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<th>Journal:</th>
<th>Canadian Journal of Microbiology</th>
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<tr>
<td>Manuscript ID</td>
<td>cjm-2016-0450.R1</td>
</tr>
<tr>
<td>Manuscript Type:</td>
<td>Article</td>
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<tr>
<td>Date Submitted by the Author:</td>
<td>15-Sep-2016</td>
</tr>
<tr>
<td>Complete List of Authors:</td>
<td>Turgeon, Nathalie; Universite Laval, Biochimie, microbiologie et bioinformatique; Centre de recherche de l'institut universitaire de cardiologie et de pneumologie de Quebec, Toulouse, Marie-Josée; Universite Laval, Departement de biochimie, microbiologie et bioinformatique; Centre de recherche de l'institut universitaire de cardiologie et de pneumologie de Quebec Ho, Jim; Universite Laval, Departement de biochimie, microbiologie et bioinformatique Li, Dongqing; University of Waterloo, Department of mechanical and mechatronics engineering Duchaine, Caroline; Universite Laval, Departement de biochimie, microbiologie et bioinformatique; Laval Hospital Research Center</td>
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<tr>
<td>Keyword:</td>
<td>aerosolization, air sampling, bioaerosols, influenza virus, neuraminidase</td>
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Neuraminidase as an enzymatic marker for the detection of airborne Influenza virus and other viruses

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Running headline: Neuraminidase air sampling resistance
Abstract

Little information is available regarding air samplers’ effectiveness to collect viruses as well as the effects of sampling processes on viral integrity. The neuraminidase enzyme is present on the surface of viruses of agricultural and medical importance. It has been demonstrated that viruses carrying neuraminidase enzyme can be detected using commercial substrate without sample processing such as RNA extraction. This project aims at evaluating the effects of three aerosol-sampling devices on neuraminidase enzyme activity of airborne viruses. The purified neuraminidase enzyme from Clostridium perfringens, a strain of Influenza A(H1N1) virus, the FluMist influenza vaccine and the Newcastle disease virus were used as models. Neuraminidase models were aerosolized in aerosol chambers and sampled with three different air samplers (SKC BioSampler, three pieces cassettes with polycarbonate filters, and Coriolis µ) to assess the effect on neuraminidase enzyme activity. Our results demonstrated that Influenza virus and Newcastle disease virus neuraminidase enzymes are resistant to aerosolization and sampling with all air samplers tested. Moreover we demonstrated that the enzymatic neuraminidase assay is as sensitive as RT-qPCR for the detection of low concentration of Influenza virus and Newcastle disease virus. Therefore, since the assay sensitivity and compatibility with air sampling methods, viruses carrying neuraminidase enzyme can be rapidly detected from air samples without sample processing using neuraminidase activity assay.

Keywords: aerosolization, air sampling, bioaerosols, Influenza virus, neuraminidase
Résumé

L’efficacité des échantillonneurs d’air pour la capture des virus de même que les effets de l’échantillonnage sur l’intégrité des virus sont peu documentés. L’enzyme neuraminidase est présente à la surface de plusieurs virus d’importance en agriculture et en médecine. Il a déjà été démontré que les virus porteurs de cette enzyme peuvent être détectés à l’aide de substrats commerciaux sans traitement préalable. Le présent projet a pour but de déterminer les effets de trois échantillonneurs d’air (SKC BioSampler, filtre de polycarbonate et Coriolis μ) sur l’activité de la neuraminidase présente à la surface de virus aéroportés. Quatre sources d’enzyme furent utilisées comme modèles (neuraminidase de Clostridium perfringens purifiée, vaccin vivant FluMist contre l’Influenza, virus Influenza A(H1N1) et virus de la maladie de Newcastle). Les virus et les enzymes modèles ont été aérosolisées en chambre et échantillonnés afin de documenter les effets de l’échantillonnage sur l’activité de la neuraminidase. Nos résultats ont montré que la neuraminidase des virus Influenza et Newcastle résiste à l’aérosolisation et à l’échantillonnage par tous les échantillonneurs testés. Nous avons également démontré que l’essai basé sur l’activité de la neuraminidase est aussi sensible que la qPCR pour la détection d’échantillons contenant de faibles concentrations des virus Influenza et Newcastle. Ainsi, étant donné la sensibilité de la méthode et sa compatibilité avec les méthodes d’échantillonnage, les virus porteurs de l’enzyme neuraminidase peuvent rapidement être détectés dans des échantillons d’air en utilisant un essai basé sur l’activité de l’enzyme.

Mots clés: aérosolisation, échantillonnage d’air, bioaérosols, virus influenza, neuraminidase
Introduction

Several viral diseases (like *Influenza virus*, *Coronavirus*, and *Porcine Circovirus*) can be transmitted through the bioaerosol route (Yu et al. 2004, Atkinson and Wein 2008, Lindsley et al. 2010a, Verreault et al. 2010a). However, the low concentration of airborne viruses constitutes an obstacle to their detection and exposure risk assessment. At this time, there is no standard method for airborne viruses sampling and only few studies have looked at air samplers efficiency to collect airborne viruses (Verreault et al. 2008). The development of a real-time method for the detection of viruses from the air would allow a quick response to eventual threats and be an helpful tool to reduce the number of infections (Effler et al. 2002).

The neuraminidase enzyme is present at the surface of many viruses causing respiratory infections in humans and animals. Indeed, this enzyme is found on several *Orthomyxoviridae*, including *Influenzaviruses*, and also the majority of *Paramyxoviridae*, including *Human Parainfluenza virus*, *Newcastle disease virus*, and *Mumps virus* (Taylor 1996). The most widely used substrate for the detection of the neuraminidase enzyme activity (NA) is the 4-methylumbelliferyl N-acetylneuraminic acid (4-MU-Neu5Ac). Modifications in positions 4, 7, 8, and 9 of this substrate are changing its specificity towards neuraminidase sources (Beau and Schauer 1980, Varki and Diaz 1983, Corfield et al. 1986, Liav et al. 1999, Shimasaki et al. 2001, Achyuthan et al. 2003, Turgeon et al. 2011). The 4-MU-4-methoxy-Neu5Ac was tested with several viruses, bacterial strains and mammalian cells and was shown to detect only viral neuraminidase (Beau and Schauer 1980, Shimasaki et al. 2001, Turgeon et al. 2011). We challenged the 4-MU-4-methoxy-Neu5Ac with swine barn air samples and demonstrated that
high dust and bacterial load do not interfere with virus detection specificity and sensitivity (Turgeon et al. 2011). In the same study we also demonstrated that the neuraminidase enzyme can be used to detect the presence of the Newcastle disease virus from air samples collected with SKC BioSampler (Turgeon et al. 2011). It is known but not well documented in the literature, that aerosolization and air sampling can structurally affect viruses (Verreault et al. 2008). More studies are needed to assess the damage caused by aerosolization and air sampling to the neuraminidase found on viruses to determine the potential usefulness of NA for airborne viruses detection.

The aim of the present study was to assess the effect of various air sampling devices on four different neuraminidase enzymes in order to validate this enzyme as a marker for the rapid detection of airborne neuraminidase positive viruses as well as to identify the best air sampling strategy for this purpose. We also compared the sensitivity of the neuraminidase enzyme detection with RTqPCR detection of Influenza A virus and Newcastle disease virus.
Materials and methods

Virus strains and neuraminidase enzyme

Four different neuraminidase sources were used in this study. We used the purified neuraminidase enzyme from *Clostridum perfringens* (N2856, Sigma-Aldrich, Oakville, Ontario, Canada), stored at 4°C as 250 µg/ml stock solution in 10 mM potassium phosphate buffer pH 6.0, 25 mM KCl. The Influenza A/Puerto Rico/8/1934 (H1N1) (American Type and Culture Collection ATCC VR-95, commonly referred to as InfA PR8) and Newcastle disease virus Hitchener B1 strain (commonly referred to as NDV B1, purchased as a live vaccine, Wyeth Animal Health) were used after two passages on embryonated chicken eggs as described previously (Turgeon *et al.* 2011). The titer of InfA PR8 and NDV B1 were $1.1 \times 10^5$ 50% egg infectious dose per ml (EID50/ml) and $1.58 \times 10^6$ EID50/ml respectively. The FluMist influenza vaccine (2012-2013 flu season vaccine, MedImmune, Gaithersburg, MD, USA) contains three live attenuated and cold adapted *Influenza virus* strains: Influenza A H1N1 (A/California/7/2009), Influenza A H3N2 (A/Victoria/361/2011), and Influenza B (B/Wisconsin/1/2010) viruses. One vaccine dose contains $10^{6.5-7.5}$ fluorescent focus units (FFU) of each *Influenza virus* strain as reported by manufacturer. The vaccine was purchased in a drugstore, stored at 4°C, and used in the aerosolization experiments. The concentrations displayed in this section were utilized to calculate the virus quantity used in each experiments in order to allow reproducibility, not to compare the results obtained for each virus.

Alexa fluor 555 (Life Technologies, Burlington, ON, Canada) was used as a fluorescent tracer during the aerosolization experiments. A 10 mM stock solution in 10 mM potassium phosphate buffer pH 6.0, 25 mM KCl was prepared and stored at -20°C until use.
Aerosol chambers description

All aerosol experiments were repeated three times. Two different chambers were used for aerosolization experiments. The GenaMini chamber (SCL Medtech Inc., Montreal, QC, Canada) is a 1.5 l chamber placed in a biosafety level 2 (BSL2) cabinet. This system was described elsewhere (Gendron et al. 2010, Verreault et al. 2010b, Turgeon et al. 2011, Turgeon et al. 2014). It can be used with air samplers with flow rate lower than 30 l/min.

A large wind tunnel was used for high flow rate air samplers (300 l/min). This wind tunnel can be used only with BSL1 organisms and is not biosafety contained. This chamber consisted of a 137 cm long tunnel of 15 cm diameter. One extremity of the tunnel was connected to a 1 m³ mixing-chamber and the other extremity was connected to the air inlet of the air sampler Coriolis μ (Bertin Technologies, France). The air was pulled through the tunnel at 300 l/min by the Coriolis μ integrated pump. The dilution air entered the system drawn through HEPA filters located on the top of the mixing-chamber.

Temperature and relative humidity (RH) cannot be controlled in these chambers but were monitored. The experiments in the GenaMini chamber were conducted at the ambient temperature inside the BSL2 cabinet (18.6°C - 28.7°C). Relative humidity was very low (3.0% to 8.9%) in this chamber because medical grade compressed air (0% RH) was used for aerosol dilution. Temperature and RH inside the GenaMini chamber were monitored using KIMO KH-210 data logger (Chevrier instrument, Montréal, QC, Canada).

In the wind tunnel, ambient air was HEPA filtered and used for aerosol dilution. Therefore the relative humidity in the tunnel was from 22.9% to 23.2%. Experiments in the wind tunnel were conducted at ambient temperature in the laboratory (17.8°C to 22.8°C). Air velocity,
temperature and RH inside the wind tunnel were monitored and recorded using KIMO CTV-210, KIMO TH-200, and Kistock KT-200 datalogger (Chevrier instrument).

Aerosol experiments with InfA PR8 and NDV B1 were performed in the GenaMini chamber only. Aerosols experiments with FluMist influenza vaccine were performed in the wind tunnel only. Aerosols experiments with *C. perfringens* purified neuraminidase enzyme were performed in both chambers.

**Aerosolization experiments in GenaMini chamber**

Aerosols were generated using an atomizer (model 9302 TSI Inc., Shoreview, MN, USA) at a dispersion rate of 3 L/min. The atomizer was filled with 70 ml of 10 mM potassium phosphate buffer, 25 mM KCl, 1X10^10 EID50 of the InfA PR8 or 1 µM Alexa fluorescent tracer and 250 µg of the *C. perfringens* neuraminidase enzyme. The aerosol stream flowed through a diffusion dryer (TSI model 306200) before entering the chamber where it was diluted with medical-grade compressed air at 30 l/min. The nebulization and dilution flows were continuous during the sampling.

Gilair Aircon II pumps (Levitt Sécurité, Montréal, QC, Canada) were used to draw air through SKC BioSamplers and PC filters. Pump calibration was performed using DryCal 2 Flow meter (SKC, Ancaster, ON, Canada). With PC filters pumps were calibrated at 10.0 l/min ±0.1. With the BioSamplers the airflow was 12.5 l/min as determined by the critical orifices of the device.

Air samples were collected for 20 minutes with an SKC BioSampler (SKC Inc., Eighty Four, PA, USA) loaded with 20 ml of 10 mM potassium phosphate buffer pH 6.0, 25 mM KCl. After sampling, the remaining liquid in the BioSampler was measured and collected. Polycarbonate (PC) filters with 0.8 µm pore size (SKC, Ancaster, ON, Canada) deposited on a
nitrocellulose support pad (SKC, Ancaster, ON, Canada) and hosted in three pieces 37 mm cassettes (SureSeal) (SKC, Ancaster, ON, Canada) were also used to collect the air samples. Readers should be aware that air filtration physics is different from liquid filtration. For explanation about how 0.8 µm filters can efficiently capture particles as small as single viruses, we are referring readers to aerosol physics textbooks (Vincent 2007). They were operated at 10 l/min for 25, 50, 100 and 200 minutes. The samples were eluted from the cassettes by adding 2 ml of phosphate buffer pH 6.0, 25 mM KCl and vortexing with a multi-pulse vortex (Glas-Col, Terre Haute, IN, USA) for 15 minutes at 70% motor speed and 100% frequency.

An Aerodynamic particle sizer (APS, model 3321, TSI Inc.) was used to monitor the concentration of the aerosols and the distribution of their aerodynamic size range. The aerosol median mass aerodynamic diameter ranged from 0.696 µm to 0.859 µm, and the particle concentration was between 1.82X10³ and 5.99X10³ particles/cm³.

InfA PR8 concentration in the GenaMini chamber was evaluated from SKC BioSamplers air samples. Total InfA PR8 virus quantity detected in the air sample was divided by total air volume collected. InfA PR8 concentration was 2.2 X10⁴ ± 1.2 X10⁴ genomes per liter of air

**Aerosolization experiments in the wind tunnel**

The aerosols were generated with a Collison 6-jet nebulizer (BGI, Waltham, MA, USA), with compressed air set at 20 psi. The nebulizer was installed into the mixing-chamber and the aerosol was mixed with dilution air when entering the tunnel. The Collison 6-jet was filled with 9 ml of of 10 mM potassium phosphate buffer pH 6.0, 25 mM KCl, 1 ml of the FluMist influenza vaccine (five doses) or with 1 µM Alexa fluor 555 tracer and 250 µg of the suspension of *C. perfringens* neuraminidase enzyme.
The aerosols were collected using the Coriolis µ filled with 15 ml of 10 mM potassium phosphate buffer pH 6.0, 25 mM KCl, at flow rate 300 l/min for 10 minutes. The Coriolis µ contains its own flowmeter. It was calibrated every year by manufacturer. After sampling, the remaining liquid volume in the sampler was measured and collected.

The concentration of the aerosols was monitored with an APS and was between $1.00 \times 10^3$ to $1.43 \times 10^4$ particles/cm$^3$ and the median mass aerodynamic diameter was between 0.790 and 0.838 µm.

**Neuraminidase stability in spiked air samplers**

The SKC BioSampler and the Coriolis µ collection buffer was spiked with 1 µM of the Alexa fluor 555 tracer and 60 µg of *C. perfringens* neuraminidase. The same experiment was also conducted by spiking the collection buffer with $2 \times 10^9$ EID50 of InfA PR8, or $2 \times 10^9$ EID50 of NDV B1 or 0.2 ml of FluMist influenza vaccine (1 dose). The SKC BioSampler was operated at 12.5 l/min for 20 minutes and the Coriolis µ was operated at 300 l/min for 10 minutes to sample clean HEPA filtered air. Experiments were replicated three times in a biosafety level 2 cabinet.

Similar experiments were conducted to assess the effect of long-term air sampling with PC filters. Five time points were tested (0h, 1h, 4h, 24h, 32h). However, since the GenaMini chamber can accommodate only three filters per experiments and to compensate any changes in aerosol concentration between experiments, the filters and time points were randomized. The aerosols containing the InfA PR8 or Alexa fluor 555 tracer and *C. perfringens* neuraminidase were generated as described in the previous section. The dilution air into the aerosolization chamber was set at 27.5 l/min. The air samples were collected on PC filters 0.8µm pore size at 10 l/min for 1 hour. After sampling, the three filters were disconnected from the chamber. One of them was eluted immediately and was considered as time 0h. The two other filters were placed
into a biosafety level 2 cabinet and connected to a pump that was driving clean HEPA filtered air through the filters for an additional 1, 4, 24, or 32 hours. The experiment was repeated with one time 0h and two other time points per experiment until all time points have been tested at least 3 times with InfA PR8 as well as *C. perfringens* purified neuraminidase. Samples were eluted from the filters as described previously before analysis.

**Neuraminidase activity detection assay and Alexa fluorescence detection**

A fluorescent substrate, the 2’-(4-Methylumbelliferyl)-α-D-N-acetylneuraminic acid (4-MU-Neu5Ac, N2856, Sigma-Aldrich, Saint Louis, MO), was used as described in a previous study to measure the neuraminidase activity (NA) (Turgeon et al. 2011) with the following modification. The buffer used was potassium phosphate buffer pH 6.0 with 25 mM KCl instead of MES pH 6.0. These two buffer have the same NA detection sensitivity for NDV B1 detection (data not shown). A stock solution of 2 mM 4-MU-Neu5Ac was prepared in Dimethylformamide (Sigma-Aldrich) and kept at -20 °C until use. The neuraminidase assay used to determine the NA was set in 96-well black plates (7701-2350, Whatman, Piscataway, NJ, USA) as follows. A volume of 45 μl of each samples were deposited in triplicates into the wells plate. To initiate the reaction 5 μl of a fresh solution of 100 μM 4-MU-Neu5Ac in potassium phosphate buffer pH 6.0, 25 mM KCl, was added to the samples. The plates were incubated at 37 °C for 40 minutes. The enzymatic reaction was stopped by adding 100 μl of 0.1 M glycine buffer pH 10.7 containing 25% ethanol. The plates were read with a spectrometer (Synergy H1 Hybrid ReaderBioTek, Highland Park, VT, USA) at respective excitation and emission wavelengths of 340 nm and 460 nm. The fluorescence of the tracer Alexa fluor 555 was read with the same apparatus at the excitation and emission wavelengths of 540 nm and 570 nm, respectively. No interference was detected between Alexa fluor 555 and neuraminidase reaction fluorescence readings.
Quantification of viral genomes

RNA was extracted from two dilutions for all samples using the QIAamp viral RNA mini kit (Qiagen, Chatsworth, CA, USA). RNA extraction protocols were optimized in previous studies (Turgeon et al. 2011, Turgeon et al. 2014). The RNA was stored at -80°C until further analysis (within the week). The viral RNA was converted into cDNA using the iScript cDNA synthesis kit (BioRad Life Sciences, Mississauga, ON, Canada) and processed for qPCR analysis immediately.

The primers and probes used in this study are listed in Table 1 and were supplied by Integrated DNA technology (Coralville, IA, USA). The probes were labeled in 5’ with FAM and with Iowa Black FQ quencher in 3’. Protocols used for the detection of Newcastle disease virus as well Influenza A and B viruses were described previously (Wise et al. 2004, World Health Organization 2009, Selvaraju and Selvarangan 2010).

Each 25 µl qPCR reaction contained: 2 µl of cDNA or standard curve plasmid DNA, 0.8µM of each primer for Influenza A virus, 1µM for Influenza B virus, and 0.5 µM for Newcastle disease virus as well as 0.2 µM of probes and 12.5 µl of the 2X iQ Supermix master mix (BioRad Life Sciences, Mississauga, Ontario, Canada). All qPCR assays were performed separately using an Opticon 2 (MJ Research, Waltham, MA, USA). The program was as followed: 2 minutes incubation at 95°C followed by 45 amplification cycles including 15 seconds denaturation at 95°C, 30 seconds annealing and elongation at 55°C for for Influenza A and B viruses, or 60°C for 60 sec for Newcastle disease virus, followed by fluorescence measurement.

Quantitative PCR reactions were performed in triplicates on two dilutions of each sample. No template controls as well as no revers transcription controls were performed on every plate.
The standard curves were constructed in triplicate. Ten-fold serial dilutions from $10^0$ to $10^8$ molecules per reaction were used. Data were analyzed with the Opticon monitor software Version 2.02.24 supplied with the unit. The background was subtracted using the average over cycle range function of the software. The detection threshold was adjusted manually. Plotting of threshold cycle ($C_T$) as a function of the logarithm of the amount of DNA template gave a straight line. The slope of this graph line gave the PCR efficiency ($E$) according to the equation $E = (10^{1/slope} - 1) \times 100$. The results obtained were considered adequate when reaction efficiency was greater than 85%, and the error between the points and the standard linear regression curve was smaller than 0.1.

Plasmid DNA for *Newcastle disease virus*, *Influenza A virus*, and *Influenza B virus* standard curves detection was obtained from previous studies (Turgeon *et al.* 2011, Turgeon *et al.* 2014). The plasmids DNA were purified from *E. coli* using the Qiagen plasmid mini kit and were quantified with a GeneQuant pro UV/Vis spectrophotometer (Biochrom Ltd, Cambridge, UK). The plasmid DNA for standard curves were stored at -20°C. Absolute genome quantitation was not used in this study. Plasmids standard curves were used for qPCR efficiency control. Nebulizer and air samples RT and qPCR were performed on the same plates. We assumed to have the same RT efficiency for the nebulizer and corresponding air samples performed on the same plate.

**Sample analysis and relative ratios calculations**

All samples were analysed for neuraminidase activity (NA) detection as described in previous sections. Samples containing *C. perfringens* purified neuraminidase and Alexa Fluor 555 were also analysed for Alexa Fluor fluorescence detection. The purified *C. perfringens* enzyme in
the samples was not concentrated enough to be quantified by direct protein dosage using micro Bradford assay (Sigma) or NanoOrange protein quantitation assay (ThermoFisher) (data not shown).

Samples containing NDV B1 or InfA PR8 were analysed using NA assay as well as RNA extraction followed by RT-qPCR detection for *Newcastle disease virus* or for *Influenza A virus* detection respectively.

Samples containing FluMist influenza virus were analysed using NA assay as well as RNA extraction followed by RT-qPCR detection for *Influenza A virus* and *Influenza B virus*. The total concentration of genomes in FluMist influenza vaccine was the addition of *Influenza A virus* genomes and *Influenza B virus* genomes detected.

Neuraminidase activity (NA) ratios were calculated for all samples, including air samples (NA<sub>sampler</sub>) as well as nebulizer content (NA<sub>nebulizer</sub>). The NA ratios of the three viruses tested (InfA PR8, NDV B1 and FluMist influenza vaccine) were calculated by dividing the fluorescence signal detected in the sample using the neuraminidase enzymatic assay by the amount of viral equivalent genomes quantified using qPCR. The NA ratio of the purified *C. perfringens* neuraminidase was calculated by dividing the NA fluorescence by the Alexa fluor signal instead of viral equivalent genomes.

The air samples relative NA ratios were calculated to compensate for variations between experiments due to differences in nebulizer content. Indeed, even if the same virus concentration, *C. perfringens* enzyme and Alaxa Fluor tracer were used for all experiments, the NA ratio in the nebulizer vary from an experiment to another due to differences in reagent or viruses dilution lots. Therefore, relative NA ratios were calculated to allow comparisons between experiments. The relative NA ratios were calculated by dividing the NA ratios in air samples (NA<sub>sampler</sub>) by the NA ratios in the nebulizer (NA<sub>nebulizer</sub>). Using relative NA ratios sampler/nebulizer also controls
for RT reaction efficiency regarding that RT and qPCR reaction of samples and nebulizers were performed on the same plate. The relative NA ratios also facilitate comparisons between neuraminidase sources.

The relative NA ratios from the spiked SCK BioSampler and Coriolis µ were calculated by dividing the NA ratios in the spiked air sampler after performing air sampling by the NA ratios in the spiked air sampler before air sampling.

The relative NA ratios from the spiked filters were calculated by dividing the NA ratios after air sampling of clean HEPA filtered air (1h, 1h, 4h, 24h, 32h) by NA ratios after 1h air sampling of aerosolized viruses (time 0h for this experiment).

In the display of the results, if relative NA ratio > 1, it means that NA ratio_{sampler} > NA ratio_{nebulizer}, so NA is more stable than genomes. If relative NA ratio = 1, it means that NA ratio_{sampler} = NA ratio_{nebulizer}, so stability of NA and genomes is similar. If relative NA ratio < 1, it means that NA ratio_{sampler} < NA ratio_{nebulizer}, so genomes are more stable than NA.

Lower limit of detection of the neuraminidase assay compared with qPCR method to detect Influenza virus and Newcastle disease virus.

Serial 10-fold dilutions of NDV B1 and InfA PR8 were prepared to obtain concentration from $10^2$ to $10^8$ EID50/ml. The titer of InfA PR8 and NDV B1 used to perform the serial dilution were 1.1 X10^{10} EID50/ml and 1.58 X10^{10} EID50/ml respectively. The diluted viruses were processed for direct detection using the neuraminidase enzyme assay as described earlier. The diluted viruses were also detected using RNA extraction, reverse transcription reaction and qPCR. The lower limit of detection (LOD) was determined from the more diluted sample that was detected in all of three replicates.
NA assay can be performed directly on the sample. Therefore, the quantity of viruses detected is equal to the virus concentration in the dilution (in EID50/ml) multiplied by the sample volume used for detection (0.045 ml). The detection with qPCR required RNA extraction prior to RT-qPCR. Therefore it introduces a dilution factor. This dilution factor can be calculated according to equation 1.

\[
160 \text{ viruses/sample} = \frac{(1 \text{ virus} / \text{qPCRwell}) \times (RNA_{volume}) \times (RT_{volume})}{(RNA_{inRT}) \times (cDNA_{volume})}
\]

In Equation 1, \(RNA_{volume}\) represents the RNA final volume after ARN extraction (0.08 ml); \(RNA_{inRT}\) represents the RNA volume used in RT (0.005 ml); \(RT_{volume}\) represents the total volume of RT reaction (0.02 ml); \(cDNA_{volume}\) is the cDNA volume used per qPCR reaction (0.002 ml). With the RNA extraction kit and RT-qPCR reaction used in this study, the dilution factor is 160: to obtain 1 genome in the qPCR reaction well, the initial sample used for RNA extraction must contain 160 genomes.

**Statistical analyses**

Data were analysed by using 2-way Anova and unpaired t-test using GraphPad Prism 6. To fulfil the normalisation and variance assumptions, variables were log transformed and P-values were reported from these transformations. The significance level that was used to assign significant differences was \(p<0.05\).
Results

Neuraminidase activity stability during aerosolization and sampling

Aerosolization into the wind tunnel and air sampling with the Coriolis µ caused 2 fold less reduction of relative NA ratios of the purified *C. perfringens* neuraminidase enzyme than the aerosolisation and air sampling from the GenaMini chamber using the SKC BioSampler despite a higher flow rate (Fig. 1) (p<0.05). The SKC BioSampler causes 10 times less damages to the relative NA/Alexa ratio of the *C. perfringens* purified enzyme when compared to PC filters (p<0.05) (Fig. 1). The relative NA ratios were < 1 for *C. perfringens* purified enzyme with PC filters, meaning that this neuraminidase model was less stable than Alexa Fluor in these conditions. The differences on the relative NA ratios between SKC BioSamplers and PC filters were not significant for InfA PR8 and NDV B1. The relative NA ratios were ≈ 1 with all air samplers tested for InfA PR8 and NDV B1 (Fig 1). It means that NA and genomes resistance of these viruses toward aerosolization and sampling in these conditions were similar.

The relative NA ratios from InfA PR8 were significantly higher than from the purified *C. perfringens* neuraminidase after air sampling on filters for time up to 200 min (Fig. 2) (p=0.0002). Moreover, there was a significant effect of sampling time on relative NA ratios for both neuraminidase sources (p=0.035). The Alexa Fluor fluorescence remains stable on PC filters over 200 min air sampling period. However, the sampling time had significant effect on the relative *C. perfringens* NA per Alexa ratio. It means that *C. perfringens* NA was less stable than Alexa Fluor fluorescence (relative NA ratio <1). On the other hand, the NA of InfA PR8 was
more stable than viral genomes as demonstrated by increasing relative NA per genome ratios over sampling time up to 200 min in this system (relative NA ratio >1) (Fig. 2).

**Neuraminidase stability in air samplers**

The variation of the relative NA ratios in the spiked collection liquid was not significant (Fig. 3): NA activity and genomes stabilities were similar in these conditions (Fig. 3). PC filters were spiked by sampling aerosols from the GenaMini for 60 min. After sampling, filters were disconnected from the chamber and clean HEPA filtered air was sampled for an additional 1h, 4h, 24h, or 32h before analysis. The HEPA filtered air was coming from ambient air in the cabinet. Therefore it was at 22.9% - 23.2% RH, and 18.6°C - 28.7°C. In these conditions, the InfA PR8 relative NA ratios were > 1, meaning that the NA was more stable than genomes in these conditions. The InfA PR8 relative NA ratios were significantly higher than the purified *C. perfringens* relative NA ratios on the spiked PC filters (p=0.0006) (Fig. 4). The *C. perfringens* relative NA ratios were < 1, meaning that this neuraminidase model was less stable than Alexa Fluor in these conditions. However, there was no significant effect of sampling time from 1 to 32 h on both enzymes tested when compared to time 0 (Fig. 4).

**Sensitivity of the neuraminidase assay to detect Influenza virus and Newcastle disease virus compared to RT-qPCR detection method.**

We compared the lower limit of detection (LOD) of the enzymatic neuraminidase assay with RT-qPCR to detect low concentration of InfA PR8 and NDV B1. We used serial ten-fold dilutions from $10^2$ to $10^8$ EID50/ml.

The lowest NDV B1 dilution detected using NA assay was $1.58 \times 10^5$ EID50/ml (Table 2). Since we used 10-fold dilutions, it means that the LOD for NDV B1 using NA assay was
between $1.58 \times 10^4$ and $1.58 \times 10^5$ EID50/ml. For InfA PR8 virus LOD using NA assay was between $1.10 \times 10^4$ and $1.10 \times 10^5$ EID50/ml (Table 2).

The RT-qPCR sensitivity of the assays used in this study were $5 \times 10^2$ genomes copies per reaction for InfA PR8 and $4 \times 10^2$ genomes copies per reaction for NDV B1. It corresponds to $5 \times 10^2$ and $1 \times 10^3$ EID50 per qPCR reaction for InfA PR8 and NDV B1, respectively. However, as calculated in equation 1, the initial sample used for RNA extraction and RT-qPCR must contain 160 more genomes copies than what is required in the qPCR reaction well. The lowest viruses dilution detected using RNA extraction followed by RT-qPCR were $1.58 \times 10^5$ EID50/ml for NDV B1 and $1.10 \times 10^6$ EID50/ml for InfA PR8 (Table 2). Since we used 0.14 ml for RNA extraction followed by RT-qPCR and 0.045 ml for NA assay, between $2.21 \times 10^3$ and $2.21 \times 10^4$ EID50 per sample were required for NDV B1 and between $1.54 \times 10^4$ and $1.54 \times 10^5$ EID50 per sample were required for InfA PR8 detection using RT-qPCR, which was 3-30 fold more than for NA detection.
**Discussion**

The purified neuraminidase from *C. perfringens* is a convenient model. Indeed, it is commercially available and can be handled in BSL1 environment. It allowed aerosol experiments in several chambers and comparisons between air samplers. However, our results showed that it might not be the most suited model for viral neuraminidase studies. Indeed, our results showed that the purified neuraminidase from *C. perfringens* is affected by the processes of aerosolization and sampling. However, the enzymes present on InfA PR8, and the FluMist influenza vaccine were not significantly affected. Similar results were observed for the NDV B1 in a previous study (Turgeon et al. 2011). Proteins, lipids, and/or carbohydrates on the virus structure may protect viral neuraminidase enzyme. Indeed, it was demonstrated that the media environment could protect proteins and virus integrity from various types of stress (Vandenheuvel et al. 2013). More studies are needed to investigate this phenomenon for *Influenza virus* and *Newcastle disease virus* neuraminidases.

In our setup, aerosolization in the wind tunnel and air sampling with the Coriolis µ caused less reduction of the purified *C. perfringens* relative NA ratios than aerosolization in the GenaMini and sampling with the SKC BioSampler despite the higher flow rate. However, our experiments with the inoculated collection liquid revealed no significant differences between these two air samplers. This finding indicates that the reduction of the NA ratios was due to the aerosolization condition in the GenaMini chamber and not the air sampler. Indeed, the temperature was higher and RH was lower in the GenaMini chamber (18.6°C to 28.7°C, 3.0% to 8.9% RH) compared to the wind tunnel (17.8°C to 22.8°C, 22.9% to 23.2% RH). Our results with
the spiked filters also indicate that dry conditions in the GenaMini chamber could affect the neuraminidase. Indeed, in this experiment, NA loss occurred mostly in the first hour when the filters were still connected to the GenaMini chamber. No significant decrease of relative NA ratios was observed during additional sampling time when the filters were disconnected from the GenaMini chamber and were sampling HEPA filtered air.

Dry condition in the aerosol chamber can also affect genome stability. In fact, for sampling time ranging between 25 and 200 minutes with the PC filters, we observed a significant increase of the InfA PR8 relative NA ratios (Fig. 2). This phenomenon might be explained by genome degradation on the PC filters. Although *Influenza virus* seem to be more stable at low RH (Schaffer *et al.* 1976), our experiments were conducted at very low RH levels (3%-8.9% RH) that do not reflect indoor air during flu season and those extremely dry conditions could have increased the damages to InfA PR8 genomes. Therefore, genome stability in air sampler must be verified when choosing air sampler for a peculiar environment, and results must be interpreted with care. In our setup, no RNA degradation was observed for 20 minutes sampling with the SKC BioSampler and the PC filters or with 10 min air sampling with the Coriolis µ.

SKC BioSamplers show good performances for the preservation of the NA in our setup. However, this air sampler can only be used for 20 min (250 l of air) when sampling using water-based buffer because of buffer evaporation. Therefore SKC BioSampler is not the most suitable air sampler to capture rare events in field sampling. The Coriolis µ is an interesting choice for field sampling. Indeed, this liquid cyclone can collect 3 000 l of air in 10 min, 24 fold more than the SKC BioSampler for the same sampling time. Finally, since they allow long-term sampling up to 32 hours, 0.8 µm PC filters would also be a good choice for air monitoring and surveillance purposes.
RT-qPCR is a very sensitive method for virus detection. Indeed, only $10^1$-$10^2$ genome copies per well are necessary for RT-qPCR detection of *Influenza virus* (de-Paris *et al.* 2012) and $10^2$-$10^4$ genomes copies per well for *Newcastle disease virus* (Wise *et al.* 2004). The qPCR detection used in this report gave similar performances than what is shown in the available literature. Indeed, we detected $5\times10^2$ genomes copies per well for InfA PR8 and $4\times10^2$ genomes copies per well for NDV B1. However, much more viruses are needed in the initial sample to allow detection. Indeed, we must consider that RT-qPCR detection is performed on only a few microliters from the original samples. We calculated that RNA extraction require 160-fold more virus quantity to obtain $10^2$ genomes copies in the 2 µl used for qPCR reaction. From a field detection point of view, it would be more realistic to use the virus quantity in the original unprocessed sample for both methods to make LOD comparison. Neuraminidase enzyme assay requires no sample processing and can be performed on 45µl of the original unprocessed sample. Our results demonstrate that 3 to 30 fold less virus quantity in the initial sample are required for detection of *Influenza virus* and *Newcastle disease virus* using NA assay compared to RT-qPCR (Table 2).

Viruses concentration in the air is influenced by several factors: nature of the virus, ventilation, nature of the sources, distance from the sources, etc. Norovirus has been detected at concentrations up to $10^3$ genomes per cubic meter of air in health care settings (Bonifait *et al.* 2015). Human cough from influenza positive patients contains 16 genomes per cough (Lindsley *et al.* 2010b). With an average of 4 l per cough, it represents a median concentration of $10^3$ genomes per cubic meter. Concentrations up to $10^7$ genomes per cubic meter of air have been reported for porcine circovirus in swine confinement buildings (Verreault *et al.* 2010a). The InfA PR8 genome concentration in the GenaMini chamber was within this range with $2\times10^7$ genomes per cubic meter.
When working with real environmental samples (air samples, surface swabs, body fluid samples, etc.) the viral concentration in the initial sample is critical for detection. Indeed, if the environmental sample is in a volume larger than the volume required for virus detection, the detection will be performed on a fraction of the sample. Therefore, unless an efficient concentration method is used to reduce the volume of the sample to the volume required for the virus detection, the sensitivity of a detection method will depend of the virus concentration in the initial sample. In our experiments, the lowest NDV B1 concentration detected was the same for both NA assay and RT-qPCR detection. However, the lowest InfA PR8 concentration detected using NA assay was 10-fold lower compared to RT-qPCR.

Our results confirmed that the viral neuraminidase can be used for the detection of viruses from air samples. Indeed, *Influenza virus* and *Newcastle disease virus* neuraminidase resistance to aerosolization and sampling with several air samplers has been demonstrated. This enzyme also remains stable over long sampling periods. Moreover, NA assay is fast, and as sensitive as qPCR for the two viruses tested in this study.

**Acknowledgments**

We are grateful to Serge Simard for statistical analysis and to Judith-Elise Marcoux for editorial revision of the manuscript. The wind tunnel chamber was kindly provided by DRDC. This work was funded by Natural Sciences and Engineering Research Council (NSERC) of Canada through a Strategic Research Project (STPGP 396451). M.-J.T. was recipient of studentships from NSERC, FQRNT, and IRSST. C.D. is a FRQ-S senior scholar and a member of the FRQ-S Respiratory Health Network.
Conflict of interest

Authors have no conflict of interest to declare.
References


Figures legends

**Fig. 1.** Comparison of purified *C. perfringens* relative NA per Alexa ratios and of relative NA per genome ratios for InfA PR8, NDV B1 and FluMist influenza vaccine remaining after air sampling. 250 l of air were collected from the GenaMini with SKC BioSamplers and PC filters (n=10). 3,000 l of air were collected from the wind tunnel with the Coriolis µ (n=3). Results with NDV B1 were obtained from a previous study using the same setup and are presented for comparison purpose (Turgeon *et al.* 2011). Dot line represents reference relative NA ratios values (before aerosolization and sampling). The bars indicate median remaining relative ratios. * There was a significant difference between all air samplers for the purified *C. perfringens* relative NA per Alexa ratios (p<0.05).

**Fig. 2.** Relative NA per Alexa ratios of purified *C. perfringens* and neuraminidase activity per genome relative ratios of InfA PR8 after air sampling from the GenaMini with PC filters (n=6). Dot line represents reference relative NA ratios values (before aerosolization and sampling). There was a significant effect of sampling time on relative NA ratios for both neuraminidase sources (p=0.035). * The differences of relative NA ratios between both neuraminidases are significant (p=0.0002).

**Fig. 3.** Comparison of purified *C. perfringens* relative NA per Alexa ratios and of relative NA per genome ratios for InfA PR8, NDV B1, and FluMist influenza vaccine into spiked air samplers. Coriolis µ and SKC BioSampler collection liquid was inoculated with 60 µg of purified *C. perfringens* neuraminidase and 10 µg of Alexa, or $2 \times 10^9$ EID50 of InfA PR8, $2 \times 10^9$ EID50 of NDV B1, or 0.2 ml (1 dose) of FluMist influenza vaccine. 250 l of clean HEPA filtered air were
passed through the SKC BioSamplers and 3,000 l were passed through the Coriolis µ (n=3). PC filters were inoculated by sampling aerosols from the GenaMini for 60 min, and clean HEPA filtered air was sampled for an additional hour (600 l) before analysis (n=4). Dot line indicates reference time 0 relative NA ratios (before air sampling for Coriolis µ and SKC BioSampler, and after 60 min air sampling for PC filters). The bars indicate remaining median ratios. The variations of the relative NA ratios were not significant.

**Fig. 4.** Comparison of purified *C. perfringens* relative NA per Alexa ratios and of InfA PR8 relative NA per genome ratios remaining on PC filters after sampling time up to 32 hours. Samples were collected on PC filters by sampling from the GenaMini for 60 min (time 0). PC filters were disconnected from the chamber and pumps continued to sampling clean HEPA filtered air at 10 l/min for time up to 32 hours before analysis. (n=3). Dot line indicates reference time 0 relative NA ratios (after 60 min air sampling). * The InfA PR8 relative NA ratios were significantly higher than the purified *C. perfringens* relative NA ratios (p=0.0006). Sampling time had no significant effect for both neuraminidase types tested when compared to time 0h.
**Table 1 Primers and probes used in this study.**

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences</th>
<th>Ref</th>
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</thead>
<tbody>
<tr>
<td>InfBfor</td>
<td>5′-TCCTCAACTCACTCTTTCGAGCG-3′</td>
<td>(Selvaraju and Selvarangan 2010)</td>
</tr>
<tr>
<td>InfBrev</td>
<td>5′-CGGTGTCTGTGACCAAATTGG-3′</td>
<td>(Selvaraju and Selvarangan 2010)</td>
</tr>
<tr>
<td>InfBprobe</td>
<td>5′-FAM/CCAATTCGAGCAGCTGAAACTGCGGTG/IABlkFQ-3′</td>
<td>(Selvaraju and Selvarangan 2010)</td>
</tr>
<tr>
<td>InfAfor</td>
<td>5′-GACCRATCCTCTGACCTCTGAC-3′</td>
<td>(World Health Organization 2009)</td>
</tr>
<tr>
<td>InfArev</td>
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<tr>
<td>InfAprobe</td>
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<td>(World Health Organization 2009)</td>
</tr>
<tr>
<td>M+4213</td>
<td>5′-TCCTCAGGTGCCAAGATAC-3′</td>
<td>(Wise <em>et al.</em> 2004)</td>
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<tr>
<td>M-4350</td>
<td>5′-TGCCCCCTCTCCAGTATAGT-3′</td>
<td>(Wise <em>et al.</em> 2004)</td>
</tr>
<tr>
<td>M-4268</td>
<td>5′-FAM/TTTTAAGCTCAGGACGCGC/IABlkFQ-3′</td>
<td>(Wise <em>et al.</em> 2004)</td>
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</table>
Table 2  LOD\textsuperscript{a} using NA assay and RT-qPCR for *Influenza virus* and *Newcastle disease virus*.

<table>
<thead>
<tr>
<th>Virus</th>
<th>NA assay\textsuperscript{b}</th>
<th>RT-qPCR\textsuperscript{b}</th>
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<tbody>
<tr>
<td><em>Influenza A(H1N1) virus</em></td>
<td>$1.10 \times 10^5$</td>
<td>$1.10 \times 10^6$</td>
</tr>
<tr>
<td><em>Newcastle disease virus</em></td>
<td>$1.58 \times 10^5$</td>
<td>$1.58 \times 10^5$</td>
</tr>
</tbody>
</table>

\textsuperscript{a} The lower limit of detection is displayed as the lowest viral dilution that was detected using both methods.
\textsuperscript{b} EID50/ml