Quantification of underlying mechanisms of classical swine fever virus transmission

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Quantification of underlying mechanisms of classical swine fever virus transmission

Kwantificering van onderliggende mechanismen van

klassieke varkenspestvirus transmissie

(met een samenvatting in het Nederlands)

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Niemand weet waarheen de wind waait….

Contents

Chapter 1

Introduction

Introduction

Classical swine fever virus

Classical swine fever (CSF) is a highly contagious disease that affects both domestic pigs and wild boar. It is caused by an enveloped RNA-virus, belonging to the family Flaviviridae, genus Pestivirus, having a genome size of approximately 12.3 kb (Lindenbach and Rice, 2001). A large number of CSFV strains exist (Paton et al., 2000), which vary considerably in their virulence (Carbrey et al., 1980; Floegel-Niesmann et al., 2003; Floegel-Niesmann et al., 2009). Although the classification of the strains according to virulence is a controversial subject of discussion, they are generally divided into either highly, moderately, and low virulent strains (Van Oirschot, 1988; Mittelholzer et al., 2000; Floegel-Niesmann et al., 2003). Highly virulent strains cause an acute haemorrhagic form of the disease that usually results in death (Van Oirschot, 1988). The acute form is further characterized by high fever, anorexia, lethargy, conjunctivitis, respiratory signs and constipation followed by diarrhea (Moennig et al., 2003). Moderately and low virulent strains produce a form of the disease that is more difficult to recognize as CSF. Infection with moderately virulent strains in particular can lead to different courses of the disease with a wide spectrum of clinical signs (Depner et al., 1996; Floegel-Niesmann et al., 2003; Uttenthal et al., 2003). Infections with these strains may result in either (sub)acute disease, resulting in death or recovery, or chronic disease, which is always fatal. Pigs infected with low virulent stains show few or no signs of disease and recover from the infection (Van Oirschot, 1988).

Epidemiology

CSF is still present in many parts of the world. It is endemic in some countries in eastern Europe, Asia, Central- and South America (www.oie.int). The situation in Africa is uncertain, but CSF has recently been reported in Madagascar and South Africa (Sandvik et al., 2005). CSF is eradicated in most countries of the European Union since the 1980's, after which a non-vaccination policy was implemented (Bendixen, 1988; Vandeputte and Chappuis, 1999; Terpstra and De Smit, 2000; Dong and Chen, 2007). The strategy then adopted was based on slaughter and disposal of all infected and potentially exposed herds (stamping out) and movement bans for animals and their products. Vaccination was allowed only in emergency situations (Anonymous, 1980b, a, 1991, 2001). After the introduction of the non-vaccination policy, several outbreaks still occurred (Koenen et al., 1996; Elbers et al., 1999; Fritzemeier et al., 2000; Sharpe et al., 2001; Allepuz et al., 2007). Due to the ban on 'prophylactic' vaccination, the population of pigs was fully susceptible to CSF. In combination with the high pig density in some areas, this resulted in rapid spread of CSF. The last major outbreak in Europe occurred in 1997-1998. Herds were affected in Germany, the Netherlands, Belgium,

Spain and Italy. In the Netherlands alone, approximately 11 million pigs had to be killed (Elbers et al., 1999; Stegeman et al., 2000; Terpstra and De Smit, 2000). High financial losses due to mass destruction of pigs and export bans were the consequence (Meuwissen et al., 1999; Terpstra and De Smit, 2000).

The introduction of CSFV into the domestic pig population of a country has mainly been the result of illegal swill feeding, contact with contaminated livestock trucks, infected pigs or wild boar (Moennig, 2000; De Vos et al., 2003; Moennig et al., 2003). CSFV has been reported in the wild-boar population in Bulgaria, Croatia, France, Germany, Hungary, Romania and Slovakia between 2005 and 2009. The danger of virus introduction due to (indirect) contact with wild boar and wild boar meat has been demonstrated. Between 1993 and 1998, 59% of the primary outbreaks in domestic pigs in Germany were assigned to this route of transmission (Fritzemeier et al., 2000).

Transmission

CSFV is spread within- and between herds through excretions and secretions from infected pigs. The most efficient and rapid transmission route of CSFV occurs via direct contact between infected and susceptible pigs (Klinkenberg et al., 2002). Also indirect transmission routes like swill feeding (Williams and Matthews, 1988; Farez and Morley, 1997; Edwards, 2000; Fritzemeier et al., 2000; Sharpe et al., 2001), artificial insemination (De Smit, 1999; Floegel, 2000; Hennecken et al., 2000), or contaminated mechanical vectors like clothing and footwear or transportation trucks, can transmit the virus (Terpstra, 1988; Stegeman et al., 2002; Ribbens et al., 2004; Ribbens et al., 2007). During the 1997-1998 epidemic in the Netherlands, in approximately 50% of the cases, no route of transmission could be identified, but because most of these infected herds were situated close to already infected herds, they were called neighbourhood infections (Elbers et al., 1999; Elbers et al., 2001). The inability to establish the origin of these neighbourhood infections may be caused by underreporting of well-known dangerous contacts or untraceable routes like transmission via arthropods, birds, pets and rodents (Terpstra, 1988; Elbers et al., 1999; Dewulf et al., 2001; Elbers et al., 2001; Kaden et al., 2003). Airborne spread has also been suggested (Elbers et al., 1999; Laevens, 1999; Dewulf et al., 2000), although no association was found between new infections and the prevailing direction of the wind during the 1997-1998 outbreak in the Netherlands (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; Mintiens et al., 2000).

Experimental studies showed that CSFV can be transmitted between groups of pigs that are not in direct contact (Hughes and Gustafson, 1960; Terpstra, 1988; Laevens et al., 1998; Laevens, 1999; Dewulf et al., 2000; Gonzalez et al., 2001). Although the most likely mechanism of virus transmission was via the air, attempts to detect CSFV in the air failed (Terpstra, unpublished, 1986; Stärk, 1998).

Scope of this thesis

Several authors found that the likelihood of occurrence of a neighbourhood infection decreased, when the distance to the primary infected herd increased (Koenen et al., 1996; Staubach et al., 1997; Crauwels et al., 2003). Based on this relationship, models have been developed that evaluate control measures like preventive depopulation of herds or emergency vaccination (Nielen et al., 1999; Mangen et al., 2001; Klinkenberg et al., 2003; Backer et al., 2009). The application of these control measures have far-reaching consequences. Preventive depopulation of pig herds in close vicinity to an infected herd resulted during previous outbreaks in the destruction of large numbers of uninfected pigs (Koenen et al., 1996; Elbers et al., 1999), which is economically and ethically undesirable. Furthermore, vaccination in EU countries could hamper international trade, which can result in high economic losses (Boklund et al., 2008). Since our knowledge of the underlying mechanisms (transmission routes) of the neighbourhood infections still has considerable gaps (Crauwels et al., 2003; Mintiens et al., 2003), the effect of more specific control measures is difficult to quantify and evaluate.

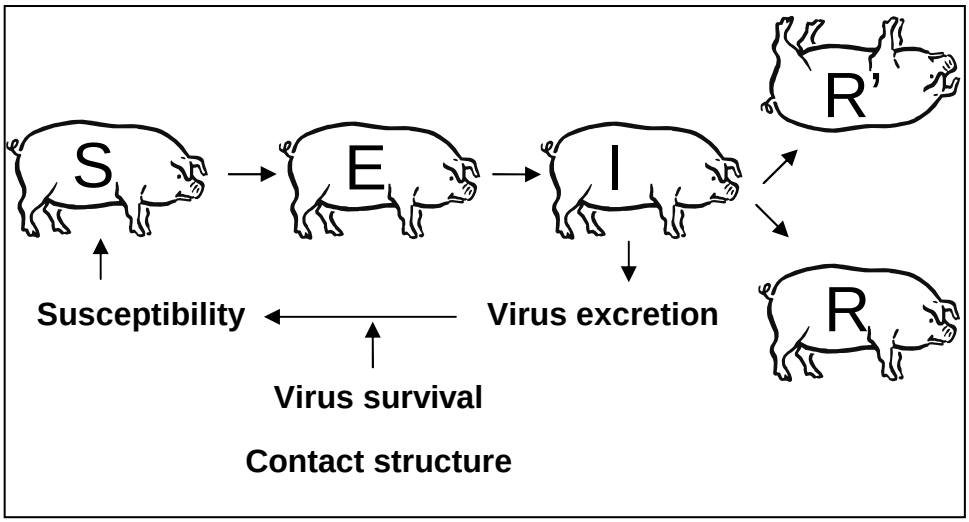

Figure 1. Underlying mechanisms of transmission, indicated in a susceptible-exposed-infectious-recovered or removed (SEIR) model. The infection of susceptible pigs (S) is dependent on virus excretion by infectious pigs (I), transfer of virus from infectious to susceptible pig, and susceptibility of the recipient pig. Successful virus transfer will depend on the contact structure between pigs and, for indirect contacts, on virus survival in the environment. When a susceptible pig (S) becomes infected, it is not infectious yet (E). After the latent period, this pig will start excreting infectious virus (I). Finally, this pig will either recover (R) or die (R').

The research in this thesis focused on quantifying underlying mechanisms of CSFV transmission and studying their contribution to transmission (Figure 1). Chapters 2, 3

and 4 focus on the infectivity of infected pigs by quantifying virus excretion in secretion and excretions, including the amounts of virus emitted in the air. Three virus strains were used that differ in their virulence; the low virulent strain Zoelen, the moderately virulent strain Paderborn and the highly virulent strain Brescia. Chapter 5 describes the survival of the virus in excretions of pigs infected with the Paderborn or Brescia strain. In Chapter 6 the effect of the virulence of CSFV strains and inoculation dose on transmission is described. In Chapter 7 this insight in the relation between virulence and transmission is further deepened by studying the effect of the clinical course of infection after inoculation with the moderately virulent strain Paderborn on transmission between pair-housed pigs. Chapter 8 uses all quantitative data of previous studies in a risk model to elucidate the role of the different secretions and excretions in transmission, and to estimate the probability of infection of a susceptible pig via these secretions and excretions. Chapter 9 ends this thesis with a summarising discussion.

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Chapter 2

Dynamics of virus excretion via different routes in pigs experimentally infected with classical swine fever virus strains of high, moderate or low virulence

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Dynamics of virus excretion via different routes in pigs experimentally infected with classical swine fever virus strains of high, moderate or low virulence

Abstract

Classical swine fever virus (CSFV) is transmitted via secretions and excretions of infected pigs. The efficiency and speed of transmission depends on a multitude of parameters, like quantities of virus excreted by infected pigs. This study provides quantitative data on excretion of CSFV over time from pigs infected with a highly, moderately or low virulent strain. For each strain, five individually housed pigs were infected. Virus excretion was quantified in oropharyngeal fluid, saliva, nasal fluid, lacrimal fluid, faeces, urine and skin scraping by virus titration and quantitative real-time reverse transcription polymerase chain reaction (qRRT-PCR). Infectious virus was excreted in all secretions and excretions of pigs infected with the highly and moderately virulent strain, while excretion from pigs infected with the low virulent strain was mostly restricted to the oronasal route. Pigs infected with the highly virulent strain excreted significantly more virus in all their secretions and excretions over the entire infectious period than pigs infected with the moderately or low virulent strains. An exception were the pigs that developed the chronic form of infection after inoculation with the moderately virulent strain. During the entire infectious period, they excreted the largest amounts of virus via most secretions and excretions, as they excreted virus continuously and for a long duration. This study highlights the crucial role chronically infected pigs may play in the transmission of CSFV. Furthermore, it demonstrates the importance of discriminating between strains and the clinical appearance of infection when using excretion data for modelling.

Introduction

Classical Swine Fever (CSF) is a highly contagious disease and affects both domestic pigs and wild boar. It is caused by an enveloped RNA-virus belonging to the family Flaviviridae, genus Pestivirus. Mortality and the severity of clinical signs depends on the virulence of the virus strain, and on characteristics of the pig such as age, breed and immune status (Moennig et al., 2003). Highly virulent strains cause an acute haemorrhagic form of the disease that usually results in death. The acute form is further characterized by high fever, anorexia, lethargy, conjunctivitis, respiratory signs and constipation followed by diarrhea (Moennig et al., 2003). Moderately and low virulent strains produce a form of the disease that is more difficult to recognize. Infection with moderately virulent strains in particular can lead to different courses of the disease with a wide spectrum of clinical signs (Floegel-Niesmann et al., 2003). Infections with these

strains may result in either (sub)acute disease, resulting in death or recovery, or chronic disease, which is always fatal. Pigs infected with low virulent stains show few or no signs of disease and recover from the infection (Van Oirschot, 1988).

In the 1980s, after successful eradication of CSF in most European Union countries of that time, a non-vaccination policy was implemented. This ban on 'prophylactic' vaccination resulted in a population of pigs fully susceptible to CSF. In combination with the high pig density in some areas this resulted in rapid spread of CSF during outbreaks. High financial losses, due to mass destruction of pigs and export bans, were the consequence (Moennig, 2000; Terpstra and De Smit, 2000; Moennig et al., 2003).

During an outbreak, CSFV is spread within- and between herds through excretions and secretions from infected pigs. The most efficient and rapid transmission route occurs via direct contact between infected and susceptible pigs. In case there is no direct contact, mechanical vectors like clothing and footwear or transport trucks, contaminated with the secretions and excretions of infected pigs, can transmit the virus (Ribbens et al., 2004). During the 1997-1998 epidemic in the Netherlands, in approximately 50% of the cases no route of transmission could be identified, but because most of these infected herds were situated close to already infected herds, they were called neighbourhood infections (Elbers et al., 1999; Elbers et al., 2001). Because the mechanisms behind neighbourhood infections are poorly understood, it is important to detect and quantify the underlying parameters of transmission, such as quantities of virus excreted by infected pigs, virus survival, contact rate, and the susceptibility of the recipient pig. More information on these parameters would provide a better understanding of these transmission mechanisms and for instance improve risk-analysis models that could indicate the importance of the different transmission routes during outbreaks.

It is likely that excretion of the virus depends on several factors, including breed, immune status and virus strain. The effect of virus strain on excretion was discussed by Terpstra (1991). According to Terpstra, pigs infected with highly virulent strains excrete large quantities of virus during the entire course of disease, while pigs infected with low virulent strains excrete virus for only a short period. However, no quantitative information was presented here, and no information was given on moderately virulent strains, which are currently the most prevalent strains in the field (Floegel-Niesmann et al., 2003). Beside the influence of the strain on the total amount of excreted virus, there is a difference between excretion routes in quantities of virus excreted. After infection with a highly virulent strain, large quantities of virus were excreted in saliva and smaller quantities in urine and faeces (Ressang, 1973). These data, however, mainly referred to the early stage of infection. Using more recent techniques like RT-PCR, virus has been detected in nasal fluid, faeces and semen, although the virus excretion was mostly not quantified (Oude Ophuis et al., 2006; Van Rijn et al., 2004). To our knowledge, no studies have been published that give an integrated overview of the dynamics of virus excretion via the different secretions or excretions of the pig. This is important information for elucidating the role of the different excretion routes in transmission. In this paper we quantified the virus excreted during the entire infectious period via saliva, oropharyngeal fluid, nasal fluid, conjunctival fluid, faeces, urine and

the skin of pigs infected with a highly, moderately or low virulent CSFV strain. Virulence as well as the course of the disease (e.g. acute or chronic), strongly influenced the quantities of virus in the secretions and excretions.

Materials & methods

Experimental setting and housing

Three experiments were conducted in succession with five male pigs each. The 8-week-old pigs were obtained from a conventional, but pestivirus free pig herd in the Netherlands. Pigs were housed in an isolation unit with five pens, separated by solid walls. Within the pens, pigs were housed individually in cages to allow individual sampling without contaminating the samples with the secretions and excretions of other pigs. To further minimize this risk of contamination, footwear and gloves were changed and materials needed for sampling and rectal temperature monitoring were provided for each pig separately. Between the experiments, the isolation unit was cleaned and decontaminated. Standard feed for finishing pigs was provided once a day, and the pigs had unlimited access to water.

To enable the calculation of total amounts of excreted virus in faeces and urine, the cages were specially designed to collect and separate these excretions. Faeces were collected in plastic bags attached to the pigs with a Velcro system. This Velcro system was glued directly on the skin around the anus (Van Kleef et al., 1994). The cages were equipped with slatted floors which allowed the collection of urine in a container attached to the tray underneath the cage. Faeces and urine production were recorded daily.

The experiments were approved by the Ethics Committee for Animal Experiments of the Central Veterinary Institute of Wageningen UR. The experiments were ended when all pigs were either dead, or when virus isolations carried out during the experiments were negative for more than 3 weeks.

Viruses

In each experiment, five pigs were inoculated with either 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) or the low virulent Zoelen strain (genotype 2.2, originally isolated during an outbreak on a Dutch farm [Van Oirschot, 1980]). 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 acute or subacute illness leading to death, recovery, or to the chronic form (a lethal clinical form leading to death 30 days or more after infection). Pigs infected with a low virulent strain show few or no signs of disease and recover from the infection.

Inoculation of animals

Pigs were inoculated intranasally with 1 ml of 100 LD_{50} (50% lethal dose) CSFV strain Brescia, which is approximately $10^{2.5}$ $TCID_{50}$ (tissue culture infectious dose 50%), with 1 ml of 10^5 $TCID_{50}$ strain Paderborn or 1 ml of 10^5 $TCID_{50}$ strain Zoelen, according to the standard infection models used in our institute (Bouma et al., 1999; Moormann et al., 2000; Klinkenberg et al., 2002). The inocula were back titrated to confirm the dose administered.

Clinical signs and body temperature

Body temperature and clinical signs were recorded daily. Fever was defined as body temperature higher than 40°C. For quantitative assessment of the severity of disease a list of ten CSF-relevant criteria, as described by Mittelholzer et al. (2000) was used. For all criteria a score was recorded of either normal (score 0), slightly altered (score 1), distinct clinical symptom (score 2), or severe CSF symptom (score 3). The scores for each pig were added up to a total score per day. Sick pigs that became moribund and unable to stand up were euthanized for reasons of animal welfare.

Sampling procedures and pre-treatment of samples

Samples were collected from blood, oropharyngeal fluid, saliva, nasal fluid, conjunctival fluid, faeces, urine, and skin scrapings to determine the virus titres. Directly after collection, the samples were stored at 5℃ to a void inactivation of the virus.

EDTA-stabilized blood samples were collected three times a week for leucocyte and thrombocyte counts, and for isolation of leucocytes. For isolation of leucocytes, 4 ml 0.84% NH_4CL solution were added to 2 ml of EDTA blood. After 10 min the samples were centrifuged at 1000 rpm and washed twice with PBS. The pellet was resuspended in 2 ml medium (Eagle minimum essential medium [EMEM] [Gibco, Invitrogen, Breda, the Netherlands] with 5% fetal bovine serum [FBS], and 10% antibiotics) and stored at -70℃ until analysis by virus titration and quantita tive real-time reverse transcription polymerase chain reaction (qRRT-PCR). Once a week, the EDTA blood (whole blood) was directly stored at -70℃ for analysis.

Oropharyngeal fluid, saliva, nasal fluid and conjunctival fluid were collected every 2 days. Pigs infected with the low virulent Zoelen strain were sampled with larger intervals from 3 weeks post-inoculation (p.i.), as virus isolations carried out in between were already negative (Figures 3 and 4 for exact sampling times). Saliva was obtained by holding a gauze tampon in the oral cavity until it was soaked with saliva. Oropharyngeal fluid was obtained with a gauze tampon held by a 30 cm long forceps, which was scrubbed against the dorsal wall of the pharynx behind the soft palatum (Ressang et al., 1972). Samples from conjunctival and nasal fluid were collected using sterile rayon swabs (Medical Wire & Equipment, Corsham, United Kingdom). Swabs and tampons were weighed before and after collection to enable the calculation of the concentration of virus per gram of fluid ($TCID_{50}$/g). The swabs and tampons were suspended in 4 ml of the same media described for the leucocyte isolation. After centrifugation (2500 rpm for 15 min) the samples were stored at -70℃ until analysis.

Faeces was collected from the rectum every 2 days by stimulation of the anus. One gram of faeces was suspended in 9 ml medium (EMEM containing 10% FBS and 10% antibiotics) and vortexed with glass beads. After centrifugation (10,000 rpm for 5 min), the supernatants were stored at -70℃ until analysis.

Urine was collected as often as possible. Only fresh urine, obtained while the pig urinated, was analysed by virus titration. In those cases where fresh samples could not be obtained, urine was collected from a container attached to the tray under the cage. This container was replaced daily, so the urine collected was maximum 24 h old. All samples, both fresh and from the container, were analysed by qRRT-PCR. A ten-fold dilution in medium (EMEM containing 10% FBS and 10% antibiotics) was prepared from the urine samples for virus titration and stored at -70℃. Undiluted urine for qRRT-PCR analysis was stored at -70℃.

Skin scrapings were taken two or three times per animal, once clinical signs were observed. Skin was scraped off from the back of the pig between the scapulas, using a plastic tube with a sharp edge, until the skin was red. The skin sample was suspended in 3 ml of medium. After 15 min, samples were vortexed, centrifuged (1000 rpm for 10 min) and stored at -70℃ until analysis.

Leucocyte and thrombocyte counts

Leucocyte and thrombocyte counts were performed using the Medonic® CA 620 coulter counter (Boule Medical AB, Stockholm, Sweden). Leucopenia was defined as $<10 \times 10^9$ cells/L blood, and thrombocytopenia as $<200 \times 10^9$ cells/L blood.

Virus isolation and titration

For virus isolation from leucocytes, nasal fluid, conjunctival fluid, or skin scrapings, a volume of 125 µl was inoculated on a monolayer of SK6 cells (permanent porcine kidney cell line) in a 24-well plate (Greiner). For virus isolation from EDTA-stabilized whole blood, oropharyngeal fluid, saliva, faeces, and urine, a volume of 250 µl was incubated for 1 h on a monolayer of SK6 cells. These plates were then washed once with PBS (phosphate-buffered saline) and medium was added to the wells. All plates were incubated at 37℃ in an atmosphere with 5% CO_2 for 4 days. After being fixated and washed, the monolayers were stained by the immuno-peroxidase technique (Wensvoort et al., 1986), using two HPRO-conjugated CSFV specific MAbs (V3/V4), and examined for stained cells.

Virus positive samples were titrated in four-fold (using five decimal dilutions) to determine the concentration of infectious virus. Virus titres were calculated as $TCID_{50}$ using the Spearman-Kärber method (Finney, 1978).

Quantitative real-time reverse transcription polymerase chain reaction (qRRT-PCR)

Virus excretion was analysed by qRRT-PCR to determine the concentration of viral RNA. For RNA isolation, 200 µl of the sample were pipetted manually into MagNA Pure sample cartridges (Roche Applied Science, Mannheim, Germany). In each run of thirty-two samples, two negative control samples and three to six dilutions of a positive control sample (standard curve) were included. The standard curves were constructed

for each sample type and each strain of virus by spiking secretions and excretions with known concentrations of infectious virus. 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 qRRT-PCR or stored at -70 ℃ in the sa mple cartridge until the PCR was carried out.

The qRRT-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). Analysis was performed with the LC software. The viral RNA concentration ($TCID_{50}$ equivalents/ 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 a high template amount, while a high Cp indicated a low template amount.

Statistical analysis

Total virus excretion per secretion or excretion of individual pigs was expressed by the area under the 'excretion against time' curve. This represents the total amount of infectious virus (virus titration data) or viral RNA (qRRT-PCR data) excreted during the entire infectious period, standardised to 1 g/day. Furthermore, total amounts of faeces and urine produced each day were recorded, which allowed for the calculation of total amount of infectious virus (in $TCID_{50}$) and viral RNA (in $TCID_{50}$ equivalents) excreted via these routes per pig during the entire infectious period. Differences between the strains were statistically analysed using an analysis of variance (ANOVA) model. Pair wise comparisons between strains were made using the least significant difference (LSD) method. To compare secretions and excretions per strain, differences were calculated within animals and analysed with an ANOVA model comprising a factor for strains. Per strain, differences were analysed with a paired t-test, with a pooled estimate for the residual variance. Calculations were performed with the statistical programming language GenStat (2007). Mean differences were considered significant when the *p* value was less than 0.05.

Results

Clinical signs

Pigs infected with the Brescia strain showed severe clinical signs and high fever (>41℃) (Figure 1). Observed clinical signs were depr ession, anorexia, constipation and/or watery diarrhea, respiratory signs, haemorrhages on the skin, a staggering gait, and convulsions. Pigs were euthanized (being moribund) between days 8 and 12 p.i., when the body temperature decreased from higher than 41℃ to a level below 40℃, and peak clinical scores (CS) of 16-23 were observed.

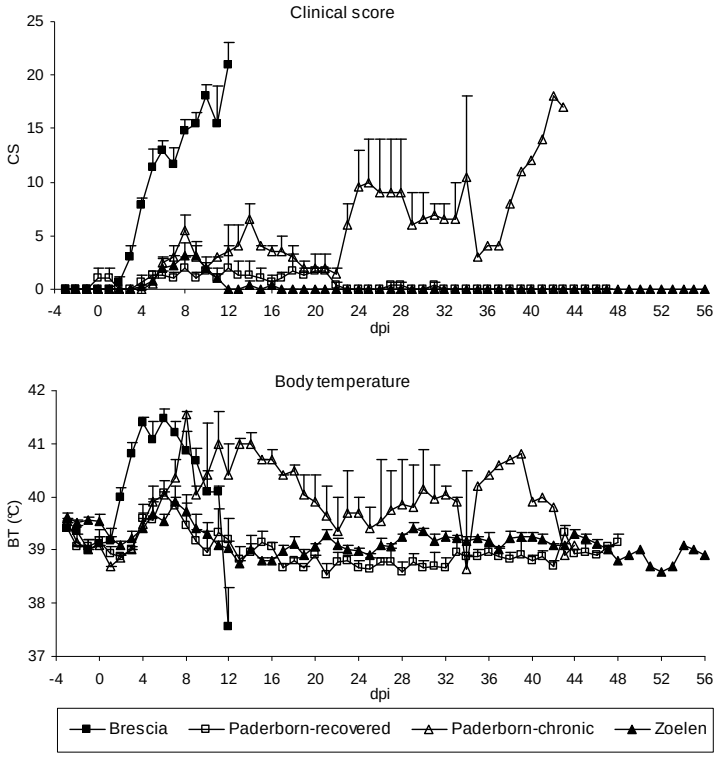

Figure 1. Clinical score (CS) values and body temperature (BT) of pigs infected with the Brescia strain (n=5), pigs infected with the Paderborn strain that recovered from the infection (n=3), pigs chronically infected with the Paderborn strain (n=2), or pigs infected with the Zoelen strain (n=5). The error bars represent the standard error of the mean (SEM). Dpi=days post-inoculation.

A wide distribution of clinical signs and fever was observed in the group of pigs infected with the Paderborn strain (Figure 1). Three pigs recovered from the infection (described as the “Paderborn-recovered” pigs), while two pigs became chronically infected (described as the “Paderborn chronically infected” pigs). Two of the three Paderborn-recovered pigs showed fever (maximum 40.4°C) and clinical signs. In these pigs lethargy, reduced appetite, stiff walking, and constipation were observed. The two chronically infected pigs showed fever for a long duration; one pig for 20 days between days 5 and 24 p.i., the other pig was intermittently feverish for 25 days between days 5 and 41 p.i. The first 3 to 5 weeks, clinical signs of limited severity were observed (depression, growth retardation, anorexia, constipation and respiratory signs), while in the terminal phase the clinical signs included petechia on the skin, a staggering gait, and weakness of the hind legs. They became moribund and were euthanized on days 34 and 44 p.i.

Of the five pigs that were infected with the Zoelen strain, two showed fever for a short duration (maximum 5 days between days 4 and 9 p.i.) and some mild clinical signs (Figure 1). The clinical signs observed were lethargy, a reduced appetite and dry faeces.

Leucocyte and thrombocyte count

The average leucocyte and thrombocyte levels during the study are shown in Figure 2.

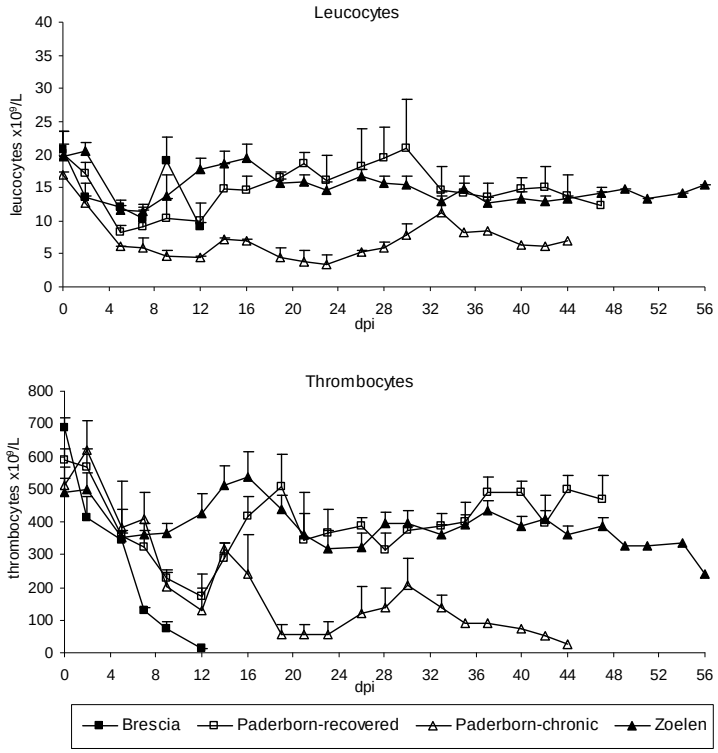

Figure 2. Leucocyte and thrombocyte counts of pigs infected with the Brescia strain (n=5), pigs infected with the Paderborn strain that recovered from the infection (n=3), pigs chronically infected with the Paderborn strain (n=2), or pigs infected with the Zoelen strain (n=5). The error bars represent the standard error of the mean (SEM). Dpi=days post-inoculation.

Pigs infected with the Brescia strain showed from day 2 p.i. a decrease in the level of leucocytes, although this decrease was only severe enough to be classified as a leucopenia in one pig at day 2 p.i., in three pigs at day 7 p.i., and in two pigs at day 12 p.i. The level decreased until day 9 p.i., when three of the four pigs showed an increase in the level of leucocytes of 8-19 x 10^9 cells/L blood compared to day 8. This might

have been due to dehydration of pigs and subsequent hypovolemia, or to secondary bacterial infections. Thrombocytopenia was observed from day 7 p.i. to death.

Pigs infected with the Paderborn strain that recovered from the infection exhibited leucopenia between days 5 and 12 p.i. Thrombocytopenia was observed in two of the three pigs between days 9 and 14 p.i. The chronically infected pigs showed leucopenia and thrombocytopenia during almost the entire study period of 34 and 44 days.

By day 5 p.i., in all pigs infected with the Zoelen strain, the level of leucocytes had decreased by 2-13 x 10^9 cells/L blood compared to day 0. In one pig the decrease in leucocytes was severe enough to be classified as leucopenia (at day 9 p.i.). Two of the five pigs showed thrombocytopenia at day 5 p.i. The other three pigs showed a small decrease in thrombocytes to a minimum level of 342 x 10^9 cells/L blood between days 7 and 12 p.i.

Virus titres in blood, secretions and excretions

Infectious virus and viral RNA were detected in blood and all secretions and excretions of pigs infected with the Brescia strain (Figures 3 and 4). Viral RNA was detected in most secretions and excretions 2 days before the infectious virus could be detected, and peak titres of viral RNA were $10^{0.9}$-$10^{2.7}$ $TCID_{50}$ equivalents/g or ml higher than infectious virus titres (Figure 4). Infectious virus was detected in skin scrapings from two pigs on day 12 p.i. (average concentration of $10^{1.9}$ $TCID_{50}/cm^2$ of skin), while viral RNA was detected in all skin scrapings obtained between days 9 and 12 p.i (average concentration of $10^{2.6}$ $TCID_{50}$ equivalents/cm^2 of skin).

Large differences in virus titres were observed between pigs infected with the Paderborn strain. The two pigs that developed the chronic form of infection had high titres of virus in blood and all secretions and excretions until death, while in samples from the three pigs that recovered from the infection, lower titres were detected for maximum 20 days (Figure 3). Virus was also detected at an earlier sampling moment in most secretions and excretions of the chronically infected pigs. Infectious virus was not detected in all sample types obtained from the pigs that recovered from the infection. It could be detected in the urine of only one pig, although viral RNA was detected in the urine of all three pigs. Viral RNA titres were higher (maximum $10^{3.8}$ $TCID_{50}$ equivalents/g or ml) than the infectious virus titres, and viral RNA could still be detected late (days 42-44 p.i.) in the infection in whole blood, oropharyngeal fluid and saliva (Figure 4). No infectious virus or viral RNA could be detected in skin scrapings from pigs that recovered at days 13 and 15 p.i. In the skin scrapings of the chronically infected pigs, viral RNA could be isolated at day 33 p.i (average concentration of $10^{2.9}$ $TCID_{50}$ equivalents/cm^2 of skin).

Infectious virus from pigs infected with the Zoelen strain was mainly detected in blood and oronasal secretions (Figure 3). No infectious virus was detected in faeces, urine or skin scrapings (taken at day 13 p.i.). Viral RNA was, however, detectable in all secretions and excretions. Furthermore, blood, oropharyngeal fluid and saliva contained virus until late in the infection (maximum 54 days p.i.) (Figure 4).

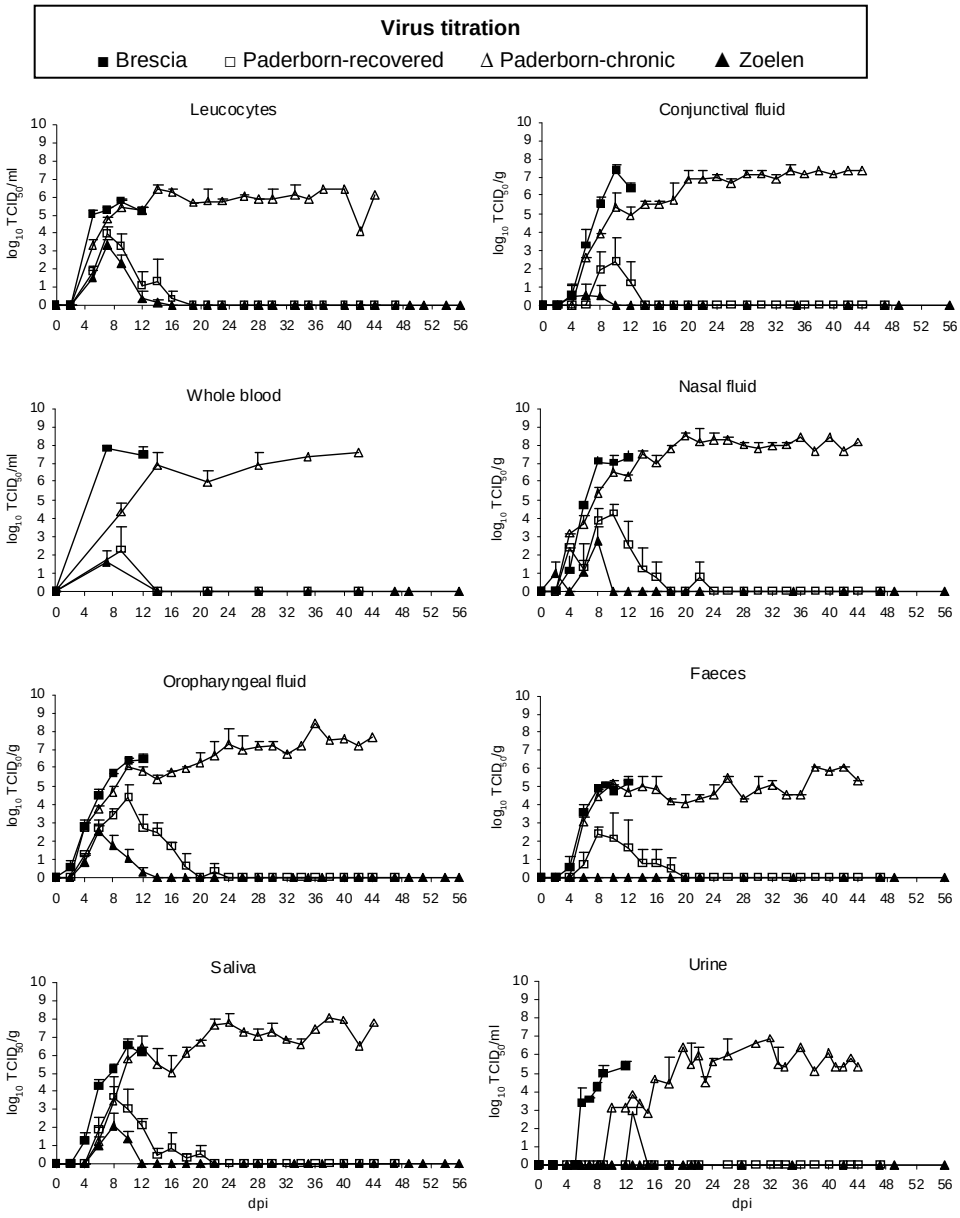

Figure 3. Infectious virus titres in eight different sample types, obtained from pigs infected with the Brescia strain (n=5), pigs infected with the Paderborn strain that recovered from the infection (n=3), pigs chronically infected with the Paderborn strain (n=2), or pigs infected with the Zoelen strain (n=5). The error bars represent the standard error of the mean (SEM). Dpi=days post-inoculation.

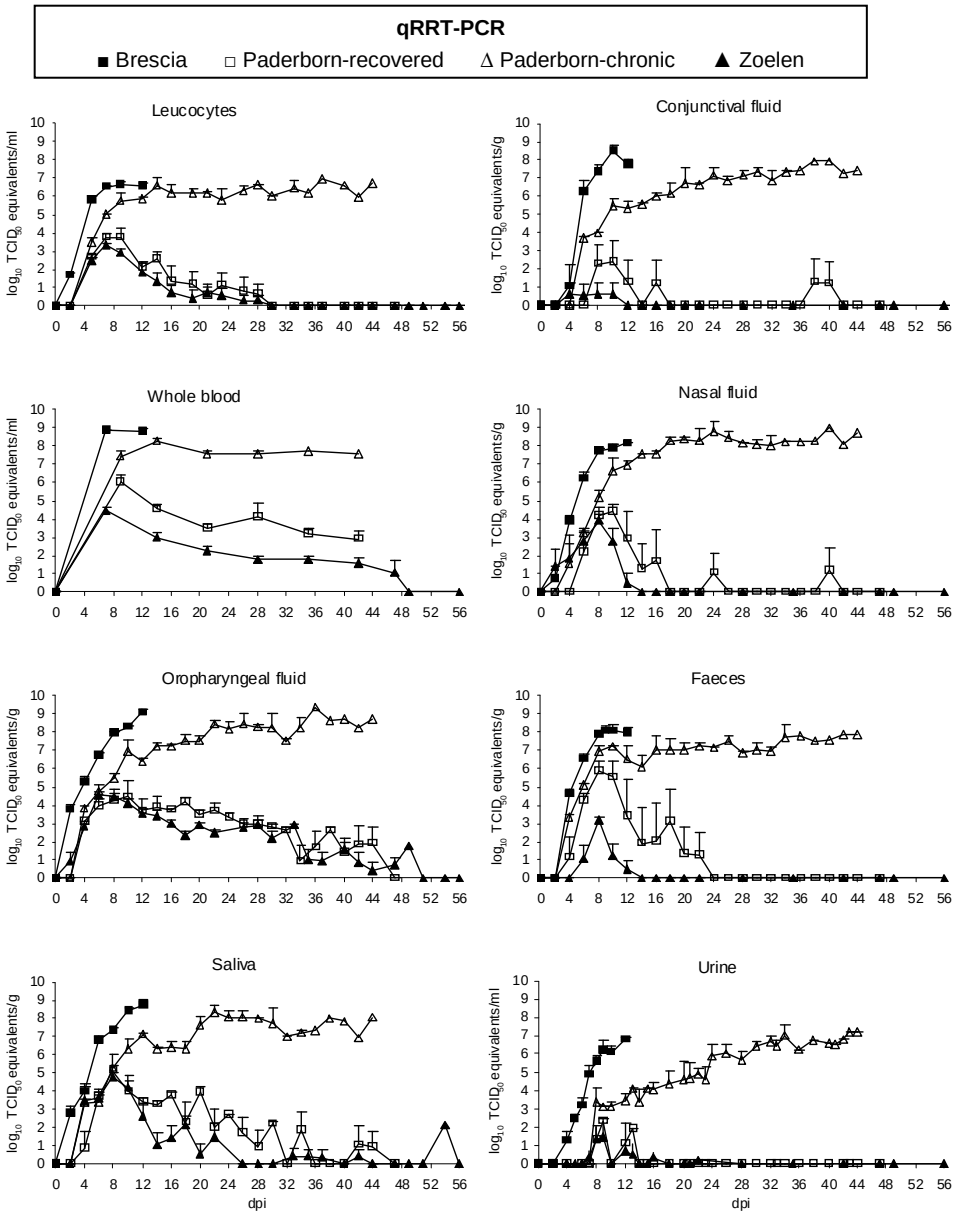

Figure 4. Viral RNA titres in eight different sample types, obtained from pigs infected with the Brescia strain (n=5), pigs infected with the Paderborn strain that recovered from the infection (n=3), pigs chronically infected with the Paderborn strain (n=2), or pigs infected with the Zoelen strain (n=5). The error bars represent the standard error of the mean (SEM). Dpi=days post-inoculation.

Actual virus excretion during the entire infectious period in faeces and urine

The total amounts of produced faeces and urine was recorded. Therefore, it was possible to calculate the actual amount of excreted virus via faeces and urine into the environment during the entire infectious period (Table 1).

Pigs infected with the Brescia strain and the chronically infected pigs (Paderborn strain) excreted the largest amounts of infectious virus and viral RNA via faeces and urine into the environment compared to the other groups (Table 1). However, at moments that these pigs showed distinct clinical symptoms of CSF (CS equal to or greater than 8), they produced less faeces (on average 154 g) and urine (on average 567 ml) than pigs with few clinical symptoms or subclinical illness (on average 442 g faeces and 1530 ml urine). Mainly during the terminal phase of the disease, a low production of these excretions was observed. Despite this lower production, virus titres in faeces and urine of these pigs were so high, that large amounts of virus were excreted via these routes. The excretion from pigs infected with the Paderborn strain that recovered from the infection was significantly lower. Excretion in faeces and urine from pigs infected with the Zoelen strain was only detectable by qRRT-PCR.

Table 1. Total amount of excreted infectious virus (quantified by virus titration [VT]) and viral RNA (quantified by qRRT-PCR) during the entire infectious period in all produced faeces and urine

	Mean total excretion (log_{10} $TCID_{50}$) analysed by VT				Mean total excretion (log_{10} $TCID_{50}$ equiv.) analysed by qRRT-PCR			
	Faeces		Urine		Faeces		Urine	
Virus strain	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Brescia	$7.26^{a,1,2}$	0.66	$7.84^{a,1}$	0.75	$10.3^{a,1}$	0.32	$9.44^{a,1}$	0.85
Paderborn-chronic	$8.93^{a,1}$	0.57	$10.3^{a,1}$	0.41	$11.1^{a,1}$	0.38	$10.7^{a,1}$	0.16
Paderborn-recovered	$6.04^{a,2}$	1.78	$1.95^{b,2}$	3.38	$8.92^{a,2}$	1.28	$5.60^{b,2}$	0.89
Zoelen	0.00*	0.00	0.00*	0.00	$6.41^{a,3}$	0.22	$3.08^{b,2}$	2.89

$^{a-b}$ Means within rows with no common superscript differ significantly (p<0.05)

$^{1-3}$ Means within columns with no common superscript differ significantly (p<0.05). Only excretions analysed by the same assay are compared.

* Differences cannot be computed as standard deviations of both groups are 0.

Standardised virus excretion during the entire infectious period

The exact amounts of oropharyngeal fluid, saliva, conjunctival fluid and nasal fluid that would end up in the environment could not be determined. Therefore, it was not possible to calculate actual amounts of virus excreted into the environment. Instead, amounts of virus excreted are represented in $TCID_{50}$ or $TCID_{50}$ equivalents standardised to 1 g or ml of secretion or excretion per day (Tables 2 and 3). This allowed comparisons among the different routes. Excretion of virus in faeces and urine was also calculated this way, to enable the comparison with other secretions and excretions.

It was shown that pigs which developed the chronic form of infection after inoculation with the Paderborn strain excreted the largest amounts of virus via most secretions and excretions during the entire infectious period compared to the other

strains (Tables 2 and 3). These pigs had high titres of virus (infectious virus up to an average of $10^{8.5}$ $TCID_{50}$/g) in their secretions and excretions for a long duration (32-42 days) (Figures 3 and 4). Although Brescia-infected pigs had also high virus titres in their secretions and excretions (infectious virus up to an average of $10^{7.4}$ $TCID_{50}$/g), they died within 2 weeks after inoculation, making the amounts of virus excreted equal to or lower than those from pigs chronically infected with the Paderborn strain. Pigs infected with the Paderborn strain that recovered from the infection excreted significantly smaller amounts of virus during their entire infectious period. These pigs had lower virus titres (infectious virus up to an average of $10^{4.4}$ $TCID_{50}$/g) in their secretions and excretions, for a shorter duration (maximum 9 days). Pigs infected with the Zoelen strain excreted the smallest amounts of virus during their entire infectious period, although they were not always significantly different from the Paderborn-recovered pigs.

In general, more infectious virus was excreted during the entire infectious period via the oronasal and conjunctival route than via faeces and urine, although there are some differences between the strains (Table 2). After infection with the low virulent Zoelen strain, no infectious virus was detected in faeces and urine. Furthermore, from only one pig that recovered from the infection with the Paderborn strain, infectious virus was detected in the urine.

Table 2. Total amount of infectious virus, quantified by virus titration, excreted during the entire infectious period, with the amount of secretion/excretion standardised to 1 g/day or 1 ml/day (urine)

Virus strain	Mean total excretion (log_{10} $TCID_{50}$ standardised to 1 g or ml/day) analysed by virus titration*											
	Oropharyngeal fluid		Saliva		Conjunctival fluid		Nasal fluid		Faeces		Urine	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Brescia	$6.54^{ab,2}$	0.47	$6.29^{bc,2}$	0.85	$7.09^{ab,1}$	0.89	$7.60^{a,1}$	0.59	$5.44^{cd,1}$	0.57	$5.08^{d,2}$	0.74
Paderborn -chronic	$8.50^{ab,1}$	0.82	$8.68^{ab,1}$	0.57	$8.50^{ab,1}$	0.00	$9.54^{a,1}$	0.32	$6.61^{c,1}$	0.54	$7.28^{bc,1}$	0.48
-recovered	$4.65^{a,3}$	1.06	$4.08^{ab,3}$	1.04	$3.46^{ab,2}$	0.94	$4.66^{a,2}$	1.02	$3.49^{b,2}$	1.72	$0.98^{c,3}$	1.70
Zoelen	$2.92^{a,4}$	0.78	$2.61^{ab,3}$	1.34	$1.19^{b,3}$	1.65	$2.89^{ab,2}$	1.74	0.00**	0.00	0.00**	0.00

$^{a-d}$ Means within rows with no common superscript differ significantly ($p<0.05$).
$^{1-4}$ Means within columns with no common superscript differ significantly ($p<0.05$).
* This is equal to the area under the curves presented in Figure 3.
** Differences cannot be computed as standard deviations of both groups are 0.

The high level of viral RNA excretion via faeces, which is for most strains as high as viral RNA excretion via the oronasal route (Table 3), is remarkable. The excretion of viral RNA via conjunctival fluid differed between strains. Pigs infected with the highly virulent Brescia strain and chronically infected pigs excreted via this excretion route amounts comparable to the oronasal route, while pigs infected with the low virulent strain excreted significantly lower amounts than via the oronasal route. Significantly

smaller amounts of viral RNA were excreted via the urine during the total infectious period (except for the chronically infected pigs).

Table 3. Total amount of viral RNA, quantified by qRRT-PCR, excreted during the entire infectious period, with the amount of secretion/excretion standardised to 1 g/day or 1 ml/day (urine)

Virus strain	Mean total excretion (log_{10} $TCID_{50}$ equiv. standardised to 1 g or ml/day) analysed by qRRT-PCR*											
	Oropharyngeal fluid		Saliva		Conjunctival fluid		Nasal fluid		Faeces		Urine	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Brescia	$8.76^{a,1}$	0.51	$8.47^{a,1}$	0.69	$8.72^{a,1}$	0.33	$8.31^{a,1}$	0.37	$8.59^{a,1}$	0.27	$6.70^{b,1}$	0.85
Paderborn -chronic	$9.66^{a,1}$	0.47	$9.22^{ab,1}$	0.37	$8.56^{ab,1}$	0.08	$9.79^{a,1}$	0.25	$8.89^{ab,1}$	0.21	$7.90^{b,1}$	0.00
-recovered	$5.94^{ab,2}$	0.98	$5.53^{bc,2}$	0.58	$4.36^{c,2}$	0.35	$5.35^{bc,2}$	1.03	$6.42^{a,2}$	1.24	$2.68^{d,2}$	0.70
Zoelen	$5.33^{a,2}$	0.53	$5.27^{a,2}$	0.37	$1.98^{d,3}$	1.81	$4.4^{ab,3}$	0.52	$3.50^{c,3}$	0.25	$1.39^{d,2}$	1.33

$^{a-d}$ Means within rows with no common superscript differ significantly ($p<0.05$).
$^{1-3}$ Means within columns with no common superscript differ significantly ($p<0.05$).
* This is equal to the area under the curves presented in Figure 4.

Discussion

In this paper, the dynamics of virus excretion by CSFV infected pigs during the entire infectious period were determined after infection with a highly, moderately or low virulent strain. Distinct differences between the strains were observed in virus excretion. Overall, virus excretion after infections with highly virulent strains can be expected to be much higher than after an infection with a moderately virulent strain, even though the infectious period may be shorter because of a relatively quick death. In our study this was up to 500 times as much, based on standardised amounts of secretions/excretions. Infections with moderately virulent strains in turn can be expected to result in a higher overall excretion of virus than infections with a low virulent strain. In our study this was up to 50 times as much. The most striking observation was however the amount of virus excreted by pigs that developed the chronic form of infection after inoculation with the moderately virulent Paderborn strain. Due to a combination of high virus titres in all excretions and secretions, and the long infectious period, they excreted up to 40,000 times more virus than acutely infected and recovered pigs did.

The chronically infected pigs showed a persistent viraemia and high virus titres in all secretions and excretions. Already in the first phase of infection, virus titres were higher and could be detected earlier than in pigs infected with the same strain, but that recovered from the infection. Relatively few clinical symptoms were observed during this first phase, that lasted from day 5 p.i. until the terminal phase (6-10 days before death). During the terminal phase, clinical symptoms increased markedly until the pigs died. The persistence of chronic CSF in the host's body during its entire lifetime and resulting in persistent viraemia was described before (Carbrey et al., 1980; Depner et

al., 1996; Moennig et al., 2003). However, the chronic form was also described as having three phases, based on severity of clinical symptoms and viraemia (Mengeling and Packer, 1969). In the first phase the spread of virus through the body resembled that in acute CSF, but was slower and virus titres in serum and organs tended to be lower. This was followed by a phase with partial clinical recovery in which virus titres were low or absent. In the terminal phase, virus spread again throughout the body with exacerbation of clinical symptoms. The observations in the present study do not confirm these observations of Mengeling and Packer (1969). Instead of the slower spread of the virus throughout the pig's body in the first phase and the lower virus titres, we observed a faster spread with higher virus titres. Also during the rest of the pig's life, titres remained higher and no phase of apparent recovery was seen. A similarity to the chronically infected pigs described by Mengeling and Packer (1969) is the persistent leucopenia during the first and second phase of illness. However, in the terminal phase the pigs studied by Mengeling and Packer developed a leucocytosis, which may reflect a response to secondary bacterial infections. In the present study, this leucocytosis was not seen, but there was also no indication that secondary bacterial infections were involved.

Development of the chronic form might depend on age, breed and immune status (Depner et al., 1997; Moennig et al., 2003). Moreover, the inoculation dose or the route of inoculation could influence this form of disease. In the present study young pigs with an undeveloped immune system were used, which might have resulted in the high frequency (40%) of chronic infection. In a recent study, using 8-week-old pigs infected with the Paderborn strain, chronic infection at a rate of only 10% was observed (Wieringa-Jelsma et al., 2006). Other moderately virulent strains are also known to induce the chronic form of CSF. For example, the moderately virulent strain 311 used by Mengeling and Cheville (1968) to infect 69 pigs, resulted in 45% of these becoming chronically infected. During virulence typing of 135 field isolates in the United States by intramuscular inoculation, 6% produced chronically infected pigs (i.e. high concentration of CSFV in the blood for a long duration and relatively free of illness). According to the authors (Carbrey et al., 1980), this was a reasonable indicator of the frequency of this event in the field. Reliable data on the frequency of chronic infections in the field is however unavailable. The prevalence of the chronic form of infection during the 1997/1998 outbreak of the Paderborn strain in the Netherlands was unknown because herds were culled immediately after infection was detected. It is questionable whether the occurrence was comparable to the present study, since pigs of different age groups and immune statuses were infected, with different doses, and via different routes. However, since chronically infected pigs excrete large amounts of virus they may have played a crucial role in spreading the virus.

The quantitative excretion data provided by this study gives some insight into the role the different secretions and excretions play in transmission. This role depends on the total amount of virus-containing secretions and excretions produced during the infection and the virus titres herein, which in turn is influenced by the virulence of the virus strain. During outbreaks caused by highly and moderately virulent strains, large amounts of virus (up to $10^{9.9}$ $TCID_{50}$ of infectious virus/per pig/day) can be excreted via

faeces and urine. Saliva and nasal fluid are expected to be excreted in the environment in smaller amounts than faeces and urine, but as virus excretion is up to 1300 times (saliva) to 5000 times (nasal fluid) higher than in faeces and urine, their contribution to transmission might be equal or even higher. These secretions and excretions together may be responsible for a major part to the transmission between- and within pens, and even between farms, as they are easily transmitted via contaminated boots, clothes or trucks. Virus excretion in conjunctival fluid is up to 300 times higher than in faeces and urine. However, this is expected to be excreted in much smaller amounts in the environment, and therefore its contribution might be limited.

The difference in virus excretion between the strains can be the result of differences in the spread of the virus throughout the pig's body. Highly virulent virus usually spreads rapidly throughout the body, resulting in high virus titres in most organs and blood, whereas with moderately virulent strains, virus titres tend to be lower. Infections with the low virulent strains result in a slow spread, with lower virus titres, mostly restricted to certain organs like the tonsil and lymphatic organs (Terpstra, 1991; Kamolsiriprichaiporn et al., 1992). As a consequence, there is a not only a difference between the strains in the first moment of excretion and the quantities of virus excreted, but also in the type of secretions or excretions that contain virus. While pigs infected with the highly virulent strain excreted virus in all secretions and excretions, virus excretion from pigs infected with the low virulent strains was mainly restricted to the oronasal excretion routes.

Virus titration and qRRT-PCR were used to determine the concentration of virus in the different secretions and excretions. It was shown that viral RNA was detected earlier and for a longer duration by qRRT-PCR than infectious virus by virus titration, which could be due to the higher sensitivity of the qRRT-PCR (Dewulf et al., 2004; Van Rijn et al., 2004). Furthermore, viral RNA titres were higher than infectious virus titres. When neutralizing antibodies are present, the virus-antibody complexes may mask infectivity in the cell culture assay. This could also explain why high viral RNA titres were detected late in the infection in blood, when infectious virus could no longer be detected. Moreover, lower infectious virus titres could be related to the toxic effect on the monolayer of SK6 cells of some of the secretions and excretions, enzymes in these samples that inhibit replication of the virus or inactivation of the virus before testing.

Proportionate and risk-based control eradication measures can be applied only if sufficient knowledge is available on virus transmission between animals and herds. To interpret and interpolate transmission data from experimental infections and outbreaks in the field, knowledge of the underlying mechanisms of transmission is needed, including quantitative excretion data as provided in this study. Based on this study, one could argue that outbreaks caused by highly or moderately virulent strains that result in a proportion of chronically infected pigs, spread faster in a population than outbreaks caused by low virulent strains. Infections caused by highly virulent strains result in large amounts of virus excreted, but the duration of virus excretion is short as most pigs die shortly after infection. Furthermore, infected pigs are relatively easy to recognize. On the other hand, during outbreaks caused by low virulent strains, less virus is excreted, and the absence of infectious virus in faeces and urine may contribute to a slower

spread of the disease. However, as clinical signs are almost absent, infected pigs are difficult to recognize and thus can act as a source of virus dissemination. With the current data on excretion dynamics, it would be interesting to study the reproduction ratio (R_0) of these strains in a transmission experiment. A correlation between virus excretion and reproduction ratio is expected. Furthermore, studies like that could give initial indications on the possible role of additional underlying mechanisms that include virus survival, contact structure and susceptibility of the recipient pigs.

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Chapter 3

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.

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

Materials & 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 [Van Oirschot, 1980]). 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%) and 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 s 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, 10 ml of demineralised water were aerosolized in 120 s 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 four-fold), 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 l 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:

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

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:

$$C_0 = C_e \times \left(\frac{V_0 + V_e}{V_0} \right) \qquad (2)$$

Co=original concentration, *Ce*=end concentration; concentration in the air sample obtained by virus titration, *Vo*=original volume of the isolator (924 l), *Ve*=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℃). After placing the Petri dishes for 5 min at 37℃ 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 8-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 milliliter 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 (p.i.), the expiration air was sampled from pigs infected with the Brescia strain, at day 9, 13, 15 and 33 p.i. from the pigs infected with the Paderborn strain, and at day 7 and 13 p.i. 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 2-5 m^3/h during 2 to 5 min. Air speed and sampling time were recorded. At day 5 p.i., cage air was sampled from pigs infected with the Brescia strain, and at days 9, 13 and 15 p.i. 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℃. 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 x *g* for 15 min) the samples were stored at -70℃. Leucocytes were isolated from EDTA-st abilized blood by adding 2 ml of EDTA blood to 4 ml 0.84% NH_4CL solution. After 10 min the samples were centrifuged at 1000 *x g* and washed twice with PBS. The pellet was resuspended in 2 ml medium and stored at -70℃.

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-well plate (Greiner) at 37℃ in an atmosphere with 5% CO_2 for 4 days. A volume of 250 µl of oropharyngeal fluid was incubated for 1 h on a monolayer of SK6 cells. After a wash procedure, medium was added to the wells and incubated for 4 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 four-fold 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℃ 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 ($TCID_{50}/m^3$) represented by Cp values. Filters were dissolved in 5 ml of medium and a twofold ($10^{-0.3}$) and ten-fold (10^{-1} to 10^{-5}) dilutions were prepared from the Brescia, Paderborn and Zoelen strain. Samples were stored at -70℃ 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 (Tables 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 30 min after inoculation resulted in a very small reduction in the VI titers compared to processing 5 min 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).

Table 1. Effect of the gelatine filters on the viability of CSFV determined by virus titration

	Treatment		Titer ($\log_{10}$ $TCID_{50}$/ml)[a]	
Virus strain	Volume (ml)[b]	Time (min)[c]	Filter solution	Stock solution
Brescia	50	30	3.60	3.85
	50	5	3.88	3.63
	5	5	4.85	4.60
Paderborn	50	30	3.35	3.60
	50	5	3.50	3.75
	5	5	4.85	4.60
Zoelen	50	30	4.38	4.75
	50	5	4.88	4.75
	5	5	5.13	5.38

Stock solutions (positive controls) were treated according to the same protocol as the filter solutions.

[a] Based on control samples, always included in the virus titration, the standard deviation (SD) of the virus titration technique is 0.43 $TCID_{50}$. This SD was calculated from 15 virus titration 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.

Table 2. Effect of the gelatine filters on the viability of CSFV determined by RRT-PCR

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

Stock solutions (positive controls) were treated according to the same protocol as the filter solutions.

[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 ten-fold dilution of the samples were tested in the RRT-PCR.

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).

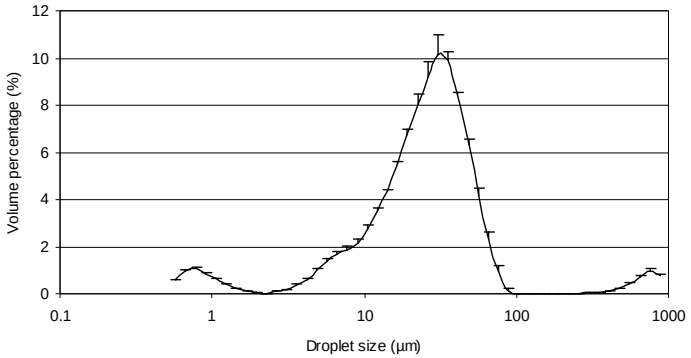

Figure 1. The average droplet size distribution with standard deviations of three aerosol spectra generated by a Walther Pilot I spray-head.

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).

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

	Brescia				Paderborn				Z oelen			
	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

[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 protocols (Tables 3 and 4).

[b] The presented half-life time values at *t* 15 min and *t* 30 min were both calculated compared to *t* 0.

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 30 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.

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

	Brescia				Paderborn				Z oelen			
	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

[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 protocols (Tables 3 and 4).

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

Clinical signs and virus isolation on oropharyngeal swabs and leucocytes

Pigs infected with the Brescia strain showed severe clinical signs and fever (>40℃) 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 p.i.), 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 p.i., fever was detected in pig number 10, and clinical signs in all animals except pig number 8. At days 13 and 15 p.i., fever was detected in pigs 6 and 10, and clinical signs in pigs 6, 9 and 10. At day 33 p.i., 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 p.i. (Table 6). Pigs 6 and 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 p.i.) than in the oropharyngeal swabs from the other three pigs (on average $10^{2.9}$$TCID_{50}$/gr between days 8 and 16 p.i.).

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

Table 5. Virus excretion of pigs infected with the Brescia strain, determined by VI, virus titration and RRT-PCR

	Virus isolation / titration ($log_{10}TCID_{50}/m^3$)									RRT-PCR (average Cp-value)			Calculated virus titration titers[a] ($log_{10}TCID_{50}/m^3$)		
	Oropharyngeal swabs			Leucocytes			Expired air		Cage air	Expired air		Cage air	Expired air		Cage air
Pig #	4 dpi	6 dpi	8 dpi	5 dpi	7 dpi	9 dpi	6 dpi	8 dpi	5 dpi	6 dpi	8 dpi	5 dpi	6 dpi	8 dpi	5 dpi
1	+	+	+	+	+	nd	-	-	-	-*	33.9[b]	36.1[c]	-	2.2	0.3
2	+	+	+	+	+	+	-	3.5	-	-*	29.9[d]	35.6[e]	-	4.0	0.6
3	+	+	+	+	+	+	-	-	-	-*	34.8[c]	-*	-	0.7	-
4	+	+	+	+	+	+	-	-	-	-*	34.1[b]	34.0[d]	-	2.1	1.6
5	+	+	+	+	+	+	-	-	-	-*	34.4[b]	-*	-	2.1	-

[a] 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 tested in the RRT-PCR.
[b] Three samples RRT-PCR positive out of four samples tested.
[c] One sample RRT-PCR positive out of four samples tested.
[d] Four samples RRT-PCR positive out of four samples tested.
[e] Two samples RRT-PCR positive out of four samples tested.
Dpi Days post-inocluation.
* Cp value >40, which is considered negative.
Nd Not done because of death.

Table 6. Virus excretion of pigs infected with the Paderborn strain, determined by VI and RRT-PCR

	Virus isolation															RRT-PCR (average Cp-value)							Calculated virus titration titers[a] ($log_{10}TCID_{50}/m^3$)						
	Oropharyngeal swabs				Leucocytes				Expired air				Cage air			Expired air				Cage air			Expired air				Cage air		
Pig #	8 dpi	12 dpi	16 dpi	32 dpi	9 dpi	12 dpi	16 dpi	33 dpi	9 dpi	13 dpi	15 dpi	33 dpi	9 dpi	13 dpi	15 dpi	9 dpi	13 dpi	15 dpi	33 dpi	9 dpi	1 3 dpi	15 dpi	9 dpi	13 dpi	15 dpi	33 dpi	9 dpi	13 dpi	15 dpi
6	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-*	-*	-*	35.3[b]	33.8[c]	36.2[d]	33.9[b]	-	-	-	2.0	0.9	0.3	1.4
7	+	+	+	-	+	+	+	-	-	-	-	nd	-	-	-	-*	-*	-*	nd	-*	-*	-*	-	-	-	nd	-	-	-
8	+	+	+	-	+	+	-	-	-	-	-	nd	-	-	-	-*	-*	-*	nd	-*	33.3[d]	-*	-	-	-	nd	-	0.5	-
9	+	+	+	-	+	+	+	-	-	-	-	nd	-	-	-	-*	-*	-*	nd	-*	-*	-*	-	-	-	nd	-	-	-
10	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-*	34.2[d]	-*	34.9[b]	35.7[c]	33.8[d]	34.2[c]	-	0.4	-	2.2	0.6	0.5	0.9

[a] 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 tested in the RRT-PCR.

[b] Three samples RRT-PCR positive out of four samples tested.

[c] Two samples RRT-PCR positive out of four samples tested.

[d] One sample RRT-PCR positive out of four samples tested.

Dpi Days post-inocluation.

* Cp value >40, which is considered negative.

Nd Not done because of death.

Table 7. Virus excretion of pigs infected with the Zoelen strain, determined by VI and RRT-PCR

	Virus isolation						RRT-PCR	
	Oropharyngeal swabs		Leucocytes		Expired air		Expired air	
Pig #	6 dpi	12 dpi	7 dpi	12 dpi	7 dpi	13 dpi	7 dpi	13 dpi
11	+	-	+	-	-	-	-*	-*
12	+	-	+	-	-	-	-*	-*
13	+	-	+	-	-	-	-*	-*
14	+	-	+	-	-	-	-*	-*
15	+	+	+	+	-	-	-*	-*

Dpi Days post-inoculation.
* Cp value >40, which is considered negative.

Virus isolation and RRT-PCR on air samples

Air samples from cages housing Brescia-infected pigs at day 5 p.i. resulted in three RRT-PCR positive samples. All pigs had RRT-PCR positive expiration air samples at day 8 p.i. One air sample was also virus titration positive with a virus titer of 103.5 TCID50/m3 (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 p.i. One expiration air sample from an infected pig was RRT-PCR positive at day 13 and two at day 33 p.i. (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 $10^{0.3}$-$10^{1.6}$ $TCID_{50}/m^3$ for the Brescia-infected pigs, and titers of $10^{0.3}$-$10^{1.4}$ $TCID_{50}/m^3$ for the Paderborn-infected pigs (Tables 5 and 6). The expiration air samples from the infected pigs showed calculated virus titration titers of $10^{0.7}$-$10^{4.0}$ $TCID_{50}/m^3$ for the Brescia-infected pigs, and titers of $10^{0.4}$-$10^{2.2}$ $TCID_{50}/m^3$ for 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.

Discussion

This is the first study describing the successful detection of CSFV in air samples originating from infected pigs. Virus was detected in air samples representing cage air,

as well as expiration air. Furthermore, in artificially created aerosols, it was shown that the virus can remain infective for at least 30 min after aerosol generation with half-life time values ranging from 4.5 to 15 min. The air-sampling device and protocols used were suitable to enable the detection and quantification of the virus in mentioned air samples.

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

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

It has been shown previously that the RRT-PCR test is more sensitive than the VI technique (Van Rijn et al., 2004). This was confirmed in the present study as many air samples from the animal experiments, negative at VI, showed positive RRT-PCR results. Besides this higher sensitivity of the RRT-PCR, negative VI results could be due to loss of infectivity of virus particles. The sample taken from the expired air of pig 2 at day 8 p.i. was the only sample positive in both the virus titration and RRT-PCR assay. The calculated virus titre in $TCID_{50}$ from the RRT-PCR result was approximately 0.5 $\log_{10}$ $TCID_{50}$ higher than the titer that was determined in the virus titration assay. Therefore, the calculated titres of excretion rates based on PCR-results could be slightly overestimated.

On the other hand, shedding rates of virus in aerosols by infected pigs under field circumstances is expected to be higher than determined in this study. Faeces were collected separately and the isolation unit had a low pig density in combination with a high ventilation rate, so subsequently dust and aerosol production was low. To assess the risk of airborne transmission, further research is necessary under circumstances

mimicking the situation in the field, where the herein described air-sampling system seems a suitable tool to determine shedding rates.

The validation studies showed that the gelatine filter processing method did not have an adverse effect on the yield and viability of the virus. Even 30 min after inoculating the virus on the filter, which was the elapsed time between air sampling and processing in the aerosol experiments, no significant inactivation was observed.

Between the different aerosolization and sampling experiments in the isolator, the isolator was not decontaminated. However, it was ventilated at a high rate (1324 m^3/h) for 15 min, which will have reduced virus concentrations to a level far below the detection limit. This was confirmed by the negative virus titration and RRT-PCR results of air samples taken before the start of each experiment and directly after ventilation.

After generating virus-containing aerosols, these aerosols are subject to both biological and physical decay. Biological decay includes factors that affect the infectivity of the virus, while the physical decay depends on the time particles remain suspended which is influenced by particle size and particle deposition (Stärk, 1999). Directly after generating the aerosols, air samples were taken and the initial loss was determined. Beside the biological and physical loss, the initial loss is dependent on the ability of the air-sampling system to recover airborne particles. The initial losses were maximum $10^{1.66}$ $TCID_{50}/m^3$. In case the inability of the sampling system to recover the particles is totally responsible for 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 since 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. Since 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 9.27-15 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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Chapter 4

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

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

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 $10^{1.2}$-$10^{3.0}$ $TCID_{50}/m^3$ of infectious virus, and $10^{1.6}$-$10^{3.8}$ $TCID_{50}$ 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.

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., 2008). These air samples were collected from cages of individually housed pigs infected with a highly or a moderately virulent strain. Isolation of CSFV 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.

Materials & methods

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 h, 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.

Housing

Pigs were housed in rooms with a volume of 42-45 m^3 with a ventilation rate of 400 m^3/h. The rooms had an average temperature of 21℃ (± 0.7℃), and a relative humidity of 50% (± 10%).

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.

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 milliliter of the virus suspension was administered per animal, 0.5 ml per nostril. The inocula were back titrated to confirm the dose administered.

Clinical symptoms and body temperature

Body temperature and clinical symptoms were recorded daily. Fever was defined as body temperature higher than 40℃, for two or more c onsecutive 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.

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, 3-8, 10-15, 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 x *g* for 15 min) the samples were stored at -70℃ until they were analysed.

Faeces were collected at days 0, 3-8, 10-15, 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 x *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 m. 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 stored at -70℃ until they were analysed.

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., 2009).

Virus isolation and titration

From oropharyngeal fluid and faeces, a volume of 250 µl was incubated for 1 h on a monolayer of SK6 cells (permanent porcine kidney cell line) in a 24-well plate (Greiner) at 37℃ in an 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 4 days, the growth medium was discarded, and the monolayers were washed in a 0.15M NaCl solution, dried for 1 h at 37℃ and frozen for 1 h at -20℃. The monolayers were fixed with 4% paraformaldehyde in PBS (4℃) 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., 2008).

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/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.

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 x 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.

Results

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.

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℃, for 2 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.

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 contact pigs in 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 contact pigs 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.

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).

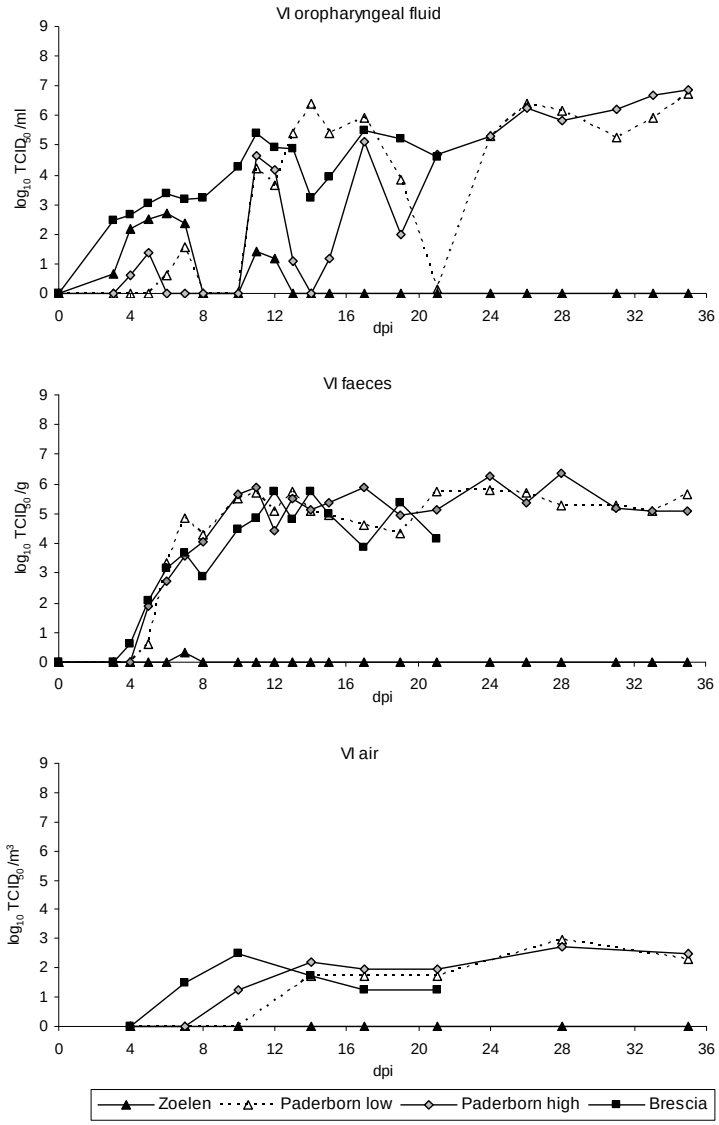

Figure 1. Average infectious virus excretion per pig in oropharyngeal fluid and faeces, as determined by virus isolation (VI) and titration, and infectious virus titres in air samples per room (dpi=days post-inoculation).

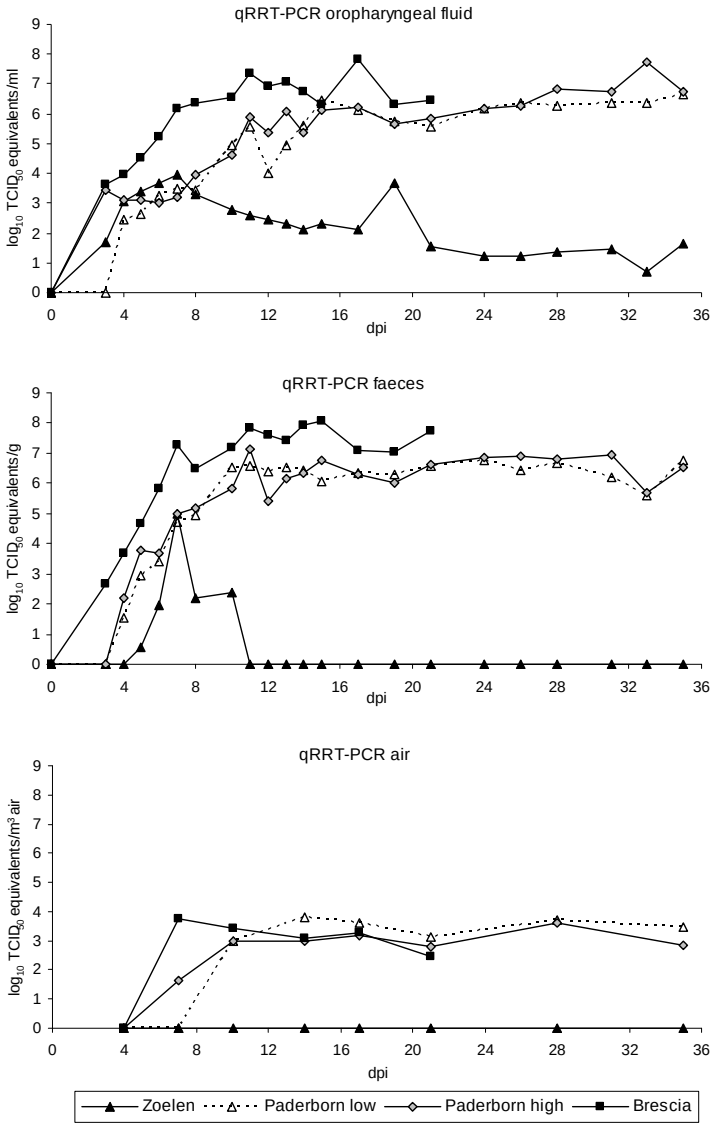

Figure 2. Average viral RNA excretion per pig in oropharyngeal fluid and faeces, as determined by quantitative real-time reverse transcription polymerase chain reaction (qRRT-PCR), and viral RNA titres in air samples per room (dpi=days post-inoculation).

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 and viral RNA were first detected between days 5 and 10 p.i., depending on sample type and test. The levels 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.

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 the room 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.

Table 2. Infectious virus titres in air samples originating from rooms housing pigs infected with different strains or different inoculation doses of the same strain, and average contribution per pig, based on the number of pigs excreting infectious virus

	Days post-inoculation							
Virus strain	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[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[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[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[b] ($TCID_{50}/m^3$)	-	1.0	1.5	1.0	0.7	0.9		

[a] OPF=oropharyngeal fluid.

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

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 inoculation doses of the same strain, and average contribution per pig, based on the number of pigs excreting viral RNA

	Days post-inoculation							
Virus strain	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[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[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[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[b] ($TCID_{50}$ equivalents/m^3)	-	2.8	2.4	2.4	2.8	2.2		

[a] OPF=oropharyngeal fluid.

[b] Based on the number (#) of pigs excreting viral RNA, which 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.

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 [*r*] 0.48, *p*=0.057) or faeces (*r*=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 (*r*=0.42, *p*=0.085) or faeces (*r*=0.31, *p*=0.21).

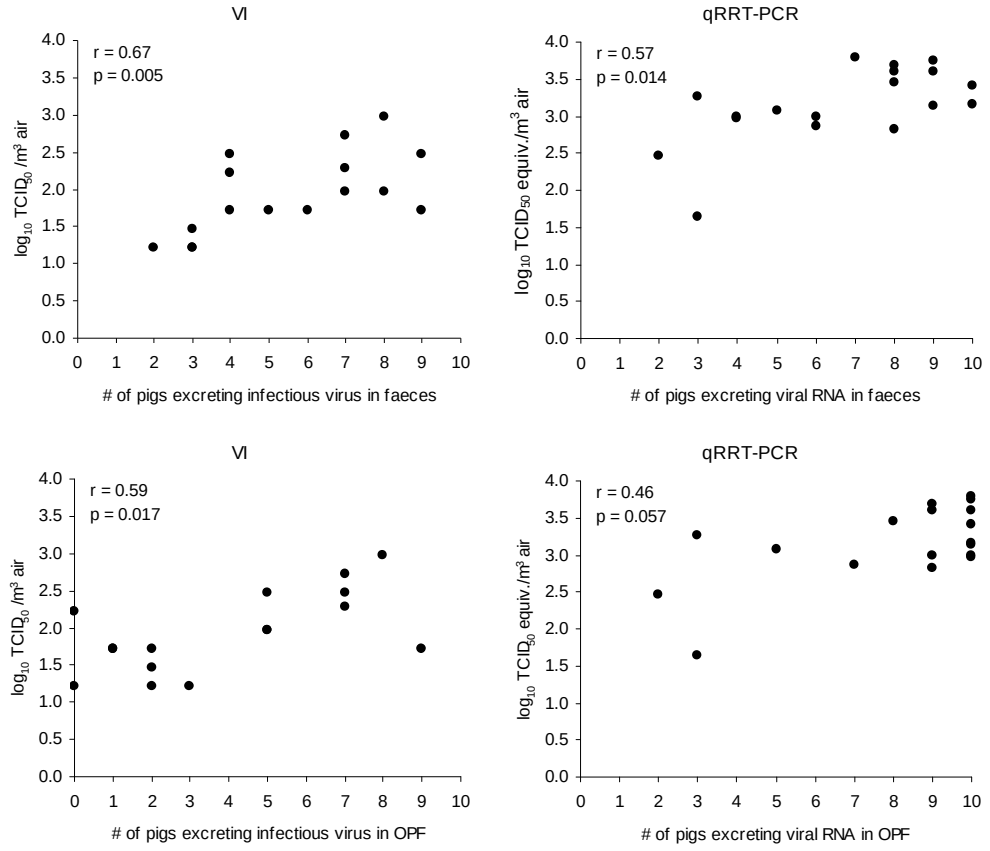

Figure 3. Relationship between virus concentration in the air, analysed by virus isolation (VI) or quantitative real-time reverse transcription polymerase chain reaction (qRRT-PCR), and the number (#) of pigs excreting infectious virus (analysed by VI) or viral RNA (analysed by qRRT-PCR) in faeces and oropharyngeal fluid (OPF) at the moments of air sampling. Negative air samples were excluded from the analysis. Spearman's rank correlation (*r*) was used for analysis of the data.

There was a significant relationship between infectious virus titre in the air and the number of pigs excreting infectious virus in faeces (r=0.67, p=0.005) or oropharyngeal fluid (r=0.59, p=0.017) (Figure 3). The relationship between viral RNA titre in the air and the number of pigs excreting viral RNA in faeces samples was also significant (r=0.57, p=0.014), but the relationship between viral RNA titre in the air and the number of pigs excreting viral RNA in oropharyngeal fluid was not significant (r=0.46, p=0.057).

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., 2008). 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., 2009). 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., 2008). 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., 2009). 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 h 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 the aerosols in this study 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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Chapter 5

Survival of classical swine fever virus at various temperatures in faeces and urine derived from experimentally infected pigs

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Survival of classical swine fever virus at various temperatures in faeces and urine derived from experimentally infected pigs

Abstract

Indirect transmission of classical swine fever virus (CSFV) can occur through contact with mechanical vectors, like clothing and footwear or transport vehicles, contaminated with the secretions or excretions of infected pigs. A prerequisite for indirect transmission is survival of the virus on the mechanical vector. Consequently, to obtain more insight into these transmission routes, it is important to know how long the virus remains viable outside the host. In this study we examined the survival of classical swine fever virus in faeces and urine derived from pigs intranasally inoculated with a highly or moderately virulent CSFV strain. Faeces and urine were collected between days 5 and 36 post-inoculation, and stored at 5, 12, 20 and 30°C. Next, the virus titres were determined in the samples by virus titration, and a random selection of these samples was also analysed by quantitative real-time reverse transcription polymerase chain reaction (qRRT-PCR) to determine the viral RNA decay. Survival curves were generated, and it was shown that the inactivation rate was inversely related to the storage temperature. Average half-life values were between 2 and 4 days at 5°C, and between 1 and 3 h at 30°C. Significant differences we re observed in survival between virus strains in faeces, however, not in urine. The reduction in viral RNA during the entire study period was limited. This study provided detailed information on survival of CSFV in excretions of infected pigs, which can be used to improve control measures or risk-analysis models.

Introduction

Classical swine fever (CSF) is a highly contagious viral disease that caused several large outbreaks in the European Union in the last 20 years (Koenen et al., 1996; Elbers et al., 1999; Fritzemeier et al., 2000; Sharpe et al., 2001). One of the most disastrous examples is the 1997-1998 outbreak in Germany, the Netherlands, Belgium, Spain and Italy. In the Netherlands alone, approximately 11 million pigs were killed, mainly for welfare reasons (Terpstra and De Smit, 2000). During this outbreak different transmission routes contributed to the spread of the disease. Direct animal contact was in 17% of the cases responsible for transmission between herds before implementing the first zoosanitary measures, including a total stand-still of transport of livestock (Elbers et al., 1999). Indirect transmission routes, however, played an important role in spread of the disease both before and after the implementation of movement restrictions (Elbers et al., 1999; Stegeman et al., 2002).

Indirect transmission can occur when susceptible pigs come into contact with mechanical vectors like clothing and footwear (Ribbens et al., 2007), or transport vehicles (Stegeman et al., 2002), contaminated with secretions or excretions of infected pigs. Saliva, nasal and lacrimal fluids, faeces, urine, and semen have been shown to contain significant amounts of classical swine fever virus (CSFV) (De Smit et al., 1999; Weesendorp et al., 2009). A prerequisite for the indirect transmission is, however, the survival of the virus on the mechanical vector. Consequently, it is important to know how long the virus remains viable outside the host.

The survival of the virus on a vector depends on different variables like the initial amount of virus, temperature, pH, humidity, presence of organic matter, exposure to various chemicals (Edwards, 2000), and most likely other factors including properties of the strain (Depner et al., 1992). Conflicting results have been observed in survival times of CSFV in the environment. After spiking slurry (a mixture of faeces and urine, which may also contain [cleaning] water, small quantities of bedding material and feed) with CSFV, it survived for at least 70 days at 17°C, and for 84 days at 4°C (Eizenberger et al., reviewed by Haas et al., 1995). However, Bøtner reported no detectable amounts of infectious virus in slurry stored at 20°C after 2 weeks (reviewed by Haas et al., 1995). Pens contaminated with secretions and excretions of infected pigs contained infectious virus for at least 10 h when the environmental temperature was around 22°C (Ribbens et al., 2004). Depending on the temperature, contaminated pens probably contain infectious virus for a few days before the virus within the pen and manure is totally inactivated (Artois et al., 2002). In certain housing systems where temperatures decrease during winter conditions, the survival time might be prolonged, and pens may contain virus in excreta and bedding for at least 4 weeks (Harkness, 1985).

These studies give useful information on survival times of CSFV in the environment, but details and conditions of the performed experiments were limited reported, or presented survival times were not specific, or only based on spiking of slurry. None of the studies on survival of CSFV in the environment contaminated with excretions or secretions provided sufficient information for the construction of survival curves. Concerning the importance of indirect transmission routes during outbreaks, specific information on virus survival is necessary (Edwards, 2000; De Vos et al., 2006). This can be used to model the risk of transmission via different transmission routes, which supports improvement of control measures.

In this report the survival of CSFV in faeces and urine from infected pigs was studied, as these excretions are produced in large amounts and are major sources of contaminating the immediate surroundings and mechanical vectors.

Materials & methods

Experimental animals and housing

Two experiments were performed in succession with 8-week-old male pigs, obtained from a conventional, but pestivirus free pig herd in the Netherlands. Pigs were individually housed in cages to collect faeces and urine. Pigs were fed once a day with commercial feed for finishing pigs, and water was provided ad libitum.

Viruses and inoculation of animals

Five pigs were inoculated intranasally with a dose of 100 LD_{50} (50% lethal dose), which is approximately $10^{2.5}$ $TCID_{50}$ (50% tissue culture infectious dose) of the highly virulent Brescia strain (genotype 1.2, strain Brescia 456610, obtained from Brescia, Italy, 1951 [Wensvoort et al., 1989]). Seven pigs were inoculated with 10^5 $TCID_{50}$ of the moderately virulent Paderborn strain (genotype 2.1, isolated in 1997 during the outbreak in the Paderborn area of Germany [Greiser-Wilke et al., 2000]). One milliliter of the virus suspension was administered per animal, 0.5 ml per nostril.

Sampling and pre-treatment of samples

Faeces and urine samples were collected from pigs infected with the Brescia strain from day 5 post-inoculation (p.i.), and from pigs inoculated with the Paderborn strain from day 10 p.i. The Brescia strain was previously isolated from faeces at day 6 p.i. (Ressang, 1973). The Paderborn strain was expected to be present in faeces and urine later, therefore no samples were collected before day 10 p.i. Samples were collected until day 12 p.i. from Brescia-infected animals (when the last one died) and until day 36 p.i. from Paderborn-infected animals, as long as they showed clinical symptoms. Samples that were negative from the start of the inactivation period were excluded from the analysis. Faeces were obtained from the rectum by stimulation of the anus, and urine was collected when pigs urinated. Directly after collection, the samples were stored in a refrigerator at 5℃ to avoid inactivation of the virus until samples were transported to the laboratory.

In the laboratory, samples were directly stored in 50 ml tubes in a refrigerator at 5℃, a thermostated water bath at 12℃, a thermostated room at 20℃, or a thermostated water bath at 30℃. Individual samples were used if enough faeces or urine could be obtained from one pig. In case not enough faeces or urine was available from one pig, samples originating from pigs infected with the same strain and on the same day p.i. were pooled. Samples were thoroughly mixed before storage. Depending on the temperature, samples were taken from the stored faeces and urine at different time intervals. These sampling moments were determined after a pilot study in which the rate of inactivation was studied on a small amount of samples. Because the pilot study showed that inactivation of virus occurs more rapidly in urine than in faeces at lower temperatures, urine samples were stored for a shorter duration at 5℃ and 12℃ than faeces samples. Faeces samples were stored for maximum 45 days at 5℃, 41 days at 12℃, and 5 days at 20℃ or 30℃. Urine samples were stored for maximum 27 days at 5℃, 15 days at 12℃, and 5 days at 20 or 30℃. The number of samples per strain-temperature combination that was ultimately tested, ranged from 5 to 10, depending on availability of faeces or urine, presence of virus in the initial sample, and (for Paderborn) inclusion of the results from the pilot study.

Faeces samples were diluted 1:10 in 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]) and vortexed with glass beads. After centrifugation (10,000 x *g* for 5 min) the supernatants were stored at -70℃ until

analysis. From urine a 1:10 dilution in medium (EMEM containing 10% FBS and 10% antibiotics) was prepared and stored at -70℃ until a nalysis. All samples were analysed by virus titration. Four randomly selected samples from both faeces and urine, and from each virus strain and each temperature, were analysed by quantitative real-time reverse transcription polymerase chain reaction (qRRT-PCR). Furthermore, the pH of two urine samples from each storage temperature was determined at each sampling moment.

Virus titration

Virus titration was performed as described by Weesendorp et al. (2008). Briefly, a volume of 250 µl of the sample was incubated for 1 h on a monolayer of SK6 cells in a 24-well plate (Greiner). After a wash procedure with PBS (phosphate-buffered saline), EMEM supplemented with 5% FBS and 10% antibiotics was added to the wells. The plates were incubated at 37℃ in an atmosphere with 5% CO_2 for 4 days. After being fixated and washed, the monolayers were stained by the immuno-peroxidase technique (Wensvoort et al., 1986), using two HRPO-conjugated CSFV specific MAbs (V3/V4), and examined for stained cells. Virus titres were calculated as $TCID_{50}$ using the Spearman-Kärber method (Finney, 1978). The detection limit with this method is $10^{1.1}$ $TCID_{50}$/ml of urine or gram faeces.

Quantitative real-time reverse transcription polymerase chain reaction (qRRT-PCR)

The concentration of viral RNA in the excretions was analysed by qRRT-PCR. For RNA isolation 200 µl of the sample were pipetted manually into MagNA Pure sample cartridges (Roche Applied Science, Mannheim, Germany). 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 by spiking faeces and urine with known concentrations of infectious virus from the Brescia or Paderborn strain. 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 RNA isolation, the nucleic acids were immediately processed for the qRRT-PCR or stored at -70 ℃ in the sa mple cartridge until the PCR was carried out.

The qRRT-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). Analysis was performed with the LC software. The viral RNA concentration ($TCID_{50}$ equivalents/ml or gram) 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 rises above the background signal. A low Cp value indicated high template amount, while a high Cp indicated a low template amount.

Statistical analysis

Per sample, the half-life (*h*) of CSFV in faeces or urine was estimated by:

$$h = -\frac{(\log_{10} 2)}{b} \quad (1)$$

where *b* is the least squares estimate of the slope of the regression of $\log_{10}$ CSFV over time for that sample. To study the effect of temperature on virus survival, the dependence of the log-transformed half-life value on temperature was modelled. Half-life values were log-transformed in order to ensure homogeneity of variance in the regression models that were used. The log-transformed half-life ln(h) was analysed as a new derived response variable with a linear mixed model (Searle et al., 1992). The model comprised random effects, to model correlation between samples from a common origin, main effects for strains, linear and quadratic effects for temperature and interaction between strains and temperature. Components of variance were estimated by restricted maximum likelihood (REML). Significance tests for interaction, slopes of linear and quadratic terms in time and main effects for strains were based on the Wald test (Cox and Hinkley, 1974). All calculations were performed with the statistical programming language GenStat (2007).

Results

Inactivation of infectious virus in faeces and urine derived from infected pigs

The mean half-life values for CSFV in faeces and urine derived from pigs infected with the highly virulent Brescia or moderately virulent Paderborn strain are shown in Tables 1 and 2.

Table 1. Survival of CSFV at different temperatures in faeces derived from pigs infected with the Brescia or Paderborn strain

Temperature (°C)	Virus strain	Mean half-life (days)	SEM[a]	Min[b]	Max[c]	*n*[d]
5	Brescia	3.50	0.58	2.41	5.69	5
	Paderborn	2.90	0.58	1.38	5.66	8
12	Brescia	2.69	0.81	0.99	6.40	6
	Paderborn	1.67	0.29	0.47	2.76	8
20	Brescia	0.28	0.05	0.14	0.42	6
	Paderborn	0.14	0.04	0.03	0.30	7
30	Brescia	0.06	0.01	0.04	0.09	6
	Paderborn	0.07	0.02	0.02	0.14	7

[a] SEM=standard error of the mean.
[b] Min=minimum half-life value observed.
[c] Max=maximum half-life value observed.
[d] *n*=number of samples tested.

The initial titres of infectious virus in faeces of pigs infected with the Brescia strain were $10^{2.6}$-$10^{5.4}$ $TCID_{50}$/g, and of pigs infected with the Paderborn strain $10^{3.6}$-$10^{5.4}$

$TCID_{50}$/g. Initial virus titres in urine of pigs infected with the Brescia strain were $10^{3.4}$-$10^{5.9}$ $TCID_{50}$/ml, and in urine of pigs infected with the Paderborn strain $10^{1.9}$-$10^{6.9}$ $TCID_{50}$/ml.

The pH of the urine samples remained 7 for the duration of the experiment. However, the urine became darker during the experiment and a granular precipitate developed on the bottom of the tube.

Table 2. Survival of CSFV at different temperatures in urine derived from pigs infected with the Brescia or Paderborn strain

Temperature (℃)	Virus strain	Mean half-life (days)	SEM[a]	Min[b]	Max[c]	*n*[d]
5	Brescia	2.82	2.25	0.39	11.81	5
	Paderborn	2.14	0.79	0.33	6.56	9
12	Brescia	0.54	0.25	0.18	1.54	5
	Paderborn	0.41	0.13	0.02	1.39	10
20	Brescia	0.19	0.11	0.06	0.61	5
	Paderborn	0.21	0.15	0.03	0.12	6
30	Brescia	0.12	0.08	0.03	0.41	5
	Paderborn	0.08	0.04	0.03	0.25	5

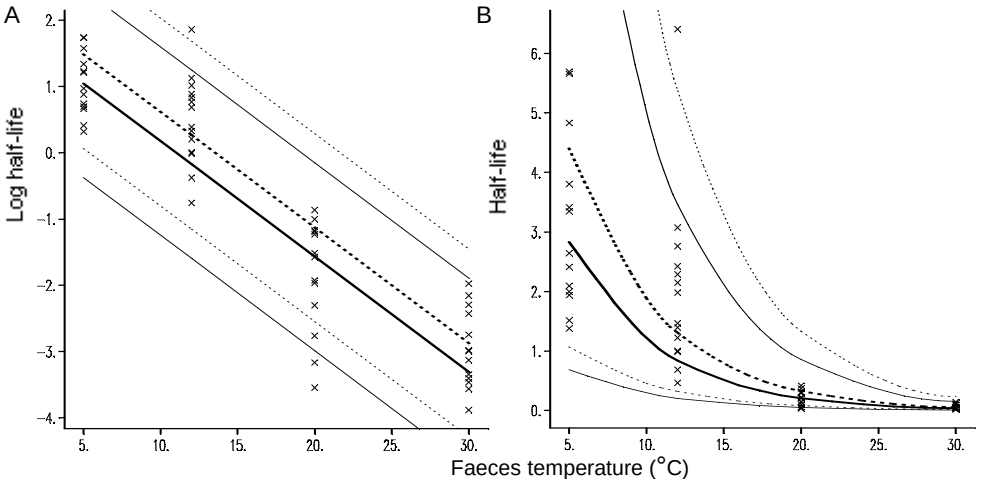
[a] SEM=standard error of the mean.
[b] Min=minimum half-life value observed.
[c] Max=maximum half-life value observed.
[d] *n*=number of samples tested.

Effect of temperature on the half-life values of the virus

The regression model comprised both linear and quadratic effects of temperature and interaction between strains and temperature. For faeces samples the quadratic effect and interaction between strains and temperature were not significant and subsequently omitted from the model, resulting in the linear relationship in Figure 1A. The Wald test showed for urine samples that the interaction between strains and temperature were not significant, but linear and quadratic effects were significant (p=0.001), which explains the parabolic shape in Figure 2A.

The analysis showed a significant difference (common slope but different intercepts on the logarithmic scale: p=0.03) between the Brescia and Paderborn strain for half-life in relation to temperature in faeces, but not in urine. For that reason, curves with a separate intercept per strain were fitted and shown in Figure 1A, while almost a single common curve was fitted and shown in Figure 2A.

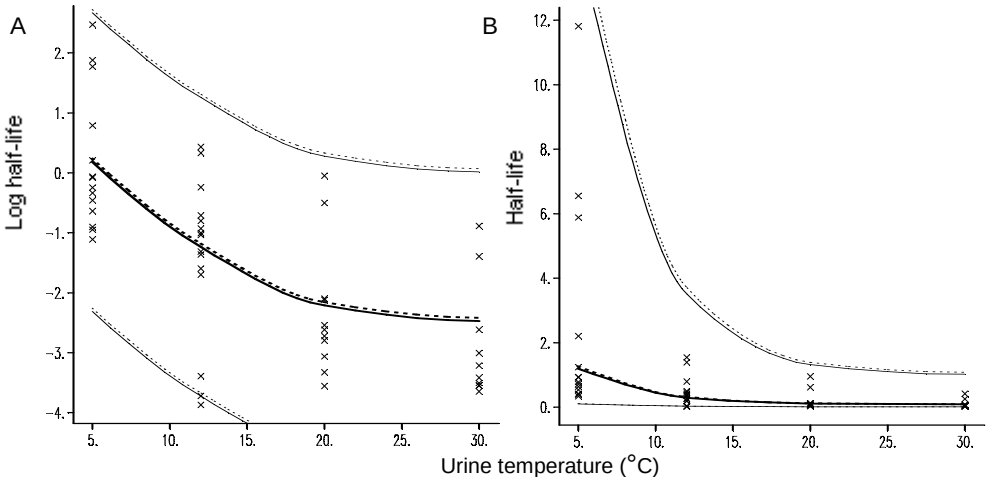
In Figures 1B (faeces) and 2B (urine) the direct relationship between half-life (untransformed) and temperature is shown. Also shown in Figures 1A and B, and 2A and B are the 95% confidence intervals around the fitted curves and the actual data. Figures 1B and 2B clearly show that variation among the data is larger for lower temperatures, while variation is reasonably constant for all temperatures after log transformation in Figures 1A and 2A.



Brescia: Log(Half-life)=2.353-0.1741*temp Half-life=$e^{2.353 - 0.1741*temp}$

Paderborn: Log(Half-life)=1.913-0.1741*temp Half-life=$e^{1.913 - 0.1741*temp}$

Figure 1. Effect of faeces temperature on virus survival. (A) Effect of faeces temperature on the log-transformed half-life values of the virus. (B) Effect of faeces temperature on the half-life values of the virus. (....) Brescia and (—) Paderborn strain with 95% confidence intervals. x= half-life values of the individual faeces samples.



Brescia: Log(Half-life)=1.562-0.2928*temp+0.005337*$temp^2$ Half-life=$e^{1.562 - 0.2928*temp + 0.005337*temp2}$

Paderborn: Log(Half-life)=1.508-0.2928*temp+0.005337*$temp^2$ Half-life=$e^{1.508 - 0.2928*temp + 0.005337*temp2}$

Figure 2. Effect of urine temperature on virus survival. (A) Effect of urine temperature on the log-transformed half-life values of the virus. (B) Effect of urine temperature on the half-life values of the virus. (....) Brescia and (—) Paderborn strain with 95% confidence intervals. x= half-life values of the individual urine samples.

Estimated survival of classical swine fever virus in faeces and urine

The presented days until inactivation of the virus (to a level below the detection limit of the virus titration assay) are calculated using the models presented in Figures 1 and 2.

Furthermore, the estimated survival of CSFV in faeces and urine (Table 3) is based on the maximum amounts of virus detected in these excretions from infected pigs (Weesendorp et al., 2009). In faeces from pigs infected with the Brescia strain, the maximum amount of virus detected was $10^{5.6}$ $TCID_{50}$/g (day 8 p.i.), and in faeces from pigs infected with the Paderborn strain $10^{6.1}$ $TCID_{50}$/g (day 38 p.i.). In urine from pigs infected with the Brescia strain a maximum of $10^{5.9}$ $TCID_{50}$/ml (day 9 p.i.) was detected, and in urine from pigs infected with the Paderborn strain $10^{6.9}$ $TCID_{50}$/ml (day 32 p.i.).

Table 3. Estimates of the duration of survival of CSFV in faeces and urine when maximum amounts of virus are present, at the peak of virus excretion[a]

Temperature(℃)	Virus strain	Mean survival in faeces (days)[b]	95% confidence interval	Mean survival in urine (days)[c]	95% confidence interval
5	Brescia	66	(16-272)	20	(1.7-243)
	Paderborn	47	(11-194)	23	(1.9-283)
12	Brescia	19	(4.7-80)	4.9	(0.41-59)
	Paderborn	14	(3.4-57)	5.6	(0.46-68)
20	Brescia	4.8	(1.2-20)	1.8	(0.15-22)
	Paderborn	3.5	(0.84-14)	2.1	(0.17-25)
30	Brescia	0.85	(0.20-3.5)	1.4	(0.12-17)
	Paderborn	0.61	(0.15-2.5)	1.6	(0.13-20)

[a] Mean survival is based on models presented in Figures 1 and 2. Survival is defined as duration of virus infectivity until virus titres were below the detection limit of the virus titration assay ($10^{1.1}$ $TCID_{50}$/ g or ml).

[b] Calculations based on maximum excretion of CSFV in faeces of pigs infected with the Brescia strain ($10^{5.6}$ $TCID_{50}$/g) or the Paderborn strain ($10^{6.1}$ $TCID_{50}$/g).

[c] Calculations based on maximum excretion of CSFV in urine of pigs infected with the Brescia strain ($10^{5.9}$ $TCID_{50}$/ml) or the Paderborn strain ($10^{6.9}$ $TCID_{50}$/ml).

Viral RNA concentration over time in faeces and urine after storage at different temperatures

The reduction of viral RNA in faeces over time was limited (Figure 3). The maximum decrease of viral RNA concentrations was $10^{1.7}$ $TCID_{50}$ equivalents at 5℃, $10^{1.0}$ $TCID_{50}$ equivalents at 12℃, $10^{1.3}$ $TCID_{50}$ equivalents at 20℃, and $10^{2.1}$ $TCID_{50}$ equivalents at 30℃. In urine the viral RNA concentrations remained quite stable, although at 30℃ in all samples a reduction (>$10^{0.7}$ $TCID_{50}$ equivalents) of the viral RNA concentrations was observed (Figure 4). The maximum decrease of viral RNA concentrations were $10^{1.1}$ $TCID_{50}$ equivalents at 5℃, $10^{1.0}$ $TCID_{50}$ equivalents at 12℃, $10^{1.4}$ $TCID_{50}$ equivalents at 20℃, and $10^{2.8}$ $TCID_{50}$ equivalents at 30℃.

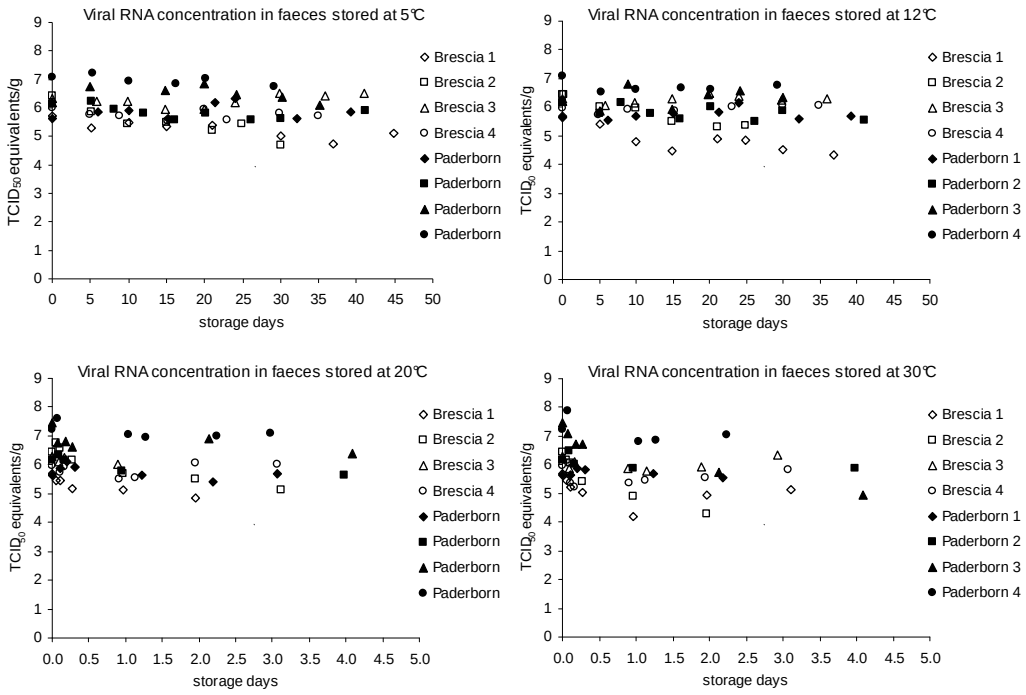


Figure 3. Viral RNA concentration over time in faeces samples from pigs infected with the Brescia or the Paderborn strain. Samples were stored at different temperatures.

Discussion

A prerequisite for indirect transmission is virus survival in the environment. Until now only limited information on virus survival was available. Furthermore, most studies focused on virus survival in medium or slurry (Depner et al., 1992; Haas et al., 1995). For indirect transmission via persons or transportation trucks, however, faeces and urine are more relevant than slurry. The present study filled these gaps by generating survival curves of CSFV in faeces and urine derived from pigs infected with a highly or moderately virulent CSFV strain.

The model presented in this study made it possible to estimate survival times of CSFV in faeces and urine for different environmental temperatures. In Table 3 results are presented of survival times when maximum amounts of virus are excreted by infected pigs (Weesendorp, 2009). Survival times of this worst-case scenario are presented with 95% confidence intervals, based on the confidence intervals of the model (Figures 1 and 2). These confidence intervals are relatively wide, due to the large variation between samples (especially at low temperatures), and the small number of samples. As this worst-case scenario is calculated using maximum amounts of virus, survival times at low temperatures are in some cases longer than the study

period of the samples, which likely resulted in an overestimation of the maximum survival time.

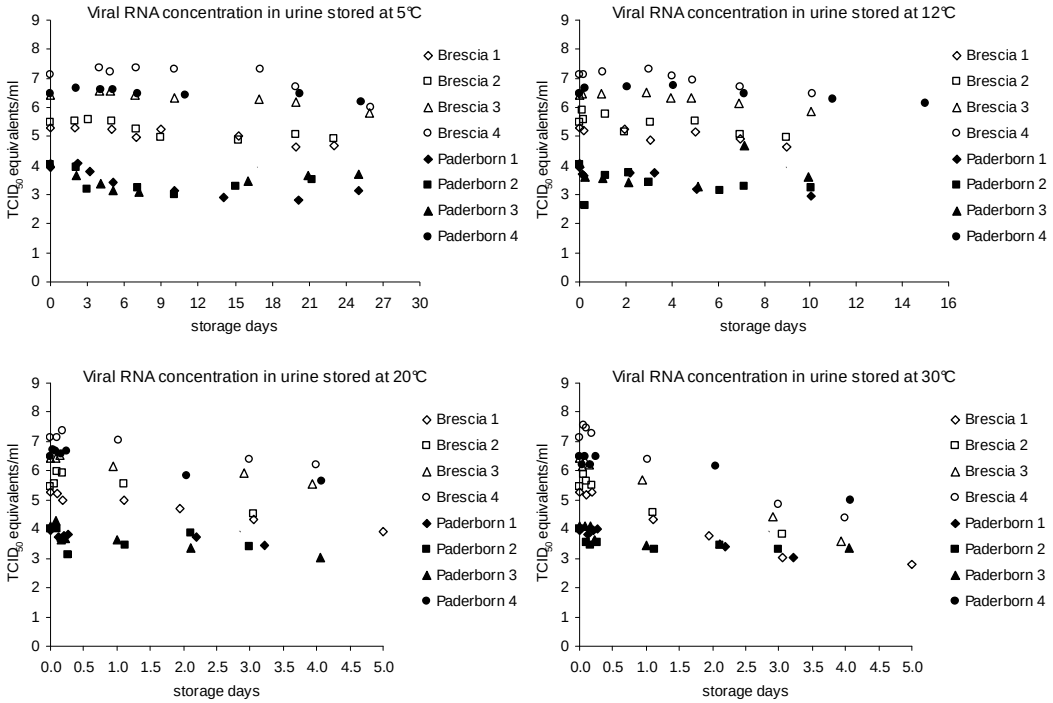


Figure 4. Viral RNA concentration over time in urine samples from pigs infected with the Brescia or the Paderborn strain. Samples were stored at different temperatures.

Within the studied temperature range, there is an inverse relationship between virus survival and temperature, which other studies already demonstrated. In spiked slurry with an initial concentration of $10^{5.5}$ $TCID_{50}$/ml the virus was not inactivated at 5°C within 42 days (Bøtner, reviewed by Haas et al., 1995). Based on this initial concentration of $10^{5.5}$ $TCID_{50}$/ml and the model presented in this study, virus in faeces produced by infected pigs would on average be undetectable within 42 days (Paderborn) or 64 days (Brescia). In urine produced by infected pigs, the virus would be inactivated to a level below the detection limit within 18 days (Paderborn and Brescia). These results would suggest that virus survival in faeces is comparable to that in slurry (Bøtner, reviewed by Haas et al., 1995). However, virus survival in slurry kept at other temperatures is different in the study of Bøtner than virus survival in faeces in the present study. Bøtner (reviewed by Haas et al., 1995) reported an inactivation time of 14 days for virus in slurry kept at 20°C, while in the present study the virus would be inactivated in faeces at this temperature within 3 days (Paderborn) or 5 days (Brescia). These differences could be due to differences in condition between slurry and faeces or urine, differences in storage condition, spiking versus using faeces and urine from

infected pigs, differences between the methods used for isolation of the virus, and differences between the detection assays.

Studies on survival of virus in excretions of infected pigs are rare. Pens contaminated with secretions and excretions of infected pigs showing clinical symptoms, contained infectious virus for at least 10 h when the environmental temperature was around 22°C (Ribbens et al., 2004). Given equal circumstances, the duration of survival will be mainly dependent on the initial concentration of virus in the pen. Pigs infected with highly virulent or moderately virulent strains of CSFV can excrete large quantities of virus when clinical signs are present. Via the faeces a maximum of $10^{9.9}$ $TCID_{50}$/Paderborn-infected pig or $10^{8.4}$ $TCID_{50}$/Brescia-infected pig was produced per day. Via the urine a maximum of $10^{9.0}$ $TCID_{50}$/Paderborn-infected pig or $10^{7.8}$ $TCID_{50}$/Brescia-infected pig was produced per day (Weesendorp et al, 2009). Based on the model presented here, it can be calculated that virus produced in one of these days would survive for 6 days (Brescia) or 4 days (Paderborn) in faeces, and 2 days (Brescia) or 3 days (Paderborn) in urine at 22°C. This agrees with previous observations that pens housing infected pigs, contain infectious virus for a few days before the virus within the pen and manure is totally inactivated (Artois et al., 2002).

The interest in the present study is not only to predict virus survival, but also its relationship to the transmission of disease to healthy pigs. To determine CSFV survival, we used the detection limit of the cell culture assay (virus titration). Information on the relationship between the detection limit of the assay and the sensitivity of the animal to infection is, however, scarce. In the present study the detection limit of the virus titration assay was 13 $TCID_{50}$/g faeces or ml of urine. The minimal infective dose that results in fatal disease after inoculation with the highly virulent strain “Alfort” is 10 $TCID_{50}$ per pig (Liess, 1987). For the Brescia virus strain a pig ID_{50} of 80 $TCID_{50}$ was determined after intranasal inoculation of virus in medium (Terpstra and Wensvoort, 1988). Thus, it seems that the detection limit of the virus titration gives a fair prediction of the transmission to healthy pigs. However, in previous studies the host animal has proven to be more sensitive than tissue culture (Stewart et al., 1979; McKercher et al., 1987; Panina et al, 1992), although it must be considered that the sample amounts tested in *in vitro* assays are mostly much smaller than in the *in vivo* assays. Furthermore, the minimum infective intranasal dose of virus in medium might be different from minimum infective oral doses of virus in faeces or urine. It is therefore difficult to predict whether the risk of infection is over- or underestimated.

Indirect transmission routes play a major role in transmission of the virus. The importance of contaminated livestock trucks in introduction of the virus into the Netherlands has been shown by De Vos et al. (2004). Their model indicated that returning livestock trucks contributed most (about 65%) to the probability of CSFV introduction in the Netherlands. However, many intermediate steps are involved in this transmission route: the livestock truck visiting an infected farm is in contact with infected pigs or infectious material (even though pigs showing clinical symptoms are not allowed to be transported), the livestock truck gets contaminated, the virus survives during transportation, the virus is not removed or inactivated by cleaning and disinfecting, there is contact between the livestock truck and susceptible pigs, and an

infective viral dose is transmitted. In spite of the small probability of all these events occurring, such worst-case scenarios have happened in the past. The 1997-1998 outbreak is supposed to be caused by a transportation truck returning from the Paderborn region in Germany. Due to the low temperatures (-10 to -20℃) the cleaning and disinfection of the truck was hampered, and the virus survived (Elbers et al., 1999). The present study showed that even at higher temperatures the virus can survive for a sufficient period to be transported over a long distance. Based on initial concentrations used in Table 3 ($10^{5.6}$-$10^{6.1}$ $TCID_{50}$), CSFV can survive for 4-5 days in faeces at 20℃, and 15-20 h in faeces at 30℃.

Differences in survival were observed between the virus strains. The Brescia strain was more resistant to inactivation in faeces than the Paderborn strain. This was also observed in urine, although these differences were small, and not significant. Differences between strains were observed before in a survival study with three cell culture-propagated strains of CSFV exposed to various temperatures and hydrogen ion concentrations (pH) (Depner et al., 1992). The Brescia strain showed the highest half-life values at 4 or 21℃ and pH 4. At lower hydrogen ion concentrations the 331/USA or Osterrode 2699/83 strains showed higher half-life values. The differences between virus strains in survival might be due to differences in the properties of the virus particle (e.g. the protein capsid and envelope proteins) or differences in the level of cell-association. Furthermore, faeces from pigs infected with the highly virulent Brescia strain could contain higher amounts of blood, as more clinical signs and haemorrhages were observed than of pigs infected with the Paderborn strain. This higher amount of blood in the faeces could have resulted in a more favourable environment for the virus.

The viral RNA level remained rather stable during the entire study period. The viral RNA reduction was in most cases within the variation of the qRRT-PCR assay. Therefore, it was not possible to calculate reliable half-life values for viral RNA. To do so, samples should be studied for a longer period. It is clear that stability of viral RNA does not predict the presence of infectious CSFV. However detection of CSFV-RNA in faeces and urine might be important from a diagnostic point of view. It will be possible to demonstrate the virus originally being present, even after a long period when samples are stored at higher temperatures.

Spiking has been used primarily to study survival of viruses (Bøtner, 1991; Haas et al., 1995; Turner et al., 2000). Based on the results of the spiking studies restrictions have been implemented after an outbreak to prevent recrudescence of the disease, like long-time storage of slurry (42 days in the European Union [Anonymous, 2001]), treatment of slurry, or decontamination of mechanical vectors like livestock trucks, boots or clothing. So far, however, it is not known whether spiking is an appropriate method to study survival in excretions produced by infected pigs. Especially with a hemorrhagic disease as CSF, faeces and urine can contain blood or infected cells, which might increase the survival time. Therefore, the approach used in the present study, using faeces and urine from infected animals, is a more realistic estimate of the behaviour of CSFV survival in excretions.

The decision whether or not to implement certain control measures, based on risk-analysis models, depends heavily on the reliability of available data. For the model

described by De Vos et al. (2004), only limited data were available to estimate the probability of CSFV survival in an empty livestock truck travelling over a distance to 900 km. It was suggested that studies should be performed to estimate this parameter more precisely (De Vos et al., 2006). These data have been generated in the present study.

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Chapter 6

Effect of strain and inoculation dose of classical swine fever virus on within-pen transmission

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Abstract

To improve the understanding of the dynamics and options for control of Classical Swine Fever (CSF), more quantitative knowledge is needed on virus transmission. In this study virus excretion and within-pen transmission of a strain of low, moderate and high virulence were quantified. Furthermore, the effect of inoculation dose on excretion and transmission were studied. The transmission was quantified using a stochastic susceptible-exposed-infectious-recovered (SEIR) model. Five transmission trials were conducted with ten pigs each. In each trail, three pigs were inoculated with the low virulent strain Zoelen, a low (10^2 $TCID_{50}$), middle ($10^{3.5}$ $TCID_{50}$), or high dose (10^5 $TCID_{50}$) of the moderately virulent strain Paderborn, or the highly virulent strain Brescia. The other seven pigs in each trial served as contact pigs. None of the pigs inoculated with the low dose of the Paderborn strain were infected. When it was assumed that the infectiousness of the pigs coincided with virus isolation positive oropharyngeal fluid and/or faeces, no significant differences in transmission rate β and basic reproduction ratio R_0 between the high inoculation dose of the Paderborn strain (β=1.62/day, R_0=35.9) and the Brescia strain (β=2.07/day, R_0=17.5) were observed. When the middle dose of the Paderborn strain was used for inoculation, the β (β=5.38/day) was not significantly higher than the Brescia strain or the high inoculation dose of the Paderborn strain, but the R_0 (148) was significantly higher. Infection with the Zoelen strain resulted in a significantly lower β and R_0 (β=0/day, R_0=0) than the other strains.

Introduction

Classical swine fever (CSF) is a highly contagious viral disease that affects domestic pigs and wild boar. For many European countries with a non-vaccination policy this is an exotic disease, but outbreaks occur occasionally. In areas with a high pig density this has resulted in severe economic losses due to mass destruction of pigs and export limitations (Meuwissen et al., 1999).

Classical swine fever virus (CSFV) is excreted by infected pigs in saliva, nasal and lacrimal fluids, faeces and urine (Ressang, 1973; Weesendorp et al., 2009). Infected pigs can transmit the virus to susceptible pigs via these secretions and excretions. This can occur by direct contact, or indirectly via contaminated clothes, livestock trucks, buildings or fomites (Ribbens et al., 2004; Ribbens et al., 2007). The efficiency and speed of transmission are dependent on several underlying parameters. For one, this includes the amounts of infectious virus excreted by infected pigs in their secretions and excretions, which may in turn depend on age and breed of the pigs, virus strain, inoculation dose or immune status. Additional important parameters include contact

structure between infectious and susceptible pigs, survival of virus in the environment and the susceptibility of contact pigs.

Direct transmission can be studied in transmission experiments where a number of pigs is inoculated with the virus and subsequently virus transmission to contact pigs within the same pen is studied (De Jong and Kimman, 1994). The transmission rate β and the basic reproduction ratio (R_0) can then be used to quantify the transmission. The transmission rate β is defined as the number of secondary infections caused by one infectious individual per unit of time. The R_0 is defined as the average number of secondary infections caused by one infectious individual during its entire infectious period in a fully susceptible population. If R_0 is smaller than 1, the infection within the population will fade out. If the R_0 is greater than 1, a large outbreak can occur. The β and R_0 can be estimated using a susceptible-exposed-infected-recovered (SEIR) model (Diekmann and Heesterbeek, 2000; Keeling and Rohani, 2008). This model describes the transmission dynamics by the change in the number of susceptible pigs (S), the number of pigs that are infected but not yet infectious (E), the number of infectious pigs (I), and the number of pigs that recovered or died (R) per unit of time. This requires assumptions regarding the start and duration of the infectious period of infected animals and the moment of infection of contact pigs. In previous studies mostly viraemia was used to determine this (Laevens, 1998; Laevens et al., 1999; Klinkenberg et al., 2002; Klinkenberg et al., 2003; Durand et al., 2009). The main problem with viraemia is that the virus is isolated from a site from which it cannot be transmitted to other pigs, and viraemia does not necessarily coincide with virus excretion in secretions and excretions (Weesendorp et al., 2009). Furthermore, for the determination of the moment of infection of contact pigs, viraemia does not seem to be the most biologically appropriate measure, since the virus replicates first in the tonsil and pharyngeal region, after which it can infect other animals via oronasal secretions. Viraemia occurs in a later stage of the infection. As a result, the transmission parameters could be over- or underestimated.

Previous studies have estimated transmission parameters, but only limited data was available on underlying parameters like virus excretion, and their relation to transmission (Klinkenberg et al., 2002; Klinkenberg et al., 2003; Durand et al., 2009). For three strains, of either high, moderate or low virulence, we have detailed information on excretion dynamics (Weesendorp et al., 2009). However, information on the transmission characteristics of these strains is limited or lacks accuracy, as described above. Knowledge on transmission mechanisms is needed to know how transmission can be reduced during an outbreak. Especially information on underlying parameters of transmission like virus excretion and their relation to transmission are needed. This will improve the understanding of the dynamics and options for control of CSF.

Therefore, we studied the virus excretion and the within-pen transmission of the same highly, moderately and low virulent strains, as we used in the previous study on excretion dynamics. Moreover, the effect of the inoculation dose on excretion and transmission was studied, since pigs are infected during outbreaks with varying doses

of virus. We used different assumptions on infectiousness of pigs to study the relationship between virus excretion and transmission.

Materials & methods

Experimental design

Five transmission experiments were carried out with ten pigs each. Each transmission experiment was performed 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 h (Bouma et al., 1999; Uttenthal et al., 2001; Kaden et al., 2004), 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 Animal Sciences Group of Wageningen UR.

Experimental animals

Eight-week-old male pigs were obtained from a conventional, but pestivirus free pig herd in the Netherlands, and were randomly divided into the five groups. The pigs were fed once a day and had unlimited access to water.

Viruses and inoculation of animals

In the first experiment, 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), used at cell passage level 3. This strain was originally isolated during an outbreak on a Dutch farm. In the second, third and fourth experiment, three pigs each were inoculated with the moderately virulent strain Paderborn, with doses of respectively 10^2 $TCID_{50}$ (Paderborn low dose), $10^{3.5}$ $TCID_{50}$ (Paderborn middle dose), and 10^5 $TCID_{50}$ (Paderborn high dose). This strain (genotype 2.1) was isolated in 1997 during the outbreak in the Paderborn area of Germany (Greiser-Wilke et al., 2000), and was used at cell passage level 5. In the fifth experiment, 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. The virus stock was heparinised blood from a pig infected with Brescia strain 456610 (genotype 1.2). This strain was derived from a strain obtained in 1951 from Brescia, Italy. The strains were classified as low, moderately or highly virulent based on the classification of CSFV strains by Van Oirschot (1988). One milliliter of the virus suspension was administered per animal (0.5 ml per nostril). The inocula were back titrated to confirm the dose administered.

Clinical symptoms and body temperature

Clinical symptoms and rectal body temperatures were recorded daily. Fever was defined as a body temperature higher than 40℃, for two or more consecutive days. For quantitative assessment of the severity of disease a list of ten CSF-relevant criteria, as described by Mittelholzer et al. (2000) was used. 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, with a maximum of 30. Only pigs with total clinical scores (CS) higher than 2 were defined as pigs having clinical symptoms due to the CSFV infection. Sick pigs that became moribund and unable to stand up were euthanized for reasons of animal welfare.

Sampling procedures

Serum and 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. Serum samples were stored at −70℃ until testing for antibodies in the ELISA and NPLA. EDTA blood samples were used for immediate leukocyte counts and isolation of leukocytes. For isolation of the leukocytes, 4 ml 0.84% NH_4CL solution were added to 2 ml of EDTA blood. After 10 min the samples were centrifuged at 300 x *g* and washed twice with PBS. The pellet was resuspended in 2 ml medium (Eagle minimum essential medium [EMEM] with 5% foetal 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]) and stored at −70℃ until analysis in the virus isolation (VI).

Oropharyngeal fluid and faeces were collected at days 0, 3-8, 10-15, 17, 19, 21, 24, 26, 28, 31, 33, and 35 p.i. Oropharyngeal fluid was obtained with a gauze tampon held by a 30 cm. long forceps, which was scrubbed against the dorsal wall of the pharynx behind the soft palatum. The tampons were not weighed, so the total amount of oropharyngeal fluid in each tampon was not exactly known, however, based on a previous experiment the average weight is approximately 1.0 g (with a standard deviation of 0.4 g) (Weesendorp et al., 2009). The oropharyngeal fluid was suspended in 4 ml of the same media as described for the leukocyte isolation. After centrifugation (1800 x *g* for 15 min) the samples were stored at −70℃ until a nalysis in the VI and real-time reverse transcription polymerase chain reaction (RRT-PCR). Faeces were collected 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 x *g* for 15 min), the supernatants were stored at −70℃ until analysis in t he VI and RRT-PCR.

Tonsils were collected from all pigs during the post-mortem examination at day 35 p.i., or at an earlier time-point when pigs died or were euthanized for reasons of animal welfare. From each tonsil 0.1 g was added to 0.5 ml of medium (same as for isolation of leukocytes), and homogenized in the MagNa Lyser (Roche Applied Science) for 30 sec at 3500 *g*. After centrifugation (9500 x *g* for 1 min), an additional one ml of medium was added. Samples were centrifuged again (9500 x *g* for 5 min) and the supernatant was stored at −70℃ until analysis in the RRT-PCR.

Leukocyte counts

Leukocyte counts were performed using the Medonic® CA 620 coulter counter (Boule Medical AB). A decrease in the number of leukocytes (leukopenia) is a typical

sign of CSF (Van Oirschot, 1988). Leukopenia was defined as $<10*10^9$ cells/L blood (Weesendorp et al., 2009).

Virus isolation and titration

Presence of infectious virus in the samples was tested by virus isolation. From oropharyngeal fluid and faeces, a volume of 250 µl was incubated for 1 h on a monolayer of SK6 cells (permanent porcine kidney cell line) in a 24-well plate (Greiner) at 37℃ in an atmosphere with 5% CO_2. Plates were then washed once with PBS (phosphate-buffered saline) and fresh medium was added to the wells. A volume of 125 µl from leukocyte samples was directly incubated on a monolayer of SK6 cells without washing. Cells were cultured at 37℃ in an atmosphere with 5% CO_2. After 4 days, the growth medium was discarded, and the monolayers were washed in a 0.15 M NaCl solution, dried for 1 h at 37℃ and frozen for 1 h at −20℃. The monolayers were fixed with 4% cold (5℃) paraformaldehyde in PBS for 10 min and then washed in 0.15 M NaCl. Monolayers were stained by the immunoperoxidase monolayer assay (IPMA) (Wensvoort et al., 1986), using two horse-radish peroxidase (HRPO)-conjugated CSFV specific MAbs (V3/V4) diluted in PBS (1:4000) with 4% horse serum. Monolayers were stained with 300 µl 0.05 M NaAc solution, containing 5% 3-amino-9-ethyl carbazole (AEC) and 0.05% H_2O_2, 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).

Real-time reverse transcription polymerase chain reaction (RRT-PCR)

The presence of viral RNA in oropharyngeal fluid, faeces and tonsils was analysed by RRT-PCR. For RNA isolation, 200 µl of the samples were pipetted manually into MagNA Pure sample cartridges (Roche Applied Science). 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 RRT-PCR or stored at -70℃ in the sample cartridge until the RRT-PCR was carried out.

The RRT-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. (Van Rijn et al., 2004). Analysis was performed with the LC software.

Serology (ELISA and NPLA)

The serum samples were tested for antibodies by the PrioCHECK® CSFV Ab (Prionics AG), an ELISA for detecting CSFV-specific (E2) antibodies (Colijn et al., 1997). The results are expressed as the percentage inhibition. A cut-off value of 30% inhibition was applied. Samples with the percentage of inhibition above 30% were considered positive.

Serum samples were tested in the direct neutralization peroxidase-linked assay (NPLA) for neutralizing antibodies against the homologous CSFV strains (Terpstra et al., 1984). Serial two-fold dilutions (1:5 to 1:10240) of serum were mixed with an equal

volume (50 µl) of CSFV (containing 100 $TCID_{50}$) in a 96-well plate (Greiner). After incubation of 1 h at 37℃ in an atmosphere with 5% CO_2, 100 µl SK6 cells (approximately 10,000 cells) in medium (Medium Earle's Balanced Salts with 0.55% lactalbumin hydrolysate, 1% MEM vitamin solution [Gibco], 5% FBS, 1% Penstrep [10,000 U/ml penicillin, 10,000 µg/ml streptomycin], 1% fungizone [250 µg/ml], 1% L-glutamin [200 mM]) were added per well. After 4 days, an IPMA was used for staining virus positive monolayers. The antibody titre was then determined as the reciprocal of the highest two-fold serum dilution that neutralized all virus.

Statistical analysis

Differences in virus excretion between the strains and doses were compared during the first days of virus excretion until the moment contact pigs were infected. This was in general within 5 days after the start of virus excretion. Differences in virus excretion of the inoculated pigs between strains and doses were determined by calculating for each individual inoculated pig the total amounts of virus excreted in faeces and oropharyngeal fluid (in $TCID_{50}$), which was expressed by the cumulative excretion (against time). Differences between the strains and doses in mean cumulative virus excretion (in $\log_{10}$ $TCID_{50}$) were statistically analysed using an analysis of variance (ANOVA) model. Pair wise comparisons between strains were made using the Bonferroni method (with an experiment wise Type I error rate of 0.05). Calculations were performed with SPSS 12 (SPSS Inc., Chicago, USA).

Estimation of transmission parameters

For the analysis of the transmission, a stochastic SEIR model was used (Diekmann and Heesterbeek, 2000; Keeling and Rohani, 2008). In a SEIR model, the transmission dynamics of infectious diseases between individuals are described by the change in the number of susceptible (S), exposed (E), infectious (I), recovered or removed (dead) (R) and total number (N) of animals. Assuming that the number of contacts per animal is independent of the herd size (frequency dependent mixing) (Begon et al., 2002), susceptible pigs become infected with a rate of $dS_t/dt=-\beta \bullet S_t \bullet I_t/N_t$. In this formula, β is the transmission rate and can be interpreted as the average number of new infections for a typical infectious animal in a susceptible population per unit of time. S_t is the number of susceptible animals, I_t is the number of infectious animals and N_t is the total number of animals at time t. The probability for a susceptible animal to become (latently) infected during period Δt is $P(S \Rightarrow E)=1-\exp(-\beta \bullet I_t \bullet \Delta t/N_t)$, and the probability for a susceptible animal to escape infection during a period Δt is given by $P(S \not\Rightarrow E)=\exp(-\beta \bullet I_t \bullet \Delta t/N_t)$. The distribution of the infectious period is modelled by a beta distribution with a maximum of 60 days, of which the mean infectious period Ti and the standard deviation are estimated from the data. Pigs infected with the strains used in this study were in previous studies death or recovered before day 60 p.i. (Klinkenberg et al., 2003; Weesendorp et al., 2009). The reproduction number R_0 is the expected number of new infections an average infectious animal causes during its entire infectious period in a fully susceptible population, i.e. β x Ti. All transmission parameters are estimated by maximizing their corresponding loglikelihood functions. The 95% confidence

intervals are obtained from the loglikelihood profile, assuming that the loglikelihood ratio is described by a chi-square distribution with one degree of freedom.

To estimate β and R_0 from the transmission experiments, the moment contact pigs became infected had to be estimated in a biologically plausible way. In previous experiments (Weesendorp et al., 2009; Weesendorp, unpublished) we observed the first PCR positive signal in oropharyngeal fluid 1 day p.i. of the highly virulent strain Brescia and 3 days p.i. of the moderately virulent strain Paderborn and the low virulent strain Zoelen. For each contact animal, this period (1 or 3 days) was subtracted from the moment the first positive RRT-PCR-signal in oropharyngeal fluid was observed to obtain the moment of infection.

Furthermore, for the estimation of β and R_0 the start and end of the infectious period of infected animals needed to be determined. The periods of infectiousness were estimated using three different assumptions. For the start and end of the infectious period, the assumption was made that infectiousness had coincided with the following: (1) virus excretion in faeces, (2) virus excretion in faeces and/or oropharyngeal fluid, (3) viraemia. Virus excretion and viraemia were determined by virus isolation. We assumed that any negative test result both preceded and followed by a positive test result was false negative.

Results

Infection and transmission- Zoelen

None of the pigs inoculated with the Zoelen strain showed fever or clinical symptoms, but infection was confirmed by leukopenia, viraemia, serology and RRT-PCR positive tonsils (Table 1). Infectious virus, as determined by virus titration, was detected mainly in oropharyngeal fluid (Tables 2 and 3). No virus transmission to any of the contact pigs was observed, since no fever, leukopenia, viraemia, and antibodies were detected. The virus isolation and RRT-PCR on oropharyngeal fluid and faeces samples were also negative. Furthermore, no viral RNA, as determined by RRT-PCR, was detected in the tonsils.

Infection and transmission- Paderborn low inoculation dose

None of the pigs inoculated with a dose of 10^2 $TCID_{50}$ became infected. This was determined by the absence of clinical symptoms, fever, leukopenia, viraemia, and antibodies in the serum. The virus isolation and RRT-PCR on oropharyngeal fluid and faeces samples were also negative. No viral RNA, as determined by RRT-PCR, was detected in the tonsils. As a consequence, no virus transmission to the contact pigs was observed either.

Infection and transmission- Paderborn middle inoculation dose

All pigs inoculated with a dose of $10^{3.5}$ $TCID_{50}$ became infected and developed clinical symptoms, leukopenia, viraemia, antibodies (ELISA) and were RRT-PCR positive in the tonsils. Infectious virus was detected in oropharyngeal fluid and faeces from day 5 p.i. A wide variety of clinical symptoms were observed in these pigs.

Table 1. Clinical symptoms, blood parameters, serology, virus isolation and RRT-PCR results

Virus strain and dose[a]	CS[b]	Fever[c]	Leukopenia[d]	VI leukocytes[e]	ELISA[f]	NPLA titre[g]	RRT-PCR on tonsils
Zoelen 10^5 $TCID_{50}$							
I	no	no	yes	5-7	+	640	+
I	no	no	no	5-7	+	320	+
I	no	no	yes	5-7	+	320	+
Paderborn middle- $10^{3.5}$ $TCID_{50}$							
I	yes	no	yes	7-12	+	80	+
I	yes	yes	yes	5-23†	+	20	+
I	yes	yes	yes	5-35	+	<5	+
C	yes	yes	yes	17-35	+	<5	+
C	yes	yes	yes	14-35	+	5	+
C	yes	yes	yes	12-35	+	<5	+
C	yes	yes	yes	17-29†	+	20	+
C	yes	yes	yes	14-35	+	<5	+
C	yes	yes	yes	14-35	+	<5	+
C	yes	yes	yes	17-28	+	80	+
Paderborn high- 10^5 $TCID_{50}$							
I	yes	yes	yes	5-32†	-	<5	+
I	yes	yes	yes	3-21	+	20	+
I	yes	yes	yes	3-21†	-	<5	+
C	yes	yes	yes	17-35	+	10	+
C	yes	yes	yes	17-35	+	<5	+
C	yes	yes	yes	17-35	+	<5	+
C	yes	yes	yes	17-24	+	80	+
C	yes	yes	yes	14-21	+	20	+
C	yes	yes	yes	10-35	-	<5	+
C	yes	yes	yes	14-31†	+	5	+
Brescia- $10^{2.5}$ $TCID_{50}$							
I	yes	yes	yes	3-14†	+	<5	+
I	yes	yes	yes	3-13†	-	<5	+
I	yes	yes	yes	3-11†	-	<5	+
C	yes	yes	yes	10-21†	+	<5	+
C	yes	yes	no	10-13†	-	<5	+
C	yes	yes	yes	10-14†	-	<5	+
C	yes	yes	yes	10-21†	+	<5	+
C	yes	yes	yes	10-18†	+	<5	+
C	yes	yes	yes	10-13†	-	<5	+
C	yes	yes	yes	10-12†	-	<5	+

[a] Contact pigs of the Zoelen trial and pigs from the Paderborn low inoculation dose trial (10^2 $TCID_{50}$) were not included in the table since none of the tests showed a positive result.
[b] Clinical symptoms: clinical scores >2.
[c] Body temperature for 2 or more consecutive days >40℃.
[d] Leukopenia: $<10*10^9$ leukocytes/L blood.
[e] Interval in days p.i. when virus isolation on leukocytes were positive.
[f] ELISA on serum samples: + positive, - negative.
[g] NPLA-titre in serum samples.
† Day of death or euthanasia (p.i.).

Table 2. Infectious virus titres in oropharyngeal fluid, in grey RRT-PCR positive samples

Virus strain/ dose[a]	Days post-inoculation																						
	0	3	4	5	6	7	8	10	11	12	13	14	15	17	19	21	24	26	28	31	33	35
Zoelen- 10^5 $TCID_{50}$																						
I	-	-[b]	1.9[c]	3.4	2.7	2.9	-	-	1.4	-	-	-	-	-	-	-	-	-	-	-	-	-
I	-	1.7	3.2	2.9	3.7	3.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
I	-	-	-	-	-	0.9	-	-	2.4	2.2	-	-	-	-	-	-	-	-	-	-	-	-
Paderborn middle- $10^{3.5}$ $TCID_{50}$																						
I	-	-	-	-	-	1.1	-	-	3.1	-	-	-	-	-	-	-	-	-	-	-	-	-
I	-	-	-	-	-	2.1	-	-	4.6	0.4	-	-	-	5.9	4.7	-	†					
I	-	-	-	0.9	-	2.4	-	0.4	5.1	4.7	6.4	7.4	6.4	6.9	4.4	-	4.7	6.4	5.7	4.7	6.7	6.4
C	-	-	-	-	-	-	-	-	-	-	-	-	-	2.4	-	-	5.2	5.4	4.9	5.9	5.9	7.2
C	-	-	-	-	-	-	-	-	0.4	-	-	1.9	-	2.7	-	-	5.2	4.9	4.2	4.4	5.4	6.9
C	-	-	-	-	-	-	-	-	0.6	-	-	-	-	4.4	-	-	5.9	6.9	6.4	3.7	4.9	6.9
C	-	-	-	-	-	-	-	-	-	-	-	-	-	2.4	1.7	-	4.7	6.9	6.4	†		
C	-	-	-	-	-	-	-	-	-	-	-	-	-	3.7	2.9	-	5.2	5.9	4.7	4.9	5.2	6.2
C	-	-	-	-	-	-	-	-	-	-	-	-	-	2.2	-	-	5.7	6.4	6.9	5.7	5.9	6.9
C	-	-	-	-	-	-	-	-	-	-	-	-	-	3.2	-	1.2	1.9	3.4	0.4	-	0.9	0.4
Paderborn high- 10^5 $TCID_{50}$																						
I	-	-	0.4	0.9	0.6	-	-	-	5.4	5.1	-	-	1.4	5.7	1.4	4.4	5.9	6.2	5.2	4.1	†	
I	-	-	-	0.9	-	-	-	-	3.9	3.4	-	-	-	4.4	-	-	-	2.2	4.9	-	-	-
I	-	-	1.6	2.4	-	-	-	-	5.4	4.1	2.1	-	2.1	5.9	2.9	†						
C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5.4	5.2	6.4	4.2	6.2	7.4	7.2
C	-	-	-	-	-	-	-	-	-	0.4	-	-	-	2.2	-	-	3.4	3.1	3.7	5.9	6.7	6.9
C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.4	2.6	4.9	4.4	6.4	4.9	6.4
C	-	-	-	-	-	-	-	-	-	2.4	-	-	-	-	-	-	-	-	-	-	-	-
C	-	-	-	-	-	-	-	-	0.4	-	-	-	-	-	-	-	-	-	-	-	-	-
C	-	-	-	-	-	-	-	-	-	0.4	-	-	-	3.2	2.4	5.2	5.9	6.9	6.7	6.9	6.7	7.4
C	-	-	-	-	-	-	-	-	-	0.4	-	-	-	-	0.6	4.4	5.2	6.7	6.2	†		
Brescia- $10^{2.5}$ $TCID_{50}$																						
I	-	2.9	3.4	3.7	3.9	4.2	3.9	3.7	5.2	4.9	3.9	-	†									
I	-	3.2	3.2	3.2	3.2	2.2	-	4.7	5.7	5.4	5.7	†										
I	-	2.9	2.9	3.7	4.2	-	3.9	5.2	6.2	†												
C	-	-	-	-	-	-	-	1.7	5.2	4.7	4.9	-	4.4	5.7	4.9	2.4	†					
C	-	-	-	-	-	-	-	-	3.9	1.9	3.9	†										
C	-	-	-	-	-	-	-	2.4	4.4	2.2	4.4	-	†									
C	-	-	-	-	-	-	1.9	2.7	4.7	4.9	3.2	3.9	1.6	5.7	5.4	4.9	†					
C	-	-	-	-	-	-	2.4	-	2.4	2.4	4.2	-	3.2	3.7	†							
C	-	-	-	-	-	-	-	-	4.9	5.4	3.7	†										
C	-	-	-	-	-	-	-	2.4	5.2	4.2	†		†									

[a] Contact pigs of the Zoelen trial and pigs from the Paderborn low inoculation dose trial (10^2 $TCID_{50}$) were not included in the table since none of these pigs were infected.

[b] RRT-PCR positive samples are indicated in grey.

[c] Log_{10} $TCID_{50}$/ml.

\- No virus was isolated.

† Death or euthanasia of the pig.

Table 3. Infectious virus titres in faeces, in grey RRT-PCR positive samples

Virus strain/ dose[a]	Days post-inoculation																					
	0	3	4	5	6	7	8	10	11	12	13	14	15	17	19	21	24	26	28	31	33	35
Zoelen- 10^5 $TCID_{50}$																						
I	-	-	-	-	-[b]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
I	-	-	-	-	-	1.4[c]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Paderborn middle- $10^{3.5}$ $TCID_{50}$																						
I	-	-	-	-	-	2.9	2.9	3.4	2.9	-	-	-	-	-	-	-	-	-	-	-	-	-
I	-	-	-	1.6	4.2	5.4	4.7	5.9	5.7	5.7	6.2	5.7	5.9	5.4	4.9	4.9	†					
I	-	-	-	1.6	3.9	5.7	5.2	6.4	6.7	5.9	6.7	5.9	4.6	5.2	4.4	4.7	6.4	5.2	5.7	4.6	6.1	6.4
C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.6	1.6	5.4	4.9	5.7	5.4	4.4	3.9
C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3.4	4.7	5.2	4.4	3.9	5.9	tox	6.4
C	-	-	-	-	-	-	-	-	-	3.2	3.2	4.4	3.4	3.4	3.4	4.2	4.2	3.7	3.7	3.9	tox	5.4
C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4.7	5.9	4.9	5.2	5.9	†		
C	-	-	-	-	-	-	-	-	-	-	-	2.9	3.7	3.4	4.2	4.9	3.9	6.4	3.6	5.7	5.2	4.9
C	-	-	-	-	-	-	-	-	-	-	-	-	-	4.2	4.7	6.7	6.4	6.2	4.9	4.6	4.7	4.9
C	-	-	-	-	-	-	-	-	-	-	-	-	-	3.7	3.9	4.4	3.4	3.4	3.4	2.9	3.2	3.7
Paderborn high- 10^5 $TCID_{50}$																						
I	-	-	tox[d]	3.3	3.7	4.4	4.7	5.4	5.2	5.4	5.9	6.2	6.2	5.2	3.9	4.5	7.2	5.9	6.2	5.2	†	
I	-	-	-	tox	2.7	3.9	4.4	2.9	3.9	-	-	2.6	3.2	3.9	4.7	3.9	3.4	4.2	3.9	-	-	-
I	-	-	-	-	2.9	3.7	4.7	6.7	6.9	4.2	6.4	3.2	6.0	6.9	5.9	†						
C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4.2	4.4	3.9	5.9	7.3	4.7	4.2	6.2
C	-	-	-	-	-	-	-	-	-	-	-	-	-	4.2	3.4	4.9	4.9	5.2	5.2	6.0	4.4	5.4
C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3.9	5.9	5.9	3.9	6.8	4.9	5.9	5.1
C	-	-	-	-	-	-	-	-	-	-	-	-	-	1.9	4.2	-	-	-	-	-	-	-
C	-	-	-	-	-	-	-	-	-	-	-	-	-	3.4	-	-	-	-	-	-	-	-
C	-	-	-	-	-	-	-	-	-	-	3.2	3.4	4.9	4.7	4.2	4.4	5.4	4.9	4.7	3.2	3.9	4.4
C	-	-	-	-	-	-	-	-	-	-	-	-	3.7	4.2	3.9	5.4	5.9	5.4	5.9	†		
Brescia- $10^{2.5}$ $TCID_{50}$																						
I	-	-	-	2.9	3.1	2.9	3.4	5.2	4.9	6.7	5.4	4.5	†									
I	-	-	1.6	2.6	2.4	3.1	3.7	4.7	4.4	4.4	4.4	†										
I	-	-	-	1.6	4.1	4.7	2.9	3.9	5.7	†												
C	-	-	-	-	-	-	-	3.9	4.8	4.7	4.4	5.5	5.5	2.7	2.7	4.2	†					
C	-	-	-	-	-	-	-	3.2	4.2	5.2	3.9	†										
C	-	-	-	-	-	-	-	3.4	4.8	4.7	2.7	4.0	†									
C	-	-	-	-	-	-	-	4.9	3.7	5.4	4.7	6.4	4.5	3.9	5.7	4.2	†					
C	-	-	-	-	-	-	-	-	3.9	2.7	5.2	3.2	4.4	4.2	†							
C	-	-	-	-	-	-	-	3.4	5.1	4.4	4.4	†										
C	-	-	-	-	-	-	-	3.4	3.7	2.7	†											

[a] Contact pigs of the Zoelen trial and pigs from the Paderborn low inoculation dose trial (10^2 $TCID_{50}$) were not included in the table since none of these pigs were infected.
[b] RRT-PCR positive samples are indicated in grey.
[c] Log_{10} $TCID_{50}$/g.
[d] Toxic sample.
- No virus was isolated.
† Death or euthanasia of the pig.

Two pigs showed severe clinical symptoms (CS of 17 and 21), while one pig showed only mild clinical symptoms (CS of 3) and no fever. One inoculated pig died at day 23 p.i. All contact pigs became infected and developed fever, clinical symptoms,

leukopenia, viraemia and antibodies (ELISA). Viral RNA was generally detected first in oropharyngeal fluid and maximum 7 days later in faeces. In oropharyngeal fluid, viral RNA was first detected either on day 8 or 10 p.i. 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 contact pig died at day 29 p.i.

Infection and transmission- Paderborn high inoculation dose

All pigs inoculated with a dose of 10^5 $TCID_{50}$ became infected and developed fever, clinical symptoms, leukopenia and viraemia. Infectious virus was detected from day 4 p.i. in oropharyngeal fluid and from day 5 p.i. in faeces. Fever started one day earlier than in the pigs inoculated with the middle dose. Two inoculated pigs died at days 21 and 32 p.i. Only the inoculated pig that recovered from the infection developed E2-antibodies higher than the cut-off of 30% inhibition. Although the other two pigs showed an increase in E2-antibody titre of 19% and 20% inhibition, they died before the antibody titre could reach the cut-off. All the contact pigs became infected and developed fever, clinical symptoms, leukopenia, and viraemia. Viral RNA was detected in oropharyngeal fluid between days 8 and 11 p.i. Six of the seven contact pigs developed E2-antibodies higher than the cut-off, including one pig that died at day 31 p.i. The observed clinical symptoms in the most severely affected pigs were comparable to (contacts of) pigs inoculated with the middle dose.

Infection and transmission- Brescia

All pigs inoculated with the Brescia strain became infected and developed fever, clinical symptoms, leukopenia, and viraemia. Infectious virus was already detected on the first sampling day (day 3 p.i.) in oropharyngeal fluid. In faeces, infectious virus was detected from day 4 p.i. All inoculated pigs died between days 12 and 15 p.i. One pig developed E2-antibodies higher than the cut-off, while the E2 ELISA of one other pig suggests an increase in E2-antibodies (17% inhibition), although the level remained under the cut-off. The contact pigs developed fever and clinical symptoms, and six of the seven contact pigs developed leukopenia. The majority of the contact pigs did not develop E2-antibodies higher than the cut-off. Viral RNA was detected in oropharyngeal fluid between days 4 and 6 p.i. All contact pigs died between days 13 and 22 p.i. The observed clinical symptoms were severe depression, emaciation, loss of appetite, cramps, ataxia, inability to stand up, large haemorrhages in the skin, and diarrhoea.

Differences in virus excretion between strains

All contact infections took place within the first five days of virus excretion by the inoculated pigs. Figure 1 shows the total virus excretion during these days by the inoculated pigs, which was expressed by the cumulative excretion (against time). Pigs inoculated with the Brescia strain excreted the highest amount of virus in oropharyngeal fluid, but this was only significantly different until the second day of virus excretion from pigs inoculated with the middle dose of the Paderborn strain. After the second day, the differences between the strains were not significant. Pigs inoculated with the Zoelen

strain excreted significantly lower amounts of virus in faeces than pigs inoculated with the Paderborn and Brescia strain. No significant differences in virus excretion in faeces were observed between pigs inoculated with the middle dose of the Paderborn strain, the high dose of the Paderborn strain or the Brescia strain.

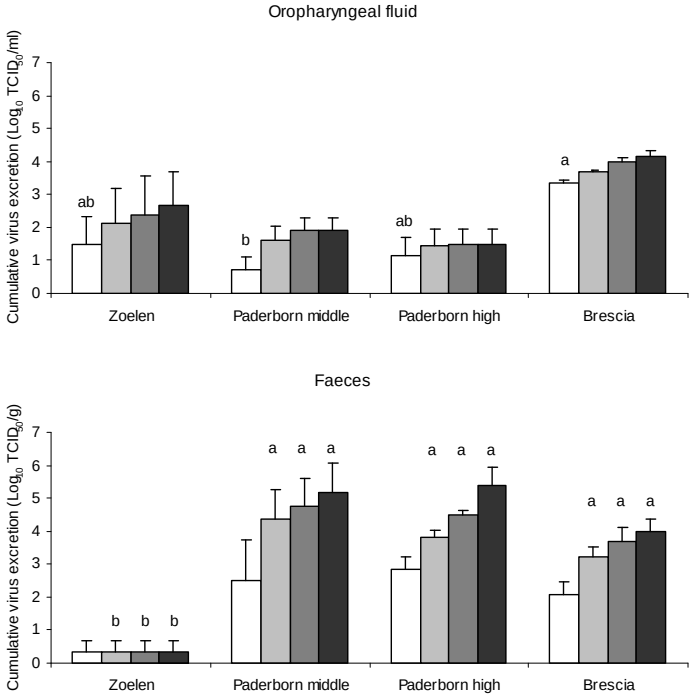

Figure 1. Total virus excretion (cumulative virus excretion [against time]) of the three inoculated pigs in faeces and oropharyngeal fluid until the second day (white bar), third day (light-grey bar), fourth day (dark-grey bar) or fifth day (black bar) of virus excretion (depending on strain and sample type, the first day of virus excretion was observed between days 3 and 5 p.i.). a,b: Bars of the same colour with no common letter differ significantly (p<0.05). Error bars represent standard error of the mean (SEM).

Quantification of transmission parameters

The transmission rate β, the infectious period *Ti*, the standard deviation of the infectious period and the reproduction ratio R_0 for all three assumptions on the infectiousness of pigs are shown in Table 4. The β of the Zoelen strain was estimated to be 0 per day, since no virus transmission occurred. The β of the Paderborn middle inoculation dose was slightly higher than that of the Paderborn high inoculation dose, although the difference was only significant when the infectious period was assumed to coincide with virus excretion in faeces and/or oropharyngeal fluid. The β's of the Paderborn and Brescia strain were comparable, except under the assumption that

infectiousness coincided with virus excretion in faeces only. In that case the β for strain Brescia was significantly higher.

Table 4. Estimation of transmission parameters with 95% confidence intervals, based on three different assumptions on the infectious period of pigs

	Virus strain and inoculation dose[1]							
Transmission parameter	Zoelen 10^5 $TCID_{50}$		Paderborn middle $10^{3.5}$ $TCID_{50}$		Paderborn high 10^5 $TCID_{50}$		Brescia $10^{2.5}$ $TCID_{50}$	
β (day^{-1})								
1. Faeces[2]	0^{c}	(0-2.74)	5.38^{b}	(2.11-12.2)	$2.83^{b,c}$	(1.17-5.84)	27.5^{a}	(10.2-63.0)
2. OPF/faeces[3]	0^{c}	(0-0.144)	5.38^{a}	(2.11-12.2)	1.62^{b}	(0.685-3.23)	$2.07^{a,b}$	(0.881-4.05)
3. Viraemia[4]	0^{b}	(0-0.203)	3.23^{a}	(1.33-6.74)	1.14^{a}	(0.486-2.24)	3.00^{a}	(1.27-5.96)
***Ti* (day)**								
1. Faeces[2]	-[5]	(- - -)	26.2^{a}	(17.4-42.6)	21.2^{a}	(13.4-34.5)	7.39^{b}	(5.54-9.99)
2. OPF/faeces[3]	6.33^{b}	(4.69-8.69)	27.5^{a}	(18.4-43.7)	22.2^{a}	(16.2-31.9)	8.47^{b}	(6.17-11.8)
3. Viraemia[4]	-	(- - -)	24.4^{a}	(17.5-36.8)	21.8^{a}	(16.7-30.3)	8.38^{b}	(6.34-11.2)
SD[6] of *Ti* (day)								
1. Faeces[2]	-	(- - -)	12.8^{a}	(7.33-21.6)	13.0^{a}	(8.14-20.9)	3.22^{b}	(2.15-5.67)
2. OPF/faeces[3]	1.23^{b}	(0.648-3.82)	13.2^{a}	(7.63-21.8)	9.32^{a}	(5.81-16.5)	4.06^{b}	(2.71-7.11)
3. Viraemia[4]	-	(- - -)	10.6^{a}	(6.27-18.9)	7.99^{a}	(4.90-14.8)	3.54^{b}	(2.36-6.13)
R_0								
1. Faeces[2]	-	(- - -)	141^{a}	(50.6-367)	60.0^{a}	(22.5-145)	203^{a}	(73.2-488)
2. OPF/faeces[3]	0^{c}	(0-0.925)	148^{a}	(53.8-382)	35.9^{b}	(14.5-77.6)	17.5^{b}	(7.13-36.9)
3. Viraemia[4]	-	(- - -)	78.8^{a}	(30.7-181)	24.8^{a}	(10.2-52.0)	25.1^{a}	(10.3-52.8)

[a-c] Means within rows with no common superscript differ significantly.

[1] Pigs from the low inoculation dose trial (10^2 $TCID_{50}$) were not included in the table since none of the pigs were infected.

[2] Assumption 1: start and end of the infectious period coincided with virus excretion in faeces.

[3] Assumption 2: start and end of the infectious period coincided with virus excretion in faeces and/or oropharyngeal fluid.

[4] Assumption 3: start and end of the infectious period coincided with viraemia.

[5] The 95% interval could not be determined due to lack of (variation in) data.

[6] Standard deviation.

The duration of virus excretion Ti was significantly higher for both inoculation doses of the Paderborn strain than for the Zoelen or Brescia strain, independent of the assumptions.

The reproduction ratio R_0 of the Zoelen strain was estimated to be 0 (0-0.925). For the Paderborn strain, the R_0 was significantly higher when the middle dose (148 [53.8-382]) was used for inoculation instead of the high dose (35.9 [14.5-77.6]), under the assumption that infectiousness coincided with virus excretion in faeces and/or oropharyngeal fluid. The R_0 was also significantly higher for the middle dose of the Paderborn strain than for the Brescia strain (17.5 [7.13-36.9]). Under the other assumptions, there was no significant difference between both inoculation doses of the Paderborn strain or the Brescia strain.

Discussion

This study quantified transmission parameters of three strains of different virulence; a low, moderately and highly virulent strain. The transmission parameters were

estimated using the maximum likelihood estimation method, which takes into account the time course of the experimental epidemic. Different assumptions were used to determine the period of infectiousness. These assumptions turned out to be rather crucial in estimating the transmission parameters β and R_0.

Transmission parameters of moderately virulent strains like the Paderborn strain have been quantified with the maximum likelihood estimation method before. For the Paderborn strain, a β of 0.65 per day (0.40-1.1) was estimated by Klinkenberg et al. (2003). Using the same method Klinkenberg applied, we actually found the same β of 0.65 per day (0.37-1.1) for the Paderborn strain with the data of our study (results not shown). This shows that the estimation of the β for the same strain is reproducible, when using the same assumptions. For our study different observations were used to determine transmission parameters, which will be discussed in more detail below.

First of all, we used another matrix (oropharyngeal fluid instead of blood) and diagnostic tool (PCR instead of VI) to determine the moment of infection of contact pigs. Using oropharyngeal fluid instead of blood as a matrix seems biologically more appropriate, since the virus replicates first in the tonsil and pharyngeal region, after which it spreads throughout the body. Viraemia occurs at a later stage of the infection, at a moment when secretions from the oropharyngeal region can already contain infectious virus (Weesendorp et al., 2009). The use of the RRT-PCR instead of the virus isolation will improve the determination of the moment of infection of the contact pigs, since the RRT-PCR has a higher sensitivity than virus isolation (Dewulf et al., 2004; Van Rijn et al., 2004; Oude Ophuis et al., 2006). Between pigs infected with the same strain, smaller differences exist in the moment after infection that the PCR on oropharyngeal swabs becomes positive than the virus isolation on blood samples is positive (this study; Uttenthal et al., 2003; Weesendorp et al., 2009).

Secondly, the start of the infectious period of pigs was determined by the first detection of infectious virus in faeces, oropharyngeal fluid or leukocytes (viraemia). The presence of infectious virus in secretions and excretions might be a better indicator of infectiousness of the pig than viraemia, since virus excretion may not coincide with viraemia (Weesendorp et al., 2009). In the present study, we used faeces and oropharyngeal fluid. Faeces is excreted in large amounts in the environment and may be the main source of infectious virus from an infected pig (Weesendorp et al., 2009). However, especially in the first days after infection, when virus cannot yet be detected from faeces, virus from other secretions and excretions, like from the oronasal region, may contribute in a relevant way to virus transmission as well. In the present study oropharyngeal fluid was used as a matrix to detect virus first, based on previous studies (Weesendorp et al., 2009). However, it cannot be excluded that virus excretion occurs even earlier in other secretions not tested in this experiment.

Differences were observed between the strains in β. The β of the Paderborn strain and Brescia strain were always significantly higher that the β of the Zoelen strain. The pigs inoculated with the Zoelen strain did not transmit the virus to the contact pigs at all. This was not expected since this strain was isolated in 1975 from foetuses of a “carrier sow” during a field outbreak (Van Oirschot, 1980a). In several experimental studies (Van Oirschot, 1980b; Weesendorp et al., 2009), virus titres in blood and secretions

and excretions were always low, which could explain the inability of this strain to transmit to contact pigs in the present experiment. This strain was, however, successful in transplacental transmission (De Smit et al., 2000). Persistently infected piglets, excreting high quantities of virus, might have been a way for this strain to maintain itself in the field. Besides, this strain has been cell passaged before inoculation of the pigs in the present experiment, which could also have reduced the virulence.

Between the Paderborn and Brescia strains, the difference in β depended on the assumptions on the start and duration of the infectious period of the pigs. Assuming that biologically the most appropriate way to determine whether a pig is infectious, is the detection of infectious virus from the combination of faeces and oropharyngeal fluid, no significant differences between the Paderborn and Brescia strains in β (and virus excretion) were observed. These results were not unexpected. In a previous study on virus excretion, it was shown that classical swine fever virus excretion is dependent on the strain and the clinical appearance of infection (Weesendorp et al., 2009). In the group of pigs infected with the Paderborn strain, both chronically and acutely infected pigs were observed. Together, these pigs did not excrete amounts of virus that were significantly different from pigs infected with the Brescia strain during the first five days of excretion, in which period all transmission events occurred (this study). Comparable excretion patterns therefore seem to result in comparable transmission rates for CSF, at least in the early infectious period. Further research using one-to-one experiments, with single infectious pigs housed with single susceptible pigs, could further clarify the correlation between the level of virus excretion and transmission.

When the infectious period was assumed to coincide with virus excretion in faeces and/or oropharyngeal fluid, the R_0 of the Paderborn strain (middle dose) was significantly higher than the R_0 of the Brescia strain. However, for strains with R_0 values significantly higher than 1, the generation interval is also an important measure in the initial phase of the outbreak (Wallinga and Lipsitch, 2007). The generation interval is the time between the infection moment of a pig and the moment of first infection of a contact animal by this pig. For the Paderborn strain this generation interval was 5 to 8 days, and for the Brescia strain 3 to 5 days, which would lead to exponential growth rates of 0.3/day (Paderborn) and 0.4/day (Brescia) in the initial phase of the outbreak (Keeling and Rohani, 2008). So even though the Paderborn strain has a higher R_0 than the Brescia strain, the growth rate of the latter strain was higher due to its shorter generation interval.

The different doses of the Paderborn strain used for inoculation resulted in differences in transmission. The low dose of 10^2 $TCID_{50}$ was not high enough to result in infection of even one of the three inoculated pigs. This was not expected since a slightly higher dose of $10^{2.5}$ $TCID_{50}$ of the Paderborn strain resulted in infection of four of five inoculated pigs (Klinkenberg et al., 2003). A higher β was observed with the middle inoculation dose than with the high dose. However, in subsequent generations, this difference will most likely disappear as there were no differences in the moment contact pigs were infected, or amounts of virus excreted by the inoculated pigs.

In conclusion, the different assumptions regarding the infectiousness of pigs resulted in differences in β and R_0. It was concluded that biologically the most

appropriate assumption is where infectiousness coincides with virus excretion in faeces and/or oropharyngeal fluid. Under this assumption it was noteworthy that β's were comparable for a moderately and highly virulent strain and that differences in transmission between these strains depend therefore on a different length of the generation interval.

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Chapter 7

Transmission of classical swine fever virus depends on the clinical course of infection which is associated with high and low levels of virus excretion

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Transmission of classical swine fever virus depends on the clinical course of infection which is associated with high and low levels of virus excretion

Abstract

Infection with moderately virulent strains of classical swine fever virus (CSFV) can lead to different courses of disease, either (sub)acute, resulting in death or recovery, or chronic disease. The virus excretion dynamics between these courses differ largely, but it is not known if this also results in differences in transmission. In this study, the excretion and transmission dynamics of the moderately virulent Paderborn strain were studied in fifteen one-to-one experiments. In these experiments, a single inoculated pig was housed with a single susceptible contact pig (C1) from day 1 post-inoculation (p.i.). Each contact pig that became infected was removed and replaced by a new contact pig at day 17 p.i. (C2) and day 26 p.i. (C3). Infection of contact pigs was monitored by quantitative real-time reverse transcription polymerase chain reaction on oropharyngeal fluid samples. Five of the inoculated pigs developed the chronic disease or died during the acute phase (high excreting pigs), while ten pigs recovered from the infection (low excreting pigs). In the first contact period, there was no significant difference in virus excretion between the high and low excreting pigs, while in the second and third contact period, high excreting pigs excreted significantly higher quantities of virus. Over the entire study period, the basic reproduction ratio differed significantly between the high (143 [56.3-373]) and low excreting pigs (23.1 [11.5-45.0]). This indicates the importance of high excreting pigs in transmission of CSFV. Furthermore, this study showed the rate of CSFV infections from a contaminated environment.

Introduction

Classical Swine Fever (CSF) is a highly contagious disease that infects both domestic pigs and wild boar. It is caused by an enveloped RNA-virus belonging to the family Flaviviridae, genus Pestivirus (Lindenbach and Rice, 2001). CSF was successfully eradicated in domestic pigs in most countries in the European Union in the 1980's, after which a non-vaccination policy was implemented (Terpstra and De Smit, 2000). This ban on 'prophylactic' vaccination resulted in a population of pigs fully susceptible to CSF. In combination with the high pig density in some areas, outbreaks with a rapid spread of CSF occurred. This resulted in high financial losses, due to mass destruction of pigs and export bans (Meuwissen et al., 1999; Terpstra and De Smit, 2000).

To eradicate CSF from a pig population, the transmission needs to be reduced to such extent that the virus cannot maintain itself in the population. This might be

obtained by efficient control measures like stamping out of infected herds, culling of herds at risk, vaccination, hygiene measures and movement restrictions. The development and evaluation of these measures requires quantitative knowledge on transmission of classical swine fever virus (CSFV). Insight in transmission dynamics of CSFV can be obtained by direct transmission experiments in which a number of pigs is inoculated with the virus and subsequently virus transmission to contact pigs within the same pen is studied (De Jong and Kimman, 1994). Most mathematical models that are used for the analysis of these transmission experiments assume equal infectiousness of all infected animals, which may be questioned for CSF. For CSFV it is known that infection with moderately virulent strains can lead to different courses of the disease, either (sub)acute disease, resulting in death or recovery, or chronic disease, which is always fatal (Van Oirschot, 1988; Floegel-Niesmann et al., 2003). The virus excretion dynamics between these courses differ largely (Weesendorp et al., 2009b). It remains to be seen to what extend these differences in clinical course and virus excretion will also result in differences in infectiousness and transmission.

In transmission experiments, animals are usually inoculated with the virus and after a defined time period, mostly 24 h for CSFV, introduced into a group of susceptible animals (Bouma et al., 1999; Uttenthal et al., 2001; Kaden et al., 2004). It takes time for the inoculated pigs to become infectious (latent period), and they start excreting increasing levels of virus until a peak or a plateau level (Weesendorp et al., 2009). The moment of infection of contact animals could be exposure dependent. When an inoculated (infectious) pig is introduced into a population of susceptible contact pigs at the peak of virus excretion, the transmission rate is expected to be higher than when the inoculated pig was introduced in the population of susceptible pigs at the beginning of its infectious period.

The aim of the present study was to investigate the association between virus excretion and transmission among pigs that developed different courses of the infection. These infection courses were divided into those of pigs that died from the CSFV infection (chronically infected or death during the acute phase) and those of pigs that recovered from the CSFV infection. Moreover, we studied whether an increasing or decreasing level of virus excretion in different phases of the infection also resulted in increasing or decreasing transmission rates. To do this, fifteen replicated one-to-one transmission trials were performed in which a single infectious pig was housed with a single susceptible pig. At two moments the latter pig was replaced by a new susceptible pig.

Materials & methods

Experimental setup

Fifteen one-to-one experiments were performed in which a single inoculated (I) pig was housed with a single susceptible (C1) pig. After infection was detected in a contact pig, it was removed to minimize CSFV contamination of the environment by this pig. Infection was determined by quantitative real-time reverse transcription polymerase chain reaction (qRRT-PCR) on oropharyngeal fluid samples. Pigs were considered to

be infected when at least two consecutive positive qRRT-PCR results were obtained. At day 17 post-inoculation (p.i) all contact pigs left were removed and replaced by new contact pigs (C2). At this moment, most inoculated pigs were still expected to be infectious, but with a large variety in virus excretion, depending on their courses of the infection (Weesendorp et al., 2009b). Next, the C2 pigs were removed at day 25 p.i., and at day 26 p.i., new contact pigs (C3) were housed with the inoculated pigs. At day 26 p.i. most of the inoculated pigs that recovered from the infection were expected to have stopped excreting infectious virus, while chronically infected pigs would still excrete virus at high levels (Weesendorp et al., 2009b). All inoculated pigs were euthanized at day 35 p.i. Contact pigs not yet confirmed to be infected at the end of the contact periods were separated from their inoculated pen mate and sampled for 7 more days (in case they were still in their latent period).

To analyze differences in virus excretion and transmission between the different courses of the infection, inoculated pigs were divided in either “high excreting pigs” or “low excreting pigs”. High excreting pigs are chronically infected pigs or pigs that die during the acute phase, and have high titres of infectious virus (as determined by virus isolation) in oropharyngeal fluid and faeces until the end of the experiment or until death. Low excreting pigs are defined as pigs that excrete infectious virus during the acute phase of the infection and subsequently recover and stop excreting virus.

Animals and housing

Sixty pigs, 8 weeks of age, were obtained from a conventional, but pestivirus free pig herd in the Netherlands. Pigs were housed in an isolation unit with four rooms. Room one contained nine solid-wall pens of 2.40 m x 1.15 m x 1.25 m (length, width, height) with a distance of minimum 1.35 m apart from each other. In this room pair 1 to 9 were housed. Room two and three contained each three pens, with pair 10 to 12 in one room, and pair 13 to 15 in the other room. In each pen, one inoculated pig was housed with one contact pig. During the first period (1 to 17 days post-inoculation [d.p.i.]) the inoculated pig was housed with the first contact pig (C1), during the second period (17 to 25 d.p.i.) with the second contact pig (C2), and during the third period (26 to 35 d.p.i.) with the third contact pig (C3). To prevent contamination from one pen to the other, clothing, footwear, gloves and hairnet were changed and materials needed for sampling and rectal temperature monitoring were provided for each pen separately. Every day the manure in the pens was removed with a shovel. No water was used for the cleaning of the pens to avoid aerosol production. The ventilation rate in the rooms was 400 m^3/h. The air inlet of the room was situated on the front side of the cages and the air outlet at the back side to reduce the possibility of virus contamination by the air from other cages. The C2 and C3 pigs were housed in groups in room four until they were placed with the inoculated pigs. In this room, a higher air pressure prevented virus contamination via the air from the other rooms.

The experiments were approved by the Ethics Committee for Animal Experiments of the Central Veterinary Institute of Wageningen UR.

Virus and inoculation

Fifteen pigs were inoculated intranasally with 1 ml containing 10^5 $TCID_{50}$ (50% tissue culture infectious dose) of the moderately virulent strain Paderborn. This strain (genotype 2.1) was isolated in 1997 during the outbreak in the Paderborn area of Germany (Greiser-Wilke et al., 2000). The inocula were back titrated to confirm the dose administered.

Clinical signs and body temperature

Body temperature and clinical signs were recorded daily. Fever was defined as body temperature higher than 40℃, for two or more consec utive days. For quantitative assessment of the severity of disease, a list of ten CSF-relevant criteria, as described by (Mittelholzer et al., 2000) was used. For all criteria a score was recorded of either normal (score 0), slightly altered (score 1), distinct clinical symptom (score 2), or severe CSF symptom (score 3). The scores for each pig were added up to a total score per day. Total scores higher than 2 were defined as clinical symptoms due to the CSFV infection.

Sampling procedures and pre- treatment of samples

EDTA-stabilized blood samples were collected three times a week from inoculated pigs for leukocyte counts, and for isolation of leukocytes. From contact pigs, EDTA-stabilized blood samples were taken at the moment of euthanasia, and three times a week from pigs that were housed for a prolonged period (see Tables 2 and 3) to determine the presence of CSF viral RNA by real-time reverse transcription polymerase chain reaction (RRT-PCR). These EDTA-stabilized blood samples were directly stored at -70℃ for analysis. For isolation of leukocytes, 4 ml 0.84% NH_4CL solution were added to 2 ml of EDTA blood. After 10 min, the samples were centrifuged at 300 x *g* and washed twice with Phosphate-Buffered Saline (PBS). The pellet was resuspended in 2 ml medium (Eagle miminum essential medium [EMEM] with 5% 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]) and stored at -70℃ until analysis by virus isolation (VI).

Serum was collected from inoculated pigs three times a week, and was stored at −70℃ until testing for antibodies in the ELISA.

Oropharyngeal fluid swabs were collected every day from the inoculated pigs and their contacts (either C1, C2 or C3). Oropharyngeal fluid was obtained with a gauze tampon held by a 30 cm long forceps, which was scrubbed against the dorsal wall of the pharynx behind the soft palate. The oropharyngeal fluid was suspended in 4 ml of the same media as described for the leukocyte isolation. After centrifugation (1800 x *g* for 15 min) the samples were stored at −70℃ until an alysis in the VI and by qRRT-PCR.

Faeces were collected every day from the inoculated pigs. It was 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 x *g* for 15 min), the supernatants were stored at −70℃ until analysis in the VI and qRRT-PCR.

Tonsils were collected from all pigs during the post-mortem examination. From each tonsil, 0.15 g was added to 0.5 ml of medium (same as for isolation of leukocytes), and homogenized in the MagNa Lyser (Roche Applied Science, Mannheim, Germany) for 30 sec at 3500 x *g*. After centrifugation (9500 x *g* for 1 min), an additional 1 ml of medium was added. Samples were centrifuged again (9500 x *g* for 5 min), and the supernatant was stored at −70℃ until analysis in the qRRT-PCR.

Leukocyte counts

Leukocyte counts were performed using the Medonic® CA 620 coulter counter (Boule Medical AB, Stockholm, Sweden). A decrease in the number of leukocytes (leukopenia) is a typical sign of CSF (Van Oirschot, 1988). Leukopenia was defined as $<10*10^9$ cells/L blood (Weesendorp et al., 2009a).

Virus isolation and titration

Presence of infectious virus in the samples was tested by virus isolation. From leukocytes, oropharyngeal fluid, and faeces, a volume of 125 µl was incubated on a monolayer of SK6 cells (permanent porcine kidney cell line). Cells were cultured in 1 ml of the same media as described for the leukocyte isolation at 37℃ in an atmosphere with 5% CO_2. After 4 days, the growth medium was discarded, and the monolayers were washed in a 0.15M NaCl solution, dried for 1 h at 37℃ and frozen for 1 h at −20℃. The monolayers were fixed with 4% cold (5℃) pa raformaldehyde in PBS for 10 min and then washed. Monolayers were stained by the immunoperoxidase monolayer assay (IPMA)(Wensvoort et al., 1986), using two horseradish peroxidase (HRPO)-conjugated CSFV specific MAbs (V3/V4) diluted in PBS (1:4000) with 4% horse serum. Monolayers were stained with 300 µl 0.05 M NaAc solution, containing 5% 3-amino-9-ethyl carbazole (AEC) and 0.05% H_2O_2, and examined for stained cells. Virus positive oropharyngeal fluid and faeces samples of inoculated pigs 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 calculated detection limits were $10^{1.65}$ $TCID_{50}$/g faeces and $10^{0.65}$ $TCID_{50}$/ml of oropharyngeal fluid or leukocytes in medium.

Quantitative real-time reverse transcription polymerase chain reaction (qRRT-PCR)

The presence of viral RNA in EDTA blood and tonsil samples was analysed by RT-PCR. For oropharyngeal fluid and faeces, the concentration of viral RNA was determined by qRRT-PCR using a standard curve. 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, one negative control sample and five dilutions of a positive control sample (standard curve) were included. The standard curves were constructed by spiking 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 RT-(q)PCR.

The RT-(q)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/ml or g) of each individual sample could be calculated using the standard curve.

Serology (ELISA)

The serum samples were tested for antibodies by the PrioCHECK® CSFV Ab (Prionics AG, Lelystad, the Netherlands), an ELISA for detecting CSFV-specific (E2) antibodies (Colijn et al., 1997). The results are expressed as the percentage inhibition. A cut-off value of 30% inhibition was applied. Samples with the percentage of inhibition above 30% were considered positive.

Statistical analysis

Differences in virus excretion were compared between the high and low excreting pigs. The excretion was compared during the first days of virus excretion of that period (when the inoculated pigs had contact with either C1, C2 or C3 pigs), until the moment contact pigs became positive in the qRRT-PCR. Only pigs were included that excreted infectious virus in that period. Differences in virus excretion of these inoculated pigs were determined by calculating for each individual inoculated pig the total amounts of virus excreted in faeces or oropharyngeal fluid (in $TCID_{50}$), which was expressed by the cumulative virus excretion (the area under the curve [(Eble et al., 2007)]). Differences between groups (high versus low excreting pigs) in mean cumulative virus excretion (in $\log_{10}$ $TCID_{50}$), and differences within groups between contact periods were statistically analysed using the non-parametric Mann-Whitney U test. Calculations were performed with SPSS 15 (SPSS Inc., Chicago, USA).

Quantification of transmission parameters

For the analysis of the transmission, a stochastic SEIR model was used (Diekmann and Heesterbeek, 2000; Keeling and Rohani, 2008). In a SEIR model, the transmission dynamics of infectious diseases between individuals are described by the change in the number of susceptible (S), exposed (E), infectious (I), recovered or removed (dead) (R) and total number (N) of animals. The rate at which susceptible pigs become infected is assumed to be $dS_t/dt=-\beta \bullet S_t \bullet I_t / N_t$. In this formula, β is the transmission rate and can be interpreted as the average number of new infections for a typical infectious animal in a susceptible population per unit of time. S_t is the number of susceptible animals, I_t is the number of infectious animals and N_t is the total number of animals at time t. The probability for a susceptible animal to become (latently) infected during period Δt is: $P(S \Rightarrow E)=1-\exp(-\beta \bullet I_t \bullet \Delta t/N_t)$, and the probability for a susceptible animal to escape infection during a period Δt is given by: $P(S \not\Rightarrow E)=\exp(-\beta \bullet I_t \bullet \Delta_t/N_t)$. The transmission rate β is estimated by maximizing the corresponding loglikelihood functions. The 95%

confidence intervals are obtained from the loglikelihood profile, assuming that the loglikelihood ratio is described by a chi-square distribution with one degree of freedom. Significant differences in the estimated transmission parameter between groups were determined by a likelihood ratio test.

To estimate β from the transmission experiments, the moment contact pigs became infected had to be estimated in a biologically plausible way. In the inoculated pigs we observed the first PCR positive signal in oropharyngeal fluid on average 4 days p.i. We expect the contact animals to have a shorter latent period due to the continuous exposure. Therefore, for each contact animal a maximum latent period of 4 days, and a minimum latent period of 0 days was used in the analysis. These periods were subtracted from the moment the first positive qRRT-PCR-signal were observed to obtain the infection interval.

The infectious period of the inoculated animals was described by a beta-distribution with a maximum of 60 days (Weesendorp et al., 2009a). Maximizing the corresponding loglikelihood function gave point estimates for the average infectious period and the standard deviation. The basic reproduction ratio R_0, which is the expected number of new infections an average infectious animal causes during its entire infectious period in a fully susceptible population, was estimated as the product of the average infectious period and an 'average' transmission rate. Here we have assumed that this constant 'average' transmission rate β reasonably reflects the transmission rate that changes over time. The 95% confidence intervals are again obtained from the loglikelihood profile.

Results

Infection of inoculated pigs

Of the fifteen inoculated pigs, ten pigs recovered from the infection, two pigs died (at days 23 and 24 p.i.), and three pigs were chronically infected (a lethal clinical form leading to death 30 days or more after infection). The chronically infected pigs (numbers 2, 4, 14) and the pigs that died (numbers 8 and 12) excreted high titres of infectious virus and viral RNA in oropharyngeal fluid and faeces until the end of the experiment or until death (Figure 1, Tables 1, 2 and 3), and are indicated as high excreting pigs. The pigs that recovered from the infection had lower infectious virus and viral RNA titres in oropharyngeal fluid and faeces and stopped excreting infectious virus between days 15 and 23 p.i. They are indicated as low excreting pigs. During the first period (days 1-17 p.i) we did not observe a difference in virus excretion between the high and low excreting pigs (Figure 2). During the second period (days 17-25 p.i.) the high excreting pigs excreted significantly higher levels of virus than the low excreting pigs. They also excreted significantly higher levels of virus in oropharyngeal fluid than during the first period, but not in faeces. Five of the ten low excreting pigs did not excrete infectious virus during the second period. In the third period (days 26-35 p.i.) only the high excreting pigs excreted infectious virus, with similar amounts of virus as in the second period.

Table 1. qRRT-PCR results ($log_{10}TCID_{50}$ equiv./g or ml) during the first contact period

			Days post-inoculation																	
Pair	Pig #	Sample	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
	1	Faeces I	-	-	-	-	-	3.8	4.3	4.9	4.9	5.9	6.0	7.2	4.5	3.5	3.8	3.4	5.2	4.1
1	1	OPF I	-	-	-	-	2.9	2.9	3.8	4.2	4.1	5.2	5.4	4.7	3.8	3.7	2.8	2.8	2.9	2.2
	16	OPF C1	-	nd	-	-	-	-	-	-	2.5	-	3.9	2.5	2.3†					
	2	Faeces I	-	-	-	-	2.5	4.0	4.4	5.3	5.7	5.9	7.1	7.4	7.3	6.6	6.9	6.9	6.3	6.5
2	2	OPF I	-	-	-	-	2.9	3.3	4.1	4.7	4.2	4.6	5.0	5.8	6.5	7.1	6.9	6.6	6.9	6.7
	17	OPF C1	-	nd	-	-	-	-	-	-	2.9	3.1	3.2	3.3†						
	3	Faeces I	-	-	-	-	2.5	3.6	4.2	5.3	4.7	4.5	3.9	3.1	3.0	-	-	-	-	-
3	3	OPF I	-	-	1.9	2.7	3.2	3.7	3.8	4.0	4.1	3.7	3.2	3.2	3.5	2.8	3.1	2.2	2.1	2.0
	18	OPF C1	-	nd	-	-	-	-	-	-	2.6	2.6	-	-	2.1	2.5	2.1	-†		
	4	Faeces I	-	-	-	-	-	-	-	3.3	4.1	5.0	6.1	6.3	7.2	7.6	6.6	6.9	6.5	5.7
4	4	OPF I	-	-	-	-	-	-	2.2	2.7	3.7	3.7	4.3	4.4	5.7	6.1	6.7	6.7	6.6	6.9
	19	OPF C1	-	nd	-	-	-	-	-	-	-	-	-	-	2.2	2.8	3.1†			
	5	Faeces I	-	-	-	-	-	3.3	4.2	4.6	5.3	5.2	6.2	4.9	4.6	4.3	5.8	4.0	4.4	4.7
5	5	OPF I	-	-	-	2.0	2.8	2.9	3.9	4.1	4.9	4.9	5.2	5.3	4.6	4.6	5.1	4.2	4.1	3.3
	20	OPF C1	-	nd	-	-	-	-	-	2.7	2.2	2.2	3.2	2.7†						
	6	Faeces I	-	-	-	-	2.8	3.3	5.0	5.5	6.3	4.6	-	-	nd	-	-	-	-	-
6	6	OPF I	-	-	-	2.5	3.6	4.3	4.4	4.4	4.6	4.6	3.7	3.1	2.7	2.8	2.8	2.3	2.7	2.4
	21	OPF C1	-	nd	-	-	-	-	-	2.0	2.2	2.2	2.0	-	1.8	1.8	-	-	-	-†
	7	Faeces I	-	-	-	-	-	3.0	3.4	4.7	5.5	4.8	5.4	nd	3.8	3.0	-	-	-	-
7	7	OPF I	-	-	-	-	2.7	3.3	3.7	4.1	4.1	5.2	4.3	4.0	3.5	2.7	2.8	3.0	2.9	2.5
	22	OPF C1	-	nd	-	-	-	-	-	-	-	2.2	2.6	1.8	2.2	2.6†				
	8	Faeces I	-	-	-	-	-	3.3	3.2	4.1	5.1	6.0	5.9	6.8	6.9	7.6	7.5	6.8	7.5	7.3
8	8	OPF I	-	-	-	-	2.6	2.6	4.5	4.7	4.5	4.6	4.9	5.8	6.2	6.6	6.9	5.3	5.9	6.4
	23	OPF C1	-	nd	-	-	-	-	-	1.8	2.0	2.1	2.2	3.1†						
	9	Faeces I	-	-	-	-	-	2.7	3.2	3.7	5.0	5.1	5.4	4.5	5.5	3.2	3.0	2.6	-	-
9	9	OPF I	-	-	-	-	1.7	2.6	3.5	4.2	4.3	4.4	4.6	4.9	4.8	4.1	3.5	3.2	3.2	3.5
	24	OPF C1	-	nd	-	-	-	-	-	-	-	-	2.2	1.8	2.1	1.8	-	1.9	2.6	1.8
	10	Faeces I	-	-	-	-	-	-	-	4.1	4.1	5.3	5.8	6.2	nd	5.9	5.2	4.3	4.7	-
10	10	OPF I	-	-	-	-	-	1.5	2.8	3.9	3.7	4.1	3.9	4.5	4.8	4.5	4.2	3.1	3.1	3.3
	25	OPF C1	-	nd	-	-	-	-	-	-	-	2.1	2.5	2.8	5.0†					
	11	Faeces I	-	-	-	-	3.0	3.8	4.4	5.1	6.0	5.9	5.1	4.6	3.3	2.6	-	-	-	-
11	11	OPF I	-	-	-	2.5	3.0	3.3	4.3	4.6	5.0	5.2	5.0	4.2	3.5	2.9	3.3	2.8	2.9	2.8
	26	OPF C1	-	nd	-	-	-	-	-	-	2.3	2.1	2.6	1.8	2.3	3.0†				
	12	Faeces I	-	-	-	-	-	-	3.3	4.0	4.8	5.5	6.2	8.0	6.7	6.7	6.9	6.6	6.7	-
12	12	OPF I	-	-	-	-	-	2.7	3.4	4.3	5.0	4.6	5.0	5.5	5.6	6.0	6.3	6.2	6.1	7.3
	27	OPF C1	-	nd	-	-	-	-	-	1.8	2.2	3.0	2.6	4.0†						
	13	Faeces I	-	-	-	-	2.4	3.6	3.8	4.9	5.0	5.3	4.2	3.9	2.9	3.2	3.1	-	-	-
13	13	OPF I	-	-	-	2.3	2.5	3.6	3.9	4.4	4.3	4.4	4.7	4.4	4.1	4.1	3.9	3.0	3.1	3.8
	28	OPF C1	-	nd	-	-	-	-	-	-	2.0	2.2	2.5	2.3	2.1	2.1†				
	14	Faeces I	-	-	-	-	2.2	3.2	4.3	5.3	6.0	6.5	6.3	7.1	6.7	6.9	6.0	6.1	5.0	-
14	14	OPF I	-	-	-	2.5	3.3	4.2	4.9	4.7	4.8	4.1	5.9	7.0	7.5	7.1	6.7	7.0	7.0	7.0
	29	OPF C1	-	nd	-	-	-	-	-	-	2.1	2.5	2.2	2.9†						
	15	Faeces I	-	-	-	-	-	3.8	3.6	4.7	4.6	4.1	3.1	-	-	-	-	-	-	-
15	15	OPF I	-	-	1.7	2.4	2.9	3.2	4.4	4.7	4.8	4.1	4.1	3.1	3.1	2.9	3.1	3.0	2.3	1.8
	30	OPF C1	-	nd	-	-	-	-	-	1.8	2.3	2.5	-	-	-	-	-	-	-	-†

Grey Virus isolation positive samples.
\- No virus was isolated.
Nd Not determined.
† Last day post-inoculation of sampling due to euthanasia of the pig.

Table 2. qRRT-PCR results ($log_{10}TCID_{50}$ equiv./g or ml) during the second contact period

			Days post-inoculation															
Pair	Pig #	Sample	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
	1	Faeces I	4.1	3.5	2.9	2.9	-	-	-	-	-							
1	1	OPF I	2.2	2.4	2.4	2.2	2.5	-	-	-	-							
	31	OPF C2	-	2.2	1.8	2.0	-	-	-	1.9	-	-[a]	-	-	2.6	3.2	4.6	3.5†
	2	Faeces I	6.5	6.7	6.9	6.7	6.2	6.4	6.6	6.0	6.7							
2	2	OPF I	6.7	6.4	6.9	7.1	7.1	7.6	6.6	7.3	6.6							
	32	OPF C2	-	2.8	3.1	2.6	3.3	2.9	3.2	3.8	4.6†							
	3	Faeces I	-	-	-	-	-	-	-	-	-							
3	3	OPF I	2.0	2.8	2.3	2.3	-	2.3	-	2.0	-							
	33	OPF C2	-	-	-	-	-	-	2.8	2.9	4.1†							
	4	Faeces I	5.7	6.6	6.2	6.7	7.3	6.5	6.7	6.7	5.9							
4	4	OPF I	6.9	7.0	6.6	6.5	6.8	6.8	7.0	7.3	7.1							
	34	OPF C2	-	3.9	5.1	2.9	4.2	3.8	3.9	3.4	4.4†							
	5	Faeces I	4.7	5.3	3.7	4.3	4.8	4.7	3.9	3.7	2.5							
5	5	OPF I	3.3	3.1	3.5	3.4	2.8	3.0	2.9	3.2	2.4							
	35	OPF C2	-	2.9	2.0	1.5	-	-	2.4	2.8	4.0†							
	6	Faeces I	-	-	-	-	-	3.4	-	-	-							
6	6	OPF I	2.4	2.7	-	2.5	-	-	2.4	-	-							
	36	OPF C2	-	-	-	-	1.6	-	-	1.8	2.9†							
	7	Faeces I	-	-	-	-	-	-	-	-	-							
7	7	OPF I	2.5	3.1	2.5	2.5	2.5	-	-	2.3	2.5							
	37	OPF C2	-	-	-	-	-	-	2.2	2.7	4.0†							
	8	Faeces I	7.3	-	6.7	6.7	7.6	7.1	7.1	‡								
8	8	OPF I	6.4	6.1	6.5	6.4	5.9	5.9	6.6	‡								
	38	OPF C2	-	3.4	3.8	3.1	3.3	3.3	2.5	3.2	3.3†							
	9	Faeces I	2.9	-	-	-	-	-	-	-	2.6							
9	9	OPF I	3.5	-	2.7	2.8	2.9	3.0	2.1	3.4	2.9							
	39	OPF C2	-	3.1	-	-	-	-	2.2	3.2	4.0†							
	10	Faeces I	3.8	-	4.0	3.0	3.4	3.4	2.7	2.2	-							
10	10	OPF I	3.3	3.3	3.1	3.1	2.8	2.5	2.2	2.3	2.1							
	40	OPF C2	-	1.9	1.9	2.8	-	-	2.3	2.8	3.1†							
	11	Faeces I	-	-	-	-	-	-	-	-	-							
11	11	OPF I	2.8	2.9	3.1	2.8	2.9	2.7	2.4	2.6	2.4							
	41	OPF C2	-	-	-	-	2.9	2.5	3.3	4.2	4.1†							
	12	Faeces I	6.8	7.0	6.6	6.7	7.9	7.1	‡									
12	12	OPF I	7.3	6.4	6.5	7.0	6.8	6.8	‡									
	42	OPF C2	-	3.8	3.7	3.6	4.2	3.5	3.6	4.2	4.3†							
	13	Faeces I	2.9	-	-	-	-	-	-	-	-							
13	13	OPF I	3.8	3.7	3.0	2.8	2.8	2.5	1.7	2.5	-							
	43	OPF C2	-	-	-	-	-	-	-	2.7	3.8†							
	14	Faeces I	5.8	5.9	7.5	6.5	7.1	7.3	7.3	7.6	8.2							
14	14	OPF I	7.0	6.9	7.2	6.8	7.2	7.0	6.3	6.2	7.0							
	44	OPF C2	-	3.8	3.8	3.1	2.2	4.1	3.9	4.6	4.9†							
	15	Faeces I	-	5.9	-	-	-	-	-	-	-							
15	15	OPF I	1.8	2.5	2.3	1.7	-	-	-	-	-							
	45	OPF C2	-	-	-	-	3.6	2.1	3.4	3.8	3.8†							

Grey Virus isolation positive samples.
\- No virus was isolated.
[a] To confirm infection, this pig was housed in a separate room until day 32 post-inoculation.
† Last day post-inoculation of sampling due to euthanasia of the pig.
‡ Day post-inoculation the pig was found dead.

Table 3. qRRT-PCR results ($log_{10}TCID_{50}$ equiv./g or ml) during the third contact period

			Days post-inoculation																
Pair	Pig #	Sample	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42
	1	Faeces I	-	-	-	-	-	-	-	-	-	-†							
1	1	OPF I	2.0	1.7	-	-	-	-	2.8	-	2.0	-†							
	46	OPF C3	-	-	-	-	-	-	2.5	3.8	3.8	3.7†							
	2	Faeces I	5.3	5.9	6.0	5.6	6.4	6.0	6.8	6.0	5.8	5.6†							
2	2	OPF I	7.0	7.0	6.8	7.5	7.4	6.8	6.9	7.1	6.6	6.4†							
	47	OPF C3	-	3.3	4.3	3.1	4.5	4.6	5.1†										
	3	Faeces I	-	-	-	-	-	-	-	-	-	-†							
3	3	OPF I	2.6	2.2	2.1	-	2.5	2.0	-	-	-	3.3†							
	48	OPF C3	-	-	-	-	-	-	-	2.9	3.8	3.3†							
	4	Faeces I	5.9	6.8	7.2	7.4	6.8	6.3	6.0	5.6	6.3	5.4†							
4	4	OPF I	7.3	7.2	7.0	6.9	7.8	7.0	7.8	7.4	7.4	7.2†							
	49	OPF C3	-	4.6	3.7	3.3	6.0	4.9	3.8†										
	5	Faeces I	-	-	-	-	-	-	-	-	2.4	-†							
5	5	OPF I	2.5	2.3	2.3	-	1.6	3.2	1.8	2.6	2.6	1.9†							
	50	OPF C3	-	-	-	-	-	2.7	2.5	2.9	3.9	4.2†							
	6	Faeces I	-	-	-	-	-	-	-	-	-	-†							
6	6	OPF I	-	2.0	-	-	-	-	-	-	-	3.4†							
	51	OPF C3	-	-	-	-	-	-	-	2.5	3.2	3.6†							
	7	Faeces I	-	-	-	-	-	-	-	-	-	-†							
7	7	OPF I	1.5	1.7	1.8	-	2.8	-	-	1.7	-	2.8†							
	52	OPF C3	-	-	-	-	-	1.7	3.2	3.4	3.9	4.2†							
	8	Faeces I																	
8	8	OPF I																	
	53	OPF C3	-	2.2	2.2	1.9	2.3	-	1.9	2.3	2.8	3.0†							
	9	Faeces I	-	-	-	-	-	-	-	-	-	-†							
9	9	OPF I	2.0	2.0	1.9	2.2	1.8	1.9	2.0	2.9	-	2.4†							
	54	OPF C3	-	-	-	-	-	-	2.3	2.9	3.2	3.7†							
	10	Faeces I	-	-	-	-	2.6	-	-	-	-	-†							
10	10	OPF I	2.7	2.5	-	1.9	2.8	2.3	-	-	2.2	1.8†							
	55	OPF C3	-	-	-	-	-	2.3	-	1.7	2.2	-	-[a]	-	-	-	-	-	-†
	11	Faeces I	-	-	-	-	-	-	-	-	-	-†							
11	11	OPF I	2.3	-	-	1.9	2.0	2.1	-	2.2	-	-†							
	56	OPF C3	-	-	-	-	-	-	-	-	-	-	-[a]	-	-	-	-	-	-†
	12	Faeces I																	
12	12	OPF I																	
	57	OPF C3	-	2.3	2.3	2.2	-	-	-	-	2.0	1.7	-[a]	-	-	1.7	-	-	2.5†
	13	Faeces I	-	-	-	-	-	-	-	-	-	-†							
13	13	OPF I	-	2.3	2.1	1.8	-	-	1.9	-	-	2.4†							
	58	OPF C3	-	-	-	-	-	-	2.5	2.9	3.5	4.4†							
	14	Faeces I	6.5	7.1	7.2	6.4	6.3	6.5	6.8	6.3	6.4	5.0†							
14	14	OPF I	7.3	6.6	6.8	6.6	7.0	7.1	6.9	7.1	7.6	7.0†							
	59	OPF C3	-	4.0	3.9	3.7	3.0	3.2	3.8†	†									
	15	Faeces I	-	-	-	-	-	-	-	-	-	-†							
15	15	OPF I	-	1.6	-	-	-	-	-	1.8	-	-†							
	60	OPF C3	-	-	-	-	-	-	2.1	2.9	3.9	4.2†							

Grey Virus isolation positive samples.
- No virus was isolated.
[a] To confirm infection, pigs were housed alone until day 42 post-inoculation.
† Last d.p.i of sampling due to euthanasia of the pig.

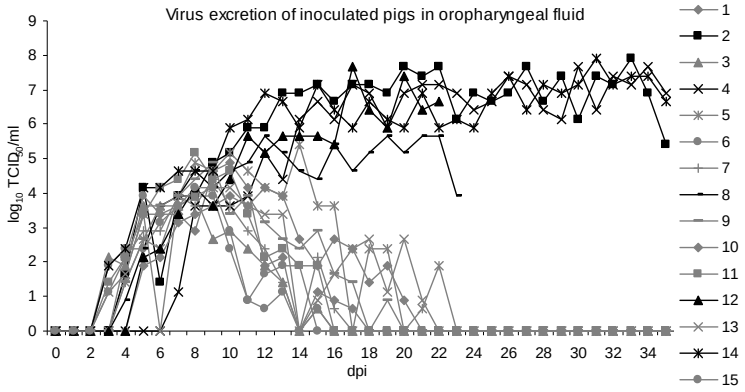

Figure 1. Infectious virus excretion of inoculated pigs in oropharyngeal fluid. High excreting pigs are indicated with black lines, low excreting pigs with grey lines.

Infection of contact pigs

The first contact pigs (C1) became positive in the qRRT-PCR on oropharyngeal fluid between days 7 and 12 p.i. (Table 1). These pigs were removed from the pens based on the number of samples positive in succession and the level of the qRRT-PCR titre (at least 3 days in a row positive and of all positive samples at least two with a viral RNA titre higher than 2.5 $\log_{10}$ $TCID_{50}$ equivalents/ml, or at least 5 days in a row positive). None of the contact pigs showed fever or clinical symptoms (Table 4), and most pigs did not excrete

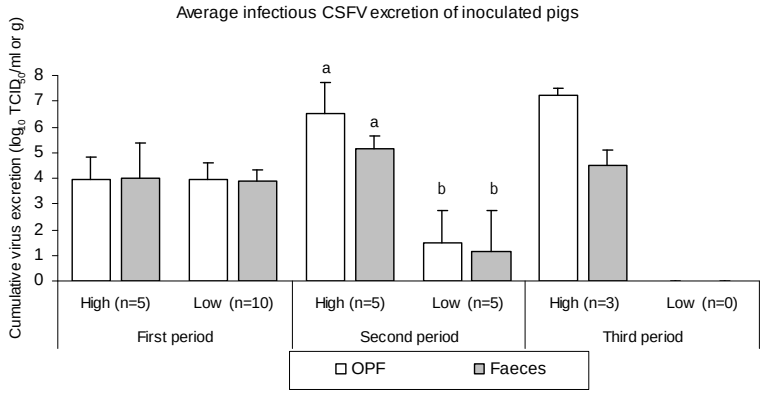

Figure 2. Average total infectious CSFV excretion (area under the curve [AUC]) in oropharyngeal fluid (OPF) and faeces of inoculated pigs, as determined by VI, from the first day of virus excretion in that period up to the day the contact pig was positive in the qRRT-PCR. The experiment was divided in three periods in which the inoculated pigs had contact with a different susceptible pig. The first period was from day 1-17 p.i, the second from day 17-26 p.i. and the third from day 26-35 p.i. The inoculated pigs were divided into “high excreting pigs” and “low excreting pigs”. a,b: Bars of the same colour with no common letter differ significantly (p<0.05). Error bars represent the standard deviation.

infectious virus at that moment. This was desired to avoid infection of C2 pigs by virus produced by the C1 pigs. Although all C1 pigs were expected to be infected based on the positive qRRT-PCR results on oropharyngeal fluid samples, they were not all positive in the RT-PCR on the tonsil and blood at the moment of euthanasia. For that reason, the C2 pigs were kept in the experiment until one day before the new contacts were added (day 25 p.i). At this moment, all infected pigs were expected to be identified, or, in case this was not certain, they were housed alone for 7 more days in a separate room of the isolation unit.

Table 4. Clinical symptoms, blood parameters, serology, VI and qRRT-PCR results

Pair	Pig # [a]	CS[b]	Fever[c]	Leukopenia[d]	ELISA[e]	VI leukocytes[f]	PCR tonsils*	PCR blood*
1	1 (I)	yes	yes	yes	18	6 - 12	+	-
	16 (C1)	no	no				+	-
	31 (C2)	no	no				+	+
	46 (C3)	no	no				+	+
2	2 (I)	yes	yes	yes	20**	6 - 35	+	+
	17 (C1)	no	no				-	-
	32 (C2)	yes	no				+	+
	47 (C3)	no	no				+	+
3	3 (I)	no	yes	yes	14	6 -12	+	+
	18 (C1)	no	no				-	-
	33 (C2)	no	no				+	+
	48 (C3)	no	no				+	+
4	4 (I)	yes	yes	yes	20	6 -35	+	+
	19 (C1)	no	no				-	-
	34 (C2)	no	yes				+	+
	49 (C3)	no	no				+	+
5	5 (I)	yes	no	yes	16	4 -18	+	+
	20 (C1)	no	no				-	-
	35 (C2)	no	no				+	+
	50 (C3)	no	no				+	+
6	6 (I)	yes	no	yes	18	6 - 14	+	-
	21 (C1)	no	no				+	-
	36 (C2)	no	no				+	+
	51 (C3)	no	no				+	+
7	7 (I)	no	yes	yes	18	6 - 12	+	+
	22 (C1)	no	no				-	-
	37 (C2)	no	no				+	+
	52 (C3)	no	no				+	+
8	8 (I)	yes	yes	yes	14	6 -22 ‡	+	-
	23 (C1)	no	no				-	-
	38 (C2)	no	no				+	+
	53 (C3)	no	no				+	+
9	9 (I)	no	no	yes	14	4 - 12	+	-
	24 (C1)	no	no				+	+
	39 (C2)	no	no				+	+
	54 (C3)	no	yes				+	+
10	10 (I)	yes	no	no	16	4 - 14	+	+
	25 (C1)	no	no				-	-
	40 (C2)	no	no				+	+
	55 (C3)	no	no				-	-

Table 4. Continued.

Pair	Pig # [a]	CS[b]	Fever[c]	Leukopenia[d]	ELISA[e]	VI leukocytes[f]	PCR tonsils*	PCR blood*
11	11 (I)	yes	yes	no	14	4 - 12	+	+
	26 (C1)	no	no				+	+
	41 (C2)	no	yes				+	+
	56 (C3)	no	no				-	-
12	12 (I)	yes	yes	yes	18	6 -22 ‡	+	-
	27 (C1)	no	no				-	-
	42 (C2)	no	yes				+	+
	57 (C3)	no	no				-	+***
13	13 (I)	no	no	yes	16	6 - 16	+	+
	28 (C1)	no	no				+	-
	43 (C2)	no	no				+	+
	58 (C3)	no	no				+	+
14	14 (I)	yes	yes	yes	18**	4 - 35	+	+
	29 (C1)	no	no				-	-
	44 (C2)	yes	no				+	+
	59 (C3)	no	no				+	+
15	15 (I)	no	no	yes	12	6 - 12	+	-
	30 (C1)	no	no				-	+
	45 (C2)	no	no				+	+
	60 (C3)	no	no				+	+

[a] Inoculated (I), first, second or third contact pig (C1, C2, C3).
[b] Clinical symptoms: clinical scores >2.
[c] Body temperature for 2 or more consecutive days >40℃.
[d] Leukopenia: $<10*10^9$ leukocytes/L blood.
[e] Day post-inoculation of first positive ELISA on serum.
[f] Interval in days post-inoculation of virus isolation positive leukocytes.
* At moment of euthanasia.
** Intermittently detected.
*** Two days prior to euthanasia.
‡ Last day post-inoculation of sampling due to death.

It was observed during the second period that the C2 pigs, housed with pigs that were still excreting infectious virus on day 17 p.i., were qRRT-PCR positive one day later (day 18 p.i.), except for pig number 43 (Table 2). Of these pigs, the ones housed with the high excreting pigs remained positive in the qRRT-PCR for several days in a row until they also started excreting infectious virus. The other pigs were positive in the qRRT-PCR intermittently, but all started excreting infectious virus, although this could take 11 days (pig number 31). The C2 pigs housed with the low excreting pigs (numbers 3, 6, 7, 11 and 15) that were not excreting infectious virus anymore at day 17 p.i. were all qRRT-PCR positive from day 21 p.i. All C2 pigs were positive in the RT-PCR on the tonsil and blood at the day of euthanasia. They were removed from the pens on day 25 p.i., and the C3 pigs were housed with the inoculated pigs from day 26 p.i. During these 24 h, virus excreted by C2 pigs that was not removed, was expected to be inactivated (Dewulf et al., 2002; Ribbens et al., 2004; Weesendorp et al., 2008). In two pens where the inoculated pig had died, C3 pigs (numbers 53 and 57) were housed alone to study the infectiousness of the environment. For pigs housed with the high excreting pigs (numbers 47, 49 and 59), the results were comparable as for the C2

pigs housed with the high excreting pigs (Table 3). One day after housing with the inoculated pig, they were positive in the qRRT-PCR. The pigs housed with the low excreting pigs became positive in the PCR between days 31 and 33, except for one pig (number 56), which was not positive in the qRRT-PCR at any time point. This pig was also not positive in the RT-PCR on the tonsil or blood at the moment of euthanasia, so it remained uninfected. The two pigs that were housed alone in the pen of the inoculated pig that died were qRRT-PCR positive one day later, but showed an intermittent pattern in qRRT-PCR test results.

Quantification of virus transmission

Based on the excretion patterns of the inoculated pigs, and the infection dynamics of the contact animals, we divided the pairs in three different groups: 1) contact with high excreting pigs, 2) contact with low excreting pigs, and 3) contact with a CSFV contaminated environment. The last group was included when pigs were housed with an inoculated pig that had died or did not excrete infectious virus during the contact period, while the contact animals became infected. In the first contact period, it is shown that the transmission rate β (Table 5) was not significantly different between the high and low excreting pigs. In the second contact period, the transmission rate β of the high excreting pigs was higher than of the low excreting pigs. The high excreting pigs infected their contacts within the first 24 h of the contact period, and the transmission rate β was estimated infinite. This was, however, not significantly higher than the transmission rate β of the low excreting pigs. In this part of the experiment there were some contact pigs infected that were housed with an inoculated pig that was not detected to be excreting infectious virus anymore. This group of pigs were expected to be infected by a CSFV contaminated environment, and the moment of contact with infectious virus was set in the model from the first day of housing these pigs together with the inoculated pig. The transmission rate β of this group was significantly lower than the high excreting pigs but not significantly different from the low excreting pigs. In the third contact period, none of the low excreting pigs did excrete detectable levels of infectious virus. The contact pigs infected were classified in the group environment, which had a significantly lower transmission rate β than the high excreting pigs.

The infectious period of the low excreting group was 14.7 (13.2-16.3) days with a standard deviation of 2.2 (1.5-3.7) days. This period was significantly (p=0.004) shorter than the infectious period of the high excreting group of 32.5 (21.2-51.8) days with a standard deviation of 11.4 (5.2-23.8) days. Also the basic reproduction ratio R_0 (assuming an 'average' transmission rate) differed significantly between the groups (p=0.002). The estimated R_0 was 23.1 (11.5-45.0) for the low excreting pigs and 143 (56.3-373) for the high excreting pigs.

Table 5. Quantification of transmission rate β

	Contact period					
Group	First (day 1-17 p.i.)		Second (day 17-26 p.i)		Third (day 26-35 p.i.)	
High excreting pigs	1.56	(0.437-5.98)	∞^{a}	(2.29-∞)	∞^{a}	(1.50-∞)
Low excreting pigs	1.15	(0.499-2.66)	2.54^{ab}	(0.870-5.94)	-	
Environment	-		1.14^{b}	(0.348-3.50)	0.612^{b}	(0.306-1.11)

[a-b] Transmission rates β within columns with no common superscript differ significantly (p<0.05)

Discussion

Infection with moderately virulent CSFV strains can lead to different courses of the disease, either (sub)acute disease, resulting in death or recovery, or chronic disease, which is always fatal (Van Oirschot, 1988; Floegel-Niesmann et al., 2003). The virus excretion dynamics between these courses differ largely (Weesendorp et al., 2009b). The present study has shown that differences in clinical course and virus excretion are also associated with differences in transmission. Pigs that developed the chronic form of infection or die from the infection in the (sub)acute phase (high excreting pigs) had a significantly higher basic reproduction ratio R_0 (143 [56.3-373]) than pigs that recovered from the infection (low excreting pigs, 23.1 [11.5-45.0]).

Differences in the reproduction ratio R_0 between high and low excreting pigs were the result of differences in both the length of the infectious period and the height of the transmission rate β. The infectious period of the low excreting pigs was on average 14.7 days, which was significantly shorter than the infectious period of the high excreting pigs of 32.5 days. This difference has been observed in experiments before (Uttenthal et al., 2003; Weesendorp et al., 2009b). The differences between high and low excreting pigs in transmission rate β depended on the phase of the infection. In the early phase of the infection, comparable transmission rates were observed between high and low excreting pigs. In the second and third phase of the infection, all high excreting pigs infected their contacts within 24 h, which resulted in higher transmission rates than of the low excreting pigs. However, this difference was not significant, possibly due to the small number of pairs in each group. Therefore, studies including more pairs are necessary to confirm these observation.

This study showed that R_0 values differ between pigs infected with the same virus strain that develop different courses of the infection, thus equal infectiousness for all infected animals is questionable. This has some consequences for the interpretation of data from previous transmission experiments. In these experiments, the transmission dynamics of moderately virulent strains like the Paderborn strain were quantified by inoculating three to five pigs and subsequently study the virus transmission to contact pigs within the same pen (Laevens et al., 1998; Laevens et al., 1999; Uttenthal et al., 2001; Durand et al., 2009; Weesendorp et al., 2009a). A SIR (Klinkenberg et al., 2002; Durand et al., 2009) or SEIR model (Weesendorp et al., 2009a) was used to quantify the transmission. In these transmission models it is assumed that all infected animals are equally infectious, and as a consequence, they contribute equally to infection of the contact pigs. Since infection with the Paderborn strain can result in different clinical course of disease with significant differences in virus excretion and reproduction ratio R_0, the presence of only a few high excreting pigs in the experiment will largely influence the transmission, and will result in an incorrect estimation of the R_0.

Another important observation in this study is the course of the transmission rate β over time. Although not significant, the transmission rates in this first phase tended to be lower than in the second and third (for high excreting pigs) phase of the infection. In most transmission experiments, the inoculated pigs are housed after a defined period, mostly 24 h, with the contact pigs. In case of increasing transmission rates over time, the results of these previous studies do not provide reliable transmission data for the

entire infectious period of an infected pig. Since transmission experiments are often used to study the effect of control measures like vaccination on transmission during outbreaks (Uttenthal et al., 2001; Klinkenberg et al., 2003; Dortmans et al., 2008), this should be considered, because the transmission could be underestimated.

The infectious period of pigs, which is used to quantify transmission, is based on virus isolation data. We observed, however, that contact pigs became infected while they were housed with an inoculated pig that excreted viral RNA, but infectious virus could not be detected. There are several possible explanations for this. Firstly, the contact pigs were infected by direct contact with the inoculated pig, but infectious virus was not detected because the detection limit of the virus isolation assay was too high, or the presence of neutralizing antibodies that masked infectivity in the cell culture assay. It has been demonstrated before that pigs are more sensitive than tissue culture cells for detecting infectious CSFV (Stewart et al., 1979).Therefore, as an alternative, the infectious period could be based on PCR data. The transmission rates β are then quite comparable to transmission rates based on VI data (for low excreting pigs for the first, second and third contact period respectively 0.985 [0.441-2.13], 1.62 [0.709-3.46] and 0.565 [0.260-1.09], and for high excreting pigs respectively 1.21 [0.371-3.66], ∞ [2.29-∞] and ∞ [1.5-∞]). Since the differences between VI and PCR in transmission rates β are small, the use of VI data was preferred given the uncertainty of PCR data regarding infectiousness. Another possible explanation why the contact pigs became infected that were housed with an inoculated pig that did not excrete infectious virus, was because of a CSFV contaminated environment. Virus could have been emitted in the air by high excreting pigs housed in other pens (Dewulf et al., 2000; Durand et al., 2009), or virus could have survived in the pen after it was excreted by the inoculated pig or a previous contact pig. This is likely to have occurred in some cases, because C3 pigs, that were housed alone in the pen due to death of the inoculated pig, became infected. This infection could only have originated from the environment

To quantify the transmission rate β, the moment of infection of the contact pigs needed to be determined. Therefore, we used the data of the qRRT-PCR on oropharyngeal fluid. This seems biologically most appropriate, as the virus replicates first in the tonsil and pharyngeal region, after which it spreads throughout the body. We observed, however, that some contact pigs were qRRT-PCR positive at several moments in oropharyngeal fluid, but not in the tonsil or blood at the moment of euthanasia. We expected those pigs to be infected, but the moment of euthanasia was too early to detect viral RNA in the tonsil, or the detection limit of the qRRT-PCR on tonsils was too high. Furthermore, the moment of euthanasia was too early to detect antibodies, since it took 12 to 20 days to detect antibodies in serum of the inoculated pigs.

The duration of the period between infection and becoming qRRT-PCR positive in oropharyngeal fluid (latent PCR period) seems to vary largely, depending on the amount of virus the pig is exposed to. The intranasal administration of 10^5 $TCID_{50}$ in one dose resulted in latent PCR periods of 3 to 7 days. Contact with a high excreting pig, resulted in maximum latent PCR periods of 24 h. This is probably due to contact with a higher dose and for a longer duration, which results in more infected cells, and

therefore a faster distribution of the virus throughout the body. This is in contrast to the susceptible pigs that were in contact with lower virus doses. The PCR on oropharyngeal fluid, although sometimes also positive within 24 h (some C2 pigs), showed intermittently positive results over time. In most cases, the contacts of high excreting pigs were also infectious sooner than contacts of low excreting pigs, which will result in faster transmission in a subsequent generation.

Several studies suggested the important role chronically infected pigs can play during an outbreak, as they excrete high amounts of virus for a long duration (Harkness, 1985; Vandeputte and Chappuis, 1999; Weesendorp et al., 2009b). The present study provides even stronger evidence for the role of both chronically infected pigs and pigs that die in the acute phase, in the spread of CSFV during an outbreak, since they have a significantly higher reproduction ratio R_0 than pigs that recover from the infection. Furthermore, a trend is observed with higher transmission rates at the moments that these pigs are at a plateau level of virus excretion, than during the initial phase of virus excretion. These differences in transmission rates over time could have resulted in an underestimation of transmission rates in previous studies (Klinkenberg et al., 2003; Durand et al., 2009; Weesendorp et al., 2009a).

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Chapter 8

Risk model for time-dependent infection probability of classical swine fever via excretions and secretions

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Abstract

Several routes contribute to the spread of classical swine fever (CSF), during outbreaks of this disease. However, for many infected herds in recent epidemics, no route of virus introduction could be indentified. Several studies tried to elucidate the routes of transmission into these herds, but they were only partly successful. To obtain more insight into the relative importance of transmission routes, a risk model was developed to study transmission from one infectious pig to one susceptible pig, using quantitative data on virus excretion from infected pigs, survival of virus and susceptibility of pigs. The model estimated the relative contribution of secretions and excretions to spread of the disease, and the probability of infection of a susceptible animal via these secretions and excretions. Results indicated that virus strains differed with respect to the time-dependent infection probabilities and the relative contribution of secretions and excretions to transmission. This supports the statement that during outbreaks control measures should ideally be based on the characteristics of the specific virus strain involved, which implies the development of tailor-made measures. This model could help to provide more insight into the effect of these specific control measures and ultimately allow for quantitative evaluation of such measures.

Introduction

Classical Swine Fever (CSF) is a highly contagious viral disease that affects both domestic pigs and wild boar. In the recent past, several outbreaks of CSF in Europe occurred that were difficult to control, especially in areas with a high pig density. During these outbreaks, CSF virus (CSFV) was spread within- and between farms through direct contact between infected and susceptible pigs, and via indirect transmission routes, like swill feeding (Williams and Matthews, 1988; Farez and Morley, 1997; Fritzemeier et al., 2000; Sharpe et al., 2001), artificial insemination (De Smit et al., 1999; Floegel, 2000; Hennecken et al., 2000), or contaminated mechanical vectors like clothing and footwear or livestock trucks (Terpstra, 1988; Stegeman et al., 2002; Ribbens et al., 2004; Ribbens et al., 2007). However, in many cases, no transmission route could be identified (approximately 50% of the cases during the 1997-1998 outbreak in the Netherlands) (Elbers et al., 1999; Fritzemeier et al., 2000; Allepuz et al., 2007). Because most of these infected herds were situated close to herds infected earlier in time, they were called neighbourhood infections (Elbers et al., 1999; Elbers et al., 2001). Subsequent studies on transmission routes involved in these neighbourhood infections, using questionnaires or by analysing outbreak data (Elbers et al., 2001; Crauwels et al., 2003; Mintiens et al., 2003), were only partly successful in elucidating

specific routes and their relative importance. As a consequence, specific measures to control neighbourhood infections can not be quantified and evaluated and are therefore difficult to implement.

However, several studies indicated that distance is an important variable in neighbourhood spread with the probability of a neighbourhood infection decreasing with increasing distance to the primary infected herd (Koenen et al., 1996; Boender et al., 2008). Measures to control CSF outbreaks are to a large extent based on this relationship, for instance, preventive depopulation of herds or emergency vaccination in a certain radius around infected farms (Nielen et al., 1999; Mangen et al., 2001; Backer et al., 2009). The application of these control measures has far-reaching consequences, both ethically, because of mass destruction of uninfected pigs due to the preventive depopulation, and economically, because of prolonged trade restrictions due to vaccination (Meuwissen et al., 1999; Boklund et al., 2008). Application of such measures should therefore ideally be avoided.

Studies on transmission routes during outbreaks often apply top-down approaches, using available outbreak data to define important contacts between herds (Elbers et al., 2001; Stegeman et al., 2002). However, also bottom-up approaches to study transmission routes between herds can be used (Ribbens, 2009). Such an approach would use quantitative data on underlying mechanisms of transmission, building a model that starts on the level of transmission from one infectious pig to one susceptible pig, ultimately reaching the level of between herd transmission.

In the present study, a model was constructed to study transmission from one infectious pig to one susceptible pig, using quantitative data on virus excretion from infected pigs, survival of virus and susceptibility of pigs. The model estimated the relative contribution of secretions and excretions to spread of the disease, and the probability of infection of a susceptible animal via these secretions and excretions. This model can be used to quantify transmission via (indirect) transmission routes to provide insight into the importance of the different routes.

Model description

A risk model was constructed in Microsoft Excel 2003 (Microsoft Corporation, Redmond, WA, USA) and @Risk 4.5.3 (Palisade, 2004) to estimate the daily probability that a susceptible pig is infected with CSFV via virus excreted in different excretions and secretions of one infected pig. The basic steps of microbial risk assessment (MRA) have been applied, i.e., exposure assessment, hazard characterisation, and risk characterisation (Anonymous, 1999; Haas et al., 1999). An outline of the model is presented in Figure 1. Experimental data (Bouma et al., 2000; Uttenthal et al., 2001; Klinkenberg et al., 2003; Wieringa-Jelsma et al., 2006; Weesendorp et al., 2008a; Weesendorp et al., 2008b; Weesendorp et al., 2009a; Weesendorp et al., 2009b; Weesendorp et al., 2009c) have been used for the exposure assessment and hazard characterisation. Model calculations were performed for three CSF virus strains that differ in virulence, i.e. the highly virulent strain Brescia, the moderately virulent strain Paderborn and the low virulent strain Zoelen. Virus excretion was evaluated in the following excretions and secretions: blood, saliva, conjunctival fluid, nasal fluid, faeces,

and urine. Furthermore, the probability of CSF infection via virus emitted in the air was evaluated.

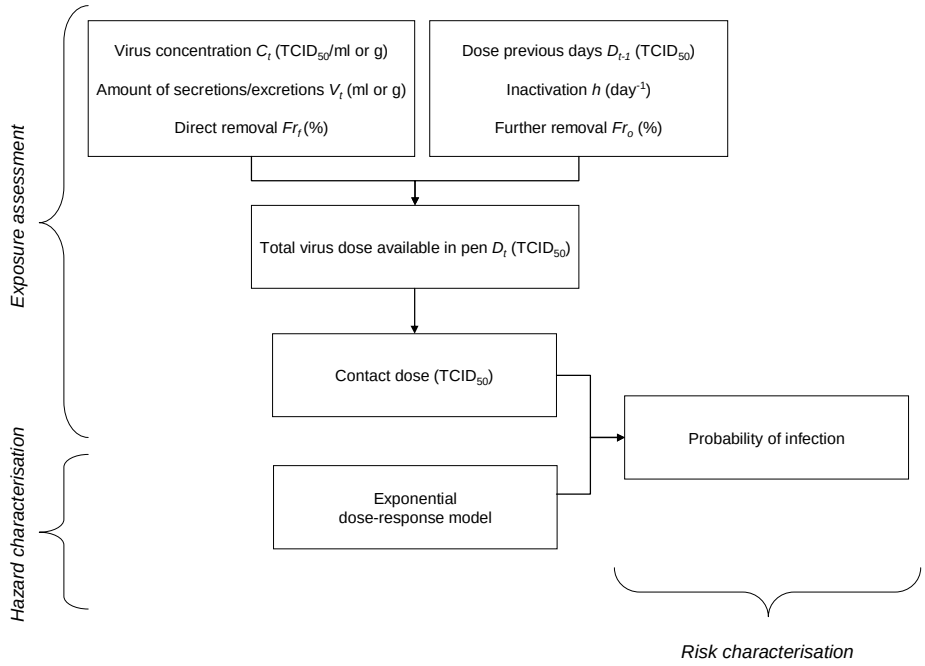

Figure 1. Schematic representation of the risk model.

Exposure assessment: time-dependent concentration of virus in secretions and excretions

Experimental data (Weesendorp et al., 2009b) were used to estimate the daily virus concentration in all secretions and excretions except air. For each virus strain, data were available from five pigs. The Paderborn-infected pigs were subdivided into two groups: low excreting pigs (pigs that recover after the acute phase of illness with low levels of virus excretion [n=3]) and high excreting pigs (pigs that die during the acute phase or pigs that are chronically infected, which results in death 30 or more days after inoculation [n=2]). The time period over which virus excretion was measured depended on the virus strain and clinical form: Brescia 12 days, Paderborn low excreting 48 days, Paderborn high excreting 44 days, and Zoelen 55 days. For each strain the mean and standard deviation of the measured virus concentrations ($\log_{10}$ TCID$_{50}$/ml or g) were calculated per d.p.i.(day post-inoculation). Excretion on days without measurement was calculated by interpolation of individual pig data. In the risk model, virus concentration of each secretion or excretion per d.p.i. was simulated using a normal distribution with the calculated means and standard deviations per d.p.i. as parameters.

Experimental data (Weesendorp et al., 2009c) for virus emitted in the air were available in a different format. Virus concentrations in the air (TCID$_{50}$/m^3) were

measured in rooms housing groups of ten pigs, in which three pigs were inoculated with either the Brescia, Paderborn or Zoelen strain. Results were recalculated to an individual pig level at each sampling moment (d.p.i. 4, 7, 10, 14, 17, 21, 28, and 35) by determining the number of infectious pigs based on virus excretion in oropharyngeal fluid or faeces. A time lag between inoculation and first virus detection in the air was observed, after which virus concentrations were on a rather constant level. The virus concentration in the air was therefore modelled by a triangular distribution taking the minimum observed concentration as minimum value (min), the average observed concentration as most likely value (ml), and the maximum observed concentration as maximum value (max). Three triangular distributions were used: for the Brescia strain from d.p.i. 5 onwards (min=0.7, ml=1.0, max=1.5 $\log_{10}$ $TCID_{50}/m^3$), Paderborn low excreting from d.p.i. 8-14 (min=0.2, ml=0.6, max=1.1 $\log_{10}$ $TCID_{50}/m^3$), and Paderborn high excreting from d.p.i. 8 onwards (min=0.8, ml=1.4, max=2.1 $\log_{10}$ $TCID_{50}/m^3$). No virus was detected in the room housing the pigs infected with the Zoelen strain. Because the ventilation rate in the experimental rooms (400 m^3/h) was high as compared to ventilation rates in conventional pig farming (12-80 m^3/h), virus concentrations in the model were set five times higher than the virus concentrations observed in the experiments (note that the mentioned values for the triangular distributions were thus multiplied by five, which is equal to an addition of 0.7 log $TCID_{50}$; e.g. Brescia min=1.4, ml=1.7, max=2.2 $\log_{10}$ $TCID_{50}/m^3$).

Exposure assessment: amount of secretions and excretions

Experimental data were available to estimate the daily production of faeces and urine (Weesendorp et al., 2009b; Weesendorp, unpublished). For each virus strain the mean and standard deviation of the measured weight of faeces (g) and the measured volume of urine (ml) were calculated per d.p.i. In the risk model, production was simulated by a lognormal distribution with the calculated means and standard deviations as parameters.

For blood, saliva, conjunctival fluid, and nasal fluid the volumes excreted by an infected pig were modelled by uniform distributions. Estimated minimum and maximum volumes were: 0.1-1 ml for blood (oral exposure in bite incidents), 1-10 ml for saliva, 0.1-1 ml for nasal fluid, and 0.05-0.1 ml for conjunctival fluid.

Volumes of air inhaled were based on an average inhalation rate of 15 L/min by pigs weighting 25 kg. The volume of air inhaled per d.p.i. was thus 21.6 m^3.

Exposure assessment: daily virus dose available for contact

The total virus dose available in the pen for contact on each d.p.i was calculated taking into account daily virus excretion, removal of excretions and secretions from the pen, and inactivation of the virus over time. Based on expert estimations, due to insufficient data from experiments and field observations, it was assumed that part of the faeces (50%) and urine (95%) produced would be removed from the pen immediately due to the (partially) slatted floor (in the Netherlands a slatted area of maximum 60% is allowed (Anonymous, 2005)). A further removal up to 99% after 24 h was assumed. For all secretions (saliva, conjunctival fluid, and nasal fluid) it was

assumed that half of the volume (50%) was directly removed and that after 24 h 99% was removed.

Temperature-dependent inactivation of CSF virus in faeces and urine was based on Weesendorp et al. (2008b). Half life-time values (*h*) (day) were estimated as:

$$h = e^{\left(x_0 - x_1 \times t + x_2 \times t^2\right)} \quad (1)$$

with *x0*, *x1*, and *x2* being constants of which the values varied per virus strain and excreta (Table 1), and *t* is temperature (℃). No temperature-dependent half- life time values were estimated for Zoelen, because no virus had been detected in the faeces and urine of Zoelen-infected pigs.

Table 1. Values of half-life time functions for inactivation of CSF virus in faeces and urine (source: Weesendorp et al., 2008).

	Virus strain[a]			
	Brescia		Paderborn	
Parameter[b]	Faeces	Urine	Faeces	Urine
X_0	2.353	1.562	1.9131	1.50826
X_1	-0.1741	-0.2928	-0.1741	-0.2928
X_2	0	0.005337	0	0.005337

[a] No values were estimated for the Zoelen strain, because no virus had been detected in the faeces and urine of Zoelen-infected pigs.

[b] x_0, x_1, and x_2 are constants, used to estimate the half life-time values (h) in $h = e^{\left(x_0 - x_1 \times t + x_2 \times t^2\right)}$. In this formula t is the temperature (℃) of the excretions.

Half-life time values of CSF virus in the air were estimated at 0.005 day for Brescia, 0.007 day for Paderborn, and 0.008 day for Zoelen at ambient temperature (Weesendorp et al., 2008a). For all other secretions, half-life time values were estimated at 0.15 day at ambient temperature for all virus strains.

For blood, no removal and inactivation were modelled, because the total volume was assumed to be ingested immediately by the contact pig in, for instance, a biting incident.

The total virus available in the pen per d.p.i (D_t) ($TCID_{50}$) was calculated by summing: (1) the fresh virus excreted minus direct removal of the excretions and secretions (Df_t) ,and (2) the old virus present in the pen, i.e., virus excreted on previous days after partial inactivation and further removal (Do_t).

$$D_t = Df_t + Do_t \quad (2)$$

The fresh virus released (Df_t) was calculated by multiplying the simulated virus concentration (C_t) and volume or weight of the excretion (V_t) on d.p.i t and then subtracting the fraction directly removed (Fr_f):

$$Df_t = C_t \times V_t \times \left(1 - Fr_f\right) \quad (3)$$

The virus load due to old virus present in the pen (Do_t) was based on the total virus dose of the previous day (D_{t-1}), adjusted for the fraction removed after 24 h (Fr_o) and temperature-dependent inactivation of CSF virus based on half-life time (h):

$$Do_t = (1 - Fr_o) \times D_{t-1} \times e^{-\frac{\ln(2)}{h}} \quad (4)$$

For blood and air we assumed that the susceptible pig had contact with all virus available (D_t). For the other secretions and excretions, based on expert estimations, it was assumed that the susceptible pig had contact with 1% of the total virus load. Median total amounts of secretions and excretions available in the pen varied from less than 100 g to more than 500 g. The median amount of secretions and excretions the pig was assumed to ingest thus varied thus from less than 1 g to approximately 5 g and was not more than 10 g (95th percentile values).

Exposure assessment: survival of pigs

Survival of pigs was based on experimental data available (Bouma et al., 2000; Wieringa-Jelsma et al., 2006; Weesendorp et al., 2009a; Weesendorp et al., 2009b). All Brescia-infected pigs died during these experiments. For high excreting Paderborn-infected pigs, only data from pigs that died before the end of these experiments were used. In the model, for each d.p.i. it was sampled whether the pig was still alive or not. For this stochastic process a binomial distribution was used with a daily probability of dying based on experimental data. The uncertainty for this daily probability was modelled by a beta distribution.

Hazard characterisation

Experimental data were available to fit an exponential dose-response model (Haas et al., 1999) for oronasal inoculations with the Brescia and Paderborn strains (Uttenthal et al., 2001; Klinkenberg et al., 2003; Weesendorp et al., 2009a; Weesendorp et al., 2009b; Terpstra, unpublished). Results are given in Table 2. The doses used for intranasal inoculation of pigs with the Zoelen strain ($10^{4.6}$ and 10^5 $TCID_{50}$) resulted in infection of all inoculated pigs (Weesendorp et al., 2009a; Weesendorp et al., 2009b). Only an estimate of the dose-response parameter could be obtained based on these data.

Table 2. Parameters of the exponential dose-response model for the oronasal infection route

Virus strain	ID_{50}[a]	r[b]
Brescia	73	9.46×10^{-3}
Paderborn	139	5.00×10^{-3}
Zoelen	770	9.00×10^{-4}

[a] Dose ($TCID_{50}$) at which 50% of the pigs is infected.
[b] Exponential dose-response parameter.

The time-dependent probability of infection (P_t) was then calculated using the exponential dose-response model:

$$P_t = 1 - e^{-rD_t} \quad (5)$$

with r being the exponential dose-response parameter, and D_t the total virus dose available for contact on d.p.i. t.

Risk characterisation

Results of the risk model gave the time-dependent probability of infection of a single susceptible pig via the different excretions and secretions if in contact with an infected pig. A 10,000 iterations were simulated for each scenario to take into account variability. A fixed seed value was used to avoid variation due to random sampling.

First model results indicated that for several secretions and excretions of the Brescia and Paderborn strain the probability of infection over the entire infectious period is 1. In order to rank the excretions and secretions for their contribution to the probability of infecting a susceptible pig, the total virus dose (D_t) was diluted to such an extent that the individual probabilities for all excretions and secretions were less than 1. Dilutions used were: 10^3 for Brescia, 10^2 for Paderborn low excreting pigs, 10^5 for Paderborn high excreting pigs, and 10^0 (i.e., no dilution) for Zoelen.

Model validation: transmission experiments

Data of fifteen one-to-one experiments (Weesendorp et al., in prep.) were used to validate the model. In each one-to-one experiment, a single pig inoculated with the Paderborn strain (I pig) was housed in a pen. Of the fifteen inoculated pigs, five pigs developed the chronic form of infection or died (d.p.i. 23 and 24) during the acute phase (high excreting pigs) (Table 3). Ten pigs recovered from the infection (low excreting pigs). At d.p.i. 1, 17 and 26, a single contact pig was placed in each pen (C1, C2, and C3 pigs). Each C1 pig that became infected was removed. At d.p.i. 17, all C1 pigs left were removed and replaced by new contact pigs (C2). These pigs were removed at d.p.i. 25. At d.p.i. 26, new contact pigs (C3) were housed with the inoculated pigs until d.p.i. 35. Infection of contact pigs was determined by quantitative real-time reverse transcription polymerase chain reaction (qRRT-PCR) on oropharyngeal fluid samples. Contact pigs were considered to have become infected three days before the first positive PCR sample (latent PCR period), since this was the median delay before inoculated pigs became positive in the PCR in these experiments and a previous experiment (Weesendorp et al., 2009a). Infection could, however, never have occurred before the moment of housing with the inoculated pig. Results of these experiments were used to calculate the probability of infection per d.p.i. by dividing the number of newly infected pigs on a day (s) by the total number of contact pigs (n) (p=s/n). Besides, the risk model was used to estimate the probability of infection of a contact pig with the Paderborn strain, depending on the period (d.p.i) of contact with the inoculated pig.

Conditional probabilities were used, which means that the probability of infection on d.p.i t [$P(I)_t$] is the probability for this day (P_t) multiplied by the probability that this contact pig was not infected on previous days:

Table 3. Results of 15 one-to-one transmission experiments used for model validation

Pair #	Excretion type[a]	Pig[b]	First contact (d.p.i)[c]	First + RT-PCR (d.p.i)[d]	Last contact (d.p.i)[c]
1	Low	C1	1	8	12
		C2	17	18	25
		C3	26	32	35
2	High	C1	1	8	11
		C2	17	18	25
		C3	26	27	32
3	Low	C1	1	8	15
		C2	17	23	25
		C3	26	33	35
4	High	C1	1	12	14
		C2	17	18	25
		C3	26	27	32
5	Low	C1	1	7	11
		C2	17	18	25
		C3	26	31	35
6	Low	C1	1	7	17
		C2	17	21	25
		C3	26	33	35
7	Low	C1	1	9	13
		C2	17	23	25
		C3	26	31	35
8	High (†24)	C1	1	7	11
		C2	17	18	25
		C3	26	27	35
9	Low	C1	1	10	17
		C2	17	18	25
		C3	26	32	35
10	Low	C1	1	9	12
		C2	17	18	25
		C3	26	31	35
11	Low	C1	1	8	13
		C2	17	21	25
		C3	26	ni	35
12	High (†23)	C1	1	7	11
		C2	17	18	25
		C3	26	27	35
13	Low	C1	1	8	13
		C2	17	24	25
		C3	26	32	35
14	High	C1	1	8	11
		C2	17	18	25
		C3	26	27	32
15	Low	C1	1	7	17
		C2	17	21	25
		C3	26	32	35

[a] High excreting pigs: chronically infected/die in the acute phase; low excreting pigs: recover.
[b] First (C1), second (C2) or third (C3) contact pig.
[c] First or last day post-inoculation (d.p.i.) of contact with the inoculated (I) pig.
[d] First + sample in the RT-PCR on oropharyngeal fluid. Infection was expected 3 days earlier.
† D.p.i. of death of inoculated pig.
ni Not infected.

$$P(I)_t = P_t \times \prod_{i=1}^{t-1} (1 - P_i) \quad (6)$$

with P_i being the probability of infection on day i (i=1, 2, …, t-1). Furthermore, in these calculations the virus concentration in the air was set at its original value, since the ventilation rate in the experiments was 400 m^3/h.

Calculations were performed to mimic the experiments and allowed for infection of C1 and C3 contact pigs by all excretions and secretions of the inoculated pigs except blood (C1 and C3). For C2 contact pigs, probabilities of infection were based on all excretions and secretions of the inoculated pigs including blood because fighting occurred to establish the hierarchical order. Infection of C3 contact pigs of low excreting inoculated pigs was furthermore possible via virus excreted by C2 contact pigs that survived in the environment. Besides, model calculations took into account infection probabilities of C2 and C3 contact pigs of low excreting inoculated pigs via virus emitted in the air by a chronically infected pig in an adjacent pen. Virus concentrations were, however, set ten times lower than the default values for virus concentrations in air, because air samples from pens housing low excreting inoculated pigs had ten to twelve-fold lower viral RNA titres than samples from pens housing high excreting inoculated pigs.

Model validation: sensitivity analysis

For further validation of the model, a simple sensitivity analysis changing one input parameter at a time was performed to investigate the impact of uncertain input parameters on the ranking of the different secretions and excretions. The sensitivity analysis was performed for three groups of input parameters of which values were not based on (animal) experiments, i.e., (a) the removal rate of excretions and secretions (Fr_f and Fr_o), (b) the half-life time values of secretions (h), and (c) the volume or weight of secretions (i.e. blood, saliva, nasal fluid, and conjunctival fluid) (V_t.) For each parameter group, two simulation runs were performed with values either set at their minimum level or at their maximum level (Table 4).

Results

Model results

The median values of the total amount of excreted virus over time in the different secretions and excretions for pigs that survive the entire infectious period are shown in Figure 2. The removal of virus due to inactivation or slatted floors is not included in this figure. For Brescia and Paderborn high excreting pigs no excretion data were available from d.p.i. 13 onwards and d.p.i. 45 onwards, respectively, because all animals in the experiment had died by that time.

The highest amounts of virus were produced by high excreting Paderborn-infected pigs, followed by Brescia-infected pigs and low excreting Paderborn-infected pigs. The lowest amounts of virus were produced by Zoelen-infected pigs.

Table 4. Description of the sensitivity analysis

Input parameter		Value		
		Default	Minimum	Maximum
Removal fraction of secretions/excretions				
Immediately (Fr_f)	Faeces, saliva, nasal fluid, conjunctival fluid	0.50	0.20	0.80
	Urine	0.95	0.80	0.99
After 24 h (Fr_o)	Faeces, saliva, nasal fluid, conjunctival fluid	0.99	0.95	0.999
	Urine	0.99	0.95	0.999
Half life-time values (days) (h) [a]				
Brescia	Faeces[a]	0.32	0.14	0.42
	Urine[a]	0.12	0.06	0.61
	Air	0.005	0.003	0.006
Paderborn	Faeces[a]	0.21	0.03	0.30
	Urine[a]	0.11	0.03	0.12
	Air	0.007	0.004	0.008
All strains	Saliva, nasal fluid, conjunctival fluid	0.15	0.05	0.25
Amount of secretions/excretions (g or ml) (V_t) [b]				
All strains	Blood	Uniform	0.1	1
	Saliva	Uniform	1	10
	Conjunctival fluid	Uniform	0.05	0.1
	Nasal fluid	Uniform	0.1	1

[a] No half life-time values included for the Zoelen strain because no virus could be detected in faeces, urine and air from pigs infected with this strain.

[b] Amount of secretions were modelled by uniform distributions. In the sensitivity analysis, the minimum and maximum value of these uniform distributions were used. Faeces and urine were not included because values were based on experimental data (Weesendorp et al., 2009b; Weesendorp, unpublished).

Based on these amounts of virus excreted, death of pigs, removal and inactivation of virus, and the fraction ingested, the probability of infection of a contact pig was calculated with the dose-response model for: (a) all secretions and excretions together, except blood (Figure 3), and (b) separately for the different secretions and excretions (Figure 4, median values presented only). Blood was not included in Figure 3, as it will only affect the probability of infection under specific conditions, i.e., when fighting occurs or when susceptible pigs are in contact with injured animals.

Based on median values, the probability of infection of a susceptible contact pig via all secretions and excretions but blood over the entire infectious period was 1 for all strains with the exception of Zoelen (median probability is 0.08) (Figure 3). The daily probability of infection for the Zoelen strain was much lower than for the other strains. When blood is excluded, the highest probability of infection with this strain was observed at d.p.i. 8 (median=0.02 [5^{th} perc.=$1.3x10^{-4}$, 95^{th} perc.=0.99]). On the other days, the median probability was lower than $2.5x10^{-3}$. For Brescia and Paderborn high excreting pigs the maximum daily median probability for a contact pig to become infected was 1 for all secretions and excretions (Figure 4). These secretions and excretions differed, however, in the moment after inoculation that they can cause infection. For pigs in contact with low excreting Paderborn pigs, larger differences existed between the different secretions and excretions.

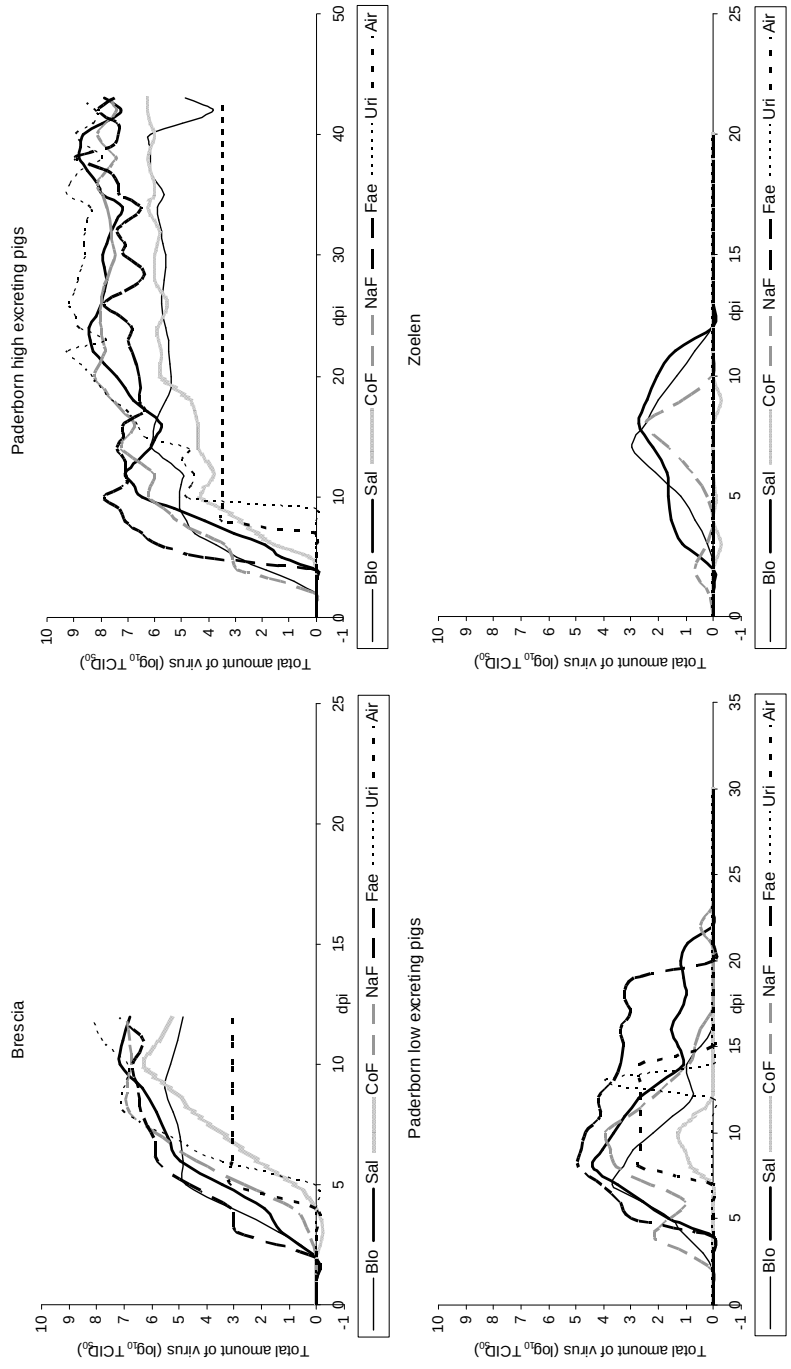

Figure 2. Median values of the total amount of virus excreted per day post-inoculation (d.p.i.) in the different secretions and excretions of pigs infected with strain Brescia, Paderborn high excreting pigs (die from the infection), Paderborn low excreting pigs (recover from the infection) or strain Zoelen. No excretion of virus was modelled beyond d.p.i. 12 for Brescia and beyond d.p.i. 44 for Paderborn high excreting pigs due to lack of experimental data.

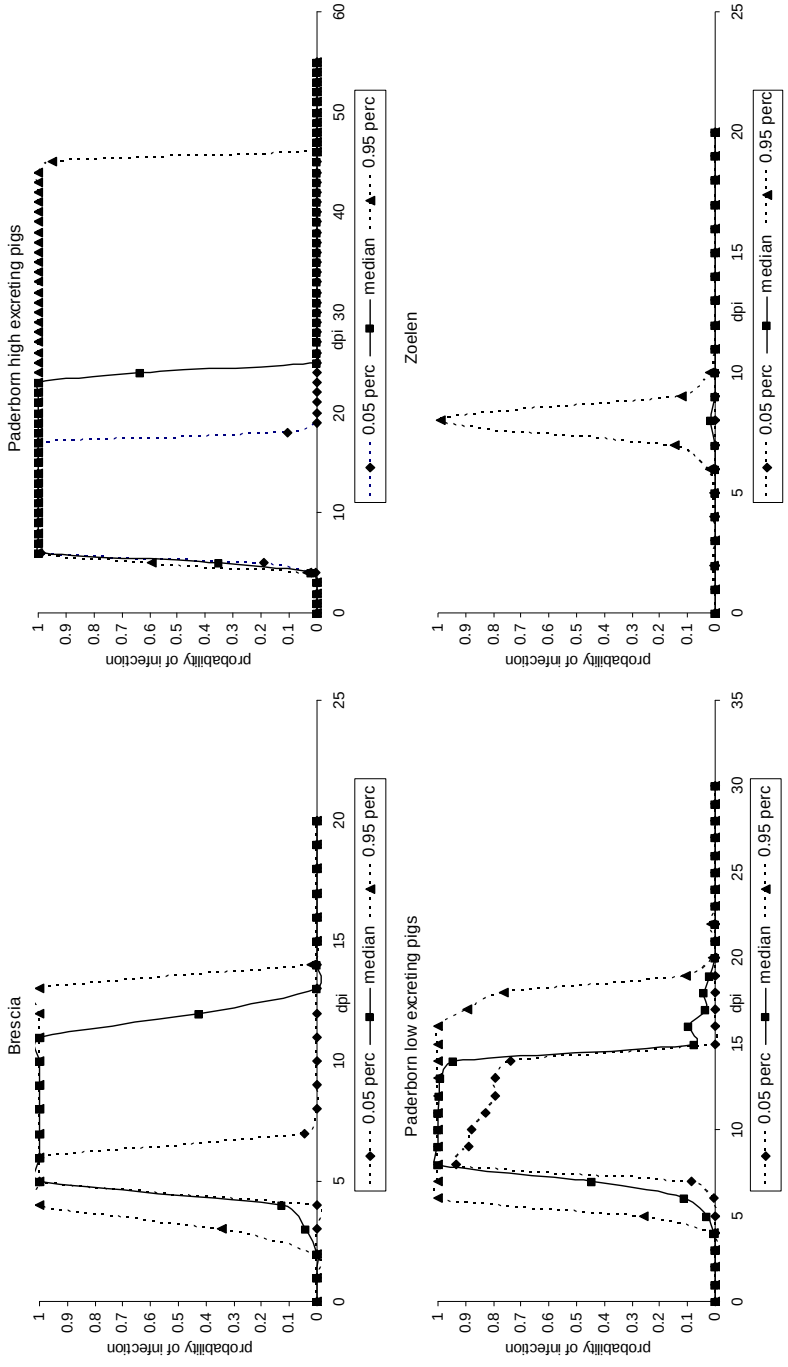

Figure 3. Daily probability of CSF infection via virus excreted by one pig in all different excretions and secretions but blood when infected with strain Brescia, Paderborn high excreting pigs (die from the infection), Paderborn low excreting pigs (recover from the infection) or strain Zoelen.

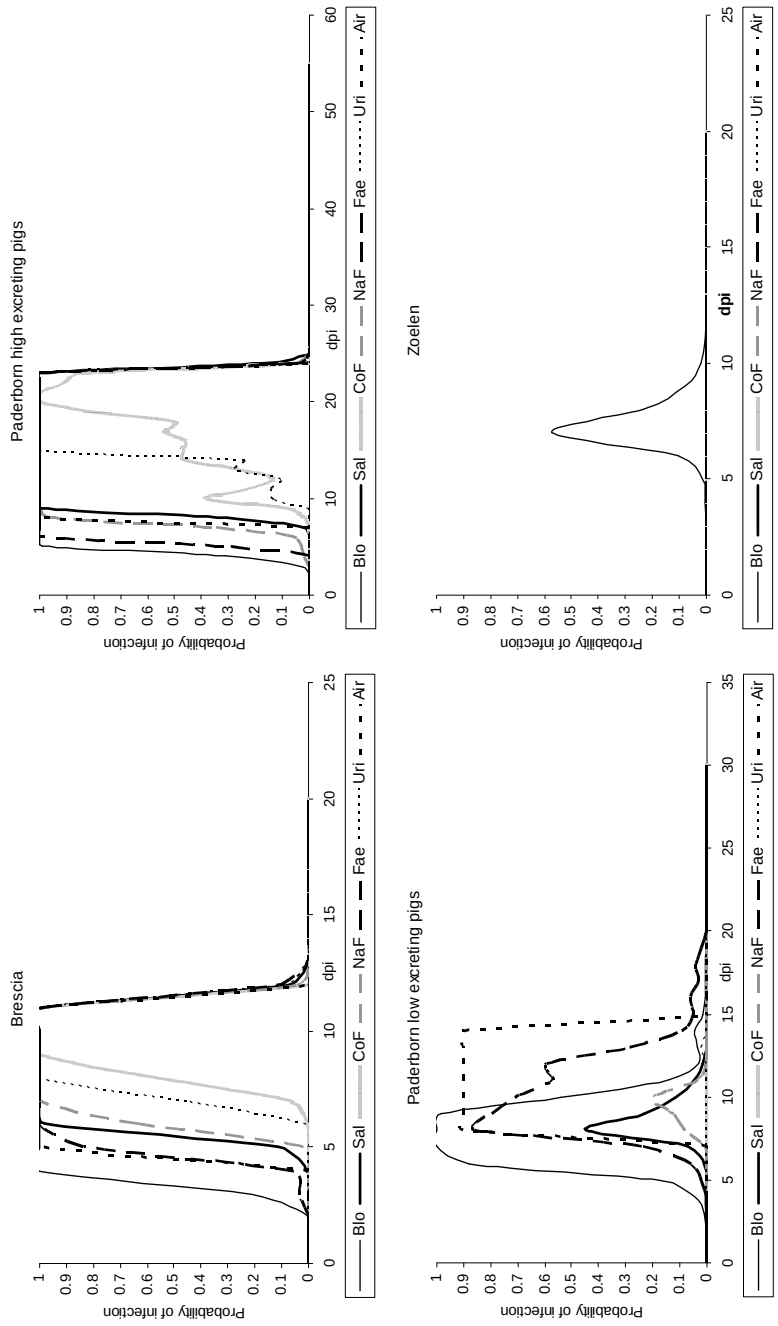

Figure 4. Median probability of CSF infection on a daily basis via virus excreted by one pig in the different excretions and secretions when infected with strain Brescia, Paderborn high excreting pigs (die from the infection), Paderborn low excreting pigs (recover from the infection) or strain Zoelen.

Air and faeces had a maximum daily median probability of respectively 0.9 (5^{th} perc.=0.69, 95^{th} perc.=0.99), and 0.86 (5^{th} perc.=0.16, 95^{th} perc.=1.0), while the maximum daily median probability of infection due to contact with conjunctival fluid was only $5.0x10^{-4}$ (5^{th} perc.=$2.9x10^{-6}$, 95^{th} perc.=0.83). For the Zoelen strain, the probability of infection was highest for blood contact with a maximum daily median probability of 0.57. The probability of infection via the other secretions and excretions (saliva, conjunctival fluid and nasal fluid) was relatively small (daily median probability <$2.5x10^{-3}$). Since blood will only contribute to infection when animals bite, the actual probability of infection of contact pigs with the Zoelen strain will depend largely on the behaviour of the pigs.

Table 5 gives a ranking of secretions and excretions per virus strain based on median probabilities to cause infection of a susceptible contact pig over the entire infectious period. These rankings were derived using a dilution factor for the total virus dose per d.p.i. (refer to Section 2.3). Between the strains, differences existed in ranking of the different secretions and excretions. In general, blood contact contributed most to infection of a susceptible pig, but this type of contact only occurs under specific circumstances, such as fighting. Saliva, nasal fluid and faeces contributed to the infection probabilities of most strains. In general, virus emitted in conjunctival fluid contributed least to infection. Urine also had a relatively low probability of infection, except for high excreting Paderborn-infected pigs. Pigs infected with the Zoelen strain and low excreting Paderborn-infected pigs excrete (almost) no virus in urine. Pigs infected with the Brescia strain excreted virus in urine, but virus excretion in urine was delayed compared to most other secretions and excretions (Figure 4). Furthermore, pigs infected with the Zoelen strain did not excrete virus in faeces and the air, or only very small amounts that were under the detection limit of the virus isolation assay. Therefore, these secretions and excretions did not contribute to the probability of infection in the model.

Table 5. Ranking of the secretions and excretions per virus strain based on the median probability of infection during the entire infectious period when using a dilution factor for the total virus dose (10^3 for Brescia, 10^2 for Paderborn low excreting pigs, 10^5 for Paderborn high excreting pigs, and 10^0 (i.e. no dilution) for Zoelen)

	Virus strain			
Secretion/excretion	Brescia	Paderborn high excreting pigs	Paderborn low excreting pigs	Zoelen
Blood	1[a]	2	2	1
Saliva	4	3	4	2
Conjunctival fluid	6	7	6	4
Nasal fluid	2	4	5	3
Faeces	3	5	1	-[b]
Urine	5	1	7	-
Air	7	6	3	-

[a] Rank 1 equals the highest probability of infection.

[b] No virus was detected in this excretion. Therefore, it does not contribute to infection.

Validation results

The probabilities of infection of contact pigs per d.p.i. based on: (a) model calculations, and (b) results of the 15 one-to-one experiments are shown in Figure 5. The model gives in general a good prediction for the infection probabilities of pigs in contact with high and low excreting infected pigs. For C1 pigs, the model estimates the highest probabilities of infection one or two days later than observed in the experiment. The first infections occurred in the experiment at 4 d.p.i., while the probabilities of infection at this day as predicted by the model were still low (95^{th} percentile value of 0.01 for low excreting pigs and 0.03 for high excreting pigs). There is only one C1 pig housed with a high excreting pig that was probably not infected within the time period the model estimated the most likely moments of infection (d.p.i. 4-6). For all C2 and C3 pigs housed with the high excreting pigs the model gave an excellent prediction, with infection within 24 h after contact with the high excreting inoculated pigs. For C2 and C3 pigs housed with the low excreting pigs the most likely moments of infection were within 24 h. For C2 pigs, this was observed in the experiment with all pigs (except one) that were housed with an inoculated pig that still excreted infectious virus. The other C2 pigs were housed with inoculated pigs that were not detected to excrete infectious virus anymore during this phase of the infection. These contact pigs were most likely infected at a later moment (d.p.i. 20 and 21) with probabilities close to the 95^{th} percentile values calculated by the model. At the start of the third contact period (C3 pigs), no infectious virus had been detected for four days in secretions and excretions of the low excreting inoculated pigs. The model indicates that it is very unlikely (median probability of 0 on d.p.i. 26, 95^{th} perc. of 7.8×10^{-15}) that the C3 pigs were infected by the inoculated pigs. However, nine of ten C3 pigs were infected. This occurred most likely via virus emitted in the air by a chronically infected pig in an adjacent pen, or virus produced by C2 pigs that survived in the environment. All C2 pigs were infected and removed from the pens 24 h before placement of the C3 pigs in the pens. When including these infection probabilities in the calculations, the most likely moment of infection of C3 pigs is at d.p.i. 26 (median probability is 0.26). In the experiment we observed infection of the pigs at a later point in time with probabilities per d.p.i. close to the 95^{th} percentile values calculated by the model.

Sensitivity analysis

Changing the values of the uncertain input variables did not result in major changes of the ranking (results not shown). The largest changes in ranking (mostly 1 ranking step difference) were observed when the minimum values were used for the amount (volume) of the secretions (V_t) and fraction of direct (Fr_f) and further (Fr_o) removal of secretions and excretions in the pens.

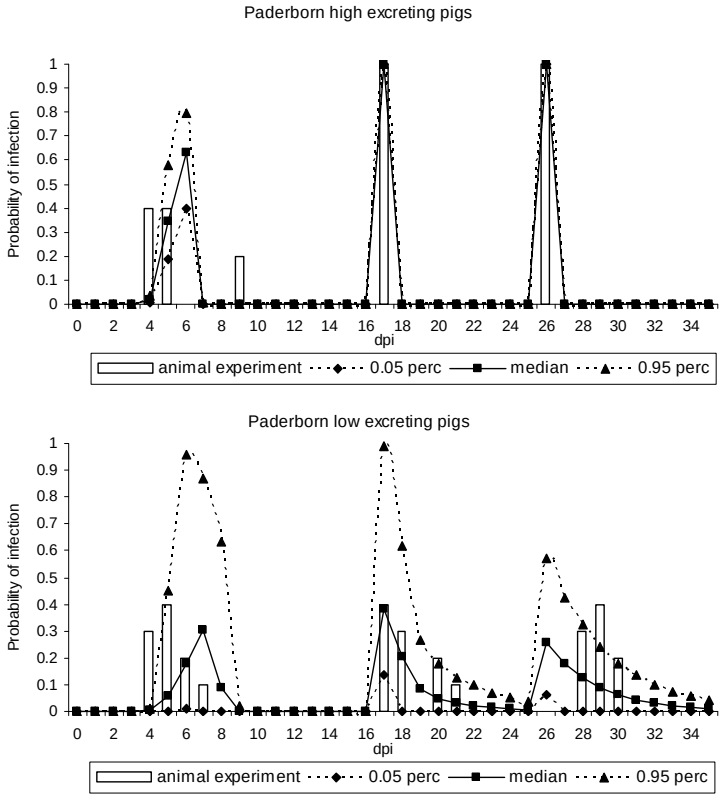

Figure 5. Probability of infection per day post-inoculation (d.p.i.) of the contact pigs housed together with Paderborn high or low excreting pigs, based on results of 15 one-to-one transmission experiments (bars) and model calculations (lines; median, 5th and 95th percentile values).

Discussion

Contribution of secretions and excretions to transmission

This study indicates that virus strains differ with respect to the relative contribution of secretions and excretions to transmission. This supports the statement that during outbreaks control measures should ideally be based on the characteristics of the specific virus strain involved, which implies the development of tailor-made measures. Implementation of such specific measures might avoid the application of general control measures with a high socio-economic impact, like preventive depopulation around an infected herd resulting in mass destruction of healthy pigs. If data would be available on virus excretion after, e.g., vaccination or hygiene measures like the cleaning and disinfection of livestock trucks, the risk model could be used for quantitative evaluation of the effectiveness of these measures.

The model estimates that it is highly probable that susceptible pigs in contact with Brescia or Paderborn-infected pigs will get infected (probability of 1). This high probability of infection was also observed in previous transmission studies (Bouma et al., 2000; De Smit et al., 2001; Uttenthal et al., 2001; Durand et al., 2009; Weesendorp et al., 2009a). Contact with two to five Brescia or Paderborn inoculated pigs resulted in infection of all three to seven contact pigs (in all studies together, 16 pigs inoculated with the Brescia strain and 25 pigs with the Paderborn strain, infected in total respectively 21 and 35 susceptible contact pigs). However, in an experiment with the Zoelen strain, none of seven pigs in contact with three inoculated pigs became infected (Weesendorp et al., 2009a). This is in accordance with model calculations, that give an overall probability of infection of 0.08 if blood is excluded.

Despite differences observed in the relative importance of secretions and excretions between the virus strains, in general, oronasal secretions had a relatively large contribution to the probability of CSFV infection of contact pigs. Due to the natural behaviour of pigs with oral and nasal exploring, these secretions are widespread in the environment. Although it was assumed that only 1 to 10 ml of saliva and 0.1 to 1 ml of nasal fluid were excreted, transmission via this route is very likely to occur. For the Brescia and Paderborn strain, faeces was also indicated as one of the most important excretions responsible for transmission. Faeces is produced in large amounts and contribute largely to contamination of the environment. Faeces and oronasal secretions together could play an important role in both direct transmission and indirect transmission routes, like CSFV contaminated environments (pens, livestock trucks), clothing and footwear (Ribbens et al., 2004; Ribbens et al., 2007).

For CSFV, the transmission via the air is assumed to be of minor epidemiological importance (Stärk, 1999). However, experimental studies showed that transmission via the air is possible over short distances (Terpstra, 1988; Dewulf et al., 2000; Gonzalez et al., 2001). This study showed that infection of susceptible pigs via virus in the air is likely to occur when these pigs are housed in the same room as pigs infected with the Paderborn or Brescia strain. Although this route contributes less to the infection probability of susceptible pigs than other secretions and excretions, the daily probability of infection of a susceptible pig in contact with a Brescia or high excreting Paderborn-infected pig via the air still reaches 1. Therefore, the role of airborne CSFV within a room housing pigs infected with a moderately or highly virulent strain should not be underestimated.

Model considerations

The purpose of this model was to provide insight into the relative contribution of secretions and excretions to spread of disease. We realise that separating virus excreted in different secretions and excretions is not conform reality, because secretions and excretions will mix in the environment. However, this separation enabled estimation of the relative contribution of secretions and excretions to CSFV infection probabilities.

The model relies heavily on excretion input parameters obtained from data of five pigs infected with either the Brescia, Paderborn or Zoelen strain (Weesendorp et al.,

2009). Clearly, the number of pigs in these experiments was low. This may have resulted in an under- or overestimate of absolute values. Nevertheless, we think that the model gives a good indication of the relative contribution of secretions and excretions to the probability of infection with CSFV. The sensitivity analysis indeed showed that the model is rather robust with only slight changes in ranking when changing values of uncertain input parameters.

The validation based on 15 one-to-one transmission experiments showed that the model predicted the infection probabilities of pigs in contact with Paderborn-infected pigs quite well. Only for some pigs the model estimated the most likely moment of infection one to two days before or after the infection moment estimated from the experimental data. Two uncertain parameters can explain this discrepancy between experimental and model results. Firstly, it is difficult to determine the moment of infection of the susceptible contact pigs in the animal experiments. A fixed latent PCR period (time between infection of pigs and the first positive sample in the PCR) of three days was assumed based on results from inoculated pigs. However, this latent PCR period seems to be dose dependent. Pigs in contact with Paderborn inoculated pigs from d.p.i. 1 onwards (C1 pigs) were subject to increasing doses of virus excreted by these inoculated pigs over time. The doses received by the susceptible pigs were most likely higher than the doses used for inoculation, which could have resulted in shorter latent PCR periods. A shorter latent PCR period would result in a better fit of model calculations to the experimental data. In contrast, pigs in contact with low excreting pigs that no longer excreted virus (C3 pigs) were most likely infected with a low dose of CSFV present in the environment. The latent PCR period could in this case be longer than three days, which would also result in a better fit of experimental data with model calculations. Secondly, the model might have overestimated the infection probabilities for some pigs because in the excretion experiments used as input data for the model low excreting Paderborn pigs excreted virus for a longer period than in the one-to-one transmission experiments used for the validation.

Model results for the Paderborn strain cannot be extrapolated to other moderately virulent strains as illustrated by calculations that we did for the “souche Lorraine” strain. The model was used to mimic experiments of Dewulf et al (2002) and Ribbens et al. (2004) in which pens housing pigs infected with this moderately virulent strain were depopulated at d.p.i 8 and restocked 20 h later with susceptible pigs, or depopulated at d.p.i 15 and restocked 10 h later. Between depopulation and restocking, the pens were neither cleaned nor disinfected. The median estimate of the probability of infection of susceptible pigs 20 h after depopulation at d.p.i. 8, is 1 for Paderborn high excreting pigs and 0.76 for Paderborn low excreting pigs. When waiting with depopulation until d.p.i. 15 and leaving the pen empty for only 10h, the probability of infection for low excreting Paderborn-infected pigs is reduced to 4.6×10^{-2}, whereas for high excreting pigs this probability is still 1. Although the “souche Lorraine” strain is moderately virulent like the Paderborn strain, results obtained with the risk model are very different from results obtained by Dewulf et al. (2002) and Ribbens et al. (2004). In their experiments, none of the susceptible pigs got infected in the 8 d.p.i., 20 h experiment, and four out of ten pigs got infected in the 15 d.p.i., 10 h experiment. Despite the

"souche Lorraine" strain being moderately virulent based on morbidity and mortality, the excretion dynamics are different from the Paderborn strain and seem to be more comparable to the Zoelen strain, with an even delayed start (Dewulf et al., 2002; Ribbens et al., 2004). This illustrates that for a good estimation of infection probabilities, input data of the model should be strain-specific. Furthermore, it emphasises the importance of providing insight into excretion dynamics of the virus strain involved when developing tailor-made control measures during an outbreak.

Practical implications

This model could help to evaluate the importance of (indirect) transmission routes and quantitatively evaluate the effect of control measures. For this purpose, the model should be extended to include transmission dynamics within a population with increasing numbers of infected pigs over time and quantitative data on the transmission routes or scenarios modelled, such as amounts of secretions and excretions transferred with (indirect) transmission routes. To analyse the effect of control measures, input data should be used that relate to these control measures, for instance excretion rates of vaccinated pigs or amounts of virus left in livestock trucks after cleaning and disinfection. Further studies are required to provide quantitative data for some of these parameters.

To make this model useful for CSFV strains for which no experimental data on excretion and secretions are available, additional quantitative knowledge on excretion patterns of other strains has to be gathered. With information on several strains with similar virulence, the predictive capacity of the model will increase. This model can then be a useful tool to assist decision makers in imposing specific control measures during an outbreak.

Conclusion

Model results indicated differences between virus strains with respect to the relative contribution of secretions and excretions to transmission, and the probability of infection of a susceptible animal via these secretions and excretions. This information can contribute to the development of tailor-made control measures based on the characteristics of a specific virus strain. The model presented in this paper can be extended to provide more insight into the effect of specific control measures and ultimately allow for quantitative evaluation of such measures.

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Chapter 9

Summarising discussion

Summarising discussion

Over the last decades, several outbreaks of classical swine fever (CSF) in Europe have demonstrated the difficulty of controlling an outbreak, especially in areas with a high pig density (Koenen et al., 1996; Elbers et al., 1999; Allepuz et al., 2007). During these outbreaks, classical swine fever virus (CSFV) was transmitted between farms through direct contact (from pig to pig) by movement of infectious pigs or via indirect contact (from pig to environment/mechanical vector to pig) (Stegeman et al., 2002; Allepuz et al., 2007). However, in a large proportion of the infected herds, no route of virus introduction could be established (Elbers et al., 1999; Sharpe et al., 2001; Allepuz et al., 2007). These herds that were located in a radius of 1 km around a herd infected earlier in time were defined as 'neighbourhood infections' (Elbers et al., 1999; Elbers et al., 2001b). Several studies have tried to elucidate the routes of virus introduction into these 'neighbourhood' farms by analysing outbreak data or by using questionnaires, but they were only partly successful (Elbers et al., 2001; Crauwels et al., 2003; Mintiens et al., 2003). Despite their inability to identify the introduction routes of a large number of farms, these studies did demonstrate that the probability of infection decreased, when the distance to the primary infected herd increased (Koenen, et al., 1996; Stegeman et al., 2002). The virus introduction routes for a large number of farms remained, however, unknown. Mainly based on the relationship between probability of infection and distance, control measures will be taken in the immediate neighbourhood of infected farms to control CSF outbreaks, such as preventive depopulation of herds or emergency vaccination around infected farms (Nielen et al., 1999; Mangen et al., 2001; Klinkenberg et al, 2003; Backer et al., 2009). The application of these control measures have far-reaching consequences, mainly ethically, due to mass destruction of uninfected pigs in case of preventive culling, or economically, due to prolonged trade restrictions in case of vaccination (Meuwissen et al., 1999; Boklund et al., 2008). More insight in the underlying mechanisms of virus transmission will allow for more specific control measures to prevent transmission of CSFV between herds.

In this thesis a bottom-up approached has been used to obtain knowledge on underlying mechanisms of transmission, instead of working top-down from outbreak data to transmission (routes) between individual pigs (Ribbens, 2009). This approach uses experimentally obtained data on the virus, environment and host to identify possible routes of transmission during outbreaks. For this purpose, several parameters that influence the speed and efficiency of transmission, like virus excreted by infected pigs (Chapters 2, 3 and 4) and survival of the virus in the environment (Chapter 5). Furthermore, additional data on susceptibility of pigs were obtained (Chapters 6 and 8). The effect of these parameters on transmission were quantified with a susceptible-exposed-infectious-recovered (SEIR) model (Figure 1, Chapters 6 and 7), which allows the calculation of the transmission rate β and the basic reproduction ratio (R_0) (Diekmann and Heesterbeek, 2000; Keeling and Rohani, 2008). The transmission rate β is defined as the number of secondary infections caused by one infectious individual

per unit of time. The R_0 is defined as the average number of secondary infections caused by one infectious individual during its entire infectious period in a fully susceptible population. The implications of the relationship between the underlying parameters and transmission will be discussed in this chapter, as well as recommendations for further research on control measures that could help to reduce virus transmission during outbreaks.

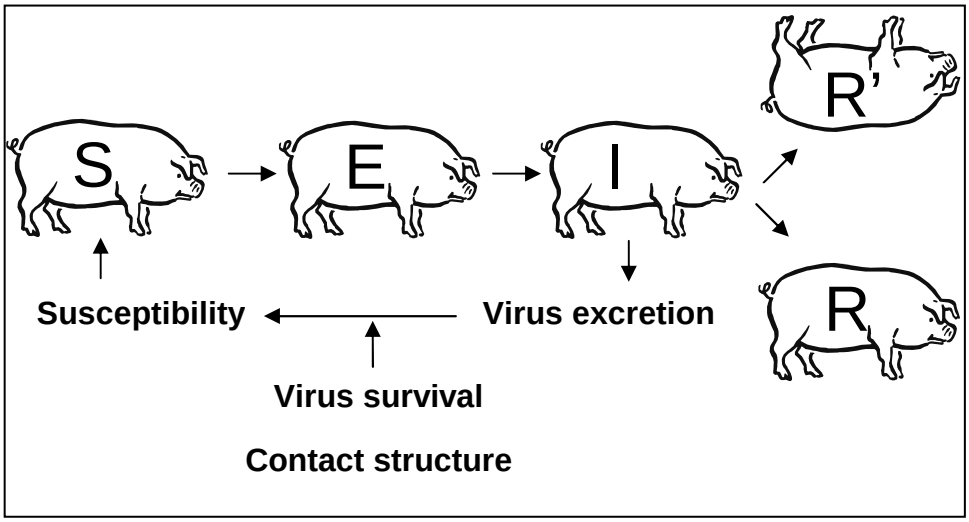

Figure 1. Underlying mechanisms of transmission, indicated in a susceptible-exposed-infectious- recovered or removed (SEIR) model. The infection of susceptible pigs (S) is dependent on virus excretion by infectious pigs (I), transfer of virus from infectious to susceptible pig, and susceptibility of the recipient pig. Successful virus transfer will depend on the contact structure between pigs and, for indirect contacts, on virus survival in the environment. When a susceptible pig (S) gets infected, it is not infectious yet (E). After the latent period, this pig will start excreting infectious virus (I). Finally, this pig will either recover (R) or die (R').

Virus excretion in relation to transmission

An important parameter that affects the transmission of CSFV is the amount of virus excreted by infectious pigs. In this thesis, virus excretion was quantified for three CSFV strains that differ in virulence: the highly virulent strain Brescia, the moderately virulent strain Paderborn and the low virulent strain Zoelen. It was demonstrated that virus excretion by infectious pigs is dependent on the virulence of the virus strain, and on the clinical course of disease (Chapters 2, 3 and 4). In general, the higher the virulence of the strain, the more virus is excreted during the entire infectious period of a pig. However, some pigs infected with the moderately virulent strain Paderborn become chronically infected or die relatively late in the (sub)acute phase. These pigs (high excreting pigs) have a significantly longer infectious period than pigs infected with the same strain that recover from the infection (low excreting pigs) or than pigs infected

with the Brescia strain. Therefore, they excrete significantly higher quantities of virus during their entire infectious period (Chapters 2, 6 and 7). The strain and clinical course affect not only the amounts of virus excreted, but also the distribution of virus across the various types of secretions and excretions that contain virus, which may result in differences in transmission characteristics (Chapters 2, 6 and 7). Ultimately, this could also affect the relevant transmission routes during an outbreak.

The results presented in this thesis show that the clinical course of the infection is strongly associated with virus excretion and transmission. Chronically infected pigs can play a major role during an outbreak, due to their high infectivity and long infectious period. This will increase the probability of virus transmission within a herd (Chapters 7 and 8), and thereby also the probability of virus transmission to other herds (Stärk et al., 2000). The mechanisms underlying the development of the chronic infections are not yet known. When multiple pigs are inoculated with the same inoculum of a (moderately virulent) strain, some pigs may die in the (sub) acute phase, while others develop the chronic course or may fully recover from the infection. Viral factors could play a role in the development of the chronic course of infection (Mengeling and Packer, 1969; Van Oirschot, 1988). It is known that virus populations are not homogeneous, i.e. they do not consist of one clone of the virus. Instead, they consist of variants, some of which may differ from the population average at one or more nucleotides, a ‘quasispecies distribution’ (Domingo et al., 1998; Kiss et al., 1999). Through selection of certain variants, quasispecies could play a role in the persistence of the virus in the host (Mengeling and Packer, 1969; Van Oirschot, 1988). Whether this kind of viral factors play a possible role could be investigated by (deep) sequencing of virus in samples obtained from chronically infected pigs and comparing the results with those obtained from pigs that recover from the infection or with virus that was used for the inoculation (Fishman and Branch, 2009).

However, the clinical course of infection is most likely not only the result of viral factors but also of host factors, or interactions between viral and host factors (Van Oirschot, 1988). Age is one example of a host factor that is expected to affect the level of virus excretion and is known to affect the clinical course of infection and the transmission (Van Oirschot, 1988; Laevens et al., 1998a; Laevens et al., 1999; Stegeman et al., 1999; Moennig, 2000; Klinkenberg et al., 2002; Loeffen, unpublished). These studies show that upon inoculation with a moderately virulent strain, the number of chronically infected pigs, as well as the transmission rate decreased with increasing age. The relationship between age and transmission is, however, not only dependent on virus excretion. Age can also affect the susceptibility of pigs (Van Oirschot, 1988). Furthermore, the contact structure within different age groups varies due to differences in housing systems and behaviour (weaner, slaughter or breeding pigs). All these factors will contribute to differences in transmission rates. In the experiments performed in this thesis, 8-week-old pigs were used (Chapters 2, 6 and 7). As a consequence, the results of this study will have the highest predictive value for that age group. Excretion and transmission dynamics observed in studies in this thesis could be different for other age groups. Furthermore, the age effect may also be different for the Brescia than for the Paderborn strain. For the Paderborn strain, it is likely that the chronic form would

occur less often in older pigs (Loeffen, unpublished). This would result in a shorter average infectious period, and in case they recover instead of becoming chronically infected, a lower level of infectivity as well. This would ultimately result in a lower R_0 value for older pigs. For the Brescia strain, on the other hand, it cannot be excluded that the chronic form might occur more often in older animals, which would result in a longer average infectious period, and therefore a higher R_0 value. To confirm these hypotheses of age on excretion and transmission, further research is required. This could start with a meta analysis on the results of available studies. If still gaps remain for specific age groups, direct transmission experiments (Chapter 6), could be performed.

The studies in this thesis on virus excretion, and the relationship to transmission, were obtained in an experimental setting. One of the advantages compared to the field situation is that excretion and transmission rates can be obtained under controlled conditions, so differences between groups can be attributed to the factor that is under investigation (e.g. virus strain or inoculation dose). Furthermore, some observations would not even be possible under field conditions. A disadvantage of an experimental setting is that other factors that may affect the excretion and transmission in the field situation are excluded or unknown. One of the factors studied in this thesis that was most likely underestimated by the experimental setting is the level of CSFV in the air of rooms housing infected pigs (Chapters 3 and 4). Under field conditions, this level is expected to be higher due to a higher pig density, with more infected pigs, and a lower ventilation rate of the stables. By correcting for the ventilation rate and the amount of infected pigs, the data obtained in this thesis may be used to estimate virus titres emitted in the air in a field situation. However, this should be validated under field conditions. Field research could be done in countries where CSF is still endemic. More relevant for the Dutch pig husbandry would be to take samples during future outbreaks in the Netherlands. The air-sampling technique developed in this thesis, would provide a valuable tool to determine the quantities of virus emitted in the air under field conditions (Chapter 3).

Contact structure and survival of virus in relation to transmission

For susceptible pigs to become infected, contact with an infectious pig is needed, either by direct contact or by indirect contact. During this contact, virus needs to be transferred from the infectious pig to the susceptible pig. With direct contact, this contact is usually abundant and intensive, while inactivation of virus during transfer is irrelevant. With indirect contacts, frequency of contacts, amounts of secretions and excretions transferred, as well as virus survival during transfer, become crucial issues. Due to these issues, transmission efficacy for indirect contact is usually lower than for direct contact, as was observed during the Dutch outbreak in 1997-1998 (Stegeman et al., 2002). Furthermore, experimental studies to provide insight into indirect transmission have been reported (Dewulf et al., 2002; Ribbens et al., 2004; Ribbens et al., 2007). Because in these studies only few susceptible pigs became infected, the authors concluded that the importance of these indirect transmission routes should not be overrated (Dewulf et al., 2002; Ribbens et al., 2004; Ribbens, 2009). However, since

some of these contacts with low transmission rates do occur often, they may still account for a considerable part to the infection of herds during an epidemic (Elbers et al., 1999; Fritzemeier et al., 2000; Stegeman et al., 2002; Allepuz et al., 2007). To determine the transmission rates associated with these routes of transmission in animal experiments, large numbers of pigs would be required. Instead, risk models could provide useful estimates of the importance of these routes (Chapter 8).

To model transmission, quantitative data on survival of virus is necessary. In this thesis a gap was filled by providing detailed data on the temperature dependent survival of virus in excretions (Chapter 5). Furthermore, quantitative data on the contact structure between pigs or herds, and the amount of virus transferred via several pathways during these contacts, are needed. Information on the contact structure between herds was obtained by tracking and tracing during outbreaks, and in several field studies (Nielen et al., 1996; Elbers et al., 1999; Fritzemeier et al., 2000; Elbers et al., 2001a; Sharpe et al., 2001; Allepuz et al., 2007; Ribbens et al., 2009). These studies depend, however, mostly on questionnaires and interviews to get information on the contact structures of herds (Nielen et al., 1996; Elbers et al., 1999; Ribbens et al., 2009). One of the disadvantages of using questionnaires is recall bias, which could result in incorrect reporting of contacts. In addition of using questionnaires, information from existing databases, like those available from the identification & registration system, rendering companies, feed companies, animal transport companies, etc. could be used. Even if the contact structure could be established in detail, a missing value would still be the amount of virus transferred during each contact. Additional research to determine these amounts of virus for specific types of contact would be very useful. For that purpose, indicator substances could be used. After release of the indicator substance in a pen of a relevant herd with well-known contact structures to other herds, it could be followed through the entire contact structure of this herd. These indicator substances would of course need to be safe and quantifiable, and detectable even in very low amounts. This approach could also allow to study the effect of specific interventions, especially aimed at hygienic measures, which are now hard to quantify.

Susceptibility of pigs

The natural route of infection of CSFV is oronasal, although also the genital, conjunctival, parenteral, and aerogenic route are described (Dunne et al., 1959; Hughes and Gustafson, 1960; Terpstra, 1988). The dose that is necessary to cause infection is often expressed as the median infectious dose (ID_{50}) (Sutmoller and Vose, 1997). This is, however, dependent on factors like the virus strain, infection route, age and immunological properties of the pig (Ward and Akin, 1984; Liess, 1987; Dahle and Liess, 1995). To determine the ID_{50} values, serial dilutions of the virus strain are inoculated in a number of pigs, and the dilution that theoretically would infect 50% of the inoculated pigs is calculated from the number of infected and number of non-infected pigs (Sutmoller and Vose, 1997). Using data of the experiments in this thesis and previous experiments, intranasal ID_{50} values were estimated for three different virus strains (Chapter 8). It was shown that the susceptibility differs for different virus strains. However, because these estimates are based on a small number of pigs and

doses, they are still quite imprecise. A more precise estimation of these ID_{50} values would require more experiments, using several serial dilutions. Whether a more precise estimation is necessary for risk-analysis models could be determined by model sensitivity analysis, which involves studying how changes in input variables will affect model outcome. Only if changing ID_{50} values results in important differences in outcome, the use of animal experiments should be considered.

The ID_{50} value gives information on the amount of virus that would infect 50% of the pigs. For risk-analysis models, it would, however, be interesting to have information on a more detailed relationship between doses and probability of infection, especially to determine the risk of indirect transmission routes, in which generally small quantities of virus are transferred that even decrease over time due to inactivation. Theoretically, even a single virion has a non-zero probability of causing infection of a pig (Sutmoller and Vose, 1997; Haas et al., 1999; Zwart et al., 2009). If one virion infects a susceptible cell, it can multiply and the newly generated virions can infect multiple other cells, leading to disease. The probability of small doses to cause infection is, typically, low. Therefore, to experimentally determine the doses required to infect a small percentage of the population (for instance ID_1 or $ID_{0.1}$) is difficult, or even impossible, because a large number of pigs has to be included in the study to obtain sufficient power (Ward and Akin, 1984). Because precise dose-response curves, especially in the area of low virus doses, can not be constructed experimentally, probabilities of infection with low doses will need to be based on modelling. Sensitivity analysis (Chapter 8) can again determine whether more precise information would be necessary.

Transmission models

In this thesis, the effect of several parameters like virus strain, inoculation dose (Chapter 6) and course of the infection (Chapter 7) on the spread of CSFV were studied in transmission experiments. The transmission was quantified using a stochastic SEIR (Susceptible-Exposed-Infectious-Recovered) model (Diekmann and Heesterbeek, 2000; Keeling and Rohani, 2008). These models require several assumptions regarding the moment of infection, and duration and level of infectiousness of pigs. Below some of these assumptions with respect to CSFV will be discussed.

Most classical SEIR models assume that all pigs are homogeneous, i.e. equally susceptible and infectious (Velthuis et al., 2007). However, for CSFV, it should be taken into account that pigs infected with moderately virulent strains can develop different courses of the disease, resulting in different levels of infectiousness. In Chapter 7 we showed that for the Paderborn strain significantly higher R_0 values were observed for high excreting pigs than for low excreting pigs. When transmission studies are performed to analyse the effect of a specific treatment or parameter on transmission (for instance groups inoculated with different doses [Chapter 6]), this can pose a problem when differences occur in the distribution of these two types of infectious pigs between treatment groups. Assuming homogeneity among the groups

may then result in wrong conclusions. As a consequence, the SEIR model should be adapted to be able to differentiate between the different types of infectious pigs.

For the estimation of transmission parameters, it is needed to determine the moment of infection of the contact pigs. In the transmission studies described in Chapters 6 and 7 it was assumed that the infection moment was one latent (PCR) period before the first positive PCR sample. The latent PCR period is the time between infection of pigs and the day of the first positive sample in the PCR on oropharyngeal fluid. This period was determined using data from the inoculated pigs, of which the exact moment of infection was known. However, in the experiments, it was observed that the latent PCR period was dose dependent (Chapter 7). This means that it will be difficult to determine the moment of infection of a contact pig because it is not known to what dose the contact pig was exposed to. To avoid using a fixed latent period in the SEIR model, Bayesian analysis could be used. Bayesian models can accommodate unobserved variables and would use results from earlier CSFV experiments to obtain prior information and combines that with observed data of the new experiment. The combination of these data then allows to compute the posterior distribution, which offers an overview of all information available (Streftaris and Gibson, 2004; Hohle et al., 2005).

Despite all constraints when using transmission models, they are a valuable tool. They allowed us to quantify transmission and study certain control measures, which is difficult to do in a field situation with a notifiable disease like CSFV. However, estimates of transmission of CSFV during field outbreaks would be a valuable addition to experimentally obtained knowledge. Data is until now limited and has been obtained in only one outbreak for one particular strain (Stegeman et al., 1999). Furthermore, control strategies in many parts of the world include depopulation of infected herds as soon as possible. As a consequence, not only within-herd transmission is hard to quantify, but also the effect of control measures like vaccination or hygiene measures. Especially when vaccination may replace the pre-emptive culling strategy in a future Dutch CSF outbreak, not only serological, but also virological data will improve our knowledge on the effectiveness of this control measures. Therefore, extensive sampling (blood, oropharyngeal and faeces swabs) on farms during outbreaks is essential to obtain valuable knowledge on transmission dynamics and control measures.

Implications

This thesis focused on increasing knowledge of underlying mechanisms of CSF virus transmission with emphasis on virus excretion and virus transfer through direct and indirect contact within a herd. The next step will be understanding and quantifying transmission routes in the field. This requires additional knowledge, mainly on contact structures and amounts of virus transferred with each type of contact. Several options for additional research to obtain this knowledge were discussed. With such information, the risk model described in Chapter 8 could be extended to infection probabilities of various transmission routes between herds. In a next step, the efficacy of specific intervention methods needs to be assessed. Relevance of intervention measures will

be determined by prevailing contact structures and probabilities of virus transmission via each type of contact. Furthermore, measures should be realistic and feasible.

It is too early to conclude that well-known dangerous contacts, such as live pigs, swill feeding, transport contact or transmission by people are indeed the main routes of virus transfer between herds. It is also not known yet if, and to which extent, other hypothesized transmission routes, like birds, pets and rodents acting as mechanical vectors, contribute to spread of CSFV. With some additional information, the risk model (Chapter 8) could be used to assess the probabilities of virus transmission through these and other possible routes.

With respect to the role of airborne transmission, studies in this thesis (Chapters 3 and 4) detected and quantified CSFV in the air of rooms housing infected pigs for the first time. Previous experimental studies, including studies using the Paderborn and Brescia strain, also showed that airborne transmission over short distances is possible (Hughes and Gustafson, 1960; Terpstra, 1988; Laevens et al., 1998b; Laevens, 1999; Dewulf et al., 2000; Gonzalez et al., 2001; Loeffen, unpublished). Airborne transmission within a herd may therefore be a relevant transmission route, resulting in more infected pigs in a shorter period of time (Laevens et al., 1998b; Laevens et al., 1999; Dewulf et al., 2000). This could then result in higher between-herd transmission, because this is dependent on the infectivity of the herd, which is likely to be correlated with the number of infectious animals (Stärk et al., 2000). Airborne transmission, even if only occurring within a herd, is therefore worthwhile to be investigated in more detail as well.

Concluding remarks

- In general, the higher the virulence of the strain, the more virus is excreted during the entire infectious period of a pig. An exception are pigs infected with a moderately virulent strain that develop the chronic form or die in the (sub)acute phase (high excreting pigs), as was shown for the Paderborn strain.
- High excreting pigs play a crucial role in the transmission of CSFV due to the high level of virus excretion, and the high reproduction ratio R_0.
- This thesis describes for the first time the detection and quantification of CSFV in the air of rooms housing infected pigs.
- In this thesis, temperature dependent survival curves of CSFV in faeces and urine, produced by experimentally infected pigs have been generated. This information is valuable for risk models to analyse the probability of infection of susceptible pigs via indirect transmission routes.
- Transmission studies showed that, in general, the transmission rate β is not significantly different between the highly virulent strain Brescia and the moderately virulent strain Paderborn. However, the moderately virulent strain can have significantly longer infectious periods (depending on the clinical course of the disease), which can result in higher R_0 values. On the other hand, the highly virulent strain has a shorter generation interval, which will result in a higher growth rate of an outbreak in the initial phase.
- Valuable knowledge has been obtained on infectivity of pigs, survival of CSFV and susceptibility of pigs that can be used in risk-analysis models to estimate the

probability of infection of susceptible pigs via different transmission routes. However, quantitative data on the contact structure and amounts of virus transferred between pigs or herds is crucial to further investigate transmission routes between herds and possible specific intervention measures.

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Samenvatting

Dankwoord

Curriculum vitae

List of publications

Samenvatting

Klassieke varkenspest (KVP) is een zeer besmettelijke virusziekte die voorkomt bij gedomesticeerde varkens en wilde zwijnen. Er bestaat een groot aantal klassieke varkenspestvirus (KVPV) stammen die verschillen in virulentie. Deze stammen worden geclassificeerd op basis van virulentie in hoog-, middel-, of laagvirulente stammen. Hoogvirulente stammen veroorzaken een acuut verlopend haemorrhagisch ziektebeeld dat meestal eindigt in de dood. Overige ziekteverschijnselen die kunnen worden waargenomen bij deze acute vorm zijn hoge koorts, verminderde eetlust, sloomheid, oogslijmvliesontstekingen, neurologische verschijnselen, luchtwegproblemen en constipatie gevolgd door diarree. Middel- en laagvirulente stammen veroorzaken een vorm van de ziekte die moeilijker te herkennen is als KVP. Vooral infecties met middelvirulente stammen kunnen leiden tot een breed scala aan klinische verschijnselen met als gevolg de dood of herstel, of de chronische vorm die altijd eindigt in de dood. Varkens geïnfecteerd met laagvirulente stammen vertonen weinig tot geen ziekteverschijnselen en herstellen over het algemeen volledig.

KVPV verspreidt zich binnen en tussen bedrijven via secreta en excreta van geïnfecteerde varkens. De snelste en efficiëntste transmissieroute gaat via direct contact tussen geïnfecteerde en gevoelige varkens. Ook indirecte transmissieroutes zoals het voeren van keukenafval gecontamineerd met KVPV, kunstmatige inseminatie of KVPV-gecontamineerde mechanische vectoren zoals kleding, schoeisel of (vee)wagens kunnen een rol spelen bij de verspreiding van het virus. Echter, tijdens uitbraken in het verleden kon bij veel geïnfecteerde bedrijven de wijze van virusintroductie niet worden achterhaald. Veel van deze bedrijven bevonden zich wel dicht bij een eerder geïnfecteerd bedrijf en werden daarom benoemd als buurtinfecties. Verschillende routes van transmissie naar deze bedrijven werden gesuggereerd, waaronder niet-traceerbare routes zoals via insecten, ongedierte, huisdieren en vogels of transmissie via de lucht. Ook werd gesuggereerd dat transmissie in een deel van deze gevallen plaatsvond via routes die wel degelijk bekend zijn, maar die niet werden gerapporteerd

Ondanks dat veel routes van virusintroductie op bedrijven niet werden opgehelderd, werd in epidemiologische studies wel een relatie gevonden tussen de kans op het optreden van een KVP-infectie en de afstand tot een eerder geïnfecteerd bedrijf. Wanneer de afstand tot een besmet bedrijf kleiner wordt, neemt de kans op besmetting van het nog niet besmette bedrijf toe. Gebaseerd op deze relatie zijn controle-maatregelen ontwikkeld als preventieve ruiming van varkens of noodvaccinatie. Deze maatregelen kunnen echter grote ethische en/of economische bezwaren hebben. Preventieve ruimingen kunnen resulteren in de vernietiging van grote aantallen gezonde dieren, terwijl vaccinatie langdurig een negatieve impact kan hebben op de export van een land. Om meer specifieke maatregelen te nemen is het noodzakelijk eerst de rol van de mogelijke transmissieroutes individueel te kennen, alsmede het effect van maatregelen die ingrijpen op deze routes. Hiervoor kunnen (wiskundige)

modellen gebruikt worden, maar vooralsnog is hiervoor onvoldoende kennis van onderliggende mechanismen van transmissie (transmissieroutes).

Het onderzoek dat in dit proefschrift beschreven wordt, richtte zich op het kwantificeren van onderliggende mechanismen van KVPV-transmissie. Dit omvatte onderzoek naar de hoeveelheid virus die varkens, geïnfecteerd met verschillende stammen, uitscheiden in hun secreta en excreta en hoeveelheden virus die in de lucht terechtkomen. Daarnaast is gekeken naar de overleving van het virus in excreta als faeces en urine. Vervolgens is de bijdrage van deze, en andere parameters, aan de transmissie onderzocht in transmissie-experimenten. Alle kwantitatieve data is vervolgens gebruikt in een risicomodel dat de kans op infectie van een gevoelig varken, in contact met een geïnfecteerd varken, berekend. Dit model maakt het mogelijk meer inzicht te krijgen in transmissie en transmissieroutes. Hieronder staan de belangrijkste conclusies beschreven.

Uit studies in dit proefschrift bleek de hoeveelheid KVPV die uitgescheiden wordt in sterke mate bepaald te worden door de virulentie van de virusstam (Hoofdstuk 2). Dit is onderzocht in varkens die waren geïnfecteerd met de hoogvirulente stam Brescia, de middelvirulente stam Paderborn of de laagvirulente stam Zoelen. Hoe virulenter de stam, hoe meer virus werd uitgescheiden. Een uitzondering waren de varkens die geïnfecteerd waren met de Paderborn stam en vervolgens een chronische infectie ontwikkelden (twee varkens). Men spreekt van een chronische infectie wanneer varkens 30 of meer dagen na infectie sterven aan KVP. Deze chronisch geïnfecteerde varkens scheidden meer virus uit dan varkens geïnfecteerd met één van de andere stammen of varkens die herstelden van de infectie. Deze varkens hadden hoge virustiters in hun secreta en excreta en scheidden virus uit gedurende een lange periode. Daarnaast was er ook een verschil tussen varkens geïnfecteerd met de verschillende stammen in de secreta en excreta die infectieus virus bevatte. Varkens geïnfecteerd met de hoog- of middelvirulente stam scheidden virus uit in speeksel, neusuitvloeiing, ooguitvloeiing, faeces en urine. Virusuitscheiding van varkens geïnfecteerd met de laagvirulente stam bleef hoofdzakelijk beperkt tot de oronasale route. Deze studie benadrukt de belangrijke rol die chronisch geïnfecteerde varkens kunnen spelen in de transmissie. Daarnaast laat het zien dat het belangrijk is om onderscheid te maken tussen stammen wanneer data gebruikt wordt voor modelleren.

In dit proefschrift is voor het eerst de hoeveelheid virus in de lucht van stallen met KVPV-geïnfecteerde varkens gekwantificeerd (Hoofdstukken 3 en 4). Transmissie via de lucht zou een rol kunnen spelen bij verspreiding van virus binnen bedrijven of mogelijk zelfs bij buurtinfecties. Hoewel was bewezen dat over korte afstand varkens via de lucht geïnfecteerd kunnen worden, was het tot nu toe niet gelukt virus in de lucht aan te tonen. In dit proefschrift wordt een methode beschreven om KVPV in de lucht aan te tonen en te kwantificeren. Om deze methode te valideren zijn de KVPV-stammen Brescia, Paderborn en Zoelen in een isolator verneveld. Op verschillende momenten is een luchtmonster genomen met behulp van een gelatinefilter. Het virus bleef infectieus in de aerosol gedurende minimaal 30 minuten met halfwaardetijden van 4.5 tot 15 minuten. Daarnaast zijn op verschillende momenten luchtmonsters genomen tijdens een dierexperiment met vijftien varkens, geïnfecteerd met de Brescia,

Paderborn of Zoelen stam (vijf varkens per stam) (Hoofdstuk 3). Deze varkens waren individueel gehuisvest en luchtmonsters werden genomen in het hok, of van de uitademingslucht. Viraal RNA werd aangetoond in zowel de hoklucht als uitademingslucht van de Brescia- en Paderborn-geïnfecteerde dieren, maar niet van de Zoelen-geïnfecteerde dieren. Een nadeel van deze studie was dat varkens individueel gehuisvest waren onder condities die erg verschillen van de praktijk. Daarom zijn in een vervolgstudie virustiters in de lucht bepaald in vier stallen met elk tien varkens, waarvan drie varkens werden geïnfecteerd met KVPV stam Brescia, Paderborn (twee dosis; $10^{3.5}$ of 10^5 $TCID_{50}$) of Zoelen (Hoofdstuk 4). Er werd zowel viraal RNA als infectieus virus aangetoond in de stallen met de varkens geïnfecteerd met de Brescia of Paderborn stam. Hoe hoger de virulentie van de stam of de inoculatiedosis, hoe sneller virus in de lucht kon worden gevonden. In deze studie werd wederom geen viraal RNA of infectieus virus aangetoond in de stallen met varkens geïnfecteerd met de Zoelen stam. Deze studies waren de eerste waarin KVPV in de lucht kon worden aangetoond en gekwantificeerd, welke eerdere bevindingen over transmissie via de lucht binnen een stal ondersteunen.

Vervolgens is onderzoek gedaan naar het kwantificeren van een mechanisme dat een rol speelt bij indirecte transmissie van KVPV: de overleving van het virus. Indirecte transmissie kan optreden doordat mechanische vectoren gecontamineerd worden met secreta en excreta van geïnfecteerde varkens. Voor het optreden van deze indirecte transmissie moet het virus echter overleven op deze mechanische vector. In dit proefschrift wordt de overleving van KVPV in faeces en urine van varkens geïnfecteerd met de Brescia of Paderborn stam beschreven (Hoofdstuk 5). Faeces en urine werden verzameld van geïnfecteerde varkens en bewaard bij 5, 12, 20 en 30℃. Vervolgens werden hiervan op verschillende momenten monsters genomen en de titer van infectieus virus en viraal RNA bepaald. Met deze data werden inactivatiecurves gemaakt. De inactivatiesnelheid was gerelateerd aan de opslagtemperatuur. Bij hogere temperaturen nam de snelheid van inactivatie toe. Gemiddelde halfwaardetijden waren tussen 2 en 4 dagen bij 5℃, en tussen 1 en 3 uur bij 30℃. Significante verschillen werden waargenomen in overleving tussen de Brescia en Paderborn stam in faeces, maar niet in urine. In tegenstelling tot de afname aan infectieusiteit van het virus gedurende de tijd, was de afname van de hoeveelheid viraal RNA beperkt. Dit is vanuit diagnostisch oogpunt gunstig, want het betekent dat KVPV zelfs na langere tijd nog aangetoond kan worden. In deze studie werd voor het eerst inactivatie bepaald in excreta van geïnfecteerde varkens, in tegenstelling tot andere studies waar virus werd toegevoegd aan excreta. Daarom is deze studie een waardevolle aanvulling op bestaande literatuur.

Meer kennis over directe transmissie en parameters die daarop van invloed zijn, is opgedaan in binnen-hok transmissie experimenten. Het effect van virulentie van een virusstam en inoculatiedosis op directe transmissie werd onderzocht en is beschreven in hoofdstuk 6. Hiervoor werden vijf groepen van tien varkens gebruikt waarvan drie varkens per groep geïnfecteerd werden met de Brescia, Paderborn (een lage 10^2 $TCID_{50}$, middel $10^{3.5}$ $TCID_{50}$ of hoge 10^5 $TCID_{50}$ dosis) of de Zoelen stam. Om tussen deze verschillende stammen en dosis onderscheid te maken in transmissie werd virus

gekwantificeerd in oropharyngeale vloeistof en faeces. De transmissieparameters β en R_0 werden gekwantificeerd met behulp van een wiskundig model. De transmissiesnelheid β is gedefinieerd als het aantal dieren dat door één infectieus dier per tijdseenheid wordt besmet. De reproductie ratio R_0 is het totaal aantal secundaire infecties dat wordt veroorzaakt door één infectieus dier gedurende zijn hele infectieuze periode, in een volledig gevoelige populatie. Uit de resultaten bleek dat geen van de varkens geïnoculeerd met de lage dosis van de Paderborn stam werd geïnfecteerd. Verder kon geconcludeerd worden dat de laagvirulente stam een significant lagere transmissiesnelheid β had, terwijl de middel- en hoogvirulente stammen niet significant verschilden. De middelvirulente stam had wel een significant langere gemiddelde infectieuze periode, waardoor de R_0 meestal hoger was. De hoogvirulente stam had daarentegen een korter generatie-interval (tijd tussen het moment van infectie van een varken en het moment van infectie van een varken dat met dit dier in contact staat). Hierdoor hebben uitbraken met hoogvirulente stammen een grotere exponentiële groeisnelheid in het begin van een uitbraak.

Uit eerdere experimenten bleek dat infectie met de middelvirulente stam Paderborn kan leiden tot verschillende ziektebeelden: (sub)acute ziekte waarvan varkens herstellen of sterven, of chronische ziekte, die altijd resulteert in de dood. De excretie-dynamiek tussen deze ziektebeelden verschilt, maar het was niet duidelijk of dit ook resulteert in verschillen in transmissie. In vijftien paartjesexperimenten is gekeken naar het effect van het ziektebeeld op de virusexcretie en -transmissie (hoofdstuk 7). In elk van deze experimenten werd een geïnoculeerd varken gehuisvest met een gevoelig contactvarken (C1), vanaf dag 1 na inoculatie. Elk contactvarken dat geïnfecteerd raakte, werd verwijderd. Op dag 17 en 26 na inoculatie werden nieuwe contactdieren bij de geïnoculeerde dieren geplaatst (C2 en C3), die vervolgens ook verwijderd werden als ze geïnfecteerd raakten. Vijf van de vijftien geïnoculeerde varkens ontwikkelden een chronisch ziektebeeld of stierven in de acute fase (hoog-uitscheidende varkens). De overige tien varkens herstelden van de infectie (laag-uitscheidende varkens). In de eerste contactperiode (dag 1-17) was er geen significant verschil tussen hoog- en laag-uitscheidende varkens in virustiters in oropharyngeale vloeistof en faeces. In de tweede en derde contactperiode waren de virustiters bij de hoog-uitscheidende varkens significant hoger dan bij de laag-uitscheidende varkens. Over de gehele studieperiode was de R_0 significant hoger voor de hoog-uitscheidende varkens dan voor de laag-uitscheidende varkens. Dit geeft aan dat deze hoog-uitscheidende varkens een belangrijke rol kunnen spelen in de transmissie van het virus tijdens een uitbraak.

Om vervolgens meer inzicht te krijgen in de verschillende routes van transmissie, en het belang van verschillende parameters op de transmissie, werd een risicomodel ontwikkeld (Hoofdstuk 8). Hierin zijn in dit proefschrift beschreven kwantitatieve data gebruikt van virusexcretie, overleving van het virus en gevoeligheid van varkens. Het model schat de relatieve bijdrage van de verschillende secreta en excreta aan transmissie en schat kansen van KVPV-transmissie van één geïnfecteerd varken naar één gevoelig varken. De resultaten lieten zien dat de virusstam erg bepalend is voor de infectiekansen gedurende de tijd en de bijdrage van de verschillende secreta en excreta aan transmissie. Daarom zouden controlemaatregelen tijdens uitbraken, ideaal

gezien, gericht moeten zijn op de karakteristieken van een specifieke stam. Dit model zou in de toekomst, met aanvullende data, kunnen helpen om inzicht te geven in het effect van deze specifieke maatregelen.

Concluderend kan worden gesteld dat dit proefschrift zich richtte op het vergroten van de kennis van onderliggende mechanismen van KVPV-transmissie, met de nadruk op virusexcretie en overdracht van virus via directe en indirecte routes. Het is nog te vroeg om te concluderen dat bekende gevaarlijke contacten als direct contact met geïnfecteerde varkens, het voeren van keukenafval, contacten met virusgecontaminneerde transportwagens of mensen de belangrijkste transmissieroutes zijn tijdens een uitbraak. Het is ook nog niet bekend of niet-traceerbare routes zoals transmissie via vogels, huisdieren of knaagdieren die optreden als mechanische vector bijdragen aan de transmissie. Het in dit proefschrift beschreven model kan met aanvullende informatie gebruikt worden om de kansen van deze, en andere mogelijke routes, te schatten. Hiervoor is wel aanvullende kennis nodig, met name van contactstructuren tussen varkens en bedrijven, en hoeveelheden virus die tijdens deze contacten worden overgebracht. In dit proefschrift zijn verschillende mogelijkheden om deze kennis te vergaren bediscussieerd. Op basis van studies in dit proefschrift kan wel geconcludeerd worden dat transmissie via de lucht binnen een bedrijf een relevante rol kan spelen. Deze transmissieroute kan resulteren in meer geïnfecteerde varkens binnen een bedrijf, in een korter tijdsbestek dan wanneer deze route geen rol zou spelen. Dit kan vervolgens weer resulteren in een hogere kans op tussen-bedrijf transmissie, omdat dit afhankelijk is van de infectieusiteit van een bedrijf, wat waarschijnlijk gecorreleerd is aan het aantal geïnfecteerde varkens. De opgedane kennis in dit proefschrift kan helpen meer inzicht te krijgen in deze route binnen en tussen bedrijven.

Concluderende opmerkingen:

- In het algemeen geldt, hoe virulenter de virusstam, hoe meer virus wordt uitgescheiden tijdens de gehele infectieuze periode van een varken. Een uitzondering zijn varkens die geïnfecteerd worden met een middelvirulente stam die de chronische vorm van infectie ontwikkelen of die in de (sub)acute fase sterven (hoog-uitscheidende varkens), zoals werd aangetoond met de Paderborn stam.
- Hoog-uitscheidende varkens spelen een cruciale rol in de transmissie van KVPV door het hoge niveau van virusuitscheiding en de hoge reproductie ratio R_0.
- Dit proefschrift beschrijft voor de eerste keer de detectie en kwantificering van KVPV in de lucht van stallen met geïnfecteerde varkens.
- In dit proefschrift zijn temperatuursafhankelijke inactivatiecurves van KVPV in faeces en urine van geïnfecteerde varkens gemaakt. Deze informatie is waardevol voor risicomodellen om de kans op infectie van gevoelige varkens via indirecte transmissieroutes te schatten.
- Transmissiestudies laten zien dat, in het algemeen, de transmissiesnelheid β niet significant verschillend is tussen de hoogvirulente stam Brescia en de middelvirulente stam Paderborn. Echter, varkens geïnfecteerd met de middelvirulente stam hebben gemiddeld een significant langere infectieuze periode,

die kan resulteren in een hogere R_0 waarde. Aan de andere kant resulteert infectie met de hoogvirulente stam in een korter generatie-interval, wat resulteert in een hogere groeisnelheid tijdens het begin van een uitbraak.

- Waardevolle kennis is vergaard over de infectieusiteit van varkens, overleving van KVPV en gevoeligheid van varkens, die gebruikt kan worden in risicomodellen om de kans op infectie van gevoelige varkens via verschillende transmissieroutes te schatten. Echter, kwantitatieve data van de contactstructuur en hoeveelheden virus die worden overgebracht tussen varkens of bedrijven is cruciaal voor verder onderzoek naar transmissieroutes tussen bedrijven en mogelijke interventie-maatregelen.

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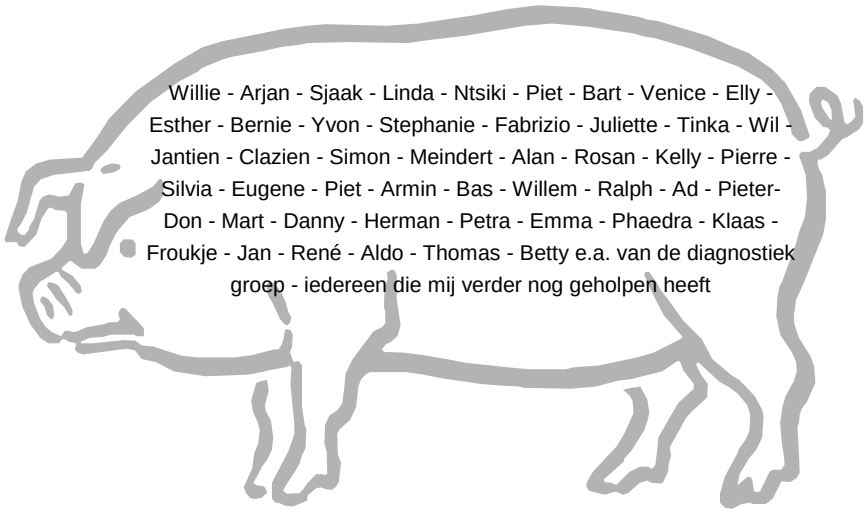

Curriculum vitae

Eefke Weesendorp werd op 25 maart 1979 geboren in Naarden. In 1996 haalde zij haar HAVO diploma en twee jaar later haar VWO-diploma. In 1998 begon zij met de studie Diergezondheidszorg aan het Van Hall Instituut in Leeuwarden. Tijdens deze studie volgde zij tevens de opleiding voor paraveterinair dierenartsassistent en artikel 12 van de Wet op de Dierproeven. Stages liep ze bij een melkveehouderijbedrijf, dierenartsenpraktijk, ID-Lelystad en Fort Dodge Animal Health. In 2002 behaalde zij haar diploma en ging voor drie maanden naar Canada om te assisteren op een dierenartsenpraktijk in Alberta. Weer in Nederland werkte ze als biotechnicus en deed onderzoek bij Fort Dodge Animal Health tot ze in 2003 aan haar studie Dierwetenschappen aan de Universiteit van Wageningen begon. Na stages bij het Rijksinstituut voor Volksgezondheid en Milieu (RIVM) en het Poultry and Rabbit Research Centre (PRRC) van Nutreco in Spanje werd deze studie in september 2005 cum laude afgerond. Na drie maanden als onderzoeksassistent op de Universiteit van Wageningen gewerkt te hebben begon ze als Assistent In Opleiding (AIO) aan haar promotie onderzoek bij het Centraal Veterinair Instituut van Wageningen UR (CVI) in Lelystad. Het onderzoek wat ze hier deed staat beschreven in dit proefschrift. Vanaf 1 januari 2010 werk zij bij het CVI als postdoc aan ‘porcine reproductive and respiratory syndrome virus’ onder leiding van Annemarie Rebel.

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