

Neuraminidase activity as a potential enzymatic marker for rapid detection of airborne viruses

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Abbreviations: 4-MU-4-methoxy-Neu5Ac, 4-methylumbelliferyl 4-*O*-methoxy *N*-acetylneuraminic acid; 4-MU-NANA, 4-methylumbelliferyl *N*-acetylneuraminic acid; AAF, amnioallantoic fluid; EID₅₀, 50% egg infectious dose; HA, hemagglutinin; HAU, hemagglutinating units; LOD, limit of detection; NA, neuraminidase; NDV, Newcastle disease virus; PCR, polymerase chain reaction; RBC, red blood cells; RFU, relative fluorescence units; RLU, relative light units; RH, relative humidity; S/N, signal-to-noise ratio; RT-PCR, real-time reverse-transcription PCR; SPF, specific pathogen-free.

ABSTRACT

Viruses offer a limited range of targets for their detection. To date, PCR and RT-PCR have been widely used for detection of viruses. In the case of environmental air sampling, the ability to detect a broad range of viruses would constitute a significant advantage for preventing outbreaks of airborne-transmitted viral infections. Given that neuraminidase is found on some respiratory virus species of medical or agricultural importance, this enzyme could theoretically be used to detect several different airborne viruses in a single assay. The aim of the present study was to evaluate the potential of neuraminidase activity as a marker for rapid detection of airborne viruses. We first validated the use of a low-pathogenic strain of Newcastle disease virus (NDV) as a model airborne virus. Our findings revealed that neuraminidase activity-based assays are almost as sensitive as RT-PCR assays currently used for detection of NDV. We also validated the utilization of a neuraminidase substrate specific to viral neuraminidase. Experiments conducted in a controlled chamber demonstrated that the neuraminidase activity is preserved after aerosolization, air sampling using impingement and handling. Finally, we tested our method with swine barn air samples. Our results demonstrate that neuraminidase activity-based assays are suitable for detection of viruses in air samples.

1. INTRODUCTION

Viral respiratory infections are in general highly contagious. In the case of avian influenza and Newcastle disease, all birds in a flock can become infected within three to four days, and the viruses can spread easily to other flocks via contaminated hands, clothes, equipment of bird handlers and possibly via the airborne route (Crossley et al. 2005; Nicholson et al. 2003). During the 2002-2003 Exotic Newcastle Disease control campaign in Southern California, more than 80,000 samples (mostly from individual birds) were tested by real-time reverse-transcription PCR (RT-PCR) (Crossley et al. 2005). In parallel to these tremendous efforts to control the outbreak, the same group of researchers evaluated air sampling as an efficient and cost-effective means of sampling flocks for detection of a circulating virus (Hietala et al. 2004). Among the advantages mentioned by the authors are the reduction of direct human contact with flocks, the elimination of individual bird handling and the providing of a time- and cost-efficient sampling technique.

Unlike bacteria, which contain ribosomal RNA and a number of conserved genes, viruses possess no “universal” markers. Assays based on viral nucleic acid amplification (such as PCR and RT-PCR) constitute the most sensitive approaches to detect specific viruses in a reasonable amount of time. However, to be applicable to continuous monitoring of environmental air samples, these methods are limited to viruses whose genome has been sequenced or for which genetic information is available. In the case of environmental air sampling, the ability to detect a broad range of viruses, as well as to detect viral particles that retained their infectious potential, would constitute significant advantages for preventing outbreaks of airborne-transmitted viral infections and for surveillance purposes.

The enzymatic activity of neuraminidase has already been exploited to detect influenza viruses in a point-of-care diagnostic test (Zstatflu™ from ZymeTx, Inc.) (Achyuthan et al. 2003;

Shimasaki et al. 2001). Neuraminidase is involved in late steps of viral replication by facilitating the release of viral particles from the host cell surface.

Several substrates can be used to detect neuraminidase activity. The most widely used is the fluorogenic molecule 4-methylumbelliferyl *N*-acetylneuraminic acid (4-MU-NANA). More recently, a chemiluminescence-based substrate for neuraminidase (1,2-dioxetane derivative of sialic acid) was developed (Buxton et al. 2000). This substrate was reported to be more sensitive than the fluorogenic substrate. But, these substrates are not specific to viral neuraminidase. Indeed, a wide range of organisms including viruses, bacteria and mammalian cells possess neuraminidase activity. However, it is known in the literature that modifications in positions 4, 7, 8 and 9 of the Neu5Ac group change the specificity of the substrate toward different neuraminidase sources (Beau and Schauer 1980; Corfield et al. 1986; Liav et al. 1999; Shimasaki et al. 2001; Varki and Diaz 1983). The 4-methoxy-Neu5Ac was demonstrated to be specific toward all viral neuraminidases tested and unrecognized by mammalian and bacterial neuraminidases (Beau and Schauer 1980; Liav et al. 1999; Shimasaki et al. 2001). The 4,7-dimethoxy-Neu5Ac substrate was found to be specific to influenza virus neuraminidase only (Liav et al. 1999; Shimasaki et al. 2001).

Given that neuraminidase is found not only on influenza but also on other respiratory viruses (Table 1), this enzyme could theoretically be targeted to detect several different airborne viruses in a single assay. However, to our knowledge, neuraminidase activity-based assays have been tested only with samples containing high levels of neuraminidase activity, such as purified neuraminidase, pure cultures and clinical samples; they might not be sensitive enough to detect neuraminidase activity in air samples with low viral concentrations. Moreover, the effect of air sampling on neuraminidase activity has not yet been addressed.

The aim of the present study was to evaluate the potential of neuraminidase activity as a marker for rapid detection of airborne viruses. We first validated the use of a low-pathogenic strain of Newcastle disease virus (NDV) as a model airborne virus. We also validated the utilization of a 4-MU-4-methoxy-Neu5Ac substrate for the specific detection of viral neuraminidase activity. The sensitivity of neuraminidase assays was then compared with that of RT-PCR, the most sensitive method for rapid detection of NDV (Wise et al. 2004). The effects of aerosolization, sampling, and handling on neuraminidase activity were then evaluated. Finally, we tested the neuraminidase assay with swine barn air samples.

2. MATERIALS AND METHODS

2.1. Bacterial and viral strains

Bacterial strains used in this study are described in table 2. Culture media were supplied by Difco (Difco Laboratories, Detroit, MI). Bacteria were grown 24h on agar plates. Colonies were removed with sterile swab and suspended in 200µl of sterile water prior to neuraminidase assays. The influenza A H1N1 pr/8/34 strain was obtained from American Type and Culture Collection (ATCC VR-95). Influenza virus was cultured on specific pathogen-free chicken eggs. The Newcastle disease virus (NDV) strains used in the experiments shown in Fig. 1 (Hitchner B1, LaSota, APMV-1/parrot/599/94, Komarov and Miyadera) (Grund et al. 2002) were part of the depositary of the OIE and national German Newcastle Disease Reference Laboratory, Friedrich-Loeffler Institute, Insel Riems, where this experiment was carried out. Every other experiment was performed at the Centre de recherche de l'Institut universitaire de cardiologie et pneumologie de Québec using the Hitchner B1 strain, which was purchased as a live vaccine (Wyeth Animal Health) and used after one passage in chicken embryos.

2.2. Inoculation of chicken embryos with NDV and influenza

Specific pathogen-free (SPF) chicken eggs were obtained from the Canadian Food Inspection Agency. Eggs were disinfected with 70% ethanol and pre-incubated (37.8°C to 38.2°C, 40% to 50% RH) in an OVA-EASY incubator (Brinsea Products Inc., Titusville, FL) for 9 to 10 days prior to injection. Eggs were inoculated at the allantoic sac according to standard diagnostic procedures as laid down by the European Union (Council of the European Communities, Directive 92/66/EEC). After inoculation, eggs were incubated for 4 days and the amnio-allantoic fluid (AAF) was collected according to standard procedures and stored at -86°C until used. The

infectious titer, expressed as 50% egg infectious dose (EID₅₀) units, was calculated according to Reed and Muench using five eggs per dilution (Reed and Muench 1938) The Reed Muench index was calculated according the equation 1. The index was applied to the dilution that give 50% infected eggs to obtain the EID₅₀/0.1ml. The EID₅₀/0.1ml was converted in EID₅₀/ml.

$$1 \quad \text{Index} = \frac{(\% \text{ Infected at dilution immediately above } 50\%) - 50\%}{(\% \text{ Infected at dilution immediately above } 50\%) - (\% \text{ infected at dilution immediately below } 50\%)}$$

2.3. Hemagglutination assays

Chicken red blood cells (RBC) were obtained from Promat-Bio (Québec, Québec, Canada) as a 1:1 preparation with Alsever's solution (114mM dextrose, 27mM sodium citrate, 29mM citric acid, 72mM NaCl). Upon reception, RBC were washed three times with Phosphate-Buffered Saline (PBS) and suspended at a final concentration of 1% in PBS. Hemagglutination assays were performed according to standard diagnostic procedures for NDV as laid down by the European Union (Council of the European Communities, Directive 92/66/EEC). To assess whether RBC hemagglutination by AAF was not due to contamination with bacteria possessing a hemagglutinating activity, all AAF were tested on blood agar plates. Plates were incubated at 37°C for 48h and bacterial growth was verified. None of the AAF used in the experiments presented here showed bacterial growth.

2.4. Synthesis of 4-methylumbelliferyl 4-*O*-methoxy *N*-acetylneuraminic acid

The 4-methylumbelliferyl 4-*O*-Methoxy *N*-acetylneuraminic acid (4-MU-4-methoxy-Neu5Ac) was synthesized and purified according to the synthetic schemes described previously (Liav 1995; Liav et al. 1999). The main features of the ¹H NMR spectrum of the product (in CD₃OD at 300 MHz) are in agreement with the structure: δ 7.65 (d, *J*=8.7 Hz, 1H, 4MU), 7.28-

7.23 (m, 2H, 4MU), 6.20 (d, $J=1.2$ Hz, 4MU), 3.42 (s, 3H,OMe, Neu5Ac), 3.18 (dd, $J_{3\text{eq},3\text{ax}}=11.6$ Hz, $J_{3\text{eq},4}=4.6$ Hz, H-3_{eq}, Neu5Ac), 2.62 (d, $J=1.2$ Hz, 3H, Me, 4MU), 2.02 (s, 3H, N-Ac, Neu5Ac), 1.68 (t, $J_{3\text{ax},3\text{eq}} = J_{3\text{ax},4}=11.6$ Hz, H-3_{ax}, Neu5Ac).

2.5. Neuraminidase assays

Chemiluminescence-based assays were performed using the NA-STAR® Influenza Neuraminidase Inhibitor Resistance Detection Kit (Applied Biosystems, Foster, CA) according to the manufacturer's instructions. Briefly, 50 μL of each sample in neuraminidase assay buffer (26 mM MES pH 6.0, 4 mM CaCl_2) were loaded in duplicate into 96-well microplates provided with the kit. As blank controls, 50 μL of NA assay buffer only was used in 8 wells (experiment from Fig. 2 and Table 3). Then, 10 μL of NA-STAR® substrate (diluted 1:1000) were added to each well. Although the assay can be carried out at room temperature, the plates were incubated at 25°C (for 1h) to ensure reproducibility. Following incubation, 60 μL of NA-STAR® enhancing solution were added to each well, the plates were shaken for 10 seconds, and luminescence was measured using the Fluoroskan Ascent FL instrument (Thermo Scientific). Results were expressed as relative light units (RLU) and were compared to the background RLU values (blank made of NA assay buffer only).

Fluorescence-based assays were adapted from two different protocols described in the literature (Matrosovich et al. 1999; Nayak and Reichl 2004). Briefly, NA activity was detected with 4-methylumbelliferyl *N*-acetylneuraminic acid (4-MU-NANA, Sigma-Aldrich, Oakville, Ontario, Canada) used as a substrate. Fifty μL of a 40 μM solution of 4-MU-NANA in calcium-TBS buffer (6.8 mM CaCl_2 , 145 mM NaCl, 20 mM Tris; pH 7.3) or 10 μM solution of 4-MU-NANA in MES buffer pH 5.5 (32 mM MES, 4 mM CaCl_2) were loaded into a 96-well microplate, and 5 μL of virus dilutions (diluted in water) were added into each well. The plate

was incubated for 45 min or 1 h at 37°C, and the reactions were stopped by adding 100 µL of 0.1 M glycine buffer (pH 10.7) containing 25% ethanol. The fluorescence of released 4-methylumbelliferone was determined with a Fluoroskan Ascent FL instrument ($\lambda_{\text{exc}} = 360 \text{ nm}$, $\lambda_{\text{em}} = 460 \text{ nm}$) (Thermo Scientific).

Fluorescence-based assay with the specific substrate (4-MU-4-methoxy-Neu5Ac) were carried out with 50µl of 10µM solution in MES Buffer pH5.5 for 45 min as described for 4-MU-NANA.

2.6. Aerosolization experiments in a controlled chamber

In each experiment, one vial containing 1.8 mL of AAF from infected chicken embryos (stored at -86°C, referred to here as infected AAF) was thawed at room temperature. As a control, one vial containing the same volume of AAF from uninfected chicken embryos (also stored at -86°C, referred to here as uninfected AAF) was also thawed at room temperature. Both AAF samples were used in parallel and were subjected to similar conditions in all experiments. Cellular debris were eliminated by filtering the AAF samples on 0.45-µm sterile filters (Millipore, Billerica, MA). One mL of each filtrate was diluted with 69 mL of NA assay buffer. NDV-containing aerosols were generated with an atomizer (model 9302, TSI Inc., Shoreview, MN, USA) at a dispersion rate of 2.2 L/min. The aerosols were then passed through a desiccator (model 306200, TSI Inc.) before entering a GenaMini chamber (SCL Medtech Inc., Montreal, QC, Canada) from which the aerosols were sampled. Aerosol inside the chamber was mixed with the dilution air at a rate of 22.6 L/min and was maintained at a continuous flow throughout the sampling procedure. Medical-grade compressed air was used for aerosol generation and dilution. The experiments were conducted at the laboratory temperature (approximately 22°C).

The aerosol size distribution and concentration was monitored with an aerodynamic particle sizer (APS, model 3321, TSI Inc. Minneapolis, USA). The mass median diameter (MMD) of the aerosols ranged between 1.3 and 1.7 μm , and the total concentration ranged between 3.6×10^3 and 6.3×10^3 particles/ cm^3 . Two sterile Biosamplers® (SKC) were loaded with 15 mL of NA assay buffer. Aerosols were sampled for 20 min at about 12.3-12.5 L/min (maximum of the critical orifice capacity of each Biosampler®) driven by a Gilair Aircon II pump (Levitt Sécurité, Montréal, Canada). Following sampling, the residual liquid in the Biosamplers® was collected and the remaining particles were eluted from the vessel wall with 5 mL NA buffer supplemented with 0.01% Tween 20. The air sample was stored at 4°C together with the remainder of the AAF that had not been aerosolized (referred to as non-aerosolized AAF). The infected AAF was then submitted to the same treatments. Both non-aerosolized and aerosolized AAF were tested, as described above, for neuraminidase activity and viral infectivity. Their viral genome equivalent concentration was also evaluated using quantitative RT-PCR. All samples were stored at 4°C 16h-20h before analysis.

2.6. Quantification of viral particles using RT-PCR

RNA was extracted from both non-aerosolized and aerosolized AAF (infected and non-infected) using the QIAamp viral RNA mini kit (Qiagen, Chatsworth, CA) according to the manufacturer's instructions and was stored at -86°C. Real-time reverse-transcription PCR for quantification of NDV was performed as described by Wise and colleagues with the NA pre-1960 genotype primers and probe set (Wise et al. 2004). Primers (M+4213 TCCTCAGGTGGCCAAGATAC, M-4350 TGCCCCTTCTCCAGCTTAGT) and dual-labeled probe (M-4268 FAM-TTTTAACGCTCCGCAGGCAC-Iowa Black) were supplied by Integrated DNA technology (Coralville, IA).

The assay components per 25 μL were: 0.5 μL of kit-supplied enzyme, 5 μL of template RNA, 12.5 pmol of each primer, 5 pmol of dual-labeled probe, and 12.5 μL of 2X master mix of the iScript One-Step RT-PCR Kit for Probes (BioRad Life Sciences, Mississauga, Ontario, Canada). The RT-PCR program was as follows: 15 min at 50°C, 5 min at 95°C followed by 40 amplification cycles including denaturation at 94°C for 15 sec, annealing and elongation at 60°C for 60 sec, and fluorescence measurement. All experiments were performed using an Opticon 2 system (MJ research, Waltham, MA).

Data were analyzed with the Opticon monitor software supplied with the apparatus. For each RT-PCR run, a standard curve was generated in duplicate with *in vitro*-transcribed RNA. Serial 10-fold dilutions from 10^2 to 10^7 molecules per reaction tube were used to establish standard curves. For each sample of unknown concentration, three 10-fold dilutions made in duplicate were analyzed, and the concentration was determined using the standard curves. The background was subtracted using the "average over cycle range" function of the software. Threshold values (Ct) were determined manually. The plotting of Ct as a function of the logarithm of DNA template gives a straight line. The slope of this graph line gives the PCR efficiency (E) according to the equation: $E=(10^{-1/\text{slope}}-1) \times 100$. Results were considered as adequate when E was over 85% and error between standard points and regression curve was lower than 0.1.

The RNA for standard curves was prepared as follows. The NA gene of NDV was amplified from cDNA by PCR using the following primers: STD4021+ CGTAGATAGGAAGGGGAAGA and STD4430- ATGATGATCTGGGTGAGTGG. The amplicon was cloned into the pCR2.1-TOPO vector using the TOPO TA cloning kit (Invitrogen, Burlington, Ontario, Canada). Another PCR amplification was performed using this plasmid construction as a template, a primer corresponding to the T7 promoter and the primer STD4021+. *In vitro* transcription was generated

from the T7 promoter on the PCR product using the MEGAshortscript T7 kit (Ambion Applied Biosystems, Austin, Tx) according to the manufacturer's instructions. The DNA template was degraded, the enzymes were inactivated and the RNA was quantified using a GeneQuant pro UV/Vis spectrophotometer (Biochrom Ltd, Cambridge England).

2.7. Field sampling

Swine barn air samples were collected in January 2010 in a building with 66% slatted floor. Sampling took place before the hogs were sent to the slaughterhouse. The animals weighed approximately 110kg (at about 6 months of age) when the barn was sampled. No animals were treated for respiratory infections or diarrhea at the time of the study. The temperature inside the barn was 20°C and relative humidity ranged from 62% to 68% during the sampling period. Air samples were taken on a stationary sampling platform, 1m above the ground. Sampling devices were placed as far away as possible from ventilation fans, doors or windows. All pumps were calibrated using a DryCal 2 flow meter (SKC, Ancaster, Ontario, Canada). All samples were kept on ice following sampling, and remained at 4°C until analysis (16h-20h).

Air samples were collected using all glass impingers with 30mm jet-to-bottom spacing (AGI-30, Ace Glass Inc., Vineland, NJ, USA) filled with 20mL sterile salt solution (145mM NaCl). The AGI-30 impingers were connected to a high-volume pump (Gast Manufacturing Inc., Benton Harbor, MI, USA) at a flow rate of 12.5L/min for 20min in triplicate. Back at the laboratory, the sample volumes were measured.

Two cubic meters of air were sampled in duplicate using a Sartorius AirPort MD8 (Sartorius Stedim Biotech S.A., Aubagne Cedex, France) samplers mounted with 80mm gelatin filters at a flow rate of 30L/min. The filters were dissolved in 15ml of sterile salt solution warmed at 37°C.

Air samples were also collected using National Institute for Occupational Safety and Health (NIOSH) 2-stages cyclone aerosol sampler (CDC/Natl. Inst. Occup. Safety & Health, Morgantown, WV) mounted with a 15ml tube (first stage), a 1.5ml tube (second stage) and a backup polycarbonate 0.8 μ M 37mm filter in an open face cassette, and connected to a high-volume pump (Gast Manufacturing Inc.) at a flow rate of 3.5L/min for 240 min in duplicate. Tubes and filters were kept on ice following the sampling. The samples were eluted using 2ml (first stage), 500 μ l (second stage) and 5 ml (filters) of sterile salt solution.

Air samples were also collected using 0.4 μ M 37mm polycarbonate filters mounted into three pieces cassette connected to a Gilair-5 pump (Levitt-Sécurité Limitée, Dorval, Quebec, Canada) at a flow rate of 2L/min for 240 min in triplicate. The filters were eluted using 5ml of sterile salt solution.

A total DNA extraction was performed from 1mL of the AGI-30, 1ml of the polycarbonate filters, 1ml of gelatin filters, 500 μ l of NIOSH first stage and 150 μ l of NIOSH second stage using the QIAamp DNA Mini kit (QIAGEN, Mississauga, ON, Canada) according to the manufacturer's instructions for tissue with the required modifications for gram-positive bacteria. The 16S rDNA of eubacteria were quantified as described previously (Letourneau et al. 2010) using the primers and probes described by (Bach et al. 2002).

3. RESULTS

3.1. Choice of a model for studying neuraminidase activity of airborne viruses

To evaluate the potential of neuraminidase activity as a marker for detection of airborne viruses, we chose as a model an avian respiratory virus, the Newcastle disease virus (NDV). NDV belongs to the *Paramyxoviridae* family. It possesses a well-described surface protein,

which carries both the hemagglutinating and neuraminidase (HN) activities of the virus. Lentogenic strains of NDV, which have only mild respiratory effects on the infected birds, are used as live attenuated vaccines in poultry to prevent more severe forms of the Newcastle disease, caused by mesogenic and velogenic strains of NDV. NDV is known to keep its immunological properties and possibly its structural integrity when aerosolized (Yadin 1980), as spraying lentogenic strains in poultry barns is one of the recommended ways to vaccinate bird flocks.

To assess whether lentogenic strains of NDV constitute good models for evaluating the potential of neuraminidase activity for detection of viruses, we compared the neuraminidase activity of several strains of NDV with different levels of virulence. Three lentogenic strains (Hitchner B1, LaSota and APMV-1/parrot/599/94), one mesogenic strain (Komarov) and one velogenic strain (Miayadera) were compared. The HN protein titer of each preparation was determined using hemagglutination assays, and the same amount of hemagglutinating units (HAU) was used for each strain in a fluorescence-based assay measuring neuraminidase activity. Results were expressed as the ratio of neuraminidase (RFU) / hemagglutinating (HAU) activities.

Values were compared with a one-way ANOVA with Tukey's comparison test (Tukey 1953), and three groups were created based on statistical differences (a, b and c). Statistical comparison of the different strains of NDV suggests that APMV-1/parrot/599/94 (group a, Fig. 1) had the highest level of neuraminidase activity (NA), followed by Komarov and Hitchner B1 strains (group b). LaSota and Miayadera strains (group c) had the lowest RFU / HAU ratios. Although these different strains of NDV showed different levels of neuraminidase activity, the differences did not exceed twofold. Moreover, neuraminidase activity does not seem to correlate with the virulence of the strain, as for instance the Hitchner B1 and LaSota strains (lentogenic) are in the same statistical group (c) as the Miayadera strain (velogenic). Thus, it seems like

lentogenic strains constitute good models for studying the potential of neuraminidase activity as a marker for detection of airborne viruses. The Hitchner B1 strain was used in all subsequent experiments and referred to as NDV B1.

3.2. Validation of 4-MU-4-methoxy-Neu5Ac substrate

Several substrates are commercially available to assess neuraminidase activity. The most widely used (4-MU-NANA) is based on a fluoregenic molecule, the 4-MU. More recently, another substrate was developed to allow a chemiluminescent detection, the NA-STAR®. This substrate has the same acetylneuraminic acid part as the 4-MU-NANA, but the 4-MU fluorescent molecule was replaced by the chemiluminescent spiroadamantyl-1,2-dioxetane. This substrate is available in the NA-STAR® Influenza Neuraminidase Inhibitor Resistance Detection Kit (Applied Biosystems). Other substrates cited in the literature were tested and these exhibited different specificity toward neuraminidase source. However, these substrates are not commercially available. We were able to synthesize one of these substrates, the 4-methoxy-Neu5Ac. We coupled the correctly modified acetylneuraminic acid with the 4-MU fluorogenic molecule to obtain a fluorescent substrate: the 4-MU-4-methoxy-Neu5Ac.

The 4-methoxy-Neu5Ac specificity to detect viral neuraminidase was already challenged in the literature with several viral strains, oral streptococci and *Vibrio cholerae* bacterial strains as well as mammalian cells (Beau and Schauer 1980; Shimasaki et al. 2001). In this study we tested the specificity of this substrate against other bacterial strains in order to justify the use of this substrate to detect viral neuraminidase from swine barn air samples contaminated with high bacterial loads. The bacterial strains were chosen for their occurrence in farm environment or their known neuraminidase activity. The 4-MU-NANA was used as a control to evaluate the neuraminidase activity of the bacterial strains tested. The 4-MU-4-methoxy-Neu5Ac was

hydrolyzed only by viral neuraminidase (Table 3). The bacterial strains with neuraminidase activity were only able to hydrolyze the 4-MU-NANA (Table 3).

3.3. Sensitivity of neuraminidase activity-based assays for viral detection

To assess whether neuraminidase activity-based assays are sensitive enough to be used for detection of airborne viruses, we selected the two most sensitive commercially available substrates described in the literature: the 4-MU-NANA and the NA-STAR®. The latter was described as being 60-fold more sensitive than the fluorescence-based assay; however, the sensitivity of these assays for viral neuraminidases has not been compared, as only purified bacterial neuraminidase was used in that study (Buxton et al. 2000). We also evaluated the sensitivity of the viral specific substrate 4-MU-4-methoxy-Neu5Ac.

We compared the sensitivity of these three assays for detection of NDV B1 (Fig. 2). NDV B1 was grown for one passage in embryonated specific pathogen-free (SPF) eggs, and the infectious titer of this virus preparation was then determined and expressed as 50% egg infectious dose (EID50) units. The crude values, initially expressed as RLU or RFU, were transformed into S/N values by dividing them by the control value. The two fluorescent substrates, the viral specific 4-MU-4-methoxy-Neu5Ac and the non-specific 4-MU-NANA, have the same sensitivity toward NDV B1 neuraminidase. The limit of detection (LOD) with the fluorescent substrates was estimated to $3,3 \times 10^4$ EID50 units for NDV B1. The LOD of the chemiluminescence-based assay for NDV B1 was estimated to 1.5×10^2 EID50 units. Thus, when setting up the detection threshold at twofold the background for both types of assays, the chemiluminescence-based assay was approximately 200-fold more sensitive than fluorescence-based assays. By comparison, the LOD of a RT-PCR assay developed to detect NDV RNA in clinical samples from birds was estimated to be 10^1 to 10^3 EID50 units (Wise et al. 2004).

3.4. Detection of neuraminidase activity on airborne viruses

Air sampling was shown to affect the integrity of viral particles (reviewed by Verreault et al. 2008). Thus, when selecting a marker for detection of airborne viruses, it is essential to verify whether it can still be detected following aerosolization and air sampling. To determine whether neuraminidase activity of NDV B1 is affected by aerosolization followed by air sampling, the virus was aerosolized in a controlled chamber, the viral particles were collected using a liquid impinger, and the effect on both neuraminidase activity and viral infectivity was evaluated. RT-PCR was used to quantify the viral particles before aerosolization and after sampling, and the results were expressed as EID50 units/genome equivalent copy (infectivity) and RLU/genome equivalent copy (neuraminidase activity). Three experiments were carried out and the results are shown in Table 4. As shown in this table, infectivity of NDV B1 was not significantly affected by aerosolization and sampling: the effect varied from a 50% decrease to a 33% increase. Even if the effect was more variable, the neuraminidase activity of NDV was not negatively affected by such a treatment; in all three experiments performed, neuraminidase activity of NDV even increased following aerosolization and sampling (by 1.8-fold to 7.2-fold depending on the experiment).

To make sure that the neuraminidase activity remains stable over extended sampling period, tubes and filters were spiked with NDV and mounted on National Institute for Occupational Safety and Health (NIOSH) 2-stage cyclone aerosol sampler. The samplers were connected to a pump calibrated at a flow rate of 3.5L/min for 1h, 2h, 4h and 6h. After the exposition, the samples were kept at 4°C over night before elution and neuraminidase activity measurement to simulate handling condition similar to field sampling. Over this sampling period, the neuraminidase activity varied from a 26% decrease to a 19% increase (Table 5). In the meantime, gelatin filters were also spiked and kept at room temperature for the same period. The

neuraminidase activity from the spiked gelatin filters was superior from 2% to 19% compared to the spiked sample kept at 4°C for the duration of the experiment (Table 5).

During the sampling in the swine barn, AGI-30 all glass impingers, polycarbonate filters and NIOSH 2-stage cyclone aerosol samplers collected 2×10^7 to 4×10^7 bacterial genomes per cubic meters of air (Table 6). The Sartorius AirPort MD8 was slightly more effective and collected 1×10^8 bacterial genomes per cubic meters. We detected neuraminidase activity in the first stage of the NIOSH 2-stages cyclone aerosol sampler and Sartorius AirPort MD8 samplers using the 4-MU-NANA substrate (Table 6). Neuraminidase activity was undetectable using 4-MU-4-methoxy-Neu5Ac substrate.

Sampling with AGI30 and polycarbonate filters collected a smaller air volume (Table 6). That could explain why no neuraminidase activity was detected using these samplers. The NIOSH 2-stage cyclone aerosol sampler fractionates aerosol particles by aerodynamic diameter. At a flow rate of 3.5 L/min, the cut off of the two stages are $1.8 \mu\text{M}$ and $1 \mu\text{M}$ respectively and the backup filter collects submicron particles (Lindsley et al. 2006). Regarding that bioaerosols median diameter in swine confinement buildings is usually $11 \mu\text{M}$ (O'Shaughnessy et al. 2002), the first stage of the NIOSH 2-stages was expected to collect more particles compared with the second stage and backup filter. Microorganisms with neuraminidase activity can be adsorbed on large particles as well as smaller particles. However, in our swine barn air samples, the microorganisms with neuraminidase activity were found on particles larger than $1.8 \mu\text{M}$ collected in the NIOSH first's stage.

Concerning neuraminidase activity, the best results were obtained with the Sartorius AirPort MD8 sampler. Even if the samples were less concentrated, the neuraminidase activity per volume of air was higher.

In order to evaluate if the high bacterial and dust load could interfere with the specific detection of viral neuraminidase activity, we spiked the swine barn air samples with several concentration of NDV. The neuraminidase activity of the spiked samples was detected using the 4-MU-4-methoxy-Neu5Ac. The neuraminidase activity detected in the spiked samples was compared to the neuraminidase activity of NDV diluted in salt solution (145mM NaCl) (Fig. 3). The neuraminidase activity detected in the MD8 spiked samples was reduced by 9% compare to NDV into salt solution. The neuraminidase detection in the NIOSH first stage was reduced by 44% on average. Nevertheless, the LOD in the spiked samples was not affected (Fig. 3).

4. DISCUSSION AND FUTURE DIRECTIONS

4.1. A substrate specific to viral neuraminidase

Several organisms, including non-pathogenic bacteria, possess neuraminidase-like enzymes (reviewed by Corfield 1992). However, the utilization of a substrate specific to viral neuraminidase such as the 4-MU-4-methoxy-Neu5Ac made possible the specific detection of viral neuraminidase without interference of bacterial load. Microorganisms without neuraminidase activity such as *E. coli*, respiratory syncytial virus and adenovirus do not interfere with the virus detection using the viral specific neuraminidase activity-based assay (Table 3). Moreover, our experiments with field samples demonstrated that the high bacterial load do not compromised the sensitivity of the assay (Fig. 3). It was previously demonstrated in the literature that bioaerosols in swine confinement buildings contain high loads of clostridia and other bacteria from pigs' feces (Nehme et al. 2008). The neuraminidase activity detected in our samples with the 4-MU-NANA substrate (Table 6) probably originated from clostridia in the samples, which posses neuraminidase activity (Tables 3). Nevertheless, the presence of high bacterial load with neuraminidase activity did not lead to false positive detection with the viral specific substrate 4-MU-4-methoxy-Neu5Ac.

4.2. A rapid, easy and sensitive assay for detection of viruses

Several characteristics of neuraminidase activity-based assays make them more suitable than RT-PCR for routine surveillance of airborne virus outbreaks. In addition to being comparable regarding sensitivity (see Fig. 2) and slightly more rapid (30-60 min), neuraminidase activity-based assays are simpler, as they require no specimen processing and are performed at constant temperature, which makes them more easily amenable to automation. Even if the NDV B1

detection using the 4-MU-4-methoxy-Neu5Ac was less sensitive, we can expect that a 4-methoxy-Neu5Ac substrate coupled with a chemiluminescent molecule would be as sensitive as the NA-STAR® detection assay and RT-PCR for the detection of viral neuraminidase. In addition, neuraminidase activity-based assays could allow detection of several distinct viruses (see Tables 1 and 3) in a single assay, which would make them a powerful tool for monitoring several threats at once. By contrast, development of multiplex PCR assays requires several considerations (Elnifro et al. 2000) such as 1- using a rational approach for the inclusion or exclusion of specific pathogens in the assay, 2- evaluating compatibility among the primers within the reaction mixture such that there is no interference, 3- using primer pairs that are inclusive for as many strains of the target pathogen(s) as possible, 4- using controls to detect false-positive results due to carryover contamination, 5- using controls to detect false-negative results due to reaction failure. Of course, not all viruses possess a neuraminidase activity, so markers for detection of other viruses should also be included in a global strategy of viral surveillance. The rationale for selecting such markers should be based on the knowledge of potential viral threats in a given environment.

4.3. A marker that can be detected on airborne viral particles

Various sampling devices have been developed for collecting airborne viruses (reviewed by Verreault et al. 2008). These methods have different properties regarding their efficiency for recovering viral particles and for preserving their infectivity. The least destructive and therefore the most commonly used samplers for the capture of airborne viruses are liquid impingers, such as all-glass impingers (AGIs) and Biosamplers®. Although the diameter of individual, enveloped virions of NDV varies between 100 nm and 200 nm, aerosol size in our aerosolization experiments was higher than 1 µm (between 1.3 µm and 1.5 µm). Thus, the droplet nuclei created

included viral particles along with other solute present in the nebulization media. In this aerosol size range, Biosamplers® are efficient in particle collection since their D50 value (diameter under which 50% of the particles are captured) is below 300 nm (Grinshpun et al. 2007). Using this device, we were able to recover aerosolized NDV particles and quantify their neuraminidase activity as well as their infectivity.

In all three experiments performed, the neuraminidase activity increased (by 1.8- to 7.2-fold) following the recovery of the airborne viral particles. This phenomenon could be at least partly due to the particle breakup caused by the aerosolization and/or sampling processes, which may have increased the contact between active sites of neuraminidase and the substrate. This constitutes an interesting observation; if it is due to either the airborne state or the sampling, the sensitivity of the assay might be higher than that determined here using liquid virus suspensions (see Fig. 2). However we cannot conclude that this increase is significant. Nevertheless, our experiments clearly demonstrate that air sampling using the Biosampler® does not negatively affect the neuraminidase activity of NDV. However, sampling with liquid impingers is performed for short periods of time (20 min). Thus the air volume collected for analysis is limited and restricts the detection to microorganism presents in high concentration. Other sampling methods are required in order to detect rare events or less concentrated particles.

Filters (PTFE or polycarbonate) are very often used for sampling over long periods. However, this method is more destructive for viruses (Fabian et al. 2009; Verreault et al. 2008). Recently, the National Institute for Occupational Safety and Health (NIOSH) developed a 2-stages cyclone sampler (Lindsley et al. 2006). This sampler was used successfully to detect influenza virus from air samples in controlled conditions (Blachere et al. 2007) and in hospital settings (Blachere et al. 2009). Influenza viruses were detected using RT-PCR, which means that

the RNA of the sample was preserved over the sampling period (Blachere et al. 2009; Blachere et al. 2007). Our results with spiked NIOSH 2-stages cyclone sampler collection tubes and filters demonstrates that the neuraminidase activity of NDV B1 is only slightly affected over a sampling period of 6h with this device.

Our results with the Sartorius AirPort MD8 sampler in the swine barn sampling suggest that this apparatus is promising for the sampling of airborne viruses in this kind of environment. Indeed, this sampler leads to the higher neuraminidase activity detection per air volume. It could mean that the collected particles contain more microorganisms with neuraminidase activity, or that the neuraminidase activity was more preserved. This sampler uses gelatin filters, which are known to be gentler than polycarbonate and PTFE filters for sensitive samples such like viruses (reviewed by Verreault et al. 2008). It could explain why more neuraminidase activity was detected using the Sartorius AirPort MD8.

4.4. A good model for studying the neuraminidase activity of airborne viruses

The potential of neuraminidase activity for detecting orthomyxoviruses has already been demonstrated with the development of a point-of-care diagnostic test for influenza infections (Zstatflu™ from ZymeTx, (Shimasaki et al. 2001)). In the present study, the potential of this marker for detection of another important family of respiratory viruses, paramyxoviruses, has been evaluated. Paramyxoviruses are responsible for several respiratory diseases in both animals and humans (particularly in infants). Airborne transmission of several paramyxoviruses, including Newcastle disease virus, has been demonstrated (Iida 1972; Li et al. 2009; Lorenz and Albrecht 1980). Thus this family is an interesting model for evaluating the potential of neuraminidase activity for detection of airborne viruses of both human and animal importance.

The Hitchner B1 strain seems to be representative of most strains of NDV in regard to its neuraminidase activity (see Fig. 1). However, experiments to estimate the LOD and the effect of aerosolization on neuraminidase activity should be performed with other strains of NDV, as well as with viruses belonging to other species (see Table 1).

Using non-pathogenic models can greatly simplify studies with viruses, especially when aerosols are produced. Bacteriophages of *E. coli* and other bacteria have been widely used to mimic the behavior of pathogenic viruses (reviewed by Verreault et al. 2008). However, the morphological characteristics of these bacteriophages often do not resemble those of mammalian viruses. Since every virus has a unique morphology and susceptibility to environmental factors, the data obtained from such studies are sometimes difficult to interpret. In the present study, we used a live vaccine strain as a surrogate for pathogenic NDV. To our knowledge, this study is a first where a naturally occurring, low-virulent strain of a pathogenic virus was used as a surrogate in aerosolization studies.

4.5. Future directions

There is at present no rapid assay for detection of infectious (viable) airborne viruses. Such assays, in combination with environmental air sampling, could allow discrimination between a current and a past event (e.g. following decontamination of a building) or between a real threat and a fake threat (e.g. following a bioterrorist act). Neuraminidase is present at the surface of viral particles. It is possible that when a viral particle is damaged by environmental stresses, neuraminidase activity is affected. For this reason, one could hypothesize that neuraminidase activity correlates with viral infectivity, i.e. that infectious viral particles show a higher neuraminidase activity than those that lost their infectivity. If it were the case, neuraminidase activity would constitute the first marker for development of rapid assays for detection of

infectious viral particles. In the experiments presented here, there was no loss of infectivity of the viral particles following aerosolization and sampling. Thus we were unable to conclude about the relationship between viability and neuraminidase activity of airborne NDV. These experiments should be repeated under harsher conditions. To our knowledge, no studies have evaluated the link between neuraminidase activity and infectivity of airborne viral particles.

In this work, we demonstrated that detection of NDV based on neuraminidase activity is slightly faster and has a sensitivity similar to that of RT-PCR assays. We also showed that neuraminidase activity is preserved after aerosolization and air sampling using the Biosampler®. Assuming that the robustness of the assay depends on the preservation of the neuraminidase activity, it will be useful to repeat the experiments in a controlled chamber with another neuraminidase-carrying virus.

We confirmed that a substrate specific to viral neuraminidases can discriminate between viral and bacterial neuraminidase activities as previously described in the literature (Beau and Schauer 1980; Liav 1995; Liav et al. 1999; Shimasaki et al. 2001). We demonstrated that this substrate could be used to detect viral neuraminidase from air samples without interference from high bacterial load. Moreover, this marker can be used to detect many types of viruses in a single reaction and requires no sample processing. These attributes make this method very attractive for the detection of viruses in environmental air samples. It would be interesting to test the method in various environments, such as waste water treatment plants and hospital settings.

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Tables and Figures

TABLE 1
Viruses known to possess a neuraminidase activity¹.

Orthomyxoviruses

Influenza virus type A²
Influenza virus type B²

Paramyxoviruses

Parainfluenza viruses²
Newcastle disease virus²
Mumps virus²
Sendai virus²
SV5 virus²
Menangle virus³
Peste des petits ruminants virus (PPRV)⁴
Rinderpest virus⁴
Murayama virus⁵

Togaviruses

Rubella virus⁶

¹ (Table adapted from Taylor 1996)

² (Taylor 1996)

³ (Bowden et al. 2001)

⁴ (Seth and Shaila 2001)

⁵ (Nishikawa et al. 1977)

⁶ (Bardeletti et al. 1975)

TABLE 2

Bacterial strains used in this study and their growth conditions.

Bacteria	Strains	Culture
<i>Actinomyces viscosus</i>	HER-1242 ^a	TSA, 37°C
<i>Clostridium perfringens</i>	ATCC 13124 ^b	TSA, 37°C, anaerobia
<i>Enterococcus faecalis</i>	CD 10 ^c	TSA, 37°C
<i>Escherichia coli</i>	ATCC 35219 ^b	TSA, 37°C
<i>Klebsiella pneumonia</i>	CD 8 ^c	TSA, 37°C
<i>Lactococcus lactis</i>	HER-1449 ^a	M17 agar 0.5% glucose, 25°C
<i>Pseudomonase aeruginosa</i>	ATCC 27853 ^b	TSA, 37°C
<i>Salmonella choleraesuis</i>	ATCC 13076 ^b	TSA, 37°C
<i>Salmonella typhimurium</i>	HER-1023 ^a	TSA, 37°C
<i>Staphylococcus aureus</i>	ATCC 29213 ^b	TSA, 37°C
<i>Streptococcus pneumoniae</i>	HER-1054 ^a	TSA Blood, 30°C
<i>Streptococcus mitis</i>	HER-1055 ^a	TSA Blood, 37°C
<i>Streptococcus salivarius</i>	ATCC 14579 ^b	TSA, 37°C
<i>Vibrio cholerae</i>	HER-1354 ^a	TSA, 37°C

a) Strain ordered at the Felix d'Hérelle reference center for bacterial viruses

b) Strain ordered at the American Type and Culture Collection

c) Strain from Institut Universitaire de Cardiologie et de Pneumologie microbiological department collection

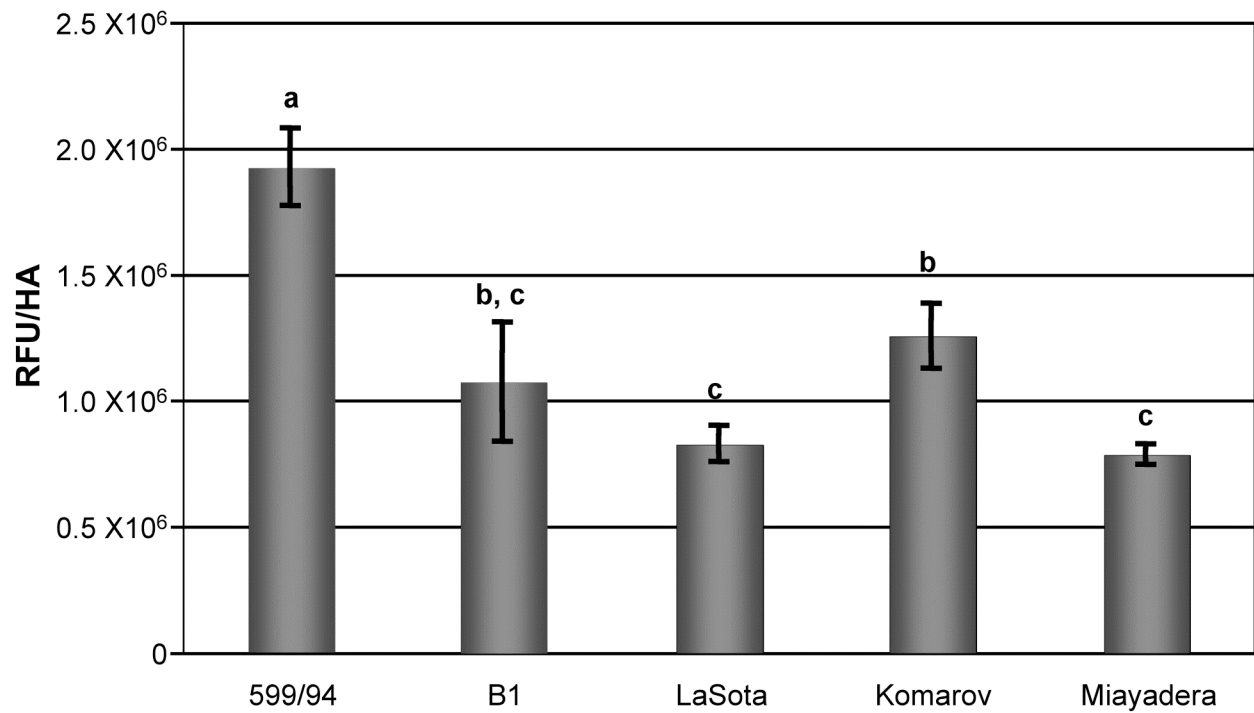


FIG. 1. Comparison of the neuraminidase activity of different strains of Newcastle disease virus.

Average RFU / HAU values for each strain were calculated from three independent experiments.

The assays were performed in 40 μ M 4-MU-NANA in calcium-TBS buffer for 1h at 37°C.

TABLE 3
Activity of viral, bacterial, and mammalian cells toward 4-MU-NANA
and 4-MU-4-methoxy-Neu5Ac.

Strains	4-MU-NANA	4-MU-4Meth Neu5Ac	Reference
Virus			
NDV B1	+	+	This study
Human influenza A	+	+	This study
	na	+ ^b	Shimasaki et al. 2001
Human influenza B	na	+ ^b	Shimasaki et al. 2001
Human parainfluenza type 1	na	+ ^b	Shimasaki et al. 2001
Human parainfluenza type 2	na	+ ^b	Shimasaki et al. 2001
Human parainfluenza type 3	na	+ ^b	Shimasaki et al. 2001
Mumps virus	na	+ ^b	Shimasaki et al. 2001
Respiratory syncytial virus	na	- ^b	Shimasaki et al. 2001
Adenovirus	na	- ^b	Shimasaki et al. 2001
Avian influenza	+ ^a	+ ^b	Beau and Schauer 1980
Bacteria			
<i>Actinomyces viscosus</i>	+	-	This study
<i>Clostridium perfringens</i>	+	-	This study
	+ ^a	- ^b	Beau and Schauer 1980
<i>Enterococcus faecalis</i>	-	-	This study
<i>Escherichia coli</i>	-	-	This study
<i>Klebsiella pneumonia</i>	-	-	This study
<i>Lactococcus lactis</i>	-	-	This study
<i>Pseudomonase aeruginosa</i>	-	-	This study
<i>Salmonella choleraesuis</i>	-	-	This study
<i>Salmonella typhimurium</i>	+	-	This study
<i>Staphylococcus aureus</i>	-	-	This study
<i>Streptococcus pneumoniae</i>	+	-	This study
<i>Streptococcus mitis</i>	+	-	This study
	+	-	Shimasaki et al. 2001
<i>Streptococcus salivarius</i>	-	-	This study
<i>Vibrio cholerae</i>	+	-	This study
	+ ^a	- ^b	Beau and Schauer 1980
<i>Corynebacterium sp.</i>	+	-	Shimasaki et al. 2001
<i>Staphylococcus hominis</i>	+	-	Shimasaki et al. 2001
type 1			
Mamalian cells			
<i>Chinese hamster ovary cells</i>	+	-	Shimasaki et al. 2001

a) Utilization of a chromogenic Neu5Ac instead of the fluorogenic substrate.

b) Utilization of a chromogenic 4-methoxy-Neu5Ac instead of the fluorogenic substrate.

c) na: data not available.

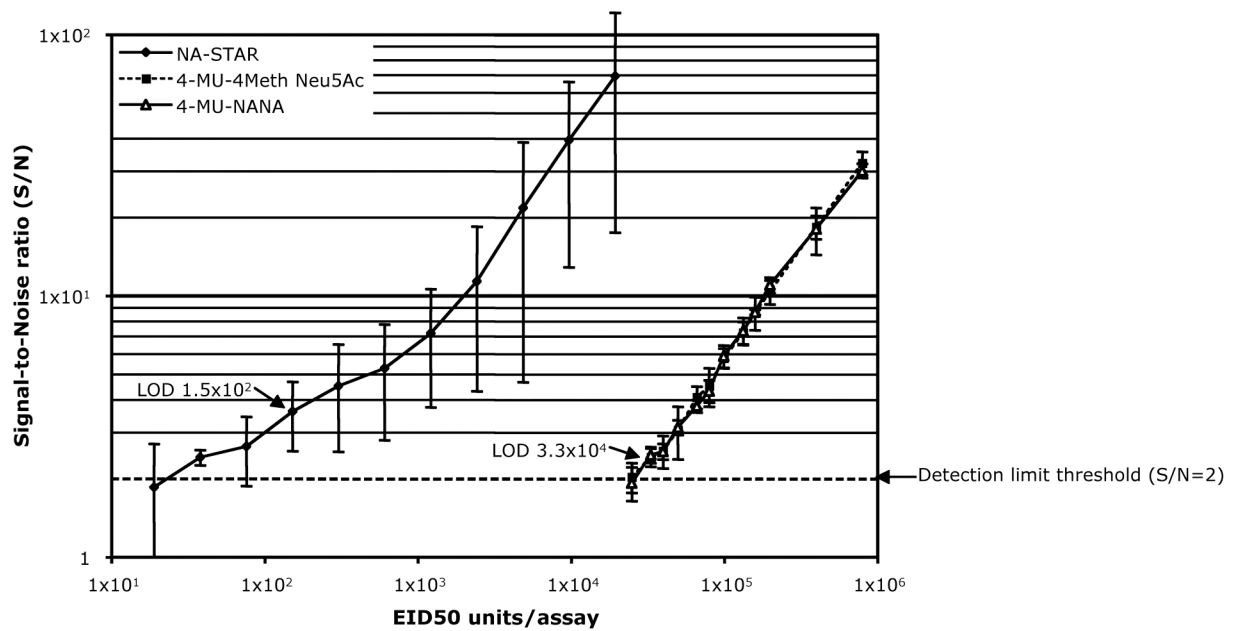


FIG. 2. Comparison of the sensitivity of neuraminidase activity-based assays for detection of NDV B1. A detection threshold of twofold the background (dashed line) was used to compare the sensitivity of the assays. The LOD corresponds to 1.5×10^2 EID50 units per assay for chemiluminescence-based assays and to 3.3×10^4 EID50 units per assay for the two fluorescence-based assays (4-MU-NANA and 4-MU-4-methoxy Neu5Ac). The fluorescence assays were performed in MES buffer pH 5.5 with $10 \mu\text{M}$ substrate for 45 min at 37°C . $n=3$.

TABLE 4
Effect of aerosolization and sampling on neuraminidase activity
and infectivity of NDV.

Experiment	Variation of infectivity (%) ^a	Variation of NA activity (Fold change) ^b
1	+32.8	+7.2
2	-50.6	+1.8
3	+20.0	+2.2

a) $\frac{(\text{infectivity after aerosolization} - \text{infectivity before aerosolization}) \times 100}{\text{infectivity before aerosolization}}$

b) $\frac{\text{NA activity after aerosolization}}{\text{NA activity before aerosolization}}$

TABLE 5

Effect of sampling on neuraminidase activity of spiked NDV^a.

Samplers	Sampling time			
	1h	2 h	4 h	6 h
Gelatin filters ^b	+2%	+19%	+8%	+16%
NIOSH first stage	-25%	-24%	-19%	-12%
NIOSH second stage	-12%	-10%	-12%	-7%
NIOSH backup filter	-1%	-26%	-14%	+19%

a) $\frac{\text{NA activity after sampling and handling} - \text{NA activity after handling without sampling}}{\text{NA activity after handling without sampling}} \times 100$

NA activity after handling without sampling

b) Gelatin filters were incubated at room temperature

TABLE 6
Neuraminidase activity in swine barn air samples detected using 4-MU-NANA substrate.

Samplers	Air volume collected (L)	Sample concentration ^a (genomes/ml)	Genomes concentration in the air (genomes/m ³)	Air volume/assay (L)	NA ^b /air volume (RFU ^c /L)	NA ^b /genome (RFU ^c /10 ⁹ genome)
AGI 30	250	3.2x10 ⁵	2.1x10 ⁷	0.07	-	-
Polycarbonate filter	480	3.6x10 ⁶	3.8x10 ⁷	0.48	-	-
MD8 gelatin filter	2000	1.6x10 ⁷	1.1x10 ⁸	0.67	5.5	1.3
NIOSH first stage	840	1.7x10 ⁷	4.2x10 ⁷ ^d	2.1	1.6	1.0
NIOSH second stage	840	3.0x10 ⁶		8.4	-	-
NIOSH backup filter	840	4.1x10 ⁴		0.84	-	-

a) Bacterial genomes concentration

b) Neuraminidase activity signal-to-noise ratio

c) Relative fluorescence unit signal-to-noise ratio

d) Total of the three stages

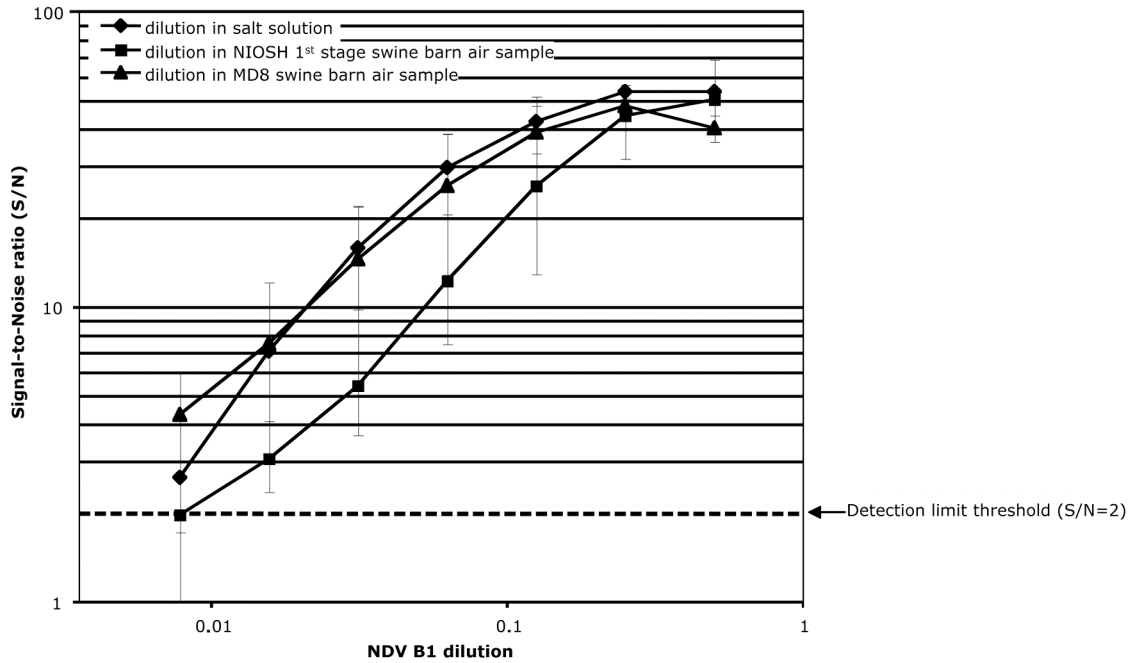


FIG. 3. Detection of NDV B1 in spiked swine barn air samples compared with dilution in salt solution. The fluorescence assays were performed in MES buffer pH 5.5 with 10 μ M 4-MU-4-methoxy-Neu5Ac for 45 min at 37°C. n=2