Characterization of pathogenic and non-pathogenic African swine fever virus isolates from *Ornithodoros erraticus* inhabiting pig premises in Portugal

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Ten African swine fever virus isolates from the soft tick *Ornithodoros erraticus* collected on three farms in the province of Alentejo in Portugal were characterized by their ability to cause haemadsorption (HAD) of red blood cells to infected pig macrophages, using restriction enzyme site mapping of the virus genomes and by experimental infection of pigs. Six virus isolates induced haemadsorption and four were non-haemadsorbing (non-HAD) in pig macrophage cell cultures. The restriction enzyme site maps of two non-HAD viruses, when compared with a virulent HAD isolate, showed a deletion of 9–6 kbp in the fragment adjacent to the left terminal fragment and of 1–6 kbp in the right terminal fragment and an insertion of 0–2 kbp in the central region. The six HAD viruses isolated were pathogenic and produced typical acute African swine fever in pigs and the four non-HAD isolates were non-pathogenic. Pigs that were infected with non-HAD viruses were fully resistant or had a delay of up to 14 days in the onset of disease, after challenge with pathogenic Portuguese viruses. Non-HAD viruses could be transmitted by contact but with a lower efficiency (42–50 %) compared with HAD viruses (100 %). The clinical differences found between the virus isolates from the ticks could have implications for the long-term persistence of virus in the field because of the cross-protection produced by the non-pathogenic isolates. This may also explain the presence of seropositive pigs in herds in Alentejo where no clinical disease had been reported.

INTRODUCTION

African swine fever (ASF) is caused by a large DNA virus (ASFV) classified as the only member of the family *Asfarviridae* (Dixon *et al.*, 2000). In Africa, ASF is endemic in many sub-Saharan countries and a reservoir of virus is maintained in wildlife hosts: warthogs, bush pigs and the soft tick vector of the virus, *Ornithodoros moubata*. ASF occurred in Portugal for the first time in 1957 and after an epizootic silence for 2 years persisted as endemic until 1993. In 1957 and 1960, the disease was described as acute causing the death of almost 100 % of the animals in the affected herds. However, subacute forms of disease were increasingly reported in the 1980s and 1990s, especially in the province of Alentejo, in southern Portugal. In addition, inapparent forms of disease were described in which no clinical signs were observed although antibodies against ASFV were detected (Boinas *et al.*, 2001b; Vigario & Caiado, 1989). The last occurrence of ASF in Portugal was a sporadic outbreak in November 1999 in Alentejo (Boinas *et al.*, 2001a; Comissão das Comunidades Europeias, 1999).

When the disease was endemic in the Iberian Peninsula, the most common transmission mechanism was considered to be direct contact with infected pigs including asymptomatic persistently infected pigs (Ordas *et al.*, 1983; Perestrelo Vieira, 1993). Other mechanisms of transmission include ingestion of infected pork products. In southern Portugal and Spain, the long-term persistence of ASFV in the field has also been associated with contact with the argasid tick *Ornithodoros erraticus* (Boinas *et al.*, 2001a; Perez-Sanchez *et al.*, 1994; Sanchez Botija, 1963; Vigario & Caiado, 1989). In one previous study, Sanchez Botija (1963) reported that the viruses they isolated from *O. erraticus* were virulent in pigs and caused haemadsorption (HAD) in pig monocyte cultures. In other studies in the 1980s, ASFV was isolated from ticks in Alentejo in southern Portugal (Louza *et al.*, 1989) and Spain (J. M. Sanchez-Vizcaino, personal communication) but the properties of these viruses were not characterized.

Most ASF virus isolates cause HAD of erythrocytes to infected cells but there are several isolates that do not and
these are referred to as non-haemadsorbing (non-HAD) isolates. This property is used as a diagnostic assay for virus isolation. The ASFV protein, CD2v (Kay-Jackson et al., 2004) responsible for haemadsorption to infected cells is encoded by a gene, EP402R, which is similar to the T-lymphocyte adhesion receptor CD2 (Borca et al., 1994; Rodriguez et al., 1993). The CD2v protein is incorporated into the membranes of virus particles as they bud through the membrane of infected cells, and is therefore probably also involved in the association of extracellular virus particles with red blood cells (Ruiz-Gonzalvo et al., 1996).

In blood from pigs infected with HAD isolates, but not non-HAD isolates, the majority of virus is associated with these findings in understanding the epidemiology of ASF are discussed.

Large deletions and insertions of DNA were found as a result of gain or loss of members of several different multigene families near the left and right genome termini (Almendral et al., 1990; Blasco et al., 1989a, b; Delavega et al., 1990, 1994; Gonzalez et al., 1990; Rodriguez et al., 1994; Yozawa et al., 1994). The European, Caribbean and Cameroon isolates of ASFV are closely related to the Lisbon 60 isolate, which was the second introduction of the virus in Portugal. Other African isolates are more diverse. Viruses resembling Lisbon 57 and 60 are distributed throughout the west and central parts of West Africa (Bastos et al., 2003; Ekue & Wilkinson, 2000; Odemuyiwa et al., 2000; Wesley et al., 1984; Wilkinson et al., 1989, 1993). Isolates from Malawi and Zambia in East Africa are very different from these (Sumption, 1992; Sumption et al., 1990).

In this study, we identified two types of viruses collected from O. erraticus ticks inhabiting pig farms in southern Portugal during the period 1988 until 1993. One type is pathogenic for domestic pigs and causes HAD, and the second type is non-HAD and non-pathogenic. The ability of the isolates to cross-protect and be transmitted by direct contact was characterized, and preliminary characterization of the genomes was carried out. The implications of these findings in understanding the epidemiology of ASF are discussed.

**METHODS**

**ASF viruses.** ASF viruses studied were isolated from O. erraticus collected in three farms in the Alentejo province in Portugal: OUR T88/2, OUR T88/3, OUR T88/4, OUR T88/5, OUR T91/1 and OUR T91/2 from farm 108 in Ourique; OUR T88/1 from farm 106 in Ourique and the isolates MAR T92/1, MAR T93/1 and MAR T93/2 from farm 103 in Castro Verde (Boinas, 1995).

The reference viruses used were a Malawi isolate (Lil 20/1) (Haresnape et al., 1988), Lisbon 57 and 60, Tomar 86 and 87 (supplied by J. Vigário, Laboratorio Nacional de Investigação Veterinaria, Lisbon).

**Preparation of virus samples from pigs.** Blood was collected from the anterior vena cava for whole blood in EDTA. The tissues collected for analysis were weighed and ground up in PBS with sterile sand in a pestle and mortar. Supernatants were collected and stored at −70°C.

**Detection of antibodies in pigs.** ELISA assay was used to detect antibodies against ASFV (Office International des Epizooties, 1996).

**Isolation and titration of virus in pig cell cultures.** Viruses isolated from ticks were characterized by infecting cultures of pig bone marrow (PBM) cells (Malmquist & Hay, 1960; Plowright et al., 1968) and pig blood leukocyte cultures (Martins et al., 1993), and observed to detect haemadsorption of erythrocytes to infected cells and cytopathic effects. Titres of virus were determined as the amount of virus causing haemadsorption (for HAD isolates) or cytopathic effects (for non-HAD isolates) in 50% of infected cultures (TCID50 ml⁻¹ or HAD50 ml⁻¹). These assays gave very similar titres.

HAD viruses isolated from ticks were passaged once, either in pigs or PBM cells. The non-HAD viruses from ticks were from the fourth passage in PBM cells.

**Isolation of ASF viral DNA.** HAD isolates obtained from ticks were used to infect pigs and viral DNA was extracted from infected pig blood (Wesley et al., 1984). PBM cell cultures were infected with the non-HAD viruses from ticks and the HAD virus isolate Tomar 87. Viral DNA was purified from supernatants from infected cultures (Wesley & Pan, 1982).

**Restriction enzyme digestion of ASF virus DNA.** Viral DNA was digested with the restriction enzyme BambH1 (Roche), according to the manufacturer’s recommendations. After digestion, DNA fragments were end-labelled with [³²P]dATP using the Klenow fragment of DNA polymerase I. Fragments were run on 0.6% agarose gels. Gels were dried and exposed to X-ray film. To confirm the genomic location of the variable fragments, Southern blotting was carried out using probes prepared from clones of the BA71V isolate (Ley et al., 1984).

**Infection of pigs.** Serum was collected from cross-bred Large White/Landrace pigs of 20–30 kg live weight, before infection. Each pig was inoculated intramuscularly with 2 ml of suspension with virus titres of log HAD50 ml⁻¹ or TCID50 ml⁻¹ 2·8 to 4·5. Clinical examination and rectal temperatures were recorded each day. Viraemia and antibodies were monitored weekly or when the temperature of pigs rose above 40°C. This was carried out until the animals died or were euthanized or for a minimum period of 3 weeks. Experiments were carried out under Home Office Licence 90/00732.

**Laboratory infection of ticks by feeding on pigs.** Feeding of O. erraticus ticks on vireamic pigs was carried out after they were anaesthetized with pentobarbital-Na (Sagatal) (Boinas, 1995).

**Contact transmission.** Donor pigs were infected as described, and placed in direct contact with healthy recipient pigs. Both groups were monitored for rectal temperature and other clinical signs, viraemia and seroconversion for periods up to 49 days. In addition to this, in two experiments with the virus OUR T88/2 pharyngeal swabs and post-mortem were performed on all the donors. Virus titres were determined in selected lymphatic organs, lungs and, in one case, an ulcer on the rear hook.
RESULTS

Characterization of virus isolates from ticks in PBM cell cultures

Ten ASF viruses were isolated in the period 1988 until 1993, from ticks inhabiting pig premises in the Province of Alentejo in southern Portugal (Boinas, 1995). Ticks were kept in the laboratory between 90 and 1085 days post-collection. The virus was isolated by grinding ticks in PBS and infecting PBM cell cultures with the supernatants obtained. Virus replication was detected either by HAD of red blood cells to infected cells or, for the non-HAD isolates, by cytopathic effect. Infection with ASFV was confirmed by using PCR or direct immunofluorescence. Non-HAD viruses were passaged up to three times in PBM cultures, whereas the HAD viruses were passaged once with the exception of one virus isolate (OUR T91/2), which was passaged twice.

Viruses isolated from the ticks were of two types: OUR T88/1, OUR T91/1, OUR T91/2, MAR T92/1, MAR T93/1 and MAR T93/2 caused HAD and the isolates OUR T88/2, OUR T88/3, OUR T88/4 and OUR T88/5 did not cause HAD (i.e. non-HAD). All of these non-HAD virus isolates were from one farm in Ourique district (farm 108) and these were isolated from ticks collected during three visits to the farm in 1988. Two HAD isolates were also obtained from farm 108 (OUR T91/1 and OUR T91/2) from ticks collected during a single visit in 1991. A single HAD isolate (OUR T88/1) was obtained in 1988 from farm 106 in Ourique and three HAD isolates (MAR T92/1, MAR T93/1 and MAR T93/2) were collected from one farm in Castro Verde district (farm 103) during two visits in 1992 and 1993. The isolates were from ticks collected between 268 and 1070 days after outbreaks of ASF had occurred.

Characterization of virus isolates by restriction enzyme site mapping of the virus genomes

Pigs were infected with HAD isolates and viral DNA was isolated from the red blood cell fraction of infected blood. BamHI digests of viral DNA were end-labelled with $^{32}$P and separated by agarose gel electrophoresis. This identified several fragments that varied in length when genomes of different isolates were compared (Fig. 1a). A BamHI restriction enzyme site map of the Lisbon 60 isolate genome has previously been prepared (Ekue & Wilkinson, 2000) and the restriction enzyme digest patterns were compared with those of the Lisbon 60 and 57 isolates. The genomes of the HAD isolates OUR T88/1 and OUR T91/1, which were isolated from ticks from different farms (farms 106 and 108), had identical fragment sizes (Fig. 1a) and the total genome length was about 179 kbp. Genome locations of

Fig. 1. BamHI restriction enzyme analysis of genomes of Portuguese ASF virus isolates. (a) ASFV DNAs from purified viruses were digested with BamHI and end-labelled with $^{32}$PdTTP. After agarose gel electrophoresis, dried gels were exposed to X-ray film. Isolates, from which DNA was obtained, are indicated. Samples in lanes 1–5, 6–10 and 11–13 were run on the same gels. Molecular mass marker run in parallel is marked M and sizes are shown in kbp. The position of genome fragments that vary from the Lisbon 60 isolate are marked with a letter indicating their genome position (see Fig. 2). (b) Southern blots of BamHI digests of DNA from isolates OUR T88/1 (lanes 1, 4 and 7), OUR T88/2 (lanes 2, 5 and 8) and Lisbon 60 (lanes 3, 6 and 9) are shown. Blots were probed with cloned ASFV DNA from clones HH (lanes 1–3), RA/SC (lanes 4–6) and RB (lanes 7–9). The sizes of molecular mass markers run in parallel are shown in kbp. Fragment genome positions are indicated with a letter (see Fig. 2).
fragments, which differed in length from those in the Lisbon 60 isolate, were confirmed by probing Southern blots of restriction enzyme digests with cloned DNA fragments (Fig. 1b). Isolates OUR T88/1, OUR T91/1 and OUR T91/2 could be distinguished from the Lisbon 60 isolate by difference in length of one fragment, J, in the right terminal region, which was 7.1 kbp in Lisbon 60 and 7.15 kbp in OUR T88/1, OUR T91/1 and OUR T91/2 isolates (Fig. 1b and Fig. 2). OUR T88/1, OUR T91/1 and OUR T91/2 could be distinguished by the mobility of four restriction enzyme fragments (B and C at the left end of the genome, L in the centre and O at the right end) from viruses isolated north of the River Tagus in 1986 from pigs (supplied by J. Vigario, Laboratorio Nacional de Investigação Veterinaria, Lisbon) (Figs 1 and 2) (P. J. Wilkinson, unpublished results). The digestion pattern of one of these isolates, Tomar 86 is shown in Fig. 1a. Isolates MAR T92/1 and MAR T93/1 had identical restriction enzyme fragments and there was no difference in the sizes of the 22 restriction fragments when compared to the Lisbon 60 isolate (Fig. 1a). Thus, the genomes analysed from the HAD isolates obtained from ticks were very similar to the Lisbon 60 isolate but differed significantly in several genome regions from isolates from north of the River Tagus including the Tomar 86 isolate. The genome of the HAD isolate MAR T93/2 was not analysed. The BamHI restriction enzyme fragment patterns of two non-HAD ASF virus isolate (OUR T88/2 and OUR T88/3) genomes differed in the size of the left terminal fragment, which was 19.4 kbp in the OUR T88/2 and 18.6 kbp in the OUR T88/3 isolate (Figs 1 and 2). Thus, it is possible that these two isolates were derived from a common ancestor by gain or loss of sequences within the left terminal fragment. Both of these isolates differed from the genomes of the HAD isolates OUR T88/1, OUR T91/1 and OUR T91/2. In addition to having a larger left terminal fragment, both non-HAD virus isolates had a deletion of 9-6 kbp in the fragment adjacent to the left terminal fragment (fragment C), an insertion of 0-2 kbp in the central region (fragment L) and a deletion of 1-6 kbp from the right terminal fragment (fragment O) (Figs 1 and 2).

The OUR T88/2 and OUR T88/3 isolate genomes were also different from the other Portuguese ASF virus isolates analysed. Southern blotting suggested that the OUR T88/4 isolate contained a mixture of the genomes from isolates OUR T88/2 and OUR T88/3 (data not shown). It is not clear whether this mixture of viruses was present in the tick prior to virus isolation or whether an additional deletion occurred during passage of virus in PBM cells. Thus, the Portuguese isolates analysed fall into three main groups. Two groups of viruses caused HAD. One of these groups of isolates was from the Alentejo region and closely resembled the Lisbon 60 isolate. Thus, these viruses had altered very little over almost 30 years. The second HAD isolate group was from north of the River Tagus. The third group consisted of the non-HAD isolates from Alentejo.

**Stability of virus genomes following passage in ticks.**

Virus was isolated from ticks that were kept alive in the laboratory for 2 years after feeding on a viraemic pig (log HAD50 ml⁻¹ 7-0), which had been infected with a virulent HAD isolate from a pig (Tomar 87). Isolates from different ticks were characterized and all retained the HAD phenotype. Following growth of these viruses in PBM cells, viral DNA was purified. The fragments produced by BamHI digestion of genomes from three of these tick isolates (ticks 9, 16 and 18) had identical sizes, when compared to the virus isolate used to infect the pig (Tomar 87).

**Table 2.** Map position and sizes of BamHI restriction enzymes fragments of Portuguese isolates of ASF virus. A BamHI restriction enzyme site of the Lisbon 60 isolate has been determined previously (Ekue & Wilkinson, 2000). The genome locations of fragments that varied in length from the Lisbon 60 isolate were determined by probing Southern blots of restriction enzyme digests with cloned DNA fragments.
This study indicated that major genome rearrangements did not occur over a 2 year period during virus replication in ticks and that the HAD phenotype was retained.

Pathogenicity of tick virus isolates in domestic pigs

HAD isolates. HAD isolates obtained from ticks were passaged once in PBM cells and inoculated intramuscularly into pigs. Clinical examination and rectal temperatures were monitored each day. Viraemia and the presence of antibodies were monitored. The four HAD viruses tested (OUR T88/1, OUR T91/1, MAR T92/1 and MAR T93/1) were highly virulent producing fever and high viraemia (up to log HAD50 ml\(^{-1}\) 8.6) from day 3 post-infection (p.i.). When the course of disease was allowed to progress, these isolates produced typical acute disease with the death of all pigs infected within 5 to 12 days p.i. (see Table 3).

Non-HAD isolates. Non-HAD isolates OUR T88/3 and OUR T88/4 were inoculated intramuscularly into five pigs and were found to be non-pathogenic (see Tables 1 and 3). Serum antibodies were detected from 14 to 28 days p.i. and persisted until the experiment was terminated. OUR T88/4 produced a transient viraemia in three pigs (RZ7, RZ8 and RZ10) (Table 1), between 4 and 27 days p.i. with low virus titres (log TCID50 ml\(^{-1}\) 1.4–3.2). No viraemia was detected in pigs inoculated with the OUR T88/3 isolate.

Four of the pigs, which were infected with OUR T88/3 and OUR T88/4, were challenged with a pathogenic HAD virus isolated from ticks (OUR T88/1) (Table 1). The challenge virus did not cause clinical signs of disease other than intermittent fever in one of the animals, and did not produce either viraemia or death.

Pigs RY14, RY15, RZ7 and RZ10, which were infected with the isolate OUR T88/3 or that were challenged with the HAD isolate OUR T88/1 and recovered from infection, were re-challenged with the virulent Lisbon 57 isolate. Pigs were either resistant (pig RY15) or there was a delay of up to 14 days in the onset of typical acute disease (pigs RY14, RZ7 and RZ10) (Table 1). The only pig (RY15) that resisted Lisbon 57 challenge had previously been inoculated twice with OUR T88/1 isolate at 35 and 49 days p.i. This pig was subsequently challenged with a pathogenic HAD isolate from Malawi (Lil 20/1). In this pig, the onset of acute ASF was delayed and the pig was moribund and killed at 25 days post-challenge. All the pigs that succumbed to challenge had high viraemia at the time of death (log HAD50 ml\(^{-1}\) 6.0–6.2) and high titres of virus were isolated from the tissues (log HAD50 g\(^{-1}\) 6.0–8.8).

Contact transmission of HAD and non-HAD virus isolates between domestic pigs

HAD isolate OUR T88/1. Two pigs were infected with the virulent HAD isolate OUR T88/1 (Fig. 4). Two non-infected pigs were exposed by contact with these infected donor pigs from the first day post-pyrexia, which was 4 days p.i. The infected donor pigs had viraemia of log HAD50 ml\(^{-1}\) 6.7 at this time. Contact was maintained between the donor and contact pigs for 2 days. Rectal temperatures of the contact pigs were monitored and, at 5 days after the first day of exposure to the infected pigs, rose above 40°C. From day 6 after the first day of exposure, both contact pigs showed typical ASF clinical...
symptoms, developed viraemia of log HAD$_{50}$ ml$^{-1}$ 7–9 and, on the post-mortem, acute ASF lesions and high virus titres were detected in the organs.

<table>
<thead>
<tr>
<th>Pig</th>
<th>Time (days p.i.)</th>
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<tbody>
<tr>
<td>RY14</td>
<td>OUR T88/3</td>
</tr>
<tr>
<td>RY15</td>
<td>OUR T88/4</td>
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<tr>
<td>RZ7</td>
<td>OUR T88/4</td>
</tr>
<tr>
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<tr>
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<tr>
<td>RZ8</td>
<td>OUR T88/4</td>
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<tr>
<td>RZ10</td>
<td>OUR T88/4</td>
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</table>

Non-HAD isolate OUR T88/2. Two separate experiments were carried out to test transmission of the low virulence non-HAD OUR T88/2 isolate to uninfected pigs (Table 2). In one experiment, eight donor pigs were infected and transmission to two contact pigs was tested by constant exposure to the infected pigs from day 3 p.i. until the end of the experiment. In the second experiment, three donor pigs were infected and in constant contact with seven contact pigs from day 0 p.i. until the end of the experiment. In the two experiments, transmission to contact pigs was observed in one of two pigs (50 %) and three of seven pigs (42 %), respectively (Table 2). Titres of virus obtained from various tissues were determined by inoculation of PBM cultures. The tissues examined included head and neck lymph nodes, tonsils, thoracic and abdominal lymph nodes, lungs, spleen, kidneys and any sores seen on limbs. Virus was recovered from the ten donor infected pigs from head and neck lymph nodes, in five donor pigs virus was recovered from prefemoral and iliac lymph nodes, in nine donor pigs from abdominal lymph nodes, in two donor pigs from lung and in one from spleen (Table 2). Viraemia was detected in only one contact pig at 33 days post-contact. With the exception of two pigs, which were killed at 7 days p.i., seroconversion occurred in all the pigs from which virus was detected.
isolated. In the donors, this was detected from day 8 p.i. and in the contact pigs was first detected 21 to 46 days post-contact. These studies show that although pigs infected with the non-pathogenic isolate develop low sporadic viraemia, the virus was transmitted to contact pigs albeit less efficiently than the high virulence HAD isolate. The mode of transmission is not clear but is unlikely to involve infected blood.

Table 2. Transmission of non-HAD virus OUR T88/2 to contact pigs

Pigs were infected with the low virulence OUR T88/2 isolate and transmission of virus to contact pigs was monitored. In experiment 1, eight donor pigs were infected and maintained in contact with two contact pigs from day 3 p.i. In experiment 2, three donor pigs were infected and maintained in constant contact with seven contact pigs from day 0. Both donor and contact pigs were monitored for development of pyrexia (DPI, days p.i.), antibodies against ASFV (seroconversion), viraemia, virus in pharyngeal swabs and virus in tissues. For seroconversion, the day that anti-ASFV antibodies were first detected is indicated as DPI of donors or days post-contact (DPC) of contact pigs. Pigs in which viraemia was detected the titre is given in log TCID_{50} ml^{-1} and DPI or DPC is also indicated. Pigs in which the virus was detected in pharyngeal swabs the titre is given in log TCID_{50} ml^{-1}. Pigs in which the virus was detected in tissues the titre is given in log TCID_{50} g^{-1}. The range of virus and when the pigs were killed are indicated. The virus was isolated from a range of tissues: +, virus was isolated and -, virus was not detected. None, No antibodies against ASFV were detected. K, Pigs were killed to assay for virus. ND, No virus was detected and NT, samples were not tested.

<table>
<thead>
<tr>
<th>Pig</th>
<th>DPI pyrexia</th>
<th>Seroconversion</th>
<th>Viraemia</th>
<th>Virus in pharyngeal swabs</th>
<th>Virus in tissues</th>
<th>Virus present in:</th>
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<td>A* B† C‡ D§ E</td>
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<tr>
<td>81</td>
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<td>86</td>
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<td>2-2 33 DPC</td>
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<tr>
<td>28</td>
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<td>K 40 DPI 1-8-4-1</td>
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<tr>
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<td>NT</td>
<td>K 40 DPI 2-1-5-0</td>
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</tbody>
</table>
| *Head and neck lymph nodes and tonsils
†Thoracic lymph nodes
‡Abdominal lymph nodes
§Spleen
||Lungs
¶Virus was recovered from ulcerative sore on rear hock.
#Some HAD was present.
**Lungs were haemorrhagic.
††One lobe of the lung consolidated and contained log 5-6 TCID_{50} g^{-1}.
Table 3. Characteristics of disease caused in pigs by pathogenic (HAD) virus isolates and non-pathogenic (non-HAD) ASFV isolated from ticks collected in Portugal

Pigs were inoculated intramuscularly with either the HAD pathogenic ASFV isolates or non-pathogenic non-HAD isolates. A summary is shown of the characteristics of the infection with each type of isolate indicating whether the virus causes haemadsorption (HAD), the maximum viraemia detected expressed as log HAD50 ml−1 (HAD isolates) or log TCID50 ml−1 (non-HAD isolates). The duration of viraemia and whether antibodies against ASFV were detected are indicated.

<table>
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<th>Disease</th>
<th>HAD +/-</th>
<th>Maximum viraemia</th>
<th>Duration of viraemia</th>
<th>Seroconversion</th>
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<td>Virulent, high mortality</td>
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<tr>
<td>Avirulent, sub-clinical, no mortality</td>
<td>−</td>
<td>2.3</td>
<td>Sporadic</td>
<td>Yes</td>
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</table>

DISCUSSION

Two very different types of ASF virus were isolated from O. erraticus ticks collected from farms in southern Portugal (Table 3). One type was highly pathogenic and produced typical acute ASF and death, whereas the other was non-pathogenic and caused no clinical signs although antibodies against ASFV were detected in all infected pigs. The pathogenic isolates caused HAD and high viraemia from 3 days p.i. and 100% mortality. Seroconversion was not observed because the pigs died between 5 and 12 days p.i. Transmission of virus from pigs infected with this pathogenic virus to contact pigs was very efficient. Following onset of fever in infected pigs, contact pigs developed acute ASF 6 days later.

The non-HAD virus isolates did not cause clinical disease but sporadic low viraemia was detected and virus was recovered from several lymph nodes (Table 2). Antibodies against ASFV were first detected from 8 days p.i. and persisted over the course of experiments (up to 49 days p.i.). Pigs infected with these isolates were protected against challenge with the pathogenic HAD virus (OUR T88/1), which was isolated from the same farm (Table 1). Less effective protection was achieved when recovered pigs were challenged with more distantly related isolates. Only one pig resisted challenge with the Lisbon 57 isolate, although the onset of clinical disease was delayed in the other pigs challenged with this virus. The onset of clinical symptoms was also delayed when recovered pigs were challenged with the distantly related Malawi LIL 20/1 virus isolate. The Malawi virus genome differs considerably from that of European isolates (Dixon et al., 1994; Sumption et al., 1990; Yanez et al., 1995). Thus, epitopes conferring protective immunity may not be conserved.

Previously non-HAD virus isolates were collected from pigs in the field from the south of Portugal where most of the herds with seropositive pigs were detected (Vigario & Caiado, 1989; Vigario et al., 1974). Non-HAD strains were also frequently reported in Spain, with a total of 206 non-HAD isolates obtained in the period between 1965 and the first semester of 1974 (Sanchez Botija et al., 1977). These non-HAD viruses are more difficult to isolate than HAD viruses since the viraemia they cause is sporadic and virus has mostly been isolated in small amounts from the pigs’ organs. Infection of pig herds with non-pathogenic non-HAD isolates may account for some of the seropositive herds detected in the field in the absence of clinical symptoms. Our experiments showed that pigs infected with non-pathogenic non-HAD isolates can be protected from challenge with closely related virulent isolates. Thus, herds infected with non-pathogenic virus may at least be partially protected from infection with pathogenic virus. Our transmission experiments showed that the non-pathogenic non-HAD isolates were less efficiently transmitted to contact pigs (~40–50% transmission) than the virulent HAD isolate (100% transmission). Nevertheless, contact of inapparent infected pigs with previously uninfected herds could have been an effective mechanism for maintaining ASF in the field in Portugal. The non-HAD isolates we obtained were from O. erraticus ticks. However, it is not clear how efficiently these viruses can infect and persist in the tick population. Pigs infected with non-HAD isolates developed only low and sporadic viraemia. Ticks, which feed on these pigs, may therefore not be efficiently infected with virus. Further studies are required to determine how efficiently the non-HAD isolates can replicate and persist in tick populations. The non-HAD isolates (OUR T88/2 and OUR T88/3) from ticks had large genomic deletions when compared with the HAD tick isolate OUR T88/1 and with the other Portuguese isolates. These deletions consisted of approximately 9 kbp from a region close to the left end of the genome and 1.6 kbp from the right terminal fragment. Although we have not precisely mapped these deletions, those on the left of the genome are in a region encoding members of multigene families 360 and 530. Variation in the number of copies of these multigene families in different ASFV isolates has previously been described (Almendral et al., 1990; Delavega et al., 1990; Gonzalez et al., 1990; Yanez et al., 1995; Yozawa et al., 1994). Members of multigene families 360 and 530 have been demonstrated to act as virulence factors for domestic pigs (Tulman & Rock, 2001; Zsak et al., 2001). Deletion of the UK and DP71L genes from the right end of the genome has also been shown to reduce virulence of the virus for domestic pigs, although these genes are found in the genomes of all isolates analysed (Zsak et al., 1996, 1998). The low pathogenicity of the non-HAD isolates may be related to loss of virulence factors because of these larger
genome deletions close to the left end of the genome or to smaller deletions or substitutions within genes encoding virulence factors elsewhere on the genome.

The loss of the HAD phenotype presumably results from mutations within the EP402R gene, which encodes the CD2v protein. The property of non-HAD does not determine the pathogenicity of an isolate. Non-HAD isolates causing up to 80–90% mortality have been isolated in outbreaks from pigs in South Africa and Madagascar (Gonzague et al., 2001; Pini & Wagenaar, 1974). Non-HAD viruses of reduced virulence have also previously been isolated in Africa (Thomson et al., 1979). Deletion of the gene encoding the CD2v protein from a virulent ASFV isolate delayed the onset of viraemia and the dissemination of virus within pigs, but did not reduce the mortality rate caused by the virus (Borca et al., 1998). This suggests that the reduction in virulence of the non-HAD isolates is because of other factors. Expression of the CD2v protein on extracellular virus particles correlates with the association of the majority of virus with red cells in infected pig blood. Loss of the HAD phenotype may therefore be a factor in the low and sporadic viraemia observed in infections with the non-HAD isolates. However, other factors, which might result in reduced virus replication in blood or tissue macrophages, could also be important.

ASFV does not readily undergo major genome modifications when passed in pigs. This was shown by the similarity of the genome following up to 20 experimental transmission in pigs or after 17–100 passages in pig macrophage cultures (Blasco et al., 1989a, b; Ekue et al., 1989). After 10 passages in PBM cells, the Cameroon virus (CAM 82) became non-HAD and remained non-HAD in the following 17 passages in PBM cells (Ekue, 1989). However, no differences were found in the BamHI and EcoRI restriction enzyme site maps of these genomes when compared to the virus before passage, and no differences in the pathogenicity of the isolate for pigs were reported.

The stability of the virus genome following passage in ticks has not previously been investigated. Here, we compared viruses obtained from three separate ticks, which had been fed on a viraemic pig 2 years previously. We did not detect differences in either restriction enzyme fragments or alteration in HAD phenotype, suggesting that the virus genome is stable in ticks. This type of analysis is relatively crude and there may be an accumulation of nucleotide substitutions in some critical genes, which we have not detected by restriction enzyme fragment analysis.

Viruses reported as attenuated have been isolated from nature in Portugal, but not further characterized other than for pathogenicity. It is known that the vaccine strain used extensively in the south of Portugal in the 1960s caused death of 3.4% of the vaccinated animals, complications in a further 7% and sometimes caused the occurrence of chronic carriers of the virus (Nunes Petisca, 1965a, b). This virus was derived from the Lisbon 60 isolate after being passaged up to 150 times in PBM cells and always showed haemadsorption (Manso Ribeiro et al., 1963).

The virus isolates characterized in this study were collected from areas where extensive vaccination took place in the 1960s (Manso Ribeiro et al., 1963). Since the introduction of the attenuated vaccine strain in the field in Portugal and Spain, chronic forms of ASF have been described and non-HAD virus isolates were also reported at this time (Vigario et al., 1974). One possibility is that the non-pathogenic isolates we obtained from ticks are derived from this attenuated vaccine strain. Alternatively, they may have been derived from the original virulent isolate present in Portugal in 1960 and selected for as a consequence of their ability to persist in the field.

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REFERENCES


