Transboundary and Emerging Diseases

ORIGINAL ARTICLE

Immunization of African Indigenous Pigs with Attenuated Genotype I African Swine Fever Virus OURT88/3 Induces Protection Against Challenge with Virulent Strains of Genotype I

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Summary

The attenuated African swine fever virus genotype I strain OURT88/3 has previously been shown to induce protection of European breeds of domestic pigs against challenge with virulent isolates. To determine whether protective immune responses could also be induced in indigenous breeds of pigs from the Kinshassa region in Democratic Republic of Congo, we immunized a group of eight pigs with OURT88/3 strain and challenged the pigs 3 weeks later with virulent genotype I strain OURT88/1. Four of the pigs were protected against challenge. Three of the eight pigs died from African swine fever virus and a fourth from an unknown cause. The remaining four pigs all survived challenge with a recent virulent genotype I strain from the Democratic Republic of Congo, DRC 085/10. Control groups of non-immune pigs challenged with OURT88/1 or DRC 085/10 developed signs of acute ASFV as expected and had high levels of virus genome in blood.

Introduction

African swine fever virus (ASFV) causes an acute haemorrhagic fever, African swine fever (ASF), in domestic pigs. This can result in very high mortality and has a severe socio-economic impact in affected countries. Currently, no vaccine is available to protect pigs against ASF, and this limits options for disease control. African swine fever is endemic in many sub-Saharan African countries, Sardinia and parts of the Trans-Caucasus region and Russian Federation (Wilkinson, 1999; Arias and Sanchez-Vizcaino, 2002; Beltran-Alcrudo et al., 2008; Penrith et al., 2013; OIE-WAHID).

Knowledge of the genotypes of ASFV isolates circulating in different regions could provide information relevant for design of vaccination campaigns as these would be expected to be more successful in regions with single or few genotypes circulating. African swine fever virus isolates have been classified into 22 different genotypes, by partial sequencing of the gene, which encodes the VP72 major capsid protein. All of these genotypes have been detected in eastern and southern Africa. In Central and West Africa and Sardinia (Wilkinson, 1999), only genotype I was described until recently although genotype IX has now been reported in the Republic of Congo and Democratic Republic of Congo (Boshoff et al., 2007; Gallardo et al., 2011a,b). In the Trans-Caucasus and Russian Federation, the virus strains described are from genotype II (Rowlands et al., 2008; Chapman et al., 2011). Genotype I is considered the predominant genotype circulating in Central and West Africa. Therefore in the current study, we tested the ability of an attenuated genotype I virus isolate, OURT88/3, to protect pigs against virulent genotype I isolates included a recent strain from DRC.

European pigs immunized with attenuated genotype I ASFV strain OURT88/3 can be protected against challenge

with related virulent strains including from genotype X (Boinas et al., 2004; King et al., 2011). The protection levels varied from 66% to 100% dependent on the pigs and the challenge virus (Boinas et al., 2004; King et al., 2011). Complete genome sequencing showed that the OURT88/3 isolate has a large deletion near the left genome end compared to virulent isolates and interruptions in three other genes involved in immune evasion. These gene deletions and interruptions are likely to be important for attenuation of the virus and induction of a protective immune response (Chapman et al., 2008). The protective response induced by immunization of pigs with this strain is dependent on CD8+ T cells as protection is abrogated if this cell subset is depleted (Oura et al., 2005).

In this study, we have determined the protection induced by OURT88/3 in indigenous African pigs from the Kinshasa Region of the Democratic Republic of Congo (DRC) against consecutive challenge with virulent genotype I strains from Europe and from DRC.

Materials and Methods

Virus isolates and cell culture

The OURT88/3 and OURT88/1 isolates have been described previously (Chapman et al., 2011). The DRC 085/10 isolate was obtained from an ASF outbreak in domestic pigs in Kimwenza/Democratic Republic of Congo in October 2010 and confirmed to be genotype I by partial sequencing of the B646L gene.

All virus isolates were passaged at maximum five times in primary pig macrophage cultures, and virus titres were obtained by limiting dilution in primary macrophage cultures and detection of virus infection by haemadsorption (HAD) assay or by immunofluorescence using an antibody against ASFV protein p30.

Pig immunization and challenge

Indigenous pigs, at least 3 months old, were bought from farms near Kinshasa and tested negative for ASFV by antibody detection ELISA (Ingenasa Spain) and PCR. To confirm their non-viraemic status, the animals were kept for more than thirty days before the beginning of the experiments. The pigs were also negative for cysticercosis and trypanosomosis. Those in which coccidiosis was detected were treated with amprolium. The animals were kept in four buildings of level three vivarium standard, within a double-fenced restricted area. The experiments were approved by the Provincial Veterinary Office of the Ministry of Agriculture and Rural Development DRC. Pigs were randomly attributed in each group. The weight of pigs by group at arrival was compared and tested. The average weight in each group was not significantly different (linear regression with group A as reference group; P value ≥ 0.28). The average and SD for each group were, respectively, group A (32.4; 13.0), group B (24.2; 8.7) and group C (24.0; 19.3). A photograph of the pigs which survived challenge is shown (Figure S1). These were representative of the groups used.

In group A, eight pigs were immunized intramuscularly (IM) with 10^4 $TCID_{50}$ attenuated OURT88/3 strain and after 21 days challenged IM with 10^4 HAD_{50} OURT88/1 virulent strain. After a further 21 days, remaining pigs were challenged IM with 10^4 HAD_{50} DRC strain 085/10.

In group B, six pigs were challenged IM with 10^4 HAD_{50} virulent strain OURT88/1 21 days after the start of the experiment, at the same time as those in group A. In group C, seven pigs were challenged IM with 10^4 HAD_{50} DRC strain 085/10 at 42 days at the same time that group A pigs were challenged with this strain.

Pigs were immunized and challenged by the intramuscular (IM) route with 10^4 HAD_{50} or $TCID_{50}$ (tissue culture infective dose 50%) of the virus isolate indicated. Pigs were monitored daily for clinical signs, and these were scored using a clinical scoring system previously reported (King et al., 2011). Blood samples were collected at different days post-immunization and challenge. At termination, tissue samples were collected from spleen, lymph nodes and kidney. The ASFV genome copy numbers in blood and tissue samples were estimated by quantitative PCR (King et al., 2003).

Results

Immunization with OURT88/3

All eight pigs in group A developed a slightly elevated temperature between 39.1 and 40°C over the first 9 or 10 days after immunization with OURT88/3. Few other clinical signs were observed. Blood samples were collected from all pigs at days 7, 14 and 21 days post-immunization. No virus DNA was detected in any of the samples.

After challenge of pigs in group A with virulent OURT88/1 strain, four pigs, 1, 3, 7 and 8, survived. Two of these pigs showed no clinical signs, one had an elevated temperature from day 1 to 6 post-challenge, and the fourth pig had a reduced food intake on days 3 and 4 (Fig. 1). Two pigs (5 and 6) were terminated at day 6 post-challenge with virulent strain OURT88/1 showing typical signs of acute ASF (high body temperature, redness, ecchymosis and cyanosis of legs and extremities, apathy and icterus of conjunctiva). Two pigs (Arias and Sanchez-Vizcaino, 2002; Penrith et al., 2013) died at days 7 or 5, respectively, without showing typical clinical signs of ASF.

Quantitative PCR was carried out to determine the level of viraemia by estimating virus genome copy numbers in blood and tissues (Tables 1 and 2). Of the pigs which survived (Wilkinson, 1999; Beltran-Alcrudo et al., 2008; Chap-

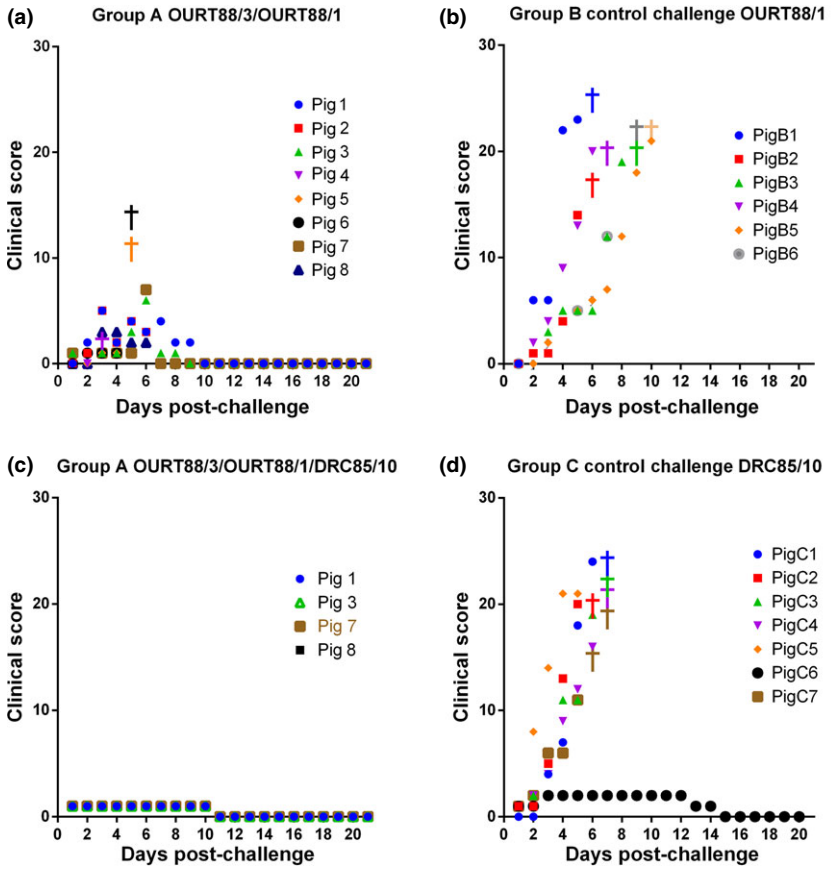

Fig. 1. Clinical scores of pigs post-immunization with OURT88/3 and challenged with OURT88/1 and DRC85/10. Clinical scores were recorded daily. Panels a and c show values recorded for pigs in Group A, which were immunized with OURT88/3 virus, at different days post-challenge with virulent virus OURT88/1 (Panel a) and DRC85/10 (Panel c). Values for control pigs in Group B, not immunized with OURT88/3, challenged with OURT88/1 are shown in Panel b and for control pigs in Group C challenged with DRC85/10 are shown in Panel d. The clinical scoring was as described previously. Values for different pigs are shown in different colours and shapes. Crosses show termination days, the colour indicates which pigs were terminated.

Table 1. Genome copy numbers of ASFV DNA in blood from pigs in Group A at different days post-challenge with OURT88/1

Pig	3	6	SD	14	21
1	2.54E+01	3.41E+04	2.84E+04	0.00	0.00
2	0.00	1.27E+06	9.53E+04	N/A	N/A
3	0.00	9.05E+00		0.00	0.00
4	0.00	N/A		N/A	N/A
5	0.00	N/A		N/A	N/A
6	0.00	N/A		N/A	N/A
7	0.00	1.04E+01		0.00	0.00
8	0.00	0.00		0.00	0.00

Pigs were immunized with OURT88/3 and at 21 days post-immunization were challenged with OURT88/1. DNA was extracted from blood samples and genome copy numbers per ml were estimated by qPCR. Two separate DNA extractions were made, and each of these samples was analysed in duplicate. Standard deviations (SD) are shown for those samples giving values >1.0E+02.

man et al., 2011; Gallardo et al., 2011b) virus DNA at 3.4×10^4 genome copies per ml of blood was detected only from pig 1 at day 6 post-challenge. Of those pigs which did not survive (Arias and Sanchez-Vizcaino, 2002; Boshoff et al., 2007; Gallardo et al., 2011a; Penrith et al., 2013), pigs 5 and 6 had high levels of ASFV DNA in tissues collected at post-mortem. Pig 5 had $>1.0 \times 10^4$ genome copy numbers per mg of tissue in lymph and spleen and pig 6 had 2.3 E+04 in lymph and 1.09E+05 per mg spleen, similar to virus levels detected in pigs from control Group B. Pig 2 had high levels of virus DNA in blood at day 6 post-challenge and high levels in tissues at termination (1E+06 genome copies per ml blood and 8.1E+03 per mg in spleen and 1.2×10^3 in lymph tissue). Pig 4 had no detectable ASFV DNA in blood or tissues. The six control pigs in Group B, which were challenged with OURT88/1 without prior immunization with OURT88/3, all exhibited high clinical scores typical of acute ASFV infection and were terminated between days 7 and 11 post-challenge. These pigs had as expected high genome copy numbers of ASFV DNA in tissues at termination (Tables 1 and 2). Therefore, we conclude that of the pigs immunized with OURT88/3, three of the eight pigs died from ASFV post-challenge with OURT88/1 and one pig died from unknown causes not related to ASFV.

Challenge with DRC085/10

The four pigs from group A (Wilkinson, 1999; Beltran-Alcrudo et al., 2008; Chapman et al., 2011; Gallardo et al.,

Table 2. Genome copy numbers of ASFV DNA from tissues collected at termination from pigs in groups A, B and C

Pig	Kidney	SD	Spleen	SD	Lymph	SD	Gang hep	SD
Group A								
1			0.00E+00		9.41E-01	1.14E+00		
2	5.43E+02	6.99E+01	8.16E+03	5.14E+02	1.23E+03	8.67E+01		
3			0.00E+00		1.05E+00	1.37E+00		
4	0.00E+00				3.94E+00	7.89E+00		
5	1.07E+04	1.15E+03	1.17E+05	1.61E+04	1.09E+05	6.97E+03		
6	6.91E+03	1.20E+03	1.09E+05	7.55E+03	2.34E+04	5.20E+03		
7			0.00E+00		0.00E+00			
8			0.00E+00		1.85E-01	1.01E+00		
Group B								
B1	5.79E+04	6.28E+03	1.50E+05	2.50E+04	1.71E+04	1.79E+03		
B2	1.18E+04	1.51E+03	4.59E+04	4.90E+03	4.29E+03	1.30E+03		
B3	1.43E+04	1.19E+03	3.88E+05	2.53E+04	3.75E+04	1.71E+03		
B4	2.29E+03	3.73E+02	1.12E+04	4.77E+02	3.42E+03	2.05E+02		
B5	2.27E+03	4.72E+01	2.97E+04	5.93E+03			8.50E+02	9.37E+0 1
B6	2.86E+02	6.19E+01	4.46E+03	7.09E+02	1.51E+01	1.64E+01		
Group C								
C1	1.51E+04	4.99E+02	3.19E+05	2.16E+04	7.83E+04	4.27E+03		
Control C3	1.37E+04	5.17E+02	7.74E+05	4.61E+04	2.79E+05	3.54E+04		
C6	6.37E+00	1.98E+00		2.74E+00	3.60E+02	1.70E+02		

Genome copy numbers of ASFV DNA detected in tissues at termination from pigs in Groups A, B and C. Values given are per mg tissue. Two separate DNA extractions were carried out, and DNA from each analysed in duplicate. The values shown are the means and standard deviation is indicated (SD). Standard deviations (SD) are shown for those samples giving values >1.0E+02.

2011b), which had survived the immunization with OURT88/3 and challenge with OURT88/1, were challenged with DRC strain 085/10 at 21 days post-challenge with OURT 88/1. All pigs survived the challenge (see Figure S1). All four pigs showed a slight temperature increase to a maximum of 39.4°C, until the 11th day post-challenge but no other clinical signs. There was a substantial increase of body weight in all of the pigs compared to when they were introduced in the experiment. No or very low levels (9.4 E+01 per mg in lymph from pig 1) ASFV DNA was detected in any tissue collected from those four pigs at termination.

A control group (C) of seven non-immunized pigs were challenged with DRC strain 085/10. Six of the pigs died between days 5 and 7 with typical clinical and post-mortem signs of acute ASF. The presence of ASFV DNA was confirmed in blood as well as other tissues by conventional PCR and for two of the pigs by qPCR. One pig from this control group (3 months of age and 10 kg) survived the challenge and was euthanized at day 21 post-challenge. In this surviving pig, a slight increase in temperature was observed between days 2 and 16 but no other clinical signs were observed. At post-mortem, this pig showed some typical lesions of ASF, consisting of ecchymosis, petechiae, small red and white infarctions on kidneys surface, fibrinous hydro-pericarditis, fibrinous hydrothorax and fibrinous adhesions to adjoining tissues/organs, but no ASFV DNA was detected in spleen and kidney and low levels (3.6×10^2) in lymph nodes.

The copy numbers of ASFV DNA in kidney, spleen and lymph nodes were compared with group C and kidney as references. This showed significantly more copy numbers of ASFV DNA in spleen (negative binomial regression; $P = 0.03$) and less for group A (negative binomial regression; $P = 0.06$) but for the later, P was close to the signification level.

Discussion

In previous experiments up to 100% of pigs immunized with OURT88/3 survived challenge with OURT88/1. The reduced survival observed in the experiment reported here may be due to differences in genetics, size, husbandry system or environment of pigs. In future experiments, the immunization and challenge regimen could be modified to improve the efficacy.

In the control non-immune group all pigs challenged with OURT88/1 developed acute ASF signs and had high levels of virus DNA in blood and tissues. Six of the seven non-immune pigs challenged with DRC085/10, died between days 5 and 7 post-challenge with typical clinical and post-mortem signs of acute ASF and presence of ASFV DNA in blood. This confirms that this is a highly virulent strain supporting field observations.

The results indicate that the OURT88/3 strain may have potential for use as a vaccine in certain regions including DRC. However, extensive further safety testing would be required to evaluate the level and duration of adverse reactions observed in immunized pigs over longer periods and the potential for the strain to persist and be transmitted between pigs. The stability of the virus during passage in pigs should also be confirmed. The high levels of mortality and socio-economic impact of ASF in DRC and other countries where disease is endemic and difficulty associated with control of ASF mean that the deployment of an effective vaccine could have a high impact in both backyard and commercial pig farming.

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References

Arias, M., and J. M. Sanchez-Vizcaino, 2002: African swine fever. In: Morilla, A., K. J. Yoon, and J. J. Zimmerman (eds), *Trends in Emerging Viral Infections of Swine*, pp. 119–124. Iowa State Press, Ames.

Beltran-Alcrudo, D., J. Lubroth, K. Depner, and S. De La Rocque, 2008: African swine fever in the Caucasus. FAO Empres Watch, 1–8. Available at http://www.fao.org/3/a-aj214e.pdf (accessed December 11, 2014).

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.

Boshoff, C. I., A. D. S. Bastos, L. J. Gerber, and W. Vosloo, 2007: Genetic characterisation of African swine fever viruses from outbreaks in southern Africa (1973–1999). *Vet. Microbiol.* 121, 45–55.

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

Chapman, D. A. G., A. C. Darby, M. Da Silva, C. Upton, A. D. Radford, and L. K. Dixon, 2011: Genomic analysis of highly virulent Georgia 2007/1 isolate of African swine fever virus. *Emerg. Infect. Dis.* 17, 599–605.

Gallardo, C., A. R. Ademun, R. Nieto, N. Nantima, M. Arias, E. Martin, V. Pelayo, and R. P. Bishop, 2011a: Genotyping of African swine fever virus (ASFV) isolates associated with disease outbreaks in Uganda in 2007. *Afr. J. Biotechnol.* 10, 3488–3497.

Gallardo, C., R. Anchuelo, V. Pelayo, F. Poudevigne, T. Leon, J. Nzoussi, R. Bishop, C. Perez, A. Soler, R. Nieto, H. Martin, and M. Arias, 2011b: African swine fever virus p72 genotype IX in domestic pigs, Congo, 2009. *Emerg. Infect. Dis.* 17, 1556–1558.

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 (R) PCR assay with internal amplification control for the detection of African swine fever virus. *J. Virol. Methods* 107, 53–61.

King, K., D. Chapman, J. M. Argilaguet, E. Fishbourne, E. Hutet, R. Cariolet, G. Hutchings, C. A. L. Oura, C. L. Netherton, K. Moffat, G. Taylor, M-F. Le Potier, L. K. Dixon, and H. H. Takakmatsu, 2011: Protection of European domestic pigs from virulent African isolates of African swine fever virus by experimental immunisation. *Vaccine* 29, 4593–4600.

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., W. Vosloo, F. Jori, and A. D. S. Bastos, 2013: African swine fever virus eradication in Africa. *Virus Res.* 173, 228–246.

Rowlands, R. J., V. Michaud, L. Heath, G. Hutchings, C. Oura, W. Vosloo, R. Dwarka, T. Onashvili, E. Albina, and L. K. Dixon, 2008: African swine fever virus isolate, Georgia, 2007. *Emerg. Infect. Dis.* 14, 1870–1874.

Wilkinson, P. J., 1999: African swine fever virus. In: Pensaert, M. B. (ed.), *Virus Infections of Porcines*, pp. 17–35. Elsevier Science Publishers BV, Amsterdam, the Netherlands.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Photo of the 4 pigs which survived immunisation with OURT88/3 and challenge with OURT88/1 and DRC85/10 virulent isolates.

