Virology 393 (2009) 319–328

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

The CD2v protein enhances African swine fever virus replication in the tick vector, *Ornithodoros erraticus*

Rebecca J. Rowlands [a], Margarida M. Duarte [b], Fernando Boinas [c], Geoff Hutchings [a], Linda K. Dixon [a,*]

[a] The Institute for Animal Health, Ash Road, Pirbright, Surrey GU24 0NF, UK
[b] Laboratório Nacional de Investigação Veterinária, Virology Dept. Estrada de Benfica, 701, Lisbon 1459-011 Lisbon, Portugal
[c] Laboratorio de Doencas Infecciosas, CISA, Faculdade de Medicina Veterinaria, Avenida da Universidade Tecnica, 1300-477, Lisbon, Portugal

ARTICLE INFO

Article history:
Received 2 July 2009
Returned to author for revision 28 July 2009
Accepted 31 July 2009
Available online 2 September 2009

Keywords:
African swine fever
Virus
CD2
Pathogenesis
Arthropod vector
Virus replication

ABSTRACT

The NH/P68 non-haemadsorbing (non-HAD) African swine fever virus (ASFV) isolate contains frameshift mutations in the EP402R and adjacent EP153R genes. These encode, respectively, the protein (CD2v) that is required for the haemadsorption (HAD) of swine erythrocytes to ASFV-infected cells and a C-type lectin protein. Two recombinant HAD viruses were constructed in this parental strain. In one of these the intact EP153R gene sequence was restored. Although restoration of the HAD phenotype did not increase virus virulence in pigs, a significant increase was observed in the number of pigs which developed viraemia. These HAD recombinant viruses replicated to titres approximately 1000-fold higher than the parental non-HAD isolate when membrane fed to *Ornithodoros erraticus* ticks. Inoculation of the non-HAD isolate across the gut wall increased viral replication to levels comparable to that of the HAD recombinant viruses. These results demonstrate a novel role for the CD2v protein in virus replication in ticks.

Introduction

African swine fever (ASF) is a severe haemorrhagic fever of domestic pigs, which is caused by a large, cytoplasmic, enveloped, double-stranded DNA virus called African swine fever virus (ASFV). ASFV is the only member the *Asfarviridae* (Dixon et al., 2000). Virulent isolates of ASFV can cause up to 100% mortality in domestic swine (*Sus scrofa*) and result in major economic losses. In Africa, ASF is maintained in a transmission cycle between its natural hosts: warthogs (*Phacochoerus* spp.), bushpigs (*Potomochorus porcus*) and soft ticks of the species *Ornithodoros*, which act as a virus reservoir (Plowright et al., 1969). The virus is highly adapted to these hosts and causes a subclinical persistent infection. The tick vector is thought to play an essential role for virus transmission between warthogs, although direct transmission can occur between domestic pigs without a tick vector (Wilkinson, 1989). ASFV is therefore a risk to pig populations, even in countries that do not contain the arthropod vector (Wilkinson et al., 1988).

Following ingestion of an infectious blood meal, ASFV replicates within the tick midgut epithelium (Kleiboeker et al., 1999) and gains entry into the haemocoel, where it can disseminate to the salivary glands and remaining organs. ASFV infection of *Ornithodoros* ticks is characterised by the establishment of long term, persistent infections. This involves replication in several tissues and is associated with minimal cytopathology (Greig, 1972; Hess et al., 1989; Kleiboeker et al., 1998; Kleiboeker and Scoles, 2001; Kleiboeker et al., 1999; Plowright et al., 1970; Rennie et al., 2000). In *Ornithodoros moubata*, the vector for ASFV in the sylvatic cycle in East and South Africa, transovarial, transtadial and transsexual transmission of virus occurs, demonstrating that the virus is very well adapted to replicate in this tick vector. It is therefore expected that the virus should encode genes which facilitate virus replication in the arthropod host. Given the importance of the tick vector in maintaining a virus reservoir and in transmission, there should be a strong selection for these genes. As yet few studies have been directed to identify these. One study demonstrated a role for members of multigene family (MGF) 360 in increasing ASFV replication in ticks (Burrage et al., 2004). ASFV also replicates in other *Ornithodoros* species and Ornithodoros erraticus acted as a virus reservoir in southern Spain and Portugal. In *Ornithodoros erraticus*, sexual transmission (Boinas, 1994; Endris and Hess, 1994) and transstadial transmission (Endris and Hess, 1992) have been demonstrated, but transovarial transmission has not (Endris and Hess, 1994) suggesting the virus may be less well adapted to replicate in this species. Experimental ASFV infection and transmission have also been demonstrated for *O. savigni* (Mellor and Wilkinson, 1985), *Ornithodoros coriaceus* and *Ornithodoros turicata* (Groocock et al., 1980; Hess et al., 1987), ticks indigenous to America and *Ornithodoros puertoricensis*, indigenous to the Caribbean (Endris et al., 1991; Hess et al., 1987).

* Corresponding author. Fax: +44 1483 232448.
E-mail addresses: rebecca.rowlands@bbsrc.ac.uk (R.J. Rowlands), margarida.duarte@lniv.min-agricultura.pt (M.M. Duarte), fboinas@fmv.utl.pt (F. Boinas), geoff.hutchings@bbsrc.ac.uk (G. Hutchings), linda.dixon@bbsrc.ac.uk (L.K. Dixon).

0042-6822/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2009.07.040

The majority of ASFV isolates cause the haemadsorption (HAD) of red blood cells to infected cells and extracellular virus particles. Less frequently, non-HAD isolates have been described (Pini and Wagenaar, 1974; Thomson et al., 1979). A viral transmembrane protein, which resembles the host CD2 protein, is required for HAD (Borca et al., 1994; Rodriguez et al., 1993). In pigs infected with HAD isolates of ASFV viraemia is primarily erythrocyte associated (Borca et al., 1998; Quintero et al., 1986). The CD2-like protein, designated CD2v (Kay-Jackson et al., 2004), is encoded by the open reading frame (ORF) EP402R. Deletion of this gene from virulent isolates did not reduce virus replication in cells and did not reduce the mortality rate in pigs (Borca et al., 1998). However, the progress of infection differed since the onset of viraemia and dissemination of virus to tissues was delayed compared to infection with the wild-type virus (Borca et al., 1998; Rodriguez et al., 1993; Thomson et al., 1979).

The similarity in structure and function of the extracellular domain of CD2v to the T-cell adhesion molecule CD2 and the observation that CD2v expression is required to suppress the proliferation of lymphocytes in response to mitogens *in vitro* suggest that CD2v plays an important role in evading the immune system of the host (Borca et al., 1998).

The interaction between CD2v and its ligand on red blood cells may be stabilised by expression of an ASFV member of the C-type lectin family of adhesion proteins encoded by the ORF EP153R (Galindo et al., 2000; Haynes et al., 1989; Neilan et al., 1999), since deletion of the EP153R gene has been reported to reduce HAD around ASFV-infected cells (Galindo et al., 2000). EP153R protein has also been shown to inhibit apoptosis (Hurtado et al., 2004).

In this report, we demonstrate that the EP153R and EP402R genes are disrupted in the genome of a natural non-HAD, low virulence ASFV isolate, NH/P68 (Leitao et al., 2001). We demonstrate that the NH/P68 isolate is less efficient in establishing a persistent infection in *O. erraticus* ticks than two recombinant viruses with the HAD phenotype restored by reinsertion of the extracellular domain of the EP402R gene. In one of these HAD recombinants the intact EP153R gene is restored (Rec 4) but not in the other (Rec 34). Furthermore, inoculation of the non-HAD NH/P68 virus across the tick gut wall directly into the haemocoel was shown to increase the titres to which this virus replicates to levels comparable to those observed with the recombinant HAD viruses. These results demonstrate that expression of the CD2v protein significantly increases virus replication in the tick vector at the step of virus uptake or replication in the tick gut. Pigs infected with this recombinant HAD virus did not show any increase in clinical scores compared to those inoculated with the parental NH/P68 virus, although the number of pigs which developed low viraemia was significantly higher. This is one of the first studies to have identified an ASFV protein which has an important role in replication in ticks.

Results

Sequence analysis of the EP153R and EP402R ORFs from the non-HAD NH/P68 isolate

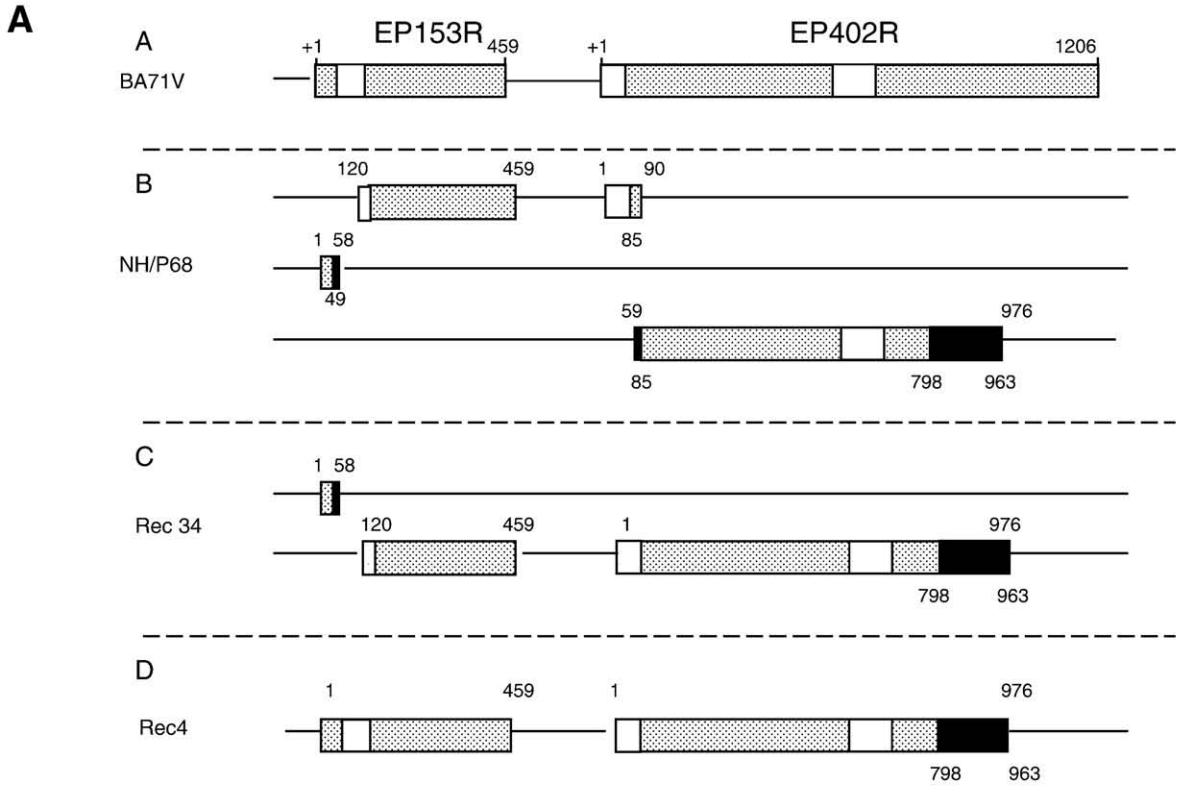
The EP153R and EP402R ORFs are adjacent to each other in the centre of the ASFV genome (positions 56166–55705 and 56242–57450, respectively, on the BA71V genome, accession number U18466). The nucleotide sequence of the fragment of the NH/P68 genome encoding these ORFs was determined and compared with the sequence in HAD isolates to establish the basis of the non-HAD phenotype of NH/P68. This showed that in the NH/P68 isolate genome both of these ORFs were interrupted by frameshift mutations. A single base pair (bp) deletion at position 49 downstream from the ATG codon of the NH/P68 EP153R ORF brings in frame a stop codon at position 58 downstream from the ATG. A second ATG, located 120 bp downstream from the first, is in frame with the remainder of the EP153R ORF and terminates at the same position (Fig. 1). The polypeptide encoded is 113-amino acid long and is truncated at the amino terminus compared to other EP153R predicted proteins. This ORF is unlikely to be efficiently translated as it is downstream of the short ORF that terminates at position 58. In the EP402R ORF of the NH/P68 isolate a single bp deletion at position 85 downstream from the first ATG causes a frameshift shortening this ORF to 90 bp (Fig. 1A). An ATG codon, located 25 bp upstream from this nucleotide deletion, is in frame with the remainder of the coding region of the EP402R protein. This ORF is truncated by 59 bp at the 5′ end compared to the ORFs in the BA71 V and Lisbon 60 isolates. Another two single-bp deletions, located 798 and 963 bp downstream from the first ATG codon, cause frame shifts. The latter deletion brings a stop codon in frame 13 bp downstream. This ORF encodes a polypeptide of 304 amino acids, which lacks the N-terminal signal sequence, has an altered sequence of 55 amino acids within the cytoplasmic domain and is also truncated by 67 amino acids at the C-terminus (Fig. 1A and B). The reason for the loss of HAD phenotype of the NH/P68 parent is most likely because just a short truncated polypeptide is expressed from the first ATG codon of the EP402R gene. The downstream larger ORF is unlikely to be translated into protein efficiently due to the upstream ATG codon and would lack the signal sequences.

Construction of recombinant HAD viruses

Recombinant ASF viruses were generated to examine the effect of restoring the HAD phenotype to the NH/P68 virus on replication in ticks and pathogenesis in pigs. Plasmids containing the full-length intact EP153R and EP402R genes and flanking regions contained on the EcoRI E′ fragment of the HAD viruses Lisbon 60 and Ba71V were transfected into swine macrophages that had been infected with the NH/P68 virus. DNA fragments from these viruses were selected because they are closely related to the NH/P68 sequences and therefore homologous recombination between the transfected plasmids and genome of the NH/P68 virus would be facilitated. Two recombinant viruses were identified by the restoration of their ability to induce HAD and were purified by 10–12 rounds of limiting dilution. Red blood cell rosettes formed in swine macrophages infected with both purified recombinants Rec 34 and Rec 4 were indistinguishable from those formed following infection with the wild-type HAD viruses (data not shown).

Comparison of the nucleotide sequences of EP153R and EP402R ORFs of recombinant HAD viruses Rec 4 and Rec 34

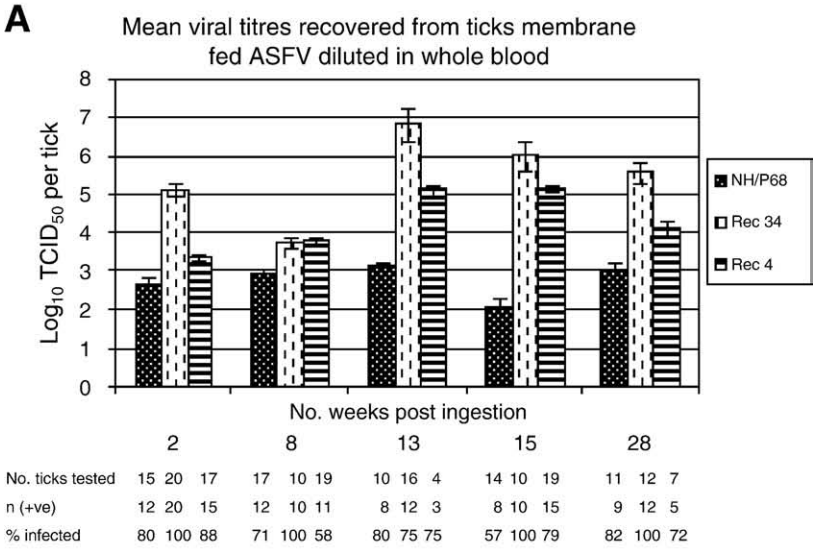
The genome regions containing the EP153R and EP402R genes from Rec 34 and Rec 4 viruses were amplified by PCR and cloned, and the nucleotide sequences were determined (Rec 34: accession number AY463915; Rec 4: accession number AY463916). As shown in Fig. 1, one of the recombinants, Rec 34, had the same N-terminal mutation observed in the EP153R gene of NH/P68 isolate, whereas the other recombinant, Rec 4, had an intact EP153R gene restored (Fig. 1). Thus, the recombination event at the 5′ end of the inserted sequences must have occurred downstream from the EP153R frameshift mutation in Rec 34 virus but upstream from this in Rec 4 virus. The EP402R genes in both recombinant viruses encoded CD2v proteins with the same N-terminal signal peptide, extracellular domain and transmembrane domain as the parental HAD sequences. However, the cytoplasmic domain encoded by both Rec 34 and Rec 4 viruses was the same as in the parental NH/P68 virus and was thus truncated and differing in sequence compared to the proteins encoded by the parental HAD proteins (Fig. 1B). Thus in both Rec 34 and Rec 4 viruses the recombination event at the 3′ end of the inserted sequence must have occurred upstream from the frameshift mutation in the cytoplasmic domain in the NH/P68 EP402R gene. It is not possible to determine the precise site of recombination due to the very close similarity of the sequences in the parental NH/P68 virus and the sequences in the plasmids used to generate recombinants.



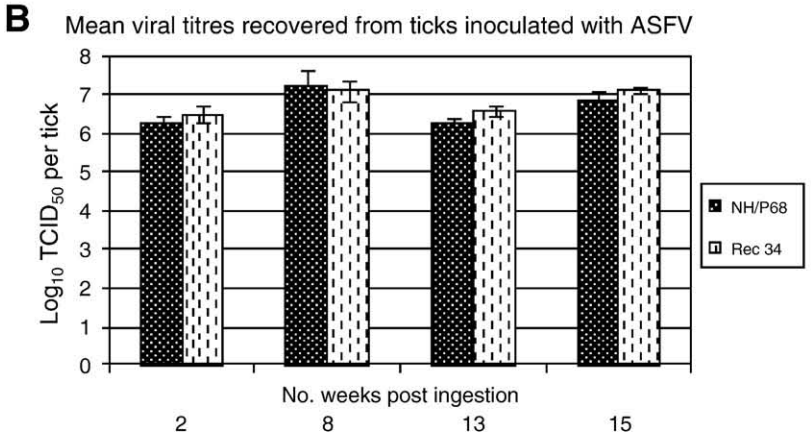
B

```
BA71V     MIIIVIFLMCLKIVLNNIIIWSTLNQTVFLNNIFTINDTYGGLFWNTYYDNNRSNFTYCGIAGNYCSCCGHNISLYNTTNNCSLI   85
rec34     ..........................................................................................  85
rec4      ..........................................................................................  85
NH/P68G                    MEYFKSNCI.....................................................................  65

BA71V     IFPNNTEIFNRTYELVYLDKKINYTVKLLKSVDSPTITYNCTNSLITCKNNNGTNVNIYLIINNTIVNDTNGDILNYYWNGNNNF  170
rec34     ..........................................................................................  170
rec4      ..........................................................................................  170
NH/P68    ....H.....................................................................................  150

BA71V     TATCMINNTISSLNETENINCTNPILKYQNYLSTLFYIIIFIVSGLIIGIFISIISVLSIRRKRKKHVEEIESPPPSESNEEDIS  255
rec34     ..........................................................................................  255
rec4      ..........................................................................................  255
NH/P68    ..........................................................................................  235

BA71V     HDDTTSIHEPSPREPLLPKPYSRYQYNTPIYYMRPSTQPLNPFPLPKPCPPPKPCPPPKPCPPPKPCPPPKPCSPPKPCRPPKPC  340
rec34     ............ENHYFLSLTVVISIIHLFTTCVQHNHSTHYLNHARHLNHVLHSHARHNLSST                          324
rec4      ............ENHYFLSLTVVISIIHLFTTCVQHNHSTHYLNHARHLNHVLHSHARHNLSST                          324
NH/P68    ............ENHYFLSLTVVISIIHLFTTCVQHNHSTHYLNHARHLNHVLHGHARHNLSST                          304

BA71V     PPPKPCPPPKPCPPPKPCPPSKPCPSPESYSPPKPLPSIPLLPNIPPLSTQNISLIHVDRII                           402
```

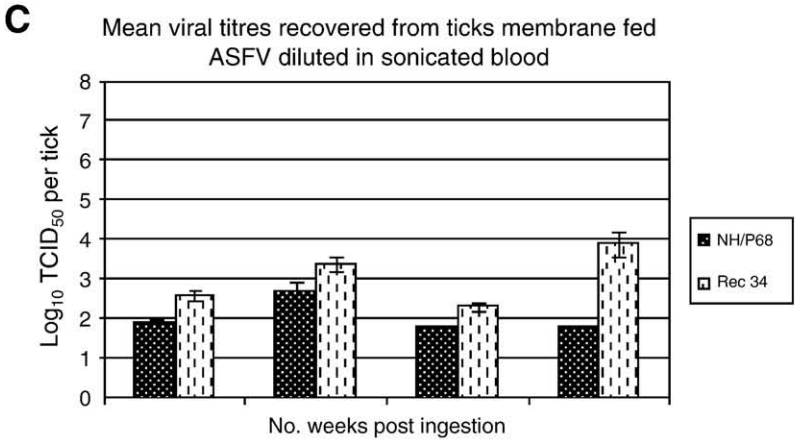
Fig. 1. Comparison of EP153R and EP402R open reading frames from BA71V, NH/P68, Rec 34 and Rec 4 viruses. (A) Predicted open reading frames (ORFs) from the genome region encoding EP153R and EP402R. (A)–(D) show these from the BA71V, NH/P68, Rec 34 and Rec 4 viruses, respectively. Boxes indicate ORFs. White boxes represent signal peptides and transmembrane coding domains. The start and stop positions of ORFs relative to those in the parental HAD BA71V isolate are numbered above the ORFs. The position of nucleotide deletions is indicated below ORFs. Black boxes in the ORFs of NH/P68, Rec 34 and Rec 4 illustrate the regions from which the deduced amino acid sequences have no homology with those encoded by BA71V isolate. (B) The deduced amino acid sequence of the EP402R proteins from BA71V, Rec 34 and Rec 4 viruses.



No. weeks post ingestion	2			8			13			15			28		
No. ticks tested	15	20	17	17	10	19	10	16	4	14	10	19	11	12	7
n (+ve)	12	20	15	12	10	11	8	12	3	8	10	15	9	12	5
% infected	80	100	88	71	100	58	80	75	75	57	100	79	82	100	72



No. weeks post ingestion	2		8		13		15	
No. ticks tested	5	4	7	5	13	10	5	2
n (+ve)	5	4	7	5	10	10	5	2
% infected	100	100	100	100	77	100	100	100



No. weeks post ingestion	2		8		13		15	
No. ticks tested	14	13	16	4	8	16	10	8
n (+ve)	2	10	6	3	2	10	1	5
% infected	14	77	38	75	25	63	10	63

Membrane feeding of O. erraticus with the non-HAD virus NH/P68 and HAD recombinant viruses Rec 4 and Rec 34

O. erraticus ticks were fed the parental non-HAD isolate NH/P68 and recombinant HAD viruses Rec 34 and Rec 4, diluted in heparanized pig blood, across an artificial membrane. Based on the average volume of blood meal ingested by *O. erraticus* ticks, the average titre of virus ingested was estimated to be 4.3 $\log_{10}$ $TCID_{50}$ $tick^{-1}$ ($\log_{10}$ 50% tissue culture infectious dose per tick) for females and 3.4 $\log_{10}$ $TCID_{50}$ $tick^{-1}$ for male and N5 stage nymphs when the blood meal contained 6 $\log_{10}$ $TCID_{50}$ ml^{-1} of virus. Despite differences in the volume of blood meal ingested by males, females and N5 stage nymphs, no difference was observed in the likelihood of a tick becoming infected (Basto et al., 2006; Boinas, 1994). The data for male, female and N5 nymph ticks were therefore grouped together.

Ticks were fed in groups of 20 with virus at a titre 6 $\log_{10}$ $TCID_{50}$ ml^{-1}. At 2, 8, 13 and 15 weeks post-ingestion (WPI) ticks were homogenised and the virus titre present in individual whole tick extracts was measured (Fig. 2A). Mean titres of virus isolated from ticks fed the non-HAD NH/P68 varied between 2.05 and 3.10 $\log_{10}$ $TCID_{50}$ $tick^{-1}$ and no significant differences were observed at the different time points.

At 2 WPI, mean virus titres of 5.13 $\log_{10}$ HAD_{50} $tick^{-1}$ were recovered from ticks fed with the Rec 34 virus. The virus present at this early stage would include virus remaining in the blood meal and virus which had entered ticks cells and initiated replication (Plowright et al., 1970). At 8 WPI, virus recovery decreased to 3.72 $\log_{10}$ HAD_{50} $tick^{-1}$, probably reflecting the disappearance of the blood meal. Persistence of the blood meal in the tick gut was observed for 3–4 weeks post-feeding as has been reported previously (Plowright et al., 1970; Encinas-Grandes, personal communication). Significant increases in virus recovered were observed from 8 to 13 and 15 WPI as estimated by one-way ANOVA followed by Tukey's multiple comparison post-test analysis. At these times mean viral titres of 6.83 and 6.01 HAD_{50} $tick^{-1}$, respectively, were recovered.

Virus titres recovered from ticks fed the recombinant Rec 4 HAD virus also increased significantly from 3.72 HAD_{50} $tick^{-1}$ at 8 WPI to 5.14–5.18 HAD_{50} $tick^{-1}$ at 13 and 15 WPI. No significant differences were observed by one-way ANOVA followed by Tukey's multiple comparison post-test analysis in virus titres recovered from ticks fed with the HAD recombinant viruses, Rec 34 or Rec 4, at each time point. However, virus titres recovered from ticks fed the HAD recombinant viruses were found to be significantly higher, by between 2 and 3 $\log_{10}$ HAD_{50} $tick^{-1}$, than those recovered from ticks fed the non-HAD NH/P68 parental virus at 13 and 15 WPI. This data suggests that the restoration of the HAD phenotype is responsible for increased virus replication in the ticks. Confirmation of this result, using two independently-obtained recombinant HAD viruses, shows that a secondary mutation, introduced during selection of the recombinant viruses elsewhere within the viral genome, was very unlikely to be responsible for these observations. No significant difference was observed in the replication in ticks of the Rec 4 virus, which encodes an intact EP153R gene, compared to the Rec 34 virus, which contains a frameshift mutation in EP153R gene. This demonstrates that restoration of the intact EP153R gene does not significantly increase virus replication in ticks. High infection rates of between 71% and 100% of ticks were generally observed with all viruses.

Inoculation of non-HAD NH/P68 and HAD recombinant Rec 34 viruses into the haemocel of O. erraticus ticks

To investigate whether bypassing the gut wall increases the ability of NH/P68 to replicate within *O. erraticus*, virus was inoculated across the gut wall into the haemocoel of *O. erraticus* ticks. Ticks were inoculated in groups of 20 with 3.55 $\log_{10}$ $TCID_{50}$ per tick of either Rec 34 or NH/P68 viruses and the virus in individual tick homogenates, harvested at 2, 8, 13 and 15 weeks post-inoculation, was measured. This titre was selected as it was similar to the minimum amount of virus estimated to be taken into the gut by a male or N5 nymph along with the blood ingested during a blood meal of titre 6 $\log_{10}$ $TCID_{50}$ ml^{-1}. The infection rates of all groups of ticks inoculated with Rec 34 virus were 100% up to 15 WPI, and three of the four groups of the NH/P68 inoculated ticks also had an infection rate of 100%. At 2, 8, 13 and 15 weeks post-inoculation, mean titres of virus recovered from ticks inoculated with both viruses varied between ~6 and 7 $\log_{10}$ $TCID_{50}$ $tick^{-1}$ (Fig. 2B).

At these times a significant difference was observed between virus titres recovered from ticks that had been membrane fed with NH/P68 virus and ticks that had been inoculated with the same isolate. Mean virus titres within the range ~2–3 $\log_{10}$ $TCID_{50}$ $tick^{-1}$ were recovered from the ticks membrane fed NH/P68 virus (Fig. 2A).

No statistically significant differences were found in virus recovery from ticks that had been inoculated with either NH/P68 or Rec 34 at any of the time points. Thus, the non-HAD NH/P68 virus is able to replicate equally well in ticks as the recombinant HAD virus Rec 34 when inoculated directly into the haemocoel.

Determination if intact red blood cells are required for enhanced virus replication by membrane feeding of HAD virus

The previous results show that the reduced replication of the non-HAD NH/P68 virus in ticks, compared to HAD Rec 34 virus, is due to enhanced uptake of the HAD virus across the tick gut, rather than more inefficient replication within the tick. This observation suggests either that virus uptake is enhanced by binding of extracellular virus particles to red blood cells in the tick gut, or that the CD2v protein enhances virus uptake by binding directly to cells within the tick gut.

To determine if the presence of red blood cells was required to enhance virus replication in ticks, whole pig blood was sonicated to disrupt cells and the membrane fraction was removed by centrifugation. Virus was then mixed with this soluble blood fraction and used to feed ticks across a membrane. The volume of sonicated blood ingested by ticks did not differ significantly from the volume of whole blood ingested (Table 1). For female ticks the average titre of virus ingested would be 4.10 $\log_{10}$ $TCID_{50}$ $tick^{-1}$ for those fed on a sonicated blood meal containing 6 $\log_{10}$ $TCID_{50}$ ml^{-1} of virus and for male and N5 ticks these values would be 3.10 $\log_{10}$ $TCID_{50}$ $tick^{-1}$ and 3.50 $\log_{10}$ $TCID_{50}$ $tick^{-1}$. Ticks were membrane-fed in groups of 20 with either the Rec 34 or NH/P68 isolates diluted in sonicated pig blood at a titre of 6 $\log_{10}$ $TCID_{50}$ ml^{-1}. At 2, 8, 13, 15 WPI ticks were homogenised and the virus titre present in individual whole tick extracts was estimated (Fig. 2C).

The infection rates of ticks membrane fed NH/P68 diluted in sonicated blood were lower than those of the ticks fed Rec 34 virus at all time points tested. The infection rate of the NH/P68 fed ticks was 14% at 2 WPI, increasing to 38% at 8 WPI before decreasing to 10% at 15

Fig. 2. Viral titres recovered from tick homogenates following infection with NH/P68, Rec 4 and Rec 34 viruses. (A) Viral titres recovered from ticks membrane-fed the HAD viruses Rec 34, Rec 4 or non-HAD NH/P68 virus diluted in whole blood. Virus was diluted to 6 $\log_{10}$ $TCID_{50}$/ml and titres recovered from whole tick homogenates ticks at 2, 8, 13 and 15 weeks post-ingestion are shown. (B) Viral titres recovered from ticks inoculated with Rec 34 or NH/P68 viruses. Ticks were inoculated with 3.55 $\log_{10}$ $TCID_{50}$ per inoculum of virus and titres recovered from whole tick homogenates were determined at 2, 8, 13 and 15 weeks post-inoculation. (C) Viral titres recovered from ticks membrane fed Rec 34 or NH/P68 viruses diluted in sonicated pig blood. Virus was diluted to 6 $\log_{10}$ $TCID_{50}$/ml and viral titres recovered from whole tick homogenates at 2, 8, 13 and 15 weeks post-ingestion. Error bars represent the standard error of the mean. The number of ticks positive by virus titration and the overall percentage infected at each time point are shown.

Table 1
Measurement of blood ingested during a blood meal by the developmental stages of *Ornithodoros erraticus*.

Tick developmental stage	Mean volume of sonicated blood ingested (μl)	SEM	Log_{10} $TCID_{50}$ units ingested per virus + sonicated blood meal (6 log_{10} $TCID_{50}$ ml^{-1})
Adult male	1.56	0.23	3.10
Adult female	15.39	2.17	4.10
Nymph N5	1.89	0.37	3.50
	Mean log_{10} HAD_{50} units ingested per sonicated blood meal		3.75

Ticks were fed in groups of 20 of the same sex or developmental stage. Ticks were marked such that they were individually distinguishable and were weighed individually before and after feeding through an artificial membrane. Twenty 50-μl aliquots of sonicated blood and virus mixtures were weighed. The average volume of blood ingested and the standard error of the mean (SEM) are indicated. The titres of virus ingested for two different virus concentrations were also calculated.

WPI. In contrast, the infection rate of ticks fed Rec 34 virus was higher, rising to 90% at 2 WPI before decreasing to 75% at 8 WPI, then 63% at 13 and 15 WPI (Fig. 2C).

Titres of virus recovered from the ticks membrane fed with virus in sonicated blood extracts varied between 1.75 and 2.66 log_{10} $TCID_{50}$ $tick^{-1}$ between 2 and 15 WPI for the NH/P68 virus and between 2.30 and 3.87 log_{10} $TCID_{50}$ $tick^{-1}$ for the Rec 34 virus. A statistically significant decrease in viral titre was observed between titres recovered from ticks that had been membrane fed Rec 34 diluted in whole blood compared to Rec 34 diluted in sonicated blood at both 2 and 15 WPI. In contrast, no statistically significant differences in virus titres recovered from ticks that had been membrane fed NH/P68 diluted in sonicated blood compared to whole blood were observed at any time point tested.

To confirm that virus ingested during feeding with the different blood/virus meals was maintained within the tick, coxal fluid was collected from ticks fed either NH/P68 or Rec 34 isolates diluted in either sonicated or whole blood. Virus was not detected in any of the pooled coxal fluid samples, showing that virus was not excreted into the coxal fluid following the ingestion of the infectious blood meal.

Our data suggests that the presence of intact red blood cells is required in the blood meal to enhance replication of the HAD Rec 34 virus in ticks. This supports the hypothesis that binding of virus particles to red blood cells is important for efficient replication.

Comparison of pathogenesis in pigs following infection with NH/P68 or Rec 4 viruses

The effect of restoring the HAD phenotype of the NH/P68 isolate, by reinserting the EP402R gene, on virus replication and pathogenesis in pigs was tested. Groups of ten pigs were infected with either the non-HAD, nonpathogenic NH/P68 virus or HAD Rec 4 recombinant. Temperatures of pigs and viraemia were monitored daily. As expected from previous experiments (Leitao et al., 2001), pigs infected with the NH/P68 isolate showed no clinical signs and only low, sporadic viraemia was detected (~$10^{2.5}$ TCID 50/ml). None of the pigs showed any of the characteristic clinical signs of acute ASF infection (high fever, loss of appetite, the appearance of reddened or cyanotic areas and bloody diarrhoea). However, there was a significant difference between the numbers of pigs infected with the NH/P68 or Rec 4 viruses that developed a viraemia during the study period (Table 2).

Only three of the NH/P68 infected pigs developed viraemia compared to nine of the Rec 4-infected pigs (Chi Square test 2 = 5.05, $p = 0.025$, assuming that pigs which had not developed viraemia by day 5 would not develop viraemia by day 10). Two of the pigs infected with the Rec 4 virus developed viraemia at day 3 after infection, whereas 4 days post-infection was the earliest day viraemia was observed in pigs infected with the NH/P68 isolate.

After 5 or 10 days, five pigs from each group were killed and a number of tissues collected and the amount of virus present titrated (Table 3). The most obvious differences between the NH/P68 and Rec 4-infected pigs were in the titres of virus from tissues collected at 5 days post-infection. From spleens collected at 5 days post-infection, all the pigs infected with Rec 4 had virus with titres varying between 4 log_{10} and 5.75 log_{10} HAD_{50}/g of tissue. In contrast, only three of the NH/P68 infected pigs had detectable virus in the spleen at 5 days post-infection, and the titre varied from 2 to 3 $TCID_{50}$/g of tissue. The gastrohepatatic lymph node and lungs from all five of the Rec 4 infected pigs sacrificed at day 5 post-infection also contained virus. Titres of between 1.75 log_{10} to 4 log_{10} HAD_{50}/g were detected in the gastrohepatatic lymph node. Of the pigs infected with the NH/P68 virus and sacrificed at 5 days post-infection, three had detectable virus of 2 log_{10} to 4.25 log_{10} $TCID_{50}$/g in the gastrohepatatic lymph node. These results are similar to previous studies on the pathogenesis of the NH/P68 isolate (Leitao et al., 2001). In contrast, in pigs infected with highly virulent ASFV isolates very high levels of viraemia and titres of virus in tissues are observed (7–8 log_{10} HAD_{50} ml^{-1}; Villeda et al., 1993).

Discussion

The aim of this study was to investigate the role of the ASFV CD2v protein, encoded by the EP402R gene, in experimental infection of ticks and pigs. The tick vector plays an essential role in ASFV transmission in the sylvatic cycle involving ticks and warthogs. The virus is well adapted to replicate in the tick vector and infection can persist over periods of years. It is therefore expected that ASFV should encode genes which can enhance virus replication in the tick vector, either by evading the arthropods defence systems or by increasing virus replication and dissemination by other mechanisms. However as yet little is known about such virus genes. In the current study we have definitively shown that the CD2v protein enhances virus replication in the tick vector. This virus protein resembles the host CD2 protein in its extracellular domain and is required for the HAD of red blood cells to ASFV infected cells and extracellular virus particles. For this study, recombinant HAD viruses were generated from a parental non-HAD isolate NH/P68, which has frameshift mutations in the EP153R and EP402R genes encoding a C-type lectin protein and the CD2v protein, respectively. Two HAD recombinant viruses, Rec 4 and Rec 34, were isolated and characterised. Both of these encoded an

Table 2
Detection of viraemia in pigs infected with NH/P68 and Rec 4 viruses at different days post infection.

A Virus	B Days to onset of viraemia	C No. of pigs showing viraemia	D Maximum titre	E Positive samples/ total samples	F Number of animals showing viraemia at each day post-infection[d]									
					1 DPI	2 DPI	3 DPI	4 DPI	5 DPI	6 DPI	7 DPI	8 DPI	9 DPI	10 DPI
NH/P68	4	3/10[c]	>3.00[b]	13/75	0/10	0/10	0/10	2/10	0/10	2/5	2/5	2/5	3/5	2/5
Rec 4	3	9/10[c]	2.25[c]	32/75	0/10	0/10	2/10	7/10	7/10	3/5	3/5	3/5	4/5	3/5

Column A shows the virus isolate used to infect pigs, column B shows the number of days until viraemia was first detected and column C shows the number of pigs which developed viraemia compared to the total number infected. [a]The maximum titre observed in each group. Column D shows the maximum titre observed. [b]The higher titre (>3.00 log_{10} $TCID_{50}$) was observed at day 4 post-infection. [c]The higher titre (2.25 log_{10} HAD_{50}) was observed at days 4, 5, 6 and 9 post-infection. Column E shows the number of positive samples compared to the total analysed. Column F shows the number of animals with viraemia at each day post-infection. [d]Five of the 10 animals were only monitored until day 5 post-infection.

Table 3
Detection of virus in different tissue of pigs infected with NH/P68 and Rec 4 viruses at days 5 or 10 post infection.

A Virus	B Day of collection	C Maximum titre	D Virus titres recovered from different organs									
			Tonsil	Lymph[rf]	Lymph[p]	Lung	Lymph[b]	Lymph[m]	Spleen	Kidney	Lymph[gh]	Lymph[me]	Lymph[f]
NH/P68	5 DPI	4.25	3/5 (2.00)	2/5 (2.60/3.25)	4/5 (2.00–3.00)	0/5	2/5 (1.60, 3.00)	1/5 (2.00)	3/5 (2.00–3.00)	0/5	3/5 (2.00–4.25)	0/5	3/5 (2.00–3.60)
NH/P68	10 DPI	4.00	1/5 (2.00)	4/5 (1.60–3.25)	3/5 (2.00–3.75)	2/5 (2.00, 3.00)	4/5 (1.60–4.00)	2/5 (2.00, 3.00)	4/5 (2.00–3.00)	0/5	1/5 (3.00)	0/5	4/5 (2.00–3.75)
Rec 4	5 DPI	5.75	1/5 (2.00)	2/5 (2.00, 3.00)	3/5 (1.60–3.60)	5/5 (1.25–2.00)	4/5 (1.60–3.00)	1/5 (1.60)	5/5 (4.00–5.75)	1/5 (1.60)	5/5 (1.75–4.00)	3/5 (1.60–3.00)	3/5 (2.00–4.25)
Rec 4	10 DPI	4.25	2/5 (2.00, 3.00)	3/5 (3.60–4.25)	4/5 (2.60–4.25)	2/5 (1.60, 2.60)	4/5 (1.25–3.00)	3/5 (2.00–2.60)	4/5 (1.25–3.00)	0/5	3/5 (2.60–4.25)	2/5 (1.60)	5/5 (2.00–4.00)

Column A shows the virus isolate used to infect pigs and column B the day post-infection (DPI) that samples were collected. Column C shows the maximum titre of virus recovered from any tissue in any pig. Titres are expressed in $\log_{10}$ $TCID_{50}$/g of tissue (NH/P68) and $\log_{10}$ HAD50/g of tissue (Rec 4). Column D shows the number of pigs from which virus was detected in each organ compared to the total number of pigs. The range of virus titres detected in different pigs is shown in brackets. Lymph node designations are as follows: rf, retropharyngeal lymph node; p, parotid lymph node; b, bronchial lymph node; m, medial lymph node; gh, gastrohepatic lymph node; me, mesenteric lymph node; f, prefemoral lymph node.

intact extracellular domain of CD2v but had a frameshift mutation which truncated the cytoplasmic domain. The recombinants differed since the Rec 34 isolate had the same frameshift mutation in the EP153R gene as the parental NH/P68 virus, which would result in a nonfunctional EP153R protein, whereas the Rec 4 virus had the intact EP153R gene restored. Although it has previously been reported that HAD is reduced in cells infected with ASFV from which the EP153R gene has been deleted (Galindo et al., 2000), we did not observe any difference in the relative efficiency of HAD in cells infected with either Rec 4 or Rec 34 compared to the parental HAD viruses Lisbon 60 or BA71V.

Pigs infected with NH/P68 and with Rec 4 viruses were observed over 10 days for clinical signs, fever, viraemia, and dissemination of virus to tissues. As expected no significant increase in clinical signs was observed in pigs infected with Rec 4 virus, confirming that rescue of the HAD phenotype in Rec 4 does not increase the virulence of the NH/P68 virus. One difference observed was that a greater percentage of pigs developed viraemia when infected with Rec 4 compared to NH/P68. In addition, higher virus titres were recovered from some tissues, including spleen and gastrohepatatic lymph nodes, from pigs infected with the Rec 4 virus compared to the NH/P68 virus. Previous data has shown that in pigs infected with HAD virus between 90% and 99% of virus is associated with the red blood cell fraction in contrast to pigs infected with non-HAD virus (Borca et al., 1998; Wardley and Wilkinson, 1977). Also deletion of the EP402R gene from a pathogenic ASFV isolate was shown not to reduce mortality in pigs, although a delay in virus dissemination to tissues was observed (Borca et al., 1998). Our studies compliment this earlier report and suggest that restoration of the HAD phenotype may enhance dissemination of the Rec 4 virus to tissues. Our data also confirm that, as predicted, factors other than loss of expression of the CD2v protein are responsible for the low virulence of the NH/P68 isolate. Sequencing of a region close to the left hand end of the NH/P68 genome showed that it has a large deletion of 8 kbp (kilobase pairs) including the same six multigene family 360 and two multigene family 530 genes that are deleted from the very closely related OURT88/3 nonpathogenic isolate (Chapman et al., 2008).

When membrane fed to *O. erraticus* ticks, Rec 34 and Rec 4 viruses were able to establish persistent infections up to at least 15 WPI and viral titres in excess of 6 $\log_{10}$ $TCID_{50}$ $tick^{-1}$ were recovered. In contrast, significantly lower mean titres of virus (2–3 $\log_{10}$ $TCID_{50}$ $tick^{-1}$) were recovered from ticks membrane fed the non-HAD parental isolate NH/P68. These results suggested that the restoration of the HAD phenotype to NH/P68 virus increased the efficiency of viral replication within *O. erraticus.* No significant difference was observed in the replication of Rec 4 or Rec 34 viruses in ticks, demonstrating that restoration of the intact EP153R gene was not important for enhancing replication in the ticks.

Following inoculation of virus through the gut wall directly into the haemocoel, the efficiency of replication of the NH/P68 isolate in ticks was increased to the same level as observed with the HAD recombinant viruses. Inoculation of NH/P68 virus resulted in recovery of mean titres of virus up to 4 $\log_{10}$ $TCID_{50}$ $tick^{-1}$ greater at each time point compared to those ticks that were membrane fed this isolate. This demonstrated that replication of the non-HAD NH/P68 virus was limited at the stage of uptake or replication in the tick gut. The inoculation of other viral isolates into the haemocel has also been shown to increase their efficiency of replication within the tick (Boinas, 1994; Kleiboeker et al., 1999), suggesting that the replication of ASFV in midgut epithelial cells is critical for establishing a generalised infection and that replication of some ASFV isolates is restricted at this step. Replication may be restricted at the stages of virus entry, replication or release from these cells, resulting in restricted spread to the secondary sites of replication.

Deletion of three multigene (MGF) 360 genes from the virulent Pr4 isolate genome reduced viral replication in infected ticks 100-fold to 1000-fold compared to infection with the parental isolate (Burrage et

al., 2004). These genes are also deleted from the NH/P68 isolate and possibly higher levels of replication may be observed if these genes were also reinserted into the NH/P68 genome.

The mechanism by which restoration of the HAD phenotype increases virus uptake is not clear. One possibility is suggested by previous observations showing, within 24 h of feeding ticks, ASFV virions adsorbed to intact erythrocytes within phagolysosomes of the midgut epithelial cells and the midgut lumen of *O. moubata* ticks. This suggested that one mechanism of initial virus entry to the midgut may be via erythrocyte phagocytosis (Kleiboeker et al., 1999). Our preliminary data suggests that uptake of virions associated with red blood cells may be one mechanism, which accounts for increased replication of the HAD recombinant viruses compared to non-HAD NH/P68 virus. We carried out experiments to compare infections in the presence and absence of red blood cells. In these experiments blood was sonicated and centrifuged to remove red blood cell membranes and ticks were infected by membrane feeding of virus mixed with this soluble blood fraction compared with whole blood. Replication of the Rec 34 HAD virus was significantly reduced when membrane fed to ticks in sonicated blood compared to whole blood whereas no significant difference was observed in replication in ticks when the non-HAD NH/P68 virus was mixed with sonicated blood compared to whole blood. However further experiments are required to verify these results and establish the mechanism by which the CD2v protein enhances virus replication in ticks.

Ticks that were membrane-fed the non-HAD isolate NH/P68 diluted in whole blood contained mean viral titres exceeding the amount of virus initially ingested at all time points tested. This indicates that some viral replication had occurred in these ticks. Furthermore, isolation of non-HAD isolates from ticks collected in warthog burrows (Thomson et al., 1983) and pig farms (Boinas, 1994; Boinas et al., 2004) suggests that non-HAD isolates can enter and replicate in ticks. This suggests that virus can also enter ticks by an alternative mechanism than by phagocytosis of virus particles adhered to red blood cells. Possibly, virus particles could attach to specific receptor sites on the luminal surface of the gut cells before entry and uncoating. The identity of receptor sites for the attachment of ASFV to arthropod cells has not been established, although their existence is partly supported by the fact that ASFV has only been shown to replicate within ticks of the species *Ornithodoros*. Virus could also pass directly into the haemocoel from the gut lumen through lesions or pores between the gut cells formed during the first phase of digestion and without entering the gut cells (Sonenshine, 1993), a phenomenon known as the leaky gut. The contents of the gut have been shown to occasionally leak into the haemocoel during or shortly after feeding (Kleiboeker et al., 1999). The leakage of midgut contents into the haemocoel following feeding is also known to occur in mosquitoes (Hardy et al., 1983). The identification of CD2v's involvement in virus uptake across the tick gut wall is the first example of an ASFV-encoded protein with a direct role in infection of *Ornithodoros spp.*. Direct transmission of ASFV between warthogs occurs infrequently, if at all, and the tick vector plays an essential role in this process. Thus virus genes, such as that encoding the CD2v protein, which increase virus replication in the tick vector must be under strong positive selection in this transmission cycle. The adherence of virus particles to red blood cells may be a more widespread mechanism enabling increased uptake and replication of virus in arthropod vectors.

Methods

Construction of recombinant viruses

ASFV viruses were generated by homologous recombination between NH/P68 parental virus and two plasmids, p1A7 and pRE′, containing the EcoRI E′ fragments of the Lisbon 60 and BA71V ASFV isolates (Ley et al., 1984) These contain the EP153R and EP402R genes and flanking regions. The fragments from these isolates were selected because they are from HAD parental strains which are closely related in sequence to the NH/P68 virus and belong to the same genotype I. Following infection, the primary swine macrophages were transfected, using the FuGene reagent (Roche). Rec 34 and Rec 4 are independent recombinant isolates that were generated by homologous recombination of genome fragments encompassing the EP153R and/or EP402R genes from either ASFV Lisbon 60 or BA71v isolates in macrophages infected with the NH/P68 isolate. The recombinant viruses were identified by rescue of the HAD phenotype and purified from macrophage cell cultures by 10–12 rounds of limiting dilution.

DNA sequencing

DNA sequences were obtained by the dideoxy chain termination method using the universal reverse and M13 primers or internal specific primers. Reactions were run on an automatic sequencer (ABI prisma). The sequences, were assembled and analysed using the FAS programs of the Wisconsin Genetics Computer Group.

Accession numbers

The sequences were deposited in the Genbank and have the following accession numbers: AY463913, AY463914, AY463915, AY463916, AF481875 and AF481876.

Tick collection and colony maintenance

O. erraticus individuals used were collected from five farms in Alentejo, Portugal, which had been unaffected by ASF outbreaks and no ASFV-infected ticks detected. Ticks were kept in screw-capped plastic containers (Sterilin) with a fine nylon cloth (16 mesh cm^{-1}) as a cover to allow equilibrium with the 85% relative humidity environment of the incubator 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 tick feeders. The method for artificial feeding of *O. moubata* (Osborne and 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.

Measurement of blood meal ingestion by O. erraticus

Ticks were anaesthetised with CO_2 gas and 20 individual ticks of each developmental stage were weighed using a precision balance before membrane feeding and again after engorgement but before the excretion of coxal fluid. Fifty-microliter mixtures of blood were weighed and the average taken to calculate the volume of 1 μl of blood so the volume of blood meal ingested could be determined.

Tick inoculation

Prior to inoculation, ticks were anaesthetised with CO_2 gas and transferred to a turntable under a dissecting microscope with a constant stream of CO_2 to maintain anaesthesia. The apparatus used for inoculation of the ticks was described by Boorman (1975). Briefly, glass capillary tunes were moulded into needles by heating and attached to 25-gauge hyperdermic needles. The virus suspension was drawn into the glass capillary needle which was then connected to a source of compressed air at a pressure of three to four pounds per square inch allowing inoculation of the virus into the tick. The inoculum was injected through a cuticular membrane above the base of the IV coxae of the tick. Ticks were inoculated with approximately 1 μl of virus.

Preparation of whole tick homogenates

Ticks were surface sterilised using a 10% sodium hypochlorite solution and followed by two washes in deionised water. Ticks were classified and separated according to the stage of development: large nymphs (nymphal stage 5) and adults (males and females). Individual whole specimens were homogenised in 500 μl RMPI media supplemented with 20% fetal calf serum and 100 μg ml^{-1} each of penicillin and streptomycin and 2.5 μg ml^{-1} of fungizone. Homogenates were flash frozen in liquid nitrogen prior to storage at −70 °C.

Virus titration

Virus titration from ticks fed HAD isolates were performed by haemadsorption assay as previously described (Malmquist and Hay, 1960). Virus titration from ticks fed non-HAD isolate were performed by direct immunofluoresence using an anti-viral p30 mouse monoclonal antibody (a gift of Prof. Jose Escribano, IMA, Madrid) and an Alexa Fluor 488 conjugated goat anti-mouse secondary antibody (Molecular Probes, Oregon).

Tick homogenate titrations were performed by inoculating limiting dilutions of experimentally infected tick homogenates on porcine alveolar macrophages in 96-well tissue culture grade microtitre plates (Sigma). Samples from pig tissue or blood were used to infect porcine bone marrow cells and titrations performed by haemadsorption assay as previously described (Malmquist and Hay, 1960) or cytopathic effect. Titres were estimated using the method of (Reed and Muench, 1938) and expressed as 50% HAD doses (HAD_{50}) or tissue culture infectious doses ($TCID_{50}$) $tick^{-1}$ or ml^{-1}. Tests were carried out to determine whether the presence of sonicated blood affected the titration assays. No difference was observed in the virus titre obtained in the presence and absence of sonicated blood (data not shown).

Statistical analysis

Differences between individual tick $\log_{10}$ virus titres obtained at each time point following experimental infection with virus were analysed by one-way ANOVA followed by Tukey's multiple comparison post-test using the Minitab Statistical Package, Version 15.0 (Coventry, UK). Analysis of the different datasets by ANOVA followed by Tukey's multiple comparison test was carried out to identify statistically significant differences. Results with $p<0.05$ were considered statistically different. No significant difference was considered to occur in groups with $p>0.05$. The same test was used to compare mean titres obtained from ticks membrane-fed with different amounts of virus.

Infection of pigs

Cross-bred Large White/Landrace pigs of 20–30 kg live weight were inoculated intramuscularly with 2 ml of suspension with virus titres of log HAD_{50}/ml or $TCID_{50}$/ml of 4. Clinical examination and rectal temperatures were recorded each day. Viraemia was measured in blood samples collected each day. Blood was collected from the anterior vena cava for whole blood in EDTA. The tissues collected for analysis were weighed and ground up in phosphate buffered saline with sterile sand in a pestle and mortar. Supernatants were collected and stored at −70 °C.

Acknowledgments

We acknowledge financial support from the EU, BBSRC and DEFRA, Dr Simon Gubbins for advice on statistical analysis, Mick Denyer for help with animal experiments and helpful discussions with Prof Philip Mellor and Eric Dennison.

References

Basto, A., Nix, R.J., Boinas, F., Portugal, R.S., Silva, M.J., Cartaxeiro, C., Leitão, A., Dixon, L.K., Martins, C., 2006. The kinetics of African Swine Fever Virus (ASFV) infection in *Ornithodoros erraticus* ticks. J. Gen. Virol. 87, 1863–1871.

Boinas, F. (1994). PhD Thesis, University of Reading.

Boinas, F., Hutchings, G., Dixon, L., Wilkinson, P., 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.

Boorman, J., 1975. Semi-automatic device for inoculation of small insects with viruses. Lab. Pract. 24, 90.

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

Borca, M.V., Kutish, G.F., Afonso, C.L., Irusta, P., Carrillo, C., Brun, A., Sussman, M., Rock, D.L., 1994. An african swine fever virus gene with similarity to the T-lymphocyte surface-antigen Cd2 mediates hemadsorption. Virology 199, 463–468.

Burrage, T.G., Lu, Z., Neilan, J.G., Rock, D.L., 2004. African swine fever virus multigene family 360 genes affect virus replication and generalization of infection in *Ornithodoros porcinus* ticks. J. Virol 78, 2445–2453.

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

Dixon, L.K., Costa, J.V., Escribano, J.M., Rock, D.L., Vinuela, E., Wilkinson, P.J., 2000. In: Regenmortel, M.H.V.V. (Ed.), Family Asfarviridae. London Academic Press, San Diego, CA, pp. 159–165.

Endris, R.G., Haslett, T.M., Hess, W.R., 1991. Experimental transmission of african swine fever virus by the Tick *Ornithodoros* (Alectorobius) *puertoricensis* (Acari, Argasidae). J. Med. Entomol. 28, 854–858.

Endris, R.G., Hess, W.R., 1992. Experimental transmission of african swine fever virus by the soft tick *Ornithodoros* (Pavlovskyella) *marocanus* (Acari, Ixodoidea, Argasidae). J. Med. Entomol. 29, 652–656.

Endris, R.G., Hess, W.R., 1994. Attempted transovarial and venereal transmission of African swine fever virus by the Iberian soft tick *Ornithodoros* (Pavlovskyella) *macrocanus* (Acari: Idodoidea: Argasidae). J. Med. Entomol. 31, 373–381.

Galindo, I., Almazan, F., Bustos, M.J., Vinuela, E., Carrascosa, A.L., 2000. African swine fever virus EP153R open reading frame encodes a glycoprotein involved in the hemadsorption of infected cells. Virology 266, 340–351.

Greig, A., 1972. The localisation of ASFV in the tick *Ornithodoros moubata porcinus*. Arch. Gesamte. Virusforsch. In, pp. 240–247.

Groocock, C.M., Hess, W.R., Gladney, W.J., 1980. Experimental transmission of ASFV by *Ornithodoros coriaceus*, an argasid tick indigenous to the U.S. Am. J. Vet. Res. 41, 591–594.

Hardy, J.L., Houk, E.J., Kramer, L.D., Reeves, W.C., 1983. Intrinsic factors affecting vector competence of mosquitoes for arboviruses. Annu. Rev. Entomol. 28, 229–261.

Haynes, B.F., Telen, M.J., Hale, L.P., Denning, S.M., 1989. Cd44 — a Molecule involved in leukocyte adherence and T-cell activation. Immunol. Today 10, 423–428.

Hess, W.R., Endris, R.G., Haslett, T.M., Monahan, M.J., McCoy, J.P., 1987. Potential arthropod vectors of African swine fever virus in North-America and the Caribbean Basin. Vet. Parasitol. 26, 145–155.

Hess, W.R., Endris, R.G., Lousa, A., Caiado, J.M., 1989. Clearance of ASFV from infected tick (Acari) colonies. J. Med. Entomol. 26, 314–317.

Hurtado, C., Granja, A.G., Bustos, M.J., Nogal, M.L., González de Buitrago, G., de Yébenes, V.G., Salas, M.L., Revilla, Y., Carrascosa, A.L., 2004. The C-type lectin homologue gene (EP153R) of African swine fever virus inhibits apoptosis both in virus infection and in heterologous expression. Virology 15, 160–170.

Kay-Jackson, P.C., Goatley, L.C., Cox, L., Miskin, J.E., Parkhouse, R.M., Wienands, J., Dixon, L.K., 2004. The CD2v protein of African swine fever virus interacts with the actin-binding adaptor protein SH3P7. J. Gen. Virol. 85, 119–130.

Kleiboeker, S.B., Burrage, T.G., Scoles, G.A., Fish, D., Rock, D.L., 1998. African swine fever virus infection in the argasid host, *Ornithodoros porcinus porcinus*. J. Virol. 72, 1711–1724.

Kleiboeker, S.B., Scoles, G.A., 2001. ASFV pathogenesis in its host *Ornithodoros porcinus porcinus*. Anim. Health Res. Rev 2, 121–128.

Kleiboeker, S.B., Scoles, G.A., Burrage, T.G., Sur, J.H., 1999. African swine fever virus replication in the midgut epithelium is required for infection of *Ornithodoros* ticks. J. Virol. 73, 8587–8598.

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

Ley, V., Almendral, J.M., Carbonero, P., Beloso, A., Vinuela, E., Talavera, A., 1984. Molecular cloning of African swine fever virus DNA. Virology 133, 249–257.

Malmquist, W.A., Hay, D., 1960. Hemadsorption and cytopathic effect produced by ASFV in swine bone marrow. Am. J. Vet. Res. 21, 104–108.

Mellor, P.S., Wilkinson, P.J., 1985. Experimental Transmission of African Swine Fever Virus by *Ornithodoros savignyi* (Audouin). Res. Vet. Sci. 39, 353–356.

Neilan, J.G., Borca, M.V., Lu, Z., Kutish, G.F., Kleiboeker, S.B., Carrillo, C., Zsak, L., Rock, D.L., 1999. An African swine fever virus ORF with similarity to C-type lectins is non-essential for growth in swine macrophages in vitro and for virus virulence in domestic swine. J. Gen. Virol. 80, 2693–2697.

Osborne, R.W., Mellor, P.S., 1985. Use of a silicone membrane feeding technique in the laboratory maintenance of a colony of *Ornithodoros moubata*. Trop. Anim. Health Prod. 17, 31–38.

Pini, A., Wagenaar, G., 1974. Isolation of a non-haemadsorbing strain of African swine fever (ASF) virus from a natural outbreak of the disease. Vet. Rec. 94, 2.

Plowright, W., Parker, J., Peirce, M.A., 1969. African swine fever virus in ticks (*Ornithodoros moubata*, Murray) collected from animal burrows in Tanzania. Nature 221, 1071–1072.

Plowright, W., Pierce, M.A., Perry, C.T., Parker, J., 1970. Experimental infection of the Argasid tick *Ornithodoros moubata porcinus*. Arch. Gesamte. Virusforsch. 31, 33–50.

Quintero, J.C., Wesley, R.D., Whyard, T.C., Gregg, D., Mebus, C.A., 1986. In vitro and in vivo association of African swine fever virus with swine erythrocytes. Am. J. Vet. Res. 47, 1125–1131.

Reed, L.J., Muench, H., 1938. A simple method of estimating fifty per cent endpoints. Am. J. Hygiene 27, 493–497.

Rennie, L., Wilkinson, P.J., Mellor, P.S., 2000. Effects of infection of the tick *Ornithodoros moubata* with African swine fever virus. Med. Vet. Entomol. 14, 355–360.

Rodriguez, J.M., Yanez, R.J., Almazan, F., Vinuela, E., Rodriguez, J.F., 1993. African swine fever virus encodes a Cd2 homolog responsible for the adhesion of erythrocytes to infected-cells. J. Virol. 67, 5312–5320.

Sonenshine, D.E., 1993. Biology of Ticks. Oxford University Press, New York. 465 Pages.

Thomson, G., Gainaru, M., Lewis, A., Biggs, H., Nevill, E., Pypekamp, H., Van Der Gerber, L., Esterhuysen, J., Bengis, R., Bezuidenhout, D., Condy, J., 1983. In: Wilkinson, P.J. (Ed.), The relationship between African swine fever virus, the warthog and *Ornithodoros* species in Southern Africa. Office for Official Publications of the European Communities, Luxembourg.

Thomson, G.R., Gaomaru, M.D., Van Dellen, A.F., 1979. ASF: pathogenicity and immunogenicity of two non-haemadsorbing viruses. Onderstepoort. J. Vet. Res. 46, 149–154.

Villeda, C.J., Williams, S.M., Wilkinson, P.J., Vinuela, E., 1993. Hemostatic abnormalities in African swine fever — a comparison of 2 virus-strains of different virulence (Dominican-Republic 78 and Malta 78). Arch. Virol. 130, 71–83.

Wardley, R.C., Wilkinson, P.J., 1977. The association of African swine fever virus with blood components of infected pigs. Arch. Virol. 55, 327–334.

Wilkinson, P.J., 1989. African swine fever virus. In: Pensaert, M.B. (Ed.), Virus Infections of Porcines, pp. Elsevier Science Publishers, Amsterdam, pp. 17–35.

Wilkinson, P.J., Pegram, R.G., Perry, B.D., Lemche, J., Schels, H.F., 1988. The distribution of African swine fever virus isolated from *Ornithodoros moubata* in Zambia. Epidemiol. Infect. 101, 547–564.