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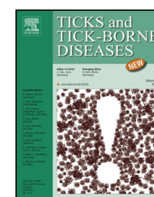

Original article

# No evidence of African swine fever virus replication in hard ticks

Helena C. de Carvalho Ferreira[a,b], Sara Tudela Zúquete[c], Michiel Wijnveld[c], Eefke Weesendorp[a], Frans Jongejan[c,d], Arjan Stegeman[b], Willie L.A. Loeffen[a,*]

[a] Virology Department, Central Veterinary Institute, Part of Wageningen UR (CVI), P.O. Box 65, 8200 AB Lelystad, The Netherlands
[b] Farm Animal Department, Faculty of Veterinary Medicine, University Utrecht, Yalelaan 7-9, 3584 CL Utrecht, The Netherlands
[c] Utrecht Centre for Tick-borne Diseases (UCTD), Faculty of Veterinary Medicine, Utrecht University, Yalelaan 1, 3584 CL Utrecht, The Netherlands
[d] Department of Veterinary Tropical Diseases, Faculty of Veterinary Sciences, University of Pretoria, Private Bag X04, Onderstepoort 0110, South Africa

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ABSTRACT

African swine fever (ASF) is caused by African swine fever virus (ASFV), a tick-borne DNA virus. Soft ticks of the genus *Ornithodoros* are the only biological vectors of ASFV recognized so far. Although other hard ticks have been tested for vector competence, two commonly found tick species in Europe, *Ixodes ricinus* and *Dermacentor reticulatus*, have not been assessed for their vector competence for ASFV. In this study, we aimed to determine whether virus replication can occur in any of these two hard tick species (*I. ricinus* and/or *D. reticulatus*), in comparison with *O. moubata* (the confirmed vector), after feeding them blood containing different ASFV isolates using an improved in vitro system. DNA quantities of ASFV in these infected hard ticks were measured systematically, for 6 weeks in *I. ricinus*, and up to 8 weeks in *D. reticulatus*, and the results were compared to those obtained from *O. moubata*. There was evidence of virus replication in the *O. moubata* ticks. However, there was no evidence of virus replication in *I. ricinus* or *D. reticulatus*, even though viral DNA could be detected for up to 8 weeks after feeding in some cases. This study presents the first results on the possible vector competence of European hard (ixodid) ticks for ASFV, in a validated in vitro feeding setup. In conclusion, given the lack of evidence for virus replication under in vitro conditions, *D. reticulatus* and *I. ricinus* are unlikely to be relevant biological vectors of ASFV.

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## Introduction

African swine fever (ASF) is a highly contagious haemorrhagic disease of swine, caused by African swine fever virus (ASFV), an enveloped double-stranded DNA virus from the family Asfarviridae, genus Asfivirus. Infection usually results in high morbidity and mortality (Costard et al., 2012). Since it was first described (1921), African swine fever has been present in most of sub-Saharan Africa, with most of the incidental introductions into Europe and the Americas eventually resulting in eradication (with the exception of Sardinia). However, after the 2007 ASFV outbreak in Georgia (Rowlands et al., 2008), the disease continued to spread, reaching European neighbouring countries. At the time of writing, ASFV is still circulating in Russia (OIE, 2013), mostly between wild boar and free-ranging domestic pigs (Gogin et al., 2013).

ASFV is a tick-borne virus (Labuda and Nuttall, 2004), and soft ticks (*Ornithodoros* spp.) have been identified as vectors of ASFV. In Africa, an intricate cycle between warthogs (*Phacochoerus africanus*), domestic swine, and *Ornithodoros* ticks (particularly *O. moubata*), is relevant in the maintenance of an endemic infection (Plowright et al., 1994; Thomson, 1985). On the Iberian Peninsula, *Ornithodoros erraticus* has also been associated with disease reoccurrence in a sporadic ASF outbreak in Portugal in 1999 (Boinas et al., 2011). Upon ingestion of blood containing ASFV, ticks may develop a persistent infection, with high virus titres in a number of tissues and organs, both in the *O. moubata/porcinus* complex (Greig, 1972; Kleiboeker et al., 1998; Plowright et al., 1970a, 1970b) and in *O. erraticus* ticks (Basto et al., 2006; Endris and Hess, 1992; Endris et al., 1992).

Moreover, ASFV-infected *Ornithodoros sonrai* ticks (Vial et al., 2007) have been found in the field, and in vitro studies have suggested that several other *Ornithodoros* species such as *O. savignyi* (Mellor and Wilkinson, 1985), *O. puertoricensis* (Hess et al., 1987), *O. turicata* (Hess et al., 1987) and *O. coriaceus* (Groocock et al., 1980) can also act as vectors of ASFV. However, none of the latter ticks

* Corresponding author. Tel.: +31 320 238696; fax: +31 320 238668.
*E-mail addresses:* helena.c.c.ferreira@gmail.com (H.C. de Carvalho Ferreira), willie.loeffen@wur.nl (W.L.A. Loeffen).

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has yet been confirmed as vector for the transmission of ASFV in the field.

ASFV vector competence of hard ticks (Ixodidae) has been assessed on *Rhipicephalus* spp. (Sanchez Botija, 1963), *Rhipicephalus simus* (Plowright, 1977; Plowright et al., 1994), *Rhipicephalus bursa* (Kovalenko et al., 1967; Plowright et al., 1994), *Amblyomma variegatum* (Plowright, 1977), *Hyalomma* spp. (Plowright et al., 1994), *Amblyomma americanum* and *Amblyomma cajennense* (Groocock et al., 1980) either by experimental infection or by field collection to determine the presence of ASFV. Field specimens were negative for ASFV, and although ASFV could be detected in *R. simus* nymphs (Plowright et al., 1994) for up to 5–6 weeks, and in both *A. americanum* and *A. cajennense* for 4–7 days after a viraemic blood meal, no hard ticks transmitted ASFV to susceptible pigs after experimental infection. Given that some hard ticks may carry ASFV for some time, it is possible that they may act as mechanical vectors; similar to the European stable fly, *Stomoxys calcitrans*, which has been shown to mechanically transmit ASFV to pigs up to 24 h post-infective meal (Mellor et al., 1987).

Of the hard ticks studied so far, only *Hyalomma* spp. and *Rhipicephalus bursa* are present in Europe (Estrada-Peña et al., 2012). However, no studies have been published addressing replication or maintenance of ASFV in other ticks commonly reported in Europe, such as *Ixodes ricinus* (Medlock et al., 2013) and *Dermacentor reticulatus* (Estrada-Peña et al., 2012). Both tick species are known to be involved in the epidemiology of other tick-borne viruses; i.e. with *I. ricinus* being a vector of tick-borne encephalitis virus (TBEV) and *D. reticulatus* being a vector of Omsk haemorrhagic fever virus (OHFV) (Jongejan and Uilenberg, 2004; Labuda and Nuttall, 2004).

However, feeding habits differ between the two tick species. *Ixodes ricinus* ticks feed on a wide range of hosts, including domestic and wild pigs (Farkas et al., 2013). In contrast, *D. reticulatus* host preference strongly depends on life stage, with adults feeding on larger mammals and nymphs mostly on small mammals (Farkas et al., 2013). Both tick species could be involved in ASFV transmission either via mechanical transmission by interrupted feeding (by adult males) or via biological transmission, either transovarially (via ASFV-infected females) or transstadially (via ASFV-infected *I. ricinus* nymphs).

In this study, *I. ricinus*, *D. reticulatus*, and *O. moubata* (one of the confirmed vector species) were fed in vitro with blood containing different ASFV isolates. DNA quantities of ASFV in these hard ticks were measured systematically, for 6 weeks in *I. ricinus* and up to 8 weeks in *D. reticulatus*, and the results were compared to those found in *O. moubata*. The purpose of this comparison was to examine if replication can occur in both species of hard ticks. Such knowledge is relevant to better understand the possible role these hard ticks could play as biological or mechanical vectors of ASFV.

## Materials and methods

### *Ticks*

*Ixodes ricinus* and *D. reticulatus* ticks originated from the Netherlands, whereas *O. moubata* was obtained from a laboratory colony maintained in Israel. Both hard tick species were maintained at the Utrecht Centre for Tick-borne Diseases (UCTD) at 23 °C and 85% relative humidity, created by a saturated potassium chloride solution, and a 12-h photoperiod. The experiments were carried out in late spring and summer. The ticks were tested by PCR and reverse line blot hybridization for pathogens prior to the start of the experiments (Gubbels et al., 1999). Specific pathogen-free, laboratory-reared *I. ricinus* nymphs ($n = 3360$), *D. reticulatus* adults ($n = 720$, 1:1 male to female ratio), age 3–6 months post moulting, and *O. moubata* nymphs of different developmental instars, namely the third nymphal instar (N3, $n = 300$) and fourth nymphal instar (N4, $n = 300$) were used. Between feeding and sample collections, hard ticks were maintained at 20–24 °C, with a relative humidity (RH) of 85%. *Ornithodoros moubata* ticks were maintained at 27–28 °C, with a RH of 85–90%.

**Table 1**
Viruses used for acquisition feeding.

| ASFV isolate | Matrix | ASFV titre ($\log_{10}$ $TCID_{50}$) | Reference |
|---|---|---|---|
| OURT 88/1 | Spleen homogenate | 5.4 | Boinas et al. (2004) |
| LIV 13/33 | Spleen homogenate | 5.5 | Rennie et al. (2000) |
| Georgia 2007/1 | Defibrinated blood | 5.9 | Rowlands et al. (2008) |
| Malta'78 | Defibrinated blood | 5.6 | Wilkinson et al. (1980) |
| Netherlands'86 | Defibrinated blood | 5.4 | Terpstra and Wensvoort (1986) |
| Brazil'78 | Defibrinated blood | 5.4 | Mebus et al. (1978) |

### *Virus*

Six ASFV isolates were used in our study. Two were originally isolated from ticks in Portugal (OURT 88/1) and in Zambia (LIV 13/33), respectively, and four were isolated from infected pigs: Georgia 2007/1, Malta'78, Netherlands'86, and Brazil'78. Virus stocks were obtained from previous animal experiments, either by collecting defibrinated blood or by preparing spleen homogenates from infected pigs (Table 1).

### *Preparation of blood for in vitro feeding*

Pig blood was collected weekly from a local slaughterhouse. Immediately after collection, the blood was stirred rapidly with a glass pipette for 15–30 min, and the fibrin clot that attached to the glass pipette was removed. The defibrinated pig blood was stored at 4 °C. During acquisition feeding, the defibrinated pig blood was spiked daily with the six different virus suspensions, resulting in a 1:10 dilution of the original ASFV titres.

### *In vitro feeding units*

The improved in vitro system used in this study, recently described by Fourie et al. (2013), results from several adaptations of a system described previously (Kröber and Guerin, 2007a, 2007b). In our system, silicone membranes were impregnated with cattle odour immediately before being fixed to the bottom of hollow plastic tubes, with a diameter of approximately 3.5 cm. Ticks were placed inside the feeding unit, on top of the feeding membrane, and forced to remain near the membrane of the feeding unit by a movable plastic lid that permitted air circulation. A volume of 3.1 ml of pig blood per well was pipetted into 6-well cell culture plates (Greiner Bio-One, Frickenhausen, Germany), and feeding units were fitted into these wells.

### *Acquisition feeding of Ornithodoros moubata (soft ticks)*

Each acquisition feeding consisted of 12 feeding units (as described above), one for each combination of ASFV isolate with each *O. moubata* nymphal instar (N3 or N4). Each feeding unit contained approximately 50 *O. moubata* nymphs. Plates with feeding

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