Transboundary and Emerging Diseases

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ORIGINAL ARTICLE

Vaccine Potential of Two Previously Uncharacterized African Swine Fever Virus Isolates from Southern Africa and Heterologous Cross Protection of an Avirulent European Isolate

R. Souto[1,2], P. Mutowembwa[1], J. van Heerden[1], G. T. Fosgate[3], L. Heath[1] and W. Vosloo[1,2,*]

[1] Transboundary Animal Diseases Programme, Onderstepoort Veterinary Institute, Agricultural Research Council, Onderstepoort, South Africa
[2] Department of Tropical Veterinary Diseases, Faculty of Veterinary Science, University of Pretoria, Onderstepoort, South Africa
[3] Department of Production Animal Studies, Faculty of Veterinary Science, University of Pretoria, Onderstepoort, South Africa

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Correspondence:
W. Vosloo. CSIRO-Australian Animal Health Laboratory, Private Bag 24, Geelong, Vic. 3220, Australia. Tel.: +61 3 5227 5015; Fax: +61 3 5227 5555;
E-mail: Wilna.vosloo@csiro.au

*Present address:CSIRO-Australian Animal Health Laboratory, Private Bag 24, Geelong Vic. 3220, Australia

Work was performed at the Transboundary Animal Diseases Programme, Onderstepoort Veterinary Institute, South Africa.

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Summary

African swine fever (ASF) is a mostly fatal viral infection of domestic pigs for which there is no vaccine available. The disease is endemic to most of sub-Saharan Africa, causes severe losses and threatens food security in large parts of the continent. Naturally occurring attenuated ASF viruses have been tested as vaccine candidates, but protection was variable depending on the challenge virus. In this study, the virulence of two African isolates, one from a tick vector and the other from an indigenous pig, was determined in domestic pigs to identify a potential vaccine strain for southern Africa. Neither isolate was suitable as the tick isolate was moderately virulent and the indigenous pig virus was highly virulent. The latter was subsequently used as heterologous challenge in pigs first vaccinated with a naturally attenuated isolate previously isolated in Portugal. Although a statistically significant reduction in death rate and virus load was observed compared with unvaccinated pigs post-challenge, all pigs succumbed to infection and died.

Introduction

African swine fever (ASF) manifests as an acute haemorrhagic fever that causes mortality approaching 100% in domestic pigs and can also lead to a moderate or chronic form of disease depending on the infecting strain (Penrith et al., 2004a). The socio-economic impact is significant in affected areas. Clinical signs of ASF are unapparent at the early stages of infection and at later stages resemble those of other swine diseases such as classical swine fever (Mebus, 1988) and bacterial septicaemias (Penrith et al., 2004a; OIE Terrestrial Manual, 2012). Difficulty in differentiating ASF is increased when infection prevails for longer and consequently develops into subacute, chronic or even subclinical forms (Penrith et al., 2004a).

African swine fever virus (ASFV) is the only known arthropod-borne DNA virus. The disease was first described in Kenya in the 1920s as an acute haemorrhagic fever of domestic pigs (Montgomery, 1921). Subsequently, it has been identified in southern, Central and West Africa (Penrith et al., 2004a) where it causes severe economic losses to rural and peri-urban subsistence pig farmers as well as to commercial enterprises. In areas where ASF is endemic, the disease impacts negatively on the sustainability of farming practices thereby limiting the availability of cheap, high-quality protein and steady income. In sub-Saharan Africa,

maintenance and transmission are by a sylvatic cycle involving *Ornithodoros* ticks and wild pigs including warthogs (*Phacochoerus africanus)* and possibly bush pigs (*Potamochoerus porcus)* (Thomson et al., 1983), with occasional spill-over to domestic pigs and pig-to-pig transmission cycles (Penrith et al., 2004a).

Due to the lack of an effective vaccine, control measures are based on movement restrictions and stamping out, measures that often encourage farmers to hide the disease thus increasing the spread of infection. A few experimental vaccines have been developed, but an effective vaccine has so far been elusive. Protection against homologous and heterologous virulent inoculums of ASFV may be acquired when pigs are first inoculated with certain non-pathogenic isolates (Lewis et al., 2000; Leitão et al., 2001; Oura et al., 2005; King et al., 2011). However, in the majority of cases there is a lack of heterologous protection that is an impediment to the development of a vaccine (Kleiboeker, 2002). There are reports from endemic regions of southern Africa that pigs show natural resistance to the disease (Penrith et al., 2004b), but it is not clear whether this is due to repeated exposure to virus with reduced virulence, low infectious doses or some innate resistance of the pig breeds in those areas.

Very little information is available on the virulence and pathogenicity of African ASFV isolates, especially those obtained from infected ticks. In this study, we determined the virulence of two African isolates compared to previously characterized strains to ascertain their potential as attenuated vaccines for use in the region. Furthermore, we tested the protective ability of a fully attenuated European strain against challenge with a highly virulent African strain.

Materials and Methods

ASF viruses

Two isolates from southern Africa were selected: a non-haemadsorbing (HAD) ASFV (MKUZI), belonging to genotype XX (Arnot et al., 2009), which was isolated from an *O. porcinus* tick collected from a warthog burrow in the Mkuzi Game reserve, South Africa (SA) in 1978 (Thomson, 1985) and MOZ 1/98 (genotype VIII), isolated from a domestic pig in Mozambique (Bastos et al., 2004). The reference viruses, a highly virulent isolate (BENIN 1/97, genotype I) isolated from a domestic pig in Benin (Chapman et al., 2008) and a non-virulent isolate from an *O. erraticus* tick (OURT 3/88, genotype I) collected in the Alentejo province in Portugal (Boinas, 1995), were obtained from the Pirbright Institute, United Kingdom. Virus isolates were cultured (4–6 passages) and titrated in bone marrow cells (BMC) using standard techniques. The titre of each virus was adjusted to 10^4 HAD_{50}/ml in growth medium (42% Earle's medium, 0.4% Hepes (UniLab), 13% swine serum, 0.52% penicillin, 0.832% streptomycin and 0.975% neomycin and distilled water).

Experimental animals

Cross-bred, Large White Landrace pigs of 20–30 kg (live weight), aged 4–6 months, were bought from commercial sources in the ASF free zone in SA and tested for ASF antibodies prior to the commencement of the trial.

Ethical considerations and disease monitoring

Animal experiments were conducted strictly according to animal welfare standards set by the ARC-Onderstepoort Veterinary Institute (OVI) and Faculty of Veterinary Science of the University of Pretoria Ethics Committees. Clinical assessment of the animals was performed twice daily for the duration of the experiment. Observations including rectal temperature, inappetance, recumbency, the degree of skin haemorrhaging, joint swelling, respiratory rate and rhythms, ocular discharges and the state of diarrhoea were noted on clinical score cards. End points for euthanasia were predetermined on a score sheet recommended by the National Society for the Prevention of Cruelty to Animals (NSPCA).

Study design to determine the virulence of ASFV isolates and vaccine trials

To determine the virulence of BENIN 1/97, OURT 3/88, MOZ 1/98 and MKUZI, pigs were allocated to four groups of three animals each. Each animal was infected by intramuscular (IM) injection with 1 ml of viral suspension at a titre of 1×10^4 HAD_{50}/ml. Sera and unclotted blood were obtained via the vena cava or the jugular vein at 0, 3, 5, 7, 14, 18 and 19 days post-infection (dpi) to detect viraemia and antibodies.

The protection provided by the live attenuated OURT 3/88 isolate was determined by vaccinating two groups of six pigs each (Group 1 and Group 2) by IM inoculation. Group 1 received a second vaccination using the same virus 21 days post-vaccination (dpv). The groups were challenged via IM injection with 1 ml MOZ 1/98 suspension (1×10^4 HAD_{50}/ml) 28 days after the second vaccination for Group 1 and 21 dpv for Group 2. The unvaccinated control group consisted of three pigs (Group 3) and was infected as previously described.

Viraemia was analysed by PCR from extracts of blood collected at 0, 3, 5, 7, 10, 14, 21, 24, 26, 28, 31, 35, 49, 52, 54, 56 and 60 dpv for Group 1 and at 21, 24, 26, 28, 31, 35, 49, 52, 54, 56 and 60 dpv for Group 2. Group 3 was sampled from day 49 onwards.

Post-mortem and virus load in organ samples

Detailed necropsies were performed when pigs died or were euthanized and organ samples were collected and immediately preserved at −80°C. Tissue samples including skin, tonsil, lung, heart, mediastinal lymph node, spleen, liver, kidney, gastro-hepatic lymph node, mesenteric lymph node, stomach and intestine were collected. Bladder samples were collected from animals in the vaccine trial.

Serology, DNA extraction and quantitative real-time PCR

Antibodies were detected using the Ingezim PPA Compac 11.PPA.K3 blocking ELISA according to manufacturer's instructions (last revision 23-01-08, Immunologia Y Genetica Aplicada, Spain), as well as an in-house OIE ELISA based on Vero cell-adapted Zaire 93 whole virus antigen. DNA extraction from whole blood and tissues was performed using the High Pure PCR Template Preparation Kit (Roche) as described by the manufacturer. Quantitative real-time PCR for ASF viral load determination was performed according to the method described by King et al. (2003).

Statistical analysis

Quantitative data for the vaccine trial were assessed for normality by calculating descriptive statistics, plotting histograms, and using the Anderson-Darling test for normality (Anderson and Darling, 1954). Data were transformed using the natural logarithm to better approximate a normal distribution and described using the median and range. Linear mixed models were used to test for group differences on the transformed data while adjusting for repeated measures within individual pigs. Viraemia post-challenge included a random effect for pig and modelled the repeated measures over time using a first-order autoregressive correlation structure. Group assignment was included as a fixed effect while adjusting for sampling day using a main effect and an interaction term with group assignment. ASFV concentration within tissues included a random effect for pig and was adjusted for the day of death or euthanasia. Significant treatment group and tissue type effect (overall) was followed by multiple pairwise comparisons to identify the levels that were different from one another using Bonferroni adjustment of *P*-values. The natural logarithm of zero is undefined so models were fit including zero values (no virus detected) and excluding these values (treated as missing data) to assess the impact of including these data points. Cox proportional hazards survival analysis was performed to estimate the rate of death in vaccinated pigs relative to the control group. Death was the event of interest and pigs that were euthanized were treated as right censored data. Statistical analyses were performed using commercially available software (IBM SPSS Statistics version 21, International Business Machines Corp., Armonk, NY, USA) and results interpreted at the 5% level of significance.

Results

Clinical outcome, serology and virus load in the virulence trial

The virulence of four different ASFV strains, representing three different p72 genotypes, was examined in domestic pigs. All pigs infected with OURT 3/88 remained apparently healthy until termination of the experiment at day 19. Pigs were sero-negative at days 0 and 5, and at day 7, one of the pigs sero-converted followed by the other two pigs by 14 dpi. There was no viral DNA detected in the blood or organs of any of these pigs by PCR (Table 1). Infection with ASFV OURT 3/88 did not produce any significant rise in temperature throughout the entire duration of the trial (results not shown).

All pigs inoculated with MKUZI had fever at 7 dpi that lasted until termination at day 18. This may have been due to infected lesions, which were observed in the hind legs of all three pigs, possibly as a result of needle trauma. All three pigs sero-converted by 14 dpi but ASFV DNA was detected in their blood only at 18 dpi. In addition to fever, the pigs demonstrated clinical signs including inappetance, depression, serous ocular discharge and diarrhoea from 10 dpi, as well as congestion, cyanosis and haemorrhages of the skin and huddling together on 18 dpi. Gross lesions detected during post-mortems included cyanotic congestion and small pinpoint type haemorrhages of the skin, generalized enlargement of lymph nodes, mild splenomegaly as well as petechiation and congestion of the stomach fundus.

All three pigs inoculated with MOZ 1/98 developed clinical signs as early as 2 dpi. Viral DNA was detected 3 dpi and all pigs died by 6 dpi without sero-converting

Table 1. General characteristics of disease caused in pigs by ASFV strains isolated from ticks and domestic pigs

	Days post-infection				
Strain	Clinical Signs	Viraemia[§]	Sero-conversion		Death
OURT 3/88	ND	ND	7[†]	14[‡]	19*
MKUZI	7	18	ND	14[‡]	18*
MOZ 1/98	2	3	–	–	6
BENIN 1/97	2	3	ND	–	7

ND: not detected; *Animals were killed at the end of the observational period but did not reach NSPCA score; [†]one animal was sero-positive; [‡]All pigs were sero-positive; – no samples were taken, [§]viral DNA detected in the blood.

(Table 1). Clinical signs included inappetance, depression and ocular discharge from 3 dpi followed by diarrhoea and lethargy, congestion of the skin of snout, tail, limbs, ears and abdomen at 6 dpi. All pigs demonstrated typical ASF lesions on post-mortem. Haemorrhages varied from small pinpoints in tissues to considerable amounts of free blood that were found in almost all organs of the body. Gross lesions included severe enlargements and haemorrhages in lymph nodes, congestive splenomegaly, blood-stained froth from nostrils, trachea and cut lung surfaces as well as severe interlobular oedema of the lungs, excess yellowish fluid in the thorax, pericardial sac and peritoneum. There was oedema and petechiation of gall bladder walls, congestion and haemorrhages of the mucosa of the stomach (fundus and pylorus) and small intestines in addition to petechiation of renal cortex, medulla and congested urinary bladder mucosa. In one pig, ASFV DNA was detected in all collected organs, while another pig had detectable DNA in all organs except the kidneys. In the third pig, all organs except the heart and intestine were positive for ASFV DNA.

In the group infected with BENIN 1/97, viral DNA was detected in the blood from 3 dpi and clinical signs were present at 4 dpi, in line with previous findings (Chapman et al., 2008). By 7 dpi, all three pigs reached terminal animal welfare scoring and were euthanized. All were seronegative at that point (Table 1). Clinical disease was similar to that observed with MOZ 1/98 infection with the exception of bloody diarrhoea. The majority of the samples collected post-mortem were ASFV DNA positive. All organs with the exception of the lungs had detectable levels of ASFV DNA in one pig, while another presented no detectable ASFV DNA in heart, kidneys and intestine. The third pig had no detectable ASFV DNA in tonsil and either mediastinal or the mesenteric lymph nodes.

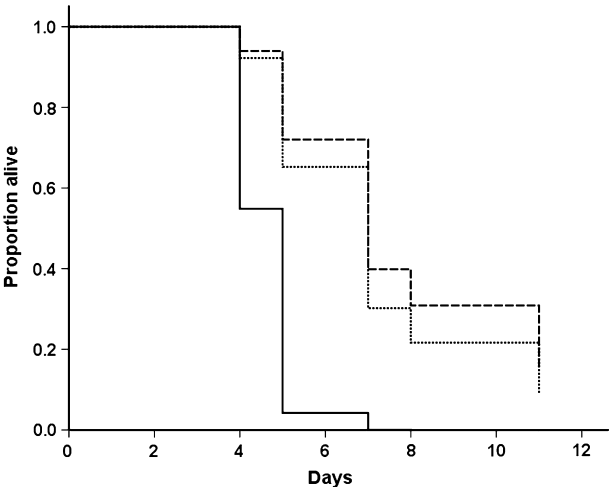

Fig. 1. The overall survival for Group 1 (long dash), Group 2 (short dash), and Group 3 (solid line) was significantly different ($P = 0.040$).

Table 2. Characteristics of disease in pigs vaccinated with a live attenuated strain (OURT 3/88) and challenged with virulent MOZ 1/98

		Days post-challenge (dpc)			
Groups	No. of pigs	Fever	Clinical Signs	Viraemia*	End point
Group 1 (vaccinated twice)	6	2	3	3	5–9
Group 2 (vaccinated once)	6	1–4	3–4	3	5–11
Group 3 (control group)	3	2	3	3	4–5

*Virus DNA detected in the blood.

Vaccination of pigs with OURT 3/88 and challenge with MOZ 1/98

General clinical outcome of the vaccination trial

As the virulence trial indicated that MKUZI is mildly virulent and therefore not suitable as a vaccine strain, it was decided to use the European OURT 3/88 as the vaccine strain and determine if infection would protect pigs against challenge with the unrelated, virulent African isolate, MOZ 1/98. In Group 1 (vaccinated twice), one pig died at 5 days post challenge (dpc), three pigs died 7 dpc while another survived until 8 dpc. The sixth pig had fever (>40°C) from 24 dpv but did not present with other typical clinical signs. This pig was killed 9 dpc for welfare reasons unrelated to ASFV infection (Fig. 1; Table 2). Two of the pigs in Group 2 (vaccinated once) died overnight at 5 dpc and another was euthanized at 6 dpc for welfare reasons. One pig died by 7 dpc, followed by another at 11 dpc, while the last pig was euthanized 11 dpc. In Group 3, 2 pigs died at 4 dpc while the third pig died at 5 dpc.

There was a significant difference in survival rates among the three groups with the mean survival time of 7.2 days (95% confidence interval (CI), 6.2–8.1) in Group 1, 8.1 days (95% CI, 5.5–10.7) in Group 2 and 4.3 days (95% CI, 3.7–5.0) in Group 3 ($P = 0.040$; Fig. 1). The rate of death in Group 1 was 7.4 times lower than in Group 3 (hazard ratio = 0.135; 95% CI, 0.023–0.790; $P = 0.026$) while that of Group 2 was 9.6 times lower than Group 3 (hazard ratio = 0.104; 95% CI, 0.016–0.654; $P = 0.016$). There was no significant difference in survival between Groups 1 and 2 ($P = 0.72$); however, combined the survival was significantly different from that of the control group ($P = 0.009$).

In general, lesions noted during necropsies were similar in all groups, namely severe generalized enlargement and haemorrhages in lymph nodes, congestive splenomegaly, blood stained froth from nostrils as well as the trachea and

cut lung surfaces and severe interlobular oedema of the lungs. There was excess yellowish fluid in the thorax, pericardial sac and peritoneum and oedema and petechiation of gall bladder walls. In addition, congestion and haemorrhages of the mucosa of the stomach (fundus and pylorus) and small intestines as well as petechiation of renal cortex and medulla, and congested urinary bladder mucosa were detected.

Virus load in organ samples from the vaccination trial

Following vaccination with OURT 3/88, viral DNA was not detected in the blood of any of the vaccinated pigs in Groups 1 or 2, except one pig in Group 1 that tested positive at 35 dpv only (results not shown). After challenge with MOZ 1/98, ASFV DNA was found in the blood of one pig from each group at 3 dpc. All pigs in Group 1, except the one with no clinical signs, were ASFV positive by PCR with increasing virus load up to death. The pigs in Group 2 were all positive by the end of the trial, when they died or were euthanized. The viral load in all samples varied over time and by treatment group ($P = 0.005$) and was lower in both Groups 1 and 2 relative to the control Group 3 ($P = 0.004$; Table 3).

The level of virus descriptively varied by treatment group, but the overall comparison (summarized for all organ specimens) was not significant (Table 3; $P = 0.199$). When combined over all three treatment groups, splenic tissues had the highest amount of viral DNA and this amount was significantly different from the intestine. Intestinal tissues had the lowest amount of viral DNA, and the amount was significantly different from lungs, skin and spleen. Group 3 had higher values than vaccinated Groups 1 and 2, but Groups 1 and 2 were not different from each other. The inclusion or exclusion of zero values did not influence overall statistical inferences and therefore results including the zero values have been reported.

Discussion

The lack of an effective vaccine critically impacts on the control of ASF in endemic areas of sub-Saharan Africa. Previous reports indicating that experimental vaccines are ineffective when animals are challenged with heterologous viruses have led to investigations into an avirulent strain of ASFV suitable to the region that could potentially be used as a vaccine. Two previously uncharacterized isolates from southern Africa were inoculated into pigs and their virulence and pathogenicity compared with that of two strains of known virulence.

The isolate from Portugal, OURT 3/88, was confirmed to have low virulence (Boinas, 1995; Boinas et al., 2004). ASFV DNA was only detected in the blood of pigs infected with MKUZI at the end of the trial (18 dpc) in line with previous findings with other non-pathogenic isolates (Leitão et al., 2001). The MKUZI virus loads in blood and organ samples were lower than for MOZ 1/98 and BENIN 1/97. Pigs inoculated with MKUZI displayed a clinically mild form of ASF defined here as chronic disease (Leitão

Table 3. Levels of ASFV (viral genome copies/ml) in blood and post-mortem tissue samples of pigs in the vaccination trial determined by quantitative real-time PCR

Tissue	Overall* Median (range)	Group 1 Median (range)	Group 2 Median (range)	Group 3 Median (range)
Blood†	22 500 (0, 2270 000)	18 300 (0, 115 000)	12 995 (543, 103 000)	955 000 (204 000, 2 270 000)
Skin	14 300[a,b,c] (0, 85 900)	4755 (0, 29 000)	12 480 (244, 85 900)	17 700 (14 300, 29 700)
Tonsil	266[a,d] (0, 506 000)	32 (0, 1620)	950 (0, 122 000)	416 000 (284, 506 000)
Lungs	21800[b,c] (0, 1 310 000)	14 650 (0, 27 900)	57 750 (1130, 183 000)	539 000 (84 400, 1 310 000)
Heart	1640[a,b,c,d] (0, 48 000)	801 (0, 6730)	1650 (0, 27 200)	16 700 (3480, 48 000)
Mediastinal LN	602[a,b,c,d] (0, 210 000)	254 (0, 14 200)	580 (125, 210 000)	112 000 (19 100, 158 000)
Spleen	53500[a,b,c] (0, 814 000)	5125 (0, 325 000)	39 650 (0, 219 000)	799 000 (394 000, 814 000)
Liver	3490[a,b,c,d] (0, 1 330 000)	1000 (0, 339 000)	76 600 (0, 379 000)	171 000 (0, 1 330 000)
Kidneys	5950[a,b,c,d] (0, 169 000)	283 (0, 31 000)	7435 (0, 113 000)	69 500 (0, 169 000)
Gastro-hepatic LN	7880[a,b,c,d] (0, 1 190 000)	332 (0, 184 000)	13 690 (0, 112 000)	49 800 (16 400, 1 190 000)
Mesenteric LN	1820[a,b,c,d] (0, 111 000)	1150 (0, 7170)	2795 (0, 111 000)	41 300 (1790, 110 000)
Stomach	2650[a,b,c,d] (0, 123 000)	161 (0, 2650)	5765 (0, 14 200)	106 000 (30 900, 123 000)
Intestine	218[d] (0, 54 300)	0 (0, 2970)	749 (137, 53 200)	21 000 (0, 54 300)
Bladder	1480[a,b,c,d] (0, 109 000)	348 (0, 12 300)	1095 (0, 109 000)	46 100 (7700, 51 300)

LN, lymph nodes.

*Viraemia was significantly different between Groups 1 & 2 compared with Group 3 ($P = 0.004$) but tissue ASFV quantification did not vary by group ($P = 0.199$). Medians without superscripts in common are significantly different ($P < 0.05$) when compared across all groups; viraemia was excluded from multiple comparisons. †Descriptive statistics present the maximum value within each individual pig from infection until death or euthanasia but statistical testing incorporated all values.

et al., 2001; Kleiboeker, 2002; Penrith et al., 2004a). It remains unclear whether the animals in this study may have developed evident ASF if they lived longer and this should be confirmed in future studies. It is probable that the antibodies that were present from 14 dpi may have provided a certain level of protection. From 8 dpi, severe inflammation at the injection site on the pigs' hind legs, with marked swelling that caused ulceration of the skin along with lameness, a characteristic lesion of chronic ASF, was observed. These lesions were not tested for the presence of ASFV DNA; however, this was the only group where high ASFV DNA loads were found in the skin of the pigs which could indicate the tropism of this virus.

Tissue samples from the swollen area in the hind leg of MKUZI infected pigs revealed bacterial infection despite the fact that the inocula were filtered and sterility tested. In addition, the inoculum was not positive when subjected to a 16S rRNA PCR (results not shown). Two pigs tested positive for *Mycoplasma hyosynoviae* infection while another pig was positive for *Fusobacterium necrophorum, Porphyromonas gingivicanis* and *Lactobacillus.* It is unclear whether the infection with ASFV caused the pigs to be more susceptible to bacterial infections.

The outcome of the MOZ 1/98 infection in domestic pigs resembled that of BENIN 1/97 with a similar time progression of clinical signs until death and presented as a general haemorrhagic syndrome. In both infections, animals survived only up to 6 or 7 dpi and viral DNA was detected in the blood from 3 dpi. Clinical signs developed 1 day earlier in the MOZ 1/98 inoculated group and pigs showed more severe lung lesions whilst those infected with BENIN 1/97 had bloody diarrhoea. According to Carrasco et al. (1996) acute ASF causes formation of alveolar oedema in the lungs of afflicted animals as well as accumulation of fibrin microthrombi in septal capillaries which may lead to congestion and haemorrhage. Therefore, taking into consideration the results seen in temporal progression of the disease and the characteristic clinical signs and post-mortem lesions observed, these two strains can be recognized as causative agents of acute ASF (Penrith et al., 2004a). No antibodies were detected in any of these animals. Moderately virulent viruses elicit increased antibody responses compared to virulent strains. Neutralizing antibody response in ASFV-challenged domestic pigs tends to appear at 10–12 or 12–14 days after exposure (Wardley et al., 1987), whereas individuals affected by acute disease have a shorter survival time and do not reach the point of antibody response.

As previously described by Oura et al. (1998), domestic pigs inoculated with high virulence ASFV presented initial high levels of virus replication in the spleen, rapidly followed by spread of the virus and high levels in other lymphoid organs. Similarly, in the present study, viral quantification in tissue samples from infected pigs revealed the spleen, tonsils and either gastro-hepatic, mediastinal or mesenteric lymph nodes as organs with the highest concentration of ASFV DNA. Interestingly, high viral loads were found in the skin of animals inoculated with MKUZI raising the possibility of slightly different viral tropism in cases of chronic ASF.

Coggins (1968) and Vigário et al. (1974) reported that viruses lacking the ability to cause haemadsorption generally tend to be non-virulent or are of reduced virulence due to the deletion of the gene encoding the CD2v protein. This deletion delays the onset of viraemia and dissemination of viruses within infected pigs (Borca et al., 1998) and was previously linked to natural attenuation of ASFV. Nevertheless, other authors have found that certain non-haemadsorbing ASFVs cause ASF (Leitão et al., 2001). The OURT 3/88 and MKUZI isolates both lack the ability to cause haemadsorption or bind red blood cells and were previously described as non-haemadsorbing viruses (Thomson, 1985 and Boinas et al., 2004). Evidence presented here demonstrates that although MKUZI is non-haemadsorbing, it can be classified as moderately virulent.

Several studies have shown that a non-fatal ASFV infection could provide some level of protection against inoculation with other virulent ASFV strains (Lewis et al., 2000; Leitão et al., 2001; Boinas et al., 2004; Oura et al., 2005). Pigs that were immunized with live attenuated ASFVs showed immunity when challenged with homologous parental virus (Lewis et al., 2000). Most domestic pigs immunized with non-virulent OURT 3/88, followed by a booster vaccination with virulent OURT 1/88 and challenged with virulent African isolates, did not show clinical signs and no viral DNA was detected (King et al., 2011). Resistance due to repeated infections may lead to acquired immunity against the pathological effects of the disease (Mendes, 1994; Penrith et al., 2004b).

Effective vaccines against ASF should prevent viral replication to minimize the effects of the virus on the reticuloendothelial system and avoid consumption coagulopathy in the infected individual (Wardley and Wilkinson, 1985). As the MKUZI isolate caused chronic ASF, it was not suitable to use as a vaccine strain. The attenuated OURT 3/88 (genotype I) was a possible candidate, but it was necessary to test its cross-immunization potential against a virulent southern African ASF isolate (MOZ 1/98 – genotype VIII). Previously, inoculation of pigs with the non-virulent OURT 3/88 was characterized by sporadic viraemia and sero-conversion and prevented clinical signs and viraemia in pigs subsequently challenged with a Portuguese pathogenic virus isolated from ticks (OURT 88/1) (Boinas et al., 2004). In this study, none of the animals vaccinated with OURT 3/88, with either one or two inoculations, showed any clinical signs indicative of ASFV infection. However, clinical signs appeared a few days after challenge with viru-

lent MOZ 1/98 and 5 of the 6 pigs in each group died. Although there was no significant difference in the virus loads in the various organs between the two vaccinated groups, there were significant differences with the unvaccinated group (Table 3). The median survival times (where fractional survival equals 50%) for Groups 1 and 2 were similar, as was the hazard ratio, indicating no significant difference in survival rates between the double and single vaccination regimen.

One pig in Group 1 demonstrated a less severe pathological outcome and survived longer than the other pigs in the same group. Only a few organs had detectable levels of viral DNA, and during post-mortem, no haemorrhages were observed. Interestingly, this pig developed a fever before inoculation with MOZ 1/98, which persisted until termination. One pig in Group 2 demonstrated more subtle signs of disease and was the last to show a fever. It did not develop haemorrhages and fewer lesions were observed at post-mortem compared with the rest of the group. Viral DNA was detected considerably later and in fewer organs in this animal than in the other pigs in Group 2.

The immunological mechanisms involved in the defence of suidae against virulent ASFV are still unclear. Antibodies against vp72 were present in pigs vaccinated with OURT 3/88 in this study. Neilan et al. (2004) previously found that neutralizing antibodies to proteins involved in ASFV attachment, vp30, vp54 and vp72, did not protect pigs from ASFV challenge but delayed the onset of disease. Clearly, a protective immune response relies on cell-mediated immunity as well.

In the current study, although single and boosted vaccination of susceptible pigs with OURT 3/88 briefly delayed the onset of disease and caused lower viral concentrations in certain organs, it had no effect on disease development, progression, or outcome. As infection with attenuated strains confers protection to pigs against homologous challenge it is conceivable that partial protection could be attained following vaccination with an attenuated African strain, genetically closer to the current circulating southern African isolates. Results described here are consistent with previous reports suggesting that in some cases, European ASFV isolates are attenuated and better adapted to domestic pigs than African field strains (Ordas-Alvarez and Marcotegui, 1987; Mebus, 1988). In the light of this, efforts to identify naturally attenuated African strains should continue. Alternatively, attenuated viruses based on African strains could be developed *in vitro* by site directed mutagenesis or deletion of immunomodulatory genes.

Conclusions

It was demonstrated that ASFV MKUZE is moderately virulent and causative of chronic ASF while ASFV MOZ 1/98 was virulent and responsible for acute ASF. In addition, this trial confirmed what was expected from reference viruses ASFV OURT 3/88 and ASFV BENIN 1/97. Although the vaccination regimen with OURT 3/88 and challenge with MOZ 1/98 did not prevent mortalities, virus load in the organs and blood of the pigs was lower than the controls and they survived longer. A more attenuated isolate than ASFV MKUZE from the same region should be trialled to verify whether or not it confers protection from virulent isolates in the area.

Conflict of interest statement

None. The funding agency was not involved in the study design, in the collection, analysis and interpretation of data; in the writing of the manuscript; and in the decision to submit the manuscript for publication.

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References

Anderson, T. W., and D. A. Darling, 1954: A test of goodness of fit. *J. Am. Statist. Assoc.* 49, 765–769.

Arnot, L. F., J. T. Du Toit, and A. D. S. Bastos, 2009: Molecular monitoring of African swine fever virus using surveys targeted at adult *Ornithodoros* ticks: a re-evaluation of Mkuzi game reserve, South Africa. *Onderstepoort J. Vet. Res.* 76, 385–392.

Bastos, A. D. S., M.-L. Penrith, F. Macome, F. Pinto, and G. R. Thomson, 2004: Co-circulation of two genetically distinct viruses in an outbreak of African swine fever in Mozambique: no evidence for individual co-infection. *Vet. Microbiol.* 103, 169–182.

Boinas, F., 1995: The role of *Ornithodoros erraticus* in the epidemiology of African swine fever in Portugal. Department of Agriculture Reading: University of Reading, pp. 240.

Boinas, F. S., G. H. Hutchings, L. K. Dixon, and P. J. Wilkinson, 2004: Characterization of pathogenic and non-pathogenic African swine fever virus isolates from *Ornithodoros erraticus* inhabiting pig premises in Portugal. *J. Gen. Virol.* 85, 2177–2187.

Borca, M. V., C. Carrillo, L. Zsak, W. W. Laegreid, G. F. Kutish, J. G. Neilan, T. G. Burrage, and D. L. Rock, 1998: Deletion of a CD2-like gene, 8-*DR*, from African swine fever virus affects viral infection in domestic swine. *J. Virol.* 72, 2881–2889.

Carrasco, L., F. C. de Lara, J. Martín de las Mulas, J. C. Gómez-Villamandos, J. Hervás, P. J. Wilkinson, and M. A. Sierra, 1996: Virus association with lymphocytes in acute African swine fever. *Vet. Res.* 27, 305–312.

Chapman, D. A. G., V. Tcherepanov, C. Upton, and L. K. Dixon, 2008: Comparison of the genome sequences of non-pathogenic and pathogenic African swine fever isolates. *J. Gen. Virol.* 89, 397–408.

Coggins, L., 1968: Segregation of a nonhemadsorbing African swine fever virus in tissue culture. *Cornell Vet.* 58, 12–20.

King, D. P., S. M. Reid, G. H. Hutchings, S. S. Grierson, P. J. Wilkinson, L. K. Dixon, A. D. S. Bastos, and T. W. Drew, 2003: Development of a TaqMan® PCR assay with internal amplification control for detection of African swine fever virus. *J. Virol. Methods* 107, 53–61.

King, K., D. Chapman, J. M. Argilaquet, E. Fishbourne, E. Hutet, R. Cariolet, G. Hutchings, C. A. Oura, C. L. Netherton, K. Moffat, G. Taylor, M. F. Le Potier, L. K. Dixon, and H. H. Takamatsu, 2011: Protection of European domestic pigs from virulent African isolates of African swine fever virus by experimental immunisation. *Vaccine* 28, 4593–4600.

Kleiboeker, B. S., 2002: Swine fever: classical swine fever and African swine fever. *Vet. Clin. North Am. Food Anim. Pract.* 18, 431–451.

Leitão, A., C. Cartaxeiro, R. Coelho, B. Cruz, R. M. E. Parkhouse, F. Portugal, J. D. Vigario, and C. L. V. Martins, 2001: The non-haemadsorbing African swine fever virus isolate ASFV/NH/P68 provides a model for defining the protective anti-virus immune response. *J. Gen. Virol.* 82, 513–523.

Lewis, T., L. Zsak, T. G. Burrage, Z. Lu, G. F. Kutish, J. G. Neilan, and D. L. Rock, 2000: An African swine fever virus *ERV1-ALR* homologue, *9GL*, affects virion maturation and viral growth in macrophages and viral virulence in swine. *J. Virol.* 74, 1275–1285.

Mebus, C. A., 1988: African swine fever. *Adv. Virus Res.* 35, 251–269.

Mendes, A. M., 1994: The history of African swine fever in Angola. *Revista Portuguesa de Ciências Veterinárias.* 89, 110–120.

Montgomery, R. E., 1921: On a form of swine fever occurring in British East Africa (Kenya Colony). *J. Comp. Pathol. Ther.* 34, 243–262.

Neilan, J. G., L. Zsak, Z. Lu, T. G. Burrage, G. F. Kutish, and D. L. Rock, 2004: Neutralizing antibodies to African swine fever virus proteins p30, p54, and p72 are not sufficient for antibody-mediated protection. *Virology* 319, 337–342.

OIE Terrestrial Manual, 2012: African Swine Fever, Chapter 2.8.1. Available at http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.08.01_ASF.pdf (accessed January 7, 2014).

Ordas-Alvarez, A., and M. A. Marcotegui, 1987: African swine fever: clinical aspects. In: Becker, Y. (ed.), *African Swine Fever*, pp. 11–20. Martinus Nijhoff Publishing, Boston, MA, USA.

Oura, C. A. L., P. P. Powell, and R. M. E. Parkhouse, 1998: African swine fever: a disease characterized by apoptosis. *J. Gen. Virol.* 79, 1427–1438.

Oura, C. A. L., M. S. Denyer, H. Takamatsu, and R. M. E. Parkhouse, 2005: *In vivo* depletion of $CD8^+$ T lymphocytes abrogates protective immunity to African swine fever virus. *J. Gen. Virol.* 86, 2445–2450.

Penrith, M.-L., G. R. Thomson, and A. D. S. Bastos, 2004a: African swine fever. Infectious diseases of livestock. In: Coetzer, J. A. W., and R. C. Tustin (eds), *Infectious Diseases of Livestock*, 2nd edn, pp. 1088–1119. Oxford University Press, South Africa.

Penrith, M.-L., G. R. Thomson, A. D. S. Bastos, O. C. Phiri, B. A. Lubisi, E. C. Du Plessis, F. Macome, B. Botha, and J. Esterhuysen, 2004b: An investigation into natural resistance to African swine fever in domestic pigs from an endemic area in southern Africa. *Rev. Sci. Tech.* 23, 965–977.

Thomson, G. R., 1985: The epidemiology of African swine fever: the role of free-living hosts in Africa. *Onderstepoort J. Vet. Res.* 52, 201–209.

Thomson, G. R., M. Gainaru, A. Lewis, H. Biggs, E. Nevill, M. Van Der Pypekamp, L. Gerbes, J. Esterhuysen, R. Bengis, D. Bezuidenhout, and J. Condy, 1983: The relationship between ASFV, the warthog and *Ornithodoros* species in southern Africa, p 85-100. In: Wilkinson, P. J. (ed.), *Proceedings of CEC/FAO Research Seminar, ASF, EUR 8466 EN*. Commission of the European Communities, Luxembourg.

Vigário, J. D., A. M. Terrinha, and J. F. Moura Nunes, 1974: Antigenic relationships among strains of African swine fever virus. *Arch. Gesamte. Virusforsch.* 45, 272–277.

Wardley, R. C., and P. J. Wilkinson, 1985: An immunological approach to vaccines against African swine fever virus. *Vaccine* 3, 54–56.

Wardley, R. C., S. G. Norley, C. V. Martins, and M. J. P. Lawman, 1987: The host response to African swine fever virus. *Prog. Med. Virol.* 34, 180–192.