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Infection of pigs with African swine fever virus via ingestion of stable flies (*Stomoxys calcitrans*)

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Abstract

Within Eastern Europe, African swine fever virus (ASFV) has unexpectedly spread to farms with high biosecurity. In an attempt to explain this process, pigs were allowed to ingest flies that had fed on ASFV-spiked blood, which had a realistic titre for an infected pig. Some of the pigs became infected with the virus. Thus, ingestion of blood-sucking flies, having fed on ASFV-infected wild boar before entering stables, represents a potential route for disease transmission.

KEYWORDSAfrican swine fever, blood-feeding flies, haemorrhagic disease, *Stomoxys calcitrans*, virus transmission

1 | INTRODUCTION

Since 2014, African swine fever virus (ASFV) has spread among wild boar and domestic pigs within the Baltic States and Poland (EFSA Panel on Animal Health and Welfare, 2015). In 2017, the virus was detected, for the first time, in the Czech Republic and Romania (Anonymous, 2017a,b), and in 2018, an outbreak has occurred in Hungary (Anonymous, 2018). Surprisingly, ASFV has also been introduced into pig farms with high biosecurity (HB; EFSA Panel on Animal Health and Welfare, 2014, 2015). However, the route of introduction into these premises is unknown. Mechanical transmission of ASFV by the stable fly, *Stomoxys calcitrans*, during feeding has been reported (Mellor, Kitching, & Wilkinson, 1987). Thus, stable flies that feed on wild boar and subsequently feed on domestic pigs represent a potential route for spreading ASFV. However, farms with HB generally have double fencing to create a buffer zone to prevent proximity of wild boar to the stables. As stable flies both breed and feed on farms (Foil & Hogsette, 1994), they may not move frequently across this zone. In contrast, blood-feeding horse flies (family *Tabanidae*) are strong flyers (Cooksey & Wright, 1987) and have been observed near ventilation openings of an ASFV-infected HB farm in Lithuania (R. B. René Bødker, unpublished data). These flies do not

breed in stables (Foil & Hogsette, 1994) but emerge from semiaquatic habitats outside the farms, where they may have contact with wild boar before entering the stables. The *Tabanidae* are known to be mechanical vectors for viral pathogens (Krinsky, 1976), but they usually do not feed inside dark stables as they are positively phototrophic (Middlekauff & Lane, 1980). *Tabanidae*, however, are large enough that pigs may chase and eat them or accidentally ingest them during feeding. It has been proposed that analysing the effect of ingestion of insect vectors should be prioritized (Guinat et al., 2016). Here, we have studied experimental ASFV transmission to pigs via oral uptake of flies previously fed on blood from ASFV-infected pigs. *Stomoxys calcitrans* has been used as a model for blood-feeding flies, as they are readily caught and fed.

2 | MATERIALS AND METHODS

This study was conducted with approval from the Danish Animal Experimentation Inspectorate (license number 2015-15-0201-00606) in accordance with Danish and EU legislation (Consolidation Act 474 15/05/2014 and EU Directive 2010/63/EU). Twelve Danish SPF pigs (Landrace × Yorkshire × Duroc hybrids), 8–9 weeks of age,

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were used. On arrival, the pigs were divided into groups of four animals within three separated BSL3 containment stables. Following acclimatization, pigs 1–4 in group 1 were inoculated orally with EDTA-stabilized blood spiked with blood obtained from pigs infected with an ASFV isolate from Poland (Olesen et al., 2017). The pigs were restrained but not sedated and swallowed the inoculum. Similarly, animals in group 2 (pigs 5–8) were inoculated orally with euthanized wild-caught stable flies (*S. calcitrans*) following their homogenization. For group 3 (pigs 9–12), intact fed flies (20 per pig) were added to ca. 100 g soft cake that was then eaten by each pig (Table 1). The flies used for inoculation of groups 2 and 3 had been wild-caught in the vicinity of a cattle herd using insect nets. Just prior to their euthanasia (by freezing), the flies had been fed for 1 hr on ASFV-spiked EDTA-stabilized blood that had a titre of $5.8 \log_{10}$ TCID₅₀/ml, which is realistic for an infected pig (Olesen et al., 2017) using a heated membrane feeder equipped with a parafilm membrane. EDTA-stabilized blood was used to prevent coagulation during the spiking and feeding procedures. Blood feeding of the flies was confirmed visually with stereo-microscopy of their abdomens following feeding and euthanasia. For group 2, the flies were homogenized in Eagle's minimum essential medium supplemented with streptomycin (Sigma-Aldrich), neomycin (Sigma-Aldrich) and 5% foetal calf serum (FCS; 200 μ l per fly) using a 3-mm stainless steel bead (Dejay Distribution Ltd., Launceston, UK) in a TissueLyser II (Qiagen, Hilden, Germany) for 1 min at 25 Hz. Note, blood-fed homogenized flies from an earlier experiment were shown to contain ASFV DNA by PCR performed as described previously (Olesen et al., 2017); the C_q-values ranged from 23.5 to 25.5 per fly, indicating that the feeding procedure was effective.

Table 1 shows the dose of virus used for each group of pigs. The titre of the blood (group 1) was obtained from back titration in porcine pulmonary alveolar macrophages (PPAM) at the time of inoculation (Olesen et al., 2017). Virus titration directly from flies has been found previously to be difficult in our laboratory. Hence, the level of virus present within the ingested flies (groups 2 and 3) was obtained by calculation assuming that each fly had consumed 11–15 μ l of the spiked blood (Schowalter & Klowden, 1979).

During the study, clinical scores, including rectal temperatures, were recorded from pigs on each day, and a total clinical score (CS) was calculated. The presence of clinical signs was defined as a CS

TABLE 1 Inoculation material and dose for each group within the study

Group no.	Pig no.	Inoculation material	Inoculation dose
1	1–4	Oral inoculation with 1 ml spiked blood	$5 \log_{10}$ TCID ₅₀
2	5–8	Oral inoculation with 20 homogenized flies	$5.1\text{--}5.3 \log_{10}$ TCID ₅₀
3	9–12	Feeding with soft cake containing 20 intact flies	$5.1\text{--}5.3 \log_{10}$ TCID ₅₀

>3 (Olesen et al., 2017). EDTA-stabilized blood and unstabilized blood were collected prior to inoculation at 0 days, postinoculation (dpi), at 2, 4, 6, 8, 10, 12, 14 and 16 dpi and at euthanasia. ASFV DNA was extracted from EDTA-stabilized blood as previously described (Olesen et al., 2017) and detected using quantitative real-time PCR (Tignon et al., 2011). The number of genome copies was determined using a standard curve based on a dilution series of plasmid pVP72 (King et al., 2003). Infectious ASFV in serum was detected by end-point titration in PPAM (Bøtner, Nielsen, & Bille-Hansen, 1994; Olesen et al., 2017), and titres were presented as TCID₅₀/ml (Reed & Muench, 1938). Serum was used for virus detection, as less background staining has been observed, when using serum compared to EDTA-stabilized blood (Olesen et al., 2017). Serum samples, obtained at euthanasia, were tested for the presence of anti-ASFV antibodies using an Ingezim PPA Compac ELISA (®Ingenasa, Madrid, Spain).

3 | RESULTS AND DISCUSSION

As a positive control, pigs (in group 1) were inoculated orally, at 0 dpi, with spiked blood containing $5 \log_{10}$ TCID₅₀ of ASFV (Table 1). Within this group, pig 2 displayed fever starting from 6 dpi. Anorexia, depression and mild convulsions were observed prior to, or at, euthanasia at 9 dpi (Figure 1a). Two other pigs within group 1 (pigs 3 and 4) appeared depressed and had a fever starting from 14 and 15 dpi, respectively. Due to severe depression and a sudden drop in rectal temperature, pig 3 was euthanized at 15 dpi (Figure 1a). Pig 4 would not eat at 16 dpi and vomiting was observed. At the morning inspection at 17 dpi, the pig had generalized convulsions and was euthanized. The clinical signs in these three pigs were accompanied by the detection of ASFV DNA and infectious virus in blood and serum, respectively (Figure 1a and 2a). Pig 1 remained clinically healthy throughout the study period (Figure 1a), but viral DNA was detected in blood obtained from this pig, after euthanasia for welfare reasons, at 17 dpi (Figure 2a).

To determine the potential for blood-fed flies to be a source of ASFV infection, pigs were inoculated orally with homogenized flies (group 2, Table 1) or allowed to ingest intact flies within soft cake (group 3, Table 1) calculated to contain $5.1\text{--}5.3 \log_{10}$ TCID₅₀ of ASFV in total. Within these groups, fever occurred in pigs 5 and 7 (from group 2) and pigs 10 and 11 (from group 3) starting from 5 and 6 dpi. On subsequent days, decreased appetite or anorexia and depression were observed. Prior to euthanasia at 7 dpi (Figure 1b,c), a sudden drop in rectal temperature was detected in these four pigs. Three remaining pigs within groups 2 and 3 (pigs 6, 8 and 9) had a fever starting from 11 or 13 dpi. These pigs and pig 12 were euthanized at 12 or 14 dpi, respectively (Figure 1b,c). Prior to euthanasia, clinical signs included depression, anorexia, vomiting, a sudden drop in rectal temperature and hyperaemic skin. Pig 12 remained clinically healthy, but was the only pig left in the pen and was therefore euthanized for welfare reasons. No ASFV

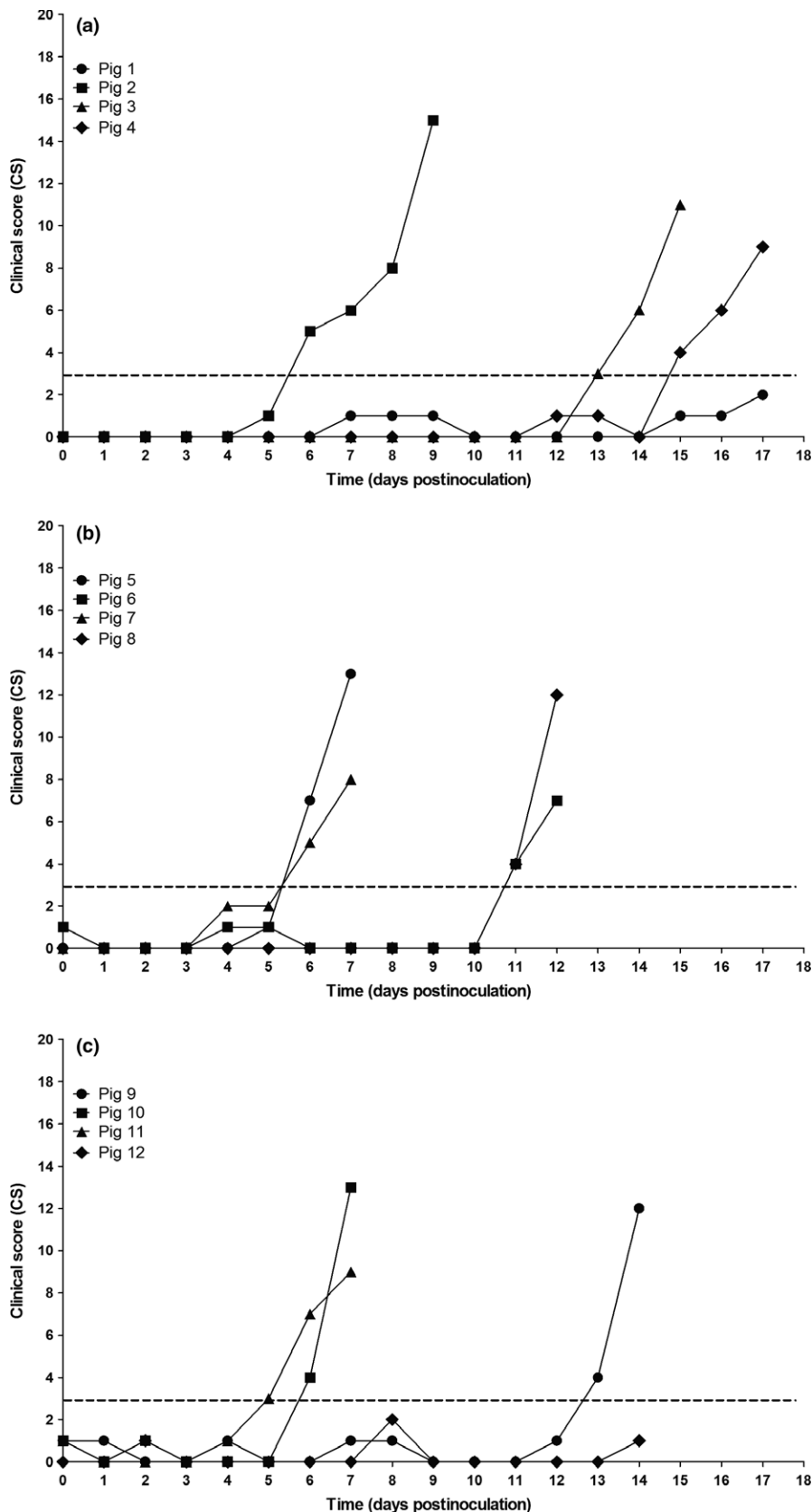


FIGURE 1 Clinical scores obtained from pigs 1–4 (group 1, panel a), pigs 5–8 (group 2, panel b) and pigs 9–12 (group 3, panel c). A clinical score (CS) above 3 is considered above the clinical threshold that is indicated by the dotted line

DNA was detected in blood obtained from this pig following euthanasia. In the other seven pigs within groups 2 and 3, clinical signs were accompanied by the detection of viral DNA and

infectious ASFV in blood and serum samples (Figures 1b,c, and 2b, c). No anti-ASFV antibodies were detected in serum from any of the pigs.

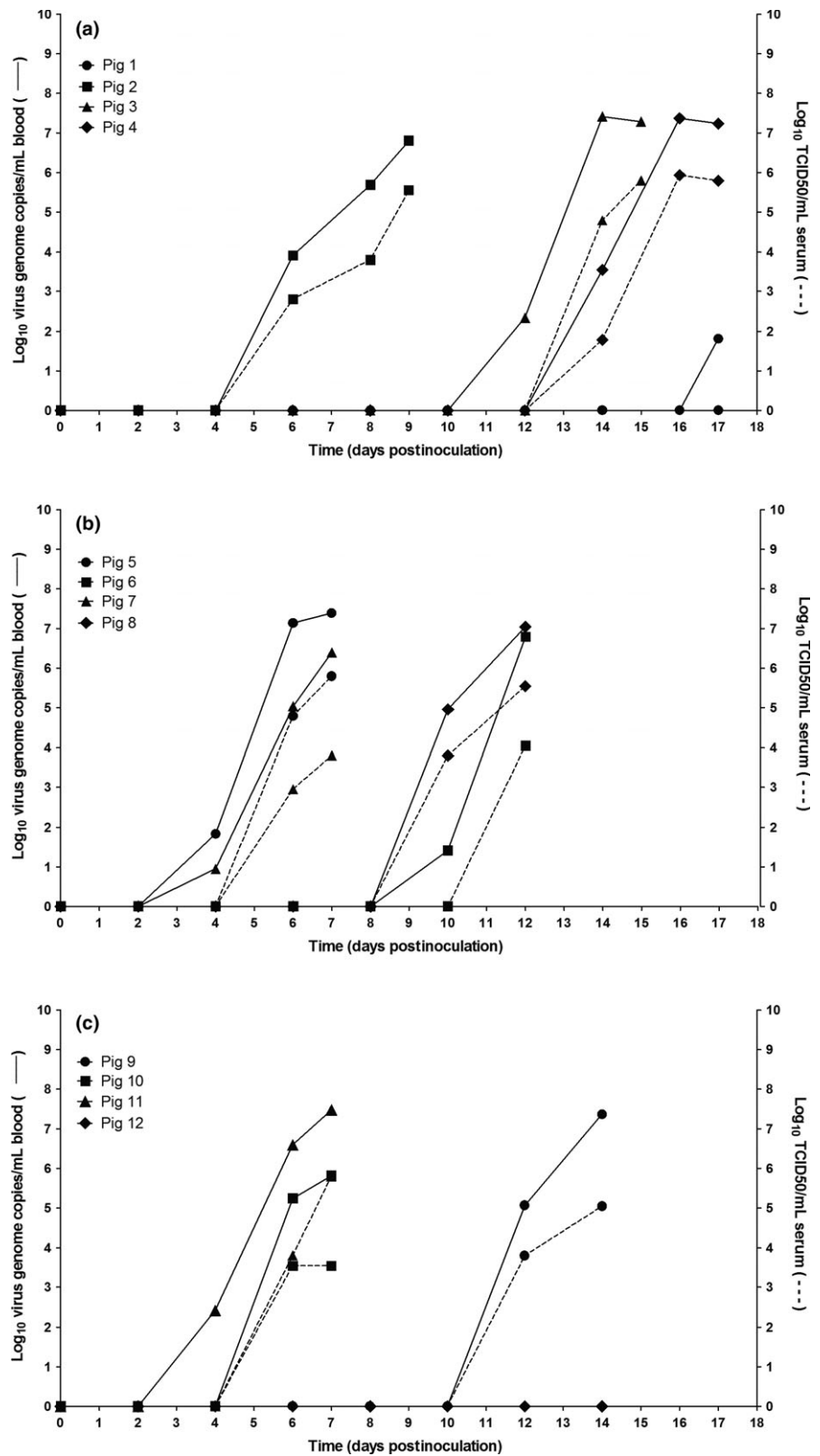


FIGURE 2 The detection of ASFV DNA and infectious virus in EDTA-stabilized blood and serum obtained from pigs 1–4 (group 1, panel a), pigs 5–8 (group 2, panel b) and pigs 9–12 (group 3, panel c). The solid lines show the detection of ASFV DNA in EDTA-stabilized blood. The dotted lines show the detection of infectious virus in serum

Within each group, the delayed time course of infection in some pigs indicates that only 25% (group 1) and 50% (groups 2 and 3) of the pigs were infected by the oral ingestion of the virus, while the remaining pigs were most likely infected subsequently via contact with the infected pig(s) within their group. Hence, the remaining pigs

started to show clinical signs of the infection and became viraemic some 5–8 days later than the pigs that were infected by oral ingestion of the virus within each group (Figures 1 and 2). A similar pattern of infection in inoculated and contact animals has been reported previously using this virus (Olesen et al., 2017). Assuming

that one 50% hemadsorbing dose (HAD₅₀) corresponds approximately to one TCID₅₀ as a measure of ASFV infectivity, then this may not be surprising. In a previous study, doses of 4 and 6 log₁₀ HAD₅₀ have been found necessary to efficiently establish ASFV infection in domestic pigs following oral inoculation with a highly virulent strain of the virus (ASFV-Malawi; Howey, O'Donnell, de Carvalho Ferreira, Borca, & Arzt, 2013), while in contrast 2 log₁₀ HAD₅₀ was insufficient.

These results indicate that, in addition to *S. calcitrans* acting as a mechanical vector for ASFV through feeding on pigs (Mellor et al., 1987), infection can also occur following oral uptake of these fed flies. In the study by Mellor et al. (1987), two healthy pigs became ASFV-infected after allowing 30 or 57 blood-fed *S. calcitrans* to feed on them, while in this study, pigs became infected with the virus following ingestion of 20 blood-fed flies. For casual ingestion, 20 flies may seem a high number. However, as previously mentioned, a relatively high dose of infectious ASFV is required to establish infection orally (Howey et al., 2013), and a calculated dose of 5 log₁₀ TCID₅₀ (in 20 flies) was chosen. As virus cultivation on fly samples had previously proven unsuccessful in our laboratory and as the stability of the virus in such samples to our knowledge has been demonstrated only once (Mellor et al., 1987), inactivation of the virus within the flies could not be ruled out. Therefore, choosing a level of virus known to cause infection via oral uptake (Howey et al., 2013) would allow us to determine whether the virus was indeed still infectious in the flies. Assuming that each fly consumes 11–15 µl of blood (Schowalter & Klowden, 1979) when feeding on an infectious pig (with a blood titre of 5.8 log₁₀ TCID₅₀), a single fly would theoretically carry some 3.8–4 log₁₀ TCID₅₀, which corresponds to the infectious dose by oral inoculation (Howey et al., 2013). In addition, other flies, in particular, the *Tabanidae*, can carry up until five times more blood than stable flies (Leprince & Foil, 1993; Salem, Franc, Jacquet, Bouhsira, & Liénard, 2012) yielding a potential level of up to 4.5 log₁₀ TCID₅₀ per *Tabanidae* fly. It has previously been demonstrated that very low doses (3 and 25 hemadsorbing units [HAU]/ml) of a Caucasian isolate of the virus can sometimes result in infection of weak animals following oronasal inoculation (Pietschmann et al., 2015). Hence, the virus present within a few blood-fed *Stomoxys* flies or *Tabanidae* flies could potentially result in infection with ASFV (and maybe even a single fly is sufficient).

It is unlikely that ingestion of blood-fed flies is a common route for transmission of ASFV between wild boars or between pigs within a stable. However, when bearing in mind the biology of the flies, the results indicate that *Stomoxys* flies could be one possible route of transmission over short distances (e.g., within farms), while larger flies, such as the *Tabanidae*, might explain some longer distance examples of ASFV transmission (e.g., into and between farms). In conclusion, blood-feeding flies could be a route for the observed, but unexplained, introduction of ASFV into farms with HB. Such transmission seems readily preventable (e.g., using ventilation filters). Keeping these findings in mind, future studies investigating the role of *Tabanidae* and other large blood-feeding flies in the transmission of ASFV are warranted.

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CONFLICT OF INTEREST

The authors declare no conflict of interests.

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