The epidemiological status of African swine fever in domestic swine herds in the Tavush Province region, Armenia

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Summary

The factors associated with the spread and persistence of African swine fever (ASF) in the Caucasus region remain to be fully identified. It is assumed that large naive populations of domestic free-ranging and wild pigs are critical to disease transmission and maintenance. Nonetheless, 11 years since its epidemic introduction into the region in 2007, African swine fever virus (ASFV) is still circulating, suggesting that an endemic cycle has been established based on contact between free-ranging domestic pigs and wild pigs, and that native *Ornithodoros* ticks probably serve as reservoirs for the virus. Therefore, research is required to gather information on the epidemiological status of ASF in the Caucasus region, focusing particularly on understanding modes of ASFV spread and persistence in this new virus environment. The authors established an ASFV survey targeting domestic pigs in the Tavush province of Northern Armenia, an area of the country considered to be at high risk of disease incursion/occurrence. All tested samples collected for this survey were negative for ASF. The probability of observing no reactors by antibody ELISA in a sample of this size (n = 1,506) from a population with an estimated disease prevalence of 1% is very low (< 0.0001). Therefore, it is possible but very unlikely for ASFV to be present among domestic pigs in the Tavush province region.

Keywords

African swine fever virus – Armenia – Prevalence – Pig – Transmission – Virulence.

Introduction

African swine fever (ASF) is a contagious viral disease of swine. The causative agent, African swine fever virus (ASFV), is a large enveloped virus containing a double-stranded (ds) DNA genome of approximately

190 kilobase pairs. The virus shares aspects of genome structure and replication strategy with other large dsDNA viruses, including the Poxviridae, Iridoviridae and Phycodnaviridae (1). Infections of immuno-naive domestic pigs with AFSV are often fatal and are characterised by fever, haemorrhages, ataxia and severe depression. However, the course of infection varies, ranging from highly lethal to subclinical, depending on host characteristics and the infecting virus strain (2).

The disease has been confirmed mainly in Sub-Saharan Africa (1). In Africa, there are at least three scenarios of ASF occurrence (3). A domestic cycle scenario is most likely occurring in the area under study in Armenia. The role of wild pigs, warthogs (*Phacochoerus africanus*) and bush pigs (*Potamochoerus porcus*) in the epidemiology of the disease is relevant in East and Southern Africa. In the case of warthogs, direct horizontal or vertical transmission of ASFV is thought not to occur, therefore maintenance of the virus is dependent on a 'sylvatic cycle'. This cycle involves transmission of ASFV to young warthogs by infected *Ornothodorus moubata* soft ticks. Infected *Ornithodoros* ticks are able to retain the virus for long periods, transmitting ASFV to susceptible hosts. Furthermore, these ticks can transmit ASFV to other ticks via sexual and transovarial transmission, allowing the virus to persist even in the absence of viraemic hosts. In West Africa, the 'sylvatic cycle' is yet to be demonstrated. In this region of Africa, Argasid ticks (such as *Ornithodoros* spp.) have not been observed in warthog burrows, and there is no clear evidence that ASFV circulates among warthogs.

The sylvatic cycle creates the potential for establishment of an endemic tick reservoir of infection upon introduction of ASFV into new regions if indigenous competent tick vectors are present. In Western Europe, ASF is still endemic on the island of Sardinia, Italy. In Sardinia and Spain (4, 5), for instance, either low prevalence or absence of ASFV antibodies in wild boars has been observed in areas where domestic pigs are free of the disease. It is likely that persistence of ASFV in wild boars is limited without contact with infected domestic pigs. In Spain, the *Ornithodoros erraticus* tick was identified as a vector and reservoir for

the virus (6), and a significant association between the presence of this tick and the occurrence of ASF outbreaks was later reported (7). Furthermore, the presence of ASF was seen to decrease only as the tick became extinct (8). Altogether, these scenarios demonstrated the important role of indigenous native ticks in disease appearance and maintenance.

The first spread of ASFV into Europe and America occurred during a period from 1957 to 1995 (9), affecting several countries. When it emerged again outside Africa, new outbreaks of ASF were reported in the Caucasus region in 2007, affecting the countries of Georgia, Armenia, Azerbaijan and Russia. Since then, isolated outbreaks of ASF have been reported in Iran, Ukraine and Belarus, and in the European Union Member States of Lithuania, Latvia and Poland. Since 2015–2016, ASF has further spread throughout Russia, Ukraine, Estonia, Latvia, Lithuania, eastern Poland, Moldova, the Czech Republic and Romania. The current ASFV epidemic affecting Europe and Asia has been caused most likely by ASFV Georgia 2007/1 or viruses derived from this isolate; ASFV Georgia 2007/1 is a highly virulent isolate belonging to genotype II (10). Genotype II is endemic in south-eastern Africa.

Outbreaks of ASF were first detected in Armenia in 2007 and early 2008 and were confined to communities in the north-eastern section of the country, in Tavush and Lori Marzes (provinces) (Fig. 1). This region borders Georgia and Azerbaijan. As a consequence of ASF emergence, domestic pig populations declined drastically in the area. This was due to the combination of acute deaths from ASF, the applied stamping out (pre-emptive slaughter) policies, and decreased rates of pig repopulation on affected farms (Figs 2 and 3). Outbreaks re-emerged in Armenia in 2010–2011, spanning six previously unaffected pig-rearing provinces across the country (Fig. 1).

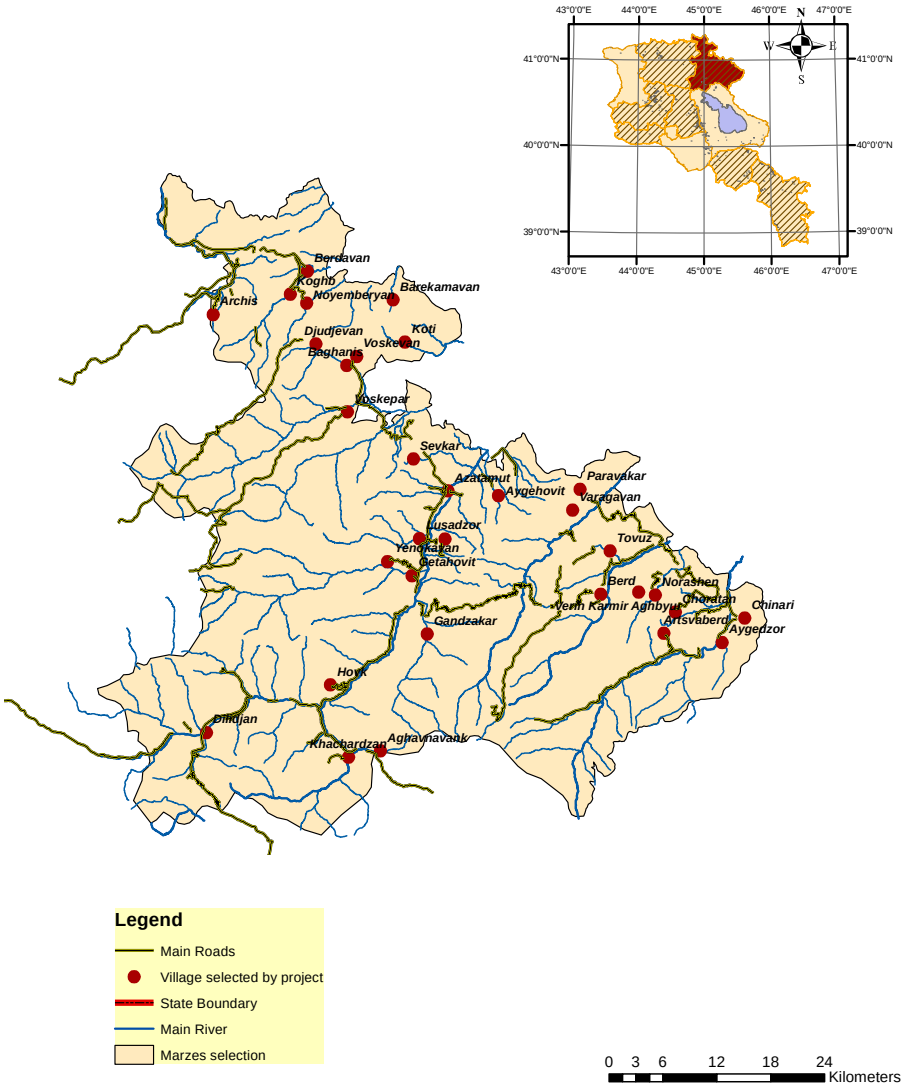

Fig. 1

In this study, samples were collected from domestic pigs at different locations (red dots) in Tavush Province, Armenia. The inset figure shows a map of Armenia indicating the location of Tavush Province (brown); diagonal lines identify provinces affected by the spread of African swine fever in the country during the period 2007–2011

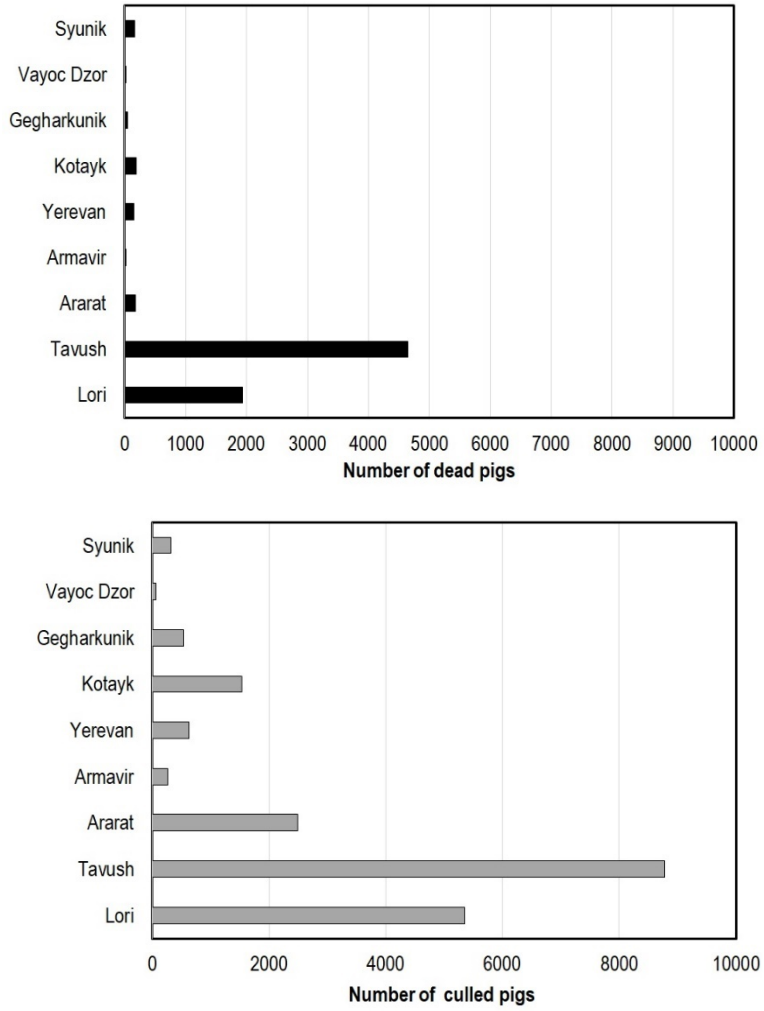

Fig. 2

Number of pigs dead (above) and culled (below) as a result of African swine fever virus outbreaks in different Marzes (provinces) of Armenia between 2007 and 2011

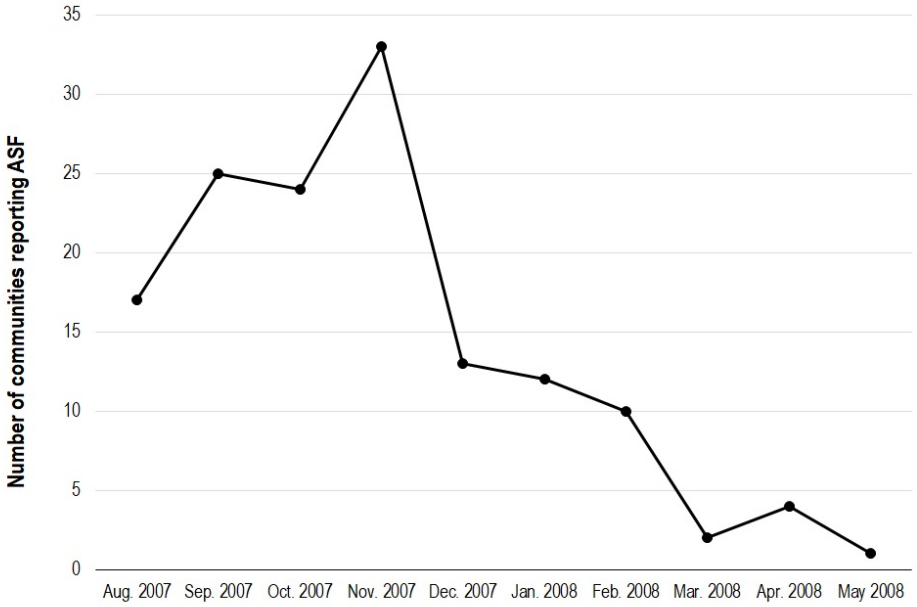

Fig. 3

Number of communities in Northern Armenia (Tavush and Lori provinces) reporting pig mortality events during the first African swine fever epidemic (2007–2008)

ASF: African swine fever

The Armenian province of Tavush, in particular, registered large numbers of ASF outbreaks in 2007. Tavush has a sizeable domestic pig population, and pigs are often released into forests to feed on fallen acorns or other nuts, a practice known as pannage. Furthermore, feral pigs, wild boar and soft-bodied ticks, including those of the *Ornithodorus* genus, are indigenous to the area (11). Tavush province borders Georgia and Azerbaijan, is crossed by major international highways and is heavily forested. All these factors make Tavush an area at high risk of ASF incursion and its current ASF status is unknown.

The current study was aimed at investigating the circulation of ASFV among domestic pig herds in 30 communities in the Armenian Province of Tavush, through an antibody and DNA survey (Fig. 1).

Materials and methods

Study area

This survey was conducted in the Tavush province, located in Northern Armenia between 40.66 N to 41.29 N and 44.77 E to 45.60 E (Fig. 1), which spans an area of approximately 2,704 km². Tavush has an estimated domestic pig population of 15,000 animals. The district was selected as a study area on the basis of a previous history of ASF outbreaks, domestic pig density, and the presence of free-ranging pigs, wild suids, soft-bodied ticks, forested land and proximity to international borders.

Sampling protocol

Study area

During the summer of 2014, ten teams of sample collectors were assembled by the Ministry of Agriculture of Armenia. Each team was tasked with obtaining 150 samples from domestic pigs in three different communities within specific geographical boundaries in Tavush province (Table I). Thus, a total of 30 communities were surveyed in the province. Several communities in the Northern provinces of Lori and Tavush had reported cases of ASF in 2007–2008 (Table II). Further outbreaks of ASF were not reported in this region until 2011 when the disease was detected across the country. Some of the communities that were initially affected by ASF in 2007 reported the disease again in 2011 (Table II). Such communities in Tavush were included in the present survey.

Table I

Location of the communities and number of samples collected from pigs in the Tavush Province region during the survey

Communities	Coordinates	# pigs sampled
Berd	40°52'51"N 45°23'30"E	
Norashen	40°52'37"N 45°27'34"E	150
Tavush	40°54'52"N 45°24'04"E	
Artsvaberd*	40°50'01"N 45°28'16"E	
Aygedzor	40°49'32"N 45°32'27"E	153
Movses	40°54'23"N 45°29'29"E	
Norashen	40°52'37"N 45°27'34"E	
N.K. Aghbyur	40°56'53"N,45°25'59"E	151
Aygepar	40°56'31"N 45°27'44"E	
Koti*	41°08'08"N 45°07'30"E	
Barekamavan**	41°10'45"N 45°06'35"E	150
Voskevan**	41°07'09"N 45°03'55"E	
Archis*	41°09'51"N 44°52'21"E	
Baghanis*	41°06'31"N 45°03'24"E	150
Jujevan*	41°07'56"N 45°00'40"E	
Noyemberyan**	41°10'21"N 44°59'37"E	
Berdavan**	41°12'10"N 45°00'12"E	152
Koghb**	41°10'57"N 44°58'33"E	
Dilijan**	40°44'27"N 44°51'47"E	
Aghavnavank	40°43'51"N 45°05'36"E	150
Khachardzan	40°43'09"N 45°03'30"E	
Hovk*	40°47'28"N 45°01'21"E	
Gandzakar*	40°50'24"N 45°09'47"E	165
Getahovit*	40°53'45"N 45°07'53"E	
Ijevan*	40°52'32"N 45°08'57"E	
Lusadzor	40°55'59"N 45°07'59"E	139
Aknaghbyur	40°57'30"N 45°09'23"E	

Khashtarak	40°56'19"N 45°10'47"E	
Lusahovit	40°54'57"N 45°11'22"E	146
Ditavan	40°57'32"N 45°12'07"E	

African swine fever virus outbreaks detected in *2007 or **2007 and 2010

Table II

African swine fever virus (ASFV) outbreaks detected in Armenia since the introduction of ASFV in 2007 as reported to the World Organisation for Animal Health (OIE)

Date	Location	Exposed	Deaths	Culled	Test	Pigs
Aug-07	Vaagnadzor, Lori	34	34	0	IFA, PCR, VI	Domestic
Aug-07	Barekamavan, Tavush	360	140	220	IFA, PCR, VI	Domestic
Aug-07	Dilijan, Tavush	406	137	269	IFA, PCR, VI	Domestic
Aug-07	Agartsni, Tavush	500	230	0	IFA, PCR, VI	Domestic
Aug-07	Vahagni, Lori	408	90	28	IFA, PCR, VI	Domestic
Aug-07	Dsegh, Lori	520	26	0	IFA, PCR, VI	Domestic
Sep-07	Tsakhkashat, Tavush	32	23	9	Clinical	Domestic
Oct-07	Nekhotc, Lori	20	10	10	qPCR	Domestic
Oct-07	Hakpat, Lori	94	18	76	qPCR	Domestic
Oct-07	Shamlukh, Lori	56	40	16	qPCR	Domestic
Oct-07	Kothi, Tavush	14	5	9	qPCR	Domestic
Oct-07	Thekhut, Lori	18	7	4	qPCR	Domestic
Oct-07	Koghb, Tavush	21	13	8	qPCR	Domestic
Mar-10	Noyemberyan, Tavush	116	3	113	AB ELISA	Domestic
Aug-10	Marts, Lori	8	3	5	AG ELISA	Feral
Aug-10	Lorut, Lori	26	19	7	AG ELISA	Feral
Oct-10	Eghegnadzor, Vayots Dzor	2	2	0	AG ELISA	Wild Boar
Jan-11	Geghashen, Kotyak	1	0	1	AG ELISA	Domestic
Jan-11	Nor Artik, Aragatson	2	0	2	AG ELISA	Domestic
Jan-11	Azatamut, Tavush	2	0	2	AG ELISA	Domestic
Feb-11	Norakert, Armavir	2	0	2	AG ELISA	Domestic
Feb-11	Mutsq, Siounik	2	0	2	AG ELISA	Domestic
Feb-11	Arinj, Kotayk	2	0	2	AG ELISA	Domestic
Jan-11	Aygedzor, Tavush	2	0	2	AG ELISA	Domestic

Jan-11	Nor Gosh, Kotayk	2	0	2	AG ELISA	Domestic
Jan-11	Kapan, Siounik	1	0	1	AG ELISA	Domestic
Jan-11	Tsaghkalanj, Armavir	4	0	4	AG ELISA	Domestic
Mar-11	Nor Egheg, Aragatsotn	2	0	2	AG ELISA	Domestic

AB ELISA: antibody detection enzyme-linked immunosorbent assay
AG ELISA: antigen detection enzyme-linked immunosorbent assay
IFA: immunofluorescence assay
PCR: polymerase chain reaction
qPCR: real-time polymerase chain reaction
VI: virus isolation

Sample collection and processing

Sampling was conducted in accordance with established guidelines. A total of 1,506 pigs were sampled during the spring and summer of 2014 from small-scale farms that bred pigs outdoors or kept pigs in small outdoor pens (Table I). The age of the animals varied between 3 and 14 months. Sera and whole blood were obtained by standard methods using the vacutainer system (red top and purple top tubes, respectively). Nasal swabs were also obtained from the animals. All samples were stored at 4°C prior to being submitted to the Risk Assessment Centre Laboratory facility in Yerevan for processing and testing. The sample size was estimated using Epitools software (http://epitools.ausvet.com.au; for details see the description below under data analysis).

Laboratory tests and data processing

Antibody detection

Serological analysis was performed using a blocking enzyme-linked immunosorbent assay (ELISA), Ingezim PPA COMPAC (Ingenasa, Spain), for detection of antibodies against ASFV. The assay uses a monoclonal antibody conjugate specific to the ASFV VP72 protein. The test and interpretation of the results were performed according to the manufacturer's instructions.

Antibody detection using an immunoperoxidase technique

A proportion of the ELISA tested serum samples (~ 5%) that showed doubtful results (optical density cut-off values between the optical

density cut-offs of positive and negative controls) were also tested for ASFV antibodies using an immunoperoxidase technique (IPT), following protocols and recommendations by the ASF European Reference Laboratory: the Centre for Animal Health Research (CISA) of the National Institute for Agricultural and Food Research and Technology (INIA) (Madrid, Spain). Briefly, serum samples were incubated in 96-well plates containing fixed ASFV-infected Vero cell monolayers (kindly provided by Dr Consuelo Carrillo at the Foreign Animal Diseases Diagnostic Laboratory [FADDL], Animal and Plant Health Inspection Service [APHIS], United States Department of Agriculture [USDA], United States of America [USA]). After repeated washes, alkaline phosphatase-conjugated protein A was added to the wells and incubated for 1 h at 37°C. The cell monolayers were then washed repeatedly and exposed to a substrate solution in the presence of hydrogen peroxide. Monolayers were examined for colour changes under a light microscope. An ASFV antibody positive serum was used as the positive control for the assay (kindly provided by Dr Carrillo at FADDL, APHIS, USDA, USA).

Virus detection in blood and nasal swab samples

Total DNA was obtained from blood and nasal swab samples using the DNeasy Blood and Tissue kit (Qiagen, Valencia, CA, USA) according to the instructions provided. An ASFV real-time quantitative polymerase chain reaction (qPCR) kit (Tetracore, Rockville, MD, USA) was used to detect viral DNA in the extracted samples. The ASFV DNA extracted from a virus-positive sample obtained during the outbreak of 2007 in Armenia was used as an additional positive control in the reaction. The reaction conditions were set according to the instructions of the manufacturer and reactions were run in a rotor gene thermal cycler (Qiagen, Valencia, CA, USA).

Data analysis

The Epitools (12, 13) program was used to estimate the prevalence of ASF for this structured survey in the Tavush province. The tool also provided sample size estimations for a range of true prevalence and precision values while considering the following: population size =

15,000 (Statistical Committee of the Republic of Armenia), test sensitivity = 0.95 and test specificity = 0.98 (Table III) (for the ELISA). A range of test sensitivity and test specificity values, assuming a population size of 15,000, a true prevalence of 0.01 and precision of 0.01, were also taken into account (Table IV).

Table III

Sample sizes required for sensitivity = 0.95, specificity = 0.98 and a range of true prevalence and precision values (http://epitools.ausvet.com.au)

	TP = 0.01	TP = 0.02	TP = 0.05	TP = 0.1	TP = 0.2	TP = 0.5
Precision = 0.01	1,166	1,486	2,330	3,434	4,895	6,378
Precision = 0.02	310	402	660	1,037	1,621	2,342
Precision = 0.05	51	66	111	177	286	432
Precision = 0.1	13	17	28	45	73	111
Precision = 0.2	4	5	7	12	19	28

TP: true prevalence

Table IV

Sample sizes required for true prevalence = 0.01, precision = 0.01 and a range of sensitivity and specificity values for the test used

	Se = 0.7	Se = 0.8	Se = 0.9	Se = 0.95	Se = 0.99	Se = 0.999
Sp = 0.7	11,581	10,271	9,026	8,446	8,005	7,909
Sp = 0.8	9,382	8,068	6,927	6,423	6,050	5,969
Sp = 0.9	6,041	4,996	4,174	3,830	3,583	3,530
Sp = 0.95	3,664	2,970	2,451	2,241	2,092	2,060
Sp = 0.99	1,231	1,011	850	785	739	729
Sp = 0.999	600	519	457	431	413	408

Se: sensitivity
Sp: specificity

Results

Antibody and viral genome detection

A total of 1,506 domestic pigs were sampled (12). The mean number of pigs sampled per team of collectors (n = 10) was 150.6 (median 150). Whole blood, serum and nasal swabs were obtained from all pigs.

The samples were processed and tested for ASFV antibodies (ELISA and IPT) and ASFV DNA (qPCR) (Fig. 4) as described in the materials and methods section. All samples tested negative for ASFV antibodies and DNA.

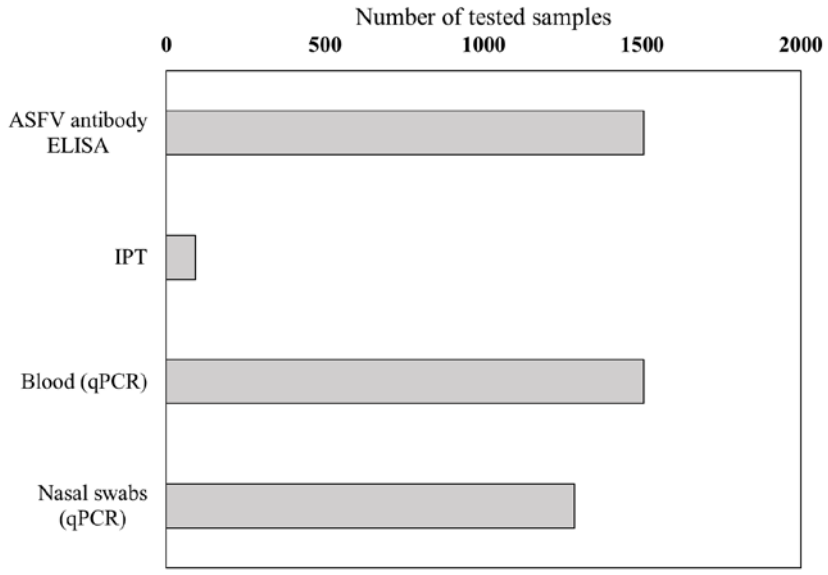

Fig. 4

Clinical samples tested for detection of African swine fever virus antibodies using enzyme-linked immunosorbent assay and/or immunoperoxidase technique and detection of ASFV deoxyribonucleic acid by real-time polymerase chain reaction

ASFV: African swine fever virus
ELISA: enzyme-linked immunosorbent assay
IPT: immunoperoxidase technique
qPCR: real-time polymerase chain reaction

Discussion

In Armenia, timely field-based research is required to gather information on the current epidemiological status of ASF. A particular focus on understanding the modes of transmission, spread and persistence of ASFV in this new environment is needed, primarily in domestic pigs, but also in feral and wild pigs, and in potential soft tick vectors. In this survey, we focused on estimating ASFV prevalence

among domestic pigs in the Tavush province, a high risk area for the disease in Armenia. All 1,506 tested serum and blood samples collected for this survey were negative for ASF antibody and DNA. The probability of observing zero reactors on ELISA in a sample of this size (n = 1,506) from a population with an estimated disease prevalence of 1% is very low (< 0.0001). Therefore, it was possible but unlikely for ASFV to have been present among domestic pigs during the span of this survey.

As ASF re-emerged outside Africa in 2007 in the Caucasus region, affecting Georgia, Armenia and Azerbaijan, new attention was aptly placed on this devastating disease of pigs. Since 2007, ASFV has spread south from the Caucasus into Iran (14) and north into Russia. In 2009, an ASF outbreak occurred in north-western Russia in pig holdings on the outskirts of Saint Petersburg (15). Rapid spread of the disease was expected at that time (1). Indeed, since then and according to reports to the World Organisation for Animal Health (OIE), ASF outbreaks have been reported in Ukraine (2012) and Belarus (2013) and in the European Union Member States of Lithuania, Latvia and Poland in 2014, affecting mainly wild boars, showing a progressive movement of the disease towards Western Europe. Since 2015–2016, ASF has further spread throughout Russia, Ukraine, Estonia, Latvia, Lithuania, eastern Poland, Moldova, the Czech Republic and Romania. In the context of the epidemic caused by ASFV Georgia 2007/1, the roles played by wild boars, feral pigs and tick vectors in the transmission, spread and persistence of ASFV are not fully understood.

Isolates of ASFV obtained from different regions of the Caucasus, ASFV Armenia 2008 and ASFV Chechen Republic 2009, have been used to challenge European wild boars via intranasal and intramuscular routes (16). Both routes of inoculation resulted in 100% mortality of the inoculated wild boars, leading to the conclusion that, since its introduction into the Caucasus region, the circulating ASFV has retained a virulent phenotype similar to that of the original Georgia 2007/1 isolate. The data suggest that, given its high virulence, the circulating virus would have a limited ability to persist in wild boar populations. Similar conclusions can be drawn from a recent study

aimed at determining the genetic changes in ASFV isolates collected in the Russian Federation from 2007 to 2011 using partial genome sequencing of these isolates. Further outbreaks of ASF were not reported in this region until 2011 when the disease was detected across the country. Some of the communities in Armenia that were first affected by ASFV in 2007 reported the disease again in 2011 (Table II). Those communities were included in the present survey. Malogolovkin *et al.* (17) observed 100% identity for the B646L and E183L genes and determined that all viruses examined that were obtained from the Russian Federation in the period 2007–2011 formed a genetic cluster within genotype II. These findings led to the conclusion that only one ASFV virus variant caused the outbreaks from 2007 to 2011 in the territory of the Russian Federation. Although virulent and genetically stable, the virus still re-emerges in the Caucasus region, reinforcing the idea that unidentified factors or reservoirs contribute to its persistence in this environment.

Most field strains of ASFV can persistently infect *Ornithodoros* ticks, allowing the arthropod to act as a reservoir for the virus. *Ornithodoros* is a genus in the soft-bodied tick family, the Argasidae. The pathogenesis of ASFV in *O. moubata* ticks is characterised by a low infectious dose, lifelong infection, efficient transmission to both pigs and other ticks, and low mortality until after the first oviposition (18). At least one European species of *Ornithodoros*, *O. erraticus*, is known to be capable of transmitting ASFV (19) and to remain persistently infected for up to 655 days (19). Furthermore, it has been proposed that *O. erraticus* surviving from 1993 were the source of the ASF outbreak in Portugal in 1999 (19). Coincidently, the last provinces to eradicate the disease in Spain were also the ones with indigenous *O. erraticus*. Diaz *et al.* (20) showed that ASFV Georgia 2007/1 can replicate effectively in *O. erraticus*, heightening the probability of this tick being an ASFV reservoir. Since *Ornithodoros* ticks also inhabit the Caucasus region, with an unknown geographical range, it will be critical to assess the role that these ticks may play in ASFV transmission and persistence. However, a recently described wild boar–habitat cycle has emerged in the Baltic countries (21), where both direct transmission between

infected and susceptible wild boar and indirect transmission through carcasses in the habitat led to ASF endemicity.

This survey showed that ASFV occurs at low prevalence, if at all, among domestic pigs in a high risk area of Armenia. It is most likely that, in Armenia, ASF is established in a low prevalence 'smouldering' endemic cycle supported by virus circulation among free-ranging domestic pigs (feral pigs) and wild pigs, and perhaps competent *Ornithodorus* ticks. The survey demonstrates that ASFV is either absent, undetectable or present at an extremely low prevalence (< 1%) in domestic pigs in the region studied. Therefore, domestic pigs do not appear to be epidemiologically relevant for ASFV maintenance and transmission in Armenia. Given that sporadic ASFV outbreaks in domestic pigs have occurred and continue to occur in the Caucuses, the study findings imply that long-term survival of ASFV in the environment and the presence of competent *Ornithodorus* ticks and feral pigs, especially wild boars, are possible transmission drivers of viral spillover to domestic pigs in Armenia.

Acknowledgements

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