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Transmission of African Swine Fever Virus via carrier (survivor) pigs does occur

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ABSTRACT

We investigated whether ASF carrier pigs that had completely recovered from an acute infection with ASFV Netherlands '86, could transmit the disease to naive pigs by direct contact transmission. For this, we used pigs that had survived an ASFV infection, had recovered from disease, and had become carriers of ASFV. These clinically healthy carriers were put together one-by-one with naive contact pigs. Two of the twelve contact pigs developed an acute ASFV infection. Using the results of the experiment we quantified the transmission parameters $\beta_{carrier}$ (0.039/day) and $T_{carrier}$ (25.4 days). With the survival rate of 0.3 for our ASFV isolate, these parameter values translate into the contribution of carriers to R_0 in groups of pigs being 0.3. Further, we placed naive contact pigs in an ASFV contaminated environment. Here, no contact infections were observed. Our findings show that clinically healthy carriers can be a source of acute new infections, which can contribute to the persistence of ASFV in swine populations. The estimates that we provide can be used for modelling of transmission in domestic pigs and, in part, for modelling transmission in wild boar.

1. Introduction

African Swine Fever (ASF) is a highly infectious viral disease, caused by African Swine Fever Virus (ASFV), a member of the Asfarviridae family (Takamatsu et al., 2011). It affects only members of the family of *Suidae*, including domestic pigs and European wild boar (both *Sus scrofa*). ASFV infection results in a wide range of clinical pictures, varying from subclinical disease to severe haemorrhagic disease with up to 100% lethality. Differences in clinical signs depend amongst others on the virulence of the involved ASFV strain (Blome et al., 2013). Outbreaks of ASF obviously have impact on the animal health, but also have major socio-economic consequences. Besides damages due to production losses, it can severely affect the trading conditions (with subsequent export losses) of a country. In 2007, ASF virus was introduced in Georgia (Rowlands et al., 2008) and subsequently spread to other Caucasian and Eastern European countries (Sanchez-Vizcaino et al., 2013). Since then also European Union (EU) member countries have reported the disease, amongst which the Baltic States, Poland, Romania, the Czech Republic, Hungary, Bulgaria and Belgium (EFSA, 2018). Recently, also China was affected (Zhou et al., 2018). Transmission of the disease has mainly been attributed to movements of infected domestic pigs and wild boar and through ingestion of contaminated pig products. There are no treatments or effective vaccines against ASF available (Zakaryan and Revilla, 2016). Therefore, outbreaks of ASF in domestic pigs have to be controlled by application of strict sanitary and biosecurity measures, such as culling of infected farms supplemented with movement restrictions (Anon, 2002). However, these type of control measures will generally not affect the movement of wild boar. The role that wild boar play in the epidemiology of ASF is not fully elucidated yet. In the current epidemic in Europe, the initial outbreaks were mainly reported in domestic pigs, mostly in backyard holdings, but as time progressed wild boar became increasingly affected. Although first seen as spill-over from infections in domestic pigs, the presence of ASFV in wild boar now seem to be one of the main factors promoting the continued spread of ASFV (Chenais et al., 2019). Many of ASFV infected pigs die because of the disease. However, if pigs recover from an ASF infection (survivors), ASF virus can persist for prolonged periods in tissues or blood of the surviving pigs (carriers) and these carrier pigs may contribute to virus transmission. The role that ASFV carriers play in transmission of the disease is still an important knowledge gap. Further, the role of an environmental contamination by infected pigs as a pathway for transmission of ASFV is not understood (Guinat et al., 2016a). Knowledge about which pathways play a role in the transmission of ASF are needed, to be able to understand the epidemiology of ASFV and to develop effective control measures. In particular, estimates of ASF transmission parameters for

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different transmission routes are essential to inform mathematical models that can be used to predict ASFV spread or to model the effect of possible control measures.

In the presented study, we investigated whether ASF carrier pigs that had completely recovered from an acute infection with ASFV Netherlands '86, could transmit the disease to naive pigs by direct contact transmission and whether an environment contaminated by ASF infected pigs could cause infection in contact pigs. With the results of the study, we quantified the transmission rate and the infectious period of the ASF carrier phase and the component of the total R_0 that is due to the ASF carrier phase. These estimates can directly be used for modelling of transmission of ASFV in domestic pigs. We discuss how the estimates can also inform transmission models for wild boar.

2. Materials and methods

2.1. Experimental design

The transmission experiment was preceded by an experiment that started with 4 groups of 5 piglets (Post et al., 2017). Ten piglets were 12 weeks old (groups I and II) and the other 10 piglets were 18 weeks old (groups III and IV). Each group of piglets was housed in a separate pen (pens I-IV) in one room of the high containment facilities of Wageningen Bioveterinary Research (WBVR). After an acclimatisation period, the piglets were inoculated with the moderately virulent strain ASFV Netherlands '86 at day 0. All pigs developed clinical disease of ASF. At 27 days post inoculation (dpi), 14 pigs had either died or had been euthanised because of ethical reasons. The six remaining pigs had completely recovered but in all a persistent viraemia was still present. Also, in all pigs ASFV was present in their oropharyngeal fluid (OPF). These carrier pigs were used for the transmission experiments that are described here.

The experiment was approved by the Animal Experiment Ethical Review Committee of WBVR in compliance with the national laws and regulations.

2.1.1. Direct contact transmission experiment

Direct contact transmission was studied in two periods of two weeks (28-41 dpi and 42-55 dpi). The carrier pigs were individually placed in new pens (pens a–f) at 27 dpi. At 28 dpi, in each pen, a healthy contact animal (C1 pigs) was introduced. At 41 dpi, the C1 pigs were removed. At 42 dpi, new contact animals (C2 pigs) were introduced in the pens. The contact pigs were 5–6 weeks younger than the carrier pigs, to limit rank order fights. Contact pigs were monitored on a daily basis, and removed as soon as they were found to be infected, so that their contribution to environmental contamination would be limited. At 55 dpi, the direct contact experiment was ended and the remaining pigs were euthanised.

2.1.2. Environmental contact transmission experiment

In the preceding experiment the pens I-IV (in which the infected pigs were housed), were not cleaned with water but instead, animal waste was removed daily with a broom and shovel, in order to create a contaminated environment. In the preceding experiment, all pigs became infected. Details about the observed virus titers in blood and survival rate of these pigs can be found in Post et al. (2017). In pen I, all infected pigs died or were euthanised for welfare reasons, where the last pig was removed at 15 dpi. In pen II, one pig survived. In pens III 3 pigs and in pen IV 2 pigs survived. The other pigs in pens II – IV died or were euthanised for welfare reasons at 15 dpi at the latest. After removal of the surviving pigs, at 28 dpi, in each pen two sentinel pigs (indirect contact pigs, IC pigs ; 8 weeks old) were introduced to investigate environmental contact transmission. The IC pigs were removed at 45 dpi.

2.2. Challenge virus and inoculation

For inoculation, we used the ASFV Netherlands'86 isolate, which is considered moderately virulent (Terpstra and Wensvoort, 1986; de Carvalho Ferreira et al., 2012), with an expected survival rate of 30–70%. Groups I and III were inoculated intranasally with 2 ml (1 ml in each nostril) virus suspension that contained $10^{3.5}TCID_{50}$/ml (titration on MARC-145 cells (de Carvalho Ferreira et al., 2012)). Groups II and IV were inoculated intranasally with 2 ml virus suspension that contained $10^{5.5}TCID_{50}$/ml.

2.3. Monitoring of clinical signs

From 28 dpi, the pigs were examined daily for clinical signs of ASF using a score table for ASF (de Carvalho Ferreira et al., 2012). The total clinical score could vary between 0 and 29. With a score of > 6, pigs were placed under analgesic treatment (Buprenorphine 10 μg/kg IM, twice a day). With a score of > 10, pigs were re-examined at the end of the day. A score of > 12 was considered as humane end point and pigs were euthanised (Euthasol 20% IV). Body temperatures of the contact pigs (C1, C2, IC) were monitored daily.

2.4. Sampling procedures

2.4.1. Pig samples

From day 28 dpi, OPF samples and EDTA samples were collected as described by Weesendorp et al. (2009). The carrier pigs that were used in this experiment were sampled twice a week. The contact pigs (C1, C2, IC pigs) were sampled daily the first week after introduction and afterwards twice a week. Serum samples from the carrier pigs were collected at the end of the preceding experiment (27 dpi) and the end of the current experiment (55 dpi).

After collection, the oropharyngeal swabs were soaked in 4 ml medium (Eagle's minimum essential medium (EMEM)) (Gibco, Invitrogen, Breda, The Netherlands) supplemented with 5% foetal bovine serum (FBS) and 10% antibiotics solution ABII (1 000 U/mL Penicillin, 1 mg/ml Streptomycin, 20 lg/mL Fungizone, 500 lg/mL; Polymixin B, and 10 mg/ml Kanamycin). Fluids were collected from the swabs by centrifugation (1800*g* for 15 min). The samples were stored at −70 °C until they were analyzed by quantitative real-time polymerase chain reaction (qPCR, all samples) and virus isolation (VI, all samples from the carrier pigs and positive qPCR samples of the contact pigs).

EDTA blood samples were stored at −70 °C until they were analyzed by qPCR (all samples) and VI (all samples from the carrier pigs and qPCR positive samples of the contact pigs).

Serum samples were centrifuged and serum was stored at −20 °C until analysis.

2.4.2. Environmental samples

From the floors of pens I-IV, swab samples were collected twice a week at 28, 31, 35, 38, 42 and 45 dpi. For this, in each pen, at two randomly chosen places in the pen (1x near the food through and 1x somewhere else in the pen) a wooden rod of 1 m long was tossed onto the floor. Gauze swabs were swiped across the floor beside the rod, each time two swabs. The swabs were placed individually in a 50 ml Falcon tube.

In the laboratory, the swabs were suspended in 4 ml medium (same as for the OPF swabs) and after vortexing, the supernatants were stored at −70 °C until they were analyzed by qPCR (all samples) and VI (qPCR positive samples).

Air samples were collected above all pens (pens a–f and I-IV) twice per week using the MD8 air scan sampling device (Sartorius) and sterile gelatine filters of 3 μm pore size and 80 mm diameter (type 17528-80-ACD; Sartorius) (Weesendorp et al., 2008; de Carvalho Ferreira et al., 2013). An air speed of 8 m^3/h and sampling period of 10 min was used. The air was sampled above the centre of the pens at a height just above

the reach of the pigs. After sampling, the filters were dissolved in 5 ml of medium (EMEM supplemented with 5% FBS and 10% ABII kept at 37 °C. Dissolved filter solutions were stored at −70 °C until they were analysed by qPCR (all samples) and VI (qPCR positive samples).

2.5. Laboratory tests

2.5.1. qPCR

Samples were analyzed by an in-house qPCR (de Carvalho Ferreira et al., 2012), with the exception that we used the Qiagen Quantifast Probe RT-PCR kit instead of the Roche DNA Master Hybridization Probes Kit. The qPCR is validated and accredited according to ISO17025 and was used to quantify the relative amount of virus particles, compared to a standard curve of a virus stock with a known titre. The viral DNA concentration of each individual sample was calculated using the standard curve and expressed as $TCID_{50}$-equivalents/ml (eq/ml). This is a relative measure of the amount of virus DNA and does not necessarily correlate to the amount of infectious virus.

2.5.2. Virus isolation

Porcine Alveolar Macrophages (PAMs) were collected from young SPF pigs as described for PRRSV (Anon, 2018) in medium (RPMI1640 + 10% FBS + 1% penicilline-streptomycine) in a concentration of 1×10^6 cells/ml. PAMs were pipetted in M24 well plates (Costar), 1 ml/well and placed for 1–4 h in a CO_2 incubator at 37 °C. EDTA blood samples were thawed and samples were pipetted in the 24 well plates (125 µl/well). In each well 80 µl of a 1% pig erythrocyte-suspension was added. The plates were placed in a CO_2 incubator at 37 °C. The samples were inspected microscopically on a daily basis (for a maximum of 10 days) for presence of characteristic rosette formation. If rosette formation was observed, the samples were considered as positive for ASFV.

2.5.3. Serology

Serum samples of the carrier pigs were tested for ASFV-specific antibodies with the INgezim PPA Compac ELISA (Eurofins Ingenasa, Madrid, Spain), according to the instructions of the manufacturer. Results are presented as percentage blocking.

2.6. Contact infections and quantification of transmission

2.6.1. Contact infections

For the detection of contact infections, qPCR results were used. Contact pigs were considered infected as soon as a blood or OPF sample tested positive with a titre of at least 10^2 $TCID_{50}$-eq/ml in the qPCR. Whether or not there was a significant difference in observed number of contact infections between groups C1 and C2 was evaluated using a Fisher Exact test (Statistics for Windows, version 7.0). To determine whether or not the contact infections that occurred were distributed equally during the time that infectious pigs were present, the homogeneity of occurrence of contact infections was evaluated using Pearson's Chi-Squared statistic. We assumed a latent period of 3 days. Because of the modest sample size, the distribution of the possible outcomes was generated by simulation (statistical software 'R', version 3.3.1).

2.6.2. Quantification of transmission parameters

An often used parameter to quantify transmission is the reproduction ratio R, which is the average number of secondary infections per infectious individual during its entire infectious period (Diekmann et al., 1990). As long as $R > 1$, an infection can spread on a large scale, whereas as $R < 1$ the infection will fade out. For diseases with a carrier state, a susceptible animal can be infected by either acutely infected individuals or carriers. In that case, the value of the basic reproduction ratio R_0 can be written as the sum of separate components from the acutely infected and chronic carriers (Keeling and Rohani, 2008)

$$R_0 = \Delta R_{0\ \text{acute phase}} + \Delta R_{0\ \text{carrier phase}}$$

where:

$\Delta R_{0\ \text{acute phase}}$ is the component of the acute state and $\Delta R_{0\ \text{carrier phase}}$ the component of the carrier state, and the latter can be written as a product of probability to become a carrier p_{carrier} times the average number of secondary infections caused by an animal in the carrier state ($M_{\text{carrier phase}}$).

With the results of our study, we quantified the $\Delta R_{0\ \text{carrier phase}}$ for ASFV. We used an SEIR (Susceptible-Exposed-Infectious-Removed) model to quantify the transmission parameter β_{carrier}. For this, for each time interval between consecutive samplings, the number of infectious and susceptible pigs was determined at the start of the interval, and the number of contact infections among these susceptible pigs during the interval. β_{carrier} was estimated from this data by maximum likelihood. A standard approach for this estimation is using a Generalized Linear Model (binomial distribution, complementary log-log link function) with the number of new contact infections as binomial totals, the number of susceptible pigs as explanatory variable, and ln (number of infectious pigs × time interval/total number of pigs) as offset (Klinkenberg et al., 2002). In our case of one-to-one experiments, this is equivalent to calculating the point estimate for β as -2 × ln ((total number of exposure days without transmission)/(total number of exposure days)). Confidence bounds for β were obtained exactly, or, if that was not possible, using the likelihood-ratio test. For the exact calculation, the ratio (total number of exposure days with transmission)/(total number of exposure days) was used as test statistic. Carrier pigs were considered to be infectious during the whole period of the study. Contact pigs were considered infected as defined above and a latent period of 3 days was assumed. The transmission parameter β_{carrier} was estimated for the first contact period of 13 days (C1 pigs) and the second period of 13 days (C2 pigs) separately and, additionally, for the whole contact period of 26 days.

The infectious period of the carrier period T_{carrier} (defined as presence of viraemia) was calculated using the VI data of the blood samples using a parametric survival analysis with an exponential distribution (statistical software 'R', version 3.3.1). A positive test period per pig was defined as the total carrier period from day 28 dpi (when all pigs tested positive) until the day before the pig tested definitely negative. The data of the pig that scored positive in the VI at the end of the experiment were treated as censored data. $M_{\text{carrier phase}}$ was estimated by multiplying the estimate for β_{carrier} by the estimated infectious period T_{carrier}. The probability to become a carrier was estimated from the results of the preceding experiment (Post et al., 2017). In our study, the probability to become a carrier equated the survival rate. $\Delta R_{0\ \text{carrier phase}}$ was calculated as this probability times $M_{\text{carrier phase}}$.

3. Results

3.1. Clinical signs

In the direct contact transmission experiment, in the carrier pigs and C1 pigs, no clinical signs were observed. In two of the C2 pigs (pens B and C) clinical signs of African Swine Fever were observed (C2 pen B: fever; C2 pen C: fever, reduced liveliness, red skin). Both pigs were euthanised at 47 dpi to avoid further spread of the virus.

In the indirect contact transmission experiment in the IC pigs no clinical signs were observed.

3.2. qPCR, VI and serology results

In the direct contact transmission experiment, the OPF samples of the carrier pigs tested positive in the qPCR during the whole test period. The qPCR titre of the OPF samples was at a constant level (1–3 ^{10}log $TCID_{50}$-eq/ml). Also the blood samples tested positive in qPCR, for 4 pigs during the whole test period and for 2 pigs until days 49 and 52

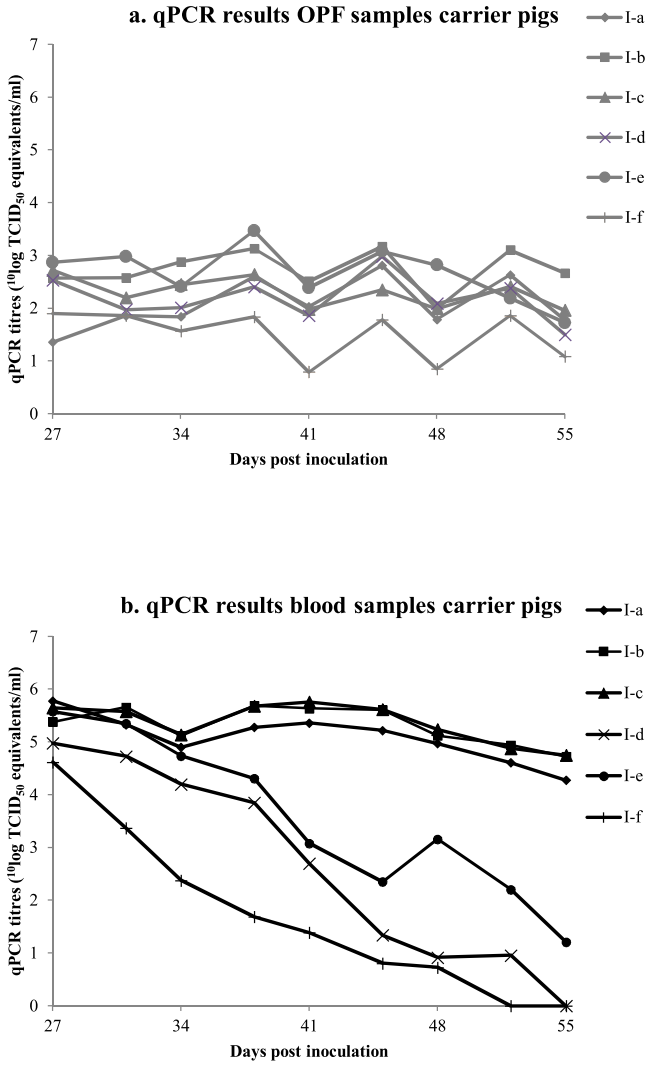

Fig. 1. qPCR results of the OPF swabs (a) and blood samples (b) of the carrier pigs.

respectively. The qPCR titre of the blood samples was at a constant high level in 3 pigs (5–6 ^{10}log TCID$_{50}$-eq/ml, pigs in pens a, b and c), whereas in the 3 other pigs the level started as high but declined during the test period (Fig. 1). The majority of the qPCR positive samples also tested positive in the VI (Table 1). The OPF and blood samples of the C1 pigs all tested negative in both qPCR and VI (Table 1). The OPF and blood samples of the C2 pigs with clinical signs (pens b and c) tested positive in both qPCR and VI. A titre of > 2 ^{10}log TCID$_{50}$-eq/ml was detected at day 47 (pen b) and 45 (pen c) respectively. The qPCR and VI results of the samples of the other 4 C2 pigs remained negative (Table 1).

In the indirect contact transmission experiment, the floor samples taken in pens I, II and IV tested (low) positive (qPCR results varying from -0.7 – 2.4 ^{10}log TCID$_{50}$-eq/ml) on several occasions in qPCR. Multiple samples taken in pen I, in which all pigs had died or had been euthanised because of acute ASF infection, tested positive from day 28-45 dpi. Samples taken in pens II and IV tested positive occasionally only. In contrast, no virus was recovered from pen III, in which three of the infected pigs survived. In pens I and IV, virus was recovered until 45 dpi, which corresponded with 32 and 18 days respectively after the last pigs had been removed from these pens. The OPF samples of the sentinel IC pigs of pen I (2 IC pigs) and pen II (1 IC pig) tested low positive in qPCR (0.7–1 ^{10}log TCID$_{50}$-eq/ml a few times, mainly samples from the IC pigs from pen I. However, in none of the positive qPCR floor swabs and OPF samples live virus could be detected in the VI (Table 2). The blood samples of the IC pigs all tested negative for ASFV in qPCR (results not shown).

The air samples that were taken in both the direct and indirect transmission experiment all tested negative in both qPCR and VI (results not shown).

The serum samples of the carrier pigs collected at 27 and 55 dpi all tested positive for presence of antibodies against ASFV. At the start of the study at 27 dpi blocking percentages varied from 70 to 90%. At the end of the study at 55 dpi blocking percentages where very similar for all carrier pigs and varied from 99 to 101%.

3.3. Contact infections and quantification of transmission parameters

3.3.1. Contact infections

In the direct contact transmission experiment, none of the C1 and two of the C2 pigs became infected. The estimated day of infection of the infected C2 pigs were day 42 dpi i.e. 1 day post contact (pig in pen c) and 44 dpi i.e. 3 days post contact (pig in pen b) respectively. The pens in which transmission was observed contained two of the three carrier pigs that still had high virus levels in their blood (Fig. 1). No significant difference in number of contact infections between groups C1 and C2 was found ($p = 0.5$, Fisher Exact test). The occurrence of contact infections was evenly distributed over the time that the infectious pigs were present ($p = 0.5$, Chi-Square test), thus no clustering in time in occurrence of contact infections was detected.

3.3.2. Quantification of transmission parameters

In Table 3, the estimates for $\beta_{carrier}$ and $T_{carrier}$ as well as their 95-percent confidence bounds are summarised and for comparison, also transmission parameter estimates for the acute phase of the disease (for pigs infected with the same ASFV Netherlands '86 strain), are shown (de Carvalho Ferreira et al., 2013).

The transmission parameter $\beta_{carrier}$ for period 28-41 dpi (C1 pigs) was estimated at 0/day, for period 42-55 dpi (C2 pigs) at 0.093/day and for the whole carrier period 28-55 dpi $\beta_{carrier}$ = 0.039/day. The average infectious period $T_{carrier}$ in this experiment was estimated to last 25.4 days. Note that this is the infectious period from 28 dpi onwards. Subsequently, the transmission component M $_{carrier\ phase}$ for the whole carrier period was $0.039 \times 25.4 = 0.99$. The probability to become a carrier was 0.3 in our case (data from preceding experiment, 6/20). $\Delta R_{0\ carrier\ phase}$ then equals $0.3 \times 0.99 = 0.3$.

4. Discussion

In this study we showed that carrier pigs can transmit ASFV to naive pigs, resulting in acute onset of disease of the contact pigs. This indicates that healthy carrier pigs can transmit the disease to other animals and subsequently cause infections in new populations / areas, which may be especially important in the epidemiology of ASFV in wild boar. In contrast to transmission caused by carriers, in our study no indirect transmission through an ASFV contaminated environment was observed.

We also quantified the transmission from carrier phase animals to naive animals. The transmission rate $\beta_{carrier}$, was estimated 0.039/day. In combination with the infectious period $T_{carrier}$ of 25 days and a probability to become carrier of 0.3 this corresponds to a $\Delta R_{0\ carrier\ phase}$ of 0.3. Previously, we determined the transmission parameter β for the acute phase of the infection. With the same ASFV Netherlands '86 strain, $\beta_{acute\ phase}$ was 0.45-0.92/day (de Carvalho Ferreira et al., 2013). Similarly, Guinat et al. (2016b) estimated for the recent ASFV Georgia strain a within pen $\beta_{acute\ phase}$ of 0.5-0.9/day. The transmission rate estimated for the carrier phase is thus approximately 10–25 times lower than the transmission rate for the acute phase. This is probably because in the acute phase of ASF more infectious virus is excreted in the environment via blood and se- and excreta such as OPF, urine and faeces.

Table 1
qPCR results of OPF swabs and blood samples of pigs used in the direct contact transmission experiment.

OPF swabs

		Days post inoculation																					
pen	pig	27	28	29	30	31	32	33	34	35	38	41	42	43	44	45	46	47	48	49	52	55
A	I	**1.36**[a,b]				**1.9**			**1.8**		**2.6**	2.0				**2.8**			**1.8**		**2.6**	1.8
	C1		–	–	–	–	–	–	–	–	–	–										
	C2												–	–	–	–	–	–	–	–	–	–
B	I	**2.6**				**2.6**			**2.9**		**3.1**	**2.5**				**3.2**			**2.0**		3.1	2.7
	C1		–	–	–	–	–	–	–	–	–	–										
	C2												–	–	–	–	–	0.7	†			
C	I	**2.7**				2.2			**2.5**		**2.6**	**2.0**				2.4			2.0		2.4	2.0
	C1		–	–	–	–	–	–	–	–	–	–										
	C2												–	–	–	2.0	**3.9**	**3.7**	†			
D	I	**2.5**				**2.0**			**2.0**		2.4	1.9				**3.0**			2.1		**2.4**	1.5
	C1		–	–	–	–	–	–	–	–	–	–										
	C2												–	–	–	–	–	–	–	–	–	–
E	I	2.9				3.0			**2.4**		**3.5**	**2.4**				**3.1**			2.8		**2.2**	1.7
	C1		–	–	–	–	–	–	–	–	–	–										
	C2												–	–	–	–	–	–	–	–	–	–
F	I	**1.9**				**1.9**			**1.6**		1.8	0.8				1.8			**0.9**		1.9	1.1
	C1		–	–	–	–	–	–	–	–	–	–										
	C2												–	–	–	–	–	–	–	–	–	–

Blood samples

		Days post inoculation																				
pen	pig	27	28	29	30	31	32	33	34	35	38	41	42	43	44	45	46	47	48	49	52	55
A	I	**5.8**				**5.3**			**4.9**		**5.3**	**5.4**				**5.2**			**5.0**		4.6	4.3
	C1		–	–	–	–	–	–	–	–	–											
	C2												–	–	–	–	–	–	–	–	–	–
B	I	**5.4**				**5.7**			**5.1**		**5.7**	**5.6**				**5.6**			**5.1**		**4.9**	4.7
	C1		–	–	–	–	–	–	–	–	–											
	C2												–	–	–	–	**1.2**	**5.8**	†			
C	I	**5.7**				**5.6**			**5.1**		**5.7**	**5.8**				**5.6**			**5.2**		**4.9**	**4.7**
	C1		–	–	–	–	–	–	–	–	–											
	C2												–	–	–	**6.9**	**7.2**	**7.2**	†			
D	I	**5.0**				**4.7**			**4.2**		**3.9**	2.7				1.3			0.9		1.0	–
	C1		–	–	–	–	–	–	–	–	–											
	C2												–	–	–	–	–	–	–	–	–	–
E	I	**5.6**				**5.4**			**4.7**		**4.3**	3.1				2.4			**3.2**		**2.2**	1.2
	C1		–	–	–	–	–	–	–	–	–											
	C2												–	–	–	–	–	–	–	–	–	–
F	I	**4.6**				3.4			2.4		1.7	1.4				0.8			0.7		–	–
	C1		–	–	–	–	–	–	–	–	–											
	C2												–	–	–	–	–	inc[c]	–	–	–	–

[a] qPCR titre (^{10}log TCID50 eq/ml); – = negative.
[b] Bold and underlined = positive in VI.
[c] Inconclusive. Sample tested positive in qPCR, but since no other indications of infection were found, this was considered a false positive.

The infectious period for the carrier period however is longer than for the acute phase: 25 days (from 28 dpi onwards) as compared to up to 8 (Guinat et al., 2014) or up to 20 days (de Carvalho Ferreira et al., 2013). Further, each naive pig that is infected by a carrier, can cause new (acute phases of) infections, and especially when wild boar, which can travel long distances, are involved, also new populations or areas can become affected.

For the total impact that carriers might have in the epidemiology of ASFV, also the fraction of animals that will survive an acute infection and subsequently become carrier is important. After infection with strain ASFV Netherlands '86, which is considered a moderately virulent strain, in our experiment 30% of the pigs survived and recovered completely from clinical symptoms and all survivors became clinically healthy carriers, with ASFV in both blood and OPF. In some of these carrier pigs the viral load in the blood decreased in time, although in all carriers virus could be detected in blood and/or OPF samples until the end of our study period at 55 dpi. Whether or not ASFV carrier pigs will remain carriers for the duration of their life, cannot be concluded from our data. Possibly, when time progresses, the pigs can recover from the carrier state and will remain serologically positive only. Petrov et al. (2018) performed a similar study as ours, where pigs were infected with ASFV Netherlands' 86 and surviving carrier pigs were put in contact with direct contact (sentinel) pigs at 90 dpi. In their study, no contact infections were observed, even though blood samples of the carrier pigs tested positive until the end of this study. It is not possible to determine whether this is due because of decreasing infectiousness of the carriers later post infection or because in experiments with a relatively small number of animals, transmission events that occur occasionally only, can be missed easily.

In contrast to the strain that we used, the strain that is currently circulating in Eastern Europe is considered highly virulent, with a lethality close to 100% (Blome et al., 2012; Gabriel et al., 2011;

Table 2
qPCR results of floor samples and OPF swabs of sentinel IC pigs used in the environmental contact transmission experiment.

			Days post inoculation											
pen	sample		28	29	30	31	32	33	34	35	38	40	42	45
I	floor samples	1	1.17[a]			1.3				1.3	0.8		–	0.4
		2	–			2.4				0.8	0.6		–	–
		3	1.4			1.1				–	1.1		–	–
		4	1.5			1.4				1.1	1.1		–	–
	OPFswabs sentinels	ICa		0.7	0.8	–	–	–	–	–	–	–	–	–
		ICb		–	0.9	0.8	1.0	–	–	–	–	–	–	–
II	floor samples	1	–			–				0.4	–		–	–
		2	0.8			–				0.2	–		–	–
		3	–			0.8				–	–		–	–
		4	−0.7			–				–	0.6		–	–
	OPFswabs sentinels	ICa		–	–	–	–	–	–	–	–	–	–	–
		ICb		–	0.8	–	–	–	–	–	–	–	–	–
III	floor samples	1	–			–				–	–		–	–
		2	–			–				–	–		–	–
		3	–			–				–	–		–	–
		4	–			–				–	–		–	–
	OPFswabs sentinels	ICa		–	–	–	–	–	–	–	–	–	–	–
		ICb		–	–	–	–	–	–	–	–	–	–	–
IV	floor samples	1	–			–				–	–		–	1.2
		2	–			0.4				–	–		–	–
		3	–			0.6				–	–		0.4	–
		4	–			–				–	–		0.4	–
	OPFswabs sentinels	ICa		–	–	–	–	–	–	–	–	–	–	–
		ICb		–	–	–	–	–	–	–	–	–	–	–

All qPCR positive samples tested negative in VI.
[a] qPCR titre (^{10}log $TCID_{50}$ eq/ml); − = negative.

Table 3
Transmission parameters for ASFV.

Phase of ASFV infection	Transmission rate		Infectious period	
	β (day-1)	95% CI	*T (days)*	95% CI
Acute phase[a]				
0-28dpi	0.45-0.92[b]	0.22-0.95 / 0.44-1.92	5.9-19.9[b]	3.3-8.5 / -0.3-40.1
Carrier phase				
28-41dpi	0	0-0.10		
42-55dpi	0.093	0.030-0.26		
28-55dpi	0.039	0.0065-0.12	25.4	11-61

[a] de Carvalho Ferreira et al., 2013, Transmission rate of African Swine Fever Virus under experimental conditions, Vet Microbiol 165:296–304.
[b] Depending on used criteria.

Gallardo et al., 2017; Guinat et al., 2016b; Pietschmann et al., 2015). However, under experimental conditions, survivors have been observed (Gallardo et al., 2017; Nurmoja et al., 2017a) and also, an increasing number of antibody-positive wild boar in the field are reported (Wozniakowski et al., 2016; Mur et al., 2016; Nurmoja et al., 2017b; Gallardo et al., 2018). Our study results show that survivor pigs that do have antibodies against ASFV can also be carriers of ASFV. The presence of antibody-positive wild boar in the field suggests that, despite the virulent nature of the virus, a number of wild boar are able to survive an ASF infection and possibly also become carriers. Previously, transmission from persistently infected carrier pigs was already postulated to play a possible role in maintaining long term ASFV infection in regions where the disease has become endemic (Sánchez-Vizcaíno et al., 2012). Our experimental data confirm this hypothesis. That ASFV can be transmitted by animals that do not die in the acute phase under experimental circumstances has been described previously by Gallardo et al. (Gallardo et al., 2015). However, in their study, the survivors did not clinically recover but showed a chronic form of ASFV infection. Also, the infected contact pigs showed either no clinical signs or very mild chronic symptoms. In contrast, our experiments show that clinically healthy carriers can be a source of virus transmission, leading to new acute infections. As clinically healthy animals will not be hampered in their behaviour, they may actively interact with other pigs and thus can contribute to regional persistence of an infection, promoting endemicity of ASFV.

As possible risk factors for transmission in the carrier phase we analysed if a) a peak in ASFV excretion or b) (time after) introduction of a new contact pig were risk factors for ASFV transmission but could not demonstrate this. Two excretion peaks around days 15 and 40 dpi in OPF (coinciding with contact period C2) had been observed in a previous experiment (de Carvalho Ferreira et al., 2012). However, similar peaks in excretion level could not be confirmed in the current experiment. Also, no significant difference was found between the number of contact infections in periods C1 and C2, although this might be caused by the limited data set (lack of power). Because transmission via an infected environment was not observed, and the viral load in the environment / viral excretion of the carriers did not significantly change throughout the experiment, we hypothesised that close inspection of a new pig / rank-order fights, resulting in contact with highly infectious blood of the carriers (as in Gabriel et al., 2011) might have triggered the contact infections that were observed. Although the two contact pigs that became infected both became infected shortly after introduction to their carrier companion, statistically, the occurrence of contact infections was not clustered in time. However, the experiment had very limited power to detect a possible underlying time-dependency.

The transmission parameters that we estimated close a data gap on transmission characteristics of carrier pigs and can be used in mathematical models for ASFV spread or to model the effect of possible control measures in groups of domestic or wild pigs (e.g. Barongo et al., 2016). We note that the above model calculation of $\Delta R_{0\ \text{carrier phase}}$ assumes that carrier animals are permanently in the close vicinity of contact animals, i.e. applies to pigs in groups. Because solitary living male wild boar will not permanently be in the close vicinity of contact animals, the calculated $\Delta R_{0\ \text{carrier phase}}$ might not be directly applied to a

whole wild boar population. Still, our transmission experiment also gives information on the transmission rate between a solitary living carrier and a contact animal during time periods that they are in close contact (quantified by $\beta_{carrier}/2$ in the standard SIR model). To obtain a complete model for wild boar, for solitary living wild boar, this transmission rate estimate needs to be supplemented with estimates of contact durations obtained from field observations.

Beside a role for persistently infected carrier animals, movement of (acutely) infected wild boar, infected wild boar carcasses and environmental contamination have been suggested as transmission mechanisms (Guinat et al., 2016a) as well as human involvement (Chenais et al., 2019). A study of Probst et al. (2017), demonstrated that carcasses can be contaminated with ASFV for a considerable length of time. However, the behaviour of free ranging wild boar towards the carcasses of dead conspecifics was monitored and no evidence of cannibalism was observed (Probst et al., 2017), although interest in the soil underneath and surrounding the carcass was detected. In the experiments described in this paper we did not see any transmission of ASFV via a contaminated environment alone, although we could detect viral DNA in the floor samples and OPF samples of the sentinel pigs. Probably, virus that had been present had already disintegrated, which is in agreement with the low survival rate of ASFV in several matrices (Davies et al., 2017) and experiments performed by Olesen et al. (2018) where transmissibility via a contaminated environment was seen during a short time window only. Interestingly, in the pen where all pigs died or were euthanised because of acute ASF infection, most viral RNA was discovered. However, since the IC pigs were placed in the contaminated pen 13 days after the last pig had been removed, the virus had probably not survived. It might be that CI pigs would have become infected when placed in the contaminated pens at an earlier time-point. However, transmission via an environment contaminated with se- / excreta is probably not a major source of new ASFV infections, which is also confirmed by results of Gabriel et al. (2011). They showed that in experiments where virus transmission to domestic pigs and wild boar was effective / induced acute disease in the recipients, blood was likely to be the main source of infection for the contact pigs as this was highly infectious whereas shedding of ASFV through nasal discharge or faeces seemed to be limited.

We conclude that clinically healthy survivors of an ASFV infection that become carriers can transmit ASFV to contact pigs, although the total impact on transmission will be dependent on the survival rate and duration of the carrier status. The transmission parameters that we estimated apply to ASFV transmission in domestic pigs and, in part, to ASFV transmission in wild boar.

Declaration of Competing Interest

None.

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