly show that the influence of RH on HAV survival on nonporous inanimate surfaces is very different from that on most other nonenveloped viruses tested (4, 30). A direct confirmation of this difference was obtained in this study by

TABLE 1. Influence of air temperature and RH on the rate of inactivation of fecally suspended HAV (HM-175) and PV (Sabin) on nonporous inanimate surfaces

Virus and incubation temp (°C)	Virus half-life (K_i)/r^a at the following relative humidity (%):			
	25 ± 5	55 ± 5	80 ± 5	95 ± 5
HAV				
5	169 (0.0041)/0.9252	151 (0.0046)/0.9041	123 (0.0056)/0.8979	103 (0.0067)/0.8952
20	187 (0.0037)/0.7841	128 (0.0054)/0.8570	71 (0.0097)/0.9157	51 (0.0136)/0.9134
35	65 (0.0106)/0.9202	50 (0.0138)/0.9130	21 (0.0329)/0.9236	2 (0.4394)/0.9454
PV, 20	2 (0.3928)/0.8786	NT[b]	NT[b]	7 (0.0984)/0.9193

[a] Half-life is expressed in hours. K_i, Loss of virus infectivity, expressed as $\log_{10}$ PFU per hour. $P < 0.05$ for all r values, where r is the square root of the coefficient of determination.

[b] NT, Not tested.

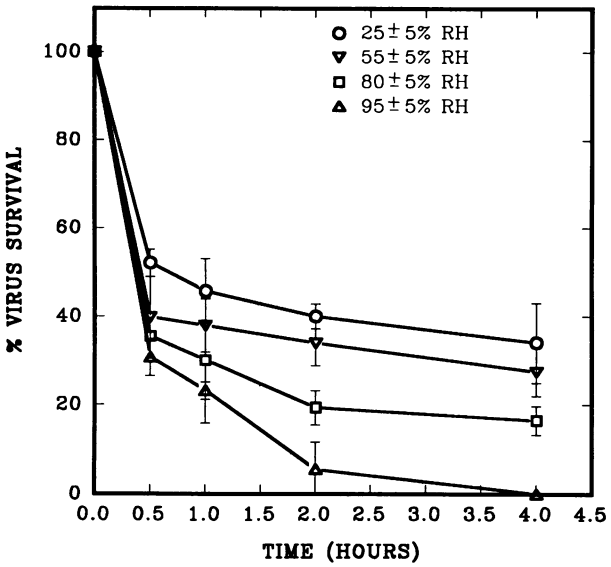

FIG. 3. Survival of HAV on stainless steel disks held at an air temperature of 35°C and at various RH levels. The vertical error bars shown represent the standard deviation from the mean of six observations for each x value.

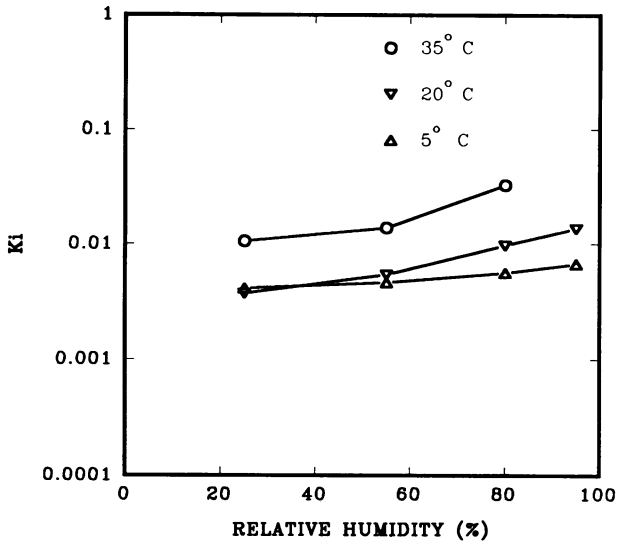

FIG. 5. Effect of air temperature on the survival of HAV on stainless steel disks held at different levels of RH.

testing PV survival under the same experimental conditions. HAV survived better than PV irrespective of the air temperature and RH level tested. It is also interesting to note that rotavirus, another nonenveloped virus, could also survive better on nonporous inanimate surfaces when the RH was at the low or medium level (31).

Previously, it has been shown that infectivity of PV and other enteric viruses decreases in parallel with evaporation of water in the inoculum (44). The mechanism of inactivation by evaporation may be accelerated by the exposure of viral particles to air at the liquid-air interface, causing inactivation by release of viral genomes (44). At the high and ultrahigh RH levels used in this study, virus suspensions placed on the disks often remained visibly moist even after 4 h. On the other hand, the inocula became visibly dry in 20 min or less when the RH was at the low or medium level. HAV survived better, however, at the lower RH levels than at the high or ultrahigh RH. Drying of the inocula, therefore, does not by itself account for the observed difference in HAV survival.

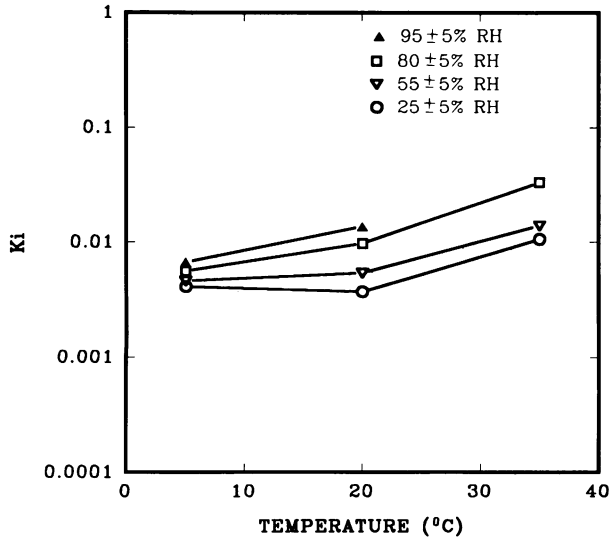

FIG. 4. Effect of RH on the survival of HAV on stainless steel disks held at different air temperatures.

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 (19). Such lipid association may confer a certain degree of resistance to environmental inactivation of the virus. In contrast to this, it has been shown by Scholz et al. (34) 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 toward the air-water interface (42) because of the hydrophobicity of the lipids; this could result in inactivation of the virus, as explained by Ward and Ashley (44). However, we believe that such a mechanism was not responsible for the HAV inactivation seen in this study.

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 in order to obtain monodisperse HAV, so that as natural a preparation as possible was presented. The presence of viral clumps, particularly if they are lipid associated, may further enhance virus survival and thus lead to aberrations in virus survival curves (3). Single virions or clumps of HAV associated with lipids 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; it is not known what effect this may have on the virus particles accumulated at the interface. Different subpopulations 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 PV. Sobsey et al. (40) have evaluated HAV survival in a variety of environmental samples. They found HAV survives much longer in waters and sediments than PV. Furthermore, when dried onto polystyrene surfaces, HAV could still be detected after 1 month (40).

The half-lives of HAV indicated in Table 1 show a marked decline at RH levels of 80% ± 5% and 95% ± 5% at 20°C and at all RH levels at 35°C relative to values found at 5°C. Such a 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 h. If enzymatic activity is indeed the cause of increased HAV inactivation, it would be interesting to know the nature of the enzymes responsible.

It is generally very difficult to determine whether 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 (6). Levy et al. (20) have suggested that the 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 (27, 33). 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 (28a). The majority of young children also show no clinical symptoms after HAV infection and may serve as asymptomatic virus transmitters (10). 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 (14).

Although classified as an enterovirus, HAV shows significant differences from other enteroviruses, and this suggests that it should be considered the prototype of a new, fifth genus in the family *Picornaviridae* (36, 41). The results of this study also show that HAV sensitivity to RH is unlike that of other enteroviruses tested. In conjunction with other observations (7, 19, 22, 34, 37, 45), our results point to the need to reclassify HAV.

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 (20) and although aerosols have been reported to initiate HAV outbreaks (1), 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 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 be either adsorbed to or embedded in particles, and this may afford greater protection to its infectivity.

The results of a recently completed study in our laboratory (22a) have shown that fecally suspended HAV can survive for several hours on human hands and could be transferred to and from other animate or inanimate surfaces. This suggests that hands could also become contaminated and spread the virus through contact with contaminated environmental surfaces. Previously, we have also shown that many commonly used chemical disinfectants are ineffective in the decontamination of HAV-contaminated hard surfaces (22). There is no information on the efficacy of currently available antiseptics against HAV.

Hepatitis A continues to be a serious human health problem in the developing world. Occurrence of this disease also appears to be increasing in many industrialized countries (5, 28). This may be due to increased travel to and from areas where hepatitis A is endemic (28), an increase in the number of children in day-care (13), changes in life-styles, and the presence of a larger pool of susceptible individuals in the community. Vaccines against hepatitis A are not yet available for general use but are being tested.

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