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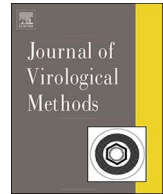
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Short communication

A method for the improved detection of aerosolized influenza viruses and the male-specific (F+) RNA coliphage MS2



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ABSTRACT

The detection of aerosolized viruses can serve as an important surveillance and control tool in agriculture, human health, and environmental settings. Here, we adapted an anion exchange resin-based method, initially developed to concentrate negatively charged viruses from water, to liquid impingement-based bioaerosol sampling. In this method, aerosolized viruses are collected in a 20 ml liquid sample contained within widely used impingers, BioSamplers (SKC Inc., Eighty Four, PA), and further concentrated via adsorption to an anion exchange resin that is suspended within this liquid. Viral nucleic acids are then extracted from the resin to facilitate molecular analyses through a reduction in the effective sample volume. For this study, various quantities of two negatively charged viruses, type A and type B influenza viruses (FluMist Quadrivalent vaccine) and the male-specific (F+) RNA coliphage MS2 (MS2), were nebulized into a custom-built bioaerosolization chamber, and sampled using BioSamplers with and without anion exchange resin. Compared to direct testing of the BioSampler liquid, detection was improved by $6.77 \times$ and $3.33 \times$ for type A and type B influenza viruses, respectively, by using the anion exchange resin. For MS2, the anion exchange resin method allowed for an average improvement in detection of $8.26 \times$.

Bioaerosols serve as a transmission vehicle for numerous and diverse types of viruses, including influenza viruses, severe acute respiratory syndrome viruses, and porcine epidemic diarrhea viruses (Alonso et al., 2014; Cowling et al., 2013; Li et al., 2005). The important role of bioaerosols in viral transmission is highlighted by recent events, such as the 2014–2015 outbreak of highly pathogenic avian influenza in U.S. poultry production. In this outbreak, multiple independent lines of epidemiological evidence suggested perpetuation within and between poultry production facilities by influenza virus-laden bioaerosols (United States Department of Agriculture, 2015). Ultimately, 48 million birds were euthanized with economic losses estimated at \$3.3 billion (Greene, 2015). Accordingly, active sampling of bioaerosols has been suggested as a tool to help mitigate viral transmission (Anderson et al., 2016; Cowling et al., 2013). Therefore, highly efficacious bioaerosol sampling strategies are needed.

The collection of airborne viruses is generally accomplished using a variety of inertia-based samplers. These samplers include cyclones, impactors, filter-based devices, and impingers (Haig et al., 2016; Verreault et al., 2008). Cyclones and impactors are typically used to size-segregate aerosols based on particle diameter while filter-based devices will collect an integrated sample across a defined range of particle sizes. The most widely used sampling strategy for viruses is liquid impingement. This technique purportedly reduces sampling-related stresses (e.g. impaction force, desiccation) compared to the other methods described above (Verreault et al., 2008). With liquid impingement, bioaerosols are drawn through nozzles to produce an impinging jet that deposits particles into a liquid-filled chamber. This liquid can then be processed using culture or molecular-based methods for viral identification and characterization. However, approximately 20 ml of liquid is required for the effective operation of many liquid

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impingers (Pepper et al., 2015), resulting in an undesirable dilutive effect that ultimately limits the sensitivity of downstream detection (Gendron et al., 2010; Hermann et al., 2006). Thus, strategies which enable additional concentration of viruses within liquid impingers would improve the overall performance of these systems.

We previously demonstrated that IRA-900 anion exchange resin was useful for concentrating male-specific (F+) RNA (FRNA) coliphages and several enteric viruses from water (Chandler et al., 2017; Perez-Mendez et al., 2014a,b). Similar to batch chromatography, anion exchange resin was suspended and mixed with aqueous samples to adsorb negatively charged viruses. The liquid was then decanted so that nucleic acids could be directly isolated from the resin in a small volume (60–80 μ l), and viruses were detected by quantitative real-time PCR (qPCR). Compared to direct testing of the water sample by qPCR, the anion exchange resin method allowed for improved detection of all viruses tested. Specifically, detection was improved 50 \times to 200 \times for FRNA coliphages, 4300 \times for human adenovirus 40, 128 \times for rotavirus, and 20 \times for hepatitis A virus (Perez-Mendez et al., 2014a). Additionally, we observed that the anion exchange resin method was functional in water samples with diverse physicochemical properties. Therefore, we hypothesized that the anion exchange resin method may be applied to air sampling methods, such as liquid impingement, to improve the detection of negatively-charged viruses.

The objective of this study was to test the ability of anion exchange resin to improve the detection sensitivity of airborne type A and type B influenza viruses and FRNA coliphage MS2 (MS2) impinged in the collection liquid of a widely used bioaerosol sampling device, the BioSampler (SKC Inc., Eighty Four, PA). Thus, the anion exchange resin would serve as a second virus concentration step within the overall sampling system. MS2 was selected for evaluation given the precedence for effective anion exchange resin-based concentration in water (Perez-Mendez et al., 2014b). Further, MS2 is considered to be a surrogate of negatively charged enteric viruses (United States Environmental Protection Agency, 2000; Michen and Graule, 2010; Vergara et al., 2015) and is used as a model organism for the evaluation of liquid impingers (Tung-Thompson et al., 2015; Turgeon et al., 2014). Similarly, influenza viruses are reported to have negative charges (Michen and Graule, 2010) and have been used in the evaluation of liquid impinger-based bioaerosol sampling (Turgeon et al., 2014).

The influenza viruses utilized were contained within the FluMist Quadrivalent vaccine 2015–2016 formulation (FluMist) (MedImmune, Gaithersburg, MD) which contains four live attenuated strains: A/Bolivia/559/2013 (H1N1), A/Switzerland/9715293/2013 (H3N2), B/Phuket/3073/2013 (B/Yamagata/16/88 lineage), and B/Brisbane/60/2008 (B/Victoria/2/87 lineage). A single 0.2 ml dose of FluMist is declared to contain between $10^{6.5}$ and $10^{7.5}$ fluorescent focus units (FFU) of virus reassortants for each of the four strains. For this study, the concentration of each type A and type B viruses within FluMist was assumed to be $1 \times 10^{6.5}$ FFU/ml. MS2 (ATCC 15597-B1) was obtained from the American Type Culture Collection (Manassas, VA), and was propagated and enumerated as described previously (Perez-Mendez et al., 2014b).

In preparation for viral aerosolization, two sterile BioSamplers were installed into a custom designed 1.4 m³ bioaerosol chamber (Fig. 1). The BioSamplers were pre-loaded with 20 ml of 0.01 M phosphate buffered saline, pH 7.5 (PBS) and were co-located inside the bioaerosol chamber using retort stands. One of the samplers also contained 0.5 g of IRA-900 anion exchange resin (Polysciences, Warrington, PA, USA) suspended within the PBS. A SidePak™ AM510 Personal Aerosol Monitor (TSI Inc., Shoreview, MN) was centrally positioned inside the chamber to measure mass concentration (mg/m³) of the bioaerosols. A Q-TRAK Indoor Air Quality Monitor 8554 (TSI Inc.) and Alnor Velometer Thermal Anemometer AVM440-A (TSI Inc.) were co-located near the samplers to measure and ensure consistency of environmental variables within the bioaerosol chamber, which included temperature, % relative humidity, carbon dioxide concentration, and wind speed

(generated using axial fans). The chamber was then sealed and purged with HEPA-filtered air. The average per-trial temperature was 24.4 °C (instantaneous range: 22.7–25.7 °C); average relative humidity was 42.8% (instantaneous range: 27.6–55.2%); average carbon dioxide was 380 ppm (instantaneous range: 58–796 ppm); average per-trial wind speed was 0.06 m/s (instantaneous range: 0.01–0.12 m/s).

For each virus, 10-fold serial dilutions ($10^{-2.5}$ to $10^{3.5}$ FFU/ml for influenza viruses; 10^{-2} to 10^6 pfu/ml for MS2) were prepared in 100 ml PBS to create the inocula used for aerosolization. Inocula were prepared within the glass vessel of a 6-jet collision nebulizer (BGI Incorporated, Waltham, MA). Filtered, dried air was delivered to the nebulizer, which operated at 6.9 kPa. Using the SidePak, 5 mg/m³ of viral bioaerosols was generated (via nebulizer) in each chamber trial. The SidePak is calibrated using the respirable fraction of the ISO 12103 A1 test dust, which contains a particle size distribution ranging from 0 to 10 μ m. Thus, the 5 mg/m³ relative mass concentration only serves to establish consistency within and between trials. After the target concentration was reached, nebulization was stopped. An air sampling pump (Vac-U-Go, SKC Inc.) for each BioSampler was then operated at a flow rate of 12.5 L/min to actively sample the chamber atmosphere over 40 min. Bioaerosolization experiments were performed in triplicate for each virus and titer tested.

Nucleic acid isolation from the anion exchange resin and from 140 μ l liquid samples (PBS contained within the BioSamplers with and without resin as well as the viral inocula used for bioaerosolization, both pre- and post-experiment) was achieved as reported previously (Perez-Mendez et al., 2014b). Quantitative real-time reverse transcription PCR (qRT-PCR) detection of influenza viruses (using CDC universal influenza A and influenza B oligonucleotides in multiplex) (Selvaraju and Selvarangan, 2010) and MS2 (Perez-Mendez et al., 2014b) was performed as previously described using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad laboratories, Hercules, CA, USA). The methods used for qRT-PCR data interpretation are reported in (Perez-Mendez et al., 2014b). qRT-PCR standard curves for each of the assays were generated using ten-fold serial dilutions of viral stocks ($10^{0.5}$ – $10^{5.5}$ FFU/reaction and 10^0 – 10^5 pfu/reaction for influenza viruses and MS2, respectively). Standard curves for the MS2 qRT-PCR were obtained from our earlier studies (Perez-Mendez et al., 2014b). Quantitative standard curves for type A and type B influenza viruses were generated using ten-fold serial dilutions of the FluMist Quadrivalent vaccine. Cycle thresholds (Cq) were plotted against the logarithm of FFU/reaction to conduct linear regression analysis ($y = mx + b$) in order to determine the correlation coefficient and slope. The standard curves for type A and type B influenza viruses are represented by the equations $y = -0.2777x + 7.8657$ and $y = -0.2748x + 7.7585$, respectively. Amplification efficiency (E) of the reaction was calculated using the formula $E = 10^{(-1/m)} - 1$, where m = slope of the regression lines of the standard curves. The amplification efficiency of the qRT-PCR for type A and type B influenza viruses was 90% and 88%, respectively. The correlation coefficient was ≥ 0.995 for all three qRT-PCR assays.

To assess the improvement in detection sensitivity afforded by the anion exchange resin within the BioSamplers' liquid, the abundance of RNA collected from BioSampler resin samples was directly compared to the corresponding BioSampler liquid samples (Table 1). These data are reported in Cq, where the difference in Cq (Δ Cq) between these two samples indicates the improvement in detection sensitivity. In a qRT-PCR that is 100% efficient, a gain of one Cq corresponds to a 2-fold increase in target concentration. Thus, improvements in detection sensitivity facilitated by the anion exchange resin were estimated using the equation 2^{Δ Cq}. The average Cq of three experimental replicates for each condition was utilized in the calculation of Δ Cq.

Compared to testing of the BioSampler liquid samples, the use of the anion exchange resin method improved qRT-PCR detection of MS2 by an average of 8.26 \times . The resin method allowed for reliable detection of MS2 when the inoculum concentration for nebulization was 10^2 pfu/

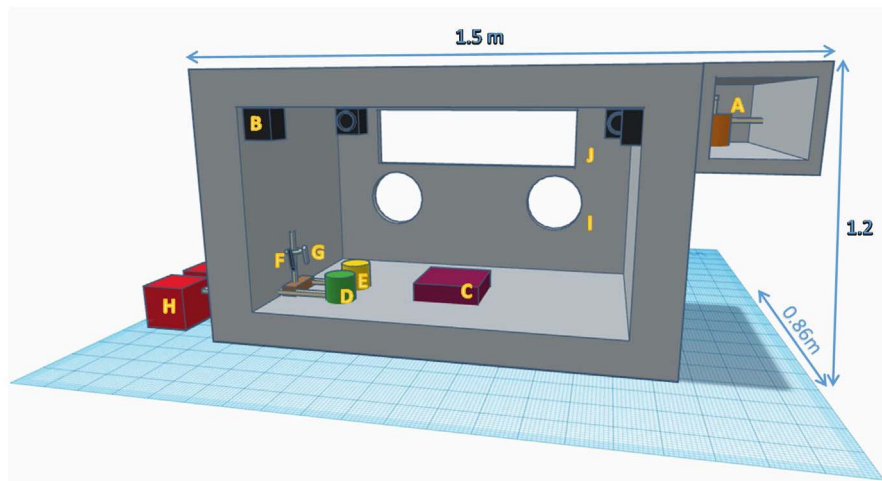


Fig. 1. Schematic and general operation of the custom bioaerosol chamber. (A) 6-jet Collison nebulizer was supplied with filtered and dried air at a generating pressure of 6.9 kPa (source not shown); (B) mixing fans were positioned in each corner of the chamber to facilitate aerosol dispersal and uniform mixing of the aerosol within the atmosphere of the chamber; (C) an aerosol monitor (SidePak AM510, TSI Inc.) was centrally located in the chamber to identify the relative concentrations of the aerosols specific to each trial; (D and E) BioSamplers (SKC Inc.) with and without resin; (F) thermal anemometer (Alnor® Velometer®, TSI, Inc.) was used to measure wind speed and (G) air quality monitor (Qtrak, TSI, Inc.) to measure other environmental variables (temperature, carbon dioxide, and percent relative humidity) inside the chamber; (H) active sampling pumps calibrated to 12.5 L/min; (I) pneumatically sealed glove ports for positioning sampling equipment within the chamber; and (J) an observation window.

ml, whereas detection in the corresponding BioSampler liquid samples was only achieved sporadically at this inoculum concentration. The improvement in MS2 detection afforded by the resin was generally consistent for all inocula concentrations in which viruses were detected in both the resin and liquid samples (10^3 – 10^6 pfu/ml).

Although MS2 was detected with $8.26 \times$ increased sensitivity in bioaerosols using the resin method, our previous experiments using resin for water sampling suggested that the improvement in sensitivity

could approach $51 \times$ (Perez-Mendez et al., 2014b). The disparity in sensitivities between these two systems is partially explained by the fact that MS2 adsorption in PBS, which is recommended for impingement by the BioSampler manufacturer, is less efficient than in dechlorinated tap water (decreased sensitivity of approximately 2 Cq). A second consideration that may account for decreased sensitivity in the bioaerosol sampling system is that the integrity of MS2 could have been compromised by factors intrinsic to the nebulization or sampling

Table 1
Comparison of the mean viral detection achieved in BioSamplers with and without resin by qRT-PCR.

Viruses	Viral concentration (pfu/ml for MS2 and FFU/ml for influenza viruses) used in the inoculum of the nebulizer	BioSampler resin		BioSampler liquid		$2^{\Delta Cq}$ fold change in detection improvement afforded by anion-exchange resin
		Cq	SD	Cq	SD	
MS2	0	ND	NA	ND	NA	NA
	10^{-2}	ND	NA	ND	NA	NA
	10^{-1}	ND	NA	ND	NA	NA
	10^0	^a 39.88	NA	ND	NA	NA
	10^1	ND	NA	ND	NA	NA
	10^2	36.85	0.46	^a 38.26	NA	NA
	10^3	35.76	1.16	38.83	1.18	8.40
	10^4	30.05	0.56	33.10	0.03	8.28
	10^5	27.80	0.84	30.76	0.25	7.80
	10^6	24.28	0.44	27.38	0.16	8.57
Type A influenza viruses	0	ND	NA	ND	NA	NA
	$10^{-2.5}$	ND	NA	ND	NA	NA
	$10^{-1.5}$	ND	NA	ND	NA	NA
	$10^{-0.5}$	ND	NA	ND	NA	NA
	$10^{0.5}$	ND	NA	ND	NA	NA
	$10^{1.5}$	34.77	0.15	36.79	0.29	4.06
	$10^{2.5}$	31.01	0.33	33.75	0.47	6.69
$10^{3.5}$	26.77	0.22	30.02	0.19	9.55	
Type B influenza viruses	0	ND	NA	ND	NA	NA
	$10^{-2.5}$	ND	NA	ND	NA	NA
	$10^{-1.5}$	ND	NA	ND	NA	NA
	$10^{-0.5}$	ND	NA	ND	NA	NA
	$10^{0.5}$	^a 39.96	NA	ND	NA	NA
	$10^{1.5}$	36.80	0.34	38.15	0.72	2.56
	$10^{2.5}$	32.37	0.20	34.44	0.41	4.20
$10^{3.5}$	28.16	0.04	30.96	0.32	6.98	

ND, not detected; NA, not applicable; SD, standard deviation.

^a One of three samples detected.

processes implemented here. Using a modified double agar overlay plaque assay described previously (Kropinski et al., 2009), the integrity of MS2 was assessed in the inocula used for nebulization and BioSampler liquid samples. Bacteriophage titers in both pre- and post-nebulized inocula were generally unchanged and were in agreement with qRT-PCR results. In contrast, only sporadic detection of MS2 was achieved in BioSampler liquid samples when the inocula concentrations were 10^6 pfu/ml or 10^5 pfu/ml, despite that qRT-PCR measurements indicated that approximately 10^4 and 10^3 pfu/ml of MS2, respectively, were present within these samples. Nevertheless, results obtained by Turgeon et al. (2014) indicated that the relative recovery of aerosolized MS2 collected within BioSamplers was on the same order of magnitude by both qRT-PCR and plaque assay when an impingement buffer consisting of 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM MgSO₄ was utilized. Thus, the formulation of an optimized impingement buffering system will be critical to achieve maximum sensitivity of the anion-exchange resin-based bioaerosol sampling method.

The anion exchange resin method also allowed for the improved qRT-PCR detection of type A and type B influenza viruses within BioSamplers compared to direct testing of the corresponding 140 μ l liquid BioSampler aliquots. On average, the resin improved detection by 6.77 \times and 3.33 \times for type A and type B influenza viruses, respectively. In contrast to MS2, both type A and type B influenza viruses were detected more effectively at higher inoculum concentrations. Detection was improved by 9.55 \times and 6.98 \times for type A and type B influenza viruses, respectively, when the viral inoculum used for nebulization was at a concentration of $10^{3.5}$ FFU/ml. Each 10-fold dilution of the inocula decreased the detection sensitivity by an average of 2 \times for both type A and type B influenza viruses. Viral adsorption to surfaces has been suggested to function in a concentration-dependent manner, where the frequencies of impacts of viruses to adsorbent surfaces are more probable at higher viral concentrations (Gerba, 1984). However, in experiments to adsorb influenza viruses spiked directly into 50 ml of dechlorinated tap water, increased viral concentrations were correlated with diminished detection improvement across all concentrations tested ($10^{1.5}$ – $10^{3.5}$ FFU/ml) (Table S2). Therefore, concentration-dependent changes in influenza detection sensitivity in the bioaerosol system are potentially an artifact of nebulization and/or the sampling system.

Viruses were detected from inocula samples both pre- and post-experiment with nearly equal efficiency (Table S1). While this metric indicated little change in viral RNA concentration over the course of experiments, it does not offer a direct evaluation of the nebulization process on viral stability. Nebulization is reported to have an impact on virus integrity. For example, decreases in viral titer between 100 \times and 1000 \times were reported for bacteriophage ϕ 6 (a surrogate used for influenza viruses) following nebulization, whereas the impact of nebulization on MS2 is reportedly less substantial (Turgeon et al., 2014). Our experimental system was not directly capable of monitoring viral survival or stability following nebulization. Factors such as desiccation, impaction on the axial fans, adsorption to the bioaerosol chamber surface, or shearing during sampling may have had a deleterious impact on viral sampling or detection. An additional limitation to this study is that environmental conditions within the bioaerosol chamber was subject to some fluctuations. Despite these environmental fluctuations, only minor variations in the Cq between experimental replicates were observed (see Tables 1 and S1).

In conclusion, we have developed an anion exchange resin-based method for viral capture from bioaerosols that increased detection sensitivity for type A and type B influenza viruses as well as the bacteriophage MS2 (surrogate of enteric viruses). The method is simple to perform, can be adapted to existing aerosol sampling methods, is cost-effective, and allows for molecular detection of viral nucleic acids directly extracted from the anion exchange resin. Future work will focus on optimizing sampling parameters to minimize potentially

adverse factors affecting lower than expected concentration/recovery efficiencies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jviromet.2017.04.004>.

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