Persistence of the 2009 Pandemic Influenza A (H1N1) Virus (pH1N1) on N95 Respirators

Coulliette, A.D.#, K.A. Perry, J. Edwards, and J. Noble-Wang

#ACoulliette@cdc.gov

National Center for Emerging and Zoonotic Infectious Diseases, Centers for Disease Control,

Atlanta, GA 30307

The findings and conclusions in this paper are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.
In the U.S., the 2009 pandemic influenza A (H1N1) virus (pH1N1) infected almost 20% of the population and caused >200,000 hospitalizations and >10,000 deaths during April 2009 to April 2010. On April 24, 2009, CDC posted interim guidance on infection control measures in healthcare settings specifically for pH1N1, which recommended filtering face respirators (FFRs) when in close contact with a suspected/confirmed infected individual and particularly when performing aerosol procedures. The persistence and infectivity of pH1N1 was evaluated on FFRs, specifically N95 respirators, under varying conditions of absolute humidity (AH; 4.1 millibar (mb), 6.5 mb, and 14.6 mb), sample matrices (2% fetal bovine serum (FBS), 5 mg/ml mucin, and viral media), and time (4, 12, 24, 48, 72, and 144 hours). pH1N1 was distributed onto N95 coupons (3.8 to 4.2 cm²), extracted by a vortex/centrifugation/filtration process, and the ability of remaining virus to replicate was quantified by ELISA to determine the log_{10} concentration of infectious virus per coupon. Overall, pH1N1 remained infectious for 6 days with approximately 1 log_{10} loss of viral concentrations over this time period. Time and AH both affected viral survival. We found significantly higher (p ≤0.01) reductions in virus concentrations at time-points beyond 24 to 72 hours (-0.52 log_{10} reduction) and 144 hours (-0.74) at AHs of 6.5 mb (-0.53) and 14.6 mb (-0.47). This research supports the discarding of respirators after close contact with a person with suspected or confirmed influenza infection due to the virus’s demonstrated ability to persist and remain infectious.

KEYWORDS: Influenza A H1N1, N95 respirators, survival, healthcare settings
INTRODUCTION

The 2009 H1N1 pandemic influenza A (H1N1) virus (pH1N1) outbreak affected over 214 countries and caused at least 18,449 deaths worldwide (WHO, August 6, 2010). The estimated impact, as extrapolated from laboratory confirmed hospitalizations in the U.S. during April 2009 to April 2010, was 60.8 million cases (range: 43.3 to 89.3 million), 274,304 hospitalizations (195,086 to 402,719), and 12,469 deaths (8,868 to 18,306) (1). The current Centers for Disease Control and Prevention (CDC) ‘Prevention Strategies for Seasonal Influenza in Healthcare Settings Guidelines and Recommendations’ state that face masks are sufficient personal protective equipment (PPE) for hospital staff, associated workers, patients, and visitors when a person is suspected or known to be infected (2). During the pandemic, the first CDC interim guidance was posted on April 24, 2009 regarding infection control measures in healthcare settings specifically for pH1N1. Filtering facepiece respirators (FFRs; i.e. N95) were recommended (in addition to standard precautions) in this guidance document as a conservative measure to protect healthcare personnel when patients are in isolation, particularly during aerosol generating procedures, and for those in close contact with patients with suspected or confirmed pH1N1 infections (3, 4).

The number of N95 FFRs used during the 2009 pandemic period is unclear and supply shortages were acknowledged in the CDC 2009 H1N1 Influenza Interim Guidance document [2]. A study by the Institute of Medicine stated that 90 million respirators would be needed for a 42 day influenza pandemic (5). Meanwhile, Murray et al. 2010 found that facial protective equipment (e.g. masks, respirators, and disposable eyewear) use more than doubled in the Vancouver Coastal Health service region during the 2009 pandemic. Specifically for respirators, the use was 51% over the historical baseline during the pandemic and the authors suggested a 1:1
ratio of respirator to mask in acute care facilities where aerosol-generating medical procedures are performed for estimating supplies in the event of a pandemic (6). The numbers of FFRs used during influenza virus outbreaks are daunting due to the protocols (i.e. ‘donning/ doffing’ for every room), while minimal direct evidence on the exclusion of influenza A during FFR use and survival after deposition remains elusive.

FFRs provide 99.5% filtration efficiency for particles larger than 0.75 µm and 95% or higher for particles between 0.1 to 0.3 µm (7). Influenza A is approximately 120 nm in diameter (8). FFRs thereby, with a proper seal, deliver protection from infectious particles ranging from large droplets (>100 µm) to inhalable droplets (10 to 100 µm) to nuclei aerosols (<10 µm) (9, 10). However, the main route of transmission of influenza virus infection continues to be a topic of debate (9-12). Some contend that airborne transmission via small particle aerosols is a feasible pathway that has not been given the appropriate attention (10, 11), while others cite the evidence for close contact and large droplets causing influenza infection (9, 12). Data regarding fomite transmission of influenza A within the hospital setting is also limited. Knowledge about survival and persistence of influenza A on the exterior of the facepiece is needed, regardless of deposition route, because of the repeated ‘donning’/’doffing’ of FFRs and subsequent hand hygiene considerations.

Influenza A virus is an enveloped virus and its’ lipid bilayer is a main determinant of survival, as viruses with greater lipid content persist better in lower humidity conditions (13). Research regarding influenza survival on surfaces has mostly focused on stainless steel (14-16). For study of survival/interactions on respirators, MS2 coliphage, a ss(+)-RNA virus that infects Escherichia coli, has been used as the surrogate (17-19). Previous studies provided insight in mostly older strains of influenza A, such as A/Brazil/11/78-like (16) and A/PR/8/34 (20),
various materials (pajamas, tissue, soft toys, surgical masks, hospital gowns) (16, 21), and single absolute humidity (AH) (16, 20, 21). We used a robust design to evaluate the persistence and infectivity, as defined by ability to infect tissue culture, of the pH1N1 virus deposited on N95 FFR material under different conditions of AH, tested in varying sample matrices (component of the sample besides pH1N1 such as mucus), and measured at time periods up to 6 days.

MATERIALS AND METHODS

The study evaluated survival and infectivity of the pH1N1 virus within three matrices: viral media (DMEM; Gibco®, Grand Island, NY), 2% fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA), and 5 mg/ml mucin (MP BioMedical, Soloni, OH) on coupons of N95 respirators (3M, Model 8210; St. Paul, MN). We studied survival at AH conditions of 4.1 millibar (mb; 18°C/20% RH), 6.5 mb (25°C/20% RH), and 14.6 mb (21°C/58.5% RH) for 0, 4, 12, 24, 48, 72, and 144 hours (h). The experiments were performed three times for all conditions with the exception of the 144 hour time-point at the 4.1 mb AH, which was performed twice. Thus, the sample size for each sample is n=9 with the following exceptions: FBS, 4.1 mb – 12 h, n=6; 144 h, n=3. Viral Media, 14.6 mb – t24 h, n=8; 144 h, n=6. Mucin, 4.1 mb – 12 to 72 h, n=6. Mucin, 6.5 mb – 72 and 144 h, n=6. Mucin, 14.6 mb – 24 and 48 h, n=8.

Experiment Parameters

Influenza pH1N1 virus and propagation: Influenza A/California/04/2009 H1N1 (type: Influenza A/ H1N1 (pdm); CDC ID# 2009712047; lot#08/13/2009) was obtained from the Influenza Division, CDC, and propagated in Madine-Darby Canine Kidney (MDCK) cells as described in Szretter et al. (22). The method is briefly detailed here. Confluent MDCK cells were washed with room temperature phosphate buffered saline (PBS Gibco®, Grand Island, NY)
twice and once with cDMEM/ 7.5% BSA (Fisher, Fair Lawn, NJ). The virus, thawed in cool water, was diluted to obtain a multiplicity of infection (ratio of influenza virus to MDCK cells) of 1:100 with viral growth media (DEM, 7.5% bovine serum albumin (BSA), 2% penicillin/
streptomycin (stock concentration: 10,000 units/ml penicillin G sodium and 10,000 µg/ml streptomycin sulfate; Life Technologies, Carlsbad, CA), HEPES buffer (Gibco®, Grand Island, NY) and TPCK-treated trypsin (ThermoScientific, Rockford, IL). One ml of the diluted virus suspension was added to the MDCK cell monolayer. The suspension was rotated to thoroughly cover the entire monolayer and incubated at 37°C for 45 minutes. Viral growth media (20 ml) was added to the monolayer and the flask (75 cm²) was not harvested until cytopathic effects (CPE) were detected in 75% of the monolayer. The monolayer supernatant was centrifuged for 15 minutes at 300 x g, where the supernatant was then divided into cryovials and stored at -80°C until the experiment. In an effort to prepare sufficient pH1N1 for the entire experiment, several flasks were prepared to propagate the virus at the same time. Once all the flasks showed the proper percentage of CPE, virus was isolated from all the flasks, combined for one large population, and rendered a stock concentration averaging 4.3 x 10⁵ Tissue Culture Infectious Dose of 50% (TCID₅₀) per ml. Infectious pH1N1 were quantified as TCID₅₀, which refers to the number of pH1N1 that produced cytopathic effect (CPE) in 50% of the cells inoculated.

Test matrices: Viral media, 2% FBS, and mucin (5 mg/ml) were used as test matrices. Viral media (detailed above) was used as a control matrix, while 2% FBS and mucin were proxies for sputum and mucous-like material generated during sneezing and coughing. The stock matrices of 4% FBS and mucin (10 mg/ml) were prepared and stored at -20°C, which were later combined with equal volumes of virus suspension during the experiment to achieve the
desired 2% FBS and 5 mg/ml of mucin. Viral media was stored at 4°C until the experiment and also was combined with equal volumes of the virus suspension for the experiments.

**N95 Respirator coupons:** The 3M Model 8210 N95 was chosen for evaluation because the respirator was listed in the Strategic National Stockpile (CDC, Atlanta, GA), approved for infection control in healthcare settings, and readily available. Additional details regarding the respirator can be found in Fisher et al. (23). Circular coupons (3.8 to 4.2 cm²) were punched from N95 respirators using a grommet and hammer, placed in six-well plates with the exterior of the mask facing upwards for the outer shell to be exposed (Costar, Corning, NY), and UV sterilized for at least 15 minutes prior to the experiment.

**Absolute humidity (AH):** AH was defined in Shaman and Kohn (2009) as the ‘actual water vapor content of air irrespective of temperature’ (24). This parameter reflects the relationship between relative humidity (RH%) and temperature, both of which are documented to influence survival of influenza (24). Absolute humidity (AH) was calculated from measured temperature (°C) and RH% conditions. The VP of water used as the measurement for AH:

\[ VPw = RH \times \left( \frac{SVP}{100} \right), \]

where \( VPw \) is the vapor pressure of water vapor, \( RH \) = relative humidity (RH %) and \( SVP \) is the saturated vapor pressure (mb), defined as \( SVP = 6.11 \text{ mb} \times e^{0.067 \times T}, \)

where \( T \) is temperature in degrees Celsius. The three AH conditions as measured via vapor pressure (VP), 4.1 mb (18°C/ 20% RH), 6.5 mb (25°C/ 20% RH), and 14.6 mb (21°C/ 58.5% RH), were maintained within an environmental chamber (Caron Model 6030, Marietta, OH) that was monitored with a temperature and RH Traceable® sensor (Control Company, Friendswood, TX). The temperature (°C) and RH % were checked at least twice a day during the experimental
time periods to assure the correct predetermined AH was obtained within the environmental chamber.

*Time-points:* Previous research on survival of influenza A virus when suspended in viral media on porous surfaces showed compelling reductions in viable virus within approximately 24-48 h (16, 20). We studied additional time-points within this 24-48 h period (4, 12, 24 h) and also extended testing to 72 h. In our initial experiments, 72 h was the final time-point at which we measured survival, similar to Bean et al. (16). However, testing at 144 h (6 days) was added after the first two experiments at 4.1 mb VP AH (mb) to detect if complete die off occurred. In summary, triplicate coupons were processed for each VP, matrix, and time-points (0, 4, 12, 24, 48, 72, and 144 h) with the exception of the 144 hr time-point for the first two experiments at 4.1 mb.

**Sample Processing**

*Cell culture:* MDCK cells (ATCC CCL-3, Manassas, VA) were maintained in tissue culture flasks (Corning®, Corning, NY) until passage 90, at which time new cells were started. A modified procedure described in Szretter et al. 2006 was followed (22). The flasks (150 cm²) were seeded with 4 x 10⁴ to 2.0 x 10⁵ cells per ml and grown to approximately 90 to 95% confluency in a 5% CO₂ atmosphere at 37°C for 24 to 72 hours. Media for cell growth consisted of DMEM, containing fetal bovine serum (10% for growth and 2% for maintenance), and 2% penicillin/streptomycin.

*N95 Respirator and pH1N1 Processing:* The UV sterilized N95 respirator coupons and required sterilized supplies (forceps, cell spreaders, pipets, pipet tips, etc.) were placed into a biosafety cabinet, in addition to the H1N1 stock inoculum and sample matrices (viral media, 2%
FBS, and 5 mg/ml mucin). The virus and sample matrices were prepared as equal parts, and mixed. The virus-matrix suspension was inoculated (100 µl) onto individual respirator coupons in triplicate for each time point (See Figure 1). The inoculated coupons dried in the biosafety cabinet for 1 hr.

Once the virus dried on the coupon, the inoculated coupon was placed inside a 15 ml conical tube (BD Falcon™, Franklin Lakes, NJ) and 5 ml of 2% BSA – 1X PBS (pH 8.5) was added. To separate pH1N1 from cell debris, the sample was vortexed for 20 minutes and centrifuged for 5 minutes at 3,000 x g to pellet cell debris. To further purify the sample, the supernatant was removed and filtered through a pre-moistened (2% BSA-1X PBS) 0.22 µm syringe filter (Fisherbrand, Pittsburgh, PA; Millex-GS, Billerica, MA) into 1.5 ml Safe Lock Tubes (Eppendorf, Hauppauge, NY). The samples were labeled and stored at -80°C until processing.

**ELISA:** MDCK cells at approximately 95% confluency were washed with 1X PBS, separated from the flask using trypsin-EDTA, concentrated by centrifugation at 500 x g for 10 minutes, and resuspended in viral media (described above). The sample (150 µl) underwent a 1:3 dilution (50 µl) in 96-well plates (CoStar®, Corning, NY) with 100 µl of viral culture media (DMEM-1%BSA) for a total of ten (10) dilutions. MDCK cells (100 µl) were then aliquoted to each well in the 96-well plates with the diluted samples. The plates were incubated in a 5% CO₂ atmosphere at 37°C overnight. The range of detection for the experiments was 1.44 x 10¹ to 3.40 x 10⁵ TCID₅₀ per ml.

Using a BioTek® ELx405 Select CW plate washer (BioTek® Instruments, Winooski, VT), the plates were rinsed with 1X PBS. Manually, 80% acetone/ 1X PBS (cold) was added to every
well and incubated for 8 minutes at room temperature. The acetone mixture was removed and the plates dried for 20 minutes. The BioTek® plate washer was used for the remainder of the wash steps during the immuno-staining steps. Mouse anti-influenza A monoclonal antibody (Millipore, Temecula, CA) diluted 1:1000 in 1X PBS-Tween 20-1% BSA was added (100 µl) to each well, incubated for an hour, and washed three times with 1X PBS-Tween 20 (200 µl). The secondary antibody, peroxidase-labeled affinity purified goat anti-mouse IgG (KPL, Gaithersburg, MD) diluted 1:1000 in 1X PBS-Tween 20-1% BSA was then added to each well (100 µl), incubated at room temperature for an hr, and washed three times with 1X PBS-Tween 20 (200 µl). A substrate development solution consisting of phosphate-citrate buffer with sodium (Sigma-Aldrich, St. Louis, MO), o-phenylenediamine dihydrochloride (ODP) tablets (10 mg; Sigma-Aldrich, St. Louis, MO), and hydrogen peroxide (Sigma-Aldrich, St. Louis, MO) was added to each well and incubated at room temperature for 10 minutes, followed by 33 µl of sulfuric acid. The samples in the 96-well plates were read by a Synergy II Plate Reader (BioTek® Instruments) with the following Gen5™ (versions 1.11 and 2.00) program set for reading a 96-well full plate at 490 λ absorbance. Data output was transferred to Microsoft Excel (version 14; Redmond, WA) and the TCID50 for each sample was calculated following the method of Reed and Muench (25).

Data Analysis: Microsoft Excel (version 14; Redmond, WA) was used for data formatting, log10 transformation, and averaging, while IBM SPSS (version 19; Somers, NY) was used for descriptive statistics (median, mean, minimum, and maximum) and the box plot graphic. The virus concentration for each coupon was log10 transformed. The triplicate coupons for each time-point were averaged and the log10 change was calculated by subtracting the log10 virus per
coupon from the samples respective time-point zero log_{10} per coupon. The log_{10} change relative to the zero time-point was used for statistical analysis.

SAS (version 9.2; Cary, NC) was used to create general linear models that assessed the potential relationships between mean log_{10} change of virus concentration and the three independent parameters under study: viral media, absolute humidity and die-off time. For these analyses, the data points were used individually and not averaged. Maximum Likelihood Estimates (MLEs) and standard errors were analyzed to determine statistical differences within the levels of each parameter (i.e. sample matrix, absolute humidity, and time-points). To account for correlation of mean log_{10} differences due to clustering of replicates over time the method of generalized estimating equations (GEE) with a compound symmetrical correlation structure was implemented. GEE parameter estimates and robust empirical standard errors were obtained using p = 0.01 as the significance-level for staying in the model.

RESULTS

The average starting inoculum was 4.3 x 10^5 pH1N1 TCID_{50} per ml (100 µl; n=27), or 4.3 x 10^4 pH1N1 TCID_{50} per coupon (100 µl), and the average recovery concentration was 1 x 10^3 H1N1 TCID_{50} per ml, or 1 x 10^2 H1N1 TCID_{50} per coupon, at time-point zero after 1 hour of drying. The loss in recovery is attributed to desiccation and/or attachment to the N95. The infectivity of pH1N1 TCID50 per coupon is represented by its respective log_{10} concentration in Figure 2. The overall trend shows a decrease in infectivity over the 6 days for each matrix and AH. For pH1N1 in viral media, the recovered median log_{10} per coupon concentration started at 1.80, 2.40, and 1.20 for 4.1 mb, 6.5 mb, and 14.6 mb, respectively, and decreased to 0.00, 0.94, and 0.16 at the 144 h end-point. In FBS (2%), the recovered TCID_{50} log_{10} per coupon
concentrations started at 1.36, 1.49, and 1.35 for 4.1 mb, 6.5 mb, and 14.6 mb, respectively, and decreased to 0.00, 0.77, and 0.42 at the 144 h end-point. In mucin (5 mg/ml), the recovered concentrations started at 1.04, 2.12, and 2.28 for 4.1 mb, 6.5 mb, and 14.6 mb, respectively, and decreased to 0.16, 0.16, and 0.72 by the 144 h end-point [except for the 4.1 mb condition where the last time-point included was 72 hours].

The log_{10} change, as compared to the zero time-point, for each of the parameters similarly illustrates the reduction in infectivity over time (Table 1). The range of log_{10} change (lowest to highest) for 4.1 mb was 0.01 to -1.33, -0.06 to -0.56, and -0.13 to -0.59 for viral media, FBS, and mucin, respectively. The range of log_{10} change for 6.5 mb was -0.85 to -1.34, 0.00 to -1.40, and -0.70 to -1.72 for viral media, FBS, and mucin, respectively. The range of log_{10} change for 14.6 mb was 0.07 to -0.97, -0.14 to -1.10, and -0.77 to -1.99 for viral media, FBS, and mucin, respectively.

The MLE univariate analysis revealed significant differences within each of the parameter groups in regards to the log_{10} change of pH1N1 influenza A virus (Table 2). The higher VP AHs (mb), 14.6 mb (p-value ≤0.01) and 6.5 mb (p-value ≤0.0001), resulted in significantly greater pH1N1 log_{10} reductions relative to the reference VP AH of 4.1 mb (p-value ≤0.0001). This translates into an overall model estimate of a -0.69 and -0.75 log_{10} reduction for 14.6 mb and 6.5 mb, respectively, when AH is evaluated alone regarding pH1N1 survival on N95 (Table 2, where the intercept is added with MLE for the individual parameter). pH1N1 survived significantly longer in viral media (p-value ≤0.0001), the reference matrix, than in FBS and mucin. However, the log_{10} changes of pH1N1 in FBS (p-value = 0.08) and mucin (p-value = 0.07) were not significantly different from one another. pH1N1 reductions in infectivity at the tested time-points significantly increased from the reference point of 4 h with the exception of
the 12 hour time-point (p-value = 0.31), where the significance levels were as follows: 24 h – p-value ≤ 0.01, 48 h – p-value = <0.01, 72 h – p-value ≤ 0.01, and 144 h – p-value ≤ 0.0001.

Meaning, there was subsequently greater loss in survival over time, where the log₁₀ reductions at 4 h, 48 h, and 144 h (6 days) estimated by the model were -0.35, -0.71, and -0.97, respectively (Table 2, intercept added with MLE for the individual parameter).

The GEE multivariate analysis data, shown in Table 3, demonstrates the overall log₁₀ change of pH1N1 for each parameter when the data is simultaneously modeled, taking into account the potential correlation between the triplicate experimental runs. The parameters that had a significant impact on the TCID₅₀ log₁₀ change of pH1N1 in the given conditions were the VP AHs (mb) of 14.6 mb (α ≤ 0.01) and 6.5 mb (α ≤ 0.01), as well as the time periods of 144 h (α ≤ 0.01) and 24 to 72 h (α ≤ 0.01). The GEE model illustrates that the log₁₀ reduction of pH1N1 of -0.53 and -0.47 was attributed to the 6.5 mb and 14.6 mb VP AH (mb), respectively, while the log₁₀ reduction of -0.74 and -0.52 could be credited to the 144 h and 24 to 72 h time periods (Table 3, intercept added with GEE for the individual parameter). The matrices (viral media, FBS, and mucin) did not have a significant impact on the survival of pH1N1.

DISCUSSION

Overall, pH1N1 (A/California/04/2009) remained infectious for 6 days with approximately 1 log₁₀ loss when deposited onto coupons of N95 respirators in the given conditions. While AH impacted survival in our experiments, the GEE multivariate analysis of factors suggested that the main component affecting survival at 6 days was elapsed time to contribute an overall -0.74 TCID₅₀ log₁₀ reduction. Although the concentration of influenza potentially transferred from respirator to hand/ fingers is unknown, understanding that influenza may remain infectious for 6
days on the exterior side of a respirator is vital for healthcare personnel, patients, and visitors to understand that there may be a risk for transmission. Healthcare personnel who are in constant contact with confirmed or suspected cases should dispose of their respirator prior leaving the patients room.

There are three papers published specifically on the influenza virus’s persistence on respirators or porous surfaces. Bean et al. researched the survival of an A/Brazil/11/78 (H1N1)-like virus on pajama’s, tissue, magazines, and handkerchiefs (16). Greatorex et al. examined survival of A/Cambridge/AHO4/2009 (H1N1) virus on various household materials including porous surfaces of a J-cloth, silver containing cloth, and soft toy (21). Sakaguchi et al. studied the survival of A/PR/8/34 (H1N1) virus on N95 respirators (Hi-Luck 350), surgical masks, and hospital gowns (20). Bean et al. and Sakaguchi et al. used cell culture to measure infectivity with an approximate absolute humidity (AH) of 14.6 mb (16, 20), while Greatorex et al. had set AH conditions around 5 mb (21). All three studies came to a similar conclusion that influenza A viruses show reductions in infectivity within 24 to 48 hours. An additional study examined the persistence of various influenza A viruses on bank notes and found that influenza A/Moscow/10/99 (H3N2) to remained infectious up to three days, and with increased survival in respiratory secretions (32).

The results from this study conflict with Bean et al. 1982 and Sakaguchi et al. 2010, which used an A/Brazil/11/78 (H1N1)-like and A/PR/8/34 (H1N1) viruses, respectively. The two studies can be compared with our study in terms of influenza virus survival within viral media at ~14.6 mb (27.8°C-28.3°C/ 35%-40% RH (16) and 25.2°C/ 55% RH (20)). A 3 log_{10} reduction in A/PR/8/34 infectivity was observed after 24 hours on N95 respirators (20), while A/Brazil/11/78 (H1N1)-like was undetectable (~ 3 log_{10} loss) within 8-12 hours on porous
surfaces (16). We studied the pH1N1 A/California/04/2009 virus and found only an overall 0.43 log\(_{10}\) reduction at 24 hours and a 0.97 log\(_{10}\) reduction after 6 days at the 14.6 VP AH (mb) (Table 1). A main component of persistence and infectivity is the interaction between the viral envelope and AH, as viruses with greater lipid content persist better in lower humidity conditions (13). Viral mutations and reassortments in the year-to-year strains provide the virus with new survival capabilities. While all three studies used cell culture infectivity methods and the three strains are all descendants of the 1918 pandemic virus, the pH1N1 virus was a reassortment of the North American swine (H3N2 and H1N2) and Eurasian H1N1 viruses (33, 34). This reassortment may be responsible for the greater persistence of pH1N1 virus as compared to the A/Brazil/11/78 (H1N1)-like and A/PR/8/34 (H1N1) viruses in the given conditions. The unusual constellation of genes from multiple lineages (34) was a factor in greater persistence due to the new, unevaluated structural components.

As previously discussed, AH significantly impacts the survival of aerosolized influenza (24, 35). Besides the present study, only one other study evaluated the survival of H1N1 on respirators (20). As previously noted, Sakaguchi et al. found a marked loss of infectivity at only 24-48 hours and although this may largely reflect differences in the HIN1 strains, the only conditions they studied consisted of relatively high AH of ~14.6 mb (25.2°C/55% RH) (20). We evaluated three different AHs and found that log\(_{10}\) reductions were significantly higher at both 6.5 mb (p<0.01) and 14.6 mb (p<0.0001) than at the lowest condition of 4.1 mb (Table 2). The multivariate GEE analysis, where all parameters are simultaneously evaluated, further confirms the important role of increased AH on overall decreased pH1N1 survival (Table 3). Humidity is controlled in healthcare facilities and the “Ventilation Standard for Health Care Facilities” lists recommended humidity for a variety of healthcare spaces (i.e. trauma room,
wound intensive care) ranging from 30 to 60% for relative humidity and 20 to 24°C for temperature (36). This equates to an absolute humidity as measured by vapor pressure at approximately 7 mb to 18.3 mb, of which this study examined two conditions (4.1 mb, 6.5 mb) at the lower end and one condition at the higher end of the scale (14.6 mb). Hence, our study approximated AH typically present in many healthcare settings. Extra caution, however, should be taken in U.S. temperate regions during the winter time to properly adjust the humidity settings to stay within this approved range.

Because influenza is dispersed via small or large respiratory droplets, mucus and saliva are the most like matrices in which the virus is deposited upon surfaces. Mucus and saliva mainly consist of water with mucous glycoproteins, free proteins, and other electrolytes being the remaining constituents [36, 37]. The substrates we used to suspend the virus were meant to simulate key constituents of phlegm or saliva, and did not appear to have an important impact the persistence of pH1N1. This suggests similar precautions for preventing fomite transmission of influenza should be taken regardless of presence or absence of visible respiratory secretions.

The strategic national stockpile contained a variety of National Institute of Occupational Safety and Health (NIOSH)-approved particulate N95 respirators (3M, Moldex, Moldex-Metrix, Kimberly Clark, and Gerson) that were authorized for release for emergency use during the 2009 H1N1 pandemic (26). The CDC’s ‘Interim Guidance on Infection Control Measures for 2009 H1N1 Influenza in Healthcare Settings, Including Protection of Healthcare Personnel’ recommended the use of a fit-tested N95 respirator (NIOSH approved) for healthcare personnel within 6 feet of a patient or within a small enclosed airspace with a suspected or confirmed H1N1 patient (2). The 3M N95 respirator meets the 95% efficiency level and N series tests with NaCl, hence the name N95, and is comprised of three layers. The details of the layers are...
proprietary; however, the electrostatically charged polypropylene fibers play an important role in protecting the user from viruses. There are publications citing potential disinfection methods for contaminated respirators (27-30) that maintain the respirators integrity (31); however, decontamination of disposable FFR for the purpose of reuse has not been recommended by the CDC.

The pH1N1 pandemic stressed the healthcare and public health infrastructure with such challenges as how to effectively disseminate vaccines and respirators in a rapid manner to healthcare personnel and facilities. Although heroic efforts were put forth in addressing these challenges, it is fortunate that the 2009 H1N1 strain was a less virulent strain than initially anticipated. Particulate respirators (i.e. N95s) were incorporated into the CDC’s “Interim Guidance on Infection Control Measures for 2009 H1N1 Influenza in Healthcare Settings, Including Protection of Healthcare Personnel” as a cautionary approach during the pandemic (2). However, as the pandemic progressed and supplies were exhausted, questions about the persistence of influenza on porous media and respirators were posed due to a desire to increase respirator supply through extended wear and re-use. This research supports the discarding of respirators after close contact with a person having a suspected or confirmed influenza infection due to the virus’s demonstrated ability to persist for 6 days on the outside of the FFR with only an approximate 1 log_{10} loss in infectivity. While this study examined the impact of AH on pH1N1 on the exterior of FFRs, it is worthy to note a person’s respiration and water vapor on the inside of the respirator may also play a role in persistence and infectivity. The starting concentration of influenza on respirators, transmission rate from fomites to hands, and the human infectious dose remain unclear. Further research is needed to determine the risk of transmission from influenza contaminated respirators.
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Table 1. Mean TCID<sub>50</sub> log<sub>10</sub> change per coupon (standard deviation) of the infectivity of pH1N1 virus on N95 coupons in relation to time-point zero for each matrix and absolute humidity (VP mb) over time, where each value represents n=9 (exceptions listed in materials and methods section) and FBS is fetal bovine serum.

<table>
<thead>
<tr>
<th>Time-point (hours)</th>
<th>Absolute Humidity (mb)</th>
<th>4.1</th>
<th>6.5</th>
<th>14.6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viral Media</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>-0.09 (1.1)</td>
<td>-1.11 (1.2)</td>
<td>0.07 (1.3)</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>-0.25 (1.5)</td>
<td>-0.94 (1.0)</td>
<td>-0.56 (0.3)</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>-0.68 (0.6)</td>
<td>-0.89 (1.5)</td>
<td>-0.43 (0.5)</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>0.01 (1.9)</td>
<td>-0.98 (0.8)</td>
<td>-0.38 (0.7)</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>-0.70 (0.8)</td>
<td>-0.85 (1.0)</td>
<td>-0.52 (0.6)</td>
<td></td>
</tr>
<tr>
<td>144</td>
<td>-1.33 (0.0)</td>
<td>-1.34 (0.8)</td>
<td>-0.97 (0.0)</td>
<td></td>
</tr>
<tr>
<td>FBS (2%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>-0.06 (0.8)</td>
<td>0.00 (2.5)</td>
<td>-0.21 (1.2)</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>-0.25 (0.5)</td>
<td>-0.94 (1.3)</td>
<td>-0.14 (1.4)</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>-0.32 (0.1)</td>
<td>-0.88 (1.3)</td>
<td>-0.54 (0.6)</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>-0.28 (0.8)</td>
<td>-1.32 (0.6)</td>
<td>-1.10 (0.0)</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>-0.56 (0.2)</td>
<td>-1.36 (0.4)</td>
<td>-0.77 (0.6)</td>
<td></td>
</tr>
<tr>
<td>144</td>
<td>-0.48 (0.0)</td>
<td>-1.40 (0.9)</td>
<td>-0.66 (0.9)</td>
<td></td>
</tr>
<tr>
<td>Mucin (5 mg/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>-0.13 (0.6)</td>
<td>-0.85 (1.2)</td>
<td>-0.94 (1.6)</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>-0.17 (0.7)</td>
<td>-0.70 (1.4)</td>
<td>-0.95 (1.5)</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>-0.58 (0.0)</td>
<td>-1.01 (1.1)</td>
<td>-1.77 (0.3)</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>-0.54 (-0.7)</td>
<td>-0.70 (1.6)</td>
<td>-1.99 (0.1)</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>-0.59 (0.3)</td>
<td>-0.92 (1.4)</td>
<td>-0.85 (1.8)</td>
<td></td>
</tr>
<tr>
<td>144</td>
<td>no data</td>
<td>-1.72 (0.4)</td>
<td>-0.77 (1.9)</td>
<td></td>
</tr>
</tbody>
</table>
Table 2. Maximum likelihood estimates (MLE) univariate analysis (standard error, SE) of the infectivity of pH1N1 virus on N95 coupons (significance limit of p-value <0.01 as determined by a regression model). The MLE results can be translated to overall mean log$_{10}$ change (Overall MLE*) by adding the MLE value for the individual parameter with its respective intercept value.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Overall MLE$^*$ log$_{10}$ change</th>
<th>Estimate (SE)</th>
<th>Confidence Limits</th>
<th>p-value (α)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Absolute Humidity (Vapor Pressure mb)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>---</td>
<td>-0.40 (0.07)</td>
<td>-0.27 to -0.53</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>4.1</td>
<td>-0.40</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>6.5</td>
<td>-0.75</td>
<td>-0.35 (0.09)</td>
<td>-0.18 to -0.53</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>14.6</td>
<td>-0.69</td>
<td>-0.29 (0.09)</td>
<td>-0.11 to -0.47</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>(b) Matrix</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>---</td>
<td>-0.63 (0.06)</td>
<td>-0.51 to -0.75</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Viral Media</td>
<td>-0.63</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Mucin (5 mg/ml)</td>
<td>-0.79</td>
<td>-0.17 (0.09)</td>
<td>0.01 to -0.34</td>
<td>0.07</td>
</tr>
<tr>
<td>FBS (2%)</td>
<td>-0.48</td>
<td>0.15 (0.09)</td>
<td>0.32 to -0.02</td>
<td>0.08</td>
</tr>
<tr>
<td>(c) Time-point (hours)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>---</td>
<td>-0.35 (0.08)</td>
<td>-0.19 to -0.51</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>4</td>
<td>-0.35</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>12</td>
<td>-0.47</td>
<td>-0.12 (0.12)</td>
<td>0.11 to -0.36</td>
<td>0.31</td>
</tr>
<tr>
<td>24 (1 day)</td>
<td>-0.66</td>
<td>-0.31 (0.12)</td>
<td>-0.07 to -0.54</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>48 (2 days)</td>
<td>-0.71</td>
<td>-0.36 (0.12)</td>
<td>-0.13 to -0.60</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>72 (3 days)</td>
<td>-0.70</td>
<td>-0.35 (0.12)</td>
<td>-0.12 to -0.58</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>144 (6 days)</td>
<td>-0.97</td>
<td>-0.62 (0.13)</td>
<td>-0.36 to -0.88</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>
Table 3. Generalized estimated equations (GEE) analysis of the infectivity of pH1N1 on N95 coupons (SE = standard error; significance limit of p-value <0.01).*

<table>
<thead>
<tr>
<th></th>
<th>Overall** log₁₀ change</th>
<th>Estimate (SE)</th>
<th>Confidence Limits</th>
<th>p-value (α)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>---</td>
<td>-0.22 (0.10)</td>
<td>-0.03 to -0.41</td>
<td>0.02</td>
</tr>
<tr>
<td>4.1 mb</td>
<td>-0.22</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>6.5 mb</td>
<td>-0.53</td>
<td>-0.31 (0.09)</td>
<td>-0.13 to -0.49</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>14.6 mb</td>
<td>-0.47</td>
<td>-0.25 (0.08)</td>
<td>-0.08 to -0.41</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Viral Media</td>
<td>-0.22</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Mucin (5 mg/ml)</td>
<td>-0.38</td>
<td>-0.17 (0.09)</td>
<td>-0.34 to 0.02</td>
<td>0.08</td>
</tr>
<tr>
<td>2% FBS</td>
<td>-0.05</td>
<td>0.17 (0.10)</td>
<td>0.36 to -0.02</td>
<td>0.08</td>
</tr>
<tr>
<td>4 to 12 h</td>
<td>-0.22</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>24 to 72 h</td>
<td>-0.52</td>
<td>-0.30 (0.08)</td>
<td>-0.14 to -0.46</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>144 h</td>
<td>-0.74</td>
<td>-0.53 (0.14)</td>
<td>-0.25 to -0.80</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

*The parameter estimates can be used to calculate mean log₁₀ change (Overall GEE**) by adding the estimate for an individual parameter with the intercept value. A further model estimation can be obtained by combining the intercept (-0.22) with parameters in a given scenario, for example at 14.6 mb absolute humidity estimate (-0.25) in 5 mg/ml mucin estimate (-0.17) for 144 hours (-0.53) results in a -1.17 mean log₁₀ reduction of Influenza A H1N1 on N95 coupons.
Figure 1. Experimental design and photographs of the procedure for inoculating pH1N1 virus on N95 respirators, where (a) pH1N1 in the sample matrix is inoculated on to the exterior layer of three N95 coupons, (b) spread evenly for homogenous distribution and to aid in drying, (c) storage of inoculated coupons in an environmental chamber at the defined parameters, and (d) after vortexing and centrifugation, the sample was filtered through a syringe and stored at -80°C until ELISA processing.

Experimental Design

- **18°C/ 20% - 4.1 mb**
  - Time points: 0, 4, 12, 24, 48, 72 hours (n=2)
  - Time points: 0, 4, 12, 24, 48, 72, and 144 hours (n=1)

- **25°C/ 20% - 6.5 mb**
  - Time points: 0, 4, 12, 24, 48, 72, and 144 hours (n=3)

- **21°C/ 58.5% - 14.6 mb**
  - Time points: 0, 4, 12, 24, 48, 72, and 144 hours (n=3)

**pH1N1 Inoculation and Processing**

(a)  (b)

(c)  (d)
Figure 2. The pH3N1 virus TCI\(_{50}\), log\(_c\) concentration per coupon over time (6 days) for different matrices and absolute humidity's, where the middle mark in each bar represents the median, the top and bottom of the bars represent the 25\(^{th}\) and 75\(^{th}\) percentiles, and the error bars represent the 95\(^{th}\) confidence intervals. The first, second, and third bars for each time group are the results from 4.5 mH, 6.5 mH, and 14.5 mH absolute humidity conditions. Each bar represents n=3 coupons.