Comparison of Five Bacteriophages as Models for Viral Aerosols Studies

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ABSTRACT

Bacteriophages are perceived as good models for the study of airborne viruses because they are safe to use, some of them display structural features similar to human and animal viruses, and they are relatively easy to produce in large quantities. Yet, only few studies have investigated them as models. It has been previously demonstrated that aerosolization, environmental conditions, and sampling conditions affect viral infectivity but this is virus-dependent. Thus, several viral models are likely needed to study their general behavior in aerosols. The aim of this study was to compare the effect of aerosolization and sampling on the infectivity of five tail-less bacteriophages and two pathogenic viruses: MS2 (ssRNA/*Leviviridae* family), Φ6 (segmented dsRNA/*Cystoviridae*), ΦX174 (ssDNA/*Microviridae*), PM2 (dsDNA/*Corticoviridae*), PR772 (dsDNA/*Tectiviridae*), human influenza A H1N1 (ssRNA/ *Orthomyxoviridae*), and poultry Newcastle disease virus (NDV, ssRNA, *Paramyxoviridae*). Three nebulizer and two nebulization salt buffers (with or without organic fluid) were tested as well as two aerosol-sampling devices, a liquid cyclone (SKC BioSampler) and a dry cyclone (NIOSH two-stage cyclone). The presence of viruses in collected air samples was detected by culture and quantitative PCR (qPCR). Our results showed that these selected five phages behave differently when aerosolized and sampled. RNA phage MS2 and ssDNA phage ΦX174 were the most resistant to aerosolization and sampling. The presence of organic fluid in the nebulization buffer protected RNA phages PR772 and Φ6 throughout aerosolization and sampling with dry cyclones. In this experimental setup, the behavior of the influenza virus resembled that of phages PR772 and Φ6 while the NDV was closer to phages MS2 and ΦX174. These results provide critical information for the selection of appropriate phage models to mimic the behavior of specific human and animal viruses in aerosols.

INTRODUCTION

The airborne route can transmit several viral diseases. Most of them can also be transmitted through other means such as direct contact with infected persons. However, the importance of the airborne route compared to the others is still less understood. While aerosols are now recognized as an important mechanism of transmission for viruses like influenza, SARS, RSV, porcine corona virus, norovirus, and FMD virus (2, 16, 27, 30, 50, 55), it has been pointed out that more standardized studies are needed to better understand the behavior of these viruses in airborne conditions (42). Indeed, little is known about virus aerosolization and persistence in aerosol state (33, 42). Moreover, the efficacy of air sampling devices to recover viruses still remains to be thoroughly analyzed (49). The field of aerovirology is aimed at, among others, studying the above (49).

Bacteriophages are believed to represent good surrogates for studies of airborne viruses. Bacterial viruses are safe for the laboratory workers and they do not require specialized biocontainment precautions. They are relatively easy to produce in large quantities and several purification procedures are available (15). Phages are highly diversified from a genetic and morphological standpoint, thereby providing a large pool of viruses to choose from (1). Interestingly, some phages also display structural features similar to eukaryotic viruses (26). Over the years, specific phages have been studied and then used as surrogates of eukaryote viruses. For example, the US Food and Drug Administration (FDA) has recognized the utilization of the phage PR772 as virus model to test filtration systems in the biopharmaceutical industry (32).

67 Tailed phages with double-stranded DNA genome (*Caudovirales* order) are by far the most
68 studied among bacterial viruses and are used in a wide range of fields, including in aerosol
69 studies (49). However, since eukaryotic viruses are tail-less, members of the *Caudovirales* order
70 such as coliphages T4 and T7 might not be the most suitable models. Tail-less phages like MS2,
71 Φ6 and ΦX174 were also explored as viral aerosol models (49). However, only few studies have
72 compared the effect of aerosolization and sampling on these tail-less phages (14, 46). Moreover,
73 culture-based methods were often used in these studies. Some authors pointed out that PCR
74 quantification of total viral particles collected by a sampler compared to the culture recovery of
75 infectious viruses is essential to assess the physical stress caused to the virus by the
76 aerosolization and air sampling (37). Considering their structural composition and their genetic
77 make-up, it is expected that very distinct viruses will react differently to the inevitable
78 mechanical stresses caused by aerosolization and sampling.
79 Virus integrity in aerosols is also influenced by environmental conditions such as
80 temperature, relative humidity, and UV light (18, 21-23, 34, 39, 53). Therefore, the behavior of
81 model viruses under various environmental conditions should be known. Finally, it is worth
82 noting that transmissible viruses are often aerosolized by natural processes like sneezing and
83 coughing and thus are found in complex mixtures containing body fluids, salts, microbial cells,
84 etc. Few studies have investigated the effect of media composition on the aerosolization process
85 and virus infectivity (37, 44, 45). These studies revealed that organic matter can have a protective
86 effect on phage T3 and Φ6 in aerosol state as well on influenza virus on dry surfaces. In these
87 latter studies, influenza virus was not tested in aerosol state and phage aerosols were analyzed
88 only by culture.

In this study, we have analyzed five tail-less phages as possible model viruses for aerovirology studies: MS2 (*Leviviridae*), Φ6 (*Cystoviridae*), ΦX174 (*Microviridae*), PM2 (*Corticoviridae*), and PR772 (*Tectiviridae*). These viruses were chosen based on their similarities (morphology, envelope, capsid size, and genome material) with known pathogenic viruses (Table 1). Their behavior during aerosolization and sampling conditions was monitored using plaque assays and quantitative PCR (qPCR) data. We also determined the ability of organic matter in aerosols to preserve viral integrity. Finally, we compared the resistance toward aerosolization and sampling of the five phage models with two pathogenic viruses, the human influenza A H1N1 (*Orthomyxoviridae*) and the poultry Newcastle disease virus (NDV, *Paramyxoviridae*).

Materials and Methods

Bacterial and viral strains. Bacteria and phages used in this study are listed in Table 1 and were provided by the Félix d'Hérelle Reference Centre for Bacterial Viruses (www.phage.ulaval.ca). Culture media were purchased from Difco Laboratories (Detroit, MI, USA). Phages MS2 and ΦX174 were amplified in trypticase soy broth (TSB) as reported (14). Phages Φ6 and PR772 were cultivated on trypticase soy agar (TSA) with TSB soft agar (0.75%) and TSB soft agarose (0.75%) respectively, as described previously (14, 32). Phage PM2 was grown on *Pseudoalteromonas espejiana* host cells in 30 ml nutrient broth containing artificial marine salt (NB-AMS) as instructed elsewhere (10). All phage lysates were titrated on there respective bacterial host using standard plaque assay (36) with TSA and TSB soft agar for Φ6, PR772, MS2 and ΦX174, as well as NB-AMS agar and NB-AMS soft agar for PM2. To assess the host specificity of the five phages, all phages were plated on all bacterial hosts used in this study. No plaque was observed with the five phages when they were plated on other bacterial hosts (data not shown).

The human influenza A H1N1 pr/8/34 strain was obtained from American Type and Culture Collection (ATCC VR-95). The Newcastle disease virus Hitchner B1 strain was purchased as a live vaccine (Wyeth Animal Health). Both strains were used after one passage in chicken embryos. Influenza and NDV strains were grown on specific pathogen-free (SPF) chicken eggs as described previously (47). The infectious titer, expressed as 50% egg infectious dose (EID50) units, was calculated according to Reed and Muench method (38) using five eggs per dilution as described elsewhere (47).

Aerosolization in a controlled chamber. The aerosolization setup is described elsewhere (51). Aerosols were generated with an atomizer (model 9302, TSI Inc., Shoreview, MN) at a dispersion rate of 3 L/min. The nebulizer was filled with 1 ml of each phage lysate (10^{10} PFU) and the volume completed to 70 ml using phage buffer (20 mM Tris-HCl pH 7.5, 100 mM NaCl, 10 mM $MgSO_4$). For the nebulization with organic fluid, phages lysates were mixed with 1 ml of allantoic fluid 16 h before the experiment. The allantoic fluid was harvest from non-infected eggs, pooled, mixed, filtered on a 0.22 µm membrane, and stored at 80°C until use. Phage buffer was added to the phage-allantoic fluid mixtures to obtain a final volume of 70 ml, immediately before aerosolization. Aerosols were also generated with a 6-jet Collison (BGI, Waltham, MA) filled with 1 ml of phage PR772 lysate (10^{10} PFU) diluted with 49 ml of phage buffer, at a dispersion rate of 7 L/min. The third nebulizer tested, the Aeroneb Lab (Aerogen Inc., Galway, Ireland), was filled with 7 ml from a phage dilution (1 ml of phage PR772 lysate at 10^{10} PFU/ml in 49 ml of phage buffer). The aerosol was driven into the chamber at a flow rate of 4 L/min.

Once produced, the aerosols were passed through a desiccator (model 306200, TSI Inc.), to form aerosol droplets nuclei, before entering a GenaMini chamber (SCL Medtech Inc., Montreal, QC, Canada) from which the aerosols were sampled (see below). Aerosols inside the chamber were mixed with the dilution air at a rate of 23 L/min with the TSI 9302 atomizers, 22 L/min with the Aeroneb Lab, and 19 L/min with the Collison 6-jet nebulizer, and were maintained in a continuous flow throughout sampling. Medical-grade compressed air was used for aerosol generation and dilution. The experiments were conducted at temperatures between 28°C to 31°C. The relative humidity in the aerosol chamber was below 20% with the Aeroneb lab and the TSI 9302 atomizer and at 30% with the 6-jet Collison. The aerosol size distribution and concentration was monitored every 30 min during each experiment with an aerodynamic particle sizer (APS, model 3321, TSI Inc.) to ensure proper nebulizer function.

Aerosols were sampled with a SKC BioSampler (SKC Inc., Eighty Four, PA) for 20 min at about 11.3-13.3 L/min (maximum of the critical orifice capacity of the SKC BioSampler) driven by a Gilair Aircon II pump (Levitt Sécurité, Montréal, QC, Canada) and loaded with 20 ml of phage buffer. Following sampling, the residual liquid in the SKC BioSampler was measured and collected. Air samples were also collected using Centers for Disease Control (CDC) National Institute for Occupational Safety and Health (NIOSH) two-stage cyclone aerosol samplers (CDC/NIOSH, Morgantown, WV) mounted with a 15 ml tube (first stage), a 1.5 ml tube (second stage) and a 0.8 μm 37 mm backup polycarbonate filter in an open face cassette (SureSeal, SKC Inc.), and connected to a Gilair Aircon II pump at a flow rate of 3.5 L/min and 10 L/min for 60 min. The samples were recovered by vortexing 15 min at 70% motor speed and 100% frequency in 5 ml (first stage), 1.5 ml (second stage) and 5 ml (filter) of phage buffer using a multi-pulse vortex (Glas-Col, Terre Haute, IN). Controls were performed to confirm that the vortex conditions did not affect viral infectivity.

For each aerosolization assay, aerosol samples and nebulizer content were collected and analyzed at the same time. When several phages were nebulized together, samples were diluted and plated on the bacterial hosts to determine the infectious titer of each specific phage. The viral genome concentration, corresponding to the total of infectious and noninfectious viral particles, was evaluated using qPCR. Every aerosolization condition was repeated at least three times in distinct experiments ($n \geq 3$). The number of experiments is displayed in the legends of the Figures.

Quantification of total viral particles using qPCR. Phages ΦX174, PM2, and PR772 genomes were quantified from two dilutions of each sample without DNA extraction. For quantification of Φ6, MS2, NDV, and influenza virus, genomic RNA was extracted from two

169 dilutions of all samples using the QIAamp viral RNA mini kit (Qiagen, Chatsworth, CA) as
170 described by Gendron et al. (14). Briefly, the RNA carrier was omitted from the AVL buffer and
171 the RNA was eluted from the column with two volumes of 40 µL TE buffer pH 8.0 (10 mM Tris,
172 0.1 mM EDTA). All RNA samples were stored at -86°C. Influenza, MS2, and Φ6 cDNA
173 synthesis was performed using the iScript cDNA synthesis kit (BioRad Life Sciences,
174 Mississauga, ON, Canada) (14).
175 Primers and dual-labeled probes listed in Table 2 were supplied by Integrated DNA
176 Technology (Coralville, IA). Probes were labeled with fluorescein (FAM) at the 5'-end and Iowa
177 Black FQ (IABlkFQ) or Black Hole Quencher 1 (BHQ) at the 3'-end. Influenza and PM2 probes
178 were also labeled with a ZEN quencher. Primers and probes for the detection of phage PM2 and
179 PR772 were designed with Beacon Designer 4.02 software (Premier Biosoft International, Palo
180 Alto, CA). The qPCR to detect all phages were done separately. Primers and probes specificity
181 was analyzed using BLAST tools at NCBI. Moreover, the five phage primers and probes sets
182 were tested with all phage models used in this study. All primers and probes sets give qPCR
183 fluorescence signal only with their designated phage (data not shown).
184 The assay components per 25 µL were: 5 µL of phage sample or 2 µl of cDNA, 12.5 pmol of
185 primers for the all phages and 20 pmol for influenza, and 12.5 µL of 2X master mix of the iQ
186 Supermix (BioRad). Five pmol of dual-labeled probe were added for phages PR772, ΦX174, and
187 PM2 detection, 7.5 pmol for Φ6, 3.75 pmol for MS2, and 4 pmol for influenza detection. The
188 PCR program for all the phages was as follows: 5 min at 94°C followed by 40 amplification
189 cycles including denaturation at 94°C for 15 sec, annealing and elongation at 60°C for 60 sec,
190 and fluorescence measurement. For influenza detection, the PCR program was 5 min at 94°C
191 followed by 40 cycles of 15 sec at 94°C and 30 sec at 55°C and then fluorescence measurement.

192 One-step detection of NDV genome was performed according to Wise and colleagues (54).
193 Briefly, the assay components per 25 µL were: 0.5 µL of the kit supplied enzyme, 5 µL of
194 template RNA, 12.5 pmol of primers, 5 pmol of dual-labeled probe, and 12.5 µL of 2X master
195 mix of the iScript One-Step RT-PCR Kit for Probes (BioRad). The RT-PCR program was as
196 follows: 15 min at 50°C, 5 min at 95°C followed by 40 cycles including denaturation at 94°C for
197 15 sec, annealing and elongation at 60°C for 60 sec, and fluorescence measurement.
198 All experiments were performed using an Opticon 2 system (MJ Research, Waltham, MA).
199 Data were analyzed with the Opticon software supplied with the apparatus. For each PCR run, a
200 standard curve was generated in duplicate with plasmid DNA (or RNA for NDV). Serial 10-fold
201 dilutions from 10^0 to 10^7 molecules per reaction tube were used to establish standard curves. For
202 each sample of unknown concentration, two 10-fold dilutions made in duplicate were analyzed,
203 and the concentration determined using the standard curves. The background was subtracted
204 using the "average over cycle range" function of the software. Threshold values (Ct) were
205 determined automatically with the software. The plotting of Ct as a function of the logarithm of
206 DNA template gave a straight line. The slope of this graph line gave the PCR efficiency (*E*)
207 according to the equation: $E=(10^{-1/\text{slope}}-1)$ x100. Results were considered accurate when *E* was
208 over 85% and error between standard points and regression curve was lower than 0.1.
209 The DNA and RNA for standard curves for phages MS2, Φ6, ΦX174, and NDV were
210 obtained in previous studies (14, 47, 51). The DNA for standard curves for influenza and phages
211 PR772 and PM2 were prepared as follows. The region of interest of each virus was amplified
212 from purified genomes (cDNA for Influenza) by PCR using the primer sets InfAfor/InfArev,
213 PM2for/PM2rev and PR772for/PR772rev. The amplification products were cloned into the
214 pDrive cloning vector using the Qiagen PCR cloning kit (Qiagen). The DNA constructions were

transformed into *E. coli* MC1061 competent cells using the rubidium chloride method (48). Plasmid DNAs 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).

Material preparation and blank controls. Air samplers were sterilized by autoclave before each experiment. After each experiment, nebulizer, tubings and air samplers were decontaminated by soaking 20 min in 1/20 Virox 5. The material was then rinsed with water and dried. Once cleaned, the tubings and nebulizer were reassembled onto the system and run with clean medical grade compressed air to purge the diffusion dryer and the GenaMini chamber. The system was purged until zero particle was counted from the chamber with the APS.

Blank aerosol experiments were performed as with aerosol experiments previously described except that the nebulizer was filed with sterile phage buffer. The samples collected in the blank aerosol experiments were processed and analyzed by culture and qPCR with the other air samples. No virus was detected by culture and qPCR from the samples collected during the blank aerosol experiments. Controls in the absence of template were performed at every qPCR run. Moreover, controls without RT were used for RNA viruses qPCR detection (MS2, Φ6, influenza and NDV).

Calculations. The number of infectious viruses collected per liter of air (PFU/L) for each sample was calculated as in equation 1.

$$I_{PFU} = \frac{c_{PFU} \times v}{f \times t} \quad (1)$$

I_{PFU} = concentration of infectious viruses collected by the air sampler (PFU/L), c_{PFU} = air sample concentration (PFU/ml), v = air sample volume (ml), f = air sampler flow rate (L/min), t = sampling time (min), c_{qPCR} = air sample concentration (genomes/ml).

The number of viral genomes per liter of air (I_{qPCR}) was also calculated for all air samples using the same method by replacing c_{PFU} by c_{qPCR} in the equation. The number of total viral particles in the GenaMini chamber as well as inside the nebulizer varied from one experiment to the other. The PFU relative recoveries were calculated as in equation 2 to compensate for the inter-experiment variation and used for the analyses.

$$\text{PFU relative recovery} = \frac{I_{PFU}}{n_{PFU}} \quad (2)$$

I_{PFU} = concentration of infectious viruses collected by the air sampler (PFU/L), n_{PFU} = concentration of infectious viruses inside the nebulizer (PFU/ml).

The qPCR relative recoveries were obtained using the equation 2 by replacing I_{PFU} and n_{PFU} by I_{qPCR} and n_{qPCR}. Relative culture/genome ratios were also calculated to allow the comparison between the viruses cultured on embryonated chicken eggs and the phages plaque assays. This ratio was calculated by dividing the infectious percentage in the samples by the infectious percentage into the nebulizer. Data were analysed using 2-way Anova or unpaired t-test (when mentioned) regarding the data. 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

Effect of the nebulizer. First, we compared three nebulizers (TSI 9302 atomizer, Aeroneb Lab, and Collison 6-jet) on the relative recovery of the dsDNA *Tectiviridae* phage PR772. We also tested two nebulization buffers (with and without organic fluid) using the TSI 9302 atomizer. In all experiments phage particles were collected using two air samplers (SKC BioSampler and NIOSH two-stage cyclone bioaerosol sampler at 10 L/min). We analyzed the phage recovery by culture (PFU) and genome copy number (qPCR). These above experiments were only conducted with the FDA approved phage PR772. The organic fluid could not be used with the Collison 6-jet and the Aeroneb Lab as the organic supplement clogged the Aeroneb Lab nebulizer as well as produced too much foam into the Collison 6-jet.

The characteristics of the aerosols delivered into the GenaMini chamber using each nebulizer are presented in Table 3. The aerosols produced with the Collison 6-jet and the TSI 9320 atomizer were similar in size and concentration. However, the aerosols delivered by the Aeroneb Lab were up to larger and times less concentrated. The addition of organic fluid into the nebulization buffer slightly increased the number of particles and decreased the particle size produced by the TSI 9320 atomizer.

In our setup, the TSI 9302 and Collison 6-jet gave similar performances when phage recovery was measured by both PFU and qPCR (Fig 1). Still, the Aeroneb Lab nebulizer led to lower phage relative recovery by PFU as well as qPCR (Fig. 1, $p<0.05$). This result can be explained by the fact that fewer particles were delivered into the aerosol chamber using this nebulizer. Overall, our results showed that qPCR detected 1000-fold more genome copies than infectious PR772 particles, suggesting that the aerosolization and/or the sampling strongly

277 affected its structural integrity. Taken altogether, the TSI 9302 nebulizer performed better in our
278 setup.

279 The PR772 relative recovery was 100-fold higher with the SKC BioSampler as compared to
280 the NIOSH two-stage sampler when the air samples were analyzed by plaque assays ($p<0.0001$).
281 However, the differences in qPCR relative recovery of phage PR772 between the two air
282 samplers were less significant ($p=0.038$). These data suggest that the NIOSH sampler is more
283 damaging to the phage PR772 integrity and/or is less efficient to collect the particle size ranges
284 generated by the nebulizers. Finally, PFU relative recovery of PR772 was higher ($p=0.03$) when
285 aerosolized in buffer containing an organic fluid (Fig. 1).

286 **Five phage models.** The five selected phages were then aerosolized all together using the
287 TSI 9302 nebulizer. Phage particles were collected using three air samplers (SKC BioSampler
288 and NIOSH two-stage cyclone bioaerosol sampler set at 3.5 L/min and 10 L/min). Again, we
289 analyzed the phage recovery by qPCR and PFU. For plaque assays, the collected air samples
290 containing the five phages were plated on each individual and phage-specific bacterial host to
291 calculate the titer of each phage contained in the samples. The qPCR for the detection of all the
292 phages were done separately. The five primer and probe sets were tested with the five phage
293 models. All primers and probes sets give qPCR fluorescence signal only with it designated phage
294 (data not shown).

295 The qPCR relative recovery was similar for all phages with all samplers (Fig. 2). However,
296 the results by plaque assays were different between phages and samplers. The most infectious
297 particles recovered were with phage MS2. Interestingly, the qPCR and PFU relative recoveries
298 were in the same order of magnitude with this phage. With phages Φ6, PR772 and ΦX174, the
299 PFU relative recovery was between 100- to 1000-fold less when compared with the qPCR

300 relative recovery ($p<0.0001$). Strikingly, infectious phage PM2 particles were poorly recovered
301 (Fig. 2). PM2 plaques were found only in three out of 18 samples analyzed. In fact, the PFU
302 relative recovery for phage PM2 was $6.3x10^7$ times lower to the qPCR relative recovery.
303 The PFU relative recoveries were similar with the NIOSH samplers at 3.5 L/min and 10
304 L/min for all phages except PM2. The SKC BioSampler led to a better recovery (100-fold higher)
305 of infectious ΦX174 when compared to the NIOSH sampler (Fig 2, $p<0.0001$). No significant
306 difference was observed between the samples collected with the two samplers for Φ6, PR772,
307 and MS2 (Fig. 2).
308 Comparison between phages PFU relative recoveries divided the five phages into four
309 clusters (Fig. 2). The PFU relative recoveries of MS2 and PM2 are different from the other
310 phages with both samplers (Fig. 2, clusters *a* and *b*). There is no statistical difference between the
311 PFU relative recovery of phages Φ6 and PR772 with both air samplers (Fig. 2, cluster *c*). There is
312 no statistical difference between PR772, Φ6 and ΦX174 PFU relative recovery with the NIOSH
313 samplers at 3.5 L/min and 10 L/min, but there is difference with the SKC BioSampler (Fig. 2,
314 cluster *d*).
315 **Phages and organic fluid.** We investigated if the use of organic fluid in the buffer used to
316 aerosolize phages could improve recovery of infectious viral particles using the NIOSH sampler
317 (Fig. 3). Our data show that the utilization of organic fluid in the nebulization buffer did increase
318 the PFU relative recovery of Φ6 by 100-fold (unpaired t-test, $p=0.0018$) but had no significant
319 impact on recovery of infectious particles for MS2, PM2 and ΦX174 (data not shown).
320 **Correlation with pathogenic viruses.** Finally, we studied the aerosolization and the
321 sampling of two pathogenic viruses, the poultry NDV as well as human influenza A H1N1. For
322 culture and biosafety reasons, these viruses were aerosolized one at the time in the same

conditions as the phage models. We compared the percentage of infectious viruses collected with the SKC BioSampler with the infectious percentage in the nebulizer at the beginning of the experiment to obtain the relative infectious ratio (Fig. 4). We used these ratios to compare with the phage models. As shown in Figure 4, NDV is the most resistant virus tested in this study. The strain of influenza A displayed an intermediate resistance to aerosolization and sampling.

DISCUSSION

Here, we have studied the behavior of five very distinct tail-less phages and two pathogenic viruses under various aerosolization and sampling conditions using culture assays and qPCR data. One clear result from this study was that the virus relative recovery was almost always higher when using qPCR as compared to culture, with the exception being phage MS2 and poultry virus NDV. It was already documented that virus relative recovery is higher when using qPCR compared to culture (7, 11, 14, 19, 51). For example, it was previously shown for RNA phages MS2 and Φ6 that the number of viral genome copies estimated by qPCR was 10 (MS2) to 1000-fold (Φ6) higher than infectious particles (14). Similar data with both phages were obtained here although minor differences were noted, likely due to the distinct sampling devices used in both studies.

qPCR is often used in aerosol chamber studies to quantify the total amount of viral particles collected and compare it with the amount of infectious viruses to assess the damage caused to the viruses by aerosolization and sampling (7, 11, 14, 47, 51). To use qPCR, one must be ascertain of the genome stability in the air sampler. Such genome degradation has been reported for influenza virus after long-term air sampling using the NIOSH two-stage air sampler (7). However, no degradation was observed in the SKC BioSampler for influenza and Porcine Reproductive and Respiratory Syndrome Virus (7, 19). In our experiments, the qPCR relative recovery was similar for the five phages when sampled with SKC BioSampler for 20 min and NIOSH two-stage air sampler at 10 L/min and 3.5 L/min for 1h, demonstrating that the genome stability of these phages was similar under all our sampling conditions.

351 The PFU relative recovery of the five phages models was remarkably different from one to
352 another (Fig. 2). Phage MS2 was the most robust among the five phages tested as it could be
353 detected by qPCR and plaque assays at similar levels. This phage demonstrated a good resistance
354 to aerosolization and sampling, which is in line with results obtained by others (14, 53). Not
355 surprisingly, phage MS2 has been used as surrogate for pathogenic viruses in several
356 sterilization, air filtration, and aerosolization studies (3, 9, 12-14, 18, 20, 24, 28, 31, 39, 40, 43,
357 53). Its facility to be detected by culture is certainly one of the reasons for its widespread use.
358 However, this phage may not be the best suitable surrogate for all pathogenic viruses. For
359 example, phage MS2 is 7-10 times more resistant to aerosolization, sampling and UV light than a
360 coronavirus (53). MS2 is also very resistant to air sampling in dry conditions as demonstrated
361 here and by others (14), while the opposite was observed for the influenza virus (11).

362 Discrepancies between plaque assay quantification and genome quantification from virus
363 preparations were previously reported for phages Φ6, MS2, and ΦX174 as well as for influenza
364 virus (5, 7, 11, 14). To shed further light into these discrepancies between our qPCR and plaque
365 assays data, we compared the amount of PFU and genome copies for the five phages in crude
366 lysates and in phage preparations purified through a CsCl gradient. Free genome can be
367 eliminated from phage preparation using a CsCl buoyant density centrifugation as the density of
368 RNA and DNA is higher than the density of phage particles (41). The CsCl gradient did not
369 decrease the ratio of genomes per PFU in pure phage preparations (data not shown). Thus, the
370 discrepancy between qPCR and culture is unlikely to come from free genomes. Moreover, we
371 observed with PR772 that the long-term storage of this phage lysate led to a decrease of PFU and
372 of genome copies, however the overall ratio of genomes/PFU increase (data not shown). These

373 data suggests that phage PR772, and possibly other phages as well, lost infectivity faster than
374 genome degradation.

375 In order to limit the difference between the number of genomes and infectious phage
376 particles into our initial material, we always worked with fresh phage lysates obtained from the
377 same stock. The phage titer was measured before each experiment and relatively the same
378 amount of each phage was used each time. Also, the concentration of genomes and PFU into the
379 nebulizer was used to calculate the culture and genome relative recoveries to make sure that if
380 differences were observed, they originated from damages caused during aerosolization and
381 sampling and not from the starting material. Such precautionary measures should be implemented
382 when testing viruses in aerosol studies.

383 The NIOSH two-stage bioaerosol sampler (29) has been previously shown to efficiently
384 sample phages (4-6, 30, 52) and influenza viruses (7). In one comparative study, the NIOSH
385 sampler outperformed the SKC BioSampler in sampling tailed phages in industrial settings (52).
386 In our experiments, this sampler set at 3.5 L/min as well as 10 L/min was as efficient as the SKC
387 BioSampler to collect phages as demonstrated by the qPCR analysis of our air samples.
388 Moreover, the operation of the NIOSH sampler at 10 L/min did not cause more damage to our
389 phage models compare to 3.5 L/min. There was no statistic difference between PFU relative
390 recovery with both samplers for phages Φ6, PR772 and MS2. On the other hand, the SKC
391 BioSampler was more efficient in recovering infectious phage ΦX174. Because the NIOSH air
392 sampler can be used for an extended period of time, collect a larger air volume, and that the
393 samples can be re-suspended in a small volume, this sampler may be more suitable to collect rare
394 events than the SKC BioSampler. This is supported by our data with phage PM2, which poorly

resisted aerosolization and sampling, but we were still able to recover infectious viruses only with the NIOSH sampler set at 10 L/min after one hour of air sampling.

Viruses aerosolized by natural process such as sneezing and coughing are in a complex and variable media containing mucus, saliva, etc. Reports have also suggested that the particles emitted by healthy or sick patients are of different size (17). Our results suggest that the aerosolization media can influence the resistance to aerosolization and sampling of some viruses. Further studies are warranted to identify the most suitable agent to improve, if needed, viral recovery.

This study illustrates the diversity of viral behaviors in aerosols. A summary of our results is presented in Table 4 as well as general recommendations to sample these five phages in aerosols and their utilization as surrogate. In our setup, the phages MS2 and ΦX174 are the best surrogates for NDV, and phages PR772 and Φ6 are the best surrogates for influenza (Fig. 4 and Table 4). As more information become available it may be possible to correlate phage behavior to human and animal viruses under various conditions.

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574
575

576 **Figure 1.** Effect of nebulizer and nebulizer buffer on phage PR772 qPCR and PFU relative
577 recoveries. Samples were taken with the SKC BioSampler (black) or the NIOSH two-stage
578 cyclone bioaerosol sampler at 10 L/min (gray). The black and gray bars indicate the median
579 relative recoveries obtained with each sampler (n=3). §: Comparison between the SKC
580 BioSampler and the NIOSH two-stage cyclone bioaerosol sampler ($p=0.03$ for qPCR relative
581 recovery and $p<0.001$ for PFU relative recovery). *: $p<0.05$ Comparison between relative
582 recoveries obtained with both air samplers when aerosolized with different nebulizers.
583
584 **Figure 2.** Comparison of qPCR relative recovery and PFU relative recovery of the five phage
585 models nebulized all together and sampled with the SKC BioSampler (black), and the NIOSH
586 two-stage cyclone bioaerosol sampler at 3.5 L/min (dark gray) and 10 L/min (light gray). The
587 bars indicate the median relative recoveries obtained with each sampler (n=6). *: Comparison
588 between the SKC BioSampler and the NIOSH two-stage cyclone bioaerosol sampler at 3.5 L/min
589 and 10 L/min PFU relative recovery. Unpaired t-test on normally distributed log transformed data.
590 $p<0.0001$ for phage ΦX174. *a*: Comparison of the qPCR relative recoveries between phages.
591 $p>0.05$ between phages for all air samplers. *a*: Comparison between qPCR and PFU relative
592 recoveries for all phages. $p<0.0001$ for phages PM2, Φ6, PR772 and ΦX174. *a*, *b, c, d* and *e*:
593 Comparison between the phages PFU relative recoveries. $p<0.05$ with the NIOSH two-stage
594 cyclone bioaerosol sampler 10 L/min when comparing phage of group *b* with phages of groups *a*,
595 *c,* and *d*. $p<0.05$ between *c* and *a* with all air samplers. $p<0.05$ between *c* and *d* with the
596 BioSampler. $p<0.05$ between *a* and *d* with all air samplers.
597

598 **Figure 3.** Comparison of the PFU relative recovery of phage Φ6 aerosolized from phage buffer
599 or phage buffer supplemented with organic fluid and sampled using the NIOSH two-stage
600 cyclone bioaerosol sampler at 3.5 L/min (dark gray) and 10 L/min (light gray). The bars indicate
601 the median relative recoveries obtained with each sampler (n=3). *: p=0.0018. Comparison of the
602 two nebulisation buffers with air sampling using the NIOSH two-stage cyclone bioaerosol
603 sampler. Unpaired t-test performed on normally distributed log transformed data.
604
605 **Figure 4.** Comparison of the relative culture/genome ratio of the five phage models with
606 influenza A H1N1 (InfA) and Newcastle disease virus (NDV). The viruses were sampled with the
607 SKC BioSampler. The bars indicate the median relative culture/genome ratio obtained for each
608 virus. The relative culture/genome ratio cannot be calculated for phage PM2 because no PFU
609 have been detected for this phage in these conditions out of six experiments. n=6 for all phages
610 and n=3 for InfA and NDV. *a, b*: Comparison of viruses relative culture genome ratio. Unpaired
611 t-test performed on normally distributed log transformed data. $p>0.05$ between viruses of cluster
612 *a* and $p>0.05$ between viruses of cluster *b*. $p<0.05$ between viruses of clusters *a* and *b*.

TABLE 1 Bacteria and viruses used in this study.

Bacterial and viral strains	Growth conditions and characteristics	Reference
Bacteria		
HER-1036	*Escherichia coli*, TSB, 37 °C, 200 rpm	(51)
HER-1102	*Pseudomonas syringae* var. *phaseolicola*, TSB, 22 °C, 100 rpm	(14)
HER-1221	*E. coli*, TSB, 37 °C, 200 rpm	(32)
HER-1254	*Pseudoalteromonas espejiana*, NB-AMS, 30 °C, 200 rpm	(10)
HER-1462	*E. coli,* TSB, 37 °C, 200 rpm	(14)
Viruses		
HER-036	Phage ΦX174, 25 nm, unenveloped, linear ssDNA, 5386 bases, bacterial host HER-1036	(51)
HER-102	Phage Φ6, 85 nm, enveloped, segmented dsRNA, 13385 bp, bacterial host HER-1102	(14)
HER-221	Phage PR772, 80 nm, unenveloped, linear dsDNA, 14492 bp, bacterial host HER-1221	(32)
HER-254	Phage PM2, 60 nm, unenveloped, circular dsDNA, 10079 bp, bacterial host HER-1254	(10)
HER-462	Phage MS2, 25 nm, unenveloped, linear ssRNA, 3569 bases, bacterial host HER-1462	(14)
ATCC VR-95	Human influenza A H1N1 A/PR/8/34, 80-120 nm, enveloped, segmented ssRNA, 13588 b, grown on SPF chicken eggs	(25, 35)
NDV B1	Newcastle disease virus Hitchner B1, 100-200 nm, enveloped, linear ssRNA, 15186 b, grown on SPF chicken eggs	(47)

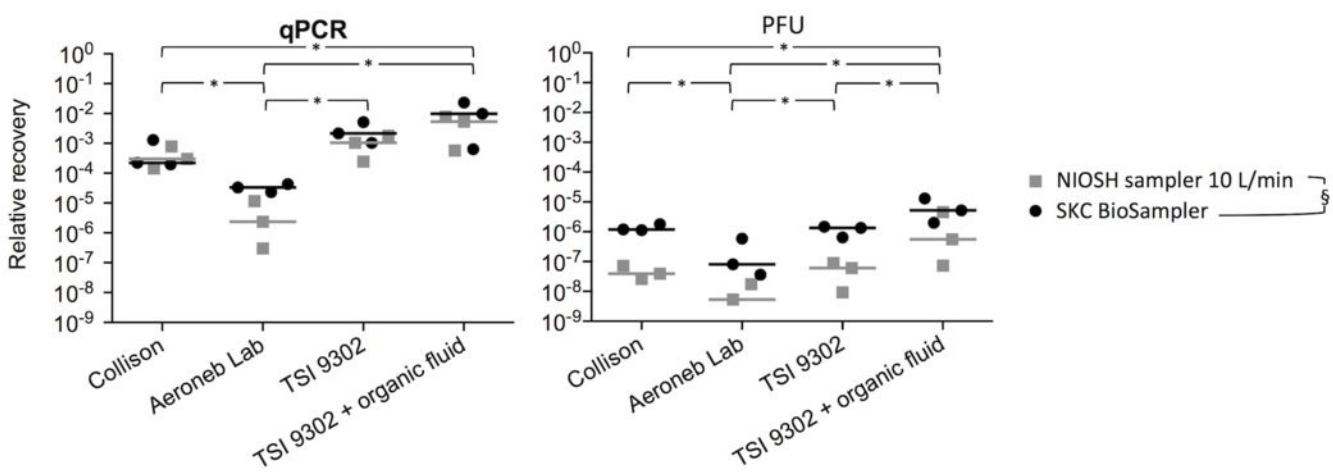
TABLE 2 Primers and probes used in this study.

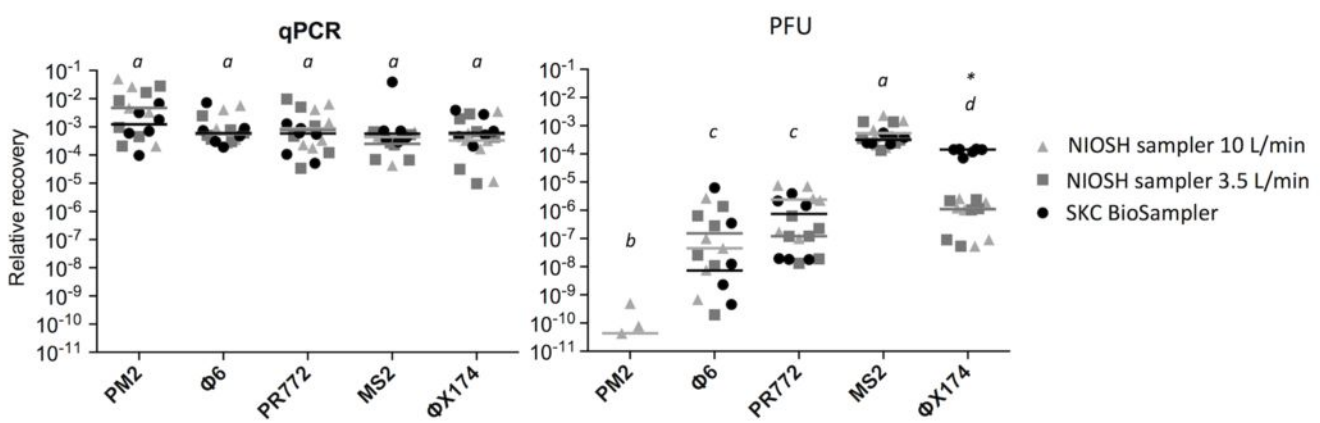
Primers	Sequences	Target	Ref
ΦX174for	5′-ACAAAGTTTGGATTGCTACTGACC-3′	ΦX174 genome 508-531	(51)
ΦX174rev	5′-CGGCAGCAATAAACTCAACAGG-3′	ΦX174 genome 630-609	(51)
ΦX174probe	5′-FAM/CTCTCGTGCTCGTCGCTGCGTTGA/BHQ-3′	ΦX174 genome 533-556	(51)
Φ6Tfor	5′-TGGCGGCGGTCAAGAGC-3′	Φ6 S segment 430-446	(14)
Φ6Trev	5′-GGATGATTCTCCAGAAGCTGCTG-3′	Φ6 S segment 530-506	(14)
Φ6Tprobe	5′-FAM/CGGTCGTCGCAGGTCTGACACTCGC/BHQ-3′	Φ6 S segment 450-474	(14)
PR772for	5′-CCTGAATCCGCCTATTATGTTGC-3′	PR772 genome 4538-4560	This study
PR772rev	5′-TTTTAACGCATCGCCAATTTCAC-3′	PR772 genome 4663-4641	This study
PR772probe	5′-FAM/CGCATACCAGCCAGCACCATTACGCA/IABlkFQ-3′	PR772 genome 4639-4614	This study
PM2for	5′-CAAGTGGTCAGGCGTTTATCAG-3′	PM2 genome 4058-4079	This study
PM2rev	5′-TGCTCGGCTTTGGCATCTTC-3′	PM2 genome 4157-4138	This study
PM2probe	5′FAM/AATTGCCGC/ZEN/ATCTTCACTCTCAACACCGTT /IABlkFQ-3′	PM2 genome 4128-4099	This study
MS2 1 for	5′-GTCCATACCTTAGATGCGTTAGC-3′	MS2 genome 1261-1284	(14)
MS2 1 rev	5′-CCGTTAGCGAAGTTGCTTGG-3′	MS2 genome 1420-1401	(14)
MS2 1 probe	5′-FAM/ACGTCGCCAGTTCCGCCATTGTCG/BHQ-3′	MS2 genome 1391-1367	(14)
M+4213	5′-TCCTCAGGTGGCCAAGATAC-3′	NDV genome 4213-4232	(54)
M-4350	5′-TGCCCCTTCTCCAGCTTAGT-3′	NDV genome 4331-4350	(54)
M-4268	5′-FAM/TTTTAACGCTCCGCAGGCAC/IABlkFQ-3′	NDV genome 4249-4268	(54)
InfAfor	5′-GACCRATCCTGTCACCTCTGAC-3′	Influenza M segment 160-181	(8)
InfArev	5′-AGGGCATTYTGGACAAAKCGTCTA-3′	Influenza M segment 242-265	(8)
InfAprobe	5′-/FAM/TGCAGTCCT/ZEN/CGCTCACTGGGCACG /IABlkFQ-3′	Influenza M segment 215-238	(8)

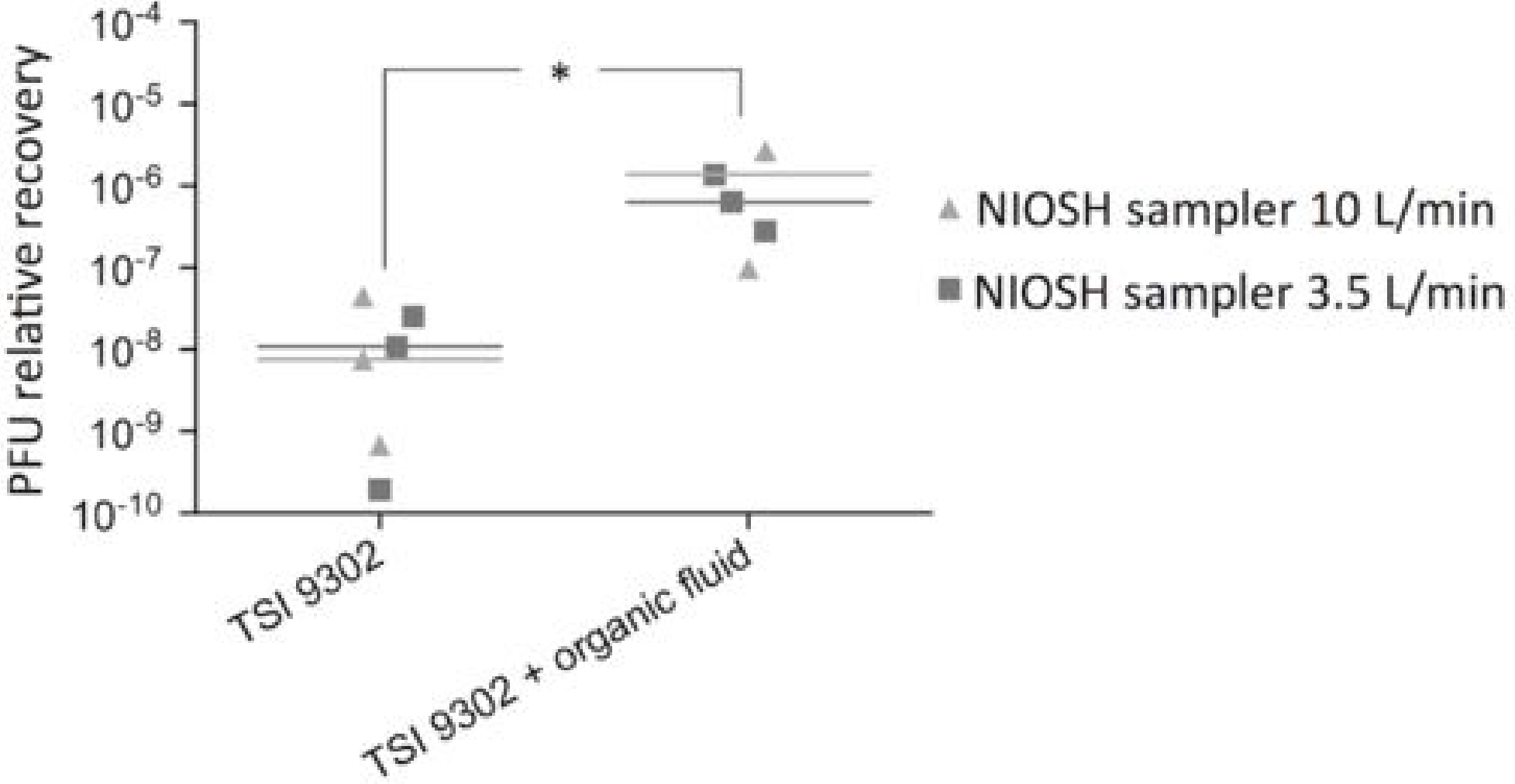
TABLE 3 Characteristics of the aerosols delivered into the GenaMini chamber by each nebulizer as measured with the Aerosol Particle Sizer*.

Nebulizer	MMAD (μm)	Total count (particles/$cm^{3)}$
Collison 6-jet	0.779 - 0.855	1.43e4 – 2.30e4
Aeroneb Lab	1.000 - 1.320	3.30e3 – 6.60e3
TSI 9302	0.890 - 1.100	1.68e4 – 4.97e4
TSI 9302 with organic fluid	0.869 - 0.877	4.18e4 – 5.51e4

* The values displayed are the minimum and maximum measured.


qPCR
PFU
Relative recovery
10^{0}
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
10^{-7}
10^{-8}
10^{-9}
*
Collison
Aeroneb Lab
TSI 9302
TSI 9302 + organic fluid
NIOSH sampler 10 L/min
SKC BioSampler
§


qPCR
PFU
Relative recovery
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
10^{-7}
10^{-8}
10^{-9}
10^{-10}
10^{-11}
a
a
a
a
a
b
c
c
a
*
d
PM2
Φ6
PR772
MS2
ΦX174
NIOSH sampler 10 L/min
NIOSH sampler 3.5 L/min
SKC BioSampler


PFU relative recovery
10-4
10-5
10-6
10-7
10-8
10-9
10-10
*
NIOSH sampler 10 L/min
NIOSH sampler 3.5 L/min
TSI 9302
TSI 9302 + organic fluid

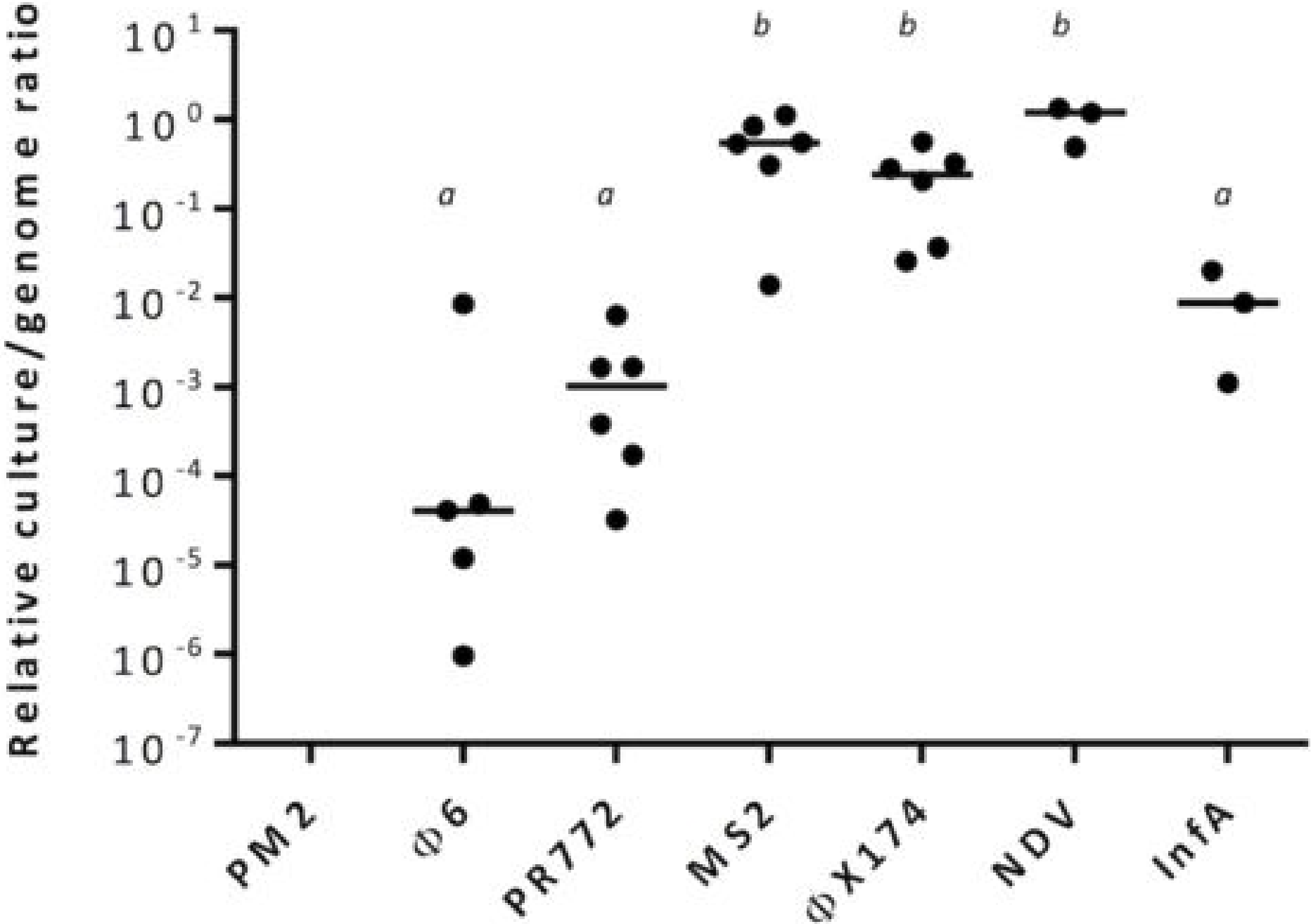

Relative culture/genome ratio
10 1
10 0
10 -1
10 -2
10 -3
10 -4
10 -5
10 -6
10 -7
a
a
b
b
b
a
PM2
Φ6
PR772
MS2
ΦX174
NDV
InfA

TABLE 4 Summary of the results obtained in this study with the recommended sampler, detection method and nebulization conditions for five phages.

Phage	MS2 (ssRNA)	Φ6 (dsRNA)	ΦX174 (ssDNA)	PR772 (dsDNA)	PM2 (dsDNA)
Recommended sampler	SKC BioSampler or NIOSH two-stage cyclone bioaerosol sampler at 3.5 and 10 L/min	SKC BioSampler or NIOSH two-stage cyclone bioaerosol sampler at 3.5 and 10 L/min	SKC BioSampler	SKC BioSampler or NIOSH two-stage cyclone bioaerosol sampler at 3.5 and 10 L/min	NIOSH two-stage cyclone bioaerosol sampler at 10 L/min
Recommended detection method	qPCR or PFU	qPCR	qPCR	qPCR	qPCR
Organic fluid in aerosolization buffer	No effect	Protective effect	No effect	Protective effect	No effect
Good model for pathogenic virus used in this study	NDV	Influenza	NDV	Influenza	_