MS2 Coliphage as a Surrogate for 2009 Pandemic Influenza A (H1N1) Virus (pH1N1) in Surface Survival Studies on N95 Filtering Facepiece Respirators

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ABSTRACT

Research on influenza viruses regarding transmission and survival has surged in the recent years due to infectious emerging strains and outbreaks such as the 2009 Influenza A (H1N1) pandemic. MS2 coliphage has been applied as a surrogate for pathogenic respiratory viruses, such as influenza, as it's safe for personnel to handle and requires less time and labor to measure virus infectivity. However, direct comparisons to determine the effectiveness of coliphage as a surrogate for influenza virus regarding droplet persistence on personal protective equipment such as N95 filtering facepiece respirators (FFRs) are lacking. Persistence of viral droplets deposited on FFRs in healthcare settings is important to discern due to the potential risk of infection via indirect fomite transmission. The objective of this study was to determine if MS2 coliphage could be applied as a surrogate for influenza A viruses for studying persistence when applied to the FFRs as a droplet. The persistence of MS2 coliphage and 2009 Pandemic Influenza A (H1N1) Virus on FFR coupons in different matrices (viral media, 2% fetal bovine serum, and 5 mg ml^{-1} mucin) were compared over time (4, 12, 24, 48, 72, and 144 hours) in typical absolute humidity conditions (4.1×10^5 mPa [18°C/20% relative humidity (RH)]). Data revealed significant differences in viral infectivity over the 6-day period (H1N1- $P < 0.0001$; MS2 - $P < 0.005$), although a significant correlation of viral $\log_{10}$ reduction in 2% FBS ($P < 0.01$) was illustrated. Overall, MS2 coliphage was not determined to be a sufficient surrogate for influenza A virus with respect to droplet persistence when applied to the N95 FFR as a droplet.

Keywords: Pandemic influenza A virus, H1N1, MS2 coliphage, infectivity, surrogate, respirator.

INTRODUCTION

Personal protective equipment (PPE) serves as the last line of defense in the hierarchy of protective measures for healthcare workers (HCWs) treating patients with influenza. For respiratory protection, the CDC recommends N95 filtering facepiece respirators (FFRs) for HCWs during aerosol generating procedures performed on patients with seasonal influenza and often recommends the use of FFRs for treating patients with novel respiratory pathogens such as 2009 Influenza A (H1N1; CDC 2013). Influenza virus transmission routes continue to be a point of discussion and fomite transmission in healthcare settings is an important route to consider. It's particularly important for HCWs to follow these guidelines in addition to proper infection control procedures (e.g. hand hygiene) as they attend their patients due to the potential transmission pathways for influenza and other infectious agents. Although direct scientific evidence is lacking, FFRs used by HCWs for respiratory protection can potentially serve as a source of infectious pathogens if contaminated by spreading from the FFR to the wearer and others via indirect contact. For routine single use, where the FFRs are immediately disposed after one patient encounter, contact transfer of pathogens should not occur if the wearer complies with proper FFR use guidance, which states that the hands of the HCW should never come in contact with the contaminated surface of the FFR. Correctly removing or 'doffing' an FFR, requires the wearer to grab the straps of the FFR located at the back of the head and avoid touching the surface (CDC). However, studies evaluating HCWs respiratory or PPE protection programs observed differing rates of correctly doffing respirators (7.2% in California hospitals during the 2009-2010 H1N1 influenza pandemic - Beckman et al. 2013; 62.5% in New York State hospitals - Hines et al. 2013) or masks (72% in Canadian acute care hospitals - Mitchell et al. 2013). Two of these studies incorporated hand hygiene observations and the percentage of HCWs that conducted proper hand hygiene after removing the PPE were comparatively low (47.1% - Beckman et al. 2013; 43% - Mitchell et al. 2013). An additional study from Canada during the 2003 SARS outbreak showed 35% of HCWs were not using proper hand hygiene after removing their protection, while hand hygiene may have been conducted before the removal (Raboud et al. 2010). It's reasonable to ascertain that contact transfer from facemask, including FFRs, is possible; however the risks associated with the contact transmission depends on many factors including pathogen contamination levels and persistence.

Although transmission of infectious respiratory viruses on FFRs used during healthcare practices is currently unknown, Coulliette et al. 2013 revealed that influenza A (H1N1) viruses may remain infectious for at least six days on a N95 FFR. While this study directly measured infectious influenza A virus, numerous studies have used surrogates for respiratory viruses due to the technical skills required, costly infectivity detection methods and biosafety risks involved with handling human respiratory viruses. MS2 coliphage, a male-specific coliphage specific to *Escherichia coli*, has been consistently used as a surrogate for respiratory viruses. For example, MS2 coliphage has been applied as a surrogate for airborne human pathogenic viruses, referring to influenza virus as a common discussion point, for the development of aerosol chambers (Woo et al. 2010), examination of re-aerosolization from N95 FFRs during simulated coughs (Fisher et al. 2012), comparison of disinfection approaches for FFRs (Vo et al. 2009, Rengasamy et al. 2010, Damit et al. 2011, Fisher et al. 2011a, and Fisher et al. 2011b), measurement of hospital air handling systems (Griffiths et al. 2005), and disinfection techniques for airborne viruses (Walker and Ko 2007). Additional studies focused on enteric and respiratory pathogens regarding fomite transfer (Lopez et al. 2013), surface survival and persistence (Liu et al. 2012), sodium hyphochlorite disinfection on stainless steel (Park and Sobsey 2011), fomite transfer from fingerpads (Julian et al. 2010), and efficacy of hand hygiene agents (Sickbert-Bennett et al. 2005) have also incorporated MS2 coliphage as a surrogate. The reliance on MS2 coliphage as a surrogate for respiratory pathogens in research aiming to examine PPE, engineering controls and recommendations for protective measures (e.g. respirator reuse, aerosolization of viruses from respirators, hospital air handling systems, fomite transfer via health practitioner hands) could impact public health decisions based upon interpretations of such data for exposure and risk values in healthcare settings.

To determine if MS2 coliphage could be applied to FFRs as droplets as a surrogate for influenza A viruses for droplet persistence, MS2 coliphage and the 2009 Pandemic Influenza A (H1N1) Virus (pH1N1) were simultaneously measured and compared on N95 FFRs at a typical indoor absolute humidity (AH) of 4.1 x 10^5 mPa [18°C/20% relative humidity (RH)] over a 6-day period in different matrices. Our null hypothesis was that there would be no difference or correlation in persistence between MS2 coliphage and pH1N1 or impact of different matrices on these parameters. Similar correlation in persistence levels would allow MS2 coliphage to be appropriately applied as a surrogate for influenza virus studies.

METHODS AND MATERIALS

The experimental design, including pH1N1 viral propagation and infectivity method, test matrices preparation, N95 FFR coupon creation, AH conditions and calculations, sample processing, ELISA, and data analysis are all fully described in Coulliette et al. 2013. Briefly, pH1N1 [(Influenza A/ California/04/2009 H1N1 (influenza A [pH1N1])] and MS2 coliphage (ATCC 15597-B1) were combined equally with the sample matrices of viral media (Dulbecco's modified Eagle media [DMEM]), 2% fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA), and 5 mg ml^{-1} mucin (BP Biomedicals, Soloni, OH), deposited one liquid droplet (100 µl) onto N95 FFR (3M model no. 8210) coupons (3.8 to 4.2 cm^2) using a cell spreader to evenly distribute the solution, allowed the droplet to air dry for 1 hour (BSC sash closed and blower off), placed in an environmental chamber (model no. 6030; Carion, Marietta, OH) at an AH of 4.1 x 10^5 mPa and removed for viral testing at time-points 0, 4, 12, 24, 48, 72, and 144 hours. The conditions of the study at 4.1 x 10^5 mPa [18°C/20% relative humidity (RH)] simulate typical indoor conditions. To recover pH1N1 and MS2 coliphage from the coupons, the coupons were placed in 5 ml of 2% BSA-1X PBS (pH 8.5) and subjected to the following steps to purify the sample: vortex - 20 min, centrifugation - 5 min at 3,000X *g*, filtration - premoistened (2% BSA-1X PBS) 0.22 m syringe filter (Fisherbrand, Pittsburgh, PA, Millex-GS, Billerica, MA). This recovery method was compared to other processed and determined to yield the highest recovery (data not shown). Some loss may have occurred during the recovery step, as Li et al. (2009) showed approximately one-half $\log_{10}$ loss for MS2 coliphage and nanofiber filters. However, in this study, viral concentrations were measured for time-point zero after this recovery process, were at high enough concentrations to measure reduction over the six days, and the authors assumed negligible loss from the vortex step. Persistence of pH1N1 was quantified by using an enzyme-linked immunosorbent assay (ELISA) to determine the $\log_{10}$ tissue culture infectious dose of 50% ($TCID_{50}$) per coupon as previously described (Coulliette et al. 2013), while MS2 coliphage was enumerated by single agar layer plaque assay using *E. coli* (ATCC 15597) as previously described (EPA 2001), using ATCC media for propagating and plating (Fisher et al. 2009). For each plate, the plaque forming units (PFU) were counted and only values between 30 and 300 PFU were recorded. The experiment was repeated three times (n=3) and triplicate samples were processed for each method. Generalized Estimating Equations (GEE) was used to account for potential correlation within replicates. Descriptive statistics were conducted using Microsoft Excel v14 (Redmond, WA), while statistical analyses for Spearman coefficients, maximum likelihood estimates (MLEs), and GEE- were processed using SAS 9.3 (Cary, NC). The significance level was set at a $P \leq 0.01$.

RESULTS

The average starting inoculum for pH1N1 and MS2 coliphage across all matrices was 8.28 x 10^3 $\log_{10}$ $TCID_{50}$ (±5.69 x 10^3) and 1.44 x 10^{10} PFU per coupon (±2.04 x 10^{10}), respectively. The initial recovery concentration (time 0) from the N95 FFR coupons for pH1N1 and MS2 coliphage across all matrices was 3.23 x 10^1 $\log_{10}$ $TCID_{50}$ (±2.33 x 10^1; 0.39% recovery) and 5.72 x 10^8 PFU (±6.74 x 10^8; 3.98% recovery) per coupon, respectively.

The mean log_{10} change in viral media from the initial concentration to the final time-point (144 hr) was -1.33 for pH1N1 and -1.28 for MS2 coliphage. The viruses showed a weak correlation in viral media (0.30, *P*=0.04; Table I). The mean log_{10} change in 2% FBS from the initial concentration to the final time-point (144 hr) was -0.48 for pH1N1 and -0.35 for MS2 coliphage. The viruses showed a significant positive correlation across time-points in 2% FBS (0.44, *P* <0.01; Table I). The mean log_{10} change of infectious virus in mucin (5 mg ml^{-1}) from the initial concentration to the final time-point (72 hr) was -0.59 for pH1N1 and to the final time-point (144 hr) -0.18 for MS2 coliphage. No association was demonstrated in mucin (0.18, *P*=0.32; Table I). Generally, greater reduction in the mean log_{10} change per coupon of infectious virus over time was determined for pH1N1 in all matrices and for MS2 coliphage in viral media, while minimal or variable reduction was seen for MS2 coliphage in 2% FBS and artificial mucus (Table I).

Univariate analysis (MLE) revealed that time was not significantly associated with the persistence of either virus (*P* >0.01), and thereby was not included in the multivariate analysis (data not shown). The GEE analysis within this study simultaneously evaluated pH1N1 and MS2 coliphage with the matrices, which demonstrated a significant difference in estimated log_{10} change of remaining infectious pH1N1 (-0.61, *P* <0.0001) and MS2 coliphage (0.25, *P* <0.005) viruses (Table 2; 'Estimated standard error (SE)' column). The matrices, viral media and 2% FBS, also contributed significantly to virus persistence characteristics (Table 2; viral media, *P* <0.0001; 2% FBS, *P* <0.01). Viral media was responsible for -0.61 log_{10} change, while 2% FBS contributed to 0.40 log_{10} change when all the model parameters are considered (Table 2; 'Estimated (SE)' column). The cumulative log_{10} change can be interpreted from the GEE estimates for developing scenarios and revealed that the lowest persistence was observed from pH1N1 in viral media with -1.22 log_{10} $TCID_{50}$ per coupon (sum of pH1N1, -0.61, and viral media, -0.61, cumulative log_{10} change parameter estimates). MS2 coliphage in 2% FBS showed the best persistence with 0.04 log_{10} PFU per coupon (sum of the intercept, -0.61, and estimated SE for MS2, 0.25, and 2% FBS, 0.40).

Table I. Concentration (standard deviation, SD) of 2009 Pandemic Influenza A H1N1 (pH1N1; $TCID_{50}$/ coupon) and MS2 Coliphage (PFU/coupon) on N95 FFR Coupons, as well as Mean Log_{10} Change per Coupon Relative to the Zero (0 h) Time-Point in (a) Viral Media, (b) 2% FBS and (c) Mucin (5 mg ml^{-1}), where each Value Represents n=9 except for 144 h (n=3). The Spearman Coefficients for pH1N1 and MS2 Coliphage within each Sample Matrix Are Noted as well.

(a) Viral Media (correlation: 0.30, *P*=0.04)

Time-point (h)	Concentration	Mean log_{10} change
pH1N1	$TCID_{50}$/coupon (SD)	
0	5.93×10^1 (1.79×10^1)	---
4	4.83×10^1 (1.36×10^1)	-0.09
12	3.32×10^1 (3.07×10^1)	-0.25
24	1.23×10^1 (4.21×10^0)	-0.68
48	6.13×10^1 (8.67×10^1)	0.01
72	1.18×10^1 ($6.10 \times 10^{0)}$	-0.70
144	2.79×10^0 (2.09×10^0)	-1.33
MS2 coliphage	PFU/coupon (SD)	
0	9.15×10^8 ($\pm 1.10 \times 10^9$)	---
4	2.29×10^8 ($\pm 3.62 \times 10^8$)	-0.60
12	1.54×10^8 ($\pm 2.16 \times 10^8$)	-0.77
24	3.69×10^8 ($\pm 5.64 \times 10^8$)	-0.39
48	3.97×10^8 ($\pm 5.61 \times 10^8$)	-0.36

72	6.23×10^8 ($\pm 9.12 \times 10^8$)	-0.17
144	4.79×10^7 ($\pm 3.70 \times 10^6$)	-1.28

(b) 2% FBS (correlation: 0.44, *P* <0.01)

Time-point (h)	**Concentration**	**Mean $\log_{10}$ change**
pH1N1	$TCID_{50}$/coupon (SD)	
0	2.60×10^1 (6.69×10^0)	---
4	2.29×10^1 (6.27×10^0)	-0.06
12	1.45×10^1 (3.09×10^0)	-0.25
24	1.24×10^1 (1.38×10^0)	-0.32
48	1.36×10^1 (6.51×10^0)	-0.28
72	7.40×10^0 (1.74×10^0)	-0.55
144	8.66×10^0 (6.00×10^0)	-0.48
MS2 coliphage	PFU/coupon (SD)	
0	3.21×10^8 (3.41×10^8)	
4	2.49×10^8 (2.52×10^8)	-0.11
12	3.49×10^8 (3.59×10^8)	0.04
24	4.06×10^8 (5.92×10^8)	0.10
48	5.06×10^8 (5.55×10^8)	0.20
72	3.05×10^8 (2.76×10^8)	-0.02
144	1.42×10^8 (8.99×10^7)	-0.35

(c) Mucin (5 mg ml^{-1}; correlation: 0.18, *P*=0.32)

Time-point (h)	**Concentration**	**Mean $\log_{10}$ change**
pH1N1	$TCID_{50}$/coupon (SD)	
0	1.15×10^1 (1.53×10^0)	---
4	8.43×10^0 (4.17×10^0)	-0.13
12	7.73×10^0 (4.84×10^0)	-0.17
24	2.99×10^0 (0.00×10^0)	-0.58
48	3.30×10^0 (2.18×10^{-1})	-0.54
72	2.98×10^0 (2.18×10^0)	-0.59
144	no data (nd)	nd
MS2 coliphage	PFU/coupon (SD)	
0	3.92×10^8 (4.20×10^8)	---
4	3.08×10^8 (2.68×10^8)	-0.11
12	2.09×10^8 (1.67×10^8)	-0.27
24	2.71×10^8 (3.34×10^8)	-0.16
48	3.86×10^8 (5.19×10^8)	-0.01
72	1.68×10^8 (1.32×10^8)	-0.37
144	2.59×10^8 (7.21×10^7)	-0.18

Table II. Generalized Estimated Equations (GEE) Analysis of the Infectivity of pH1N1 and MS2 Coliphage on N95 Coupons[a]

Parameter	Cumulative log_{10} change	Estimated (SE)	Confidence Limits	*P*-value (α)
Intercept	---	-0.61 (0.13)	-0.86 to -0.36	<0.0001
pH1N1[b]	-0.61	---	---	---
MS2	-0.36	0.25 (0.09)	0.08 to 0.43	<0.005
Viral Media[b]	-0.61	---	---	---
2% FBS	-0.21	0.40 (0.15)	0.10 to 0.70	<0.01
Mucin (5 mg ml^{-1})	-0.33	0.28 (0.16)	-0.04 to 0.59	0.09

[a]The parameters can be used to calculate mean log_{10} change (cumulative generalized estimated equation [GEE]) by adding the estimate for an individual parameter with the intercept value. A further model estimation can be obtained by combining the intercept (-0.61) with the parameters in a given scenario; for example, MS2 coliphage (0.25) in 2% FBS (0.40) results in a 0.04 mean log_{10} change of coliphage MS2 coliphage on N95 coupons within a six day time period at 4.1 x 10^5 mPa AH (set conditions of the study, where the AH represents typical indoor hospital air humidity).
[b]This group is the referent group and is reflective of the intercept.

DISCUSSION

The Recent aerosol studies have acknowledged the limitations of MS2 coliphage as a surrogate for human and animal respiratory viruses due to different viral behaviors (Turgeon et al. 2014, Zuo et al. 2013b). Noting that our study examined porous surface survival, not viral aerosols, our findings are in agreement as the analysis for this study reveals that pH1N1 and MS2 coliphage infectivity characteristics were significantly different in the tested conditions on N95 FFRs. The significant difference is likely attributed to pH1N1 being an enveloped virus and MS2 coliphage being a non-enveloped virus. The authors also acknowledge that viruses of the *Cystoviridae* family (e.g. Φ-6), which are enveloped but consist of segmented double-stranded RNA, have been shown as an adequate influenza virus surrogate regarding virus aerosol models (Turgeon et al. 2014), water survival and chlorine disinfection (Adcock et al. 2009), and thermal inactivation through composting (Elving et al. 2012). However, MS2 coliphage is still used in environmental microbiology research as an influenza surrogate due to (1) being readily available, (2) the coliphage being a safer, non-pathogenic microorganism (Griffiths et al. 2005), and (3) requiring only a BSL-I facility (Woo et al. 2010); thereby becoming a default influenza virus surrogate during the heightened awareness of potential influenza A virus (H1N1) transmission in healthcare settings. While using MS2 coliphage alone as a surrogate for influenza A virus for studies regarding infectivity, persistence, or viral behaviors is not recommended, using MS2 coliphage as a viral tracer for physical determinations (e.g. fomite transfer, measuring filtration, re-aerosolization, etc.) may be appropriate.

This concise viral comparison also provides valuable insight regarding methodology and persistence of MS2 coliphage and pH1N1 on N95 FFRs, a porous surface material. The authors recognize the limitations but being transparent can assist in future research. The differences in starting concentrations were an intrinsic result of differing propagating techniques that the authors were limited to using. While there was approximately 6 log_{10} difference between pH1N1 and MS2 coliphage inoculum, the viruses were well mixed to create a homogenous sample with the matrix. The inoculum concentration

differences could have played a role in the higher persistence of the MS2 coliphage virus. In addition, the recoveries of the viruses (0.39% to 2.65%) were lower as compared to other studies extracting avian influenza (approximately 20% to 80%) from similar polypropylene respirator materials after spike tests (Zuo et al. 2013a). It is unclear whether the methodologies for distributing influenza viruses were similar between studies, as the presented study used cell spreaders to evenly distribute the virus on the coupons and the coupons were not processed for time-point zero until the coupons were visibly dry. While other studies have recovered influenza viruses from various materials, the efficiency of recovery was not mentioned and future work to determine the most efficient method for recovering influenza from hospital PPE would be a significant contribution to this field.

Future surface survival and fomite transmission studies should incorporate several types of bacteriophages, such as non-enveloped MS2 coliphage to relate to previously conducted research and enveloped bacteriophages from the *Cystoviridae* family (e.g. Φ-6). There is a need for this type of evaluation of various hospital surfaces and PPE, porous and non-porous, as well as the potential transmission routes relative to influenza and other pathogenic viruses. Such research will identify adequate surrogates, or at least outline the limitations of the chosen surrogate. Overall, the authors recommend that a direct comparison between the pathogenic virus and surrogate should be conducted before relying on the surrogate as a model; and especially when calculating risk or making public health decisions based upon the surrogate data.

CONCLUSIONS

This study revealed significant differences in viral infectivity over the 6-day period (H1N1- P <0.0001; MS2 - P <0.005), although a significant correlation of viral $\log_{10}$ reduction in 2% FBS (P <0.01) was illustrated. Overall, MS2 coliphage was not determined to be a sufficient surrogate for influenza A virus with respect to droplet persistence when applied to the N95 FFR as a droplet.

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