Factors affecting the infectivity of tissues from pigs with classical swine fever: Thermal inactivation rates and oral infectious dose

Lucie Cowan [a,c], Felicity J. Haines [a], Helen E. Everett [a], Bentley Crudgington [a], Helen L. Johns [a], Derek Clifford [b], Trevor W. Drew [a], Helen R. Crooke [a,*]

[a] Virology Department, Animal and Plant Health Agency, Woodham Lane, New Haw, Surrey KT15 3NB, United Kingdom
[b] Animal Sciences Department, Animal and Plant Health Agency, Woodham Lane, New Haw, Surrey KT15 3NB, United Kingdom
[c] Department of Medical and Veterinary Sciences, University of Bristol, Bristol BS8 1TD United Kingdom

ABSTRACT

Outbreaks of classical swine fever are often associated with ingestion of pig meat or products derived from infected pigs. Assessment of the disease risks associated with material of porcine origin requires knowledge on the likely amount of virus in the original material, how long the virus may remain viable within the resulting product and how much of that product would need to be ingested to result in infection. Using material from pigs infected with CSFV, we determined the viable virus concentrations in tissues that comprise the majority of pork products. Decimal reduction values (D values), the time required to reduce the viable virus load by 90% (or $1\log_{10}$), were determined at temperatures of relevance for chilling, cooking, composting and ambient storage. The rate of CSFV inactivation varied in different tissues. At lower temperatures, virus remained viable for substantially longer in muscle and serum compared to lymphoid and fat tissues. To enable estimation of the temperature dependence of inactivation, the temperature change required to change the D values by 90% (Z values) were determined as 13 °C, 14 °C, 12 °C and 10 °C for lymph node, fat, muscle and serum, respectively. The amount of virus required to infect 50% of pigs by ingestion was determined by feeding groups of animals with moderately and highly virulent CSFV. Interestingly, the virulent virus did not initiate infection at a lower dose than the moderately virulent strain. Although higher than for intranasal inoculation, the amount of virus required for infection via ingestion is present in only a few grams of tissue from infected animals.

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1. Introduction

Incursions of classical swine fever (CSF) into disease-free regions has significant consequences, resulting in ban of international trade and costly control measures (Moennig, 2000). Spread of CSF virus (CSFV) occurs via direct contact between pigs, via indirect contact with virus-contaminated fomites or via ingestion of products from infected pigs. The oral route, via swill feeding, has been responsible for primary disease introductions (Fritzemeier et al., 2000; Paton and Greiser-Wilke, 2003) and although currently banned in the EU, the practice continues in many parts of the world, both with and without mandatory cooking and the possibility of illegal activities cannot be ruled out.

Factors that affect whether or not a pig becomes infected upon ingestion of infected pork-derived material are important for assessing the risks that pork products

may have for introduction and spread of CSFV (Farez and Morley, 1997). These factors include; the likely level of viral contamination within a product, how long the pathogen may survive within the product under the conditions to which it is exposed, what is the likelihood that a susceptible animal will ingest a contaminated product and, finally, how much product needs to be ingested to instigate an infection (Wooldridge et al., 2006).

The level of viable virus in CSFV infected animals, and hence the level of contamination of a product derived from an infected animal, varies according to the virulence of the infecting virus and the stage and course of disease (Weesendorp et al., 2009b). The tissue tropism of the virus will also affect the viral load found in different pork products. The main target cells for CSFV are leukocytes, myeloid, epithelial and endothelial cells and high concentrations of CSFV are found in lymphoid tissue, spleen and blood (Belak et al., 2008; Liu et al., 2011; Weesendorp et al., 2010). Few studies have quantified viable CSFV in muscle and fat which constitute the major proportion of pork products. Available data indicates the levels in muscle and fat are low, and are often either undetectable or at the limit of detection (Mebus et al., 1997; Thur and Hofmann, 1998), although levels as high as $10^{4.9}$ TCID$_{50}$/g have been reported (Wood et al., 1988).

After slaughter, pork tissues are subjected to various processes such as chilling of carcases, butchery, transport and cooled storage. Products, which may or may not be cured and/or cooked prior to generation of waste, will then be subjected to varying conditions depending on whatever route leads to subsequent ingestion by a susceptible animal, such as landfill, composting or storage of scraps prior to illegal swill feeding (Gale, 2004; Wooldridge et al., 2006). Temperature, pH, the tissue matrix and processing will affect the rate at which virus viability decays (Depner et al., 1992; Edwards, 2000; Farez and Morley, 1997). Studies have demonstrated the times at which virus can no longer be detected in tissues held at different temperatures (Edwards, 2000) and survival kinetics in media (Depner et al., 1992), slurry (Botner and Belsham, 2012), faeces, urine (Weesendorp et al., 2008) and diagnostic tissues (Weesendorp et al., 2010). However, there is a paucity of data on the rate of thermal inactivation of CSFV in tissues that comprise pork products.

Assessment of the risks associated with processes which may result in the ingestion of pork products by pigs requires knowledge of the oral infective dose. A review by Farez and Morley (1997) highlighted that experimental inoculation of pigs with less than 10 TCID$_{50}$ CSFV could initiate infection in a proportion of pigs (Dahle and Liess, 1995; Farez and Morley, 1997). These studies used intranasal inoculation and thus the virus was likely to have had direct contact with the tonsils, the primary site of CSFV replication. The situation is very different when a pig ingests a potentially CSFV – contaminated pork product, where the degree of mastication and rapidity of swallowing will influence if the virus can gain entry by contact with the tonsils or via oral abrasions (Farez and Morley, 1997). It is therefore expected that the oral pig infective dose via ingestion will be higher than 10 TCID$_{50}$.

To improve assessment of the risk of pork products for the introduction and spread of CSF we have determined the rate of CSFV inactivation in tissues found in pork products and gained a more accurate estimate of the pig oral infective dose.

2. Materials and methods

2.1. Viruses and cells

PK15 cells were propagated in EMEM with 10% v/v Foetal Bovine Serum (FBS) and penicillin, streptomycin and Nystatin supplement. CSFV strain UK2000/7.1 (Genotype 2.1) was isolated in the UK, the CBR/93 strain (Genotype 3.3) was kindly provided by Dr. S. Parchariyanon, National Institute of Animal Health, Bangkok and the Brescia strain (Genotype 1.1) was kindly provided by Dr. Alexandra Meindl-Böhmer, University of Veterinary Medicine, Hannover.

2.2. Tissues

Viral loads and rates of CSFV inactivation were determined in muscle (longissimus dorsi or bicep femoris), lymph node (mandibular, Ileocecal, retropharyngeal, ventral superficial cervical), fat and serum samples which had been stored at −80 °C after harvest from animals experimentally infected with CSFV CBR/93, UK2000/7.1 or Brescia, for other purposes, as described below or previously (Everett et al., 2010; Graham et al., 2012). Material from pigs exhibiting the acute form of CSF, between 7 and 21 days post infection (dpi), were used for the study.

2.3. Methods of quantitation of virus

Viable virus in tissues and oral dose inocula was quantified by titration on PK15 cells. Samples were serially diluted (1 in 2, 1 in 3, 1 in 5 or 1 in 10 depending on the viral load present in the sample) in EMEM with 10% (v/v) FBS and antibiotics and 50 μl of each dilution added to 8 wells of a 96 well plate. Plates were incubated for 4–5 days at 37 °C with 5% CO_2 prior to fixing with 4% (v/v) paraformaldehyde and detection of virus by a modified immunoperoxidase staining method (Anonymous, 2012). Briefly after washing with PBS cells were incubated with 3% (v/v) H_2O_2 in methanol for 10 min and permabilised with 1% (v/v) Tween 20, 10% (v/v) normal goat serum in PBS. Virus was detected using CSF-specific Mab WH303 or WH304 (AHVLA), rabbit anti-mouse HRP-conjugated secondary antibody (DAKO) and 3,3′-diaminobenzidine tetrahydrochloride (DAB) chromogenic substrate (Sigma).

2.4. Decimal reduction values of CSFV in porcine tissues, serum and cell cultures

Thawed tissues were cut into approximately 2 mm^3 sections and sections mixed to ensure homogeneity. Aliquots of serum, cell culture medium, or sectioned tissue (250 mg) were incubated in micro-centrifuge tubes, at 25 °C, 56 °C or 68 °C in a circulating water bath or at 4 °C

in a refrigerator. For tissues held at 4 °C, 1 ml of PBS plus antibiotic supplement (Mycostatin/Nystatin (500 U/ml), Streptomycin 2.5 mg/ml, polymyxinB sulphate (100 U/ml), benzyl Pencillin (100 U/ml), and Neomycin sulphate (1.4 mg/ml)) was added to prevent microbial growth. To compare thermostability of the three different strains, for which tissues from experimentally infected animals was available, cell culture stocks of virus were incubated at 50 °C in EMEM with 10% v/v FBS plus antibiotics. In all cases, triplicate aliquots were removed at each of eight time points and tissues homogenised with 1 ml of PBS supplemented with antibiotics in a Precellys 24 homogeniser and 2.8 mm ceramic zirconium oxide beads (Precellys CK28-R), using two, 10-s pulses at 6000 rpm separated by a 15-s pause. After clarification of homogenates by centrifugation at $1000 \times g$ for 10 min at 4 °C, viable virus was quantified by titration as above on PK15 cells. Due to cell toxicity observed with muscle homogenates, media was replenished after 24 h. Infection was detected by immunoperoxidase staining as above and the 50% tissue culture infectious dose ($TCID_{50}$) calculated using the Spearman–Karber method (Hamilton et al., 1977).

2.5. Statistical analysis, calculation of D and Z values and the 50% oral pig infectious dose

Statistical analysis, graphs and linear regression were prepared using GraphPad Prism 6. D_t values were calculated from linear regression of virus titres versus time at temperature t (D value $= -1/$slope). The temperature change needed to change the D value by 90% (z values) and 95% confidence intervals for each tissue were determined from the slopes of linear regression of $\log_{10} D$ values (minutes) versus temperature. Differences between mean values were analysed by one-way ANOVA with Tukey's multiple comparison test.

Estimate of the dose required to infect 50% of pigs inoculated via the oral dose, together with 95% confidence intervals (CI) was determined using the trimmed Spearman–Karber method (Hamilton et al., 1977, 1978).

2.6. Determination of the pig oral infectious dose

Sixty-six, 10-week old Large White/Landrace cross male pigs, which were free from antibodies to all pestiviruses, were purchased from a local commercial supplier. Five groups of six pigs were inoculated with serial ten-fold dilutions of UK2000/7.1 and in a separate experiment six groups of six pigs were inoculated with similar ten-fold dilutions of Brescia. Pigs weights ranged between 12.8 kg and 24.5 kg and animals were allocated into groups such that the mean weights and standard deviations were 15.4 kg (SD 0.1) or 19.0 kg (SD 0.1) for the UK2000/7.1 or Brescia inoculated animals, respectively. Groups were housed in separate rooms.

Corn-covered blister pack baits, kindly provided by IDT Biologika GmbH, were filled with 1.5 ml of EMEM containing the required doses of either UK2000/7.1 or Brescia and fed individually to pigs. The viral inocula were back-titrated on the day of inoculation on PK15 cells to confirm the actual dose of virus fed.

Temperatures and clinical signs were recorded daily throughout the experiment using a clinical scoring scheme as described previously (Everett et al., 2010). Blood and nasal swabs were collected at 2–3-day intervals. Leucocyte counts and viral RNA copy numbers were determined by flow cytometry and qRT-PCR, respectfully, as described previously (Graham et al., 2012). Animals were euthanised by administration of 20% pentobarbitol solution either at the end of the experiment, 28–32 dpi, or when clinical scores exceeded 12.

The project was approved by the Animal and Plant Health Agency Ethics Review Panel. Procedures were in accordance with the UK Animals (Scientific Procedures) Act 1986, and conducted under project license permit number PPL70_6559.

3. Results

3.1. Amount of virus in tissues forming major components of pork products

To identify samples from animals infected with CSFV with viral loads sufficient to determine rates of CSFV inactivation, the viral concentrations in muscle, fat, skin and lymph node samples were obtained from separate CSFV infection studies from pigs infected with moderately (UK2000/7.1, CBR/93) or highly virulent (Brescia) CSFV (Everett et al., 2010; Graham et al., 2012). As expected, high levels of virus were present in lymph nodes: mean $10^{5.5}$ $TCID_{50}$/g, range $10^{4.8}$–$10^{7.1}$ $TCID_{50}$/g. The levels of virus in fat and muscle were lower and no viable virus could be detected in skin samples. The mean viral load in fat was $10^{4.3}$ $TCID_{50}$/g with titres ranging from less than the quantifiable limit of the assay ($10^{2.8}$ $TCID_{50}$/g) to as high as $10^{5.9}$ $TCID_{50}$/g. Whilst virus could be detected in most muscle samples, many of the animals tested had levels that were either only just above or below the quantifiable limit of the assay. However, one animal, that had clinical signs not consistent with CSF shortly after inoculation and which responded to antibiotic treatment (Graham et al., 2012), had $10^{5.1}$ $TCID_{50}$ of virus per g of muscle tissue.

3.2. There is little or no difference in the thermal inactivation rate of different genotypes of CSFV of different virulence

Differences in virus thermal stability between different CSFV have been reported (Aynaud et al., 1972; Depner et al., 1992; Weesendorp et al., 2008), although these differences were not always statistically significant. To determine if there are differences between the thermal stability of strains for which tissues from infected animals were available, the three strains used in this study, from three different genotypes of CSFV, were incubated in cell culture media at the mid-range temperature of 50 °C and the amount of virus remaining after different time intervals was determined and D values calculated for that temperature (D_{50}). There was no significant difference ($P = 0.48$) in thermal stability between the strains at this temperature. Mean D_{50} values and standard deviations were 51.6 (SD 21.8), 61.5 (SD 17.2), and 75.8 (SD 32.1) min,

for CSFV Brescia (Genotype 1.1), UK2000/7.1 (2.1), and CBR/93 (3.3), respectively.

3.3. The thermal inactivation rate of CSFV in porcine tissues varies dependent on the matrix

To determine the rate at which CSFV is inactivated in tissues found in pork products, samples of fat, lymph node and muscle were incubated at the four key temperatures of 68 °C, 56 °C, 25 °C and 4 °C. EC legislation (European Union, 2003) states that heating to a minimum temperature of 70 °C, which must be reached throughout the meat, is effective at eliminating the risk to animal health due to CSFV. Therefore 68 °C was selected to determine the extent of virus survival if treatments do not reach the required temperature. Similarly, 56 °C was selected as a temperature that would inform the ability of virus to survive in composted material that may contain meat products which does not reach the desired composting temperature of 60 °C. The remaining temperatures were selected to inform on the survival of CSFV in pork products at ambient and refrigerated temperatures. Serum samples were also incubated to assess the stability of CSFV in diagnostic samples and biological reagents. For the majority of samples, triplicate experimental repeats were completed and mean $TCID_{50}$ at eight incubation time points were obtained. For muscle tissues, only samples from the single animal that had an underlying infection had a sufficient titre to determine inactivation rates. Due to the limited quantities of muscle samples from this animal, fewer replicate experiments were completed. Some inactivation curves had a slightly biphasic shape but the data were largely a reasonable fit for a linear model (median $r^2 = 0.85$) and D values were calculated from linear regression analysis (Table 1, Supplementary Figs. S1–S4). At the highest temperature (68 °C) there was no difference in virus inactivation rates between the different tissue matrices except for fat samples: The virus survived twice as long in fat as in the other tissues (Fat vs LN or Serum: $P < 0.01$). At lower temperatures, a substantial difference was observed between matrices. At 56 °C a log_{10} reduction in the amount of virus was achieved in lymph nodes, fat and muscle within 3 min. However, in serum, virus survival

was significantly longer compared to other tissues ($P < 0.001$). In three of four replicate assays, D_{56} values of about 30 min were obtained for serum. In the fourth assays performed at this temperature, over 1.5 h were required to achieve a 1 log_{10} reduction in the virus load. This 4th experiment appears to be an outlier, although no difference in the experimental parameters could be identified. At ambient temperature (25 °C), virus survival was similarly much longer in serum compared to lymph node and fat. Interestingly, virus survival in muscle at 25 °C was also substantially greater than in lymph node or fat ($P < 0.05$). A greater virus survival in muscle was also observed at chilling temperature: a 1 log reduction occurred within 1.1–2.8 weeks in lymph nodes and fat whereas 4.7 or 6.5 weeks were required for a similar reduction in muscle ($P < 0.05$).

Supplementary Figs. S1–S4 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetmic.2014.12.003.

3.4. Calculation of Z values

To enable interpolation of the virus inactivation rates at different temperatures from those tested, the log_{10} D values were plotted versus temperature for all tissues (Fig. 1). Analysis of r^2 values (>0.95) and runs tests indicated the data do not significantly deviate from linearity and so Z values were calculated from linear regression analysis (Table 2). The line equation of the linear regression of D values and the Z values can be used to predict D values at different temperatures. For example, it can be calculated that the D_{60}-value for CSFV in lymph node, fat and muscle tissue will be 1.7 min, 1.5 min and 2.3 min, respectively. Conservative estimates of the D value at different temperatures can be achieved using the upper limit of the 95% confidence interval so, for example, a conservative estimate of the D_{60} value in lymph node tissue would be 4.5 min. Similarly at 70 °C, the temperature that EU legislation indicates is sufficient to eliminate health risks linked to meat, the following D_{70} values and conservative estimated based on the upper 95% confidence interval (in brackets) can be calculated: muscle 0.33 (19.2) min, fat 0.26 (2.1) min, lymph node 0.29 (0.89) min and serum 0.76 (10.6) min.

Table 1
Mean decimal reduction values (D values) for inactivation of CSFV in tissues at key temperatures.

Sample	D values (standard deviation)			
	68 °C (min)	56 °C (min)	25 °C (h)	4 °C (weeks)
Lymph node	0.47 (0.01)	2.38 (1.1)	32.24 (2.52)	2.22 (0.49)
Fat	1.15 (0.24)	2.99 (0.76)[a]	36.55 (13.80)	1.51 (0.39)
Muscle	0.41[b]	2.11[b]	148.01[c] (87.67)	5.6[c] (1.27)
Serum	0.53 (0.07)	48.38[a] (35.75)	219.92 (7.60)	nd

Mean D values were determined from linear regression analysis of triplicate inactivation experiments. Only a limited quantity of material was available from the one animal identified with high viral loads in muscle. A smaller number of replicate experiments where therefore used to determine the inactivation rates in muscle.

[a] $n = 4$.
[b] $n = 1$.
[c] $n = 2$.
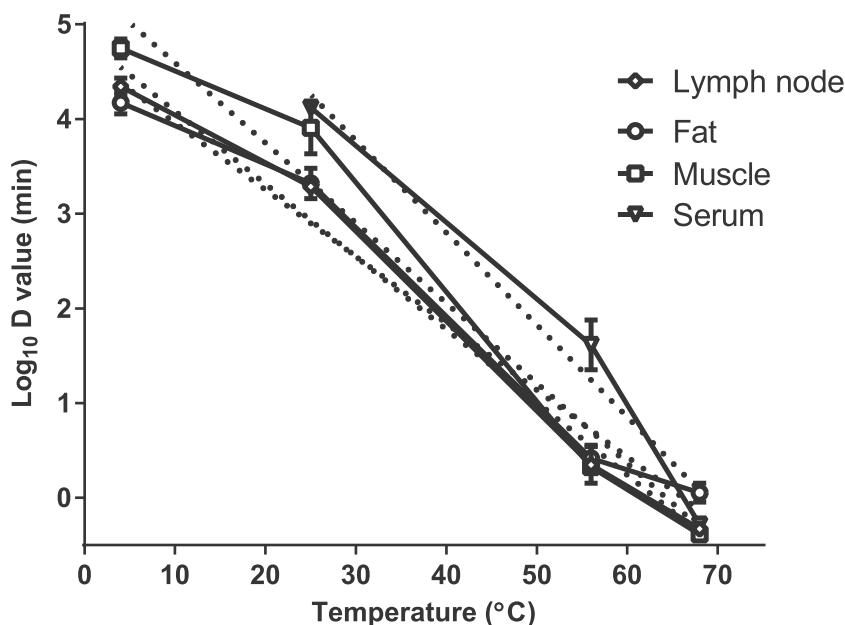
Lymph node	Y = -0.07686*X + 4.852
Fat	Y = -0.07049*X + 4.662
Muscle	Y = -0.08459*X + 5.440
Serum	Y = -0.09726*X + 6.690

Fig. 1. Linear regression analysis of log D values (minutes) versus temperature for CSFV in tissues. Values are mean D values for all tissue types. Error bars represent standard deviation of the mean. Solid lines connect the data points. Dotted lines indicate the linear regression best fit lines for which the line equations are included.

3.5. The amount of CSFV required to infect pigs via ingestion is higher than for intranasal inoculations and independent of strain virulence

To establish the amount of virus required to initiate CSF by the oral route, groups of six animals were fed doses of virus ranging from 10^1 to 10^6 TCID$_{50}$ of the moderately virulent UK2000/7.1 or the highly virulent Brescia. In the UK2000/7.1 experiment, all the animals fed blister packs containing 10^1–10^3 TCID$_{50}$ remained healthy throughout the experiment except for one animal which was eutha-nized for welfare reasons at 11 dpi due to signs unrelated

to CSF. RT-PCRs on blood and nasal swab samples and blood leucocyte levels in all animals in these three groups indicated that none had become infected with CSFV (Table 3). Viral RNA was first detected at 4 or 6 dpi in the blood of three of the six animals fed 10^4 TCID$_{50}$ of UK2000/7.1 (Fig. 2A). One of these animals developed

Table 2
Z-values for inactivation of CSFV in tissues.

Sample	Z value (°C)	95% confidence interval (°C)
Lymph node	13.0	12.0–14.3
Fat	14.2	12.7–16.1
Muscle	11.8	9.1–16.8
Serum	10.3	8.8–12.4

The temperature change required to change the $\log_{10} D$ value by 1 for CSFV in different tissues was determined from the slope of linear regression of $\log_{10} D$ values versus temperature. The 95% confidence intervals were also determined from the best fit line of linear regression analysis.

Table 3
Number of pigs infected after ingestion of different doses of CSFV.

UK2000/7.1		Brescia	
Dose (TCID$_{50}$)	Number of pigs infected (out of 6)	Dose (TCID$_{50}$)	Number of pigs infected (out of 6)
$10^{1.2}$	0	$10^{1.3}$	0
$10^{2.2}$	0	$10^{2.3}$	0
$10^{3.2}$	0	$10^{3.3}$	0
$10^{4.2}$	3	$10^{4.3}$	0
$10^{5.2}$	5	$10^{5.3}$	2
$10^{6.2}$	nt	$10^{6.3}$	6

Number of pigs designated to have been infected by ingestion of oral inoculum.
Groups of pigs were fed 10-fold serial dilutions of the highly virulent Brescia or moderately virulent UK2000/7.1 CSFV and infection by inoculum versus contact infection determined due to the timing of onset of viraemia and clinical signs.
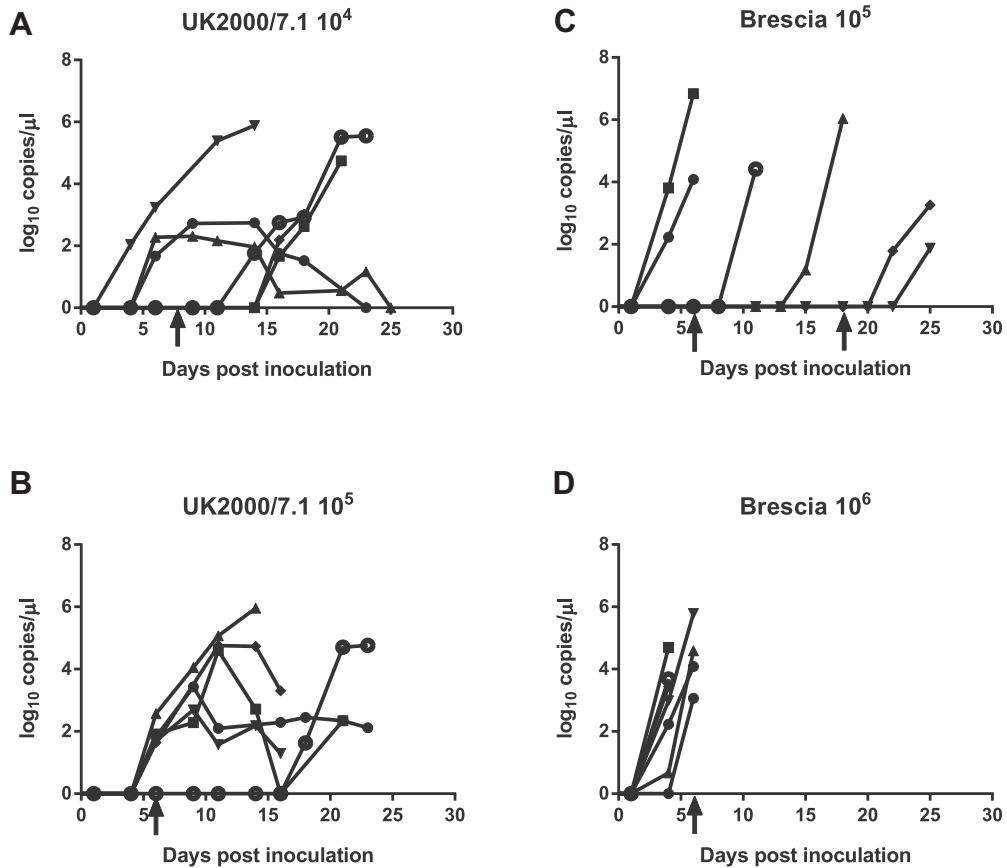nt = not tested.

Fig. 2. Discrimination of animals infected via ingestion of inoculum versus contact infection. Viral RNA detected in the blood of animals inoculated with the two highest doses of either UK2000/7.1 or Brescia. Different lines and symbols represent the individual animals within each group of six. Arrows indicate the day post inoculation that CSFV viral RNA was first detected in nasal secretions. In the group inoculated with $10^{5.3}$ TCID$_{50}$ Brescia RNA was detected in two nasal swab samples only (both indicated by arrow).

clinical signs of CSF and was euthanized at 15 dpi. The other two pigs had only mild signs, with clinical scores not exceeding 6. RNA copy numbers in blood samples only reached moderate levels, which decreased after 14 dpi. Given the time between bait administration and onset of clinical signs and viraemia, these three animals were considered to have been infected directly through ingestion of the oral inoculum. Viral RNA was first detected in nasal swab samples from this group at 8 dpi, with high levels detected in swabs from the animal that was euthanized for ethical reasons. Viral RNA was only detected in blood of the remaining three animals 6 days later, at 14–16 dpi, and these animals were considered to have become infected indirectly, through contact infection, rather than directly from ingestion of the inoculum. Similarly, in the group fed 10^5 TCID$_{50}$ UK2000/7.1, five of the six animals were designated to have been infected by the inoculum due to viral RNA levels detected in the blood from 6 dpi; the remaining animal only became viraemic 10 days after viral RNA was detected in nasal secretions (Fig. 2B). Interestingly, the levels of RNA in blood decreased in a high proportion of animals considered to have been infected directly by the oral inoculum, indicating that these animals were clearing the infection.

It was anticipated that, compared with moderately virulent viruses, lower amounts of highly virulent strains would be able to initiate infection. However, all animals fed doses of 10^1–10^4 TCID$_{50}$ of the highly virulent CSFV Brescia remained healthy and no virus was detected in blood, nasal swabs and no decrease in leucocyte numbers was observed. Viral RNA was detected at 4 dpi in the blood of two animals fed 10^5 TCID$_{50}$ which were euthanized at 5 and 6 dpi due to clinical signs of CSF (Fig. 2C). Viral RNA was detected at 6 dpi in nasal swab samples from one of these two animals. Detection of viral RNA and development of clinical signs in the remaining four animals in this group occurred in phases, with animals being euthanized due to clinical disease on 12, 19, 24 and 26 dpi. It was concluded that only two animals in this group became infected via the oral inoculum. All animals fed 10^6 TCID$_{50}$ developed CSF signs within 4 or 5 dpi (Fig. 2D).

The infectious dose that resulted in infection of 50% of pigs via the oral route (oral PID$_{50}$) for the two strains was determined as $10^{4.2}$ TCID$_{50}$ (95% CI $10^{3.6}$–10^5) for UK2000/7.1 and $10^{5.5}$ TCID$_{50}$ (95% CI $10^{5.1}$–$10^{5.8}$) for Brescia. The difference in oral PID$_{50}$ between the two strains is significant ($P < 0.05$)

4. Discussion

Assessment of the risks of products from animals which may be infected with exotic diseases requires estimation of the likely pathogen load. Quantification of the viable CSFV in tissues from experimentally infected animals confirmed the viral loading is lower in tissues that form the majority of pork products (muscle and fat) than in lymphoid tissues, which have higher virus concentrations but constitute only a small proportion of total carcass weight. The levels of virus detected in muscle and fat are consistent with studies which detected CSFV less frequently in striated muscle compared to other tissues (Thur and Hofmann, 1998) and previous attempts to titrate virus loads in fat and muscle indicated that levels are often close to or below the quantifiable limit of cell culture assays (McKercher et al., 1987; Mebus et al., 1993; Wood et al., 1988). No viable virus could be detected in skin samples. However, other studies have detected CSFV in skin (Kaden et al., 2007; Wood et al., 1988) and so, although the levels are likely to be low, the risk due to CSFV may not be negligible in products containing porcine skin.

Whilst a comprehensive quantification of viral loads in animals infected with CSFV of different virulence and at all stages of CSF disease was beyond the scope of this study, the mean viral loadings reported here are representative of levels in tissues from most weaner pigs with acute CSF. The high viral loads obtained in one animal with underlying disease highlights that viral loads are influenced by the health status of an animal. For consideration of the worst case scenario, conservative estimates can be made using the upper range of values reported here.

In contrast to other studies, we did not detect a significant difference in the stability in cell culture media of the three isolates tested. Notably, the greatest difference in thermal stability between isolates reported (Depner et al., 1992) was observed at pH3, whereas variation between strains at pH7, the pH use in this study, was minimal. The absence of significant difference between the three isolates tested allowed use of material from animals infected with these strains for investigation of thermal stability in tissues. However, the pH of muscle tissues used in this study was 6.1 and so we cannot exclude the possibility that small differences in thermal stability might exist at this slightly lower pH, at different temperatures or between other strains.

This study has generated data that can be used to estimate the length of time required to inactivate all virus present in a product. The data indicate that CSFV remains viable for a considerable time under chilled conditions. For example, a product with a starting titre of 10^4 TCID$_{50}$/g in muscle tissue would be predicted to contain viable virus for ~22 weeks at 4 °C, which is longer than products are likely to be kept refrigerated.

Similarly, for an infected product containing a portion of fat with a starting titre of 10^4 TCID$_{50}$/g, it is predicted that heating to 68 °C for 6 min could be required to inactivate the viable virus within the fat portion. This highlights that failure of a process to reach 70 °C throughout the product, as required by EU legislation 2002/99/EC (European Union, 2003) in order to eliminate

the risk of CSF due to introduction of products of animal origin, could allow viable virus to remain. Indeed, even if the target temperature of 70 °C is reached the extent that all CSFV that could be present would be inactivated is questionable. The legislation does not specifiy a time for which the 70 °C target must be applied for, but estimation of D_{70} values indicate that the target temperature would have to be achieved for over 1 min to reduce material with a titre of 10^4 TCID$_{50}$/g to zero. Much longer periods would be required if conservative estimates based on the upper 95% CI are applied. A reduction of the level of virus to zero TCID$_{50}$ may be insufficient to give confidence that the risk of CSF is eliminated. For canned goods, a sterilising process is one that achieves a 12-log reduction in the most resistant microorganism. At 70 °C, a 12-log reduction would require heating for 4 min (12 times the D value) for muscle tissues. Council directive 2202/99/EC also lists 80 °C as an acceptable treatment to eliminate CSF. From our data it can be estimated that the D_{80} value for muscle is 2.8 s, indicating that adopting this temperature as a minimum for acceptable treatments would eliminate the possibility for viable CSFV to remain. Alternatively, the legislation could be revised to define a minimum time required for treatments at 70 °C.

There was a significant difference in virus stability in the different tissue matrices which were more pronounced at lower temperatures. Under ambient temperature conditions, muscle with 10^4 TCID$_{50}$/g would theoretically still contain viable virus for up to 24 days. Although lymphoid tissue from the same CSFV infected animal will have a higher virus titre than muscle tissues the virus is less stable in this tissue type compared to muscle at 25 °C. Therefore, if lyphoid tissue with a starting titre of, for example, 10^6 TCID$_{50}$ was also held at 25 °C, it would be predicted that virus in this matrix would only remain viable for up to 8 days. Faster inactivation rates for CSFV in pig slurry compared to tissue culture media have been reported by others (Botner and Belsham, 2012) highlighting the importance of considering if available survival data has been generated from a relavent matrix when estimating risk. The mechanisms behind matrix dependent differences in inactivation rate are unclear. We speculate that at higher temperatures the denaturing effect of heat on viral proteins, particularly disruption of capsid, is less affected by the matrix whereas, at lower temperatures, the mechanism of virus inactivation may be influenced by the activity of enzymes. For example, lipases that could result in disruption of the viral envelope may be present in higher levels in lymph node and fat matrices than muscle. Hepatitis C virus, which, like CSFV, is an enveloped member of the flaviridae, is inactivated in milk by a lipase dependent mechanism (Pfaender et al., 2013), most likely due to the release of fatty acids which inactivate enveloped viruses (Thormar et al., 1987).

At 56 °C, CSFV is inactivated rapidly in muscle, lymph node and fat samples and our data give confidence that composting processes which require a temperature of 60 °C for 2 days, are more than adequate to inactivate CSFV. However, in serum the virus is substantially more stable at this temperature. Treatment of serum at 56 °C for 30 min, which is used to inactivate complement prior to serological

tests, will, at most, reduce viral titre by only one $\log_{10}$ and so is insufficient to inactivate all CSFV that could be present and is not appropriate as a method to release serum samples that may contain CSFV from biocontainment.

The generation of Z values allows estimations to be made on virus survival in these tissues at other temperatures. Such estimations are useful when no data exists at a given temperature for a particular matrix. However, due caution should be applied as the variation between actual and estimated D values could have a substantial practical impact. The results reported here indicate CSFV is more stable at room temperatures in lymph node than has been reported by others (Weesendorp et al., 2010), highlighting the high level of variablity between studies and hence the impact that slight variations in the conditions of a process involving pork products could have. It should also be noted that various differences exist between the samples from experimental infections used in this study compared to processed pork products. For example, the method of slaughter, exangination and hanging/chilling of carcase prior to butchering, processing of product such as curing and packaging to maintain shelf life are likely to affect virus stability.

The amount of a virus required to cause infection varies according to the route. For porcine respiratory and reproductive virus (PRRSV), the oral PID_{50} is estimated as $10^{5.3}$ $TCID_{50}$ whereas a lower value of 10^4 was estimated for intranasal exposure (Hermann et al., 2005). Virulence can also affect the dose required to instigate infection. For example, a low intranasal dose of moderately virulent CSFV failed to instigate infection whereas all animals inoculated with a similar dose of highly virulent virus became infected (Weesendorp et al., 2009a). We therefore determined the oral ID_{50} of both a virulent and moderately virulent CSFV. Blister packs designed for oral vaccination of wild boar were used to encourage chewing, rather than swallowing, of the inoculum to allow some contact with tissues in the mouth and tonsils, rather than likely inactivation by the low pH in the stomach. Whilst this route is not an exact representation of what might happen if an animal were to eat an infected pork product, it allowed inoculation of accurate dilution of doses in a standard volume, which would not have been possible by attempting to feed pigs with tissues from infected donor animals. The dose required to infect 50% of pigs via this oral route was much higher than via intranasal inoculation (Farez and Morley, 1997; Weesendorp et al., 2009a). Although, dose response data have not been published on oral live attenuated CSFV vaccines administered using blister baits, our data are in accordance with the stated dose require for oral vaccination (Kaden et al., 2010).

Surprisingly, a slightly higher dose of the highly virulent virus was required for infection than the moderately virulent virus. It is unclear if this higher dose represents a true difference between the two strains tested, as the data are from a limited number of experiments performed at different times. Whilst we cannot exclude the possibility that other highly virulent strains may instigate infection via ingestion at lower doses, our data indicates that viruses of higher virulence may not represent an increased risk of infection via the oral route compared with moderately virulent viruses.

5. Conclusion

Our data provide information to estimate how much CSFV may remain viable in a porcine product at different temperatures and how much of a product would need to be ingested to instigate infection in 50% of animals. Although higher than previously stated, the amount required is still present in a few grams of tissue from infected pigs and the different stabilities between tissues indicates the importance of basing estimates on stability data generated in a relevant matrix.

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