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# Quantification of classical swine fever virus in aerosols originating from pigs infected with strains of high, moderate or low virulence

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**Abstract**

During epidemics of Classical Swine Fever (CSF), the route of virus introduction into a farm is often unclear. One of the suggested routes is via the air. Under experimental conditions, airborne transmission over a short distance seems possible, but analysis of outbreak data is still inconclusive. For a better understanding of the role of airborne transmission, quantitative information is needed on concentrations of virus emitted by infected pigs. This was studied in four groups of ten pigs in which three pigs were inoculated with either a low virulent strain (Zoelen), a low or high dose of a moderately virulent strain (Paderborn), or a highly virulent strain (Brescia). The other seven pigs in each group served as contact pigs. At several moments after infection, air samples were obtained using gelatine filters. Infectious virus and viral RNA were detected in the air of rooms housing the pigs infected with the moderately and highly virulent strains with titres of $10^{1.2}$ to $10^{3.0}$ $TCID_{50}/m^3$ of infectious virus, and $10^{1.6}$ to $10^{3.8}$ $TCID_{50}$ equivalents/$m^3$ of viral RNA. It was observed that the higher the dose or virulence of the virus strain used for inoculation of the pigs, the sooner virus could be detected in the air samples. This is the first study describing the quantification of (infectious) CSFV in air samples of rooms housing infected pigs, enabling to quantify the contribution of individual infected pigs to virus concentrations in aerosols. This can be used as input for quantitative models of airborne spread over large distances.

*Key words*: Air sampling; Airborne transmission; Classical swine fever virus; Virus excretion

## 1. Introduction

Epidemics of classical swine fever (CSF), a highly contagious viral disease, have resulted in huge economic losses and the destruction of large amounts of pigs (Moennig, 2000; Terpstra and De Smit, 2000; Moennig et al., 2003). During one of the most recent and disastrous epidemics in Europe, the 1997-1998 outbreak in Germany, The Netherlands, Belgium, Spain and Italy, the virus was often transmitted between farms through contact with pigs, people or transport vehicles (Elbers et al., 1999; Elbers et al., 2001; Stegeman et al., 2002). However, in approximately 50% of the cases the route of virus introduction into a farm remained unknown. Most of these farms that were infected via an unknown route, were located in the immediate vicinity of a previously infected herd. These infections that occurred within a radius of 1 km of this previously infected herd were called “neighbourhood

infections" (Elbers et al., 1999; Stegeman et al., 2002). The inability to establish the origin of these neighbourhood infections may be caused by underreporting of well-known dangerous contacts (Elbers et al., 1999, Elbers et al., 2001) or untraceable routes like transmission via arthropods, birds, pets and rodents (Elbers et al., 1999; Dewulf et al*.,* 2001; Kaden et al*.,* 2003). Airborne spread has also been suggested (Elbers et al., 1999; Dewulf et al*.,* 2000), although its role during the 1997-1998 outbreak in the Netherlands was unclear. There was no association found between new infections and the prevailing direction of the wind (Crauwels et al., 2003). However, during other outbreaks there were indications that airborne transmission may have contributed to the spread of the disease (Laevens, 1999; Sharpe et al., 2001).

The role of virus transmission via the air over short distances has been studied experimentally by connecting two isolation chambers with a pipe. One isolation chamber housed one to four infected pigs, and the other isolation chamber housed susceptible pigs (Hughes and Gustafson, 1960; Terpstra, 1987; González et al., 2001). It was shown that transmission occurred, and that the most likely route was via the air. Also within an isolation unit, transmission occurred when the air current was flowing from one compartment housing infected pigs to another compartment housing susceptible pigs (Dewulf et al*.,* 2000).

Although transmission through the air may occur, attempts to detect CSFV in the air failed initially (Terpstra, unpublished, 1986; Stärk, 1998). However, recently, both viral RNA and infectious virus were detected in air samples for the first time (Weesendorp et al., 2008a). These air samples were collected from cages of individually housed pigs infected with a highly or a moderately virulent strain. Isolation from cages of pigs infected with a low virulent strain failed. From the cages housing the pigs infected with the highly or moderately virulent strain, viral RNA was detected in the air at several moments, infectious virus only once. However, these pigs were housed individually under artificial conditions and, as a consequence, it is still unclear what virus concentrations will be present in the air under field circumstances. Such knowledge can help to predict the airborne spread of CSFV. In this paper we describe the detection and quantification of infectious CSFV and viral RNA in air samples taken from rooms housing pigs infected with a low, moderately or highly virulent strain. Furthermore, it is analysed whether there is an association between the virus concentration in the air, and the number of infected pigs or quantities of virus excreted in faeces or oropharyngeal fluid.

## 2. Materials and methods

### *2.1 Experimental design*

Four groups of ten pigs were used. Each group was housed in a separate room of an isolation unit. At the start of the experiment, three pigs were removed from each group and intranasally inoculated. After 24 hours, the inoculated pigs were returned to their original groups, allowing contact exposure of the remaining seven pigs. Each group was inoculated with a different virus strain or dose. The experiment was terminated 35 days post-inoculation (p.i.).

This experiment was approved by the Ethics Committee for Animal Experiments of the Central Veterinary Institute of Wageningen UR.

### *2.2 Housing*

Pigs were housed in rooms with a volume of 42 to 45 $m^3$ with a ventilation rate of 400 $m^3$ per hour. The rooms had an average temperature of 21°C (± 0.7°C), and a relative humidity of 50% (± 10%).

### *2.3 Experimental animals*

Eight-week-old male pigs were obtained from a conventional, but pestivirus free pig herd in the Netherlands, and randomly divided over the four groups. Pigs were fed once a day and water was provided ad libitum.

### *2.4 Viruses and inoculation of animals*

In the first group, three pigs were inoculated with a dose of $10^5$ $TCID_{50}$ (50% tissue culture infectious dose) of the low virulent strain Zoelen (genotype 2.2). This strain was originally isolated during an outbreak on a Dutch farm (Van Oirschot, 1980). In the second and third group, three pigs per group were inoculated with the moderately virulent strain Paderborn. In the second group with a dose of $10^{3.5}$ $TCID_{50}$ (low dose group), and in the third group with a dose of $10^5$ $TCID_{50}$ (high dose group). The Paderborn strain (genotype 2.1) was isolated in 1997 during the outbreak in the Paderborn area of Germany. In the fourth group, three pigs were inoculated with a dose of 100 $LD_{50}$ (50% lethal dose), which is approximately $10^{2.5}$ $TCID_{50}$, of the highly virulent strain Brescia. This strain (genotype 1.2) was derived from a strain obtained in 1951 from Brescia, Italy (Wensvoort et al., 1989).

The strains were classified as low, moderately or highly virulent based on the classification of CSFV strains by Van Oirschot (1988). 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.

*2.5 Clinical symptoms and body temperature*

Body temperature and clinical symptoms were recorded daily. Fever was defined as body temperature higher than 40°C, for two or more consecutive days. The severity of the clinical symptoms was determined using a list of ten CSF-relevant criteria, as described by Mittelholzer et al. (2000). For each criterion a score was recorded of either normal (score 0), slightly altered (score 1), distinct clinical sign (score 2), or severe CSF symptom (score 3). The scores for each pig were added up to a total score per day. Only pigs with total clinical scores (CS) higher than 2 were defined as pigs having clinical symptoms due to the CSFV infection. Pigs showing severe clinical symptoms, becoming moribund and unable to stand up, were euthanatized for reasons of animal welfare.

*2.6 Sampling procedures*

EDTA blood samples were collected from each pig at days 0, 3, 5, 7, 10, 12, 14, 17, 19, 21, 24, 28, and 35 p.i. to determine the number of leucocytes.

Samples from oropharyngeal fluid were collected at days 0, 3, 4, 5, 6, 7, 8, 10, 11, 12, 13, 14, 15, 17, 19, 21, 24, 26, 28, 31, 33, and 35 p.i. Oropharyngeal fluid was obtained with a sterile gauze tampon held by a 30-cm long forceps (Ressang et al*.,* 1972), which was scrubbed against the dorsal wall of the pharynx behind the soft palatum. The oropharyngeal fluid was suspended in 4 ml medium (Eagle minimum essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS) and 10% antibiotics solution ABII (1000 U/ml Penicillin, 1 mg/ml Streptomycin, 20 µg/ml Fungizone, 500 µg/ml; Polymixin B, and 10 mg/ml Kanamycin)). After centrifugation (1800 *g* for 15 min) the samples were stored at -70℃ until they were analysed.

Faeces were collected at days 0, 3, 4, 5, 6, 7, 8, 10, 11, 12, 13, 14, 15, 17, 19, 21, 24, 26, 28, 31, 33, and 35 p.i. Faeces were obtained from the rectum by stimulation of the anus. One gram of faeces was suspended in 9 ml medium (EMEM containing 10% FBS and 10% antibiotics solution ABII) and vortexed with glass beads. After centrifugation (2500 *g* for 15 min) the supernatants were stored at -70℃ until they were analysed.

Air samples were collected at days 4, 7, 10, 14, 17, 21, 28, and 35 p.i. in each room. Samples were taken with the MD8 airscan sampling device (Sartorius). Air was sampled with a speed of 8 $m^3$/h for 10 min within the pig pen at a height of 1 meter. Samples were taken using sterile gelatine filters of 80 mm diameter and 3 µm pore size (type 17528-80-ACD; Sartorius). After sampling, the filters were dissolved in 5 ml of medium (EMEM supplemented with 5% FBS and 10% antibiotics solution ABII) kept at 37℃. Dissolved filter solutions were store d at -70℃ until they were analysed.

*2.7 Laboratory tests*

*2.7.1 Leucocyte counts*

Leucocyte counts were performed using the Medonic® CA 620 coulter counter (Boule Medical AB). Leucopenia was defined as <10x $10^9$ cells/l blood (Weesendorp et al., 2008b).

*2.7.2 Virus isolation and titration*

From oropharyngeal fluid and faeces, a volume of 250 µl was incubated for one hour on a monolayer of SK6 cells (permanent porcine kidney cell line) in a 24-wells plate (Greiner) at 37℃ in a n atmosphere with 5% $CO_2$. Plates were then washed once with PBS (phosphate-buffered saline) and medium was added to the wells. From air samples, a volume of 125 µl was directly incubated on a monolayer of SK6 cells without washing. Cells were cultured at 37℃ in an atmosphere with 5% $CO_2$.

After four days, the growth medium was discarded, and the monolayers were washed in a 0.15M NaCL solution, dried for 1 hour at 37℃ and f rozen for 1 hour at -20℃. The monolayers were fixed with 4% paraformaldehyde in PBS (4°C) for 10 min. After being washed, the monolayers were stained by the immuno-peroxidase technique (Wensvoort et al., 1986), using two horse-radish peroxidase (HRPO)-conjugated CSFV specific MAbs (V3/V4), and examined for stained cells. Virus positive samples were titrated in four-fold after making five decimal dilutions. Virus titres were calculated as $TCID_{50}$ using the Spearman-Kärber method (Finney, 1978).

The detection limits of the different sample types in the virus titration assay were calculated. For faeces and oropharyngeal fluid the detection limits were respectively, $10^{1.1}$ $TCID_{50}$/g faeces and $10^{1.1}$ $TCID_{50}$/ml of oropharyngeal fluid in medium. For air samples the detection limit was $10^{1.22}$ $TCID_{50}/m^3$ air (Weesendorp et al., 2008a).

*2.7.3 Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction*

The concentrations of viral RNA in air samples, oropharyngeal fluid and faeces were analysed by quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (qRRT-PCR). For RNA isolation 200 µl of the sample were pipetted manually into MagNA Pure sample cartridges (Roche Applied Science)). In each run of thirty-two samples, two negative control samples and five dilutions of a positive control sample (standard curve) were included. The standard curves were constructed for each strain of virus by spiking gelatine filter solutions, medium (for the oropharyngeal fluid), or faeces suspensions with known concentrations of infectious virus. The RNA was extracted with the Total Nucleic Acid Isolation Kit (Roche Applied Science) according to the manufacturer's instructions using the automated MagNA Pure LC instrument (Roche Applied Science). After the MagNA Pure completed the RNA isolation, the nucleic acids were removed from the MagNA Pure LC and immediately processed for the qRRT-PCR.

The qRRT-PCR was performed with a LightCycler (LC) instrument (Roche Applied Science) using the RNA Master Hybridization Probes Kit, as described by Van Rijn et al. (2004). Analysis was performed with the LC software. The viral RNA concentration ($TCID_{50}$ equivalents per ml or g) of each individual sample could be calculated using the standard curve. The standard curves were constructed based on Cp (crossing point) values for all dilutions of the positive control. The Cp value is the cycle number at which the fluorescence emission from a PCR reaction rises above the background signal. A low Cp value indicated high template amount, while a high Cp indicated a low template amount.

*2.8 Statistical analysis*

The relationship was studied between virus concentration in the air and: a) the total quantities of virus in oropharyngeal fluid or faeces per room (= average virus titre in $TCID_{50}$/g or ml * number of pigs excreting virus) at the moment of air sampling, and b) number of pigs excreting virus in oropharyngeal fluid or faeces at the moment of air sampling. This relationship was analysed using the Spearman's rank correlation tests (SPSS 12; SPSS Inc.). Virus negative air samples were excluded from the analysis. A *p* value less than 0.05 indicated a significant relationship.

## 3. Results

*3.1 Fever, clinical symptoms and leucopenia*

None of the pigs inoculated with the Zoelen strain showed fever or clinical symptoms. Leucopenia was observed in two inoculated pigs at day 5 p.i. (Table 1). One of the contact pigs in the Zoelen group showed clinical symptoms (lethargy and reduced appetite) for one day at day 31 p.i. (CS of 3). However, as no virus or viral RNA could be isolated from samples of this pig during the entire experiment, and no antibodies were detected in the serum, the clinical symptoms could not be attributed to an infection with CSFV.

All pigs inoculated with the low dose of the Paderborn strain developed clinical symptoms and leucopenia. Fever was observed in two of the three inoculated pigs. All contact pigs developed fever, clinical symptoms and leucopenia. A wide variety of clinical symptoms were observed, ranging from subclinical to severe. Observed clinical symptoms in the most severely affected pigs were depression, loss of appetite, emaciation, increased frequency of breathing, cramps, paralysis of the hindquarters, inability to stand up, haemorrhages in the skin, and diarrhoea. One inoculated pig died at day 23 p.i., and one contact pig at day 29 p.i.

All pigs inoculated with the high dose of the Paderborn strain developed fever, clinical symptoms and leucopenia. Fever started one day earlier than in the pigs inoculated with the low dose. All the contacts of the high dose group developed fever, clinical symptoms and leucopenia. Clinical symptoms were similar to those observed in the group of pigs inoculated with the low dose. Two inoculated pigs died at days 21 and 32 p.i., and one contact pig at day 31 p.i.

All pigs inoculated with the Brescia strain developed fever, clinical symptoms and leucopenia and died between days 12 and 15 p.i. All contacts developed fever and clinical symptoms, and six of the seven contact pigs developed leucopenia. The contact pigs died between days 13 and 22 p.i. Observed clinical symptoms were severe depression, emaciation, loss of appetite, cramps, ataxia, inability to stand up, large hemorrhages in the skin, and diarrhoea.

*3.2 Virus titres in oropharyngeal fluid and faeces samples*

In oropharyngeal fluid of pigs inoculated with the Zoelen strain, infectious virus (determined by virus isolation) was detected intermittently from days 3 to 12 p.i. (Figure 1). Viral RNA (determined by qRRT-PCR) was detected constantly from day 3 p.i. until the end of the experiment (Figure 2). Infectious virus was detected in faeces of one inoculated pig at day 7 p.i., and viral RNA was detected in faeces of all Zoelen inoculated pigs between days 5 and 11 p.i. In none of the samples of the

contact pigs infectious virus or viral RNA could be detected. The level of infectious virus and viral RNA was on average lower than in samples from pig infected with the Paderborn or Brescia strains.

Infectious virus was detected in oropharyngeal fluid and faeces of all pigs inoculated with the low or high dose of the Paderborn strain, and their contacts. In general, infectious virus and viral RNA were detected one day earlier, or with a higher dose, in samples of the pigs inoculated with the high dose of the Paderborn strain than in samples of pigs inoculated with the low dose. However, excretion of contact pigs started in general on the same days p.i. (depending on the sample type and test between days 8 and 13 p.i.). Infectious virus titres, and viral RNA titres were comparable between both groups.

Infectious virus and viral RNA were detected in oropharyngeal fluid from pigs inoculated with the Brescia strain from day 3 p.i., and in faeces from day 4 p.i. All contact pigs were infected, and infectious virus was detected between days 5 and 10 p.i., depending on sample type and test. The level of infectious virus titres in samples from the Brescia infected pigs were equal to the titres from pigs of the Paderborn groups, while viral RNA titres were on average higher.

*3.3 Virus titres in air samples*

Infectious virus was not detected in air samples taken from the room housing the pigs infected with the Zoelen strain (Figure 1). In samples from the rooms housing the Paderborn and Brescia infected pigs, it was observed that the higher the dose or virulence of the virus strain used for inoculation of the pigs, the earlier virus could be detected in the air samples. From the room housing the pigs inoculated with the low dose of the Paderborn strain, infectious virus was detected from day 14 p.i., while in the rooms housing pigs inoculated with the high dose, infectious virus could be detected in air samples from day 10 p.i. From rooms housing the Brescia infected pigs, infectious virus was already detected from day 7 p.i.

Viral RNA titres in the air samples were higher than infectious virus titres (Figure 2). Furthermore, in air samples from the pigs infected with the Paderborn strain, viral RNA was detected one sampling moment before infectious virus was detected. From the room housing the pigs infected with the Zoelen strain, no viral RNA could be detected.

The virus excretion in the air per pig was calculated, based on the number of pigs excreting infectious virus (Table 2) or viral RNA (Table 3). The average infectious virus titres in the air per pig

were between $10^{0.7}$ and $10^{2.1}$ $TCID_{50}/m^3$, and differed maximum $10^{0.6}$ $TCID_{50}/m^3$ between the Paderborn and Brescia groups at sampling moment when a plateau is reached (from day 14 p.i.). Average viral RNA titres per pig were slightly higher, between $10^{1.2}$ and $10^{2.8}$ $TCID_{50}$equivalents/$m^3$. Differences between the Paderborn and Brescia groups at sampling moment when a plateau was reached (from day 10 p.i.) were maximum $10^{0.8}$ $TCID_{50}$ equivalents/$m^3$.

*3.4 Factors associated to the virus titres in the air*

There was no significant relationship between infectious virus titres in the air and total quantities of infectious virus per room in oropharyngeal fluid (Spearman's rank correlation coefficient 0.48, p=0.057) or faeces (Spearman's rank correlation coefficient 0.36, p=0.167). There was also no significant relationship between viral RNA titres in the air and total quantities of viral RNA per room in oropharyngeal fluid (Spearman's rank correlation coefficient 0.42, p=0.085) or faeces (Spearman's rank correlation coefficient 0.31, p=0.21).

There was a significant relationship between infectious virus titre in the air and the number of pigs excreting infectious virus in faeces (Spearman's rank correlation coefficient 0.67, p=0.005) or oropharyngeal fluid (Spearman's rank correlation coefficient 0.57, p=0.014) (Figure 3). The relationship between viral RNA titre in the air and the number of pigs excreting infectious virus in faeces samples was also significant (Spearman's rank correlation coefficient 0.57, p= 0.014), but the relationship between viral RNA titre in the air and the number of pigs excreting infectious virus in oropharyngeal fluid was not significant (Spearman's rank correlation coefficient 0.46, p= 0.057).

**4. Discussion**

This paper confirmed our previous observations that CSFV is emitted in the air by infected pigs. Furthermore, it adds important new information on the quantities emitted by groups of infected pigs, which also enabled us to quantify the contribution of individual infected pigs to virus concentrations in the air. It was shown that both infectious virus and viral RNA could be detected for a considerable time in the air of rooms housing pigs infected with the highly virulent Brescia strain or the moderately virulent Paderborn strain. The first moment that virus in the air could be detected seems to depend on the strain and dose used for inoculation of the pigs. Virus was detected earlier in rooms of pigs infected with higher virulent strains or higher inoculation doses.

In the present study, infectious virus was isolated with maximum titres of $10^3$ $TCID_{50}/m^3$ from the air of rooms housing pigs infected with the Brescia or Paderborn strain. Infectious virus was isolated from the air continuously until all pigs died (strain Brescia) or until the end of the animal experiment at day 35 p.i. (strain Paderborn). In a previous study we were able to detect viral RNA in air samples from pigs infected with the Paderborn and Brescia strain, however, infectious virus was detected only once (Weesendorp et al., 2008a). Moreover, viral RNA titres in the air samples were in general lower than in the present study. The difference in housing system and number of infected animals are most likely the reasons for the lower titres and inability to detect infectious virus in the previous study. In the present study air samples were obtained from rooms housing ten pigs, with at least three infected pigs, while in the previous study samples were taken from individually housed pigs. Other studies performed before failed to isolate infectious CSFV or viral RNA from the air entirely (Terpstra, unpublished, 1986; Stärk, 1998). As in these studies also the Brescia strain was used for infection of the pigs, virus excretion in the air is likely to have occurred, although a smaller number of pigs (two to four) were infected. The limited sensitivity of their test system is probably the reason for their inability to detect CSFV. In the study of Stärk (1998), the detection limit of the air sampling system in combination with RT-PCR was $10^{4.1}$ $TCID_{50}$/filter. Because 5225 l of air was sampled, this is equal to $10^{3.4}$ $TCID_{50}/m^3$, which is not only much higher than the detection limit of our sampling system in combination with the virus titration assay, but also higher than the amounts of virus we detected in the air. More important, their detection limit was apparently also higher than the minimum infectious dose, as transmission through the air from infected to susceptible pigs has been demonstrated before (Terpstra, 1987; Dewulf et al., 2000).

Between the strains and doses used for inoculation of the pigs, differences were observed in the first moment of detectable virus or viral RNA in the air. The higher the virulence of the strain, or the higher the dose used for inoculation, the sooner infectious virus was detected, or the higher the viral RNA titres were at the first sampling moment. With the highest virulent strain in this study, the Brescia strain, this is in agreement with the fact that contact animals became infected earlier than contact pigs of the lower virulent Paderborn strain. Besides this larger number of infected pigs, higher virus titres were observed, particularly in oropharyngeal fluid, but most likely also in other secretions and excretions (Weesendorp et al., 2008b). The differences between numbers of infected pigs and differences in excretion patterns between the two doses of the Paderborn strain were small. But even

here the results with respect to virus excretion, but also timing of clinical symptoms and fever, agree with the finding that with a high inoculation dose virus can be isolated sooner from the air. It is however questionable whether this effect will continue in subsequent generations of infections within a herd, and therefore also whether the initial virus dose that enters a herd will have a relevant effect on virus spread through the air.

In air samples taken from the room housing pigs infected with the low virulent Zoelen strain, no infectious virus or viral RNA was detected. This was not unexpected, as in a previous experiment we were also unable to detect virus in air samples from individually housed pigs (Weesendorp et al., 2008a). Furthermore, transmission from pigs inoculated with this strain to contact pigs failed, and therefore only three pigs became infected with the Zoelen strain. These three pigs excreted much lower quantities of virus in oropharyngeal fluid and faeces than pigs infected with the Brescia and Paderborn strain. Most likely, the same applies to other secretions and excretions (Weesendorp et al., 2008b). The combination of low numbers of infected pigs, with low excretion levels, has resulted in low levels of virus in the air, at least below the detection level. This suggests that transmission of CSFV by the air is less likely to occur with low virulent strains than with strains of higher virulence, as has been observed before for other viruses like Porcine reproductive and respiratory syndrome virus (Cho et al., 2007).

Infectious aerosols are generated when pigs excrete virus in breathing air, or other secretions and excretions that could end up in an aerosol, like splashes of faeces and urine or nasal fluid and saliva after sneezing and coughing. The concentrations of virus in the air are directly proportional to the strength of the aerosol source. This is most likely dependent on the number and concentration of infectious animals (Stärk, 1999). The present study confirmed this relationship between virus concentration in the air and number of pigs that excreted virus. However, it was expected that the concentrations of virus in the air were also dependent on the concentrations of virus excreted in the secretions and excretions. Such a relationship between virus concentration in the air, and the virus concentration in secretions has been observed before for Aujeszky's disease virus. After challenging vaccinated pigs with Aujeszky's disease virus, a correlation (r =0.83) was found between virus titres in the air and in nasal fluid (Bourgueil et al., 1992). In the present study the relationship between virus concentration in the air, and virus quantities in secretions or excretions (faeces or oropharyngeal fluid) were not observed, at least not statistically significant. Due to the limited number of observations the

power of the comparison is, however, low and a relationship between virus concentration in the air, and virus quantities in faeces or oropharyngeal fluid can not be ruled out yet. Furthermore, it is possible that other secretions or excretions would reflect virus concentrations in the air better, and would have a significant correlation with virus in the air, even with the small number of observations.

The number of pigs that excreted virus in faeces at the moments of air sampling showed a better relationship with virus concentration in the air than the number of pigs that excreted virus in oropharyngeal fluid. The number of pigs that excreted infectious virus in faeces increased until days 17-21 p.i (Paderborn), or day 10 (Brescia), and then decreased due to death or recovery of pigs, while the number of pigs that excreted virus in oropharyngeal fluid showed a rather irregular pattern. In series of samples taken on consecutive days from the same pigs, virus titration on oropharyngeal fluid sometimes gave a negative result, even though the day before or the day after, high virus titres could be found. What is more, the results of the qRRT-PCR remained at the same level, suggesting that an equal amount of virus particles was present in these samples. Apparently the virus was inactivated for some reason, either already in the oropharyngeal cavity of the animal, during sampling, or shortly after sampling. The inability to isolate infectious virus from oropharyngeal fluid in sampling series over time has been observed before for classical swine fever (Bouma et al., 2000). Therefore, the number of pigs excreting virus in faeces has a better correlation with the virus concentration in the air than the number of pigs excreting virus in oropharyngeal fluid.

The present study showed that aerosols containing infectious CSFV of up to $10^{3.0}$ $TCID_{50}/m^3$ are produced even by small groups of maximum ten infected pigs. With a ventilation rate of 400 $m^3/h$, the maximum amount of virus emitted in the air per pig per day was estimated to be approximately $10^{6.1}$ $TCID_{50}$. Assuming that a 25 kg pig in the same room inspires about 15 l/min (Alexandersen and Donaldson, 2002), the dose received is approximately $10^{4.3}$ $TCID_{50}$ in a 24 hour period. This is more than sufficient to induce infection, based on estimated minimal infectious intranasal doses of 10 $TCID_{50}$ for the highly virulent Alfort strain (Liess, 1987), or pig $ID_{50}$ of 80 $TCID_{50}$ for the Brescia strain (Terpstra, 1988).

When the distance of susceptible animals to the virus source increases, several additional factors will be important to determine whether transmission through the air is feasible (Donaldson, 1978; Stärk, 1999). First and foremost, dilution of the air will occur with increasing distance, also depending on directions of wind and air turbulence. Second, the amount of infectious virus reaching

susceptible animals will depend on amounts of virus excreted at the source (in turn depending on virus strain and number of animals infected) and biological or physical decay of virus along the way. Finally, susceptibility of target animals will depend on virus dose and exposure time. Over short distances it is likely that the virus concentrations found in this study in the aerosols will be sufficient in inducing infection in susceptible pigs. Short distance transmission through aerosols was demonstrated experimentally, even though one can question in some of these experiments whether movement and dilution of virus in the air, mimics that under field conditions (Hughes and Gustafson, 1960; Terpstra, 1987; Dewulf et al*.,* 2000; González et al., 2001).

The present study provides important and quantitative information on virus concentrations in aerosols per infected pig over time. This information could be used in models that simulate the spread of CSFV via the air over large distances, like this has been done for foot-and-mouth disease virus and Aujeszky's disease virus (Casal et al., 1997; Sørensen et al., 2000). To really quantify the possible role of airborne virus transmission, additional information will however be needed, like quantitative information on inactivation of CSFV in aerosols (for instance on the effect of temperature, relative humidity, UV-radiation or fluid in which the virus is suspended) as well as data on susceptibility, and especially on minimum doses necessary to induce infection via the inhalation route. For further insight into airborne transmission, more research into all these parameters will be necessary.

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Table 1. Day after inoculation that fever, clinical symptoms or leucopenia were observed for the first time in one of the inoculated or contact animals

| | First day post inoculation of: | | |
|---|---|---|---|
| Virus strain | Fever [a] | Clinical symptoms [b] | Leucopenia [c] |
| **Zoelen** | | | |
| Inoculated | no | no | 5 |
| Contact | -[d] | -[d] | -[d] |
| **Paderborn low** | | | |
| Inoculated | 6 | 11 | 5 |
| Contact | 15 | 17 | 14 |
| **Paderborn high** | | | |
| Inoculated | 5 | 11 | 3 |
| Contact | 14 | 16 | 14 |
| **Brescia** | | | |
| Inoculated | 3 | 5 | 3 |
| Contact | 8 | 9 | 7 |

[a] Fever was defined as body temperature higher than 40°C, for two or more consecutive days.

[b] Clinical symptoms were defined as clinical scores higher than 2.

[c] Leucopenia was defined as <10x $10^9$ cells/l blood.

[d] Contact pigs were not infected.

543 Table 2. Infectious virus titres in air samples originating from rooms housing pigs infected with different strains or different initial doses of the same strain, and

544 average contribution per pig, based on the number of pigs excreting infectious virus

| Virus strain | Days post inoculation | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 4 | 7 | 10 | 14 | 17 | 21 | 28 | 35 |
| **Zoelen** | | | | | | | | |
| Virus titration titre in air sample ($TCID_{50}/m^3$) | - | - | - | - | - | - | - | - |
| # of pigs excreting infectious virus in OPF [a]/ total # of pigs | 2/10 | 3/10 | 0/10 | 0/10 | 0/10 | 0/10 | 0/10 | 0/10 |
| # of pigs excreting infectious virus in faeces/ total # of pigs | 0/10 | 1/10 | 0/10 | 0/10 | 0/10 | 0/10 | 0/10 | 0/10 |
| Average titre/pig based on # of pigs excreting infectious virus [b] ($TCID_{50}/m^3$) | - | - | - | - | - | - | - | - |
| **Paderborn low dose** | | | | | | | | |
| Virus titration titre in air sample ($TCID_{50}/m^3$) | - | - | - | 1.7 | 1.7 | 1.7 | 3.0 | 2.3 |
| # of pigs excreting infectious virus in OPF [a]/ total # of pigs | 0/10 | 3/10 | 1/10 | 2/10 | 9/10 | 1/10 | 8/9 | 7/8 |
| # of pigs excreting infectious virus in faeces/ total # of pigs | 0/10 | 3/10 | 3/10 | 4/10 | 6/10 | 9/10 | 8/9 | 7/8 |
| Average titre/pig based on # of pigs excreting infectious virus [b] ($TCID_{50}/m^3$) | - | - | - | 1.1 | 0.8 | 0.8 | 2.1 | 1.4 |
| **Paderborn high dose** | | | | | | | | |
| Virus titration titre in air sample ($TCID_{50}/m^3$) | - | - | 1.2 | 2.2 | 2.0 | 2.0 | 2.7 | 2.5 |
| # of pigs excreting infectious virus in OPF [a]/ total # of pigs | 2/10 | 0/10 | 0/10 | 0/10 | 5/10 | 5/9 | 7/9 | 5/7 |
| # of pigs excreting infectious virus in faeces/ total # of pigs | 0/10 | 3/10 | 3/10 | 4/10 | 8/10 | 7/9 | 7/9 | 4/7 |
| Average titre/pig based on # of pigs excreting infectious virus [b] ($TCID_{50}/m^3$) | - | - | 0.7 | 1.6 | 1.1 | 1.1 | 1.9 | 1.8 |
| **Brescia** | | | | | | | | |
| Virus titration titre in air sample ($TCID_{50}/m^3$) | - | 1.5 | 2.5 | 1.7 | 1.2 | 1.2 | **x** | **x** |
| # of pigs excreting infectious virus in OPF [a]/ total # of pigs | 3/10 | 2/10 | 7/10 | 1/5 | 3/3 | 2/2 | | |
| # of pigs excreting infectious virus in faeces/ total # of pigs | 1/10 | 3/10 | 9/10 | 5/5 | 3/3 | 2/2 | | |
| Average titre/pig based on # of pigs excreting infectious virus [b] ($TCID_{50}/m^3$) | - | 1.0 | 1.5 | 1.0 | 0.7 | 0.9 | | |

545 [a] OPF = oropharyngeal fluid

546 [b] The number (#) of pigs excreting infectious virus is determined by virus titration positive results on oropharyngeal fluid and/or faeces.

547 **x** End of the experiment due to death of all pigs.

Table 3. Viral RNA titres in air samples originating from rooms housing pigs infected with different strains or different initial doses of the same strain, and average contribution per pig, based on the number of pigs excreting viral RNA

| Virus strain | Days post inoculation | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 4 | 7 | 10 | 14 | 17 | 21 | 28 | 35 |
| **Zoelen** | | | | | | | | |
| qRRT-PCR titre in air sample ($TCID_{50}$ equivalents/$m^3$) | - | - | - | - | - | - | - | - |
| # of pigs excreting viral RNA in OPF [a]/ total # of pigs | 3/10 | 3/10 | 3/10 | 3/10 | 3/10 | 3/10 | 2/10 | 3/10 |
| # of pigs excreting viral RNA in faeces/ total # of pigs | 0/10 | 3/10 | 3/10 | 0/10 | 0/10 | 0/10 | 0/10 | 0/10 |
| Average titre/pig based on # of pigs excreting viral RNA [b] ($TCID_{50}$ equivalents/$m^3$) | - | - | - | - | - | - | - | - |
| **Paderborn low dose** | | | | | | | | |
| qRRT-PCR titre in air sample ($TCID_{50}$ equivalents/$m^3$) | - | - | 3.0 | 3.8 | 3.6 | 3.1 | 3.7 | 3.5 |
| # of pigs excreting viral RNA in OPF [a]/ total # of pigs | 3/10 | 3/10 | 10/10 | 10/10 | 10/10 | 10/10 | 9/9 | 8/8 |
| # of pigs excreting viral RNA in faeces/ total # of pigs | 2/10 | 3/10 | 4/10 | 7/10 | 9/10 | 9/10 | 8/9 | 8/8 |
| Average titre/pig based on # of pigs excreting viral RNA [b] ($TCID_{50}$ equivalents/$m^3$) | - | - | 2.0 | 2.8 | 2.6 | 2.1 | 2.7 | 2.6 |
| **Paderborn high dose** | | | | | | | | |
| qRRT-PCR titre in air sample ($TCID_{50}$ equivalents/$m^3$) | - | 1.6 | 3.0 | 3.0 | 3.2 | 2.8 | 3.6 | 2.9 |
| # of pigs excreting viral RNA in OPF [a]/ total # of pigs | 3/10 | 3/10 | 9/10 | 10/10 | 10/10 | 9/9 | 9/9 | 7/7 |
| # of pigs excreting viral RNA in faeces/ total # of pigs | 1/10 | 3/10 | 4/10 | 6/10 | 10/10 | 8/9 | 8/9 | 6/7 |
| Average titre/pig based on # of pigs excreting viral RNA [b] ($TCID_{50}$ equivalents/$m^3$) | - | 1.2 | 2.0 | 2.0 | 2.2 | 1.9 | 2.6 | 2.0 |
| **Brescia** | | | | | | | | |
| qRRT-PCR titre in air sample ($TCID_{50}$ equivalents/$m^3$) | - | 3.8 | 3.4 | 3.1 | 3.3 | 2.5 | **x** | **x** |
| # of pigs excreting viral RNA in OPF [a]/ total # of pigs | 4/10 | 10/10 | 10/10 | 5/5 | 3/3 | 2/2 | | |
| # of pigs excreting viral RNA in faeces/ total # of pigs | 3/10 | 9/10 | 10/10 | 5/5 | 3/3 | 2/2 | | |
| Average titre/pig based on # of pigs excreting viral RNA [b] ($TCID_{50}$ equivalents/$m^3$) | - | 2.8 | 2.4 | 2.4 | 2.8 | 2.2 | | |

[a] OPF = oropharyngeal fluid

[b] The number (#) of pigs excreting infectious virus is determined by quantitative Real-Time Reverse Transcription Polymerase Chain Reaction positive results on oropharyngeal fluid and/or faeces.

**x** End of the experiment due to death of all pigs.

554 Figure 1. Average infectious virus excretion per pig in oropharyngeal fluid and faeces, as determined
555 by virus isolation (VI) and titration, and infectious virus titres in air samples. Dpi= days post inoculation.
556
557 Figure 2. Average viral RNA excretion per pig in oropharyngeal fluid and faeces, as determined by
558 quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (qRRT-PCR), and viral
559 RNA titres in air samples. Dpi= days post inoculation.
560
561 Figure 3. Relationship between virus concentration in the air, analysed by virus isolation (VI) or
562 quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (qRRT-PCR), and the
563 number (#) of pigs excreting infectious virus (analysed by VI) or viral RNA (analysed by qRRT-PCR) in
564 faeces and oropharyngeal fluid (OPF) at the moments of air sampling. Negative air samples were
565 excluded from the analysis. Spearman's rank correlation (r) was used for analysis of the data.

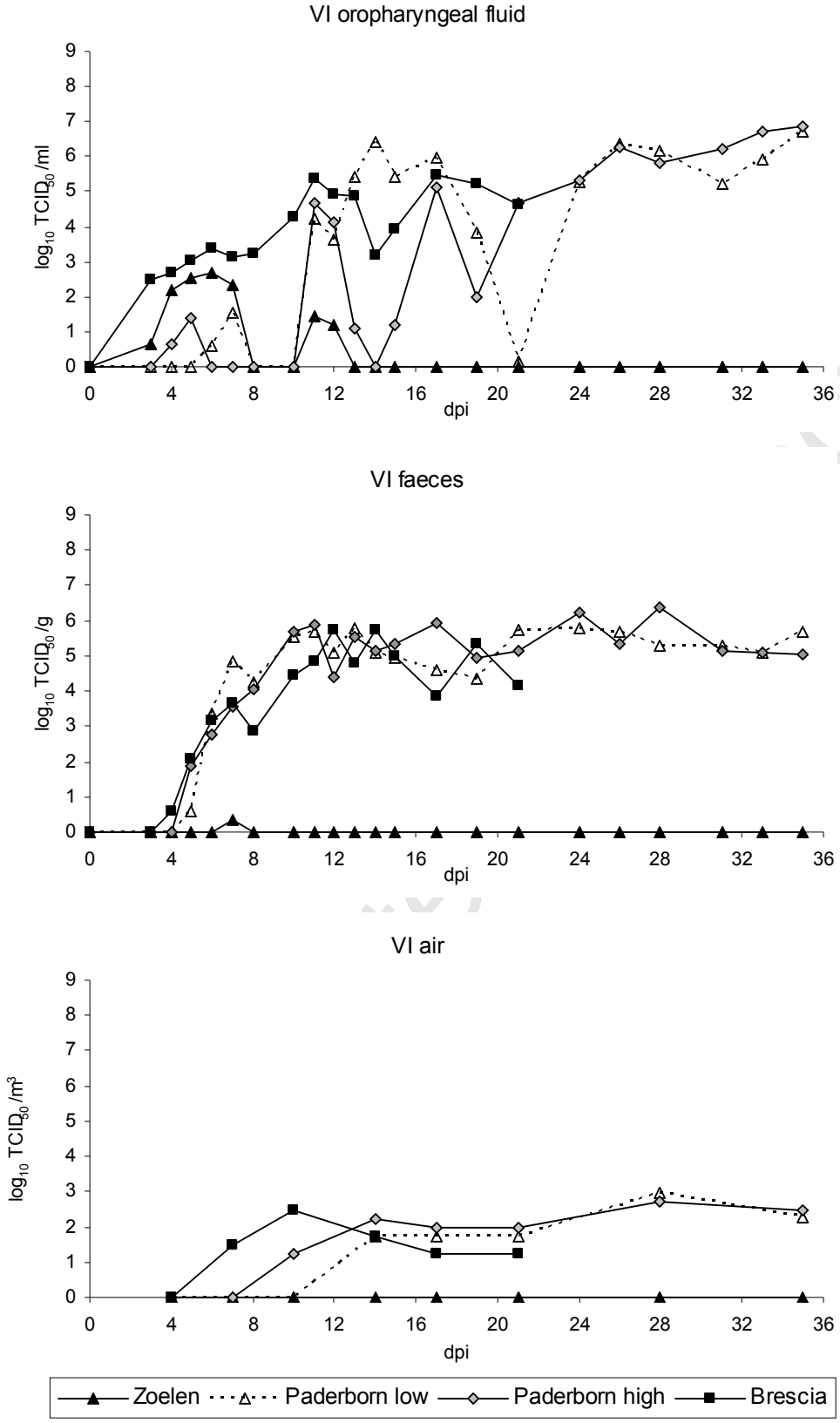


Figure 1.

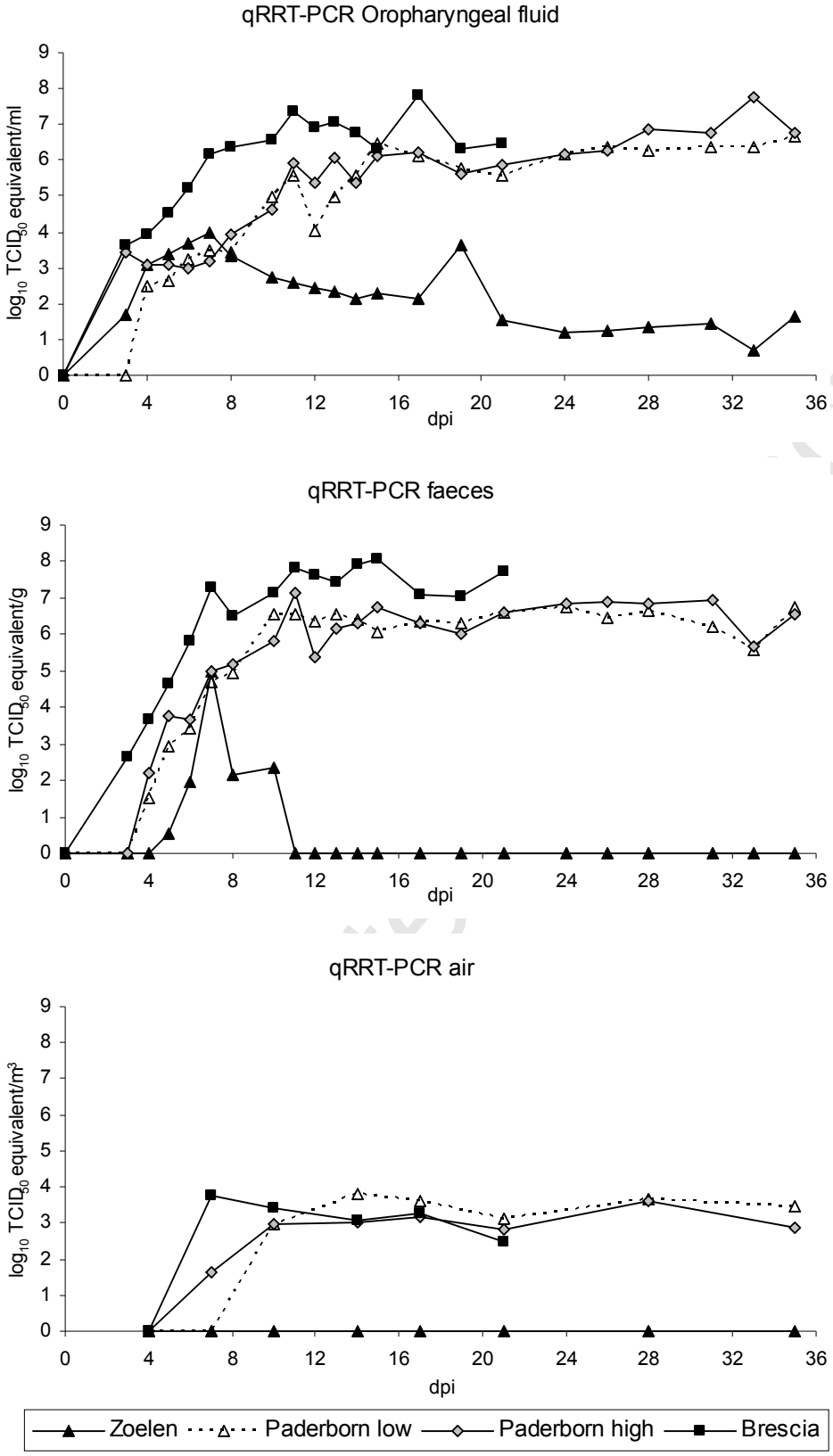


Figure 2.

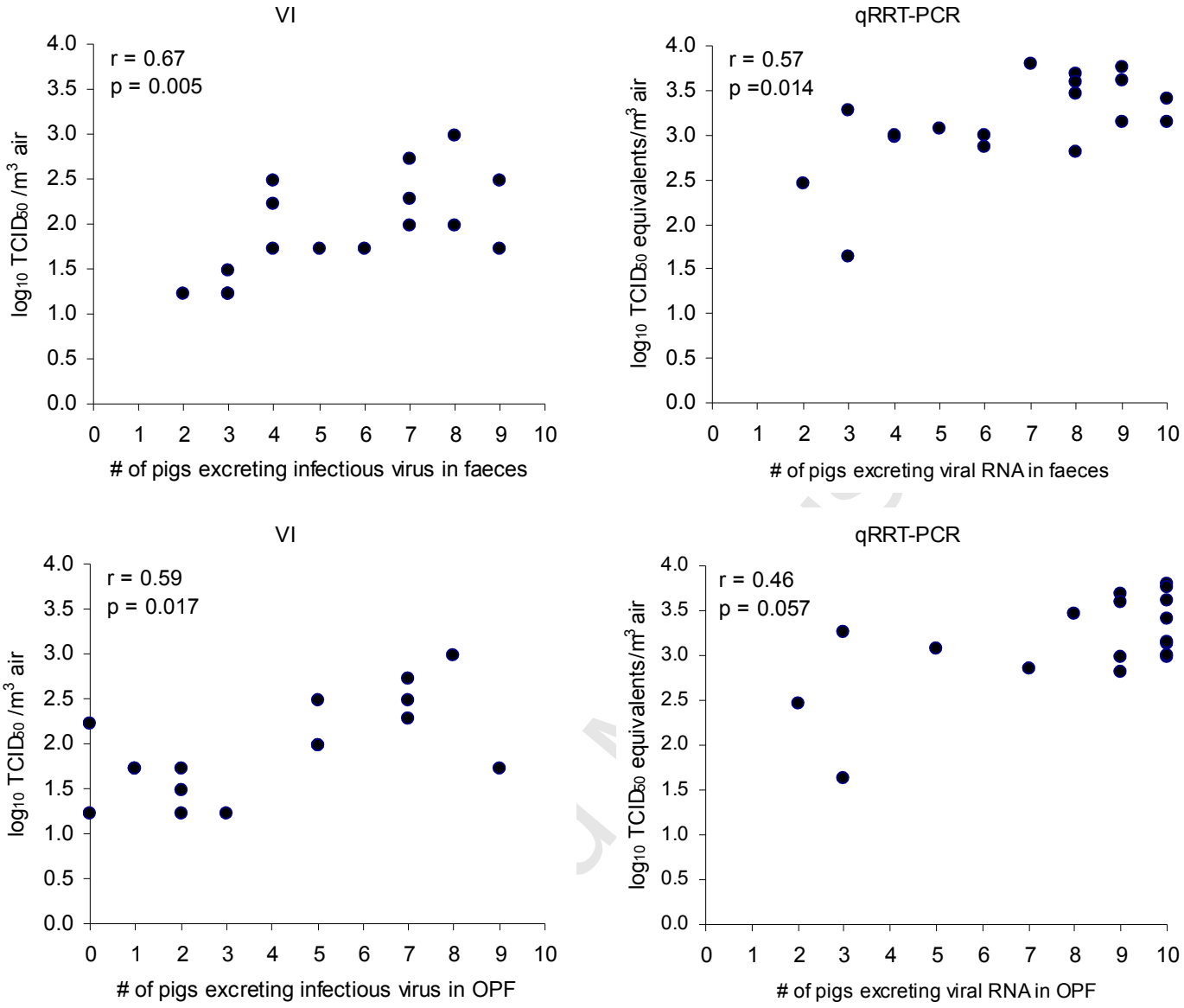


Figure 3.