Kinetics of African swine fever virus infection in *Ornithodoros erraticus* ticks

Afonso P. Basto, Rebecca J. Nix, Fernando Boinas, Susana Mendes, Maria J. Silva, Clara Cartaxeiro, Raquel S. Portugal, Alexandre Leitão, Linda K. Dixon and Carlos Martins

1Laboratório de Doenças Infecciosas, CIISA, Faculdade de Medicina Veterinária, Avenida da Universidade Técnica, 1300-477 Lisboa, Portugal
2Institute for Animal Health, Pirbright Laboratory, Ash Road, Pirbright, Surrey GU24 0NF, UK
3Instituto de Investigação Científica Tropical, CVZ, CIISA, Avenida da Universidade Técnica, 1300-477 Lisboa, Portugal

The kinetics of African swine fever virus (ASFV) infection in *Ornithodoros erraticus* ticks were investigated in specimens collected in the field at different times following an outbreak of the disease in Portugal in 1999 and in ticks infected experimentally with a virus isolated from a tick collected during this outbreak. In ticks collected from the field, initial screening for ASFV was carried out by PCR, followed by attempts to isolate the virus in macrophage cultures. Considering total numbers of ticks tested independently of developmental stages, ASFV DNA was detected in 42-3, 26-4 and 22-4 % of specimens collected at weeks 0, 32 and 63 following the outbreak, respectively. Although virus was not isolated from most of these ticks, the proportion of isolations from large nymphs and adults increased between weeks 0 and 32 from 2 to 9 % and from 5 to 11.5 %, respectively. These results, together with the higher virus titres at week 32, suggest that virus replication occurred. In contrast, virus isolations from small nymphs decreased over this period, from 5 to 1.3 %. At week 63, infection rates decreased for all stages. Experimental infections showed the occurrence of virus replication within 4 weeks post-feeding and maintenance of high titres in almost 100 % of ticks until 20 weeks post-infection. At weeks 41 and 61, a drop in virus titres and infection rates was observed. Relevant to the understanding of African swine fever epidemiology, our results show that ASFV replicates and persists in *O. erraticus*, but a viral clearance occurs at later times in both natural and experimental infections.

INTRODUCTION

African swine fever virus (ASFV) is an icosahedral, double-stranded DNA virus classified as the only member of the family Asfivirusidae, genus Asfivirus (Dixon et al., 2005). It infects vertebrate hosts of the family Suidae and argasid ticks of the genus Ornithodoros, and is the only known DNA arbovirus. ASFV causes a highly contagious disease of domestic pigs with a wide range of clinical forms, varying from hyperacute to chronic or unapparent. No vaccine has been developed and disease control is based on stamping out and implementation of rigorous sanitary measures.

In most parts of sub-Saharan Africa, where the disease is enzootic, ASFV persists in nature by a sylvatic cycle of transmission between wild suids (mainly the warthog, *Phacochoerus aethiopicus* and *Ornithodoros moubata* ticks, which infest their burrows (Wilkinson, 1984). Relevant for the maintenance of the virus in the ticks, it was shown previously that ASFV can replicate to high titres in *O. moubata* (Greig, 1972; Kleiboeker et al., 1998, 1999; Parker et al., 1969; Plowright et al., 1970b), and transtadial (Parker et al., 1969), transovarial (Kleiboeker et al., 1999; Plowright et al., 1970a; Rennie et al., 2001) and sexual (Plowright et al., 1974) transmissions were demonstrated.

Outside of Africa, African swine fever (ASF) was first reported in 1957 in Portugal. The disease re-emerged in 1960 (Manso Ribeiro & Azevedo, 1961) and became enzootic in the Iberian peninsula until the early 1990s. During this period, different studies provided evidence that ticks of the species *Ornithodoros erraticus* were associated with persistence and recurrence of the disease in some regions of Portugal and Spain. The first demonstration of a connection between ASFV and *Ornithodoros* ticks was established in Spain by Sánchez Botija (1963), who isolated the virus from *O. erraticus* collected 4 months after an outbreak of disease. Later, an epidemiological study attributed 5 % of the responsibility of ASF cases in Spain to arthropods (Ordás et al., 1983), and Pérez-Sánchez et al. (1994), using serological methods for the screening of farms infested by
O. erraticus showed a statistically significant association between the presence of the argasid and the persistence of ASF in the province of Salamanca. In Portugal, ASFV strains of different virulence have been isolated from ticks collected in the field (Boinas, 1994; Boinas et al., 2004; Louzã et al., 1989).

Experimental studies aiming at characterizing the vector competence of O. erraticus for ASFV confirmed its ability to transmit the virus to susceptible pigs (Boinas, 1994; Endris & Hess, 1992; Sánchez Botija, 1982) and demonstrated virus replication (Boinas, 1994; Endris & Hess, 1992), as well as transtadial (Endris & Hess, 1992) and sexual, but not transovarial (Endris & Hess, 1994), transmissions in O. erraticus. Furthermore, the long-term persistence of ASFV in these argasids was demonstrated. Endris & Hess (1992) showed that O. erraticus is able to harbour the virus and transmit it to pigs for at least 588 days after infection. Boinas (1994) isolated ASFV from ticks collected 2 years after an outbreak on a depopulated farm in Portugal and kept in the laboratory without feeding for a further 3 years. Reporting unpublished results, Sánchez Botija (1982) referred to the persistence of the virus in O. erraticus for up to 8 years. Oleaga-Pérez et al. (1990) suggested around 5 years as the longest period for which tick populations were observed to survive in total fasting after the depopulation of affected pig pens and Encinas et al. (1999) reported that the lifespan of adult ticks might be prolonged for more than 15 years if they occasionally fed on other animal species.

The above-mentioned studies strongly emphasize the need to clarify the risk posed by O. erraticus for the maintenance and recurrence of ASF in the Iberian peninsula. Portugal has been considered free of the disease since 1993 but, in November 1999, a sporadic outbreak occurred in the southern Portuguese region of Alentejo on a farm with no history of ASF for the 9 years prior to the outbreak. All pigs were slaughtered when the outbreak was initially detected. All pigs were slaughtered when the outbreak was finally 63 weeks after the outbreak, when pig pens were destroyed (January 2001, designated week 63). Samples were transported to the laboratory and stored at −70°C until further use. Ticks used for laboratory infections were from an ASFV-free colony started from ticks collected in 2002 on farms in the region of Alentejo, southern Portugal, with no history of ASF for the 9 years prior to the collection.

**Methods**

**Virus isolate.** The ASFV/P99 isolate used for experimental infection was originally obtained from a tick collected on a farm infected during the last ASF outbreak in Portugal in 1999. The virus was isolated and passaged once in blood-derived pig macrophage cultures (PMCs) prior to three passages in bone-marrow PMCs, both obtained as described previously (Leitão et al., 2001; Malmquist & Hay, 1960).

**Tick collection.** O. erraticus individuals used for the assessment of ASFV persistence in the field were collected by using CO₂ traps (Caiado et al., 1990) from the farm where the outbreak in 1999 was initially detected. All pigs were slaughtered when the outbreak was declared and the farm was not restocked. Ticks were first collected at the time of the outbreak (November 1999, hereafter designated week 0), again 32 weeks later (June 2000, referred as week 32) and finally 63 weeks after the outbreak, when pig pens were destroyed (January 2001, designated week 63). Samples were transported to the laboratory and stored at −70°C until further use. Ticks used for laboratory infections were from an ASFV-free colony started from ticks collected in 2002 on farms in the region of Alentejo, southern Portugal, with no history of ASF for the 9 years prior to the collection.

**Colonial maintenance and membrane feeding.** Ticks used for laboratory infections were kept in separate screw-capped plastic containers (Sterilin) with a fine nylon cloth (16 mesh cm⁻²) as a cover to allow equilibrium with the 85% relative-humidity environment of the incubator at a temperature of 27–28°C. Up to 30 ticks were kept in each container with strips of filter paper (Whatman No. 5) folded multiple times. Adult and nymphal stage 5 (N5) ticks were infected by feeding on pig blood containing diluted virus by using sterile glass tick feeders. The method for artificial feeding of O. mouheta (Osborne & Mellor, 1985) was adapted by replacing the silicone membrane with a Parafilm membrane (M'; American National Can Company). The feeder had a water jacket connected to a temperature-controlled water pump to maintain the temperature of the virus suspensions in the inner compartment at 37°C.

**Sample preparation.** Ticks collected from the outbreak farm were classified and separated according to the stage of development into small nymphs (nymphal stages 1–3, N1–N3), large nymphs (stages 4 and 5, N4–N5) and adults (males and females). Individual specimens were ground in porcelain grinders with 1 ml cold PBS supplemented with 1% fetal calf serum (FCS) and antibiotics (100 IU penicillin ml⁻¹ and 100 μg streptomycin ml⁻¹). Suspensions were clarified by centrifugation (5000 g, 1 min, 4°C) and the supernatants were stored at −70°C until further use. Laboratory-fed ticks were surface-sterilized prior to harvesting by using 10% hypochlorite solution. Individual specimens were homogenized by using a syringe and needle in 0.5 ml RPMI medium supplemented with 20% FCS and 100 μg ml⁻¹ each of penicillin and streptomycin and 2.5 μg fungizone ml⁻¹. Homogenates were flash-frozen in liquid nitrogen prior to storage at −70°C.

**Detection of ASFV DNA in field-collected ticks by PCR.** Detection of ASFV DNA in field-collected ticks was performed by using protocols described previously (Basto et al., 2006). Briefly, DNA was extracted from 200 μl of each tick supernatant and recovered in a final volume of 65 μl by using a High Pure Viral Nucleic Acid kit (Roche) following the manufacturer’s instructions. DNA was stored at −20°C until further use. An initial screening was then carried out by PCR using 15 μl DNA extracted from each tick and the pair of primers for the VP72 gene, 72ARs (5' - GAGGCAAGTGATCTTGACAT-3') and 72Ars (5'-TTTCAGGGGTATACAAAGG-3'). In order to evaluate the occurrence of false-negative results due to the presence of PCR inhibitors in DNA samples purified from tick supernatants, some of the PCR-negative ticks were tested again by PCR, but using an internal positive control that is co-amplified with the same primers for VP72. Samples assayed by PCR with internal controls were further tested by transferring 0.5 μl amplification product to a nested PCR in which the primers 72Ns (5'-TACTATCAGCCCCCTCTTGC-3') and 72As (5'-AATGACCTCGGGAT-AAACCAT-3') were used to assess the presence of small amounts of ASFV DNA. To eliminate the possibility of false-positive results caused by carry-over of amplification products, ticks with a PCR-positive result in initial screening from which virus was not isolated on PMCs were further tested by PCR using a set of primers targeting the VP32 gene [VP32s, 5'-CGTGAATTCTTGTTACGGCCG-3', and
VP32as, 5’-GCTTTCGATGATGCTGGG-3’; sequences kindly supplied by Margarida Duarte, Laboratório Nacional de Investigação Veterinária (LNIV), Lisbon, Portugal.

Virus isolation and titration. Virus isolation and titration were performed by using a haemadsorption assay (Malmquist & Hay, 1960) by inoculating limiting dilutions of supernatants either from experimentally infected ticks on bone-marrow PMCs or from PCR-positive field-collected ticks on blood-derived PMCs, as described previously (Martins et al., 1988). Titres were estimated by using the method of Reed & Muench (1938) and expressed as 50% haemadsorbing doses (HAD50) per tick. Isolates of known titre were used as positive controls to ensure no significant variation in susceptibility to infection of cells from different pigs. Uninfected cells were used as a negative control. Samples collected from the field that were initially negative for viral isolation were further tested by up to three sequential passages on PMCs cultivated in 24-well plates (two wells per sample; 100 μl inoculum per well; 5 × 10^5 cells per well). Viral isolation in macrophages showing cytopathic effect (CPE) and/or haemadsorption was confirmed by direct immunofluorescence (DIF) using fluorescein isothiocyanate-conjugated anti-ASFV polyclonal pig sera (kindly supplied by Benedita Cruz, LNIV, Lisbon, Portugal).

Statistical analysis. Differences between means of log_{10} virus titres obtained at each time point following experimental infections were analysed by one-way ANOVA with Tukey’s multiple-comparison post-test. Results with P<0.05 were considered statistically significant.

The same test was used to compare mean titres obtained from ticks membrane-fed with different amounts of virus.

RESULTS

Characterization of O. erraticus populations collected on the farm in Portugal where an ASF outbreak occurred in 1999

The numbers and distribution by developmental stage of ticks collected at the three time points (weeks 0, 32 and 63) from the farm in Alentejo where a sporadic outbreak of ASF occurred in 1999 are summarized in Table 1. Using similar collection procedures during fieldwork, the total number of ticks captured decreased from 1468 at the time of the outbreak to 1084 at week 32 and 512 at week 63 after the outbreak. The proportion of each developmental stage also varied. In the two initial collections, small nymphs were clearly more abundant (51 and 58%, respectively), but at week 63, a significant decrease in specimens of this developmental stage was observed (32%). Numbers of adult ticks were the lowest in all three collections (23, 17 and 20% at weeks 0, 32 and 63, respectively).

Table 1. Analysis of ASFV infection rates in different developmental stages of O. erraticus ticks collected from a farm at different times following an outbreak of ASF

<table>
<thead>
<tr>
<th>Collection</th>
<th>Stage</th>
<th>No. ticks collected</th>
<th>PCR</th>
<th>Isolation</th>
<th>Nested PCR*</th>
<th>ASFV titre (HAD_{50} per tick) on initial titration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. ticks tested</td>
<td>No. positive (%)</td>
<td>No. positive (%)</td>
<td>No. ticks tested</td>
<td>No. positive (%)</td>
<td></td>
</tr>
<tr>
<td>Week 0</td>
<td>N1–N3</td>
<td>752</td>
<td>100</td>
<td>33 (33-0)</td>
<td>5 (5-0)</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>N4–N5</td>
<td>382</td>
<td>100</td>
<td>45 (45-0)</td>
<td>2 (2-0)</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Adult</td>
<td>334</td>
<td>100</td>
<td>49 (49-0)</td>
<td>5 (5-0)</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>1468</td>
<td>300</td>
<td>127 (42-3)</td>
<td>12 (4-0)</td>
<td>60</td>
</tr>
<tr>
<td>Week 32</td>
<td>N1–N3</td>
<td>631</td>
<td>160</td>
<td>26 (16-3)</td>
<td>2 (1-3)</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>N4–N5</td>
<td>265</td>
<td>100</td>
<td>23 (23-0)</td>
<td>9 (9-0)</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Adult</td>
<td>188</td>
<td>156</td>
<td>61 (39-1)</td>
<td>18 (11-5)</td>
<td>20</td>
</tr>
<tr>
<td>Week 63</td>
<td>N1–N3</td>
<td>164</td>
<td>100</td>
<td>13 (13-0)</td>
<td>0 (0-0)</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>N4–N5</td>
<td>246</td>
<td>162</td>
<td>41 (25-3)</td>
<td>3 (1-9)</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Adult</td>
<td>102</td>
<td>100</td>
<td>27 (27-0)</td>
<td>5 (5-0)</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>512</td>
<td>362</td>
<td>81 (22-4)</td>
<td>8 (2-2)</td>
<td>60</td>
</tr>
</tbody>
</table>

*Samples tested by nested PCR were chosen randomly among ticks with a negative result on initial screening PCR.
†Percentage of positive isolations refers to the number of isolations in relation to the total number of ticks initially tested by PCR in each group.
‡Three more isolations on subsequent passages: two on second passage and one on third passage.
§One more isolation on first subsequent passage.
‖Two more isolations on subsequent passages; one on first passage and one on second passage.
Presence of ASFV in field-collected ticks

To assess ASFV infection in ticks collected in the field, individual specimens were tested for the presence of virus DNA by PCR and for the presence of replicative virus by inoculation on cell cultures by using the strategy shown in Fig. 1.

Detection of ASFV DNA from ticks by PCR. To detect ASFV DNA, at least 100 ticks from samples of each developmental stage collected at each of the three time points were initially screened by PCR (Table 1). A high proportion of ticks collected at the time of the outbreak contained ASFV DNA (33·0 % for small nymphs, 45·0 % for large nymphs and 49·0 % for adult ticks). At later collections, a general decrease in the number of ticks positive for ASFV DNA was observed. At 63 weeks after the outbreak, the proportion of positive ticks was 13·0 % for small nymphs, 25·3 % for large nymphs and 27·0 % for adults. At each of the three time points, the proportion of positive ticks was always higher for the later stages of development.

Negative results obtained on initial screening were confirmed by testing 180 samples (20 of each stage from each collection) chosen among the 760 ticks showing an initially negative PCR result. These ticks were retested by PCR in which an internal positive control was used to assess the presence of inhibitors. Internal-control amplification occurred in all of the samples, confirming the initial negative PCR results. Amplification products of these PCRs were further assayed by using a nested PCR for the detection of low ASFV DNA amounts. By this method, 30 out of 180 ticks that previously tested negative on the first-round PCR showed a positive result (Table 1). Although these results were not confirmed by a second method, they point to an advantage of the nested PCR to complement the detection of residual viral DNA in ticks.

ASFV recovery from field-collected ticks with a positive result by PCR. Attempts to isolate ASFV were carried out by inoculation of individual PCR-positive samples onto PMC cells. The results are summarized in Table 1. A decrease of infection rate with time was observed in small nymphs (5 % at week 0, 1·3 % at week 32 and no isolations at week 63), whilst for large nymphs and adults, an increasing proportion of isolations was found from week 0 to week 32 (from 2 to 9 % for large nymphs and from 5 to 11·5 % for adults). From week 32 to week 63, however, a decrease in the number of isolations was also observed on the later developmental stages (1·9 % for large nymphs and 5 % for adults at last collection). Ticks negative on initial PCR, but showing a positive result on nested PCR, were also assayed on macrophage cultures, but no virus isolations were obtained after four passages (data not shown).

Virus titres isolated from individual ticks are summarized in Table 1. In general, titres were low at week 0 (seven ticks with $<10^{2·5}$ HAD$_{50}$ per tick, one tick with $10^{3·1}$ HAD$_{50}$ per tick and four ticks positive only at subsequent passages), higher at week 32 (16 ticks with $<10^{2·5}$ HAD$_{50}$ per tick, 10 ticks with titres ranging from $10^{2·7}$ to $10^{4·3}$ HAD$_{50}$ per tick and three ticks positive only at subsequent passages) and lower again at week 63 (seven ticks with $<10^{2·5}$ HAD$_{50}$ per tick and one tick with $10^{2·9}$ HAD$_{50}$ per tick). The highest titres, almost all obtained at week 32, were observed in...
ticks at the later stages of development. All of the isolates obtained were haemadsorbing and all isolations were confirmed by DIF.

As shown in Table 1, a large majority of PCR-positive ticks were negative by isolation on cells. In order to eliminate the possibility of false-positive results by PCR caused by carry-over contamination with amplification products, PCR results were all confirmed by retesting these samples with primers targeting a different region of the genome (VP32 gene). This confirmed that all PCR results were genuine positives.

**Evaluation of the amount of infectious blood meal ingested by experimentally infected *O. erraticus* ticks**

For experimental infections, *O. erraticus* individuals from a colony maintained in the laboratory were used. Groups of 20 males, females or the last large nymph stage (N5) were membrane-fed on blood meals containing $10^4$ or $10^6$ HAD$_{50}$ ml$^{-1}$ of the ASFV/P99 isolate. These amounts of virus fit the range of viraemia levels observed in infected pigs, which may vary from $<10^0$ to $10^5$ CPE$_{50}$ and/or HAD$_{50}$ ml$^{-1}$, depending on the virulence of the isolate (Forman et al., 1982; Genovesi et al., 1988; Leitão et al., 2001; Villeda et al., 1993). To determine the amount of virus ingested by individual ticks, the mean volume of blood meal ingested was estimated. With this purpose, 20 individual ticks of each of the stages were weighed before and after feeding, but before excretion of coxal fluid. The mean blood meal of the females was 20-21 $\mu$l (SD 18-42), of the males it was 2.47 $\mu$l (SD 2.43) and of the N5 it was 1.86 $\mu$l (SD 3.06). The largest variation between the partially and fully engorged ticks was observed in the females.

Based on the results obtained for the mean volume of blood meal ingested by each stage, the amount of virus fed during experimental infections was estimated. For female ticks, the mean titre of virus ingested would be $10^{4.3}$ HAD$_{50}$ per tick for those fed on a blood meal containing $10^6$ HAD$_{50}$ ml$^{-1}$ and $10^{5.3}$ HAD$_{50}$ per tick for those fed on $10^4$ HAD$_{50}$ ml$^{-1}$. The corresponding figures for male ticks would be $10^{1.4}$ and $10^{1.3}$ HAD$_{50}$ per tick and for N5 would be $10^{3.3}$ and $10^{1.3}$ HAD$_{50}$ per tick.

**ASFV recovery from experimentally infected ticks at various times post-feeding**

At 4, 6, 10, 20, 41 and 61 weeks post-ingestion (w.p.i.), ticks were homogenized and the virus titre present in individual whole-tick extracts was estimated by limiting-dilution inoculation in bone-marrow PMCs.

**Infection rates.** Infection rates for membrane-fed ticks were generally 100% until 20 w.p.i. (Table 2), independent of the virus titre in the blood meal. However, infection rates of between 50 and 88% were recorded for four out of the 23 groups analysed. From the eight groups of ticks analysed at either 41 or 61 w.p.i., infection rates of 100% were recorded in only three groups and the remaining groups of ticks had infection rates of between 0 and 67%. Thus, there was a tendency for the proportion of infected ticks to decrease at 41 and 61 w.p.i., but there was no obvious difference in infection rates among males, females and N5s.

**Viral titres.** Table 2 records the means of log$_{10}$-transformed virus titres recovered from males, females and N5s at different times post-feeding. For ticks of the three developmental stages, membrane-fed with either $10^4$ or $10^6$ HAD$_{50}$ ml$^{-1}$, the amount of virus recovered per tick at the first time point (4 w.p.i.) was in general higher than the amount of virus estimated to be ingested per blood meal, as described above.

Considering that viral titres obtained at each time point were very similar among males, females and N5 nymphs, data from the different developmental stages were combined (Table 2).

A similar pattern in the progression of virus titres with time was observed for ticks fed both titres of virus (blood meal with $10^4$ or $10^6$ HAD$_{50}$ ml$^{-1}$). Mean titres fluctuated from 4.32 to 5.08 log$_{10}$ HAD$_{50}$ per tick between 4 and 20 w.p.i., with a multiple-comparison test showing no significant difference between groups ($P>0.05$). From 20 to 41 w.p.i., a decrease in mean titres to around 3 log$_{10}$ HAD$_{50}$ per tick was observed in ticks infected with both virus titres. A multiple-comparison test showed a statistically significant difference ($P<0.05$) between each of the mean titres obtained in the earlier time points and those of 41 w.p.i. The lower titre-fed ticks were not tested at 61 w.p.i., but for ticks fed the higher titre of virus, the results obtained at this time point were almost identical to those obtained at week 41.

At each time point tested, there was no statistically significant difference ($P>0.05$) between mean titres from ticks fed different blood meals.

**DISCUSSION**

This work comprises two studies aiming at characterizing the kinetics of ASFV infection in *O. erraticus*. One study used ticks collected from a farm at different times (weeks 0, 32 and 63) after the occurrence of the sporadic outbreak of ASF in Portugal in 1999. The pigs were sacrificed at the time of the outbreak and the farm was not restocked during the period of collection. The other study involved experimental infection of *O. erraticus* with the virus isolate obtained from this outbreak (ASFV/P99).

It is known that the ability of ASFV to infect *Ornithodoros* ticks varies widely, depending on the virus isolate and origin of the ticks (Greig, 1972; Kleiboeker et al., 1999; Kleiboeker & Scoles, 2001; Parker et al., 1969; Plowright et al., 1970b). The data from our experimental infections demonstrate clearly that the ASFV/P99 isolate is able to replicate and
Table 2. Analysis of infection rates and ASFV titres recovered at various times post-ingestion from O. erraticus ticks fed on blood meals containing different titres of virus

Ticks were membrane-fed in groups of 20 on pig blood containing two different titres of virus by using sterile tick feeders. Up to the time of harvesting, ticks were maintained at 27–28 °C with a relative humidity of 85%. Only ticks alive at the time of harvesting were used for titration. Titres were estimated by using the method of Reed & Muench (1938). ND, Not done; NA, not applicable.

<table>
<thead>
<tr>
<th>Week p.i.*</th>
<th>Stage</th>
<th>Initial titre of blood meal</th>
<th>Initial titre of blood meal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10⁶ HAD₅₀ ml⁻¹</td>
<td>10⁴ HAD₅₀ ml⁻¹</td>
</tr>
<tr>
<td></td>
<td>No. ticks tested</td>
<td>No. ticks positive (%)</td>
<td>Mean titre†</td>
</tr>
<tr>
<td>4</td>
<td>Female</td>
<td>4</td>
<td>4 (100)</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>2</td>
<td>2 (100)</td>
</tr>
<tr>
<td></td>
<td>N5</td>
<td>7</td>
<td>7 (100)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>13</td>
<td>13 (100)</td>
</tr>
<tr>
<td>6</td>
<td>Female</td>
<td>2</td>
<td>2 (100)</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>2</td>
<td>2 (100)</td>
</tr>
<tr>
<td></td>
<td>N5</td>
<td>5</td>
<td>5 (100)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>9</td>
<td>8 (89)</td>
</tr>
<tr>
<td>10</td>
<td>Female</td>
<td>5</td>
<td>5 (100)</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>2</td>
<td>2 (100)</td>
</tr>
<tr>
<td></td>
<td>N5</td>
<td>5</td>
<td>4 (80)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>12</td>
<td>11 (92)</td>
</tr>
<tr>
<td>20</td>
<td>Female</td>
<td>3</td>
<td>3 (100)</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>1</td>
<td>1 (100)</td>
</tr>
<tr>
<td></td>
<td>N5</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>4</td>
<td>4 (100)</td>
</tr>
<tr>
<td>41</td>
<td>Female</td>
<td>6</td>
<td>6 (100)</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>2</td>
<td>1 (50)</td>
</tr>
<tr>
<td></td>
<td>N5</td>
<td>3</td>
<td>1 (33)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>11</td>
<td>8 (73)</td>
</tr>
<tr>
<td>61</td>
<td>Female</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>1</td>
<td>1 (100)</td>
</tr>
<tr>
<td></td>
<td>N5</td>
<td>3</td>
<td>2 (67)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>4</td>
<td>3 (75)</td>
</tr>
</tbody>
</table>

*Week post-ingestion.
†Log₁₀ HAD₅₀ per tick.

Persist in O. erraticus and that, under laboratory conditions, the threshold for establishment of an active infection in N5 and adult ticks is surpassed when this virus is ingested from a blood meal with a titre equal to or above 10⁴ HAD₅₀ ml⁻¹. The titres of virus recovered from infected ticks at 4 weeks post-experimental feeding increased when compared with the initial amount of virus ingested, indicating that 4 weeks or less is sufficient for virus replication. These higher titres (around 10⁴–10⁵ HAD₅₀ per tick) were then maintained in almost 100% of ticks during the 20 weeks post-feeding, demonstrating the establishment of a persistent infection in the large majority of the tick population. The virus was still present in ticks until the end of the study; however, between 20 and 41 w.p.i., mean viral titres decreased from around 10⁷ to about 10⁴ HAD₅₀ per tick and were maintained at this level at week 61. Depending on the amount of virus initially ingested in blood meals containing 10⁶ versus 10⁴ HAD₅₀ ml⁻¹, infection rates of ticks decreased at 41 w.p.i. to 73 and 29%, respectively, and remained almost identical for the first group at 61 w.p.i. Thus, our results suggest that 20–41 weeks is a critical period when reduction of virus in infected ticks is observed.

The fieldwork enabled, for the first time, a study of ASFV infection in a population of O. erraticus under natural conditions. In this case, PCR was used for the initial detection of ASFV DNA in individual ticks. As shown previously (Basto et al., 2006), in addition to enabling easier and more rapid screening of the population, this method can detect amounts of virus below the limits of what can be detected by virus isolation in cell cultures. Moreover, PCR also enables non-replicating viral DNA to be detected. Screening by PCR detected the presence of viral DNA in a high proportion of ticks collected at the time of the outbreak (33% for small
nymphs, 45% for large nymphs and 49% for adults), indicating that a significant proportion of the tick population had the opportunity to feed on viraemic pigs during this occurrence. However, at this time point, virus could only be isolated from a low proportion of ticks (5% for small nymphs, 2% for large nymphs and 5% for adults). Interestingly, 32 weeks following the outbreak, despite a decrease in numbers of ticks with a positive result by PCR, the proportion of large nymphs and adult ticks from which virus was isolated increased to 9 and 11.5%, respectively, and viral titres recovered also increased. These observations strongly suggest the establishment of an active infection in some ticks of these later developmental stages, with sufficient virus replication taking place between weeks 0 and 32 to enable virus to be isolated in cell cultures. In contrast, a reduction in virus isolations to 1.3% was observed for small nymphs at week 32. The lower likelihood of these stages to become infected was reported previously in *O. erraticus* (Boinas, 1994) and *O. moubata* (Parker et al., 1969; Plowright, 1977; Thomson et al., 1983; Wilkinson et al., 1988). In both situations, the authors suggest that this may be related to the low volume of infected blood meal ingested during feeding. In fact, at the last tick collection, which took place 63 weeks after the outbreak when pig pens were destroyed, virus was not isolated from small nymphs, but it was still present in large nymphs and adults. Infection rates had, however, decreased to 1.9 and 5%, respectively, and the amounts of virus recovered were also lower than at week 32.

Considering the data from our two studies, the low proportions of isolations and low viral titres obtained from PCR-positive ticks collected in the field clearly contrast with the efficiency of infection observed in the laboratory. In fact, positive results by first-round and nested PCR in field-collected ticks from which virus was not isolated suggest that, although ticks ingested viraemic blood, infection was not established. A possible explanation for this may be that the majority of the ticks in the field, with the exception of a small fraction mainly composed of large nymphs and adults, fed on infected pig blood with titres below the minimum dose used in experimental infections (10^4 HAD_{50} ml^{-1}). This might have occurred because ticks did not feed during the peak of viraemia or because the viraemia in pigs had not reached a high level. This may depend on the virulence of the isolate, which is unknown for ASFV/99. Further explanations for the differences found in both contexts may rely on other, as-yet-unknown factors that, under natural conditions, may interfere with the interaction between the virus and ticks, thus affecting the efficiency of the infection.

Despite the above-mentioned differences, both studies agree in two relevant aspects that allow important conclusions about the potential role of *O. erraticus* in the epidemiology of ASF to be drawn. First, considering the fact that virus titres of 10^4–10^5 HAD_{50} per tick persisted up to 20 weeks after experimental feeding and titres of up to 10^4.3 HAD_{50} per tick were found in ticks collected 32 weeks after the outbreak, it is clear that *O. erraticus* may represent a significant risk of viral transmission to pigs when zoosanitary measures are not implemented effectively following ASF outbreaks. Thus, our results support the hypothesis that *O. erraticus* contributed to recurrence of disease in the Iberian peninsula before eradication took place. Second, the results from both studies indicate that, when ticks do not have the opportunity to feed on viraemic pigs for a long time, a reduction in virus titres and infection rates in *O. erraticus* populations is observed.

This phenomenon has been observed previously in experimentally infected *O. erraticus* (Boinas, 1994; Endris & Hess, 1992, 1994), as well as in other *Ornithodoros* species (Greig, 1972; Hess et al., 1987; Haresnape & Wilkinson, 1989). Different factors relevant for this clearance may include loss of infection in individual ticks, increased mortality of infected ticks (Endris et al., 1992; Groocock et al., 1980; Hess et al., 1987, 1989; Rennie et al., 2000) and the absence or inefficiency of transovarial transmission in *O. erraticus* (Endris & Hess, 1994). These factors, together with the vulnerability of ticks to prolonged starvation (Oleaga-Pérez et al., 1990), which is also suggested by the marked decrease in the numbers of ticks captured in the successive collections of our field study, contribute to a reduction in the risk posed by the ticks when pig pens are depopulated for long periods. However, in order to establish whether or when it is safe to restock pig pens after the occurrence of ASF outbreaks, additional studies are required to clarify how ASFV infection evolves further in ticks that remain infected at later times with low amounts of virus, as well as to characterize the capacity of the virus to be transmitted to pigs. In this respect, previous studies have suggested that a titre of 10^4 HAD_{50} per tick in total extracts from *O. moubata* ticks may indicate a threshold for virus transmission to pigs (P. J. Wilkinson & P. S. Mellor, unpublished observations; cited by Haresnape & Wilkinson, 1989) and it is interesting to note that, at the later time points of our studies, all of the positive ticks have shown either a titre below this level or a PCR-positive result with no virus isolation.

In conclusion, our data suggest strongly that *O. erraticus* is to be considered relevant for the maintenance of ASFV in rural pig pens following disease outbreaks. However, in the absence of pigs, this danger is reduced in the long term. Further studies are required to establish safe quarantine periods for restocking after ASF outbreaks, although our results indicate that periods of less than around 40 weeks are likely to present a significant risk of pigs becoming infected by bites from infected ticks.

**ACKNOWLEDGEMENTS**

We would like to thank Professor Philip Mellor and Mr Eric Denison for advice and assistance with laboratory maintenance of ticks and to Dr Benedita Cruz from LNIV, Lisbon, Portugal, for providing some of the tick specimens used in this work. This work was supported by contract no. QLK2-CT-2001-02216, EU, and CIISA, FMV.
REFERENCES


