The importance of experimental design for studying the vector competence of *Ornithodoros* ticks for African swine fever virus: a meta-analysis of published evaluations

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Abstract

African Swine Fever (ASF) is a highly economically devastating viral disease for swine. Soft ticks of the genus *Ornithodoros* are involved in its epidemiology, as vectors and natural reservoirs of African Swine Fever Virus (ASFV). This constitutes a significant problem as it is currently challenging to eliminate endophilous soft ticks living in inaccessible, sheltered habitats. The vector competence of *Ornithodoros* ticks for ASFV has been mainly studied by mimicking natural tick-to-pig and tick-to-tick transmission pathways through experimental infections in the laboratory. However, experimental designs are anticipated to exert significant influence on the results, owing to inherent technical limitations of the approaches. By reviewing the original research studies dating back to the 1960s on the vector competence of *Ornithodoros* for ASFV, we clarified the processes involved in ASFV maintenance and transmission by ticks, emphasized the extensive range of protocol designs employed and subsequently analysed the all-encompassing factors impacting vector competence. Our study demonstrated that the success of tick infection is primarily impacted by experimental factors, whereas the impact of natural determinants is inconclusive. Our results offer valuable insights into how future trials can be refined by curtailing potential experimental biases. They call for caution when interpreting conclusions drawn from studies on ASFV vector competence.

Keywords: *Ornithodoros* soft ticks, African swine fever virus, vector competence, methodology, meta-analysis

Introduction

African swine fever (ASF) ranks among the most damaging viral diseases affecting swine farming. The etiologic agent is a large DNA virus belonging to the Asfaviridae family (Dixon *et al.*, 2013). African Swine Fever Virus (ASFV) causes haemorrhagic fever in susceptible infected swine, with up to mortality rates of up to 100% in naïve domestic pigs. In Africa, wild suids (warthogs, and possibly bushpigs and giant forest hogs) are natural vertebrate reservoirs that are resistant to the disease (Jori et al. 2013). In Europe, wild boars are susceptible to ASFV and exhibit clinical symptoms and mortality rates comparable to those of domestic pigs. In certain countries, soft ticks of the genus *Ornithodoros* act as vectors or natural reservoirs of ASFV. The virus can persist for several years, potentially leading to epizootic outbreaks in pig farms that have become colonized by ticks, even with the implementation of ASF control measures. (i.e., pig slaughtering, disinfection, and quarantine) (Boinas et al. 2011, Jori *et al.*, 2013). Once ASFV is introduced into a new free area where the presence of *Ornithodoros* ticks is highly suspected, as it occurred in the Caucasus in 2007 or more recently in the Caribbean, the potential for long-term persistence of ASFV in tick reservoirs and ASFV transmission via tick bites raises concern (Beltran Alcrudo *et al.*, 2008, EFSA, 2010).

Vector competence generally refers to the vector's capacity to be infected by a pathogen, to maintain and multiply this pathogen, and finally to transmit it to a new susceptible host (Nuttal, 1994; Weaver et al., 2004). The biological steps taking place within the arthropod vector can be broken down into four stages:: (i) the ingestion of an infected blood meal in sufficient amount, (ii) the infection of the vector upon crossing of its intestinal midgut by the pathogen, (iii) the persistence and multiplication of the pathogen within the vector despite the vector's immune response, and (iv) the dissemination of the pathogen inside the vector by crossing organic barriers and ultimately reaching organs necessary for transmission to vertebrate hosts, typically the salivary glands. Sexual transmission of pathogens between males and females of vectors as well as transovarial transmission from female vectors to their progeny can occur through the infection of reproductive organs. Although not strictly included in the formal definition of the vector competence, these pathways of transmission will also be considered in this review.

All these biological steps are observed in the interaction between ASFV and *Ornithodoros* tick although results reported varied greatly between studies. In addition to historical demonstration of tick vector competence for ASFV in African *O. moubata* sensu lato and Iberian *O. erraticus*, several other *Ornithodoros* tick species succeed to maintain or transmit ASFV under experimental conditions (Endris et al., 1987, Endris et al., 1991, Hess et al., 1987, Mellor & Wilkinson, 1985). This suggests that most *Ornithodoros* ticks might be able to maintain and transmit ASFV (EFSA, 2010b). Based on available results, some researchers have proposed to predict the global role of each tick species as a vector and reservoir for ASFV (Kleiboeker & Scoles, 2001; EFSA, 2010a, 2010b, 2014; Burrage, 2013). However, these comprehensive reports do not account for the evident disparities in vector competence results, which likely rely on intricate tick-virus adaptations as proposed by Pereira de Oliveira (2019, 2020) and other yet-to-be-identified biological and experimental factors. No paper has considered the substantial diversity of techniques employed to test vector competence except for a study on *O. erraticus* infected with two Portuguese ASFV strains (Ribeiro et al., 2015). Nonetheless, the importance of experimental methodology has been demonstrated in other models like mosquitoes with Rift Valley fever, Dengue, Zika or Japanese Encephalitis viruses. (Azar, 2019; Auerswald, 2021; Drouin, 2022). We hypothesize that the experimental design, including the choice of biological materials and the methods used to infect ticks and detect the virus, is an important factor in explaining variation in vector competence, in addition to the influence of tick species, ASFV strains and their combinations.

To investigate this hypothesis, a systematic review of studies published up to 2022 was conducted, examining the vector competence of Ornithodoros ticks for ASFV. The present paper focuses on the diverse experimental factors that may impact vector competence and determines the overall vector competence of the various associations between *Ornithodoros* tick and ASFV. We provide recommendations on how experimental trials can be refined to minimize biases and obtain more comparable and reliable results on *Ornithodoros* vector competence for ASFV.

Material and Methods

Collection of bibliographical resources

The first objective of the study was to identify the bibliographic resources related to vector competence of *Ornithodoros* ticks for ASFV, published in English, in peer-reviewed journals or under high publication standards. This was achieved following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (Annex 1). Our bibliographic review was conducted until July 2022, using three different search systems: EThOS (http://ethos.bl.uk/Home.do), PubMed (http://www.ncbi.nlm.nih.gov/) and Scopus (http://www.scopus.com/). Queries were built under the keywords "African swine fever" AND "tick". The topics not related with tick competence (e.g., immunology, genetic, vaccinology, epidemiology) were excluded during the screening phase. Then, during the eligibility phase, studies were excluded if quantitative reliable data could not be extracted from publications. Purely descriptive reviews without any original vector competence results were also excluded. Apart from published scientific papers, some authors presented their results in their PhD manuscript. These data were also considered but all duplicated results were removed to avoid biases in the analyses. Finally, the references cited at the end of the included papers were checked, in order to identify further relevant studies. The final list of included papers that were used to extract all data in soft tick vector competence for ASFV are shown in Table 1.

Table 1 - Included papers providing original results on the vector competence of *Ornithodoros* ticks for ASFV, with number attributed to each paper by years of publication.

N°	Included papers
1	Parker J, Plowright W, Pierce MA. The epizootiology of African swine fever in Africa. Vet Rec. 1969 Dec 13;85(24):668-74
2	Plowright W, Perry CT, Peirce MA, Parker J. Experimental infection of the argasid tick, *Ornithodoros moubata porcinus*, with African swine fever virus. Arch Gesamte Virusforsch. 1970;31(1):33-50.
3	Plowright W, Perry CT, Peirce MA. Transovarial infection with African swine fever virus in the argasid tick, *Ornithodoros moubata* porcinus, Walton. Res Vet Sci. 1970 Nov;11(6):582-4.
4	Greig A. The localization of African swine fever virus in the tick *Ornithodoros moubata porcinus*. Arch Gesamte Virusforsch. 1972;39(1):240-7.
5	Plowright W, Perry CT, Greig A. Sexual transmission of African swine fever virus in the tick, *Ornithodoros moubata* porcinus, Walton. Res Vet Sci. 1974 Jul;17(1):106-13.
6	Groocock CM, Hess WR, Gladney WJ. Experimental transmission of African swine fever virus by *Ornithodoros coriaceus*, an argasid tick indigenous to the United States. Am J Vet Res. 1980 Apr;41(4):560-4.
7	Thomson GR. The epidemiology of African swine fever: the role of free-living hosts in Africa. Onderstepoort J Vet Res. 1985 Sep;52(3):201-9. Review.
8	Mellor PS, Wilkinson PJ. Experimental transmission of African swine fever virus by *Ornithodoros savignyi* (Audouin). Res Vet Sci. 1985 Nov;39(3):353-6.
9	Hess WR, Endris RG, Haslett TM, Monahan MJ, McCoy JP. Potential arthropod vectors of African swine fever virus in North America and the Caribbean basin. Vet Parasitol. 1987 Dec;26(1-2):145-55.
10	Endris RG, Haslett TM, Geering G, Hess WR, Monahan MJ. A hemolymph test for the detection of African swine fever virus in *Ornithodoros coriaceus* (Acari: Argasidae). J Med Entomol. 1987 Mar;24(2):192-7.
11	Hess WR, Endris RG, Lousa A, Caiado JM. Clearance of African swine fever virus from infected tick (Acari) colonies. J Med Entomol. 1989 Jul;26(4):314-7.
12	Haresnape JM, Wilkinson PJ. A study of African swine fever virus infected ticks (*Ornithodoros moubata*) collected from three villages in the ASF enzootic area of Malawi following an outbreak of the disease in domestic pigs. Epidemiol Infect. 1989 Jun;102(3):507-22.
13	Endris RG, Haslett TM, Hess WR. Experimental transmission of African swine fever virus by the tick *Ornithodoros* (Alectorobius) *puertoricensis* (Acari: Argasidae). J Med Entomol. 1991 Nov;28(6):854-8.
14	Endris RG, Hess WR, Caiado JM. African swine fever virus infection in the Iberian soft tick, *Ornithodoros* (Pavlovskyella) *marocanus* (Acari: Argasidae). J Med Entomol. 1992 Sep;29(5):874-8.
15	Endris RG, Hess WR. Experimental transmission of African swine fever virus by the soft tick *Ornithodoros* (Pavlovskyella) *marocanus* (Acari: Ixodoidea: Argasidae). J Med Entomol. 1992 Jul;29(4):652-6.
16	Endris RG, Haslett TM, Hess WR. African swine fever virus infection in the soft tick, *Ornithodoros* (Alectorobius) *puertoricensis* (Acari: Argasidae). J Med Entomol. 1992 Nov;29(6):990-4.
17	Endris RG, Hess WR. Attempted transovarial and venereal transmission of African swine fever virus by the Iberian soft tick *Ornithodoros* (Pavlovskyella) *marocanus* (Acari: Ixodoidea: Argasidae). J Med Entomol. 1994 May;31(3):373-81.
18	Boinas, FS. The role of *Ornithororos erraticus* in the epidemiolohy af ASFV in Portugal. PhD thesis manuscript. 1994
19	Rennie L. The Persistence of African swine fever virus in the Argasid Tick *Ornithodoros moubata*. PhD thesis manuscript. 1998.
20	Anderson EC, Hutchings GH, Mukarati N, Wilkinson PJ. African swine fever virus infection of the bushpig *(Potamochoerus porcus*) and its significance in the epidemiology of the disease. Vet Microbiol. 1998 Apr 30;62(1):1-15.

21	Kleiboeker SB, Burrage TG, Scoles GA, Fish D, Rock DL. African swine fever virus infection in the argasid host, *Ornithodoros porcinus porcinus*. J Virol. 1998 Mar;72(3):1711-24.
22	Kleiboeker SB, Scoles GA, Burrage TG, Sur J. African swine fever virus replication in the midgut epithelium is required for infection of Ornithodoros ticks. J Virol. 1999 Oct;73(10):8587-98.
23	Rennie L, Wilkinson PJ, Mellor PS. Effects of infection of the tick *Ornithodoros moubata* with African swine fever virus. Med Vet Entomol. 2000 Dec;14(4):355-60.
24	Rennie L, Wilkinson PJ, Mellor PS. Transovarial transmission of African swine fever virus in the argasid tick *Ornithodoros moubata*. Med Vet Entomol. 2001 Jun;15(2):140-6.
25	Burrage TG, Lu Z, Neilan JG, Rock DL, Zsak L. African swine fever virus multigene family 360 genes affect virus replication and generalization of infection in *Ornithodoros porcinus* ticks. J Virol. 2004 Mar;78(5):2445-53.
26	Nix-Rowlands RJ. Interaction of ASFV with its tick vector. PhD thesis manuscript. 2006.
27	Basto AP, Nix RJ, Boinas F, Mendes S, Silva MJ, Cartaxeiro C, Portugal RS, Leitão A, Dixon LK, Martins C. Kinetics of African swine fever virus infection in Ornithodoros erraticus ticks. J Gen Virol. 2006 Jul;87(Pt 7):1863-71.
28	Vial L, Wieland B, Jori F, Etter E, Dixon L, Roger F. African swine fever virus DNA in soft ticks, Senegal. Emerg Infect Dis. 2007 Dec;13(12):1928-31.
29	Rowlands RJ, Duarte MM, Boinas F, Hutchings G, Dixon LK. The CD2v protein enhances African swine fever virus replication in the tick vector, Ornithodoros erraticus. Virology. 2009 Oct 25;393(2):319-28.
30	Ravaomanana J, Michaud V, Jori F, Andriatsimahavandy A, Roger F, Albina E, Vial L. First detection of African Swine Fever Virus in *Ornithodoros porcinus* in Madagascar and new insights into tick distribution and taxonomy. Parasit Vectors. 2010 Nov 30;3:115.
31	Boinas FS, Wilson AJ, Hutchings GH, Martins C, Dixon LJ. The persistence of African swine fever virus in field-infected *Ornithodoros erraticus* during the ASF endemic period in Portugal. PLoS One. 2011;6(5):e20383.
32	Diaz AV, Netherton CL, Dixon LK, Wilson AJ. African swine fever virus strain Georgia 2007/1 in *Ornithodoros erraticus* ticks. Emerg Infect Dis. 2012 Jun;18(6):1026-8.
33	de Carvalho Ferreira HC, Tudela Zúquete S, Wijnveld M, Weesendorp E, Jongejan F, Stegeman A, Loeffen WL. No evidence of African swine fever virus replication in hard ticks. Ticks Tick Borne Dis. 2014 Sep;5(5):582-9.
34	Ribeiro R., Otte J., Madeira S., Hutchings G.H., Boinas F. Experimental Infection of Ornithodoros erraticus sensu stricto with Two Portuguese African Swine Fever Virus Strains. Study of Factors Involved in the Dynamics of Infection in Ticks. PLoS One. 2015 Sep 14;10(9):e0137718.
35	Pereira de Oliveira R, Hutet E, Paboeuf F, Duhayon M, Boinas F, Perez de Leon A, Filatov S, Vial L, Le Potier MF. Comparative vector competence of the Afrotropical soft tick Ornithodoros moubata and Palearctic species, *O. erraticus* and *O. verrucosus*, for African swine fever virus strains circulating in Eurasia. PLoS One. 2019 Nov 27;14(11):e0225657.
36	Pereira De Oliveira, R., Hutet, E., Duhayon, M., Paboeuf, F., Le Potier, M. F., & Vial, L. No experimental evidence of co-feeding transmission of african swine fever virus between Ornithodoros soft ticks. Pathogens 2020; 9(3), 168.
37	Pereira De Oliveira, R., Hutet, E., Duhayon, M., Guionnet, J. M., Paboeuf, F., Vial, L., & Le Potier, M. F.. Successful infection of domestic pigs by ingestion of the European soft tick *O. erraticu*s that fed on African swine fever virus infected pig. Viruses, 2020 12(3), 300.
38	Pereira De Oliveira, R., Hutet, E., Lancelot, R., Paboeuf, F., Duhayon, M., Boinas, F., ... & Vial, L. (2020). Differential vector competence of Ornithodoros soft ticks for African swine fever virus: What if it involves more than just crossing organic barriers in ticks?. Parasites & vectors 2020 13(1), 1-15.
39	Forth, J. H., Forth, L. F., Lycett, S., Bell-Sakyi, L., Keil, G. M., Blome, S., ... & Beer, M. Identification of African swine fever virus-like elements in the soft tick genome provides insights into the virus' evolution. BMC biology, 2020 18(1), 1-18.

Extraction of data about tick competence from bibliographical resources

Bibliometric general information on authors and years of publication were collected to describe the general interest in tick vector competence for ASFV and to identify scientific experts. We considered the experts as the persons in first and last position of the included papers. Information on tested *Ornithodoros* tick-ASFV associations was systematically reported. A challenge was to deal with missing or incomplete data for ASFV strains, as well as the ambiguities in tick species identification.

Regarding virus, we recorded the strain, the genotype (on the basis of p72 gene sequences, Qu *et al*. 2022), the geographical origin, and the host (vertebrate animals or ticks) from which the ASF virus was isolated. The original names of some ASFV strains were retrieved by crossing information from several papers, according to known authors' collaborations, or by directly contacting the authors. When the determination of strains remained impossible, the strain was identified by indicating the country where the ASFV strain was isolated, the year of the study, and a letter if duplicates existed. One exception was a strain used to infect *O. turicata (*Hess *et al.* 1987*)* without any indication of the country of origin which was named Ourt87. Six recombinant ASFV strains, genetically modified and obtained from two natural strains (from papers n°25 and n°26), were excluded from this review since they cannot infect ticks in nature. Genotypes were not available for some strains of ASFV, but they could be inferred based on their geographical origin and ASFV genotype distribution published by Lubisi (Lubisi *et al.*, 2005).

Concerning ticks, the species, their geographical origin, their colony origin and status were reported. When ticks were reared for less than five years in the laboratory, they were considered comparable to ticks sampled from the field due to the limited number of generations occurring during this period. Some

Ornithodoros ticks belong to species complexes and soft tick systematics has greatly changed during the last decades. Tick species identification was updated in accordance with the revision of valid tick names by Guglielmone (Guglielmone *et al.*, 2010) and the mapping of their known distribution (Vial, 2009). The more recent taxonomy study of southern African *Ornithodoros* ticks (Bakkes, 2018) could not be used, due insufficiently precise location to attribute a species name among the *O. moubata* complex of species. Finally, when determining tick geographical origin, 6 studies involving 4 different tick species (*O. coriaceaus, O. parkeri, O. moubata*, and *O. porcinus*) did not report this information but since *O. parkeri* and *O. coriaceus* are known to be limited to North America, especially the United-States (de la fuente et al. 2008), they were assigned to this country.

We reviewed the various measurements used by authors to assess the tick vector competence for ASFV (Annex 2). Some authors reported numbers of ticks positively detected with ASFV, while others scrutinized the virus titers in ticks or monitored ASFV kinetics in ticks for several weeks or months, providing more comprehensive information on the virus persistence within its tick reservoir. Some authors preferred to detect ASFV in tick transmission organs (salivary or coxal glands) or secretions (saliva or coxal fluid). Such measurements suggest possible successful transmission to pigs although only transmission experiments can confirm it. In these transmission experiments, clinical signs observed in pigs following tick bites confirm the ability of ticks to transmit the disease. Detection of ASFV in pig blood is usually associated to confirm infection. Transmission between ticks is either tested directly by measuring the proportion of transmitting ticks or the proportion of infected tick or indirectly through the detection of ASFV in male reproductive organs (for sexual transmission) or female ovaries (for transovarial transmission). However, this was not considered sufficient evidence to confirm such transmission pathways. Conversely, the detection of ASFV in transmission secretions (e.g., saliva or coxal fluids) was considered sufficient evidence, as the virus is already excreted in this case.

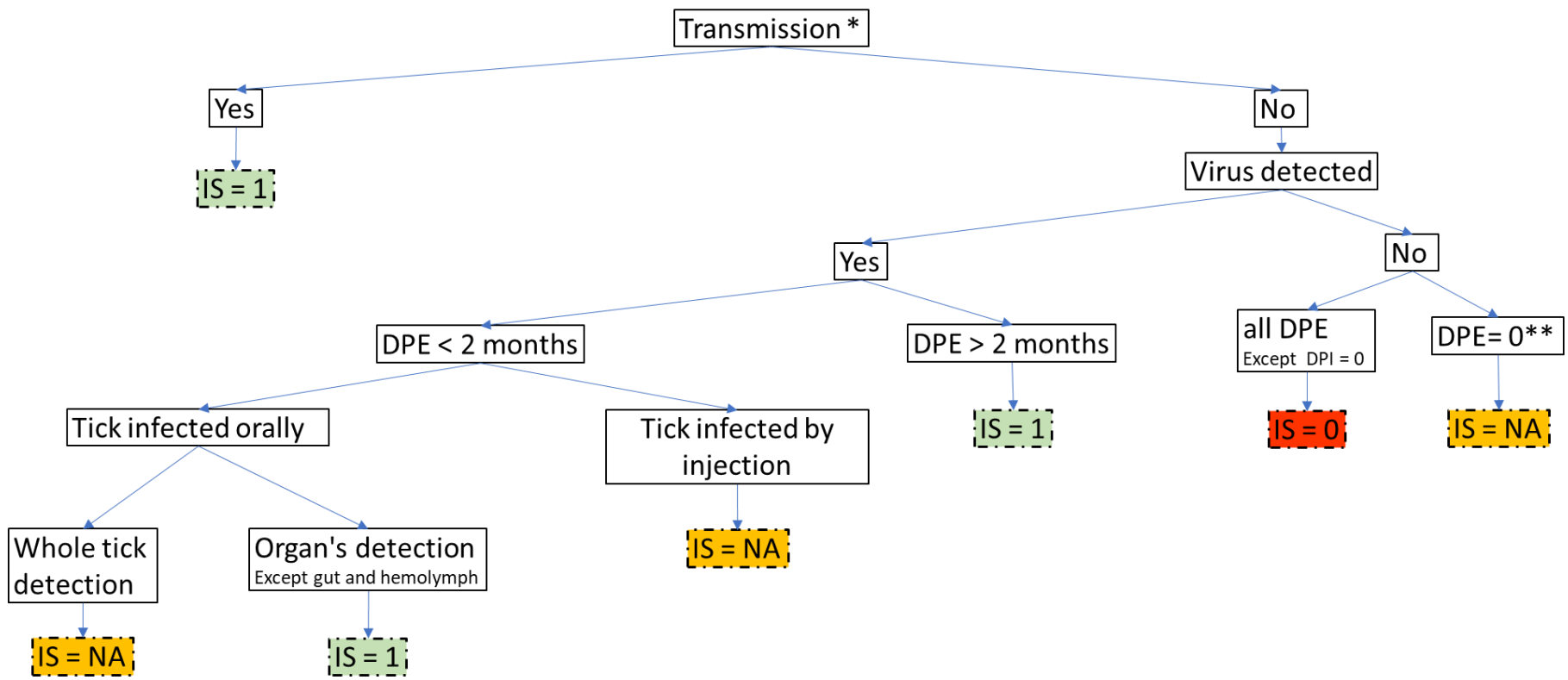
Based on these data, it was possible to evaluate three major steps of the vector competence of the ticks: 1) the tick infection (revealed by the crossing of the tick midgut by ASFV), 2) the ability of ticks to transmit ASFV to pigs (named horizontal transmission) and 3) their ability to transmit ASFV to other ticks through sexual or transovarial vertical transmission (named tick-to-tick transmission). Although these steps are not necessarily gradual, transmission to vertebrate hosts through tick bite or from ticks to ticks cannot occur without the previous infection of the tick, which was thus considered as the main step to be assessed. In addition, tick ability to transmit ASFV to pigs or other ticks are less frequently reported (N= 160 and N=54 over 900 experiments, respectively) likely because they are somewhat more difficult to assess than tick infection. Therefore, for each experiment, we computed a binary variable, the infection status, according to any measurements providing direct information on tick infection as well as indirect transmission measurements that imply previous tick infection (Figure 1). More precisely, if ASFV transmission to pigs or ASFV transmission from ticks to other ticks were reported in papers, the ticks were considered to be infected as the virus has necessarily crossed the tick organic barriers to reach transmission organs and be transmitted.

If transmission failed or was not tested, the detection of ASFV in ticks was used as an indication of infection status. Obviously when ASFV was not detected, ticks were considered as not infected. The only exception was when the detection occurred on the same day as virus exposure, in which case no virus detection indicates a failure of experimental infection or a too low detection threshold.

Different methods have been used by authors to detect ASFV in ticks, namely ASFV isolation/titration, observation of ASFV particles, and viral DNA or RNA detection using multiple designs of Polymerase Chain Reaction (PCR). ASFV isolation/titration is based on haemadsorption (HAD), which is the ability of many ASFV strains to aggregate on red blood cells when they infect macrophages and create typical “rosettes” (Malmquist & Hay 1960); this implies that the virus is alive but does not necessarily confirm its entry in ticks. Similarly, detection of viral RNA also indicates active viral replication. Conversely the detection of viral DNA can be due to residual virus from blood meal and does not mean that the virus is still alive. However, we considered that those methods brought equivalent results to assess infection status once the duration after exposure was taken into account.

Indeed, when ASFV was detected in ticks, a minimum duration of 61 days between tick exposure to ASFV and detection of ASFV in tick (hereafter DPE for duration post exposure) was chosen to distinguish successful viral replication inside the tick from detection of residual virus after the infectious blood meal or virus inoculation. Indeed, the minimum duration for blood meal digestion and clearance of ingested blood in soft ticks is approximately 4 weeks (Rowlands *et al.*, 2009). Furthermore, if tick infection is unsuccessful, ASFV can be detected during 4 weeks by isolation (Ribeiro et al., 2015) and after at least 49 days by qPCR (de Carvalho et al., 2014, see also Bernard 2015 for a review on ASFV kinetics inside soft ticks). If tick infection is successful, virus generalization in ticks takes about 30 to 56 days (Basto *et al.*, 2006, Burrage, 2013, Kleiboeker *et al.*, 1998, Plowright *et al.* 1970). Therefore, if ASFV was tested and detected before 61 DPE, the infection status was considered unknown unless virus was detected in organs other than the midgut. In this case, this indicates that the virus has crossed the midgut. In the specific case of injection into the haemocoel, virus is spread throughout the tick. Therefore, the detection of virus in the organ was doubtful and the infection status was recorded as unknown.

The experimental design used in the different studies was also reported, including: i) the exposure route for the tick infection, ii) the ASFV titer in the inoculum used to infect the ticks (infectious dose), iii) the development stage of the ticks tested in the study, iv) the DPE, v) the number of ticks tested in the study. Except for the DPE and the number of ticks monitored, all parameters were categorized in different classes. In field studies, viral titers in the hosts were not necessarily reported but they were considered to be high regarding the viraemia usually observed in ASF diseased pigs (Plowright et al., 1968). Tick stage was classified as early from larva to nymph 3 and as late from nymph 4 to adult.



*Virus detection in the transmission organs is not a proof of transmission but the detection in the transmission fluids is a proof of transmission.
** we suspect in this case a failure in the experiment.

IS = infection status
DPE = date post exposure

Figure 1 - Criteria used to determine the infection status of ticks after their virus exposure, with three classes (IS: Infection Status; DPE: Date post exposure; NA: Not Applicable, 1: Infected, and 0: Not Infected).

Statistical analysis of the tick infection status

A statistical analysis was carried out to identify factors potentially affecting the tick infection status. More precisely, we investigated if factors related to the nature of tick-virus association or the experimental design influenced the infectious status (see above for details) of the ticks in published experimental trials. The explanatory variables were: the ASFV strain and genotype, the tick species, the potential geographical co-occurrence of tick and virus as an indication of possible tick-virus adaptation (ASFV and ticks from the same country versus from different countries), the viral titer in infection blood meal and the exposure route for tick infection, the tick stage and the tick colony status, the host from which the ASFV strain was sampled. Using a dataset where all ticks were adults, we also tested the influence of tick sex on infectious status (along with all other factors except tick stage). All statistical analyses were performed on R 4.2.2 (R Core Team, 2018). To assess which factors influenced the infection status, we applied a generalized mixed model with a binomial family. The tick species, virus genotype and virus strain were set as random effects to account for the fact that only a random fraction of soft tick species and virus genotypes and strains were sampled. All other factors were set as fixed effects. The significance of random effects was tested using a likelihood ratio test (Zuur et al., 2009). We then selected the best parsimonious models (with the remaining 6 fixed effects) based on the AIC (Akaïke Information Criterion). In this best model, the significance of fixed effects was tested using Chi^2 tests between models and post-hoc tests, with Tukey HSD correction, were performed.

Assessment of the overall tick vector competence for ASFV

Although our main assumption is that vector competence results are influenced by the experimental design, we attempted to assess the overall vector competence of the various *Ornithodoros* tick-ASFV associations. Based on the three main steps described above, we scored each tick-ASFV association, according to the following ranking: “score 0” for ticks that were unable to become infected or for which it was impossible to conclude (see flow chart figure 2) ; “score 1” when ASFV was able to cross tick midgut and replicate in ticks; “score 2” when transmission from tick to pig was successful ; and, “score 3” for ticks validating all steps of vector competence, including ASFV transmission from ticks to other ticks. The attribution of a score was based on all data available from the different included papers related to the considered tick-ASFV association, and thus the longest period of monitoring and the best vector competence performance for this association.

Attributing graduate steps of vector competence sounds as if that associated transmission events are also graduate. This is not the case: even if horizontal transmission scored 2 and sexual or transovarial transmissions scored 3, the success of tick-to-tick transmission does not necessarily imply the efficiency of horizontal transmission. For example, *O. erraticus* showed vertical transmission with the viral strain OURT88/1 but no transmission to pig (Pereira de Oliveira *et al*. 2019, Pereira de Oliveira *et al.* 2020)

Results

Bibliometric description

The initial bibliographic search generated 236 references using Scopus and 138 by Pubmed, with 2 articles detected only by Pubmed. The ETHOS database of thesis manuscripts and our manual bibliography search allowed identifying 8 more resources. Among the final 246 references selected, 75 were reviews, 163 scientific papers and 8 thesis manuscripts. In this pool of bibliographic references, 39 resources presenting vector competence original results were included in the final dataset. They consisted of 5 field studies monitoring natural tick infections, 34 articles of laboratory experiments on ticks and 2 mixed papers (Table 1). As shown in figure 1, the number of publications pertaining to the vector competence of *Ornithodoros* ticks for ASFV was consistently low over the years, while the total number of papers related to ticks and ASFV progressively increased from the 1970s to 2022.

Thirty-nine authors were reported as first and/or last authors of the selected publications and were considered as “experts”. Their period of expertise, including all their publications on ASFV & ticks, varied from 1 to 34 years. After 2015, 9 out of 39 experts continued to publish in this field and can be considered the current core of experts on the vector competence of ticks for ASFV.

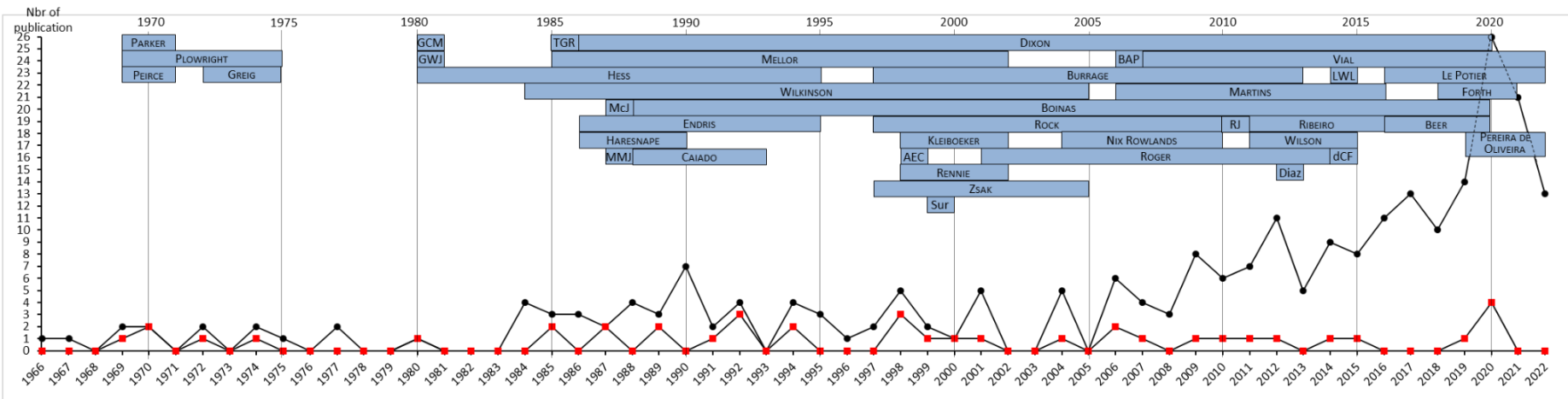


Figure 1 - Number of bibliographic resources published each year, from 1966 to 2022, on ticks and ASFV (black dots = 246 screening papers) and on the vector competence of *Ornithodoros* ticks for ASFV (red dots = 39 included papers). The names of first and last authors of the 39 included papers were reported in blue boxes at the top of the graphic. The length of boxes indicating their respective period of expertise. It is the period where they appear in the list of authors from the 246 screening papers. list of the abbreviation author name: AEC: Anderson E.C.; BAP: Basto A.P.; dCF: de Carvalho Ferreira H.C.; GWJ: Gladney W.J.; GCM: Groocock C.M.; LWL: Loeffen W.L.; McJ: McCoy J.P.; MMJ: Monahan M.J.; RJ: Ravaomanana J.; TGR: Thomson G.R.

Data related to the biological material: ticks and ASFV

A database was created to record all the extracted data. Each record comprises a precise combination of any modalities of informed indicators concerning the publication, the ticks, the ASFV strain and the experimental design. From the 39 included papers, 2037 records corresponding to 900 unique experiments were extracted. For some papers, authors detailed the results of their experiment for each tick or with multiple records over time for the same ticks. Each experiment was characterized by a combination of tick species, virus strain and experimental conditions (see Annexes 3 and 4). In total, the tick species and ASFV strains represented 51 tick-virus associations and more than half (34/51) were studied only once.

For the ticks, 10 species were tested for their vector competence for ASFV. The Iberian tick *O. erraticus* was the most studied (representing 44% of the reported experiments), followed by the *O. moubata* complex of species, including *O. porcinus* and *O. moubata* sensu stricto (43%). The North American *O. coriaceus* (6.4%) were third, then they were far followed by the other species *O. puertoricensis, O. verrucosus, O. savignyi, O. parkeri, O. sonrai, O. turicata* (3.7% - 1.0% - 0.8% - 0.4% - 0.4% - 0.2 % respectively). The geographical origins of ticks specified in the included papers are presented in figure 2. All *O. erraticus* ticks tested came from Portugal, and most of them from the same region of Alentejo, while *O. moubata* and *O. porcinus* ticks came from diverse countries, which is representative of their large distribution range in East and Southern Africa.

A total of 38 different ASFV strains were tested with ticks. Most ASFV strains used in the studies were isolated from ticks and pigs (43.1% and 40.4% of the reported experiments, respectively). Only 4 strains studied in 16.2% of the experiments were isolated from warthogs and 0.2% are from undetermined hosts. ASFV strains were geographically distributed over 17 different countries, mostly in Africa, and belonged to 8 genotypes over the 23 genotypes described sofar, with a large representation of the genotype I (63.7% Figure 2). The genotype of the 114a and Transvaal CV strains from South Africa remains unknown.

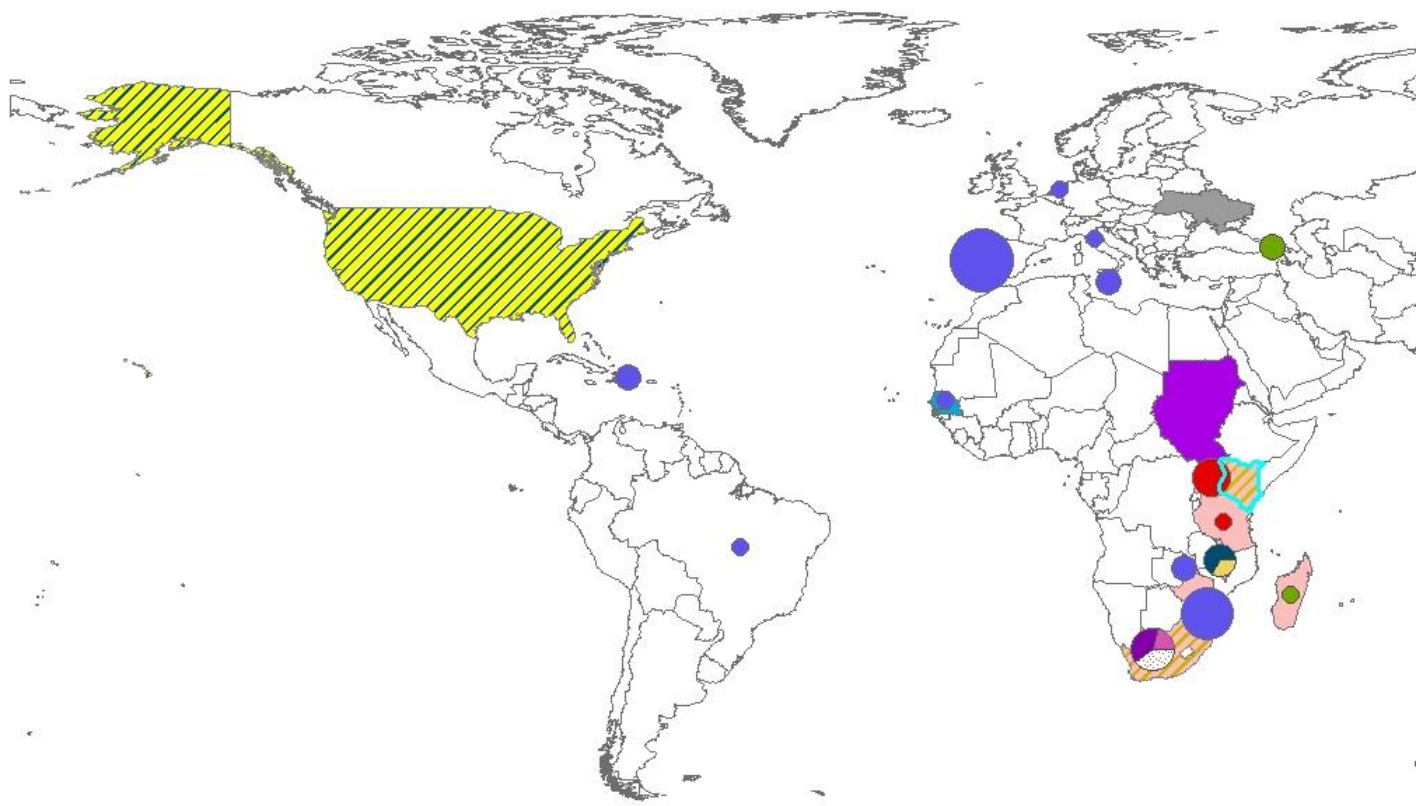


Figure 2 - Geographical origins of *Ornithodoros* tick species (represented by colored countries) and ASFV strains (indicated by pie charts on each country, with size of pies indicating the number of different strains and colors the genotype of each strain) involved in natural tick-ASFV associations studied in included papers. The origin of the tick *i* was not indicated but should be the United-States, as described in the literature.

Data related to the experimental design

On the 900 experiments recorded, half used ticks directly sampled from the field (especially *O. erraticus* and *O. porcinus*) or reared in insectary for less than 5 years (48.7%). The other experiments used ticks reared in the laboratory for more than 5 years with some reared until 25 years for one study on *O. turicata*) (47.2%). It was not possible to find information for the remaining experiments. The majority of experiments (57.9%) involved ticks in their later developmental stages, ranging from nymph 4 to adults. Early stages, from larva to nymph 3, were employed in 23.3% of the experiments. In the remaining experiments, authors either utilized a

combination of early and late stages (14.7%) or did not provide information about the specific developmental stage used (4.1%). The number of ticks tested for each experiment was fairly low especially in laboratory experiments (median=7 ticks tested, mean=30.03, range=[1;1141]).

Four methods were used to perform viral ASF inoculation to the ticks. Natural blood feeding on vertebrate hosts was reported either in field studies, or in some laboratory experiments using experimentally infected pigs. This method represented almost half of the total experiments conducted (47.4%). Two artificial feeding methods are reported: the longest established method uses capillary tubes inserted on tick mouthparts (8.8%) while a more recent approach uses animal skin, parafilm, or silicone membranes (35.7%). Another artificial infection route was the syringe inoculation of ASFV directly in the tick heamoceal (8.1%). ASFV infectious dose used to infect ticks was very heterogeneous and thus classified in three categories. Most experiments used medium to high viral doses (between 10^4 and 10^6 or over10^6 HAD_{50}/mL of blood). The DPE was very variable, ranging from 1 month to up to 8 years (median value=58.0 days and mean value = 115,5 days), meaning that half experiments reported a DPE lower than the duration required to differentiate a successful viral replication inside the tick from a detection of residual virus after the infection.

Factors influencing the likelihood of tick infection

On the initially 900 recorded experiments, we obtained a dataset of 408 observations with full information regarding tick infection status and experimental conditions (Annex 5 and 6). Among the random effects tested, the tick species and virus genotype had no effect on the infection status but the viral strain had (χ_1^2=4.787 p_value= 0.027). Model selection based on AIC ended up with three variables displaying large and comparable importance across all models: virus titer, tick stage and tick colony status (Figure 3 left). All those three variables had significant effects on infectious status (Figure 3 right).

In more details, a higher viral titer corresponded to a higher likelihood of tick infection (χ_2^2X²2= 38.497, p-value=4.369×10-9). Bigger nymphs and adults were more prone to get infected than small nymphs (χ_1^2=17.888, p_value=2.343×10-5). And ticks from laboratory colonies had higher probability of infection than ticks recently sampled in the field (χ_1^2= 14.046, p_value=1.784×10-4). The potential geographical co-occurrence of tick and virus, the exposure route for tick infection and the virus host were not significantly linked with infection status even if they were present in some of the best models (Annex 7). Using the dataset containing only experiments with adult ticks showed that sex had no influence on infection status.

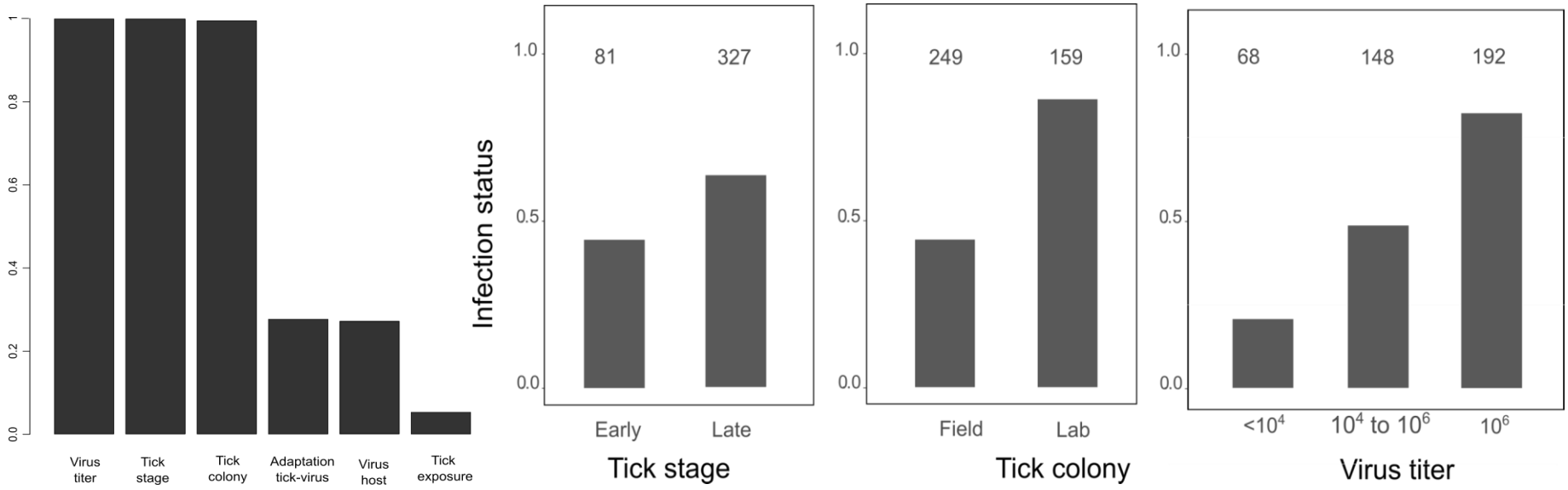


Figure 3 - *Left*: Relative importance of model terms across all models explaining infection status. The importance value for a particular predictor is equal to the sum of the weights for the models in which the variable appears. *Right*: Effect of tick stage, tick colony and virus titer on the infection status (all three effects are significant). Mean of infection status (i.e., proportion of experiments where ticks were infected). Values above the bars are the number of experiments in each level. Virus titer in HAD50/mL of blood

Vector competence scores for ASFV

Figure 4 summarizes the vector competence score for each of the 51 associations between soft ticks and ASFV virus tested so far. In more details, 23.5% of the tick-ASFV associations presented a vector competence score null (score 0) i.e., ticks were unable to become infected or it was impossible to conclude on their infection status (Figure 2). For 27.5% of tick-ASFV associations, only tick infection (score 1)) was achieved. Transmission from ticks to pigs was tested in 64.7% of

tick-ASFV associations and was successful in 29.4% (score 2). Finally, 33.3% of tick-ASFV associations were assessed for ASFV transmission from ticks to ticks and 19.6% succeeded to transmit (score 3).

Only 27.5 % of tick-ASFV associations were fully tested for vector competence (including tick infection, transmission to pig, and transmission to other ticks) and 15.7% were fully competent, meaning that they reached scores 1, 2 and 3. Conversely, 3 associations reached score 3, without being tested for horizontal transmission (O. erraticus-Zimbabwe/83/a and O. erraticus-Tomar/87) or by failing to transmit to pigs when tested (O. erraticus-Ourt/88/1). In one tick-ASFV association (*O. porcinus*- MwL1/20/1) transmission capacity was suggested by virus secretion in saliva and in coxal fluid only via virus direct injection into tick hemolymph.

At the species level, when considering only scores 1 and 2, most tick species tested were confirmed to be competent vectors for at least one ASFV strain. This competence remains unclear for 3 species that were either tested with only one ASFV strain and failed to transmit the virus to pig and/or to ticks (*O. parkeri* and *O. verrucosus*) or never tested for transmission *(O. sonrai)*. Regarding ASFV transmission to other ticks, 6 tick species were tested and 4 (*O. moubata*, *O. porcinus, O. puertoricensis*. and *O. erraticus)* succeeded for at least one ASFV strain whereas 2 (*O. coriaceus* and *O. verrucosus)* that were tested with respectively three and one ASFV strains, failed. Finally, high heterogeneity of vector competence scores was observed within a same tick species depending on ASFV strains used (e.g. score 3 for *O. moubata*-LIV13/33 and score 0 for *O. moubata*-Brazil/78), and sometimes for a same tick-ASFV association (e.g. scores 0/1 and 2 for two *O. turicata* colonies of different ages with the same ASFV strain).

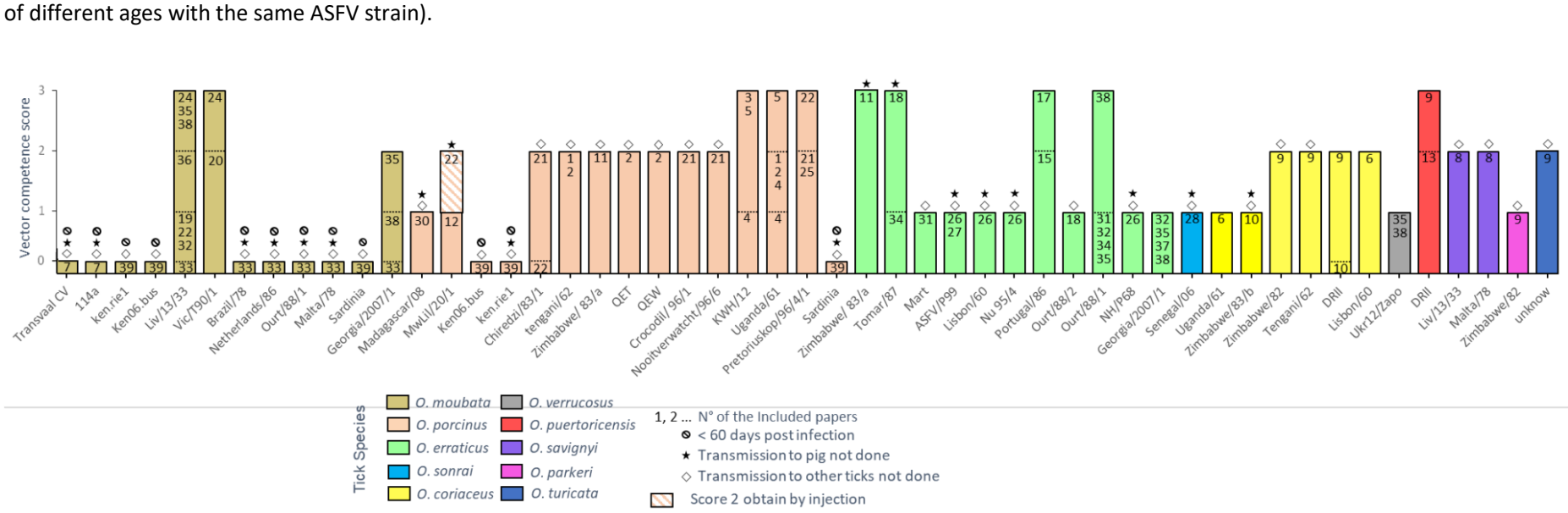


Figure 4 - ASFV vector competence scores for the different tick-ASFV associations studied in the included papers (score 0: no tick infection or no information to conclude, score 1: successful tick infection, score 2: successful ASFV transmission to pigs, score 3: successful ASFV transmission to other ticks). In each histogram bar, the number(s) are related to the included papers recorded in the table n°1. For each included paper, an individual score is given according to available results in this paper. The overall vector competence score for a given tick-virus association is the best vector competence result reported from all included papers related to this tick-ASFV association.

Discussion

Bibliographic ressources

This paper is the first exhaustive review of all the original studies published on the vector competence of soft ticks for ASFV until 2022. Despite an exhaustive screening of bibliographic resources, only 39 papers were considered and the expert panel had always been limited as exemplified by only 9 publishing experts since 2015. For a comparison, in the case of the Rift Valley fever virus, which generated a similar number of articles on Pubmed as ASFV (2020 articles and 2622 respectively) an EFSA systematic literature review identified 219 publications on the competent infected mosquitoes (Versteirt, *et al.* 2013**).** This paucity of bibliographic resources regarding soft tick competence for ASFV may be attributed to the difficulty of investigating their vector competence, both in laboratory and in field conditions. Indeed, sampling endophilous *Ornithodoros* ticks and developing relevant methods to detect ASFV in natural tick populations (Jori *et al.*, 2013, Oura 2013) or rearing soft ticks and amplifying ASF virus in the laboratory conditions are long and laborious tasks (Vial 2009, Carrascosa 2011). In addition, although ASF is of high veterinary and economic concern, vectorial transmission is seen as occasional with a relatively minor role in disease spread at least in some regions (Guinat et al., 2016). Nevertheless, soft ticks play a key role in the maintenance of the virus and this is why characterizing competence for tick-virus associations is required to assess the risk of ASFV introduction and spill over.

Vector competence and tick-virus co-adaptation

Although all papers included in this study aimed to evaluate the vector competence of soft ticks for ASFV, we noticed a considerable diversity of vector competence measurements that yield distinct information. As a consequence, vector competence was only partially assessed for most tick-ASFV associations, with a noticeable decrease of studies along the successive biological steps of vector competence, from the tick infection to ASFV transmission by tick to pigs, and then by tick to other ticks. In addition, more than half of tick-ASFV associations were characterized by only one single article. Heterogeneous vector competence scores were observed within species between viral strains. For instance, *O. moubata* showed efficient vector competence for only 3 viral strains out of the 12 tested thus far. This could be interpreted either as a lack of sufficient reliable studies or high tick-virus specificity. This latest hypothesis is in agreement with our results showing a significant effect of viral strain on tick infection status

Adaptation between arthropod vectors and viruses is based on genetic features defining susceptibility in both organisms and the selection of susceptibility may be mediated partly by detrimental effects of arboviruses on vector physiology (Weaver et al., 2004). In soft tick-ASFV model, the presence of ASFV MGF360 genes, the lectin-like EP153R gene, or the CD2-like EP402R gene enhance the entry and the replication of ASFV into ticks (Burrage et al., 2004; Rowlands et al., 2009). Leaving their original sylvatic cycle involving warthogs and ticks, some ASFV strains naturally lost these genes through long-term circulation among Iberian domestic pigs, or by multiple passages on pig cell cultures for vaccine production (Portugal et al., 2015). Concerning these vector susceptibility genes, some of them, such as MGF 360, are involved in the suppression of the immune response of vertebrate and arthropod hosts against ASFV (Portugal et al., 2015). In addition, among the large panel of immune responses described in arthropods against transmitted pathogens [44], arthropods are known to display RNA interference as an important immune response against virus infection. The involvement of such molecular pathway has recently been suggested in soft ticks with ASFV- like integrated elements coding for small interfering RNA in *O. moubata* (Forth et al. 2020). More specifically, it appears that a greater number of these small matched with ASFV genomes from genotype II than with genotype I, contributing to the variation in vector competence between viral strains.

We can suppose that field ticks, coevolving or not with ASFV, may develop a much more heterogeneous and adaptive response against ASFV infection and show a lower success of infection, as it was already shown in some mosquito-virus models (Weaver et al., 2004). Indeed, some authors previously reported very low ASFV infection rates in field ticks (Plowright 1977, Haresnape & Wilkinson 1989, Boinas et al 2011), as well as over-mortality in laboratory ASFV highly infected ticks (Endris & Hess, 1994, Kleiboeker & Scoles, 2001). In our study, laboratory tick colonies reared for more than five years were shown to be significantly much more infected by ASFV than field ticks or recently established laboratory ticks. In addition, tick from old laboratory colonies may be more prone to efficient artificial blood feeding, increasing the blood volume and ASF virus intake.

Despite heterogeneous vector competence according to studies and viral strain tested, experimental evidence shows that 7 *Ornithodoros* species from Africa (*O. moubata, O. porcinus and O. savignyi*), Europe (*O. erraticus*) and North America (*O. coriaceus, O. turicata and O. puertoricensis*) can be infected by at least one strain of ASFV and then transmit it to pigs.

Effect of experimental factors on tick competence

Our analysis of vector competence papers demonstrated the importance of the experimental design on vector competence results. Several factors related to the choices of biological material and methodologies were tested. The ASFV titer in the inoculum for infecting ticks appears as a key parameter in the vectorial competence experiments, with likelihood of tick infection above 50% when ASFV load was higher than 10^6 HAD_{50}/mL. Accordingly, a threshold of $10^{5.75}$ HAD_{50}/mL was previously determined by Ribeiro (Ribeiro *et al.* 2015) for the specific ability of *O. erraticus* to maintain and multiply two Portuguese ASFV strains. Similar assumption was also proposed for the likelihood of ASFV transmission by ticks, with a threshold of 10^4 HAD_{50}/mL as a sufficient virus load per tick to insure the success of ASFV dissemination in ticks and the colonization of tick transmission organs (Kleiboeker & Scoles, 2001, Plowright, 1977, Ribeiro *et al.*, 2015). Forth *et al.* (2020) also demonstrated an impact of ASFV titer on tick infection probability and load. Soon after ingestion, ASFV has to cross the tick midgut barrier to be amplified in replication units localized in tick endothelial cells and then enter the tick hemolymph (Burrage et al., 2013). These results may suggest that the tick midgut and other tick membranes act as porous surfaces; the probability of ASFV entry would thus depend on the number of available pores (Franz *et al.*, 2015, Gutiérrez *et al.*, 2012). Tick immune system such as RNAi could also block low level of ASFV infection (Forth *et al.*, 2020).

The development stage of ticks had a significant impact on tick infection status, with better results for late stages (from nymph 4 to adults). This pattern confirms previous results on *O. erraticus*-Tomar and *O. erraticus*-OURT88/1 from Ribeiro (Ribeiro *et al.* 2015). This was explained by higher blood volume intake for late and large stages, and therefore highest ASFV titre inside the tick after bloodmeal digestion. In addition, we found no effect of observed sexual dimorphism in adults on the likelihood of tick infection which is in agreement with Ribeiro et al (2015). In this study, the authors suggest that, in adults, the number of ASFV particles already reached a certain threshold necessary to cause tick infection.

The route for tick infection was expected to play a role on tick competence. Natural feeding on vireamic pigs may provide higher viral titer (usually $>10^6$ HAD_{50}/mL) than artificial feeding on membrane or with capillary (from 10^4-10^6 HAD_{50}/mL) due to the difficulty for amplifying ASFV on pig cell cultures). Injection of virus into the haemocoel spread the virus throughout tick body and remains traumatic for the tick (Bonnet & Liu 2012). Despite differences between inoculation methods, this does not seem to translate into any significant differences on the success rate of infection in our dataset. This could be attributed to our dataset being incomplete and imbalanced, with the virus titer being a confounding factor. The impact of inoculation methods could be specifically addressed by conducting dedicated studies comparing the various methods with a similar virus titer. Similarly, we did not find any effect of the host in which viral strain was isolated on tick competence.

Our review highlighted the huge diversity of measurements used in the included papers. They differ in their value for informing the various steps of tick competence. In our analysis, the main issue was to differentiate “residual” infection that consisted of remaining virus or viral DNA in the tick midgut before complete digestion, from “true” infection where live virus crossed the tick midgut barrier and began its replication in tick cells. Reverse Transcription quantitative PCR seems very appropriate as it detects ASFV gene duplication and expression, indicating ASFV crossing of tick midgut barrier where replication units are located. Apart from this method, all the other techniques reported from included papers show some weakness to assess tick infection. For instance, ASFV isolation and titration on pig alveolar macrophages was largely used by authors, this technique may suffer from inhibition issues due to cytotoxic effect of tick components on pig macrophages and its achievement in at least 6 days is very time-consuming (Oura et al., 2013). Also, some degree of standardization of research protocols for evaluation of vector competence for soft ticks, would be useful.

Conclusion and perspectives

The examination of 39 papers on the vector competence of *Ornithodoros* soft ticks for ASFV highlighted factors influencing vector competence results. For the same tick-ASF association, vector competence results may vary according to the methodology used for infecting ticks (ASFV titer in the inoculum), the choice of biological material (viral strain, tick development stage of tick, tick colony), and the techniques used to monitor and assess the likelihood of tick infection. Although comparisons were difficult due to the scarcity of complete vector competence assessment for many tick-ASFV associations, our analysis highlighted some standards that could be used for further investigations on the vector competence of *Ornithodoros* ticks for ASFV. Preferring blood from vireamic pigs for infecting ticks would insure sufficient virus load and ideal virus hemadsorption to erythrocytes. Favoring oral inoculation of the virus, instead of virus injection to the heamolyph, would avoid doubtful detection of ASFV. Using Reverse-Transcription quantitative PCR to quantify replicative virus inside ticks, tick organs or tick fluids is certainly the best option to assess the infectious status of ticks.

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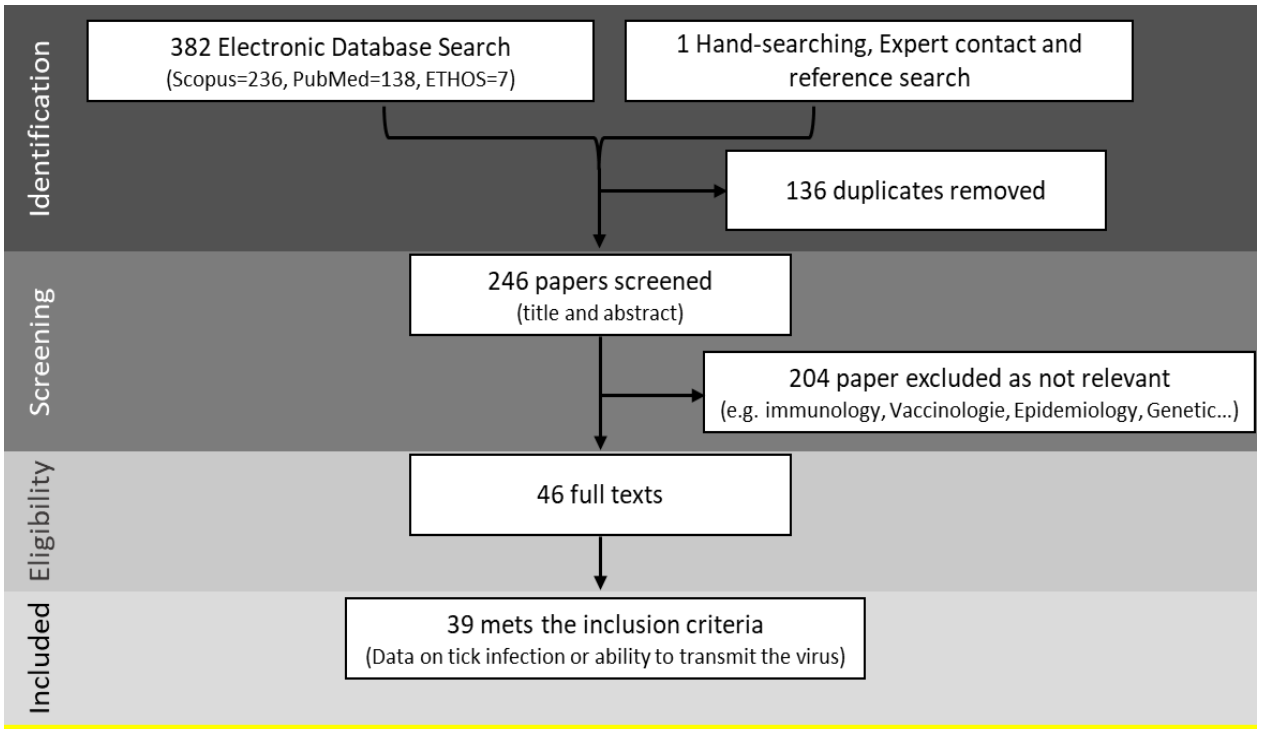
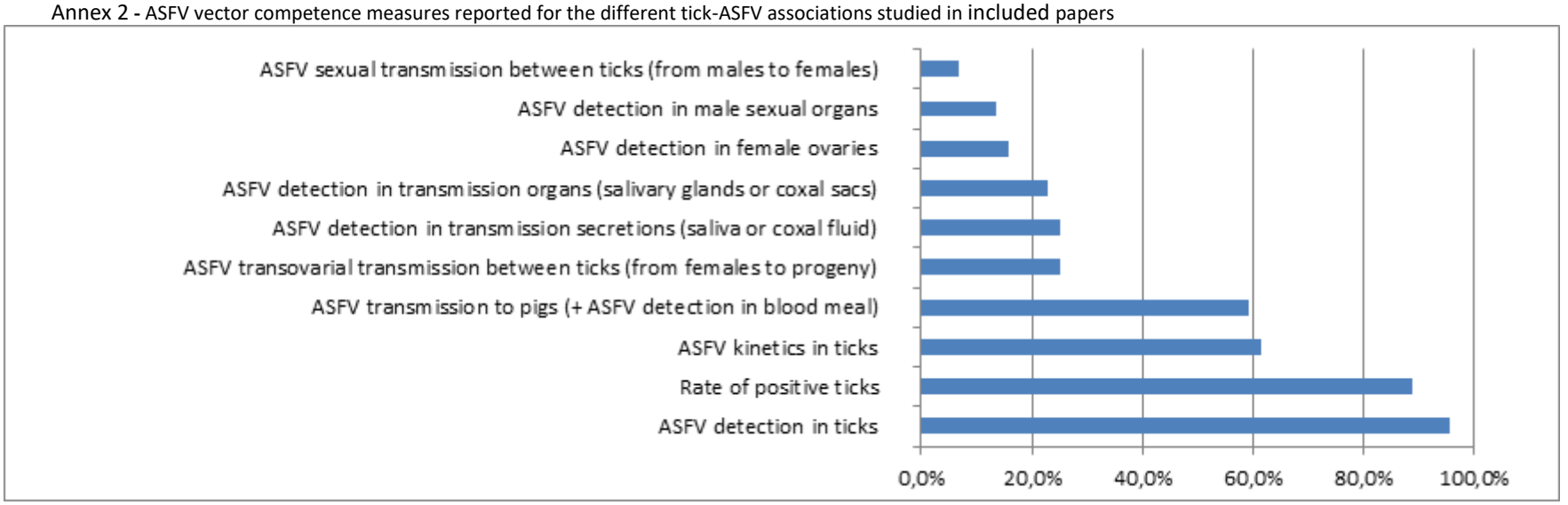
Appendix



Annex 1: Article selection process according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines

Annex 2 - ASFV vector competence measures reported for the different tick-ASFV associations studied in included papers



Annex 3: Number of experiments reported for each tick species, virus strain and virus genotype in the whole dataset (900 experiments)

Virus strain		Virus genotype		Tick species	
114a2	1	1	574	coriaceus	58
asfvp99	30	2	62	erraticus	396
brazil78	4	5	19	moubata	107
chiredzi831	38	8	22	parkeri	4
crocodil961	4	9	10	porcinus	279
dr2	56	10	176	puertorice	33
georgia200	42	19	8	savignyi	8
ken06bus	10	20	19	sonrai	4
kenrie1	20	NA's	10	turicata	2
kwh12	30			verrucosus	9
lisbon60	10				
liv1333	46				
madagasca	1				
malta78	9				
mart	5				
mwLil201	22				
netherland	4				
nhp68	30				
nooitverwa	4				
nu954	5				
Otu87	2				
ourt881	110				
ourt882	40				
portugal86	51				
pretoriusk	19				
qet	12				
qew	17				
sardinia	10				
senegal06	4				
tengani62	19				
tomar87	93				
transvaalcv	7				
uganda61	97				
ukr12zapo	9				
vict901	4				
zimbabwe8	7				
zimbabwe8	17				
zimbabwe8	11				

Annex 4: Number of experiments reported in each category of the studied experimental factors in the whole dataset (900 experiments)..

Exposure route		Tick stage		Tick colony		Virus host		Viral titer		Geographic co-occurrer	
artificial	319	early	210	field	438	pig	364	<10^4	109	yes	747
capillary	79	late	522	lab	425	tick	389	10^4 to 10^6	296	no	153
natural	427	mix	131	NA's	37	warthog	145	>10^6	495		
inoculation	75	NA's	37			NA's	2				

Annex 5: Number of experiments reported for each tick species, virus strain and virus genotype in the dataset analyzed.

Virus strain		Virus genotype		Tick species	
asfvp99	20	1	287	coriaceus	2
chiredzi831	16	2	30	erraticus	244
dr2	3	5	6	moubata	44
georgia20071	20	8	6	parkeri	1
ken06bus	5	9	5	porcinus	104
kenrie1	4	10	73	puertoricer	3
kwh12	21	20	1	savignyi	4
lisbon60	2			verrucosus	6
liv1333	29				
malta78	1				
mwLil201	6				
nhp68	26				
nu954	5				
ourt881	61				
ourt882	21				
portugal86	25				
pretoriuskop96	1				
qet	7				
qew	8				
sardinia	4				
tengani62	6				
tomar87	69				
uganda61	33				
ukr12zapo	6				
vict901	4				
zimbabwe82	1				
zimbabwe83a	2				
zimbabwe83b	2				

Annex 6: Number experiments reported in each category of the studied experimental factors in the dataset analyzed.

Exposure route		Tick stage		Tick colony		Virus host		Viral titer		Geographic co-occurrence	
artificial	169	early	81	field	249	pig	172	$<10^4$	68	yes	370
capillary	47	late	327	lab	159	tick	174	10^4 to 10^6	148	no	38
natural	154					warthog	62	$>10^6$	192		
inoculation	38										

Annex 7: 5 best models with the included variables influencing infection status (note that all models have « virus strain » as random effect), corrected Akaike Information Criteria and weight of the model (probability that the model is the best out of all models considered).

Adaptation tick/virus	Exposure	Colony type	Tick stage	Viral titer	Viral host	AICc	delta
		+	+	+		393.6	0.00
+		+	+	+		395.6	2.01
		+	+	+	+	395.7	2.07
+		+	+	+	+	397.4	3.84
	+	+	+	+		399.6	5.97