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Detection and quantification of classical swine fever virus in air samples originating from infected pigs and experimentally produced aerosols

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Abstract

During epidemics of Classical Swine Fever (CSF) neighbourhood infections occurred where none of the 'traditional' routes of transmission like direct animal contact, swill feeding, transport contact or transmission by people could be identified. A hypothesized route of virus introduction for these herds was airborne transmission. In order to better understand this possible transmission route, we developed a method to detect and quantify classical swine fever virus (CSFV) in air samples using gelatine filters. The air samples were collected from CSFV infected pigs and after experimental aerosolization of the virus. Furthermore, we studied the viability of the virus with time in aerosolized state. Three strains of CSFV were aerosolized in an empty isolator and air samples were taken at different time intervals. The virus remained infective in aerosolized state for at least 30 min with half-life time values ranging from 4.5 to 15 min. During animal experiments concentrations of $10^{0.3}$ $TCID_{50}/m^3$ to $10^{1.6}$ $TCID_{50}/m^3$ CSFV were detected in air samples originating from the air of the pig cages and $10^{0.4}$ $TCID_{50}/m^3$ to $10^{4.0}$ $TCID_{50}/m^3$ from the expired air of infected animals. This is the first study describing the isolation and quantification of CSFV from air samples originating from infected pigs and their cages, supporting previous findings that airborne transmission of CSF is feasible.

Key words: Aerosol; Air sampling, Airborne transmission, Classical swine fever virus, Quantification

Introduction

During epidemics of classical swine fever (CSF) the route of virus introduction into a farm is often unclear (Koenen *et al.*, 1996; Elbers *et al.*, 1999). If newly infected farms with an unknown route of virus introduction are located within a 1 km radius of a previously infected farm, they are usually classified as neighbourhood infections, because farms within this radius have a significantly higher risk of infection than other farms (Laevens, 1999; Stegeman *et al*., 2002). Although underreporting of well-known dangerous contacts, such as live pigs, swill feeding, transport contact or transmission by people may be a reason for being unable to elucidate the route of virus introduction, (Elbers *et al*., 2001), alternative routes of virus introduction have also been hypothesized for these neighbourhood infections. These included introduction by arthropods, birds, pets and rodents (Stewart *et al.,* 1975; Terpstra, 1987; Dewulf *et al.,* 2001; Kaden *et al.,* 2003), but also airborne spread through aerosols

produced by infected animals (Terpstra, 1987; Laevens *et al.,* 1999; Dewulf *et al.,* 2000; Ribbens *et al.,* 2004). Even aerosols produced during high-pressure cleaning of the electrocution equipment, used to kill the pigs on a neighbouring infected herd, were taken into consideration (Elbers *et al.,* 2001).

Moreover, Laevens (1999) concluded on the basis of studies of the Belgian CSFV epizootic of 1993-1994 that the likelihood of a neighbouring herd to become infected was, among others, dependent on the frequency with which it was downwind of the primary infected herd. In contrast, Crauwels *et al. (*2003), using data of the Dutch epidemic of 1997-1998, could not associate new infections with the prevailing direction of the wind. Instead, they observed a decreasing infection rate with increasing radial distance from the primary infected herd.

Experimental studies showed that CSFV can be transmitted between groups of pigs that are not in direct contact (Hughes and Gustafson, 1960; Terpstra, 1987; Laevens *et al*., 1998; Laevens *et al*., 1999; Dewulf *et al.,* 2000; González *et al*., 2001). Likely the mechanism of virus transmission is by air. Animals can generate aerosols containing virus after sneezing and coughing or during normal breathing. Also splashes of urine and faeces or sources like bedding and feed can generate aerosols (Stärk, 1999). However, until now to our knowledge no reports on the successful isolation of CSFV from the air have been published. Terpstra (1987) was able to induce indirect transmission of CSFV between infected donor pigs and susceptible recipients, but his attempts to isolate the virus from air samples using both an all-glass cyclone and a May three stage liquid impinger were unsuccessful (May, 1966; Terpstra, unpublished, 1986). Also using polyethersulfon membrane filters isolation of CSFV from the air of rooms housing experimentally infected pigs failed (Stärk, 1998). These failures were explained by a low excretion rate of CFS virus in aerosols by the infected animals and/or a lack of sensitivity of their sampling system and/or virus detection method. As a consequence, the amount of virus shed by infected pigs in the air is still unknown. This knowledge, combined with knowledge of the virus survival in aerosols, is however of great value in understanding the contribution of airborne transmission in general and to neighbouring herds more specifically.

In this paper we describe the detection and quantification of CSFV in air samples originating from infected pigs and experimentally produced aerosols. The aerosolized CSFV was studied using an air sampling technique with gelatine filters and the MD8 airscan, as it proved to be successful at the isolation of a number of airborne pathogens affecting poultry such as *Mycoplasma gallisepticum*, *M. synoviae*, *Enterococcus faecalis*, Newcastle disease virus and Gumboro disease virus (Aarnink *et al.,*

2004; Landman and van Eck, 2001; Landman *et al*., 2004). After aerosolization the initial loss and the viability of the virus with time were analyzed. Next, shedding rates of CSFV in air samples originating from infected pigs and their cages were studied.

Materials and Methods

Viruses

Three CSFV strains were used in the experiment: the highly virulent Brescia strain (genotype 1.2, derived from a strain obtained from Brescia, Italy, 1951), the moderately virulent Paderborn strain (genotype 2.1, isolated in 1997 during the outbreak in the Paderborn area of Germany) and the low virulent strain Zoelen (genotype 2.2, originally isolated from pigs in a Dutch farm in 1974). According to the classification of CSFV strains by Van Oirschot (1988) infection with a highly virulent strain results in death of nearly all pigs, infection with a moderately virulent strain results in subacute or chronic illness leading to death or recovery while pigs infected with a low virulent strain show few or no signs of disease and recover from the infection.

Experimental aerosol production

Virus stocks for aerosolization were prepared in cell culture medium (Eagle minimum essential medium (EMEM) supplemented with 10% antibiotics and 10% fetal bovine serum (FBS)). One ml of the Brescia and Paderborn virus stocks contained approximately 10^5 $TCID_{50}$ (tissue culture infectious dose 50%), the Zoelen virus stock contained approximately 10^6 $TCID_{50}$, as determined by virus titration.

The three different CSFV strains were aerosolized successively in an empty isolator (Beyer & Eggelaar, Utrecht, the Netherlands) with a volume of 0.924 m^3. An air compressor (Compact 102/3 OF, Creemers compressors, Eindhoven, the Netherlands) was coupled to a spray-head (Walther Pilot I spray-head with 0.5 mm diameter; Walther Spritz- and Lackiersysteme, Wuppertal, Germany) to generate the aerosols. A volume of 10 ml of virus stock was aerosolized in 95-180 seconds at a pressure of 2 bar. The isolator temperature was 21-22°C and the relative humidity after aerosol production was 60-74%. Aerosolization of each virus and for each sampling protocol (see below) was carried out in duplicate. After each aerosol experiment the ventilation, with a rate of 1324 m^3/h, was

switched on for 15 min before the next aerosolization. Control air samples were taken to ensure that virus concentrations were reduced to a level below the detection limit.

Characterization of the aerosol spectrum

The droplet size distribution of the aerosol, produced by the Walther Pilot I spray-head, was determined with a laser diffraction particle size analyzer (Mastersizer-S long bed; Malvern Instruments Ltd., Malvern, UK). The test was performed in triplicate as described by Landman *et al.* (2004). Briefly, ten ml of demineralised water were aerosolized in 120 seconds at a pressure of 2 bar. The aerosol spectra were determined holding the spray-head 4 cm distant to the laser beam and approximately 2 cm from the lens. A 300 mm lens (measuring range: 0.5 – 990 µm) with open bench was used. The temperature during the experiments was 22.8°C and the relative humidity 45.8%.

Air sampling after experimental aerosol production

During the aerosol experiments the concentration of CSFV (per m^3 air) with time was studied. The air was sampled immediately after aerosol production to determine the initial loss, and then after 15 and 30 min to calculate the half-life time values of the airborne virus. Samples were taken using the MD8 airscan sampling device (Sartorius, Nieuwegein, the Netherlands) and sterile gelatine filters of 3 µm pore size and 80 mm diameter (type 17528-80-ACD; Sartorius). Two different sampling protocols were used, sampling with an air speed of 2 m^3/h for 2 min or with an air speed of 8 m^3/h for 10 min. After sampling, the filters were dissolved in 5 ml of medium (EMEM supplemented with 10% antibiotics and 5% FBS) kept at 37°C. Virus titration was used to determine virus concentrations.

The detection limit of the air sampling procedure varied depending on the amount of air sampled and was calculated as demonstrated next for the 2 m^3 of air/h during 2 min sampling protocol. In order to obtain a positive result in the virus titration (performed in fourfold), at least one of the four wells containing the undiluted sample should be tested virus positive. The corresponding virus titer is $10^{0.65}$ $TCID_{50}$/ml. As 67 liters of air were sampled and the filter was dissolved in 5 ml, the detection limit was $10^{2.52}$ $TCID_{50}/m^3$ air. If sampling was performed for 10 min at 8 m^3/h, the detection limit was $10^{1.22}$ $TCID_{50}/m^3$ air.

The half-life time value of the virus in the aerosols (the time required for the quantity of infectious virus to be reduced to half of its initial value) was calculated using the formula:

$$t_{1/2} \equiv \frac{(\log_{10} 2) \times T}{\log_{10} \frac{C}{E}}$$

T = time interval in min

C = virus concentration at the start (t 0)

E = virus concentration after 15 or 30 min (t 15 or t 30)

During the 8 m^3/h sampling protocol 1333 l of air were extracted from the isolator, which resulted in a too high underpressure. Therefore, the air inlet of the isolator was left open to allow the entrance of the same amount of air as was detracted with sampling. Hence, the virus concentrations obtained with this sampling protocol had to be corrected. The corrections were made assuming that the dilution, due to the air that was allowed to enter passively into the isolator, was linear. The original concentrations were calculated using the formula:

$$C_o \equiv C_e \times \left(\frac{V_o + V_e}{V_o} \right)$$

C_o = original concentration

C_e = end concentration; concentration in the air sample obtained by virus titration

V_o = original volume of the isolator (924 l)

V_e = extracted amount of air

Effect of the gelatine filter on the viability of CSFV

To determine if the gelatine filters have a negative effect on the viability of the captured virus particles, a validation was carried out. Gelatine filters were placed in Petri dishes and inoculated with 0.5 ml of the same virus stock that was used for the aerosolization (containing 10^5 $TCID_{50}$/ml of the Brescia or Paderborn strain and 10^6 $TCID_{50}$/ml of the Zoelen strain). Three filters were assayed for each CSFV strain. The first filter was dissolved 30 min after inoculation, in a total of 50 ml of medium. This was done by initially adding 20 ml of medium to the filter (EMEM supplemented with 10% antibiotics and 5% FBS, kept at 37°C). After placing the Petri dishes for 5 min at 37°C and gently mixing, the fluid was pipetted into flasks containing another 30 ml of medium. These 30 min represented the time interval of transport from the isolator to the laboratory. The second filter was dissolved 5 min after inoculation, in the same way as the first filter. The third filter was also dissolved 5 min after inoculation, however, only in 5 ml of medium, in order to get a higher concentration of virus in the sample. As control, the virus

stocks were kept for the same time period at room temperature as the filters and were diluted in the same volumes of medium. Virus titration and Real-Time Reverse Transcription Polymerase Chain Reaction (RRT-PCR) were used to determine virus concentrations.

Experimental animals and design

Three animal experiments were conducted with eight-week-old male pigs, obtained from a conventional, but pestivirus free pig herd in the Netherlands. In each experiment five pigs were inoculated with either the highly virulent Brescia, moderately virulent Paderborn, or low virulent Zoelen strain. Samples were taken from the expiration air of the pigs and from their cages at different time points to determine shedding rates of CSFV by virus titration and RRT-PCR.

Housing of animals

Pigs were individually housed in cages in one isolation unit. Each cage was placed in a pen separated by solid walls. The cages were designed to separately collect faeces and urine for CSF studies. Faeces were collected in plastic bags attached to the pigs with a Velcro system, while urine was collected in a tray underneath the cage. Each time before cage air sampling was started, a cover was placed on top of the cage. This resulted in an air flow from the front side of the cage to the back where the MD8 was placed. The MD8 was attached to the cage with a PVC tube of 10 cm to prevent pigs from touching the filter.

Inoculation of animals

Pigs were inoculated intranasally with a dose of 10^2 $TCID_{50}$ CSFV strain Brescia, 10^5 $TCID_{50}$ strain Paderborn or 10^5 $TCID_{50}$ strain Zoelen, according to the standard infection models used in our institute. One ml of the virus suspension was administered per animal (0.5 ml per nostril). The inocula were back titrated to confirm the dose administered.

Sampling procedures

At day 6 and 8 post inoculation the expiration air was sampled from pigs infected with the Brescia strain, at day 9, 13, 15 and 33 post inoculation from the pigs infected with the Paderborn strain, and at day 7 and 13 post inoculation from the pigs infected with the Zoelen strain. Sampling was performed

on moments when maximum amounts of virus in leucocytes and oropharyngeal swabs were expected. Thus, different sampling schemes were used for the different virus strains. During sampling the gelatine filter was kept at a distance of approximately 10 cm of the pig's nose. Most animals were laying and were breathing quietly. Pigs that did not lay down (the ones infected with the Zoelen strain) were restrained in order to avoid touching the filter. The expired air was sampled with an air speed of 2 to 5 m^3/h during 2 to 5 min. Air speed and sampling time were recorded. At day 5 post inoculation cage air was sampled from pigs infected with the Brescia strain, and at days 9, 13 and 15 post inoculation of the pigs infected with the Paderborn strain. The cage air was sampled at an air speed of 8 m^3/h during 10 min. Filters were dissolved in 5 ml of medium (EMEM supplemented with 10% antibiotics and 5% FBS) kept at 37°C. Virus concentration was determined by virus titration and RRT-PCR.

Every two days samples were collected from oropharyngeal fluid and three times a week from EDTA stabilized blood, which were analysed in the virus isolation (VI) assay. Oropharyngeal swabs were weighed before and after sampling to be able to calculate $TCID_{50}$ per gram oropharyngeal fluid ($TCID_{50}$/gr). The oropharyngeal fluid was suspended in 4 ml medium. After centrifugation (2500 ***g*** for 15 minutes) the samples were stored at -70°C. Leucocytes were isolated from EDTA stabilized blood by adding 2 ml of EDTA blood to 4 ml 0.84% NH_4CL solution. After 10 minutes the samples were centrifuged at 1000 ***g*** and washed twice with PBS. The pellet was resuspended in 2 ml medium and stored at -70°C.

Virus isolation and titration

A volume of 125 µl of the air samples or leucocytes were incubated on a monolayer of SK6 cells (permanent porcine kidney cell line) in a 24-wells plate (Greiner) at 37°C in an atmosphere with 5% CO_2 for four days. A volume of 250 µl of oropharyngeal fluid was incubated for one hour on a monolayer of SK6 cells. After a wash procedure, medium was added to the wells and incubated for four days. After being fixated and washed, the monolayers were stained by the immuno-peroxidase technique (Wensvoort *et al.*, 1986) and examined for stained cells. Virus positive air samples were titrated in fourfold after making five decimal dilutions (10^{-1} to 10^{-5}) from the filter solution. Virus titers were calculated as $TCID_{50}$ using the Spearman-Kärber method (Finney, 1978).

Real-Time Reverse Transcription Polymerase Chain Reaction (RRT-PCR)

RNA isolation was performed by pipetting 200 µl of the sample manually into MagNA Pure sample cartridges (Roche Applied Science, Mannheim, Germany). In each run three negative samples (PBS) and three dilutions of a positive control sample were included which were spread throughout the cartridge. The RNA was extracted with the Total Nucleic Acid Isolation Kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions using the automated MagNA Pure LC instrument (Roche Applied Science, Mannheim, Germany). After the MagNA Pure completed the RNA isolation, the nucleic acids were removed from the MagNA Pure LC and immediately processed for the RRT-PCR or stored at -70 °C in the sample cartridge until the RRT-PCR was carried out.

The RRT-PCR was performed with a LightCycler (LC) instrument (Roche Applied Science, Mannheim, Germany) using the RNA Master Hybridization Probes Kit, as described by Van Rijn *et al.* (2004). Crossing points (Cp) values were determined for each sample. The Cp value is the cycle number at which the fluorescence emission from a RRT-PCR reaction rises above the background signal. A low Cp value indicates high template amount, while a high Cp indicates a low template amount.

Filter samples from the animal experiment were tested in four-fold, as low RNA levels were suspected. Samples were considered positive in case one or more test results showed a RRT-PCR reaction rising above the background signal.

Correlation between virus titration and RRT-PCR

The correlation between Cp value and virus titration was determined. This enabled the quantification of the viral load ($TCID_{50}$ per m^3) represented by Cp values. Filters were dissolved in 5 ml of medium and a twofold ($10^{-0.3}$) and tenfold (10^{-1} to 10^{-5}) dilutions were prepared from the Brescia, Paderborn and Zoelen strain. Samples were stored at -70°C until analysis by virus titration and RRT-PCR.

Statistical analysis

The effect of the gelatine filter on the viability of the virus was analysed using a paired-sample T-test (SPSS 12; SPSS Inc., Chicago, Illinois). Therefore, the differences in virus titration and RRT-PCR value between the filter and stock solution were compared. *p*-values < 0.05 denoted a significant difference in virus viability between the filter and stock solution.

Results

Effect of the gelatine filter on the viability of CSFV

The effect of the gelatine filter on the viability of the virus was studied by comparing it with the stock solution, which was given the same treatment (Table 1 and 2). The filter and stock solutions for the different CSFV strains showed comparable results in both the virus titration (p=0.56) and the RRT-PCR (p=0.73), although there may be a small effect on the viability of the virus with increased time before processing. Processing the filter thirty minutes after inoculation resulted in a very small reduction in the VI titers compared to processing five minutes after inoculation.

In addition, it was possible to dissolve the filter in only 5 ml of medium without negative effect on the virus, as in general 1 $\log_{10}$ $TCID_{50}$/ml higher titers were observed compared to filters dissolved in 50 ml. Because of this, a higher virus concentration in the sample could be obtained.

Characterization of the aerosol spectrum

The particle size distribution (D) is expressed as a volume diameter below which a certain percentage of the particle volume is contained. The particle diameter below which 10% of the particle volume is contained; D(v, 0.1), was 6.52 µm. A diameter of 24.43 µm was found for D(v, 0.5) (below which 50% of the particle volume is contained) and a diameter of 49.55 µm for D(v, 0.9). The average droplet size distribution of three aerosol spectra is shown in Figure 1. In previous experiments (data not shown) different types of medium were tested during aerosol spectrum analysis of the spray head with similar results to those achieved with demineralized water. Therefore, the reflected aerosol spectrum was considered representative for the medium used in this experiment (EMEM supplemented with 10% antibiotics and 10% FBS).

Assessment of the yield and viability of CSFV in air samples from experimental aerosols

Three different CSFV strains were aerosolized in duplicate (experiments 1 and 2) and the concentration of the CSFV was studied by means of two different sampling protocols; sampling for 2 min at 2 m^3/h (Table 3) or sampling for 10 min at 8 m^3/h (Table 4). When sampling was performed directly after aerosolization at an air speed of 8 m^3/h, the filters clogged before the sampling time was completed. This was due to the high relative humidity after aerosol generation in combination with the

high air speed. As in these cases less air was sampled, the results of the virus titration were corrected accordingly.

The reproducibility of the aerosols and sampling results were good, with only small differences between experiments 1 and 2. Before each aerosol experiment and after ventilation control air samples were taken in which no CSFV was detected.

The initial loss was determined by taking air samples immediately after aerosol generation and comparing it with the aerosol virus dose. These losses were similar for both sampling protocols and were on average $10^{1.1}$ $TCID_{50}/m^3$ for the Brescia strain, $10^{0.5}$ $TCID_{50}/m^3$ for the Paderborn strain and $10^{0.8}$ $TCID_{50}/m^3$ for the Zoelen strain. Fifteen and thirty min after the aerosol production concentrations had decreased, with slightly lower concentrations for the 10 min at 8 m^3/h protocol than the 2 min at 2 m^3/h protocol, except for the first experiment of the Zoelen strain.

The half-life time values, which give an indication of the viability of the virus with time, ranged between 4.5 and 12 min. The half-life time values for the Brescia and Paderborn strain determined at t 30 min when sampling during 2 min at 2 m^3/h were slightly higher than when sampling was performed with the other protocol. The half-life time values for the Zoelen strain determined at this time point were slightly higher when sampling was performed with the 10 min at 8 m^3/h protocol.

Clinical signs and virus isolation on oropharyngeal swabs and leucocytes

Pigs infected with the Brescia strain showed severe clinical signs and fever (>40°C) at all air sampling moments. Virus isolation on oropharyngeal swabs was positive at all air sampling moments (on average $10^{4.5}$ $TCID_{50}$/gr at day 6 and $10^{5.6}$ $TCID_{50}$/gr at day 8 post inoculation), as was virus isolation on leucocytes (Table 5).

All pigs infected with the Paderborn strain showed mild to severe clinical signs of CSF after inoculation. At day 9 post inoculation fever was detected in pig number 10, and clinical signs in all animals except pig number 8. At days 13 and 15 post inoculation fever was detected in pigs 6 and 10, and clinical signs in pigs 6, 9 and 10. At day 33 post inoculation fever was detected in pig 6, and clinical signs in pigs 6 and 10. Virus isolation on oropharyngeal swabs and leucocytes was positive at all sampling moments, except for pig 8 at day 16 post inoculation (Table 6). Pig 6 and pig 10 were chronically infected (more than 30 days virus positive).Virus concentrations in oropharyngeal swabs

from these pigs were higher (on average $10^{6.3}$ $TCID_{50}$/gr from day 8) than in the oropharyngeal swabs from the other three pigs (on average $10^{2.9}TCID_{50}$/gr between day 8 and 16 post inoculation).

Amongst pigs infected with the Zoelen strain only pig number 12 showed fever at day 7 post inoculation. None of the pigs showed fever at day 13 post inoculation. Mild clinical signs were only observed at day 7 post inoculation in pigs number 11, 12 and 13. Virus isolation on oropharyngeal swabs from all pigs was positive at day 6 post inoculation (on average $10^{2.2}TCID_{50}$/gr between day 6 and 8 post inoculation), as was virus isolation on leucocytes (Table 7). At day 13 post inoculation, when the second air sampling was performed, only one pig tested positive in the VI on oropharyngeal swabs and leucocytes.

Virus isolation and RRT-PCR on air samples

Air samples from cages housing Brescia infected pigs at day 5 post inoculation resulted in three RRT-PCR positive samples. All pigs had RRT-PCR positive expiration air samples at day 8 post inoculation. One air sample was also virus titration positive with a virus titer of $10^{3.5}$ $TCID_{50}/m^3$ (Table 5). Paderborn infected pigs yielded two RRT-PCR positive cage air sample at day 9, three at day 13, and two at day 15 post inoculation. One expiration air sample from an infected pig was RRT-PCR positive at day 13 and two at day 33 post inoculation (Table 6). The positive air samples mainly originated from pigs 6 and 10, which were chronically infected and excreted high amounts of virus in oropharyngeal swabs. None of the expiration air samples from pigs infected with the Zoelen strain were virus positive (Table 7).

Correlation virus titration and RRT-PCR

A good correlation was observed between the virus titration and the RRT-PCR technique (R^2=0.993 for the Brescia strain, R^2=0.985 for the Paderborn strain and R^2=0.991 for the Zoelen strain). Based on this correlation the samples of cage air had calculated virus titration titers of $10^{0.3}$ $TCID_{50}/m^3$ to $10^{1.6}$ $TCID_{50}/m^3$ for the Brescia infected pigs, and titers of $10^{0.3}$ $TCID_{50}/m^3$ to $10^{1.4}$ $TCID_{50}/m^3$ for the Paderborn infected pigs (Tables 5 and 6). The expiration air samples from the infected pigs showed calculated virus titration titers of $10^{0.7}$ to $10^{4.0}$ $TCID_{50}/m^3$ for the Brescia infected pigs, and titers of $10^{0.4}$ $TCID_{50}/m^3$ to $10^{2.2}$ $TCID_{50}/m^3$ for the Paderborn infected pigs(Tables 5 and 6). None of the air samples from the Zoelen infected pigs were positive in the RRT-PCR.

356

357 **Discussion**

358 This is the first study describing the successful detection of CSFV in air samples originating from
359 infected pigs. Virus was detected in air samples representing cage air, as well as expiration air.
360 Furthermore, in artificially created aerosols, it was shown that the virus can remain infective for at least
361 30 min after aerosol generation with half-life time values ranging from 4.5 to 15 min. The air sampling
362 device and protocols used were suitable to enable the detection and quantification of the virus in
363 mentioned air samples.

364 We were able to detect CSFV in air samples obtained from pigs infected with the Brescia or
365 the Paderborn strain, however, not from pigs infected with the low virulent Zoelen strain. This was not
366 unexpected, as in general, looking at other secreta and excreta, pigs infected with the Zoelen strain
367 excreted much lower quantities of virus than pigs infected with the Brescia and Paderborn strain (data
368 not shown). Transmission of CSFV by the air seems therefore more likely to occur during outbreaks
369 caused by highly and moderately virulent strains than by low virulent strains.

370 Classical swine fever virus shedding rates of pigs in aerosols were low compared to pigs
371 infected with for instance foot-and-mouth disease virus. The CSFV infected pigs excreted up to $10^{5.2}$
372 $TCID_{50}$ of infectious virus per day in expiration air while foot-and-mouth disease infected pigs excrete
373 up to $10^{8.6}$ $TCID_{50}$ per day (Donaldson and Alexandersen, 2002). The minimal infective dose that
374 results in fatal disease after inoculation with the highly virulent strain "Alfort" is 10 $TCID_{50}$ per pig
375 (Liess, 1987). For the Brescia virus strain a pig ID_{50} of 74 $TCID_{50}$ after intranasal inoculation was found
376 by Terpstra (C.Terpstra, unpublished, 1984 and 1987). Based on the intranasal dose it can be
377 concluded that a sentinel pig, exposed to the expired air of an infected pig, receives a dose that is
378 most likely sufficient to induce infection. Whether this is also enough to transmit the virus over larger
379 distances (e.g. farms in the neighbourhood of an infected farm) will depend on additional factors.
380 These factors may include total virus excretion by a population of infected animals (which in turn
381 depends on number of infected animals, and virus type), weather conditions, ventilation rate, etc.
382 Further research into these parameters will be necessary to move forward with respect to the question
383 of neighbourhood infections caused by aerosols.

384 It has been shown previously that the RRT-PCR test is more sensitive than the VI technique
385 (Van Rijn *et al*., 2004). This was confirmed in the present study as many air samples from the animal

386 experiments, negative at VI, showed positive RRT-PCR results. Besides this higher sensitivity of the
387 RRT-PCR, negative VI results could be due to loss of infectivity of virus particles. The sample taken
388 from the expired air of pig 2 at day 8 post inoculation was the only sample positive in both the virus
389 titration and RRT-PCR assay. The calculated virus titre in $TCID_{50}$ from the RRT-PCR result was
390 approximately 0.5 $\log_{10}$ $TCID_{50}$ higher than the titer that was determined in the virus titration assay.
391 Therefore, the calculated titres of excretion rates based on PCR-results could be slightly
392 overestimated.
393 On the other hand, shedding rates of virus in aerosols by infected pigs under field circumstances is
394 expected to be higher than determined in this study. Faeces were collected separately and the
395 isolation unit had a low pig density in combination with a high ventilation rate, so subsequently dust
396 and aerosol production was low. To assess the risk of airborne transmission, further research is
397 necessary under circumstances mimicking the situation in the field, where the herein described air
398 sampling system seems a suitable tool to determine shedding rates.
399 The validation studies showed that the gelatine filter processing method did not have an
400 adverse effect on the yield and viability of the virus. Even 30 min after inoculating the virus on the filter,
401 which was the elapsed time between air sampling and processing in the aerosol experiments, no
402 significant inactivation was observed.
403 Between the different aerosolization and sampling experiments in the isolator, the isolator was
404 not decontaminated. However, it was ventilated at a high rate (1324 m^3/h) for 15 min, which will have
405 reduced virus concentrations to a level far below the detection limit. This was confirmed by the
406 negative virus titration and RRT-PCR results of air samples taken before the start of each experiment
407 and directly after ventilation.
408 After generating virus containing aerosols, these aerosols are subject to both biological and physical
409 decay. Biological decay includes factors that affect the infectivity of the virus, while the physical decay
410 depends on the time particles remain suspended which is influenced by particle size and particle
411 deposition (Stärk, 1999). Directly after generating the aerosols air samples were taken and the initial
412 loss was determined. Beside the biological and physical loss the initial loss is dependent on the ability
413 of the air-sampling system to recover airborne particles. The initial losses were maximum $10^{1.66}$
414 $TCID_{50}/m^3$. In case the inability of the sampling system to recover the particles is totally responsible for
415 the initial losses, only 2.2% of the infectious particles were recovered from the air. However, the

manufacturer claims high absolute retention rates for micro-organisms and viruses, like 99.9995% for *Bacillus subtilis niger* spores and 99.94% for T3 coliphages. Therefore, the represented data are unlikely to be underestimated.

The physical decay after generation of the aerosols was expected to be similar in all experiments as the same aerosol generator and isolator were used and climatic conditions did not change. However, the highly virulent Brescia strain seems more susceptible to aerosolization or sampling than the moderately and low virulent Paderborn and Zoelen strain, as shown by higher initial losses and shorter half-life time values than the Paderborn and Zoelen strain. It is not clear whether these differences are reproducible and significant because of the limited number of observations. Any difference could be caused by differences in their susceptibility to shear forces of the spraying device, to droplet evaporation and differences in susceptibility of the virus to the stress caused by the sampling technique.

Sampling at higher air speed (8 m^3/h for 10 min) resulted for the Brescia and Paderborn strain in slightly lower half-life time values. The higher air speed could have caused mechanical stress to the virus adhered to the filter and favoured inactivation by enhancing evaporation. Furthermore, during sampling at 8 m^3/h the air inlet was left open and it was assumed that the air entering the isolator mixed adequately, however, in case the entering and isolator air did not mix well, fresh air could have been sampled in relatively higher amounts, yielding lower half-life time values. As the same conditions were applied when sampling the Zoelen strain, this explanation is however less likely.

Schwarte and Mathews (1954) demonstrated that aerosols of powdered lyophilized CSFV obtained from blood can infect susceptible pigs up to 30 min after aerosolization. The current study not only confirmed that the virus can remain infective in aerosols for at least 30 min after aerosol generation, but also gives information about the half-life time values of the virus in aerosols which can be used to assess the risk of airborne transmission during an outbreak of CSFV. In the present work, the aerosol fluids were however prepared from virus in medium containing 10% fetal calf serum, known to protect the virus. This may also have been the case in the study of Schwarte and Mathews, where masses of desiccated serum and blood cells could have prevented inactivation of the virus. Therefore, the infectivity of the virus in aerosols produced after sneezing and coughing or in splashes from faeces or urine could be different.

The aerosols produced in the current study could be used to infect pigs in further experimental studies. If a susceptible pig of 25 kg would be exposed during 30 min to the aerosols, assuming the decrease in concentration of CSFV is linear, the uptake of virus from the Brescia, Paderborn or Zoelen strain would be $10^{4.7}$ $TCID_{50}$, $10^{5.1}$ $TCID_{50}$ or $10^{6.0}$ $TCID_{50}$, respectively. This is based on reported tidal air volumes of 9.27 to 15 l/min for 25 kg pigs with a mean respiratory rate of 35 breaths per minute (reviewed by Stärk, 1999; Alexandersen and Donaldson, 2002). These calculations were made assuming that not only the part of the aerosol that reaches the bronchi and lungs (with particle sizes <6 μm), but also larger particles deposited in the upper airways and oro-nasal cavity will result in infection (Sellers and Parker, 1969). Based on the intranasal dose it can be concluded that the aerosols in this study contain a sufficient virus dose to infect pigs.

In conclusion, although the possibility to infect pigs with aerosols containing CSFV has been demonstrated in the past, and airborne transmission has been shown in experimental studies, there was no information available on the shedding rates of CSFV from infected pigs and half-life time values of different aerosolized CSFV strains. These data have been generated in the present study after developing an air sampling technique, which might be used to unravel the role of airborne transmission in outbreaks of CSFV.

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571 **Tables**

572

573 Table 1. Effect of the gelatine filters on the viability of CSFV determined by virus titration. Stock
574 solutions (positive controls) were treated according to the same protocol as the filter solutions.

	Treatment		Titer ($TCID_{50}$/ml)[a]	
Virus strain	Volume[b]	Time[c]	Filter solution	Stock solution
Brescia	50 ml	30 min	3.60	3.85
	50 ml	5 min	3.88	3.63
	5 ml	5 min	4.85	4.60
Paderborn	50 ml	30 min	3.35	3.60
	50 ml	5 min	3.50	3.75
	5 ml	5 min	4.85	4.60
Zoelen	50 ml	30 min	4.38	4.75
	50 ml	5 min	4.88	4.75
	5 ml	5 min	5.13	5.38

575 [a]) Based on control samples, always included in the virus titration, the standard deviation (SD) of the
576 virus titration technique is 0.43 $TCID_{50}$. This SD was calculated from 15 virus titration tests performed
577 in the lab in the course of this study.

578 [b]) Volume of medium used for dissolving the filter or stock.

579 [c]) Time interval between inoculation and processing of the filter or stock.

580

Table 2. Effect of the gelatine filters on the viability of CSFV determined by RRT-PCR. Stock solutions (positive controls) were treated according to the same protocol as the filter solutions.

	Treatment		Cp value[a]	
Virus strain	Volume[b]	Time[c]	Filter solution[d]	Stock solution[d]
Brescia	50 ml	30 min	29.8	30.2
	50 ml	5 min	30.1	29.8
	5 ml	5 min	26.3	27.1
Paderborn	50 ml	30 min	29.0	29.9
	50 ml	5 min	29.6	29.3
	5 ml	5 min	26.3	25.2
Zoelen	50 ml	30 min	25.9	25.5
	50 ml	5 min	25.2	25.6
	5 ml	5 min	21.9	22.2

[a]) Cp = crossing point; the standard deviation of the crossing points in the RRT-PCR has been calculated for the positive control samples and is 0.9, based on 9 RRT-PCR tests performed in the lab in the course of this study.

[b]) Volume of medium used for dissolving the filter or stock.

[c]) Time interval between inoculation and processing of the filter or stock.

[d]) A tenfold dilution of the samples were tested in the RRT-PCR.

589 Table 3. The aerosol concentration determined by virus titration of three different CSFV strains with time and their half-life time values. Air samples were
590 collected using the 2 min at 2 m^3/h protocol.
591

	Brescia				Paderborn				Zoelen			
	Experiment 1		Experiment 2		Experiment 1		Experiment 2		Experiment 1		Experiment 2	
	Log_{10} $TCID_{50}$/ m^3 air	Half-life (min)	Log_{10} $TCID_{50}$/ m^3 air	Half-life (min)	Log_{10} $TCID_{50}$/ m^3 air	Half-life (min)	Log_{10} $TCID_{50}$/ m^3 air	Half-life (min)	Log_{10} $TCID_{50}$/ m^3 air	Half-life (min)	Log_{10} $TCID_{50}$/ m^3 air	Half-life (min)
Aerosol dose[a]	6.43		6.68		6.18		6.43		7.93		7.18	
Control filter	0		0		0		0		0		0	
Initial loss	0.91		1.66		0.91		0.41		1.16		0.41	
t 0	5.52		5.02		5.27		6.02		6.77		6.77	
t 15[b]	4.52	4.5	5.02	∞	4.52	6.0	5.27	6.0	6.27	9.0	6.27	9.0
t 30[b]	4.27	7.2	4.02	9.0	4.52	12	5.27	12	5.77	9.0	6.02	12

592 [a]) A slightly different aerosol dose was used in the duplicate experiments (experiment 1 and 2), however, the same aerosol doses were used for both sampling
593 protocols (Table 3 and 4).
594 [b]) The presented half-life time values at t 15 min and t 30 min were both calculated compared to t 0.

595 Table 4. The aerosol concentration determined by virus titration of three different CSFV strains with time and their half-life time values. Air samples were
596 collected using the 10 min at 8 m^3/h protocol.
597

	Brescia				Paderborn				Zoelen			
	Experiment 1		Experiment 2		Experiment 1		Experiment 2		Experiment 1		Experiment 2	
	Log_{10} $TCID_{50}$/ m^3 air	Half-life (min)	Log_{10} $TCID_{50}$/ m^3 air	Half-life (min)	Log_{10} $TCID_{50}$/ m^3 air	Half-life (min)	Log_{10} $TCID_{50}$/ m^3 air	Half-life (min)	Log_{10} $TCID_{50}$/ m^3 air	Half-life (min)	Log_{10} $TCID_{50}$/ m^3 air	Half-life (min)
Aerosol dose[a]	6.43		6.68		6.18		6.43		7.93		7.18	
Control filter	0		0		0		0		0		0	
Initial loss	0.61		1.30		0.15		0.61		1.15		0.65	
t 0[b]	5.82		5.38		6.03		5.82		6.78		6.53	
t 30	4.11	5.8	3.36	4.6	4.36	6.1	4.86	10	5.86	11	5.86	15

598 [a]) A slightly different aerosol dose was used in the duplicate experiments (experiment 1 and 2), however, the same aerosol doses were used for both sampling
599 protocols (Table 3 and 4).
600 [b]) Due to the high relative humidity in combination with high air speed after aerosol generation, the filters taken at t 0 min clogged before the sampling time
601 was completed. As in these cases less air was sampled, the results of the virus titration were corrected accordingly.

602 Table 5. VI, virus titration and RRT-PCR results of pigs infected with the Brescia strain

Animal number	Virus isolation / titration ($log_{10}TCID_{50}/m^3$)									RRT-PCR (Average Cp-value)			Calculated virus titration titers[b] ($log_{10}TCID_{50}/m^3$)		
	Oropharyngeal swabs			Leucocytes			Samples of expired air		Samples of cage air	Samples of expired air		Samples of cage air	Samples of expired air		Samples of cage air
Dpi[a]:	4	6	8	5	7	9	6	8	5	6	8	5	6	8	5
1	+	+	+	+	+	nd[a]	-	-	-	Neg	33.9[3]	36.1[1]	-	2.2	0.3
2	+	+	+	+	+	+	-	3.5	-	Neg	29.9[4]	35.6[2]	-	4.0	0.6
3	+	+	+	+	+	+	-	-	-	Neg	34.8[1]	Neg	-	0.7	-
4	+	+	+	+	+	+	-	-	-	Neg	34.1[3]	34.0[4]	-	2.1	1.6
5	+	+	+	+	+	+	-	-	-	Neg	34.4[3]	Neg	-	2.1	-

603 [a]) nd = not done because of death; dpi = days post inocluation.

604 [b]) Calculation based on the correlation between the virus titration assay and the RRT-PCR assay. The represented titers are the average of the four samples

605 tested in the RRT-PCR.

606 [1]) one sample RRT-PCR positive out of four samples tested;

607 [2]) two samples RRT-PCR positive out of four samples tested;

608 [3]) three samples RRT-PCR positive out of four samples tested;

609 [4]) four samples RRT-PCR positive out of four samples tested.

610 Table 6. VI and RRT-PCR results of pigs infected with the Paderborn strain

	Virus isolation															RRT-PCR (Average Cp-value)							Calculated virus titration titers [b] ($log_{10}TCID_{50}/m^3$)						
Animal number	Oropharyngeal swabs				Leucocytes				Samples of expired air				Samples of cage air			Samples of expired air				Samples of cage air			Samples of expired air				Samples of cage air		
Dpi[a]:	8	12	16	32	9	12	16	33	9	13	15	33	9	13	15	9	13	15	33	9	13	15	9	13	15	33	9	13	15
6	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	Neg	Neg	Neg	35.3[3]	33.8[2]	36.2[1]	33.9[3]	-	-	-	2.0	0.9	0.3	1.4
7	+	+	+	-	+	+	+	-	-	-	-	nd[a]	-	-	-	Neg	Neg	Neg	nd[a]	Neg	Neg	Neg	-	-	-	nd[a]	-	-	-
8	+	+	+	-	+	+	-	-	-	-	-	nd[a]	-	-	-	Neg	Neg	Neg	nd[a]	Neg	33.3[1]	Neg	-	-	-	nd[a]	-	0.5	-
9	+	+	+	-	+	+	+	-	-	-	-	nd[a]	-	-	-	Neg	Neg	Neg	nd[a]	Neg	Neg	Neg	-	-	-	nd[a]	-	-	-
10	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	Neg	34.2[1]	Neg	34.9[3]	35.7[2]	33.8[1]	34.2[2]	-	0.4	-	2.2	0.6	0.5	0.9

611 [a]) nd = not done; dpi = days post inoculation.

612 [b]) Calculation based on the correlation between the virus titration assay and the RRT-PCR assay. The represented titers are the average of the four samples

613 tested in the RRT-PCR.

614 [1]) one sample RRT-PCR positive out of four samples tested;

615 [2]) two samples RRT-PCR positive out of four samples tested;

616 [3]) three samples RRT-PCR positive out of four samples tested;

617 [4]) four samples RRT-PCR positive out of four samples tested.

618 Table 7. VI and RRT-PCR results of pigs infected with the Zoelen strain

	Virus isolation						RRT-PCR	
Animal number	Oropharyngeal swabs		Leucocytes		Samples of expired air		Samples of expired air	
Dpi[a]:	6	12	7	12	7	13	7	13
11	+	-	+	-	-	-	Neg	Neg
12	+	-	+	-	-	-	Neg	Neg
13	+	-	+	-	-	-	Neg	Neg
14	+	-	+	-	-	-	Neg	Neg
15	+	+	+	+	-	-	Neg	Neg

619 [a]) dpi = days post inoculation.

620

621 Figure 1. The average droplet size distribution with standard deviations of three aerosol spectra
622 generated by a Walther Pilot I spray-head.