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ORIGINAL ARTICLE

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Re-emergence of genotype I of African swine fever virus in Ivory Coast

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Abstract

In July 2014, an outbreak of severe haemorrhagic disease in a domestic pig population, was reported in San-Pedro, the second seaport city of Ivory Coast. Animals of all age groups developed clinical signs consistent with African swine fever (ASF). Tissue and serum samples from dead pigs were sent to the laboratory for diagnostic confirmation and molecular characterization based on the partial *B646L* (*p72*), the full *E183L* (*p54*) gene and the central variable region of the *B602L* gene. The PCR results confirmed the outbreak of ASF. Phylogenetic analyses based on *p72* and *p54* sequences showed that the San-Pedro 2014 outbreak virus strain belongs to *p72* genotype I. The Analysis of the tetrameric amino acid repeat regions of the *B602L* gene showed two repeat signatures which differ by an extra A = CAST in the second signature. The ASFV sequence of the San-Pedro 2014 outbreak strain is closely related to historical and recent ASFV strains collected in Angola and Cameroon whose ships have repeatedly visited the seaport of San-Pedro from March to June 2014. The 2014 viruses are distinct from the strains involved in the previous ASF wave in 1996 in Ivory Coast.

KEYWORDS
African swine fever, domestic pigs, genotyping, Ivory Coast, phylogenetic analysis, San-Pedro

1 | INTRODUCTION

African swine fever (ASF) is a highly lethal and economically important viral disease of domestic pigs and European wild boar causing an acute haemorrhagic fever with case fatality rates of up to 100% (Costard, Mur, Lubroth, Sanchez-Vizcaino, & Pfeiffer, 2013; Plowright, Thomson, & Neser, 1994). Nevertheless, ASF clinical signs and the case fatality rate are known to change as the disease progresses. For instance, following the second introduction of ASF genotype I in the Iberian Peninsula in 1960, the disease presentation was similar to that observed in Africa with mainly acute cases. As ASF became endemic, an increased number of ASF outbreaks with low mortality rates was observed (Wilkinson, 1984).

The disease is caused by African swine fever virus (ASFV) which is the sole member of the Asfivirus genus in the Asfarviridae family (Alonso et al., 2018; Dixon et al., 1994; Tulman, Delhon, Ku, & Rock, 2009; Yanez et al., 1995). ASFV has a complex DNA genome ranging in length from 170 to 193 kbp depending on the virus strain. Until now, 24 genotypes of ASFV have been identified based on the C-terminal end of the major protein *p72* (Achenbach et al., 2017; Bastos et al., 2003; Boshoff, Bastos, Gerber, & Vosloo, 2007; Lubisi, Bastos, Dwarka, & Vosloo, 2005; Quembo, Jori, Vosloo, & Heath, 2018). In addition to the major protein *p72* gene, the tandem repeats sequences (TRS) of the central variable region (CVR) within the *B602L* gene (Gallardo et al., 2009; Lubisi, Dwarka, Meenowa, & Jaumally, 2009; Nix, Gallardo, Hutchings, Blanco, & Dixon, 2006) and the *E183L* gene encoding *p54* protein (Gallardo et al., 2009; Gallardo et al., 2011) are the common targets for the molecular epidemiological study of ASF to characterize the diversity of ASFV isolates.

African swine fever virus transmission occurs through direct contact with an infected domestic pig, ingestion of contaminated

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feed, contact with infected fomites, or bites from infected soft ticks of the *Ornithodoros* genus (Penrith, Thomson, & Bastos, 2004). The disease affects domestic and wild pigs including giant forest hogs (*Hylochoerus meinertzhageni*), warthogs (*Phacochoerus africanus*) and bushpigs (*Potamochoerus porcus*). In eastern and southern Africa, the virus is maintained through a sylvatic cycle involving warthogs and *Ornithodoros* soft tick vectors that inhabit the warthog burrows, (Burrage, 2013; Gallardo et al., 2015; Jori & Bastos, 2009). In Western Africa, where soft ticks of the *Ornithodoros moubata* complex are absent and where wild pigs do no act as a natural virus host, the transmission is mainly due to a domestic cycle by direct contact between the source of virus and naive domestic pigs. This source can be infected pigs or pig products, contaminated wastes, premises, fomites, other facilities such as vehicle (Gallardo et al., 2015). No effective treatment or vaccine is available for ASF control.

The first outbreak of ASF was reported in 1907. However, it is possible that this disease had caused losses in central and eastern Africa before the first description in Kenya (Montgomery, 1921). Later, ASF escaped from Africa to infect Europe and America (South America and the Caribbean countries). The disease was eventually eradicated with great economical losses everywhere except Sardinia (Italy) where it became enzootic in wild boars (Oura, 2015; Penrith, 2009). Recent studies to identify risk factors contributing to the maintenance of the disease in Sardinia have demonstrated the need to adapt ASF preventive and control strategies; all risk factors such as social-cultural practices, productive and economic conditions of the region including the management of wild boar population and rapid laboratory examination of samples have to be considered in order to eradicate the disease (Cappai, Rolesu, Coccollone, Laddomada, & Loi, 2018; Jurado et al., 2018; Martínez-López et al., 2015; Mur et al., 2016).

In the southern and eastern regions of Africa, Madagascar reported ASF for the first time in 1997/98 (Ravaomanana et al., 2010; Rousset et al., 2001). Then, the disease continued to expand to Mauritius, in 2007 and Ethiopia in 2012 (Achenbach et al., 2017; Couacy-Hymann, 2014; FAO-AU/IBAR-ILRI, 2017).

African swine fever also emerged in the Republic of Georgia in 2007 and spread to some Near-East countries and Eastern Europe including Russia and Belarus. ASF is not yet under control in Eastern Europe, thus, putting the large pig populations in both Eastern and Western Europe at high risk (FAO-AU/IBAR-ILRI, 2017; Gallardo et al., 2014; Oura, 2015). The isolates from Eastern and Western Europe outbreaks were characterized as genotype II, like isolates from Mozambique, Madagascar and Zambia (Bastos, Penrith, Macome, Pinto, & Thomson, 2004). The source of infection in this region is probably due to contaminated ship waste. Free-range pigs and wild boars were both involved, complicating the epidemiological situation (Penrith, 2009). More recently, in July 2018, an outbreak of ASF was reported for the first time in China and is still ongoing. The molecular characterization of outbreak samples showed that isolates belong to genotype II (Ge et al., 2018). Similarly, in September 2018, Belgium reported ASF in two wild boars near the village of Etalle in the province of Luxembourg caused by isolates belonging to genotype II (Garigliany et al., 2019).

African swine fever is currently enzootic in most sub-Saharan African countries. The epizootic wave of ASF in West Africa, caused by a genotype I ASF virus, started in Ivory Coast in 1996 and spread to Benin, Cape Verde, Togo, and Nigeria in 1997, Senegal and Ghana in 1999 (Babalobi et al., 2007; El Hicheri et al., 1998; Odemuyiwa et al., 2000) and Burkina-Faso in 2003 (OIE, 2004). However, prior to 1996, the disease was already endemic in southern Senegal and Nigeria since 1979 and there are multiple records of ASF in Cameroon in the years 1980s caused by genotype I viruses.

The first ASF crisis in Ivory Coast killed 135,000 pigs which represented 29% of the national pig population and 80% of the commercial production pig farming system. Following the investigation on the first pig farm where the disease started suggests the source of infection in Ivory Coast is probably due to contaminated ship waste used to feed pigs without any heat treatment. The global cost of the epizootic was estimated at US$ 18 million in Ivory Coast (El Hicheri et al., 1998) and US$ 6 million in Benin (FAO, 1997). Fortunately, the disease was eradicated in 1998. From the date of eradication, a thorough surveillance was conducted along the eastern border close to Ghana and Burkina-Faso (two enzootic countries) and the district of Abidjan, which has the highest concentration of commercial pig farms, to monitor the ASF situation in the country. Ivory Coast remained free from ASF until June 2014 (Couacy-Hymann, Kouakou, & Gnabro, 2016; Kouakou et al., 2017). In June 2014, a disease, causing the death of pigs of all ages and sexes was reported in the city of San-Pedro, the second seaport of the country, located in the southwest, 350 km from Abidjan. The infection began in free-ranging pigs and spread to commercial pig farms where it caused a fatality rate of 100%. The clinical and laboratory diagnosis confirmed that the Ivory Coast was infected for the second time by ASF virus.

The aim of this study was to investigate this ASF outbreak and characterize molecularly, the ASFV strain responsible for this new outbreak, to determine the probable source of infection. In this study, we carried out field investigations of the disease, followed by laboratory confirmation and molecular characterization of the virus strain using a conventional multi-locus genotyping approach.

2 | MATERIALS AND METHODS

2.1 | ASF outbreak locations and sampling sites

Beginning in April 2014, the regional field veterinary office for the southwest recorded mortalities of free-range pigs in the city of San-Pedro, but the information was not reported to the National Laboratory for Agricultural Development (LANADA)/Virology Laboratory of Bingerville until June 2014 when a high number of pigs continued to die. A team was sent by the Central Veterinary Services based in Abidjan to further investigate the outbreak areas.

Suspicious outbreaks were investigated in San-Pedro city in both free-range pigs, as well as commercial pig farms located in Moussadougou and Sassandra, 37 and 70 km from San-Pedro, respectively (Figure 1). Clinical signs were recorded for dead, sick and contact animals.

The authority took the decision to compensate farmers as done previously during the ASF epizootic crisis in 1994 based on the local pig market price.

2.2 | Sample collection

On autopsy, tissue samples were collected from tonsil, spleen, mesenteric lymph nodes, kidney, lung and liver and were kept in a box containing icepacks. From both sick and recently dead animals, whole blood was collected and processed to collect serum. Samples were transported within 24 hr to the Virology Laboratory in Bingerville. All samples were collected from domestic pigs and came from the site of the index outbreak. At the laboratory, serum samples were stored at −20°C and tissue samples at −80°C until further analyses.

2.3 | Molecular detection of ASFV genome

For each tissue sample, a 5% w/v tissue homogenate was prepared in phosphate buffered saline (pH 4, 0.01 M) and clarified by centrifugation at 6,000 *g* for 5 min. DNA was extracted from 100 µl of the tissue homogenate supernatants, or from serum samples (100 µl) using the DNeasy Blood &Tissue kit (Qiagen, USA) following the manufacturer's instructions.

Conventional PCR was performed, using primers PPA/PPA2, targeting the B646L gene (Agüero et al., 2003) to confirm the presence of ASFV. PCR products were separated by gel electrophoresis followed by ethidium bromide staining to visualize the expected 257 bp amplicon using this set of primers.

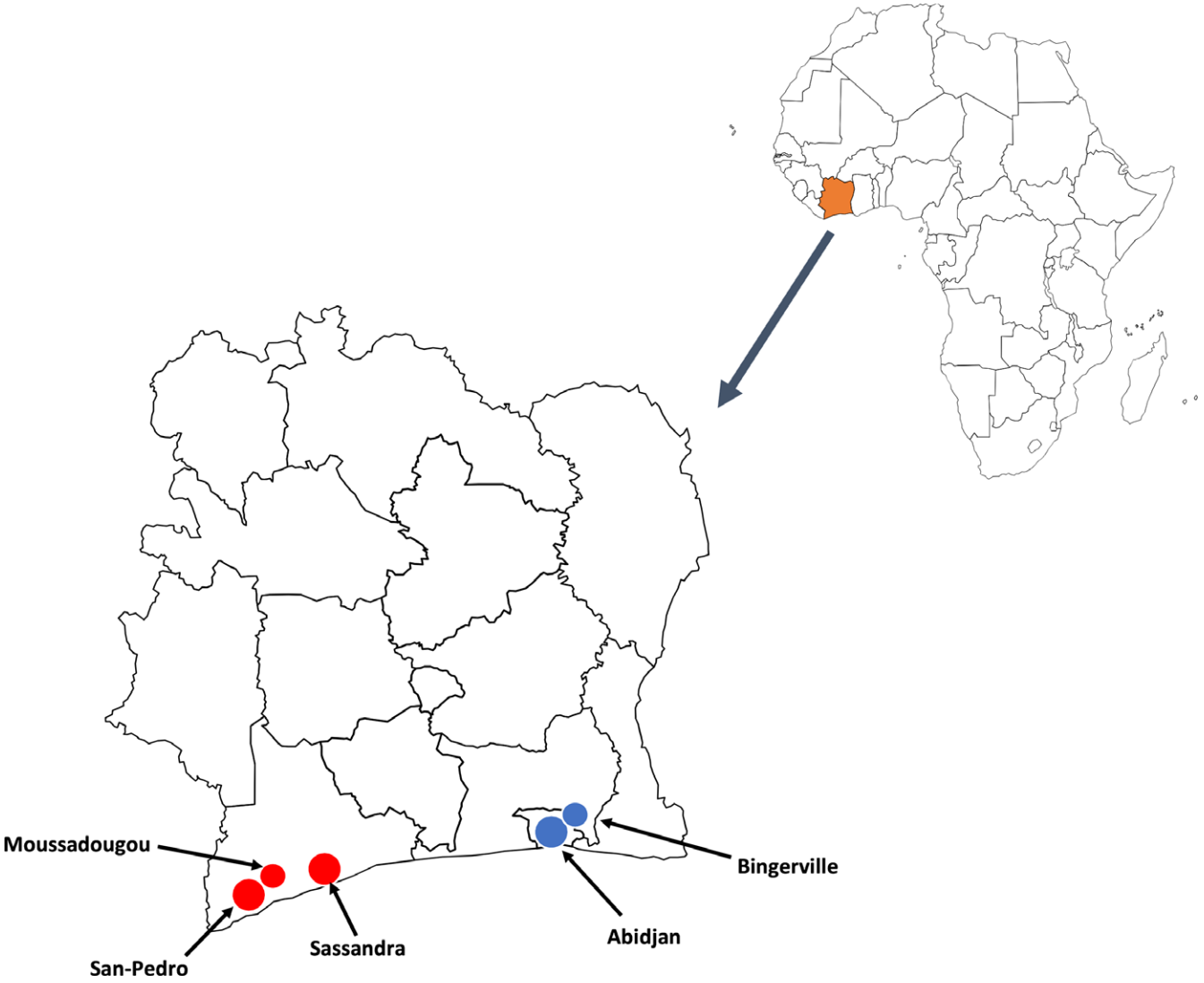

FIGURE 1 Map of Ivory Coast showing the area of the 2014 ASF outbreak: San-Perdro, Moussadougou and Sassandra. Abidjan hosts Central Veterinary Services. Bingerville hosts LANADA/Virology Laboratory. Ivory Coast is located in the Gulf of Guinea in West Africa [Colour figure can be viewed at wileyonlinelibrary.com]

2.4 | Molecular characterization of ASFV using the B646L (*p72*), E183L (*p54*) and CVR

For the molecular characterization of ASFV isolates, the following targets were amplified as follows: (a) the C-terminal-end of the *B646L* gene encoding the major capsid protein *p72* using primers *p72*U and *p72*D (Bastos et al., 2003) (b) the complete *E183L* gene encoding the inner envelope transmembrane protein *p54* using primers PPA89/PPA722 (Gallardo et al., 2009) and (c) the hypervariable central region of the *B602L* gene, containing the tetramer amino acid repeats, using primers ORF9L-F and ORF9L-R (Nix et al., 2006). In total, the complete *p54*, the partial *p72* and the partial *B602L* (CVR) genes of 12 samples were amplified and sequenced. The PCR products were purified using the Wizard ® SV Gel and PCR Clean Up kit (Promega, USA) per the manufacturer's protocol and sequenced commercially by LGC Genomics (Berlin, Germany). The raw data were edited and assembled using the Staden software package version 2.0.0b8. For each of the sequenced genes, the nucleotide sequences of the San Pedro ASFV isolates were compared to publicly available sequences using the Basic Local Alignment Search Tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple sequence alignments were performed using the CLUSTALW algorithm implemented in BioEdit 7.5 software package (Ibis Biosciences, Carlsbad, CA, USA). For phylogenetic reconstructions, the MEGA software version 7.0 (Kumar, Stecher, & Tamura, 2016) was used. Two datasets were produced for phylogenetic analyses. The *p72*-gene dataset comprising 84 taxa (398 characters) consisting of 12 *p72* nucleotide sequences generated in this study, together with 72 homologous sequences from public databases. At least one representative of each of 24 known *p72* genotypes (Quembo et al., 2018) was included (Table 1). ASVF genotype I sequences from neighbouring countries (Benin, Burkina, Ghana, and Togo) as well as those from countries whose boats visited the seaport of San-Pedro around the outbreak period (Ghana, Angola and Cameroon) were included as well. Up to 62 sequences of ASFV collected from Angola, Cameroon, Ghana, Benin, Togo, Nigeria and Burkina Faso between 2015 and 2010 were initially collected: 31 were from Genbank and 31 other were from the supplement material of a paper by Alkhamis et al., 2018. However, the sequence alignment showed that all were 100% identical to the partial *p72* gene sequence of isolate Ang72 (FJ174378), therefore, only 12 were kept in the final data set for this study. A Neighbour-Joining (NJ) tree and a Maximum Likelihood (ML) tree were produced for the *p72* gene using MEGA 7. For the NJ tree the evolutionary distances were computed using the Maximum Composite Likelihood method. For the ML tree, the Tamura 3-parameter model with a discrete Gamma distribution, selected as the best substitution model using MEGA 7, was used to produce the tree. For both NJ an ML trees, the data were re-sampled 1,000 times using the bootstrap method.

The dataset for the *p54* gene consisted of 54 taxa (453 characters) including 12 *p54* gene sequences generated in this study and an additional 42 sequences retrieved from GenBank, including sequences of isolates from Western Africa (Table 2). The sequences included at least one representative sequence from 18 different genotypes of ASFV, for which some *p54* sequences are available. For the *p54* sequences of AFSVs from genotype I, at least one representative for each of know sub-types (1a, 1b, 1c and 1d) was included. A Minimum Evolution tree was constructed using the p-distance substitution model and the Close-Neighbour-Interchange (CNI) algorithm. The initial tree was generated using the Neighbour-joining algorithm. The position with gaps, which were present in triplets, was removed. The data were re-sampled 1,000 times using the bootstrap method.

For each PCR-positive sample, the CVR nucleotide sequence was translated into amino acid and the amino acid tetramers were matched with previously reported codes (Achenbach et al., 2017; Boshoff et al., 2007; Gallardo et al., 2009; Lubisi, Bastos, Dwarka, & Vosloo, 2007; Nix et al., 2006). The CVR sequences were analyses together with those of 48 historical and recent isolates of genotype I from neighbouring countries (Benin, Burkina, Ghana, and Togo) and Nigeria, as well as those from countries whose boats visited the seaport of San-Pedro around the outbreak period (Ghana, Angola and Cameroon).

2.5 | Investigating the source of ASFV introduction in San-Pedro

To determine the route of entry of ASFV in San-Pedro, an investigation was carried out with the Regional Department for Livestock Production and Fisheries Resources, which is in charge of livestock farming and all its ancillary activities, the Regional Department for the Environment and the Captaincy office at the seaport of San-Pedro.

At the Livestock Production and Fisheries Resources level, information was sought regarding the pig and pig products trade circuit and sources in San-Pedro city and the San-Pedro region.

Then at the level of the Department for the Environment, information was collected to know which companies oversee city sanitation and the treatment of household waste from boats and the airport.

Registers were then viewed at the Captaincy office in San-Pedro, to verify which ships had docked in the seaport between March and June 2014, their place of origin and the treatment of household waste from the boats.

Finally, a letter was sent to the United Nations Operation in Ivory Coast to ascertain the nationality of the military contingent stationed at San-Pedro at the time of the epizootic and the source of their food.

3 | RESULTS

3.1 | ASF outbreak investigation

From April to September 2014, an outbreak was first reported in free-ranging pigs scavenging on food wastes in the city of San-Pedro with clinical signs suggestive of ASF. This was followed by reports of disease in commercial farms in Moussadougou, 37 km from San-Pedro. The disease killed 6,599 pigs in total, including 2,050 contact animals slaughtered for sanitary measures from 20 commercial pig farms.

TABLE 1 African swine fever viruses *p72* sequences used for comparative analyses

Virus designation	Country of origin	Year of sampling	Genbank accession No	Reference	*p72* Genotype
Ang72	Angola	1972	FJ174378	Gallardo et al. (2009)	I
Nig01	Nigeria	2001	FJ174382	Gallardo et al. (2009)	I
ANG/70	Angola	1970	AF301542	Bastos et al. (2003)	I
Georgia/2007	Georgia	2007	AM999764	Rowlands et al. (2008)	II
MAD/1/98	Madagascar	1998	AF270706	Bastos et al. (2004)	II
MOZ-60/98	Mozambique	1998	AY274455	Bastos et al. (2004)	II
MOZ/2002/2	Mozambique	2002	AY351518	Lubisi et al. (2005)	II
MOZ_12/2006	Mozambique	2006	KY353991	Quembo et al. (2018)	II
MOZ_9/2006	Mozambique	2006	KY353988	Quembo et al. (2018)	II
LUS93/1	Zambia	1991	AY351563	Lubisi et al. (2005)	II
SPEC/257	South Africa	1993	DQ250120	Boshoff et al. (2007)	III
RSA/5/95	South Africa	1995	DQ250124	Boshoff et al. (2007)	III
BOT/1/99	Botswana	1999	AF504886	Bastos et al. (2003)	III
RSA/1/99/W	South Africa	1999	AF449477	Bastos et al. (2003)	IV
MAL/2002/1	Malawi	2002	AY494553	Lubisi et al. (2005)	V
MOZ/1960	Mozambique	1960	AF270708	Bastos et al. (2004)	V
MOZ/1979	Mozambique	1979	AF270709	Bastos et al. (2004)	V
MOZ/94/1	Mozambique	1994	AF270711	Bastos et al. (2003)	VI
SPEC/265	Mozambique	1994	AF270710	Bastos et al. (2003)	VI
MOZ/94/8	Mozambique	1994	AF270712	Bastos et al. (2004)	VI
RSA/1/98	South Africa	1998	AF302818	Bastos et al. (2003)	VII
SPEC/154	Botswana	1987	DQ250113	Boshoff et al. (2007)	VII
SPEC/260	South Africa	1993	DQ250121	Boshoff et al. (2007)	VII
KIRT/892	Malawi	1978	AY351511	Bastos et al. (2003)	VIII
MAL/1978	Tanzania	1989	AF270707	Lubisi et al. (2005)	VIII
MOZ-A/98	Mozambique	1998	AY274452	Bastos et al. (2004)	VIII
Ug03H.1	Uganda	2003	FJ154440	Gallardo et al. (2009)	IX
Ken06.Kis	Kenya	2006	FJ154428	Gallardo et al. (2009)	IX
UGA/1/95	Uganda	1995	AF449475	Bastos et al. (2003)	IX
TAN/09/Longido	Tanzania	2009	JX262383	Misinzo et al. (2012)	X
MWHOG/9	Kenya	1959	AY351565	Lubisi et al. (2005)	X
Ken09Tk.15/6	Kenya	2009	HM745280	Gallardo et al. (2011)	X
Ug64	Uganda	1964	FJ174383	Gallardo et al. (2009)	X
KIRW/891	Tanzania	1989	AY351514	Lubisi et al. (2005)	X
KAB/62	Zambia	1983	AY351522	Lubisi et al. (2005)	XI
MFUE6/1	Malawi	1992	AY351561	Lubisi et al. (2005)	XII
MZI/921	Zambia	1982	AY351543	Lubisi et al. (2005)	XII
SUM/1411	Zambia	1983	AY351542	Lubisi et al. (2005)	XIII
NYA/12	Zambia	1986	AY351555	Lubisi et al. (2005)	XIV
TAN/1/01	Tanzania	2001	AY494552	Lubisi et al. (2005)	XV
TAN/08/MAZIMBU	Tanzania	2008	GQ410765	Misinzo et al. (2011)	XV
TAN/2003/1	Tanzania	2003	AY494550	Lubisi et al. (2005)	XVI
TAN/2003/2	Tanzania	2003	AY494551	Lubisi et al. (2005)	XVI
ZIM/90/1	Zimbabwe	1990	KC662376	Unpublished	XVII
ZIM/92/1	Zimbabwe	1992	DQ250119	Boshoff et al. (2007)	XVII
NAM/1/95	Namibia	1995	DQ250122	Boshoff et al. (2007)	XVIII

(Continues)

TABLE 1 (Continued)

Virus designation	Country of origin	Year of sampling	Genbank accession No	Reference	*p72* Genotype
RSA/3/96	South Africa	1996	DQ250127	Boshoff et al. (2007)	XIX
SPEC120	South Africa	1987	AF302812	Boshoff et al. (2007)	XIX
SPEC/125	South Africa	1987	DQ250112	Boshoff et al. (2007)	XIX
SPEC/251	South Africa	1992	DQ250118	Boshoff et al. (2007)	XIX
RSA/1/95	South Africa	1995	DQ250123	Boshoff et al. (2007)	XX
Lillie	South Africa	1979	DQ250109	Boshoff et al. (2007)	XX
24823	South Africa	1975	DQ250110	Boshoff et al. (2007)	XX
RSA/1/96	South Africa	1996	DQ250125	Boshoff et al. (2007)	XXI
SPEC/53	South Africa	1985	DQ250111	Boshoff et al. (2007)	XXI
SPEC/245	South Africa	1992	DQ250117	Boshoff et al. (2007)	XXII
ETH/1	Ethiopia	2011	KT795354	Achenbach et al. (2017)	XXIII
ETH/3	Ethiopia	2011	KT795360	Achenbach et al. (2017)	XXIII
ETH/017	Ethiopia	2014	KT795355	Achenbach et al. (2017)	XXIII
ETH/1a	Ethiopia	2011	KT795359	Achenbach et al. (2017)	XXIII
MOZ/11/2006	Mozambique	2006	KY353990	Quembo et al. (2018)	XXIV
MOZ/19/2006	Mozambique	2006	KY353998	Quembo et al. (2018)	XXIV
Pretorisuskop/96/4	South Africa	1996	AY261363	Unpublished	XX
ANG_2011/01	Angola	2011	JX310041	Unpublished	I
BEN/1/97	Benin	1997	AF302816	Bastos et al. (2003)	I
Burkina_Faso/09	Burkina Faso	2009	KT368178	Kouakou et al. (2017)	I
Cam_2009/02	Cameroon	2009	JX310034	Unpublished	I
CAM/82	Cameroon	1982	AF301544	Bastos et al. (2003)	I
CV97	Cape Verde	1997	FJ174380	Gallardo et al. (2009)	I
Togo/09	Togo	2009	KT368176	Kouakou et al. (2017)	I
IC96	Ivory Coast	1996	FJ174379	Gallardo et al. (2009)	I
Ghana/09	Ghana	2009	KT368177	Kouakou et al. (2017)	I

Note. Seventy-Two *p72* partial gene sequences from public databases including at least one representative of each of 24 known *p72* genotypes were included.

Pigs of all age groups and sex were affected and presented the same clinical signs including hind leg weakness, recumbence, dyspnoea, anorexia, erythema and cyanosis of the skin. Abortions were occasionally observed in pregnant sows. At postmortem, blood-tinged fluid was observed in the pleura, pericardium and peritoneal cavities. In addition, splenomegaly, petechiae of the heart and kidney, and haemorrhagic heart, kidneys, liver, intestines and lymph nodes especially the mesenteric lymph nodes, were observed (Figure 2).

In total, 48 tissue samples were collected from 12 dead animals and six serum samples were obtained from six recently dead and sick animals. All collected samples came from domestic pigs and from the site of the index outbreak.

3.2 | Cost of the epizootic

The global cost based on the total number of pigs which died or were slaughtered for sanitary measures (n = 6,599) was estimated at 803,827.4 Euros using 1/3 of the indicated market price of each category of pig, at that time, on an agreement between the Ministry of Animal resources and the Pig farmers Association.

3.3 | Laboratory confirmation

The samples collected from this ASF outbreak in San-Pedro were analyzed using conventional PCR as described in the methods section. All tissue and serum samples were positive showing an expected amplicon of 257 bp following gel electrophoresis. There was no attempt for virus isolation on cell culture.

3.4 | Molecular characterization of ASFV targeting the *B646L* (*p72*), *E183L* (*p54*) genes and the CVR

3.4.1 | *p72* gene phylogeny

The C-terminal end of the *p72* gene was amplified as described in the methods section and sequenced to classify the San-Pedro 2014 ASFV isolates into one of the known 24 genotypes. The NJ tree showed that all San-Pedro outbreak isolates belong to *p72* genotype I (Figure 3). The 2014 strains from Ivory Coast clustered with historical and recent strains from Angola, Benin, Burkina Faso, Cameroon, Cape Verde, Ghana, Togo and Nigeria, as well as the 1996 isolate of

TABLE 2 Summary of African swine fever virus *p54* sequences used for subtyping

Virus designation	Country of origin	Year of sampling	Genbank accession No	Reference	*p72* Genotype
Co62	Spain	1962	FJ174387	Gallardo et al. (2009)	Ia
Malta	Malta	1978	FJ174419	Gallardo et al. (2009)	Ia
E75	Spain	1975	FJ174394	Gallardo et al. (2009)	Ia
Benin 97/1	Benin	1997	AM712239	Chapman, Tcherepanov, Upton, & Dixon (2008)	Ia
Nig14_KAF37_14	Nigeria	2014	KT961351	Unpublished	Ia
NIG_IMOWR02	Nigeria	2014	KT150963	Unpublished	Ia
NIG_KidJosAb	Nigeria	2014	KT150966	Unpublished	Ia
Kat67	Democratic Republic of the Congo	1967	FJ174423	Gallardo et al. (2009)	Ib
IC96	Cote d'Ivoire	1996	FJ174429	Gallardo et al. (2009)	Ib
CV97	Cape Verde	1997	FJ174427	Gallardo et al. (2009);	Ib
LISB60	Portugal	1960	X84889	Sun, Jacobs, Smith, Dixon, & Parkhouse (1995)	Ic
ANG70	Angola	1970	EU874327	Unpublished	Ia
Mkuzi_79	South Africa	1979	AY261362	Unpublished	Id
MAD198	Madagascar	1998	KC662387	Unpublished	II
Warm	South Africa	Unknown	AY261365	Unpublished	III
NAMWart	Namibia	Unknown	AY261366	Unpublished	IV
Teng62	Malawi	1962	AY261364	Unpublished	Va
MOZ60	Mozambique	1960	EU874371	Unpublished	Va
Moz64	Mozambique	1964	FJ174422	Gallardo et al. (2009)	Vb
KAL881	Zambia	1988	KF736412	Unpublished	VIII
Ug03P.6	Uganda	2003	FJ174436	Gallardo et al. (2009)	IX
Ug64	Uganda	1964	FJ174430	Gallardo et al. (2009)	Xa
Kenya 1950	Kenya	1950	AY261360	Unpublished	Xb
KAB/62	Zambia	1983	EU874331	Unpublished	XI
SUM/1411	Zambia	1983	EU874357	Unpublished	XIII
NYA/1/2	Zambia	1986	EU874330	Unpublished	XIV
TAN08	Tanzania	2008	GQ410768	Misinzo et al. (2011)	XV
TAN2003/2_GXVI	Tanzania	2003	KF015947	Unpublished	XVI
ZAM01/2_XVII	Zambia	2001	KF015915	Unpublished	XVII
Pretorisuskop/96/4	South Africa	1996	AY261363	Unpublished	XXa
Lillie_GXX	South Africa	1979	X84888	Sun et al. (1995)	XXb
SPEC/53	South Africa	1985	KC662384	Unpublished	XXI
SPEC/245	South Africa	1992	EU874381	Unpublished	XXII
ETH/5a	Ethiopia	2011	KT795370	Achenbach et al. (2017)	XXIII
ETH/3a	Ethiopia	2011	KT795365	Achenbach et al. (2017)	XXIII
ETH/2a	Ethiopia	2011	KT795364	Achenbach et al. (2017)	XXIII
ETH/1a	Ethiopia	2011	KT795363	Achenbach et al. (2017)	XXIII
ETH/004	Ethiopia	2014	KT795368	Achenbach et al. (2017)	XXIII
ETH/017	Ethiopia	2014	KT795369	Achenbach et al. (2017)	XXIII
ETH/3	Ethiopia	2011	KT795367	Achenbach et al. (2017)	XXIII
ETH/1	Ethiopia	2011	KT795366	Achenbach et al. (2017)	XXIII
ETH/AA	Ethiopia	2011	KT795362	Achenbach et al. (2017)	XXIII

Note. Forty-two sequences retrieved from GenBank, including sequences of isolate from Western Africa.

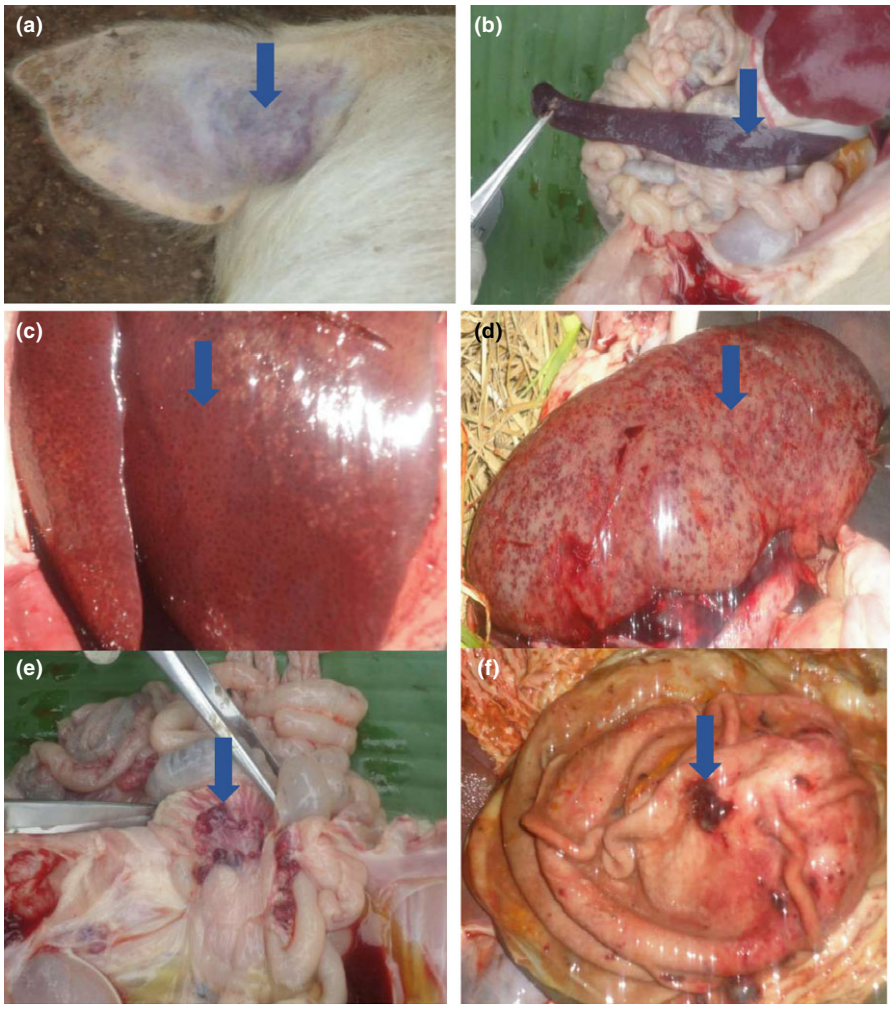

FIGURE 2 Clinical and postmortem findings in infected pigs in San-Pedro. (a) Image of an ear with cutaneous congestion common in ill pigs. Postmortem lesions include hypertrophied spleen (b), haemorrhagic liver (c), petechiation on the kidney (d), haemorrhagic mesenteric lymph node (e) and in the stomach (f) were observed in pigs with ASF [Colour figure can be viewed at wileyonlinelibrary.com]

Ivory Coast. Similar patterns were observed with the ML tree except that several branches collapsed into a polytomy.

The multiple sequence alignments of the partial *p72* gene sequences of all ASFV isolates from the five sampling locations of the study showed that they are all identical at the nucleotide level and completely identical to the strains collected in Angola, Benin, Burkina Faso, Cameroon, Cape Verde, Ghana, Togo and Nigeria, as well as the 1996 isolate of Ivory Coast. The *p72* gene sequences were submitted to GenBank with the accession numbers MG674296, MG674297 and MH836343 to MH836352.

3.4.2 | *p54* gene phylogeny and CVR analysis

We have also analyzed the San-Pedro ASFV isolates by comparing their full *p54* gene sequences to those of other isolates available in GenBank. The phylogenetic analysis of the *p54* gene showed that all 2014 strains from Ivory Coast clustered in genotype Ia (Figure 4), together with historical isolates from Angola, Benin and Malta, Spain and recent isolates from Nigeria while those of the previous 1996 outbreaks in Ivory Coast belong to the genotype Ib.

The multiple sequence alignments of the full *p54* gene sequences of all ASFV isolates from San-Pedro ASFV 2014 outbreaks showed that they are all identical at the nucleotide level. There were also completely identical to isolate Ang70, however, contrasting with the *p72* gene, they were different from isolate Nig01 and Ang72, mainly, by a deletion of six nucleotides in their *p54* gene sequence. The *p54* gene sequences of these San-Pedro isolates also differed from isolates IC96 collected in Ivory Coast 1996.

The *p54* sequences were submitted to GenBank with the accession numbers MG674298, MG674299 and MH836353 to MH836362.

Regarding the analysis of the CVR, the nucleotide sequences were translated into amino acids and each amino acids tetramers were matched with codes described in previous studies.

The CVR analysis revealed two distinct tetrameric repeat amino acid signatures (Table 3) differing by an insertion A (CAST). Isolates (IC-SP/2014/3 and IC-SP/2014/1 from San-Pedro shared common CVR profiles with isolates Cam10/YGT9; Cam10/GGT5, Cam10/OGT13 collected in 2010 in Cameroon (Alkhamis et al., 2018). All isolates from Moussadougou and the remaining isolates from San-Pedro shared common CVR profiles with isolates CAM/1/86 (AF513047) and CAM/82 (AF513046) collected in 1986 and 1982 in Cameroon. Sequences representing each profile were submitted to

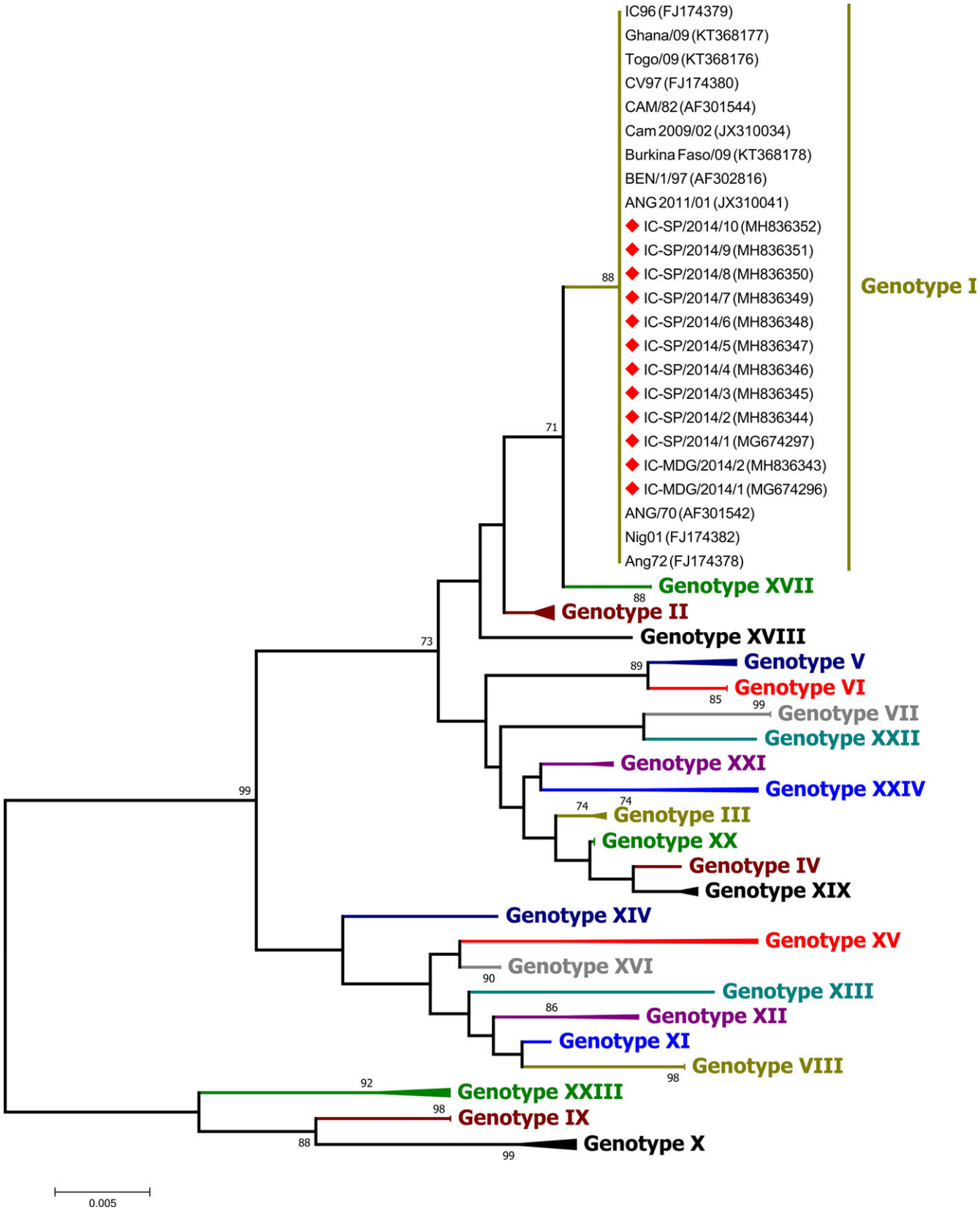

FIGURE 3 Neighbour-joining tree, of the partial *p72* gene, depicting genetic relationships of the San Pedro 2014 isolates with representatives of the 24 known ASFV genotypes. The evolutionary distances were computed using the Maximum Composite Likelihood method. Only the bootstrap values >70% are shown. The isolates of this study are highlighted with red diamond before the name [Colour figure can be viewed at wileyonlinelibrary.com]

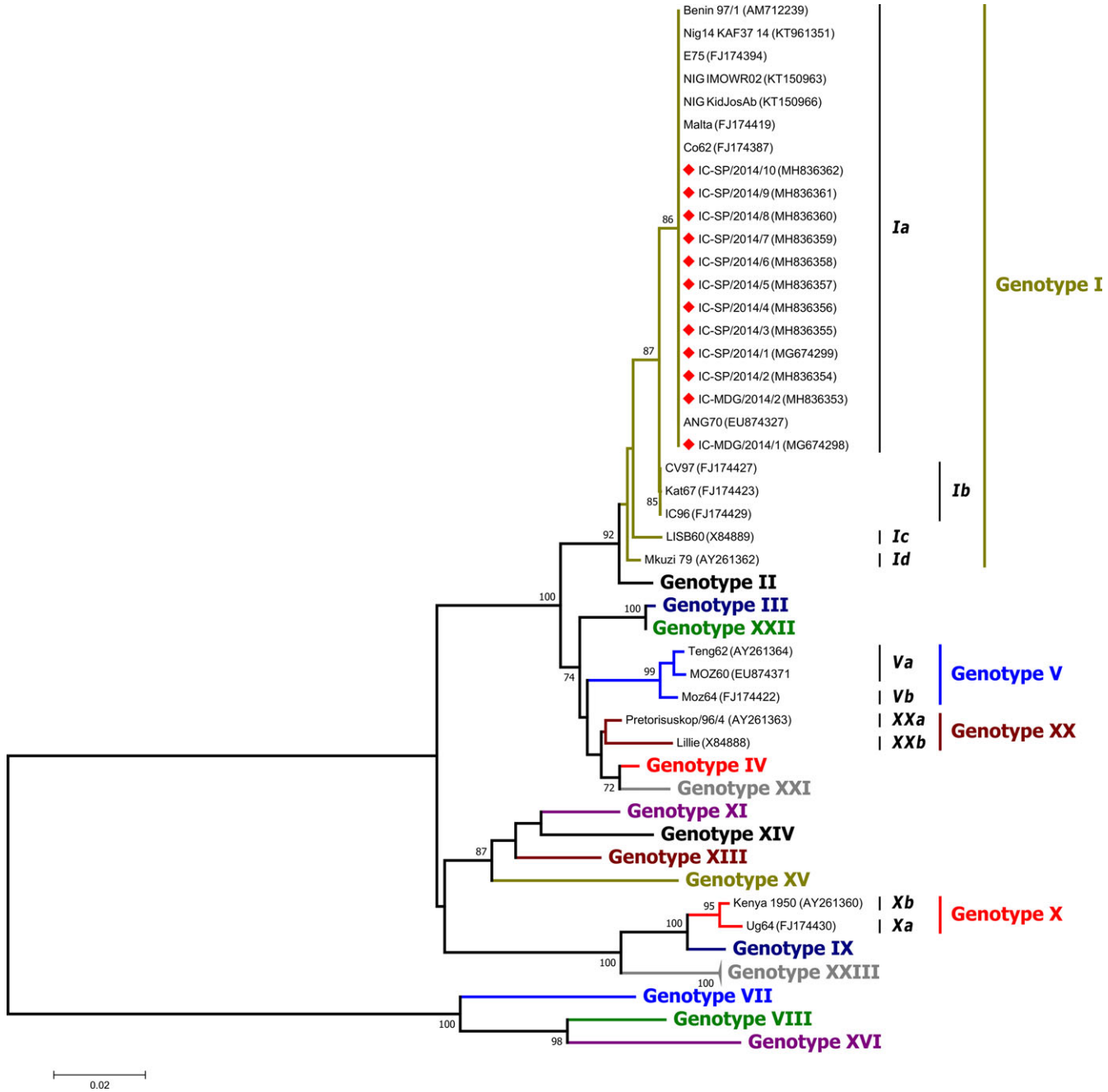

FIGURE 4 Minimum Evolution tree, based on the full *p54* gene, depicting genetic relationships of the San Pedro 2014 isolates with representatives of the 18 out of 24 known ASFV genotypes. The evolutionary distances were computed using the p-distance method. Only the bootstrap values >70% are shown. The isolates of this study are highlighted with a red diamond before the name [Colour figure can be viewed at wileyonlinelibrary.com]

GenBank and given accession numbers MG674300, MG67430 and MH836363 to MH836372.

3.5 | Investigating the source of ASFV introduction in San-Pedro

Following our investigation, the information collected from the different regional directions shows that:

The pig meat and pig products consumed in San-Pedro came partly from local production (local breed pigs from traditional free-range farms in the city and modern commercial pig farms) and from imports from European countries exclusively (Germany, Spain, France, Italy, Poland, the Netherlands and Sweden). No pig meat or pig products consumed in San- Pedro came from neighbouring countries like Liberia.

Five companies were responsible for collecting household waste from boats at the seaport. The agreement between these companies and the city of San-Pedro as well as the Department of the environment allows them to remove household waste from boats then deliver the waste to the municipal garbage dump without any treatment.

TABLE 3 Comparison of the CVR profiles of Ivory Coast ASFV to those of foreign strains

Virus designation	Country of origin	Year of sampling	Genbank accession No	Reference	CVR profile
Ang72	Angola	1972	AM259410	Nix et al. (2006)	AABNABTDBNAAAA
IC/1/96	Ivory Coast	1996	AF513036	Unpublished	ABNAAAACBNAAAAACBNAAAAACBNAAAACBNAFA
IC/3/96	Ivory Coast	1996	AF513053	Unpublished	AABNABTDBNAAAA
CV97	Cape Verde	1997	AM259462	Nix et al. (2006)	AABNABNABTDBNAAAA
CV98	Cape Verde	1998	AM259463	Nix et al. (2006)	AABNABNABTDBNAAAA
GH05/ASOb24	Ghana	2005	NA	Alkhamis et al. (2018)	ABNAAAAACBNAAAAACBNAAAAAACBNAAAAAACBNAAAAAACBNAFA
Nig06/PLJs16	Nigeria	2006	NA	Alkhamis et al. (2018)	ABNAAAACACBNAAAACBNAFA
Nig06/PLJs42	Nigeria	2006	NA	Alkhamis et al. (2018)	ABNABNAAAAACBNAFA
GH06/NE26	Ghana	2006	NA	Alkhamis et al. (2018)	ABNAAAAAACBNAAAAAACBNAAAAAACBNAFA
BF07/IpB1	Burkina Faso	2007	NA	Alkhamis et al. (2018)	ABNAAAACBNAAAAACBNAAAAACBNAAAAAA
BF07/CkAB	Burkina Faso	2007	NA	Alkhamis et al. (2018)	ABNAAAAAACBNAAAAAACBNAAAAACBNAFA
GH07/GAPk4	Ghana	2007	NA	Alkhamis et al. (2018)	ABNAAAACBNAAAAAAACBNAFA
Nig08/BNGb10	Nigeria	2008	NA	Alkhamis et al. (2018)	ABNABNAAAAACBNAFA
Nig08/BNGb15	Nigeria	2008	NA	Alkhamis et al. (2018)	ABNAAAACBNAAAACBNAAAACBNAFA
Nig08/BNGb2	Nigeria	2008	NA	Alkhamis et al. (2018)	ABNAAAACBNAAAACBNAAAACBNAAAACBNAFA
Nig08/BNGb24	Nigeria	2008	NA	Alkhamis et al. (2018)	ABNAAAAACBNAAAAACBNAFA
Nig08/BNGb4	Nigeria	2008	NA	Alkhamis et al. (2018)	ABNAAAACBNAAAACBNAAAACBNAFA
Nig08/BNGb9	Nigeria	2008	NA	Alkhamis et al. (2018)	ABNAAAACACBNAAAACBNAFA
Nig08/Cr15	Nigeria	2008	NA	Alkhamis et al. (2018)	ABNAAAAACBNAAAAAACBNAFA
Nig08/LAOk1	Nigeria	2008	NA	Alkhamis et al. (2018)	ABNAAAACBNAAAAACBNAAAAACBNAAAACBNAFA
Nig08/LAOk2	Nigeria	2008	NA	Alkhamis et al. (2018)	ABNAAAACBNAAAAACBNAAAAACBNAAAAACBNAFA
Nig08/NW10	Nigeria	2008	NA	Alkhamis et al. (2018)	ABNAAAAACACBNAAAACACBNAACBNAAAACBNAFA
Nig08/NW12	Nigeria	2008	NA	Alkhamis et al. (2018)	ABNAAAAACACBNAAAACACBNAACBNAAAACBNAFA
GH08/BASu17	Ghana	2008	NA	Alkhamis et al. (2018)	ABNAAAACBNAAAAAAAACBNAFA
GH08/GADg2	Ghana	2008	NA	Alkhamis et al. (2018	ABNAAAACBNAAAACBNAAAAAAAAACBNAFA
Ben09/Z8	Benin	2009	NA	Alkhamis et al. (2018)	ABNAAAAAAACBNAAAAACBNAAAAACBNAFA
BF09/BAG1	Burkina Faso	2009	NA	Alkhamis et al. (2018)	ABNAAAACBNAAAAAAAACBNAFA
BF09/K1	Burkina Faso	2009	NA	Alkhamis et al. (2018)	ABNAAAACAAACABNAABNAAAAAACBNAAAAAACBNAAAAAACBNAFA
BF09/S1	Burkina Faso	2009	NA	Alkhamis et al. (2018)	ABNAAAACBNAAAAAACBNAAAAAACBNAAAAAACBNAAAAAACBNAFA
Ben09/AGL1	Benin	2009	NA	Alkhamis et al. (2018)	ABNAAAAACBNAAAAACBNAFA
Ben09/Ak3	Benin	2009	NA	Alkhamis et al. (2018)	ABNAAAAACBNAAAAACBNAFAACBNAFA
BF09/DO1	Burkina Faso	2009	NA	Alkhamis et al. (2018)	ABNAAAACBNAAAAAAAAAAAACBNAFA
BF09/SeA1	Burkina Faso	2009	NA	Alkhamis et al. (2018)	ABNAAAACBNAAAAACBNAAAAAACBNAFA
Tog09/P1	Togo	2009	NA	Alkhamis et al. (2018)	ABNAAAAACBNAAAAACBNAFA
Cam10/YGT9	Cameroon	2010	NA	Alkhamis et al. (2018)	ABNAAAAACBNABTDBNAFA
Cam10/GGT5	Cameroon	2010	NA	Alkhamis et al. (2018)	ABNAAAAACBNABTDBNAFA
Cam10/OGT13	Cameroon	2010	NA	Alkhamis et al. (2018)	ABNAAAAACBNABTDBNAFA
BF10/DalO22	Burkina Faso	2010	NA	Alkhamis et al. (2018)	ABNAAAAACBNAAAAAAAACBNAFA
BF10/NanB9	Burkina Faso	2010	NA	Alkhamis et al. (2018)	ABNAAAACBNAAAAAACBNAAAAAACBNAFA
BF10/NiouK34	Burkina Faso	2010	NA	Alkhamis et al. (2018)	ABNAAAACBNAAAAAACBNAAAAAAAACBNAFA
Angola 70	Angola	1970	AM259411	Nix et al. (2006)	AABNABTDBNAAAA
Ben_97/6	Benin	1997	AM259416	Nix et al. (2006)	ABNAAAACBNAAAAACBNAAAAACBNAAAACBNAFA
Ben_97/3	Benin	1997	AM259415	Nix et al. (2006)	AAABNABA

(Continues)

TABLE 3 (Continued)

Virus designation	Country of origin	Year of sampling	Genbank accession No	Reference	CVR profile
CAM/1/86	Cameroon	1986	AF513047	Unpublished	ABNAAAACBNABTDBNAFA
CAM/82	Cameroon	1982	AF513046	Unpublished	ABNAAAACBNABTDBNAFA
CAM/89	Cameroon	1989	AF513045	Unpublished	ABNAAAACBNAAAACBNAFA
Cam_82	Cameroon	1982	AM259413	Nix et al. (2006)	ABNAAAACBNABTDBNAAAAANA
GHA/1/00	Ghana	2000	AF513038	Unpublished	ABNAAAAACBNAAAACBNAAAACBNAAAAACBNAFA
IC-MDG/2014/1	Ivory Coast	2014	This study	This study	ABNAAAACBNABTDBNAFA
IC-MDG/2014/2	Ivory Coast	2014	This study	This study	ABNAAAACBNABTDBNAFA
IC-SP/2014/2	Ivory Coast	2014	This study	This study	ABNAAAACBNABTDBNAFA
IC-SP/2014/4	Ivory Coast	2014	This study	This study	ABNAAAACBNABTDBNAFA
IC-SP/2014/5	Ivory Coast	2014	This study	This study	ABNAAAACBNABTDBNAFA
IC-SP/2014/6	Ivory Coast	2014	This study	This study	ABNAAAACBNABTDBNAFA
IC-SP/2014/7	Ivory Coast	2014	This study	This study	ABNAAAACBNABTDBNAFA
IC-SP/2014/8	Ivory Coast	2014	This study	This study	ABNAAAACBNABTDBNAFA
IC-SP/2014/9	Ivory Coast	2014	This study	This study	ABNAAAACBNABTDBNAFA
IC-SP/2014/10	Ivory Coast	2014	This study	This study	ABNAAAACBNABTDBNAFA
IC-SP/2014/1	Ivory Coast	2014	This study	This study	ABNAAAAACBNABTDBNAFA
IC-SP/2014/3	Ivory Coast	2014	This study	This study	ABNAAAAACBNABTDBNAFA

Notes. CVR codes as previously described: CAST/CVST/CTST = A, CADT/CTDT = B, GAST/GANT = C, CASM = D, CANT = F, CTNT = G, NEDT = M, NVDT/NVGT/NVNT = N, NANI/NADI/NASI = O, RAST = H, SAST = S, NVNT = T, NAST/NADT/NANT = V and SADT/SVDT = W.
The CVR sequences of additional 50 ASFV of genotype I from Western and Central Africa as well as Angola where retrieved from Genbank (15) or the supplement file of the paper by Alkhamis et al. (2018) and compared with 2014 ASFVs from Ivory Coast.
The lighter orange color indicates the isolates with the ABNAAAAACBNABTDBNAFA profile in their CVR while the lighter green color indicates the isolates with the ABNAAAACBNABTDBNAFA profile.

At the Captaincy office, 122 boat entries at the seaport were recorded between April and June 2014 originating from several countries. It should be noted that some of these boats came from the Gulf of Guinea including Cameroon (12 entries from the seaport of Douala), Ghana (nine entries from Takorady [7] and Tema [2] seaports), Nigeria (14 entries from Lagos [6], Onne [8] seaports) and Angola (three entries from the seaport of Luanda), all countries with confirmed ASF.

Finally, the United Nations contingent consisted of soldiers from Niger. Their food was sourced from Argentina (frozen meat), Europe (dairy product) and Asia (dry food and rice). This contingent does not consume pork or any of its derived products.

4 | DISCUSSION

The second ASF epizootic in Ivory Coast started in April 2014 in free-ranging pigs in the city of San-Pedro, and lasted until May 2015, causing the death of 4,549 pigs and the culling of 2,050 others for sanitary measures to curb the spread of infection. The direct losses from this ASF epizootic wave were estimated to be around 803, 827.4 Euros.

The suspicion of the disease, based on clinical signs and lesions was confirmed by laboratory analyses. Following these results, the Ivorian Veterinary Services notified the World Organisation for Animal Health (OIE) on 27/08/2014 (REF OIE 15914).

During this period the disease affected two main neighbourhoods in the southwestern region: the city of San-Pedro, and a nearby region, Sassandra, with one affected village. Following the confirmation of the disease, the Veterinary Services along with the LANADA/Virology Laboratory, the field staff members and local farmers, promptly reacted to implement appropriate control measures to stop the spread of the disease. The following main control measures were implemented to eradicate the disease: quarantine of the city of San-Pedro and a ban on the movement of animals. The application of these measures was entrusted to law enforcement personnel (Police, Army) stationed in San-Pedro. All pigs in the city of San-Pedro were slaughtered, carcasses chemically denatured with quicklime and buried deep within the ground. The affected farms were disinfected and a moratorium was observed before the introduction of sentinel animals 6 months later. Farmers with slaughtered animals were compensated at 1/3 of the market purchase price per animal.

The prompt control of this epizootic event was due to rapid laboratory confirmation, low numbers of pig populations in the affected area and an effective involvement of pig farmers, who through their association, took the lead to conduct and implement appropriate policies. Their support is most likely due to the dramatic consequences of the first epizootic wave of ASF in Ivory Coast in 1996. Indeed, during the first ASF crisis in 1996, approximately 100,000 pigs representing 1/3 of the national pig population at that time were lost. The spread of disease over many regions was facilitated

by pig farmers who were reluctant to implement the appropriate disease control policies and moved their infected flocks from Abidjan to naïve regions spreading the infection to other pig farms and free-ranging animals. The control of this first epizootic wave took almost 2 years to reach the eradication phase in 1998 (Couacy-Hymann, 2012; Kouakou et al., 2017).

To determine the genetic diversity of ASFV strains involved in the 2014 ASF crisis in Ivory Coast and explore the possible origin of the epizootic, the *p72* gene, *p54* gene and CVR of the *9RL* gene (Atuhaire et al., 2013; Bastos et al., 2003; Gallardo et al., 2009) were analyzed. As expected, all ASFV isolates from the San-Pedro outbreak clustered within genotype I in the *p72* tree and genotype Ia in the *p54* gene tree. This agrees with previous findings that only ASFV genotype I is present in West Africa since the onset of this disease in the 1960s in this region (Costard et al., 2013).

The CVR analysis revealed two types of profiles which are very close to those of isolates from Cameroon (Alkhamis et al., 2018). *p72* analysis combined with the *p54* and CVR characterization has been shown to be an effective approach for determining a relationship between ASF outbreaks and to determine the possible sources of infection (Gallardo et al., 2009; Penrith et al., 2004). Based on the analysis of these three genes, it is evident that the 2014 strains from Ivory Coast are different from those involved in the 1996 outbreaks.

Similarly, the CVR analyses were used during the ASF outbreak in Ivory Coast in 1996 to confirm the *p72* genotyping results and demonstrate that two genetically distinct viruses were circulating in the country. One of these was identical to a historical Senegal isolate and closely related to a virus recovered from the outbreak in Gambia in 2000. The other isolates of the 1996 Ivory Coast outbreak could be directly linked to subsequent outbreaks occurring in Benin in 1997, in Nigeria from 1998 to 2000 and in Ghana in 2000. Interestingly, viruses recovered from outbreaks in Cameroon in the 1980s were genetically distinct from those occurring in other West Africa countries in 1990s (Penrith et al., 2004).

There are many different routes for virus entry into a country, however, the most frequent are through seaports, airports and the exchanges of infected live animals or contaminated pig products from infected area to ASF free zones.

Due to the geographical position of San-Pedro, informal exchanges between the inhabitants of this city and those of an infected neighbouring country (Liberia) are very rare. San-Pedro also has a small airfield but it was not used for international traffic at the time of the epizootic. Another source that could have been incriminated is the United Nations (UN) contingent that was stationed in the city. At the time of the epizootic, the UN contingent present in San-Pedro was composed of soldiers from Niger who do not eat pork, and all their food was imported: meat from Argentina, rice from Asia and dry food and dairy products from Europe.

San-Pedro, a seaport city, is mainly dedicated to the transportation of agricultural products, especially cocoa and is extremely active, receiving boats from all over the world. Between March and June 2014, our survey at the Captaincy office showed that several boats from ASF infected African countries such as Angola, Benin, Cameroon, Ghana, and Togo visited the San-Pedro seaport. The household wastes from those boats, especially swill were removed and sent to the municipal city waste dump. This is a place where free-ranging pigs scavenge and thus, a probable source of the 2014 ASF epizootic that began in free-ranging pig populations before spreading to intensive pig farms. In addition, several factors contributed to the spread of the disease in the city such as trade of pig meat from dead pigs and infected or contaminated pigs at low prices, gift of pig meat from infected pigs to others in a neighbourhood, gift of remaining pig feed from an infected farm to a disease free-farm or introduction of infected or contaminated animals from infected areas to disease free farms.

The 2014 ASF wave suggests that continuous surveillance should be in effect and remain a high priority for Veterinary Services and include cooperation with pig farmers and stakeholders. Veterinary Services should take actions including adequate education of all the stakeholders focusing on prevention measures such as the importance of treating all swill prior to disposal at municipal sites.

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