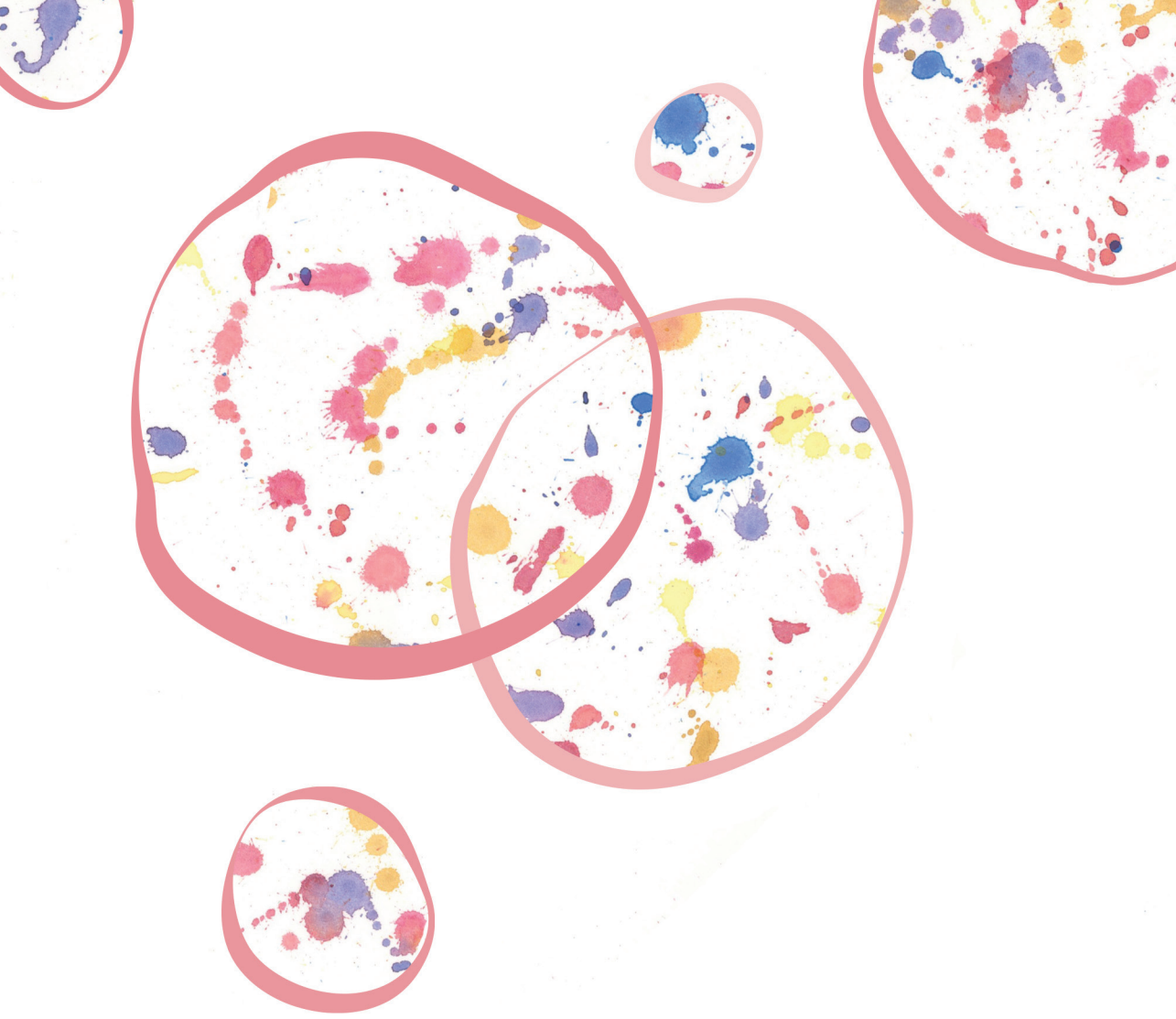

EXPERIMENTAL METHODS TO ASSESS THE AIRBORNE TRANSMISSION OF RESPIRATORY VIRUSES

Jasmin S Kutter

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The research described in this thesis was performed at the Department of Viroscience, Erasmus University Medical Center, Rotterdam, the Netherlands and within the Postgraduate School of Molecular Medicine.

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Experimental Methods to Assess the Airborne Transmission of Respiratory Viruses

Experimentele methoden ter bestudering van de luchtoverdraagbaarheid van luchtwegvirussen

Thesis

to obtain the degree of Doctor from the
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by command of the
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and in accordance with the decision of the Doctorate Board.

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by

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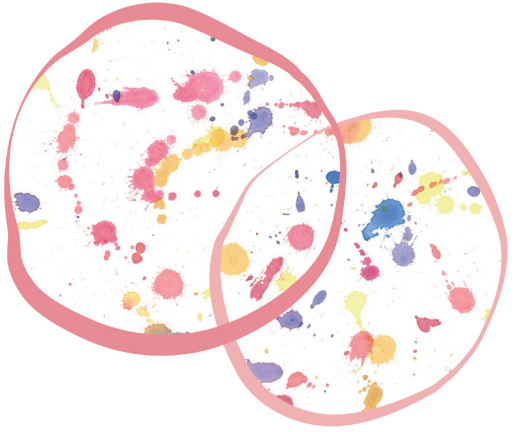

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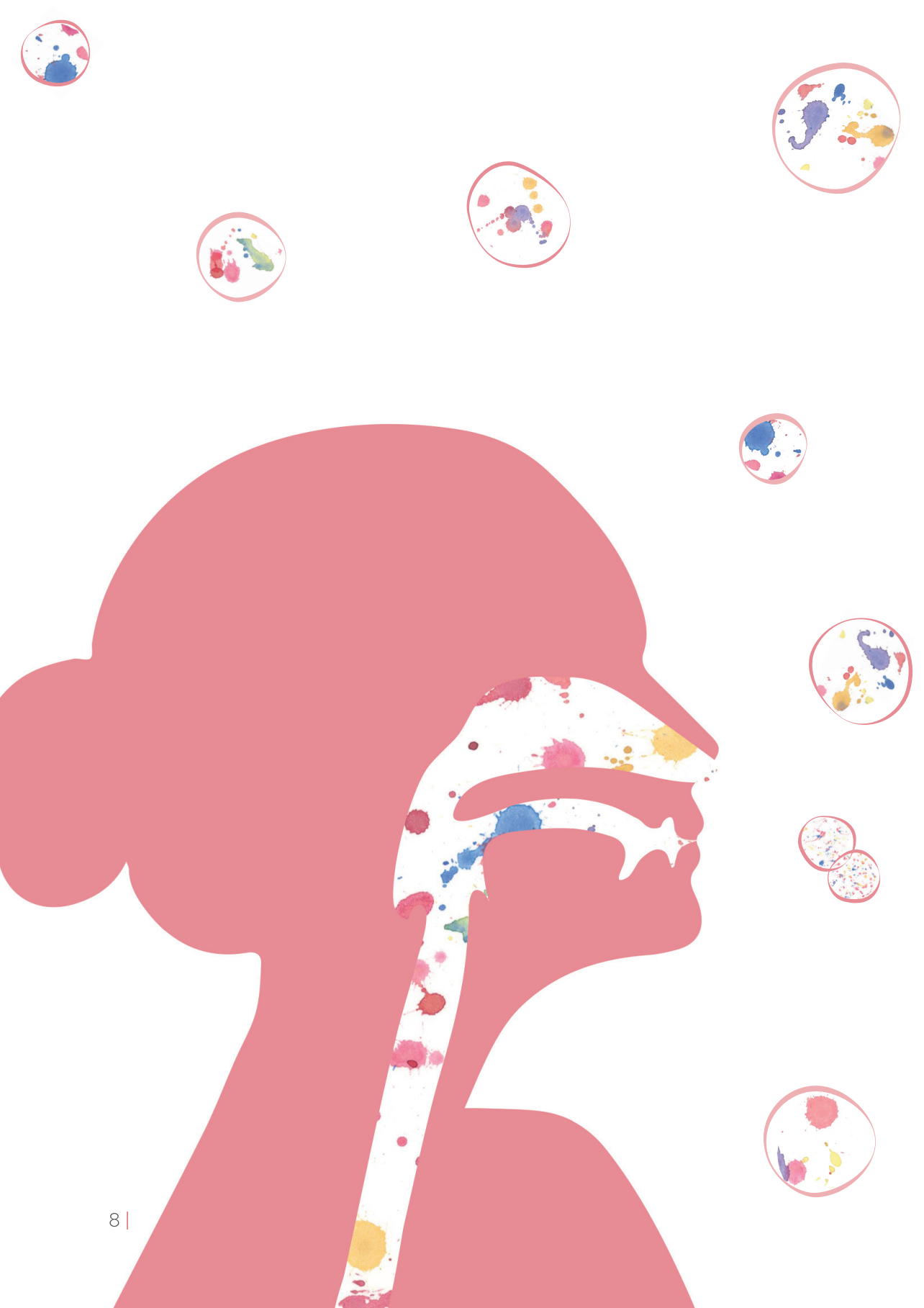

1

GENERAL INTRODUCTION & OUTLINE OF THE THESIS

Partially based on

Transmission routes of respiratory viruses among humans

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Global impact of respiratory viruses

Viral respiratory tract infections cause significant morbidity and mortality worldwide. People of all ages are affected by respiratory virus infections, experiencing several upper respiratory tract infections each year[1–3]. However, children, the elderly and immunocompromised people are at the greatest risk of developing severe lower respiratory tract infections[4–8]. Respiratory viruses are responsible for 43-67% of community-acquired pneumonia in children and for over 90% of bronchiolitis cases in infants[9,10]. In addition, (re-) emerging respiratory viruses have the potential to become pandemic and thus pose an additional threat to global health and the economy[11,12]. To date, it is still unclear for several respiratory viruses via which routes they are predominantly transmitted and how viral properties (e.g., virus stability in the air) and factors such as shedding kinetics contribute to the efficient spread of respiratory viruses in the human population. Gaining a deeper insight into these aspects is crucial to assess the risks of emerging viruses to cause outbreaks and to improve non-pharmaceutical intervention strategies to control and prevent the spread of respiratory viruses among humans.

Transmission of respiratory viruses

Respiratory viruses infect and replicate in the human respiratory tract from which they can be shed to other individuals via contact or the air[13,14] (**Table 1**). Contact transmission refers to direct virus transfer from an infected person to a susceptible individual (e.g., via contaminated hands) or indirect virus transfer via fomites on intermediate objects such as door knobs or water taps. Transmission of virus through the air can occur via droplets or aerosols. A commonly used arbitrary cut-off size between droplets and aerosols is 5 µm, although this varies considerably between studies, ranging up to 12 µm[15–18]. Droplets do not remain suspended in the air for prolonged periods of time and generally travel less than 1 m before settling on the mucosa of close contacts or environmental surfaces. Aerosols have a slow settling velocity and therefore remain suspended in the air longer and can travel further[19,20].

Transmission via each of the above-mentioned routes is complex and depends on many variables, such as environmental factors (e.g., humidity and temperature) including the behavior of people like crowding in cold and wet seasons, but also on host factors, such as receptor distribution throughout the respiratory tract[19,21]. The fact that these variables affect the transmission routes of different respiratory viruses in a dissimilar way makes it very difficult to investigate them experimentally[19,21]. For that reason, up to now, knowledge on

the transmission routes of respiratory viruses is mainly based on observational studies such as outbreak and case reports, and household or intervention studies.

Table 1. Commonly accepted transmission routes of respiratory viruses

Transmission Route	Source	Characteristics/Definition of Transmission
Contact		
Direct	Virus contaminated skin of infected individual.	Virus transfer via skin of infected individual and subsequent self-inoculation of susceptible individual by touching mucous membranes.
Indirect	Virus contaminated objects (fomites).	Virus transfer via fomites and subsequent self-inoculation of susceptible individual by touching mucous membranes.
Air		
Droplet	Droplets. Remain only shortly in air and are dispersed over short distances. Deposition mainly on mucous membranes and upper respiratory tract.	Short range transmission. Direct inoculation of naïve person through exhalatory activities of infected person.
Aerosol	Aerosols. Remain in air for a prolonged period of time and are dispersed over large distances.	Long range transmission. Inhalation of aerosols in respirable size range. Deposition along the respiratory tract, including the lower airways.

Evidence from experimental and observational studies on the transmission routes of important respiratory viruses

Adenovirus

Human adenoviruses comprise more than 50 serotypes causing a variety of diseases with the most common ones being conjunctivitis, respiratory and gastrointestinal tract infections. In particular, types 1-5, 7, 14, and 21 are associated with respiratory illness and pneumonia in children[22–25], whereas infections are generally asymptomatic in adults[22]. Adenoviruses cause nosocomial outbreaks, especially in pediatric care facilities, where they spread rapidly[24,26,27]. Moreover, adenovirus types 4 and 7 are responsible for large outbreaks of acute respiratory disease, especially in crowded conditions. This is illustrated by, for example, outbreaks among military recruits for which spread through the air was suggested[22,28,29]. It is difficult to eliminate adenovirus from the skin, fomites, and environmental surfaces[30]. An outbreak in a mental care facility

was probably enhanced by spending the day mainly in a crowded room while sharing cigarettes and soda cans, suggesting indirect fomite spread[31]. In a study published in 1966, experimental infections with adenovirus administered as aerosols (0.3-2.5 µm) or droplets (15 µm) to healthy, male inmates, resulted in infection of all volunteers, although the resulting illness resembled a natural infection only in the aerosol group[32]. During a military training period, increased numbers of adenovirus infections occurred over time, which correlated with increased detection of PCR-positive air filters. Additionally, a correlation between disease and the extent of ventilation was observed, with more ventilation resulting in fewer disease cases[33]. In a more recent study in military recruits, positive viral DNA samples were mainly obtained from pillows, lockers, and rifles, although adenovirus DNA was also detected in air samples. No consistent correlation between increased positive environmental samples and disease was observed[34].

Influenza A virus

Due to the severity of the yearly influenza epidemics and the potential of zoonotic influenza A viruses to cause severe outbreaks, there have been many studies on influenza A virus transmission among humans. Different kinds of studies, such as air sampling[35–37] and intervention studies[38], as well as human challenge studies[39–41], have been conducted. In addition, transmission events have been described after outbreaks in aircrafts[42,43], households[44–46], and hospital settings[47]. However, it is still unclear how important transmission via the air is in the spread of influenza A viruses among humans compared to other routes and hence several reviews intensely discuss this issue[48–53].

Already in the mid-1900s, human challenge models were used to assess the transmission route of influenza virus[39–41,54]. It was shown that individuals can be infected by inhalation of aerosolized influenza A viruses and that illness outcome tends to be milder in intranasally infected volunteers in comparison to inoculation through inhalation[40,41]. Increasing numbers of studies focused on the detection and quantification of influenza viruses contained in droplets and aerosols expelled into the air through breathing, sneezing, and coughing of infected individuals[19,35–37,55–58]. Influenza virus RNA was detected in the air up to 3.7 m away from patients with the majority of viral RNA contained in aerosols (<5 µm)[56]. The presence of virus in aerosols could indicate potential transmission through the air, although many studies only quantified the amount of viral RNA[37,58,59]. A few studies quantified viable virus, although this was only recovered from a minority of samples[19,35,36,56].

Rhinovirus

Extensive human rhinovirus transmission experiments have not led to a widely accepted view on the transmission route[60–65]. Inhalation of aerosols (0.2-3 μm) resulted in efficient rhinovirus infection[66], but little to no infectious rhinovirus could be demonstrated in sneezes and coughs as detected by virus titration[67]. Rhinovirus can survive on stainless steel, plastic, and skin for a couple of hours[67,68]. Additionally, virus was detected in saliva, occasionally on hands, and could be recovered from the skin of recipients after rubbing either a contaminated fomite or hand[67,69]. When rubbing of fomites was followed by auto-inoculation this resulted in infection of the volunteers[60]. In a three-day rhinovirus experiment with healthy volunteers different exposure modes were used to investigate the rhinovirus transmission route: 1) small-particle exposure (separating donor and recipients by wire mesh), 2) large particle exposure (encouraging contact, coughing, and sneezing while wearing gloves) and 3) direct contact exposure (hand contact followed by self-inoculation). From the results, it was concluded that direct contact was the main transmission route[61]. Furthermore, rhinovirus RNA was detected in offices by air sampling studies and subsequent sequencing resulted in a matched air-mucus pair[62]. In a miniature field trail, experimentally infected donors with severe colds participated in a card game with susceptible recipients for ~12 hours[63–65]. A restraining device, preventing touching of the head and face, was used in the aerosol condition and heavily contaminated cards and exaggerated hand-to-face movements in the fomite condition. In these experiments, aerosol transmission was suggested[64]. In general, transmission rates and exposure time varied between studies, which may contribute to the different routes of transmission that were observed. Therefore, the donor-hours of exposure were determined using donors with severe rhinovirus infections. At 200 hours of exposure to donors, transmission had occurred to 50% of the susceptible recipients, though the transmission route itself was not investigated[65].

Coronavirus (CoV)

In humans, alpha coronaviruses (229E and NL63) and beta coronaviruses (OC43, HKU1, SARS, and MERS) are associated with respiratory disease[70,71]. Alpha coronaviruses have a high attack rate early in life and spread rapidly during outbreaks, indicating efficient human-to-human transmission[71]. Furthermore, samples obtained from staff and patients of a neonatal and pediatric intensive care unit showed a high incidence of human coronaviruses HCoV-229E and HCoV-OC43, suggesting staff-to-patient and patient-to-staff transmission[72]. Unfortunately, there is very little data to corroborate on the HCoV-229E, HCoV-NL63,

Table 2. Overview of the methods to study human-to-human transmission and their respective pros and cons

Study design	Pro	Con	Reference
Virus stability	• Can provide indirect evidence for transmission route • Easy to perform	• Not conclusive as transmission itself is not investigated.	73,78,92,113,123
Air sampling	• Noninvasive for patients. • Quantification of viable virus in the air • Characterization of droplet/aerosol size • Can be used in parallel with human studies or outbreaks • Can gain information on possible aerosol spread	• In a nosocomial setting aerosol-generating procedures can play a major role • Frequently only detection by PCR • Direct human-to-human transmission is not studied (circumstantial) • Technical issues (procedure may affect virus viability) or false interpretation	37,58,59,98,114, 115,125
Outbreak (household or hospital) reports	• Study natural infections • Includes the most susceptible patients who are difficult to include in experimental studies	• Retrospective • Usually not conclusive on transmission route or relative importance of transmission routes	44,127–129
Outbreak report aircraft	• Relatively easy to perform • Outbreak in closed setting	• Retrospective which can result in recall-bias and hard to trace back passenger movements • Inconclusive • Only reported in case of secondary infections and in these cases, infections may also occur before or after the flight	43,126,130,131,156

Study design	Pro	Con	Reference
Experimental infection	• Controlled environment • Donor selection and control • Real-time data collection • Repeatable • Various parameters can be studied at the same time • Possibility to study different inoculation routes	• Ethical obstacles • Infectivity and disease can differ from that in a natural infection (attenuated strains) • Difficult to create ideal and comparable circumstances • Many factors have to be taken into account: duration, influence of superspreaders, sampling methods • Difficult to get naïve or risk group participants who are interesting to study	40,41,64,65,67, 132,133
Miniature Field Trial	• Can discriminate between contact transmission and transmission through the air	• Ethical obstacles • Exposure time may not be sufficient • Difficult to create ideal and comparable circumstances	63–65
Non-pharmaceutical Intervention	• Can help to discriminate between transmission routes if performed properly	• Usually no controlled environment • Difficult to determine ideal time-point of the intervention • Risk of drop-out or perseverance	38,60,134–136
Pharmaceutical intervention	• Can help to identify relative importance of transmission routes • Controlled environment	• Difficult to include enough patients to obtain statistically significant results	137

Study design	Pro	Con	Reference
Computational Modelling/ Simulation	• Describes transmission in a greater context • Can account for heterogeneity of transmission within a population • Human mannequins can be used as replacement for humans	• Theoretical (for mathematical modelling) • Artificial setting	90,138–144
Air tracer studies	• Monitoring airflow pattern can indicate possible transmission through the air (if not done retrospectively) • Visualize airstream	• Usually performed retrospectively and not during outbreaks	145,146,157

and HCoV-OC43 transmission routes. HCoV-OC43, HCoV-229E, and HCoV-NL63 infectivity was lost between 0-72 hours on non-absorptive surfaces, although it can survive several days in medium or PBS[73–75]. Aerosolized HCoV-229E had a half-life of 67 hours in a rotating steel drum (at 20˚C and 50% relative humidity)[76]. SARS-CoV and MERS-CoV appeared to have an unusual capacity to survive on dry surfaces as compared to HCoV-229E, HCoV-OC43, and HCoV-NL63[77,78].

The SARS outbreak was primarily linked to healthcare settings, with ≥49% of the cases linked to hospitals[79], most probably caused by aerosol-generating procedures on severely ill patients[80,81]. Aerosol-generating procedures like intubation, the use of continuous positive-pressure ventilation, and drug delivery via nebulizers are likely to produce "fine infectious droplets", which travel further than droplets from coughs[82]. Additionally, superspreading events contributed to the dispersion of the SARS outbreak[81,83–85], particularly in the Hotel Metropole and the Prince of Wales Hospital in Hong Kong[84]. Moreover, a link with transmission to healthcare workers was observed when they were in close proximity (<1 m) to an index patient, suggesting direct contact or droplet transmission[81,86,87]. Air samples and swabs from frequently touched surfaces in a room occupied by a SARS patient tested positive by PCR, although no virus could be cultured from these samples[88]. In the Amoy gardens outbreak, fecal droplet transmission was suggested[89,90].

To date, there is little data on the human-to-human MERS-CoV transmission route[91]. MERS-CoV remained stable on non-absorptive surfaces for 8 up to 48 hours and for 10 minutes at 20 ˚C and 40% relative humidity in aerosols[92]. MERS-CoV outbreaks in humans are, like those with SARS-CoV, primarily linked to healthcare settings, with a link to hospitals in ≥31% of the cases[79,93,94] and healthcare-associated human-to-human transmission was observed[95,96]. Superspreader events were shown to play an important role in nosocomial outbreaks[79,97]. Virus was isolated from environmental samples in hospital rooms, suggesting direct contact or fomite transmission. Moreover, the potential of MERS-CoV to transmit through the air was investigated by air sample analysis[98,99]. Viral RNA was detected on the inlet of air ventilation equipment and virus was isolated from air samples and surfaces from inaccessible areas like the ventilator exit, implicating potential aerosol transmission[99].

Measles virus (MV)

Measles is one of the most contagious viral diseases in humans that has been associated with aerosol transmission for a long time[100–106]. In the late 1970s and early 1980s, data from retrospective observational studies obtained during

outbreaks in pediatric practices, a school, and a sporting event suggested transmission through aerosols[101–105]. Indeed, those studies showed that most secondary cases never came in direct contact with the index patient and some were never even simultaneously present in the same area as the index case[101,105]. Examination of airflow in the pediatricians' offices showed that aerosols were not only dispersed over the entire examination room but also accumulated in the hallway and other areas[101,105]. Furthermore, based on the investigation of air circulation in a sports stadium, in which an MV outbreak occurred, the authors suggested that MV had been dispersed through the ventilation system[103]. Thus, it was concluded that MV can be transmitted via aerosols. Although coughing is a common symptom associated with measles disease, index patients were described to cough frequently and vigorously in the outbreak reports of pediatric practices. Remington et al. calculated the infectious dose of MV produced by the index case through coughing, using a mathematical model based on transmission through the air. They found that the index case produced a very high infectious dose compared to cases from other outbreaks and mentioned a phenomenon called superspreading[105]. Superspreaders are individuals who are able to infect a disproportionally large number of susceptible contacts when compared to a typical individual[107–109], which may contribute to the efficient transmission of MV[110].

Respiratory Syncytial Virus (RSV)

Transmission of RSV among humans is thought to occur via droplets and fomites[111]. In the 1980s three potential transmission routes of RSV were studied in humans by dividing infected infants and healthy volunteers into three groups, representing: 1) all transmission routes, 2) transmission via fomites, and 3) transmission through the air by allowing the volunteers to have either 1) direct contact with infants (cuddlers), 2) touching potential fomites (touchers) or 3) sitting next to the infant (sitters). Volunteers in the group of the cuddlers and touchers but not the sitters became infected, suggesting that direct contact and droplet transmission were the probable routes for efficient infection of the volunteers and that transmission via aerosols was less likely[112]. Another study on the transmission via fomites showed that RSV could be recovered from countertops for several hours, but only for several minutes from absorptive surfaces such as paper tissue and skin[113]. Later on, in the late 1990s, Aintablian et al. detected RSV RNA in the air up to 7 m away from a patient's head[114]. In spite of that, since virus infectivity could not be demonstrated, potential transmission of RSV through the air has been considered negligible and transmission of RSV was thought to occur mainly

through contact and droplet transmission. However, in a recent study authors were able to collect aerosols that contained viable virus from the air around RSV-infected children[115]. Although the detection of viable virus in the air is by itself not enough to confirm aerosol transmission, the general presumption that RSV exclusively transmits via droplets should be confirmed and explored further.

Parainfluenza virus (PIV) and human metapneumovirus (HMPV)

There is a substantial lack of (experimental) evidence on the transmission routes of PIV types 1-4 and HMPV. For these viruses, contact and droplet transmission are commonly accepted transmission routes[116–118]. However, only virus stability on various surfaces has been investigated so far and it has been shown that PIV and HMPV are stable on non-absorptive surfaces and can barely be recovered from absorptive surfaces[119–123].

Knowledge gaps on respiratory virus transmission

Studies on the transmission routes of respiratory viruses have been performed under many different (experimental) conditions since the beginning of the 20th century[124]. Despite this, the relative importance of the transmission routes of respiratory viruses is still unclear, depending on the heterogeneity of many factors like the environment (e.g., temperature and humidity), pathogen, and host[19,21]. Consequently, every study design has its strengths and weaknesses (**Table 2**). The stability of respiratory viruses on surfaces and in the air is frequently investigated in the laboratory, as these experiments are easy to perform and provide indirect evidence for a transmission route[73,78,92,113,123]. However, virus transmission itself is not investigated in these experiments, and therefore results remain inconclusive about the actual infection risk via either of the routes. To gain more information on the viral load present in the air of different environments and around infected individuals, air sampling is often performed[37,98,115,125]. With air samplers, the quantity of infectious virus in the air and the size of virus-containing aerosols and droplets can be determined[58], which is crucial information for the implementation of infection prevention measures. However, viruses collected from the air are frequently detected by PCR only[58,59], which does not provide information on the infectivity of a virus. Moreover, infectious virus in the air is not a direct correlate of infection and does not provide conclusive results on the relative importance of virus transmission via the air. Hence results should be interpreted with care and in relation to the evidence of other studies. Overall, it is also still difficult to collect (infectious) viruses from the air due to technical constraints of air samplers, which may lead

Table 3. Overview of the evidence on transmission routes of respiratory viruses*

Virus	Human-to-human transmission	Route (suggested)	Stability		Setting	
			Surface	Air	Experimental	Household
Adenovirus	X	Contact				
		Aerosol			X	
		Droplet/aerosol				
Influenza virus		Droplet/aerosol				
		Droplet/aerosol				
		Droplet/aerosol				
		Droplet/aerosol				X
Rhinovirus	X	Contact			X	
	X	Contact			X	
	X	Contact			X	
		Aerosol				X
	X	Aerosol			X	
HCoV		Contact	X		X	
		Contact	X		X	
SARS-CoV		Contact	X		X	
		Contact	X		X	
	X	Contact/Droplet				
	X	Contact/Droplet				
	X	Droplet				
	X	Droplet				
	X	Aerosol				
	X	Aerosol				
MERS-CoV		Contact/Aerosol				
		Contact	X	X	X	
	X	Contact/Droplet				
	X	Contact				
Measles virus	X	Aerosol				
	X	Aerosol				
	X	Aerosol				
	X	Aerosol				
RSV	X	Contact/Droplet				
		Contact	X		X	
		Aerosol				
		Aerosol				
HMPV		Contact	X		X	
		Contact	X		X	
PIV		Contact	X		X	
		Contact	X		X	

Setting								
Community	Clinical	AGP[¥]	SSE[‡]	Air Sampling	Surface sampling	Reference	Isolation guidelines
	X					31	Contact[14.153–155], droplet[14.153.154], aerosol[154.155]
						32	
X				X		33	
	X			X		37	Contact[153–155], droplet[14.153–155], aerosol[14.153–155]
X				X		17	
X				X		36	
				X		56	
						60	Contact[153–155], droplet[153–155], aerosol[153–155].
						61	
						67	
				X		62	
						64	
						73	Contact[14.154.155], droplet[14.154.155].
						74	
						78	Contact[14.154.155], droplet[14.154.155], aerosol[14.154.155].
						158	
	X	X				81	
	X					87	
	X	X	X			86	
	X					159	
X			X			126	
X			X			90	
	X	X		X	X	99	Contact[155], droplet[14.155]
						92	
	X		X			97	
	X				X	98	
	X					101	Contact[14.154], droplet[14.153–155], aerosol[14.153–155]
X						103	
X					X	104	
	X					105	
	X					112	Contact[14.153–155], droplet[14.153.154], aerosol[153.155].
						113	
	X			X		114	
	X	X (some patients)		X		115	
						123	Contact[14.154.155], droplet[14.154.155].
						74	
						119	Contact[14.153–155], droplet[14.153–155], aerosol[14.153]
						120	

*based on experimental data and the transmission route according to infection prevention guidelines from National (Working Group Infection Prevention (WIP)[152]), European ("The Blue Book")[153], American ("The Red Book"[154] and the Centers for Disease Control (CDC)[14]) and International (UpToDate)[155] organizations. ¥ Aersosol generating procedure; ǂ Superspreading event;

to a false interpretation of results. Outbreak reports provide valuable information on the transmission dynamics of a virus in real-life settings. Natural infections can be studied in the most susceptible people, which are difficult to include in experimental studies[43,44,126–131]. However, such studies often rely on retrospective reporting and questionnaires, which can result in a recall bias and consequently in conflicting data. In addition, movements and contacts of infected individuals are often difficult to trace back. In these settings, a single transmission route cannot be excluded with certainty and hence results remain inconclusive about the relative contribution of any of the transmission routes. In contrast to outbreak reports, human challenge studies are performed in a controlled environment in which various parameters can be studied at the same time[40,41,64,65,67,132,133]. As such experiments are repeatable, they can be carried out under different conditions and with various inoculation routes to investigate the relative importance of the transmission routes. However, often (attenuated) laboratory strains are studied in healthy adults[39,40], which does not reflect the natural circumstances and target group and can result in a lower secondary attack rate[133]. Another way of investigating the relative importance of the transmission routes of respiratory viruses is performing (non-) pharmaceutical intervention studies[38,134–137]. These studies can include naturally infected individuals and their respective contacts. However, such studies are frequently performed in uncontrolled settings like households or schools in which adherence to the given tasks may be low[38,134,135]. It is also difficult to include enough participants to obtain statistically significant results, especially as subjects may drop out during the studies. To study virus transmission on a greater scale, often computational modeling and simulation studies are performed[138–143]. These studies have the advantage to account for the heterogeneity of transmission within a population. In simulation studies, human mannequins are used as a replacement for humans to assess the potential spread of viruses between individuals in different settings using tracer gas[144]. Tracer gas is a great tool to visualize the airflow between individuals, in rooms or buildings to assess the airflow through ventilation systems and thus can give an estimation on the dispersal of viruses within rooms and buildings[145,146]. However, these are artificial studies that can only theoretically predict the spread of viruses among individuals.

With different conditions and circumstances, the relative importance of the transmission routes can change, and consequently, viruses cannot be categorized into one of the transmission routes. Rather respiratory viruses move on a sliding scale depending on the setting and situation (**Table 3**). Differences in virus shedding between individuals can contribute to the transmissibility rate, especially in the case of superspreaders[83,147]. In addition, the SARS-CoV outbreak highlighted the impact of aerosol-generating procedures on the increased risk of human-to-human transmission[82,148], demonstrating that for these procedures additional containment measures are necessary. In contrast, in absence of such procedures, SARS-CoV transmission was predominantly mediated by close contact[81,86,87]. This suggests that under natural conditions, the risk of SARS-CoV transmission via small aerosols was negligible, while this became an increased infection risk when medical procedures were performed. Large amounts of infectious RSV were detected in the air around hospitalized infants and children in the absence of aerosol-generating procedures[115]. RSV was contained in aerosols of respirable size, overall indicating that aerosolized RSV may pose an infection risk[115]. However, a study on the transmission routes of RSV in humans demonstrated that RSV transmission predominantly occurs via close contact[112]. This is also supported by the fact that room-sharing of RSV infected and non-infected patients did not seem to influence the risk of nosocomial infections[149]. In addition, wearing gowns and gloves, and adhering to strict hygiene has been shown to reduce the risk of nosocomial RSV transmission considerably, further indicating that aerosol transmission is not efficient and possibly negligible in this context[150,151]. Thus, while infectious viruses can be aerosolized in the air, this does not necessarily pose an actual infection risk for every virus. In the future, it is important to separate epidemiological from clinical data and investigate if theoretical infection risks (e.g., infectious virus in the air) pose an actual threat in practice.

Infection prevention and virus transmission

In the light of the limited availability of antivirals and effective vaccines, the prevention of respiratory virus transmission via non-pharmaceutical methods becomes even more crucial. Although most infections are community-acquired, respiratory viruses are an important cause of nosocomial infections, as well. Therefore, the implementation of infection prevention guidelines is crucial. A comparison of the isolation guidelines of National[152], European[153], American[14,154] and International organizations[155] (**Fig. 1**) for their information on transmission routes (**Table 2**), showed that terms and definitions of respiratory transmission routes and

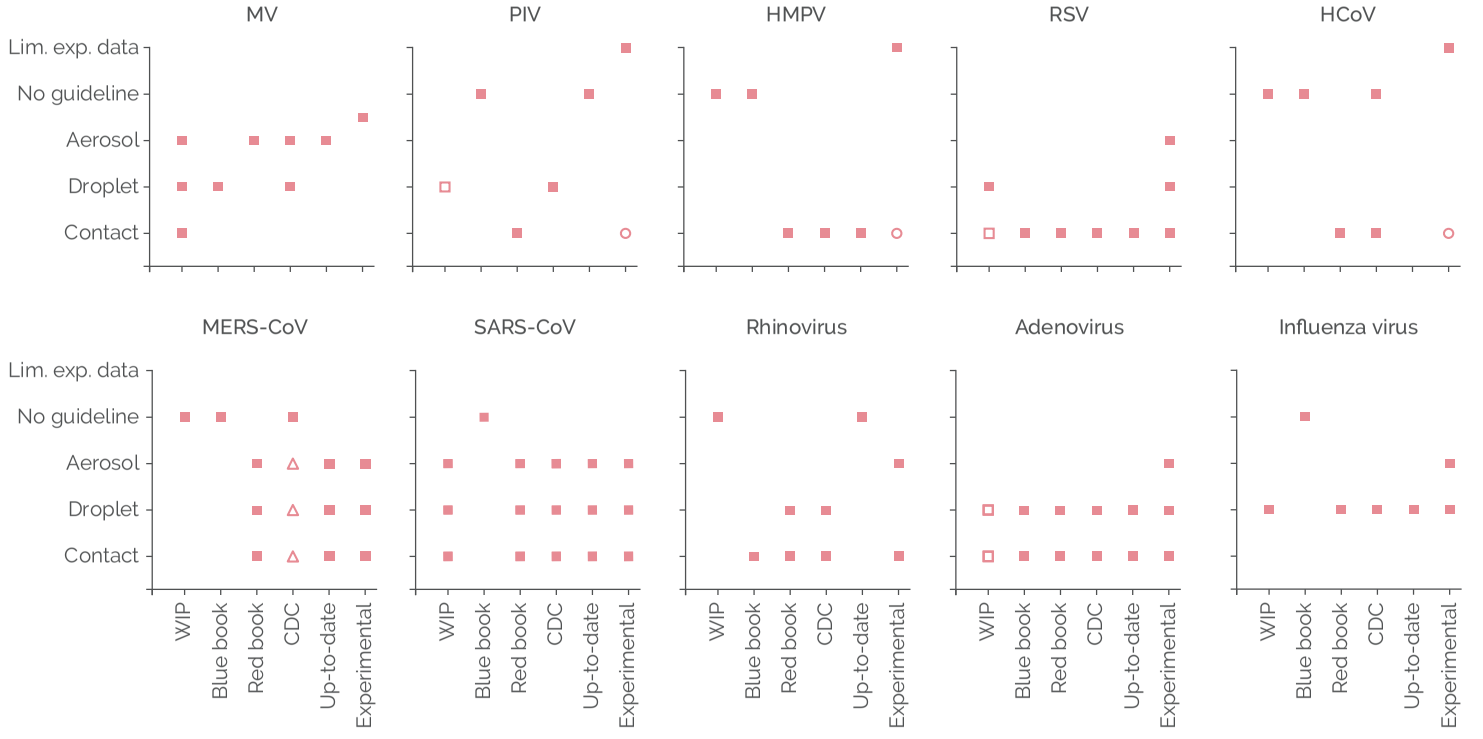
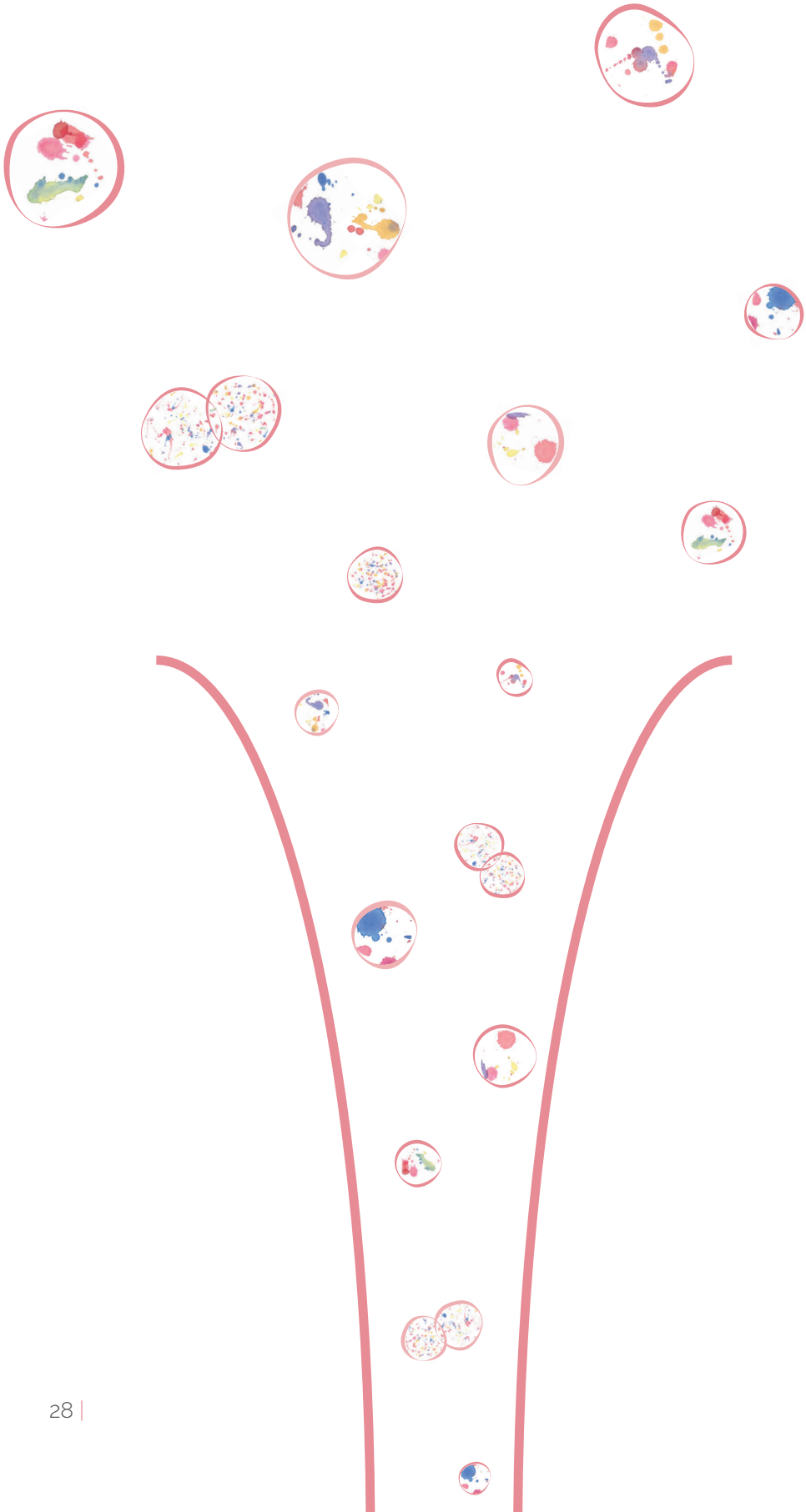


Figure 1. Isolation guidelines for respiratory virus infections in comparison to experimental evidence on transmission routes. Isolation guidelines for all respiratory viruses discussed in this review from National (Working Group Infection Prevention (WIP)[152], from the Netherlands National Institute for Public Health and the Environment (RIVM)), European ("The Blue Book")[153], American ("The Red Book"[154] and the Centers for Disease Control (CDC)[14]) and International (UpToDate)[155] organizations are shown on the X-axis, together with the experimental evidence on transmission routes (**Table 3**). The categories on the Y-axis are the different transmission routes (contact, droplet or aerosol), the absence of guidelines for infection prevention ('No guideline'), or the limited availability of experimental data ('Lim. exp. data'). The information shown for influenza virus reflects the guidelines on seasonal influenza virus. Closed squares (■): isolation guidelines for the respective respiratory virus. Open squares (□): guidelines are only for children ≤6 years old. Open circles (○): data from stability experiments only. Open triangles (△): specific CDC guidelines for Healthcare Professionals[160] (not the isolation guideline[14] used in this chapter).

isolation guidelines are not always used in a uniform way, leaving room for personal interpretation. But more importantly, information on the transmission route does not always reflect the isolation guidelines (e.g., for PIV and rhinovirus, **Fig. 1**). As a proxy for transmission route, virus stability is often referred to in the guidelines, however, as mentioned above, this can only imply a role for indirect contact transmission but is by no means conclusive on the transmission route. In hospital settings, prevention of contact transmission is generally implemented in standard infection prevention precautions such as strict hand hygiene and cough etiquette. It is important to note differences in isolation guidelines between different organizations and the lack of correlation to scientific data. The variation in the described transmission routes and associated isolation guidelines among the different organizations underscores the lack of convincing data and the urgent need for new knowledge on respiratory virus transmission routes. This new knowledge has to be implemented in infection control guidelines to advance intervention strategies for currently circulating and newly emerging viruses and to improve public health.

Scope and outline of the thesis

The scope of this thesis is to gain a better understanding of the possible transmission routes of respiratory viruses and to identify viral and host properties that drive the efficient transmission of respiratory viruses. Air samplers are an important tool to quantify the amount of virus in the air and determine the size of virus-containing aerosols and droplets. In **Chapter 2**, existing and newly developed air samplers are optimized and evaluated for their efficiency to collect viable respiratory viruses from the air. In **Chapter 3**, the Andersen cascade impactor, one of the best performing air samplers in Chapter 2, was employed in a hospital to determine the amount of RSV in the air around infants hospitalized with acute respiratory infections. In **Chapter 4**, the genetic and phenotypic determinants of A/H2N2 virus adaptation during the first year of circulation in humans are characterized using *in vitro* assays and animal transmission experiments. During the course of this PhD trajectory, a newly emerging respiratory virus, SARS-CoV-2, rapidly spread around the world to cause the Covid-19 pandemic. To start to address potential routes through which SARS-CoV-2 is spread, in **Chapter 5**, the transmissibility of this virus via the air was assessed between ferrets over more than one meter distance in a newly developed experimental transmission set-up. A summarizing discussion of the studies described in this thesis in the context of the work of others is presented in **Chapter 6**.



2

COMPARISON OF THREE AIR SAMPLERS FOR THE COLLECTION OF FOUR NEBULIZED RESPIRATORY VIRUSES

- Collection of respiratory viruses from air -

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ABSTRACT

Viral respiratory tract infections are a leading cause of morbidity and mortality worldwide. Unfortunately, the transmission routes and shedding kinetics of respiratory viruses remain poorly understood. Air sampling techniques to quantify infectious viruses in the air are indispensable to improve intervention strategies to control and prevent spreading of respiratory viruses. Here, the collection of infectious virus with 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 straight-forward 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.

PRACTICAL IMPLICATIONS

- Respiratory viruses pose a continuous health threat, and knowledge on their transmission routes will help to prevent their spread in the population.
- Knowledge on the quantity of an infectious respiratory virus in the air will help to predict the importance of transmission of this virus through the air.
- Most currently available air sampling devices have not been designed to collect infectious viruses from the air, hence the optimization and comparison of their ability to collect different respiratory viruses from the air is of utmost importance.

KEY WORDS

air sampling | collection efficiency | electrostatic precipitator | impactor | impinger

INTRODUCTION

Lower respiratory tract infections are the 4th leading cause of death globally[161]. Next to bacteria, respiratory viruses are the most common etiology of respiratory tract infections and pose a substantial burden on public health worldwide[7,8,162]. 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, animal transmission experiments, and virus stability studies) and observational epidemiological studies during outbreaks[163].

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[56,57,59,125,164,165], however, collecting infectious virus from the air remains challenging[166,167]. 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)[165,168]. 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[59,169]. 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[88,125]. In only a few studies, infectious respiratory viruses were collected from the air as demonstrated by virus isolation in cell cultures[17,36,56,164,165], whereas in most studies the presence of virus in the air was solely determined by the detection of viral RNA by (quantitative) RT-PCR[37,55,170].

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 airflow. 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[171–173].

For the cascade impactor and BioSampler, the collection efficiency is low for aerosols in the submicron range[174–176]. 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[177–180]. 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[179,180].

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.

RESULTS

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 $log_{10}TCID_{50}$ lower than without nebulization. The virus titers of HMPV, PIV3, and RSV were reduced by 0.58, 0.55, and 0.54 $log_{10}TCID_{50}$, respectively (**Table 1**). The loss of virus infectivity during nebulization may be due to incomplete nebulization of virus suspensions or due to viruses 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.

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

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

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 $log_{10}TCID_{50}$ (**Fig. 1a**). 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 (**Fig. 1b**). 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 (**Fig. 1c**). 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 (**Fig. 1c**). 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. (**Fig. 1d**).

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 (**Fig. 1e** and **1f**). 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 (**Fig. 1g** and **1h**). Overall, the infectivity of both viruses was well conserved with semi-solid gelatin, which was therefore used in subsequent experiments.

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 (**Fig. 2a**). For all four viruses, only low amounts of infectious virus were collected with the in-house developed electrostatic precipitator (**Fig. 2a**). 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 (**Fig. 2b**).

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 (**Fig. 2c** and **2d**).

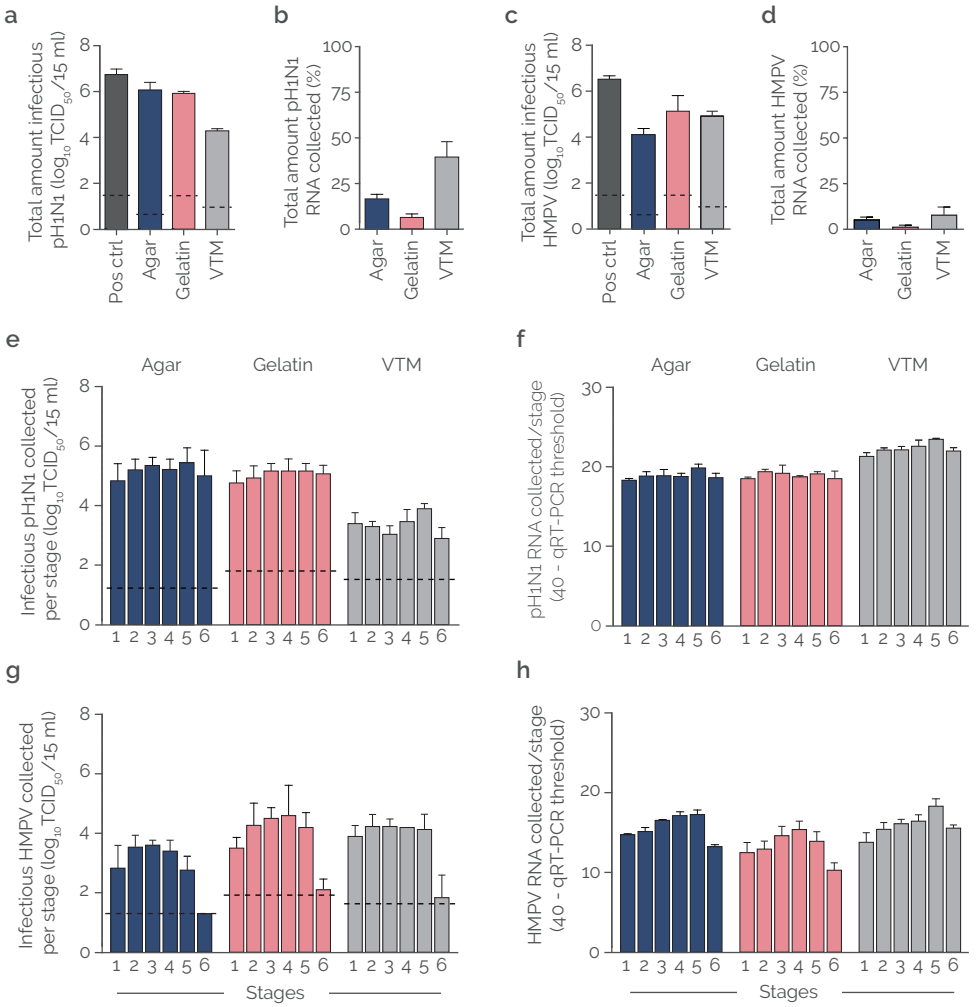

Figure 1. Evaluation of different collection media for the cascade impactor. pH1N1 virus and HMPV were collected on agar, semI-solid gelatin, or VTM to compare the collection efficiency of the cascade impactor with each medium. For both viruses and the different collection media, the total amount of collected infectious virus (**a** and **c**) and viral RNA (**c** 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.

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 $10^{5.7}$ and $10^{3.7}$ $TCID_{50}$ of virus was nebulized, respectively (**Fig. 3a** and **3b**). 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 $10^{5.7}$ and $10^{3.7}$ $TCID_{50}$ were nebulized, respectively (**Fig. 3a** and **3b**).

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[181–183]. 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 studies[184]. 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[37,115,185,186]. 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[187]. 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 spil-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[185,187]. 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

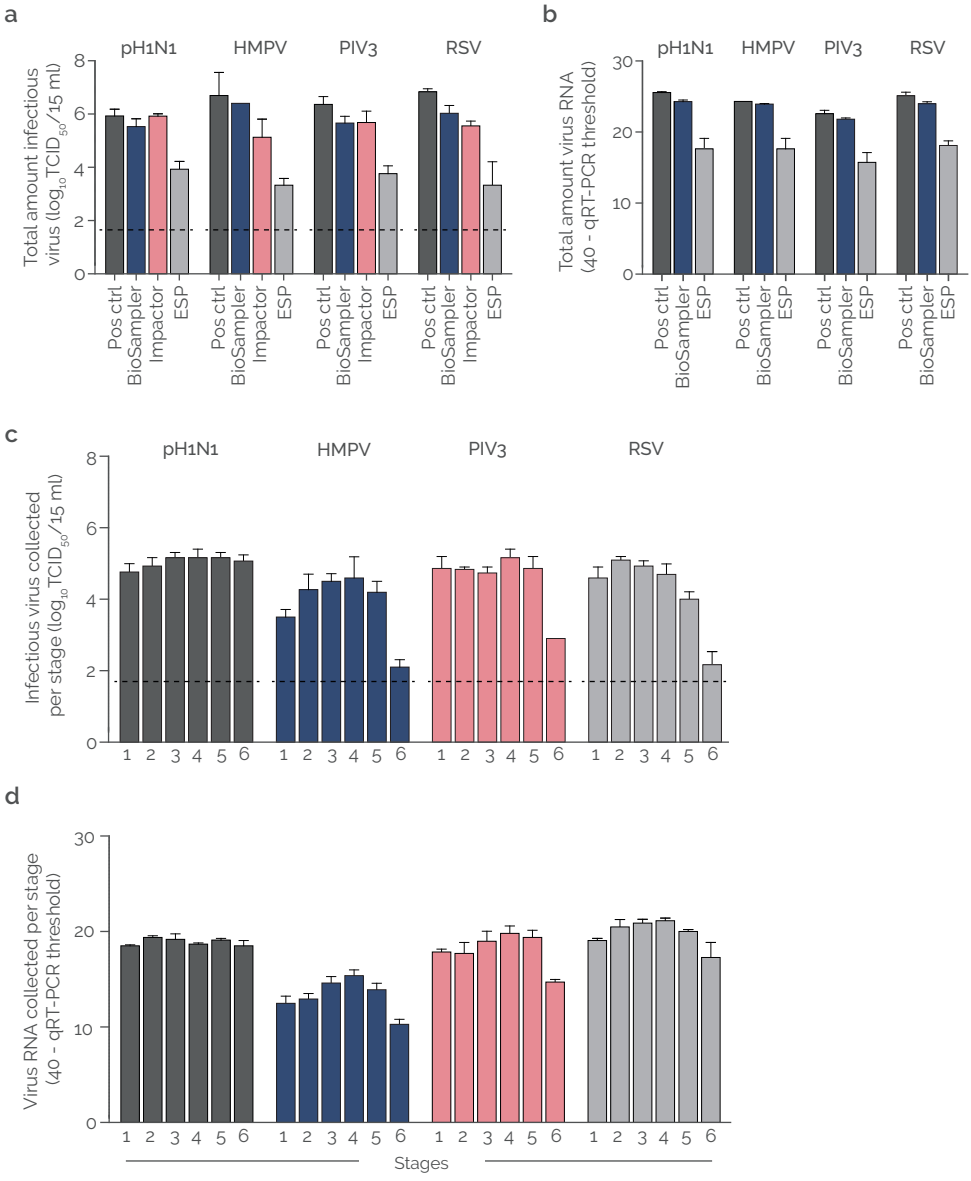

Figure 2. Performance of all air samplers with different respiratory viruses. To compare the performance of the three air samplers, pH1N1 virus, HMPV, PIV3, and RSV were each nebulized and collected with the BioSampler, cascade impactor (with semi-solid gelatin), and electrostatic precipitator. For all 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.

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 (**Fig. 1**). 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.[187] 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.[185], 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[56,188]. 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 Paramyxo- and Pneumoviridae were contained in aerosols of size 1.1–0.6 µm. 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

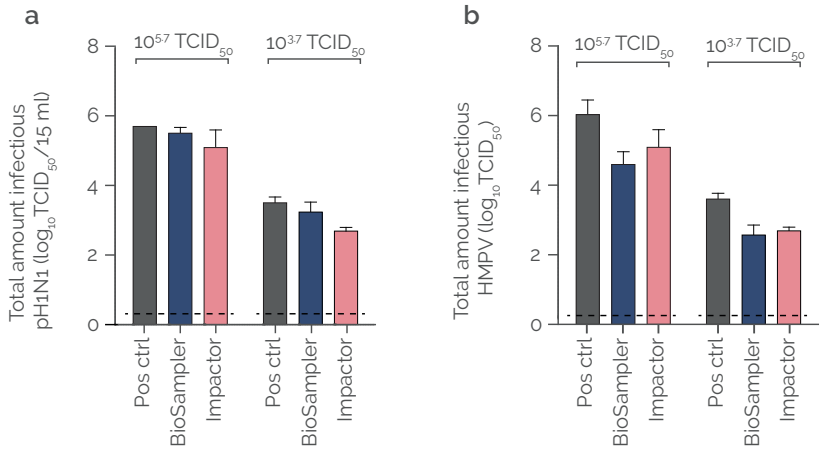

Figure 3. Collection efficiency of the BioSampler and cascade impactor for diluted virus stocks. $10^{5.7}$ and $10^{3.7}$ TCID$_{50}$ of pH1N1 virus (**a**) and HMPV (**b**) were nebulized and the total amount of infectious virus was determined by virus titration. Dotted lines indicate the detection limit of virus titrations. Bars represent mean values of 3 experiments. Error bars indicate SD of 3 experiments.

of mainly spherical particles of around 200 nm[189,190]. 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[186–188,191]. For example, Raynor et al.[192] 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[193]. 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)[193]. 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[186]. 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[193]. 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[174,177,194,195]. 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.

MATERIAL AND METHODS

Cells and viruses

Human H1N1 influenza A virus A/Netherlands/602/2009 (pH1N1) was propagated in MadinDarby 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 Hepes (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^{-1} penicillin-100 µg ml^{-1} streptomycin mixture (Lonza)[196,197]. For HMPV-GFP, 3.75 µg ml^{-1} 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-GFP) 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^{-1} penicillin-100 µg ml^{-1} streptomycin mixture (Lonza).

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^{-1} penicillin-100 µg ml^{-1} streptomycin mixture (Lonza), 2 mM L-glutamine (Lonza), 1.5 mg ml^{-1} sodium bicarbonate (Lonza), and 10 mM Hepes (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^{-1} penicillin-100 µg ml^{-1} streptomycin mixture (Lonza), 2 mM L-glutamine (Lonza), 1.5 mg ml^{-1} sodium bicarbonate (Lonza), 10 mM Hepes (Lonza). All cells were cultured at 37˚C and 5% CO_2.

Virus stock concentrations were calculated as described below and were 6.8, 6.6, 6.9, and 7.5 $\log_{10}TCID_{50}ml^{-1}$ for pH1N1, HMPV, PIV3, and RSV, respectively.

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^{-1} penicillin-100 µg ml^{-1} streptomycin mixture (Lonza), 2 mM L-glutamine (Lonza), 1.5 mg ml^{-1} sodium bicarbonate (Lonza), 10 mM Hepes (Lonza) and 1x nonessential amino acids (Lonza) and 20 µg ml^{-1} N-tosyl-l-phenylalanine chloromethyl ketone (TPCK) treated trypsin (Sigma Aldrich) for MDCK cells, serum-free IMDM supplemented with 100 IU ml^{-1} penicillin-100 µg ml^{-1} streptomycin mixture (Lonza) and 2 mM L-glutamine (Lonza) and 3.75 µg ml^{-1} trypsin (BioWhittaker) for Vero-118 cells and serum reduced (2%) DMEM 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 Hepes (Lonza), and 0.25 mg ml^{-1} fungizone (Invitrogen) for Hep-2 cells. For pH1N1 virus, supernatants of cell cultures were tested for agglutination activity using turkey erythrocytes after 3 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 ($TCID_{50}$) by the Spearman-Karber method.

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[198,199]. 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.

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[200] (**Fig. 4a**). With increasing stage number, the size of the orifices

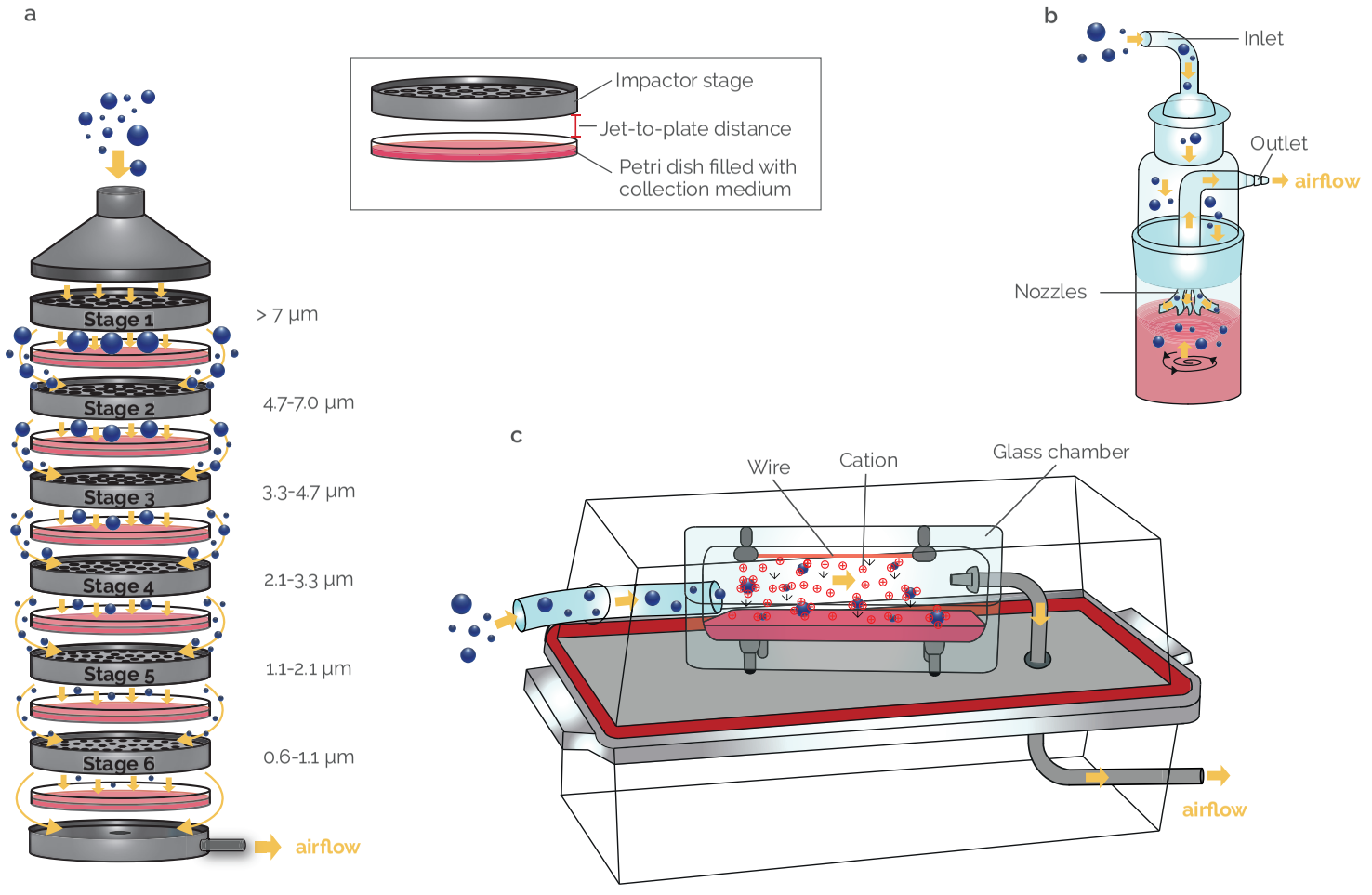
a
Impactor stage
Jet-to-plate distance
Petri dish filled with collection medium
Stage 1
> 7 µm
Stage 2
4.7-7.0 µm
Stage 3
3.3-4.7 µm
Stage 4
2.1-3.3 µm
Stage 5
1.1-2.1 µm
Stage 6
0.6-1.1 µm
airflow
b
Inlet
Outlet
airflow
Nozzles
c
Wire
Cation
Glass chamber
airflow

Figure 4. 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. Yellow arrows indicate airflow. Blue spheres indicate aerosols and droplets of different sizes.

decreases, and hence, impaction velocity increases, enabling the collection of sizefractionated 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 inhouse developed semisolid gelatin layer were compared with the conventional agar. VTM consisted of Minimum Essential Medium (MEM)-Eagle with Hank's BSS and 25 mM Hepes (Lonza), glycerol 99% (Sigma Aldrich), lactalbumin hydrolysate (Sigma Aldrich), 10 MU polymyxin B sulfate (Sigma Aldrich), 5 MU nystatin (Sigma Aldrich), 50 mg ml^{-1} gentamicin (Gibco), and 100 IU ml^{-1} penicillin-100 µg ml^{-1} streptomycin mixture (Lonza), while the semi-solid gelatin layer was prepared from commercial gelatin sheets (Dr. Oetker) dissolved in VTM (10 mg ml^{-1}). 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 prewarmed (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 (**Fig. 4b**). 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 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 (**Fig. 4c**). 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 (**Fig. 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[201,202]. A positive charge was used in the electrostatic precipitator because it produces less ozone than a negative charge[203,204]. The electrostatic precipitator is operated at 4 LPM.

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[193,205]. The vacuum pump was switched on just before nebulization and air was drawn through the air samplers for a total of 5 min (**Fig. 5a**). 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.

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, **Fig. 5b**). Once the virus suspension was completely nebulized (30–90 s), the nebulizer

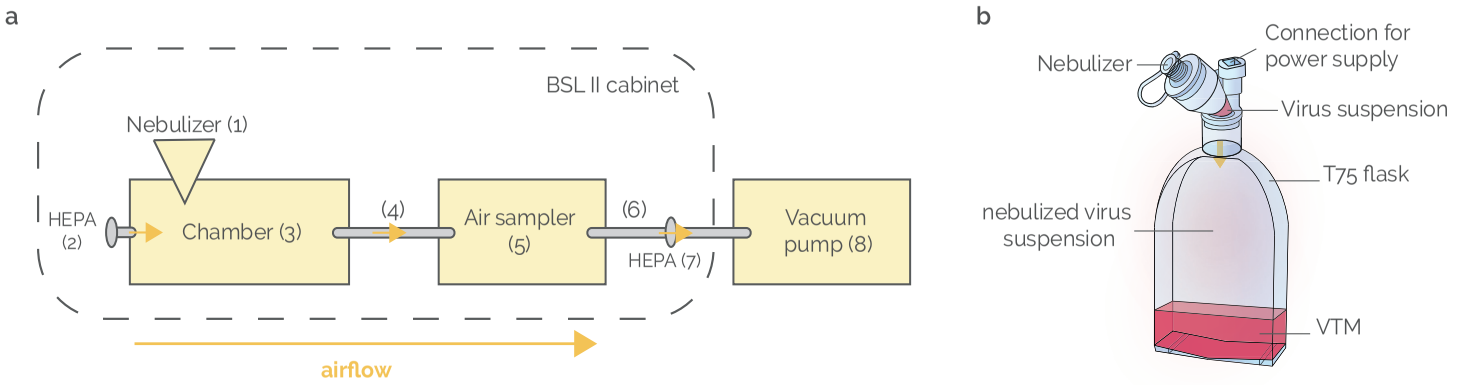

Figure 5. Schematic representation of the nebulizer control and experimental air sampling set-up. (**a**) Virus suspensions were directly nebulized into 15 ml VTM in a T75 cell culture flask. (**b**) Virus suspensions were nebulized (1) to generate aerosols and droplets containing virus particles in 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 yellow 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.

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 $\log_{10}TCID_{50}ml^{-1}$. 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.

DECLARATIONS

Data availability

All data are available from the corresponding author (S.H.) on reasonable request.

Acknowledgements

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Conflict of interest statement

The authors declare no competing interests.

Authors' 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.

SUPPLEMENTARY INFORMATION

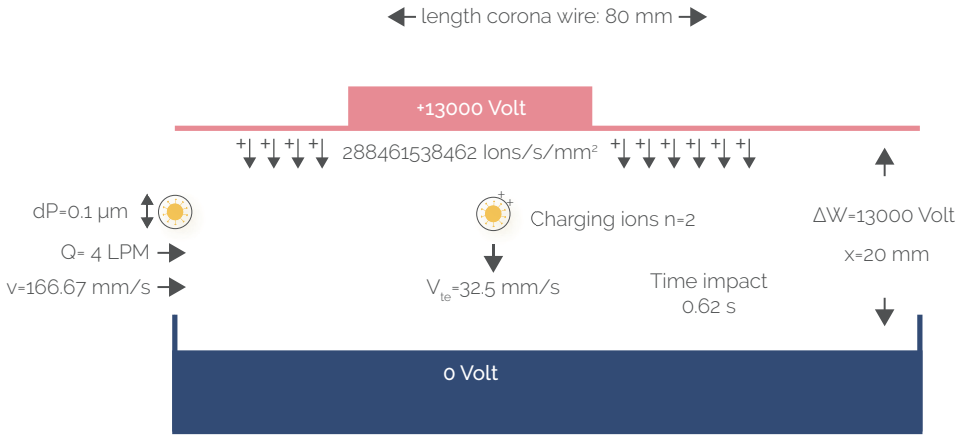
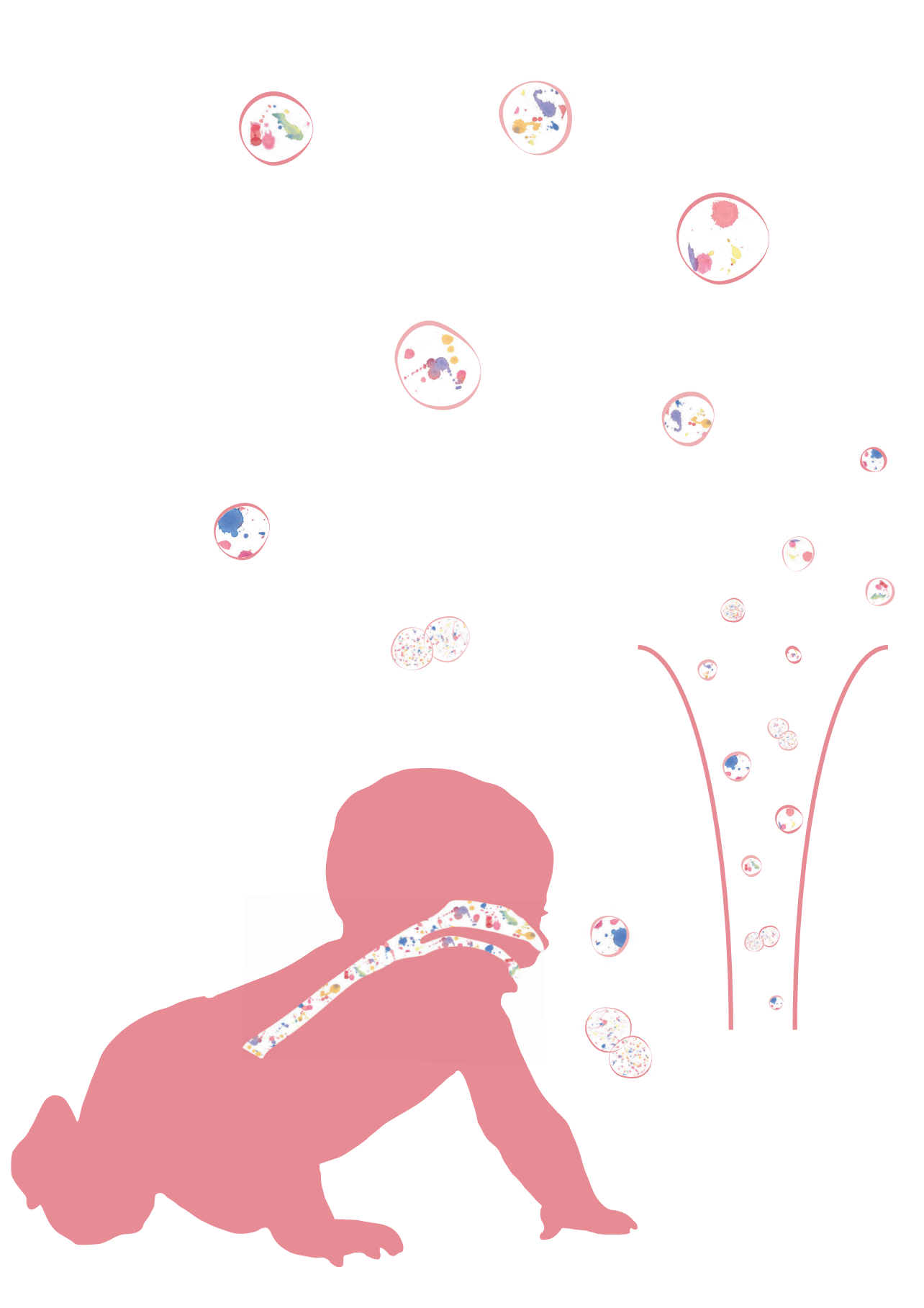


Figure S1. Design and operating conditions of the electrostatic precipitator. dP: particle size; Q: airflow rate; v: particle velocity; V_{te}: Terminal electrostatic drift velocity of a charged particle; x: distance;

Table S1. Theoretical collection efficiency of the electrostatic precipitator for submicron particles.

Particle size (µm)	0.2	0.25	0.3	0.5
Calculated collection efficiency (%)	65	64	62	43



SMALL QUANTITIES OF RESPIRATORY SYNCYTIAL VIRUS RNA ONLY IN LARGE DROPLETS AROUND INFANTS HOSPITALIZED WITH ACUTE RESPIRATORY INFECTIONS

Jasmin S Kutter, Dennis de Meulder, Theo M Bestebroer, Jeroen J van Kampen, Richard Molenkamp, Ron AM Fouchier, Jérôme O Wishaupt, Pieter LA Fraaij, Sander Herfst

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ABSTRACT

Background: Respiratory syncytial virus (RSV) is a major cause of respiratory tract infections in young children. The predominant transmission routes for RSV are still a matter of debate. Specifically, it remains unclear if RSV can be transmitted through the air and what the correlation is between the amount of RSV in nasopharynx samples and in the air.

Methods: The amount of RSV in the air around hospitalized RSV infected infants in single-patient rooms was quantified using a six-stage Andersen cascade impactor that collects and fractionates aerosols and droplets according to size. RSV shedding in the nasopharynx of patients was followed longitudinally by quantifying RSV RNA levels and infectious virus in nasopharyngeal aspirates. Nose and throat swabs of parents and swabs of the patient's bedrail and a datalogger were also collected.

Results: Patients remained RSV positive during the air sampling period and infectious virus was isolated up to 9 days post onset of symptoms. In three out of six patients, low levels of RSV RNA, but no infectious virus, were recovered from impactor collection plates that capture large droplets >7 µm. For four of these patients, one or both parents were also positive for RSV. All surface swabs were RSV-negative.

Conclusions: Despite the prolonged detection of infectious RSV in the nasopharynx of patients, only small amounts of RSV RNA were collected from the air around three out of six patients, which were primarily contained in large droplets which do not remain suspended in the air for long periods of time.

KEYWORDS

air sampling | droplet transmission | respiratory syncytial virus | transmission routes | viable six-stage Andersen cascade impactor

BACKGROUND

Respiratory syncytial virus (RSV) is a major cause of lower respiratory tract infections (LRTIs) in young children, that may be severe[8,206]. Two antigenically different subtypes of RSV, A and B, often co-circulate, but usually one subtype predominates[207–211]. By the age of two, nearly all children have been infected with RSV at least once[212,213]. For 2015 it was estimated that approximately 33 million children under the age of 5 suffered from an LRTI caused by RSV worldwide, of which 3.2 million required hospitalization, resulting in almost 30.000 in-hospital deaths[8].

Respiratory viruses can be transmitted via different transmission routes: via direct contact, e.g., through handshaking with an infected person, via indirect contact by touching contaminated surfaces, or via the air through droplets and/or aerosols that are expelled by an infected person[214]. Droplets quickly settle on the ground or objects in near vicinity of the source, while aerosols are small enough to remain suspended in the air for prolonged periods of time and can infect susceptible individuals further away from the source. For this reason, depending on the transmission properties of the pathogen, droplet or aerosol precautions are implemented[214].

To date, it has been widely accepted that short distances or close contact between individuals are needed for efficient RSV transmission, and as a result, contact and droplet precautions are implemented in infection prevention guidelines globally[214]. However, the scientific data to support these guidelines is scarce, and often contrasting[214]. In the 1980s it was demonstrated that healthy individuals only became infected upon self-inoculation after touching contaminated surfaces, or through close contact with infected infants, but not by solely sitting in the same room at a distance of >1.8 m away from the patient's bed[112]. These observations were later supported by several air sampling studies, in which RSV was detected infrequently, or not at all, in the air around infected patients[58,215–217]. In contrast to these studies, Aintablian and colleagues were able to collect RSV RNA from the air around RSV infected patients between 0.3 and 7 m away from the patient's head, with a higher likelihood of RSV detection close to the patient[114]. Other researchers recently collected large quantities of infectious RSV in the air around RSV-infected children in a pediatric ward, up to 5 m from the head of an index case[115].

Because of these conflicting experimental data, the likelihood of RSV being transmitted through the air is still unknown. Here, the amount of RSV in the air around infants (<2 yrs.) hospitalized with RSV infections was quantified longitudinally and correlated to the RSV load in upper respiratory tract samples of these patients.

With a six-stage Andersen cascade impactor that collects droplets and aerosols according to size, RSV RNA was collected from the air around three out of six infants and was found to be predominantly present in droplets >7 µm. We did not detect infectious virus in any air fractions and did not detect RSV RNA in finer aerosols, whereas such finer aerosol fractions did contain rhinovirus RNA.

RESULTS

Clinical setting and patient demographics

Six infants with ages ranging from 10 days to 7 months (median age 2 months) were included over the course of three consecutive winter seasons (Nov 2017 – Mar 2020), of whom two were female and four were male (**Table 1**). No comorbidities were reported, however, patient 1 was born at a gestational age of 36 weeks. All patients had RSV-B confirmed infections with the majority of infections manifesting as bronchiolitis and/or dyspnea. Two patients were diagnosed with a rhinovirus (RV) co-infection on the day of hospital admission. All infants received non-invasive positive airway pressure ventilation with supplemental oxygen. Standard treatment was only supportive aimed at relieving symptoms (**Table 1**). All infants were hospitalized in single patient rooms together with one in-rooming parent. Ambient room temperature and relative humidity were monitored in each room and were on average 21.7˚C and 45.5%, respectively (**Table 2**).

RSV load in patients and parents

To study the virus shedding kinetics in patients, both the RSV RNA levels, as well as the amount of infectious virus were determined in the nasopharyngeal aspirates that were collected over time. For patient 2, only one nasopharyngeal aspirate was obtained, because the parents did not consent to obtain an aspirate solely for the purpose of this study. For this reason, it is unknown if this patient remained RSV positive during the sampling period. However, the other five patients were qRT-PCR positive during the entire sampling period (**Fig. 1**). In four patients, infectious RSV was isolated from the nasopharyngeal aspirates on more than one day. For RV co-infected patients, aspirates were RV-positive by qRT-PCR on all days except on day 5 (Patient 4) and day 2 and 3 (Patient 5) after hospital admission (**Fig. 1**).

Since parents stayed with their children during hospitalization, it was possible that they became infected as well. For this reason, nose or throat swabs were collected from the parents. For four patients, at least one parent tested positive for RSV-B by qRT-PCR (**Fig. 1**). For patient 1, both parents were found to be RSV-B-positive by qRT-PCR. All parents of RV co-infected patients tested negative for RV. High levels of RSV RNA, similar to those in the patients, were detected in swabs collected from the mother of patient 1 and the father of patient 5, but no infectious virus was detected (**Table 2**).

Table 1. Patient demographics and medical information

Patient	Sex	Age	Weight (kg)	Co-infection	Symptom	Started with vaccination program*
1	M	2 months¥	4.7	-	Dyspnea	No
2	F	1 month	5.0	-	Bronchiolitis	No
3	M	10 days	3.8	-	Sinusitis	No
4	M	2 months	6.4	RV	Dyspnea	No
5	M	7 months	11.1	RV	Bronchiolitis & Tachypnea	Yes
6	F	3 months	6.0	-	Bronchiolitis & Dyspnea	Yes

Standard medical treatment was only supportive and aimed at relieving symptoms. (¥) born premature,

Suppl. oxygen	Medication[$]	Antibiotics	Other[§]
Yes	Ipratropium (0.25 mg) Salbutamol (2.5 mg) Xylometazoline (0.25 mg ml^{-1}) Paracetamol (60 mg)	No	No
Yes	Xylometazoline (0.25 mg ml^{-1}) Paracetamol (60 mg)	No	Vitamin K oil droplets
Yes	Xylometazoline (0.25 mg ml^{-1}) Paracetamol (60 mg)	Amoxicillin (500 mg ml^{-1}) Cefotaxime (500 mg ml^{-1})	No
Yes	Xylometazoline (0.25 mg ml^{-1}) Paracetamol (120 mg)	No	Vitamin K oil droplets Cholecalciferol (10 µg)
Yes	Xylometazoline (0.25 mg ml^{-1}) Paracetamol (240 mg) Hypertonic saline inhalation (2.5 mg ml^{-1})	No	Cholecalciferol (10 µg)
Yes	Ibuprofen (20 mg ml^{-1}) Paracetamol (120 mg) Hypertonic saline inhalation (0.9 mg ml^{-1})	No	Cholecalciferol (10 µg)

(*) according to Dutch vaccination program, ($) was given if needed, (§) was given once a day.

RSV in air and on surfaces

Air sampling in patient rooms was started 4 to 7 days (median 6 days, **Table 2**) after the onset of symptoms. In three out of six patient rooms, RSV RNA was collected from the air on at least one day (**Fig. 1**). For patients 1 and 3, RSV RNA was detected on days 3 and 2 after hospital admission, respectively. In the room of patient 2, RSV RNA was detected in air samples intermittently on days 2 and 5 after admission. All RSV RNA positive air samples originated from stage 1 with the largest droplet particle size (>7 µm). The quantities of virus RNA retrieved from the RSV positive samples were low (Ct values ranging between 33.5 and 35.8) and no infectious virus was detected (**Table 2**).

For one of the two patients co-infected with RV, air samples were positive for RV RNA (patient 4). Stage 1 (particles >7 µm) was positive for RV RNA on days 2 and stage 3 (particles between 4.1 and 3.3 µm) was positive on day 2, 4, and 5 after admission. RV RNA levels were also low, with Ct values ranging between 35.3 and 37.8 (**Table 2, Fig. 1**).

To investigate if RSV and RV were also present on surfaces, the bedrail on the side of the cascade impactor and the datalogger which was placed approximately 1.5 - 2 meters from the patient's head were swabbed on the day of discharge (**Table 3**). A bedrail swab of patient 6 is missing because the bedrail had already been disinfected and cleaned before a sample could be obtained. No RSV or RV RNA was detected in any of the surface swabs by qRT-PCR.

Table 2. Overview of environmental conditions and Ct values in air samples and samples from patients and parents.

Patient	Days between symptom onset and 1st day of sampling	Ambient room temperature (˚C)		Room relative humidity (%)		Ct value of RSV air sample (day)	Ct value of RV air sample (day)	Ct value of father for RSV (day)	Ct value of mother for RSV (day)
		Average	SD	Average	SD				
1	Unknown	21.8	0.1	47.9	3.9	34.9 (2)	-	28.2 (4) 31.4 (5)	21.4 (1) 33.6 (5)
2	4	22.0	0.2	48.8	3.9	33.5 (2) 35.8 (5)	-	Neg (1) Neg (4)	Neg (1) 35.5 (4)
3	6	21.7	0.1	42.2	2.1	34.9 (2)	-	Neg (1)	Neg (1) Neg (5)
4	6	21.8	0.4	43.5	1.9	Neg	35.7 (2) 35.3 (2) 37.8 (4) 35.6 (5)	31.8 (1) 33.3 (5)	Neg (1) Neg (5)
5	5	21.5	1.0	45.6	3.4	Neg	Neg	22.4 (1) 20.4 (1)	Neg (1) Neg (3)
6	7	21.7	0.1	44.6	3.9	Neg	-	Neg (3)	Neg (2)

DISCUSSION

Despite the substantial impact of RSV globally, it is still unclear via which routes RSV is primarily transmitted and if and how long infectious virus is shed by infected individuals. Here, we determined the amount of RSV in the air around hospitalized infants, in correlation with the viral load in their upper respiratory tract over time. We demonstrated that despite the presence of infectious RSV in nasopharyngeal samples of infants, only low amounts of RSV RNA, but no infectious virus, were detected in the air around three out of six patients. RSV RNA was only detected in large (>7 μm) droplets.

For two of these patients, one or both parents also tested positive for RSV, so they may have contributed to the RSV RNA quantities collected from the air. For the third patient in whose room RSV was collected from the air, both parents were RSV negative, and therefore the RSV RNA must have been expelled by the patient.

For one patient who was co-infected with RV, low amounts of RV RNA were collected from the air on multiple days. Except for one positive sample that was recovered from stage 1 (droplets >7 μm), the remaining three RV positive air samples were consistently recovered from stage 3 of the cascade impactor. This stage collects aerosols in the size range of 3.3 μm to 4.7 μm, indicating that at the time of air sampling RV RNA was contained in smaller particles than RSV RNA. Remarkably, on a day that the airway samples collected from the patient and both parents were negative for RV RNA, an air sample turned out to be positive. It is unclear if virus was still shed by the patient or the parents but from an anatomical site of the respiratory tract that was not sampled, or that the air was contaminated by hospital personnel that was present that day.

Air sampling was only started a few days to one week after symptom onset during the late phase of infection, which may explain the low quantities of RSV and RV RNA collected from the air (**Table 2**). In several other studies, in which various air samplers were used, also low numbers of RSV RNA positive air samples were reported, with a detection rate ranging from 2.3 to 31.8 %[58,114,215,216]. Moreover, in a recent study by Chamseddine et al., none of the collected air samples around RSV-infected patients were positive for RSV RNA, while half of the air samples collected around influenza A virus-infected patients were positive for influenza virus RNA[217]. However, attempts to isolate infectious influenza virus from these samples were not successful[217].

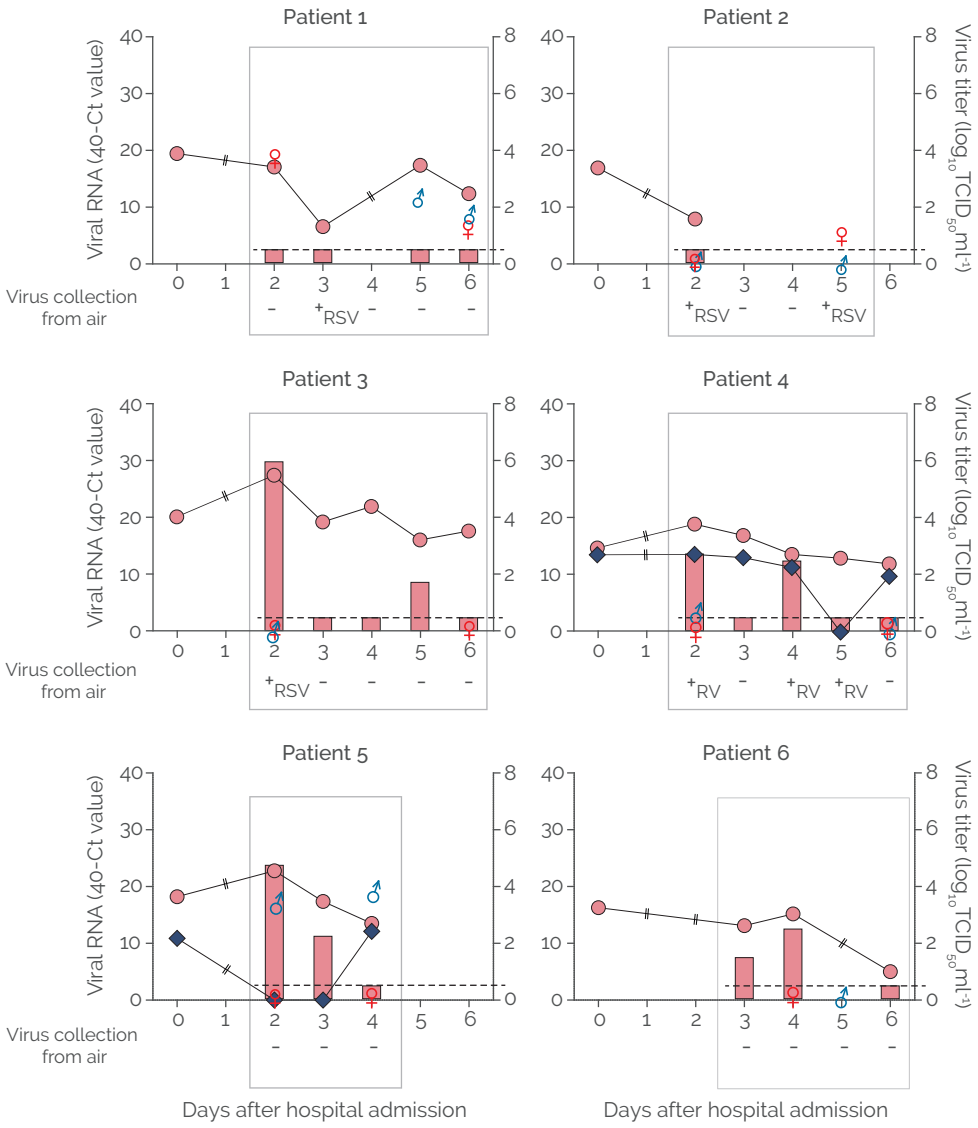

Figure 1. Graphs representing the viral load in 6 infants, their parents, and air samples. Pink circles represent RSV-B RNA, dark blue diamonds RV RNA (left Y-axis) and pink bars RSV-B titers (right Y-axis) of nasopharyngeal aspirates of patients. Circles and diamonds are replaced by a double line at days on which no sample from the patient was obtained. Gender symbols represent total RSV-B RNA of the mother (red venus) and father (blue mars). Dotted lines indicate the detection limit of virus titrations. Grey rectangles mark the period during which air sampling was performed. Plus and minus signs indicate if air samples were positive or negative on the day of air sampling.

Contrasting results were previously reported by Kulkarni et al., where air sampling around infants with RSV-confirmed bronchiolitis in general wards of a pediatric hospital resulted in the collection of high amounts of infectious RSV from the air[115]. Although viral quantities in the air decreased with increasing distance to the patient's head, up to $10^{5.6}$ plaque forming units (PFU) of infectious RSV were still collected 5 meters away from the patient's head. As in the present study, Kulkarni and colleagues used a six-stage Andersen cascade impactor, however, liquid medium was used as a collection medium. We have recently shown in an *in vitro* set-up that the collection of infectious virus using liquid medium is less efficient than when semi-solid gelatin is used (as in the current study), so this does not explain the differences in collected amounts of RSV between the studies[218]. It should be noted that the Andersen cascade impactor was designed and validated with solid impaction media rather than liquids[200].

The small amounts of RSV RNA detected in large droplets and the total absence of infectious RSV in the air around infected infants as presented here, and the low detection rates of RSV RNA in the air in most other studies using various air samplers, indicate that transmission via the air is unlikely to be a route by which RSV spreads in the population. This observation is also supported by the fact that room-sharing of RSV infected and non-infected patients did not seem to influence the risk of nosocomial infections[149]. In addition, wearing gowns and gloves, and adhering to strict hygiene has been shown to reduce the risk of nosocomial RSV transmission considerably, further indicating that aerosol transmission is not efficient and possibly negligible in this context[150,151].

To investigate the possibility of RSV transmission through fomites, we also took surface swabs of the bedrails and dataloggers on the last day of air sampling. In none of the surface swabs, RSV RNA was detected by qRT-PCR, which is in contrast to the study of Wan et al., where RSV RNA was detected on various objects[215]. A reason for the conflicting results might be the timepoint when the surface swabs were taken. Wan and colleagues took surface swabs shortly after the admission of patients. In our study, surface swabs were only taken on the last day of air sampling, during the late stage of RSV infection when RSV RNA levels in the patients had already decreased.

Table 3. Detection rate of RSV and RV in all collected samples.

Sample	# of samples obtained	# of samples positive	
		Total viral RNA	Infectious virus
Nasopharyngeal aspirates	21	21	8
Nose swabs parents	21	8	-
Mother	11	3	-
Father	10	5	-
Air samples	34	7	-
RSV	26	4	-
RV	8	3	-
Surface swabs	11	-	-
Bedrail	5	-	-
Datalogger	6	-	-

CONCLUSION

In conclusion, we here demonstrate that despite the shedding of infectious RSV from the nasopharynx of hospitalized infants, no or only low amounts of RSV RNA were detected, but no infectious RSV was detected in any of the collected air samples. RSV RNA was only detected in large droplet samples on only a limited number of days. These results suggest that in the current hospital setting, RSV transmission through the air at later stages of infection was negligible and that the implementation of contact and droplet precautions, as currently employed in most hospitals, is sufficient.

MATERIALS AND METHODS

Patients

The study was conducted during three consecutive winter seasons (November 2017 - April 2020) at the department of pediatrics at the Reinier de Graaf Hospital, Delft, The Netherlands. Hospitalized children aged between 0 and 2 years with laboratory-confirmed RSV-A or RSV-B infections were both eligible. However, during these three seasons, only RSV-B positive patients were detected in the study. Patients were excluded if they were older than 2 years, or if signed informed consent was not obtained. For practical reasons, immunocompromised patients and patients that were experiencing symptoms for more than a week prior to hospitalization were not included. Patients with other co-morbidities, viral co-infections, or use of medication, including antivirals, were not excluded, but this information was documented together with other clinical data obtained from the patient's medical record using a standardized case record form, specifically developed for this study. Patients were hospitalized in single patient rooms with an additional bed for parents. All patient rooms had a ventilation rate of 2 air changes per hour (ACH) with a fresh air transportation rate of 100 m^3h^{-1} Temperature and relative humidity were recorded every five minutes during the whole air sampling period using EL-GFX-2 dataloggers (Lascar Electronics Inc.).

Sample collection

For the collection of RSV from the air, the six-stage Andersen cascade impactor (Thermo Scientific™) was used[200]. The cascade impactor operates at a flow rate of 28.3 liters per minute (LPM) and collects droplets and aerosols according to size in six different stages: Stage 1 (>7.0 µm), stage 2 (7.0 - 4.7 µm), stage 3 (4.7 - 3.3 µm), stage 4 (3.3 - 2.1 µm), stage 5 (2.1 - 1.1 µm) and stage 6 (1.1 - 0.65 µm). The air inlet of the cascade impactor was positioned approximately 1 m away from the patient's head at a height of 109 cm. Air was sampled daily for 30 minutes, starting the day after the informed consent was obtained, until discharge or a maximum of five days. To prevent contamination of samples, air sampling was performed in the morning, prior to routine care that may involve aerosol-generating procedures like nasopharyngeal aspiration.

Droplets and aerosols were impacted onto collection plates filled with an in-house developed semi-solid gelatin layer, as previously described[218]. The gelatin layer was prepared from commercial gelatin sheets (10 mg ml^{-1}; Dr. Oetker)

dissolved in virus transport medium (VTM). VTM consisted of Minimum Essential Medium (MEM) – Eagle with Hank's BSS and 25 mM Hepes (Lonza), glycerol 99% (Sigma Aldrich), lactalbumin hydrolysate (Sigma Aldrich), 10 MU polymyxin B sulfate (Sigma Aldrich), 5 MU nystatin (Sigma Aldrich), 50 mg ml^{-1} gentamicin (Gibco) and 100 IU ml^{-1} penicillin 100 µg ml^{-1} streptomycin mixture (Lonza). To avoid high dilution factors, each collection plate was first filled with 32 ml of 2% agarose (Roche) as a bottom layer on which 9 ml of the semi-solid gelatin was pipetted. Subsequently, plates were stored at +4˚C for a maximum of 4 days.

On the last day of air sampling, surface swabs were taken using Copan flocked swabs (Copan Diagnostics Inc.) from the datalogger and the bed rail on the side where the cascade impactor was located. From the first day of air sampling onwards, nasopharyngeal aspirates (for decongestion) were obtained from patients during routine clinical care if available, and otherwise aspirates or nose swabs were taken specifically for this study if consent was given by the parents. From each parent, nose or throat swabs (either Copan eSwab® or Copan flocked swabs (Copan Diagnostics Inc.)) were taken on the first and last day of air sampling when possible, to estimate their contribution to viral shedding in the air.

Sample processing

The collection plates with gelatin from the cascade impactor were processed after sampling by adding 6 ml of prewarmed VTM, followed by a 30 min incubation at 37˚C to dissolve the gelatin layer and harvesting of the samples[218] Nose and throat swabs of parents were collected in Amies (eSwab®; Copan Diagnostics Inc.) or universal transport medium (UTM; flocked swabs; Copan Diagnostics Inc.). VTM was added to the nasopharyngeal aspirates of patients and nose and throat samples of parents to reach a total volume of 6 ml, after which nasopharyngeal aspirates were centrifuged at 500 G for 5 min to remove cell debris. All samples were subsequently aliquoted and stored at +4˚C for a maximum of 4 days until further analysis. An additional vial of each sample was stored at -80˚C. The surface swabs were collected in UTM (flocked swabs; Copan Diagnostics Inc.), and stored in 25% sucrose at -80˚C. At the end of the study, the samples were thawed and RNA was extracted followed by qRT-PCR analysis.

Cells

Hep-2 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Lonza or Gibco) 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 0.25 mg ml^{-1} fungizone (Invitrogen). The cells were maintained at 37˚C and 5% CO_2.

RNA extraction and qRT-PCR

RNA was extracted from the samples using the MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche) and subjected to qRT-PCR analysis for the detection of RSV-B RNA, and in the case of the rhinovirus (RV) co-infected patients also for RV RNA[199]. For this purpose, 20 µl of extracted 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[199]. 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. For all samples, a cycle threshold (Ct) value of >40 was considered negative.

Virus titration

RSV-B-positive samples were titrated on Hep-2 cells for the quantification of infectious virus, as defined by the culturability of RSV. Briefly, Hep-2 cells were grown to confluency in 96 well plates overnight. Subsequently, cells were spin-inoculated (15 min, 2000 rpm) with 100 µl of 10-fold serial dilutions of RSV-B-positive samples and incubated at 37˚C, 5% CO_2. One hour after inoculation, cells were washed once and cultured in serum-reduced (2%) Dulbecco's Modified Eagle Medium (DMEM, Lonza) supplemented with 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 0.25 mg ml^{-1} fungizone (Invitrogen). After 7 days of incubation, positive samples were identified by immunofluorescence assays using a FITC labeled polyclonal antibody directed against RSV (Fisher Scientific). Infectious virus titers were calculated from four replicates as tissue culture infective dose ($TCID_{50}$) by the Spearman-Karber method.

DECLARATIONS

Ethics approval and consent to participate

This study was approved by the regional medical ethics committee "Medisch Ethische Toetsings Commissie (METC)" at the Erasmus Medical Center, Rotterdam, The Netherlands (MEC-2017-013/NL59998.078.16).

Consent for publication

Not applicable.

Availability of data and materials

All data and materials are available from the corresponding author (S.H.) on reasonable request.

Competing interests

The authors declare that they have no competing interests

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Authors' contribution

JSK, JOW, RAMF, PLAF and SH conceptualized and designed the study. JSK and JOW planned and completed data collection. JSK, DM, TMB JJAK and RM conducted the sample processing and laboratory analysis. JSK, RAMF, PLAF and SH interpretated the data, and drafted the manuscript, which was critically reviewed and revised by all other authors. All authors read and approved the final manuscript.

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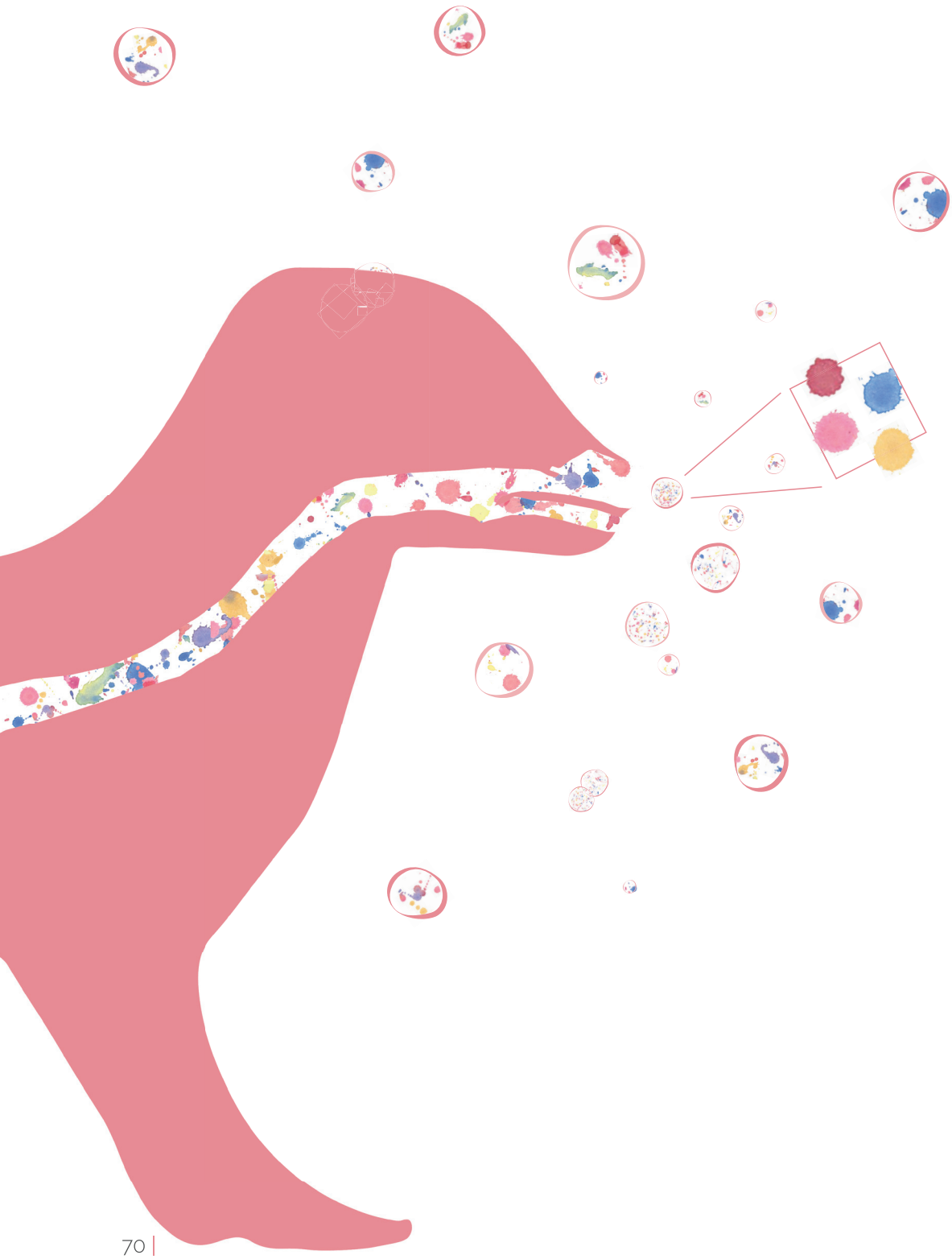
None.



4

CONTINUED ADAPTATION OF A/H2N2 VIRUSES DURING PANDEMIC CIRCULATION IN HUMANS

- A/H2N2 adaptation in humans -

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ABSTRACT

Influenza A viruses of the H2N2 subtype sparked a pandemic in 1957 and circulated in humans until 1968. Because A/H2N2 viruses are still circulating in wild birds worldwide and human population immunity is low, they can possibly be reintroduced into humans. To assess the risk of avian A/H2N2 viruses, the transmissibility of six avian A/H2N2 viruses isolated between 1999 and 2007 was investigated in the ferret model. None of the avian A/H2N2 viruses was transmitted between ferrets via direct contact or via the air, suggesting that their pandemic risk may be low. The transmissibility, receptor binding preference, and HA stability of human A/H2N2 viruses was also investigated. Human A/H2N2 viruses from 1957 and 1958 were found to preferentially bind to human-type α2,6-linked sialic acid receptors. However, the virus from 1958 had a more stable HA as compared to the virus from 1957, suggestive for adaptation to replication and spread in the new host. This increased stability was caused by a previously unknown stability substitution G205S in the HA of the A/H2N2 virus from 1958, which became fixated in A/H2N2 viruses after 1958. Although individual substitutions were identified that affected the HA receptor binding and stability properties, they were not found to have a substantial effect on transmissibility of A/H2N2 viruses via the air in the ferret model. Our data demonstrate that A/H2N2 viruses continued to adapt during the first year of pandemic circulation in humans, similar to what was previously shown for the A/H1N1pdm09 virus.

IMPORTANCE

Although A/H2N2 influenza viruses disappeared from the human population in 1968, they are still circulating in wild birds and could potentially re-emerge in humans. With waning population immunity against A/H2N2 viruses, continuous surveillance as a component of pandemic risk assessment is warranted. In previous studies, changes in the hemagglutinin receptor binding site were identified to be crucial for virus transmission between mammals. Here, we identified a previously unknown substitution that stabilized the hemagglutinin of a human A/H2N2 virus from 1958 that was not yet present in a human A/H2N2 virus from 1957, indicating that human A/H2N2 viruses continued to adapt in humans during their first year of circulation. This adaptation, however, did not substantially affect the transmission efficiency of human A/H2N2 viruses between ferrets via the air. A/H2N2 viruses isolated from different avian species do not have any of these mammalian adaptation markers and we here showed that these viruses were not transmissible between ferrets via direct contact or via the air.

KEYWORDS

A/H2N2 virus | airborne transmissibility | HA | receptor specificity | stability

INTRODUCTION

In 1957, a newly emerging influenza A virus of the H2N2 subtype caused significant morbidity and mortality in humans, an event known as the 'Asian influenza' pandemic[219–221]. The pandemic A/H2N2 virus emerged upon reassortment of a previously circulating seasonal human A/H1N1 virus that acquired the H2 hemagglutinin (HA), the N2 neuraminidase (NA), and basic polymerase 1 (PB1) genes of avian origin[222–224]. The A/H2N2 virus was replaced in 1968 by the A/H3N2 'Hong Kong influenza' virus after 11 years of circulation in humans. Nowadays, over 50 years after the last detected A/H2N2 virus infection in humans, immunity against A/H2N2 viruses is low and waning in the human population[225]. Meanwhile, A/H2N2 influenza viruses and viruses containing other viral gene combinations of H2 and N2 segments have been isolated frequently from waterfowl and pigs around the globe[226–231], possibly allowing reintroduction of A/H2N2 viruses into the human population, with or without the need of prior reassortment with other virus subtypes[232].

It was previously shown that a switch in binding preference from avian- to human-type receptors, that was associated with amino acid substitutions in the HA receptor binding site at positions 226 and 228, contributed to the emergence of the 1957 H2N2 and 1968 H3N2 pandemics[233,234]. In general, avian A/H2 and A/H3 influenza viruses with 226Q and 228G in HA preferentially bind to α2,3-linked sialic acids (SIA, avian-type receptor), whereas human A/H2 and A/H3 viruses with 226L and 228S in HA preferentially bind α2,6-linked SIAs (human-type receptor). However, it has been described that A/H2N2 viruses isolated during the first year of the pandemic did not yet have a human-type receptor binding preference[233–235]. Unfortunately, those viruses had been passaged multiple times in eggs, so it could not be excluded that as a result, these viruses had adapted to binding to avian-type receptors[233–237].

Interestingly, the Q226L and G228S substitutions were also found to be required for the transmission of avian A/H5N1 viruses via the air between ferrets[12,238]. However, in these studies, a stabilized HA was also a prerequisite for efficient transmission between ferrets. Recently, it was also shown that the HA stability of A/H1N1pdm09 viruses increased during the evolution from precursors in swine, to the early pandemic A/H1N1pdm09 human cases and the later human virus isolates[239]. A possible role of HA stability in the emergence of A/H2N2 and A/H3N2 viruses in humans has not been described to date.

To assess the risk of A/H2N2 viruses that currently circulate in birds to cause a pandemic in humans, the transmissibility of six avian A/H2N2 viruses, isolated

between 1999 and 2007, was investigated in the ferret model. In addition, we studied the transmissibility via the air in the ferret model of three human A/H2N2 viruses, isolated in 1957, 1958, and 1968, and compared their receptor binding preference and HA acid and temperature stability. We identified a novel amino acid substitution G205S in the HA that emerged during the first year of circulation in humans and that not only stabilized the A/H2N2 HA but also seemed to compensate for the small increase in α2,3-linked SIA binding induced by G228S. Altogether, our data demonstrate that A/H2N2 viruses continued to adapt to the new host during circulation in humans in the first pandemic year, as previously also described for A/H1N1pdm09 virus[239].

RESULTS

Avian A/H2N2 viruses were not transmitted between ferrets through direct contact or via the air.

To assess the threat of avian A/H2N2 influenza viruses to mammals, we investigated the transmissibility of the A/H2N2 viruses A/chicken/Jena/4705/1984, A/chicken/New York/Sg-00425/2004, A/mallard/Netherlands/14/2007, A/mallard/Netherlands/31/2006, A/mallard/Sweden/68735/2007 and A/white-fronted goose/Netherlands/22/1999 in a ferret transmission model. For each virus, two individually housed donor ferrets were inoculated intranasally and after 6 h direct contact animals were added to each of the cages. One day after inoculation, indirect recipient ferrets were added to adjacent cages that were separated from the donor cages by two steel grids, 10 cm apart, allowing viruses to be transmitted only via the air (**Fig. 1a**). All donor ferrets were productively infected, as shown by the successful virus isolation from the throat and nose swabs from all animals (**Fig. 2**). Overall, virus titers were higher in throat swabs than in nose swabs. Peak virus titers reached up to 5.0 $\log_{10}TCID_{50}ml^{-1}$ in the throat and 3.0 $\log_{10}TCID_{50}ml^{-1}$ in the nose, with no substantial differences between the viruses. No infectious virus was recovered from nose or throat swabs obtained from direct contact and indirect recipient ferrets. These animals did also not seroconvert, confirming the lack of transmission of these viruses between ferrets (data not shown).

A/H2N2 virus from 1958, but not from 1957 and 1968, was transmitted via the air between ferrets.

To compare the transmissibility of early and late human A/H2N2 virus isolates via the air, transmission experiments were performed in the ferret model with three human A/H2N2 viruses isolated in 1957 (the start of the pandemic), 1958, and 1968 (the last year that A/H2N2 viruses were detected in humans). One day after intranasal inoculation of donor animals with either A/Netherlands/M1/1957 (M1/57), A/Netherlands/M1/1958 (M1/58), or A/Netherlands/B1/1968 (B1/68), indirect recipient ferrets were placed in a cage adjacent to the donor ferret. Again, both cages were 10 cm apart and separated by steel grids (**Fig. 1b**). All three viruses replicated well in the upper respiratory tract (URT) of donor ferrets, as shown by the successful virus isolation from throat and nose swabs of all ferrets on multiple days (**Fig. 3**). B1/68 displayed a delay in replication with low or undetectable virus titers at 1 day post inoculation (dpi), and peak titers only at 3 or 5 dpi, as compared to M1/57 and M1/58 which replication immediately peaked at 1 dpi. Peak virus titers

reached up to 5.75 $log_{10}TCID_{50}ml^{-1}$ in the throat and 5.0 $log_{10}TCID_{50}ml^{-1}$ in the nose, with no substantial differences between the viruses. Of the three viruses tested, only M1/58 was transmitted via the air to indirect recipient ferrets, as demonstrated by virus isolation from throat and nose swabs from all four indirect recipient animals, between 3 and 9 days post exposure (dpe) (**Fig. 3b**). The lack of transmission of M1/57 and B1/68 was confirmed by the absence of seroconversion in all indirect recipient ferrets as determined by hemagglutination inhibition (HI) assay (data not shown).

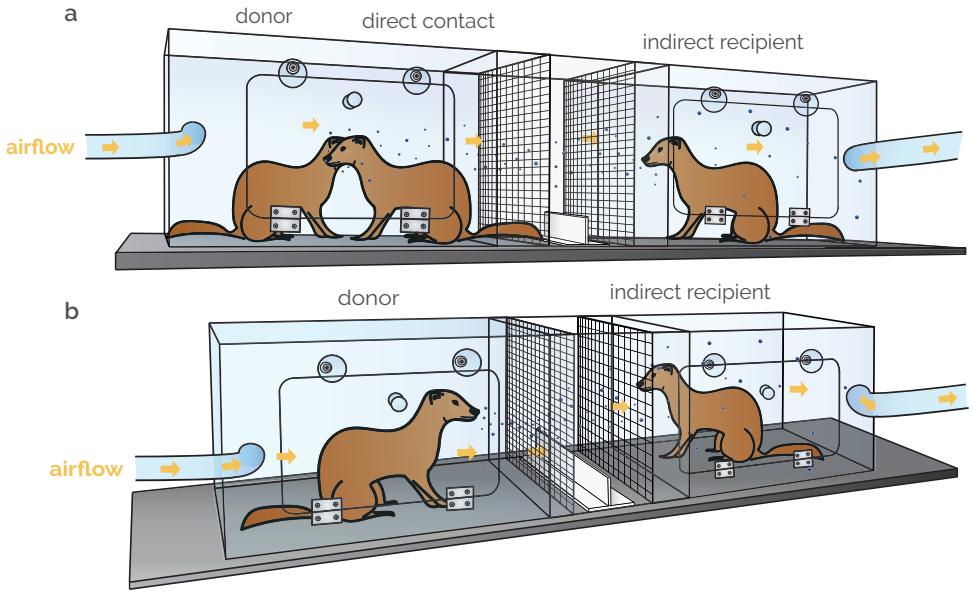


Figure 1. Experimental transmission set-ups. Schematic representation of the set-ups used to assess the transmissibility of A/H2N2 influenza viruses. (**a**) Transmission of avian A/H2N2 viruses via direct contact and through the air. Six hours after intranasal inoculation of a donor ferret, a direct contact animal is placed in the same cage as the donor ferret. The next day, an indirect recipient ferret is placed in an opposite cage. To avoid contact transmission with the indirect recipient ferret, the cages are separated by two steel grids, 10 cm apart. In addition, a 5 cm high plate is placed between the cages to avoid spill-over of food, feces, and other large particles. (**b**) Transmission of human A/H2N2 viruses through the air. A similar procedure was followed as under (**a**), but the direct contact animal was not included. Yellow arrows indicate the direction of airflow (100 L min^{-1}). Set-ups were placed in class III isolators in a biosafety level 3+ laboratory.

The double substitution Q226L/G228S did not facilitate a complete switch to human-type receptor specificity of A/H2 viruses.

Sequence analysis of the HA consensus of the human A/H2N2 virus stocks showed three amino acid differences in M1/58 HA as compared to M1/57 HA: G205S (trimer interface), G228S (receptor binding site), and I289L (on the surface of the HA head, but outside of the receptor binding site, **Table 1**). The absence of 228S in M1/57 HA suggests that this HA was not fully adapted to binding to human-type receptors yet. The phenotypic effect of the G205S and I289L substitutions detected in the HA of M1/58 has not been described in the literature and thus their potential effect on functional properties is unknown. In the HAs from all A/H2 viruses that were sequenced after 1958, so including the B1/68 HA, the I289L substitution had reverted to 289I, whereas 205S and 228S were present in most of these viruses (**Table 1**). Furthermore, the B1/68 HA carried 25 additional substitutions as compared to the M1/58 HA.

To investigate if G228S, G205S, and I289L affect the receptor binding preference of the M1/57 and M1/58 HAs and hence may explain the difference in transmissibility between M1/57 and M1/58, the three single amino acid substitutions or a combination thereof were introduced into the HA of M1/57. As 228S and 205S were already present in the wild-type B1/68 HA, only the I289L substitution was introduced in this HA to investigate if this substitution affected receptor binding and would explain the lack of transmission of this virus isolate.

A/Puerto Rico/8/1934 (PR8) viruses harboring a wild-type or mutant H2 HA were generated and glycan microarray analyses were performed with 12 different linear and bi-antennary N-glycans[240]. The linear glycans consisted of 3 galactose-N-acetyllactosamine (LacNAc) repeats, whereas biantennary glycans contained 1 to 3 LacNAc repeats. All glycans were either nonsialylated (#1-4), serving as controls, or capped with α2,3- (#5-8) or α2,6-linked (#9-12) SIAs (**Fig. 4a**). A/Netherlands/213/2003 (human H3) and A/Indonesia/5/2005 (avian H5) were used as controls as these viruses selectively bind to α2,6- and α2,3-linked SIAs, respectively. All three wild-type A/H2 viruses bound to α2,6 receptors. However, whereas M1/57 and M1/58 only showed low levels of binding to α2,3 receptors, B1/68 showed dual receptor binding preference with substantial binding to α2,3 receptors (**Fig. 4b**). Introduction of G228S in M1/57 HA resulted in an increase in α2,3-linked SIA binding. Interestingly, although introduction of G205S alone in M1/57 HA did not change the receptor binding phenotype, the addition of G205S to G228S seemed

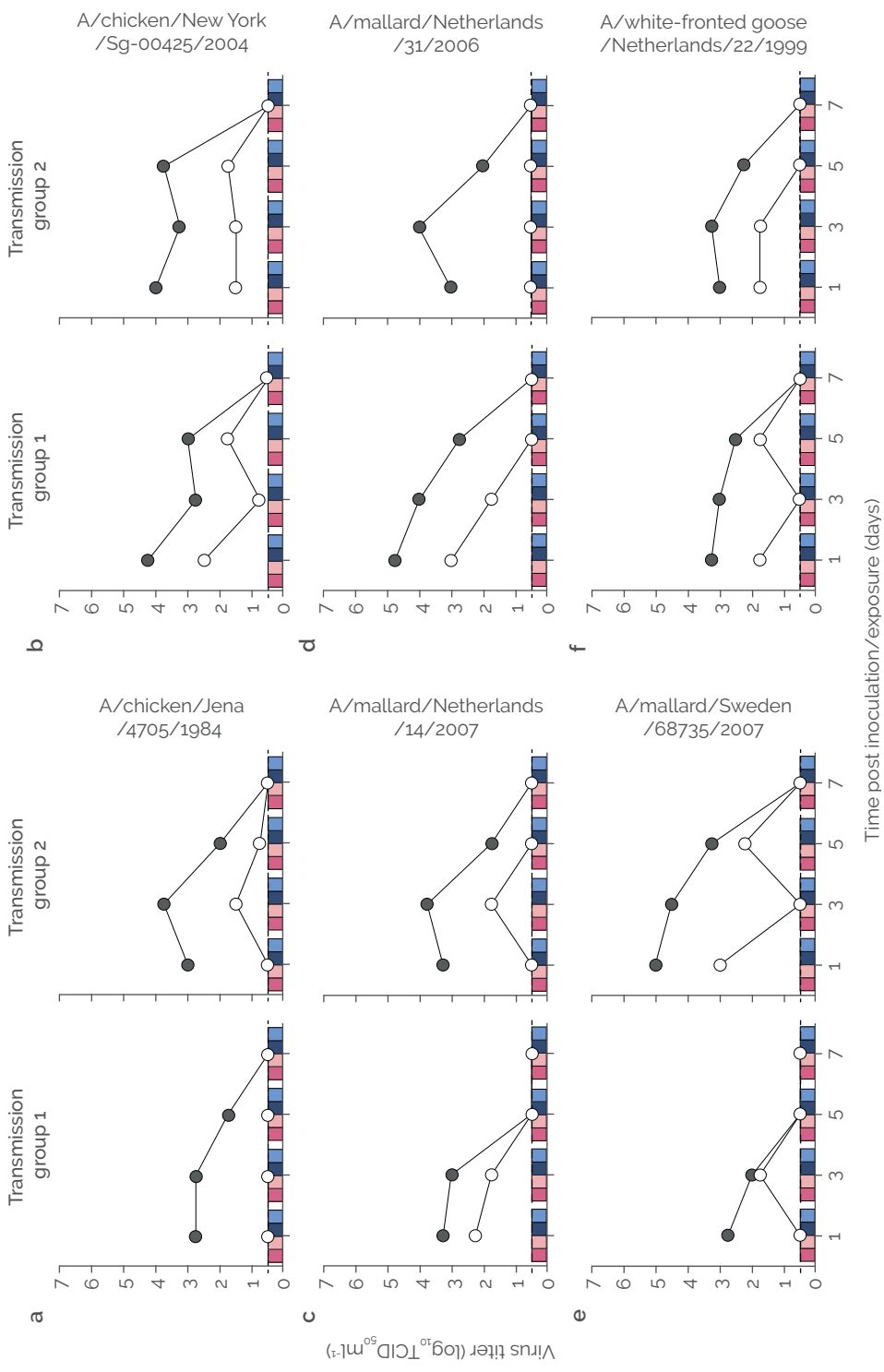

A/chicken/New York /Sg-00425/2004
A/mallard/Netherlands /31/2006
A/white-fronted goose /Netherlands/22/1999
A/chicken/Jena /4705/1984
A/mallard/Netherlands /14/2007
A/mallard/Sweden /68735/2007
Transmission group 1
Transmission group 2
a
b
c
d
e
f
Virus titer ($log_{10}TCID_{50}ml^{-1}$)
Time post inoculation/exposure (days)

Figure 2. Lack of transmission of avian A/H2N2 viruses via direct contact or air between ferrets. Transmission experiments are shown for six avian A/H2N2 virus isolates in two individual ferret groups: A/chicken/Jena/4705/1984 (**a**), A/chicken/New York/Sg-00425/2004 (**b**), A/mallard/Netherlands/14/2007 (**c**), A/mallard/Netherlands/31/2006 (**d**), A/mallard/Sweden/68735/2007 (**e**) and A/white-fronted goose/Netherlands/22/1999 (**f**). Dark grey and white circles indicate virus titers in throat and nose swabs of donor ferrets, respectively. Dark and light pink, and dark and light blue bars each represent virus titers in throat and nose swabs of direct contact and indirect recipient animals, respectively. Dotted lines indicate the detection limit of virus titrations.

to reverse the effect of G228S, resulting in low binding to α2,3-linked SIAs, similar to wild-type M1/57 and M1/58 HAs. Furthermore, the double substitution G205S and G228S in M1/57 increased binding to short linear glycans with α2,6-linked SIAs (#9). Amino acid substitution I289L in the M1/57 HA, either in the presence or absence of 228S, did not affect the receptor binding preference of these HAs. The I289L substitution also did not affect the receptor binding preference of the B1/68 HA. To investigate the binding properties in a different assay, the binding specificity of the A/H2N2 HAs was also assessed in a modified red blood cell assay. In brief, turkey red blood cells (TRBCs) were treated with Vibrio cholerae neuraminidase (VCNA) to remove SIAs followed by resialylation with solely α2,3- or α2,6-linked SIAs. The glycans that are present on the turkey erythrocytes are heterogenic and consist primarily of biantennary glycans containing 1 or 2 LacNAc repeats[241]. Consequently, the results of this assay are expected to be most comparable with the shorter glycan structures on the array, but not entirely. As expected, the human A/H3 and avian A/H5 control viruses bound exclusively to α2,6- or α2,3-linked SIAs, respectively (**Table 2**). In agreement with the glycan array analysis, introduction of 228S in M1/57 overall led to a slight increase in binding to α2,3 receptors, while maintaining binding to α2,6 receptors. In addition, also in this assay, the B1/68 HA showed a dual binding preference to both α2,6- and α2,3-linked SIAs.

A newly identified substitution G205S stabilized the HA of A/H2 viruses.

Upon attachment to SIA receptors and receptor-mediated endocytosis, the HA protein is exposed to a low pH in the endosome which triggers a conformational change that mediates fusion of the viral and endosomal membranes, resulting in the release of the viral genome in the cytoplasm[242]. Previous work has shown that the HAs of influenza A viruses that are transmissible via the air between mammals are comparatively stable, as demonstrated in fusion and temperature stability assays[12,238,243]. To assess if the HA acid stability of A/H2N2 viruses had changed during circulation in humans, fusion assays were performed. For this purpose,

M1/57
M1/58
B1/68
Transmission pair 1
Transmission pair 2
Transmission pair 3
Transmission pair 4
a
b
c
Virus titer ($log_{10}TCID_{50}ml^{-1}$)
Time post inoculation/exposure (days)

Figure 3. Transmission of human A/H2N2 viruses between ferrets via aerosol or respiratory droplets. Transmission experiments are shown for three human A/H2N2 virus isolates: A/Netherlands/M1/1957 (M1/57) (**a**), A/Netherlands/M1/1958 (M1/58) (**b**) and A/Netherlands/B1/1968 (B1/68) (**c**). An individual donor-recipient pair is shown in each panel. Dark and light blue circles represent virus titers in throat and nose swabs of donor ferrets, respectively. Dark and light blue bars each represent virus titers in throat and nose swabs of indirect recipient ferrets. Dotted lines indicate the detection limit of virus titrations.

Vero-118 cells were transfected with HA-expression plasmids and exposed to trypsin to cleave and activate the HA, followed by exposure to a pH gradient to induce membrane fusion. Cell cultures were subsequently examined under a microscope for the presence of syncytia (multinucleated cells) to determine the pH threshold of fusion for each HA (**Fig. 5a**). Human H3 (stable) and avian H5 (unstable) HAs were included as controls, for which the pH threshold for fusion was 5.5 and 5.8, respectively. The H2 HA of M1/57, M1/58, and B1/68 required a pH for fusion of 5.6, 5.4, and 5.8, respectively, demonstrating that the H2 HA stability had changed during circulation in humans. Introduction of G228S in the M1/57 HA did not change the pH of fusion. In contrast, introduction of G205S alone or in combination with G228S increased the acid stability of the M1/57 HA from 5.6 to 5.4, comparable to that of M1/58. The substitution I289L did not affect the acid stability of the M1/57 and B1/68 HA, suggesting that one or more of the 25 additional substitutions may be responsible for the decreased stability of the B1/68 HA.

The switch of influenza virus HA from a metastable non-fusogenic to a stable fusogenic conformation can also be triggered at neutral pH when the HA is exposed to increasing temperature. This conformational change of HA is biochemically indistinguishable from the change triggered by low pH and results in a loss of the ability to bind the receptor[244]. To assess the thermostability of the H2 HAs, viruses were exposed for 30 min to increasing temperatures ranging from 52 to 62˚C at neutral pH. Subsequently, the HA titer of each A/H2 virus was recorded (**Fig. 5b**). In agreement with the fusion assay, M1/57 HA was less stable than M1/58 HA but more stable than B1/68 HA. Introduction of G228S did not affect the thermostability of M1/57, whereas introduction of G205S was found to stabilize the M1/57 HA. This stability was further increased when G228S was added to G205S in the M1/57 HA. Introduction of I289L alone or in combination with G228S did not affect the thermostability of M1/57 HA, neither did introduction of I289L in the B1/68 HA. Overall, the assay results on acid stability and temperature stability yielded mirror images for all viruses and amino acid substitutions.

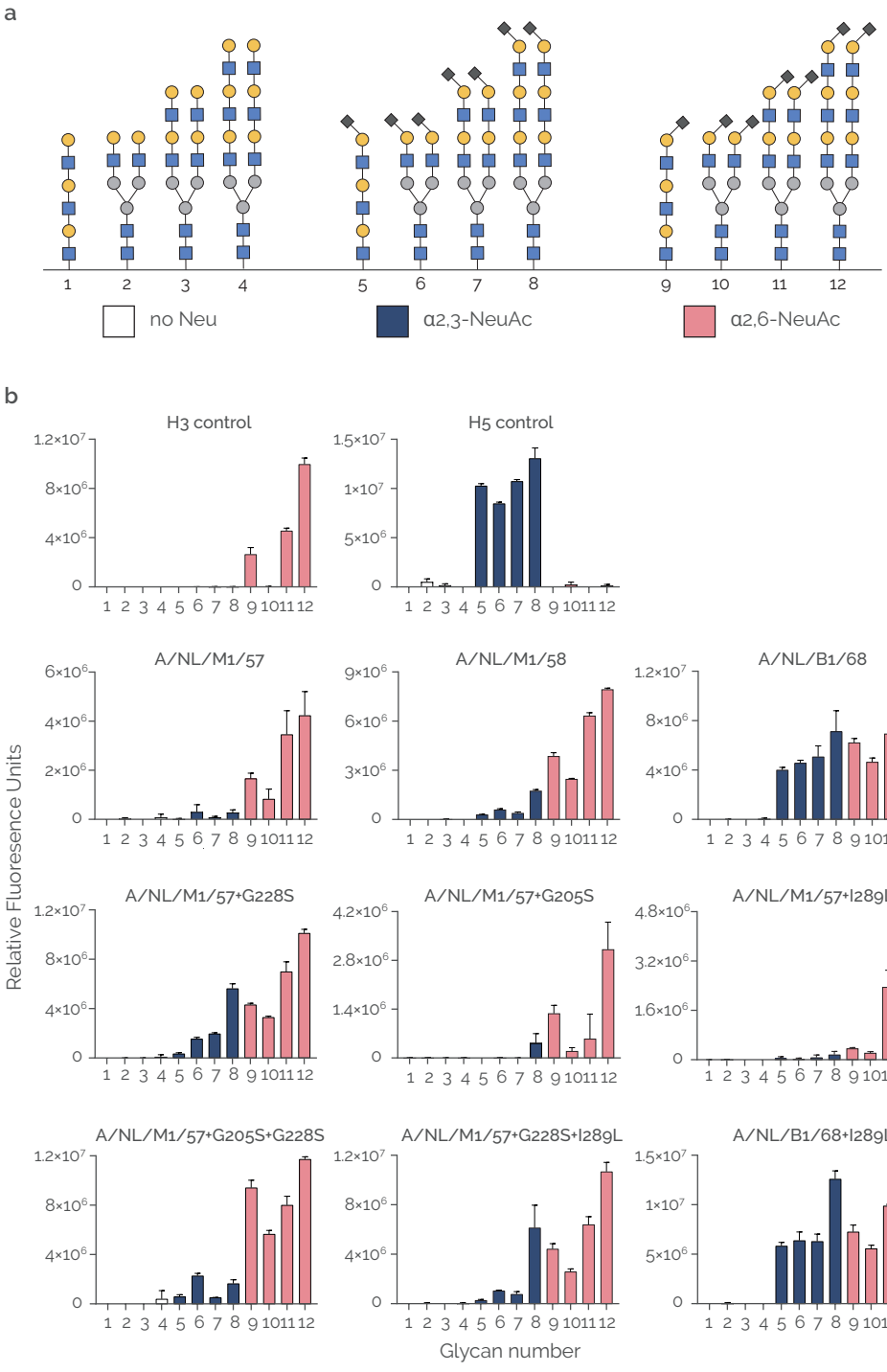
Table 1. Amino acids at positions 205, 226, 228, and 289 in the HA of human A/H2N2 viruses

A/H2N2 viruses	Amino acid position in HA				Passage history	Genbank accession number
	205	226	228	289		
A/El Salvador/2/1957[235]	G	Q	G	I	E_9MDCK_X	HM204767
A/El Salvador/2/1957†,[235]	G	L	G	I	$MDCK_X$	HM204775
A/Netherlands/M1/1957 (M1/57)	G	L	G	I	tMK_2MDCK_2	KM402801
A/Netherlands/M2/1957	G	L	G	I	$MDCK_3$	KM885170
A/Singapore/1/1957	G	L	S	I	E_xMDCK_1	CY125894
A/Netherlands/M1/1958 (M1/58)	S	L	S	L	tMK_2MDCK_2	CY077741
A/Albany/6/1958[235]	G	L	S	I	E_{10}	AF270723
A/Netherlands/N1/1959	V	L	G	I	tMK_2MDCK_2	CY077904
A/Netherlands/H1/1960	S	L	S	I	$E_1tMK_1MDCK_3$	CY077786
A/Netherlands/K1/1963	S	L	S	I	$E_1tMK_1MDCK_2$	CY077733
A/England/12/1964	S	L	S	I	$MK_1E_{11}MDCK_3$	AY209967
A/Sydney/2/1964	G	L	S	I	E_7MDCK_3	KP412320
A/Taiwan/1/1964	S	L	S	I	$YMDCK_2$	DQ508881
A/England/1/66	S	L	S	I	$MK_1E_1A_{16}MDCK_2$	KP412318
A/England/10/67	S	L	S	I	E_2MDCK_3	AY209980
A/Tokyo/3/67	S	L	S	I	$YMDCK_3$	AY209987
A/Netherlands/B1/68 (B1/68)	S	L	S	I	E_4MDCK_3	KM402809
A/Netherlands/B2/68	S	L	S	I	E_xMDCK_4	KM885174

Viruses marked in grey were used in the current study. † this variant of A/El Salvador/2/1957 was plaque-purified from the nasal wash of a ferret during transmission experiments with A/El Salvador/2/1957[235]. A, amniotic cavity; E, allantoic cavity; MDCK, Madin-Darby kidney cells; MK, monkey kidney cells; tMK, tertiary monkey kidney cells; X, unknown passage number; Y, unknown passage history in eggs;

A stabilized HA and human receptor binding did not promote robust airborne transmission of A/H2N2 viruses.

To assess whether the substitution G228S alone was sufficient, or the addition of the stabilizing substitution G205S to G228S was required for the airborne transmissibility of M1/58, recombinant viruses M1/57, M1/58, and M1/57 with G228S, or G205S and G228S in HA were generated and tested in the ferret transmission model. Donor ferrets inoculated with each virus were productively infected, as shown by the successful isolation of infectious virus until 5 or 7 dpi, and all four viruses replicated equally well, with peak titers reaching up to 6.25 $\log_{10}TCID_{50}ml^{-1}$ in ferrets inoculated with M1/57 (**Fig. 6**). M1/57 and M1/57 harboring the G228S substitution were transmitted to one out of four indirect recipient ferrets, whereas M1/58 and M1/57 containing the double substitution G205S/G228S were transmitted to two out of four indirect recipient ferrets. The lack of transmission in the A/H2N2 virus-negative indirect recipient animals was confirmed by the absence of seroconversion (data not shown). Transmission of recombinant M1/57 was not expected based on the previous transmission experiments with the virus isolate (**Fig. 3**) and M1/57 was also only detected from 7 dpe onwards in one recipient ferret. To investigate if the M1/57 virus had acquired substitutions that favored transmissibility, as previously observed in another A/H2N2 transmission study[235], viruses from the throat and nose samples of the M1/57-positive indirect recipient ferret were sequenced. However, no mutations were detected in any of the HA sequences (data not shown).


a
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H3 control
H5 control
A/NL/M1/57
A/NL/M1/58
A/NL/B1/68
A/NL/M1/57+G228S
A/NL/M1/57+G205S
A/NL/M1/57+I289L
A/NL/M1/57+G205S+G228S
A/NL/M1/57+G228S+I289L
A/NL/B1/68+I289L
Relative Fluorescence Units
Glycan number

Figure 4. Binding specificity of human A/H2 viruses to selected glycans. Synthesized glycans (**a**) were used to assess the binding specificity of A/H2 viruses to α2,3- and α2,6-linked SIAs on a glycan microarray (**b**). Glycans 1-4 are nonsialylated controls (white), structures 5-8 (blue) and 9-12 (pink) represent α2,3- and α2,6-linked glycans, respectively. Human A/Netherlands/213/2003 (H3 control, α2,6-linked SIA specificity) and avian A/Indonesia/5/2005 virus (H5 control, α2,3-linked SIA specificity) were used as controls. The mean intensity and standard deviation (SD) were calculated from 4 independent replicates. Neu, neuraminic acid; NeuAc, N-acetyl neuraminic acid;

Table 2. Binding specificity of human A/H2 viruses in a modified turkey red blood cell assay

	HA titer (HAU[a]/25µl)		
Virus	Untreated TRBCs[b]	α2,3 resialylated	α2,6 resialylated
H3 control	24	-	48
H5 control	32	24	-
M1/57	24	0.5	24
M1/58	48	6	64
B1/68	48	16	64
M1/57 + G228S	32	6	32
M1/57 + G205S	48	-	24
M1/57 + G205S + G228S	48	8	64
M1/57 + I289L	32	0.5	32
M1/57 + G228S + I289L	32	3	32
B1/68 + I289L	48	6	64

[a] Hemagglutination units
[b] Turkey red blood cells

DISCUSSION

As a contribution to the risk assessment of A/H2N2 influenza viruses for humans, we here evaluated the transmissibility of avian A/H2N2 viruses, isolated from various bird species, in the ferret model. The six avian A/H2N2 virus isolates tested, replicated in the URT of ferrets, but did not transmit to recipient animals either through direct contact or via the air. Similar results were previously obtained in a study by Jones et al., in which the transmissibility of nine avian H2N2 isolates was assessed in the ferret model[245]. Of these, five virus isolates replicated in the URT of donor animals of which three isolates were transmitted to one or two out of two direct contact animals. In this study, the isolate A/chicken/Jena/4705/1984, which was also used in the present study, replicated in donor animals, but just as described here, was not transmitted via direct contact. None of the five A/H2N2 isolates were transmitted between ferrets via the air. Moreover, in a study by Pappas et al. a chicken A/H2N2 virus was transmitted to two out of three direct contact animals, but was also not transmitted between ferrets via the air[246]. This indicates that, despite limited transmission via direct contact in the ferret model, the risk of avian A/H2N2 viruses without prior reassortment or adaptation is low for mammals.

Of the three human A/H2N2 viruses tested, only the A/H2N2 virus isolated in 1958 was transmitted via the air between ferrets, but not the viruses isolated in 1957 and 1968. To investigate the observed difference in transmissibility of the 1957 and 1958 viruses, phenotypic assays were performed to study the HA receptor binding preference and the HA stability, two phenotypes that were previously shown to affect transmissibility[243]. Both viruses were found to preferentially bind α2,6-linked SIAs, without an additive effect of the G228S substitution that was present in M1/58, but not in M1/57. Furthermore, M1/58 had a more stable HA as the result of a G205S substitution that was absent in M1/57. However, when M1/57 recombinant viruses harboring HAs with substitutions from M1/58 were tested in transmission experiments, no major differences in transmissibility were observed. The transmission results hinted that those viruses harboring both G205S and G228S (M1/57 with both substitutions and M1/58) may be transmitted slightly more efficient than the viruses without these substitutions (M1/57 and G228S mutant), but the small number of animals used in this study and the limited overall power of experiments in ferrets do not allow a statistically sound conclusion (**Fig. 6**).

Previously, Pappas et al. described a similar analysis as shown here, in which the human A/H2N2 strain A/Albany/6/1958 was transmitted via the air between

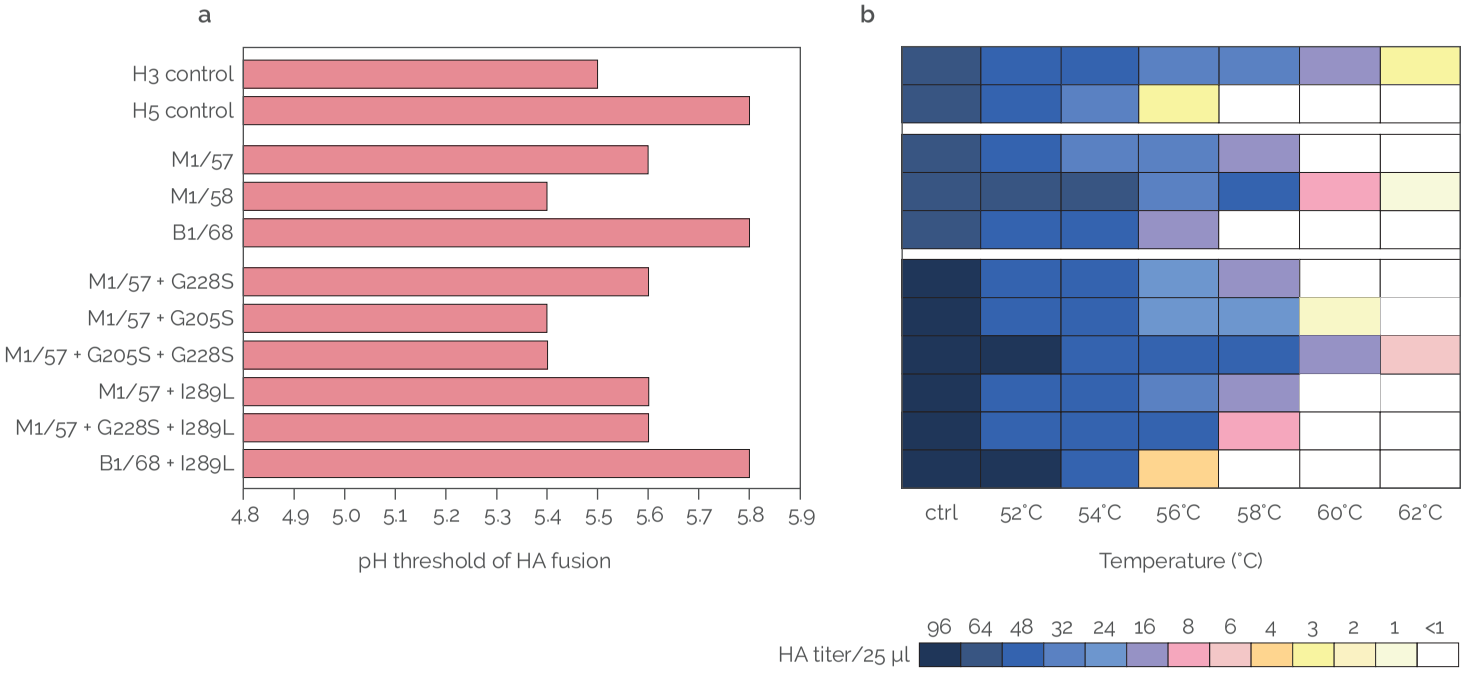

Figure 5. HA stability of recombinant wild-type and mutant A/H2 viruses. Fusion stability of HAs upon acidification of Vero-118 cells at different pH (**a**). Vero-118 cells were transfected with wild-type and mutant HAs and subsequently exposed to PBS with different pH. The next day, cells were observed for the presence of syncytia (multinucleated cells). Bars indicate the pH threshold at which syncytia formation was detected microscopically. The HA of A/Netherlands/213/2003 (stable H3) and of A/Indonesia/5/2005 virus (unstable H5) were included as controls. Thermostability of recombinant viruses containing wild-type and mutant HAs (**b**). A/Puerto-Rico/8/1934 (7+1) viruses with A/H2 HA were incubated at different temperatures for 30 min after which the HA titer of viruses was measured for each temperature in an HA assay with TRBCs. As indicated by the color key, rectangles represent the HA titer/25 µl of the viruses before (first column, ctrl.) and after exposure to various temperatures. A/Netherlands/213/2003 (stable H3) and A/Indonesia/5/2005 virus (unstable H5) were included as controls.

ferrets, whereas the human A/H2N2 strain A/El Salvador/2/1957 was only transmitted via the air in 1 out of 6 ferret pairs, late during the experiment. Sequence analysis of a nasal wash sample collected from the indirect contact ferret showed that the transmission event was associated with a natural variant containing the Q226L substitution in HA, similar to M1/57 used in the current study. The Q226L substitution in A/El Salvador/2/1957 was found to change the receptor binding preference from α2,3-linked to α2,6-linked SIAs[235].

Based on the transmission data from multiple avian and human influenza viruses collected over the past years, transmission of a virus with a predominant α2,3-linked SIA binding preference between mammals is highly unlikely. In addition, it was previously shown that passaging of human influenza viruses in eggs may result in egg-adaptation and the ability to bind to α2,3-linked SIAs[236,237]. Therefore, it may be possible that due to the 8 passages in eggs, the binding preference of A/El Salvador/2/1957 shifted to α2,3-linked SIAs. In contrast, M1/57 that was used in our study has only been passaged in mammalian cells (2 passages in tertiary monkey kidney cells and 2 in MDCK cells, **Table 1**), and may therefor better represent the viruses that circulated during the first year of the A/H2N2 pandemic. Although the possibility that α2,3-linked SIA binding A/H2N2 viruses were circulating early in the pandemic cannot be excluded, our binding data of A/M1/57 suggests that the viruses that started the pandemic had already acquired α2,6-linked SIA binding.

Introduction of G228S in M1/57 HA did not change the binding to α2,6-linked SIAs, however, an increase in α2,3-linked SIA binding was observed. This α2,3-linked SIA binding was also observed for A/El Salvador/2/1957 upon introduction of G228S, and in A/Albany/6/1958 that already contained Q226L and G228S[235]. This effect of G228S in M1/57 was reversed when G205S was also introduced (**Fig. 4**). Both A/El Salvador/2/1957 and A/Albany/6/1958 did not contain 205S, which may explain the residual α2,3-linked SIA binding of those HAs.

In addition to preferential binding to α2,6-linked SIAs, a stable HA is required for transmission of influenza A viruses via the air between mammals, as previously demonstrated for A/H5N1 viruses[238,239,243]. While the early A/H2N2 virus M1/57 had a pH threshold for fusion of 5.6, the pH threshold of M1/58 was 5.4 (**Fig. 5a**). A comparative analysis between M1/57 and M1/58 identified a stabilizing substitution, G205S, which lowered the pH threshold for fusion of the M1/57 HA from 5.6 to 5.4. To our knowledge, this amino acid position has so far not been described to affect the acid or temperature stability of influenza A virus HA of any subtype[247]. Interestingly, this serine at position 205 became fixated, because most A/H2N2 viruses isolated

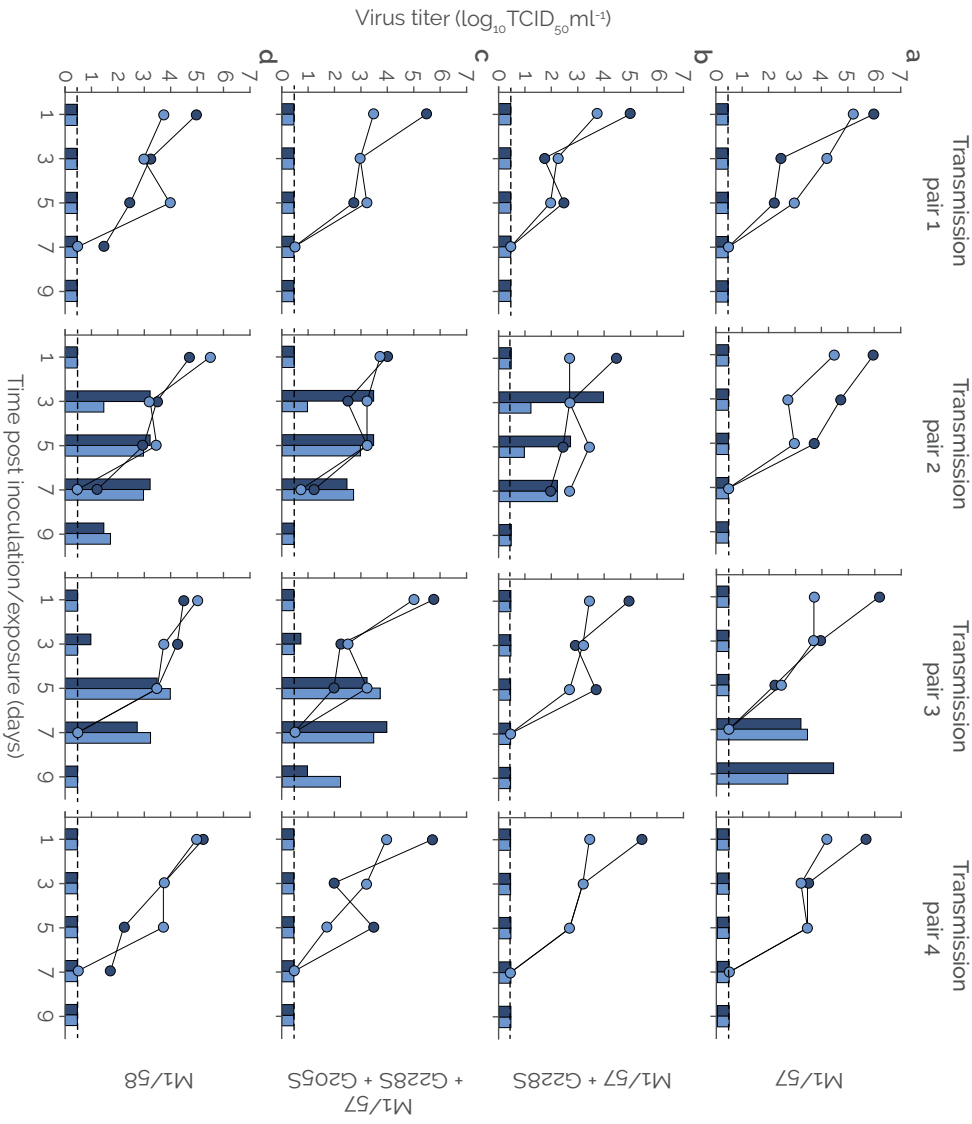

Figure 6. Limited transmission of recombinant human wild-type and mutant A/H2N2 viruses via aerosol or respiratory droplets. Transmission experiments are shown for 4 viruses: M1/57 (**a**), M1/57 + G228S (**b**), M1/57 + G205S + G228S (**c**) and M1/58 (**d**). An individual donor-recipient pair is shown in each panel. Dark and light blue circles represent virus titers in throat and nose swabs of donor ferrets, respectively. Dark and light blue bars each represent virus titers in throat and nose swabs of indirect recipient ferrets. Dotted lines indicate the detection limit of virus titrations.

after 1958 had a serine at position 205 (**Table 1**). This indicates that the increased HA stability was an advantage for the virus for circulation in the human population. Such an increase in stability over time during circulation in humans has previously also been described for A/H1N1pdm09 virus, where early isolates from the 2009 pandemic had a pH threshold for fusion of around 5.5 whereas viruses isolated in later years displayed lower pH threshold values ranging from 5.2 to 5.4[239].

Surprisingly, however, during the subsequent 10 years of circulation in humans, the H2 HA stability decreased again, as demonstrated by the B1/68 HA with a pH threshold for fusion of 5.8, similar to the unstable A/H5 HA control. In addition, the B1/68 showed a dual receptor binding preference for both α2,3- and α2,6-linked SIAs, despite the presence of G205S, Q226L, and G228S, and was not transmitted via the air between ferrets. In contrast, a previously studied A/H2N2 virus from 1967 (A/England/10/1967) was shown to be transmitted via the air in three out of three ferret pairs and this virus bound preferentially α2,6-linked SIAs as shown in a modified TRBC assay[246]. The B1/68 HA carried 25 substitutions as compared to the M1/58 HA, and it cannot be excluded that these substitutions and their potential phenotypic effects were the result of egg adaptation since this virus was propagated four times in eggs, before being passaged in mammalian cells (**Table 1**).

In conclusion, the data presented here indicate that circulating A/H2N2 avian viruses are not directly transmissible between ferrets. Assuming that ferrets represent a reasonable model for humans, this would suggest that the risk of circulating A/H2N2 viruses to cause a pandemic without prior adaptation or reassortment is low. However, in the absence of immunity against A/H2N2 viruses and a sufficiently large naïve population, the virus may not require to spread efficiently via the air to initiate a pandemic. In the current study, A/H2N2 viruses were less efficiently transmitted via the air between ferrets as compared to other human influenza viruses[243]. This may either indicate that ferrets are a suboptimal model for human-to-human transmission of A/H2N2 viruses or that A/H2N2 viruses may have caused a pandemic despite lower transmissibility compared to other pandemic strains. Nevertheless, continuous surveillance as a component of pandemic risk assessment is warranted. Our data on HA receptor binding, HA stability, and transmissibility in the ferret model demonstrated that A/H2N2 viruses continued to adapt to replication and spread in humans during the first years of pandemic circulation, resulting in the acquisition of the hitherto unknown G205S stability substitution in HA.

MATERIALS AND METHODS

Biocontainment

All experiments involving A/H2N2 viruses were conducted at enhanced animal biosafety level 3 (ABSL3+). The ABSL3+ facility of Erasmus MC consists of a negative pressurized (30 Pa) laboratory in which all *in vivo* and *in vitro* experimental work is carried out in class 3 isolators or class 3 biosafety cabinets, which are also negative pressurized (<-200 Pa). Although the laboratory is considered "clean" because all experiments are conducted in closed class 3 cabinets and isolators, special personal protective equipment, including laboratory suits, gloves, and FFP3 facemasks is used. Air released from the class 3 units is filtered by High Efficiency Particulate Air (HEPA) filters and then leaves the facility via a second set of HEPA filters. Only authorized personnel that received the appropriate training can access the ABSL3+ facility. All personnel working in the facility is vaccinated against seasonal influenza viruses. For animal handling in the facilities, personnel always work in pairs. The facility is secured by procedures recognized as appropriate by the institutional biosafety officers and facility management at Erasmus MC and Dutch and United States government inspectors. Antiviral drugs (oseltamivir and zanamivir) and personnel isolation facilities are directly available to further mitigate risks upon incidents.

Viruses

Avian A/H2N2 virus isolates were obtained from surveillance efforts of wild birds in the Netherlands and Sweden (A/mallard/Netherlands/14/2007 (ACR58574); A/white-fronted goose/Netherlands/22/1999 (AFM82566); A/mallard/Netherlands/31/2006 (ACR58563); A/mallard/Sweden/68735/2007 (AFM82544). Avian isolates A/chicken/Jena/4705/1984 (AFJ12827) and A/chicken/New York/Sg-00425/2004 (ACJ69315) were shared within the NIH/NIAID CEIRS network. A/mallard/Sweden/68735/2007 was a kind gift of Jonas Waldenström. These viruses were propagated in the allantoic cavity of embryonated eggs. Human A/H2N2 influenza virus isolates A/Netherlands/M1/1957 (M1/57) and A/Netherlands/M1/1958 (M1/58) were passaged twice in tertiary rhesus monkey kidney (tMK) and twice in Madin-Darby kidney (MDCK) cells, from freeze-dried samples that were collected from individuals presenting between 1957 and 1968 with flu-like symptoms and that tested positive for the presence of influenza virus. A/Netherlands/B1/1968 was first propagated four times in the allantoic cavity of embryonated eggs, followed by three passages in MDCK cells. Gene segments of M1/57, M1/58, and B1/68 were amplified by reverse transcription

polymerase chain reaction (RT-PCR) and cloned in a modified version of the bidirectional reverse genetics plasmid pHW2000[248]. In addition, the HA gene segment of these three A/H2N2 viruses was cloned in the pCAGGs expression plasmid and amino acid substitutions of interest (G205S, G228S, I289L in HA of A/Netherlands/M1/1957 and I289L in HA of A/Netherlands/B1/1968) or a combination thereof were introduced by site-directed mutagenesis. Recombinant A/H2N2 viruses and recombinant viruses (7+1) containing 7 gene segments of A/Puerto-Rico/8/1934 and wild-type A/H2N2 HA or mutant HA containing the substitutions G228S, G205S, I289L, G228S + G205S, and G228S + I289L were generated upon transfection of 293T cells followed by one or two passages in MDCK cells. Recombinant 7+1 viruses were concentrated using Amicon® Ultra-15 Centrifugal Filter Units (Merck Millipore). Virus rescue and production of virus stocks of recombinant A/H2N2 viruses were done in a class III biosafety cabinet in a negatively pressurized ABSL3+ facility as described previously[12].

Cells

tMK cells were cultured in Minimal essential medium with Hank's salts (MEM; Lonza) supplemented with 5% fetal bovine serum (FBS; Greiner or Atlanta Biologicals), 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) and 10 mM Hepes (Lonza). MDCK cells (ATCC) were cultured in Eagle's minimal essential medium (EMEM; Lonza) supplemented with 10% FBS (Greiner or Atlanta Biologicals), 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 Hepes (Lonza) and 1x nonessential amino acids (Lonza). 293T cells were cultured in Dulbecco modified Eagle's medium (DMEM; Lonza) supplemented with 10% FBS, 100 IU ml^{-1} penicillin-100 µg ml^{-1} streptomycin mixture (Lonza), 2 mM glutamine, 1 mM sodium pyruvate (Gibco), and non-essential amino acids. Subclone 118 of Vero-WHO (Vero-118) cells were cultured in Iscove's modified Dulbecco's medium (IMDM; Lonza) supplemented with 10% FBS and 100 IU ml^{-1} penicillin-100 µg ml^{-1} streptomycin mixture (Lonza)[196,197]. All cell lines were maintained at 37˚C and 5% CO_2.

Transmission experiments in ferrets

Animals were housed and experiments were performed in strict compliance with the Dutch legislation for the protection of animals used for scientific purposes (2014, implementing EU Directive 2010/63). Influenza virus and Aleutian Disease

Virus seronegative 6-month-old female ferrets (Mustela putorius furo), weighing 735–1095 g, were obtained from a commercial breeder (TripleF, USA). Research was conducted under a project license from the Dutch competent authority (license number AVD101002015340) and the study protocols were approved by the institutional Animal Welfare Body (Erasmus MC permit number 122-10-17, 122-11-15, and 15-340-18). Animal welfare was monitored on a daily basis. Virus inoculation of ferrets was performed under anesthesia with a mixture of ketamine/medetomidine (10 and 0.05 mg kg^{-1} respectively) antagonized by atipamezole (0.25 mg kg^{-1}). Swabs were collected under light anesthesia using ketamine to minimize animal discomfort. For each experiment, two (avian A/H2N2 viruses) or four (human A/H2N2 viruses) donor ferrets were inoculated intranasally with 10^6 $TCID_{50}$ (250 µl instilled dropwise in each nostril) of A/H2N2 virus. For avian A/H2N2 virus isolates, direct contact animals were placed in the same cage as donor animals 6 hpi (**Fig. 1a**). One day later, for both avian and human A/H2N2 viruses, indirect recipient ferrets were added in an adjacent cage separated by two steel grids, 10 cm apart, to avoid contact transmission (**Fig. 1b**). The contribution of transmission via fomites was not investigated [248]. Throat and nasal swabs were collected from the ferrets every other day until 7 dpi and 9 dpe, respectively, to prevent cross-contamination. All swabs were stored at −80°C in virus transport medium consisting of MEM Eagle with Hank's BSS and 25 mM Hepes (Lonza), glycerol 99% (Sigma Aldrich), lactalbumin hydrolysate (Sigma Aldrich), 10 mU polymyxin B sulfate (Sigma Aldrich), 5 mU nystatin (Sigma Aldrich), 50 mg ml^{-1} gentamicin (Gibco) and 100 IU ml^{-1} penicillin 100 µg ml^{-1} streptomycin mixture (Lonza). Ferrets were euthanized by heart puncture under anesthesia. All animal experiments were performed in class III isolators in a negatively pressurized ABSL3+ facility as described previously[12].

Virus titrations

Throat and nasal swabs were titrated in MDCK cells. Briefly, confluent cells were inoculated with 10-fold serial dilutions of sample in serum-free EMEM 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 Hepes (Lonza), 1x nonessential amino acids (Lonza) and 20 µg ml^{-1} trypsin (Lonza). At 1 hpi, the first three dilutions were washed twice with media and 200 µl fresh media was subsequently added to the whole plate. Supernatants of cell cultures were tested for agglutination activity using turkey erythrocytes three days after inoculation. Infectious virus titers ($TCID_{50}$ ml^{-1}) were calculated from four replicates of each throat and nasal swab using the Spearman-Karber method.

Glycan microarray

Glycan microarray analysis was performed as described previously[240]. As controls, the plant lectins Erythrina cristagalli agglutinin (ECA, specific for terminal Gal), Sambuca nigra agglutinin (SNA, specific for α2,6-linked Neu5Ac), and Maackia Amurensis Lectin I (MAL-I, specific for α2,3-linked Neu5Ac) were used. Recombinant A/Puerto-Rico/8/1934 (7+1) viruses with A/H2 HA (25 µl) were diluted with PBS-T (PBS + 0.1% Tween, 25 µl) and applied to the array surface in the presence of oseltamivir (200 nM) in a humidified chamber for 1 h, followed by successive rinsing with PBS-T (PBS + 0.1% Tween), PBS and deionized water (2x) and dried by centrifugation. The virus-bound slide was incubated for 1 h with the CR6261 A/H2 influenza HA stem-specific antibody (100 µl, 5 µg ml^{-1} in PBS-T), which was kindly provided by Dr. Dirk Eggink. Quality control of the CR6261 A/H2 influenza HA stem specific antibody specificity was performed by incubation of the antibody to the array in the absence of a virus. Afterwards, the slide was washed again as described above. Subsequently, a secondary goat anti-human Alexa-647 antibody (100 µl, 2 µg ml^{-1} in PBS-T, Thermo Fisher) was applied, incubated for 60 min in a humidified chamber, and washed again as described above. The control lectins containing a biotin tag were visualized with Streptavidin Alexa Fluor-635 (Thermo Fisher). Washed slides were dried by centrifugation and immediately scanned using an Innopsys Innoscan 710 microarray scanner at the appropriate excitation wavelength. To ensure that all signals were in the linear range of the scanner's detector and to avoid any saturation of the signals various gains and PMT values were employed. Images were analyzed with Mapix software (version 8.1.0 Innopsys) and processed with an in-house developed Excel macro. The average fluorescence intensity and standard deviation (SD) were measured for each compound after exclusion of the highest and lowest intensities from the spot replicates (n=4) and displayed using GraphPad Prism 9.

Modified TRBC agglutination assay

To assess the receptor binding specificity of recombinant A/Puerto-Rico/8/1934 (7+1) viruses with A/H2 HA, a modified TRBC assay was performed. Briefly, all α2,3-, α2,6-, α2,8-, and α2,9-linked sialic acids were removed from the surface of TRBCs by incubating 62.5 µl of 1% TRBCs in PBS with 50 mU Vibrio cholerae NA (VCNA; Roche) in 0.1 mM calcium chloride at 37 °C for 1 h. Removal of sialic acids was confirmed by observation of complete loss of haemagglutination of the TRBCs by the tested influenza A viruses. Subsequently, resialylation was

performed using 5 mU of α2,3-(N)-sialyltransferase (Sigma-Aldrich) or 0.5 µg of α2,6-(N)-sialyltransferase (R&D Systems) and 1.5 mM CMP-sialic acid (Merck millipore) at 37 ˚C in 75 µl for 2 h to produce α2,3-TRBC and α2,6-TRBC, respectively. After a washing step, the TRBCs were re-suspended in PBS containing 1% bovine serum albumin to a final concentration of 0.5% TRBCs. Resialylation was confirmed by haemagglutination (HA) of viruses with known receptor specificity: recombinant viruses with seven gene segments of influenza virus A/Puerto Rico/8/1934 and the HA of H3N2 A/Netherlands/213/2003 and the HA of H5N1 A/Indonesia/05/2005. The receptor specificity of recombinant A/Puerto-Rico/8/1934 (7+1) viruses with A/H2 HA was tested by performing a standard HA assay with untreated and modified TRBCs. In brief, serial twofold dilutions of virus in PBS were made in a 50 µl volume; 50 µl of 0.5% TRBCs were added, followed by incubation for 1 h at 4 ˚C before determining the HA titer. Two independent experiments were performed for each assay. Results are shown from one representative experiment.

Fusion assay

Influenza virus HA-induced cell fusion was tested in Vero-118 cells. Approximately 106 Vero-118 cells were transfected in 100 mm petri dishes (Greiner) with 5 µg pCAGGS-HA using Xtremegene transfection reagent (Roche). One day after transfection, cells were collected using trypsin-EDTA (Gibco) and plated in 6-well plates. The next morning, cells were washed and medium was replaced with IMDM supplemented with 100 IU ml^{-1} penicillin-100 µg ml^{-1} streptomycin mixture (Lonza) and 20 µg ml^{-1} trypsin (Lonza). After 1h incubation at 37˚C, cells were washed once with PBS and exposed to PBS at pH 4.8-5.9 in 0.1 increments for 10 min at 37˚C. Subsequently, the PBS was replaced by IMDM supplemented with 10% FBS (Greiner or Atlanta Biologicals) and 100 IU ml^{-1} penicillin-100 µg ml^{-1} streptomycin mixture (Lonza). Eighteen hours after the pH shock, cells were fixed using 80% ice-cold acetone, washed, and stained using a 20% Giemsa solution (Merck Millipore) for microscopic analyses. Two independent experiments were performed for each assay. Results are shown from one representative experiment.

Temperature stability assay

The HA stability of wild-type and mutant recombinant 7+1 viruses was assessed by exposing the viruses to a temperature gradient. Briefly, viruses were set to similar HA units using PBS. Subsequently, the viruses were incubated in a thermal cycler for 30 min at temperatures ranging from 50-62˚C in 2˚C increments. Afterwards, the HA titer was determined by performing an HA assay using turkey erythrocytes, as described above. Two independent experiments were performed for each assay. Results are shown from one representative experiment.

DECLARATIONS

Data availability

All data and materials are available from the corresponding author (S.H.) on reasonable request.

Acknowledgements

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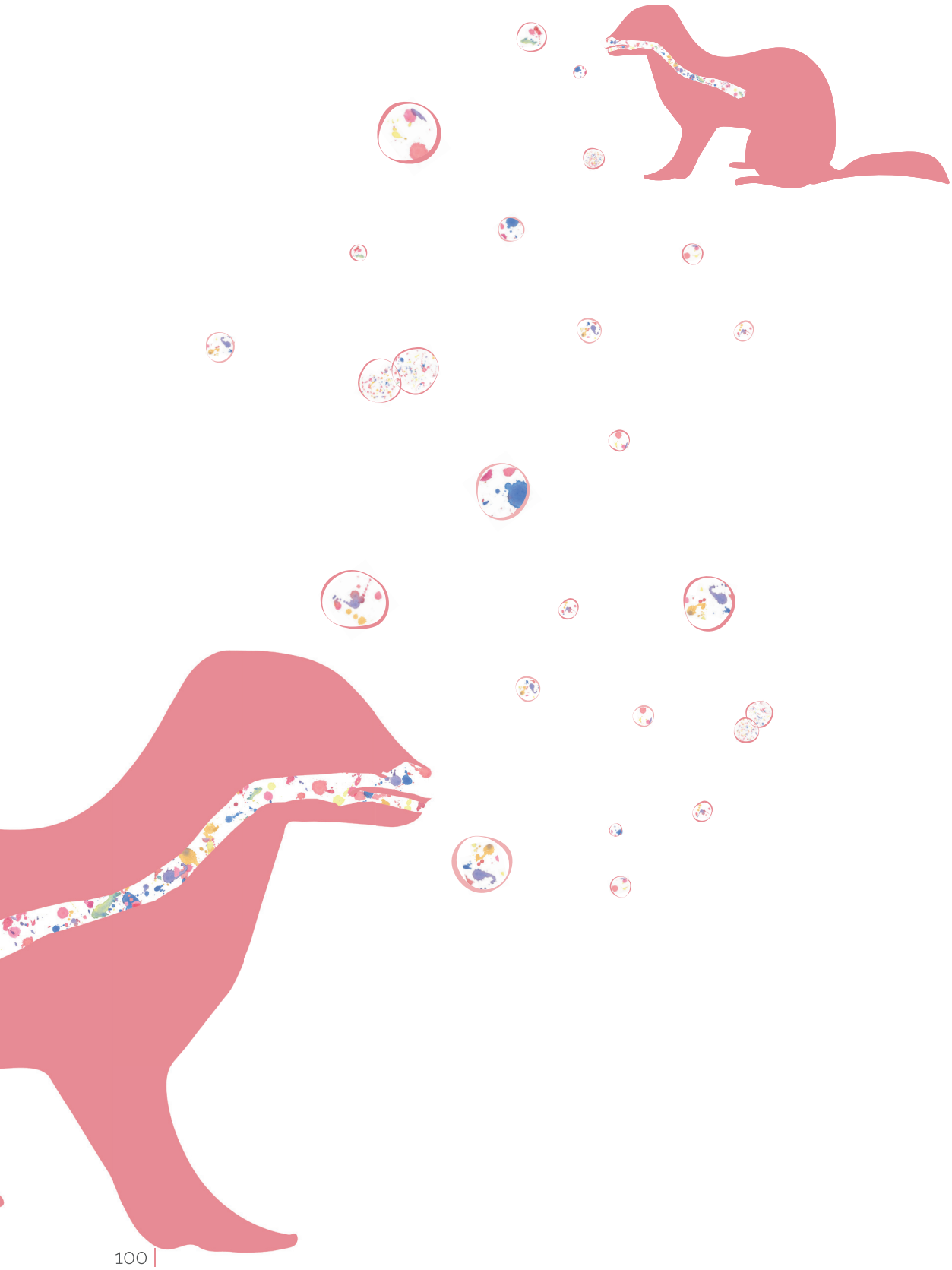
Conflict of interest statement

The authors declare no competing interests.



SARS-COV AND SARS-COV-2 ARE TRANSMITTED THROUGH THE AIR BETWEEN FERRETS OVER MORE THAN ONE METER DISTANCE

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ABSTRACT

SARS-CoV-2 emerged in late 2019 and caused a pandemic, whereas the closely related SARS-CoV was contained rapidly in 2003. Here, an experimental set-up is used to study transmission of SARS-CoV and SARS-CoV-2 through the air between ferrets over more than a meter distance. Both viruses cause a robust productive respiratory tract infection resulting in transmission of SARS-CoV-2 to two of four indirect recipient ferrets and SARS-CoV to all four. A control pandemic A/H1N1 influenza virus also transmits efficiently. Serological assays confirm all virus transmission events. Although the experiments do not discriminate between transmission via small aerosols, large droplets, and fomites, these results demonstrate that SARS-CoV and SARS-CoV-2 can remain infectious while traveling through the air. Efficient virus transmission between ferrets is in agreement with frequent SARS-CoV-2 outbreaks in mink farms. Although the evidence for virus transmission via the air between humans under natural conditions is absent or weak for SARS-CoV and SARS-CoV-2, ferrets may represent a sensitive model to study interventions aimed at preventing virus transmission.

INTRODUCTION

In December 2019, pneumonia cases were reported in China, caused by a virus that was closely related to the severe acute respiratory syndrome coronavirus (SARS-CoV)[250,251]. In 2003, the SARS-CoV outbreak affected 26 countries and resulted in more than 8000 human cases of infection of whom almost 800 died[252]. In contrast to SARS-CoV, the new coronavirus, named SARS-CoV-2, spread around the world in only a few months, with over 30 million cases and more than 900.000 deaths by the end of September 2020[253]. So far there is no unambiguous experimental or observational evidence on the main mode of transmission of SARS-CoV-2. However, given that most outbreaks occurred in clusters of people in close contact and in household settings, international health authorities conclude that SARS-CoV-2 is primarily transmitted within a short distance between individuals via direct and indirect contact, or respiratory droplets with little support for an important contribution of transmission via the air[254]. To prevent transmission via both routes, the World Health Organization and governments have advised control measures such as frequent hand washing and physical distancing to mitigate the rapid spread of SARS-CoV-2. In addition, in many countries, the use of face masks is encouraged or enforced in public buildings or public transportation where physical distancing is not always possible.

We and others previously used ferret models to show that SARS-CoV can be transmitted via direct contact and that SARS-CoV-2 can be transmitted via the air over 10 cm distance and others previously used ferret models to show that SARS-CoV can be transmitted via direct contact and that SARS-CoV-2 can be transmitted via the air over 10 cm distance[255–257]. To study if SARS-CoV and SARS-CoV-2 can maintain their infectivity when bridging a distance of more than one meter through the air, an experimental ferret transmission set-up was developed. After validation of the set-up with the A/H1N1 influenza virus, we subsequently demonstrated that both SARS-CoV and SARS-CoV-2 can be transmitted over a one-meter distance via the air.

RESULTS

Transmission of A/H1N1 virus between ferrets

To investigate coronavirus transmission via the air over more than a meter distance, a transmission set-up was built in which individual donor and indirect recipient ferret cages were connected through a hard duct system consisting of horizontal and vertical pipes with multiple 90˚ turns. The airflow was directed upwards from the donor to the indirect recipient animal and air traveled on average 118 cm through the tube (**Fig. 1**). A steel grid was placed between each cage and tube opening to prevent spill-over of food, feces, and other large particles.

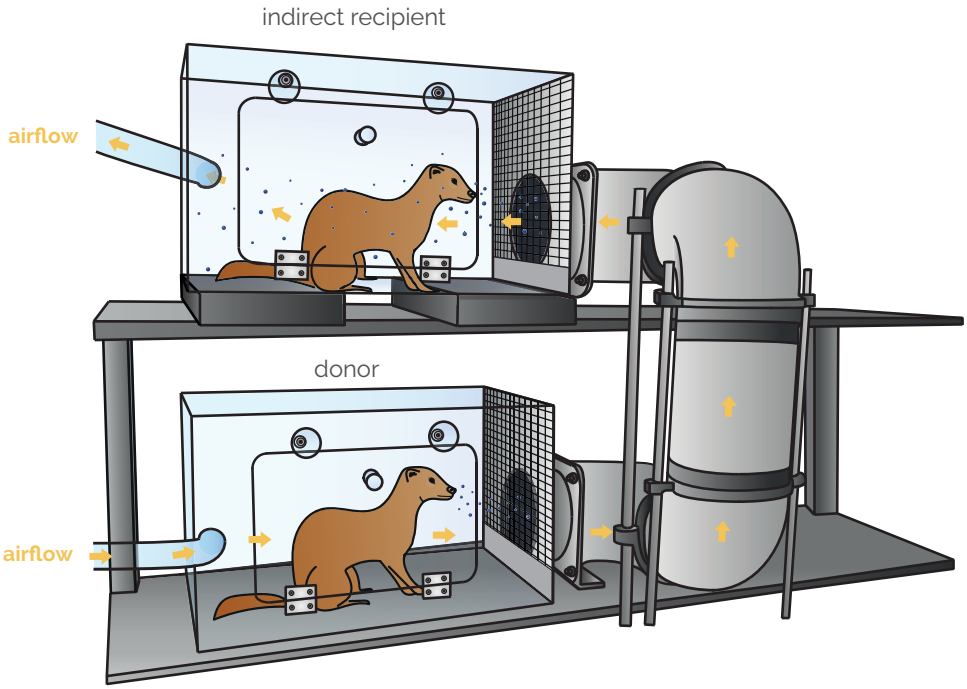

Figure 1. Experimental transmission set-up. Schematic representation of the set-up to assess transmission over >1 m distance. An inoculated donor ferret is housed in the bottom cage and the next day, an indirect recipient ferret is added to the top cage. The cages are connected through a hard duct system consisting of four 90˚ turns. The system is built of several horizontal and vertical 15 cm wide PVC pipes that allow upward airflow from the donor to the indirect recipient animal. The average length of the duct system is 118 cm with the shortest and longest length 73 and 163 cm, respectively. A steel grid is placed over the inlet and outlet of the duct system. The bottom five cm of the grid was closed to prevent spill-over of food, feces, and other large particles into the tube system. Yellow arrows indicate the direction of airflow (100 L min^{-1}). Set-ups were placed in class III isolators in a biosafety level 3+ laboratory.

The transmission set-up was first tested using A/H1N1 influenza virus A/Netherlands/602/2009, which was previously shown to be transmitted efficiently through the air between ferrets over 10 cm distance[258] (**Table 1**). Four individually housed donor animals were inoculated intranasally with 10^6 $TCID_{50}$ (median tissue culture infectious dose) of A/H1N1 virus and the next day indirect recipient ferrets were placed in separate cages above those of the donor ferrets. Throat and nasal swabs were collected from the donor and indirect recipient animals on alternating days to prevent cross-contamination, followed by virus detection by qRT-PCR and virus titration. Swabs were collected from the donor and indirect recipient animals until 7 days post-inoculation (dpi) and 13 days post-exposure (dpe), respectively. A/H1N1 virus was detected until 7 dpi in donor animals, with the highest RNA levels until 5 dpi (**Fig. 2a**). Attempts to isolate infectious virus were successful in all four animals until 5 dpi and in one animal until 7 dpi (**Fig. 3b**). A/H1N1 virus was transmitted to indirect recipient ferrets in four out of four independent transmission pairs between 1 and 3 dpe onwards, as demonstrated by the presence of viral RNA in throat and nose swabs. Infectious A/H1N1 virus was isolated from three out of four indirect recipient animals with similar peak virus titers and duration of virus shedding as observed in the donor animals. In these three animals, virus titers ranged from $10^{1.5}$ to $10^{6.0}$ $TCID_{50}ml^{-1}$, showing that these indirect recipient ferrets were productively infected (**Fig. 3a**). Besides nasal discharge, no other signs of illness were observed in the A/H1N1 virus-positive donor and indirect recipient animals (**Fig. 2a** and **3a**). Three of four A/H1N1 virus-positive animals seroconverted 15 dpi/dpe, and the hemagglutination inhibition titers were similar in donor and indirect recipient animals. The indirect recipient animal with low RNA levels and no infectious virus did not seroconvert (**Fig. 4a**).

Transmission of SARS-CoV and SARS-CoV-2 between ferrets over a one-meter distance

Upon validation of the experimental transmission set-up with the A/H1N1 virus, the transmissibility of SARS-CoV and SARS-CoV-2 over more than one meter distance was assessed, using the same procedures as for the A/H1N1 virus. Four donor animals were inoculated intranasally with either 6×10^5 $TCID_{50}$ of SARS-CoV-2 (isolate BetaCoV/Munich/BavPat1/2020) or 1.6×10^6 $TCID_{50}$ of SARS-CoV (isolate HKU39849). All donor animals were productively infected, as demonstrated by the robust and long-term virus shedding (**Fig. 2, Fig. 3**). SARS-CoV-2 RNA levels peaked around 3 and 5 dpi and were detected up to 13 dpi in one animal and up to 15 dpi, the last day of sample collection, in the

Table 1. Virus transmission to recipient ferrets over various distances.

			Recipient ferrets		
Virus	Distance between donor and recipient	Trans-mission	Onset shedding (dpe)	peak virus shedding (dpe)	peak virus titer ($log_{10}TCID_{50}mL^{-1}$)
A/H1N1	10 cm[258]	4 / 4	3, 3, 1, 3[a]	3, 3, 5, 5	4.8, 5.3, 4.5, 5.0
	> 1 m	4 / 4	5, 1, 3, -[a]	7, 3, 3, -	5.3, 5.5, 6.0, -
SARS-CoV-2	DC[255]	4 / 4	3, 3, 1, 3[b]	9, 7, 5, 7	3.5, 2.9, 2.3, 3.1
	10 cm[255]	3 / 4	7, 3, 3[b]	11, 9, 5	4.3, 3.0, 1.7
	> 1 m	2 / 4	1, 3[b]	7, 5	1.6, 3.7
SARS-CoV	DC[256c]	2 / 2	2, 2[b]	8, 8	4.1[d]
	> 1 m	4 / 4	1, 1, 1, 3[b]	5, 3, 5, 3	4.0, 3.6, 3.4, 2.6

[a] based on virus titers; [b] based on qRT-PCR Ct-value. DC: direct contact. [c] different transmission set-up and inoculation route (intratracheally); [d] average of two animals; $TCID_{50}$ equivalent was calculated from a standard curve of serial dilutions of the SARS-CoV virus stock.

other three animals. In contrast, SARS-CoV RNA levels peaked immediately at 1 dpi. Whereas SARS-CoV-2 inoculated animals did not display any symptoms of the disease, SARS-CoV donor animals became less active and exhibited breathing difficulties from 7 dpi onwards, warranting euthanasia by 9 dpi, when all animals were still SARS-CoV RNA positive in the throat and nasal swabs (**Fig. 2**).

Interestingly, both SARS-CoV-2 and SARS-CoV transmitted to indirect recipient animals via the air over more than one-meter distance. SARS-CoV-2 was transmitted in two out of four independent transmission pairs at 3 dpe, with peak viral RNA levels at 7 dpe and throat and nasal swabs still positive for viral RNA at 15 dpe, the last day of the experiment (**Fig. 2b**). Similar to the donor animals, the indirect recipient ferrets did not show any signs of illness. SARS-CoV was transmitted to four out of four indirect recipient ferrets on 1 or 3 dpe, with peak viral RNA levels at 3 to 5 dpe (**Fig. 2c**). Similar to the donor animals, indirect recipient animals exhibited breathing difficulties and became less active and were consequently euthanized for ethical reasons at 11 dpe, at which time the throat and nasal swabs were still positive for SARS-CoV RNA.

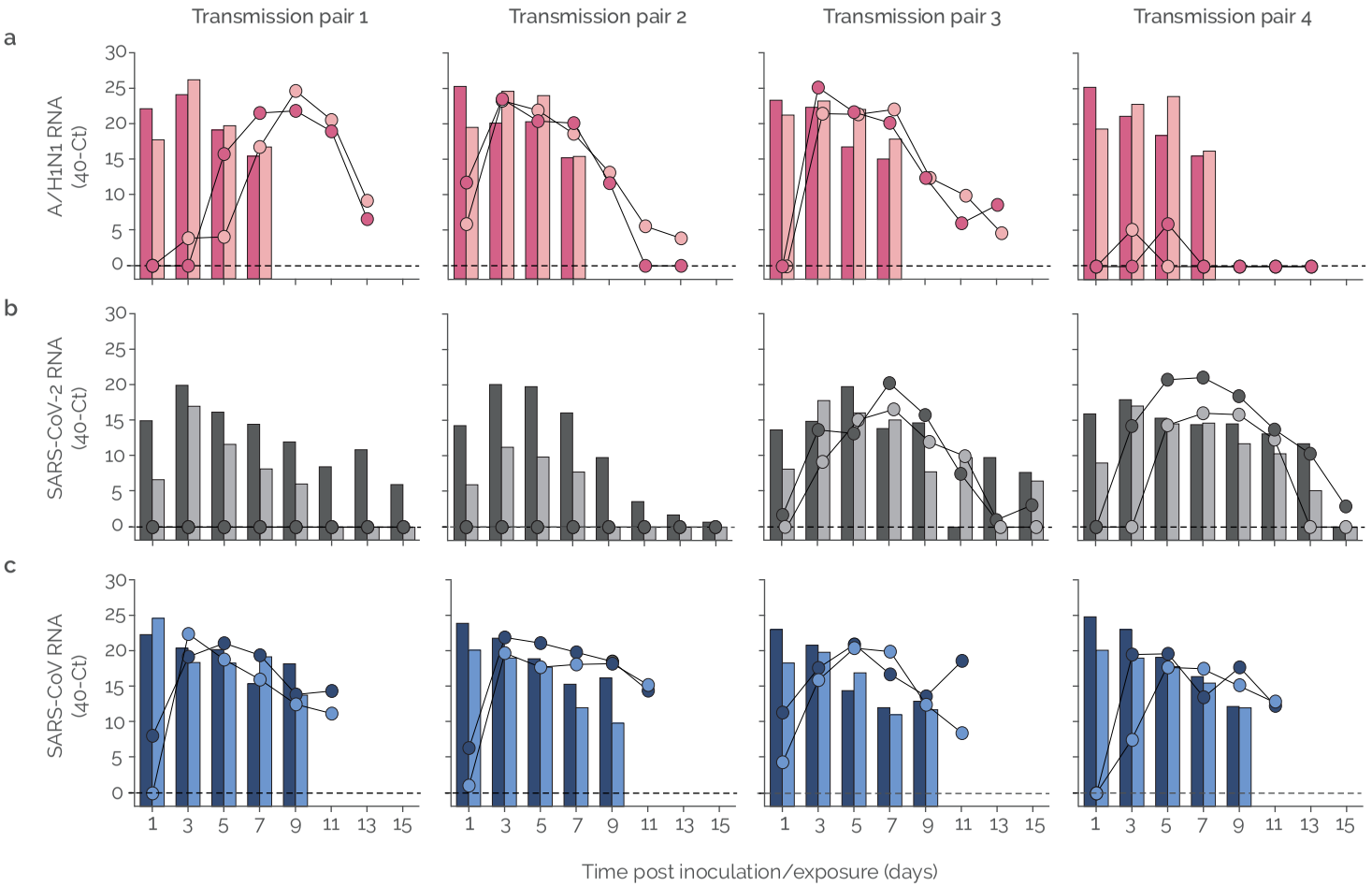
Transmission pair 1
Transmission pair 2
Transmission pair 3
Transmission pair 4
a
A/H1N1 RNA (40-Ct)
b
SARS-CoV-2 RNA (40-Ct)
c
SARS-CoV RNA (40-Ct)
0
5
10
15
20
25
30
1 3 5 7 9 11 13 15
Time post inoculation/exposure (days)

Figure 2. Virus RNA shedding in ferrets. A/H1N1 (**a**), SARS-CoV-2 (**b**), and SARS-CoV (**c**) RNA were detected by qRT-PCR in the throat (dark pink, grey, and blue) and nasal (light pink, grey, and blue) swabs collected from donor (bars) and recipient (circles) ferrets every other day. An individual donor-recipient pair is shown in each panel.

All SARS-CoV and SARS-CoV-2 positive indirect recipient ferrets had seroconverted at 11 and 17 dpe, respectively (**Fig. 4**). The two indirect recipient ferrets, in which no SARS-CoV-2 was detected, did not seroconvert. Despite the different inoculation routes and doses of the donors that were given a high virus dose in a large volume of liquid and indirect recipient animals that likely received a lower infectious dose via the air, the kinetics of virus shedding was similar in all animals, both in terms of duration and virus RNA levels. This indicated a robust replication of both SARS-CoV-2 and SARS-CoV upon transmission via the air, independent of the infectious dose and route. In general, SARS-CoV and SARS-CoV-2 RNA levels were higher in the throat swabs as compared to the nasal swabs. From each SARS-CoV and SARS-CoV-2 RNA-positive animal, infectious virus was isolated in VeroE6 cells from the throat and nasal swabs for at least two consecutive days (**Fig. 3**).

Investigating the potential of fomite transmission via the fur of ferrets

In SARS-CoV-2 outbreaks on mink farms in the Netherlands, a potential route of virus transmission through aerosolized fomites originating from bedding, fur, and food has been suggested[259]. Although the current transmission set-up was designed to prevent spill-over of large pieces like food and feces from donor to recipient cages, smaller particles such as aerosolized fur or dust from the carpet tiles in the cages, could potentially still be transmitted to the recipient cage. This has very recently been demonstrated in the guinea pig model where a virus-immune animal, whose body was contaminated with influenza virus, transmitted the virus through the air to an indirect recipient animal[249]. Indeed, measurements with an aerodynamic particle sizer in our set-up showed that particles >10 µm were present in the outlet of donor cages, but also at the inlet of the recipient cages, suggesting that despite the distance between the cages, larger particles were carried to the recipient animals due to the high flow rate (**Fig. S1**). To study if fur could serve as a carrier for infectious virus, fur swabs from the left and right flank of SARS-CoV inoculated donor ferrets were also collected in the last experiment from 3 to 9 dpi. SARS-CoV RNA was detected in fur swabs of all donor ferrets indicating that the fur of ferrets was contaminated with the virus and therefore can be a potential source for aerosolized fomite transmission (**Fig. 5**). Given the observed grooming behavior

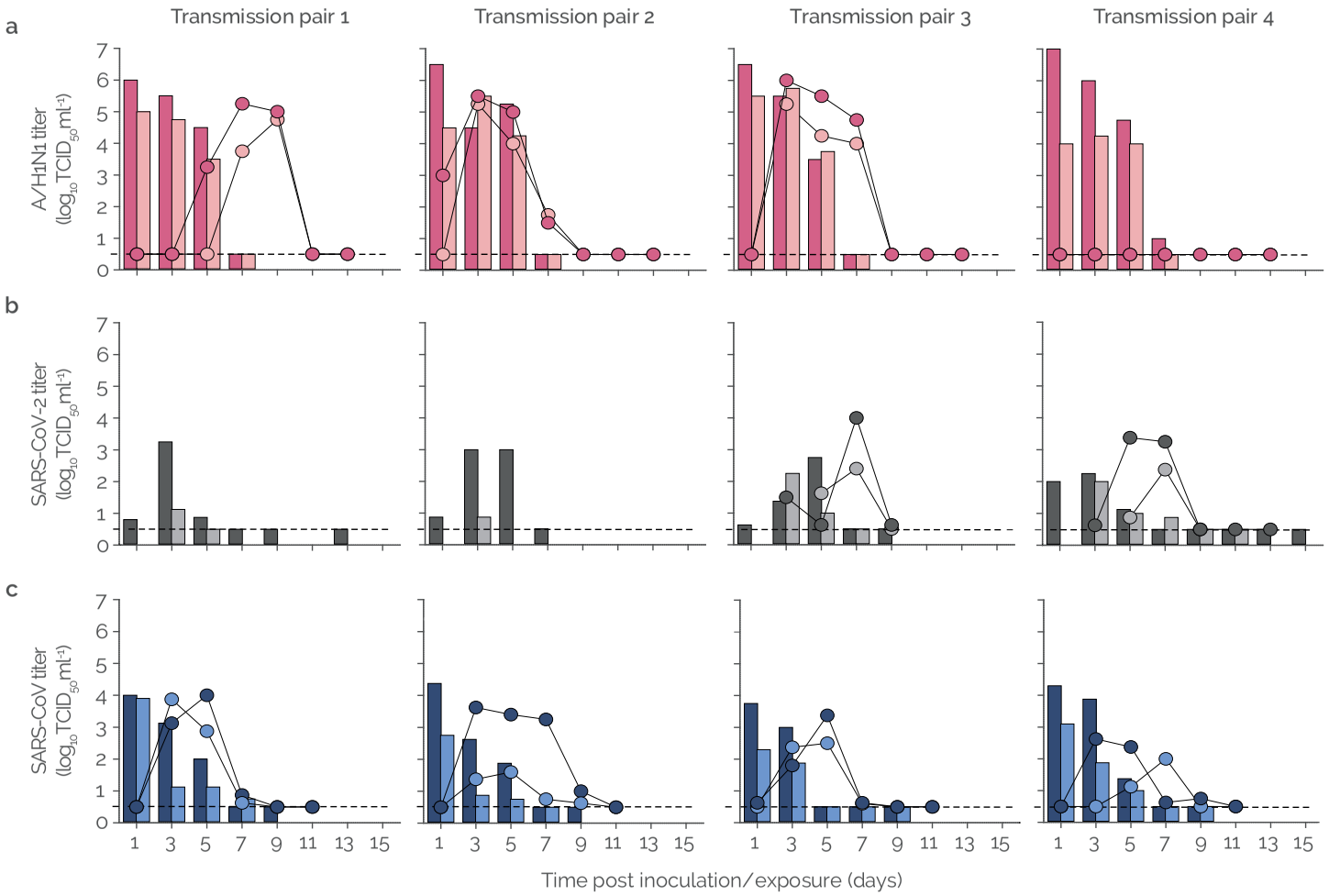
a
b
c
Transmission pair 1
Transmission pair 2
Transmission pair 3
Transmission pair 4
A/H1N1 titer (log10TCID50 ml-1)
SARS-CoV-2 titer (log10TCID50 ml-1)
SARS-CoV titer (log10TCID50 ml-1)
0
1
2
3
4
5
6
7
1 3 5 7 9 11 13 15
Time post inoculation/exposure (days)

Figure 3. Infectious virus shedding in ferrets. A/H1N1 virus (**a**), SARS-CoV-2 (**b**), and SARS-CoV (**c**) titers were detected in the throat (dark pink, grey, and blue) and nasal (light pink, grey, and blue) swabs collected from inoculated donor (bars) and indirect recipient (circles) ferrets. An individual donor-recipient pair is shown in each panel. Dotted lines indicate the detection limit.

of ferrets and the high viral loads detected in the URT, grooming was most likely the dominant route of virus transfer to the fur. SARS-CoV RNA levels were on average 240-fold (7,9 Ct) lower than those in the throat and nasal swabs of the same donor ferrets. Importantly, no infectious virus was isolated from these fur samples. The inability to detect infectious virus in fur samples was in agreement with the inability to detect infectious virus in respiratory samples with similarly low viral RNA levels.

Sequence analysis of viruses isolated from ferrets

To study if genetic changes might have contributed to the transmission of SARS-CoV-2 via the air between ferrets, Illumina next-generation sequencing was performed on the virus inoculum and on throat swabs of all four donors (3 dpi) and two indirect recipient ferrets (5 and 7 dpe). Single nucleotide polymorphisms (SNP) which were present in >5% of the total number of reads were called (**Table S1a**). Mainly, two substitutions were detected in the sequence of all virus isolates: N501T and S686G. Both residues are located in the spike protein and have been detected previously in ferrets[255]. N501T and S686G were present in all donor ferrets in the majority of reads (53–99%) and in both indirect recipient ferrets in >99% of reads. The SNP analysis of the virus isolate used to inoculate ferrets (passage 3 of the virus stock) revealed that only the substitution S686G was present in >5% of the reads (**Table S1b**). Given that these substitutions were already present in the donor ferrets, it is likely that they were selected due to adaptation to the new host, rather than having substantial importance for transmission. A replacement of asparagine by threonine at position 501 decreases the binding affinity of the spike protein to human ACE2 but might favor binding to ferret ACE2[260]. An additional L1035F substitution was detected in Nsp3 in the throat swab of a donor ferret and a synonymous mutation C10757T in Nsp5 in the throat swab of an indirect recipient ferret.

DISCUSSION

Here, it is shown that SARS-CoV can be transmitted through the air between ferrets and that both SARS-CoV and SARS-CoV-2 are transmissible through the air between ferrets over more than a meter distance, similar to a control A/H1N1 influenza virus.

In the transmission set-up described here, ferret cages were connected by a hard duct system with four 90° turns and a flow rate of approximately 100 L min^{-1}. The shortest and longest distance between the inlet and outlet of the duct system was 73 and 163 cm, respectively, so that viruses shed by the donor animal had to bridge an average distance of 118 cm before reaching the cage of the indirect recipient ferret. Based on airflow fundamentals, it is anticipated that the minimal distance of the path followed by the particles through the duct is 1 m. The duct system was designed to have an upward airflow, with the aim to prevent large particles to reach the outlet of the duct system. Unfortunately, particles >10 µm that originated from the donor cage were detected in the indirect recipient cage, which was likely due to the relatively high flow rate. As a consequence, the set-up described here does not allow the discrimination between transmission of viruses via aerosols, droplets, and aerosolized fomites, and therefore transmission between ferrets can occur via either route.

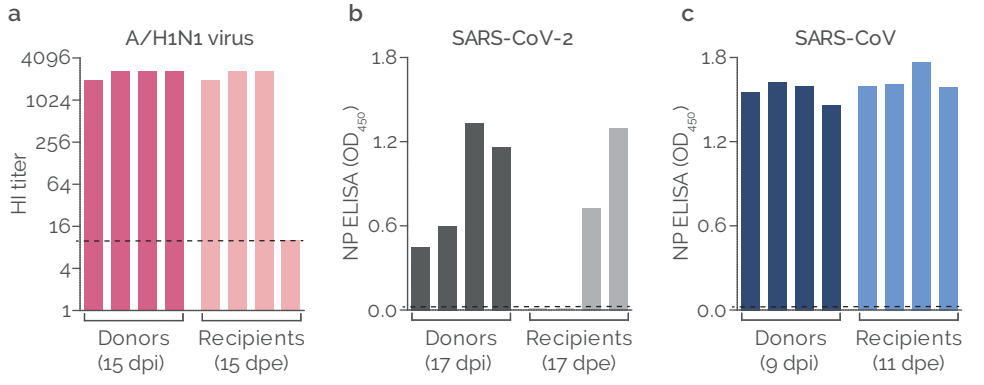

Figure 4. Antibody responses in donor and recipient ferrets. Sera were collected from donor and recipient ferrets on the indicated days. Antibody responses against A/H1N1 virus (**a**) were measured by hemagglutination inhibition (HI) assay, whereas responses against SARS-CoV-2 (**b**) and SARS-CoV (**c**) were assessed using a nucleoprotein (NP) ELISA. Dotted lines indicate the detection limit of each assay. OD: Optic density.

Ferrets and minks both belong to the Mustelinea subfamily of the Mustelidae family. Minks are the first animal species for which SARS-CoV-2 outbreaks have been reported, and to date, outbreaks have been detected on 53 mink farms in the Netherlands and on several mink farms in Denmark, Spain, and the USA[259,261]. In investigations of the first two outbreaks, 119 out of 120 serum samples collected from minks were positive, indicating that SARS-CoV-2 had spread readily through the population[259]. The high infection rate among minks together with the productive SARS-CoV-2 infection in ferrets suggests that mustelids are highly susceptible to infection with SARS-CoV-2, perhaps even more so than humans.

Epidemiological studies in humans in 2003 demonstrated that SARS-CoV transmission occurred often during the second week of illness. Virus excretion in respiratory secretions and stool followed a Gaussian distribution and peaked approximately 10 days after symptom onset when patients were often already hospitalized[262–265]. Hence, most cases of SARS-CoV human-to-human transmission occurred in healthcare settings, predominantly when adequate infection control precautions were absent. Virus transmission via the air was limited to hospital procedures where mechanical aerosol formation could not be prevented. The fact that SARS-CoV was transmitted efficiently via the air between ferrets thus does not align well with the lack of evidence for efficient SARS-CoV virus transmission via the air between humans under natural conditions. In the four indirect recipient animals that became infected with SARS-CoV upon transmission via the air, virus replication peaked as early as 3 to 5 dpe (**Fig. 3**). This demonstrated that SARS-CoV replicates remarkably faster to peak titers in ferrets as compared to the 10 days after symptom onset in humans, and indicated that ferrets are also highly susceptible for SARS-CoV as observed for SARS-CoV-2, which may have contributed to the observed high efficiency of transmission in the ferret model.

Distinctive from what was described for SARS-CoV, infection with SARS-CoV-2 is characterized by long-term shedding of virus RNA in patients, characterized by peak RNA levels on the day of symptom onset or earlier and infectious virus has primarily been successfully isolated in the initial phase of illness[265–268]. During several outbreaks in churches, nursing homes, call centers, cruise ships, and restaurants a potential role for SARS-CoV-2 transmission via the air has been debated but remained inconclusive as other transmission routes could not be excluded[269–273]. In a few studies, low concentrations of SARS-CoV-2 RNA were detected in air samples collected in healthcare settings[274–277]. However, in only one study infectious SARS-CoV-2 was isolated from air samples

collected in a hospital room, 2–4.8 m away from patients[165]. Despite the lack of evidence that exposure to SARS-CoV-2 over substantial distances poses a high infection risk, the debate about the potential role of small aerosols and large droplets in SARS-CoV-2 transmission through the air remains.

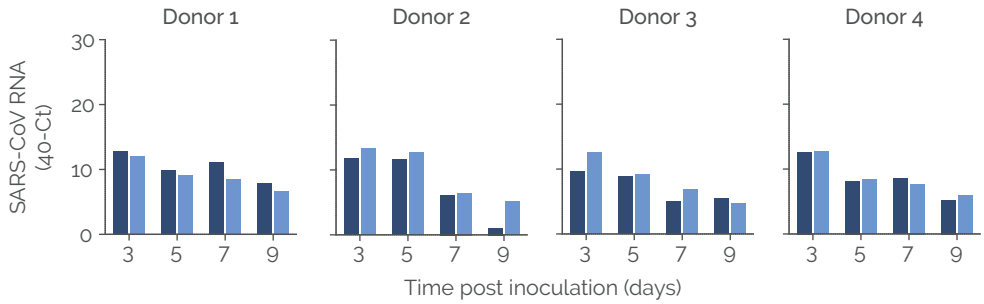

Figure 5. Detection of SARS-CoV RNA on the fur of donor ferrets. SARS-CoV RNA was detected by qRT-PCR in swabs collected from the fur on the left (dark blue) and right (light blue) flank of all four donor ferrets. Infectious virus was not detected in these samples.

It was recently shown for influenza virus in the guinea pig model that virus transmission through the air is also possible via aerosolized fomites originating from fur; animals transmitted the virus to 25% of the indirect recipient animals when 108 PFU of influenza virus was applied on fur, compared to 88% via airways and fur upon intranasal inoculation[249]. In this study, SARS-CoV RNA was detected on fur swabs from four out of four donor animals but no infectious virus was isolated. In contrast, in the guinea pig study of Asadi et al., up to 650 PFU of infectious influenza virus was recovered from the fur of intranasally inoculated animals, which is not a surprise since influenza viruses replicate to much higher titers than SARS-CoV-2 as also shown in **Fig. 3**. Thus, although transmission via aerosolized fomite particles cannot be excluded in this study, the low amounts of viral RNA and undetectable levels of infectious virus in fur as compared to those in the guinea pig studies make this a less likely route here.

The efficiency of transmission via the air depends on the anatomical site of virus excretion, the amount and duration of infectious virus shedding in the air, the ability of the virus to remain infectious in the air, and the infectious dose required to initiate an infection in an individual. It was recently shown that influenza A viruses are transmitted via the air from the nasal respiratory epithelium of ferrets[278]. In this study, SARS-CoV and SARS-CoV-2 RNA were detected in nose and throat swabs of all infected ferrets. In COVID-19 patients, SARS-CoV-2 RNA

was also easily detected in upper respiratory tract (URT) specimens, however, the detection rate of SARS-CoV RNA in URT specimens of SARS patients was low, with SARS-CoV RNA detection by RT-PCR in only 32% to 68% of the tested patients[262,266,279,280]. This lower detection rate, likely as a result of lower or no replication of SARS-CoV in the upper respiratory tract, may explain why SARS-CoV was less efficiently transmitted between humans than SARS-CoV-2.

The RNA levels and infectious SARS-CoV-2 titers detected in respiratory swabs collected from ferrets and humans were similar[281]. However, the duration and moment of peak virus shedding are different, as described above. The susceptibility to infection is probably different between ferrets and humans, especially given the difference in the efficiency of spread observed in ferrets and minks on one hand and humans on the other hand. With respect to the ferret model, it should be noted that in the experimental set-up with uni-directional airflow described here, indirect recipient animals are constantly at the right place at the right moment, which may contribute to the relatively high efficiency of virus transmission via the air. It is also important to note that superspreading events played a critical role in the epidemiology of SARS-CoV and SARS-CoV-2. Several superspreading events were identified during the SARS-CoV outbreak and there is growing evidence for such events during the COVID-19 pandemic[85,282–284]. However, it is still unknown which transmission route is predominantly involved in these events[285].

Altogether, our data on the transmissibility of SARS-CoV and SARS-CoV-2 demonstrate qualitatively that SARS-CoV and SARS-CoV-2 can remain infectious when transmitted through the air over more than one-meter distance. However, quantitatively, the data should be interpreted with caution and no conclusions can be drawn about the importance of transmission via the air in the spread of SARS-CoV-2 in the human population. Although the evidence for virus transmission via the air between humans under natural conditions is absent or very weak for both SARS-CoV and SARS-CoV-2, ferrets may represent a sensitive model to study intervention strategies aimed at preventing virus transmission.

METHODS

Viruses and cells

Influenza A/H1N1 virus (isolate A/Netherlands/602/2009) was passaged once in embryonated chicken eggs followed by two passages in Madin-Darby Canine Kidney (MDCK) cells (ATCC) 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 Hepes (Lonza), 1× nonessential amino acids (Lonza) and 20 µg ml^{-1} trypsin (Lonza). MDCK cells were inoculated at an moi of 0.01. The 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 (Greiner), 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 1× nonessential amino acids (Lonza).

SARS-CoV-2 (isolate BetaCoV/Munich/BavPat1/2020; kindly provided by Prof. Dr. C. Drosten) and SARS-CoV (isolate HKU39849, kindly provided by Prof. Dr. M. Peiris) were propagated to passage 3 and 9, respectively, in Vero E6 cells (ATCC) in Opti-MEM (1×) + GlutaMAX (Gibco), supplemented with penicillin (10,000 IU ml^{-1}, Lonza) and streptomycin (10,000 IU ml^{-1}, Lonza). Vero E6 cells were inoculated at an moi of 0.01. The supernatant was harvested at 72 hpi, cleared by centrifugation, and stored at −80 ˚C. Vero E6 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, Gibco or Lonza) supplemented with 10% fetal bovine serum (Greiner), 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) and 10 mM Hepes (Lonza). Both cell lines were maintained at 37˚C and 5% CO2.

Ferret transmission experiment

Animals were housed and experiments were performed in strict compliance with the Dutch legislation for the protection of animals used for scientific purposes (2014, implementing EU Directive 2010/63). Influenza virus, SARS-CoV-2, and Aleutian Disease Virus seronegative 6-month-old female ferrets (Mustela putorius furo), weighing 640–1215 g, were obtained from a commercial breeder (TripleF, USA). The research was conducted under a project license from the Dutch competent authority (license number 248 AVD1010020174312) and the study protocols were approved by the institutional Animal Welfare Body (Erasmus MC permit

number 17-4312-03, 17-4312-05, and 17-4312-06). Animal welfare was monitored on a daily basis. Humane endpoint criteria were defined as follows: animal does not eat or drink anymore (1), >20% loss of body weight (2), moderate to serious circulation problems or breathing difficulties (3), moderate to serious behavioral and motor changes (4) and display of moderate to serious clinical symptoms (5).

Virus inoculation of ferrets was performed under anesthesia with a mixture of ketamine/medetomidine (10 and 0.05 mg kg^{-1}, respectively) antagonized by atipamezole (0.25 mg kg^{-1}). Swabs were taken under light anesthesia using ketamine to minimize animal discomfort. Four donor ferrets were inoculated intranasally with 10^6 $TCID_{50}$ of A/H1N1 virus, 6×10^5 $TCID_{50}$ of SARS-CoV-2, or 1.6×10^6 $TCID_{50}$ of SARS-CoV (250 µL instilled dropwise in each nostril) and were housed individually in a cage. One day later, indirect recipient ferrets were added to a cage placed above the donor cage. Both cages were connected by a 15 cm wide duct system with four 90˚ turns. The average length of the duct system was 118 cm long, with an upward airflow of 100 L min^{-1} from the donor to the indirect recipient cage (**Fig. 1**). Throat and nasal swabs were collected using dry swabs (Copan, cat. 155CS01) from the ferrets every other alternating day to prevent cross-contamination. For the assessment of A/H1N1 virus transmission between ferrets, swabs of donor and indirect recipient animals were collected until 7 dpi and 13 dpe, respectively. Swabs of donor and indirect recipient animals for the SARS-CoV-2 experiment were collected until 15 dpi/dpe. Swabs of SARS-CoV inoculated donor animals were collected until 9 dpi and of indirect recipient animals until 11 dpe. For SARS-CoV inoculated animals, fur samples were collected from 3 dpi onwards by swabbing the left and right flank of animals with swabs (Copan, cat. 155CS01) wetted in virus transport medium (VTM) consisting of Minimum Essential Medium (MEM)–Eagle with Hank's BSS and 25 mM Hepes (Lonza), glycerol 99% (Sigma Aldrich), lactalbumin hydrolysate (Sigma Aldrich), 10 MU polymyxin B sulfate (Sigma Aldrich), 5 MU nystatin (Sigma Aldrich), 50 mg ml^{-1} gentamicin (Gibco) and 100 IU ml^{-1} penicillin 100 µg ml^{-1} streptomycin mixture (Lonza). All swabs were stored at –80˚C in VTM for end-point titration in Vero E6 cells as described below. Ferrets were euthanized by heart puncture under anesthesia. Blood was collected in serum-separating tubes (Greiner) and processed according to the manufacturer's instructions. Sera were heated for 30 min at 60˚C and used for the detection of virus-specific antibodies as described below. All animal experiments were performed in class III isolators in a negatively pressurized ABSL3+ facility. Average temperature and relative humidity in the isolators were 22.9 ˚C (±0.2˚C) and 53.2% (±9.0%), respectively (**Fig. S2**). After

each experiment, transmission set-ups were disassembled in the class III isolators and subsequently decontaminated by two rounds of formaldehyde fumigation. After the second fumigation, the formaldehyde gas was neutralized with ammonia. The effectiveness of the formaldehyde fumigation was validated each round with biological indicator strips (EZTest, MesaLabs). After successful fumigation, isolators and transmission set-ups were thoroughly cleaned with soap and water.

RNA isolation and qRT-PCR

Virus RNA was isolated from swabs using an in-house developed high-throughput method in a 96-well format. Sixty microliters of the sample were added to 90 µl of MagNA Pure 96 External Lysis Buffer. A known concentration of phocine distemper virus (PDV) was added to the sample as an internal control for the RNA extraction[286]. The 150 µl of sample/lysis buffer was added to a well of a 96-well plate containing 50 µl of magnetic beads (AMPure XP, Beckman Coulter). After thorough mixing by pipetting up and down at least 10 times, the plate was incubated for 15 min at room temperature. The plate was then placed on a magnetic block (DynaMag™–96 Side Skirted Magnet, ThermoFisher Scientific) and incubated for 3 min to allow the displacement of the beads towards the side of the magnet. Supernatants were carefully removed without touching the beads and beads were washed three times for 30 s (sec) at room temperature with 200 µl/well of 70% ethanol. After the last wash, a 10 µl multi-channel pipet was used to remove residual ethanol. Plates were air-dried for 6 min at room temperature. Plates were removed from the magnetic block and 50 µl of elution buffer (Roche) was added to each well and mixed by pipetting up and down 10 times. Plates were incubated for 5 min at room temperature and then placed back on the magnetic block for 2 min to allow separation of the beads. Supernatants were pipetted in a new plate and RNA was kept at 4˚C. Eight microliters of RNA were directly pipetted into a mix for qRT-PCR, containing 0.4 µl of primers and probe mix targeting the M gene of A/H1N1 virus[199], the E gene of SARS-CoV-2[287], or the NP gene of SARS-CoV[196], 0.4 µl of primers and probe mix targeting the HA gene of PDV[199], 4 µl of TaqMan™ Fast Virus 1-Step Master Mix (ThermoFisher Scientific) and 6.2 µl of PCR grade water (for primer and probe sequences see **Table S2**). Amplification and detection were performed on an ABI7700 (Thermo Fisher Scientific) using the following program: 5 min 50˚C, 20" 95˚C, [3" 95˚C, 31" 58˚C] × 45 cycles.

Virus titrations

Throat and nasal swabs were titrated in quadruplicates in either MDCK or VeroE6 cells. Briefly, confluent cells were inoculated with 10-fold (A/H1N1 virus) and 3-fold (SARS-CoV-2 and SARS-CoV) serial dilutions of the sample in serum-free EMEM supplemented with 20 µg mL^{-1} trypsin (Lonza) for MDCK cells, or Opti-MEM I (1×) + GlutaMAX, supplemented with penicillin (10,000 IU ml^{-1}), streptomycin (10,000 IU ml^{-1}), primocinTM (50 mg ml^{-1}, Invivogen) for Vero E6 cells. At one hpi, the first three dilutions were washed twice with media, and 200 µl fresh media was subsequently added to the whole plate. For swabs of ferrets from the A/H1N1 virus experiment, supernatants of cell cultures were tested for agglutination activity using turkey erythrocytes three days after inoculation. For swabs of ferrets from the SARS-CoV and SARS-CoV-2 experiments, virus positivity was assessed by reading out cytopathic effects in the cell cultures. Infectious virus titers ($TCID_{50}$ ml^{-1}) were calculated from four replicates of each throat and nasal swab using the Spearman-Karber method.

Serology

Sera of ferrets from the A/H1N1 virus experiment were tested for virus-specific antibodies using the hemagglutination inhibition (HI) assay[287]. Briefly, ferret antisera were treated with receptor destroying enzyme (Vibrio cholerae neuraminidase) and incubated at 37˚C overnight, followed by inactivation of the enzyme at 56 ˚C for 1 h. Twofold serial dilutions of the antisera, starting at a 1:10 dilution, were mixed with 25 µl phosphate-buffered saline (PBS) containing four hemagglutinating units of virus and were incubated at 37˚C for 30 min. Subsequently, 25 µl 1% turkey erythrocytes were added, and the mixture was incubated at 4˚C for 1 h. HI titers were read and expressed as the reciprocal value of the highest dilution of the serum that completely inhibited agglutination of virus and erythrocytes. Sera of ferrets from the SARS-CoV and SARS-CoV-2 experiments were tested for virus-specific antibodies using a receptor-binding domain (RBD) enzyme-linked immunosorbent assay (ELISA) as described previously, with some modifications[289]. Briefly, ELISA plates were coated overnight at 4˚C with 100 ng/well with SARS-CoV NP protein (Sino Biological Inc.). After blocking with BlockerTM BLOTTO in TBS (Life technologies) + 0.01% of Tween-20 (Sigma-Aldrich), heat-inactivated sera (diluted 1:100) were added and incubated for 1 h at 37˚C. Bound antibodies were detected using horseradish peroxidase (HRP)-labeled goat anti-ferret IgG (1:10,000; ab112770, Abcam) and

3,3',5,5'-Tetramethylbenzidine (TMB, Life Technologies) as a substrate. The absorbance of each sample was measured at 450 nm. OD-values were higher than two times the background value of negative serum (0.02) were considered positive.

Next-generation sequencing

Amplicons were generated by a SARS-CoV-2 specific multiplex PCR46 (for primer sequences, see **Table S3**). Amplicons were purified with 0.8x AMPure XP beads (Beckman Coulter) and 100 ng of DNA was converted into paired-end Illumina sequencing libraries using the KAPA HyperPlus library preparation kit (Roche), following the manufacturer's recommendations, to enable subsequent sequencing of multiple libraries in a single Illumina V3 MiSeq flowcell (2 × 300 cycles). Multiplex Adaptors (KAPA Unique Dual-Indexed Adapters Kit (Roche)) with indexes were used. FASTQ files were then imported to the CLC Genomics Workbench v20.0.3 (QIAGEN) for analysis. First, sequences were trimmed off 33 base pairs on both the 3 and 5 ends to remove primer sequences and also using Phred quality score threshold of 20. The trimmed sequences were mapped to the reference sequence (GISAID ID EPI_ISL 406862) with the following default parameters (match score = 1, mismatch cost = 2, insertion cost = 3, length fraction = 0.5, and similarity fraction = 8). Variants were called with the Basic Variant Detection tool. Single nucleotide polymorphisms that were present in both the forward and reverse reads with a 100x minimum coverage and a minimum variant count of 5 (5%) were called.

Particle size measurements

To determine the number and particle size distribution of droplets and aerosols entering and exiting the tubing system, particles at the outlet of the donor and the inlet of the indirect recipient cage were measured with an aerodynamic particle sizer (APS; Model Solair 1100+, Lighthouse Worldwide Solutions Benelux BV, **Fig. S1**). Measurements were recorded every minute for a total of 60 min. To determine the number and size of particles produced by the caging environment, as well as by a ferret, measurements were performed with and without an uninfected ferret present in the bottom cage. In addition, the activity of the ferret was observed visually and recorded in 5-min intervals for 60 min.

DECLARATIONS

Data availability

All data are available from the corresponding author (S.H.) on reasonable request. The source data underlying **Figs. 2, 3, 4, 5, Supplementary Figs. 1** and **2** are provided as a Source data file. The sequencing raw data were deposited in the NCBI Sequence Read Archive (SRA) under the BioProject PRJNA700531. Source data are provided with this paper.

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Author contributions

J.K. and S.H. conceived, designed, analysed and performed the work and wrote the manuscript. A.M. helped with the design of the transmission set-up. D.M., T.B., P.L. and M.R. helped with performing the work. R.A.M.F. helped with the design of the work, interpretation of the data and manuscript revision. All authors read and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Table S1. Detection of single nucleotide polymorphisms by Illumina sequencing

a. Single nucleotide polymorphisms in the ferret samples present at >5%

	Time after inoculation (dpi)/exposure (dpe)	Nt change	Aa change	Protein	Reads with the specified substitution (%)
Donor ferret 1	3 dpi	A23032C	N501T	Spike	95.0
		A23586G	S686G	Spike	96.8
Donor ferret 2	3 dpi	G228T	-	NA (5'UTR)	8.3
		A23032C	N501T	Spike	53.0
		A23586G	S686G	Spike	84.3
Donor ferret 3	3 dpi	A23032C	N501T	Spike	98.0
		A23586G	S686G	Spike	98.3
		T29552A	F9L	ORF10	15.2
Donor ferret 4	3 dpi	G228T	-	NA (5'UTR)	6.1
		C5790T	L1035F	Nsp3	34.0
		A23032C	N501T	Spike	98.8
		A23586G	S686G	Spike	99.0
Indirect recipient ferret 3	7 dpe	A23032C	N501T	Spike	99.6
		A23586G	S686G	Spike	99.6
		G29719T	-	NA (3'UTR)	5.8
Indirect recipient ferret 4	5 dpe	G228T	-	NA (5'UTR)	5.3
		C10757T	D245D	Nsp5	99.3
		A23032C	N501T	Spike	99.8
		A23586G	S686G	Spike	99.2

b. Presence of the ferret sample single nucleotide polymorphisms in the virus stocks (>1%)

			Reads with the specified substitution (%)	
Nt change	Aa position	Protein	Virus stock P1	Virus stock P3 VeroE6
G228T	-	5'UTR	<1	<1
C5790T	L1035F	Nsp3	<1	<1
C10757T	D245D	Nsp5	<1	<1
A23032C	N501T	Spike	2.0	<1
A23586G	S686G	Spike	15.2	8.1
T29552A	F9L	ORF10	<1	<1
G29719T	-	3'UTR	<1	<1

Nt: nucleotide; Aa: amino acid; NA: not applicable

Table S2. Sequences of primers and probes used for qRT-PCR analysis

Virus	Oligo name & orientation	Sequence (5'-3')
A/H1N1 virus	Forward primer	CTTCTRACCGAGGTCGAAACGTA
	Reverse primer	TCTTGTCTTTAGCCAYTCCATGAG
	Probe	FAM-TCAGGCCCCCTCAAAGCCGAGA-BHQ
SARS-CoV-2	Forward primer	ACAGGTACGTTAATAGTTAATAGCGT
	Reverse primer	ATATTGCAGCAGTACGCACACA
	Probe	FAM-ACACTAGCCATCCTTACTGCGCTTCG-BHQ
SARS-CoV	Forward primer	CAAACATTGGCCG CAAATT
	Reverse primer	CAATGCGTGACA TTCCAAAGA
	Probe	FAM-CACAATTTGCTCCAAGTGCCTCTGCA-BHQ
PDV	Forward primer	CGGGTGCCTTTTACAAGAAC
	Reverse primer	TTCTTTCCTCAACCTCGTCC
	Probe	Cy5-ATGCAAGGGCCAATTCTTCCAAGTT-BHQ

Table S3. Sequences of primers used for the generation of amplicons for whole-genome sequencing and next-generation sequencing

Mix 1

Name	Sequence	Orientation	Volume
SARS-CoV-2_1_LEFT	ACCAACCAACTTTCGATCTCTTGT	FWD	10µl
SARS-CoV-2_1_RIGHT	CGAGCATCCGAACGTTTGATGA	REV	10µl
SARS-CoV-2_3_LEFT	ACGAGCTTGGCACTGATCCTTA	FWD	10µl
SARS-CoV-2_3_RIGHT	GGTTGCATTCATTTGGTGACGC	REV	10µl
SARS-CoV-2_5_LEFT	TGTCCAGCATGTCACAATTCAGA	FWD	10µl
SARS-CoV-2_5_RIGHT	TACAACACGAGCAGCCTCTGAT	REV	10µl
SARS-CoV-2_7_LEFT	TGGCACTGTTTATGAAAAACTCAAACC	FWD	10µl
SARS-CoV-2_7_RIGHT	TTTCGAGCAACATAAGCCCGTT	REV	10µl
SARS-CoV-2_9_LEFT	TCACTTTTGAACTTGATGAAAGGATTGA	FWD	10µl
SARS-CoV-2_9_RIGHT	GATTGTCCTCACTGCCGTCTTG	REV	10µl
SARS-CoV-2_11_LEFT	AAACATGGAGGAGGTGTTGCAG	FWD	20µl
SARS-CoV-2_11_RIGHT	TCTTGTTTTCTCTGTTCAACTGAAGGT	REV	20µl
SARS-CoV-2_13_LEFT	GCTCCATATATAGTGGGTGATGTTGT	FWD	10µl
SARS-CoV-2_13_RIGHT	AGCCATGTGTTACATAGCCAAGTG	REV	10µl
SARS-CoV-2_15_LEFT	CTATTCTGGACAATCTACACAACTAGGT	FWD	10µl
SARS-CoV-2_15_RIGHT	AGCATCTTGTAGAGCAGGTGGA	REV	10µl
SARS-CoV-2_17_LEFT	GCTGTTATGTACATGGGCACACT	FWD	10µl
SARS-CoV-2_17_RIGHT	GCTTGCGTTTGGATATGGTTGG	REV	10µl
SARS-CoV-2_19_LEFT	TGTTACATAAACCTATTGTTTGGCATGT	FWD	10µl
SARS-CoV-2_19_RIGHT	TCCCAAGGGACACTATTAACAGCA	REV	10µl
SARS-CoV-2_21_LEFT	AGCAAAGAATACTGTTAAGAGTGTCGG	FWD	20µl
SARS-CoV-2_21_RIGHT	TCGGGGCCATTTGTACAAGATT	REV	20µl
SARS-CoV-2_23_LEFT	ACTATTGTTAATGGTGTTAGAAGGTCCT	FWD	10µl
SARS-CoV-2_23_RIGHT	GCAACTTCCGCACTATCACCAA	REV	10µl
SARS-CoV-2_25_LEFT	TGTCTTAAATTGTCACATCAATCTGACAT	FWD	10µl
SARS-CoV-2_25_RIGHT	TCACGAGTGACACCACCATCAA	REV	10µl
SARS-CoV-2_27_LEFT	CTGGTTTGCCTGGCACGATATT	FWD	10µl
SARS-CoV-2_27_RIGHT	TCTACACCACAGAAAACTCCTGGT	REV	10µl
SARS-CoV-2_29_LEFT	ACAGTCATGTAGTTGCCTTTAATACTTTAC	FWD	10µl
SARS-CoV-2_29_RIGHT	GAGCCTTTGCGAGATGACAACA	REV	10µl
SARS-CoV-2_31_LEFT	AACGGTCTTTGGCTTGATGACG	FWD	10µl
SARS-CoV-2_31_RIGHT	ACCTTCTAAGTCTGTGCCAGCA	REV	10µl
SARS-CoV-2_33_LEFT	TGTGGCTATGAAGTACAATTATGAACCT	FWD	10µl
SARS-CoV-2_33_RIGHT	AGCTACAGTGGCAAGAGAAGGT	REV	10µl
SARS-CoV-2_35_LEFT	TGGTGCTAGGAGAGTGTGGACA	FWD	10µl
SARS-CoV-2_35_RIGHT	GGCTACTTTGATACAAGGTTTGCC	REV	10µl
SARS-CoV-2_37_LEFT	CTTTCCATGCAGGGTGCTGTAG	FWD	10µl
SARS-CoV-2_37_RIGHT	GTTTGGCTGCTGTTGTAAGAGGT	REV	10µl
SARS-CoV-2_39_LEFT	ACAATTCACCTAATTTAGCATGGCCT	FWD	10µl
SARS-CoV-2_39_RIGHT	TTGGTTGTCCCCCACTAGCTAG	REV	10µl
SARS-CoV-2_41_LEFT	AGTATGTACAAATACCTACAACTTGTGCT	FWD	10µl
SARS-CoV-2_41_RIGHT	AGCATAGACGAGGTCTGCCATT	REV	10µl
SARS-CoV-2_43_LEFT	TGGTATTGTTGGTGTACTGACATTAGA	FWD	10µl
SARS-CoV-2_43_RIGHT	AGGGTCAGCAGCATACACAAGT	REV	10µl

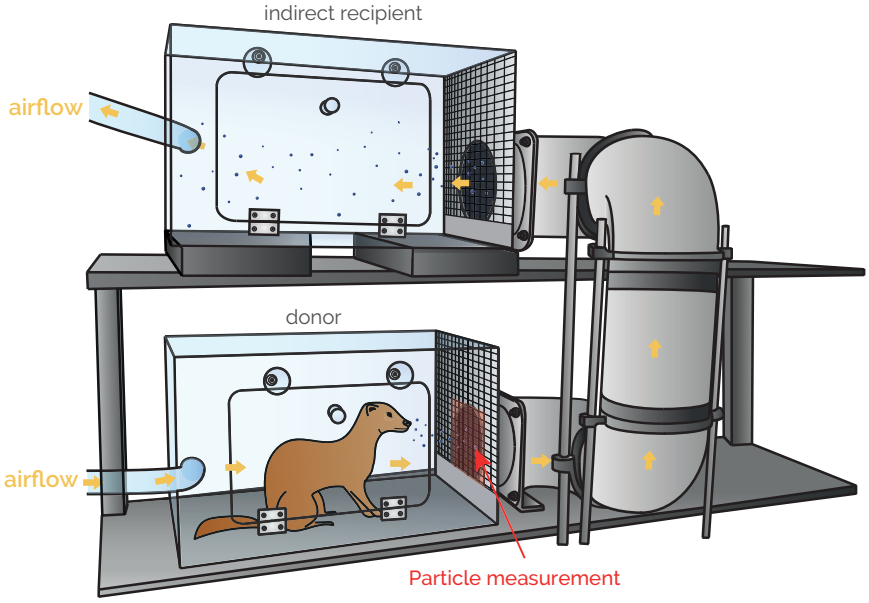
Name	Sequence	Orientation	Volume
SARS-CoV-2_45_LEFT	CACTTCTTCTTTGCTCAGGATGGT	FWD	10µl
SARS-CoV-2_45_RIGHT	AACATGTTGTGCCAACCACCAT	REV	10µl
SARS-CoV-2_47_LEFT	TGTAGCTTGTCACACCGTTTCT	FWD	10µl
SARS-CoV-2_47_RIGHT	AGTAAGGTCAGTCTCAGTCCAACA	REV	10µl
SARS-CoV-2_49_LEFT	AGGAGTATGCTGATGTCTTTCATTTGT	FWD	10µl
SARS-CoV-2_49_RIGHT	CACCAGCATTTGTCCAGTCACA	REV	10µl
SARS-CoV-2_51_LEFT	TCTTTCATGGGAAGTTGGTAAACCT	FWD	20µl
SARS-CoV-2_51_RIGHT	TCACATAGTGCATCAACAGCGG	REV	20µl
SARS-CoV-2_53_LEFT	TGTCAATGCCAGATTAYGTGCT	FWD	10µl
SARS-CoV-2_53_RIGHT	CAAGAGTGAGCTGTTTCAGTGGT	REV	10µl
SARS-CoV-2_55_LEFT	TGTTGACACTAAATTCAAAACTGAAGGT	FWD	20µl
SARS-CoV-2_55_RIGHT	TGTCAACTCAAAGCCATGTGCC	REV	20µl
SARS-CoV-2_57_LEFT	CGTTTATGATTGATGTTCAACAATGGGG	FWD	10µl
SARS-CoV-2_57_RIGHT	ACAACCAGGCAAGTTAAGGTTAGA	REV	10µl
SARS-CoV-2_59_LEFT	AGTCTCATGGAAAACAAGTAGTGTCA	FWD	10µl
SARS-CoV-2_59_RIGHT	ATTAGCAGCAATGTCCACACCC	REV	10µl
SARS-CoV-2_61_LEFT	AGAAATGCCCGTAATGGTGTTCT	FWD	10µl
SARS-CoV-2_61_RIGHT	TGAACCTGTTTGCGCATCTGTT	REV	10µl
SARS-CoV-2_63_LEFT	TGGCCATGTAGAAACATTTTACCCA	FWD	10µl
SARS-CoV-2_63_RIGHT	ATAGCCACGGAACCTCCAAGAG	REV	10µl
SARS-CoV-2_65_LEFT	GGCAAACCACGCGAACAAATAR	FWD	10µl
SARS-CoV-2_65_RIGHT	ACCTCTTAGTACCATTGGTCCCA	REV	10µl
SARS-CoV-2_67_LEFT	CAATTTTGTAATGATCCATTTTTGGGTGT	FWD	10µl
SARS-CoV-2_67_RIGHT	GGTCAAGTGCACAGTCTACAGC	REV	10µl
SARS-CoV-2_69_LEFT	ACTGTGTTGCTGATTATTCTGTCCT	FWD	20µl
SARS-CoV-2_69_RIGHT	TAGGTCCACAAACAGTTGCTGG	REV	20µl
SARS-CoV-2_71_LEFT	ACTTCTAACCAGGTTGCTGTTCTT	FWD	10µl
SARS-CoV-2_71_RIGHT	CAGCTATTCCAGTTAAAGCACGGT	REV	10µl
SARS-CoV-2_73_LEFT	CTTGCAGATGCTGGCTTCATCA	FWD	10µl
SARS-CoV-2_73_RIGHT	TGCACTTCAGCCTCAACTTTGT	REV	10µl
SARS-CoV-2_75_LEFT	CTTCCCTCAGTCAGCACCTCAT	FWD	10µl
SARS-CoV-2_75_RIGHT	CAAGCCAGCTATAAAACCTAGCCA	REV	10µl
SARS-CoV-2_77_LEFT	TGGAACTGTAACTTTGAAGCAAGGT	FWD	10µl
SARS-CoV-2_77_RIGHT	GACTTGTTGTGCCATCACCTGA	REV	10µl
SARS-CoV-2_79_LEFT	GGTGTTGAACATGTTACCTTCTTCAT	FWD	10µl
SARS-CoV-2_79_RIGHT	GTACCGTTGGAATCTGCCATGG	REV	10µl
SARS-CoV-2_81_LEFT	CTTGTTTTGTGCTTGCTGCTGT	FWD	10µl
SARS-CoV-2_81_RIGHT	ACTGCTACTGGAATGGTCTGTGT	REV	10µl
SARS-CoV-2_83_LEFT	TGAAGAGCAACCAATGGAGATTGA	FWD	10µl
SARS-CoV-2_83_RIGHT	TGTTCGTTTAGGCGTGACAAGT	REV	10µl
SARS-CoV-2_85_LEFT	AGCACCTTTAATTGAATTGTGCGTG	FWD	10µl
SARS-CoV-2_85_RIGHT	CGTCTGGTAGCTCTTCGGTAGT	REV	10µl
SARS-CoV-2_87_LEFT	AAAAGATCACATTGGCACCCGC	FWD	10µl
SARS-CoV-2_87_RIGHT	CGACATTCCGAAGAACGCTGAA	REV	10µl
SARS-CoV-2_89_LEFT	AGGCTGATGAAACTCAAGCCTT	FWD	20µl
SARS-CoV-2_89_RIGHT	AAAATCACATGGGGATAGCACTACT	REV	20µl

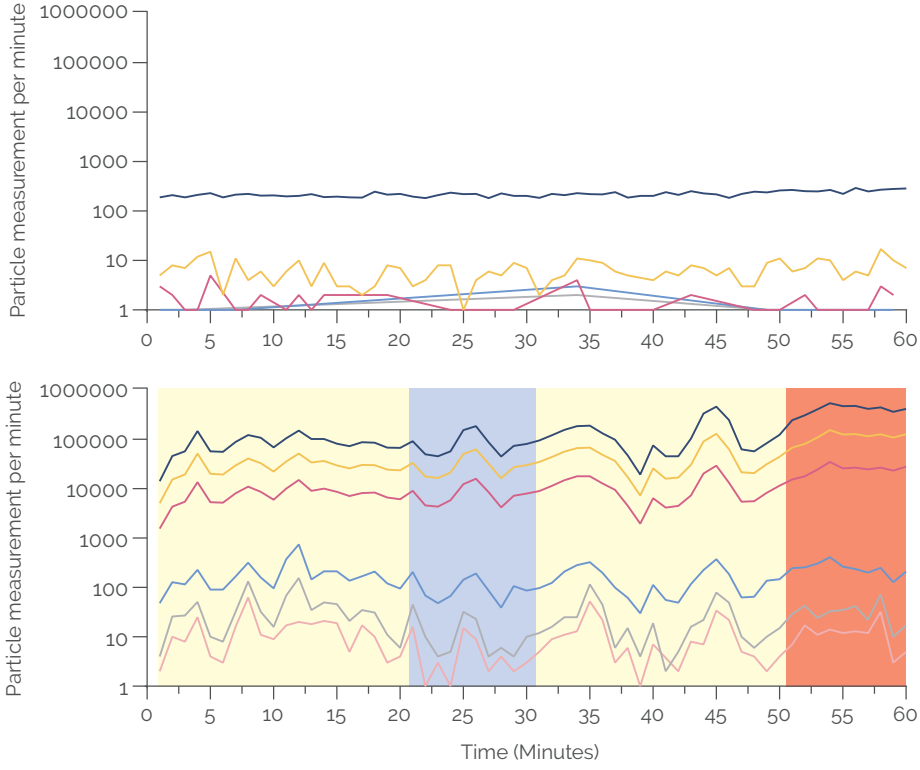
Mix 2

Name	Sequence	Orientation	Volume
SARS-CoV-2_2_LEFT	TCGTACGTGGCTTTGGAGACTC	FWD	10µl
SARS-CoV-2_2_RIGHT	ATGCACTCAAGAGGGTAGCCAT	REV	10µl
SARS-CoV-2_4_LEFT	ACACCTTCAATGGGGAATGTCC	FWD	10µl
SARS-CoV-2_4_RIGHT	AGGCACACTTGTTATGGCAACC	REV	10µl
SARS-CoV-2_6_LEFT	TGTGAAAGGTTTGGATTATAAAGCATTCA	FWD	10µl
SARS-CoV-2_6_RIGHT	ACAGGTGACAATTTGTCCACCG	REV	10µl
SARS-CoV-2_8_LEFT	AGGGAGAAACACTTCCCACAGA	FWD	10µl
SARS-CoV-2_8_RIGHT	AATCAATGCCCAGTGGTGTAAGT	REV	10µl
SARS-CoV-2_10_LEFT	TGAGTATGGTACTGAAGATGATTACCAAG	FWD	10µl
SARS-CoV-2_10_RIGHT	GCCGACAACATGAAGACAGTGT	REV	10µl
SARS-CoV-2_12_LEFT	ACTGTTCGCACGAAYGTCTACT	FWD	10µl
SARS-CoV-2_12_RIGHT	CCTGACCCGGGTAAGTGGTTAT	REV	10µl
SARS-CoV-2_14_LEFT	GTTTCAACTATACAGCGTAAATATAAGGGT	FWD	20µl
SARS-CoV-2_14_RIGHT	CGTGTGGAGGTTAATGTTGTCTACT	REV	20µl
SARS-CoV-2_16_LEFT	AGGTACATGTCAGCATTAAATCACACT	FWD	10µl
SARS-CoV-2_16_RIGHT	AGTTCATACTGAGCAGGTGGTG	REV	10µl
SARS-CoV-2_18_LEFT	CAGTTACACAACAACCATAAAACCAGT	FWD	10µl
SARS-CoV-2_18_RIGHT	GATTATCCATTCCCTGCGCGTC	REV	10µl
SARS-CoV-2_20_LEFT	TACAGAAGAGGTTGGCCACACA	FWD	7.5µl
SARS-CoV-2_20_RIGHT	AACACYTAAAGCAGCGGTTGAG	REV	7.5µl
SARS-CoV-2_22_LEFT	AGTTGCAGAGTGGTTTTTGGCA	FWD	10µl
SARS-CoV-2_22_RIGHT	ACTGTAGTGACAAGTCTCTCGCA	REV	10µl
SARS-CoV-2_24_LEFT	AGCTAATAACACTAAAGGTTCATTGCCT	FWD	10µl
SARS-CoV-2_24_RIGHT	TGACTTTTTGCTACCTGCGCAT	REV	10µl
SARS-CoV-2_26_LEFT	TGGTTGAAGCAGTTAATTAAAGTTACACT	FWD	10µl
SARS-CoV-2_26_RIGHT	TTCAGCAGCCAAAACACAAGCT	REV	10µl
SARS-CoV-2_28_LEFT	CCTTGAAGGTTCTGTTAGAGTGGT	FWD	10µl
SARS-CoV-2_28_RIGHT	AGGTGTGAACATAACCATCCACTG	REV	10µl
SARS-CoV-2_30_LEFT	AGAAATGTATCTAAAGTTGCGTAGTGATG	FWD	10µl
SARS-CoV-2_30_RIGHT	CCCTGAGTTGAACATTACCAGCC	REV	10µl
SARS-CoV-2_32_LEFT	TACCAATGTGCTATGAGGCCCA	FWD	10µl
SARS-CoV-2_32_RIGHT	GCACTACCCAATATGGTACGTCC	REV	10µl
SARS-CoV-2_34_LEFT	GTCCAGAGTACTCAATGGTCTTTGT	FWD	10µl
SARS-CoV-2_34_RIGHT	ACCTCTGGCCAAAAACATGACA	REV	10µl
SARS-CoV-2_36_LEFT	CGCTACTTTAGACTGACTCTTGGTG	FWD	10µl
SARS-CoV-2_36_RIGHT	ATCACCATTAGCAACAGCCTGC	REV	10µl
SARS-CoV-2_38_LEFT	AGATCTGAGGACAAGAGGGCAA	FWD	10µl
SARS-CoV-2_38_RIGHT	TGTCATCAGTGCAAGCAGTTTGT	REV	10µl
SARS-CoV-2_40_LEFT	GGTATGGTACTTGGTAGTTTAGCTGC	FWD	10µl
SARS-CoV-2_40_RIGHT	ACGATTGTGCATCAGCTGACTG	REV	10µl
SARS-CoV-2_42_LEFT	TCTCTAACTACCAACATGAAGAAACAATTT	FWD	20µl
SARS-CoV-2_42_RIGHT	GCAGTTAAAGCCCTGGTCAAGG	REV	20µl
SARS-CoV-2_44_LEFT	TGGACCACTAGTGAGAAAAATATTTGTTG	FWD	10µl
SARS-CoV-2_44_RIGHT	ACAGCCACCATCGTAACAATCA	REV	10µl
SARS-CoV-2_46_LEFT	TGCAAAGAATAGAGCTCGCACC	FWD	10µl
SARS-CoV-2_46_RIGHT	TGCATTAACATTGGCCGTGACA	REV	10µl

Name	Sequence	Orientation	Volume
SARS-CoV-2_48_LEFT	CTCTCTGACGATGCTGTTGTGT	FWD	10µl
SARS-CoV-2_48_RIGHT	TGCGGTGTGTACATAGCCTCAT	REV	10µl
SARS-CoV-2_50_LEFT	AGGAGGTATGAGCTATTATTGTAAATCACA	FWD	10µl
SARS-CoV-2_50_RIGHT	GTTGTACCTCGGTAAACAACAGCA	REV	10µl
SARS-CoV-2_52_LEFT	TGCAAATTATCAAAAGGTTGGTATGCA	FWD	10µl
SARS-CoV-2_52_RIGHT	CCGAGGAACATGTCTGGACCTA	REV	10µl
SARS-CoV-2_54_LEFT	TGGAGAAAAGCTGTCTTTATTTCACCT	FWD	10µl
SARS-CoV-2_54_RIGHT	GCTTCTTCGCGGGTGATAAACA	REV	10µl
SARS-CoV-2_56_LEFT	ACCACCGCCTGGAGATCAATTT	FWD	10µl
SARS-CoV-2_56_RIGHT	CGCTTAACAAAGCACTCGTGGA	REV	10µl
SARS-CoV-2_58_LEFT	GCCTTGTAGTGACAAAGCTTATAAAATAGA	FWD	10µl
SARS-CoV-2_58_RIGHT	AAACCCACAAGCTAAAGCCAGC	REV	10µl
SARS-CoV-2_60_LEFT	CAGGGTGAAGTACCAGTTTCTATCATT	FWD	10µl
SARS-CoV-2_60_RIGHT	GAGTAAAGTAAGTTTCAGGTAATTGTTGG	REV	10µl
SARS-CoV-2_62_LEFT	TCGTTTATGGAGATTTTAGTCATAGTCAGT	FWD	10µl
SARS-CoV-2_62_RIGHT	TTGCGACATTCATCATTATGCCTTT	REV	10µl
SARS-CoV-2_64_LEFT	CTGTACATACAGCTAATAAATGGGATCTCA	FWD	10µl
SARS-CoV-2_64_RIGHT	TTTGACCTTCTTTTAAAGACATAACAGCA	REV	10µl
SARS-CoV-2_66_LEFT	ACCCCCTGCATACACTAATTCTYT	FWD	10µl
SARS-CoV-2_66_RIGHT	ACCCTGTTTTCCTTCAAGGTCC	REV	10µl
SARS-CoV-2_68_LEFT	ACATAGAAGTTATTTGACTCCTGGTGA	FWD	10µl
SARS-CoV-2_68_RIGHT	CCCTGGAGCGATTTGTCTGACT	REV	10µl
SARS-CoV-2_70_LEFT	CCGGTAGCACACCTTGTAATGG	FWD	10µl
SARS-CoV-2_70_RIGHT	CCCCTATTAAACAGCCTGCACG	REV	10µl
SARS-CoV-2_72_LEFT	TGTTACCACAGAAATTCTACCAGTGT	FWD	10µl
SARS-CoV-2_72_RIGHT	TACCCGCTAACAGTGCAGAAGT	REV	10µl
SARS-CoV-2_74_LEFT	GTGCACTTGGAAAACTTCAAGATGT	FWD	10µl
SARS-CoV-2_74_RIGHT	TGTTACAAACCAGTGTGTGCCA	REV	10µl
SARS-CoV-2_76_LEFT	GTTGATTTAGGTGACATCTCTGGCA	FWD	7.5µl
SARS-CoV-2_76_RIGHT	AGCGCTCTGAAAAACAGCAAGA	REV	7.5µl
SARS-CoV-2_78_LEFT	CTTTGGCTTTGCTGGAAATGCC	FWD	10µl
SARS-CoV-2_78_RIGHT	GTGCTTACAAAGGCACGCTAGT	REV	10µl
SARS-CoV-2_80_LEFT	ACGTGAGTCTTGTAAAACCTTCTTTTT	FWD	10µl
SARS-CoV-2_80_RIGHT	AATGACCACATGGAACGCGTAC	REV	10µl
SARS-CoV-2_82_LEFT	GGACCTGCCTAAAGAAATCACTGT	FWD	10µl
SARS-CoV-2_82_RIGHT	TGCCCTCGTATGTTCCAGAAGA	REV	10µl
SARS-CoV-2_84_LEFT	CTTCACACTCAAAAGAAAGACAGAATGA	FWD	10µl
SARS-CoV-2_84_RIGHT	ACGAACAACGCACTACAAGACT	REV	10µl
SARS-CoV-2_86_LEFT	GGCCCCAAGGTTTACCCAATAA	FWD	10µl
SARS-CoV-2_86_RIGHT	CTGTTGCGACTACGTGATGAGG	REV	10µl
SARS-CoV-2_88_LEFT	TAACACAAGCTTTCGGCAGACG	FWD	10µl
SARS-CoV-2_88_RIGHT	GTGGTCTGCATGAGTTTAGGCC	REV	10µl

a


indirect recipient
airflow
donor
airflow
Particle measurement


Particle measurement per minute
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1000
100
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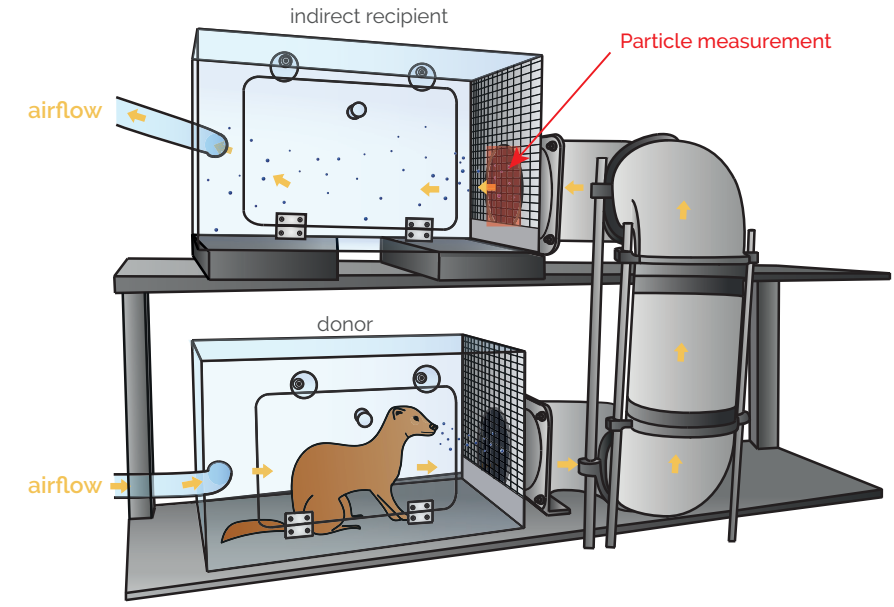
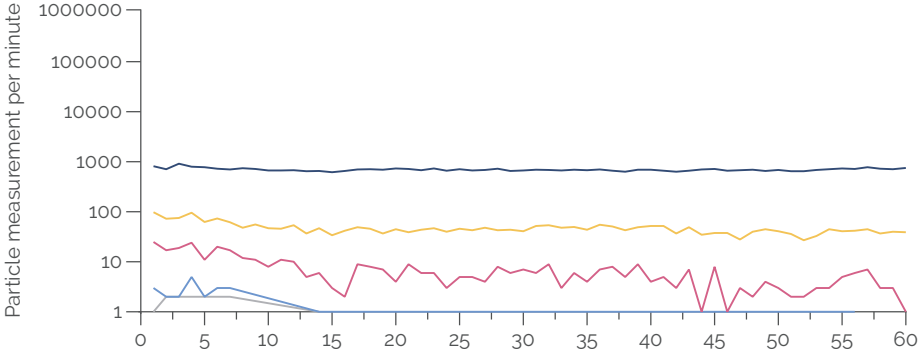
55
60
Particle measurement per minute
Time (Minutes)

b


indirect recipient
Particle measurement
airflow
donor
airflow


Particle measurement per minute
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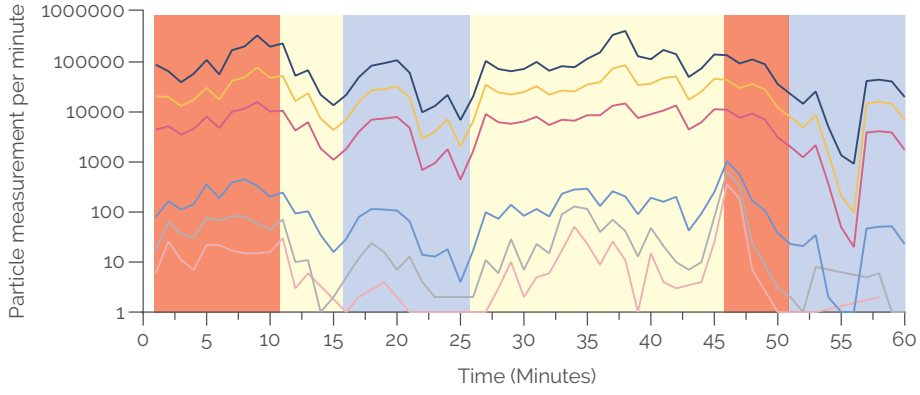
Particle measurement per minute
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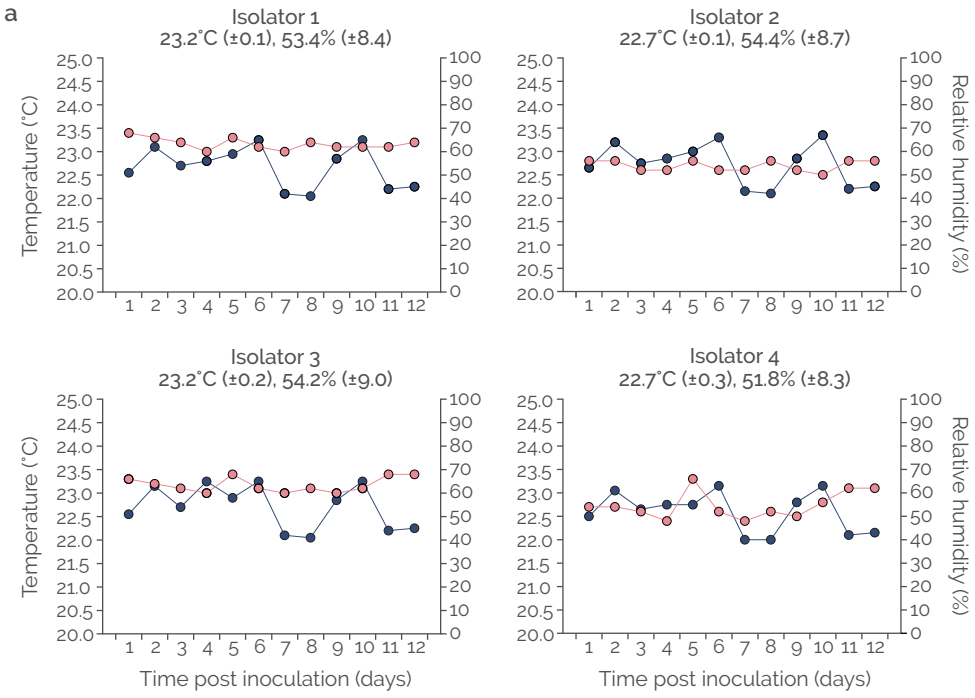
55
60
Time (Minutes)

Figure S1. Number and particle size distribution of aerosols and droplets in the experimental set-up. The number and size of particles entering (**a**) and exiting (**b**) the tubing system was recorded continuously with an aerodynamic particle sizer (APS) every minute for a total of 60 min. Measurements were performed without (upper graph) and with (bottom graph) one uninfected ferret present in the bottom cage to determine the number and size distribution of particles (0.3 µm: dark blue, 0.5 µm: yellow, 1.0 µm: dark pink, 3.0 µm: light blue, 5.0 µm: grey and 10.0 µm light pink) originating from the caging environment itself and from the ferret. In order to correlate the amount and size of particles with the activity of the ferret, the animal's behavior was recorded every 5 minutes. The activity was scored based on three categories and shown as color shading in the bottom graphs: calm (blue), active (yellow) and agitated (red). Calm included resting, occasional grooming; active included moving around, exploring, occasional scratching of walls and carpet; agitated included agitated movement, intense scratching of walls and carpet.


a
Isolator 1
23.2°C (±0.1), 54.4% (±8.4)
Isolator 2
22.7°C (±0.1), 54.4% (±8.7)
Isolator 3
23.2°C (±0.2), 54.2% (±9.0)
Isolator 4
22.7°C (±0.3), 51.8% (±8.3)
Temperature (°C)
Relative humidity (%)
Time post inoculation (days)

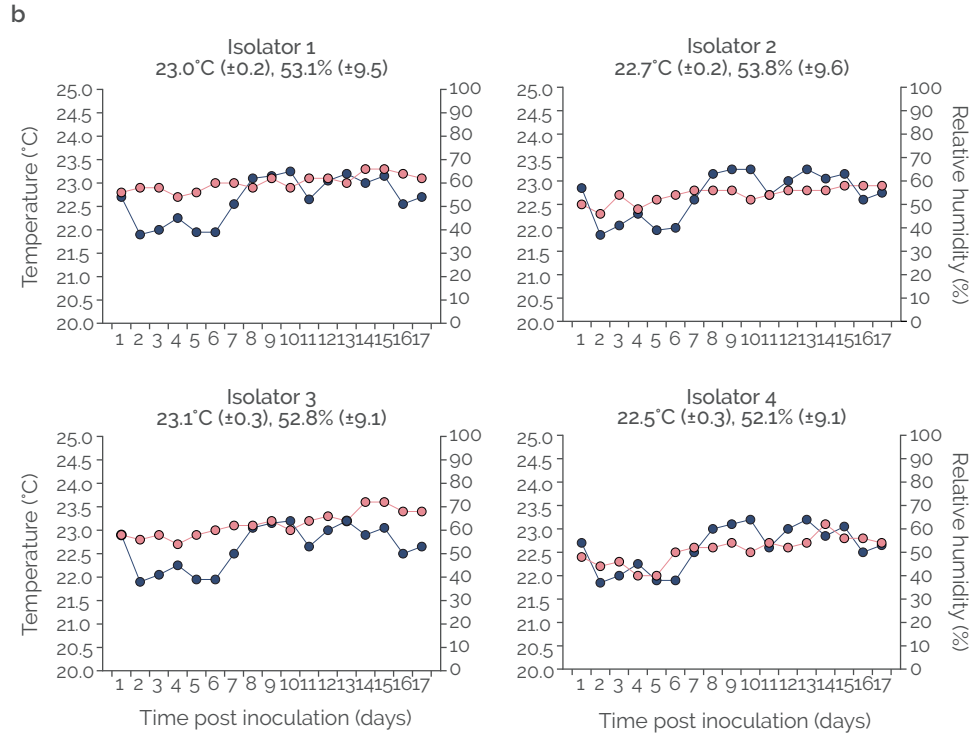
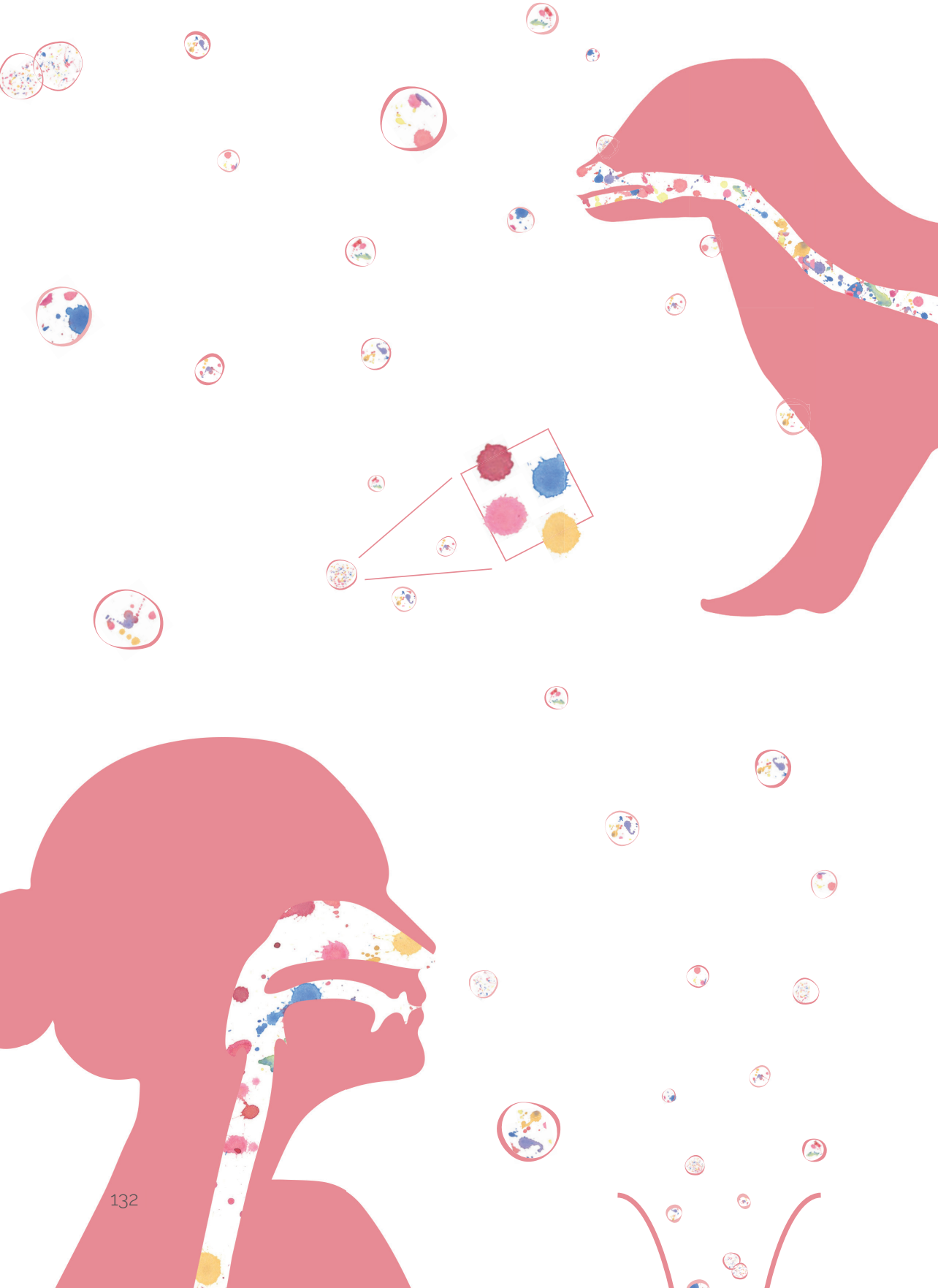

b
Isolator 1
23.0°C (±0.2), 53.1% (±9.5)
Isolator 2
22.7°C (±0.2), 53.8% (±9.6)
Isolator 3
23.1°C (±0.3), 52.8% (±9.1)
Isolator 4
22.5°C (±0.3), 52.1% (±9.1)
Temperature (°C)
Relative humidity (%)
Time post inoculation (days)

Figure S2. Environmental parameters in class III isolators. Graphs show temperature and relative humidity recordings in each isolator during SARS-CoV (**a**) and SARS-CoV-2 (**b**) transmission experiments. Pink and dark blue circles and lines represent temperature and relative humidity measurements, respectively. Average values and standard deviation (±SD) are calculated from n=12 and n=17 recordings for SARS-CoV and SARS-CoV-2, respectively and shown below the title.



SUMMARIZING DISCUSSION & FUTURE PERSPECTIVES

Respiratory viruses are transmitted via direct or indirect contact, or via the air in the form of fine aerosols or larger respiratory droplets. Many variables affect the transmission routes of respiratory viruses[19,21] and these variables can differ for each virus. For this reason, it is very difficult to gain a thorough understanding of the relative importance of each of the above-mentioned routes in virus transmission. Consequently, there is no one-size-fits-all experiment, but multiple approaches are necessary to acquire a better understanding of respiratory virus transmission via a specific route. Air sampling is a valuable tool with which quantitative data such as the number of respiratory virus particles in the air of different environments and their infectivity can be obtained, with or without size-fractionation of the air samples. Animal models are used for fundamental research on the viral and environmental properties that may affect the transmission routes and allow qualitative analyses of the transmissibility of respiratory viruses. Ultimately, a thorough understanding of respiratory virus transmission between humans via a specific route can only be gained by studying respiratory virus transmission in humans themselves.

Air sampling

To date, a variety of air samplers is available that employ different collection methods[290,291]. In air samplers such as the BioSampler or Andersen cascade impactor, particles are accelerated through narrow holes and impacted in either a liquid or solid collection medium. This is a very efficient collection method for robust particles in the micron range (e.g., bacteria and fungi). For viruses, however, such a collection method is less efficient as viruses are less resistant to the high collection forces than bacteria and fungi and consequently can get damaged during the collection process, or may not be collected at all due to their small size. In **Chapter 2,** we focused on the optimization and comparison of three existing or newly developed air samplers to efficiently collect four different infectious respiratory viruses from the air. A comparison of three different collection media in the Andersen cascade impactor showed that the infectivity of all four respiratory viruses was equally well preserved in a semi-solid gelatin layer, while the infectivity of viruses was substantially decreased with agar or liquid transport medium depending on the respiratory virus tested. In addition, the BioSampler also performed well, collecting high amounts of infectious virus for each of the viruses tested. An advantage of the Andersen cascade impactor is that it allows size-fractionation of the virus-containing aerosols and droplets, which is not possible with the BioSampler.

As the Andersen cascade impactor in combination with the semi-solid gelatin layer was one of the best performing air samplers in the *in vitro* studies, and since it provided information on the size of virus-containing aerosols and droplets, in **Chapter 3**, this air sampler was employed in a pediatric hospital setting. Here, the RSV load in the air around RSV-infected infants was quantified in relation to the viral load in their upper respiratory tract (URT) over time. While infectious RSV was present in the nasopharynx samples of infants, only low amounts of RSV RNA but no infectious virus was collected from the air. Interestingly, the RSV RNA was collected in the first stage of the sampler, indicating that it was present in droplets >7 µm. These results were in agreement with several other studies[58,215,216] and suggest that currently implemented droplet and contact precautions in hospitals are sufficient. In contrast to these findings, Kulkarni et al. reported high amounts of infectious RSV in the air around infants and children with RSV-confirmed bronchiolitis in general wards of a pediatric hospital[115]. Although viral concentrations in the air decreased with increasing distance to the patient's head, a substantial amount was found to be contained in aerosols that are small enough to remain in the air for hours, and thus, could be transmitted over long distances. Kulkarni et al. employed the same viable six-stage cascade impactor as we did (**Chapter 3**), but used liquid medium as a collection surface. We and others have observed that the use of a liquid collection medium resulted in lower collection efficiencies as compared to a more solid collection surface[187] and that it did not preserve virus infectivity well[185]. Furthermore, we and others[185,187] observed that the use of liquid medium can result in spill-over into other stages or even the pump itself, which substantially increases the chances of cross-contamination. In addition, the airflow pushes aside liquid medium in the petri dish, thereby increasing the jet-to-plate distance that can result in a shift of particle size distribution in which large droplets, which should normally be collected in the first stages, will then be collected in lower stages. Consequently, this would lead to a misinterpretation of the size of particles containing RSV. It is important to note that the results of Kulkarni et al. are not in line with epidemiological data; if RSV would efficiently be transmitted via the air, there would be a considerably higher incidence of nosocomial RSV outbreaks, as the currently implemented precautions would be ineffective in preventing RSV transmission. Overall, the evidence base on RSV transmission via the air does not support the data reported by Kulkarni et al. and it remains unclear why in this study a high amount of infectious virus was detected in aerosols and larger droplets in the air.

In other recent studies, various air samplers including the BioSampler and Andersen cascade impactor have been employed in hospitals[37,125,216,292,293], but also in households[56] and animal experiments[185] to quantify the amount of different respiratory viruses in the air. However, independent of the virus of interest, low detection rates and/or low amounts of virus RNA were frequently reported[58,59,125,215,217,294,295]. Consequently, virus isolation from air samples is often not included and the few studies that have attempted it could recover little to no infectious virus[88,125,169,217]. This suggests that, although air samplers collect infectious viruses well *in vitro*[192] (**Chapter 2**), their application in field settings, where low amounts of virus may be dispersed over a large volume of air, might be limited due to the constraints in the design of air samplers. Many air samplers have a high cut-off size, which prevents the efficient collection of submicron aerosols. In addition, the strong collection forces generated in such air samplers cause a loss of virus infectivity, re-aerosolization, and particle bounce from the collection medium, overall resulting in low collection efficiencies[181,182,296]. Moreover, in the BioSampler and Andersen cascade impactor, viruses are collected in large volumes of collection medium, although they should ideally be collected in a small volume to avoid high dilution factors. To overcome the above-mentioned limitations of air samplers, the VIVAS air sampler that gently collects viruses through condensational growth of aerosols and droplets was developed[168]. With the condensational growth method, particles as small as a few nanometers were efficiently collected and concentrated in a small sample volume while retaining the infectivity of viruses. Infectious influenza A viruses[297] and SARS-CoV-2[165] were already successfully collected from the air around infected individuals with the VIVAS air sampler. Unfortunately, this air sampler does not size-fractionate collected aerosols and droplets and hence no information on the size of virus-containing aerosols and droplets can be obtained. However, this information is necessary to design and implement appropriate infection control and prevention measures. For this reason, an ideal air sampler should size-fractionate droplets and aerosols, including those in the submicron range, in two or three stages. As mentioned, cascade impactors size-fractionate incoming droplets and aerosols over multiple stages. However, processing multiple samples is rather laborious and costly, especially if this requires multiple steps before the samples can be analyzed. In addition, the small amounts of virus collected are divided over large-volume samples, thereby increasing the already high dilution factors. More importantly, a refined size-fractionation of more than two or three stages does not provide more information on the infection risk of viruses than when droplets and aerosols are collected in two or three stages to discriminate

particles that can remain suspended in the air for long periods of time from those that do not. Although cascade impactors are available with only two or three stages, these devices cannot efficiently collect aerosols in the submicron range. An alternative is the NIOSH air sampler, in which particles >4 μm are separated from particles between 1-4 μm by collecting them in two separate plastic tubes[298]. In addition, submicron particles are trapped in a filter that is installed after the two tubes. However, collecting viruses by impacting them on the walls of plastic tubes is sub-optimal for the successful recovery of infectious virus. Moreover, while some groups have reported the collection of infectious viruses[185], usually recovery of infectious virus from filters is very poor. In all of the above-mentioned air samplers, the airflow rate is too low to collect a representative amount of virus from the air in a short time, necessitating longer sampling times that may affect the infectivity of viruses. In turn, air samplers with a high flow rate, such as the Coriolis μ sampler[299], are capable of sampling a large volume of air in a short amount of time and hence collect more virus, but this also may come at the cost of virus infectivity due to the sampling stresses that are created by the strong airflow.

Overall, although some advancements have been made in recent years towards the development of air samplers that also allow the collection of viruses, currently existing air samplers still have major shortcomings that limit their application in field settings. Therefore, a better air sampler for the collection of viruses is urgently needed (**Table 1**). In general, an ideal air sampler should collect viruses from the air in a short time and should provide accurate information on the amount of infectious virus present in the air. Air samplers should employ a collection mechanism that exerts less stress on viruses than an inertia-based collection mechanism but still should operate with a sufficiently high flow rate to sample large volumes of air. In addition, air samplers should be capable of collecting particles as small as 10 nm and should separate large from small particles. Ideally, collected viruses should be concentrated in a small volume of a universal collection medium. This medium should preserve the infectivity of different viruses equally well not only during the sampling process itself but also during transportation to the laboratory. In addition, to prevent a loss of collected viruses and a decrease in virus infectivity after air sampling, post-processing of the collection medium should be kept to a minimum or, ideally, be avoided completely. Moreover, air samplers should be re-usable, hence the material should be cleanable to eliminate the risk of cross-contamination. Furthermore, as air samplers may be employed in field settings, they should be portable, i.e., built from lightweight but robust material and run on batteries. In

addition, air samplers should be applicable in many different environmental conditions and therefore should withstand varying humidity and temperature.

Animal models

Animal models are used to study the transmissibility of respiratory viruses between hosts. For example, influenza A viruses that are transmissible between humans are generally also transmissible between ferrets, whereas avian influenza viruses are generally not transmissible via the air between either humans or ferrets. Therefore, the ferret model has been of great value in understanding the transmissibility of influenza A viruses and has become indispensable in the estimation of the pandemic potential of influenza A viruses[258,300]. In combination with *in vitro* analyses, the ferret model can further be used to study the genetic and phenotypic properties of avian influenza A viruses that drive virus transmission between mammals via the air[12,238,243,300–306]. As a contribution to the pandemic risk assessment of A/H2N2 viruses, in **Chapter 4**, we showed that despite replication in the URT of ferrets, avian A/H2N2 viruses were not transmitted via direct contact or the air between ferrets. Furthermore, from ferret transmission experiments and *in vitro* phenotyping assays that we performed with human H2N2 influenza viruses isolated in 1957, 1958, and 1968, we concluded that A/H2N2 viruses continued to adapt to humans during the first year of pandemic circulation. However, in contrast to other human influenza A viruses[243], these human H2N2 viruses had limited ability of airborne transmission between ferrets. Thus, it cannot be excluded that the ferret model may be less suitable for A/H2N2 viruses than for other human influenza A viruses. On the other hand, these results may indicate that when population immunity against emerging viruses is low or absent, viruses such as H7N9 that also displayed limited airborne transmissibility in the ferret model[300,306,307], may not need to be efficiently transmitted via the air to cause a pandemic. It is important to understand if the newly identified phenotypes of A/H2N2 viruses are subtype-specific or are general characteristics of multiple subtypes. As a component of risk analysis and mitigation, it should be further investigated if specific phenotypes associated with influenza virus adaptation and transmission between mammals can also be translated to other emerging respiratory viruses such as coronaviruses and paramyxoviruses.

In addition to risk assessment studies with avian influenza A viruses, as done in **Chapter 4**, animal models can further be used to characterize the transmissibility of zoonotic viruses that emerge in the human population. During the course of this PhD trajectory a newly emerged respiratory virus, SARS-CoV-2, spread

rapidly around the globe. After it was shown that SARS-CoV-2 transmission via the air over 10 cm was possible[255], in **Chapter 5**, we designed a new experimental set-up with which virus transmission via the air over more than one meter could be studied. With this newly developed set-up, we demonstrated that SARS-CoV-2 remained stable while traveling through the air over more than one meter distance and was capable of causing a robust infection in indirect recipient ferrets. These results show that, in principle, transmission of SARS-CoV-2 between ferrets via the air is possible. However, epidemiological data suggest that the contribution of transmission via the air in the spread of SARS-CoV-2 in the human population is relatively small. SARS-CoV RNA has been detected in the air in several cases[274,275,277]. However, in only one study infectious virus was successfully recovered from air samples[165]. Some reported outbreaks may have been associated with airborne transmission of SARS-CoV-2, but as transmission via other routes cannot be excluded, it remains inconclusive if airborne transmission played a major role in the spread of SARS-CoV-2 in these outbreaks[271,308]. Based on a recent analysis of the SARS-CoV-2 outbreak on a cruise ship, authors dismissed the possibility of efficient long-range airborne transmission of SARS-CoV-2 and suggested that transmission more likely occurred through close contact[309]. Overall, it still proves difficult to identify the main transmission route of a respiratory virus. In the future, (quantitative) data on the transmission routes of a virus need to be obtained quickly in an effort to mitigate and prevent outbreaks through specific non-pharmaceutical interventions. With the continuous threat of newly emerging viruses also more specific studies are needed that focus on the identification of factors that facilitate sustained onward transmission of various respiratory viruses between humans. Ultimately, these studies should result in a better understanding of the emergence of novel transmissible virus strains.

Role of fomites in animal transmission experiments

The transmission set-ups used in **Chapter 4** and **5** can give information about the transmission efficiency of viruses via the air over short and long distances, however, they do not allow the discrimination between droplets, aerosols, or re-aerosolized dust particles, and thus no conclusion can be drawn about the particle source and/or size that predominantly governs the transmission of respiratory viruses in these set-ups. Up to now, it was generally assumed that the transmission of viruses via the air in animal models is solely mediated by aerosols and droplets directly emitted from the respiratory tract of an infected host. Recently, a study warned for a contribution of fomites to virus transmission via the air in animal

experiments by showing that influenza A virus can be transmitted through the air between guinea pigs via re-aerosolized particles originating from the fur of animals and fomites[248]. In addition, transmission of SARS-CoV-2 via fomites has been demonstrated with naïve hamsters that were each placed in a cage that had previously housed a SARS-CoV-2 infected hamster[310]. In both studies, infectious virus was recovered from several objects in the cage. In **Chapter 5**, we showed that SARS-CoV RNA was present on the fur of all donor animals, albeit the RNA levels were low and no infectious virus was detected. These data highlight the need to better understand the role of re-aerosolized fomites in virus transmission via the air in animal models. This is important to elucidate how much of the transmissibility can actually be directly attributed to the respiratory secretions of an infected host. Therefore, to identify the source of virus transmitted via the air, existing experimental set-ups need to be optimized and new set-ups designed. In animal models, the donor animal can be placed in an empty cage for a certain amount of time, from which particulate matter in the air is filtered using a material that does not emit dust particles itself and does not interfere with animal behavior, to assess virus transmissibility through exhaled droplets and aerosols. The donor animal can also be removed from its cage, before placing a naïve animal into an adjacent cage, to assess virus transmission through re-aerosolized fomites only.

Investigating factors that drive virus transmission between animals via the air

The quantity and size distribution of aerosols and droplets emitted by an infected host can vary substantially[57,311–313], which might also affect the relative importance of virus transmission via the air. Therefore, the variation in the amount and distribution of droplets and aerosols emitted by individual animals, and the difference in an infected versus a non-infected state, should be investigated. For this purpose, particle measurements should be carried out on donor animals prior to and during the course of infection. In addition, it is also necessary to study the size of aerosols and droplets that govern virus transmission. For this purpose, transmission set-ups should be designed with which the contribution of aerosols and droplets to the transmission via the air (at various distances) can be studied. For example, Zhou et al. installed impactor stages between influenza A virus inoculated animals and naïve animals to allow only virus-containing aerosols and droplets of certain sizes to pass through to the naive animals[314]. To better understand how the transmission efficiency via the air changes with varying humidity and temperature and hence might explain the seasonality of viruses, transmission experiments

should also be conducted under different environmental conditions as it was done for influenza A viruses in the guinea pig model[315]. In the future, the set-ups used in the studies by Zhou et al.[314] and Lowen et al.[315] can be combined to further investigate how the transmission efficiency of viruses contained in aerosols and droplets of certain sizes is affected by humidity and temperature. In addition, as the transmissibility of respiratory viruses can only be qualitatively studied in animals, the implementation of nebulizers and air samplers in such experiments is warranted to obtain quantitative data on the infectious dose and transmission kinetics. For example, by replacing a donor animal with a nebulizer the infectious dose can be quantified. In addition, recipient animals can either be replaced or complemented with air samplers, thereby quantifying virus shedding in the air. Implementing these devices helps to better control animal transmission experiments and thereby these experiments become more standardized and quantitative in the future.

Differences in virus transmissibility between animals and humans

Virus transmission is a complex interplay between many factors that differ between hosts. As shown in **Chapter 5**, SARS-CoV shedding kinetics in ferrets was completely different from that observed in humans. While in ferrets, the peak of virus shedding was reached within one day post inoculation, peak virus shedding in humans has been reported to be around 10 days after symptom onset[262–265]. In addition, the efficient spread of SARS-CoV-2 on mink farms might be indicative of a higher susceptibility of animals belonging to the family of Mustelidae, which also includes ferrets. Minks are housed in large groups in cages partially filled with bedding material, which in addition to the grooming behavior of animals, gives the virus enough opportunities to spread via fomites, contact, and air. Since animal and human contact behavior is completely different, contact transmission in animal models should be interpreted with caution anyway. A study that analyzed the correlation of influenza virus transmission between ferrets and humans also found that direct contact transmission in animal experiments did not significantly correlate with the secondary attack rate in humans, indicating that these experiments are less valuable for the prediction of human-to-human transmission of influenza viruses than experiments assessing virus transmission via the air[316]. In addition, the efficient transmission of SARS-CoV and SARS-CoV-2 between ferrets via the air over more than one meter distance does not align with the transmission dynamics of either of the two viruses observed in the human population.

Despite all limitations, animal models are an invaluable tool to conduct fundamental research on the viral properties and environmental conditions that determine the transmission efficiency of respiratory viruses. The animal models can further be used to assess the efficacy of intervention methods including the potential of vaccines and therapeutic targets to reduce or prevent virus transmission as was shown for SARS-CoV-2 where intranasal administration of a lipopeptide prevented direct contact transmission of SARS-CoV-2 between ferrets[317].

Virus transmission via the air

The SARS-CoV-2 pandemic sparked a fierce debate about the transmission efficiency of SARS-CoV-2 via the air over long distances. As a consequence, also longstanding definitions of virus transmission routes via the air were discussed. In general, there is agreement that expiratory activities such as breathing, sneezing, and coughing of an infected individual can lead to the emission of (respiratory virus-containing) aerosols and droplets. However, the size definition of aerosols and droplets still causes considerable debate and hinders the interpretation of research results and the implementation of accurate infection prevention measures. Historically, aerosols and droplets were separated by an arbitrary cut-off size of 5-12 µm[13,15,18]. Based on the behavior of particles in still air, which is affected by gravity and Brownian motion, droplets larger than this cut-off size were considered to fall quickly on the ground or on objects in close vicinity of the source, while aerosols smaller than this cut-off size were considered to have a slow settling velocity and hence remained in the air for prolonged periods of time and traveled further away from the source[13]. This definition led to the distinction between droplet (short-range) and aerosol (long-range) transmission of pathogens[13]. However, as subsequent research showed, this is an oversimplified model of virus transmission via the air that should be revisited. It has been shown that particles <100 µm evaporate when emitted in the air and are thus capable of remaining in air and traveling beyond 1 m from the source of emission[318–320]. The travel distance of a particle is not solely determined by its size but is also influenced by the force with which particles are expelled (e.g., sneezes), ventilation rates, and environmental parameters (e.g., humidity and temperature)[320,321]. Studies on the emission of respiratory secretions showed that upon sneezing, particles are expelled as a "multiphase turbulent gas cloud" containing a broad spectrum of different particle sizes and are thus not expelled as isolated droplets and aerosols[319,322,323]. This cloud can travel 7 to 8 m through the air before remaining particles evaporate and further disperse in the air[319,322,323]. Taken together, as particles up to 100 µm may be considered

relevant for airborne transmission, a cut-off size of 100 µm should be used to distinguish aerosols from droplets instead of the traditional cut-off size of 5-12 µm[324].

As the gas cloud also contains small particles close to the source of emission, virus-containing aerosols can pose an infection risk in close proximity to the source as well, which is an important consideration for infection prevention guidelines. It should be noted, however, that the volume of very few large droplets is substantially greater than the volume of many small aerosols which is why a few large droplets may still pose a higher infection risk than aerosol spray. Up to now, it is also unknown, if and to what degree the stability and hence infectivity of viruses contained in large droplets is affected when these droplets evaporate and disseminate into smaller ones. It is also unclear if the shrinking of larger droplets affects all virus families equally, or if some viruses are more resistant to factors like shear stress and hence remain infectious in shrunken droplets and aerosols. Therefore, although collectively the above-mentioned studies contribute greatly to our understanding of aerosol and droplet behavior in the air, the data provide little information on the actual infection risk of a virus at various distances, if it is not investigated if these aerosols and droplets contain infectious virus.

Respiratory virus transmission between humans

With the COVID-19 pandemic, the importance of superspreading in the transmission of respiratory viruses between humans has resurfaced. It is estimated that superspreading events, i.e., where some individuals infect an unproportionally high number of susceptible people, comprise approximately 20% of infected people that are responsible for 80% of the transmission events[107,325]. However, it remains unclear if superspreading can be attributed to a person itself, particular circumstances and environments, or to a combination of both. There are some indications that the amount of (virus-containing) aerosols and droplets emitted by an individual varies considerably between people[57,312,313] and that this may correlate with biological factors, such as age and body mass index[326]. High emission of aerosols and droplets consequently could result in the shedding of an unproportionally high amount of virus in the environment which may also change in the course of an infection. Also, a higher viral load in the URT of an infected individual in combination with an increased emission of droplets and aerosols may contribute to a more efficient spread of respiratory viruses. In addition to these individual factors, particular environments and circumstances may contribute to superspreading, as well. During the COVID-19 pandemic,

superspreading events were often associated with indoor areas in which a large number of people came together[327,328]. Nearby examples are apres-ski parties in the Alps and carnival in the Netherlands, where hygiene is sometimes not taken seriously. Crowding in places with limited space makes it difficult to completely prevent close contact between individuals. In addition, the duration of exposure time in such settings may increase the infection risk, especially when the indoor areas are poorly ventilated. Also, the activity performed during events such as singing or sporting may lead to increased emission of particles which in turn may increase the risk of infection even further[52]. During the SARS-CoV outbreak in 2003, it became evident that the relative contribution of transmission routes may also depend on the situation. For example, virus transmission via the air due to aerosol-generating procedures (AGPs) can pose a high infection risk as was observed with SARS-CoV[82,329], while SARS-CoV transmission via the air in e.g., household settings was negligible[330]. Filling the knowledge gaps around superspreading events and potential superspreaders and risk environments may prevent such epidemiologically important transmission events in the future.

Future perspectives

The COVID-19 pandemic showed that despite many advancements, it is still extremely challenging to identify via which route a respiratory virus is predominantly transmitted and what the contribution of other routes might be under particular circumstances. In my thesis, existing and newly developed experimental methods have been employed to assess the airborne transmission of various important respiratory viruses. In the future, these methods should be employed further to obtain more quantitative data on the airborne transmission of the different respiratory viruses and to identify environmental and viral determinants that influence virus stability in the air and hence drive efficient airborne transmission. For example, air samplers specifically designed for the collection of viruses from the air (**Table 1**) should be employed in various environments such as hospitals and schools to identify which infectious respiratory viruses are present in the air. Furthermore, the association between the amount of infectious virus in the URT of infected people and the amount of virus in the air remains to be determined. Air sampling next to AGPs can also help to estimate the risk these procedures pose in a clinical setting. As a contribution to surveillance efforts, air samplers could also be employed in environments such as live animal markets or next to farmhouses to monitor the air for (re-) emerging respiratory viruses. In addition, appropriate animal models may be selected for each respiratory virus so that the

transmissibility of viruses, factors that drive the efficient airborne transmission of these viruses and (non-) pharmaceutical intervention methods can be studied. In the future, new experimental methods have to be designed and employed with which the above-mentioned aspects can be studied in a more quantitative manner and with reduced numbers of laboratory animals or completely without them. As mentioned above, nebulizers and air samplers could replace donor and/or recipient animals in transmission studies. More importantly, systems should be developed, in which the stability of viruses in the air, including the factors that determine their stability can be quantitatively assessed. For example, in aerosol chambers, viruses can be nebulized and subsequently exposed to various environmental conditions before they are collected with air samplers to determine the number and size of aerosols and droplets that still contain infectious viruses. In these systems, air-liquid interface cell cultures can be further implemented mimicking the environment of the respiratory tract to study virus infectivity. In addition, the effect of the pH, mucus composition, and salinity in aerosols and droplets on virus infectivity, can be studied in such a system.

To date, the viral determinants that affect the stability of a respiratory virus in the air are largely unknown and need to be elucidated in the future. For example, a stabilized HA protein, among other factors, contributes to transmission of influenza A viruses between mammals. However, it is still unclear how a stabilized HA facilitates influenza A virus stability in the air. We show in **Chapter 4** that in contrast to other influenza A virus subtypes, a stabilized HA in A/H2N2 viruses was not sufficient to promote a robust transmission between ferrets via the air. These data could also hint towards a yet unidentified factor that in addition to a stabilized HA determines the transmissibility of influenza A viruses via the air. Here, the aerosol chambers described above can be used to study the HAs of different influenza A virus subtypes regarding their stability in the air. These studies should also include other surface proteins such as the neuraminidase (NA) to elucidate if these proteins or a combination of determinants in more than one surface protein affects virus stability in the air and virus transmissibility.

Altogether it would be of indispensable value that in the event of a future outbreak, epidemic, or pandemic, reliable information on the transmission routes of respiratory viruses becomes quickly available so that prompt measures to control and prevent the spread of respiratory viruses can be implemented.

Table 1. Specifications of an ideal air sampler

Specification	Explanation
Precise and fast	• Provides accurate information on the amount of infectious viruses present in the air • Collects viruses from the air in a short amount of time
Gentle collection mechanism	• Preserving infectivity of viruses
High flow rate	• Sampling large volumes of air
Low cut-off size	• Collection of particles as small as 10 nm
Coarse size fractionation	• Separation of large from small particles
Small collection volume	• Concentration of collected viruses from air
Universal collection medium	• Equal collection efficiency for a wide variety of respiratory viruses • No post-sampling processing • Perseverance of virus infectivity while transporting samples to the laboratory
Cleanable	• Reusable without cross-contamination
Portable	• Light weight, robust material • Running on batteries
Applicable in many environmental conditions	• Can withstand varying humidity and temperature

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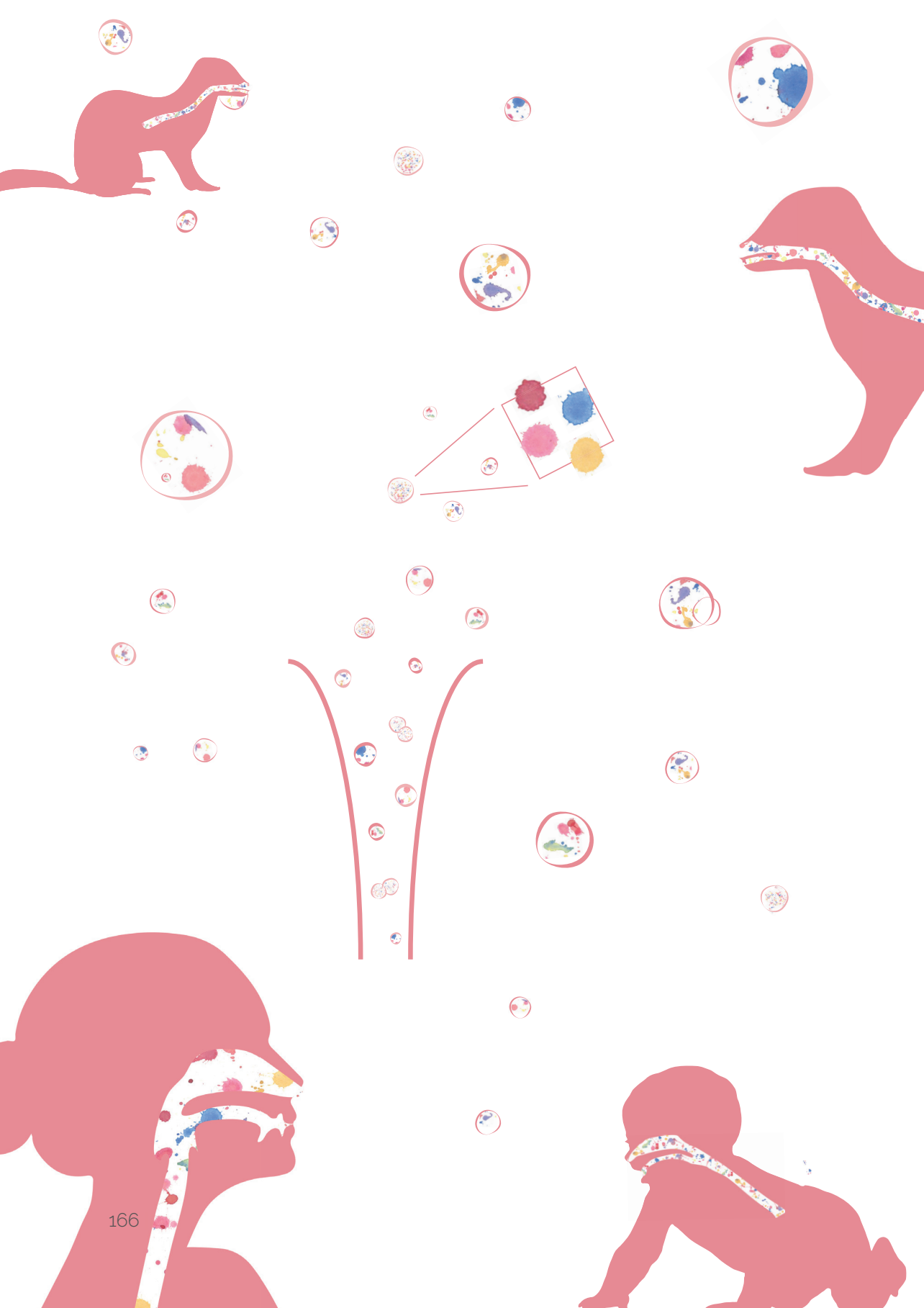

SUMMARIES

- Samenvatting -

- Zusammenfassung -

SAMENVATTING

Luchtwegvirussen zijn een groep virussen die de luchtwegen van mensen binnen dringen en een breed spectrum aan ziekteverschijnselen kunnen veroorzaken, van simpele verkoudheidsklachten tot ernstige infecties zoals een longontsteking. Bij de meeste mensen zijn dit soort infecties ongevaarlijk en gaan de klachten vanzelf weer over. Echter, bij mensen met onvoldoende weerstand zoals jonge kinderen, ouderen of personen met een onderliggende ziekte kunnen deze virussen ernstige infecties van de lagere luchtwegen veroorzaken, die in het ergste geval fataal kunnen zijn.

Luchtwegvirussen kunnen via verschillende routes overgedragen worden naar een vatbaar persoon. Virussen kunnen overgedragen worden via direct contact, bijvoorbeeld door elkaar de hand te schudden of via indirect contact door het aanraken van besmette objecten zoals deurklinken. Als de vatbare persoon vervolgens met de besmette handen de binnenkant van de neus of mond aanraakt, kan het virus via de slijmvliezen binnendringen en vervolgens een infectie veroorzaken. Virussen kunnen ook via de lucht overgedragen worden, in druppels en aerosolen, zonder direct contact met een besmet persoon. Grote druppels blijven slechts gedurende korte tijd in de lucht aanwezig en kunnen alleen korte afstanden afleggen voordat ze op de slijmvliezen in de mond en neus, van een vatbaar persoon terecht kunnen komen. Aerosolen daarentegen zijn druppels die klein genoeg zijn om lang in de lucht te blijven hangen en virussen kunnen op deze manier over grote afstanden overgedragen worden. Doordat aerosolen zo klein zijn, kunnen ze ook diep in de longen worden ingeademd. Voor veel luchtwegvirussen is het tot op heden nog niet duidelijk via welke van deze routes ze zich voornamelijk verspreiden, omdat dit verschilt per soort virus. Ook is het onduidelijk hoe variabelen zoals de stabiliteit van het virus in de lucht, de mate van virusuitscheiding, de temperatuur en de luchtvochtigheid de relatieve bijdrage van deze routes beïnvloeden. Deze kennis is echter wel nodig om de dreiging van opkomende virussen, die in de toekomst mogelijk uitbraken veroorzaken, in te kunnen schatten. Daarnaast zal deze kennis bijdragen om niet-farmaceutische infectiepreventie-maatregelen zoals afstand houden, het sluiten van openbare ruimtes en het dragen van mond-neusmaskers te verbeteren en zo de verspreiding van luchtwegvirussen tussen mensen te beheersen of zelfs te voorkomen. Om fundamentele kennis te vergaren over de overdrachtsroutes via welke virussen zich kunnen verspreiden, is in dit proefschrift met bestaande en nieuw ontwikkelde experimentele methodes de overdraagbaarheid via de lucht van een aantal belangrijke luchtwegvirussen bestudeerd.

Het opvangen van luchtwegvirussen met behulp van "lucht samplers"

Om de hoeveelheid infectieus virus in de lucht in een bepaalde omgeving, zoals bijvoorbeeld een ziekenhuis, te bepalen wordt vaak gebruik gemaakt van zogenaamde lucht samplers. Lucht samplers zijn apparaten die via een aangesloten pomp lucht uit de omgeving aanzuigen en de virussen die in de lucht aanwezig zijn in een bepaald medium opvangen. Er bestaan verschillende lucht samplers die verschillende opvangmethodes gebruiken om virussen uit de lucht op te vangen. Echter, de meeste lucht samplers zijn niet specifiek voor het opvangen van virussen ontwikkeld. Een voorbeeld van zo'n lucht sampler is de Andersen cascade impactor. Een groot voordeel van de Andersen cascade impactor ten opzichte van de andere lucht samplers is dat aerosolen en druppels, afhankelijk van hun grootte, in verschillende fracties worden opgevangen. Informatie over de grootte van de aerosolen en druppels die virus bevatten is belangrijk om te kunnen beoordelen of een virus over een lange of alleen korte afstand kan worden overgedragen. Deze informatie kan gebruikt worden om te bepalen welke infectiepreventie-maatregelen toegepast moeten worden om verspreiding van het virus tegen te gaan. De Andersen cascade impactor was oorspronkelijk ontwikkeld voor het opvangen van bacteriën, schimmels en sporen uit de lucht op een hard opvangmedium. Dit opvangmedium is helaas niet geschikt voor virussen. Om die reden hebben we in **hoofdstuk 2**, naast het harde opvangmedium, ook een vloeibaar en een zelf ontwikkeld semi-vloeibaar medium in de Andersen cascade impactor getest om te bestuderen welk medium het meest geschikt is voor het opvangen van infectieus virus. Hiervoor hebben we in eerste instantie twee verschillende luchtwegvirussen, een griepvirus en het humaan metapneumovirus, gebruikt. Deze virussen werden in een afgesloten ruimte verneveld en vervolgens weer opgevangen met de Andersen cascade impactor in combinatie met één van de drie verschillende opvangmedia. We vonden dat in tegenstelling tot het harde en het vloeibare opvangmedium, met het zelf ontwikkelde semi-vloeibaar medium wel grote hoeveelheden van beide virussen efficiënt werden opgevangen waarbij de infectiviteit behouden bleef. Om die reden hebben we de Andersen cascade impactor met het geoptimaliseerde semi-vloeibare opvangmedium vervolgens vergeleken met twee andere lucht samplers, de BioSampler en een zelf ontwikkelde elektrostatische precipitator. De BioSampler vangt virussen op in vloeibaar medium, maar daarbij kan geen onderscheid gemaakt worden tussen de grootte van aerosolen en druppels zoals

bij de Andersen cascade impactor. Zowel met de Andersen cascade impactor als de BioSampler worden deeltjes opgevangen op basis van hun massatraagheid. Als gevolg kunnen deze lucht samplers kleine aerosolen helaas heel slecht opvangen. Om die reden hebben we een elektrostatische precipitator ontwikkeld waarbij inkomende aerosolen en druppels positief geladen worden en vervolgens aangetrokken worden door een neutraal geladen vloeibaar opvangmedium. Theoretisch gezien zouden kleine aerosolen met deze opvangmethode beter moeten worden opgevangen dan met de Andersen cascade impactor of de BioSampler. Om de opvangefficiëntie van deze drie lucht samplers met elkaar te vergelijken, hebben we vier verschillende luchtwegvirussen, een griepvirus, humaan metapneumovirus, respiratoir syncytieel virus en humaan parainfluenza virus type 3, verneveld en met elk van de drie lucht samplers opgevangen. Uit deze studie bleek dat de BioSampler en Andersen cascade impactor in staat waren om van alle vier virussen grote hoeveelheden op te vangen, terwijl met de elektrostatische precipitator slechts heel kleine hoeveelheden virus uit de lucht opgevangen konden worden en daarom dus minder geschikt is.

Detectie van RSV RNA in de lucht bij kinderen met acute luchtweginfecties

Luchtwegvirussen bestaan uit genetisch materiaal (RNA of DNA) dat is omhuld door eiwitten en vaak ook door een membraan waar eiwitten op zitten. De oppervlakte eiwitten zijn onder andere nodig om een gastheercel binnen te kunnen dringen. Alleen een intact virusdeeltje, maar niet het RNA zelf, kan een infectie in de gastheer veroorzaken. Wanneer er wordt gesproken over een infectieus virus, wordt daarmee een intact virusdeeltje bedoeld dat in staat is om een gastheercel te infecteren. Het is belangrijk om na te gaan of de druppels of aerosolen die uit de lucht worden opgevangen wel of niet infectieuze virussen bevatten. Dat is mogelijk door niet alleen te testen op de aanwezigheid van het genetisch materiaal in een luchtmonster, maar ook te testen of het virus een geschikte cellijn kan binnendringen en zich daar kan vermenigvuldigen.

Respiratoir syncytieel virus (RSV) is een luchtwegvirus dat bij mensen over het algemeen milde verkoudheidsklachten veroorzaakt. Bij zeer jonge kinderen en fragiele ouderen kan RSV echter ernstigere infecties veroorzaken die kunnen leiden tot bronchiolitis of een longontsteking, waarbij soms zelfs een ziekenhuisopname nodig is. Er werd jarenlang aangenomen dat RSV voornamelijk via nauw contact met een besmet persoon wordt overgedragen en daarom worden in ziekenhuizen

contact- en druppelisolatie maatregelen toegepast bij kinderen met RSV-infecties. Echter, in een recente studie werd gerapporteerd dat er grote hoeveelheden infectieus virus in de lucht aanwezig was rond geïnfecteerde kinderen in een ziekenhuis. Het is dus nog steeds onduidelijk of RSV zich ook efficiënt via de lucht kan verspreiden. Daarom hebben we in **hoofdstuk 3** de Andersen cascade impactor, de lucht sampler die tijdens het laboratoriumonderzoek het best presteerde, in een ziekenhuis gebruikt om de hoeveelheid infectieus RSV in de lucht te bepalen in de nabijheid van jonge kinderen met een RSV-infectie. In onze studie lieten we met behulp van neus monsters zien dat er gedurende lange tijd infectieus virus in de neus van kinderen aanwezig was, maar dat er desondanks slechts bij 3 van de 6 kinderen een kleine hoeveelheid virus-RNA kon worden opgevangen. Bij geen van de kinderen kon er infectieus virus in de lucht worden aangetoond. Deze resultaten wijzen erop dat de kans dat RSV efficiënt via de lucht wordt overgedragen, in deze ziekenhuissetting, heel klein is. Wel moet worden opgemerkt dat slechts een klein aantal kinderen in deze studie kon worden geïncludeerd. Bovendien kon er pas enkele dagen na de eerste ziekteverschijnselen begonnen worden met de bemonstering van lucht. Het is dus mogelijk dat er in de eerste dagen van het ziekteverloop meer virus in de lucht werd uitgescheiden en dat we deze dagen in onze studie hebben gemist. Desalniettemin komen onze resultaten overeen met de bevindingen uit het merendeel van soortgelijke studies en bevestigen ze dat de isolatiemaatregelen die op dit moment in ziekenhuizen worden toegepast voldoende zijn. Waarom in één studie zeer grote hoeveelheden infectieus RSV werd gevonden in de lucht maar in vele andere studies niet blijft onduidelijk.

Adaptatie van H2N2 influenzavirus aan de mens

Griepvirussen, oftewel influenzavirussen, zijn luchtwegvirussen die in de mens jaarlijks tijdens de winter griepuitbraken veroorzaken. Influenzavirussen worden onderverdeeld in type A, B, C en D. Influenza A virussen worden verder onderverdeeld in verschillende subtypes gebaseerd op twee eiwitten die zich op het oppervlak van deze virussen bevinden, het hemagglutinine (HA) en het neuraminidase (NA). Tot nu zijn er 16 verschillende HA en 9 verschillende NA subtypes bekend die voorkomen in wilde vogels en twee HA en NA subtypes die voorkomen in vleermuizen. De combinatie van de HA en NA subtypes op het virus oppervlak wordt gebruikt voor de naamgeving, zoals bijvoorbeeld de humane H1N1 en H3N2 influenza A virussen. Influenza A virussen komen voornamelijk in wilde vogels voor, maar deze virussen springen soms over naar de mens en kunnen dan een infectie veroorzaken. Meestal kunnen influenza A virussen uit

vogels niet tussen mensen overgedragen worden, maar in sommige gevallen muteren deze virussen tijdens de infectie van mensen, waardoor ze efficiënt van mens tot mens kunnen worden overgedragen. In het ergste geval kan dit tot pandemieën leiden zoals in de jaren 1918 (H1N1), 1957 (H2N2), 1968 (H3N2) en 2009 (H1N1). Omdat het erfelijk materiaal van influenza A virussen uit 8 verschillende gensegmenten bestaat (PB2, PB1, PA, HA, NP, NA, M en NS) en deze segmenten uitgewisseld kunnen worden bij een gelijktijdige infectie van een gastheer met twee verschillende influenzavirussen (reassortering), kunnen nieuwe influenza A virus subtypes ontstaan. Het pandemische H2N2 virus uit 1957 is ontstaan door reassortering van het toen circulerende humane H1N1 virus met de HA, NA en PB1 gensegmenten afkomstig van één of meerdere H2N2 virussen uit vogels. Het H2N2 virus circuleerde vervolgens in mensen van 1957 tot 1968, toen het na reassortering vervangen werd door een H3N2 virus. H2N2 virussen komen op dit moment niet meer bij mensen voor, maar zijn nog wel aanwezig in wilde vogels en zouden dus opnieuw naar mensen kunnen worden overgedragen. Omdat de afweer tegen deze virussen in de bevolking steeds verder afneemt zouden H2N2 virussen uit vogels opnieuw een pandemie kunnen veroorzaken, mogelijk met ernstige gevolgen.

Om de overdracht, oftewel transmissie van influenza A virussen te bestuderen worden vaak fretten als diermodel gebruikt, omdat de luchtwegen van fretten fysiologisch gezien vergelijkbaar zijn met die van de mens. Daarnaast komt de anatomische verdeling van receptoren (structuren op cellen waar het virus aan hecht om een cel binnen te kunnen dringen) in de luchtwegen van fretten overeen met die van mensen. Zo worden bijvoorbeeld de humane H1N1 en H3N2 influenza A virussen efficiënt tussen fretten door de lucht overgedragen, terwijl influenza A virussen uit vogels niet door de lucht overdraagbaar zijn tussen fretten. Om overdracht van virussen via direct contact tussen fretten te bestuderen, wordt het virus in de neus van een fret (donor dier) gedruppeld, waarna de fret in een kooi wordt geplaatst. Een dag later wordt een niet geïnfecteerde fret (contact dier) in dezelfde kooi als de donor geplaatst om te kijken of het virus door direct contact tussen fretten kan worden overgedragen. Om virus overdracht door de lucht te bestuderen, wordt een niet-geïnfecteerde fret (ontvanger dier) in een aparte kooi geplaatst die op 10 cm afstand staat van de kooi met het donor dier. De dieren worden gescheiden door metalen roosters, zodat de dieren niet in direct contact kunnen komen en het virus dus alleen via de lucht kan worden overgedragen. Deze transmissieproeven worden in ons laboratorium altijd met vier donor-ontvanger paren uitgevoerd. Met behulp

van zulke experimenten is bijvoorbeeld voor het H5N1 vogelgriepvirus eerder aangetoond dat een klein aantal genetische veranderingen (mutaties) in het virus RNA voldoende is om dit virus via de lucht overdraagbaar te maken tussen fretten.

Om de mogelijke pandemische dreiging van H2N2 virussen uit vogels te bestuderen, is in **hoofdstuk 4** de overdraagbaarheid van zes H2N2 virussen uit vogels in het transmissiemodel bestudeerd. We laten zien dat geen van deze virussen via direct contact of via de lucht overdraagbaar was tussen fretten. Deze resultaten wijzen erop dat de pandemische dreiging van H2N2 virussen uit vogels zonder voorafgaande adaptatie, door mutatie of reassortering met andere influenza A subtypes, heel klein is. Vervolgens hebben we in het frettenmodel de luchtoverdraagbaarheid van drie humane H2N2 virussen met elkaar vergeleken. Deze virussen werden in 1957 (begin van de pandemie), 1958, en 1968 (het laatste jaar waarin H2N2 virussen in mensen gedetecteerd werden) verzameld uit monsters afkomstig van de bovenste luchtwegen van geïnfecteerde personen. Uit dit onderzoek bleek dat alleen het virus uit 1958, maar niet de virussen uit 1957 en 1968 efficiënt via de lucht overdraagbaar waren tussen fretten. Daarom hebben we in een vervolgonderzoek bestudeerd of H2N2 virussen na de introductie in mensen in 1957 nog verder zijn gemuteerd om efficiënter te vermenigvuldigen en verspreiden in de mens, aangezien dit het verschil in overdracht van het 1957 en 1958 virus tussen fretten zou kunnen verklaren. Eerdere studies aan het H5N1 vogelgriepvirus hebben laten zien dat onder andere een verbeterde binding aan humane receptoren en een stabiel HA-eiwit nodig zijn voor vermenigvuldiging en verspreiding in fretten. Deze receptoren bevinden zich op het oppervlak van gastheercellen en bestaan uit suikerketens met zogenaamde siaalzuren die door het HA-eiwit gebonden worden. Binding aan receptoren wordt gevolgd door penetratie van de cel waarna het virus zich kan vermenigvuldigen. Griepvirussen afkomstig uit vogels binden 2,3-gekoppelde siaalzuren (aviaire receptoren) die voornamelijk op cellen in het darmkanaal van vogels zitten maar ook op cellen in de lagere luchtwegen bij mensen aanwezig zijn. Humane griepvirussen daarentegen binden 2,6-gekoppelde siaalzuren (humane receptoren) die voornamelijk op cellen in de bovenste luchtwegen van mensen aanwezig zijn. Als een vogelgriepvirus in een mens terecht komt kan het virus in eerste instantie slecht aan cellen in de bovenste luchtwegen binden en zich niet efficiënt vermenigvuldigen en tussen mensen worden overgedragen. Het virus moet eerst muteren om te kunnen binden aan de receptoren die in de bovenste luchtwegen van mensen aanwezig zijn. Door het verschil in overdraagbaarheid tussen fretten was het onze verwachting dat het

1957 virus nog niet volledig was aangepast aan de mens en het 1958 virus wel. Om dit te onderzoeken hebben we de binding aan receptoren en de HA-stabiliteit van de drie humane H2N2 virussen met behulp van verschillende *in vitro* (proeven met cellen) proeven geanalyseerd. Deze analyse liet zien dat de virussen uit 1957 en 1958 allebei goed aan humane receptoren konden binden en dat daar dus geen verdere aanpassing nodig was. Wel hebben we een nieuwe substitutie in het HA-eiwit van het virus uit 1958 geïdentificeerd dat het HA-eiwit stabieler maakte ten opzichte van het virus uit 1957. Dit suggereert dat H2N2 virussen in het eerste jaar na introductie bij de mens verder zijn gemuteerd in de nieuwe gastheer. Vervolgens hebben we onderzocht of het stabielere HA-eiwit de luchtoverdraagbaarheid van het virus uit 1958 zou kunnen verklaren. Hieruit bleek dat een stabieler HA-eiwit in het 1957 virus niet tot een verbeterde luchtoverdraagbaarheid van het virus leidde in het frettenmodel. Wel moet hierbij worden opgemerkt dat het frettenmodel niet geschikt is om kleine verschillen in luchtoverdraagbaarheid vast te stellen.

Het virus uit 1968 bleek de receptoren van zowel vogels als mensen even goed te binden. Verder was het HA-eiwit van dit virus in vergelijking met dat van de twee vroegere virussen instabiel. De combinatie van binding aan zowel humane als vogel receptoren en een instabiel HA-eiwit zou mogelijk kunnen verklaren waarom dit virus niet overdraagbaar was tussen fretten. Echter, moet worden opgemerkt dat dit virus ten opzichte van de virussen uit 1957 en 1958 veel mutaties had. De virussen uit 1957 en 1958 zijn alleen in zoogdiercellen gekweekt, terwijl het virus uit 1968 ook in kippeneieren was opgegroeid. Dit kan tot gevolg hebben gehad dat dit virus tijdens de vermenigvuldiging in het ei is gemuteerd en daardoor vogelreceptoren beter herkent. We weten dus niet of het virus dat gebruikt is in onze experimenten identiek is aan het virus dat vroeger in mensen heeft gecirculeerd.

Wij concluderen uit dit werk dat H2N2 virussen uit wilde vogels niet zonder voorafgaande mutatie of reassortering in staat zijn tot transmissie tussen zoogdieren. Substituties in H2 HA die receptorspecificiteit en stabiliteit bepalen kunnen echter leiden tot beter overdraagbare virusvarianten. Het zou kunnen dat door de totale afwezigheid van immuniteit tegen H2N2 virussen in mensen, de vroege virussen uit 1957 zich misschien niet efficiënt door de lucht hoefden te kunnen te verspreiden om een pandemie te kunnen veroorzaken en dat de efficiëntie van transmissie pas later toenam. Het is echter ook mogelijk dat het frettenmodel niet geschikt is om betrouwbare uitspraken te doen over de efficiëntie van overdracht van H2N2 influenzavirus. Wij achten een continue monitoring van H2N2 virussen in vogels en andere dieren als onderdeel van een pandemische risicoanalyse gerechtvaardigd.

Onderzoek aan de luchtoverdraagbaarheid van SARS-CoV-2 tussen fretten over meer dan een meter afstand

Coronavirussen, zoals het humane coronavirus (HCoV)-229E, HCoV-NL63, HCoV-OC43 en HCoV-HKU1, zijn luchtwegvirussen die jaarlijks vooral in de winter verantwoordelijk zijn voor een groot percentage van de verkoudheidsklachten bij mensen. Er zijn echter ook coronavirussen die een fatale longontsteking kunnen veroorzaken. Severe acute respiratory syndrome coronavirus (SARS-CoV) is in 2003 voor het eerst opgedoken in China en heeft vervolgens een uitbraak van longontstekingen veroorzaakt. SARS-CoV werd voornamelijk door nauw contact tussen mensen overgedragen en werd naar 26 landen verspreid voordat de uitbraak kon worden ingedamd. Sinds 2004 zijn er geen infecties met SARS-CoV meer waargenomen. In 2019 werd een verwant coronavirus, SARS-CoV-2, gedetecteerd in China. In tegenstelling tot SARS-CoV heeft dit virus zich razendsnel over de hele wereld verspreid. SARS-CoV-2 veroorzaakt een breed spectrum aan ziekteverschijnselen variërend van milde verkoudheidsklachten tot zware longontstekingen die fataal kunnen zijn. Echter, een infectie met SARS-CoV-2 kan ook asymptomatisch, dus zonder enige symptomen, verlopen. Dat maakt het heel moeilijk om verspreiding van het virus te voorkomen, omdat mensen niet, of te laat, merken dat ze geïnfecteerd zijn en ondertussen het virus wel verder kunnen verspreiden. In een eerste transmissie studie bleek dat SARS-CoV-2 zowel via direct contact als via de lucht kan worden overgedragen tussen fretten. Omdat we met deze experimentele opstelling alleen de luchtoverdraagbaarheid van virussen tussen fretten over korte afstand (10 cm) konden aantonen, hebben we in **hoofdstuk 5** een nieuwe opstelling ontwikkeld waarbij de overdracht van virussen door de lucht over meer dan één meter kan worden bestudeerd. Hiervoor werd eerst een ontvanger kooi boven een donor kooi geplaatst. Vervolgens werden deze twee kooien met elkaar verbonden door een 15 cm breed en 119 cm lang buizensysteem dat vier hoeken van 90 graden bevat. In tegenstelling tot de oude opstelling moeten virussen dus nu met de luchtstroom mee tegen de zwaartekracht door het buizensysteem heen om bij het ontvanger dier terecht te komen. Door deze langere afstand worden virussen langer blootgesteld aan de luchtstroom, temperatuur en luchtvochtigheid wat de stabiliteit van het virus kan beïnvloeden en dus als gevolg zou kunnen hebben dat het virus infectiviteit verliest. De nieuwe transmissieopstelling hebben we eerst getest met een pandemisch influenza A H1N1 virus, waarvan we weten dat het efficiënt via de lucht tussen fretten of mensen wordt overgedragen en dus ook in dit nieuwe systeem zou moeten worden

overgedragen naar ontvanger dieren. Het pandemisch H1N1 virus bleek inderdaad overdraagbaar tussen fretten in de nieuwe opstelling. Daarom hebben we in een vervolgonderzoek de overdracht van SARS-CoV-2 in dit systeem bestudeerd en ook dit virus werd overgedragen naar ontvanger dieren. Omdat het er vanuit de epidemiologische data niet op lijkt dat SARS-CoV-2 zich in mensen efficiënt via de lucht over grotere afstanden kan verspreiden, waren deze resultaten verrassend. Om dit verder uit te zoeken, hebben we vervolgens besloten om ook SARS-CoV, het virus dat zich in 2003 nauwelijks via de lucht tussen mensen verspreidde, in het nieuwe systeem te testen. De resultaten van dit experiment waren onverwacht, aangezien ook SARS-CoV de ontvanger dieren via de lucht kon infecteren. Deze resultaten die verkregen werden met SARS-CoV en SARS-CoV-2 laten zien dat beide virussen zich goed tussen fretten door de lucht kunnen verspreiden. Noemenswaardig is dat SARS-CoV-2 tientallen uitbraken in nertsen, die net als fretten tot de familie van de marterachtigen behoren, heeft veroorzaakt. Het lijkt er dus op dat SARS-CoV-2 makkelijk binnen deze diersoorten overgedragen wordt en dat fretten en nertsen misschien vatbaarder zijn voor deze virussen dan mensen. Deze resultaten moeten met voorzichtigheid geïnterpreteerd worden en kunnen niet direct vertaald worden naar de situatie bij mensen. We hebben met behulp van een nieuw ontwikkelde transmissie opstelling wel voor de eerste keer aangetoond dat SARS-CoV en SARS-CoV-2 in de lucht infectieus blijven en via de lucht over een afstand van meer dan één meter overgedragen kunnen worden tussen fretten. Dit betekent dat de overdracht van SARS-CoV-2 door de lucht tussen mensen in principe mogelijk is, hoewel de epidemiologische data aangeven dat de bijdrage van overdracht via de lucht bij de verspreiding van SARS-CoV-2 relatief klein is.

Conclusie

Samengevat geven de resultaten in dit proefschrift, door gebruik te maken van verschillende experimentele methodes, meer inzicht in de luchtoverdraagbaarheid van een aantal belangrijke luchtwegvirussen. Met behulp van een in het laboratorium geoptimaliseerde lucht sampler hebben we aangetoond dat de lucht om RSV-geïnfecteerde kinderen heen geen infectieus RSV bevat, terwijl infectieus RSV wel in de neus van kinderen kon worden gedetecteerd. Verder hebben we laten zien dat H2N2 virussen uit vogels niet overdraagbaar zijn tussen fretten en dus geen directe pandemische dreiging vormen voor de mens. We hebben ook aangetoond dat H2N2 virussen zich in het eerste jaar van circulatie in de mens verder hebben aangepast aan de mens, maar dat deze aanpassingen in ons frettenmodel niet tot een betere luchtoverdraagbaarheid

heeft geleid. Verder hebben we door gebruik te maken van een nieuw ontwikkelde transmissieopstelling, voor het eerst aangetoond dat SARS-CoV-2 via de lucht tussen fretten over een afstand van meer dan een meter kan worden overgedragen.

In de toekomst moeten lucht samplers in verschillende omgevingen zoals ziekenhuizen of scholen worden gebruikt om te bestuderen welke infectieuze luchtwegvirussen in de lucht aanwezig zijn. Deze lucht samplers kunnen mogelijk ook als middel voor monitoring in bijvoorbeeld veehouderijen gebruikt worden om opkomende virussen snel te kunnen detecteren. Daarnaast moet er voor elk luchtwegvirus onderzocht worden wat het beste diermodel is en wat een geschikte transmissieopstelling is voor deze diersoorten. Zo kunnen de overdraagbaarheid van virussen, de factoren die de efficiëntie van luchtoverdraagbaarheid van virussen bepalen en (niet-) farmaceutische interventiemaatregelen bestudeerd worden. Het is belangrijk dat nieuwe methodes ontwikkeld worden waarmee dit soort factoren in de toekomst met een verminderd aantal of zelfs helemaal zonder dieren kan worden bestudeerd. Zo kunnen bijvoorbeeld vernevelaars en lucht samplers mogelijk als vervanging voor donor en ontvanger dieren gebruikt worden. Verder kunnen *in vitro* systemen ontwikkeld worden waarmee de stabiliteit van virussen in de lucht, inclusief factoren die dit beïnvloeden, kan worden onderzocht. De geproduceerde data moet uiteindelijk betrouwbare informatie over de relatieve bijdrage van de transmissieroutes in verschillende omgevingen opleveren. Met het snel beschikbaar komen van dergelijke informatie bij uitbraken, epidemieën en pandemieën kunnen maatregelen tegen de verspreiding van bestaande en opkomende luchtwegvirussen snel geïmplementeerd worden, met een goede wetenschappelijke onderbouwing.

ZUSAMMENFASSUNG

Bei Atemwegsviren handelt es sich um eine Gruppe Viren, die in menschliche Atemwege eindringen und dort ein breites Spektrum an Krankheitssymptomen hervorrufen. Das kann von einfachen Erkältungsbeschwerden bis hin zu schweren Infektionen, wie einer Lungenentzündung, reichen. Für die meisten Menschen sind diese Arten von Infektionen harmlos und klingen von selbst wieder ab. Bei Abwehrgeschwächten, wie beispielsweise Kleinkindern, älteren Menschen, oder Personen mit Grunderkrankungen, können diese Viren jedoch schwere Infektionen der unteren Atemwege verursachen. Im schlimmsten Fall können diese tödlich sein. Einmal in die Atemwege eingedrungen, vermehren sich die Viren und werden durch Atmen, Sprechen, Husten und Niesen einer infizierten Person in die Umgebung ausgeschieden. Atemwegsviren können dann über verschiedene Wege auf andere Menschen übertragen werden. Sie können durch direkten Kontakt über beispielsweise Händeschütteln mit einer infizierten Person, oder durch indirekten Kontakt über das Anfassen kontaminierter Oberflächen wie Türklinken, übertragen werden. Wenn eine Person anschließend mit kontaminierten Händen seine Nase oder seinen Mund anfasst, kann das Virus über die Schleimhäute in die Atemwege eindringen und dort eine Infektion auslösen. Diese Viren können auch ohne jeglichen Kontakt mit einer infizierten Person in Form von Tröpfchen und Aerosolen durch die Luft übertragen werden. Große Tröpfchen können nur kurze Zeit in der Luft verbleiben und nur innerhalb kurzer Abstände auf die Nasen- und Mundschleimhäute einer Person gelangen. Aerosole hingegen sind Tröpfchen, die klein genug sind, um für einen langen Zeitraum in der Luft zu verbleiben und können daher über größere Abstände übertragen werden. Da Aerosole so klein sind, können sie auch tief in die Lungen eingeatmet werden. Bei vielen Atemwegsviren weiß man bis heute nicht genau, über welche dieser Übertragungswege sie sich hauptsächlich ausbreiten, da dies per Virus unterschiedlich ist. Zudem ist unklar, inwiefern Faktoren, wie die Virusstabilität in der Luft, das Ausmaß der Virusausscheidung, die Temperatur oder die Luftfeuchtigkeit den relativen Beitrag der einzelnen Übertragungswege beeinflussen. Dieses Wissen ist jedoch dringend notwendig, um die Gefahr von neuen und wiederkehrenden Viren, die einen Ausbruch verursachen könnten, einschätzen zu können. Darüber hinaus kann dieses Wissen dazu beitragen, nicht-pharmazeutische Infektionspräventionsmaßnahmen, wie Abstand halten, öffentliche Bereiche schließen und das Tragen von Gesichtsmasken, zu verbessern, und so die Verbreitung von Atemwegsviren zwischen Menschen zu kontrollieren oder sogar

zu verhindern. Um grundlegende Kenntnisse der Übertragungswege, über die sich Atemwegsviren ausbreiten können, zu erlangen, wurde in dieser Dissertation, unter Verwendung bestehender und neu entwickelter experimenteller Methoden, die Übertragbarkeit einer Reihe wichtiger Atemwegsviren über die Luft untersucht.

Sammeln von Atemwegsviren aus der Luft unter Verwendung von Luftprobenehmern

Um die Anzahl an infektiösem Virus in der Luft in einer bestimmten Umgebung zu ermitteln, beispielsweise in einem Krankenhaus, werden oftmals sogenannte Luftprobenehmer verwendet. Diese Geräte saugen über eine angeschlossene Vakuumpumpe Luft aus der Umgebung an und sammelt die darin enthaltenen Viren in einem bestimmten Medium. Es gibt verschiedene Luftprobenehmer, mit unterschiedlichen Methoden, um Viren aus der Luft zu sammeln. Die Meisten sind jedoch nicht speziell für das Sammeln von Viren konzipiert. Ein solcher Luftprobenehmer ist beispielsweise der Andersen Kaskadenimpaktor. Ein großer Vorteil dessen gegenüber anderen Luftprobenehmern besteht darin, dass Aerosole und Tröpfchen je nach Größe in sechs Stufen gesammelt werden. Mit Informationen über die Größe der virushaltigen Aerosole und Tröpfchen kann man beurteilen, ob ein Virus über eine große oder nur geringe Entfernung übertragen werden kann. Diese Informationen helfen dabei Maßnahmen zur Infektionsprävention anzuwenden und die Ausbreitung des Virus zu verhindern. Der Andersen Kaskadenimpaktor wurde ursprünglich für das Sammeln von Bakterien und Pilzen aus der Luft in ein festes Sammelmedium entwickelt. Ein festes Medium ist jedoch für das Sammeln von Viren nicht geeignet. Aus diesem Grund haben wir in **Kapitel 2** neben dem harten Sammelmedium im Kaskadenimpaktor auch ein flüssiges und ein selbstentwickeltes halbfestes Medium getestet. Damit sollte ermittelt werden, welches Medium am besten zum Sammeln von infektiösem Virus geeignet ist. Zu diesem Zweck verwendeten wir zunächst zwei verschiedene Atemwegsviren, ein Grippevirus und das humane Metapneumovirus, das Infektionen hauptsächlich bei Kindern und älteren Menschen verursacht. Diese Viren wurden in einer luftdichten Box vernebelt und anschließend im Andersen Kaskadenimpaktor mit einem von den drei verschiedenen Medien gesammelt. Wir fanden heraus, dass im Gegensatz zum harten und flüssigen Sammelmedium, im halbfesten Medium eine große Menge an infektiösem Virus gesammelt werden konnte. Daraufhin haben wir den Andersen Kaskadenimpaktor mit dem halbfesten Medium mit zwei anderen Luftprobenehmern, dem BioSampler und einem selbstentwickelten Elektrofilter, verglichen. Der BioSampler sammelt Viren in einem flüssigen Medium, kann

jedoch nicht, wie der Andersen Kaskadenimpaktor, die Größe von virushaltigen Aerosolen und Tröpfchen unterscheiden. Sowohl der Andersen Kaskadenimpaktor, als auch der BioSampler sammeln Aerosole und Tröpfchen basierend auf der Masseträgheit dieser Teilchen. Infolgedessen können diese Luftprobenehmer kleine Aerosole nur sehr schwierig sammeln. Aus diesem Grund haben wir einen Elektrofilter entwickelt, bei dem Aerosole und Tröpfchen beim Eintreffen im Luftprobenehmer positiv geladen werden und dann von einem neutral geladenen, flüssigem Sammelmedium angezogen werden. Theoretisch sollten durch diese Methode kleine Aerosole besser gesammelt werden als mit dem Andersen Kaskadenimpaktor oder dem BioSampler. Um die Sammlungseffizienz dieser drei Luftprobenehmer zu vergleichen, haben wir vier verschiedene Atemwegsviren vernebelt – ein Grippevirus, das humane Metapneumovirus, das respiratorische Synzytial-Virus und das humane Parainfluenzavirus Typ 3 – und jeweils mit einem der Luftprobenehmer gesammelt. Diese Studie zeigte, dass sowohl der BioSampler, als auch der Andersen Kaskadenimpaktor, große Mengen aller vier Viren sammeln konnte. Weniger effizient war dagegen der Elektrofilter, der nur sehr geringe Mengen an Viren aus der Luft sammelte und daher ungeeignet ist.

Nachweis von RSV RNA in der Luft bei Kleinkindern mit akuten Atemwegsinfektionen

Atemwegsviren bestehen aus genetischem Material (RNA oder DNA), das von Eiweißen und häufig auch von einer Lipidmembran umhüllt ist. Diese Lipidmembran trägt verschiedene Eiweiße auf ihrer Oberfläche, sogenannte Oberflächenproteine. Diese sind unter anderem für das Eindringen des Virus in eine Wirtszelle nötig. Nicht die nackte Virus RNA, sondern nur intakte Virusteilchen, können in eine Wirtszelle eindringen und eine Infektion auslösen. Wenn also von infektiösem Virus gesprochen wird, ist damit jenes intakte Virusteilchen gemeint, welches im Stande ist, einen Organismus zu infizieren. Es ist daher wichtig zu bestimmen, ob die aus der Luft gesammelten Tröpfchen und Aerosole infektiöse Viren enthalten oder nicht. Dies ist möglich, indem nicht nur das Vorhandensein des genetischen Materials in einer Luftprobe ermittelt wird, sondern auch zu testen, ob das Virus in eine geeignete Zelllinie eindringen und sich dort vermehren kann.

Das respiratorische Synzytial-Virus (RSV) ist ein Atemwegsvirus, das beim Menschen im Allgemeinen leichte Erkältungssymptome verursacht. Bei sehr kleinen Kindern kann RSV jedoch schwerwiegendere Infektionen verursachen, die zu einer Bronchiolitis oder Lungenentzündung führen können und manchmal

einen Krankenhausaufenthalt erfordern. Es wurde jahrelang angenommen, dass RSV hauptsächlich durch engen Kontakt mit einer infizierten Person übertragen wird. Daher werden bei Kindern mit RSV Infektionen in Krankenhäusern Isolationsmaßnahmen zur Verhinderung der Kontakt- und Tröpfcheninfektion eingesetzt. In einer kürzlich veröffentlichten Studie wurde jedoch berichtet, dass in einem Krankenhaus große Mengen an infektiösem RSV in der Luft um infizierte Kinder herum nachgewiesen wurden. Es ist daher noch unklar, ob sich RSV auch effizient über die Luft ausbreiten kann. Aus diesem Grund haben wir in **Kapitel 3** den Andersen Kaskadenimpaktor, der beim Labortest am besten abgeschnitten hatte, in einem Kinderkrankenhaus eingesetzt, um die Menge an infektiösem RSV in der Luft rund um RSV-infizierte Kleinkinder zu bestimmen. In unserer Studie zeigten wir auf, dass infektiöses RSV zwar für einen langen Zeitraum in den oberen Atemwegen der Kleinkinder vorhanden war, jedoch bei drei von sechs Kindern nur eine geringe Menge RSV RNA aus der Luft gesammelt werden konnte. Bei keinem der Kinder wurde infektiöses RSV in der Luft festgestellt. Diese Ergebnisse weisen darauf hin, dass die Wahrscheinlichkeit einer RSV-Übertragung durch die Luft in einer solchen Krankenhauseinrichtung sehr klein ist. Jedoch muss darauf hingewiesen werden, dass nur eine sehr kleine Anzahl an Kleinkindern in diese Studie aufgenommen werden konnten. Darüber hinaus konnte erst ein paar Tage nach dem Auftreten der ersten Krankheitssymptome mit der Luftprobenentnahme begonnen werden. Daher besteht die Möglichkeit, dass zu Beginn einer Infektion mehr infektiöses Virus durch infizierte Kleinkinder in die Luft ausgeschieden wird und wir diese Tage in unserer Studie nicht berücksichtigen konnten. Nichtsdestotrotz stimmen unsere Ergebnisse mit den Erkenntnissen der Mehrheit solcher Studien überein und deuten darauf hin, dass die im Moment in Krankenhäusern eingesetzten Isolationsmaßnahmen ausreichend sind. Es bleibt unklar, warum in dieser einen Studie sehr große Mengen an infektiösem RSV in der Luft gefunden wurden, in vielen anderen, vergleichbaren Studien jedoch nicht.

Anpassung von H2N2 Grippeviren an Menschen

Influenzaviren, besser bekannt als Grippeviren, sind Atemwegsviren die für die jährlichen Grippeausbrüche im Winter verantwortlich sind. Influenzaviren werden in die Typen A, B, C und D unterteilt. Influenza A Viren werden zusätzlich basierend auf die zwei Oberflächenproteinen Hemagglutinin (HA) und Neuraminidase (NA), in verschiedene Subtypen aufgeteilt. Die Kombination aus den HA und NA Proteinen auf der Oberfläche eines Grippevirus wird für die Namensgebung dieser Viren verwendet. So werden beispielsweise die Viren, die die saisonale

Grippe in Menschen verursachen H1N1 und H3N2 Viren genannt. Bis jetzt sind 16 verschiedene HA und 9 verschiedene NA Subtypen bekannt, die in Wildvögeln zirkulieren und zwei HA- und NA-Subtypen, die in Fledermäusen zirkulieren. Influenza A Viren kommen hauptsächlich in Wildvögeln vor, daher der Name Vogelgrippe. In einzelnen Fällen springen diese Vogelgrippeviren jedoch auf den Menschen über und können dort schwere Atemwegsinfektionen auslösen. Meistens können Influenza A Viren, die von Vögeln stammen, nicht zwischen Menschen übertragen werden. In einzelnen Fällen verändern sich diese Viren während einer Infektion beim Menschen jedoch, wodurch sie dann effizient von Mensch zu Mensch übertragen werden können. Im schlimmsten Fall kann dies zu Pandemien führen wie in den Jahren 1918 (H1N1), 1957 (H2N2), 1968 (H3N2) und 2009 (H1N1). Da Influenza A Viren aus acht einzelnen Genabschnitten bestehen (PB2, PB1, PA, HA, NP, NA, M und NS), können diese Genabschnitte bei einer gleichzeitigen Infektion mit zwei unterschiedlichen Influenza A Viren in einer Wirtszelle ausgetauscht werden (Neuzusammenstellung), wodurch neue Influenza A Virus Subtypen entstehen können. Das pandemische H2N2 Virus aus dem Jahr 1957 ist durch eine Neuzusammenstellung eines damals zirkulierenden humanen Grippevirus mit den HA, NA und PB1 Genabschnitten aus ein oder mehreren Vogelgrippeviren entstanden. Das H2N2 Virus zirkulierte von 1957 bis 1968 anschließend zwischen Menschen, bis es durch eine Neuzusammenstellung vom H3N2 Virus ersetzt wurde. H2N2 Viren sind unterdessen vollständig aus der Bevölkerung verschwunden, werden jedoch immer noch in wilden Vögeln nachgewiesen und könnten daher erneut in der Bevölkerung auftauchen. Da die Abwehr gegen H2N2 Viren in der Bevölkerung stetig weiter abnimmt, könnten H2N2 Viren eine erneute Pandemie mit ernsten Folgen verursachen.

Um die Übertragbarkeit von Influenza A Viren zu untersuchen, werden oftmals Frettchen als Versuchstier verwendet, da sie eine ähnliche Lungenphysiologie wie Menschen besitzen. Des Weiteren besitzen die Atemwege der Frettchen eine vergleichbare Rezeptorverteilung (Strukturen auf den Wirtszellen, an die Viren anbinden, um in eine Zelle einzudringen) wie die menschlichen Atemwege. Daher werden beispielsweise die humanen H1N1 und H3N2 Viren sehr effizient zwischen Frettchen durch die Luft übertragen, während Vogelgrippeviren zwischen Frettchen nicht durch die Luft übertragbar sind. Um also eine Pandemie auszulösen, müssen sich Influenza A Viren erst durch Veränderungen in ihrem genetischen Material (Mutationen) oder durch eine Neuzusammenstellung an Menschen anpassen, damit sie effizient zwischen Menschen übertragen werden können. Um die

Übertragbarkeit von Viren durch direkten Kontakt zwischen Frettchen untersuchen zu können, wird das entsprechende Virus in die Nase eines Frettchens, das Donortier, getropft, welches anschließend in einen Käfig gesetzt wird. Einen Tag später wird ein nicht-infiziertes Frettchen, das Kontakttier, in denselben Käfig zum Donortier gebracht, um zu untersuchen, ob das Virus durch direkten Kontakt zwischen Frettchen übertragen werden kann. Um die Übertragbarkeit eines Virus durch die Luft zu ermitteln, wird ein nicht-infiziertes Frettchen, das indirekte Empfängertier, in einen separaten Käfig gesetzt. Die beiden Käfige stehen 10 cm auseinander und sind durch Metallgitter getrennt, sodass die Kontaktübertragung zwischen den Tieren verhindert wird und nur eine Übertragung des Virus durch die Luft stattfinden kann. Diese Übertragungsstudien werden in unserem Labor immer mit vier solcher Donor-Empfänger Paaren durchgeführt. Studien am Vogelgrippevirus H5N1 in einer solchen Einrichtung haben aufgezeigt, dass einige wenige Mutationen ausreichen, um das Virus zwischen Frettchen luftübertragbar zu machen.

Um das pandemische Risiko, das von H2N2 Viren aus Vögeln ausgeht, abschätzen zu können, haben wir in **Kapitel 4** sechs H2N2 Viren, die aus verschiedenen Vögeln stammten, im Übertragungsmodell untersucht. In dieser Studie konnten wir zeigen, dass diese Viren weder durch direkten Kontakt, noch durch die Luft zwischen den Frettchen übertragen wurden. Diese Ergebnisse weisen darauf hin, dass die pandemische Gefahr die von diesen H2N2 Viren ausgeht ohne vorhergehende Anpassung durch Mutation oder Neuzusammenstellung mit anderen Influenza A Subtypen sehr gering ist. Anschließend haben wir die Luftübertragbarkeit von drei menschlichen H2N2 Viren im Übertragungsmodell untersucht. Diese Viren wurden 1957 (Beginn der Pandemie), 1958 und 1968 (das letzte Jahr, in dem H2N2 Viren beim Menschen nachgewiesen wurden) aus Proben der oberen Atemwege infizierter Personen gewonnen. Diese Studie ergab, dass nur das Virus aus dem Jahr 1958, nicht aber die Viren aus den Jahren 1957 und 1968 zwischen Frettchen durch die Luft übertragen wurde. In einer Folgestudie untersuchten wir daher, ob sich H2N2 Viren nach ihrer Einführung in die Bevölkerung im Jahr 1957 noch weiter angepasst haben. Dies könnte erklären, warum das Virus aus dem Jahr 1958, aber nicht das aus 1957 zwischen Frettchen übertragen wurde. Frühere Studien am Vogelgrippevirus H5N1 haben aufgezeigt, dass unter anderem eine verbesserte Bindung an menschliche Rezeptoren und ein stabiles HA Protein für die Vermehrung und Ausbreitung bei Frettchen erforderlich sind. Diese Rezeptoren befinden sich auf der Oberfläche von Wirtszellen und bestehen aus Zuckerketten mit sogenannten Sialinsäuren, woran das HA Protein

anbindet. Nach der Bindung an Rezeptoren folgt dann das Eindringen des Virus in die Zelle, worin sich das Virus vermehren kann. Vogelgrippeviren binden 2,3-verkettete Sialinsäuren (aviäre Rezeptoren), welche sich auf den Zellen im Darmtrakt von Vögeln, aber auch auf Zellen der unteren Atemwege von Menschen befinden. Humane Grippeviren binden dagegen 2,6-verkettete Sialinsäuren (humane Rezeptoren), die hauptsächlich auf Zellen der oberen Atemwege des Menschen vorhanden sind. Wenn ein Vogelgrippevirus also erstmals in einen Menschen eindringt, bindet es möglicherweise zunächst schlecht an Zellen in den oberen Atemwegen und kann sich daher nicht effizient vermehren und in der Bevölkerung ausbreiten. Hierfür muss sich das Virus zunächst an Rezeptoren anpassen, die in den oberen Atemwegen des Menschen vorhanden sind. Aufgrund des Unterschieds in der Übertragbarkeit der Viren aus den Jahren 1957 und 1958 zwischen Frettchen, erwarteten wir, dass das Virus aus dem Jahr 1957 noch nicht vollständig an den Menschen angepasst war, während sich das Virus aus dem Jahr 1958 bereits angepasst hatte. In verschiedenen *in vitro* Experimenten (Experimente mit Zellen) analysierten wir daher die Neigung der drei menschlichen H2N2 Viren an aviäre und humane Rezeptoren anzubinden, sowie auch ihre HA Stabilität. Diese Analyse zeigte, dass die Viren aus den Jahren 1957 und 1958 bereits gut an menschliche Rezeptoren binden konnten, sodass keine weitere Anpassung an humane Rezeptoren erforderlich war. Darüber hinaus identifizierten wir eine neue Mutation im HA Gen des Virus aus dem Jahr 1958, die das HA Protein dieses Virus gegenüber dem HA Protein des Virus aus dem Jahr 1957 stabiler machte. Dies deutet darauf hin, dass sich H2N2 Viren im ersten Jahr nach der Einführung in die Bevölkerung weiter im Menschen verändert haben. Danach untersuchten wir, ob das stabilere HA Protein die Übertragbarkeit des Virus aus dem Jahr 1958 durch die Luft erklären könnte. Dies zeigte, dass im Frettchenmodell ein stabileres HA Protein im Virus aus dem Jahr 1957 nicht zu einer verbesserten Luftübertragbarkeit des Virus führte. Hierbei muss jedoch vermerkt werden, dass das Frettchenmodell nicht zur Bestimmung kleiner Unterschiede in der Luftübertragbarkeit von Viren geeignet ist. Das Virus aus 1968 konnte aviäre und humane Rezeptoren gleich gut binden. Darüber hinaus war das HA Protein dieses Virus im Vergleich zu dem der anderen beiden Viren instabil. Die Kombination aus der gleichguten Bindung an humane und aviäre Rezeptoren und einem unstabilen HA Protein könnte erklären, wieso das Virus aus dem Jahr 1968 zwischen Frettchen nicht übertragbar war. Es muss jedoch vermerkt werden, dass dieses Virus im Vergleich zu den Viren aus den Jahren 1957 und 1958 viele Mutationen im HA Gen aufwies. Die Viren aus den Jahren 1957 und 1958 wurden ausschließlich in Säugetierzellen gezüchtet,

während das Virus aus dem Jahr 1968 zusätzlich in Hühnereiern gezüchtet wurde. Dies könnte dazu geführt haben, dass das Virus während der Vermehrung im Ei mutiert ist und dadurch besser an aviäre Rezeptoren bindet. Wir wissen nicht, ob das in unseren Experimenten verwendete Virus mit dem im Jahr 1968 in Menschen zirkulierenden Virus identisch ist. Wir schließen aber aus dieser Studie, dass sich H2N2 Viren von Wildvögeln ohne vorhergehende Mutationen oder Neuzusammenstellung nicht in Säugetieren ausbreiten können. Veränderungen im H2 HA Protein, die die Rezeptorspezifizität und Stabilität bestimmen, können jedoch zu übertragbareren Virusvarianten führen. Daher kann es sein, dass sich frühe H2N2 Viren aus dem Jahr 1957, aufgrund der fehlenden Immunität gegen H2N2 Viren, nicht so effizient durch die Luft ausbreiten, um eine Pandemie zu verursachen und, dass die Übertragbarkeit dieser Viren erst in späteren Jahren zunahm. Das Frettchenmodell ist jedoch möglicherweise auch nicht geeignet, verlässliche Aussagen über die Effizienz der Übertragung von H2N2 Viren zu treffen. Im Rahmen einer Pandemie-Risikoanalyse, halten wir eine kontinuierliche Überwachung von H2N2 Viren bei Vögeln und anderen Tieren für sinnvoll.

Untersuchung der Übertragung von SARS-CoV-2 durch die Luft zwischen Frettchen über eine Entfernung von mehr als einem Meter

Coronaviren wie das humane Coronavirus HCoV-229E, HCoV-NL63, HCoV-OC43 und HCoV-HKU1 sind Atemwegsviren, die insbesondere im Winter für einen großen Prozentsatz an Erkältungen verantwortlich sind. Es gibt jedoch auch Coronaviren, die tödliche Lungenentzündungen verursachen können. Das schwere akute Atemwegssyndrom Coronavirus (SARS-CoV) ist erstmals 2003 in der chinesischen Bevölkerung aufgetreten und löste folglich einen Ausbruch an Lungenentzündungen aus. SARS-CoV wurde über engen Kontakt mit infizierten Personen übertragen und breitete sich in über 26 Ländern aus bevor der Ausbruch eingedämmt werden konnte. Seit 2004 wurden keine Infektionen mehr mit SARS-CoV festgestellt. Im Jahr 2019 wurde ein sehr ähnliches Coronavirus, SARS-CoV-2, in der chinesischen Bevölkerung erstmals nachgewiesen. Im Gegensatz zu SARS-CoV breitete sich dieses Virus rasend schnell über die ganze Welt aus. SARS-CoV-2 löst ein sehr breites Spektrum an Krankheitssymptomen aus, variierend von milden Erkältungsbeschwerden bis hin zu schweren Lungenentzündungen, die tödlich sein können. Eine Infektion mit SARS-CoV-2 kann jedoch auch ganz asymptomatisch, also ohne jegliche Krankheitssymptome, verlaufen. Dies hat zur Folge, dass das Virus nur sehr schwer eingedämmt werden kann, da einige

Menschen gar nicht oder erst zu spät merken, dass sie infiziert sind und in der Zwischenzeit das Virus weiterverbreiten. In einer ersten Übertragungsstudie zeigte sich, dass SARS-CoV-2 zwischen Frettchen durch sowohl direkten Kontakt als auch durch die Luft übertragen werden kann. Da wir mit diesem experimentellen Modell die Luftübertragbarkeit von Viren zwischen Frettchen nur über eine geringe Entfernung (10 cm) nachweisen konnten, entwickelten wir in **Kapitel 5** ein neues Modell, bei welchem die Übertragbarkeit von Viren durch die Luft über eine Entfernung von mehr als einem Meter ermittelt werden kann. Hierfür wurden die Käfige des Donor- und Empfängertiers übereinandergestellt. Die beiden Käfige wurden dann über ein 15 cm breites und 119 cm langes Rohrsystem, das vier 90 Grad Winkel besitzt, miteinander verbunden. Im Gegensatz zum vorherigen Übertragungsmodell müssen Viren nun mit dem Luftstrom gegen die Gravitationskraft durch das Rohrsystem transportiert werden, um beim Empfängertier anzukommen. Aufgrund der größeren Entfernung sind die Viren länger dem Luftstrom, der Temperatur und der Luftfeuchtigkeit ausgesetzt, was ihre Stabilität beeinträchtigt und dazu führen kann, dass die Viren ihre Infektiosität verlieren. Das neue Übertragungsmodell testeten wir zunächst mit einem pandemischen H1N1 Virus, von welchem wir wissen, dass es effizient zwischen Frettchen und Menschen durch die Luft übertragen wird und daher im neuen Modell auch auf Empfängertiere übertragen werden sollte. Das pandemische H1N1 Virus war in der Tat auch im neuen Modell zwischen Frettchen übertragbar. Nachfolgend ermittelten wir in diesem Modell die Übertragung von SARS-CoV-2; auch dieses Virus wurde auf Empfängertiere übertragen. Diese Ergebnisse waren überraschend, da aus epidemiologischen Daten nicht hervorgeht, dass SARS-CoV-2 effizient über größere Entfernungen zwischen Menschen durch die Luft übertragbar ist. Um dies weiter zu untersuchen, haben wir beschlossen auch SARS-CoV, das Virus, das sich 2003 kaum zwischen Menschen durch die Luft ausbreitete, im neuen Übertragungsmodell zu testen. Auch die Ergebnisse dieses Experiments waren unerwartet, da auch SARS-CoV die Empfängertiere durch die Luft infizieren konnte. Diese Ergebnisse zeigen, dass SARS-CoV und SARS-CoV-2 gut zwischen Frettchen durch die Luft übertragen werden können. Es ist erwähnenswert, dass SARS-CoV-2 dutzende Ausbrüche in Nerzen verursacht hat, die wie Frettchen zur Familie der Marder gehören. Es scheint daher, dass SARS-CoV-2 leicht zwischen solchen Tieren übertragen wird und Frettchen sowie Nerze möglicherweise für eine Infektion mit diesen Coronaviren anfälliger sind als Menschen. Daher müssen diese Ergebnisse vorsichtig interpretiert werden; sie können nicht direkt verwendet werden, um die Ausbreitung in der Bevölkerung einzuschätzen. Wir

haben unter Verwendung eines neu entwickelten Übertragungsmodells erstmals gezeigt, dass sowohl SARS-CoV als auch SARS-CoV-2 in der Luft infektiös bleiben und über eine Entfernung von mehr als einem Meter durch die Luft zwischen Frettchen übertragen werden können. Dies bedeutet, dass die Übertragung von SARS-CoV-2 zwischen Menschen über größere Entfernungen durch die Luft prinzipiell möglich ist. Epidemiologische Daten legen jedoch nahe, dass der Beitrag der Übertragung durch die Luft zur Ausbreitung von SARS-CoV-2 relativ klein ist.

Schlussfolgerung

Zusammenfassend bieten die Ergebnisse dieser Dissertation unter Verwendung verschiedener experimenteller Methoden einen besseren Einblick in die Übertragbarkeit einer Reihe wichtiger Atemwegsviren durch die Luft. Unter Verwendung eines im Labor optimierten Luftprobenehmers konnten wir zeigen, dass die Luft rund um RSV-infizierte Kleinkinder kein infektiöses RSV enthält, obwohl in diesen Kindern für einen längeren Zeitraum infektiöses RSV in den oberen Atemwegen nachgewiesen wurde. Des Weiteren haben wir aufgezeigt, dass H2N2 Viren, die in wilden Vögeln zirkulieren, nicht zwischen Frettchen übertragbar sind und daher keine direkte pandemische Gefahr für die Bevölkerung darstellen. Zusätzlich haben wir nachgewiesen, dass sich H2N2 Viren im ersten Jahr ihrer Ausbreitung weiter an Menschen angepasst haben, doch diese Anpassungen haben in unserem Frettchenmodell nicht zu einer verstärkten Übertragung durch die Luft geführt. Darüber hinaus konnten wir unter Verwendung eines neu entwickelten Übertragungsmodells zum ersten Mal aufzeigen, dass SARS-CoV-2 über eine Entfernung von mehr als einem Meter zwischen Frettchen durch die Luft übertragen werden kann.

Zukünftig sollten Luftprobenehmer in verschiedenen Umgebungen wie Krankenhäusern oder Schulen eingesetzt werden, um zu ermitteln, welche infektiöse Atemwegsviren in der Luft vorhanden sind. Diese Luftprobenehmer können auch zur Überwachung auf Viehmärkten und Tierfarmen eingesetzt werden, um neu auftretende Viren schnell zu erkennen. Darüber hinaus muss für jedes Atemwegsvirus ermittelt werden, welches Tiermodell das Beste ist und welche Übertragungsmodelle für diese Tierarten geeignet sind. So können die Übertragbarkeit dieser Viren, Faktoren, die effiziente Übertragung von Viren durch die Luft steuern, sowie (nicht-)pharmazeutische Interventionsmaßnahmen untersucht werden. In Zukunft müssen auch Methoden entwickelt werden, um diese Faktoren mit einer reduzierten Anzahl oder gar ohne Labortiere untersuchen

zu können. So können beispielsweise Vernebler und Luftprobenehmer als Ersatz für Donor- und Empfängertiere verwendet werden. Zusätzlich müssen *in vitro* Systeme entwickelt werden, mit denen die Stabilität von Viren einschließlich der Faktoren, die diese Stabilität beeinflussen, untersucht werden können. Letztendlich müssen die erzeugten Daten verlässliche Informationen über den relativen Beitrag der einzelnen Übertragungswege in verschiedenen Umgebungen liefern. Im Falle von Ausbrüchen, Epidemien und Pandemien können mit der raschen Verfügbarkeit solcher Informationen und mit fundierten wissenschaftlichen Erkenntnissen Maßnahmen gegen die Ausbreitung bestehender und neu auftretender Atemwegsviren rasch umgesetzt werden.

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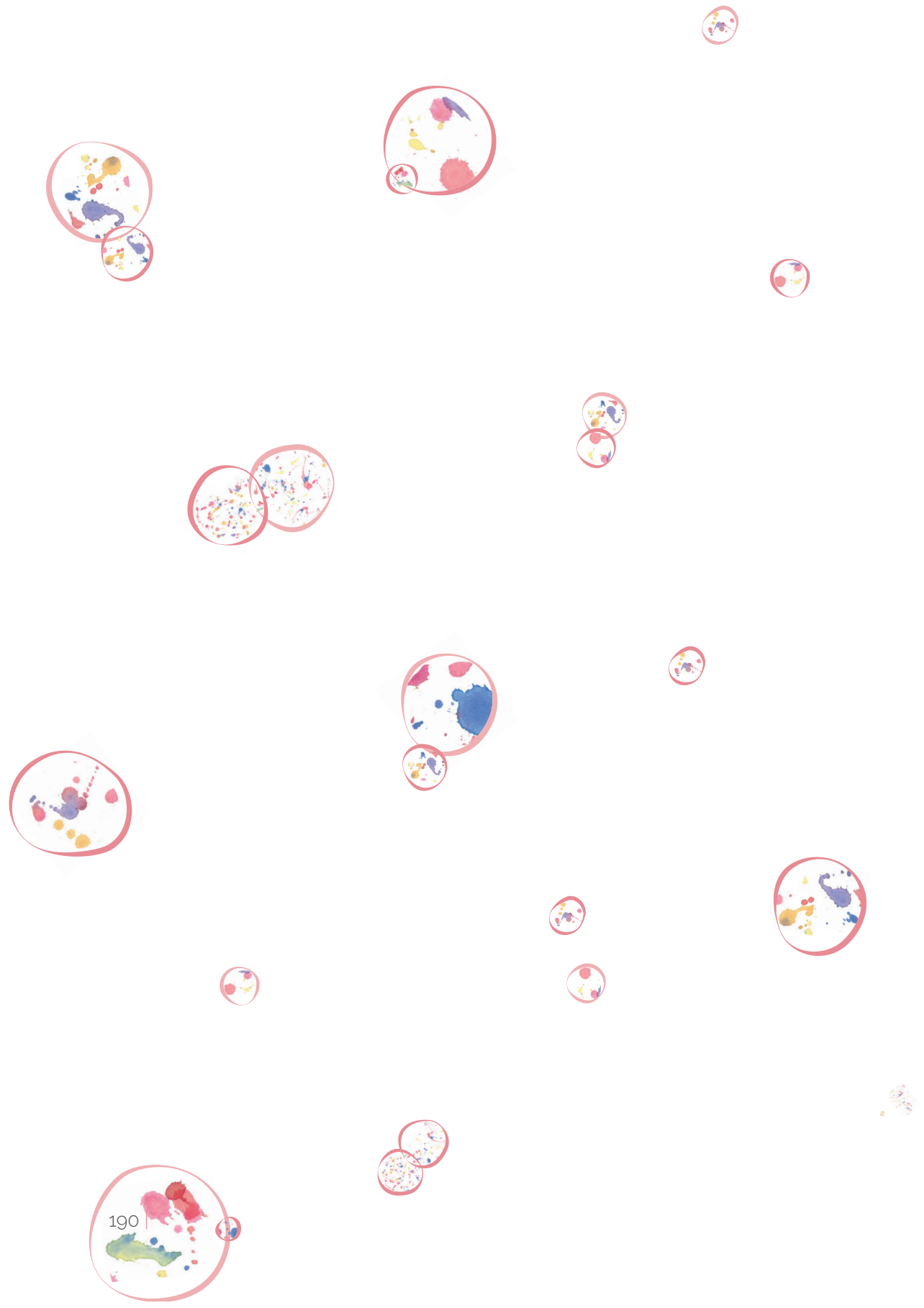

ADDENDUM

- About the author -

- Acknowledgements/Danksagung -

ABOUT THE AUTHOR

CURRICULUM VITAE

Jasmin Susanne Kutter was born on the 3rd of March 1990 in Munich, Germany. After she obtained her Abitur in 2010 from the Sophie-Scholl Gymnasium in Munich, she moved to Wageningen, the Netherlands to study Molecular Life Sciences at Wageningen University. During her Bachelor internship at the Cell Biology and Immunology Group, she studied the sensitivity of infectious bronchitis virus to type I interferon and investigated potential evasion mechanisms under the supervision of Dr. Joeri Kint and Dr. Maria Forlenza. After receiving her Bachelor of Science in 2013, she moved back to Munich, where she continued her education in Molecular Biotechnology at the Technical University of Munich. During her research internship at the Helmholtz Zentrum München, she focused on the characterization of the Ebola glycoprotein and its interaction with tetherin and tetraspanin-enriched microdomains under the supervision of Dr. Julia Nehls and Prof. Dr. Michael Schindler. After obtaining her Master's degree, in 2016, she continued her studies in the field of Virology as a PhD student under the supervision of Dr. Sander Herfst and Prof. Dr. Ron A. M. Fouchier in the department of Viroscience, Erasmus MC, Rotterdam, the Netherlands. Her research focused on experimental methods to assess the airborne transmission of respiratory viruses. The results of this research are presented in this thesis.

PORTFOLIO

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2019	Biomedical English Writing and Communication	MolMed
2018	Species-specific Laboratory Animal course (Carnivores)	Utrecht University
2017	Annual Molecular Medicine Course	MolMed
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2016	Laboratory animal science (Art. 9)	MolMed

Specific courses and workshops

2017	Programming with Python	MolMed
2016	Bayesian statistics and JASP	MolMed
2016	Workshop on SPSS and statistics	MolMed
2016	Introduction to Graphpad prism	MolMed
2016	Workshop on Indesign	MolMed
2016	Workshop on Photoshop and Illustrator	MolMed
2016	Course in Virology	MolMed

Attended meetings / seminars

2016-2021	Viroscience department seminars	Dept. Viroscience
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2018	EPAR Young researchers career day	EPAR
2016	PhD day	Erasmus MC

Attended symposia / conferences

2021	Dutch Annual Virology Symposium	Amsterdam, the Netherlands
2021	13th Annual CEIRS Network meeting	Rotterdam, the Netherlands
2019	OPTIONS X for the Control of Influenza	Singapore
2019	European Congress of Virology	Rotterdam, the Netherlands
2019	23rd Molecular Medicine Day	Rotterdam, the Netherlands
2018	Negative Strand RNA Virus meeting	Verona, Italy
2018	22nd Molecular Medicine Day	Rotterdam, the Netherlands
2017	Transmission of respiratory viruses	Hong Kong
2017	Dutch Annual Virology Symposium	Amsterdam, the Netherlands
2017	21st Molecular Medicine Day	Rotterdam, the Netherlands
2016	Young Antigone meeting	Cambridge, UK

Poster presentations

2019	OPTIONS X for the Control of Influenza	Singapore
2019	European Congress of Virology	Rotterdam, the Netherlands
2019	23rd Molecular Medicine Day	Rotterdam, the Netherlands
2018	22nd Molecular Medicine Day	Rotterdam, the Netherlands
2017	21st Molecular Medicine Day	Rotterdam, the Netherlands

Oral presentations

2021	13th Annual CEIRS Network meeting	Rotterdam, the Netherlands
2017	Transmission of respiratory viruses	Hong Kong
2016	Young Antigone meeting	Cambridge, UK

Teaching

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LIST OF PUBLICATIONS

Kutter JS, Linster M, de Meulder D, Bestebroer TM, Lexmond P, Rosu ME, Boons G-J, Richard M, de Vries RP, Fouchier RAM, Herfst S.
Continued adaptation of A/H2N2 viruses during pandemic circulation in humans.

In preparation

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