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The effect of tissue degradation on detection of infectious virus and viral RNA to diagnose classical swine fever virus

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## Abstract

A considerable part of tissue samples that are collected for the monitoring of classical swine fever (CSF) from the wild boar population or from domestic pigs are unsuitable for virus detection using the fluorescent antibody test (FAT) or virus isolation (VI), due to tissue degradation. Reverse transcription polymerase chain reaction (RT-PCR) has a higher sensitivity than the FAT and VI, and is supposed to be less sensitive to sample degradation. Reliable and quantitative information on how long viral RNA and infectious virus can be detected in organs and which organs are most susceptible to degradation is, however, lacking. In the present study we generated survival curves of infectious CSF virus (CSFV) and viral RNA in the tonsil, mesenteric lymph node, spleen and kidney, obtained from twenty-four pigs infected with a moderately virulent CSFV strain. Tissue samples were stored at room temperature and tested by VI and RT-PCR, directly after storage and 1, 2, 3, 4, 7, 14 and 21 days later. It was shown that the RT-PCR is not only more sensitive than VI on fresh tissue samples, but RT-PCR is also less vulnerable to sample degradation. Average half-life values of viral RNA in the tissues ranged from 0.95 to 2.55 days, while half-life values of infectious virus ranged from 0.21 to 0.31 days. The tonsil and spleen are regarded as the most appropriate organs for the detection of infectious virus and viral RNA, not only in fresh samples, but also in samples that suffer from tissue degradation.

Key words: Classical swine fever virus; Diagnosis, Organs, Survival, Tissue degradation

## 1. Introduction

Classical swine fever (CSF) is a highly contagious viral disease in domestic pigs and wild boar. It is included in the list of diseases notifiable to the OIE. Several large outbreaks of CSF occurred in the European Union in the last twenty years (Koenen et al., 1996; Elbers et al., 1999; Fritzemeier et al., 2000; Sharpe et al., 2001), causing high economic losses, mainly due to restrictions in the trade of animals and animal products (Terpstra and de Smit, 2000). The main sources of these outbreaks have been illegal swill feeding, contact with contaminated livestock trucks, infected pigs or wild boar (Moennig, 2000; Moennig et al., 2003). In Europe, CSFV was reported from the wild-boar population in Bulgaria, Croatia, France, Germany, Hungary, Romania and Slovakia between 2005 and 2009. The danger of virus introduction due to (indirect) contact with wild boar and wild boar meat was demonstrated in Germany. Between 1993 and 1998, 59% of the primary outbreaks in domestic pigs were due to this route of transmission (Fritzemeier et al., 2000).

Surveillance in domestic pigs, but also wild boar, is the key to quickly detect classical swine fever upon a new introduction in a country. For early diagnosis of CSF, the detection of the virus is more suitable than the detection of antibodies, as antibodies can be found only in a later stage of the infection (Koenen et al., 1996; Dewulf et al., 2004). Virus can be detected with high titres in blood and several organs like the spleen, bone marrow, lymph nodes, tonsil, kidney, ileum and lung (Ressang, 1973; Van Oirschot, 1988; Kamolsiriprichaiporn et al., 1992). Samples that are being used for the detection of the virus, or viral genome, are therefore generally blood or tissue samples. In this paper we will focus on the latter.

The traditional techniques to detect virus in tissue samples are virus isolation (VI) and the fluorescent antibody test (FAT). These techniques require good sample quality, however, obtaining fresh tissue samples may be difficult or impossible because of postmortem alterations (haemolysis, putrefaction or virus destruction), that will increase in time. Especially in wild boar that are found dead, with an unknown length of time between death and sampling, this will quickly reduce the suitability of such samples for a reliable diagnosis of CSF. According to the experiences of several research groups, up to 30%, and even exceptionally 50% of field samples from wild boar may be unsuitable for laboratory tests (Artois et al., 2002; Rossi et al., 2005). The same may apply for samples from domestic pigs. Three percent of all samples (18,000) from domestic pigs taken during the 1997-1998 outbreak in The Netherlands were deteriorated to such an extent that they were unsuitable for testing in the FAT (De Smit et al., 1999). In a long-running Dutch surveillance program for CSFV, for which tonsils were collected from all domestic pigs that were sent in for a post-mortem examination, approximately 15% of the tonsils were deteriorated to such an extent that they were unsuitable for testing in the FAT (authors' own observation).

More recently developed techniques enabled the detection of the viral genome by reverse transcription polymerase chain reaction (RT-PCR). This method is not only more sensitive than VI and FAT (Blome et al., 2006; Depner et al., 2007; Greiser-Wilke et al., 2007) but may also be less susceptible to the deterioration of tissue and degradation of virus than both conventional techniques. If so, RT-PCR may be more suitable to detect CSFV in deteriorated tissue, therefore allowing for a more reliable surveillance in wild boar and domestic pigs, in case tissue samples are collected for testing.

In this study the effect of tissue degradation on virus infectivity and viral RNA stability in a range of commonly used organs was studied to find out how this affects the possibility to diagnose CSF by VI and RT-PCR.

## **2. Materials and Methods**

### **2.1 Experimental animals and housing**

Because of animal welfare concerns and the need to limit the number of experimental animals, organs were used from pigs that were included in a transmission study. For that study, twenty-four male pigs, eight weeks of age, were obtained from a conventional, but pestivirus free pig herd in the Netherlands. Pigs were housed in six pens, with four pigs each, within one room of an isolation unit. Two pigs were inoculated at the start of the study (pigs 23 and 24), and within the 31 days the study lasted, the other 22 pigs became infected through contact infections (Table 1). All pigs were euthanized at day 31 post-inoculation (p.i.). The experiment was approved by the Ethics Committee for Animal Experiments of the Animal Sciences Group of Wageningen UR.

## 2.2 Viruses and inoculation of animals

The two pigs (number 23 and 24) were inoculated with the moderately virulent strain Paderborn (genotype 2.1). This strain was isolated in 1997 during the outbreak in the Paderborn area of Germany (Greiser-Wilke et al., 2000). The virus suspension, with a dose of  $10^5$  TCID<sub>50</sub> (50% tissue culture infectious dose), was administered intranasally, 1 ml per animal, 0.5 ml per nostril.

## 2.3 Collection and treatment of samples

Oropharyngeal fluid samples were collected from all pigs at days -4, 3, 5, 7, 10, 12, 14, 17, 19, 21, 24, 26, 28 p.i. to determine the approximate moment of infection of each animal (Table 1). Oropharyngeal fluid was obtained with a sterile gauze tampon held by a 30-cm long forceps, which was scrubbed against the dorsal wall of the pharynx behind the soft palate. The oropharyngeal fluid was suspended in 4 ml medium (Eagle minimum essential medium [EMEM], supplemented with 5% fetal bovine serum [FBS] and 10% antibiotics solution ABII [1000 U/ml Penicillin, 1 mg/ml Streptomycin, 20 µg/ml Fungizone, 500 µg/ml; Polymixin B, and 10 mg/ml Kanamycin]). After centrifugation (1800 g for 15 min) the samples were stored at -70°C until they were analysed by reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR).

From each pig, entire tonsils, several mesenteric lymph nodes, and a piece of at least 5 grams of spleen and kidney were collected at day 31 p.i. Tissue samples were stored in petri-dishes at room temperature for 21 days. Directly after collection and at days 1, 2, 3, 4, 7, 14 and 21, a piece of 0.15 g was cut off and added to 0.5 ml of the same medium as described for the oropharyngeal fluid samples. The tissue was homogenized in the MagNa Lyser (Roche Applied Science) for 30 sec at 3500 g. After centrifugation (9500 g for 1 min), an additional one ml of medium was added. Samples were centrifuged again (9500 g for 5 min) and the supernatant was stored at -70 °C until they were analysed by VI and RT-qPCR.

From the tonsil, mesenteric lymph node, spleen and kidney of six randomly selected pigs, additional samples were taken from fresh organs to test for homogeneity of the virus in the organs. Four samples were taken from different locations of each organ. These samples were analysed by RT-qPCR.

## 2.4 Tests

### 2.4.1 Virus isolation and titration

Virus titration was performed to detect the presence of infectious virus in the tissue samples. Ten-fold dilutions of the tissue supernatant were prepared. Four replicates of 125  $\mu$ l of each serial dilution ( $10^{-1}$  to  $10^{-6}$ ) were incubated for four days on a monolayer of SK6 cells in a 24-wells plate (Greiner). After four days, the growth medium was discarded, and the monolayers were washed in a 0.15M NaCl solution, dried for 1 hour at 37°C and frozen for 1 hour at -20°C. The monolayers were fixed with 4% cold (5°C) paraformaldehyde in Phosphate-Buffered Saline (PBS) for 10 min. After fixation, the plates were washed in 0.15 M NaCl. Monolayers were stained by the immunoperoxidase monolayer assay (IPMA) (Wensvoort et al., 1986), using two horseradish peroxidase (HRPO)-conjugated CSFV specific MAbs (V3/V4) diluted in PBS (1:4000) with 4% horse serum. Monolayers were stained with 300  $\mu$ l 0.05 M NaAc solution, containing 5% 3-amino-9-ethylcarbazole (AEC) and 0.05%  $H_2O_2$ , and examined for stained cells. Virus titres were calculated as TCID<sub>50</sub> using the Spearman-Kärber method (Finney, 1978). The detection limit was  $10^{2.4}$  TCID<sub>50</sub>/g of tissue, since tissue samples were tested 100 times diluted in fourfold.

### 2.4.2 Reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR)

The concentration of viral RNA in the tissue samples was analyzed by RT-qPCR. For RNA isolation, 200  $\mu$ l of the sample were pipetted manually into MagNA Pure sample cartridges (Roche Applied Science, Mannheim, Germany). In each run of thirty-two samples, one negative control sample and five dilutions of a positive control sample (standard curve) were included. The standard curves were constructed as a dilution series in medium with a well known concentration of infectious virus (Paderborn strain). The RNA was extracted with the Total Nucleic Acid Isolation Kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions, using the automated MagNA Pure LC instrument (Roche Applied Science, Mannheim, Germany). After RNA isolation, the nucleic acids were immediately processed for the RT-qPCR or stored at -70°C in the sample cartridge until the PCR was carried out.

The RT-qPCR 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<sub>50</sub> equivalents per gram) of each individual sample could be calculated using the standard curve. It has to be stressed that TCID<sub>50</sub> equivalents are only a relative measure for the amount of RNA and that no correlation exists with the amount of infectious virus.

## 2.5 Statistical analysis

### 2.5.1 Differences between organs in initial infectious virus and viral RNA titres

Significant differences between organs in initial infectious virus and viral RNA titres were calculated with the non-parametric Wilcoxon matched-pairs test. For the multiple comparisons, Bonferroni's correction was applied. Virus or viral RNA negative tissue samples were excluded from the calculation. Due to the Bonferroni's correction, a *p* value less than 0.05/3 indicated a significant relationship.

### 2.5.2 Homogeneity of viral RNA distribution in the organs

From each of the six tonsils, mesenteric lymph nodes, spleens and kidneys, the average viral RNA titre and standard deviation of the four locations was calculated. Per organ type an average standard deviation was calculated.

### 2.5.3 Calculation of half-life values

For all tissue samples, the decay of infectious virus (log<sub>10</sub> TCID<sub>50</sub>) or viral RNA (log<sub>10</sub> TCID<sub>50</sub> equivalents) as a function of time after storage at room temperature was determined using a random coefficient regression model. The dispersion parameters were estimated by restricted maximum likelihood (REML), employing GenStat (Eleventh edition, copyright 2008) software. The half-life (*h*) of infectious virus or viral RNA in the kidney, spleen, tonsil and mesenteric lymph nodes was then estimated by:  $h = -\log_{10} 2 / b$ . Here, *b* is the estimated (expected) slope in the regression of log<sub>10</sub> CSFV on time for a random sample of the specific tissue.

Differences in half-life values between organs were statistically analysed using a paired T-test. Mean differences were considered significant when the *p* value was less than 0.05.

## 3. Results

### 3.1 Initial infectious virus and viral RNA titres in fresh organs

Seventy-nine out of the 96 fresh tissue samples tested positive in the VI with initial infectious virus titres ranging from below the detection limit (lower than 10<sup>2.4</sup> TCID<sub>50</sub>/g) to 10<sup>8.9</sup> TCID<sub>50</sub>/g (Table 1). Only one of the pigs

was VI-negative in all four organs. This was one of the two inoculated pigs, which by then had fully recovered from the infection. The initial infectious virus titres were on average the highest in the spleen and the tonsil (on average  $10^{7.0}$  and  $10^{6.7}$  TCID<sub>50</sub>/g). With the exception of the recovered pig, all fresh tonsils were VI-positive. Significantly lower titres than in the tonsil were observed in the mesenteric lymph node (on average  $10^{6.2}$  TCID<sub>50</sub>/g) and the lowest titres in the kidney (on average  $10^{5.6}$  TCID<sub>50</sub>/g). However, variations in virus titres were large, depending of the estimated time of infection of the pig.

Ninety-five out of the 96 tissue samples tested positive in the RT-qPCR with initial viral RNA titres in the 95 positive samples ranging from  $10^{2.4}$  to  $10^{9.1}$  TCID<sub>50</sub> equivalents/g (Table 2). The only negative result originated from the kidney sample of the recovered pig that was completely VI-negative. The initial viral RNA titres were highest in the tonsil and the mesenteric lymph node (on average  $10^{7.1}$  and  $10^{6.9}$  TCID<sub>50</sub> equivalents/g). Significant lower titres were observed in the spleen (on average  $10^{6.3}$  TCID<sub>50</sub> equivalents/g) and the lowest titres in the kidney (on average  $10^{5.1}$  TCID<sub>50</sub> equivalents/g). As seen with infectious virus titres, variations in viral RNA titres were also large, depending of the estimated time of infection of the pig.

### 3.2 Homogeneity of viral RNA distribution in the organs

The viral RNA was least homogeneously distributed in the kidney, as the viral RNA titres at the different locations of the organ showed an average standard deviation of 0.25 log<sub>10</sub> TCID<sub>50</sub> equivalents/g (minimum 0.09 and maximum 0.53 log<sub>10</sub> TCID<sub>50</sub> equivalents/g). In the tonsil the average standard deviation was 0.15 log<sub>10</sub> TCID<sub>50</sub> equivalents/g (minimum 0.07 and maximum 0.30 log<sub>10</sub> TCID<sub>50</sub> equivalents/g), in the spleen 0.11 log<sub>10</sub> TCID<sub>50</sub> equivalents/g (minimum 0.06 and maximum 0.19 log<sub>10</sub> TCID<sub>50</sub> equivalents/g), and in the mesenteric lymph node 0.10 log<sub>10</sub> TCID<sub>50</sub> equivalents/g (minimum 0.01 and maximum 0.24 log<sub>10</sub> TCID<sub>50</sub> equivalents/g).

### 3.3 Decay of infectious virus and viral RNA in tissue samples

The average half-life values for infectious virus ranged from 0.21 to 0.31 days (Table 3). There were small differences in half-life values between the organs, however, these were not statistically significant. After 7 days of storage at room temperature, virus could still be isolated from only 6 out of the 96 organs. Four of these were spleen samples and two were lymph node samples.

The average half-life values for viral RNA ranged from 0.95 to 2.55 days (Table 4). The shortest average half-life value was observed in the lymph node, while the longest average half-life value was observed in the spleen. After 7 days of storage at room temperature, viral RNA could still be detected in 74 out of the 96 organs, and even

at day 21, viral RNA could still be detected in 48 of the 96 organs. At 21 days the highest recovery rates were observed in the tonsil and the spleen.

#### 4. Discussion

In the present study, survival curves of infectious CSFV and viral RNA in tissue samples from tonsils, mesenteric lymph nodes, spleens and kidneys were generated. These survival curves showed that the RT-PCR is not only more sensitive than VI on fresh tissue samples, but also that viral RNA can be detected much longer in autolytic samples than infectious virus. Average half-life values of viral RNA in the tissues, as detected with RT-qPCR, ranged from 0.95 to 2.55 days, and in 50% of the organs, viral RNA could still be detected after 21 days of storage at room temperature. On the other hand, half-life values of infectious virus, as detected with VI, ranged from 0.21 to 0.31 days, and in only 6% of the cases, infectious virus could still be detected after 7 days of storage at room temperature.

The results of the present study are not unexpected and confirm previous observations that the RT-PCR is more sensitive than virus isolation (Dewulf et al., 2004; Van Rijn et al., 2004), and is supposed to be less influenced by tissue degradation. However, half-life values of viral RNA in tissue samples were not known. Information on half-life values of infectious CSFV have been published before, but focused mainly on survival of the virus in medium, faeces and urine (Depner et al., 1992; Weesendorp et al., 2008). In medium, a mean half-life of 2 days at neutral pH and a temperature of 20°C was observed (Depner et al., 1992). In faeces and urine of pigs infected with the Paderborn strain, half-life values of 0.14 days (faeces) and 0.21 days (urine) were more comparable to the results of the present study (Weesendorp et al., 2008). Differences between the results of the present study and others could be due to differences in condition between tissues and faeces, urine or medium, differences in storage condition, spiking versus using tissues from infected pigs, or differences in virus strain.

The tonsil and the spleen are the organs of preference to detect viral RNA. The spleen has the highest half-life value in deteriorating samples, when the RT-qPCR is used. The tonsil on the other hand, has a significantly higher initial viral RNA titre than the spleen. In both the tonsil and spleen, viral RNA was still detectable in the majority of the samples at the end of the storage period of 21 days. For the detection of infectious virus by VI, the tonsil and spleen are also the preferential organs. From fresh samples, tonsils gave the highest rate of positive VI, with significantly higher initial virus titres than in kidney and mesenteric lymph node samples. Half-life values for infectious virus in deteriorating tissue samples were, however, not significantly different.

231 The tissue samples in the present study were stored at room temperature, which is approximately 20-24  
 232 degrees Celsius. Average temperatures over a whole day will rarely exceed this in most European countries, even  
 233 during the summer. It is therefore expected that the deterioration that was simulated in this study will represent the  
 234 worst case scenario in the field, both for wild boars and domestic pigs. However, during the day in the summer,  
 235 temperatures may occasionally be higher, which might result in a faster deterioration of tissue and degradation of  
 236 virus and viral RNA. In these cases the spleen might be somewhat less appropriate than the tonsils as it may be  
 237 more susceptible to decomposition and contain a higher level of endogenous RNases, as was suggested by  
 238 Blacksell et al. (2004). To extend the duration of virus detectability a stabilization reagent like RNA<sup>later</sup><sup>TM</sup> or Trizol®  
 239 could be used, as it has been shown that this prevents or reduces the RNA decomposition (Blacksell et al., 2004;  
 240 Hofmann et al., 2000). However, this will only apply for stabilizing samples during transport, and may therefore be of  
 241 limited value.

242 In this study, organs were kept at room temperature for up to 21 days. This resulted in severe deterioration  
 243 of some of the organs, almost liquefying them. In reality, it will be very rare for samples to be subject to such a long  
 244 period of deterioration before they are being tested. In the field, carcasses of wild boar are quickly consumed by  
 245 scavengers. Piglets may decompose in less than 48 hours, but this depends on the climatic conditions, body mass  
 246 and location of animals (Sophie Rossi, personal communication). At that time, viral RNA could still be detected in  
 247 94% of the samples used in this study. What is more, wild boar, and also domestic pigs, dying of CSF, and therefore  
 248 usually showing severe clinical symptoms and fever shortly before death, are likely to have high or very high initial  
 249 virus titres in their organs. Even with the lowest half-life values observed in this study in the spleen and tonsil  
 250 samples, only an initial viral RNA titre of  $10^{4.4}$  TCID<sub>50</sub> equivalents/g is required to detect the virus after 48 hours (with  
 251 a detection limit of the RT-qPCR of  $10^2$  TCID<sub>50</sub> equivalents/g).

252 Depending on the technique of preparation of samples for either VI or RT-PCR, only a very small piece of  
 253 an organ may be sampled to detect the virus. This is actually a general trend as a result of ongoing robotisation and  
 254 the use of smaller sample volumes in diagnostic tests. Distribution of the virus in the organ may therefore become  
 255 crucial to obtain a sufficiently high sensitivity. If the virus is heterogeneously distributed, it would be possible to miss  
 256 infected animals by simply taking the wrong small piece of tissue. Homogeneity was tested in the fresh organs by  
 257 taking samples from multiple places. It was shown that the virus was distributed very homogeneously in the tonsils,  
 258 spleen, and lymph nodes. The viral RNA titres at the different locations of these organs showed an average  
 259 standard deviation between 0.10 and 0.15 log<sub>10</sub> TCID<sub>50</sub> equivalents/g. The standard deviation of the RT-qPCR itself  
 260 was between 0.07 and 0.17 log<sub>10</sub> TCID<sub>50</sub> equivalents/g. This was calculated by testing the reproducibility of five

261 samples, representing the standard curve of decreasing viral RNA concentrations, that were included in 36 RT-  
 262 qPCR-tests performed in this study. Thus, the variation of the viral RNA titres found in the tonsil, spleen and lymph  
 263 nodes, can be attributed as much to just test variation as it can to a non-homogeneous distribution over the organ.  
 264 Taking one small sample from these organs is therefore likely to give a representative and reproducible test result. A  
 265 somewhat larger variation between individual samples was observed in the kidney, with an average standard  
 266 deviation of 0.25 log<sub>10</sub> TCID<sub>50</sub> equivalents/g. It may be concluded that distribution of virus in the kidney may not be  
 267 as homogeneous, therefore giving an additional argument why kidney samples are less suitable than tonsil and  
 268 spleen. Whether the homogeneity that was found in fresh organs will last during the deterioration process was not  
 269 investigated.

270 In conclusion, this study showed the advantage of the RT-PCR over virus isolation as a diagnostic assay on  
 271 fresh organs, but also on deteriorated samples. A reliable detection of viral genome can be expected in deteriorated  
 272 samples within a reasonable period that these samples may be subject to deterioration in the field. The organs of  
 273 preference to detect CSF infections in dead pigs, being either domestic pigs or wild boar, are the tonsil and the  
 274 spleen.

275

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Table 1. Overview of all 24 pigs from which organs were collected at day 31 after inoculation of two of them (pigs 23 and 24). Time of infection will be related to the first day the pigs tested positive in the oropharyngeal fluid (OPF). Infectious virus titres are given for all organs, including the last day after storage at room temperature that the samples were still positive.

Pig	D.p.i of first positive OPF sample *	Initial infectious virus titre in log <sub>10</sub> TCID <sub>50</sub> /g (last day of virus detection in tissue sample)							
		kidney		spleen		tonsil		lymph node	
1	24	3.2	(0)	b.d.l		4.4	(1)	4.4	(1)
2	21	6.9	(3)	7.7	(4)	7.4	(2)	7.4	(4)
3	21	7.2	(1)	8.9	(3)	8.9	(3)	b.d.l	
4	21	6.7	(3)	8.2	(2)	8.4	(3)	7.7	(2)
5	21	5.7	(3)	7.9	(7)	7.4	(3)	6.9	(7)
6	17	b.d.l		b.d.l		4.4	(1)	2.7	(1)
7	21	b.d.l		2.7	(1)	5.4	(4)	4.7	(3)
8	24	4.4	(4)	4.4	(1)	6.9	(2)	5.7	(1)
9	19	2.7	(3)	2.7	(3)	5.9	(4)	3.7	(1)
10	21	3.7	(3)	b.d.l		5.9	(1)	3.4	(2)
11	21	3.7	(2)	b.d.l		4.4	(2)	3.2	(2)
12	21	6.4	(4)	8.7	(3)	7.2	(2)	7.4	(3)
13	21	5.7	(3)	7.7	(3)	7.2	(3)	7.7	(4)
14	21	6.7	(4)	8.2	(7)	8.7	(2)	8.2	(4)
15	21	4.4	(2)	7.2	(7)	6.4	(2)	6.9	(7)
16	21	6.2	(4)	7.7	(7)	7.4	(3)	7.7	(2)
17	14	5.9	(2)	8.4	(4)	8.7	(2)	8.4	(4)
18	19	4.7	(1)	5.7	(4)	7.2	(2)	5.9	(1)
19	14	b.d.l		b.d.l		3.4	(1)	b.d.l	
20	21	5.2	(4)	7.2	(7)	6.4	(3)	6.4	(4)
21	10	8.4	(1)	8.7	(2)	8.2	(2)	7.9	(2)
22	10	b.d.l		b.d.l		4.2	(1)	b.d.l	
23	3	b.d.l		b.d.l		b.d.l		b.d.l	
24	5	8.4	(2)	7.7	(2)	8.9	(2)	8.2	(2)
Average		5.6 <sup>c</sup>		7.0 <sup>ab</sup>		6.7 <sup>a</sup>		6.2 <sup>b</sup>	
Standard deviation		1.7		2.0		1.7		1.9	

<sup>a-c</sup> Means within row with no common superscript differ significantly ( $p < 0.05$ ). b.d.l = below the detection limit of the virus isolation (2.4 log<sub>10</sub> TCID<sub>50</sub>/g). \* First day after inoculation of the first two pigs that the oropharyngeal fluid (OPF) was positive in the qRRT-PCR.

Table 2. Overview of all 24 pigs from which organs were collected at day 31 after inoculation of two of them (pigs 23 and 24). Time of infection will be related to the first day the pigs tested positive in the oropharyngeal fluid (OPF). Viral RNA titres are given for all organs, including the last day after storage at room temperature that the samples were still positive.

Pig	D.p.i of first positive OPF sample *	Initial viral RNA titre in log <sub>10</sub> TCID <sub>50</sub> equivalents/g (last day of virus detection in tissue sample)							
		kidney		spleen		tonsil		lymph node	
1	24	3.7	(3)	4.5	(4)	5.7	(4)	6.4	(7)
2	21	6.8	(14)	8.4	(21)	8.0	(21)	8.0	(21)
3	21	6.8	(21)	8.3	(21)	8.0	(21)	8.4	(21)
4	21	6.8	(14)	8.4	(21)	8.4	(21)	8.1	(21)
5	21	5.9	(14)	7.8	(21)	8.1	(21)	7.9	(21)
6	17	3.0	(7)	4.3	(7)	5.9	(7)	5.9	(14)
7	21	3.1	(3)	4.3	(7)	5.5	(21)	5.1	(7)
8	24	4.0	(4)	5.1	(21)	7.0	(7)	7.0	(14)
9	19	3.1	(4)	3.9	(21)	6.5	(21)	6.7	(21)
10	21	3.3	(1)	3.9	(4)	5.9	(7)	4.6	(4)
11	21	2.4	(3)	4.0	(4)	5.6	(7)	5.2	(7)
12	21	7.1	(21)	8.9	(21)	8.9	(21)	8.4	(14)
13	21	6.2	(21)	7.9	(21)	7.8	(21)	8.2	(14)
14	21	6.6	(14)	8.1	(21)	7.9	(21)	8.1	(7)
15	21	4.7	(4)	6.9	(21)	7.2	(21)	7.8	(7)
16	21	6.0	(21)	8.4	(21)	8.2	(21)	7.7	(21)
17	14	6.5	(21)	8.5	(21)	8.7	(21)	8.6	(21)
18	19	4.8	(2)	5.8	(21)	7.4	(14)	6.8	(7)
19	14	3.5	(3)	3.4	(1)	4.7	(7)	4.4	(7)
20	21	5.2	(21)	6.9	(21)	6.9	(21)	7.4	(7)
21	10	7.6	(21)	8.1	(21)	9.0	(21)	9.1	(21)
22	10	2.5	(0)	3.4	(1)	5.6	(7)	3.9	(4)
23	3	b.d.l		3.2	(4)	4.6	(4)	3.9	(1)
24	5	7.8	(21)	7.8	(21)	7.9	(21)	8.2	(21)
Average		5.6 <sup>c</sup>		6.3 <sup>b</sup>		7.1 <sup>a</sup>		6.9 <sup>a</sup>	
Standard deviation		1.7		2.1		1.3		1.6	

<sup>a-c</sup> Means within row with no common superscript differ significantly ( $p < 0.05$ ). b.d.l = below the detection limit of the qRRT-PCR (unknown). \* First day after

inoculation of the first two pigs that the oropharyngeal fluid (OPF) was positive in the qRRT-PCR.

Table 3. Decay of infectious virus, as determined by virus isolation, in organs from pigs infected with the Paderborn strain

Organ	Average half-life (days)	se <sup>1</sup>	Min <sup>2</sup>	Max <sup>3</sup>
Kidney	0.31	0.051	0.10	1.00
Spleen	0.28	0.042	0.14	2.41
Tonsil	0.21	0.019	0.11	0.57
Lymph node	0.29	0.047	0.08	2.41

<sup>1</sup> se = standard error

<sup>2</sup> Min = minimum half-life value observed.

<sup>3</sup> Max = maximum half-life value observed.

Table 4. Decay of viral RNA, as determined by qRRT-PCR, in organs from pigs infected with the Paderborn strain

Organ	Average half-life (days)	se <sup>1</sup>	Min <sup>2</sup>	Max <sup>3</sup>
Kidney	1.83 <sup>ab</sup>	0.210	0.26	4.75
Spleen	2.55 <sup>a</sup>	0.237	0.25	4.96
Tonsil	1.68 <sup>b</sup>	0.116	0.42	3.29
Lymph node	0.95 <sup>c</sup>	0.088	0.21	4.74

<sup>a-c</sup> Means within column with no common superscript differ significantly ( $p < 0.05$ )

<sup>1</sup> se = standard error

<sup>2</sup> Min = minimum half-life value observed.

<sup>3</sup> Max = maximum half-life value observed.