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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

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Abstract

Classical swine fever virus (CSFV) is transmitted via secretions and excretions of infected pigs. The efficiency and speed of the 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 qRRT-PCR (quantitative Real-Time Reverse Transcription Polymerase Chain Reaction). 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.

Keywords: Excretion routes; Classical swine fever virus, Chronic infection; Transmission

1. 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

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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

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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.

2. Materials and Methods

2.1 Experimental setting and housing

Three experiments were conducted in succession with five male pigs each. The eight-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

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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 three weeks.

2.2 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.

2.3 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.

2.4 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

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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.

2.5 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°C to avoid 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 was added to 2 ml of EDTA blood. After 10 minutes 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°C until analysis by virus titration and quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (qRRT-PCR). Once a week the EDTA blood (whole blood) was directly stored at -70°C for analysis.

Oropharyngeal fluid, saliva, nasal fluid and conjunctival fluid were collected every two 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 centimetre 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 minutes) the samples were stored at -70°C until analysis.

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Faeces was collected from the rectum every two 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 minutes) the supernatants were stored at -70°C 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 hours 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°C. Undiluted urine for qRRT-PCR analysis was stored at -70°C.

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 minutes samples were vortexed, centrifuged (1000 rpm for 10 minutes) and stored at -70°C until analysis.

2.6 Tests

2.6.1 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 x 10^9 cells/l blood, and thrombocytopenia as <200 x 10^9 cells/l blood.

2.6.2 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-wells plate (Greiner). For virus isolation from EDTA stabilized whole blood, oropharyngeal fluid, saliva, faeces, and urine, a volume of 250 µl was incubated for one hour 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°C in an atmosphere with 5% CO_2 for four days.

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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 fourfold (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).

2.6.3 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 was 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 °C in the sample 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 per ml or g) of each individual sample could be calculated using the standard curve. The standard curves were constructed based on Cp (crossing point) values for all dilutions of the positive control. The Cp value is the cycle number at which the fluorescence emission from a PCR reaction rises above the background signal. A low Cp value indicated a high template amount, while a high Cp indicated a low template amount.

2.7 Statistical analysis

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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.

3. Results

3.1 Clinical signs

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

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 (max. 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 day 5 and 24 p.i., the other pig was intermittently feverish for 25 days between day 5 and 41 p.i. The first three to five weeks clinical signs of limited severity were

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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 day 4 and 9 p.i.) and some mild clinical signs (Figure 1). The clinical signs observed were lethargy, a reduced appetite and dry faeces.

3.2 Leucocyte and thrombocyte count

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

3.3 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 two days before the infectious virus could be detected, and peak titres of viral RNA were

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0.9-2.7 log_{10} $TCID_{50}$ 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 3.8 log_{10} $TCID_{50}$ 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).

3.4 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

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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.

3.5 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 to 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 two 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

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entire infectious period, although they were not always significantly different to 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.

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 differs 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).

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.

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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 to 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 eight 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

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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 pigs 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

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lymphatic organs (Terpstra et al., 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

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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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References

Bouma, A., De Smit, A.J., De Kluijver, E.P., Terpstra, C., Moormann, R.J.M., 1999. Efficacy and stability of a subunit vaccine based on glycoprotein E2 of classical swine fever virus. Vet. Microbiol. 66, 101-114.

Carbrey, E.A., Stewart, W.C., Kresse, J.I., Snyder, M.L., 1980. Persistent hog cholera infection detected during virulence typing of 135 field isolated. Am. J. Vet. Res. 41, 946-949.

Depner, K.R., Rodriguez, A., Pohlenz, J., Liess, B., 1996. Persistent classical swine fever virus infection in pigs infected after weaning with a virus isolated during the 1995 epidemic in Germany: clinical, virological, serological and pathological findings. Eur. J. Vet. Path. 2, 61-66.

Depner, K.R., Hinrichs, U., Bickhardt, K., Greiser-Wilke, I., Pohlenz, J., Moennig, V., Liess, B., 1997. Influence of breed-related factors on the course of classical swine fever virus infection. Vet. Rec. 140, 506-507.

Dewulf, J., Koenen, F., Mintiens, K., Denis, P., Ribbens, S., De Kruif, A., 2004. Analytical performance of several classical swine fever laboratory diagnostic techniques on live animals for detection of infection. J. Virol. Methods 119, 137-143.

Accepted Manuscript

ACCEPTED MANUSCRIPT

Elbers, A.R.W, Stegeman, J.A., Moser, H., Ekker, H.M., Smak, J.A., Pluimers, F.H., 1999. The classical swine fever epidemic 1997-1998 in the Netherlands: descriptive epidemiology. Prev. Vet. Med. 42, 157-184.

Elbers, A.R.W., Stegeman, J.A., De Jong, M.C.M., 2001. Factors associated with the introduction of classical swine fever virus into pig herds in the central area of the 1997/98 epidemic in the Netherlands. Vet. Rec. 149, 377-382.

Finney, D.J., 1978. Statistical Methods in Biological Assay, Charles Griffin& Company LTD, London, pp. 394-401.

Floegel-Niesmann, G., Bunzenthal, C., Fischer, S., Moennig, V., 2003. Virulence of recent and former classical swine fever virus isolates evaluated by their clinical and pathological signs. J. Vet. Med. 50, 214-220.

GenStat Committee, 2007. The guide to GenStat® Release 10. (Payne R.W., Ed). VSN International, Hemel Hempstead, UK.

Kamolsiriprichaiporn, S., Morrissy, C.J, Westbury, H.A., 1992. A comparison of the pathogenicity of two strains of hog cholera virus. 2. Virological studies. Aust. Vet. J. 69, 245-248.

Klinkenberg, D., Moormann, R.J.M, De Smit, A.J., Bouma, A., De Jong, M.C.M., 2002. Influence of maternal antibodies on efficacy of a subunit vaccine: transmission of classical swine fever virus between pigs vaccinated at 2 weeks of age. Vaccine 20, 3005-3013.

Mengeling, W.L., Cheville, N.F.,1968. Host response to persistent infection with hog cholera virus. Proc. 72nd Ann. Meet. US Anim. Hlth Assoc., 283-296.

Mengeling, W.L., Packer, R.A, 1969. Pathogenesis of chronic hog cholera: host response. Am. J. Vet. Res. 30, 409-417.

Accepted Manuscript

ACCEPTED MANUSCRIPT

Mittelholzer C., Moser C., Tratschin J., Hofmann, M.A., 2000. Analysis of classical swine fever virus replication kinetics allows differentiation of highly virulent from avirulent strains. Vet. Microbiol. 74, 293-308.

Moennig, V., 2000. Introduction to classical swine fever: virus, disease and control policy. Vet. Microbiol. 73, 93-102.

Moennig, V., Floegel-Niesmann, G., Greiser-Wilke, I., 2003. Clinical signs and epidemiology of classical swine fever: A review of new knowledge. Vet. J. 165, 11-20.

Moormann, R.J.M., Bouma, A., Kramps, J.A., Terpstra, C., De Smit, H.J., 2000. Development of a classical swine fever subunit marker vaccine and companion diagnostic test. Vet. Microbiol. 73 209-219.

Oude Ophuis, R.J.A., Morrissy, C.J., Boyle, D.B., 2006. Detection and quantitative pathogenesis study of classical swine fever virus using a real time RT-PCR assay. J. Virol. Meth. 131, 78-85.

Ressang, A.A., van Bekkum, J.G., Bool, P.H., 1972. Virus excretion in vaccinated pigs subject to contact infection with virulent hog cholera strains. Zbl. Vet. Med. B. 19, 739-752.

Ressang, A.A., 1973. Studies on the pathogenesis of hog cholera. I. Demonstration of hog cholera virus subsequent to oral exposure. Zbl. Vet. Med. B. 20, 256-271.

Ribbens, S., Dewulf, J., Koenen, F., Laevens, H., De Kruif, A., 2004. Transmission of classical swine fever. A review. Vet.Q. 26, 146-155.

Terpstra, C., 1991. Hog cholera: an update of present knowledge. Br. Vet. J. 147, 396-406.

Accepted Manuscript

ACCEPTED MANUSCRIPT

Terpstra, C., De Smit, A.J., 2000. The 1997/1998 epizootic of swine fever in the Netherlands: Control strategies under a non-vaccination regimen. Vet. Microbiol. 77, 3-15.

Van Kleef, D.J., Deuring , K., Van Leeuwen, P., 1994. A new method of faeces collection in the pig. Lab. Anim. 28, 78-79.

Van Oirschot, J.T., 1980. Comparison of virulence of three field isolates of swine fever virus. Persistent and inapparent infections with swine fever virus of low virulence. Their effects on the immune system. Ph.D. Thesis. University of Utrecht, The Netherlands, pp. 120-125.

Van Oirschot, J.T., 1988, Description of the virus infection, In: Liess, B., Classical swine fever and related viral infections. Martinus Nijhoff Publishing, Dordrecht, pp. 1-25.

Van Rijn, P.A., Wellenberg, G.J., Hakze-van der Honing, R., Jacobs, L., Moonen, P.L., Feitsma, H., 2004. Detection of economically important viruses in boar semen by quantitative RealTime PCR technology. J. Virol. Meth. 120, 151-160.

Wensvoort, G., Terpstra, C., Boonstra, J., Bloemraad, M., Van Zaane, D., 1986. Production of monoclonal antibodies against swine fever virus and their use in laboratory diagnosis. Vet. Microbiol. 12, 101-108.

Wieringa-Jelsma, T., Quak, S., Loeffen, W.L.A., 2006. Limited BVDV transmission and full protection against CSFV transmission in pigs experimentally infected with BVDV type 1b. Vet. Microbiol. 118, 26-36.

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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.

Virus strain	Mean total excretion ($TCID_{50}$) analysed by VT				Mean total excretion ($TCID_{50}$equivalents) analysed by qRRT-PCR			
	Faeces		Urine		Faeces		Urine	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Brescia	$7.26^{a,12}$	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

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

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

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

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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 ($TCID_{50}$ 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
Paderborn-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

* 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.

$^{1-4}$ Means within columns with no common superscript differ significantly ($p < 0.05$)

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

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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 ($TCID_{50}$ equivalents 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
Paderborn-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

* This is equal to the area under the curves presented in Figure 4.

$^{1-4}$ Means within columns with no common superscript differ significantly ($p < 0.05$)

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

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Figure legends

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.

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.

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.

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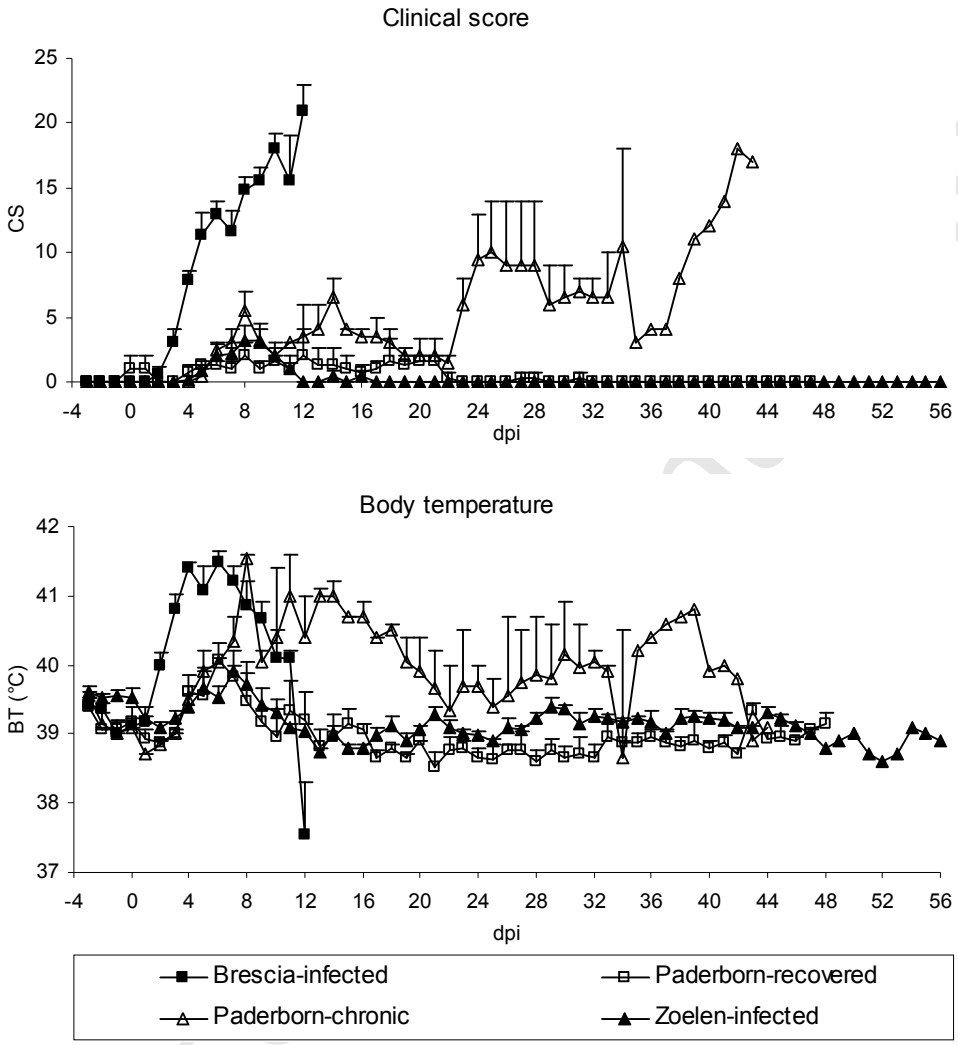

Figure. 1

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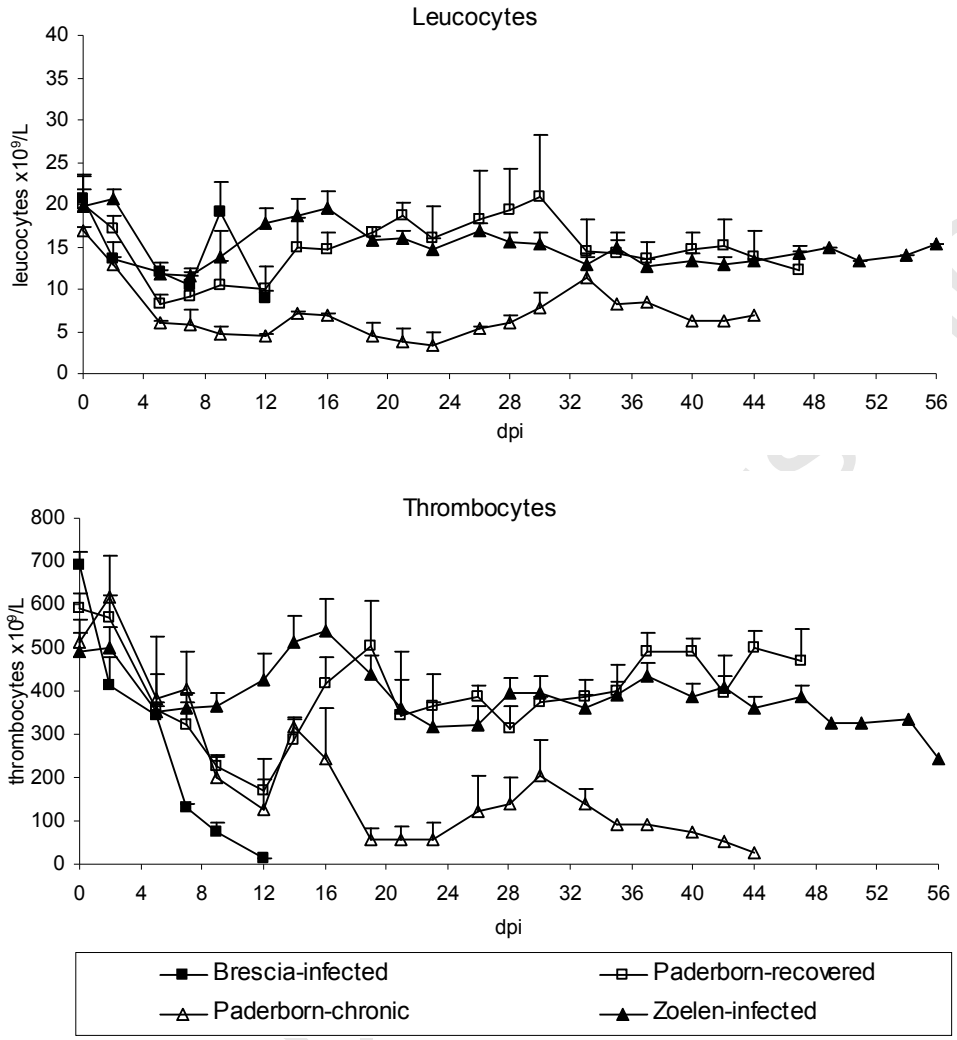

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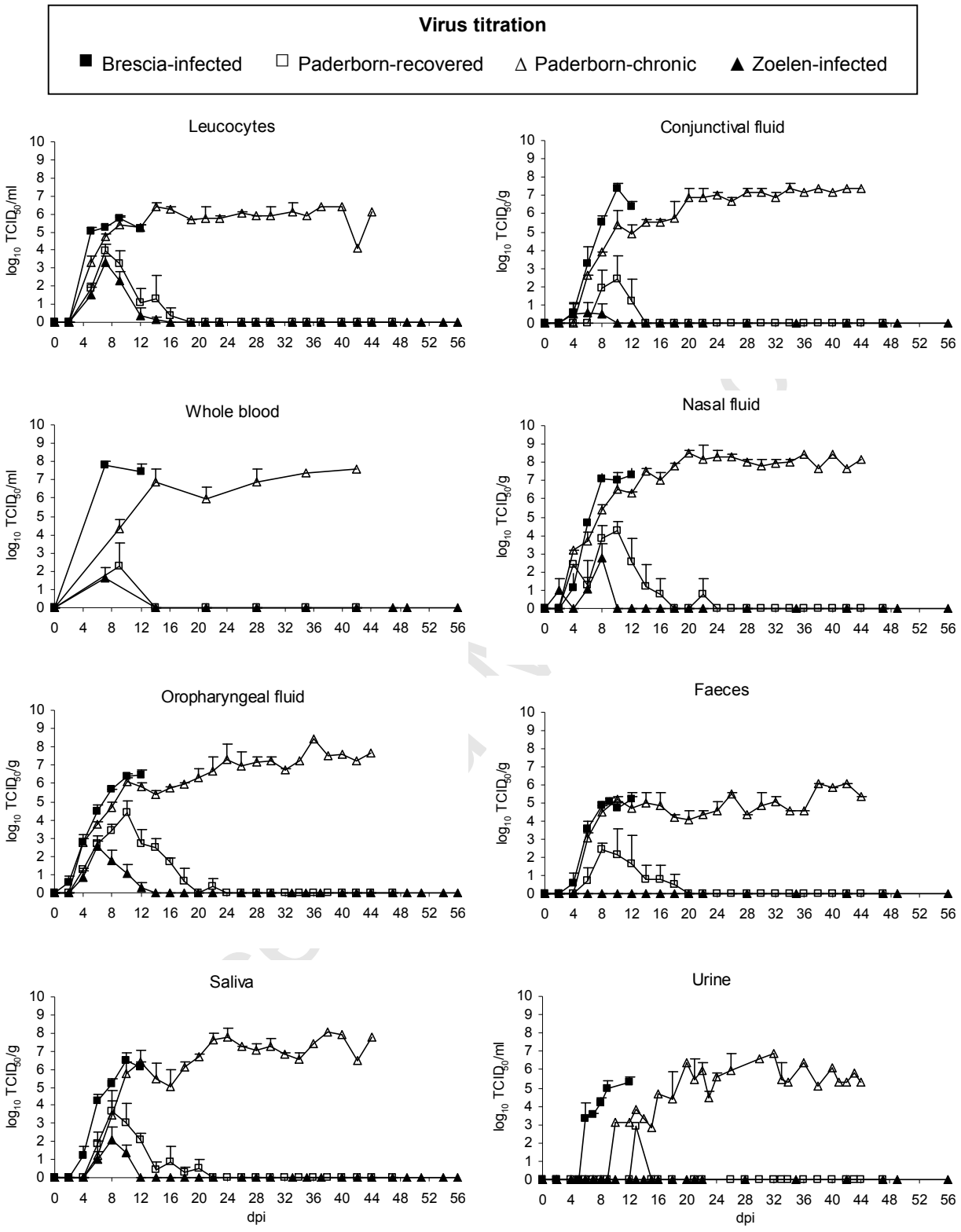

Figure 3

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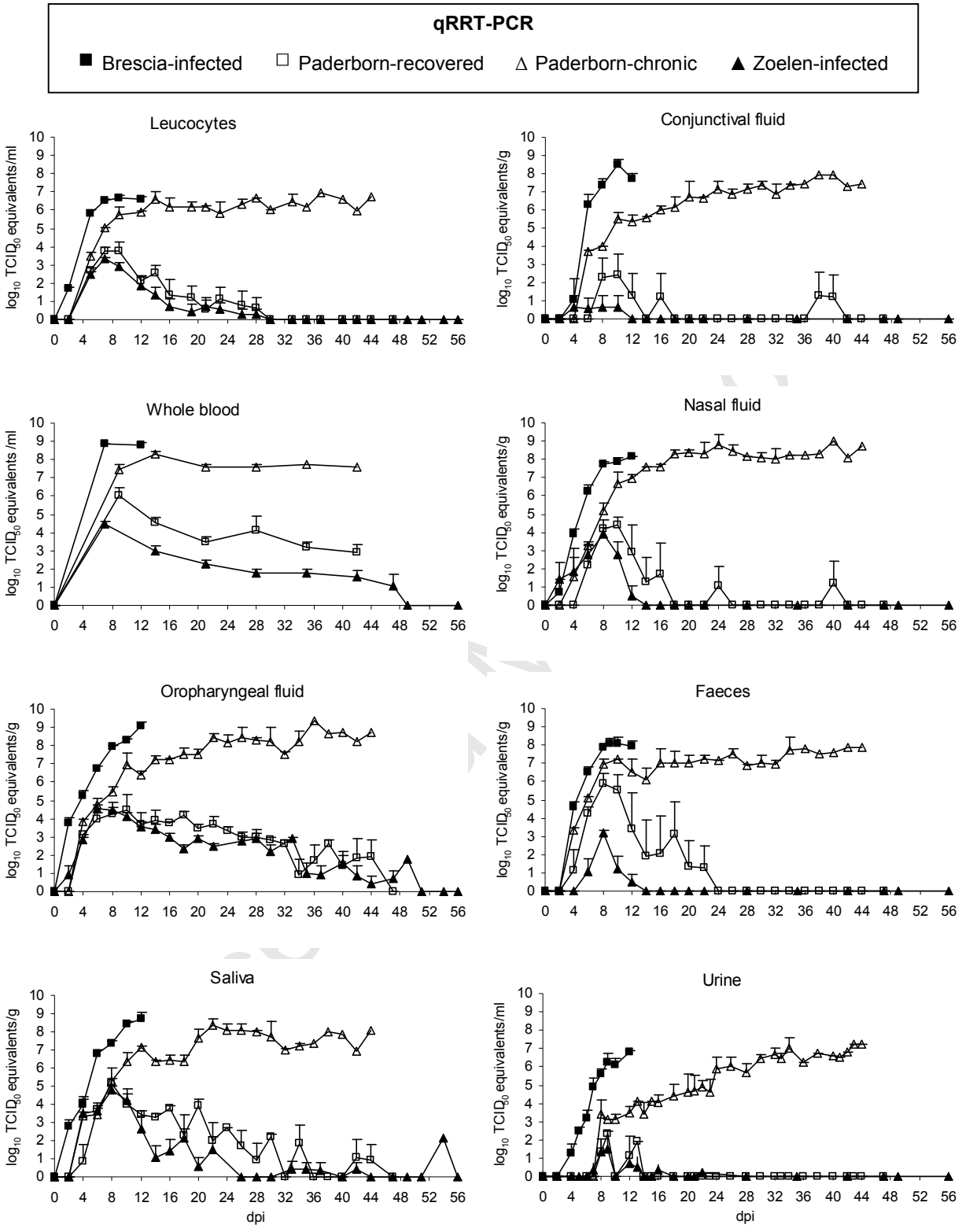

Figure 4.