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

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30 **Abstract**

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

49

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

51

52 **1. Introduction**

53 Classical Swine Fever (CSF) is a highly contagious disease and affects both domestic pigs and
54 wild boar. It is caused by an enveloped RNA-virus belonging to the family Flaviviridae, genus
55 *Pestivirus*. Mortality and the severity of clinical signs depends on the virulence of the virus strain,
56 and on characteristics of the pig such as age, breed and immune status (Moennig et al., 2003).
57 Highly virulent strains cause an acute haemorrhagic form of the disease that usually results in
58 death. The acute form is further characterized by high fever, anorexia, lethargy, conjunctivitis,
59 respiratory signs and constipation followed by diarrhea (Moennig et al., 2003). Moderately and low

60 virulent strains produce a form of the disease that is more difficult to recognize. Infection with
61 moderately virulent strains in particular can lead to different courses of the disease with a wide
62 spectrum of clinical signs (Floegel-Niesmann et al., 2003). Infections with these strains may result
63 in either (sub)acute disease, resulting in death or recovery, or chronic disease, which is always
64 fatal. Pigs infected with low virulent stains show few or no signs of disease and recover from the
65 infection (Van Oirschot, 1988).

66 In the 1980s, after successful eradication of CSF in most European Union countries of that
67 time, a non-vaccination policy was implemented. This ban on 'prophylactic' vaccination resulted in
68 a population of pigs fully susceptible to CSF. In combination with the high pig density in some
69 areas this resulted in rapid spread of CSF during outbreaks. High financial losses, due to mass
70 destruction of pigs and export bans, were the consequence (Moennig, 2000; Terpstra and De Smit,
71 2000; Moennig et al., 2003) .

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

86 It is likely that excretion of the virus depends on several factors, including breed, immune
87 status and virus strain. The effect of virus strain on excretion was discussed by Terpstra (1991).
88 According to Terpstra, pigs infected with highly virulent strains excrete large quantities of virus
89 during the entire course of disease, while pigs infected with low virulent strains excrete virus for

90 only a short period. However, no quantitative information was presented here, and no information
91 was given on moderately virulent strains, which are currently the most prevalent strains in the field
92 (Floegel-Niesmann et al., 2003). Beside the influence of the strain on the total amount of excreted
93 virus, there is a difference between excretion routes in quantities of virus excreted. After infection
94 with a highly virulent strain, large quantities of virus were excreted in saliva and smaller quantities
95 in urine and faeces (Ressang, 1973). These data, however, mainly referred to the early stage of
96 infection. Using more recent techniques like RT-PCR, virus has been detected in nasal fluid,
97 faeces and semen, although the virus excretion was mostly not quantified (Oude Ophuis et al.,
98 2006; Van Rijn et al., 2004). To our knowledge, no studies have been published that give an
99 integrated overview of the dynamics of virus excretion via the different secretions or excretions of
100 the pig. This is important information for elucidating the role of the different excretion routes in
101 transmission. In this paper we quantified the virus excreted during the entire infectious period via
102 saliva, oropharyngeal fluid, nasal fluid, conjunctival fluid, faeces, urine and the skin of pigs infected
103 with a highly, moderately or low virulent CSFV strain. Virulence as well as the course of the
104 disease (e.g. acute or chronic), strongly influenced the quantities of virus in the secretions and
105 excretions.

106

107 **2. Materials and Methods**

108 *2.1 Experimental setting and housing*

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

118 To enable the calculation of total amounts of excreted virus in faeces and urine, the cages
119 were specially designed to collect and separate these excretions. Faeces were collected in plastic

120 bags attached to the pigs with a Velcro system. This Velcro system was glued directly on the skin
121 around the anus (Van Kleef et al., 1994). The cages were equipped with slatted floors which
122 allowed the collection of urine in a container attached to the tray underneath the cage. Faeces and
123 urine production were recorded daily.

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

128

129 *2.2 Viruses*

130 In each experiment, five pigs were inoculated with either the highly virulent Brescia strain
131 (genotype 1.2, derived from a strain obtained from Brescia, Italy, 1951), the moderately virulent
132 Paderborn strain (genotype 2.1, isolated in 1997 during the outbreak in the Paderborn area of
133 Germany) or the low virulent Zoelen strain (genotype 2.2, originally isolated during an outbreak on
134 a Dutch farm (Van Oirschot, 1980)). According to the classification of CSFV strains by Van
135 Oirschot (1988), infection with a highly virulent strain results in death of nearly all pigs. Infection
136 with a moderately virulent strain results in acute or subacute illness leading to death, recovery, or
137 to the chronic form (a lethal clinical form leading to death 30 days or more after infection). Pigs
138 infected with a low virulent strain show few or no signs of disease and recover from the infection.

139

140 *2.3 Inoculation of animals*

141 Pigs were inoculated intranasally with 1 ml of 100 LD₅₀ (50% lethal dose) CSFV strain Brescia,
142 which is approximately 10^{2.5} TCID₅₀ (tissue culture infectious dose 50%), with 1 ml of 10⁵ TCID₅₀
143 strain Paderborn or 1 ml of 10⁵ TCID₅₀ strain Zoelen, according to the standard infection models
144 used in our institute (Bouma et al., 1999; Moormann et al., 2000; Klinkenberg et al., 2002). The
145 inocula were back titrated to confirm the dose administered.

146

147 *2.4 Clinical signs and body temperature*

148 Body temperature and clinical signs were recorded daily. Fever was defined as body temperature
149 higher than 40°C. For quantitative assessment of the severity of disease a list of ten CSF-relevant

150 criteria, as described by Mittelholzer et al. (2000) was used. For all criteria a score was recorded of
151 either normal (score 0), slightly altered (score 1), distinct clinical symptom (score 2), or severe CSF
152 symptom (score 3). The scores for each pig were added up to a total score per day. Sick pigs that
153 became moribund and unable to stand up were euthanized for reasons of animal welfare.

154

155 *2.5 Sampling procedures and pre- treatment of samples*

156 Samples were collected from blood, oropharyngeal fluid, saliva, nasal fluid, conjunctival fluid,
157 faeces, urine, and skin scrapings to determine the virus titres. Directly after collection the samples
158 were stored at 5°C to avoid inactivation of the virus.

159 EDTA stabilized blood samples were collected three times a week for leucocyte and
160 thrombocyte counts, and for isolation of leucocytes. For isolation of leucocytes, 4 ml 0.84% NH₄CL
161 solution was added to 2 ml of EDTA blood. After 10 minutes the samples were centrifuged at 1000
162 rpm and washed twice with PBS. The pellet was resuspended in 2 ml medium (Eagle minimum
163 essential medium (EMEM) (Gibco, Invitrogen, Breda, The Netherlands) with 5% fetal bovine serum
164 (FBS), and 10% antibiotics) and stored at -70°C until analysis by virus titration and quantitative
165 Real-Time Reverse Transcription Polymerase Chain Reaction (qRRT-PCR). Once a week the
166 EDTA blood (whole blood) was directly stored at -70°C for analysis.

167 Oropharyngeal fluid, saliva, nasal fluid and conjunctival fluid were collected every two
168 days. Pigs infected with the low virulent Zoelen strain were sampled with larger intervals from 3
169 weeks post inoculation (p.i.), as virus isolations carried out in between were already negative
170 (Figures 3 and 4 for exact sampling times). Saliva was obtained by holding a gauze tampon in the
171 oral cavity until it was soaked with saliva. Oropharyngeal fluid was obtained with a gauze tampon
172 held by a 30 centimetre long forceps, which was scrubbed against the dorsal wall of the pharynx
173 behind the soft palatum (Ressang et al., 1972). Samples from conjunctival and nasal fluid were
174 collected using sterile rayon swabs (Medical Wire & Equipment, Corsham, United Kingdom).
175 Swabs and tampons were weighed before and after collection to enable the calculation of the
176 concentration of virus per gram of fluid (TCID₅₀/g). The swabs and tampons were suspended in 4
177 ml of the same media described for the leucocyte isolation. After centrifugation (2500 rpm for 15
178 minutes) the samples were stored at -70°C until analysis.

179 Faeces was collected from the rectum every two days by stimulation of the anus. One
180 gram of faeces was suspended in 9 ml medium (EMEM containing 10% FBS and 10% antibiotics)
181 and vortexed with glass beads. After centrifugation (10.000 rpm for 5 minutes) the supernatants
182 were stored at -70°C until analysis.

183 Urine was collected as often as possible. Only fresh urine, obtained while the pig urinated,
184 was analysed by virus titration. In those cases where fresh samples could not be obtained, urine
185 was collected from a container attached to the tray under the cage. This container was replaced
186 daily, so the urine collected was maximum 24 hours old. All samples, both fresh and from the
187 container, were analysed by qRRT-PCR. A ten-fold dilution in medium (EMEM containing 10%
188 FBS and 10% antibiotics) was prepared from the urine samples for virus titration and stored at
189 -70°C. Undiluted urine for qRRT-PCR analysis was stored at -70°C.

190 Skin scrapings were taken two or three times per animal, once clinical signs were
191 observed. Skin was scraped off from the back of the pig between the scapulas, using a plastic tube
192 with a sharp edge, until the skin was red. The skin sample was suspended in 3 ml of medium. After
193 15 minutes samples were vortexed, centrifuged (1000 rpm for 10 minutes) and stored at -70°C
194 until analysis.

195

196 2.6 Tests

197 2.6.1 Leucocyte and thrombocyte counts

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

201

202 2.6.2 Virus isolation and titration

203 For virus isolation from leucocytes, nasal fluid, conjunctival fluid, or skin scrapings, a volume of 125
204 μ l was inoculated on a monolayer of SK6 cells (permanent porcine kidney cell line) in a 24-wells
205 plate (Greiner). For virus isolation from EDTA stabilized whole blood, oropharyngeal fluid, saliva,
206 faeces, and urine, a volume of 250 μ l was incubated for one hour on a monolayer of SK6 cells.
207 These plates were then washed once with PBS (phosphate-buffered saline) and medium was
208 added to the wells. All plates were incubated at 37°C in an atmosphere with 5% CO₂ for four days.

209 After being fixated and washed, the monolayers were stained by the immuno-peroxidase technique
210 (Wensvoort et al., 1986), using two HPRO-conjugated CSFV specific MAbs (V3/V4), and examined
211 for stained cells.

212 Virus positive samples were titrated in fourfold (using five decimal dilutions) to determine
213 the concentration of infectious virus. Virus titres were calculated as TCID₅₀ using the Spearman-
214 Kärber method (Finney, 1978).

215

216 *2.6.3 Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (qRRT-PCR)*

217 Virus excretion was analysed by qRRT-PCR to determine the concentration of viral RNA. For RNA
218 isolation, 200 µl of the sample was pipetted manually into MagNA Pure sample cartridges (Roche
219 Applied Science, Mannheim, Germany). In each run of thirty-two samples two negative control
220 samples and three to six dilutions of a positive control sample (standard curve) were included. The
221 standard curves were constructed for each sample type and each strain of virus by spiking
222 secretions and excretions with known concentrations of infectious virus. The RNA was extracted
223 with the Total Nucleic Acid Isolation Kit (Roche Applied Science, Mannheim, Germany) according
224 to the manufacturer's instructions using the automated MagNA Pure LC instrument (Roche Applied
225 Science, Mannheim, Germany). After the MagNA Pure completed the RNA isolation, the nucleic
226 acids were removed from the MagNA Pure LC and immediately processed for the qRRT-PCR or
227 stored at -70 °C in the sample cartridge until the PCR was carried out.

228 The qRRT-PCR was performed with a LightCycler (LC) instrument (Roche Applied
229 Science, Mannheim, Germany) using the RNA Master Hybridization Probes Kit, as described by
230 Van Rijn et al. (2004). Analysis was performed with the LC software. The viral RNA concentration
231 (TCID₅₀ equivalents per ml or g) of each individual sample could be calculated using the standard
232 curve. The standard curves were constructed based on Cp (crossing point) values for all dilutions
233 of the positive control. The Cp value is the cycle number at which the fluorescence emission from a
234 PCR reaction rises above the background signal. A low Cp value indicated a high template
235 amount, while a high Cp indicated a low template amount.

236

237 *2.7 Statistical analysis*

238 Total virus excretion per secretion or excretion of individual pigs was expressed by the area under
239 the 'excretion against time' curve. This represents the total amount of infectious virus (virus titration
240 data) or viral RNA (qRRT-PCR data) excreted during the entire infectious period, standardised to 1
241 g/day. Furthermore, total amounts of faeces and urine produced each day were recorded, which
242 allowed for the calculation of total amount of infectious virus (in TCID₅₀) and viral RNA (in TCID₅₀
243 equivalents) excreted via these routes per pig during the entire infectious period. Differences
244 between the strains were statistically analysed using an analysis of variance (ANOVA) model. Pair
245 wise comparisons between strains were made using the least significant difference (LSD) method.
246 To compare secretions and excretions per strain, differences were calculated within animals and
247 analysed with an ANOVA model comprising a factor for strains. Per strain, differences were
248 analysed with a paired t-test, with a pooled estimate for the residual variance. Calculations were
249 performed with the statistical programming language GenStat (2007). Mean differences were
250 considered significant when the *p* value was less than 0.05.

251

252 **3. Results**

253 *3.1 Clinical signs*

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

260 A wide distribution of clinical signs and fever was observed in the group of pigs infected
261 with the Paderborn strain (Figure 1). Three pigs recovered from the infection (described as the
262 "Paderborn-recovered" pigs), while two pigs became chronically infected (described as the
263 "Paderborn chronically infected" pigs). Two of the three Paderborn-recovered pigs showed fever
264 (max. 40.4°C) and clinical signs. In these pigs lethargy, reduced appetite, stiff walking, and
265 constipation were observed. The two chronically infected pigs showed fever for a long duration;
266 one pig for 20 days between day 5 and 24 p.i., the other pig was intermittently feverish for 25 days
267 between day 5 and 41 p.i. The first three to five weeks clinical signs of limited severity were

268 observed (depression, growth retardation, anorexia, constipation and respiratory signs), while in
269 the terminal phase the clinical signs included petechia on the skin, a staggering gait, and
270 weakness of the hind legs. They became moribund and were euthanized on days 34 and 44 p.i.

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

274

275 *3.2 Leucocyte and thrombocyte count*

276 The average leucocyte and thrombocyte levels during the study are shown in Figure 2. Pigs
277 infected with the Brescia strain showed from day 2 p.i. a decrease in the level of leucocytes,
278 although this decrease was only severe enough to be classified as a leucopenia in one pig at day 2
279 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.,
280 when three of the four pigs showed an increase in the level of leucocytes of 8 to 19 x 10⁹ cells/l
281 blood compared to day 8. This might have been due to dehydration of pigs and subsequent
282 hypovolemia, or to secondary bacterial infections. Thrombocytopenia was observed from day 7 p.i.
283 to death.

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

288 By day 5 p.i., in all pigs infected with the Zoelen strain, the level of leucocytes had
289 decreased by 2 to 13 x 10⁹ cells/l blood compared to day 0. In one pig the decrease in leucocytes
290 was severe enough to be classified as leucopenia (at day 9 p.i.). Two of the five pigs showed
291 thrombocytopenia at day 5 p.i. The other three pigs showed a small decrease in thrombocytes to a
292 minimum level of 342 x 10⁹ cells/l blood between days 7 and 12.

293

294 *3.3 Virus titres in blood, secretions and excretions*

295 Infectious virus and viral RNA were detected in blood and all secretions and excretions of pigs
296 infected with the Brescia strain (Figures 3 and 4). Viral RNA was detected in most secretions and
297 excretions two days before the infectious virus could be detected, and peak titres of viral RNA were

298 0.9-2.7 log₁₀ TCID₅₀ equivalents/g or ml higher than infectious virus titres (Figure 4). Infectious
299 virus was detected in skin scrapings from two pigs on day 12 p.i. (average concentration of 10^{1.9}
300 TCID₅₀/cm² of skin), while viral RNA was detected in all skin scrapings obtained between days 9
301 and 12 p.i (average concentration of 10^{2.6} TCID₅₀ equivalents/cm² of skin).

302 Large differences in virus titres were observed between pigs infected with the Paderborn strain.
303 The two pigs that developed the chronic form of infection had high titres of virus in blood and all
304 secretions and excretions until death, while in samples from the three pigs that recovered from the
305 infection, lower titres were detected for maximum 20 days (Figure 3). Virus was also detected at an
306 earlier sampling moment in most secretions and excretions of the chronically infected pigs.
307 Infectious virus was not detected in all sample types obtained from the pigs that recovered from the
308 infection. It could be detected in the urine of only one pig, although viral RNA was detected in the
309 urine of all three pigs. Viral RNA titres were higher (maximum 3.8 log₁₀ TCID₅₀ equivalents/g or ml)
310 than the infectious virus titres, and viral RNA could still be detected late (days 42-44 p.i.) in the
311 infection in whole blood, oropharyngeal fluid and saliva (Figure 4). No infectious virus or viral RNA
312 could be detected in skin scrapings from pigs that recovered at days 13 and 15 p.i. In the skin
313 scrapings of the chronically infected pigs viral RNA could be isolated at day 33 p.i (average
314 concentration of 10^{2.9} TCID₅₀ equivalents/cm² of skin).

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

320

321 *3.4 Actual virus excretion during the entire infectious period in faeces and urine*

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

325 Pigs infected with the Brescia strain and the chronically infected pigs (Paderborn strain)
326 excreted the largest amounts of infectious virus and viral RNA via faeces and urine into the
327 environment compared to the other groups (Table 1). However, at moments that these pigs

328 showed distinct clinical symptoms of CSF (CS equal to or greater than 8), they produced less
329 faeces (on average 154 g) and urine (on average 567 ml) than pigs with few clinical symptoms or
330 subclinical illness (on average 442 g faeces and 1530 ml urine). Mainly during the terminal phase
331 of the disease, a low production of these excretions was observed. Despite this lower production,
332 virus titres in faeces and urine of these pigs were so high, that large amounts of virus were
333 excreted via these routes. The excretion from pigs infected with the Paderborn strain that
334 recovered from the infection was significantly lower. Excretion in faeces and urine from pigs
335 infected with the Zoelen strain was only detectable by qRRT-PCR.

336

337 *3.5 Standardised virus excretion during the entire infectious period*

338 The exact amounts of oropharyngeal fluid, saliva, conjunctival fluid and nasal fluid that would end
339 up in the environment could not be determined. Therefore, it was not possible to calculate actual
340 amounts of virus excreted into the environment. Instead, amounts of virus excreted are
341 represented in TCID₅₀ or TCID₅₀ equivalents standardised to 1 g or ml of secretion or excretion per
342 day (Tables 2 and 3). This allowed comparisons among the different routes. Excretion of virus in
343 faeces and urine was also calculated this way, to enable the comparison with other secretions and
344 excretions.

345 It was shown that pigs which developed the chronic form of infection after inoculation with
346 the Paderborn strain excreted the largest amounts of virus via most secretions and excretions
347 during the entire infectious period compared to the other strains (Tables 2 and 3). These pigs had
348 high titres of virus (infectious virus up to an average of $10^{8.5}$ TCID₅₀/g) in their secretions and
349 excretions for a long duration (32 to 42 days) (Figures 3 and 4). Although Brescia-infected pigs had
350 also high virus titres in their secretions and excretions (infectious virus up to an average of $10^{7.4}$
351 TCID₅₀/g), they died within two weeks after inoculation, making the amounts of virus excreted
352 equal to or lower than those from pigs chronically infected with the Paderborn strain. Pigs infected
353 with the Paderborn strain that recovered from the infection excreted significantly smaller amounts
354 of virus during their entire infectious period. These pigs had lower virus titres (infectious virus up to
355 an average of $10^{4.4}$ TCID₅₀/g) in their secretions and excretions, for a shorter duration (maximum 9
356 days). Pigs infected with the Zoelen strain excreted the smallest amounts of virus during their

357 entire infectious period, although they were not always significantly different to the Paderborn-
358 recovered pigs.

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

364 The high level of viral RNA excretion via faeces, which is for most strains as high as viral
365 RNA excretion via the oronasal route (Table 3), is remarkable. The excretion of viral RNA via
366 conjunctival fluid differs between strains. Pigs infected with the highly virulent Brescia strain and
367 chronically infected pigs excreted via this excretion route amounts comparable to the oronasal
368 route, while pigs infected with the low virulent strain excreted significantly lower amounts than via
369 the oronasal route. Significantly smaller amounts of viral RNA were excreted via the urine during
370 the total infectious period (except for the chronically infected pigs).

371

372 **4. Discussion**

373 In this paper, the dynamics of virus excretion by CSFV infected pigs during the entire infectious
374 period were determined after infection with a highly, moderately or low virulent strain. Distinct
375 differences between the strains were observed in virus excretion. Overall, virus excretion after
376 infections with highly virulent strains can be expected to be much higher than after an infection with
377 a moderately virulent strain, even though the infectious period may be shorter because of a
378 relatively quick death. In our study this was up to 500 times as much, based on standardised
379 amounts of secretions/excretions. Infections with moderately virulent strains in turn can be
380 expected to result in a higher overall excretion of virus than infections with a low virulent strain. In
381 our study this was up to 50 times as much. The most striking observation was however the amount
382 of virus excreted by pigs that developed the chronic form of infection after inoculation with the
383 moderately virulent Paderborn strain. Due to a combination of high virus titres in all excretions and
384 secretions, and the long infectious period, they excreted up to 40.000 times more virus than
385 acutely infected and recovered pigs did.

386 The chronically infected pigs showed a persistent viraemia and high virus titres in all
387 secretions and excretions. Already in the first phase of infection virus titres were higher and could
388 be detected earlier than in pigs infected with the same strain, but that recovered from the infection.
389 Relatively few clinical symptoms were observed during this first phase, that lasted from day 5 p.i.
390 until the terminal phase (6 to 10 days before death). During the terminal phase, clinical symptoms
391 increased markedly until the pigs died. The persistence of chronic CSF in the host's body during its
392 entire lifetime and resulting in persistent viraemia was described before (Carbrey et al., 1980;
393 Depner et al., 1996; Moennig et al., 2003). However, the chronic form was also described as
394 having three phases, based on severity of clinical symptoms and viraemia (Mengeling and Packer,
395 1969). In the first phase the spread of virus through the body resembled that in acute CSF, but was
396 slower and virus titres in serum and organs tended to be lower. This was followed by a phase with
397 partial clinical recovery in which virus titres were low or absent. In the terminal phase virus spread
398 again throughout the body with exacerbation of clinical symptoms. The observations in the present
399 study do not confirm these observations of Mengeling and Packer (1969). Instead of the slower
400 spread of the virus throughout the pig's body in the first phase and the lower virus titres, we
401 observed a faster spread with higher virus titres. Also during the rest of the pig's life titres remained
402 higher and no phase of apparent recovery was seen. A similarity to the chronically infected pigs
403 described by Mengeling and Packer (1969) is the persistent leucopenia during the first and second
404 phase of illness. However, in the terminal phase the pigs studied by Mengeling and Packer
405 developed a leucocytosis, which may reflect a response to secondary bacterial infections. In the
406 present study, this leucocytosis was not seen, but there was also no indication that secondary
407 bacterial infections were involved.

408 Development of the chronic form might depend on age, breed and immune status (Depner
409 et al., 1997; Moennig et al., 2003). Moreover, the inoculation dose or the route of inoculation could
410 influence this form of disease. In the present study young pigs with an undeveloped immune
411 system were used, which might have resulted in the high frequency (40%) of chronic infection. In a
412 recent study, using eight week-old pigs infected with the Paderborn strain, chronic infection at a
413 rate of only 10% was observed (Wieringa-Jelsma et al., 2006). Other moderately virulent strains
414 are also known to induce the chronic form of CSF. For example, the moderately virulent strain 311
415 used by Mengeling and Cheville (1968) to infect 69 pigs, resulted in 45% of these becoming

416 chronically infected. During virulence typing of 135 field isolates in the United States by
417 intramuscular inoculation, 6% produced chronically infected pigs (i.e. high concentration of CSFV
418 in the blood for a long duration and relatively free of illness). According to the authors (Carbrey et
419 al., 1980), this was a reasonable indicator of the frequency of this event in the field. Reliable data
420 on the frequency of chronic infections in the field is however unavailable. The prevalence of the
421 chronic form of infection during the 1997/1998 outbreak of the Paderborn strain in The Netherlands
422 was unknown because herds were culled immediately after infection was detected. It is
423 questionable whether the occurrence was comparable to the present study, since pigs of different
424 age groups and immune statuses were infected, with different doses, and via different routes.
425 However, since chronically infected pigs excrete large amounts of virus they may have played a
426 crucial role in spreading the virus.

427 The quantitative excretion data provided by this study gives some insight into the role the
428 different secretions and excretions play in transmission. This role depends on the total amount of
429 virus-containing secretions and excretions produced during the infection and the virus titres herein,
430 which in turn is influenced by the virulence of the virus strain. During outbreaks caused by highly
431 and moderately virulent strains, large amounts of virus (up to $10^{9.9}$ TCID₅₀ of infectious virus/per
432 pig/day) can be excreted via faeces and urine. Saliva and nasal fluid are expected to be excreted
433 in the environment in smaller amounts than faeces and urine, but as virus excretion is up to 1300
434 times (saliva) to 5000 times (nasal fluid) higher than in faeces and urine, their contribution to
435 transmission might be equal or even higher. These secretions and excretions together may be
436 responsible for a major part to the transmission between- and within pens, and even between
437 farms, as they are easily transmitted via contaminated boots, clothes or trucks. Virus excretion in
438 conjunctival fluid is up to 300 times higher than in faeces and urine. However, this is expected to
439 be excreted in much smaller amounts in the environment, and therefore its contribution might be
440 limited.

441 The difference in virus excretion between the strains can be the result of differences in the
442 spread of the virus throughout the pigs body. Highly virulent virus usually spreads rapidly
443 throughout the body, resulting in high virus titres in most organs and blood, whereas with
444 moderately virulent strains, virus titres tend to be lower. Infections with the low virulent strains
445 result in a slow spread, with lower virus titres, mostly restricted to certain organs like the tonsil and

446 lymphatic organs (Terpstra et al., 1991; Kamolsiriprichaiporn et al., 1992). As a consequence,
447 there is a not only a difference between the strains in the first moment of excretion and the
448 quantities of virus excreted, but also in the type of secretions or excretions that contain virus. While
449 pigs infected with the highly virulent strain excreted virus in all secretions and excretions, virus
450 excretion from pigs infected with the low virulent strains was mainly restricted to the oronasal
451 excretion routes.

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

462 Proportionate and risk-based control eradication measures can be applied only if sufficient
463 knowledge is available on virus transmission between animals and herds. To interpret and
464 interpolate transmission data from experimental infections and outbreaks in the field, knowledge of
465 the underlying mechanisms of transmission is needed, including quantitative excretion data as
466 provided in this study. Based on this study one could argue that outbreaks caused by highly or
467 moderately virulent strains that result in a proportion of chronically infected pigs, spread faster in a
468 population than outbreaks caused by low virulent strains. Infections caused by highly virulent
469 strains result in large amounts of virus excreted, but the duration of virus excretion is short as most
470 pigs die shortly after infection. Furthermore, infected pigs are relatively easy to recognize. On the
471 other hand, during outbreaks caused by low virulent strains, less virus is excreted, and the
472 absence of infectious virus in faeces and urine may contribute to a slower spread of the disease.
473 However, as clinical signs are almost absent, infected pigs are difficult to recognize and thus can
474 act as a source of virus dissemination. With the current data on excretion dynamics, it would be
475 interesting to study the reproduction ratio (R_0) of these strains in a transmission experiment. A

476 correlation between virus excretion and reproduction ratio is expected. Furthermore, studies like
477 that could give initial indications on the possible role of additional underlying mechanisms that
478 include virus survival, contact structure and susceptibility of the recipient pigs.

479

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484

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586 protection against CSFV transmission in pigs experimentally infected with BVDV type 1b. *Vet.*
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588

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

591

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

592

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

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

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

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

598

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

599

600 * This is equal to the area under the curves presented in Figure 3.

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

602 ¹⁻⁴ Means within columns with no common superscript differ significantly ($p < 0.05$)

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

604

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

607

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

608

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

610 ¹⁻⁴ Means within columns with no common superscript differ significantly ($p < 0.05$)

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

612

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.

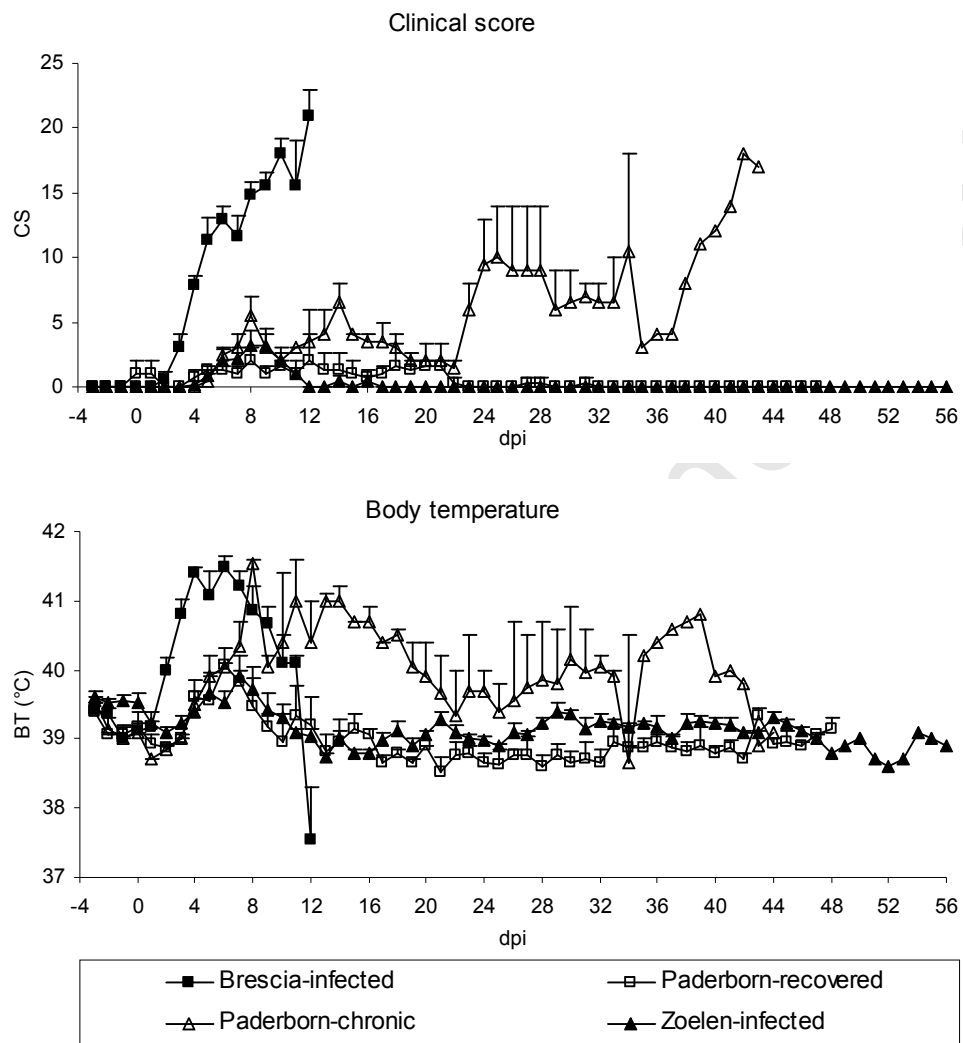


Figure. 1

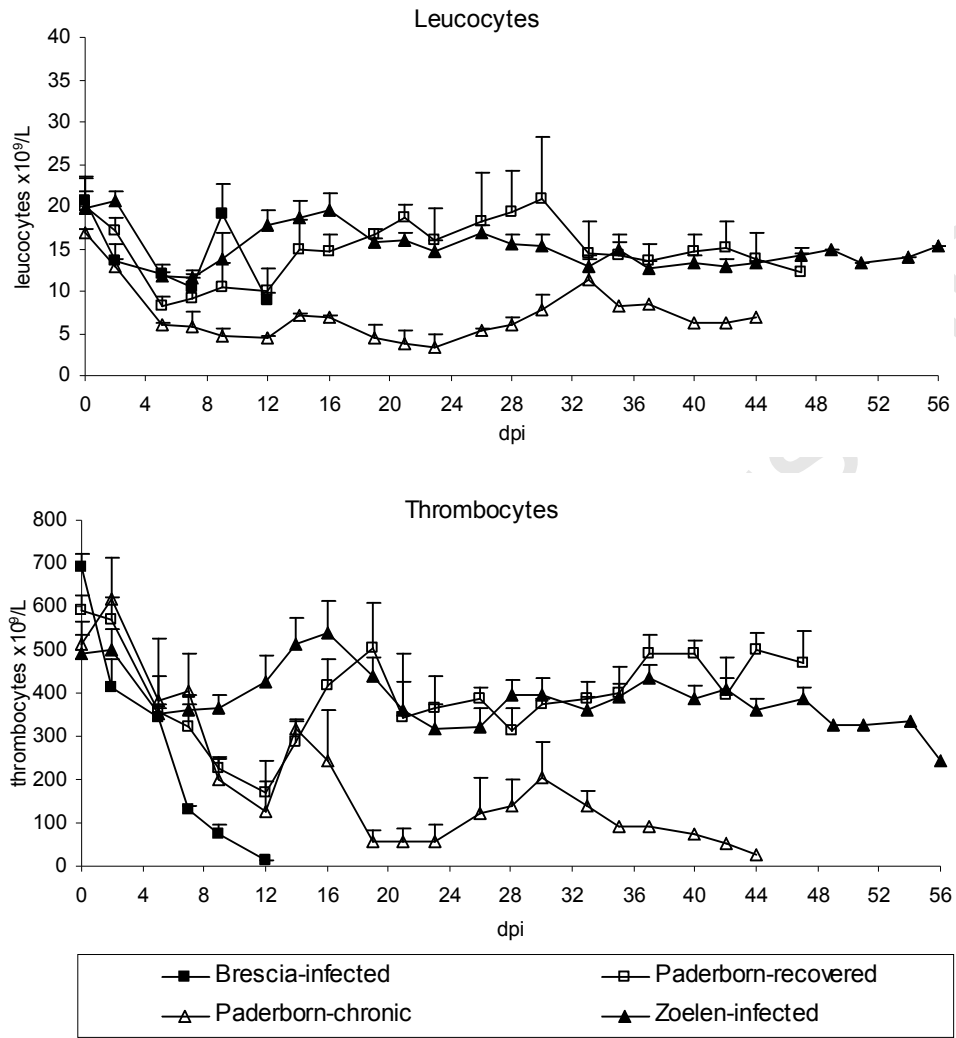


Figure 2.

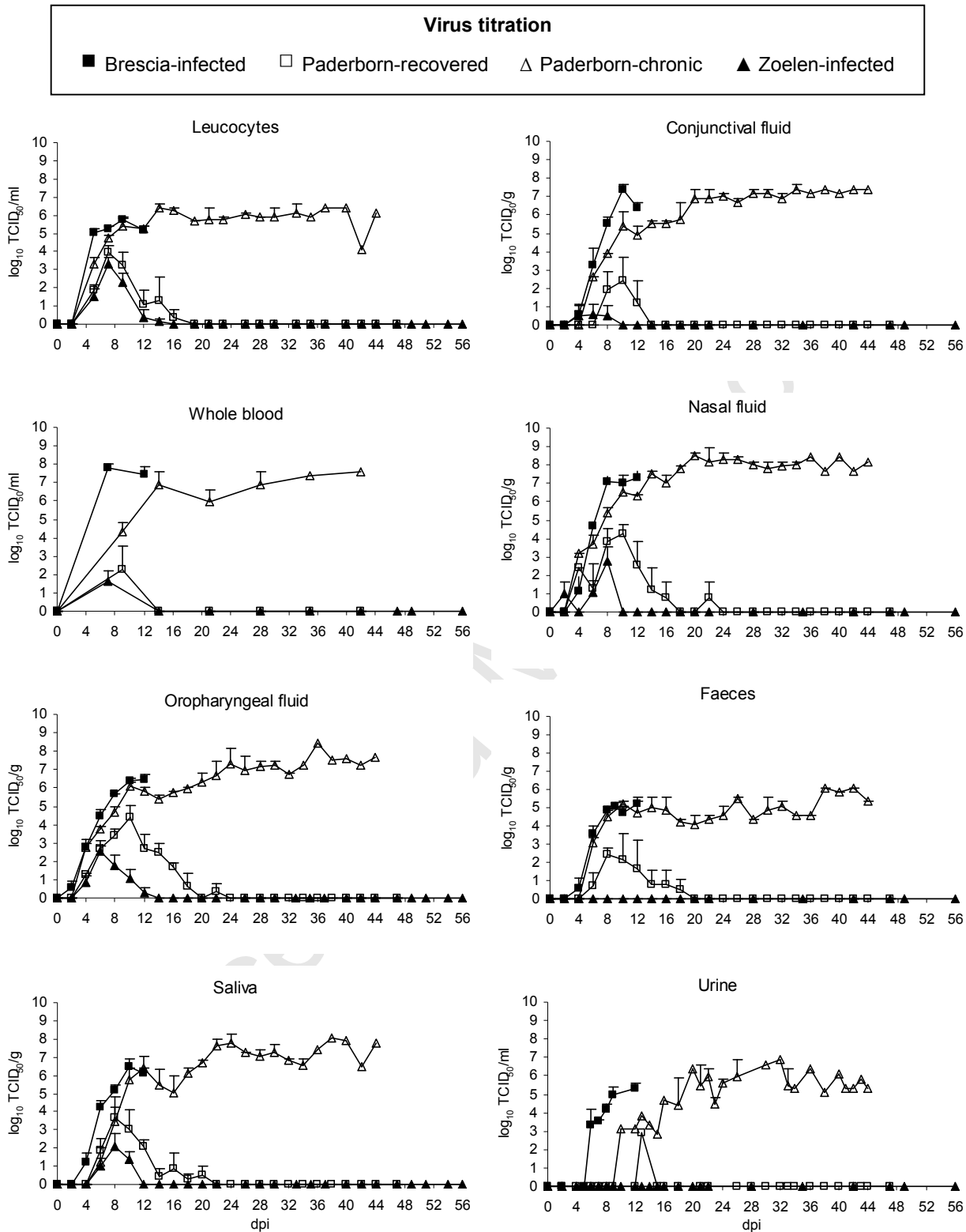


Figure 3

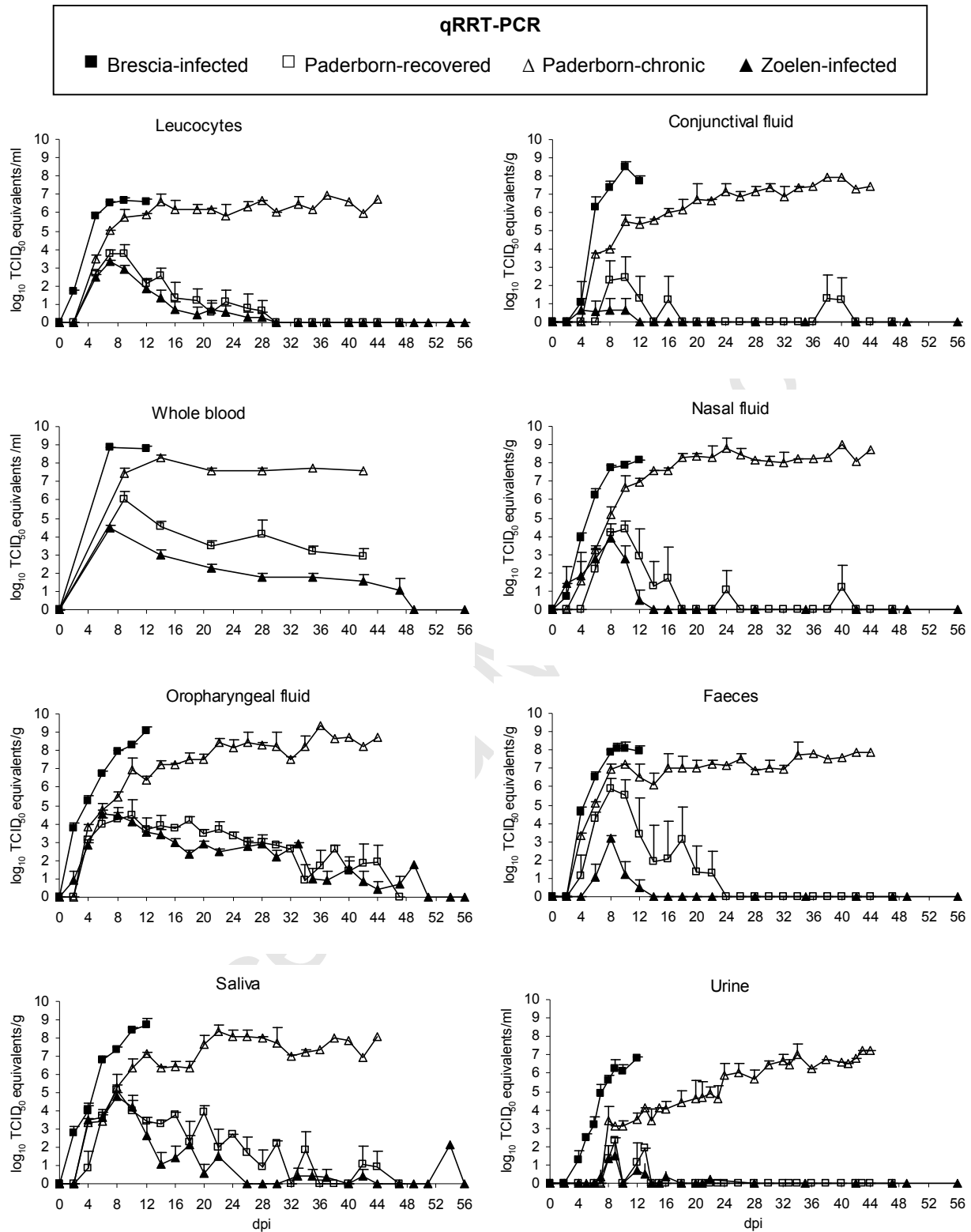


Figure 4.