INTRODUCTION

Lower respiratory tract infections are the 4th leading cause of death globally. Next to bacteria, respiratory viruses are the most common etiology of respiratory tract infections and pose a substantial burden on public health worldwide. Despite their impact on global health, for most respiratory viruses little is known about the relative contribution of various routes of transmission. Most of the current knowledge of respiratory virus transmission has been derived from experimental studies (e.g., human challenge studies, experimental infections). This gap in knowledge hinders the development of effective control measures.

The goal of this study is to compare the collection efficiency of three air samplers for the collection of four nebulized respiratory viruses: influenza A virus, human metapneumovirus, parainfluenza virus type 3, and respiratory syncytial virus. The comparison is made to determine which sampler is best suited for different research needs.

The six-stage Andersen cascade impactor was optimized with semi-solid gelatin as collection surface. Subsequently, the collection efficiency of the cascade impactor, the SKC BioSampler, and an in-house developed electrostatic precipitator was compared. In an in vitro set-up, influenza A virus, human metapneumovirus, parainfluenza virus type 3, and respiratory syncytial virus were nebulized and the amount of collected infectious virus and viral RNA was quantified with each air sampler. Whereas only low amounts of virus were collected using the electrostatic precipitator, high amounts were collected with the BioSampler and cascade impactor. The BioSampler allowed straightforward sampling in liquid medium, whereas the more laborious cascade impactor allowed size fractionation of virus-containing particles. Depending on the research question, either the BioSampler or the cascade impactor can be applied in laboratory and field settings, such as hospitals to gain more insight into the transmission routes of respiratory viruses.
animal transmission experiments, and virus stability studies) and observational epidemiological studies during outbreaks.

Respiratory viruses can spread via different routes: direct contact (e.g., via handshaking), indirect contact (via contaminated surfaces), or through the air via droplets and/or aerosols. We define droplets as particles that can only travel short distances through the air before they settle onto the mucosa of individuals or nearby surfaces, and aerosols as particles that are small enough to remain suspended in the air for prolonged periods of time and cover large distances. To understand the relative importance of various transmission routes, knowledge on the viral load and infectivity of viruses in the air, as well as the size of virus-containing droplets and aerosols is warranted. To meet this need, the collection of viruses from the air with air samplers has gained increasing attention. Such air samplers can be applied in environments such as hospital settings, animal experiments, or livestock farms, however, collecting infectious virus from the air remains challenging.

Apart from air samplers that employ a water-condensation growth method to capture particles, a major limitation of air samplers that use physical forces to separate aerosols and droplets from the air stream, is their high cut-off size, which prevents the efficient collection of small aerosols (<1 µm). To overcome this limitation, filters are sometimes installed after air samplers to capture aerosols and droplets that are too small to be collected by the air sampler itself. In addition, the high collection forces and sampling velocities applied inside these air samplers can damage virus particles, resulting in the collection of mainly non-infectious virus. In only a few studies, infectious respiratory viruses were collected from the air as demonstrated by virus isolation in cell cultures, whereas in most studies the presence of virus in the air was solely determined by the detection of viral RNA by (quantitative) RT-PCR.

Here, existing and newly developed air samplers with different collection methods were improved and compared: the six-stage Andersen cascade impactor, the SKC BioSampler, and an in-house developed electrostatic precipitator. The BioSampler and cascade impactor are commercially available air samplers that employ inertial forces to remove aerosols and droplets from the air flow. The cascade impactor was originally developed to collect airborne bacteria and fungi onto petri dishes filled with bacteriological agar. However, agar is less suitable as collection medium for viruses because of the high impaction forces and desiccation effects on aerosols and droplets.

For the cascade impactor and BioSampler, the collection efficiency is low for aerosols in the submicron range. To overcome the poor collection efficiency of small aerosols, we developed an electrostatic precipitator. Electrostatic precipitators are widely used to remove small particles such as dust from the air, and they are being increasingly explored for air sampling of airborne microorganisms. In electrostatic precipitators, the air around a conductor is ionized through the application of high voltage. Incoming droplets and aerosols get charged and attracted into a neutral or oppositely charged collection medium. These air samplers have a low flow rate and subject aerosols and droplets to less physical stress, thereby yielding higher recovery rates of infectious microorganisms.

In this study, the collection of infectious virus with the cascade impactor was first improved by optimizing the collection medium. Subsequently, the efficiency to collect infectious virus and viral RNA of the BioSampler, cascade impactor and electrostatic precipitator for nebulized pandemic H1N1 influenza A virus (pH1N1), human metapneumovirus (HMPV), human parainfluenza virus type 3 (PIV3), and respiratory syncytial virus (RSV) was compared in an in vitro set-up. Finally, the sensitivity of the BioSampler, and cascade impactor for low virus concentrations was evaluated.

2 MATERIAL AND METHODS

2.1 Cells and viruses

Human H1N1 influenza A virus A/Netherlands/602/2009 (pH1N1) was propagated in Madin-Darby canine kidney (MDCK) cells in Eagle’s minimal essential medium (EMEM; Lonza) supplemented with 100 IU ml⁻¹ penicillin-100 µg ml⁻¹ streptomycin mixture (Lonza), 2 mM L-glutamine (Lonza), 1.5 mg ml⁻¹ sodium bicarbonate (Lonza), 10 mM Hapes (Lonza), 1x nonessential amino acids (Lonza), and 20 µg ml⁻¹ trypsin (Lonza). MDCK cells were inoculated at an moi of 0.01. Supernatant was harvested at 72 hpi, cleared by centrifugation, and stored at −80°C. MDCK cells were maintained in EMEM supplemented with 10% fetal bovine serum (FBS, Greiner, or Atlanta Biologicals), 100 IU ml⁻¹ penicillin-100 µg ml⁻¹ streptomycin mixture (Lonza), 200 mM L-glutamine (Lonza), 1.5 mg ml⁻¹ sodium bicarbonate (Lonza), 10 mM Hepes (Lonza), and 1x nonessential amino acids (Lonza).

Recombinant HMPV NL/1/00 expressing green fluorescent protein (GFP) and GFP-expressing PIV3 (ViraTree) were propagated in subclone 118 of Vero-WHO cells (Vero-118 cells) in Iscove’s Modified Dulbecco’s Medium (IMDM) supplemented with 100 IU/ml penicillin-100 µg/ml streptomycin mixture (Lonza). For HMPV-GFP, 3.75 µg/ml trypsin (BioWhittaker) was added to the infection
medium. Vero-118 cells were inoculated with either HMPV-GFP or PIV3-GFP at an moi of 0.01. Cells and supernatant were harvested 7 (HMPV-GFP) or 5 (PIV3) dpi and stored at −80°C. After one freeze-thaw cycle, cell-free supernatants were concentrated and purified on a 30/60% sucrose cushion and subsequently stored in 25% sucrose at −80°C. Vero-118 cells were maintained in IMDM supplemented with 10% FBS and 100 IU/ml penicillin-100 µg/ml streptomycin mixture (Lanza). Hep-2 cells were inoculated at an moi of 0.01. Supernatant was harvested 6 dpi and stored at −80°C. After one freeze-thaw, cycle cell-free supernatants were concentrated and purified on a 30/60% sucrose cushion and subsequently stored in 25% sucrose at −80°C. Hep-2 cells were maintained in DMEM supplemented with 10% FBS, 100 IU/ml penicillin-100 µg/ml streptomycin mixture (Lanza), 2 mM L-glutamine (Lonza), 1.5 mg/ml sodium bicarbonate (Lonza), and 10 mM Hapes (Lonza). Hep-2 cells were inoculated at an moi of 0.01. Supernatant was harvested 6 dpi and stored at −80°C. After one freeze-thaw, cycle cell-free supernatants were concentrated and purified on a 30/60% sucrose cushion and subsequently stored in 25% sucrose at −80°C.

Respiratory syncytial virus A2 (ATCC) was propagated in human epithelial 2 (Hep-2) cells in Dulbecco’s Modified Eagle Medium (DMEM; Lonza or Gibco) supplemented with 2% FBS, 100 IU/ml penicillin-100 µg/ml streptomycin mixture (Lanza), 2 mM L-glutamine (Lonza), 1.5 mg/ml sodium bicarbonate (Lonza), and 10 mM Hapes (Lonza). Hep-2 cells were inoculated at an moi of 0.01. Supernatant was harvested 6 dpi and stored at −80°C. After one freeze-thaw, cycle cell-free supernatants were concentrated and purified on a 30/60% sucrose cushion and subsequently stored in 25% sucrose at −80°C. Hep-2 cells were maintained in DMEM supplemented with 10% FBS 100 IU/ml penicillin-100 µg/ml streptomycin mixture (Lanza), 2 mM L-glutamine (Lonza), 1.5 mg/ml sodium bicarbonate (Lonza), 10 mM Hapes (Lonza). All cells were cultured at 37°C and 5% CO₂. Virus stock concentrations were calculated as described below and were 6.8, 6.6, 6.9, and 7.5 log₁₀ TCID₅₀/ml for pH1N1, HMPV, PIV3, and RSV, respectively.

2.2 | Virus titrations

For endpoint titration of viruses, cells were grown to confluency in 96-well-plates overnight. Subsequently, cells were inoculated with 100 µl of 10-fold serial dilutions of collected air samples or controls. One hour after inoculation, cells were washed once and cultured in infection medium consisting of either serum-free EMEM supplemented with 100 IU/ml penicillin-100 µg/ml streptomycin mixture (Lanza), 2 mM L-glutamine (Lonza), 1.5 mg/ml sodium bicarbonate (Lonza), 10 mM Hapes (Lonza) and 1x nonessential amino acids (Lonza) and 20 µg/ml N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) treated trypsin (Sigma Aldrich) for MDCK cells, serum-free IMDM supplemented with 100 IU/ml penicillin-100 µg/ml streptomycin mixture (Lanza) and 2 mM L-glutamine (Lonza) and 3.75 µg/ml trypsin (BioWhittaker) for Vero-118 cells and serum reduced (2%) DMEM supplemented with 100 IU/ml penicillin-100 µg/ml streptomycin mixture (Lanza), 2 mM L-glutamine (Lonza), 1.5 mg/ml sodium bicarbonate (Lonza), 10 mM Hapes (Lonza), and 0.25 mg/ml fungizone (Invitrogen) for Hep-2 cells. For pH1N1 virus, supernatants of cell cultures were tested for CPE after 7 days of incubation. For RSV, cell cultures were observed for CPE after 7 days of incubation. For GFP-expressing HMPV and PIV3, wells were screened for GFP positive cells using an inverted fluorescence microscope at 7 and 5 days of incubation, respectively. Infectious virus titers were calculated from four replicates as tissue culture infective dose (TCID₅₀) by the Spearman-Karber method.

2.3 | Real-time quantitative RT-PCR

Viral RNA was extracted from 200 µl sample and eluted in a total volume of 50 µl using the MagNA Pure LC Total Nucleic Acid Isolation Kit, according to instructions of the manufacturer (Roche). Twenty µl of virus RNA was amplified in a final volume of 30 µl, containing 7.5 µl 4xTaqMan Fast Virus 1-Step Master Mix (Life Technologies), and 1 µl Primer/Probe mixture. Amplification was performed using the following protocol: 5 min 50°C, 20 sec 95°C, 45 cycles of 3 sec 95°C, and 31 sec 60°C.

2.4 | Air samplers

The viable six-stage Andersen cascade impactor (Thermo Scientific) operates at 28.3 liters per minute (LPM) and consists of six stages, with 400 orifices each, and 6 petri dishes (Figure 1A). With increasing stage number, the size of the orifices decreases, and hence, impaction velocity increases, enabling the collection of size-fractionated droplets and aerosols over the different petri dishes. Based on solid impaction, bacteria and fungi were originally captured onto petri dishes filled with bacteriological agar. For virus collection, virus transport medium (VTM) and an in-house developed semi-solid gelatin layer were compared with the conventional agar. VTM consisted of Minimum Essential Medium (MEM)—Eagle with Hank’s BSS and 25 mM Hapes (Lonza), glycerol 99% (Sigma Aldrich), lactalbumin hydrolysate (Sigma Aldrich), 10 MU polymyxin B sulfate (Sigma Aldrich), 5 MU nystatin (Sigma Aldrich), 50 mg/ml gentamicin (Gibco), and 100 IU/ml penicillin 100 µg/ml streptomycin mixture (Lanza), while the semi-solid gelatin layer was prepared from commercial gelatin sheets (Dr. Oetker) dissolved in VTM (10 mg/ml). For all collection media, polystyrene 100 mm petri dishes (Greiner) were used. To maintain an optimal jet-to-plate distance with the polystyrene dishes, a total volume of 41 ml was used to fill the plates based on manufacturer instructions. To avoid high dilution factors of the samples, petri dishes were first filled with 32 ml of 2% agarose (Roche) as a bottom layer on which the actual collection medium, 9 ml VTM, or 9 ml semi-solid gelatin, was placed. For the agar impaction surface, 41 ml of 1.5% w/v bacteriological agar NO.1 (Thermo Scientific™) was used. To quantify collected infectious virus and total virus RNA, samples were processed in liquid form. Semi-solid gelatin was liquefied directly after sampling by adding 6 ml of pre-warmed (37°C) VTM to each plate followed by incubation for 30 min at 37°C. Agar plates were carefully scraped with a cell scraper after adding 6 ml VTM. VTM samples were simply aspirated from the petri dishes. Samples were aliquoted and titrated or stored at −80°C for subsequent RNA isolation and qRT-PCR analysis.

The SKC BioSampler (SKC Inc) is an all-glass impinger that utilizes a liquid collection medium to capture droplets and aerosols (Figure 1B). It consists of an inlet, a collection vessel, and an outlet. The inlet contains three 0.63 mm tangential nozzles through which air is drawn at a flow rate of 12.5 LPM, thereby creating a swirling motion in the liquid collection medium. The swirling motion...
FIGURE 1  The different samplers that were compared in this study (A) Six-stage Andersen cascade impactor. Aerosols and droplets are collected from the air according to their size in 10 cm dishes filled with semi-solid gelatin, agar, or VTM. An accurate jet-to-plate distance is important to ensure a correct size fractionation. (B) SKC BioSampler (all-glass impinger). Air is drawn in and accelerated in the three nozzles. Particles are subsequently collected into swirling VTM by impingement. (C) Electrostatic precipitator. Inside a box, air is drawn into a glass chamber in which air is ionized. Cations bind to the particles and drag aerosols and droplets to the bottom reservoir which is filled with VTM. Orange arrows indicate airflow. Blue spheres indicate aerosols and droplets of different sizes.

FIGURE 2  Schematic representation of the nebulizer control and experimental air sampling set-up. (A) Virus suspensions were directly nebulized into 15 ml VTM in a T75 cell culture flask. (B) Virus suspensions were nebulized (1) to generate aerosols and droplets containing virus particles in an air-tight chamber (3), which was connected via a tube (4) to an air sampler (5). A second tube (6) was placed between the air sampler and a vacuum pump (8) that was placed outside the BSL II cabinet. High-efficiency particulate air (HEPA) filters (2,7) were installed on both sides of the air sampling set-up to guarantee that clean air entered the box and to prevent contamination of the environment. The direction of airflow is indicated with orange arrows. The flow rate through the system equals the recommended flow rate of the different samplers. For each experiment, nebulized viruses were collected from the chamber with air samplers for 5 min.
minimizes the chances of particle re-nebulization and maintains the infectiousness of collected particles. When aerosols and droplets exit the nozzles they get impinged into the liquid medium, while the remaining air exits the air sampler through the outlet. As collection medium, 15 ml virus transport medium (VTM) was used. Twenty µl antifoam B emulsion (Sigma Aldrich) was added to prevent the generation of bubbles and foam due to the swirling motion of the collection medium during air sampling.

The in-house developed electrostatic precipitator is made of a glass chamber consisting of an upper and bottom part (Figure 1C). A voltage of 13 kV is applied to a 80 mm long corona wire that is attached to the upper part of the glass chamber with a distance of 20 mm between the wire and the bottom part (Figure S1). Application of high voltage produces an ion discharge which ionizes the air in the chamber. Upon collision of incoming aerosols and droplets with ionized air molecules, aerosols and droplets become charged and attracted by the neutral bottom part of the glass chamber which is filled with 20 ml VTM. As a side effect, corona discharges also generate ozone, which is known to inactive viruses.37,38 A positive charge was used in the electrostatic precipitator because it produces less ozone than a negative charge.39,40 The electrostatic precipitator is operated at 4 LPM.

2.5 | Experimental air sampling set-up

Air samplers were connected to a chamber (Nalgene BioTransport Carrier box, 11.5 L, dimensions 36.8 × 18.4 × 17.0 cm (L × W × H)), in which 500 µl of a virus suspension was nebulized using the Aerogen Solo (Medicare Uitgeest B.V.). The Aerogen Solo is a vibrating mesh nebulizer that uses electricity to produce aerosols and droplets between 1 and 5 µm, with a reported mass median aerodynamic diameter of 3.05 µm, when nebulized in a 12 L chamber.41,42 The vacuum pump was switched on just before nebulization and air was drawn through the air samplers for a total of 5 min (Figure 2B). Subsequently, air samples were retrieved from the samplers, agar and semi-solid gelatin samples were processed as described above and all samples were subjected to further analysis. All experiments were performed in a class 2 biosafety cabinet.

2.6 | Air sampling experiments

Prior to the air sampling experiments, the performance of the nebulizer was assessed, as the mechanical nebulization process may affect virus infectivity. For this purpose, 500 µl of the pH1N1 virus, HMPV, PIV3, or RSV stock was directly nebulized into 15 ml VTM in a T75 cell culture flask (nebulizer control, Figure 2A). Once the virus suspension was completely nebulized (30–90 s), the nebulizer was removed, the flask closed and aerosols and droplets allowed to settle into the medium for another 5 min. Afterward, the flask was gently swirled to collect residual particles. In addition, 500 µl of the same virus suspension was directly pipetted into 15 ml of VTM in a 50 ml tube (positive control), representing the amount of virus before nebulization. Subsequently, both samples were subjected to virus titration and qRT-PCR to determine the amount of infectious virus and viral RNA, respectively. The amount of virus in VTM before nebulization was then compared to the amount of virus in VTM after nebulization. Average virus titers (±SD) were calculated from six replicates. No statistical analyses were performed.

The virus collection efficiency of the cascade impactor was optimized with an in-house developed gelatin layer and VTM, as agar was expected to be less suitable as collection medium for viruses. For this purpose, 500 µl of the pH1N1 virus and HMPV stock was nebulized into the chamber and subsequently collected from the air using the cascade impactor containing either petri dishes filled with agar, semi-solid gelatin, or VTM. As the collection of viruses was most efficient in combination with the in-house developed semi-solid gelatin layer, this collection medium was used in subsequent experiments. After improving the collection efficiency of the cascade impactor, the collection efficiency of all three air samplers was compared using pH1N1 virus, HMPV, PIV3, and RSV. Five hundred µl of each virus stock was nebulized and subsequently collected with each air sampler. In the last experiment, the sensitivity of the BioSampler and cascade impactor, the two samplers with the highest collection efficiency in this study, was assessed for collecting infectious viruses from the air. For this purpose, the pH1N1 virus and HMPV stocks were diluted in VTM to virus concentrations of 6.0 and 4.0 log10 TCID50/ml. Subsequently, 500 µl of these virus suspensions were nebulized and collected by both air samplers.

To determine the collection efficiency of the air samplers, samples of each experiment were subjected to virus titration and qRT-PCR to determine the amount of infectious virus and viral RNA collected by each air sampler. Subsequently, the amount of virus collected by each air sampler was compared to the amount of virus in the positive control which was 15 ml of VTM containing the same amount of virus as was nebulized and collected by the air sampler. For the cascade impactor, the total collection efficiency (i.e., the sum of collected virus of all six stages) and the collection efficiency per stage was assessed. Average virus titers and Ct values (±SD) of each air sampling experiment were calculated from three replicates. No statistical analyses were performed.

3 | RESULTS

3.1 | Loss of virus infectivity due to mechanical nebulization

A direct comparison of the titers with and without nebulization demonstrated that pH1N1 virus infectivity was barely affected by this process, as the virus titer after nebulization was only 0.03 log10 TCID50 lower than without nebulization. The virus titers of HMPV, PIV3, and RSV were reduced by 0.58, 0.55, and 0.54 log10 TCID50 respectively (Table 1). The loss of virus infectivity during nebulization may be due to incomplete nebulization of virus suspensions or due to viruses...
TABLE 1  Loss of virus infectivity due to nebulization with the Aerogen solo nebulizer

| Virus  | Virus titer before nebulization (log$_{10}$TCID$_{50}$ +/-SD) | Virus titer after nebulization (log$_{10}$TCID$_{50}$ +/-SD) |
|--------|-------------------------------------------------------------|--------------------------------------------------|
| pH1N1  | 6.36 +/- 0.46                                              | 6.33 +/- 0.58                                    |
| HMPV   | 6.20 +/- 0.7                                               | 5.62 +/- 0.73                                    |
| PIV3   | 6.53 +/- 0.3                                               | 5.98 +/- 0.2                                     |
| RSV    | 6.86 +/- 0.16                                              | 6.32 +/- 0.43                                    |

Note: Values represent average virus titers (±standard deviation, SD) of six replicates.

FIGURE 3  Evaluation of different collection media for the cascade impactor. pH1N1 virus and HMPV were collected on agar, semi-solid gelatin, or VTM to compare the collection efficiency of the cascade impactor with each medium. For both viruses and the different collection media, the total amount of collected infectious virus (A and C) and viral RNA (B and D), as well as the distribution of the amount of infectious virus (E and G) and viral RNA (F and H) over the six stages, is shown. Dotted lines indicate the detection limit of the virus titrations. Bars represent mean values of 3 experiments. Error bars indicate SD of 3 experiments.
not being resistant to the mechanical forces applied during nebulization. Overall, the loss of virus infectivity due to nebulization was only marginal, hence, this method was used in subsequent experiments.

### 3.2 | Optimization of the virus collection efficiency of the cascade impactor

For pH1N1 virus, collection of infectious virus was equally efficient when agar or semi-solid gelatin was used, and only 0.6 and 0.8 log_{10} TCID_{50} were lost, respectively, as compared to the positive control. Collection of infectious pH1N1 virus in VTM was much less efficient and resulted in a reduction of 2.4 log_{10} TCID_{50} (Figure 3A). The total collection efficiency of the cascade impactor for pH1N1 virus RNA as compared to the positive control was 16.4% and 6.4% for agar and semi-solid gelatin, but interestingly 39.4% for VTM, despite the substantial loss of virus infectivity (Figure 3B). Also, for HMPV, the amount of infectious virus collected with each medium varied. The collection efficiency was highest with semi-solid gelatin and VTM, where 1.4 and 1.6 log_{10} TCID_{50} less infectious virus was recovered after air sampling as compared to the positive control (Figure 3C). When agar was used as collection medium, considerably less infectious HMPV was collected with a reduction of 2.4 log_{10} TCID_{50} as compared to the positive control (Figure 3C). The total collection efficiency of the cascade impactor for HMPV RNA varied from 1.4%, 5.4%, to 12.3% for semi-solid gelatin, agar, and VTM, respectively. Thus, also for HMPV, the highest physical collection efficiency was obtained with VTM (Figure 3D).

To size fractionate virus-containing particles in the cascade impactor, the air velocity and impaction forces increase with increasing stage number. As a consequence, viruses may be subjected to more physical stress in stage 6 as compared to stage 1. Therefore, to investigate if the virus infectivity was differently conserved over all stages, the amounts of infectious virus and virus RNA collected in the individual stages were also compared. When agar, semi-solid gelatin, or VTM was used to collect pH1N1 virus, the amounts of infectious virus and virus RNA were evenly distributed over all stages, suggesting that the infectivity of pH1N1 virus was not more affected in the higher stage numbers as compared to the lower stage numbers (Figure 3E, F). The distribution of infectious HMPV and HMPV RNA over the six stages was slightly more variable than that of pH1N1 virus. Interestingly, substantially lower amounts of infectious HMPV and viral RNA were captured in stage 6 as compared to pH1N1 (Figure 3G, H). Overall, the infectivity of both viruses was well conserved with semi-solid gelatin, which was therefore used in subsequent experiments.

### 3.3 | Comparison of the collection efficiency of three air samplers for four common respiratory viruses

The highest collection efficiency of infectious virus was obtained with the cascade impactor for pH1N1 virus and the BioSampler for HMPV and RSV, whereas similar amounts of PIV3 were collected with the cascade impactor and BioSampler (Figure 4A). For all four viruses, only low amounts of infectious virus were collected with the in-house developed electrostatic precipitator (Figure 4A). The collection efficiency of the electrostatic precipitator for virus RNA was also very low demonstrating that the overall collection of viruses with this sampler was poor (Figure 4B).

When the distribution patterns of the four viruses over the six stages of the cascade impactor were investigated, the amounts of collected infectious pH1N1 virus and pH1N1 RNA were found to be similar in all stages. In contrast, considerably lower amounts of infectious virus and virus RNA were collected in stage 6 for HMPV, PIV3, and RSV as compared to the other stages (Figure 4C, D).

### 3.4 | Sensitivity of the BioSampler and the cascade impactor

Despite the lower amount of nebulized virus compared to the high amount of virus used in earlier experiments, both air samplers were still able to collect infectious virus as efficient as when high amounts of virus were nebulized. Air sampling with the BioSampler resulted in a reduction of 0.2 and 0.3 log_{10} TCID_{50} for pH1N1 virus, and 1.4 and 1.0 log_{10} TCID_{50} for HMPV, as compared to the positive control, when 10^{3.7} and 10^{3.7} TCID_{50} of virus was nebulized, respectively (Figure 5A, B). The cascade impactor collected 0.6 and 0.8 log_{10} TCID_{50} less pH1N1 virus, and 0.9 and 0.9 log_{10} TCID_{50} less HMPV, as compared to the positive control, when 10^{3.7} and 10^{3.7} TCID_{50} were nebulized, respectively (Figure 5A, B).

### 4 | DISCUSSION

Air sampling is increasingly recognized as an important tool for the characterization and quantification of respiratory viruses in the air in different environments, such as hospital settings, epidemiological investigations, and laboratory experiments. Information on the amount of infectious virus in the air, the ability of a virus to remain infectious in the air, and the size distribution of droplets and aerosols that contain infectious viruses will help to identify the relative contribution of the possible transmission routes of the respiratory virus under investigation. For this purpose, here, the performance of three air samplers which employ different collection methods and use different collection media was compared in an in vitro set-up by evaluating their efficiency to collect and preserve the infectivity of four artificially nebulized respiratory viruses.

In cascade impactors, originally agar was used to collect bacteria from the air. However, agar is generally considered less suitable as a collection surface for viruses, given the possibility of desiccation and increased particle bounce. In addition, it was demonstrated for infectious bursal disease virus, that virus recovery from agar is reduced significantly when petri dishes are processed at later time points, and immediate processing is not always possible in field
FIGURE 4 Performance of all air samplers with different respiratory viruses. To compare the performance of the three air samplers, pH1N1 virus, HMPV, PIV3, and RSV were each nebulized and collected with the BioSampler, cascade impactor (with semi-solid gelatin), and electrostatic precipitator. For all viruses, the amount of collected infectious virus (A and C) and viral RNA (B and D) is shown for each air sampler. Dotted lines indicate the detection limit of the virus titrations. Bars represent mean values of 3 experiments. Error bars indicate SD of 3 experiments.
F I G U R E  5 Collection efficiency of the BioSampler and cascade impactor for diluted virus stocks. $10^{5.7}$ and $10^{3.7}$ TCID$_{50}$ of pH1N1 virus (A) and HMPV (B) were nebulized and the total amount of infectious virus was determined by virus titration. Dotted lines indicate the detection limit of virus titrations. Bars represent mean values of 3 experiments. Error bars indicate SD of 3 experiments.

As an alternative to agar, liquid medium is also frequently used in the cascade impactor, since the chances of virus desiccation are smaller and sample processing after collection is not needed. However, the high flow velocities within the sampler push aside liquid medium where the air stream hits the surface, creating a dent, thereby increasing the jet-to-plate distance. This may result in a shift of size fractionation, with larger particles being collected in lower stages and smaller particles escaping from collection by the cascade impactor. Furthermore, liquid spill-over into other stages increases the chances of cross-contamination and VTM can be spilled easily, making the transport of petri dishes challenging, as also reported by others. The results of the present study show that the collection of infectious pH1N1 virus in agar was equally efficient as in semi-solid gelatin and more efficient than in VTM, demonstrating that both, agar and semi-solid gelatin but not VTM are suitable media for the collection of infectious pH1N1. In contrast, the opposite was true for HMPV, where collection of infectious virus was least efficient in agar, while it was comparably efficient in semi-solid gelatin and VTM. These results indicate that different collection media may be required depending on the respiratory virus under investigation (Figure 3). This is a disadvantage when different viruses are collected from the air simultaneously. With semi-solid gelatin, infectious pH1N1 virus and HMPV were equally well collected. In addition, with semi-solid gelatin, a correct jet-to-plate distance is maintained for accurate size fractionation of aerosols and droplets and is also sufficiently solid for it to be safely transported, which is an enormous advantage over VTM.

Although the present study is the first to compare the use of solid, semi-solid, and liquid media in the impactor in terms of viral collection efficiency, our findings that a semi-solid collection surface works better than a pure solid or liquid one, are in agreement with the findings of other studies. For example, when Kesavan et al. compared the collection efficiency of the impactor with liquid and solid media as collection surface, efficiencies using liquid medium were considerably lower than those using wax-filled petri dishes with wet membrane filters on top. In a study by Bekking et al., high amounts of influenza virus RNA were collected from infected ferrets with the cascade impactor, however, attempts to recover infectious virus from air samples were unsuccessful. In the current study, collection of pH1N1 virus with VTM in the impactor also resulted in the collection of high amounts of pH1N1 virus RNA, however, the least viable virus could be recovered compared to the use of agar and gelatin.

When the collection efficiency of the three air samplers for different respiratory viruses was compared, the BioSampler performed best by collecting infectious virus and viral RNA of all four viruses with high efficiency. The BioSampler also showed superior performance over other tested air samplers in a study by Fabian et al. and successfully collected infectious H3N2 virus from the air in an apartment of infected occupants. Also the cascade impactor collected high amounts of infectious virus, however, the amount of virus captured in each stage varied for the different respiratory viruses. The largest difference was observed in stage 6 of the cascade impactor, where substantial lower amounts of HMPV, PIV3, and RSV were collected as compared to pH1N1 virus, suggesting that fewer virus particles of the Paramyxoviridae and Pneumoviridae were contained in aerosols of size 1.1–0.6 µm. A possible explanation for this observation might be the pleomorphic character, size differences, or aggregation of these viruses. Despite the fact that influenza viruses also form filamentous virus particles, it has been shown that passaging the viruses on eggs and cells results in the formation of mainly spherical particles of around 200 nm. Although the influenza strain used here was a low-passage clinical isolate, the ratio of spherical and filamentous particles in the influenza virus stock is not known, also not for the HMPV, PIV3, and RSV stocks. In addition, the composition of the different media in which the viruses were stored and nebulized, may have affected the number and distribution of virus particles inside aerosols and droplets as well as the size of the droplets and aerosols.

The BioSampler and cascade impactor have a cut-off size of approximately 300 and 650 nm, respectively, meaning that only particles larger than the cut-off size are collected with high efficiency.
Therefore, to collect smaller aerosols more efficiently, an electrostatic precipitator was developed in-house and also tested with all four respiratory viruses. Although the collection efficiency of the electrostatic precipitator, as determined by using an aerodynamic particle sizer, ranged from 65% to 43% for particles between 0.2 and 0.5 µm (Table S1), its overall performance was disappointing as only very low amounts of infectious virus and viral RNA was collected. A possible reason may be that droplets and aerosols were insufficiently charged with cations resulting in droplets and aerosols moving with the air through the sampler, rather than precipitating in the collection medium.

Several other studies have also evaluated the collection efficiency of the BioSampler and cascade impactor or have also employed electrostatic precipitation to collect microorganisms from the air.\textsuperscript{49–51}\textsuperscript{,}49–51\textsuperscript{,}56 For example, Raynor et al. recently compared several impingers and cyclones including the BioSampler and a non-viable cascade impactor, for their efficiency to collect nebulized avian and swine influenza A viruses from the air in an experimental room (3.24 × 2.34 × 4.11 m). Also in this set-up, the BioSampler and the non-viable cascade impactor performed well. In general, it should be mentioned, however, that experimental set-ups including nebulizer type, collection medium, and the applied flow rate are not uniform among studies. As an alternative to a jet nebulizer, which is often used in similar studies, here, a vibrating mesh nebulizer was used, as this nebulizer was shown to preserve the infectivity of viruses better than a jet nebulizer.\textsuperscript{42}\textsuperscript{,}42 Comparing the amount of infectious virus and viral RNA before and after nebulization in the present study showed that the virus solution was almost completely nebulized by the device and that the infectivity of viruses was well preserved. It should be noted that the nebulizer creates a higher relative humidity compared to a jet nebulizer and that in the current study aerosols and droplets were directly nebulized into a small chamber (11.5 L).\textsuperscript{42}\textsuperscript{,}42 In other studies, a dryer is often added to the experimental set-up that further dries aerosols and droplets before entering a collection chamber and thereby further decreasing particle sizes.\textsuperscript{49}\textsuperscript{,}49 Despite in the current set-up particles were not dried and therefore particle sizes potentially larger as compared to other studies, Bowling et al. showed that the average particle size produced by the nebulizer was 3.1 µm in a 12 L chamber with 80.89% and 59.03% of the generated particles being ≤5 µm and ≤3 µm, and that the average humidity was not significantly higher in that chamber than when particles were produced with the Collison nebulizer.\textsuperscript{42}\textsuperscript{,}42 Also, in the current experimental set-up high amounts of virus were nebulized, whereas, under field conditions, it is more likely that lower amounts of virus are dispersed over larger volumes of air. For the collection of representative samples under field conditions therefore air samplers likely need to run for longer time periods, whereas in the experimental set-up presented here, air samplers only run for five minutes. Particularly in the case of electrostatic precipitation, no device is commercially available yet that is specifically designed to collect infectious respiratory viruses from the air, and hence, air samplers are custom made designs.\textsuperscript{25,28,57,58}\textsuperscript{25,28,57,58} This makes it very difficult to directly compare the performance of the air samplers evaluated here, with other studies.

In conclusion, in the present study, the commercially available BioSampler and cascade impactor are both capable of collecting artificially generated droplets and aerosols containing respiratory viruses while maintaining their infectivity during the sampling process. With the cascade impactor, quantitative data on the sizes of virus-containing particles can be obtained, and in combination with a semi-solid gelatin layer as collection surface, the cascade impactor is also easy to use in various field settings such as hospitals. With the BioSampler size fractionation of the collected aerosols and droplets is unfortunately not possible. However, collection is more facile, since only one air sample is obtained per collection moment and no post-air sampling processing is needed. The choice for either of the two air samplers therefore also depends on the environment in which it is to be used and on the research questions to be addressed. Overall, implementation of these air samplers in field studies will help to obtain more quantitative data on the amount of infectious respiratory virus that is present in the air, thereby generating a better understanding of respiratory virus transmission.

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CONFLICT OF INTEREST
The authors declare no competing interests.

AUTHOR CONTRIBUTIONS
J.K. and S.H. conceived, designed, and analyzed the work and wrote the manuscript. A.M. helped with the design of the electrostatic precipitator. J.K. and D.M. performed the work. T.B. helped with performing the work. R.F. helped with the interpretation of the data and manuscript revision. All authors read and approved the final manuscript.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.

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