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Role of Absolute Humidity in the Inactivation of Influenza Viruses on Stainless Steel Surfaces at Elevated Temperatures▽

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Influenza virus has been found to persist in the environment for hours to days, allowing for secondary transmission of influenza via inanimate objects known as fomites. We evaluated the efficacy of heat and moisture for the decontamination of surfaces for the purpose of preventing of the spread of influenza. Aqueous suspensions of influenza A virus were deposited onto stainless steel coupons, allowed to dry under ambient conditions, and exposed to temperatures of 55°C, 60°C, or 65°C and relative humidity (RH) of 25%, 50%, or 75% for up to 1 h. Quantitative virus assays were performed on the solution used to wash the viruses from these coupons, and results were compared with the solution used to wash coupons treated similarly but left under ambient conditions. Inactivation of influenza virus on surfaces increased with increasing temperature, RH, and exposure time. Reductions of greater than 5 logs of influenza virus on surfaces were achieved at temperatures of 60 and 65°C, exposure times of 30 and 60 min, and RH of 50 and 75%. Our data also suggest that absolute humidity is a better predictor of surface inactivation than RH and allows the prediction of survival using two parameters rather than three. Modest amounts of heat and adequate moisture can provide effective disinfection of surfaces while not harming surfaces, electrical systems, or mechanical components, leaving no harmful residues behind after treatment and requiring a relatively short amount of time.

In a recent publication, Shaman and Kohn concluded that absolute humidity, which can be calculated if temperature and relative humidity (RH) are known, is the controlling factor in both the inactivation of influenza virus and the transmission of influenza (27). To arrive at this conclusion, Shaman and Kohn reanalyzed experimental data collected by Lowen et al. and Harper (12, 17, 18), which covered a rather narrow range of temperatures typical of normal weather conditions, that is, in the range of 5 to 30°C. For this temperature range, the maximum absolute humidity that can occur is 24 g/m^3. One question that comes to mind is whether this trend of decreasing influenza virus survival with increasing absolute humidity (AH) persists as temperature increases. If it does, then AH may also be the controlling factor when heat and moisture are used to decontaminate surfaces. In the present work, we tried to answer this question through a series of experiments in which absolute humidity was sufficiently high to result in effective surface decontamination.

Effective and easily implemented public health interventions are needed to prevent the spread of infectious diseases such as influenza. Transmission of influenza virus, especially in the event of a pandemic with a highly virulent strain of influenza virus, such as avian influenza H5N1 virus or 2009 H1N1 influenza A virus, is of great concern due to widespread mortality and morbidity (7, 23). The significant morbidity and mortality associated with seasonal influenza should also not be discounted. There is compelling evidence for transmission of influenza viruses from infected individuals to uninfected individuals by direct contact, via fomites (inanimate objects capable of carrying infectious organisms), and through large droplets expelled during forceful exhalation, such as during coughing and sneezing (2–4, 19). Virtually any exposed surface can become contaminated with infectious viruses and can be a potential source of secondary virus transmission. The probability for transmission increases in situations where many people are in close proximity and in locations with highly transient populations, such as public transportation, air transportation, classrooms, theaters, and other public venues (16, 20, 22). Influenza virus has been found to persist in the environment for hours to days; it has been found on surfaces in day-care centers, hands, laboratory gowns, and in surface dust (3, 4, 9, 29). Efforts to prevent the spread of flu through contact transmission via fomites require methods of decontamination that are easy to use and will not disrupt critical services and operations.

Laboratory research has shown that the moisture content of the air is an important factor for antimicrobial survival. Past research on influenza virus has focused on airborne viruses and generally suggests that survival and/or transmission is facilitated by low RH (10, 12, 15, 17, 26, 30). A limited number of surface inactivation studies have been performed at environmentally relevant temperatures, ranging from 7 to 32°C (30), and have shown modest reductions in numbers of influenza viruses (5, 21).

We evaluated the efficacy of heat and moisture for decontaminating surfaces and controlling the spread of influenza virus infection. In this study, temperatures were maintained well above room temperature (55 to 65°C) but were still not expected to cause harm to most surfaces, mechanical compo-

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nents, or electrical systems. Furthermore, heat does not leave behind potentially harmful residues, as do most chemical decontaminants, and the use of heat requires a relatively brief period of time for surface treatment and allows resources to be quickly returned to service. To our knowledge no previous measurements of influenza virus inactivation rates on surfaces at these temperatures have been made.

MATERIALS AND METHODS

A suspension of influenza virus (A/PR/8/34 H1N1), which was purchased from Advanced Biotechnologies Inc. (Columbia, MD), was thawed, divided into single-use portions, and stored at −80°C until needed. Stainless steel coupons measuring 7.6 by 2.5 by 0.4 cm were used as test surfaces. Prior to use the coupons were cleaned with soap and water, rinsed with 70% ethanol, allowed to dry, and then autoclaved. Fifty microliters of virus suspension was pipetted onto a clearly marked section of each coupon. All coupons were allowed to sit in a biological safety cabinet at ambient temperature (24 ± 2°C [mean ± standard deviation]) and relative humidity (35% ± 20%) until the liquid evaporated, which required approximately 30 min. Exposed coupons were placed horizontally into a VWR model 9005 humidity test chamber (West Chester, PA) that was fitted with a custom-made shroud to minimize air infiltration into the chamber when the chamber door was opened. A triplicate set of coupons were removed from the chamber after 15, 30, and 60 min of exposure for each RH (25%, 50%, and 75%) and temperature (55, 60, and 65°C) combination. Testing for each combination of temperature and RH was carried out independently and considered an experimental run. Three experimental runs were carried out for each of the nine temperature-RH combinations. For each experimental run a triplicate set of seeded coupons were left under ambient conditions for the duration of the experiment and were considered "unexposed." Coupons with shorter exposure times were removed from the exposure chamber and set aside with the control coupons. The viruses on all coupons, including the controls, were extracted after the last coupon was removed from the exposure chamber. Extraction consisted of rinsing the coupon by forcefully dispensing a 500-μl portion of wash solution (phosphate-buffered saline containing 0.1% bovine serum albumin) from a Rainin P-1000 pipette (Oakland, CA) onto the clearly marked portion of the slide where the virus had been initially deposited. This rinsing process was repeated with the same 500-μl portion of wash solution 20 times for each slide. Used coupons were placed into a 10% bleach solution, washed with soap and water, wiped down with 70% ethanol, and autoclaved prior to reuse.

A fluorescent focus reduction assay, which labels nucleoproteins expressed by infected Madin-Darby canine kidney (MDCK) cells with fluorescent antibodies, was used to enumerate the number of infective viruses and has been described previously (13, 25). Briefly, triplicate wells on a 96-well plate containing monolayers of MDCK cells (ATTC CCL-34) were infected with 50 μl of rinsate from each coupon and allowed to incubate for approximately 8 h. Then, infected cells containing influenza A virus nucleoproteins were labeled with mouse monoclonal antibody A-1 (catalog no. VS2366; Centers for Disease Control and Prevention, Atlanta, GA) and subsequently labeled with rhodamine-labeled goat anti-mouse IgG (catalog number 115026062; Jackson ImmunoResearch Laboratories, West Grove, PA). The number of cells with resulting fluorescent foci, which are referred to as fluorescent focus units (FFU), were then counted at 200× total power using an Olympus CKX-41 inverted fluorescence microscope (Olympus, Center Valley, PA). Each well was scanned in a standard pattern with 10 fields chosen at random for counting (about 30% of the well). For samples with less than 2 FFU per viewing field, the entire well was counted. We totaled the counts for the nine wells associated with each test (three wells × triplicate coupons). The number of FFU per slide was then computed based on dilution factors and the fraction of the well counted.

Calculations. Relative humidity (R_H in equation 1) is generally defined as the ratio of the partial pressure of water vapor (p_w) to its saturation vapor pressure (P^o), that is, the partial pressure at which an increase in partial pressure will cause water vapor to condense (11, 24):

$$R_H = \frac{p_w}{P^o} \tag{1}$$

Relative humidity can be expressed as a fraction, as in equation 1, or more commonly as a percentage. The saturation vapor pressure of water can be obtained from handbooks or, for the temperature range from 0° to 200°C, from the following equation taken from the ASHRAE Handbook (1):

$$P^o = \exp\left(-\frac{5{,}800}{T} + 1.391 - 0.04864T + 4.176 \times 10^{-5}T^2 - 1.445 \times 10^{-8}T^3 + 6.546\ln T\right) \tag{2}$$

where T is absolute temperature in degrees Kelvin and P^o is in pascals. For this research, equation 1 was used to calculate the partial pressure of water vapor from the measured RH and the saturation vapor pressure at the measured temperature, which was calculated from equation 2.

Absolute humidity is generally defined as the mass of water vapor per volume of moist air (1). It is a parameter used to quantify the concentration of water vapor in air. Because water vapor behaves like an ideal gas (24), the absolute humidity can be calculated from the ideal gas law if the temperature and the partial pressure of the water vapor are known, as in equation 3:

$$p_w V = n_w R T \tag{3}$$

where V is volume, n_w is moles of water vapor, and R is the universal gas constant. Substituting the mass of water vapor (m_w) divided by the molecular weight of water (M_w) for n_w and rearranging the factors, as in equation 4, provides the absolute humidity (H_A).

$$H_A = \frac{m_w}{V} = \frac{M_w p_w}{RT} = 0.00217\frac{p_w}{T} \tag{4}$$

Equation 4 was used to calculate absolute humidity (in g/m^3) based on the temperature (in degrees Kelvin) and the partial pressure of water vapor (in pascals), which was determined from equation 1.

The number of FFU per volume of coupon rinsate is a measure of the quantity of infective viruses present on the coupon. The ratio of the number of FFU/μl of rinsate from an exposed coupon (U) to the number from an unexposed coupon (U_o) is defined as the fraction of virus surviving (f) and can be calculated with equation 5:

$$f = \frac{U}{U_o} \tag{5}$$

The number of log reductions (base 10) of virus (n) is defined by equation 6:

$$n = \log U_o - \log U = -\log f \tag{6}$$

Thus, $n = 4$ corresponds to 4 log reductions in virus, which is equivalent to 0.01% of the virus surviving and a 99.99% virus reduction.

For each experimental run, nine coupons were exposed (three for 15 min, three for 30 min, and three for 60 min), and three control coupons were not exposed. Because the unexposed and exposed coupons could not be separated into pairs, the mean number of log reductions of virus ($\bar{n}$) was calculated for each experimental run by using equation 7:

$$\bar{n} = \overline{\log U_o} - \overline{\log U} \tag{7}$$

where $\overline{\log U_o}$ and $\overline{\log U}$ are the means of the logarithms of U_o and U, respectively.

Log reductions were reported as above a specific value for data points in which no viruses were detected (i.e., the limit of detection). The number of log reductions was calculated based on detecting 1 FFU on one of the nine stainless steel coupons used for the three identical experimental runs.

Statistical analysis was performed using Excel 2003 (Microsoft, Redmond, WA).

RESULTS

The average number of log reductions of influenza viruses measured for the three exposure times (15, 30, and 60 min) for all combinations of the three temperatures (55°C, 60°C, and 65°C) and three RH levels (25%, 50%, and 75%) are listed in Table 1. Surface inactivation of influenza virus increased with increasing temperature, RH, and exposure time. The limit of detection was not reached for any of our experiments at 25% RH. All experiments at 75% RH reached the limit of detection within 15 min. A >5-log reduction of influenza virus on surfaces was achieved at temperatures of 60 and 65°C, exposure times of 30 and 60 min, and RH of 50 and 75%. The mean

TABLE 1. Reductions of influenza virus numbers on stainless steel surfaces at various exposure times, temperatures, and relative humidity levels

Temp (°C)	Time (min)	Mean (SD) log reduction at RH of:		
		25%	50%	75%
55	15	0.9 (0.2)	2.9 (0.1)	>4.4
	30	1.1 (0.3)	3.4 (0.3)	>4.4
	60	1.5 (0.1)	4.1 (0.1)	>4.4
60	15	1.2 (0.1)	4.1 (0.7)	>5.2
	30	1.5 (0.4)	>5.0	>5.2
	60	1.8 (0.2)	>5.0	>5.2
65	15	1.8 (0.2)	>5.1	>5.1
	30	2.2 (0.1)	>5.1	>5.1
	60	3.1 (0.2)	>5.1	>5.1

concentration of viruses measured on the control slides after drying was 1.6×10^5 FFU per slide and ranged from 1.3×10^4 to 2.9×10^5 FFU/slide. The mean number of viruses applied to the slide, based on the titer of the stock measured for each experiment, was 5.3×10^5 FFU/slide and ranged from 7.5×10^4 to 1.5×10^6 FFU/slide over the course of all experiments. Comparisons of the titer of the virus stock used to inoculate the test and control coupons with the concentration of virus recovered from the control coupons showed that there was an average loss of 63% of viruses during the drying process. These drying losses were assumed to be the same for the control and test coupons.

The relationship between the log reduction of influenza virus and RH for 15-, 30-, and 60-min exposure times, which are shown in Table 1, are simplified if temperature and RH are replaced with absolute humidity. The absolute humidity is plotted in Fig. 1 as determined based on equations 1, 2, and 4 and using the RH and temperature test parameters previously described. Surface inactivation of influenza virus increased with increasing absolute humidity and increasing exposure time. The data within our experimental range were linear with a high degree of fit, with all r^2 values exceeding 0.91 for each exposure time. A multiple linear regression model of the log reduction of influenza virus was developed that included independent variables for AH (in g/m^3) and time (in minutes) giving an adjusted r^2 of 0.93 ($P < 0.001$), as shown with equation 8:

$$n = -2.2 + 0.096AH + 0.020t \quad (8)$$

When temperature or RH was added to the regression model, there was no statistical significance at the 95% level as measured by a partial F-test, but the data did show a significant difference ($P < 0.001$) when time was removed from the model. The multiple linear regression model including independent variables for RH, temperature, and time ($n = -10 + 0.11RH + 0.14T + 0.018t$) has a slightly lower adjusted r^2 value of 0.90 ($P < 0.001$) and provides a reasonable means of predicting virus survival, but it does so at the expense of adding an additional parameter to the model.

DISCUSSION

This is the first investigation to our knowledge that systematically measured the role of moderately high temperatures and moisture content in air on influenza virus inactivation on surfaces. The results of our experimental tests demonstrate unequivocally that the thermal inactivation of influenza virus on surfaces is a useful method to significantly reduce the number of viruses and that AH and exposure times are strong predictors of virus inactivation. Our data also suggest that AH is a better predictor of surface inactivation than RH. This finding supports those of Shaman and Kohn (27), who showed

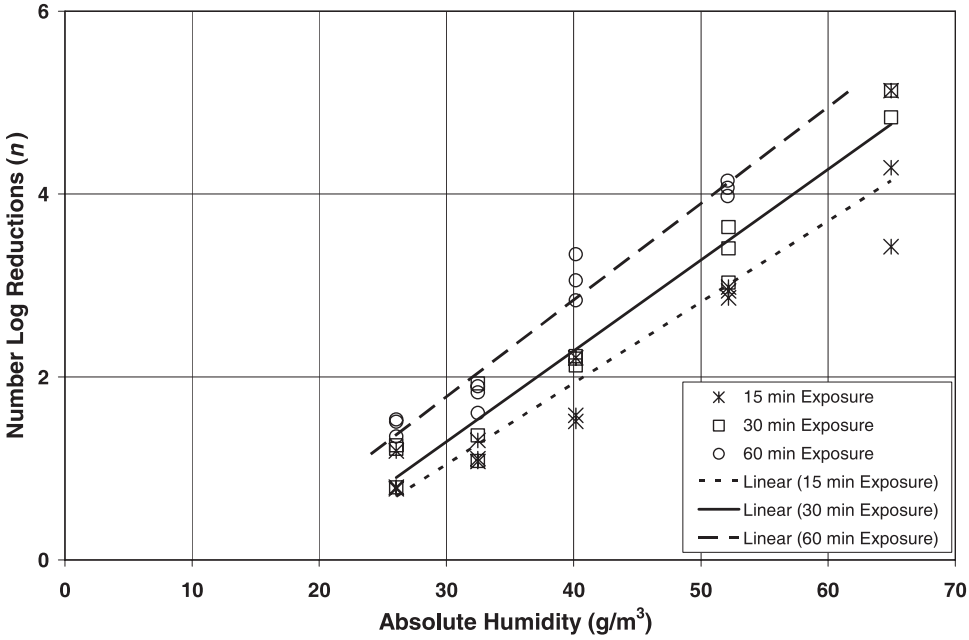

FIG. 1. Plot of log reductions of influenza virus on stainless steel surfaces as a function of absolute humidity for 15-, 30-, and 60-min exposure times.

that AH was a better predictor of influenza virus survival in their reanalysis of studies that originally found RH affects influenza survival and transmission. Analysis of our experiments, which were done at a >25°C higher temperature than those in the analyses of Shaman and Kohn, shows 93% of the experimental variability was accounted for by the AH and time parameters. The addition of temperature as a covariate did little to improve the fit of the model. Thus, survival can be predicted using the two parameters rather than three and greatly simplifies analysis and interpretation of virus survival data.

The data presented in Fig. 1 show that a doubling of AH from 30 to 60 g/m^3 is associated with a 3.9- and 2.5-fold increase in the number of log reductions of influenza virus for 15 and 60 min of exposure, respectively. Since AH is a function of RH and temperature (equations 1, 2, and 4), these parameters can be adjusted to fit the needs of a specific application; that is, temperature can be traded with RH and vice versa to achieve specific reductions. For example, at 55°C and 30% RH, the AH is 31 g/m^3. Doubling the AH from 31 to 62 g/m^3 results in 3.0-log reduction based on equation 8. If this doubling of AH were desired, it could be facilitated by either increasing the RH to 60% while holding temperature constant at 55°C or by increasing the temperature to 71°C while holding RH at 30%, or increasing both temperature and RH so as to yield 62 g/m^3. This relationship would be very useful in situations where either a high RH or temperature could not be tolerated.

Despite considerable research on the effect of humidity on influenza virus aerosols, actual mechanisms of action or damage have not been addressed experimentally and are not fully understood. Enveloped viruses, such as influenza virus, are thought to be less stable in the environment than nonenveloped viruses and more sensitive to higher relative humidity (6, 8, 28, 30). Surface inactivation for viruses with structural lipids may be due to denaturing of the lipoproteins found in enveloped viruses. Phase changes in the phospholipid bilayer, which lead to cross-linking of associated proteins, have been proposed as a mechanism of action (6). However, the findings of some studies are not consistent with these observations, and experimental data supporting these proposed mechanisms are lacking (8, 28, 30). More research is needed on the mechanisms of virus inactivation associated with humidity.

The majority of previous studies on the influence of RH on influenza virus survival have been largely focused on survival of influenza virus aerosols rather than influenza virus on surfaces (10, 12, 14, 15, 17, 26). A single study by Buckland and Tyrrel report a 1.7-log reduction of influenza virus deposited on slides for 2.5 h at room temperature at 20% RH and up to a 3.5-log reduction when RH was increased to 84% (5). This study, along with the aerosol studies, largely concluded that virus survival is highest at low RH. Our data are consistent with these studies, albeit with much higher temperatures.

We chose stainless steel as a testing surface because (i) the surface texture is relatively smooth and facilitates removal of exposed viruses, (ii) the surface was easily decontaminated and reused, and (iii) the frequency of use of stainless steel surfaces on public transportation. However, virus inactivation rates on surfaces are likely a function of the surface on which the virus is deposited. Noyce et al. report much higher log reduction rates on copper surfaces than on stainless steel (21). Bean et al. reported that influenza virus survived up to 24 to 48 h on stainless steel and plastic surfaces but only from <8 to 12 h on porous, fabric-type surfaces (3). Further research is needed on heat inactivation and moisture content in air on surfaces other than stainless steel, but this was beyond the scope of this study.

Although the use of heat inactivation appears to be an effective and simple means of inactivating influenza virus on surfaces, there are issues that must be addressed for effective implementation as a method of controlling disease transmission. Although actual exposure times to heat were minimal (<1 h), it may take a significant amount of time to bring surfaces up to required temperatures. Factors such as ambient temperature, insulation values, and thermal mass must be taken into consideration. Furthermore, condensation may occur upon cooling heated spaces in air with a high moisture content. Precautions would have to be taken to ensure moisture levels are reduced prior to cooling to prevent condensation. The data presented in this research are limited to a single strain of influenza A virus and were obtained under artificial conditions. Other strains or types of influenza virus may respond differently to moist thermal inactivation. The methods used herein for evaluating heat inactivation on viruses on surfaces can easily be applied to other microbes to investigate these differences. However, the impacts of buffers and methods of surface deposition, among other factors, used under artificial conditions may play a significant role in virus survival. Thus, additional research is needed to validate these findings under "natural" conditions.

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