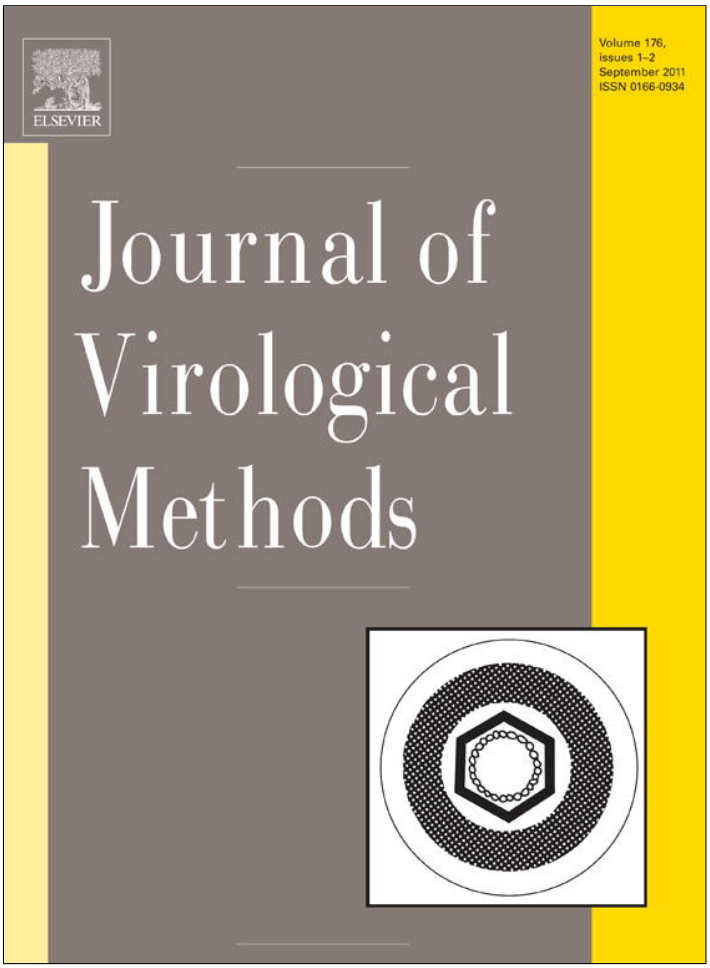

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Short communication

Enhanced detection of infectious airborne influenza virus

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A B S T R A C T

Current screening methodologies for detecting infectious airborne influenza virus are limited and lack sensitivity. To increase the sensitivity for detecting infectious influenza virus in an aerosol sample, the viral replication assay was developed. With this assay, influenza virus is first amplified by replication in Madin–Darby canine kidney (MDCK) cells followed by detection with quantitative PCR (qPCR). Spanning a 20-h replication period, matrix gene expression levels from infectious virus were measured at several time points using qPCR and found to exponentially increase. Compared with the traditional culture-based viral plaque assay, the viral replication assay resulted in a 4.6×10^5 fold increase in influenza virus detection. Furthermore, viral replication assay results were obtained in half the time of the viral plaque assay. To demonstrate that the viral replication assay is capable of detecting airborne influenza virus, dilute preparations of strain A/WS/33 were loaded into a nebulizer, aerosolized within a calm-air settling chamber and subsequently collected using NIOSH Two-Stage Bioaerosol Samplers. At the most diluted concentration corresponding to a chicken embryo infectious dose 50% endpoint ($CEID_{50}$) of 2.8E+02/ml, the viral replication assay was able to detect infectious influenza virus that was otherwise undetectable by viral plaque assay. The results obtained demonstrate that the viral replication assay is highly sensitive at detecting infectious influenza virus from aerosol samples.

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1. Introduction

Influenza is a highly contagious respiratory virus that causes severe morbidity and extensive mortality each year world-wide. A wide body of evidence shows that influenza spreads at least in part by contact and droplet transmission; however the amount of airborne transmission that occurs remains to be determined. Reviews on aerosol transmission by Tellier (2009) and Weber & Stilianakis (2008) suggest that airborne transmission plays a critical role in the spread of influenza. In contrast, Brankston et al. (2007) conclude that airborne transmission is an unlikely route in the spread of influenza virus. Despite conflicting reports, the role of airborne transmission cannot be over-looked.

Small airborne particles and/or droplet nuclei that are generated during respiratory activities such as coughing, breathing or sneezing can remain airborne for prolonged periods and dispersed by circulating air currents. Several studies have reported that influenza virus RNA is detectable in the exhaled breath and coughs of influenza patients (Fabian et al., 2008; Huynh et al., 2008; Stelzer-Braid et al., 2009; Lindsley et al., 2010a). The airborne distribution of influenza virus RNA was also examined in a hospital emergency department (Blachere et al., 2009) and in an urgent care clinic (Lindsley et al., 2010b). Results established that influenza virus was present and over half of the collected particles were detected within the respirable aerosol fraction. However, the question remains as to whether these airborne particles have the ability to cause acute infection in susceptible individuals. Due to inherent difficulties of stress-sensitivity, small particle size and low concentration, there is limited research that demonstrates the likelihood of airborne influenza transmission. Further studies are greatly needed to resolve the debate of short and long-range airborne influenza virus transmission in healthcare and other indoor settings.

Methods of detection play a critical role in evaluating airborne transmission. Although a number of detection methods are available, limitations such as specimen type, turnaround time, sensitivity and specificity exist. As with most bioaerosols, influenza-laden aerosol samples pose a particular challenge and require optimal methods of detection. Real-time quantitative PCR (qPCR) analysis has proven to be a sensitive method for quickly and accurately detecting influenza virus from aerosol samples (Blachere et al., 2009; Lindsley et al., 2010a,b; Hermann et al., 2006; Chen

Abbreviations: MDCK, Madin–Darby canine kidney; $CEID_{50}$, chicken embryo infectious dose 50% endpoint; $TCID_{50}$, tissue culture infectious dose 50% endpoint; qPCR, quantitative real-time polymerase chain reaction; PBS, phosphate-buffered saline; TPCK, N-*p*-tosyl-L-phenylalanine chloromethyl ketone; BSA, bovine serum albumin; PFU, plaque forming units.

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et al., 2009). However, qPCR alone does not distinguish between infectious and non-infectious virus. On the other hand, the viral plaque assay, tissue culture infectious dose 50% endpoint ($TCID_{50}$) assay, and chicken embryo infectious dose 50% endpoint ($CEID_{50}$) assay are important methods for establishing viral titers but are time consuming, labor intensive and limited with regards to detection threshold levels (Cottey et al., 2001; LaBarre and Lowy, 2001; Matrosovich et al., 2006). In order to enhance detection levels and reduce turnaround time, the purpose of this study was to develop a sensitive and specific assay that would detect infectious influenza virus from aerosol samples.

2. Materials and methods

2.1. Cell and virus stock

Madin–Darby canine kidney (MDCK) cells (ATCC CCL-34) were purchased from the American Type Tissue Culture (ATCC), Manassas, VA. Cells were propagated and maintained in 75-cm^2 Corning CellBind Surface flasks (Corning, NY). Complete growth medium for MDCK cells consisted of Eagle's Minimum Essential Medium (EMEM) (ATCC) containing 10% fetal bovine serum (Hyclone Laboratories, Inc., Logan, UT), 200 units/ml penicillin G/200 μg/ml streptomycin (Invitrogen, Carlsbad, CA). Cells were incubated at 35 °C in a humidified 5% CO_2 incubator until 90% confluent.

Influenza virus strain A/WS/33 (H1N1, ATCC VR-825, Lot # 58772128, 2.8×10^6 $CEID_{50}$/ml) was purchased from ATCC. Ten-fold serial dilutions of strain A/WS/33 were prepared in supplemented Hank's Balanced Salt Solution (HBSS) (Invitrogen) containing 0.1% bovine serum albumin (BSA) (Sigma–Aldrich, St. Louis, MO), 100 units/ml penicillin G and 100 units/ml streptomycin (Invitrogen).

2.2. Viral replication assay

To assess infectivity, the viral replication assay was performed as follows: MDCK cells cultured to approximately 90% confluence were detached with 0.25% Trypsin–EDTA (Invitrogen), washed and re-suspended in complete EMEM at a density of 2.5×10^5 cells/ml. Thereafter, 200 μl of the cell suspension was plated in triplicate in a CoStar 96-well plate (Corning, NY) and incubated overnight at 35 °C in a humidified 5% CO_2 incubator. The following day, cell monolayers were washed twice with 200 μl of phosphate-buffered saline (PBS), inoculated with 50 μl serial dilutions of strain A/WS/33 and incubated for 45 min at 35 °C in a humidified 5% CO_2 incubator. Mock infected cells (negative control) were inoculated with 50 μl PBS alone. Inoculated cells were then washed once with 150 μl of PBS and next overlayed with 200 μl supplemented Dulbecco's modified Eagle's medium (DMEM)/F12 containing 100 units/ml penicillin G/100 μg/ml streptomycin (Invitrogen), 2 mM L-glutamine (Invitrogen), 0.2% BSA (Sigma–Aldrich), 10 mM HEPES (Invitrogen), 0.22% sodium bicarbonate (Invitrogen), 0.01% DEAE-dextran (MP BioMedicals, LLC, Solon, OH), and 2 μg/ml N-*p*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) (Sigma–Aldrich). Treated cells were subsequently incubated at 35 °C in a humidified 5% CO_2 incubator. Following 20 h of incubation, the media was removed and cell monolayers were washed once with 200 μl of PBS. Upon removal of the PBS, 77 μl of Lysis/Binding Solution Concentrate (Ambion, Austin, TX) was added to each well and the plate was shaken at maximum speed for 1 min on a Titer Plate Shaker (Thermo Scientific, Asheville, NC). The 96-well plate containing the cellular lysate was next stored at −20 °C until RNA extraction and cDNA reverse transcription were performed. Matrix gene expression from the generated cDNA was analyzed with qPCR.

2.3. RNA isolation and cDNA transcription

For the viral replication assay, total RNA was isolated from MDCK cells infected with strain A/WS/33 using the MagMax™-96 Total RNA Isolation Kit (Ambion). Briefly, upon thawing of the cellular lysate containing the Lysis/Binding Solution Concentrate, 63 μl of 100% isopropanol (Sigma) was added to each well to complete the Lysis/Binding Solution preparation. Samples were thoroughly mixed by shaking at moderate speed for 1 min on a Titer Plate Shaker. The samples were next transferred to the provided U-bottom 96-well processing plate and total RNA was extracted according to the MagMax™-96 Total RNA Isolation Kit instructions. The final eluted total-RNA volume was 32 μl.

For qPCR determination of total virus, (Chamber nebulization experiments), viral RNA was isolated from the collected aerosol samples using the MagMax™-96 Viral RNA Isolation Kit (Ambion) as previously described (Blachere et al., 2009). The final eluted viral RNA volume was 32 μl. Total RNA (viral replication assay) and viral RNA (qPCR) were immediately transcribed into cDNA using the High Capacity RNA to cDNA Master Mix in accordance with the manufacturer's instructions (Applied Biosystems). The final cDNA volume was 40 μl.

2.4. Real-time qPCR analysis

qPCR analysis was performed using the following matrix-specific primers: Forward 5′AGATGAGTCTTCTAACCGAGGTCG 3′, Reverse 5′TGCAAAAACATCTTCA AGTCT CTG 3′ and probe: 6FAM-TCAGGCCCCCTCAAAGCCGA-MGBNFQ (Spackman et al., 2002). All primers and probes were synthesized by Applied Biosystems and used at a final concentration of 0.8 μM and 0.2 μM, respectively. Reactions were performed and analyzed using the Applied Biosystems 7500 Fast Real-Time PCR System under the following cycling conditions: 95 °C for 20 s followed by 40 cycles at 95 °C for 3 s and 60 °C for 30 s. To determine the relative gene copy number from strain A/WS/33-infected MDCK cells (viral replication assay) and the collected aerosols, a standard curve was generated from 10-fold serial dilutions of the cloned influenza H1N1 matrix gene and analyzed concurrently with all qPCR reactions. A negative control without template was also included in all real-time PCRs. All reactions were run in duplicate.

2.5. Influenza growth kinetic experiments

Utilizing the viral replication assay, influenza matrix gene expression was monitored over time. Following treatment of MDCK cells with strain A/WS/33, hourly samples were taken for qPCR analysis (see above total RNA isolation, cDNA transcription and qPCR analysis).

2.6. Viral plaque assay

MDCK cells cultured to approximately 90% confluence were detached with 0.25% Trypsin–EDTA, washed and re-suspended in complete EMEM at a density of 1.0×10^6 cells/ml. Next, 2 ml of the cell suspension was added to each well of a 6-well CoStar tissue culture plate (Corning) and incubated overnight at 35 °C in a humidified 5% CO_2 incubator. Confluent cell monolayers were next washed twice with 2 ml of PBS, inoculated with 800 μl of strain A/WS/33 (from the above serially diluted stock or from the collected aerosol sample from the calm-air chamber experiments below) and incubated for 45 min at 35 °C in a humidified 5% CO_2 incubator. Inoculated cells were then washed once with 2 ml of PBS, over-layed with supplemented DMEM/F12 containing 0.6% agarose (Oxoid Ltd., Hampshire, England) and incubated at 35 °C for 60 h in a humidified 5% CO_2 incubator. The cells were then fixed with 2 ml

of 10% formalin for 15 min and the agarose overlay was removed by washing with tap water. Plaques were stained with 2 ml of 1% crystal violet/0.19% methanol for 15 min, rinsed with tap water, dried and plaque forming units (PFU) were calculated. Viral plaque assay results with fewer than 10 PFU's per well were considered below the limit of detection (Szretter et al., 2006).

2.7. Calm-air chamber nebulization experiments

Diluted strain A/WS/33 was aerosolized by an Aeroneb nebulizer (Aerogen, Galway, Ireland) for each experiment. The generated aerosols were mixed in a mixing chamber with 30 L/min mixture of dry filtered air and humid air and flowed into the top center of a 40 L calm-air chamber through a dispersion nozzle. Air flow and gravity carried the aerosols to the bottom of the chamber where NIOSH two-stage bioaerosol samplers are located (Lindsley et al., 2006; Blachere et al., 2007). The NIOSH sampler collects air samples and separates airborne particles into three size fractions (>4 μm, 1–4 μm, and <1 μm). The chamber air was drawn by a Model 3321 Aerodynamic Particle Sizer (TSI, Shoreview, MN) at 5 L/min through a vertical probe at the same height as the sampler inlets to monitor the aerosol concentration and size distribution. The NIOSH samplers were connected to Model 224-PCXR4 personal air sampling pumps (SKC, Eighty Four, PA). To collect the airborne influenza virus, vacuum pumps for the NIOSH samplers were switched on simultaneously once the aerosol concentration in the chamber stabilized after operation of the Aeroneb nebulizer for 10 min. The nebulizer remained on during the entire experiments to load the aerosols continuously in the chamber. The NIOSH samplers collected the aerosols for 30 min at 3.5 L/min. After collection, the nebulizer, the pumps and the vacuum line were turned off. The exterior of the samplers were wiped off to remove the deposited particles, NIOSH samplers were dissembled and each collected aerosol fraction was suspended in 1 ml of supplemented HBSS.

3. Results

3.1. Optimization of infection

In the viral replication assay, influenza virus is analyzed after initially incubating the viral sample with MDCK cells for 45 min to allow adsorption and entry of the virus. Following infection, viral RNA replicates to several orders of magnitude within the cell. A time course experiment analyzing matrix gene expression was, therefore, performed to determine the optimal incubation time for maximum amplification of viral copy number. During the initial 2 h of sampling (Fig. 1), the total matrix copy number per well of infected MDCK cells was 1.5×10^5, similar to that detected in the initial viral inoculums. Thereafter, matrix copy number increased exponentially up to 20 h to a maximum of 4.0×10^9 copies per well of infected MDCK cells. After 20 h of incubation, matrix copy numbers declined (results not shown). All subsequent viral replication assays incorporated a 20 h incubation period.

3.2. Limits of detection

The dilute nature of bioaerosols poses a particular challenge with regards to detection and quantification. To assess the detection limits for infectious influenza virus, 10-fold serial dilutions of strain A/WS/33 ranging from 2.8×10^5 $CEID_{50}$/ml to 2.8×10^0 $CEID_{50}$/ml were incubated with MDCK cells and evaluated using both the viral plaque assay and viral replication assay. Following 60 h of incubation, plaque formation was detectable in viral samples diluted up to 2.8×10^2 $CEID_{50}$/ml. However, a further 10-fold dilution to 2.8×10^1 $CEID_{50}$/ml resulted in insufficient

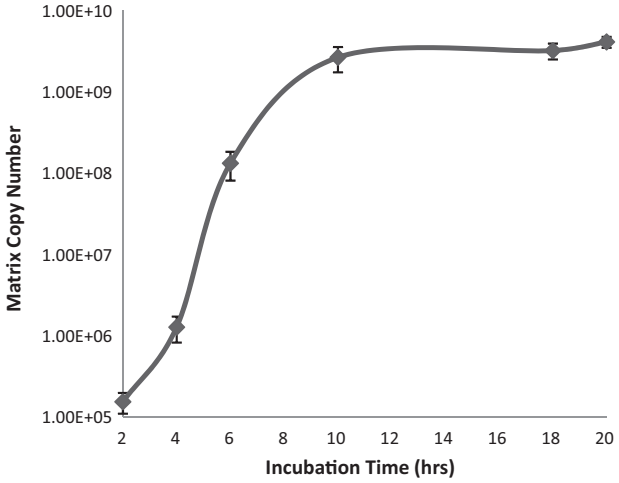

Fig. 1. Optimization of infection. Following infection of MDCK cells with strain A/WS/33 (2.8×10^4 $CEID_{50}$/ml) the replicate cultures were incubated at 35 °C in a 5% CO_2 incubator for up to 20 h before processing and analysis by qPCR to determine the matrix copy number of each replicate. The error bars show the standard deviation (2 experiments × 5 replicates/experiment).

plaque formation and was below the limit of detection. In contrast, using the viral replication assay, influenza virus was detectable in samples diluted up to 2.8×10^0 $CEID_{50}$/ml (Fig. 2). Mock-infected MDCK cells failed to generate any detectable fluorescence signal during qPCR analysis (results not shown), thus demonstrating the specificity of the viral replication assay.

3.3. Calm-air chamber studies

To show proof of concept, strain A/WS/33 was loaded into a nebulizer at 2.8×10^3 $CEID_{50}$/ml and 2.8×10^2 $CEID_{50}$/ml, the lowest dilution that yielded quantifiable plaques in the previous experiment, and aerosolized within a calm-air settling chamber for 30 min. Samples were collected using NIOSH bioaerosol cyclone samplers and infectivity was evaluated with the viral plaque assay and viral replication assay. To measure the total collected virus, samples were also analyzed with qPCR. When nebulized at 2.8×10^3 $CEID_{50}$/ml, influenza virus could be detected in all three sample size fractions when evaluated by qPCR (Table 1). Using the

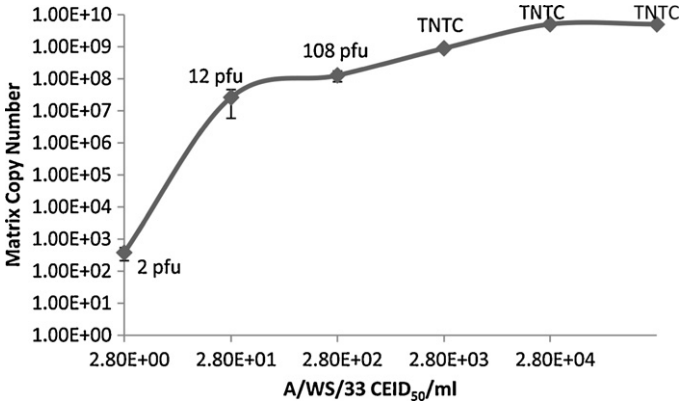

Fig. 2. Limits of detection. Serial dilutions ($CEID_{50}$/ml) of strain A/WS/33 were assessed for infectious virus by the viral replication assay and viral plaque assay. The matrix copy number indicates the number of amplified virus obtained after performing the viral replication assay on each diluted sample. With the viral plaque assay, plaque forming units (PFU) were too numerous to count (TNTC) at a $CEID_{50}$ > 2.80E+02 and below the limit of detection at a $CEID_{50}$ < 2.80E+02. The error bars show the standard deviation of the viral replication assay (2 experiments × 3 replicates/experiment). The PFUs per well at each dilution point are indicated.

Table 1
Calm-air chamber studies. Strain A/WS/33 was aerosolized into a calm-air chamber and collected by NIOSH aerosol samplers positioned at the bottom of the chamber. The concentration of strain A/WS/33 in the nebulizer prior to aerosolization is shown. Total virus collection in each aerosol fraction was determined by amplification of the matrix gene using qPCR. Infectious virus was determined by either the viral replication assay (VRA) or viral plaque assay (VPA), b.d. = below detection.

Aerosol fraction (particle size)	$CEID_{50}$/ml (initial viral suspension in nebulizer)	VRA matrix copy number (infectious virus)	qPCR matrix copy number (total virus)	VPA plaque forming units (infectious virus)
>4 μm	2.8×10^3	1.9×10^4	9.7×10^2	b.d.
1–4 μm		1.3×10^7	3.6×10^2	2.0×10^1
<1 μm		2.9×10^6	1.2×10^3	1.5×10^1
Total		1.6×10^7	2.5×10^3	3.5×10^1
>4 μm	2.8×10^2	1.7×10^5	b.d.	b.d.
1–4 μm		3.7×10^6	1.5×10^1	b.d.
<1 μm		2.6×10^5	4.9×10^1	b.d.
Total		4.1×10^6	6.4×10^1	N/A

viral plaque assay, plaques were detected from the 1 to 4 μm and <1 μm fractions but not from the >4 μm fraction. The sum of the fractions collected as found by viral plaque assay was 3.5×10^1 PFU, revealing that only 1.4% of the virus was infectious. In contrast, using the viral replication assay, virus was detected from all three aerosol fractions. Further, expression levels detected by the viral replication assay was 1.6×10^7 matrix copies and corresponds to a 4.6×10^5 fold amplification of infectious virus in the sample.

When aerosolized at the lowest concentration of 2.8×10^2 $CEID_{50}$/ml, the total virus detected by qPCR in the collected fractions was sharply reduced to only 6.4×10^1 matrix copies and undetectable in the >4 μm fraction. Plaque formation was found to be below the detection limits of the viral plaque assay. In contrast, influenza virus was readily detected from all three fractions (4.1×10^6 total infectious virus) with the viral replication assay.

4. Discussion

The extent by which airborne transmission plays a role in the spread of influenza remains to be determined. A number of factors limit the recovery and detection of infectious influenza virus from aerosol samples. In this study, an enhanced method of viral detection that employs both standard culture techniques and quantitative real-time PCR is reported. Prior to RNA isolation and qPCR analysis, MDCK cells are washed to remove residual virus and therefore, detection of the matrix gene by qPCR is attributed to amplification of infectious virus within the cell. Using laboratory experiments to compare the viral replication assay to the viral plaque assay, the viral replication assay was found to be far more sensitive at detecting infectious influenza virus from dilute samples. Unlike the viral plaque assay, which requires an ample viral titer for plaque formation, the viral replication assay was able to detect influenza virus from dilute samples with a $CEID_{50}$ as low as 2.8. Further, results were obtained in about half the time it would take with the viral plaque assay. To investigate the ability of the viral replication assay to detect infectious airborne influenza, strain A/WS/33 was aerosolized within a calm-air chamber system and samples were collected using the NIOSH two-stage bioaerosol sampling device. At a nebulized concentration of 2.8×10^2 $CEID_{50}$/ml, the viral replication assay detected influenza virus and yielded a total matrix copy number of 4.1×10^6. In contrast, using the viral plaque assay, plaque formation was below detection limits.

While there are many advantages to using the viral replication assay for detecting infectious influenza virus from aerosol samples, limitations exist. Unlike the viral plaque assay and qPCR which are quantitative methods, the viral replication assay is a qualitative assay and cannot be used to determine the initial viral concentrations. Due to cell death and detachment from the plate surface, we found that samples with an extremely high viral titer negatively affected the viral replication assay (results not shown). Although aerosol samples tend to be dilute in nature, serial dilutions can be utilized in the viral replication assay to over-come this limitation, especially when working with concentrated specimens such as nasopharyngeal swabs. Likewise, we found that there was no longer a linear relationship between high infectious doses and matrix copy numbers during qPCR analysis. This observation is most likely caused by issues with RNA isolation from MDCK cells that generate extremely high viral titers as the amount of viral RNA produced may exceed the RNA-binding capacity of the magnetic beads used in the RNA isolation step. However, this is easily corrected by performing RNA isolations from serially diluted lysates of infected cells. Another limitation of the viral replication assay is the inability of the qPCR analysis step to discriminate between non-infectious immature particles and mature virions within MDCK cells. Therefore, the total number of matrix copies is not an absolute measure of the number of newly synthesized virions.

Despite the aforementioned limitations, the viral replication assay has a clear advantage over other detection methodologies. During the viral aerosolization and collection process, there is a significant decline in infectivity from 9.8% to 1.4%. Such low levels of infectious virus fall below the limit of detection when using the viral plaque assay. Further, plaquing efficiencies for the viral plaque assay vary significantly with the kind of components that comprise the overlay medium (Matrosovich et al., 2006) and reduce detection sensitivity. Finally, it should be noted that the performance of aerosol samplers varies greatly with respect to their collection efficiency and infectious virus preservation.

When comparing the collection performance of four different aerosol sampling devices, research by Fabian et al. (2009) demonstrated a significant difference in the recovery of infectious virus. The SKC Biosampler, which collects virus directly into liquid media, was shown to recover and maintain essentially 100% of influenza virus infectivity. However, the large sample volume dilutes the viral concentration and may reduce detection of infectious virus. Regardless of which aerosol sampler is used, the viral replication assay increases the detection of potentially low amounts of infectious virus likely to be present in real-world environmental air samples.

In conclusion, the ability to determine the magnitude of a potential epidemic would likely depend on whether virus collected by an air sampler is infectious, and such information would dictate the precautionary measures for prevention of an outbreak. The viral replication assay described in this report is a highly sensitive and specific method that is able to detect infectious influenza virus from dilute aerosol samples and provides an alternative, expedited method of detection from dilute influenza-positive aerosol samples. Viral replication assay detection of influenza virus from other environmental or clinical samples such as exhaled respiratory aerosols should be further explored. Such studies may shed light on the role of airborne transmission of influenza.

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