HHS Public Access

Author manuscript

J Virol Methods. Author manuscript; available in PMC 2015 July 29.

Published in final edited form as:

J Virol Methods. 2015 June 1; 217: 14–17. doi:10.1016/j.jviromet.2015.01.008.

Equivalence of influenza A virus RNA recovery from nasal swabs when lysing the swab and storage medium versus storage medium alone

Maya Nadimpalli[a,*], **Nora Pisanic**[b], **Christopher D. Heaney**[b], and **Jill Stewart**[a]

[a] Department of Environmental Sciences and Engineering, Gillings School of Global Public Health, University of North Carolina at Chapel Hill, 1301 Michael Hooker Research Center, 135 Dauer Drive, CB #7431, Chapel Hill, NC 27599, USA

[b] Departments of Environmental Health Sciences and Epidemiology, Johns Hopkins Bloomberg School of Public Health, Room W7033B, 615 N. Wolfe St, Baltimore, MD 21205, USA

Abstract

Surveillance of healthy individuals at high risk for zoonotic influenza A transmission is important for tracking trends in influenza A epidemiology. Practical measurement methods that maximize viral recovery and produce low variability are essential when low viral loads are expected. For this study, lysing both a nasal swab and its storage medium was compared to lysing the storage medium alone to determine which method results in greater influenza A virus recovery. Independent results from two laboratories suggest that including the swab in the lysis step does not lead to higher influenza A virus recovery, and that recovery is less variable when only the swab storage medium is extracted. These results indicate that simply lysing the swab storage medium is an effective extraction method for nasal swabs collected during studies of influenza A virus exposure among healthy populations.

Keywords

Influenza A virus; Nasal swabs; Viral RNA extraction; Surveillance

Influenza A virus can infect humans, avian species, swine, and other mammals. Seasonal influenza epidemics are estimated to cause between 250,000 and 500,000 fatalities world-wide each year (World Health Organization, 2003). Influenza epidemics and pandemics tend to be caused by strains that originate in animals but adapt to human-to-human transmission (Reperant et al., 2012). Healthy individuals who have frequent contact with swine and poultry, such as livestock workers, may be at increased risk of exposure to influenza A virus and may serve as a bridging population for cross-species exposure, adaptation, and

* Corresponding author. Tel.: +1 978 407 6756; fax: +1 919 966 7911. mnadim@live.unc.edu (M. Nadimpalli)..

Conflict of interest

None declared.

Disclaimer

The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

transmission of novel reassortant subtypes. As a result, increased surveillance of healthy swine and poultry production workers has been recommended for pandemic influenza surveillance (Gray et al., 2007).

Maximum recovery of influenza A virus from human specimens is essential in surveillance studies of healthy individuals, as viral loads may be lower than among symptomatic individuals. For surveillance among populations with high exposure potential (Nichol and Hauge, 1997), nasal swabs are a more practical sampling method than nasopharyngeal swabs as they require little specialized training, are more readily accepted, and can be obtained quickly with minimum discomfort. Using nasal swabs can therefore facilitate increased participation in active surveillance studies to detect novel influenza viruses. When PCR is used for viral detection, nasal swabs are comparable in sensitivity to nasopharyngeal swabs (Irving et al., 2012; Sung et al., 2008). Previous work has examined the impact of storage temperature and time on viral recovery from nasal swabs (Fereidouni et al., 2012). However, the effect of extraction method on recovery of influenza A virus from nasal swabs has not been examined.

The goal of this study was to compare two extraction methods – lysis of both the storage media and the nasal swab versus lysis of only the media in which nasal swabs are stored (as is routine in clinical settings) – to determine which method maximizes recovery of influenza A virus RNA from nasal specimens along with lowest variability (measurement error). It was hypothesized that lysis including the swab could result in higher recovery, since virus particles present on a nasal swab may not completely elute into storage media prior to extraction.

A methods comparison study was conducted at the Johns Hopkins University (Laboratory A) and at the University of North Carolina at Chapel Hill (Laboratory B) following the same study design.

In each laboratory, a frozen stock of deactivated influenza A H1N1 (A/WSN/1933) virus containing 6.8×10^8 infectious units/mL (provided courtesy of Dr. Andrew Pekosz, Johns Hopkins Bloomberg School of Public Health) was thawed and four ten-fold dilutions (labeled dilutions 1–4) were prepared in sterile phosphate buffered saline. These dilutions were selected to reflect the range of influenza A virus concentrations that may be detected during epidemiologic studies of healthy populations; swabs seeded with the lowest dilution (dilution 4) were projected to absorb approximately 1400 infectious units per swab and result in only 1.8 infectious units/qPCR reaction (on average, between Laboratories A and B). A summary of the dilutions used in these experiments is provided in Table 1.

Twenty μL of each dilution were seeded onto six regular flocked nasal swabs (Diagnostic Hybrids, Athens, OH), allowed to absorb, inserted into 1 mL of Universal Transport Medium (UTM, Copan Diagnostics, Murietta, CA), and stored at 4 °C until analysis. To determine the concentration of virus in each of the dilutions, 20 μL of each dilution were spiked into three 1 mL vials of UTM (without nasal swabs), which were also stored at 4 °C until analysis. In total, 24 nasal swabs and 12 vials of UTM were seeded for these experiments in each laboratory.

All extractions were performed using the QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA) following the spin column protocol within 5–6 h of viral seeding. One hundred and forty μL of each sample were extracted. Spiked UTM solutions were vortexed for 30–60 s before extraction following manufacturer protocols. Seeded swabs were vortexed for 60 s and then "expressed" prior to RNA extraction, i.e. swabs were removed from the medium by their handle, and pressed on the side of the sample collection tube while rolling back and forth to expunge any virus-containing liquid that may have been absorbed. For three of the six the nasal swabs of each dilution, only the UTM containing the expressed virus was used for RNA extraction. To determine whether extracting only the UTM and not the nasal swab influenced influenza A virus RNA recovery, the (clipped off) nasal swab was included in the viral lysis step for the remaining three seeded nasal swabs of each dilution. After the lysis step was compete and as much lysis solution as possible was transferred to the spin column, the remaining sample and swab were briefly centrifuged. The swab was expressed using sterile forceps and discarded. The remaining sample was then also transferred to the spin column. All other steps of the extraction procedure followed manufacturer protocols.

Quantitative PCR was used to selectively target a conserved region of the influenza A virus matrix protein gene using primer and probe sequences developed by the U.S. Centers for Disease Control and Prevention (World Health Organization, 2009). This target was quantified in each laboratory using five-point, plasmid-derived standard curves. Plasmid inserts were derived from deactivated influenza A H1N1 (A/WSN/1933) virus.

The WHO/CDC protocol for influenza A detection (World Health Organization, 2009) was modified for use in a planned multi-lab surveillance study of livestock workers in the United States. First, the protocol was modified for two-step rather than one-step amplification of the target. Livestock workers in the United States are difficult to access for surveillance studies (Villarejo, 2003). Since samples collected from livestock workers are precious, multiple downstream analyses may be run on the same sample; each analysis may require the sample to be thawed, then re-frozen. Converting virus RNA to cDNA prior to beginning analysis ensures the sample will have sufficient long-term stability once archived to allow multiple downstream analyses to be performed. Previous work suggests that any increase in variability among sample replicates as a result of using two-step versus one-step amplification may be negligible (Wacker and Godard, 2005). Second, the protocol was modified by using different reverse transcription kits, PCR kits, and real-time PCR instrumentation between labs. These changes were reflective of potential real-life conditions during a multi-lab surveillance study.

At Laboratory A, 5 μL of each 60 μL RNA extract were reverse transcribed into first-strand cDNA (20 μL) using the High Capacity cDNA Reverse Transcription Kit (Invitrogen, Carlsbad, CA). Two μL of cDNA were then added to a 20 μL qPCR reaction containing 1× TaqMan® Fast Universal PCR Master Mix (Applied Biosystems, Foster City, CA), 250 nM probe, and 500 nM each of forward and reverse primers. All samples were analyzed on a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA) using 40 cycles of amplification. At Laboratory B, 5 μL of each 60 μL RNA extract were reverse transcribed into cDNA (20 μL) using the SuperScript® VILO™ cDNA synthesis kit (Invitrogen, Carlsbad, CA). 2.5 μL of cDNA were then added to a 25 μL qPCR reaction

containing: 1× Platinum qPCR SuperMix (Invitrogen, Carlsbad, CA), 4 mM MgCl, 200 nM probe and 300 nM each of forward and reverse primers. All samples were run on a SmartCycler II (Cepheid, Sunnyvale, CA) using 40 cycles of amplification. Negative template controls were included with every run in both laboratories.

Difference scores were calculated for all extracted nasal swabs by subtracting the concentration of virus RNA (copies/reaction) recovered from each nasal swab from the average concentration of virus RNA seeded onto each nasal swab. A generalized linear model with a generalized estimating equation (GEE) (Rogers, 1993) (to account for the non-independence of observations at each dilution) was used to determine whether there was an overall difference in recovery of virus RNA between extraction methods. Two-sided student t-tests were used to examine (a) differences between extraction methods by dilution and (b) interlaboratory variation, by examining whether the overall, average difference score calculated for each method was equivalent between laboratories. Analyses were conducted using SAS version 9.3 (SAS Institute, Cary, NC).

The concentration of RNA in dilutions 1–4 and the concentration recovered using each extraction method is depicted in Fig. 1. Overall, there was no statistical difference ($p > 0.05$) between extraction methods in recovery of influenza A virus RNA from seeded nasal swabs (Table 2). This finding was independently observed in both laboratories. Average difference scores were significantly different between extraction methods ($p < 0.05$) for two of four dilutions in Laboratory A and one dilution in Laboratory B; however, average difference scores were equivalent in both laboratories for dilution 4, the lowest concentration for which influenza A RNA was quantified. Swabs seeded with dilution 4 corresponded to approximately 1.8 infectious units/reaction and produced cycle threshold (C_t) values near 38 in each laboratory, close to the limit of detection for a 40-cycle PCR amplification protocol. The virus RNA concentrations on swabs seeded with dilution 4 were at least three times lower than observed among clinical samples from individuals with influenza-like illness (Ward et al., 2004). The range of dilutions examined in these experiments is informative for epidemiologic studies of healthy populations where influenza A virus concentrations may be close to the limit of detection.

Overall, the average difference scores for each extraction method were statistically equivalent between laboratories (Table 3), indicating that minimal interlaboratory variation in virus RNA recovery was observed for either extraction method. Since the modifications made to the WHO/CDC protocol for influenza A detection (World Health Organization, 2009) were different in each laboratory, it would not have been possible to pinpoint the exact source of interlaboratory variation, if such variation had occurred. However, no such variation was observed for either extraction method. Previous work has found that the differences in methodology between laboratories in this study (different PCR kits and real-time PCR instrumentation) are not major contributors of interlaboratory variation in quantitative results; rather, the most significant contributor is differences in reference material (Cao et al., 2013). This finding has not been examined in the context of influenza A virus RNA or the qPCR platforms used here. However, both laboratories that participated in this study used the same influenza A reference material and followed the same study design.

Average difference scores were significantly different between laboratories ($p < 0.05$) for two of four dilutions when the swab was included during lysis (Table 3). Additionally, when examining average difference scores by dilution within each laboratory, it was observed that scores were largest in magnitude when including the swab during lysis, suggesting that recovery of influenza A virus RNA from nasal swabs is more variable using this method. Greater variability may be due to the additional sample handling steps required when including the swab during lysis, which could introduce a greater opportunity for error during sample processing.

Recovery of influenza A virus RNA was equivalent when the swab was included in the viral lysis step versus when it was not (medium only). However, larger difference scores were observed for lysis with the swab than for lysis without the swab (Table 2), indicating there was greater overall variability in recovery when swabs were included in the lysis step. Overall, lysis of only the storage media into which swabs are expressed results in high recovery of influenza A virus RNA and low variability. Thus this method is preferable for surveillance studies of individuals who may be at high risk for influenza exposure, but have low viral loads.

Acknowledgement

The authors thank the Odum Institute at the University of North Carolina at Chapel Hill for help with statistical analyses.

Funding

M.N. was supported by the Royster Society of Fellows and an EPA Science to Achieve Results fellowship, N.P. by the National Institute of Environmental Health Sciences (award no. 5T32ES007141-30), and C.D.H. by the National Institute for Occupational Health and Safety (award no. 5K01OH010193-02). Funding sources had no role in this study.

References

Cao Y, Sivaganesan M, Kinzelman J, Blackwood AD, Noble RT, Haugland RA, Griffith JF, Weisberg SB. Effect of platform, reference material, and quantification model on enumeration of Enterococcus by quantitative PCR methods. Water Res. 2013; 47:233–241. [PubMed: 23123048]

Fereidouni SR, Globig A, Starick E, Harder TC. Effect of swab matrix, storage time, and temperature on detection of avian influenza virus RNA in swab samples. Avian Dis. 2012; 56:955–958. [PubMed: 23402118]

Gray GC, Trampel DW, Roth JA. Pandemic influenza planning: shouldn't swine and poultry workers be included? Vaccine. 2007; 25:4376–4381. [PubMed: 17459539]

Irving SA, Vandermause MF, Shay DK, Belongia EA. Comparison of nasal and nasopharyngeal swabs for influenza detection in adults. Clin. Med. Res. 2012; 10:215–218. [PubMed: 22723469]

Nichol KL, Hauge M. Influenza vaccination of healthcare workers. Infect. Cont. Hosp. Ep. 1997; 18:189–194.

Reperant LA, Kuiken T, Osterhaus AD. Adaptive pathways of zoonotic influenza viruses: from exposure to establishment in humans. Vaccine. 2012; 30:4419–4434. [PubMed: 22537992]

Rogers WH. sg17: regression standard errors in clustered samples. Stata Tech. Bull. 1993; 13:19–23.

Sung RY, Chan PK, Choi KC, Yeung AC, Li AM, Tang JW, Ip M, Tsen T, Nelson EA. Comparative study of nasopharyngeal aspirate and nasal swab specimens for diagnosis of acute viral respiratory infection. J. Clin. Microbiol. 2008; 46:3073–3076. [PubMed: 18614661]

Villarejo D. The health of U.S. hired farm workers. Annu. Rev. Public Health. 2003; 24:175–193. [PubMed: 12359914]

Wacker MJ, Godard MP. Analysis of one-step and two-step real-time RT-PCR using SuperScript III. J. Biomol. Tech. 2005; 16:266–271. [PubMed: 16461951]

Ward CL, Dempsey MH, Ring CJ, Kempson RE, Zhang L, Gor D, Snowden BW, Tisdale M. Design and performance testing of quantitative real time PCR assays for influenza A and B viral load measurement. J. Clin. Virol. 2004; 29:179–188. [PubMed: 14962787]

World Health Organization. CDC protocol of real-time RTPCR for swine influenza A (H1N1). 2009

World Health Organization. Influenza, fact sheet no. 211. 2003

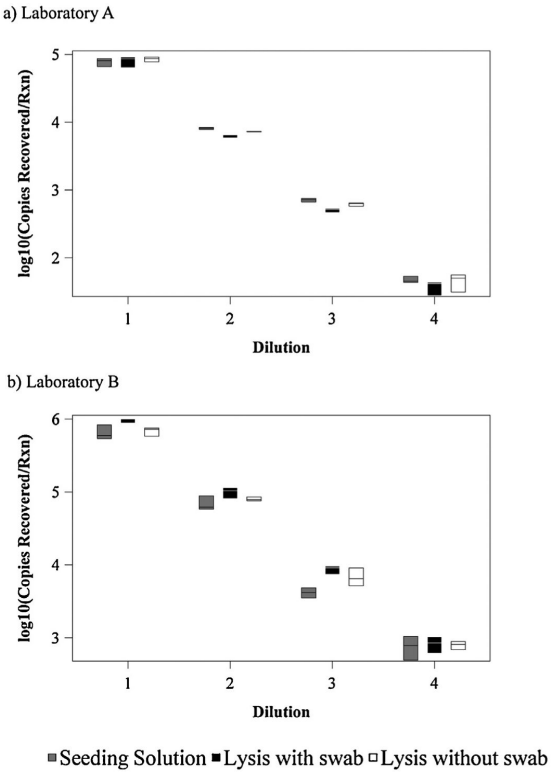

Fig. 1.

Recovery of influenza A virus RNA from nasal swabs using two extraction methods.[a,b] [a] Height of box plots indicates range of observed values; horizontal line indicates median. [b] qPCR reaction volume is 20 μL (Laboratory A) or 25 μL (Laboratory B). The amount of cDNA in each qPCR reaction corresponds to less than 1.5 μL of the original sample.

Table 1

Influenza A virus dilutions examined in this study.

Dilution	Infectious units[a]	Infectious units per qPCR reaction		No. of replicates seeded[b]	
		Laboratory A	Laboratory B	UTM vials	Nasal Swabs
1	1,400,000	1600	2000	3	6
2	140,000	160	200	3	6
3	14,000	16	20	3	6
4	1400	1.6	2.0	3	6

[a] Determined using the 50% tissue culture infectious dose ($TCID_{50}$) assay by the provider of the influenza A stock used in these experiments.

[b] Refers to the number of replicates seeded in each laboratory. Seeded swabs were evenly divided between the two extraction methods compared in this study.

Table 2

Differences between extraction methods in recovery of influenza A virus RNA from seeded nasal swabs by laboratory.[a]

Dilution	Laboratory A			Laboratory B		
	Lysis with swab Avg. difference score	Lysis without swab Avg. difference score	*p*-Value[b]	Lysis with swab Avg. difference Score	Lysis without swab Avg. difference Score	*p*-Value[b]
1	−2353.6	−7171.1	0.6303	−308812.49	−31900.56	0.0143
2	2024.41	865.66	0.0038	−30804.80	−10342.74	0.1138
3	205.7	95.02	0.0164	−4535.75	−2719.54	0.2425
4	9.72	1.74	0.4226	−58.37	−20.83	0.7918
Overall	−28.44	−1552.16	0.2261	−86052.85	−11245.92	0.3249

[a] Average difference scores were calculated by subtracting the average number of copies recovered from the average number of copies seeded. Average difference scores are presented as copies/qPCR reaction.

[b] *p*-Value comparing difference scores between methods.

Table 3

Interlaboratory variation for each influenza A virus RNA extraction method.[a]

Dilution	Lysis with Swab			Lysis Without Swab		
	Laboratory A Avg. difference score	Laboratory B Avg. difference score	*p*-Value[b]	Laboratory A Avg. difference score	Laboratory B Avg. difference score	*p*-Value[b]
1	−2353.59	−308812.49	0.0134	−7171.06	−31900.56	0.6907
2	2024.41	−30804.80	0.0766	865.66	−10342.74	0.0684
3	205.70	−4535.75	0.0190	95.02	−2719.54	0.1335
4	9.72	−58.37	0.6257	1.74	−20.83	0.7399
Overall	−28.44	−86052.85	0.0542	−1552.16	−11245.92	0.4413

[a] Average difference scores were calculated by subtracting the average number of copies recovered from the average number of copies seeded. Average difference scores are presented as copies/qPCR reaction.

[b] *p*-Value comparing difference scores between labs.