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ORIGINAL ARTICLE

Course and transmission characteristics of oral low-dose infection of domestic pigs and European wild boar with a Caucasian African swine fever virus isolate

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Abstract In 2007, African swine fever virus (ASFV) was introduced into the Transcaucasian countries and Russia. Since then, it has spread alarmingly and reached the European Union. ASFV strains are highly virulent and lead to almost 100 % mortality under experimental conditions. However, the possibility of dose-dependent disease courses has been discussed. For this reason, a study was undertaken to assess the risk of chronic disease and the establishment of carriers upon low-dose oronasal infection of domestic pigs and European wild boar. It was demonstrated that very low doses of ASFV are sufficient to infect especially weak or runted animals by the oronasal route. Some of these animals did not show clinical signs indicative of ASF, and they developed almost no fever. However, no changes were observed in individual animal regarding the onset, course and outcome of infection as assessed by diagnostic tests. After amplification of ASFV by these animals, pen- and stablemates became infected and developed acute lethal disease with similar characteristics in all animals. Thus, we found no indication of prolonged or chronic individual courses upon low-dose infection in either species. The scattered onset of clinical signs and pathogen detection within and among groups confirms moderate contagiosity that is strongly linked with blood contact. In conclusion, the prolonged course at the "herd level" together with the exceptionally low dose that proved to be sufficient to infect a runted wild boar could be important for disease dynamics in wild-boar populations and in backyard settings.

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Introduction

African swine fever (ASF) is one of the most complex and important viral diseases of pigs. Clinical signs depend on several host and virus factors and may vary from almost inapparent disease to a hemorrhagic-fever-like illness [16, 26]. At this time, no vaccine is available and control is based on rapid laboratory diagnosis and strict, mandatory sanitary measures [29]. The disease is notifiable to the World Organization for Animal Health (OIE).

The causative agent of ASF is African swine fever virus (ASFV), a large, complex, double-stranded DNA virus that is the sole member of the genus *Asfivirus* and the family *Asfarviridae* [30]. The vertebrate hosts of this virus are members of the family *Suidae*, e.g. warthogs (*Phacochoerus* spp.), bush pigs (*Potamochoerus* spp.), European wild boar (*Sus scrofa scrofa*), and domestic pigs (*Sus scrofa domestica*) [6, 21]. Warthogs especially can be regarded as reservoir hosts in Africa. These animals do not

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show marked clinical signs and are part of a sylvatic cycle with soft ticks of the genus *Ornithodoros* [6, 23].

African swine fever is endemically present in several countries of Sub-Saharan Africa but has also caused long-lasting outbreaks on the Iberian Peninsula and short epidemics in other European and American countries in the second half of the 20th century [1]. African swine fever is also endemic on Sardinia, Italy [9, 18]. In 2007, ASFV was introduced into Georgia and subsequently into other Transcaucasian Countries and the Russian Federation [14]. Since then, it has spread at an alarming rate towards ASF-free areas, and only recently have outbreaks been confirmed in Belarus, Ukraine, and in the European Union member states Poland, Lithuania, Latvia, and Estonia (WAHID, status report as of January 20, 2015). From the beginning of the outbreak, ASFV has infected both domestic pig and wild boar populations [12], and it now affects all sectors of the Russian pig industry and production [10, 14]. These ASFV strains belong to genotype II and are closely related to strains that have been found in Madagascar, Zambia, and Mozambique [27]. Based on partial sequences, strains from 2007 to 2011 are almost identical [19]. Experimental infections have shown that these strains are highly virulent for domestic pigs and European wild boar [8], irrespective of their age [2]. In all experimental infections, 100 % mortality was observed within less than 12 days.

However, there have been reports (Kolbasov, D., Alonso, C., personal communication) that low-dose ASFV infections may lead to prolonged incubation times and altered clinical courses. Dose dependence was also seen with virulent strains in recent pathogenesis studies, with different doses and routes of inoculation [13].

Especially in wild boar, oral low-dose infections are likely to occur by intake of small pieces of pig carcasses or ASFV-containing refuse. The same may hold true for domestic pigs in backyard settings. If this could lead to chronic forms of disease or the development of a carrier status, the impact on disease dynamics would be high: prolonged courses may facilitate the establishment of an endemic transmission cycle and hamper timely diagnosis. For this reason, a study was undertaken to assess the risk of chronic disease and the establishment of carriers upon oronasal infection with low doses of ASFV strain "Armenia08" in domestic pigs and European wild boar (100 and 10 haemadsorbing units, HAU, respectively). Parameters recorded upon inoculation included clinical signs, levels of viraemia in blood and oropharyngeal swabs, serological responses, pathomorphological changes, and virus distribution in selected organs. Moreover, transmission characteristics were assessed.

Materials and methods

Experimental settings

The study included a total of fifteen European wild boar with an age of four to five months, and fifteen domestic pigs with an age of eight to twelve weeks. Upon arrival in the high-containment facilities of the Friedrich Loeffler Institute (FLI), all animals were individually ear-tagged and divided into three experimental groups. Each group was placed in a separate stable unit. All applicable animal-welfare regulations, including EU Directive 2010/63/EC and institutional guidelines, were taken into consideration. The animal experiment was approved by the responsible authority under reference number 7221.3-1.1-015/12.

Group I consisted of six wild boar and six domestic pigs. It had originally been planned to allow the animals to comingle. However, due to unforeseen hierarchic encounters among these animals, domestic pigs and wild boar had to be placed in separate pens within the stable (shared room, ventilation, tools, and trough). In group II, six wild boar and six domestic pigs were allowed to commingle in one pen. The same applies for the group of negative controls (group III), which consisted of three animals of each subspecies.

During the acclimatization phase, the wild boar received metaphylactic treatment with enrofloxacin (Baytril 10 %, Bayer Animal Health) and ivermectin (Ivomec S, Merial GmbH) to eliminate parasites and to control inapparent bacterial infections. Thereafter, animals of groups I and II were inoculated oronasally with 2 ml of a diluted spleen suspension containing 100 and 10 hemadsorption units (HAU), respectively, of ASFV strain "Armenia08". Group III was mock-inoculated with the same amount of cell culture medium.

Clinical signs and rectal temperatures of domestic pigs were evaluated daily. In contrast, rectal temperatures of the wild boar were measured only on sampling days or with special indication. Fever was defined as a body temperature above 40.0 °C for at least two consecutive days. For a harmonized evaluation, and to ensure comparability with other animal trials within the ASFORCE project (http://asforce.org/), clinical signs were documented based on a scoring system as follows: temperature (<39 °C = 0 points; 40.0-40.5 °C = 3 points; 40.6-41 °C = 4 points; >41.1 °C = 5 points), anorexia (reduced eating = 1 point; only picking at food = 4 points; not eating = 6 points), recumbency (lethargic = 1 point; animal gets up only when touched = 2 points; gets up only slowly when touched = 4 points; remains recumbent when touched = 6 points), skin (score from 0 to 3 points depending on combined assessment of skin erythema, edema, and

hemorrhages), swelling of joints (joint swelling = 1 point; severe swelling with lameness = 4 points), breathing (labored and/or coughing = 1 point; severe = 3 points), ocular discharge (mild = 1 point; moderate = 2 points), digestive findings (mild diarrhea for less than 24 h = 1 point; moderate findings, e.g., diarrhea and vomiting for more than 24 h = 3 points; severe, bloody diarrhea and/or bloody urine = 4 points). Neurological disorders (ataxia, paralysis, convulsion) were assigned scores from 0 to 6 points depending on the occurrence and severity of signs. The sum of the points was recorded as the clinical score (CS).

Levels of viraemia, virus distribution, virus shedding, and immune responses were assessed. For this purpose, sera and EDTA blood samples were collected along with oropharyngeal swabs. Those samples were collected prior to inoculation and at 2, 4, 6, 8, 10, 14, 17, 21, 28, 34, and 36 days post-inoculation (dpi).

Moribund animals and animals experiencing unjustifiable suffering as assessed by the responsible veterinarian were euthanized through intracardial injection of embutramide (T61, Merck) after deep anesthesia with tiletamine/zolazepam (Zoletil®, Virbac). Necropsy was performed on all animals, and at the same time, tissue samples (lymph nodes, spleen, tonsil, salivary gland, lung, and liver) were collected.

Viruses

The virus used in this trial was isolated from a diagnostic specimen sent by the Central Veterinary Laboratory, Yerevan, Armenia, in February 2008. This isolate belongs to genotype II and is closely related (identical based on the genome fragments used routinely for phylogenetic analysis) to strains circulating in Russia and other affected countries in Eastern Europe. For experimental infection, a spleen suspension was produced and diluted with cell culture medium to 10 and 100 HAU/ml. The dilutions were based on an end-point virus titration of the original material on macrophages derived from peripheral blood monocytic cells (PBMCs). Upon application, back titration was carried out to confirm the administered dose.

Cells

Blood for the preparation of PBMC-derived macrophages was collected from domestic donor pigs. In brief, PBMCs were obtained from anticoagulated blood using Ficoll-Paque density gradient medium (GE Healthcare Life Sciences), and the remaining erythrocytes were lysed by treatment with buffered ammonium chloride solution (155 mM NH_4Cl, 10 mM $KHCO_3$, 1 mM EDTA/pH 7.4). Cells and viruses were grown in cell culture medium (RPMI-1640 with 10 % foetal calf serum [FCS] or, alternatively, RPMI-1640 with HEPES and 10 % FCS) at 37 °C in a humidified atmosphere containing 5 % CO_2. To facilitate maturation of macrophages, GM-CSF (granulocyte macrophage colony-stimulating factor) was added to the cell culture medium at 2 ng/ml.

Laboratory investigations

Processing of samples

Oropharyngeal swabs were soaked in 1 ml of medium, vortexed for approximately 15 seconds, incubated for one hour at room temperature, and decanted in microcentrifuge tubes.

Serum samples, which were obtained from native blood by centrifugation at 3300 × g at 20 °C for 20 min, were aliquoted and stored at -70 °C. Tissue samples of tonsil, spleen, salivary gland, liver, lung, and lymph nodes were collected at necropsy and stored at -70 °C until further use. For real-time polymerase chain reaction (qPCR) and virus isolation (hemadsorption tests), tissue samples were homogenized in 1 ml of DMEM using a TissueLyser II (QIAGEN® GmbH).

Virus detection

For quantitative real-time PCR (qPCR), viral DNA was extracted from anticoagulated whole blood samples and oral swabs using a QIAamp® Viral RNA Mini Kit (QIAGEN) with 75 µl of blood and 140 µl of swab material, respectively. For purification of DNA from tissues, a QIAamp® DNA Mini Kit (QIAGEN) was used according to the manufacturer's instructions. Both extraction methods were modified through the addition of an internal control DNA (5 µl per reaction with 2×10^5 copies per µl). Subsequently, qPCR was performed according to the protocol published by King et al. [15]. A dilution series of a synthetic standard with known copy numbers was used to quantify genome copies in the respective samples.

To detect ASFV in blood and tissue samples, a haemadsorption test (HAT) was carried out using PBMC-derived macrophages according to slightly modified standard procedures [4]. In brief, 200 µl of a PBMC preparation (5×10^6 cells/µl) was seeded into 48-well microplates. After 16-24 hours, non-adherent cells were removed and cell culture medium containing GM-CSF was replenished (200 µl). The culture was then incubated for 24 to 48 h to allow initial maturation of macrophages. Subsequently, 100 µl of serum or organ suspension was added. After another 24 h, 40 µl of a 1 % homologous erythrocyte suspension was added. All samples were tested in duplicate. For

readout, cultures were analysed for haemadsorption phenomena over a period of two to four days.

Virus back titration was performed by endpoint titration of the diluted spleen suspensions. For read-out, haemadsorption (haemadsorbing units, HAU) was utilized. In this case, the PBMC preparation was seeded into 96-well microplates, the test volume was 100 µl per dilution step and 20 µl of a 1 % homologous erythrocyte suspension was added. All samples were tested in quadruplicate.

Antibody detection

Sera were tested for the presence of ASPV p30-specific antibodies using a SVANOVIR ASFV-Ab ELISA in the confirmatory plate format (Boehringer Ingelheim Svanova); and for ASPV p73-specific antibodies, using the INGEZIM PPA COMPAC ELISA (Ingenasa) according to the manufacturer's instructions.

Sera taken on the day of euthanasia were tested in indirect immunoperoxidase tests according to standard protocols provided by the European Union Reference Laboratory for ASF.

Estimation of transmission parameters

A limited number of animals became infected by the initial oral low-dose inoculation, but contact transmission was observed in all experimental groups. Consequently, transmission parameters such as the basic reproduction number (R_0) (defined as the average number of newly infected cases caused by one infectious individual during its infectious period in a susceptible population [31]) were estimated for each contact scenario. Based on clinical scores and laboratory results, quantification of within-pen (R_{0w}) and between-pen (R_{0b}) basic reproduction numbers was performed using the stochastic SEIR (susceptible-infected-infectious-dead) model fitted to the experimental data using the maximum-likelihood method [7, 17].

Data and statistical analysis

All data were recorded and evaluated using Microsoft Excel 2010 (Microsoft Deutschland GmbH), SigmaPlot for Windows version 11.0 (Systat Software, Inc.), and R statistical software (http://www.r-project.org/).

Results

Back titration of challenge virus dilutions

In order to determine the true virus titers administered, back titrations of challenge virus dilutions were conducted. A suspension with an estimated titer of 100 HAU/ml showed a mean titer of 25 HAU/ml in back titration. The second suspension with an assumed titer of 10 HAU/ml had a mean virus titer of 3 HAU/ml in back titration. Both values are within the expected biological variation for virus dilutions and titrations.

Clinical findings and virus detection

Clinical findings and virus detection group I

Wild boar: The smallest wild boar in this group, WB7, tested positive in qPCR using EDTA blood (Fig. 1) and in HAT using serum, starting from 4 dpi. Oral swabs from this animal were found positive in qPCR starting from 6 dpi (Fig. 2). These laboratory findings were accompanied by reduced feed intake from 5 dpi and watery diarrhea from 8 dpi. Slightly elevated body temperatures were observed at 2 dpi, 6 dpi, and 8 dpi (temperatures between 40.0 and 40.5 °C, see Supplementary Table 1). In general, WB7 appeared runted and was found dead at 11 dpi. By that time, it showed (postmortal) bite lesions around the left eye. The other wild boar appeared slightly depressed at 11 dpi. One day later, three out of five wild boar (WB3, WB10 and WB11) still showed slight depression, which in one case (WB3) was accompanied by frequent laboured breathing. To link these clinical observations with laboratory results, an additional blood sample was collected on day 12 from two of the wild boar that showed depression on that day. These samples were positive in qPCR. From 13 dpi, all wild boar showed worsening clinical signs (Fig. 3). These included depression, anorexia, conjunctivitis, accelerated and labored breathing, vomiting, and slight ataxia. On 14 dpi, all of the remaining wild boar showed febrile temperatures (40.3 °C to 41.7 °C, see Supplementary Table 1). One animal had to be euthanized due to severe dyspnoea upon blood sampling on 14 dpi (WB10). All samples were positive on that day by qPCR using blood and swabs and by HAT using serum, except for a negative swab from WB10 (Fig. 2). Animals WB2 and WB3 were found dead at 17 dpi, whereas WB1 and WB11 were in a moribund state and were therefore euthanized. Spleen and blood samples taken on the day of death or euthanasia were strongly positive in qPCR (ct <22) and HAT. Maximum clinical scores ranged from 11 to 13 points (Fig. 3), and overall mortality reached 100 % within 17 days (Fig. 4).

Domestic pigs: While the wild boar showed clinical ASF, all domestic pigs (kept in the same stable but in another pen) remained clinically healthy up to 23 dpi (Fig. 3 and Supplementary Table 1). On that day, one of the domestic pigs (DP26) developed fever for the first time (see Supplementary Table 1), but this was not

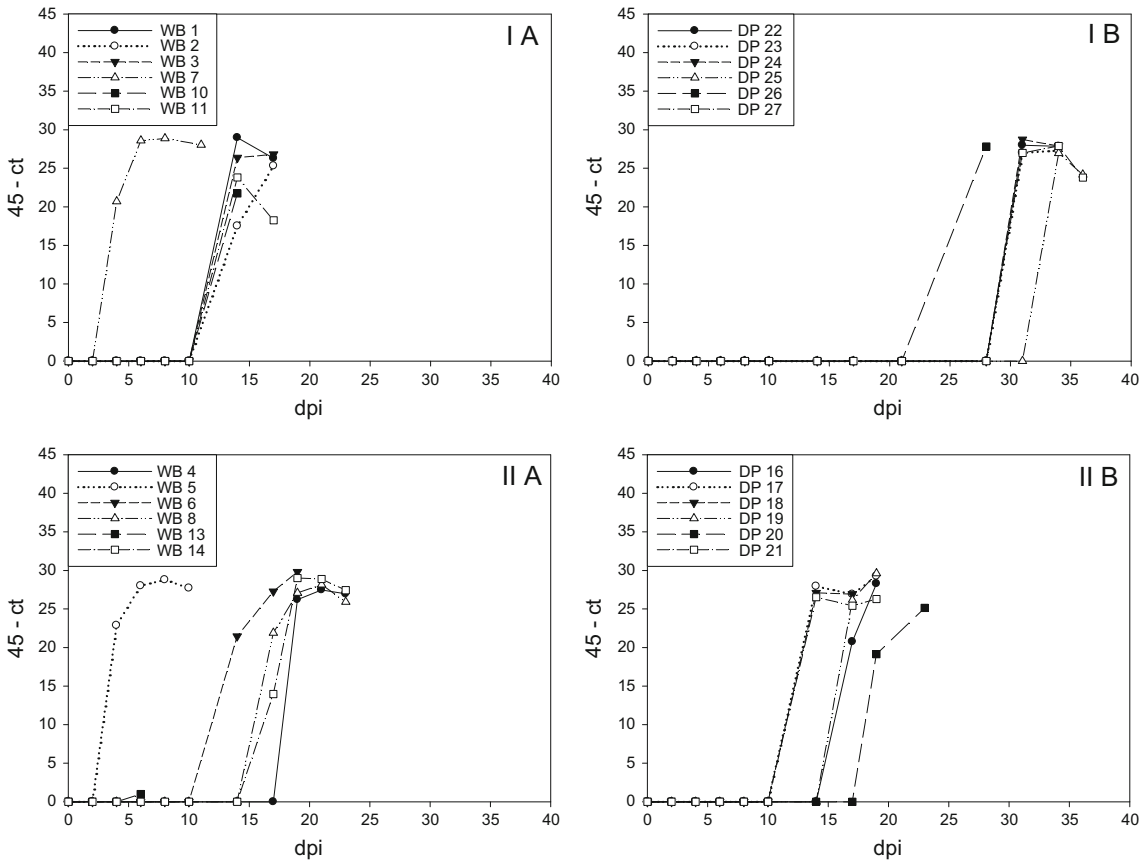

Fig. 1 PCR results for EDTA-blood samples depicted as 45-ct. IA and B, results for animals of group I, which were inoculated with 100 HAU; IIA and B, results for animals of group II, which received 10 HAU. A, wild boar; B domestic pigs. WB, wild boar; DP, domestic pig; dpi, days postinfection

accompanied by any other clinical signs. However, fever continued up to day 28 postinfection, when this animal had to be euthanized, showing severe depression, labored breathing, anorexia, conjunctivitis, and neurological signs (ataxia, convulsions, nystagmus). On that day, all other domestic pigs were healthy (Fig. 3) and had normal physiological body temperatures (Supplementary Table 1). These clinical findings were in line with virus detection. While animal DP26 was positive in all virus detection tests, the other animals were still negative in qPCR using EDTA blood (Fig. 1) and in HAT. However, weakly positive reactions were observed in swabs (ct 34 to 35) of all but one of the remaining animals (Fig. 2). At 30 dpi, two additional pigs showed fever and slight depression (DP22 and DP24). These animals showed high fever (Supplementary Table 1) and worsening clinical signs (Fig. 3) over the next few days and were euthanized 34 dpi. Two other animals (DP23 and DP27) first showed febrile temperatures and reduced liveliness at 31 dpi. On that day, four of the five remaining pigs were positive by qPCR using blood and swab samples (see figures 1 and 2). These findings were accompanied by positive HAT. On the following sampling day (34 dpi), all pigs were found positive by qPCR (see Figures 1 and 2) and HAT and had to be euthanized at 34 dpi (DP23) and 36 dpi (DP27), respectively. The remaining animal (DP25) first showed febrile temperatures at 33 dpi and was euthanized at 36 dpi. Maximum clinical scores ranged from 10 (DP23) to 20 (DP26) (Fig. 3). Mortality reached 100 % within 36 days (Fig. 4).

Clinical findings and virus detection group II

Wild boar: One of the wild boar (WB13) showed a runted appearance from the beginning of the trial and received additional antibiotic and antiparasitic treatment. This animal nevertheless developed diarrhea, and its condition declined between 0 dpi and 6 dpi, when it was found dead. No fever was observed in this animal at any time point (see Supplementary Table 1). In blood and spleen samples taken during necropsy, WB13 yielded weakly positive results in several repetitions. Another small animal (WB5) showed declining health from 9 dpi (Fig. 3), at which time reduced feed intake and labored breathing were observed, but again, fever did not occur (see Supplementary Table 1).

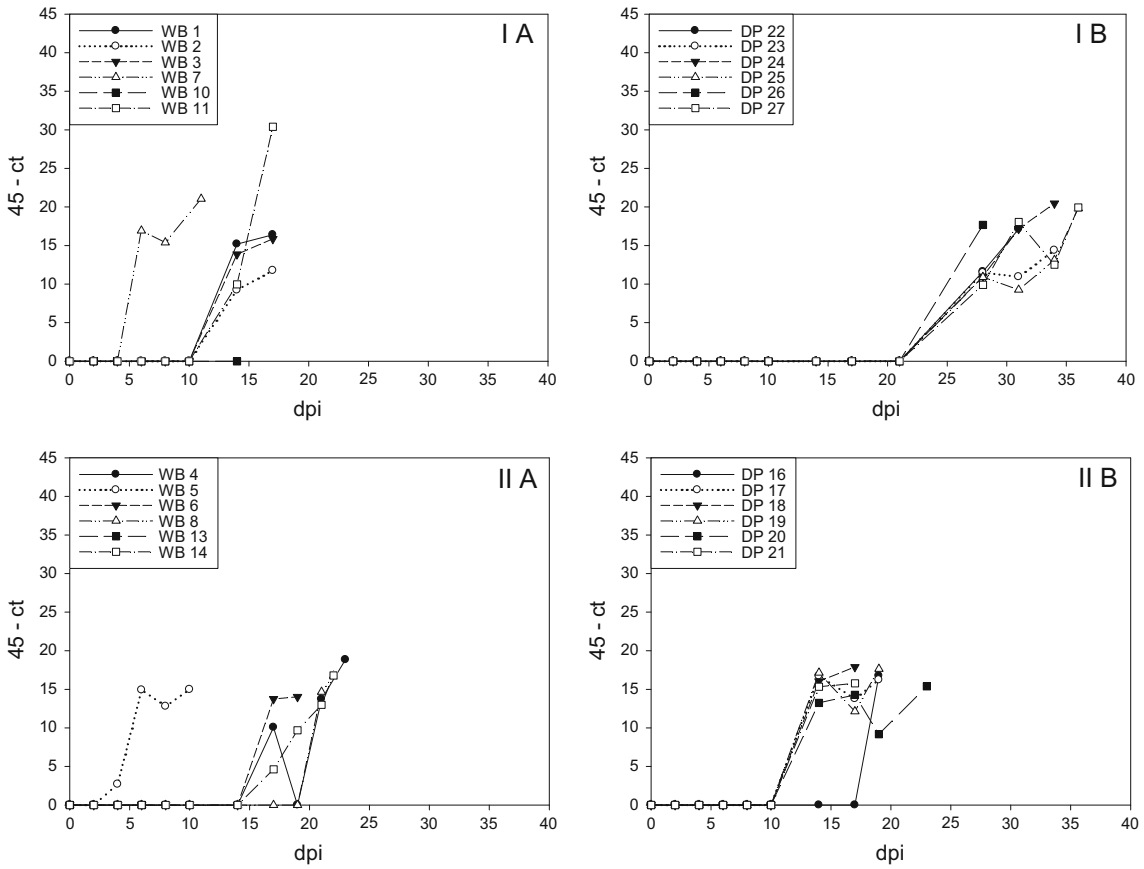

Fig. 2 PCR results for oro-pharyngeal swabs depicted as 45-ct. IA and B, results for animals of group I, which were inoculated with 100 HAU; IIA and B, results for animals of group II, which received 10 HAU. A, wild boar; B, domestic pigs. WB, wild boar; DP, domestic pig; dpi, days postinfection

At 10 dpi, this animal developed severe dyspnoea, which became more severe upon blood sampling. By that time, dyspnoea was accompanied by bloody foam at the nostrils (lung edema). The animal was euthanized in a moribund state. This animal was positive in qPCR using EDTA blood (Fig. 1) and oropharyngeal swabs (Fig. 2), as well as in HAT, beginning at 4 dpi. At 10 dpi, all remaining animals of this group were negative in qPCR (see Figures 1 and 2) and HAT. The remaining wild boar developed fever between 14 dpi and 19 dpi (see Supplementary Table 1). The first qPCR- and HAT-positive results were obtained from animal WB6 at 14 dpi. On the subsequent sampling day (17 dpi), 3 out of 4 remaining wild boar were found positive in qPCR using EDTA blood (Fig. 1), of which two were also positive in HAT. Swab samples were found positive for 2 of the 3 animals that showed positive reactions in blood (Fig. 2). From 19 dpi onward, all wild boar yielded positive results in all pathogen detection tests. Fever was again accompanied by depression, reduced feed intake, labored breathing, and ataxia. The animals had to be euthanized approximately 5 to 7 days after the onset of first clinical signs, i.e., between 18 dpi and 23 dpi. Spleen and blood samples taken on the day of death or euthanasia were found positive in qPCR and HAT. Maximum clinical scores ranged from 14 to 18 (Fig. 3), and 100 % mortality was observed within 23 days (Fig. 4).

Domestic pigs: Up to day 10, all domestic pigs remained negative in all pathogen detection tests, and elevated in body temperature (see Supplementary Table 1) was only observed in combination with inflammatory joint lesions in three animals (DP19, DP20 and DP21). ASF-related fever was observed starting between 12 dpi (DP21) and 19 dpi (DP20). At 13 dpi, the animals began to develop progressive depression, anorexia, labored breathing, and skin reddening. At 14 dpi, 3 out of 6 animals showed strong positive reactions in qPCR assays of EDTA blood samples (Fig. 1). Two of these animals were also found positive by HAT. At that time point, 5 out of 6 animals showed positive reactions in oropharyngeal swabs (Fig. 2). At 17 dpi, 5 out of 6 animals yielded positive results in qPCR from EDTA blood (Fig. 1) and in HAT. Swab samples were positive for 5 out of 6 animals, but these findings did not completely match the above-mentioned results: While the negative animal had a weak positive swab result (65

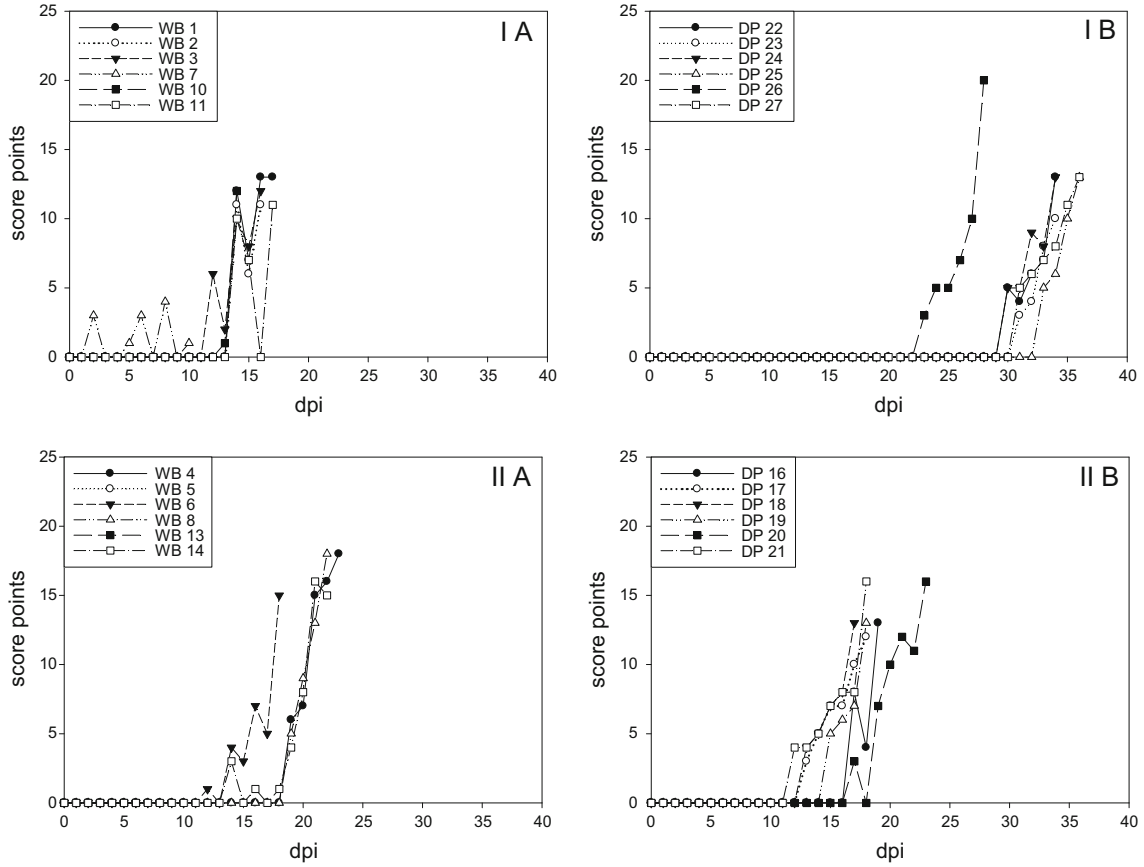

Fig. 3 Daily summation of clinical scores (excluding neurological findings) for each individual animal. The clinical signs were documented based on a harmonized scoring system. In brief, the parameters temperature, anorexia, recumbency, skin, swelling of joints, breathing, ocular discharge, digestive findings, and neurological disorders were assigned a score from 0 to 6 depending on the occurrence and severity of clinical signs. The sum of the scores was recorded as clinical score (CS). WB, wild boar; DP, domestic pig; dpi, days postinfection; IA, group I, wild boar; IB, group I, domestic pig; IIA, group II, wild boar; IIB, group II, domestic pig

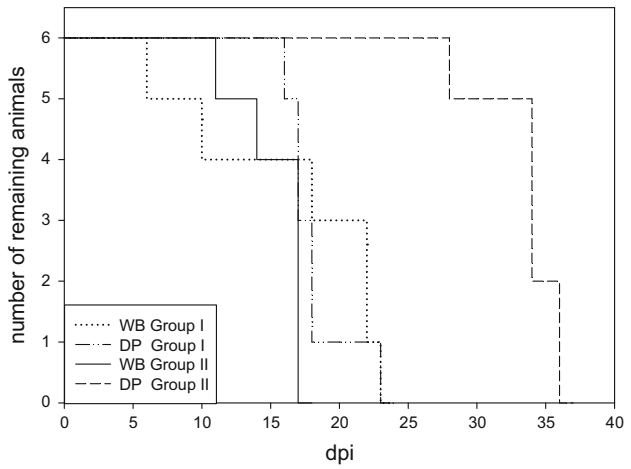

Fig. 4 Survival analysis for all inoculated groups. WB, wild boar; DP, domestic pig; dpi, days postinfection

genome copies per µl), one positive animal remained negative. At the subsequent time points, all of the remaining domestic pigs were positive in all pathogen detection tests. Blood and spleen samples taken on the day of death/euthanasia were found positive in qPCR and HAT. Clinical courses were comparable among all animals of this group, with maximum clinical scores ranging from 12 to 16 (Fig. 3). All animals had to be euthanized between 17 dpi and 23 dpi (100 % mortality, see Fig. 4).

Pathomorphological changes

Pathomorphological changes were comparable among all infected animals. Lesions included, to different degrees of severity, enlarged, hemorrhagic and edematous lymph nodes (gastrohepatic lymph nodes were often ebony-coloured and severely enlarged), splenomegaly, pulmonary edema, gall bladder edema, petechiae in the kidneys, and hemorrhagic gastritis. Most animals showed signs of coagulation disorders, e.g., large hematomas in connection with small bite lesions and missing clot formation in the heart and in large vessels. The latter was more severe in wild boar. In individual animals, secondary infections of the respiratory and gastro-intestinal tract were found.

Clinical and pathomorphological findings group III

The control group remained healthy throughout the experiment and did not show any pathomorphological changes related to ASF. Moreover, all animals remained negative in all pathogen and antibody detection tests throughout the trial.

Antibody detection

With only a few exceptions, all antibody detection techniques yielded negative results for the samples taken prior to inoculation and at the end of the trial. Two sera (from the runted animals WB5 and WB7) gave doubtful results in the INGEZIM PPA COMPAC ELISA (Ingenasa), which detects p73-specific antibodies. These doubtful results could not be confirmed using the indirect immuno-peroxidase test. The latter gave three inconclusive results for domestic pigs (repeatedly weak reactivities together with high background). However, no reactivity was seen for these sera (DP20, DP21, DP27) in any of the ELISAs.

Transmission parameters

Estimation of transmission parameters was based on infection time assumptions (see Fig. 5). Animals were considered infectious when ASFV genome copies were recovered from blood samples. The latent period, i.e., the time period between the date of infection and the onset of infectiousness, was assumed to be 4 days. The infectious period, i.e., the time period between the onset of infectiousness and the date of death, ranged from 2 to 9 days. Based on these assumptions deduced from clinical and laboratory diagnostic findings, the R_{0w} (within-pen) was estimated for the wild boar in group I to be 6.1 (95 % confidence interval [CI]: 0.6-14.5), while it was 5.0 (95 % CI: 1.4-10.7) within the mixed group II. The basic reproduction number R_{0b} (between-pen) for the complete group I, which indicates the rate of transmission between wild boar and domestic pigs, was estimated to be 0.5 (95 % CI: 0.1-1.3).

Discussion

African swine fever virus is currently circulating in Russia and shows a clear tendency to move toward ASFV-free areas. Recent outbreaks in eastern member states of the European Union (WAHID, January 2015) show that the virus is spreading to the northwest, but the presence of positive wild boar in Iran [25] shows that there is also a tendency for the virus to spread in a southeasterly direction. Despite the high virulence of the ASFV strains involved [3, 8], the virus is continuously present in backyard settings and in the wild boar population [14]. Especially the latter raises concerns for regions with high wild boar density (e.g., Central Europe), as endemic infections may hamper disease control and lead to substantial losses to the domestic pig industry. It is currently believed that ASF outbreaks in wild boar fade out unless infection is reinforced or sustained by contact with infected domestic pigs or other sources of infection [5, 18, 22, 24, 28]. However, disease dynamics are still far from being understood, and a role of chronic infections, carrier pigs, or long-term presence of infectious materials cannot be excluded.

Here, we investigated the impact of oral, low-dose ASFV infection on the course and outcome of the disease in domestic pigs and European wild boar. Inoculation doses were chosen to correspond to those obtained through contact with fomites, swill, excretions of infected animals, or contact with carcasses.

Upon infection with less than 10 HAU (3 HAU based on repeated back titrations), only the weakest animals of each group became directly infected. One of the animals apparently died from a concomitant gastrointestinal infection and general exhaustion prior to the onset of ASF-related signs. However, it was found to be virus-positive in spleen and blood samples taken at the day of death (6 dpi). The other directly infected animal had a frail and runted appearance but ran and fed with the group. While all previous experimental infections had always led to marked fever in all infected wild boar [3], fever was not a noticeable sign in the runted animals. Comparable results are known for CSF infections of wild boar (personal observations) and are worrisome with regard to the timely recognition of the disease. However, disregarding the clinical presentation, the course of infection was still comparable with previous experiments: Virus was first detected 4 dpi, and the animal died by 10 dpi. Four days after euthanasia of the second runted animal, four additional animals (two wild boar and two domestic pigs) were found positive in pathogen detection and developed an acute-lethal course of ASF over the next few days. It was probably not by chance that these findings were exactly four days after the possible contact to bloody discharge from the nose and mouth of the runted wild boar. If infection is assumed four days prior to the first virus detection, the course and duration of infection was again comparable to the first case and to previous studies. The remaining animals followed similar courses. In all secondary cases, clinical signs were accompanied by fever in both domestic pigs and wild boar. Virus was always found in blood and, to a lesser extent, in oro-pharyngeal swabs. Some results obtained from swab samples did not match the results obtained from blood (positive results in otherwise negative animals). This could be due either to the

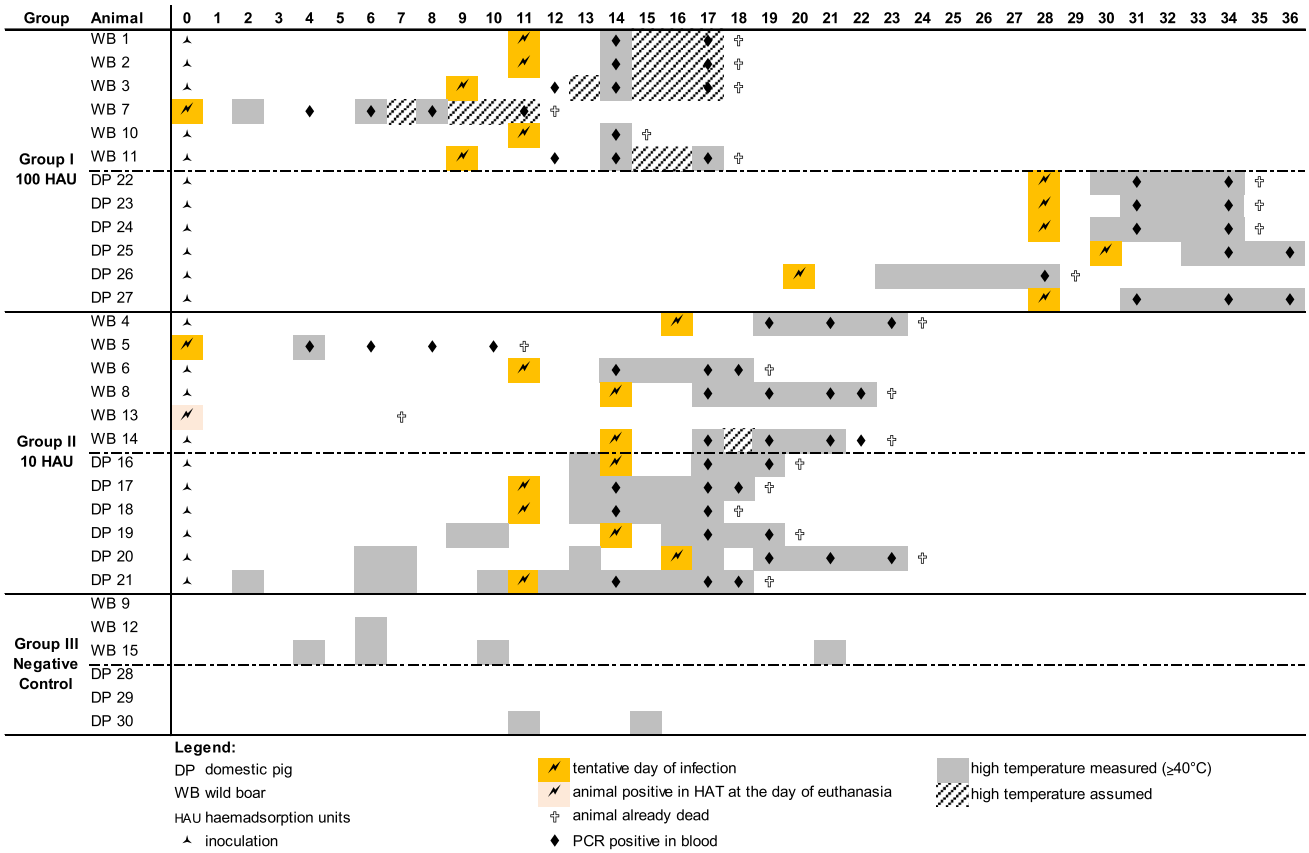

Fig. 5 Tentative course of infection based on clinical and laboratory diagnostic findings. Upon inoculation on day 0, only three animals got directly infected. All remaining animals were infected through contact with a probability bordering on certainty. The tentative time point of infection was estimated based on the assumption that the first positive results in PCR are obtained between days 3 and 4 postinfection and that the onset of viraemia coincides with fever. Body temperatures of wild boar were only measured on sampling days. In cases where high temperatures were recorded at two consecutive samplings, high temperatures were also assumed for the remaining time

more or less ubiquitous presence of virus in the stable or to shedding prior to viraemia. The latter could be explained by the fact that tonsils and retropharyngeal lymph nodes are the primary replication sites after oral infection [21]. While all individual courses confirmed high virulence and acute-lethal illness lasting about 10 to 11 days, the group course lasted 23 days.

Interestingly, a similar picture was observed upon infection with 100 HAU. One frail and runted animal was directly infected and was positive by pathogen detection methods at 4 dpi. This animal showed mild and nonspecific clinical signs including remittent increases in body temperature. However, temperature increases were only marginal compared to previous experiments (40.0 to 40.5 °C). Only after amplification of ASFV in this animal did immediate group mates became infected. The infection of at least three additional wild boar was probably linked to contact with highly infectious blood from the initially infected runted wild boar. These animals were found positive four days after the group had nibbled at the carcass of the directly infected animal that had succumbed to infection during the night. After euthanasia of the wild boar, several days elapsed without noticeable signs in the subgroup of domestic pigs that were not allowed to commingle but were kept in a neighboring pen in the same stable. However, infection also established in this subgroup, and all animals developed acute-lethal disease. It was again seen that infection was confirmed in additional animals about four days after possible blood contact. How the subgroup of domestic pigs got infected can only be hypothesised. One possible explanation is that virus remained on water hoses or other fomites within the stable. At a certain time, probably 20 dpi, one animal got in contact with enough virus to get infected. When this animal (DP26) had again amplified the virus to very high titers, the rest of the group became infected. All courses followed the pattern described above. The complete lack of responses in genome and antibody detection assays may indicate acute infection, but personal observations have shown that incubation periods may be as long as 21 days, even with a Caucasian ASFV isolate (D. Kolbasov, personal communication).

To summarize the tentative transmission within and between groups, a hypothetical course of infection is shown in Figure 5. Here, the following assumptions underlie the graphs: first positive results are likely at 4 dpi (between 3 and 4 dpi). Usually, the onset of viraemia coincides with first fever reactions or other noticeable clinical signs [11, 20]. Following this hypothesis, sufficiency of very low doses of virus is combined with moderate contagiosity and leads to a herd course that is a multiple of the individual duration. In this study, the last animals were euthanized 36 days after first introduction of the virus. Under field conditions, diagnosis and timely intervention might be hampered and would be in contrast to the experiences with high-dose inoculation experiments. The estimated basic reproduction numbers reflect moderate contagiosity with quite high numbers within a pen but low numbers between pens. Compared to studies by Guinat et al. (manuscript submitted) that showed R_{0w} numbers of 2.8 (95 % CI: 1.3-4.8), the estimated parameters for within-pen transmission are high. This could be due to the use of different humane endpoint criteria and a greater frequency of contacts within the groups. However, it could also reflect high efficiency of ASF transmission by wild boar.

To further elucidate pathogenetic aspects, cellular responses of primarily infected, runted animals will be studied, since the reason for the obvious higher susceptibility of the runted wild boar remains unknown.

Conclusions

It was demonstrated that very low doses of ASFV are sufficient to infect especially weak or runted animals by the oronasal route. Some of these animals did not present any clinical signs indicative of ASF and had almost no fever. However, no changes were observed regarding the onset, course and outcome of infection for individual animals. After amplification of ASFV by these animals, pen- and stablemates got infected and developed acute lethal disease that was similar in all animals. Thus, no indications exist for prolonged or chronic individual courses upon low-dose infection in either species.

The scattered onset of clinical signs and pathogen detection within and among groups confirms the moderate contagiosity of this virus, which is strongly linked with blood contact.

In conclusion, the prolonged course at the "herd level", together with the exceptionally low dose that proved to be sufficient to infect a runted wild boar, could be important for disease dynamics in wild boar populations and in backyard settings.

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