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SERVICE D'EPIDEMIOLOGIE ET ANALYSE DES RISQUES APPLIQUÉS AUX SCIENCES VETERINAIRES

CONTRIBUTION TO THE STUDY OF AFRICAN SWINE FEVER IN THE DEMOCRATIC REPUBLIC OF CONGO: EPIDEMIOLOGICAL AND VIROLOGICAL APPROACHES

CONTRIBUTION A L'ETUDE DE LA PESTE PORCINE AFRICAINE EN REPUBLIQUE DEMOCRATIQUE DU CONGO : APPROCHES EPIDEMIOLOGIQUES ET VIROLOGIQUES

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“ Je ramasserai toutes les pierres avec lesquelles j’ai été lapidé pour me bâtir une cabane ”

Anonyme.

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Acronyms and abbreviations List

9RL: specific designation of coding gene in one of the two variable genomic regions of African swine fever virus
ABC: avidin biotin complex
Abs: antibodies
ABTS: 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid
ADN: acide désoxyribonucléique
AITVM: association of institutions for tropical veterinary medicine
AP: apparent prevalence
ASF: African swine fever
ASFV: African swine fever virus
AUC: area under the curve
BAD: Banque Africaine de Développement
Bacp30: protein 30 baculovirus vector
BMC Vet Res: biomedical central veterinary research
BSA: Bovine Serum Albumin
Buffer AL: specific designation of a buffer used in the QIAGEN extraction kit, lysing the cells and exposing DNA
CD: cluster of differentiation
CDC: Center for Disease Control and prevention
Chi2: Chi square test
CI: confidence interval
CISA: centro de investigacion en Sanidad animal (Center for Animal Health Research, Madrid, Spain)
CSF: classical swine fever
CVL: Central Veterinary Laboratory
CVR: central variable region
DAB: 3, 3-diaminobenzidine
ddH_2O: double distilled water
DNA: deoxyribonucleic acid
dNTPs: dexyriboNucleotide Tri-Phosphate (exclusively including adenine, cytosine, guanine thymine)
DRC: Democratic Republic of Congo

DP: domestic pig

DR: Democratic Republic

dUTPase: dUTP diphosphatase

E. coli: *Escherichia coli*

EDTA: Ethylene Diamin Tetra Acid

EFSA: European Food Safety Authority

ELISA: Enzyme Linked Immunosorbent Assay

EP: enzootic pneumonia

Epidem Infect: Epidemiology and Infection (Journal)

ESACWA: Europe, South America, Caribbean and West Africa

FAO: Food and Agriculture Organisation

FAOSTAT: Food and Agriculture Organisation Statistic

FASTA: DNA and protein sequence alignment software package

GPS: global positioning system

HAD: haemadsorption

HPMPA: a broad spectrum antiviral

HRP: horseradish peroxidase

IAEA: International Atomic Energy Agency

IAH: Institute for Animal Health

IAP: Intracisternal A-type particle

IC: intervalle de confidence

ID: identity

ICCN: Institut Congolais pour la Conservation de la Nature

iELISA: indirect ELISA

IFAD: International Fund for Agricultural Development

IFN: Interferon

Ig G: immunoglobin G

INIA: Instituto Nacional de Investigacion y Technologia Agraria y Alimentaria

ISBN: International Standard Book Number

ISSN: International Standard Serial Number

IU: international unit

Kb: kilobase

Kbp: kilo base - pair

KDa: kilodalton

LW: Large White

mAb: monoclonal antibody

MEGA: Molecular Evolutionary Genetics Analysis

MGF: multigene family

mRNAs: messenger ribonucleic acid

rRNA: ribosomial ribonucleic acid

MWG: specific designation of "eurofins | mwg operon" which is a factory specialised in DNA and RNA oligonucleotides supplying

NK: natural killer cells

NT: non tested

O: *Ornithodoros*

OD: optical density

OIE: Office International des Epizooties (Organisation Mondiale de la Santé Animale)

OPD: Orthophenylenediamine

ORF: open reading frame

ORF9RL: open reading frame 9RL

OURT: Ourique (Portuguese village) ticks

P: prevalence

p30: protein 30

p72: protein 72

PAGE: PolyAcrylamyl Gel Electrophoresis

pAR3038: plasmid AR3038

PBMM: pig bone marrow macrophages

PBS: phosphate buffer saline

PCR: Polymerase Chain Reaction

PPA: Peste Porcine Africaine

pSG72a: plasmid SG72a 5

QIAamp: QIAGEN amplification system (specific designation from the manufacturer)

QMS: quality management system

qPCR: quantitative PCR

R.: *Rhipicephalus*

RDC: République Démocratique du Congo

RNA: ribonucleic acid

ROC: Receiver Operating Characteristic

rp30: recombinant protein 30

rp72: recombinant protein 72

rpm: round per minute

RSA: Republic of South Africa

rtPCR: real time PCR

SDS: Sodium Dodecyl Sulphate

Se: sensitivity

SEM: Scanning Electron Microscope

SOP: standard operating procedure

Sp: Specificity

SSA: sub-Saharan Africa

T7 (bacteriophage): specific identification of T7 infecting the majority E. coli serotypes

TAE: Tris, Acetate, EDTA buffer

TaqMan: acronym from the factory, to be understood as thermo - aquaticus enzyme acting as a man, i.e. hydrolysing probe designed to increase qPCR specificity

TBED: Transboundary and Emerging Disease (Journal)

TCID: tissue culture infecting dosis

TDA: Transvaal Department of Agriculture

Tet-type: amino acid tetramers type

TMB: tetramethylbenzidine

TNF-E: tumor necrosis factor E

TP: true prevalence

TRs: tandem repeats

UK: United Kingdom

UREAR-Ulg: Unité de recherche en épidémiologie et analyse de risques appliquées aux sciences vétérinaires-Université de Liège

USA: United States of America

USD: United States Dollars

UV: Ultraviolet

VP54: Virus Protein 54

VP72: Virus Protein 72

VPPA : virus de la Peste Porcine Africaine

WB: World Bank

WOAH: World Organisation for Animal Health

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Résumé

Contexte et justification de la recherche

A partir de la fin des années 1980 et pendant les années 1990, une maladie fatale, mais insidieuse apparut dans les exploitations porcines des zones annexes de la Ville de Kinshasa et ses environs qui dépendent des provinces voisines du Bas-Congo et de Bandundu. Cette maladie qui affectait surtout les porcs à partir de l'âge de 5 mois, tuait entre un et cinq sujets par semaine et ce, jusqu'à l'extermination de tout l'effectif d'une porcherie. Cliniquement, la toux, la dyspnée, la fièvre, l'anorexie, de discrets piquetés hémorragiques qui pouvaient être confondus avec de la saleté cutanée avec parfois la présence de vésicules sur l'abdomen autour des glandes mammaires étaient observés. En plus de ces observations épidémiologiques et cliniques, à l'Est de Kinshasa, il avait été constaté que les exploitations porcines d'où partaient ces alertes étaient pour la plupart situées le long de la rivière Ndjili, petit affluent du fleuve Congo, qui tire sa source dans la province voisine du Bas-Congo.

Vers avril et mai 1984, les informations reçues des mêmes zones, notamment du village Kingantoko, avaient fait état de l'occurrence d'une maladie qui, après avoir tué, tous les porcs dans une ferme porcine commerciale d'une vingtaine des porcs se serait propagée dans tout le village d'où elle avait frappé même les porcs indigènes errants, ne laissant que de rares survivants. Officiellement, y compris même par le passé, aucune déclaration de foyer de la peste porcine africaine n'avait été faite dans cette partie de la RDC (**Figure 1**). Dans le même temps, des alertes similaires provenaient de l'Ouest de Kinshasa, plus précisément de Lutendele, une vallée abritant plusieurs autres exploitations porcines, non loin de la rive gauche du fleuve Congo (**Figure 2**).

L'augmentation des pertes associées au syndrome décrit ci-dessus, le malentendu et la confusion entretenus par les professionnels de l'élevage sur le terrain autour du diagnostic, en pointant du doigt la maladie du Rouget, malgré l'échec du traitement avec des antibiotiques à large spectre et, le manque d'une unité spécifique de diagnostic au sein du laboratoire national de référence constituent des faits qui ont justifié cette recherche. Le but de ce travail était de permettre une maitrise de la situation de la peste porcine Africaine en RDC à travers des enquêtes de terrain et des analyses de laboratoire.

Ainsi, six études de terrain ont été menées pouvant être regroupées en quatre thématiques :

- la confirmation ou infirmation des cas suspects de peste porcine Africaine dans les zones à problèmes (une étude) ;
- l'étude de la prévalence, la vérification de l'état d'endémicité et l'évaluation de tests de diagnostic utilisés sur terrain (trois études) ;
- la caractérisation moléculaire des souches du virus de la peste porcine africaine et l'évaluation de leur distribution (une étude) ;
- le testage de l'efficacité d'un candidat vaccin pour la protection des porcs natifs de l'Afrique (une étude).

Stratégie et méthodologie

Pour les enquêtes de terrain, les zones concernées par les investigations ont été choisies en fonction de l'information épidémiologique disponible, leur lien commercial avec la ville ou province de Kinshasa, leur importance en cheptel porcin, la présence d'une interface entre porcs domestiques et porcs sauvages (interactions) ainsi que leur accessibilité. C'est ainsi que les provinces de Bas-Congo, Equateur, Katanga, Orientale, Kasaï Oriental et Maniema ont été sélectionnées. Parmi ces provinces, celles qui ont été choisies en raison de la présence d'une interface entre les porcs domestiques et porcs sauvages étaient : le Bas-Congo, l'Equateur et le Kasaï Oriental.

Pour des raisons logistiques, les données épidémiologiques et les prélèvements des échantillons ont été effectués en deux périodes (1997 à 2006 et de 2005 à 2012). Les données de terrain et les prélèvements étaient enregistrés sur un fichier Excel renseignant l'origine, le lieu de chaque prélèvement, la date et la nature du prélèvement, les coordonnées géographiques (longitude et latitude), les facteurs de risque et les résultats de laboratoire. S'agissant des facteurs de risque, les principaux étaient les suivants : l'espèce (porc domestique ou sauvage), l'âge, le poids, la race, le sexe, le système d'élevage, la présence et le type d'ectoparasites trouvés sur la peau, les lésions hémorragiques, l'état de nutrition et la distance avec la ferme la plus proche. D'autres observations étaient notées telles que, par exemple, la température pendant le transport vers le laboratoire. Les porcs adultes inclus dans l'étude étaient de toute race et âgés de plus de cinq mois. Au niveau de laboratoire, les

méthodes suivantes ont été mises en œuvre : l'identification des tiques, la détection des anticorps dirigés contre le virus de la peste porcine Africaine (PPA), la détection des antigènes et la détection de l'ADN du virus de la PPA, le séquençage et la construction des phylogrammes. En outre, un candidat vaccin atténué a également été testé et les outils statistiques adaptés ont été utilisés.

Pour la détection des anticorps spécifiques (Ac) dirigés contre le virus de la PPA et l'évaluation des caractéristiques des tests sérologiques les plus utilisés sur terrain, trois formats d'ELISA ont été évalués. C'est ainsi que pour la sensibilisation des plaques en rapport avec lesdits formats, trois types des protéines étaient utilisés comme antigènes (Ag), à savoir:

- la protéine totale et cytosoluble p72 (cp72), utilisée dans un test ELISA indirect approuvé comme test de référence par l'Organisation mondiale pour la Santé Animale (OIE) et la FAO ;
- la forme recombinante de la même protéine p72 (rp72) utilisée dans un test ELISA de compétition ;
- la forme recombinante de la protéine p30 (rp30) du même virus aussi utilisée dans un test ELISA indirect.

Les tests de confirmation des résultats révélés positifs (douteux) consistaient en deux tests d' « immunoblott » (IB) basés sur la p72 et la p30 et un test d'immunohistochimie (IHC).

Pour l'estimation relative au test de référence de la sensibilité (Se) et de la spécificité (Sp) des tests ELISA basés sur la rp72 et la rp30, quelques outils statistiques ont été utilisés dont le calcul du coefficient Kappa, la courbe ROC (*Receiver Operating Characteristic*), l'estimation par noyau (*Kernel density*) et le test de Chi2.

Les tiques prélevées sur les porcs domestiques et sauvages étaient identifiées par scannage grâce à un microscope électronique adapté (SEM, *Scanning Electron Microscope*) et un microscope stéréoscopique Olympus SZX16. Cette identification a été complétée par l'exploitation d'une clé taxonomique.

Pour la caractérisation moléculaire des souches circulantes, l'ADN viral a été extrait au moyen d'un kit QIAGEN (protocole préconisé pour le sang et les autres tissus). Trois régions cibles du génome du virus de la PPA comprenant les gènes B646L, K183L et B602L

(CVR) ont été amplifiés par PCR (Aguero et al., 2003 ; Bastos et al., 2003, Nix, et al., 2006). Les amplicons ont été purifiés en utilisant le kit Wizard ® SV Gel et le kit PCR Clean Up System, en respectant le protocole préconisé par le fabricant (Promega Corporation, USA). Les arbres phylogéniques ont été construits sur base des logiciels FASTA et MEGA (Tamura et al., 2013). Les séquences des nucléotides de tous les isolats collectés dans cette étude ont été fournies par LGC Genomics (Berlin, Allemagne) chez qui les amplicons purifiés avaient été soumis.

Pour la protection des porcs indigènes contre la PPA, le candidat vaccin utilisé est une souche naturellement atténuée OURT88/3, isolée sur une tique *Ornithodoros* au Portugal. Huit porcs villageois ont été vaccinés avec cette souche et inoculés successivement par les souches virulentes de génotype I, OURT88/1 provenant du Portugal et DRC085/10, provenant de la RDC. Chaque étape d'infection était séparée de l'autre par 21 jours d'intervalle. Un score clinique était établi et un suivi sérologique était réalisé successivement aux jours 3 (J3), 6, 14 et 21 post-inoculation (pi). La réponse immune ainsi que la charge virale étaient suivies grâce à une PCR quantitative (qPCR). Comme témoins, 7 porcs non vaccinés ont été utilisés lors du premier challenge et, 6 porcs lors du deuxième. Les porcs vaccinés qui ont survécu après les challenges ont été sacrifiés, en adéquation avec les règles éthiques, au 21eme jour après le deuxième challenge, soit au 63eme jour de l'expérimentation. L'essai était autorisé par les Services vétérinaires officiels de la Ville de Kinshasa.

Résultats de la recherche

1. Par rapport aux incertitudes et confusions à propos du diagnostic de la maladie qui était à la base des pertes enregistrées à partir des années 1998-90 dans les exploitations porcines à Kinshasa et dans les environs, au lieu du Rouget, c'est la PPA qui a été confirmée. Les Ac dirigés contre le virus de la PPA ont été détectés par les tests ELISA et confirmés par les tests d'IB et d'IHC. De plus, l'ADN du virus de la PPA a été détecté par PCR dans des échantillons de tissus prélevés dans toutes les localités où les foyers avaient été suspectés. La caractérisation moléculaire des souches impliquées a permis de savoir qu'elles appartenaient toutes au génotype I, dans un même taxon que les souches du Nigeria [Nig. 98/99] et du Ghana [Gha] (Mulumba-Mfumu et al., 2013). Nous avons déduit que la confusion du diagnostic était plus liée à la faible virulence des souches impliquées; en effet, la PPA a toujours été abusivement considérée comme une maladie

exclusivement hémorragique suivie de 100% morbidité et 100% de mortalité. Ce qui n'est pas le cas dans les zones endémiques de l'Afrique sub-Saharienne. Cette croyance empirique a souvent retardé la riposte et le contrôle de la part des autorités vétérinaires et a contribué à la persistance et l'endémicité de la PPA en Afrique.

2. Concernant l'étude de la prévalence, la vérification de l'état d'endémicité et l'évaluation de tests de diagnostic utilisés sur terrain :

   a) L'endémicité de la maladie a été établie, du fait de la détection des anticorps dirigés contre le virus de la PPA et des antigènes de celui-ci, autant chez les porcs domestiques apparemment sains que chez les Potamochères (**Figure 5**). L'évaluation de la présence des anticorps chez les sujets apparemment sains a révélé une prévalence apparente de 27 %.

   b) Concernant l'épidémiologie de la maladie, deux approches ont été envisagées lors des investigations menées à l'interface où les interactions entre les porcs domestiques et les suidés sauvages étaient possibles, notamment dans les provinces de l'Equateur (Mbandaka et Boende) et du Kasaï-Oriental (Kole). La première approche visait la confirmation de la circulation de la maladie chez les suidés sauvages et la deuxième visait la détection et prélèvement des tiques *Ornithodoros* dans l'écosystème de la forêt où vivent les potamochères. La première approche a permis la détection d'anticorps dirigés contre le virus de la PPA dans les sérums prélevés de potamochères de la forêt de Lingonda (2^{o}.35' N, 18 ^{o}.55'E), en Province de l'Equateur et la détection des antigènes dans les tissus lymphoïdes de potamochères de la savane en provenance de Kole (3 ^{o}.28'S, 22 ^{o}.28'E) et de Boma (5^{o} 83 S, 13^{o}07'E), respectivement dans les Provinces du Kasaï Oriental et du Bas-Congo. Par rapport avec la deuxième approche, aucune tique *Ornithodoros* n'a pu être identifiée. Ceci pourrait être mis en relation avec la méthode utilisée. En effet, plutôt que d'aller chercher les tiques dans les terriers des potamochères (difficiles à repérer dans une forêt inondée), les tiques avaient été prélevées sur les corps des suidés (domestiques et sauvages). Par contre, parmi les tiques prélevées, plusieurs variétés des tiques ont été identifiées telles que les genres *Ixodes*, *Amblyoma*, et *Rhipicephalus* (**Table 1**). Parmi les *Rhipicephalus*, une nouvelle espèce a été découverte, non encore décrite dans la littérature et celle-ci a été nommée "*Rhipicephalus congolensis*" (Apanaskevich et al., 2013). Par ailleurs, nous n'avons pas pu amplifier de l'ADN du

virus de la PPA à partir d'un broyat de toutes les tiques prélevées y compris celles provenant des bêtes séropositives (données non détaillées dans cette thèse).

c) L'évaluation des caractéristiques des tests sérologiques utilisés sur terrain a révélé, dans nos conditions de laboratoire et de terrain, une sensibilité (Se) relative de 93.48 % (95 % IC : 82.10-98.63) et une spécificité (Sp) relative de 87.98 % (95 % IC : 83.62-91.53) pour l'ELISA de compétition utilisant la protéine recombinant p72 (rp72). En outre, sur base des trois études indépendantes menées dans différentes zones et systèmes d'élevage, la prévalence apparente en anticorps a varié de 20 à 27 %. Par ailleurs, en prenant en considération les données de l'étude dans laquelle les autres tests de diagnostic ont été réalisés (3 tests ELISA susmentionnés et test PCR), le nombre de porcs positifs confirmés par au moins un autre test de diagnostic était de 55/328 (17%), ce qui constitue une bonne estimation de la prévalence dans la région et qui est très proche de 16,9 % noté au Sénégal par le passé (Etter et al., 2011).

3. Concernant les souches circulantes du virus de la PPA et leur distribution, une étude a été menée sur une période de sept années (2005 à 2012). Un séquençage basé sur trois gènes choisis comme marqueurs (B646L, K183L, B602L CVR) a permis de mettre en évidence, dans la zone d'étude, la circulation de trois génotypes p72 (I, XI, et XIV) et 19 variants. La co-circulation de différents génotypes en un même endroit (même foyer) a également été révélée. Ceci avait déjà été démontré auparavant (Carmina G. et al., 2010 ; Bastos, 2004). En outre, deux autres observations originales ont pu être faites, à savoir : la co-infection d'un même porc par deux variants du virus de la PPA, au cours d'un même foyer et dans une même ferme ainsi que la présence de répétions en tandems de tétramères d'acides aminés chez des isolats appartenant à différents génotypes. Ce dernier constat fait après analyse des séquences du "locus hypervariable" du génome viral (B602L-CVR) incrimine soit une homoplasie, soit une possible recombinaison génétique de plusieurs souches circulantes dans un foyer ; hypothèse déjà posée dans d'autres études antérieures *in vivo* (Smith, 1976). Cette étude a dans le même temps aussi démontré que le génotype I était de loin le plus dominant dans la zone d'étude, avec 40 séquences sur 62 (64,5 %), suivi du génotype IX avec 20 séquences sur 62 (32,3%) et enfin le génotype XIV avec 2 séquences sur 62 (3,2%).

4. Concernant les perspectives de protection des porcs natifs de l'Afrique contre la PPA, un

candidat vaccin basé sur une souche naturellement atténuée, OURT88/3, isolée sur une tique *Ornithodoros* au Portugal a été testé. Ce vaccin a été inoculé à 8 porcs natifs de l'Afrique. Les sujets inoculées ont ensuite été challengés deux fois à 21 jours d'intervalle avec deux souches virulentes, OURT88/1 et DRC85/10 /08 provenant respectivement du Portugal et de la RDC. A la fin de cette expérimentation, 50% des porcs soit 4 sur 8 ont été protégés, un porc était mort d'une autre cause et, 3 porcs ont été tués par les souches du virus utilisées pour le challenge. Six porcs sur six, soit 100% et, six sur sept, soit 86%, utilisés respectivement comme animaux témoins dans les challenges 1et 2 sont morts (Mulumba-Mfumu et al., 2015). Les sujets protégés ont présenté des titres élevés en anticorps dirigés contre le virus de la PPA (test ELISA) et étaient négatifs au test de PCR quantitative (qPCR). Les porcs protégés ont été euthanasiés selon les normes éthiques au $21^{ième}$ jour après le deuxième challenge, soit au $63^{ième}$ jour de l'expérimentation. Ces résultats indiquent que l'espoir de trouver un vaccin efficace contre le virus de la PPA pourrait venir de souches naturellement atténuées (Sanchez-Vizcaino, 2012).

Summary

Background and justification of the research

By the end of the 1980s and during the 1990s, occurrence of fatal but, insidious disease of swine was reported within a couple of pig exploitations in the Kinshasa's city hinterlands, including the areas of the neighbouring provinces of Bas-Congo and Bandundu. This disease in which, adult pigs starting from the winning age were the most involved was reported and killing between 1 to 5 animals per week until extermination of the total population of piggeries. Syndromically, cough, dyspnea, pyrexia, loss of food intake, discrete skin petechiae sometimes confused with skin dust, with or without vesicles on abdomen around the mammary glands were the most observed signs. Apart from these epidemiological and clinical particularities, in the East of Kinshasa, it was also observed that the majority of concerned farms were located on the edge of Ndjili river or within the surrounding areas. Ndjili is a tiny affluent of Congo River, with its source located within a hilly area southward within the neighbouring province of Bas-Congo.

Between May and July 1984, alerts received from aforementioned areas, namely from the Kingantoko village, were incriminating a severe disease of swine that killed all the pigs within a commercial farm and that, finally spread to all of the mentioned village where it kills a huge number of free ranging pigs leaving only few survivors. ASF occurrence has never been reported within these areas of DRC (**Figure 1**). Similar alerts were also received from the Western part of Kinshasa, namely from Lutendele, a farming valley with several piggeries, located on the East bond of Congo River (**Figure 2**). Finally, progressive increase of losses associated with this syndrome, misunderstanding of diagnosis by field veterinary technicians, incriminating Erysipelas despite failure of treatment with large spectrum antibiotics and, lack of local specific laboratory for ASF diagnosis formed the basis of this research work. A better understanding of ASF situation in the DRC, chiefly within the above mentioned areas was the long term objective of this research work which was carried out through field investigations and laboratory analyses.

In total, six studies grouped in four themes were conducted as follows:

- confirmation of circulation of ASF within the suspected areas (one study);
- prevalence study, assessment of the endemicity of the disease and determination of

screening tests characteristics (three studies);

- genetic assessment of ASFV isolates' dynamics (one study);
- vaccine trial for protection of African indigenous pigs (one study).

Methodology

For the field work, the surveyed areas were: peri-urban areas of Kinshasa city, and other provinces selected on the basis of available epidemiological information, their commercial links with Kinshasa, their importance in pigs populations, the presence of interfaces with interactions between wild and domestic suids and, their accessibility. Therefore, the provinces of Bas-Congo, Equateur, Katanga, Orientale, Kasai Oriental and Maniema were selected. Among these provinces those chosen for interfaces with interactions between domestic and wild suids were Bas-Congo, Equateur and Kasai Oriental. Because of logistic constraints epidemiological events and samples were documented during two sequences of time (from 1997 to 2006 and from 2005 to 2012). Field data and samples were recorded in Excel file informing about origin, sampling location and dates, geographical location (latitude and longitude), critical risk factors and laboratory results. Regarding critical risk factors, the most important were the following: species (whether domestic or bush pigs), age, weight, race (breed), sex, farming system, presence of skin ectoparasites, hemorrhagic clinical signs, health status, nearest distance with other pigs' farms, suspicion of ASF within the area and other remarks such as temperature during transportation of samples, type of preservation of specimens in the laboratory. Adult pigs of any breeds starting from the age of 5 months were targeted.

At the laboratory level, the following methods were used for various types of needed results: ticks identification, anti-ASFV antibodies detection, confirmatory diagnostics, ASFV's antigens detection, ASFV DNA detection, nucleotides sequencing, phylogenetic trees construction, vaccine trial and statistical analysis. For serological screening of collected sera samples we used 3 ELISAs' formats for both detection of antibodies (Abs) and assessment of characteristics of the two screening tests exploited for both, active and passive surveillance. Coating Antigens (Ag) for the 3 ELISAs were crude cytosoluble p72 (cp72), recombinant protein p72 (rp72) and recombinant p30 (rp30) respectively. For confirmation of some positive results obtained with serological tests, p30 or p72 immunoblotting (IB) and immunohistochemistry (IHC) were used as confirmatory tests. For assessment and validation

of relative sensitivity (Se) and specificity (Sp) of the two compared screening tests as regard to the reference test (cp72). Some statistical tools were used also like the Kappa coefficient, the Receiver Operating Characteristic (ROC) curve, the Kernel density estimation, and chi-square test. Taxonomy of collected ticks' specimens was examined using scanning electron microscopy (SEM) and stereoscopic microscope (Olympus SZX16, Olympus Corporation, Tokyo, Japan). ASFV DNA was extracted using QIAGEN kits (blood and tissues protocol). For molecular characterization, three target genomic regions comprising B646L, K183L and B602L (CVR) genes were amplified using PCR (Aguero et al., 2003, Bastos et al., 2003, Nix et al., 2006). Nucleotides sequences from amplicons purified using Wizard ® SV Gel and PCR Clean Up System kit, according to manufacturers' protocol (Promega Corporation, USA) were provided by LGC Genomics (Berlin, Germany). Phylogenetic trees were generated using FASTA and MEGA software (Tamura et al., 2013). For vaccine trial, eight pigs were inoculated with the naturally attenuated ASFV OURT88/3 strain isolated from *Ornithodoros* tick in Portugal, and challenged two times at 21 days post-inoculation (pi) interval, using two virulent genotype I strains, OURT88/1 from Portugal and DRC085/10 from DRC. Vaccinated pigs were compared with non-vaccinated ones, used as controls during the assay, comprising six pigs for the first challenge and seven pigs for the second. Clinical score and sera samples were successively collected at each days 3, 6, 14 and 21 pi. Viral charge and immune response were measured using quantitative PCR and rp72 ELISA respectively. More than 50% of vaccinated pigs were protected. One pig was killed by other cause and three pigs were killed by the challenge viruses. Protected pigs were euthanised at the humane end point which was fixed at day 21 post challenge 2, corresponding to day 63 post trial. The overall experimental trial was officially allowed by the chief provincial veterinary authority of the Kinshasa City.

Research results

1. With regard to uncertainty and confusion which have been raised about ASF and Erysipelas or other so-called "*red diseases of pigs*", ASF occurrence has been confirmed as source of persistent mortalities and heavy losses that were reported in the 1980-90s within the peri-urban areas of Kinshasa and the neighbouring provinces. Anti-ASFV antibodies were detected serologically by ELISAs and confirmed by both, immunoblotting (IB) and immunohistochemistry (IHC) techniques. Furthermore, ASFV DNA was detected in samples collected in pigs from all the suspected areas. The

molecular characterization of involved strains revealed that they were all belonging to p72 genotype I, where they were all clustering in the same taxa with Nig 98/99 from Nigeria and Gha from Ghana (Mulumba-Mfumu et al., 2013). The diagnosis confusion was mainly due to low virulence of involved strains. In fact ASF has been always known by the field professionals as a swine disease that is strictly hemorrhagic and that kills 100% of infected pigs. This empiric knowledge of ASF contributed largely to delays in the implementation of control strategies and to endemicity of this disease.

2. Concerning the prevalence study, the determination of the endemicity status and screening tests characteristics in the field:

   a) ASF endemicity was established given the fact that anti-ASFV Abs and ASFV Ags were detected both, in apparently healthy pigs and wild suids (**Figure 5**). Apparent Abs prevalence in these symptomless animals, revealed by three independent studies ranged between 20 to 27%, with 16 % as the very minimum in terms of reproduced results. (Barbara and Mulumba, AITVM Proceedings, 2007 ; Mulumba-Mfumu et al., 2013; and article in press)

   b) Regarding ASF epidemiology, field investigations within interface villages in the Equateur province (Mbandaka and Boende) and Oriental Kasaï province (Kole), two objectives were aimed: the first was the determination of ASF circulation in wild suids and, the second was the identification of *Ornithodoros* ticks within that forest ecological scenario. The former survey revealed anti-ASFV antibodies in sera samples of two *Red river hogs* from Mbandaka (Lingonda) (2^{o}.35’ N, 18 ^{o}.55’E) and ASFV antigens (p72 protein) in lymph nodes tissues from two *Potamocherus porcinus* sampled in Kole (3 ^{o}. 28’S, 22 ^{o}. 28’E) and Boma (5^{o} 83 S, 13^{o}07’E) from the provinces of Kasai Oriental and Bas-Congo respectively. The survey dealing with the second objective failed to detect *Ornithodoros* ticks, may be owing to collection strategy. Rather than digging borrows or nets (not evident to be identified in such a flooded forest), available tick’s specimens were those found on the bodies of wild suids. Finally, instead of *Ornithodoros* ticks, other variety of ticks including *Ixodes*, *Amblyoma* and *Rhipicephalus* genera (**Table 1**) were identified. Amongst *Rhipicephalus* ticks, new species, never described in existing literature were discovered and named "*Rhipicephalus congolensis*" (Apanaskevich et al., 2013). In regard to detection of ASFV within these ticks specimens, we failed to amplify ASFV DNA

from a mixture of crushed ticks including those collected from positive wild suids, this finding supported the previous one reported by other workers (Plowright et al, 1994; Owolodun et al., 2010).

c) The assessment of screening tests characteristics, revealed that the rp72 blocking ELISA (Ingenasa) behaved better, with a relative sensitivity (Se) of 93.48 % (95 % CI: 82.10-98.63) and relative specificity (Sp) of 87.98 % (95 % CI: 83.62-91.53) and, with respect to overall prevalence, our third study in which four different tests were combined revealed that 55 out of 328 tested pigs were positive in at least two of the four used tests. This value represents 17% of "objective" apparent prevalence; very closed to 16.9 % previously reported in Senegal upon a similar study (Etter et al., 2011).

3. Genotyping and ASFV isolates dynamics study based on the sequencing of B646L, K183L and B602L CVR genes revealed existence of three p72 genotypes comprising genotypes I, IX, XVI as well as 19 variants of ASFV in the surveyed areas of DRC between 2005 and 2012. It also revealed co-circulation of different genotypes within the same farm and during the same outbreak. This finding has been already reported in previous studies (Carmina Gallardo et al., 2010; Bastos et al., 2004). The same study reveals two other critical events: co-infection of the same pig by two ASFV variants and identity of amino-acids tetramers types in ASFV isolates belonging to different genotypes, which suggests either homoplasy or genetic recombination of co-circulating or co-infecting isolates, as already hypothised *in vivo* by previous authors (e.g. Smith, 1976). This survey revealed also that, with 40 sequences out of 62 (64.5%), the genotype I (ESACWA genotype) was the most dominant in those surveyed areas of the DRC, which confirmed the findings of previous similar studies on Central Africa (Ekwe, 2000, Bastos, 2003; Lubisi, 2007). Genotype I was followed by genotype IX with 20 sequences out of 62 (32.3 %) and genotype IX was followed by genotype XIV, with 2 sequences out 62 (3.2%).

4. Concerning the prospective study on protection of African indigenous pigs; a naturally attenuated ASFV strain OURT88/3 isolated from *Ornithodoros* ticks in Portugal was used as vaccine candidate. Eight African native pigs were inoculated and challenged twice at 21 days of interval from each post-inoculation step with two highly virulent isolates, OURT88/1 and DRC085/10 from Portugal and DRC respectively. At the end of

the trial, four pigs out eight (50 %) were protected, one pig died from another cause and three pigs were killed by challenge viruses (Mulumba-Mfumu et al., 2015). Six pigs out of six (100%) used as control for challenge one were all killed by challenge viruses and six pigs out the seven (86%) used as control for challenge two were as well killed by the challenge viruses. Protected pigs showed high titer of anti-ASFV antibodies, by rp72 blocking ELISA with zero copies of ASFV as demonstrated by quantitative PCR (qPCR). Protected pigs were euthanised at day 21 post-challenge two (63th day upon the trial), which was fixed as humane end point. These encouraging results suggest that, the future of protective vaccines against ASF could be expected from naturally attenuated vaccines (Sanchez-Vizcaino, 2012). This trial data were published as original article in *Transbound Emerg Dis*, 2015, doi10.1111/tbed.12303.

General Preamble

The overall framework of this thesis is a compilation of 13 chapters grouped in 5 parts detailed as follows: - part I, general introduction (4 chapters); - part II, description the objectives (1 chapter); - part III, experimental work (6 chapters); - part IV, general discussion, conclusions and perspectives (2 chapters) and, - part V, bibliographical references.

I. **Part I-General Introduction**:

1. **Chapter 1-General Background**: this section is briefly describing some basic data of the DRC including geographical, ecological, and main socio-economic features, husbandry background in general, pig sector and ASF occurrences in particular. The focus of this chapter is to provide the reader with a broad overview that will enable a better understanding of logical links between ecosystems, risk factors, risk drivers and, epidemiological events such as ASF occurrences;
2. **Chapter 2-African swine fever, update and literature review**: in this chapter I am trying to recall or to provide the reader with basic and update scientific information that deals with various approaches of ASF in general and ASFV infection in susceptible suids and vectors. That is to say, the specific introduction about the disease, its aetiology, epidemiology, pathogenesis, symptomatology, diagnosis, immunology, socio-economic impact, treatment and vaccine prospects will be discussed in this section.
3. **Chapter 3**: **African swine fever virus serodiagnosis: a general review with focus on the analyses of African serum samples.** This chapter is a specific literature review which is describing the use of ASFV p30 and its exploitation as a coating antigen for detection of anti-ASFV antibodies in routine diagnostic. It is demonstrated in this section that the ELISA based on this protein is suitable detection of the disease in pre-clinical situations or for detection of ASFV signals in the early stages of infection. As a matter of fact, it is demonstrated that ASFV p30 is important for internalisation of virus, just after attachment to infected cells and during resorbative

endocytosis which is allowing it detectable in early stages of infection. This literature review is subject of our co-authored and published article. As outcome of the paper, the diagnostic method was exploited during Eurasia's ASF outbreaks, where it behaved very well as field investigation tool. In fact, in this situation of lack of vaccine and treatment, rapid and accurate diagnosis of ASF is the only means that can enable early and effective implementation of control strategies.

II. **Part II-Objectives**

Chapter 4: Objectives. The overall and specific objectives of the research work, as main pathways to address scientific questions are described in this section.

Chapter 5: Research strategies and methodology. This chapter is depicting, the investigation strategies and analysis methods that were exploited during our field and laboratory studies. The usefulness of this chapter is that it will enable the reader to detect the strengths and weakness of this research work and if possible to come back to us with constructive remarks that could be applied during future similar work.

III. **Part III-Experimental work**: this part is devoted to results and outcome of the research work. This comprises six completed articles of which four already published in internationally indexed journals of which two published as first author, two co-authored as main contributor, and two original as first author, under peer reviewing process within internationally recognised scientific journals with good impact factor. The components of this third part are the following:

1. **Chapter 6**: **Molecular characterisation of African Swine Fever Virus isolates involved in infection persistence in Central Africa: Democratic Republic of Congo.** This chapter devoted to a prospective study which not only confirmed and reported for the first time ASF occurrences in the research areas within the DRC, but also differentiated this epizootic from other so-called "*red diseases of pig*". This was a pilot study which generated other subsidiary ones. This study was subject of original article (*Annales Africaines de Medecine*, 2013, 6(3), 1390-1472).
2. **Chapter 7: Estimation of the prevalence of African swine fever in the Democratic Republic of Congo and assessment of sensitivity and specificity of the ELISA tests in field conditions.** This chapter is covered

by an original paper which is an outcome of a combined study carried out between 2007 and 2008, reporting on field screening tests characteristics, endemicity and ASF prevalence in the DRC in addition to assessment of relative sensitivity and specificity of two serological tests used for ASF diagnosis. This article has been peer reviewed in "*Transboundary and Emerging Infectious Diseases*" where it is under publication process.

3. **Chapter 8**: **Genetic assessment of African swine fever isolates involved in outbreaks in the Democratic Republic of Congo between 2005 and 2012 reveals circulation of p72 genotypes I, IX and XIV and 13 variants.** This original paper is dealing with genetic characterisation dataset of all ASFV isolates recovered during this research work. This was the first extensive study to be conducted on ASFV isolates' dynamics in the DRC. These data are presented in an original article which is currently submitted to *Emerging infectious diseases*.
4. **Chapter 9**: **Immunisation of African indigenous pigs with attenuated Genotype I African swine fever virus OURT 88/3 induces protection against challenge with virulent strains of the same genotype.** This chapter reports novel findings on protection of African indigenous pigs using a naturally attenuated ASFV isolate as vaccine candidate. During this trial, the protection was more than 50 % of vaccinated pigs that were challenged twice with two different strains of the same genotype, while control pigs were killed. The experimental pigs were immunised with a vaccine candidate naturally attenuated in *Ornithodoros* tick from Portugal where it has been collected. This has been already published in *Transboundary and Emerging Infectious Diseases*.

IV. **Part-IV**: **General discussion, conclusions and perspectives.**

Chapter 10: **General discussion.** This chapter is devoted to the general discussion comparing current results with the ones previously published about the same topic.

Chapter 11: Conclusions and recommendations.

V. Part-V: **Bibliographical references.**

VI. **PART VI Appendixes**

Appendix 1

Co – published article about identification of new ticks, titled "*A new species of*

Rhipicephalus (Acari: Ixodidae), a parasite of Red River Hogs and Domestic Pigs in the Democratic Republic of Congo". This chapter dealing with a survey on the transmission cycle of ASF in wildlife. Rather than identification of *Ornithodoros* ticks in both, red river hogs and domestic pigs, other variety of ticks including *Ixodes, Amblyoma and Rhipicephalus* genus (Table 1) were recovered. Among the recovered *Rhipicephalus* ticks, new species of *Rhipicephalus* never described in existing literature was found in our research areas. This new species was analysed and named "*Rhipicephalus congolensis"*. This novel investigation was subject of specific article co-authored and published (J. Med. Entomol., 2013, 50(3), 479-484).

Appendix 2

Co – published article about: "The contribution of molecular epidemiology to informing control measures for African swine fever". This chapter is covered by a paper which is dealing with partial data, as outcome of active surveillance conducted between 2005 and 2006, in the same research area. The findings were presented as full article in an international congress and published in related proceedings. Interestingly, prevalence revealed by this partial study was very close to the one revealed by the Chapter 8 paper.

PART 1 - GENERAL INTRODUCTION

Chapter 1 - General background

Chapter 1 - General background

1. 1 - Overview of agricultural sector and animal production.

The Democratic Republic of Congo (DRC), with 2,345,409 square kilometres (Km^2), is the second largest country of Africa (just after Algeria), the largest in Central Africa and Congo Basin and one of the largest states of the world (Siradou et al., 2012) (**Figure 1**). Just behind Amazonia basin, the Congo basin is the second largest of the world, representing 13% of the African Continent.

The DRC national border, 9,165 Kms long, is shared with 9 neighbouring countries as follows: Republic of Central Africa and Sudan in the North, Uganda, Rwanda, Burundi and Tanzania in the East, Zambia and Angola in the South and the Republic of Congo and the Cabinda enclave (Angola) in the West. The DRC is characterised by huge, rainy and virgin forest of 135.2 million Km^2 representing 21 % of the continental forest and 64.5 % of the overall surface of the country (Kitengie, et al., 2013). With several natural reserves and protected areas of which 8 national parks (Virunga, Kahuzi-Biega, Maiko, Salonga South, Salonga North, Upemba, Kundelungu, and the marine Mangroves Park of Moanda) beside some hunting domains like Swakibula, Bili-Uele, Bombo Lumene and the Elephant domain of Bushimaie, including the specific park for Okapi within the Oriental Province (Kabeya et al., 2010; Siradou et al., 2013), the DRC is internationally recognised for its cornucopia of biodiversity. As a matter of fact, compared to other countries, the DRC has diverse biospheres with other types of ecosystems comprising tropical, mangroves and mountains forests, grasslands savannas and dambos that harbour many wildlife species. Globally, at least 409 mammal species (ICCN, 2010) among which wild suids species have been identified in this country. The Congo river (CR) which is crossing nine provinces including Katanga, Maniema, Oriental, Equateur, Bandundu, Kinshasa, and Bas Congo with its ten effluents of which Mongala, Rubi, Aruwimi, Lomami, Lindi, Lowa, Ulindi, Lukuga, Luvua and Lufira is an important communication means also used for traffic and trade of live animals which sometimes making it involved in the spread of epizootics.

The Congo river's length is 4,700 Km which is ranking it as second behind Nil in Africa, as the fifth in the world after Nil, Amazon, Mississippi and Yang-Tsé (Siradou, D. et al., 2012). The DRC communication network represents: 16,000 km of navigable rivers, 1,300 km of lakes, 156,000 Km of land roads and 5,138 Km of rail ways, which, in terms of length is ranging

Figure 1. Map of Africa: Democratic Republic of Congo (in white) and neighbouring countries

it as the first of Africa and the thirtieth of the world (Kitengie et al., 2013). DRC population is nowadays estimated to 80,000,000 inhabitants (Kitengie et al., 2013) with a growth rate of 3% per year. However, in 2004, this population was estimated to 55,000,000 inhabitants (WOAH/OIE, 2008). Kinshasa, the administrative capital, has currently about 8,000,000 of inhabitants, which is ranking in the second position behind Cairo and then two times more populated than Addis-Ababa. The basic commodities demand in terms of meat, eggs, milk, fish and pork is also increasing. The DRC population is mostly rural, with 75 % of inhabitants basically living in country side. A total of 60% of this population is composed of young persons (WOAH/OIE, 2008).

The DRC agricultural system is basically traditional. This country is harbouring 80% of arable areas of which only 10 % currently exploited for husbandry. The contribution of agriculture to country's economy was estimated to 60 % in 2007. The husbandry contribution to this economy was estimated to 26 % representing 52 % of the overall agriculture contribution. The DRC's potential in terms of grasslands is able to support at least 40 million heads of cattle and 54 million of small ruminants. Unfortunately, the local production of meat is based only on 900,000 heads of cattle; 20,000,000 of goats; 967,000 of sheep; 960,000 of pigs and 34,000,000 of poultry.

The national need in meat is complemented with 180,000 tonnes of fishery and aquaculture products per year. Nine per cent of animal production of the country is provided by insects and larvae (Kitengie et al., 2013). Therefore, the animal proteins production is very low representing only 2 % of basic needs and, the relevant deficit is estimated to 130,000 tonnes per year (WOAH/OIE, 2008). In order to address this deficit, about 550,000 tonnes of meat are imported each year. Locally, in order to resorb this gap, intensive husbandry activities consisting in exploitation of short cycle animals such as poultry and pigs have been initiated in peri-urban areas, this notably observed around some important cities of the country like Kinshasa, Lubumbashi, Mbuji-Mayi, Matadi, Mwene-Ditu, Likasi, Kolwezi and Kolwezi (WOAH/OIE, 2008).

Historically, some unfortunate events which negatively impacted on the local production of meat such as the "zaïrianisation" of non-citizens' properties in the 1970s, the looting of private and State's properties in the 1990s, the security crisis that followed genocide in the neighbouring Rwanda (1994) and the collapse of the second republic in 1997 (WOAH/OIE, 2008; FAO, 2012b) are still being incriminated among the causes of the aforementioned deficit. In order to boost local production in the agricultural sector, some major initiatives were finally initiated by the government in partnership with some grant organisations like the World Bank (WB), the

FAO, the Belgium government and, the Development African Bank (DAB). The majority of the national programmes were basically taken in compliance with some global and regional development strategies such as the WHO millennium objectives and the African renaissance initiatives in terms of poverty reduction, food security promotion and the employment of vulnerable classes of population. Some of these programmes were national and are still running and comprise: the Multi-sectorial and Urgent Programme for Rehabilitation and Reconstruction (in connection with war episodes, PMURR), the Strategic Document for Growth and Poverty Reduction (DSCRP), the Urgent Programme for Food Auto-sufficiency (PUAA), de Rehabilitation of the Presidential Farm of Nsele (DAIPN) and the implementation of the industrial Park of Bokanga-Lonzo.

1. 2 - Pig sector and African swine fever

Historically, it was not before colonisation, when in the 19th century, European Christian evangelists brought European breeds and introduced another system of pig exploitation in the country (FAO, 2012b). Before that period of time, only local breeds of the mentioned species were raised. With poultry, more than other livestock species, the DRC pig sector is for the moment the most growing and the most promoted, especially in peri-urban areas of growing cities as described above (FAO, 2012b).

Three types of pig exploitation systems are currently remarked in the country (i.e): i) industrial or modern clustered facilities, supplemented with veterinary drugs, mineral premixes, anti-helminthics, well composed feedings and efficient biosafety policy; ii) familial or back yarded exploitation, supplemented with household food waste and iii) traditional or free-ranging farming practice, with pigs scavenging during the day for feeding and returning back home in the evening (FAO, 2012b). Ninety per cent of the DRC population is mostly Christian and, pig husbandry is one of the main sources of animal proteins and income, especially in poor rural populations. Women and young people are the main owners of pigs in the villages. Traditional pig's production represents 65 % of local production and, industrial system comprising modern premises represents 35 % (FAOSTAT, 2010). Nowadays, estimated at 960, 000 animals (FAOSTAT, 2010), the DRC pig population reached its peak in 1998 when 1,154,000 were counted. Unfortunately, due to abovementioned security crisis, the majority of villages were emptied in their pig populations. The huge movement of internal populations and refugees from Rwanda and Burundi was induced and, this was incriminated as the main risk factor which favoured the spread of ASF within the country (Mulumba-Mfumu et al., 2013). The DRC pig

population declined tremendously during the wars period that broke out during the 1990s.

In terms of biomass, imported pork and by-products represents nowadays, 1000 Tons per year as the local production decreased at 24,000 Tons since 1998 (FAO, 2012b). The average price of a live pig is currently estimated at 4 USD per Kg of live pig in the villages i.e., 5 USD in urban markets. The majority of the pig exploitations of the country are located in 5 Provinces: Equateur, Bas-Congo, Bandundu, Kinshasa and Kasaï-Occidental. Due to the lack of grasslands in the forest areas, pigs and, to some extent goats are the most exploited livestock species in the Province of Equateur. The most raised breeds of pigs in the DRC are improved breeds like Large White, Pietrain (stress negative), Landrace and Duroc; apart from other cross-breeds pigs and African native pigs, so-called indigenous pigs like Ganda breed, the African large black breed (Nsalambi, 1987).

Apart from domestic pigs production, hunting of wild suids is allowed but, it is very often running in government hunting regulations, depending on year's period or whether the concerned area is protected. In conclusion, we may nowadays confirm that the pig production has become the first of the local production in the DRC, largely before cattle (FAO, 2012b).

1. 3 - ASF within the DRC

ASF has been recognised as endemic in the SSA, an area which largely covers the DRC, by many workers (Haresnape, J. M. et al., 1987; Wilkinson, P. J., 1989). One of the major weaknesses dealing with ASF in the DRC is the lack of alerts, the timely disease reporting and, the lack of records of outbreaks by official veterinarians. According to data collected from different written reports, historical occurrences of ASF in the DRC can be presented as follows: in 1939 (Saliki et al., 1985), 1954 (DeTray, 1963), 1963 and 1967 in Katanga (Boshoff, C. I., et al., 2007), 1977 presented as Zaïre 77 (Anonymous), 1978, 79, 80, 81, 82, 83 84, 86 and 87, ASF was confirmed to be present within the country according to OIE reports (Plowright et al., 1994). During the 1990s and specifically 1997, ASF-like disease was reported in a commercial farm in Ndjili, in the East of Kinshasa, the majority of pigs were naturally dying but, some survivors were observed two weeks or even later in some lodges of the concerned farm.

Some of the pigs showing growth delay with fever were slaughtered, tissues samples were collected from these pigs; ASFV DNA was detected in kidney tissues and, anti-ASFV antibodies were as well detected in the sera samples from some survivors (Mulumba-Mfumu et al., 2013). Since then, in 2001, 2002, 2003, 2005, 2007, 2008, 2009, 2010, 2011, and 2012 more alerts about outbreaks were claimed in many locations in Kinshasa and other provinces of the country.

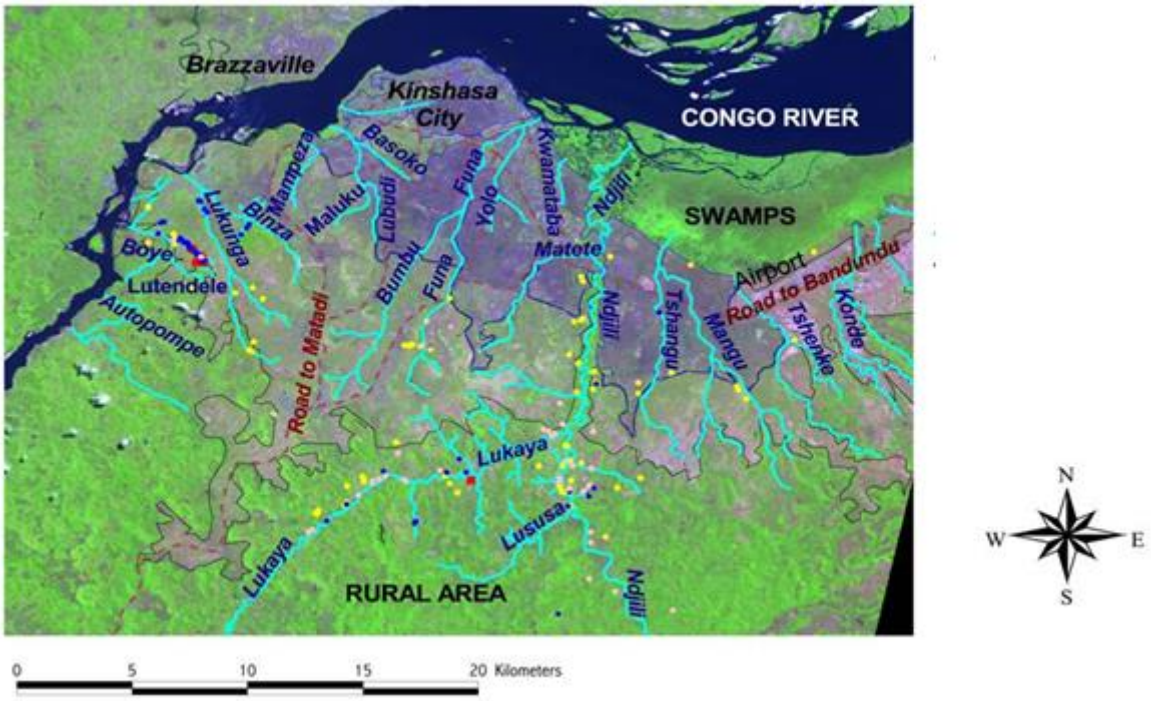
1. 4 - Risk factors and drivers at the country level

As previously reviewed by several authors about the SSA, a broad range of risk factors that lead to exposure of health pigs to ASF have been characterised. Based on these previous investigations, quantification of exposure pathways as well as ASF transmission models was already developed by some other researchers (Costard et al., 2007). A major outcome from such studies is a confirmation that, risk assessment is a good and efficient tool for identification of ecologically-based risk factors. Then, given the surface and the number of sub-areas of the country, a specific risk assessment framework should be recommended for the Democratic Republic of Congo. A variety of exposure pathways may be gathered:

1) Contact between natural reservoirs of sylvatic cycle (*Ornithodoros* ticks and wild suids) and domestic pigs;
2) Within domestic pigs, contact between infected and non-infected animals;
3) Contact between domestic pigs and ticks (Jori et al., 2013);
4) Farming practices;
5) Movements of suspected or exposed pigs including derivative products and pork;
6) Risk drivers, involving a wide range of administrative gaps comprising lack of veterinary services on the national borders, weakness of veterinary services, lack of good salaries, lack of data collection, storage and management, lack of strong surveillance systems, lack of diagnostic facilities, etc.

Main risks factors are specifically addressed in the conclusions, recommendations and perspectives chapter.

Figure 2. Map of Kinshasa City Province: ecological profile and commercial pig farms in the 1990s at the beginning of this research study (yellow spots: ASF free farms; red spots: ASF suspected farms; blue spots: pig farms being implemented or under construction)



Chapter 2 - African Swine Fever: an update

Chapter 2 - African swine fever: an update

2.1. – Preamble

Between 1921, year of ASF description by Montgomery, and to date, a broad range of relevant articles aiming on important approaches have been published. In order to obtain consistent background as well as accurate scope of disease situation, we have been obliged to consult several libraries and catalogues for further knowledge (EFSA, 2014). As a matter of fact, ASF data about the DRC have been missing (Penrith et al., 2013).

2.2. – The following article is devoted to literature review describing the disease on the basis of field realities.

This section is subject of a review article already submitted.

ARTICLE 1:

Mulumba – Mfumu, L. K.[1, 4], Dixon, L. K. [3], Madimba, Y. [1], Kazadi, E.[1], Mutalakat, N. **T.** [1], Thiry E.[2] and Saegerman, C.[4]

African swine fever: an update.

Mulumba – Mfumu, L. K.[1, 4], Dixon, L. K. [3],, Madimba, Y. [1], Kazadi, E.[1], Mutalakat, N. **T.** [1], Thiry E.[2] and Saegerman, C.[4]

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Abstract

African swine fever virus (ASFV) genome is unique; it is 170 to 190kbp long, both left and right terminal ends of its two DNA strands are covalently cross – linked by hair – loops nucleotides that are not perfectly base paired. Beside the presence of inverted terminal repeats, ASFV genome contains multigene family members, recognised for losing or gaining genomic copies depending on isolate. Instability of ASFV genetic structure has then been established. On the other hand, African swine fever virus (ASF) epidemiology within the majority of sub-Saharan Africa (SSA) countries was recognised as being complex, due to the natural history of the disease which is comprising sylvatic cycle, in addition to domestic cycle and, a wide range of risk factors. Diversity of clinical and pathological pictures of ASF, depending on isolate and host is frequently observed in many endemic ASF areas in the SSA. The acute form of the disease which is highly lethal, with hundred percent of mortality cases within infected pig population is the most stressed and the most frequently reported in published papers. Empirical understanding of ASF has tremendously contributed to persistence of infection, development of carrier status and endemicity of the disease in the SSA. Confusion with the so-called "red diseases of pigs" has as well been a source of delay in implementation of prevention and control strategies. This review is trying to recall some neglected approaches that could be of much help for optimisation of prevention and control strategies. With respect to the lack of neutralising antibodies, description of molecular basis of ASFV evasion to host immune response system is enhanced.

Introduction

African swine fever (ASF) is a complex and highly contagious disease of domestic pigs, occurring in forms ranging from peracute, acute, subacute, chronic and unapparent, with mortality rates ranging from 100% to as little as 3% (Hess, 1987). ASF is a major constraint to poverty reduction in rural areas of less developed countries where vulnerable pigs owners are the most affected.

ASF is as a threat to world pig industry and, to international trade of pigs and pork products (EFSA, 2010; FAO, 1998). Described for the first time by Montgomery in Kenya on introduced improved breeds of pigs in 1921 (DeTRAY, 1963), ASF clinical and epidemiological features were already observed on the continent before that formal description (TDA, 1905). As destructive transboundary animal disease, ASF, as other major devastative epizootics, is categorised as an old list A disease by the World Organisation for Animal Health (OIE) and thus, should be notified when and where it is occurring (Cubillos et al., 2013; Rowlands et al., 2007).

ASF is endemic in the majority of the Sub-Saharan countries (Haresnape, 1985 and 1987; Plowright et al., 1994; Wilkinson, 1989), in Sardinia and some countries of the Caucasus and East Europe where it is has been reported in dead wild boars (OIE, 2014; Giammarioli et al., 2011; Solenne Costard et al., 2009; Wilkinson, 1989).

Outside of Africa, ASF broke out widely three times, in 1957, 1960, and 2007 (Sanchez-Vizcaino et al., 2014). The first and the second time were to Portugal probably from Angola and Senegal respectively, through Atlantic Ocean (DeTray, 1963; Sanchez-Vizcaino, 2014). The third time was to Georgia in the Caucasus and, from there to the Russian Federation including the neighbouring countries like Armenia, Azerbaijan and Ukraine (EFSA, 2010; Sanchez-Vizcaino, 2014; Rowlands et al., 2007).

Due to genetic similarity with some isolates involved in previous outbreaks in Zambia, Malawi, Mauritius, Madagascar and Mozambique (Lubisi et al., 2005, 2007 and 2009; Sanchez-Vizcaino, 2012), the link between the third escape and initial outbreaks in those countries of Southeast Africa and Indian Ocean Islands was established. Illegal imports of pork by ships through the Black sea were suspected as determinative risk factors (Sanchez-Vizcaino, 2014).

With these occurrences close to the Arctic Circle, far from the African source, the ability of ASF to spread across the world was demonstrated (FAO, 2012). ASFV was already identified in wild boars in the Russian Federation and, recently massive mortalities were observed in wild boars in Poland, 9 Km from its border with Belarus and Lithuania (Sanchez-Vizcaino, 2014). Apart from wild boars, ASFV was also detected in a backyard farm in Poland (Pejsak et al.

2014). The infection of wild suids in these newly affected areas is an indication that ASF will remain endemic in this concerned region (Gogin et al., 2013), which as Sardinia will make it difficult to eradicate.

Aetiology

Historically, taxonomic assignment of ASFV was achieved in two steps. Initially, based on gross morphological features, ASFV was classified in the *Iridoviridae* family, based on the fact that it is a large icosahedral cytoplasmic deoxyribovirus (ICDV). In fact, apart from swine, ASFV multiplies in *Ornithodoros* (O) ticks and thus, is the only DNA-containing arbovirus (Plowright et al., 1994; Vinuela, 1986).

Advanced studies on molecular biology of ASFV (Vinuela, 1987) led the International Committee on the Taxonomy of Viruses to assign ASFV by itself to a new genus, the Asfivirus and *Asfarviridae* as family where it is the alone member (Dixon et al., 2004). As a matter of fact, based on morphological properties, genomic structure features, virion-associated enzymes, and the replication mode, ASFV behaves like Poxvirus (Huaichang et al., 1995, Vinuela, 1986).

ASFV mature virion structure consists of a nucleocapsid core of about 80 to 90 nm diameter, successively covered by a lipoprotein inner membrane, a capsid double layer, and a lipid envelope, acquired from budding through the plasma membrane when escaping the infected cell (Plowright et al., 1994). The external diameter of ASFV virion measures between 175 to 220 nm. The capsid double layer shell consists of interdigitating caspomeres that are hexagonal-like prisms, of about 13 nm by 5-6 nm showing a central hole. The overall numbers of caspomeres varies between 1,892 and 2,172 units; they are separated between themselves by about 8 nm of distance (Plowright et al., 1994).

Basically, ASFV replicates in van Furth mononuclear phagocyte system cells (Pan, I. C., 1987). Mainly infected cells *in vivo* are macrophages, even if at the latest stages of the disease, the virus is detected in many other types of cells (Pan, 1987; Wilkinson, 1989). *In vitro*, ASFV replicates in both macrophages and aortic endothelial cells (Dixon et al., 2004; Pan, 1987; Wilkinson, 1989) and in other non-lympoid cells such as vero-cells (Galindo et al., 2015, Vinuela et al., 1986).

Three proteins, p12, p72, and p54 are known to play an important role in the binding and entry process of virion and, one protein within the infected cell including the p30 are involved in the cellular internalisation of virus during the infection process (Cubillos et al., 2013).

Furthermore, it has been demonstrated that antibodies against these four proteins block both, the binding and the internalisation of ASFV, respectively (Dixon et al., 2004). More than 100

proteins are induced in ASFV-infected cells. Extracellular virion contains more than 50 structural and non-structural proteins but, none of them is glycoprotein (Huaichang et al, 1995; Plowright et al., 1994).

Advanced studies using high resolution polyacrylamide gel electrophoresis (PAGE), optimised by treatment with detergents and NaCl was carried out to analyse ASFV polypeptides within infected cells; the experiment allowed a recovery of 34 proteins in extracellular virus (Tabares, 1987; Vinuela, 1987) and, revealed that the majority of structural polypeptides, were corresponding to those found in intracellular virus (Tabares, 1987). According to some similar studies, at least 17 ASFV proteins inducing antibodies in naturally infected cells were detected (Plowright, 1994). Amongst these target antigens, the major structural p72, located within the outer capsid layer, was identified as the most stable and immunodominant, which suggested it as an effective coating antigen for ELISA test (Tabares, 1987; Vinuela, 1987) for detection of the majority of isolates from different geographical areas, during both, surveillance and disease control operations.

Unfortunately, all the attempts to immunise pigs using these aforementioned proteins failed (Plowright et al., 1994, Wilkinson, 1989). Other critical polypeptides, such as p220 and p150 were revealed as not being stable *in vitro,* depending on the type of infected cells, should they be Vero or macrophages (Garcia-Barreno et al., 1986; Vinuela, 1987). On the other hand, p220 and p62 have been revealed as polyproteins and precursor forms of some virus mature proteins such as p35 and p15 respectively, presumably in association with p72 (Cubillos et al., 2013). The same polyproteins were detected in the virus core shell, which is suggesting their processing as playing an important role in the core assembly and virus infectivity (Andrés, 2002; Dixon and Zhang, 2007). Other important proteins and genomic factors were demonstrated as being involved in ASFV morphogenesis, infectivity, replication and survival. That is case with TNF-E which is inducing vasodilation, increase of vascular permeability and, activation of the vascular endothelium which is ending in pro-coagulation and generation of micro-thrombi. In addition to that, TNF-E can also lead to apoptosis which is ending up in destruction of endothelial cells (Dixon et al., 2005, Zakaryan et al., 2015), such that virus progeny is stopped. The virus-encoded thymidine kinase (K196R) as well as dUTPase (E165R) genes are non-essential for virus replication in dividing tissue culture cells but, their deletion dramatically reduces virus replication in macrophages (Dixon et al., 2005). Other advanced studies carried on in natural and experimental conditions of the disease revealed some critical functions of some ASFV encoded proteins, for instance, the structural polyprotein p220 could be involved in packing of nucleoprotein core and host cell proteins; A224L and A179L were revealed as inhibitors of

apoptosis, while B602L Chaperone would be involved in the folding of capsid protein p72 and EP402R (CD2v) causes haemadsorption to infected cells (Linda et al., 2004).

On the other hand, reactive production of oxygen in infected macrophages which has been demonstrated as resulting in microbial DNA, might induce the same type of damage for ASFV DNA through, either miscoding properties or blocking of DNA or RNA polymerase, which consequently should block viral DNA replication. Repair mechanism of such damages is completed through a Base Excision Repair (BER) process where three enzymes, type X DNA glycosylase (O174L), ATP dependent-DNA ligase (NP419L) and AP endonuclease (E296R) could be involved (Dixon and Zhang, 2007).

ASFV genome is the only recognized with encoding multigene families (MGF) (Vinuela, 1987). Five MGFs have been identified: MGF 360, MGF 110, MGF 300, MGF 530/505 and MGF 100 (Chapman et al., 2008). Some of ASFV mutants missing several copies in MGFs 360 and 530 have been identified as inducing early macrophages death (Zsak et al., 2001), which might be suggesting their involvement in host defense system evasion (Linda et al., 2004).

ASFV particles develop 6 to 8 hours after, within the host cell cytoplasm where they can be ultra-structurally recognised as electron dense materials concentrating either as circular forms or as 5-6 sided polygonal shells (Plowright et al., 1994). While the latter become closed and acquire nucleoids, the circular do not apparently reach the virion level. Cytoplasmic sites of replicating virus correspond to DNA-positive cytoplasmic inclusions-bodies watchable within the para-nuclear locations of infected cells as they are positively stained by Acridine orange and Feulgen methods (Plowright et al., 1994).

Virus materials are transported to perinuclear sites of assembly through cyto-microtubules (Valdeira et al., 1998). Synthesis of less than viral genome length has been detected in the nucleus at early stages post-infection (Dixon et al., 2005). This suggests that cell nucleus might be the exact replication site and that cytoplasm might be the site of viral materials assembly. This hypothesis seems to be supported by the fact that the replication of ASFV DNA has never been observed in enucleated cells (Costa, 1990; Vinuela, 1987).

ASFV specific-enzymes which could be divided in two groups, virion enzymes (VE) and induced enzymes (IE) (Costa, 1990), comprise DNA dependent RNA polymerase, Poly-A polymerase, Nucleoside triphosphatase, RNA guanylyltransferase, RNA (guanine-7-) methyltransferase, RNA (nucleoside -2'-) methyltransferase, Deoxyribonuclease (optimum pH of 4.5), Deoxyribonuclease (optimum pH of 7.5), DNA topoisomerase, Nucleoside Triphosphatase and Protein kinase and Single-stranded DNA nuclease, DNA Polymerase, Thymidine Kinase and Ribonucleotide reductase (Linda et al., 2004; Plowright et al., 1994;

Costa, 1990; Vinuela, 1987; Saliki et al., 1985).

Genomically, following penetration of ASFV and release of DNA steps, the first transcription step seems to be achieved by virion-associated DNA dependent RNA polymerase synthesised in infected cells (Plowright et al., 1994) which give rise to other RNA species coding for about 12 early proteins. The synthesised RNA polymerase in the presence of protein or DNA synthesis inhibitors hybridise preferentially with four genomic regions, the E1 (0-51.9 Kb), E3 (63.7-75.2 Kb), E5 (100.1-111.6 Kb) and E7 (150-170 Kb) (Vinuela, 1987). According to the genomic map established *in vitro* using some restriction endonucleases (RE), it is explained that, after DNA replication, new RNAs may arise that hybridise with other DNA regions not transcribed *in vitro*, these might be late and complementary RNAs. Those DNA regions might be, L2 (51.6-63.7 kb), L4 (75.2-100.1) and L6 (111.6-150 kb) (Vinuela, 1987). In general, it is recognised that production of viral progeny occurs within the cytoplasm of the infected cell and that it is catalysed by DNA polymerase (Vinuela, 1987), which is phosphonoacetic acid sensitive (Plowright et al., 1994) but, this sensitivity is fundamentally more related to the synthesis of DNA polymerase than to DNA cleavage.

ASFV contains a long linear double stranded DNA, varying in length from 170 to 193 Kbp, depending on the isolate. This DNA molecule is close-ended by imperfectly base-paired and covalent terminal cross links; it is characterised by the presence of terminal inverted repeats tandems of nucleoid sequences containing between 150 and 167 open reading frames (ORF) (Dixon et al., 2013 and 2004; W. Plowright, 1994). The mapping of ASFV genome using restriction enzymes indicated the existence of a large highly conserved genomic region of 125 Kbp (to which belongs the p72 gene) flanked by two highly variable regions at the left and right ends, with the left region having more inverted tandems repeats than the right one (Plowright, 1994). These variable terminal regions are recognised as sites of MGFs. Insertion and deletions of members in these MGFs regions form the basis of genomic divergence among ASFV field isolates (O'Donnell, et al., 2015). Genomic, antigenic and pathogenic variability among different ASFV isolates are largely associated with these deletions and insertions (Pan, 1988, Chapman et al., 2008a; Dixon et al., 2004; Plowright, 1994).

Post-infection modulations in infected cells, such as Vero cells and macrophages or in case of susceptible hosts like domestic pigs, wild suids and *Ornithodoros* ticks, were also identified as more source of genomic modifications (Krug et al., 2015, Vinuela, 1986b; Plowright, 1994). The whole genome sequencing of Georgia 2007/1 isolate, revealed a 189, 344 Kbp genome containing 166 ORFs (Chapman et al., 2011) while is known as having 170 to 193 Kbp and containing 150 to 167 ORFs. The cross-links are partially base-paired hair loops into a

polynucleotide chain of about 37 nucleotides (Dixon and Zhang, 2007; Vinuela, 1987) consisting entirely of A and T residues. The outline of ASFV genome is estimated at 58 nm, with a density of 1.7 g / cm^3 and 41 % of GC content (Plowright, 1994).

In terms of stability, it has been established that ASFV is resistant to common inactivants, conditions and factors, such as extreme alkaline and acid pH values, proteases, detergents and that the required temperature for its destruction in cooked ham ranges from more than 60° C (Wilkinson, 1989). Further parameters on ASFV stability under different circumstances and biological substances were also described in details by Plowright, 1994. ASFV may be maintained 18 months, in serums; 6 years at the refrigerator temperature in blood; 1 month at 37^o C, 30 minutes at 55^o C; 11 days in faeces and at the room temperature and in putrefied carcasses the virus is not quickly destroyed. For preservation of infectivity at the laboratory level, -70^o C is more suitable than -20^o C. When in contact with detergents, other chemicals, and enzymes, ASFV is only sensitive to lipid solvents, hypochloride, phenols, and pancreatic lipases. Formalin 0.5 % kills ASFV after 4 days.

Some ASFV proteins are exploited as target and novel antigens for diagnosis purpose. Monoclonal antibodies induced by these antigens including their encoding genes are used for serological and molecular diagnostic of ASF, respectively. The major structural capsid protein 72, and others such as p30, p54, and B602L (orf9rl) which were detected as immunodominant are nowadays the most used for diagnostic and field investigations purpose (Cubillos et al., 2013; Fernandez-Pinero et al., 2013; OIE, 2012; Freidje, 1992; Oura et al., 1998a, 1998b and 2013; Tabares, 1987).

ASFV isolates circulating so far across the world have been delineated in 22 genotypes.

Historical background

Before formal description of ASF in 1921, Montgomery first observed strange clinical signs incriminating in what has been supposed by him as a viral disease on newly introduced pigs of European breeds in Kenya, in 1910, which he first presented as "Eastern Africa swine fever" (DeTray, 1963) but, which he named later "African swine fever ". Due to similarity of clinical signs, the disease was initially confused with an acute form of Classical Swine Fever (CSF) (Plowright, 1994). Also, before ASF formal description, a killing swine fever with similar clinical picture was reported in the Republic of South Africa (RSA) (TDA, 1905). At that period of time, differentiation between ASF and CSF could not be made as ASF was not yet described as a separated pathological entity and, CSF was poorly known (Plowright et al., 1994). ASF was

then existing on the African continent without being detected until 1921 (Costa, 1988). After description, ASF outbreaks were finally recognised in several areas surrounding Kenya (DeTRAY, 1963). Tanganyika was then recognised to be affected since 1914; Republic of South Africa in 1933 and 1934, Northern Rhodesia in 1954 and 1956, Nyasaland in 1960 and 1962, Mozambique in 1960, Senegal in 1960, Angola where the disease was described in details by Conceição, in 1949. The Belgian Congo (DRC) in 1954, 1961 (DeTRAY, 1963) and even before in 1939 (Saliki et al., 1985), 1963 (Bastos et al., 2003), 1967 (Gallardo et al., 2011), 1972, 1978 and 1985 (Plowright et al., 1994).

Years 1957 and 1960 are critical dates for ASF outbreaks history as this disease escaping from Africa, reached two times respectively, the Iberian Peninsula in Europe, through Portugal probably from Angola (DeTRAY, 1963) and from Senegal (Sanchez-Vizcaino, 2014) respectively. From Portugal, taking advantage of illicit movement of pigs and pork products, ASF spread out successively to France (1964, 1967, 1977); Madeira (1965, 1974, 1976); Italy (1967, 1980); Malta (1978); Sardinia (1978); Belgium (1985) and Holland (1986).

Apart from Sardinia where ASF is still persisting in wild boars (Giammarioli et al., 2011), the disease was successfully eradicated in all these European countries by stamping out and other drastic, but costly measures (Plowright, 1994). Outside of Europe and apparently from Iberian Peninsula, Cuba in the Caribbean was challenged in 1971, 1977 and 1978. ASF resurged back in Iberian Peninsula, and this might have been the source of another series of occurrences in Caribbean and Latin America, Dominican Republic and Brazil in 1978, Haiti in 1979, Cuba again in 1980 and possibly a way back to Africa, Cameroun in 1982. As a matter of fact, the Cameroonian, Dominican and Haitian isolates were genetically similar (Plowright, 1994). ASF outbreaks in Latin America, Caribbean, and Europe, were successfully eradicated for the second time.

Another episode of ASF occurrences happened in the second half of the 1990s in West Africa where the majority of affected countries were undergoing ASF outbreaks for the first time (Walter, 2003), except Senegal, Gambia, Guinea-Bissau and Cape-Verde where the disease was already enzootic (FAO, 1998b). Among these concerned countries, Cote d'Ivoire was affected in 1998, Ghana in 1999, Gambia with sporadic outbreaks in 2000, Togo in 1997, 1998, and 2001, Benin in 1997, 1998, and 2000, Nigeria, the Ibadan Christian area in 2001 (FAO, 1998a and 1998b).

In Eastern Africa, Kenya and Mozambique were re-challenged in 1994; and in Southern Africa, Zambia in 2001. The potential of ASF to spread to new areas outside of the continent, was demonstrated for the second time when the Indian Ocean islands when touched for the first

time, Madagascar was affected 1997 (Roger et al., 2001) by a severe outbreak which was confirmed in 1998 and which killed about half of pigs population. Mauritius was touched in 2007 (Lubisi et al., 2009).

Again outside of Africa, and this was for the third time, Georgia in the Caucasus was affected in 2007 (Rowlands et al., 2007); the disease spread then to the Russian Federation (RF) and its neighbouring countries including Armenia and Azerbaijan southward of RF. Ukraine was affected in 2012 (Chapman et al., 2011; EFSA, 2014; FAO, 2012), Belarus in 2013; Lithuania, Latvia and Poland in 2014 (Sanchez-Vizcaino, 2014; Jemeršic et al., 2014). The presence of ASF in wild boars will complicate its control and eradication in these regions.

Genetically, the isolates from the Indian Ocean Islands, the Caucasus, and the Russian Federation were found with 100 % similarity of nucleotids sequences with isolates involved in previous outbreaks in Zambia, Mozambique, Madagascar and Mauritius, all of them belonging to p72 Genotype II (FAO, 2012; Lubisi et al., 2009; Rowlands et al., 2007).

Dynamically, ASFV isolates circulating outside of Africa, reflect two watersheds that might be considered as corresponding to two ecosystems, one trans-Atlantic, represented by the Iberian virus including relevant variants, assigned to the p72 Genotype I, also called "ESACWA (Europe-South America-Caribbean and West Africa) genotype" (Bastos et al., 2003); and an another, trans-Indian Ocean, represented by isolates of the p72 Genotype II (Lubisi et al., 2005, Rowlands et al., 2008, Chapman et al., 2011). This latter genotype has been circulating over 23 years in domestic pigs in Mozambique, Malawi and Zambia, in the Southeast Africa.

Epidemiology

Basically, the natural history of ASFV infection involves reservoirs comprising wild suids and *Ornithodoros* ticks, which is making that the whole cycle of the disease can be basically divided in two components, comprising invertebrates and vertebrates hosts (Plowright et al., 1994). Nowadays, five genera of wild suids are known as being involved in this cycle; namely warthogs (*Phaecochoerus Africanus and Aethiopicus), Potamocherus larvatus or porcus* to which belong "red river hogs" (**Cover sheet**), wild boars (*Sus scrofa*), giant forest hog (*Hylochoerus*) (DeTRAY, 1963) and feral pigs. Susceptibility of Babyrousa babyrussa, another wild suid type (Fowler, M. E., 1996), also called pig-deer, has never been established.

ASF transmission to susceptible suids is achieved through two main cycles, sylvatic and domestic cycles. Practically, the transmission is achieved under three main pathways as follows, internal sylvatic cycle, sylvatic cycle-domestic cycle and, internal domestic cycle which is

involving pig to pig contacts (Lubisi et al., 2995). The fourth pathway involving domestic pig-*Ornithodoros* ticks have been recently confirmed (Rovaomanana et al., 2010, Jori et al., 2013).

The sylvatic cycle comprises transmission of ASFV between *Ornithodoros* ticks, eyeless, burrow dwelling Argasid ticks (**Figure 3**), and wild suids, although the alone presence of *Ornithodoros* ticks and wild suids within a given geographical area is not enough to establish the presence of sylvatic cycle (Jori, et al., 2013). *Ornithodoros* ticks transmit the disease between themselves through trans-sexual, trans-ovarian and trans-stadial paths and the transmission of ASF from *Ornithodoros* ticks to warthogs (**Figure 4**) is completed through biting when feeding on the latter (Plowright et al., 1994). Transmission to adult warthogs is not followed by clinical disease. Infected wild suids are symptomless (**Figure 5**) but, potential healthy carriers and source of infection to domestic pigs (DeTRAY, 1963). On the other hand, if bitten warthogs are infants, they will develop fever with transient viraemia, which finally will disappear when they become adults (Plowright et al., 1994). The transmission link between sylvatic cycle and domestic cycle involves *Ornithodoros* ticks and, possibly, contact with infected bush pigs. Unfortunately, the presence of virus of reduced virulence which may naturally occur in both, wild and domestic suids obscures the division between the two cycles (Plowright et al., 1994).

ASF transmission between domestic pigs themselves is made by contact favoured by many contamination factors among which urine, faeces, meat, swills and raw garbage are the most known. That is usually happening through ingestion or nuzzling when pigs are eating together (Wilkinson, 1989). Some professional acts related to veterinary practice or husbandry may

Figure 3 – Partial taxonomic key of ticks: a summary (Horak et al., 2003).

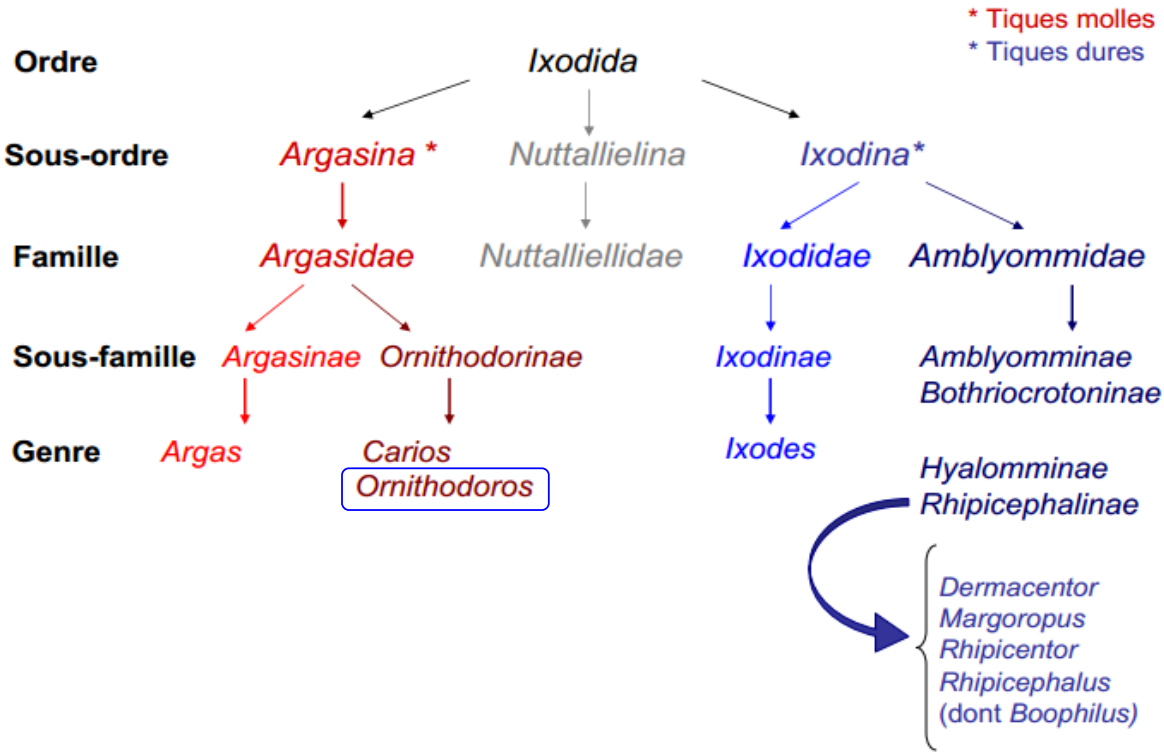


Figure 4 – Sylvatic cycle: *Ornithodoros* ticks and warthogs component (Plowright et al., 1994)

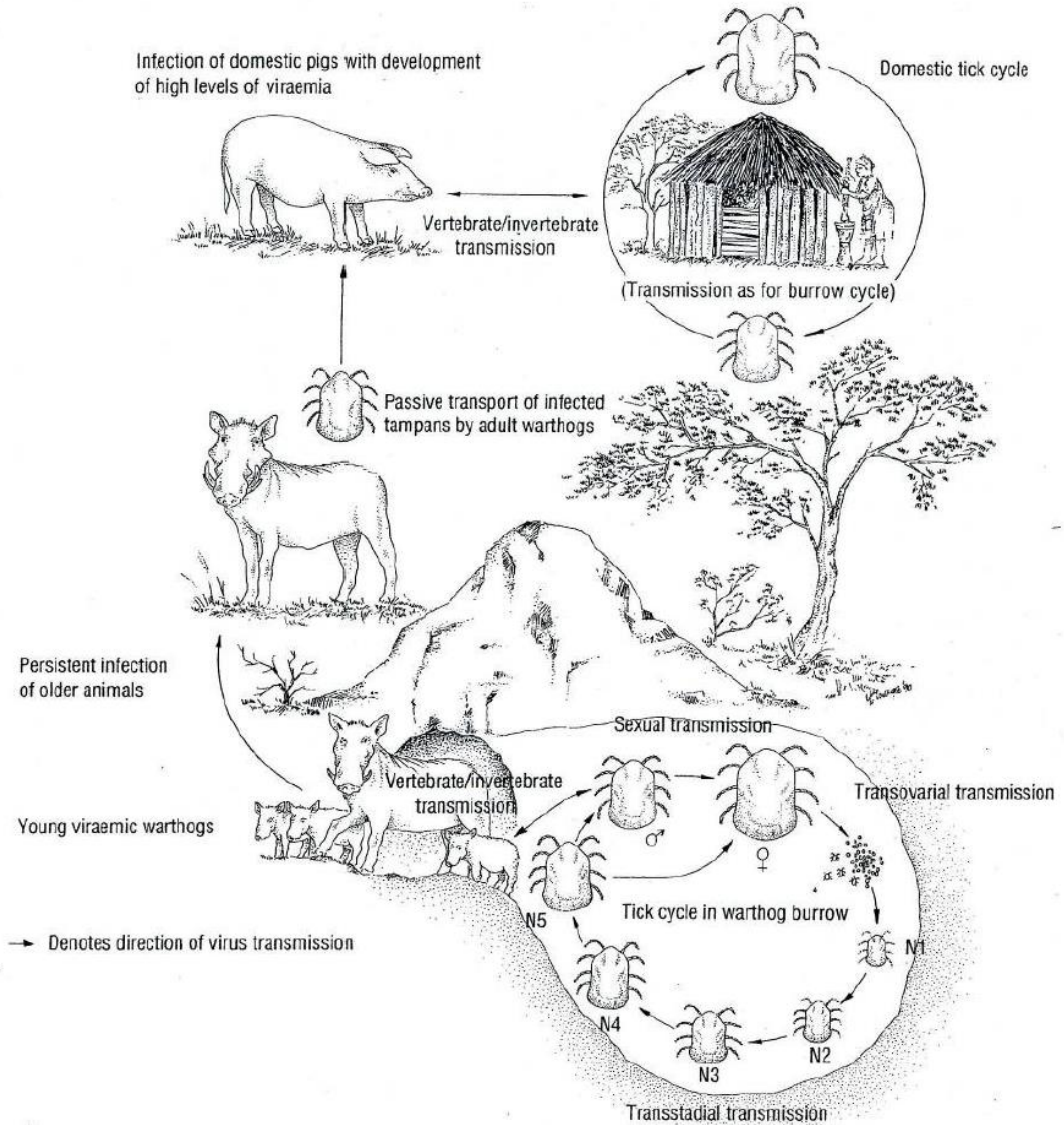
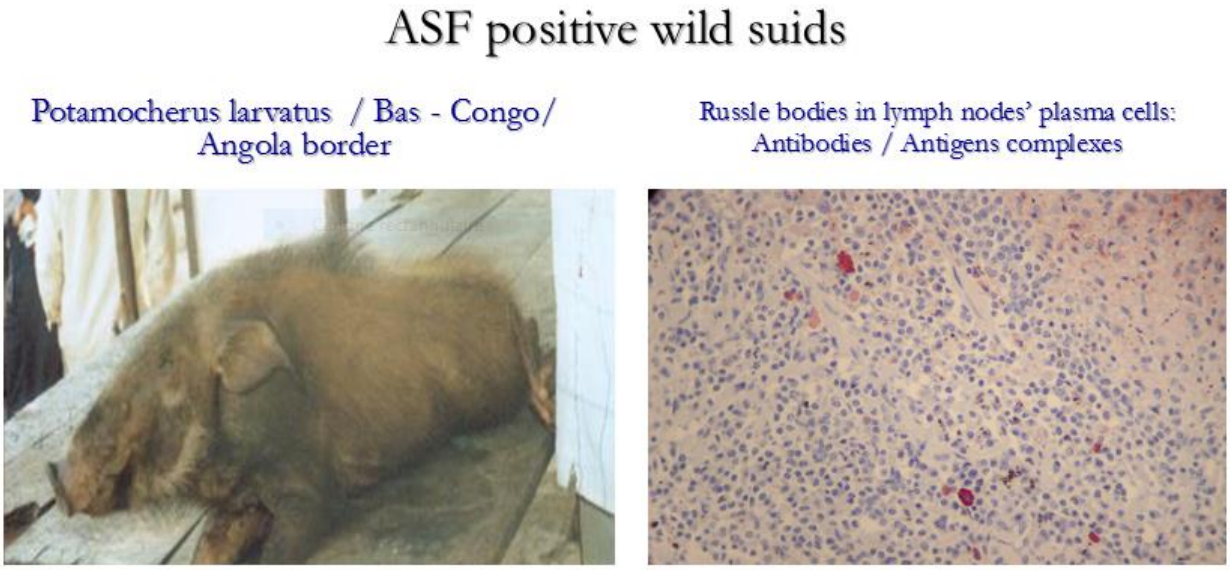


Figure 5 - Naturally infected feral pig killed by the hunters within an island on the Congo river on the border with Angola; antigens-antibodies complexes were detected within lymph nodes by immunohistochemistry. They are some evidence of ASF endemicity within the country.



also play an important role in the transmission of the disease. Domestic pigs that survive ASFV infection may become carriers and then, potential source of the spread of the disease (DeTray, 1963). Another possible source of infection is the ingestion lymphoid tissues from infected carcasses of dead suids.

Basically, the sylvatic cycle becomes a potential source of disease transmission to domestic pigs only when ASFV is maintained in at least one of the three components of this cycle: *Ornithodoros* ticks, feral or bush pigs and warthogs where they are existing. The presence of ASFV in these reservoirs as in the case of the Southern and Eastern African countries (Jori et al., 2007), is an important determinant for the maintenance of sylvatic cycle. ASFV may persist for at least one year in ticks without reinfection and, the virus can be cleared from infected ticks if there is no reinfection (Hess, 1989, Boinas et al., 2011).

Invertebrate transmission of ASF, or arthropod-borne ASF, within the sylvatic cycle is completed by argasid ticks of the *Ornithodoros moubata* complex (Ferreira et al., 2014; Penrith et al., 2013), they are classically known as host and reservoirs (Kleiboeker, 2001). Regarding ASF transmission by *Ornithodoros* (O) genus, it has been confirmed that distribution of involved species is related to geographical regions. As a matter of fact, ASF presence in the wildlife in the Iberian Peninsula was associated with the presence of *O. erraticus* (Fernando S. Boinas, 2011). This species is nowadays known as *O. marocanus* (Louza, 1989). In the sub-Saharan Africa, *O. moubata* is recognised as the most involved in ASF transmission but, *O. savignyi* in Western Southern Africa and *O. sonrai* in Sahel, were also described as susceptible hosts and vectors which means that they may as well be involved in the spread of the disease (FAO, 2009, Plowright, 1994) within the mentioned regions. For the Western Hemisphere, other members of the *Ornithodoros* genus were identified; those include *O. rostratus* reported in Bolivia, Paraguay, Argentina and Brazil (Costa et al., 2015) and *O. turicata*, *O. coriaceous* and *O. puertoricensis* reported in Dominican Republican and Haiti (Mc Daniel, 1986). These two countries were previously infected in the 1970s, but the disease was drastically eradicated. Other species of ticks might be suggested as mechanic and occasionally vectors of ASF, as it was already reported for *Amblyoma variegatum* and *Rhipicephalus sanguineus* (Badaev, 1992) but, this seems to be achieved only mechanically and only after fresh blood meal on infected suids. In fact, ASFV replication has never been reported in hard ticks (Ferreira et al., 2014). This type of mechanical transmission of ASF may also be made by other arthropod bloodsucking insects (FAO, 2009, Plowright et al., 1994, Zakrzewski, 1981) such as mosquitoes and horseflies upon feeding freshly on viraemic pigs which means that other swine herds living in the vicinity may become victims (Mc Daniel, 1986).

In order to avoid the existing divergence in literature regarding the taxonomy of *Ornithodoros* ticks, this update has adopted the classification suggested by Walton (1979). In fact, the *Ornithodoros moubata* complex was divided in 4 species (Walton, 1979), comprising *O. compactus*, *O. apertus*, *O. moubata* (sensu Walton) and *O. porcinus* (p) which was as well divided in 2 subspecies, *O. p. domesticus* and *O. p. porcinus*. The latter is the most encountered in warthog burrows, it is then supposed to be responsible of virus maintenance in enzootic cycle and seems to be the best ASFV amplifier amongst others (Kleiboeker, 2001).

Geographically, the DRC, which is among the first largest countries of the sub-Saharan Africa is also recognised as one of the most endemic areas; due to complex ecological profile comprising a large rainy forest, abundant savannas that are harbouring a wide range of ticks species and, wild suids. Both sylvatic and domestic cycles of ASF are expected to coexist (Costard et al., 2009). A short–term survey on ticks identification conducted in 2007 and aiming Red River Hogs and domestic pig ticks collected in two provinces, Equateur and Kasai provinces, revealed that 100 % of collected ticks were hard ticks, comprising *Ixodes muniensis*, *Rhipicephalus compositus*, *R. complanatus*, *R. planus*, *R. ziemani*, *Amblyoma paulopunctatum* and a new species of *Rhipicephalus* (R), never described in literature which finally was named "*R. congolensis*" (Apanaskevich et al., 2013) (**Table 1**). No soft ticks were identified among the ticks collected during that survey, possibly due to two main reasons. Firstly due to inaccessibility to wild suids habitats in the rainy forest, and secondly this might be due to the fact that *Ornithodoros* ticks do not stay longer on suids skins, just the feeding period of time. To detect possible contact between *Ornithodoros* ticks and domestic pigs, ELISA method consisting of the detection of anti-*Ornithodoros* bites antibodies has been suggested as a promising approach (Roger, 2001). This technique is based on the detection of antibodies against *Ornithodoros* tick's salivary glands antigens. Among, the target antigens purified from both, *O. erraticus* and *O. moubata,* the protein 20A1 of *O. moubata* was found more stable and immunogenic, with increased sensitivity and specificity and, without interference with α-chain of the mammals' haemoglobin (Baranda, 2000).

According to available literature, ASFV has been circulating over years in DRC but, the outbreaks were not recorded and even reported by the past, apart from those communicated by colonial industrial farms when submitting suspected samples to regional reference laboratories. The advanced initial date existing in the literature for this country is 1939 (Saliki, 1985). Free ranging farming system with scavenging pigs, restocking of back yards and piggeries without depopulation after

Figure 6 - First case to be detected with genotype XIV; severe clinical picture incriminating naïve animal and introduction of new virus within endemic ASF area. Co-circulation of genotype I and genotype XIV was detected within the same backyard farm.

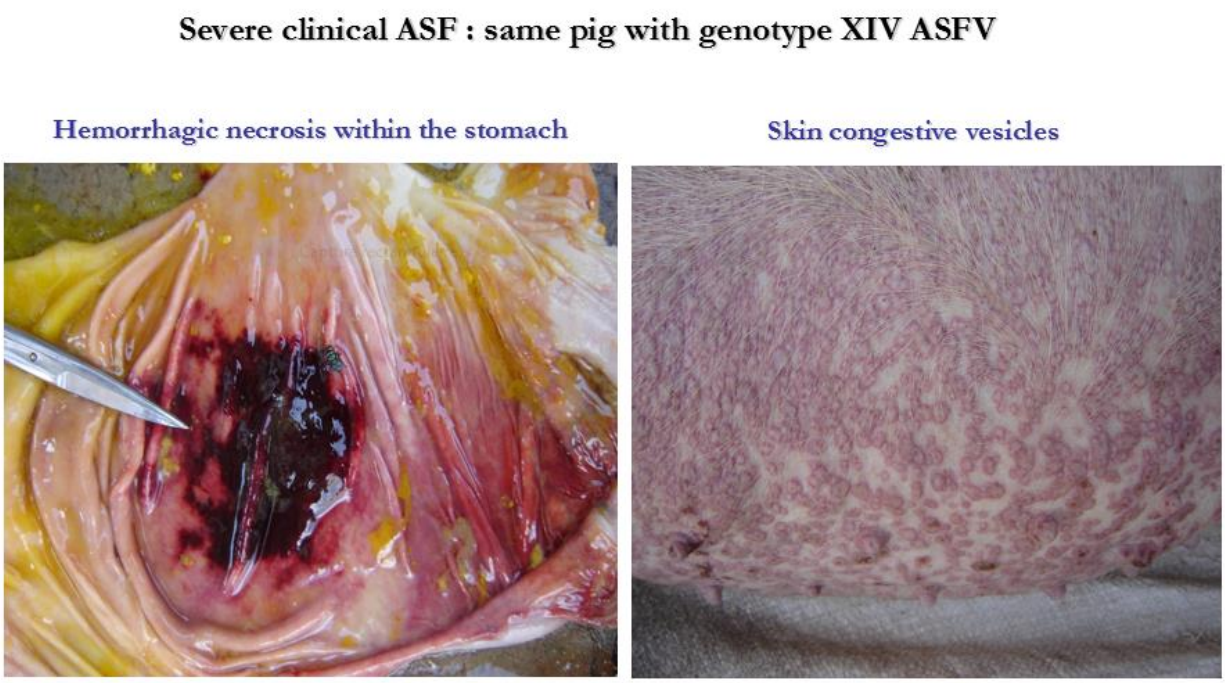


Table 1- Collected hard ticks (rather than *Ornithodoros*) during field investigation of sylvatic cycle within the interfaces of the Equateur province. The new *Rhipicephalus* ticks was assigned as *Rhipicephalus congolensis* and, a specific article is referred to (**Appendix 1**).

N°	Origin and Code	Overall number	Number of ticks / host		Identified ticks / host		Comments
			Wild suids	Domestic pigs	Wild suids	Domestic pigs	
1	BOENDE (*COBT*)	79	2	77	■	■ ●	Plus 61 new (◘)
2	LODJA (*COLOT*)	43	11	32	▼□■	□■	Plus 10 new (◘)
3	KIN – GAMBELA (*COKGT*)	1	0	1	-	■	-
4	KOLE IDJOLO (*COKIT*)	55	55	0	▼□■♦	-	All from potamocherus
5	MBANDAKA VILLE (*COMVT*)	152	152	0	▼■◙	-	All from potamocherus
OVERALL		330	220	110	▼ □ ■ ● ♦ ◙ ◘		7 types

▼ – Ixodes muniensis,
□ - Rhipicephalus compositus
■ – Rhipicephalus complanatus
● - Rhipicephalus planus
♦ - Rhipicephalus ziemani
◙ - Amblyomma paulopunctatum
◘ - New ticks ??? = Rhipicephalus. Congolensis

outbreaks, poor management of carcasses disposal, weakness of veterinary governance (OIE, 2008) as already reported in other African countries (Penrith et al., 2007), movements of pigs and pork products through a very long and porous border shared with nine countries, can be cited among potential risk factors that contribute to ASF spread and maintenance in the DRC. Low virulence isolates, such as the one already described from DRC by Haresnape in the 1980s (Haresnape, 1985), decrease of pathogenicity of newly introduced strains and possible reactivation of outbreaks strains (Wilkinson, 1989) could be as well mentioned as other sources of ASF occurrence within endemic ASF countries. Beside introduction of new genotypes (Lubisi et al., 2005), genomic variations and mutations leading to different subgroups within the existing genotypes have been demonstrated as another important source of severe outbreaks in endemic ASF areas (Odemuyiwa et al., 2000). Based on the central variable region (CVR) of the pB602L gene sequencing, the presence of more than one p72 Genotype I subgroups, has been demonstrated in DRC (Mulumba-Mfumu et al., 2015; article in press).

Clinical and Pathological signs

ASF clinical and pathological signs depend on involved strains, intensity of exposure, disease course, route of infection and infected host (Blome et al., 2013; Burrage et al., 2013; Guinat et al., 2014; Saliki, 1985; Wilkinson, 1989). In newly affected locations, or when new strains are involved specifically in case of endemic ASF areas (**Figure 2**), due to fact that pig populations are naïve, clinical and pathological profiles of the disease are similar to those described in acute forms (Saliki, 1985; Wilkinson, 1989), even if asymptomatic animals were also reported during the acute form ot the disease (Gallardo et al., 2015). In such circumstances, the most clinical signs observed are dramatic including pyrexia (40.5-42.2°C), hyperaemia of the skin, abortions in pregnant females, melena, haematochezia, epistaxis, petechiae, oedema and haemorrhage in many internal organs, especially in lymph nodes (Gómez-Villamandos et al., 2013; Hess, 1981; Howey et al., 2013). As a consequence dying animals are frequent. A mortality rate of 100% may also happen between 4 to 11 days post infection (Howey et al., 2013). In these conditions, usually observed clinical signs are similar to viral haemorrhagic fever syndrome (Gómez-Villamandos et al., 2013). Acute clinical form of ASF is usually the most presented in many published papers and text books, which does not fit in with the reality in the sub-Saharan Africa (SSA) where the disease is characterised by long-term infection. Within the SSA background, ASF is dominantly enzootic and, the subacute, chronic and even unapparent disease are more prevalent, the mortality rate is decreased (Kleiboeker, 2001) and overinfections are often present.

Usually, mild or long term forms of the disease are associated with remittent fever, anorexia, loss of condition, dyspnoea, cardiac insufficiency, polyarthritis, skin lesions, and growth delay (**Figure 7**). ASF should be suspected in case of association of some of these cited signs (Plowright et al., 1994; Wilkinson et al., 1989), especially if they last more than 14 days.

Pathologically, the most observed gross changes comprise serofibrinous types of hydrothorax, ascites, hydropericarditis, hydroarthrosis which, depending on quantity and duration of produced proteins, pathological adhesions with adjacent tissues may be present (Maurer, 1985; Plowright, 1994). Caseous pneumonia is also one of the most encountered gross changes in subacute and chronic forms of ASF (Mc Daniel, 1986).

Microscopically and, depending on the virulence of involved strain, host susceptibility, or modulating factors, histological damages involve mononuclear phagocytic system cells, fixed in lymphoid tissues or circulating in blood, which usually undergo picnosis or apoptosis (Blome et al., 2013; Gómez-Villamandos et al., 2013; Oura et al., 1998a), karyorhexis or karyolysis of lymphocytes, which will finally ends in generalised lymphopenia, thrombocytopenia, and occlusion of small vessels due to fibrineous micro-thrombi (Maurer, 1985; Mc Daniel, 1986). Depletion of lymphocytes related to apoptotic lymphoid tissues is the most pathognomonic microscopic change in endemic ASF areas.

Importantly, differential diagnosis should be performed with "ASF-like diseases also called red diseases of pigs" as basically the clinical picture is similar. Interference with ASF-like diseases in enzootic ASF areas was already reported even in Europe (Alvarez, 1987; Sanchez-Vizcaino and Mur, 2013). The most encountered "red diseases of pigs" in the DRC comprise Erysipelas, Pasteurellosis, Salmonellosis, and some forms of porcine Trypanosomosis.

Pathogenesis

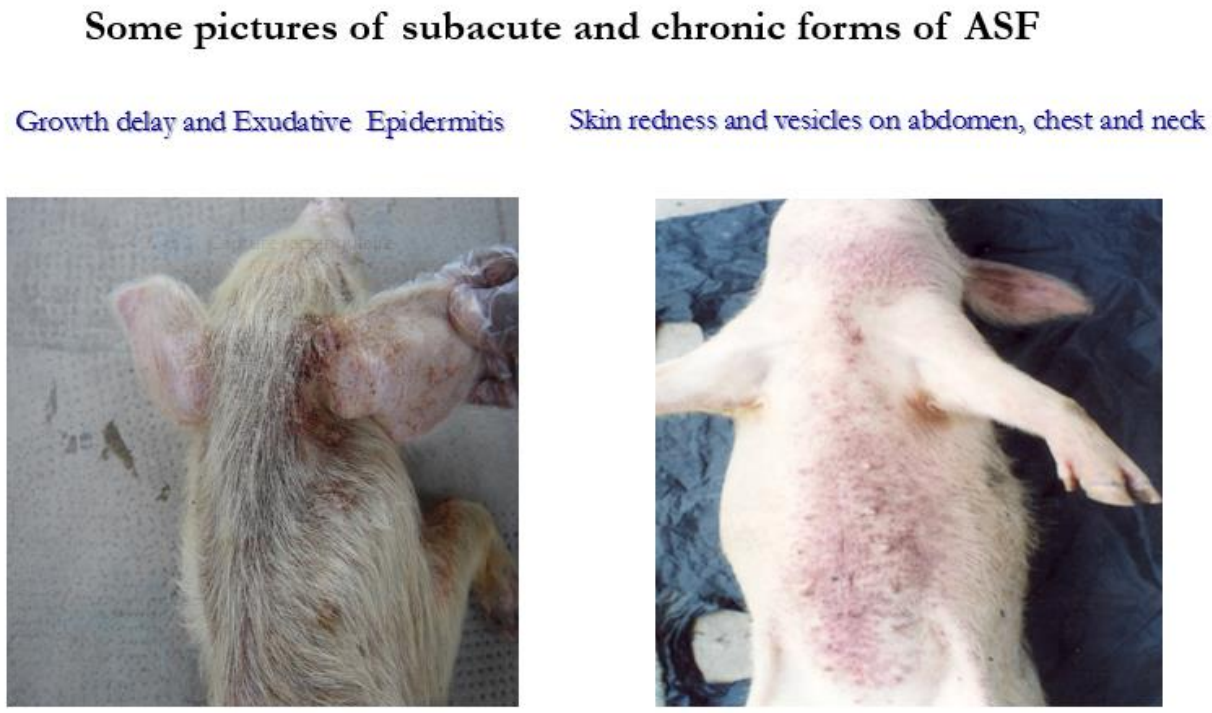
Depending on the infection route, which may be parenteral, intradermal in case of inoculation by ticks or hematophagous flies, ingestion, or nuzzling of virus from contaminated food or fomite when feeding; ASF incubation period varies between 24 hours and 8 days post-infection with minimal required doses (Plowright et al., 1994). In case of oral or nasal routes, ASFV replicates first in the pharyngeal mucous membrane and tonsils before being drained to head lymph nodes within 24 hours after exposure, which justifies the fact that saliva could be also exploited as goo sample containing ASFV. In case of airborne transmission, primary infection may start in the lower respiratory tract. Generalisation of infection through lymphatic or blood streams is usually completed in 48 hours post-exposure, with high level of virus concentration in target tissues, such as lymph nodes, spleen, liver, lungs and kidneys where the replication of

virus take place.

The main replication sites of ASFV are the target cells of the phagocytic mononuclear system (PMS), mainly monocytes and macrophages (Carrasco et al., 1996). Other type of cells like Vero cells is also adaptable for growth of ASF virus (Garcia Barreno et al., 1986). Apart from the PMS cells, and based on the use of fluorescent dyes in cell culture, reticulo-endothelial cells of lymphoid tissues, mesangial cells of renal glomeruli and, reticulo-epithelial cells of thymus have been reported as other spontaneous ASFV susceptible cells (Pan, 1987). Due to apoptosis and cytopathic effect of ASFV consisting chiefly in picnosis, karyorhexis and karyolysis, there will be decrease total population of white cells involving notably lymphocytes as well as neutrophil populations which definitely give rise to lymphopenia (Oura et al., 1998a; Wilkinson, 1989). Dramatic vascular changes undergone by endothelial cells, thrombocytopenia, and coagulation cascade will end up in impairment of haemostasis mechanism which will certainly give rise to blood leakage and impairment of the immune function (Blome et al., 2013; Gomez-Villamandos et al., 2003). Macrophages and aortic cells are primarily the most infected cells before detection of ASFV in many other cells of target tissues.

Penetration of ASFV in infected macrophages is mainly achieved through a receptor-mediated endocytosis (Dixon et al., 2004; Vinuela, 1986a) where the release of viral vesicles into infected cell cytoplasm is made through fusion of the virus envelope and those of endocytic vesicles (Dixon, 2004). Biochemically, apart from dynamin dependence, Clathrin mediation and Cholesterol presence within the cell membrane, Actin dependence, and phosphoinositide-3-Kinase activity are other cellular factors that are required for the viral to be successful (Galindo et al., 2015), even if it is not so far confirmed that this process is the only determining virus tropism. Other pathways such as micropinocytosis have been described during endocytosis. ASFV viral particles undergo fragmentation in various pieces that will be moved to replication sites through cytoskeleton’s microtubules (Alonso et al., 2013). Fragmentation or disassembly of viral particles relies on acid pH of late endosomes in association with other interactions between virus and host cells which are supposed to be important for optimization of replication environment (Alonso et al., 2013).

Figure 7 - Atypical clinical signs observed in subacute and long-term ASF within endemic disease areas within sub-Sahara Africa (DRC).



Diagnosis

In terms of disease recognition, clinical, pathological and epidemiological information form the basis of suspicion of ASF occurrence and, first alerts should be started in both, newly affected areas and, sporadic outbreaks in endemic disease areas. For confirmation of ASF outbreak, a wide range of diagnostic techniques have been smartly described in many published papers (Cubillos et al., 2013; Fernandez-Pinero, 2013; Maurer, 1985; Mc Daniel, 1986; Oura, 2013, 1998a and 1998b; Pastor et al., 1990; Perez-Filgueira, 2006; Plowright, 1994; Saliki, 1985; Sánchez-Vizcaíno and Mur, 2013; Steiger Yves, 1992; Zsak, 2005).

Basic diagnostic strategies exploited in described techniques are basically aimed either on the identification of the causal agent by isolation of the strain, detection of virus antigen and virus DNA, or detection of anti-ASFV antibodies using serums or liquid samples.

Apart from the fact that the majority of the described techniques are not cheap, simple, fast, and practical, all the existing diagnostic tools do not have the same performance, which is justifying international recognition through thorough validation policy. Conventional methods for ASFV diagnostic approved FAO (OIE, 2012) for ASF investigations across the world comprise several techniques among which a serological assay based on anti-ASFV Ig G detection ELISA (Pastor et al., 1990). This technique is based on the VP72 cytosoluble antigen for detection antibodies against ASFV during large-scale surveillance operations. It is cheap, simple and practical with good a sensitivity and specificity coefficient, which justifies its use in the majority of regions across the world.

Highly sensitive and specific commercial kit based on a competitive format using recombinant VP72 is as well available but, it is very costly for less developed countries, which is limiting its use for large scale screening, beside the issue of background in microplates when using haemolytic sera. The cheaper alternative way for less developed countries would be to prepare the soluble antigen for use as indirect ELISA (iELISA). As confirmatory test, other methods such as immunoblotting (IB) (Escribano et al., 1990; Pastor et al., 1989), indirect immunofluorescence (IFA) (Pan et al., 1974) or Immunoperoxidase (IMP) assays are recommended. Usually at least two tests are recommended for confirmation of positive cases (Oura, 2013).

Several new ELISAs techniques based on ASFV recombinant (r) proteins have been validated among which the rp30 based ELISA assay (Cubillos et al., 2013; Freidje, 1992; Gallardo, 2009; Perez - Filgueira, 2006). For ASFV identification, the cell culture assay, based on haemadsorption effect of blood red cells adhering to infected cells of mononuclear phagocytic

system (Malmquist and Hay, 1960) is unique but, in case of non-haemadsorbing isolates as those demonstrated in Madagascar and DRC, fluorescent antibody assay (FAT) as described by Bool et al, 1969 could be alternatively used (Oura, 2013).

Detection of ASFV genome by polymerase chain reaction (PCR) using primers that hybridise the highly conserved genomic region encoding VP72, was recommended for all the isolates including low virulence strains, and both haemadsorbing and non-haemadsorbing viruses. The PCR assay is effective even for samples undergoing putrefaction process. Two formats of this molecular strategy comprising, the standard PCR (described by Agüero et al., 2003) and the real time PCR (rtPCR) using TaqMan probes described by King et al., 2003 have been approved for ASFV DNA detection.

Currently, the rtPCR assay is being considered as the gold standard test for ASFV genome detection and the one developed by King et al., 2003 is being used in all the OIE reference laboratories (Oura, 2013). Furthermore, as ASFV p72 protein is histologically detectable, embedded tissues or cell cultures samples from ASFV infected pigs are suitable for other more specific and sensitive assays like Immunohistochemistry exploiting IMP-based strategy (Oura, 1998c) or *in situ* hybridisation (Galo Alexandre, 1990; Oura et al., 1998c)

Immune response

The absence of neutralizing antibodies (Cubillos et al., 2013; Vinuela, 1987), the lack of structural glycoproteins and, the requirement of the cell nucleus for viral DNA replication are some of important ASFV properties (Vinuela, 1987); although the presence of neutralising antibodies were recently demonstrated in convalescent pigs including the nature of their specificity and neutralisation mechanism (Escribano et al., 2013). Protective reaction against ASFV has been previously described by several workers, unfortunately this seems to be only limited to resistance to homologous strains to which concerned hosts are refractory (Wilkinson, 1989).

Experimentally, neutralising antibodies were produced against ASFV p54 and p30 (Paulino Gomez-Puertas, 1998). These viral target proteins play an important role in productive infection through biding and internalisation of viral particles, respectively and, during a receptor-mediated endocytosis process (Cubillos et al., 2013; Paulino Gomez-Puertas et al., 1998). Strong total Ig G antibody responses were also observed against viral proteins E183L (assigned as p54), A104R/histone-like, K205R and B602L (9RLORF) (Chapman et al., 2008b) and in pigs experimentally infected with a non-haemadsorbing isolate ASFV/NH/P68 (Ana Luisa Reis et al.,

2007). During the same trial, it was observed that K205R protein was stimulating Ig M antibodies, which is suggesting that this protein may be useful for detection of recently infected animals. Furthermore, antibody response to A104R/histone-like protein showed high titres in asymptomatic pigs, which is an indication of possible effective immune response to ASFV (Ana Luisa Reis et al., 2007). During a different trial, after inoculation of pigs with the same non-haemadsorbing and avirulent isolate ASFV/NH/P68, it was observed that viraemia and fever disappeared at the day 14 post infection (pi) in some pigs and within these asymptomatic animals, there was a normal plasma Ig concentrations, low level of specific anti-virus antibody but, with highest NK cells activity, which is suggesting a good cell-mediated protection.

The same group of asymptomatic pigs resisted to subsequent challenge with the highly virulent ASFV/60 isolate (Alexandre Leitao et al., 2001). Non-haemadsorbing ASFV isolates have been investigated in Madagascar (Gonzague, 2001) and in the DRC (Haresnape, 1985). Other similar experimental studies, revealed high proliferation of $CD8^+$ T cells associated to high frequency of ASFV encoded antigenic epitopes, which is supporting the concept that cellular immunity to this virus may play an important role in resistance to ASF (Jenson, 2000). It was also confirmed that the huge decrease of the total number of mononuclear phacogytic system cells which is happening during acute ASFV infection, due to apoptosis or karyorrhexis of monocytes, macrophages and lymphocytes that are destroyed and removed from blood and lymphatic circulation should negatively impact on cell-mediated immunity and finally on the host immune response (Wilkinson, 1989).

Blocking antibodies competing with some neutralising monoclonal antibodies have been demonstrated in convalescent pigs (Gomez-Puertas and Escribano, 1997), although this mechanism was shown during neutralisation assays, the protective role of these antibodies need to be investigated. Passively administered antibodies can result in reduction of virus replication level and decrease in severity of clinical signs as watched when maternal antibodies are administrated (Wilkinson, 1989). In fact, piglets receiving clolostral antibodies showed delayed onset of clinical signs, brief period of fever, low levels of viraemia and, higher survival rate than those receiving normal colostrum (Wilkinson, 1989).

Some limitations in protective reaction against ASFV, observed during some experimental assays carried out by Norley and Wardley (1982, 1983, and 1984) were reported by Wilkinson. Antibody dependent cell-mediated cytotoxicity in which neutrophils are involved as major effectors resulted in reduction of virus yields, with delay in production of responsible antibodies normally expected to happen as early as at about 13 days pi. Another important limitation that need to be clarified was observed with anti-ASFV antibodies binding the complement; the

attachment process could happen only at 14 or 15 days pi., which sounds to be very later for prevention of virus lethality in case of acute disease (Wilkinson, 1989). Furthermore, it was observed that complement-mediated antibodies induced by one isolate do not induce lysis of cells infected with other isolates (Wilkinson, 1989).

On the other hand and, on the ground of innate immunity the lack of neutralising antibodies has been attributed to some ASFV proteins involved in host immune response evasion (Chapman et al., 2008). Nowadays, the majority of proteins known as being involved in that evasion are encoded by unassigned genes located in multigene families (MGF) genomic regions (Correia et al., 2013). Five MGFs including MGF 360, MGF 110, MGF 300, MGF 530 (or 505) and MGF 100 were incriminated as being involved in this host immune response evasion (Dixon, 2004), which was apparently confirmed during a study where the Georgian isolate loosing members in MGF 360 and MGF 505/530 showed a protective activity against challenge with the parental virus (O'Donnell et al., 2015). ASFV proteins involved in host immune response evasion are also using modulation of different intracellular signalling pathways, such as manipulation or invasion of interferon (IFN), which was demonstrated with ASFV A276R, A528R, and I329L genes encoded proteins from MGF360 that were demonstrated as inhibiting induction of IFN β, type I IFN and IFN at the level of TRIF respectively (Correia et al., 2013). It has been also revealed in some studies that during ASFV infection decrease of IFN α correlated strongly with decrease of blood white cells (Karalyan et al., 2012). Other ASFV proteins non-depending on MGFs, comprise the potential immunomodulatory protein A238L, which has been demonstrated as inhibiting some host transcription factors and calcineurin phosphatase activity, as well the CD2v protein which is expressed in T and NK cells where it is responsible of adsorption of red blood cells and extracellular virus particles (Goatley and Dixon, 2011). The same protein has been recognised as being responsible of inhibition of bystander lymphocytes proliferation (Goatley and Dixon, 2011). Other two ASFV proteins, IAP and a Bcl2 homologue were identified as inhibitors of apoptosis of infected cells and thus favouring production of virions' progeny (Dixon, 2004). In the absence of sustainable and complete neutralising antibodies, on the basis of these existing data, further cell-mediated immunity studies aiming on ASFV unassigned genes of MGFs regions are required.

Another observation which could be placed on the ground of immune response concerns low or lack of serological response to ASFV infection in pigs from East African countries when using the OIE recommended tests. This might be due to the immunogenetics of indigenous pigs of this region, notably to polymorphism of some target immunodominant antigens supposed to interact with some host proteins, further studies on these interactions issue, involving differences

in both innate and acquired immune responses are required (Perez-Filgueira et al., 2006).

Socio-economic impacts

According to 2008 World Bank report, as an important source of income, animal proteins and employment, livestock is an important economic means exploited in developing countries for maintenance of livelihoods. Approximately 85 % of poor small scale farmers, consumers, traders, butchers and laborers depend on agriculture and livestock (IFAD, 2000).

In Africa, pork is the choice meat for wedding, funerals, and other major social events (FAO, 1998a). The animals of poor people are very vulnerable to disease due to costly inputs in the sub-sector of animal-health and production (Swai, 2014). ASF is a major constraint to poverty alleviation and food security in rural populations of less developed countries due to the fact it is affecting their valuable source of income and animal proteins (FAO, 1998a). Furthermore, ASF is a bottle neck to international trade and, a threat to world swine production (Sanchez-Vizcaino, 2014), which becomes a great concern even for developed countries (**Table 2**).

Table 2 – Some indicative socio- economic impact data related to some outbreaks.

Outbreak year	Country	Type/nature of losses	Observation / Level	Reference
1980	Spain	92, 000,000 USD	5 years of eradication	Arias & Sanchez Vizcaino, 2002
1980	Cuba	9.400 000 USD	5 years eradication	S.-Negrin & F.-Lepoureau, 2002
1982	Cameroon	1, 000,000 pigs	Outbreak was ongoing	FAO, 1998b
1994	Kenya	6,000 pigs	Faced another outbreak in 2000	FAO (Penrith, M. L.)
1994	Mozambique	20,000 pigs	In the South of the country	FAO (Penrith, M. L.)
1997	Nigeria	1, 016,000	Apparently from Benin	FAO, 1998b
1997	Benin	363,000 pigs	Highly severe outbreak	FAO, 1998b
1997	Togo	3,000 pigs	Outbreak was ongoing	FAO, 1998b
1998	Madagascar	500,000 pigs	50% of pig population	Roger et al., 2001
1998	Cote d'Ivoire	135,000 pigs	Stamping out for eradication	FAO, 1998b
1999	Ghana	6,700 pigs	-	FAO (Penrith, M. L.)
2000	Benin	Heavy losses	Including 1998 outbreak	FAO (Penrith, M. L.)
2000	Gambia	8,000 pigs	Within poorest Christian area	FAO (Penrith, M. L.)
2001	Nigeria	20,000 pigs	Only for the South (Ibadan)	FAO (Penrith, M. L.)
2001	Zambia	2000 pigs	412,238 ZK (Isoka District)	Samui et al., 1996
2005-2012	Tanzania (Kombo)	160, 632,000 TS	84 % of pigs' population	Swai et al., 2014
2007	Armenia	7,900 pigs	In only 3 months of outbreak	Beltrán-Alcrudo, 2008
2007-2012	Russian Federation	1,000,000,000 USD	In 5 years	Sanchez-Vizcaino, 2012
2008	D. R. Congo (Tshuapa)	4500 pigs (900,000 USD)	In Boende Village/ Equateur P.	CVL, 2010
2011	D. R. Congo	105, 614 pigs	Lost in 84 outbreaks	El-Sawalhy et al., 2011
2011	Zambia (Isoka)	-	50% of pigs'population	Komba et al., 2014

Prevention and Control strategies

To combat successfully ASF, in the absence of vaccine and treatment, control strategies should basically depend on the local epidemiological situation in association with regional and global approaches in order to prevent cross-countries and transcontinental spread of the disease (Costard et al., 2013 and 2009). Categorisation of affected countries in: at high risk, low risk, and disease-free areas would be a good approach for both, fruitful surveillance programme and efficient control strategies (FAO, 2012).

ASF is still spreading in Eastern Europe and, control measures applied so far seem to be unsuccessful. Classical knowledge of ASF in terms of disease recognition, early and accurate confirmation at the laboratory level combined with a good identification and assessment of risks factors are the major required assets to address the spread of ASF (Sanchez-Vizcaino, 2014). Some regions of the world including relevant countries, such as America, Canada, East and Southeast Asia, New Zealand, Australia, Japan and South Korea are still ASF-free (Costard, 2009).

Due to direct vicinity with infected areas, non-affected countries of East Europe, and other European community, may be currently categorised as countries at high risk. Given the intensive development of commercial links between the SSA countries with China and due to fact that the latter is currently holding 50 % of the world pig population; this country and surrounding regions such as Southeast Asia could classified as areas at high risk. Asia is currently the biggest importer of pork products representing 57% of the global pork trade especially destined to three countries, China, Japan and South Korea (Sanchez-Vizcaino, 2014).

Prevention and control of ASF in both areas at high and low risk should be based on active surveillance using serological screening (Toma et al., 1999) in both wild and domestic suids. Differential diagnostic with other haemorrhagic diseases of swine should be completed in order to provide correct results. Among the recommendations formulated for countries at high risk, availability of good diagnostics facilities, preparation of contingency plans at the regional level and simulations exercises (FAO, 1998b and 2012) are crucial. Early warning and public awareness based on good communication network, disease recognition and vulgarisation are also recommendable for this category of countries.

A good control programme needs to be risk-targeted, well-structured and harmonised with involvement of other stakeholders among which pigsties owners, farmers, veterinarians, officials, butchers, hunters, traders and carriers (FAO, 1998b; Sanchez-Vizcaino, 2014; Costard, 2009). In addition, it is profitable if all the stakeholders are organised in professional

organisations which should facilitate a good circulation of information and, effective coordination of strategies (FAO, 1998b). A good explanation of programme to each component of stakeholders I also required as it should enable a same understanding of the objectives and final benefits (Costard, 2009).

Timely and accurate information should be provided to stakeholders, some critical data such as source of infection, movement of pigs and pork products, swill feeding practice, movements of boats, ships and trucks, reservoirs animals, including any including risks drivers should be communicated (Sanchez-Vizcaino, 2012). Several prophylactic measures focused on safe importation of pork and pork products including other risk materials have been established by the World Organisation for Animal Health within the Terrestrial Animal Health Code. Guidelines on quarantine policy, zonage, stamping out, carcasses disposal, cleaning and disinfection of infected premises, wildlife and ticks control, restocking and sentinel animals and, compensation have been also described (FAO, 2012). Control of pigs *Ornithodors* and other haematophagous ectoparasites should be applied on a routine basis during outbreaks (CIRAD, 2015) but in respect of environmental requirements, and having in the mind the fact that, *Ornithoros* ticks may stay several years without feeding and, are burrow and cracks dwelling such that they are not easily reached by acaricides (FAO, 2012). Depopulation of affected premises including any outbreak survivors should be applied. The lack of timely and sufficient compensation for culled animals may lead to dismissing of information on ASF occurrences which finally will end up in consumption, commercialisation of infected meat on local or outside markets and, inappropriate disposal of carcasses (FAO, 2012). Strict application of biosafety standards is crucial (Chapman et al., 2008).

Prohibition of pig and pork products from infected countries is strictly recommended at the international level (Sanchez-Vizcaino, 2012), which means that careful attention should be paid on transcontinental movements of aircrafts, ships and trucks. Governments 'goodwill for application of contingency plans and giving priority to control programmes is very important, which means that networking is required at both, national and regional levels (FAO, 1998b).

To protect China, in regard to ASF challenge from outside, due to high density of pig population, construction of animal and plant quarantine posts including reinforcement of port law were suggested (Li et al., 2013). In regard to movements of wild boars through its very long land border shared with Eurasia countries, reinforcement of the old Great Wall using wire and new electric devices was also suggested to protect China (Ozawa, 2014).

Genotyping of circulating isolates, for a better understanding of source of outbreaks and links at regional and international levels is strongly recommended. Specific funds from governments

and grant organisations are required to assist the African countries to carry out molecular epidemiology studies on ASF. Intensive research works aiming on molecular characterisation of isolates collected in different endemic ASF areas in SSA are required. Introduction of new genotypes and genomic variations of existing isolates induces complication of control programmes in SSA.

Although control and eradication programmes are not easy in endemic ASF areas, they are unique and the only available solution (Sanchez-Vizcaino, 2012), particularly due the lack of vaccine and treatment. In order to prevent infection of free-ranging pigs, contact between components of sylvatic cycle and domestic pigs should be avoided. Domestic pigs should be kept in pig-proof enclosures, double fenced (Penrith, 1998); this strategy yielded good results in the prevention of ASF in Kenya and South Africa.

The ability of African countries to comply with conventional ASF control strategies is facing a broad range of constraints among which, lack and weakness of veterinary services, administrative difficulties, lack of funds, illegal imports of pork products even in passengers luggage, corruption at customs barriers, porous terrestrial borders very dominated by tribal connections, poor communication systems, lack of good diagnostic laboratories and, lack of compensation in case of stamping out, should be cited.

As it is unrealistic to radically change pig husbandry methods in Africa, alternative strategies consisting mainly in improvement of farming systems such as raising pigs in intensive or semi-intensive pigs farms were suggested (Penrith, 1998) due to the fact that free-ranging pigs are usually the central source of virus propagation in endemic areas (Nantima et al, 2015). There are plenty of ASF-free areas within the SSA, and in order to protect them as it has been demonstrated in South Africa, strict application of conventional control strategies is required for localised disease prevention and eradication in endemic areas (Costard, 2009).

Intensification of international cooperation efforts from veterinary authorities aiming on prohibition of infected pigs, including movement of derivate products and vectors is a pre-requisite for the global control of the disease (Fraser, 1986; Maurer, 1985; Costard et al., 2009).

Furthermore, research aimed at the development of effective vaccine as well as antiviral drugs should be continued (Sanchez-Vizcaino, 2014; Costard et al., 2009).

Vaccine and drugs prospects

Basic principles for production of vaccine against ASF were theoretically suggested (Escribano et al., 2013, Dixon et al., 2013). Natural or experimental infection of pigs with ASFV

is basically followed by antibodies production but, unfortunately, they are not effective to sustainably neutralise infectious virus. All the attempts to produce inactivated vaccine have failed (Hess, 1987). Based on the fact that recovered pigs are resistant to challenge infection with homologous virus, attenuated virus vaccines that were experimentally tried in regard to this observation, seemed promising but, when used at large-scale in vaccination programmes in Spain and Portugal, they showed disastrous side-effects, beside the production a wide range of clinical signs, chronic disease and even unapparent carriers (Wilkinson, 1989). None of vaccine inactivated using detergent or other adjuvant was able to prevent ASFV infection and replication (Kihm, U., et al., 1987).

Alternative strategy consisting in simultaneous use of immune serum and virus provided only a 192 days protection, which unfortunately disappeared after 283 days and, when the animals were hyperimmunised with additional doses, they still were susceptible to other ASFV strains (Maurer, 1985). The tendency of recovered animals to remain carriers is very dangerous, as it is leading to the maintenance of natural virulent virus under unapparent form of the disease among the immunised animals.

Other formats of vaccine, based on virus proteins, genomic deletions, and DNA were either ineffective or provided only very limited protection (Sanchez-Vizcaino, 2014). To date, promising results were obtained with a naturally attenuated and non-virulent strain OURT 88/3, assigned to the VP72 genotype I, isolated from *Ornithodoros* tick in Portugal, intramuscularly (IM) inoculation of this strain was followed 21 days after, by a second inoculation with a closely related virulent strain OURT 88/1 of the same VP72 genotype I; the two inoculations conferred protection to European domestic pigs against challenge with other virulent African isolates, the p72 genotype I Benin 97/1 and the p72 genotype X Uganda 1965 (King et al., 2011).

To determine whether protective immune response could also be induced in African indigenous pigs, the same VP72 genotype I strain OURT 88/3 was inoculated to local breeds of pigs from the DRC, the first inoculation was followed 21 days later by a second inoculation with the same VP72 genotype I virulent strain OURT 88/1; the two inoculations were followed 21 days after by a second challenge with the VP72 genotype I, highly virulent strain DRC 085/10 which killed hundreds of pigs during a severe outbreak within an industrial pig farm in the peri-urban area of Kinshasa; at the end of this trial, 50 % of tested animals were protected (Mulumba-Mfumu et al., 2015) and among the pigs that died, 25% were not killed by ASFV. It was also found that this vaccine reduced viraemia, which was even eliminated in some pigs, although ASFV was detected in some lymphoid tissues. The big issue is to determine the basis of protective mechanisms and specific antigens involved; several epitopes have been identified as

stimulating cytotoxic lymphocytes (Sanchez-Vizcaino, 2014). It was also found in similar studies that mRNAs levels for chemokines and chemokines receptors were higher in the macrophages infected with OURT 88/3 than in those infected with Benin 97/1 (Fishbourne et al., 2013).

To prevent the transmission of ASFV from *Ornithodoros* ticks, vaccination of pigs against these invertebrate hosts has been tried (Dias-Martin et al., 2015; Neelakanta et al., 2014; Astigarraga et al., 1995). The basic strategy of this vaccine is to induce immune response against some salivary glands proteins of adult ticks that enable these ectoparasites to feed on vertebrate hosts (Astigarraga et al., 1995). Encouraging immune response was obtained when using *O. moubata* salivary glands extracts; it was also observed that the potential of ticks' fecundity was negatively affected.

With respect to treatment prospects, during some experimental trials, several substances representing diverse biochemical pathways were reported as showing antiviral activity against ASFV (Costa, 1990). Chloroquine was reported as being inhibitor of ASFV infectivity; Colchicine as inhibitor of ASFV particles migration to the cell membrane; derivatives of both Actinomycin D and Rifamycin as inhibitors of ASFV RNA polymerase activity; and Amanitin, as inhibitor of cellular RNA polymerase. On the other hand, when used as treatment for cells enucleation, Cytochalasin prevented ASFV growth within the used specific media (Costa, 1990) and according to the same author, Coumermycin A1 *in vitro* replication of ASFV.

Extracts and fractions of *Ancistrocladus uncinatus*, a plant described by Hutch and Dalziel as belonging to Ancistrocladaceae family proved potential antiviral activity against ASFV isolate Nig. 99, apart from its cytotoxic effect on the primary cells culture used in the assay (Fasina et al., 2013). The therapeutic potency of this plant empirically reported by the farmers during some ASF outbreaks in Nigeria; this needs to be demonstrated through clinical experimentations.

The (S)-9-(3-hydroxy-2-phosphonylmethoxypropyl) adenine ((S)-HPMPA) is a new compound, that was reported as having potential and selective anti-viral activity against a broad spectrum of DNA viruses including ASFV (De Clercq, E., et al., 2013).

Conclusion

Based on a wide range of demonstrated parameters among which, genomic instability of the causal agent, lack of neutralising antibodies, progressive dynamics of the causal agent in terms of spreading to new areas and generation of new variants, tendency of the disease to become endemic in newly affected areas, it is not hazardous to confirm that ASF is a real threat to world

pig industry and, to rural economy of less developed countries. Advanced virus genomic and proteomic studies and further field investigations are required. The spread of ASF should be addressed at its homeland in the sub-Saharan Africa, the most endemic disease area even in the future, according to recent predictive studies (de Glanville et al., 2014).

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Chapter 3 - African swine fever virus serodiagnosis: a general review with a focus on the analyses of African serum samples

Chapter 3 - African swine fever virus serodiagnosis: a general review with a focus on the analyses of African serum samples.

3.1. – Preamble

For easy detection of pathogens signals during field investigations, serological tests, especially for massive screening, have been advertised as the appropriate tools (Toma et al., 1999). Furthermore, in actual practice, the most recommended serological technique, are those recognised as simple, practical, fast and cheaper as it is the case for ELISA, given the high level of automation and objective reading (Toma et al., 1999). Unfortunately, depending on geographical origins of sera specimens, all the recommended ELISA techniques do not behave in the same ways in all the fields, due probably to immunogenetics of the indigenous pigs of each region (Gallardo et al., 2011).

3.2. – The following article, is once again a review one, which is describing a validation exercise in which a part of this research work samples were involved, in order to first check whether the technique was effective and, secondly to know whether it was appropriated to our ground. This was determinative for the choice to be made by us between a couple of proposed coating proteins and ELISA formats during our field operations.

The following section is devoted to the above mentioned article in which I was as well active co-author.

ARTICLE 2:

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Review

African swine fever virus serodiagnosis: A general review with a focus on the analyses of African serum samples

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ABSTRACT

African swine fever (ASF) is an infectious disease that causes heavy mortality in domestic pigs. At present there is no vaccine against ASF, and eradication in countries where the disease is endemic is based only on competent diagnosis programs and the sacrifice of infected animals. Due to the presence of natural attenuated strains, certain infection conditions may result in reduced mortality. In these situations, the disease can be diagnosed by detection of specific antibodies. The use of classical and validated diagnosis assays, such as ELISA and Indirect Immunofluorescence or Immunoblotting, allowed the eradication of ASF in the Iberian Peninsula in the 1990s. However, given that conventional tests include the use of antigens obtained from ASF virus (ASFV)-infected cells, they have several disadvantages, such as difficulties to achieve standardization and also the risks associated with the manipulation of live virus. Such drawbacks have led to the development of alternative and more robust systems for the production of ASFV antigens for use in anti-ASFV antibody detection systems. In the present review, we provide an update on current knowledge about antigen targets for ASFV serodiagnosis, the significant progress made in recombinant antigen production, and the refinement of ASF serological diagnostic assays. Moreover, we describe the accuracy of an ELISA developed for the serodiagnosis of ASFV in Africa. This assay is based on a novel p30 recombinant protein (p30r) obtained from an Eastern African viral isolate (Morara strain), which shares 100% amino acid sequence identity with the Georgia virus isolate. That study included the analyses of 587 field sera collected from domestic pigs and warthogs in Senegal (West Africa), the Democratic Republic of Congo (Central Africa), Mozambique (South-East Africa), and South Africa. The results revealed that the novel p30r-based ELISA allows the accurate detection of antibodies against ASFV, independently of the geographical origin of the sera.

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1. Introduction

Classified as a notifiable disease by the World Organisation for Animal Health (OIE), African Swine Fever (ASF) is a highly contagious disease of domestic and wild pigs and causes major economic losses for the pig industry in affected countries. In addition, in Africa, where the disease is endemic in most sub-Saharan countries, ASF also has serious implications for food safety, thus limiting the availability of an important source of human dietary protein (Costard et al., 2009). Since there is no vaccine available for ASF, the detection of specific antibodies against the virus are indicators of historic infection, or current infection only if the presence of antibodies coincides with the presence of viable virus. Therefore, rapid serodiagnosis procedures can contribute to the complete eradication of the disease in certain affected regions. For example, the strategy for eradication of the ASF in the Iberian Peninsula was carried out by detection and slaughter of seropositive pigs. This strategy cannot be recommended for Africa, where the countries are not able to compensate for pigs that are culled. However, in these areas the great value of highly sensitive and specific serological tests is to improve the understanding of the disease and on that bases be able to identify high risk areas and to develop appropriate recommendations for prevention of the disease.

ASF virus (ASFV) causes inapparent persistent infections in its natural hosts, namely warthogs (*Phacochoerus africanus*), bushpigs (*Potamochoerus porcus, P. larvatus*), and soft ticks (*Ornithodoros moubata*) (Anderson et al., 1998; Kleiboeker et al., 1999). In domestic pigs, ASF was originally described to cause an acute hemorrhagic fever, leading the death of all animals infected. However, less virulent isolates have emerged during the circulation of the virus in domestic pigs, thereby increasing the prevalence of subacute and inapparent infections (De Kock et al., 1940; Mebus and Dardiri, 1980; Bech-Nielsen et al., 1995; Penrith et al., 2004). Pigs that survive natural infection usually develop antibodies against ASFV from 7 to 10 days post-infection which persist for long periods. Therefore, the detection of specific antibodies against ASFV should be performed for the diagnosis of subacute and inapparent forms of the disease.

In this context, the present review focuses on current knowledge about ASFV serological tests and immunodeterminant antigens used for disease control. The progress made in these fields may have great impact on the development of more reliable and accurate serological assays for the diagnosis of ASFV infections. Moreover, we describe the accuracy of an enzyme-linked immunoabsorbent assay (ELISA), based on a novel recombinant p30 (p30r) protein obtained from a viral isolate from East Africa (Madagascar), for ASFV serodiagnosis in Africa and potentially in Europe. Serum samples from domestic and sylvatic hosts, collected in Senegal (West Africa), the Democratic Republic of Congo (Central Africa), Mozambique (South-East Africa), and South Africa, were successfully analyzed by this ELISA. The novel p30r-based assay allowed the accurate detection of antibodies against ASFV, independently of the geographical origin of the sera. This has special relevance taking into account that ASFV isolates from Europe and West Africa are closely related to each other (Genotype I), while South and East Africa isolates are more diverse (21 different genotypes).

2. ASF serodiagnostic protein targets

Knowledge of the protein composition of ASFV virion structures is crucial because some might have immunological significance. Furthermore, identification of the most antigenic viral proteins is highly relevant for the improvement of serological diagnostic tests. ASFV is the only known DNA arbovirus and the only member of the Asfarviridae family (Dixon et al., 2011). ASFV is a large virus showing tropism for macrophages and monocytes, where it induces approximately 100 polypeptides (Alcaraz et al., 1992). About 40 of these molecules have been reported to be incorporated into the viral particle (Carrascosa et al., 1985). The viral DNA encodes for a number of novel genes not present in other virus families. The capacity of ASFV to persist in its natural hosts and in domestic pigs recovered from infection and carrying low virulence isolates shows that the virus has effective mechanisms to evade host defense systems (Dixon et al., 2004). The virion core is composed of a nucleoid, enclosed by a protein layer (core shell) which contains several viral proteins (Andrés et al., 1997, 2002). Surrounding the core are two lipid bilayers, called the inner membrane and, outside this membrane, the capsid. Additional complete or disrupted membranes, acquired during virus budding, can also be found in the viral particle. The major component of the viral capsid is protein p72, one of the first viral proteins identified as responsible for the induction of antibodies after a natural infection (Tabarés et al., 1980). The development of a semi-purification process for this protein from viral particles first led to the use of ELISA for antibody screening, thus reducing the number of false positive reactions found with previously developed antigens (Tabares et al., 1981). In addition, the two structural proteins p30 (also denominated p32) and p54 were clearly identified as highly antigenic during infection (Pastor et al., 1989; Afonso et al., 1992; Alcaraz et al., 1990, 1995; Oviedo et al., 1997; Kollnberger et al., 2002; Gallardo et al., 2006). Furthermore, antibodies against these three proteins are involved in virus neutralization, inhibiting the attachment (p72 and p54) and internalization (p30) (Borca et al., 1994; Gómez-Puertas et al., 1996, 1998) of virus. However, despite the potential of p72, p54 and p30 as serodiagnostic targets, these ASFV proteins are not sufficient for antibody-mediated protection against different virus strains (Gómez-Puertas et al., 1998; Neilan et al., 2004).

The recognition of ASFV p54 and p30 by porcine field sera collected in Spain has been compared to that of polyprotein pp62 (encoded by the gene CP530R) (Gallardo et al., 2006). Sera from infected pigs recognized the three recombinant proteins (p54r, p30r and pp62r) by immunoblotting (IB). The similar reactivity of these three proteins was also found when they were used as antigens in ELISA. In this assay p54 requires solubilization in 7 M Urea to reach optimal signals. This observation suggests that antibodies induced against this protein during ASFV infection recognize mainly linear epitopes. Polyprotein pp62 is the precursor protein of mature products p35 and p15, structural proteins localized at the core shell (Andrés et al., 2002). Unpublished data from our lab at CISA identify p15 as the mature protein responsible for the antigenicity of pp62.

Interestingly from the point of view of ASF serodiagnosis, the recognition of pp62 by sera from ASFV-infected pigs was maintained even in poorly preserved sera, while less reactivity was detected against p30 and p54 (Gallardo et al., 2006). These results

may indicate that antibodies against pp62 are more stable or display higher affinity than others. More experiments with field sera are needed to confirm this hypothesis. This property of pp62 could be very useful to overcome one of the disadvantages of using crude preparations from infected cells as antigens in ELISA, namely the lack of analytical reliability for poorly preserved samples (Arias et al., 1993).

The identification of other new serological immunodeterminants of ASFV was achieved by screening a viral cDNA expression library of the ASFV Ba71V isolate, using immune antisera from infected domestic pigs and from bush pigs (Kollnberger et al., 2002). That study identified 14 viral open reading frames (ORFs) encoding antigenic epitopes of the virus. Five of these corresponded to the following structural proteins: the previously identified p30 (CP204L), p54 (E183L) and p72 (B646L), the bacterial histone-like protein (A104 R), and p10 (K78R). In addition, 3 non-structural (F334L, K196R and NP419L) and 4 unassigned proteins (B602L, C44L, Cp312R and K205R) also showed significant reactivity with immune antisera. Strong reaction of convalescent pig serum has been described against a protein yielded from the in vitro translation of ORF B602L (Irusta et al., 1996). In a longitudinal antibody response analysis against the 12 abovementioned recombinant proteins using sera from experimentally infected pigs, strong and sustained antibody titers were confirmed against 4 of them: p54, K205R, A104R and B602L (Reis et al., 2007). Later on, the antigenicity of these recombinant proteins was further confirmed by testing porcine serum from animals naturally infected with ASFV (Gallardo et al., 2009a).

3. Conventional procedures for ASFV antigen production and serological assay formats

One of the earliest serological assays used for laboratory diagnosis of ASF was the immunoelectroosmophoresis (IEOP) test (Pan et al., 1972). This assay was much more sensitive than the agar gel double-diffusion precipitation test and even more sensitive than the complement fixation technique (Ferris et al., 1980), and was soon established as a screening test. The conventional antigen used in the IEOP test was prepared from extracts of Vero cells infected with ASFV (Pan et al., 1974).

Nevertheless, in infectious diseases where diagnosis is based on antibody determination, confirmatory tests are usually required to confirm positive reactions, especially when crude antigens are used in the diagnostic techniques. In this regard, the ASF serodiagnosis strategy used in the past included IEOP as a screening test and indirect immunofluorescence (IIF) to confirm positive reactions (Botija, 1970; Pan et al., 1974). This confirmatory assay is run with cell cultures in which only 10–20% of cells are infected with the virus. Therefore, typical positive reactions can be seen as fluorescent intracellular corpuscules, which correspond to viral factories. Positive and false positive reactions are easily distinguished when analyzing pig serum against infected and uninfected cell cultures. Although these assays played a critical role in ASF serosurveillance and eradication programs (Pan et al., 1974), they were laborious and not readily adaptable to large-scale surveys.

Therefore, the IEOP assay was soon replaced by the ELISA, considered then the most sensitive and suitable method available for testing large numbers of sera. This test is the most widely used technique for the diagnosis of subacute and inapparent ASF carriers (Wardley et al., 1979; Tabares et al., 1981; Pastor et al., 1990). ELISA shows greater sensitivity than the IEOP test, although in both assays the quality of the antigen preparation influences the final sensitivity and specificity of the test. Indeed, the use of crude antigens in ELISA, such as those described for the IEOP test, did not provide sufficient specificity to be included in an acceptable routine diagnostic test for ASF. Thus, Tabares et al. (1981) described the preparation of a semi-purified ASFV major capsid protein VP73 (p72), which greatly increased the reliability of ELISA when it was used as antigen.

Later on, the production of ASF antigen for ELISA was improved in order to make the technique economically feasible in large-scale surveys. At present, the cytoplasmic soluble antigen used in this assay (Escribano et al., 1989) is obtained from MS cells (monkey kidney cell line), grown in the presence of pig serum infected with an ASFV isolate passaged 48 times on MS cells. The use of pig serum in cell cultures instead of bovine serum circumvented antigen contamination with albumin from the latter, which was the main factor responsible for false positive reactions in ELISA until then (Escribano et al., 1989). The soluble protein fraction from infected cells is prepared by cell disruption, elimination of nuclei, and sedimentation of cellular debris by a 20% (w/w) sucrose cushion. The supernatant above the sucrose layer is used as the ELISA antigen. This crude antigen is currently recommended as a detector reagent in the prescribed screening test for international trade (OIE, 2012).

Despite the sensitivity of ELISA, one of the disadvantages continues to be the number of false positive reactions obtained with field sera and the standardization of the technique in laboratories. The main consequence of these limitations is that positive serum samples require confirmation by a second serological test. Although the IIF test can be used for this purpose, it has been replaced by IB, which provides increased specificity and a similar sensitivity to that of ELISA. Additionally, it has been demonstrated that sera lose reactivity in ELISA earlier than in IB (recognition of linear epitopes instead of conformational), thereby allowing improved detection of antibodies in poorly preserved sera (Arias et al., 1993). Furthermore, IB presents a simple and objective interpretation of the results (Pastor et al., 1989; Alcaraz et al., 1990; OIE, 2012). This test is performed using the same antigen as in ELISA and described above. The antigenic proteins are resolved in 17% acrylamide gels and transferred onto a nitrocellulose filter. Strips approximately 4 cm long, containing proteins of 23–35 kDa, constitute the antigen strips used for individual sera samples. This test has the additional advantage that the filter strips show stability throughout storage and transportation at room temperature in a dry atmosphere. No observable loss in the reactivity of transferred proteins is observed in at six months (Pastor et al., 1989). Another alternative confirmatory test to IIF and IB is the indirect immunoperoxidase plaque-staining method (IIPS) (Pan et al., 1982). This technique shows sensitivity and specificity comparable to that of the IIF test, but it is more suitable for large-scale analyses.

Despite the satisfactory performance of the classical assays described here (based on the use of virus-induced proteins in infected cells as antigen reagent), they have several limitations, such as the standardization of techniques and the need to manipulate the infectious agent and thus the consequent requirement of approved facilities for the biocontainment of Groups 3 and 4 pathogens (OIE, 2012).

4. Recombinant serological tests for ASF

Advances in molecular biology have greatly improved opportunities to upgrade the selection and production of immunoreagents and their application for the development of new assays. In this regard, during recent years extensive research has focused on the development of recombinant antigens to be introduced into serological diagnostic tests for ASF.

The use of recombinant proteins as reagents offers many advantages over antigen production based on virus-infected cells. In the case of ASFV, the use of recombinant proteins circumvents the

need to manipulate potentially dangerous live viruses and allows the standardization and scale-up of their production. In addition, these antigens improve the homogeneity of the results obtained in different laboratories and may increase sensitivity and specificity, thus reducing the false positive reactions produced by cellular culture compounds that contaminate antigens (Escribano et al., 1989). In some cases, such as IB, recombinant antigens also facilitate the interpretation of diagnostic results (Alcaraz et al., 1995).

Many potentially useful virus protein candidates in diagnosis have been described; however, only a small number have been tested and validated in various techniques. One of the first recombinant assays described for ASFV was a Western blot technique to confirm positive results obtained in ASV antibody detection by ELISA (Alcaraz et al., 1995). This confirmatory test was based on the use of protein p54 expressed in *Escherichia coli* fused to the N-terminus of the MS2 polymerase. The recombinant Western blot assay was highly specific and equally sensitive for the detection of pigs with antibodies to ASFV as the conventional Western blot using crude antigens obtained from infected cells and described above.

The same prokaryotic expression system, *E. coli*, was used by Reis et al. (2007) to produce 12 viral proteins previously identified as serological targets (Kollnberger et al., 2002). Serum samples from pigs infected experimentally with a non-fatal ASFV isolate and collected at various days post-infection (d.p.i.) were analyzed by ELISA using the 12 recombinant proteins individually. Strong antibody responses were found to 4 of them (p54, Histone-like, pK205R and pB602L), all showing 100% sensitivity at 21 days d.p.i. Interestingly, that study found that recombinant p73 expressed in bacteria is a not an adequate serological target for ASFV serodiagnosis as it failed to detect serologically positive pigs with inapparent symptoms.

The suitability of these four candidate proteins as diagnostic tools was further evaluated with European and African porcine field sera (Gallardo et al., 2009a). Purified *E. coli*-derived recombinant protein-based ELISAs proved effective for the analysis of pig sera from Europe when proteins p54 and pB602L were used as antigens, obtaining high sensitivity and specificity (among 95 and 98%, respectively). Therefore, these recombinant ELISAs performed as well as the OIE-approved diagnosis technique (conventional ELISA plus confirmation by Western blot).

Serological differentiation of ASF field isolates is not possible due to the lack of discernible serotypes. However, the sequence variability reported among viral isolates from various geographic locations can affect the recognition of specific antigens in serological tests. The accurate selection of ASFV antigens with a low ratio of antigenic variability among virus isolates is prerequisite for their use in serological assays. Although a limited number of positive sera from West Africa were tested, the 9 positives identified by OIE-approved assays in that study were confirmed by the p54- and pB602L-based recombinant ELISAs. Given their performance, these recombinant assays would also be suitable for testing sera samples from West Africa. Regarding sera from East Africa, since very few ASF-positive samples were analyzed by these recombinant ELISAs, the sensitivity of the test was not calculated (Gallardo et al., 2009a). However, the frequency of positive sera detected by these assays was lower than achieved by the OIE-approved tests. This observation may thus indicate that these recombinant ELISAs provide unacceptable sensitivity for diagnosis purposes in East Africa.

In addition to the analyses of porcine serum samples, the studies of sera collected from wild animals, such as the warthog (*Phacochoerus africanus*), are particularly interesting in East and Southern Africa, were wild pigs have been described as reservoir hosts of ASFV. Therefore the control of the disease in warthogs is highly relevant for the management of sporadic outbreaks in domestic pigs in contact with these wild animals and for determining the exposure status of warthogs to ASFV, where the sylvatic cycle plays a crucial part in the epidemiology of the disease (Lubisi et al., 2005; Jori and Bastos, 2009). A small number of warthog samples collected in Uganda were tested by the recombinant ELISAs using the proteins expressed in *E. coli*. A total of 23 out of 26 positive sera by OIE-approved approaches were also positive with the B602L- and p54-based ELISAs (Gallardo et al., 2009a). These results contrast with the low sensitivity found when testing domestic pig sera from East Africa. This discrepancy may be related to the distinct ASFV genotypes responsible for the induction of antibodies. ASFV isolates belonging to genotype IX have been reported in Uganda (Gallardo et al., 2009b), whereas mainly genotype II occurred in Mozambique in outbreaks in 1998–2005 (Bastos et al., 2004; Lubisi et al., 2005). Therefore if this hypothesis were confirmed, the recombinant ELISA results would suggest that the antigenicity of proteins B602L and p54 is conserved in ASFV isolates from genotype IX but not in those from genotype II.

Eukaryotic systems, such as the insect cell-baculovirus recombinant system, provide an alternative system for the production of recombinant antigens. The advantage of this expression system with respect to *E. coli* is that the proteins are likely to be produced in a native conformation as they can post-translationally modify the proteins that are expressed efficiently. ASFV proteins p30, p54 (Oviedo et al., 1997; Gallardo et al., 2006) and polyprotein pp62 (Gallardo et al., 2006) were produced as recombinant antigens expressed in insect cells (Sf9 or Hi5 cells) using a baculovirus expression system. These recombinant proteins were used in ELISA and Western blot for ASF antibody detection in sera from experimentally inoculated pigs and field sera from European inapparent ASF serologically positive animal. These analyses showed that the sensitivity and specificity of p30-r, p54-r and pp62-r based ELISAs, using the baculovirus-expressed proteins, were highly efficient for ASF serodiagnosis, with sensitivity and specificity ranging between 96 and 99%. Furthermore, in agreement with results obtained using p54 and pB602L proteins expressed in *E. coli* (Gallardo et al., 2009a), recombinant ELISAs using the baculovirus-expressed p30, p54 and pp62 showed improved sensitivity over than the conventional OIE-approved ELISA (based on cell extracts from infected cells) for the analyses of poorly preserved samples (Gallardo et al., 2006). Therefore, the use of these baculovirus-expressed proteins as reagents in ELISA reduces the number of false positives detected, thus allowing a more accurate diagnosis.

The use of insects as living biofactories is a cost-efficient alternative with respect to the baculovirus-based protein production in insect cells (Barderas et al., 2000; Pérez-Filgueira et al., 2007). This is highly relevant for developing countries. Heterologous protein production by the combination of recombinant baculovirus and *Trichoplusia ni* (*T. ni*) insect larva has been denominated improved baculovirus expression system technology (IBES®technology) and represents one of the best production alternatives based on baculovirus vectors. This inexpensive platform has been used to efficiently produce several recombinant antigens as diagnostic reagents for other diseases (Gomez-Sebastian et al., 2008; Pérez-Martín et al., 2008; Encinas et al., 2011; Todolí et al., 2009). Furthermore, ASFV p30 has been generated at very high levels by IBES®technology (Barderas et al., 2000; Pérez-Filgueira et al., 2006). ELISA and immunoblotting assays have been validated using insect extracts containing p30 without further purification. The insect-derived p30 presented very low levels of background reactivity when used as an ELISA reagent, discriminating accurately between positive and negative sera and reducing false positive reactions (Pérez-Filgueira et al., 2006). These results contrast with those obtained with p30 expressed in *E. coli*, which provided a very high background reactivity, even when the protein was extensively purified (Reis et al., 2007). Purification processes dramatically increase the production costs of any protein and cause considerable loss of recombinant protein yields. A single infected insect larva may produce enough p30 reagent to carry out more than 40,000 ELISA

Fig. 1. Sequence analysis of p30 proteins from a Morara/Georgia ASFV or the E70 ASFV isolates. (A) Alignment of p30 sequences obtained from the two ASFV isolates. The residues in which they differed are indicated with an arrow. The number of the residue is also indicated in brackets. (B) Correlation between antigenicity, structure, and variability in the p30 protein from the two ASFV isolates. Charts display the variation of the antigenic index as a function of amino acid position for the Spanish E75 and the African isolate Malagasy. Horizontal black bars indicate regions of predicted intrinsic disorder.

determinations and 2000 confirmatory IB tests (Pérez-Filgueira et al., 2006).

The recombinant ELISA with p30 produced in *T. ni* insects was validated testing Spanish ASF porcine field sera. This assay showed 98.2% sensitivity, a value similar to that of the conventional OIE-approved ELISA (Escribano et al., 1989). However, the specificity with the recombinant protein was greatly improved compared to conventional ELISA (97.4% versus 87.8%).

5. Accuracy of a Morara/Georgia-derived p30r- ELISA for serodiagnosis of African sera of distinct geographical origin

The recombinant ELISA using p30 produced in larvae was also tested with serum samples from Africa. Recombinant proteins p54 and pB602L (Gallardo et al., 2009a,b) and the recombinant p30 produced in larvae all derived from a Spanish isolate. Insect-derived p30 was accurate when tested with a limited number of samples from West Africa but performed with less effectiveness with samples from East Africa (Pérez-Filgueira et al., 2006).

Comparison of the p30 sequences from Spanish isolates and isolates from West, Southern and East Africa, showed that identity scores decreased from West to East, with those of the Southern isolates falling between the two (Pérez-Filgueira et al., 2006). These results may indicate that antigenic divergences regarding p30 are responsible for the distinct performance of p30r-ELISA for African sera from various geographical origins.

On the basis of these results, we produced an additional version of p30r from an ASFV isolate more distant to genotype I (European, South American, Caribbean and Western Africa isolates), and used it as antigen in an ELISA. We selected the CP204L gene encoded by the virus strains Morara and Georgia (Morara/Georgia). DNA sequences of the CP204L genes from Morara/Georgia and Spanish (E70) ASFV isolates were translated, and alignments and identity scores were obtained using the Clustal-W program (European Bioinformatics Institute). Antigenicity profiles along the sequence were determined using the algorithm described by Hopp and Wood (1981), whereas the hydropathicity pattern was analyzed by the ProtScale tool, using the Kyte and Doolittle scale (Kyte and Doolittle, 1982). The amino acid sequence of the two p30r proteins (Morara/Georgia and E70) presented a high degree of identity (97%). However, the Morara/Georgia p30r protein had three amino acid changes compared to p30r from the Spanish isolate. These changes were found in position 67 (Histidine by Arginine), 131 (Glutamic by Valine) and 172 (Histidine by Tyrosine) (Fig. 1A) and were responsible for variations in the hydropathicity pattern of the African p30r protein (Fig. 1B).

The Morara/Georgia CP204L gene, which encodes for p30, was amplified from a plasmid kindly provided by Drs. E. Albina and V. Michaud (CIRAD, Montpellier, France). This gene was cloned into the pFastBac1™ (Invitrogen) under the control of the polyhedrin promoter for high protein expression, and a recombinant baculovirus was obtained following the Bac to Bac™ system (Invitrogen). The resulting recombinant baculovirus containing the gene encoding for Morara/Georgia p30 was used to express the recombinant protein by IBES®technology. The Morara/Georgia p30r was efficiently expressed in *T. ni* larvae and accumulated in the inoculated insects in a dose- and time-dependent manner (data not shown), as was found previously for p30r from the Spanish isolate E70 (Pérez-Filgueira et al., 2006). Total p30r protein extracts obtained from infected larvae with the baculoviruses expressing the p30 derived from E70 or Morara/Georgia viruses were analyzed by SDS-PAGE electrophoresis. Both recombinant proteins were clearly identified in Coomassie blue-stained gels as a single band,

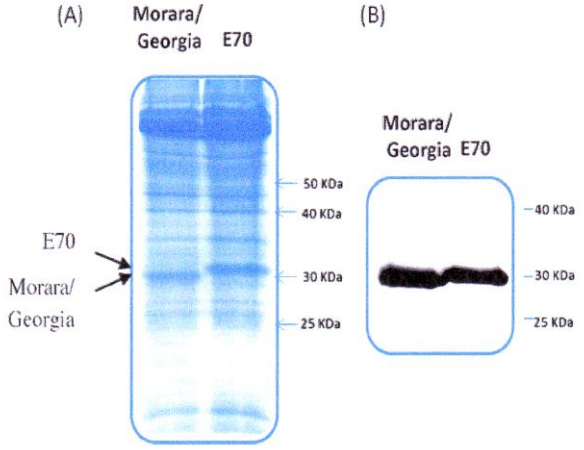

Fig. 2. Production of the Morara/Georgia recombinant p30 in *T. ni larvae*. Total protein extracts from larvae infected with the Bacp30 baculovirus expressing the Morara/Georgia or the E70 p30r proteins were analyzed by (A) Coomassie brilliant blue staining of SDS-page gels and (B) Western blotting using a positive reference pig serum as a probe. Red arrows indicate the position of the recombinant p30.

with slightly different electrophoretic moieties, but both around the expected size of 30 kDa (Fig. 2A). This distinct electrophoretic moiety of Morara/Georgia p30r could be attributable to its higher hydropathicity. Both p30r proteins reacted with a pool of sera from ASFV-seropositive swine used as probe in a Western blot (Fig. 2B). Total soluble protein extracts from insect larva were obtained basically as described by Pérez-Filgueira et al. (2006) (see details in Supplementary material) and tested to demonstrate improvement in ASF serodiagnosis in African countries, adapting the tools available so far for detection of this disease.

An initial panel of 92 pig serum samples collected in Mozambique were tested by OIE-approved assays (ELISA plus IB) as gold-standard, and against both Morara/Georgia and E70 p30r proteins by ELISA to compare the performance of the latter two. Twenty-one out of 92 samples were classified as ASFV-positive by OIE-approved tests. Pig sera from Mozambique did not present significant background reactivity against control (uninfected) larva protein extracts (OD values < 0.2), as also found for Spanish serum samples (Pérez-Filgueira et al., 2006). Therefore, the results were expressed as the ratio between the mean OD obtained for each sample against positive antigen (p30r protein) and negative antigen (larva extracts). (Details on ELISA procedures are given in Supplementary material.)

With an OD ratio of 2.5 as the cut-off level, 21 out of 92 pig sera were seropositive by Morara/Georgia p30r-ELISA, whereas only 19 were seropositive by E70 p30r-ELISA (data not shown). Furthermore, 2 out of the 19 seropositive samples detected by the E70 p30r-assay were in the cut-off interval. Positive sera in ELISA were all confirmed by p30r-Western blot (data not shown). Comparative results between p30r-ELISAs and OIE-approved tests indicated that the specificity of these recombinant proteins (Morara/Georgia and E70) for the analysis of pig sera from Mozambique was equivalent. However, differences were detected between Morara/Georgia and E70 p30r proteins with regard to sensitivity, which was slightly lower for the E70 p30r-ELISA (90.4% of seropositives detected) than for the Morara/Georgia p30r-ELISA (100% of seropositives detected). The results obtained by the Morara/Georgia p30r-ELISA for sera samples from Mozambique were expressed as ODs ratios and are shown in a Box-plot (Fig. 3).

The characteristics of the Morara/Georgia p30r-ELISA for ASF serodiagnosis of samples from Mozambique were calculated by evaluating the results of this assay by means of a Diagnostic test 2×2 table, using OIE-approved assays (ELISA + IB) as reference tests. The sensitivity of the test reached 100% (95% CI, 83.89% to 100%) and the specificity 97.18% (95% CI, 90.19% to 99.66%).

Given the satisfactory performance of the Morara/Georgia p30r-ELISA in the analysis of pig serum samples from Mozambique, we extended the study and evaluated this assay for the detection of antibodies against ASFV in sera from other geographical locations in Africa. For this purpose, we used the Morara/Georgia p30r-ELISA and OIE-approved tests to test domestic pig serum samples collected in the Democratic Republic of Congo (DRC) ($n = 303$) and in Senegal ($n = 109$). Ratio values obtained for sera from these two countries are shown in Figs. 4 and 5 respectively.

One hundred and thirty-four serum samples out of 135 DRC seropositive samples (tested by OIE-assays) were correctly diagnosed by the Morara/Georgia p30r-ELISA. Regarding the reactivity of OIE-negative sera to the recombinant ELISA, 166 out of 168 DRC serum samples were correctly detected. For samples from Senegal, 20 out of 22 OIE-seropositive samples were correctly detected by the Morara/Georgia p30r-ELISA, while all the samples from this country tested negative by OIE-approved assays were also negative by the recombinant ELISA.

Therefore, the sensitivity of the Morara/Georgia p30r-ELISA for the sera samples from DRC and Senegal was 97.78% (95% CI, 93.64% to 99.54%) and 90.91% (95% CI, 70.84% to 98.88%) respectively. With respect to the specificity of the recombinant test, it was 98.81% (95% CI, 95.77% to 99.86%) and 97.75% (95% CI, 92.12% to 93.73%) for samples from the DRC and Senegal respectively.

Together, the results obtained testing pig sera from Africa indicate that the Morara/Georgia p30r-ELISA has a specificity of around 98%, independently of the origin of the sera (East, Central or West Africa), whereas the sensitivity varied between 100% (East Africa)

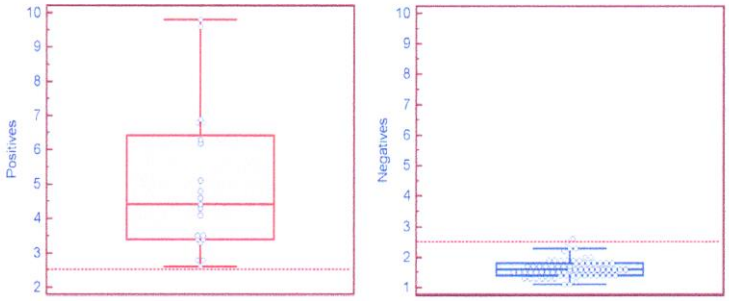

Fig. 3. Detection of ASFV-specific antibodies in pig serum samples from Mozambique by the Morara/Georgia p30r-ELISA. Ratio OD distribution of OIE ASFV-positive (red box at left) and -negative (blue box at right) sera are shown in a Box-and-whisker plot graph. Each dot corresponds to media of duplicate analyses of an individual sample. Cut-off value is shown as dotted line. The central box represents the values from the lower to upper quartile (25–75 percentile). The middle line shows the median and the vertical line extends from the minimum to the maximum value. Among seropositive sera detected by OIE-approved tests, the highest value recorded was 9.8.

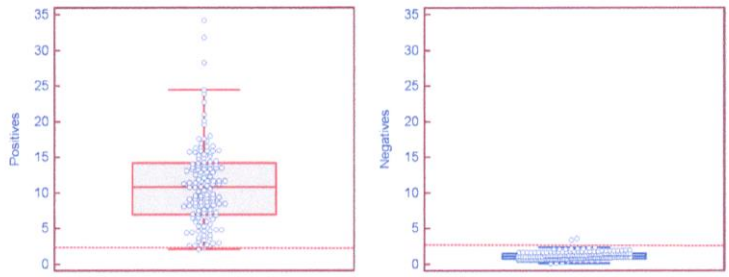

Fig. 4. Detection of ASFV-specific antibodies in pig serum samples from DRC by the Morara/Georgia p30r-ELISA. Results are expressed as explained in figure 4. Among seropositive sera detected by OIE-approved tests, the highest value recorded was 34, 2.

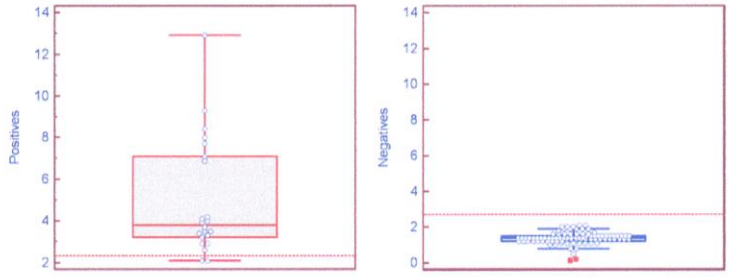

Fig. 5. Detection of ASFV-specific antibodies in pig serum samples from Senegal by the Morara/Georgia p30r-ELISA. Results are expressed as explained in Fig. 4. Among seropositive sera detected by OIE-approved tests, the highest value recorded was 12, 9.

and 90% (West Africa). These variations are probably due to the high genetic variability between ASFV isolates from these locations. However, these variations in sensitivity were clearly lower than those described previously using the E70 p30r protein, which provided sensitivity of 70% and 100% for samples from East and West Africa respectively (Pérez-Filgueira et al., 2006). Therefore, we can conclude that the Morara/Georgia p30r-ELISA provides acceptable specificity (97–98%) and sensitivity (90–100%) for ASF serodiagnosis in domestic pig samples from South-Eastern (Mozambique), Central (DRC) and Western (Senegal) African countries. A combination of p30r from E70 and Morara/Georgia would probably provide a universal ELISA test for ASF.

ASF diagnosis in wild species is especially relevant in Africa, where these animals are reservoirs of the virus. Thus, in this study we used the recombinant ELISA and OIE-approved tests to analyze warthog serum samples collected in Senegal ($n = 73$), the Gorongosa National Park (Mozambique) ($n = 5$), and the Kruger National Park (South Africa) ($n = 1$), and also from a Classical Swine Fever challenge trial in Mozambique ($n = 4$).

Six out of the 83 warthog samples tested were positive by OIE-approved tests and also by the Morara/Georgia p30r-ELISA, while the rest of the samples were negative by both analyses (Fig. 6). Unfortunately, due to the limited number of ASF-positive serum samples included in this panel, it was not possible to determine the characteristics of the recombinant assay for the ASF diagnosis in warthogs. However, since the six ASFV-positive serum samples were detected by the recombinant ELISA, we can expect that this assay has the capacity to provide acceptable sensitivity for warthog diagnosis.

In summary, this report describes a feasible and inexpensive serological test able to accurately detect antibodies against ASFV, independently of the geographical origin of the sera. It should be highlighted that, to the best of our best knowledge, this is the first evaluation of a recombinant ELISA test with such a high number of ASF-seropositive samples from the three main African locations in terms of virus variability (representative of South-Eastern, Central and Western regions).

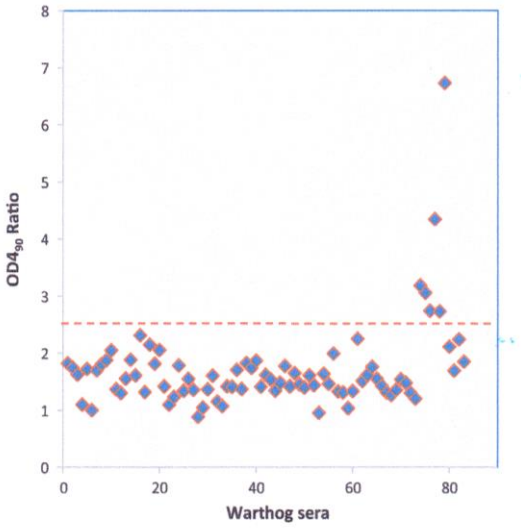

Fig. 6. Detection of ASFV-positive and -negative warthog serum samples using the Morara/Georgia p30r-ELISA. Results are expressed as ratio ODs where each dot corresponds to media of duplicate analyses of individual samples. Cut-off value is shown as dotted line.

6. Conclusions

ASF is a devastating disease caused by a large and complex virus. Given its extremely high potential for transboundary spread, escaping from Africa to Asia (Georgia, Armenia) and Europe (Russia, Ukraine), this virus is a threat to as yet unaffected African countries and other continents. In the absence of any vaccine, the control of ASF relies on rapid diagnosis and implementation of sanitary measures and domestic pigs movement restrictions. However, ASF diagnosis is complicated by the varying pathogeneses and

epidemiological scenarios, as well as by its similarity to other hemorrhagic diseases, such as Classical swine fever.

The detection of ASF-specific antibodies is indicative of previous infection, and as antibodies are produced from the first week of infection and persist for long periods they are appropriate markers for the diagnosis of the disease. Classical assays such as ELISA or IB, based on the use of crude extracts from ASFV-infected cells, are very useful for the diagnosis of subacute or inapparent forms of the disease, but present several disadvantages, mainly related to biosecurity, diagnostic interpretation and standardization concerns.

Therefore, measurement of the presence serum antibody using a recombinant protein would be a reproducible and safe alternative to conventional methods, allowing the standardization of antigen production and eliminating the need for the manipulation of infectious material. Several studies focusing on the use of recombinant ASFV proteins for serological diagnosis have shown promising results. Furthermore, these proteins have the additional advantage that they simplify the interpretation of tests, improve the reproducibility of the assays, and provide high sensitivity for poorly preserved samples.

The utility of the newly developed tests for ASF diagnosis requires validation. As reflected by the studies discussed in this review, recombinant assays have shown differences in performance depending on the origin of the samples. Only the new Morara/Georgia p30r-ELISA appears to present adequate characteristics for ASF serodiagnosis throughout Africa. Furthermore, the Morara/Georgia (Genotype II) p30r sequence corresponds to the circulating viruses in Eastern Europe (Georgia 2007/1 isolate; Gene Bank accession number: FR682468.1; Rowlands et al., 2008), thus constituting the best diagnostic option for these regions. This protein also is recognized by all positive sera previously collected in Spain (data not shown).

Finally, the data shown here demonstrate that the production of ASFV proteins, such as p30, in insect larvae (IBES®technology) is a reliable alternative to other methods, especially considering that p30r antigen can be used in diagnostic tests without any previous purification. This advantage implies a reduction of cost and antigen loss during antigen production. Worthy of note, three companies are currently commercializing ELISA tests for ASF serodiagnosis, and two of these assays (SVANOVIR® ASFV-Ab assay from Boehringer Ingelheim Svanova and ID Screen® African Swine Fever Indirect ELISA kit from ID.vet) use p30 antigen as a reagent.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.virusres.2012.10.021.

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PART II - OBJECTIVES

Chapter 4 - Objectives

The general objective of the thesis consists in the improvement of epidemiological and virological knowledge of African swine fever in the Democratic Republic of Congo.

The specific objectives are the following:

1) To confirm whether or not ASFV infection was present in domestic and wild suids within the surveyed areas;
2) To assess the endemicity and the prevalence of the disease through the serological status of domestic and wild suids pigs within the surveyed areas;
3) To genotype circulating strains of ASFV and to determine phylo-geographical links and the dynamics in order to know the source of outbreaks in DRC and
4) To suggest prevention and control strategies.

Chapter 5 - Research strategies and methods

Chapter 5 – Strategy and methodology

5. 1- Field operations:

Based on this research work objectives, as aforementioned, the choice of strategies targeted some key variables of which the surveillance type (pro-active), the ecological scenario (forest), the farming system (free-ranging, thus rural and peri-urban), the health status (apparently healthy pigs), the antibody detection for estimation of prevalence, the data and samples collection using markets (as best assembly locations; thus half participatory, half classic), the geographical position and, some key biological risk factors like age, sexe, weight, race, etc. A specific Excel file was created for storage of different collected data.

Importantly and given semantic discrepancy of epidemiological terminology, the active surveillance terminology as used for our field investigations is respective of the following criteria: temporo-spatially limited, close contact with the field and stakeholders in order to get as many and as correct data as possible, direct and real time collection of samples by ourselves as investigators for instance when pigs were being slaughtered in the markets.

5. 1. 1– Study areas selection

Six provinces out of eleven that form the Democratic Republic of Congo were covered by this study. All the samples analysed in this study were collected in Bas-Congo, Equateur, Katanga, Kinshasa, Oriental Province, Kasai Oriental and Maniema, some of these provinces that harbor the majority of pig population of the country. Five main approaches were conducted:

- prospective approach aimed on confirmation of exposure to ASF or ASF occurrence;
- several cross sectional studies aimed to estimate the prevalence of ASF were conducted;
- determination of sylvatic cycle, oriented both to identification of *Ornithodoros* ticks and detection of ASFV in wild suids samples;
- genotyping of ASFV isolates collected in this research work and finally
- prospects of protection of African indigenous pigs against ASF.

Apart from Kasai Oriental, other remaining provinces aimed in this study are linked by the Congo River which as pointed out above, seems to be the main communication way of the DRC.

In regard to the justification of sites, Kinshasa, was selected for high population of pigs, frequent alerts in the 1990s and as important platform which is receiving live pigs for trade from other provinces; Equateur for pig population, direct river link with Kinshasa, and interfaces with wild suids, Kasaï Oriental for domestic populations beside the presence of interfaces with wild suids, Bas-Congo for pig population and vicinity with Kinshasa; Katanga for high population of pigs, Province Orientale for high population and direct river link with Kinshasa.

For genetic characterisation, samples from any ASF outbreak that occurred within the country during the survey period were received. For vaccine trial study, indigenous pigs were randomly purchased in the villages of the neighbouring provinces of Bandundu and Bas-Congo.

5. 1. 2 - Samples and collection

For serology, 5 ml or at least 2 ml of whole blood sample were directly collect either in vacutainer tubes containing EDTA, or in plain tubes. For PCR, cell culture or immunohistochemistry (IHC), given ASFV tropism for lymphoid tissues, 5 gram of spleen, gastro-hepatic lymph node or other haemorrhagic tissues including, kidney, lung, liver, heart, stomach, where collected. Tissues samples were directly collected in separated and sealed plastic bags for PCR and cell culture. For IHC, tissues were placed in 50 ml bottles within 10 % of buffered formalin. All the samples were transported to laboratory at 25°C and, once in the laboratory after labelling and documented in Excel file; sera samples were separated from clots and directly placed in 1,5 ml cryotubes; for PCR or cell culture, tissues samples were preserved either in-20°C for analysis the next day, or in -90°C freezer for long-term storage. For tick's identification, Acari specimens collected on the bodies of domestic and bush pigs were directly placed in Ethanol within 50 ml plastic bottles.

5. 2-Laboratory analyses

5. 2. 1-Anti - ASFV antibodies detection

5. 2. 1. 1- Indirect ELISA using whole viral p72 cytosoluble antigen (Ag), conventionally recommended by OIE and FAO

Freeze dried viral crude Ag (0.5 ml) was rehydrated with 0.5 ml of double distilled water (ddH2O) in order to allow a titre of 1/1600, or an equivalent of 8,000 tests per vial. The working concentration of 1/125 with 9.6 Bicarbonate/Carbonate buffer. Nunc-Immunopolysorb microplates were coated with 100 µl per well over night at + 4o C, or during one hour at + 37° C. The test sera were registered on a plate layout sheet with reference numbers (labels) corresponding to microplates 'wells for both test and control sera. The washing step was achieved 4 times with washing buffer. Unspecific bindings were prevented by adding 2 % of skimmed milk or Bovine Serum Albumin (BSA) to dilution buffer of which 100 µl was added to each microplate well. Microplates were then incubated during 1 hour at 37o C and then washed 4 folds as at the first step. The sera samples to be tested and the positive and negative controls diluted at 1/30 concentration with the blocking buffer were finally added in duplicate to microplates that were directly incubated for the second time during 1 hour at 37°C. After this second step of, microplates were washed for the 3d time, 4 folds as achieved in the two first steps. Then a Protein A/G peroxidase conjugate, diluted according to manufacturer's recommendations was added to microplate wells, which allowed them to be incubated for the 3d time at 37°C, during 1 hour. The microplates were finally washed for the last time. A substrate, 200 µl of OPD (Orthophenylenediamine) was added to microplates and incubated in dark room at the ambient temperature during 10 minutes. The reaction was stopped with 100 µl of 1.25 M sulphuric acid solution added within each well. One extra microplate was prepared as blank. Microplates with both test and control sera samples, including the blank were read using a Thermo-Ascent Mutliskan EX reader. The microplates were read using 492 nm filter. HP desktop computer equipped with a printer was used to allow printing out and visualisation of ODs values, respectively. Calculation of final results was performed using a specific equation recommended by the supplier of the kit (INIA, Madrid, Spain). PBS tween or NaCl tween was used as washing buffer and all, the incubation steps for control and test sera were performed in incubator shakers at the required duration and temperature.

5. 2. 1. 2- Indirect ELISA using the recombinant p30 (rp30) as coating antigen supplied by the manufacturer (Algenex, Madrid, Spain)

This is a type of iELISA based on the same format than the one using crude p72 cytosoluble Ag, described above. The same steps are applied apart from some specific changes that needed to be respected, i.e. :

a) instead of using 1 antigen, the Algenex kit was using 2 Ags, the positive Ag (Ag +) and the negative one Ag (Ag -). The two antigens were supplied as freeze-dried reagents.

b) to coat microplates, the two Ag were rehydrated with 50mM, pH 9.6 Bicarbonate/Carbonate buffer solution, according to manufacturer's protocol. They were after diluted at concentration of 1/20 with the blocking buffer before distribution on microplates using 100 µl per well as follows: the microplate was coated with the Ag + from column 1 to 12 but, only in the wells of rows A, C, E, and H. Following the same direction, the Ag-was only used to coat the microplate's wells of rows B, D, F, and G, such that when distributing test and control sera on the microplate, the duplicate should have one copy above with Ag + and one copy below with Ag -.

c) instead of using skimmed milk in order to prevent unspecific binding of proteins, this kit is using BSA but, at the same concentration (2 %);

d) test and controls sera were diluted at 1/100;

e) as conjugate, this test was using Proteins A and G combined with horseradish peroxidase (HRP) at the concentration of 1/2000 dilution;

f) as substrate, ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)) was used;

g) to stop the reaction, SDS (Sodium Dodecyl Sulphate) was used;

h) microplates were read using 405 nm.

5. 2. 1. 3- Blocking ELISA (cELISA) using recombinant p72 antigen supplied by the manufacturer (Ingenasa, Madrid, Spain)

This blocking ELISA kit of which the format is totally different than the cp72 and rp30 described above presented a couple of innovations:

- Recombinant protein 72 was used as Ag instead of cp72;
- Pre-coated microplates are delivered along with others reagents such as controls sera, conjugate, diluent, washing buffer, enzyme's substrate and stop solutions within the same package;
- Within the conjugate, the enzyme is directly coupled with a specific monoclonal antibody (mAb), prepared against the coating Ag and

- To hydrolyse the enzyme, instead of OPD or ABTS, TMB (tetramethylbenzidine) was supplied as substrate.

The main principle of the kit, is based on the fact that the mAb coupled with the conjugate will be in competition with the test sera antibodies should they be present within the sample. This means that when the conjugate will be added to the reaction, no binding of the mAb will occur, due to anticipation of binding by the anti-ASFV antibodies present in the test sera. Thus, the lack of release of mAb will explain the lack of hydrolyse of the enzyme and then the lack of colour, and thus positive sample. Colour is an indication of hydrolysed enzyme by the substrate or the binding of mAb to coating Ag, and then negative sample.

Apart from the above strategy exploited in the kit, the main steps of the standard operating procedure (SOP) comprise:

- addition of 50 µls of dilution buffer to each well of the pre-coated microplate;
- addition of 50 µls of test sera to the same wells but, with wells A1 and B1, and A2 and B2 of the microplate reserved for positive and negative controls respectively;
- test sera are distributed following the same scheme as for the control sera;
- incubation of sealed microplate at 37 o C during 1 hour;
- washing 4 folds the microplate;
- addition of conjugate;
- incubation of microplate at 37o C during 30 minutes;
- washing 5 folds;
- addition of 100 µls of TMB to the microplate;
- incubation in dark at the room temperature during 7 minutes;
- addition of 100 µls of SDS to each well to stop the reaction and;
- Reading of microplates using Multiscan EX reader equipped with Thermo-Ascent software for calculation and storage of results.

5. 2. 1. 4- Detection of ASFV p72 antigen in formalin fixed, paraffin embedded Tissues.

The strategy used in this technique is based on different principles:

- the ASFV p72 protein is histologically detectable;
- the optimization of the conjugate by the use Avidin-Biotin-Complex labelled with horseradish peroxidase (HRP);
- the use of two antibodies: primary antibody directed to ASFV p72 antigen, and goat or rabbit anti-ASFV Ig G and,

- the exploitation of the histological technique consisting of preparation of formalin-fixed, paraffin-embedded tissues sections, rehydration of prepared sections, digestion of tissues using trypsin, dehydration of sections, dewaxing of sections with Xylene and staining of sections using eosin-hematoxylin technique for contrast, hydrolysis of HRP using DAB and finally observation of antigen-antibody complexes within the preparation in case of positive specimens if the specimens are positive.

This technique was especially used for detection of ASFV signals in wild suids tissues (Oura et al., 1998c). The hunters were supplied with buffered 10 % formalin. Detailed SOP can be briefly described as follows: specimens of target tissues (spleen, lymph nodes, kidney, tonsils or other hemorrhagic organs were immersed in buffered 10 % formalin, tissues were embedded in paraffin as per histological routine technique; tissues' sections were prepared using microtome, paraffin-embedded tissues sections were rehydrated; rehydrated tissues were submitted to trypsin, tissues sections were covered with BSA (bovine serum albumin) for quenching of unspecific bindings, at the recommended dilution, and then covered with the primary anti-ASFV p72 Ab, and covered after with the secondary Ab (goat anti-ASFV p72 Ig G), then covered with the conjugate (ABC labelled with HRP [Vector Laboratories, Ontario, Canada]), and finally covered with DAB as substrate which also counterstained haematoxylin. Immuno-complexes were then watched at the light microscope, in case of positive tissues were positive. Washing steps were carefully respected as recommended in protocols.

5. 2. 2-Cell culture

Cells of Van Furth mononuclear phacocytic system have been demonstrated as target tissues for ASFV (Pan, 1987; Carrasco et al., 1996). Porcine bone marrow macrophages (PBMM) were produced, grown, stored and infected as described by previous authors (Lubisi, et al., 2005; Pan, 1987; Malmquist and Hay, 1960). The basic strategy exploited in this method is that, infected macrophages of fixed lymphoid tissues or peripheral blood white cells attract blood red cells through haemadsorption (HAD) (Malmquist and Hay, 1960; Carrasco et al., 1996; Goatley et al., 2011). Unfortunately due to very long-term storage at -20o C, ASFV isolates could not be maintained alive in many of our clinical samples, but positive ASFV DNA was still detected by PCR. But even though, it has been already demonstrated that for tissues originating from long term infections PCR technique is the appropriated technique rather than Cell culture (Carrillo et al., 1993).

5. 2. 3-Polymerase Chain Reaction (PCR)

This was the basic and the most used tool in our research work, as ASFV DNA detection was our most purpose. Standardization of PCR as diagnostic tool for ASFV DNA detection was achieved since the 1990s (Steiger at al., 1992). Depending on availability of logistics and the number of samples to test, this method can be used for both, laboratory confirmation of ASF outbreaks, and field massive screening in order to check exposure to ASF.

In this research work, PCR was used not only for confirmation of ASF occurrences but also as field investigation tool. Positive tissues temporo-spatially collected were stored at -20° C either as supernatants or as solid organs. ASFV DNA was extracted using QIAGEN kits and amplified according to previously described protocols (Steiger et al, 1992; Agueiro et al., 2003; Bastos et al., 2003), but, given the difficulty of amplification of ASFV isolates from clinical and long term infected (Haresnape, 1985 and 1986; Wilkinson, 1989), primers with more than 50 % of GC content were preferred (Mulumba-Mfumu et al., 2013). Depending on genomic region, different oligonucleotides were used as primers for ASFV DNA amplification during our field investigations (Table 2).

Table 3 - Primers used for amplification of ASFV DNA.

Gene	Primer name	Primer sequence (5' to 3')	GC content
VP72	VP72 3 F	CTGTAACGCAGCACAGCTGAACCGTTCTG	55 %
	VP72 4 R	ATAGGATTAAAACCTACCTGGAACATCTCCG	42 %
J13l	J13L 3 F	GGTTGGTTTTCAAATGTTGGCGAAAGTAG	41 %
(p54)	J13L 4 R	CCATAAATTCTGTAATTTCATTGCGCCACAAC	38 %
CD2v	CD2v 1 F	GGGTTAACAATACGGTAATATTAC GTGGTG	43 %
	CD2v 2 R	GATTCAGAGGGTGGTGGAATTTCTATTTC	41 %
EP	ep152 F 5'	CCAAAGCAGTTTAACTTTCTGTTAGACAGCGCAGAAGG	42 %
	ep364R 5'2	CATTGGATTATGTTGCATATCATGAAAATCGG	34 %
9RL	9rl (F)	AAT GCG CTC AGG ATC TGT TAA ATC GG,	46 %
	9rl (R)	TCT TCA TGC TCA AAG TGC GTA TAC CT,	42 %

The amplification profile was done in 30 cycles as follow: step 1, denaturation: 96°C, 4 minutes; step 2, denaturation: 94°C, 1 minute, Annealing: 57°C, 15 seconds, Elongation: 72°C, 15 seconds and repetition of step 2, 29 times and, PCR products were cooled at + 4°C. As Taq polymerase, Eppendorf Mastermix 2.5 x as Taq master was used for amplification. Details for mastermixing of reagents, separation, sizing and visualisation of DNA bands are those described in chapters 6 and 8.

5. 2. 4- Molecular characterisation

Targeted amplicons excised from the Agarose gel were purified using Wizard SV Gel and PCR Clean Up System Kit using Promega Corporation Quick protocol, part # 9FB072 revised 11/09, supplied by Promega Corporation, USA. Purified amplicons were sent to LGC Genomics in Berlin, who provided us with sequences. Sequences edition, trimming and assembly were achieved using Edit software, sequences alignment and phylogenetic trees were constructed using Neighbour Joining (NJ), using MEGA package, as described by previous researchers (Boshoff et al., 2007). SOP details are provided in the relevant article.

5. 3- Vaccine trial

The trial was aimed on African indigenous pigs. Experimental pigs were purchased in the villages of the country side, in Bandundu and Bas-Congo provinces. Before purchasing these experimental indigenous pigs, two tests were performed: the first is a rapid test using a chromogenic rapid kit supplied by Ingenasa (Madrid, Spain) and the second test is a blocking ELISA performed with reagents supplied by the same manufacturer. This second test was performed before the transfer of animals to the quarantine. Two weeks minimum were the duration of animals in the quarantine before introduction in the vivarium for vaccination and challenges. For vaccination of experimental animals, a naturally attenuated ASFV strain isolated from *Ornithodoros* tick was used.

All the 3 main steps: vaccination, first and second challenge were separated each one from another by 21 days. After each step, at day 3, 6, 14, and 21 post-inoculation, clinical parameters including chiefly body temperature, skin hemorrhage, joints ‘swilling, food intake jaundice, or other typical signs were recorded. At the same time, blood samples were collected. At the end of the trial, all the blood samples collected during the trial and tissues samples collected were tested using a quantitative PCR (qPCR) and a blocking ELISA for

quantification of ASFV DNA copies and determination of anti-ASFV antibodies, respectively. These tests concern all animals including survivors (Katherine et al., 2011). The specific details dealing with this trial are developed in the relevant section in the chapter 11.

5. 4- Statistical tools

For assessment of field screening serological tests, some statistical tools comprising calculation of confidence interval for the sensitivity and the specificity, kappa coefficient, Chi-square test, Receiver Operating Characteristic (ROC) curve, Kernel density and *P*-value. were used. Specific details dealing with this trial are presented in the chapter 7.

PART III - EXPERIMENTAL WORK

Chapter 6: Molecular characterisation of African Swine Fever Virus isolates involved in infection persistence in Central Africa: Democratic Republic of Congo

Chapter 6: Molecular characterisation of African swine fever virus isolates involved in infection persistence in Central Africa: Democratic Republic of Congo.

6.1. – Preamble

For confirmation of ASF occurrence, and for effective implementation of control strategies, and, due to presence of other hemorrhagic diseases of pigs in the region, the laboratory analyses were very important for accurate diagnosis (Sánchez-Vizcaino, 2012). For that parallel detection of ASFV antigen and antibodies was crucial (Sanchez-Vizcaino, 2006).

6.2. – The following section of this research work is devoted to ASF exposure confirmation.

ARTICLE 3:

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Molecular characterization of African swine fever Virus isolates involved in infection persistence in Central Africa: Democratic Republic of Congo

Caractéristiques moléculaires du virus de la peste porcine africaine en Afrique centrale :
cas de la République Démocratique du Congo

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Résumé

Contexte : La Peste Porcine Africaine (PPA) est un réel frein à l'allègement de la pauvreté et à la sécurité alimentaire des populations les plus démunies en milieu rural africain. Le porc domestique y représente, non seulement une source d'épargne ambulante pour les frais médicaux, scolaires et diverses taxes, mais aussi une source précieuse de protéine animale. Cette affection est endémique en Afrique Sub-saharienne, notamment en République Démocratique du Congo.
Objectif : Genotyper, à travers une étude rétrospective, une des souches les plus dévastatrices, et établir des liens phylogéographiques pour une meilleure compréhension de l'épidémiologie en vue d'une stratégie de contrôle adaptée.
Matériel et méthodes : prélèvements (sang et organes) sur les porcs domestiques des sites les plus touchés, évaluation de la prévalence apparente, détection de l'ADN viral et caractérisation respectivement par les tests d'ELISA indirect, PCR et Séquencage appuyés par la culture cellulaire et l'immunohistochimie.
Résultats : Les Anticorps Anti –VPPA ont été détectés chez 73 porcs sur 350 (20.8%), l'ADN du virus a été détecté dans les échantillons provenant de 5 fermes, la séquence du gène d'immunodominance d'une souche impliquée dans le foyer le plus sévère a été décryptée et, la comparaison de ces dernières avec les isolats d'autres zones géographiques a permis d'établir une similarité génétique.
Conclusion: Les souches identifiées en RDC montrent une homologie avec celles isolées en Afrique de l'ouest.

Mots clé: Virus PPA, caractérisation moléculaire, homologie génétique, RDC, Afrique de l'ouest

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Summary

Background: African swine fever (ASF) is a major threat to poverty alleviation and to food security especially for vulnerable rural populations in Africa. For these communities, domestic pig is not only a cash provider for medical bills, school fees, and taxes, but also an affordable source of animal proteins. ASF is endemic in Sub-Saharan Africa, chiefly in the Democratic Republic of Congo (DRC).
Objective: Genotyping of circulating strains involved in the most severe outbreak in order to come up with phylogeographical link in terms of origin, for a better understanding of epidemiological pattern and then, for choice of a proper control strategy.
Material and methods: Blood and tissues samples collected on pigs from challenged locations; empiric assessment of apparent prevalence; ASF virus (ASFV) DNA detection, and genotyping using respectively indirect ELISA, PCR and Sequencing techniques supported by confirmatory diagnostics such as cell cultures and immuno-histochemistry.
Results : Anti-ASFV antibodies were detected in 73 animals out of 350 (20.8%), ASFV DNA was detected in specimens originating from 5 locations, genomic sequence was depicted from a severe outbreak isolate and different isolates sequences alignment allowed genetic relatedness establishment.
Conclusion: Genetic relationship was established between DRC and West Africa'isolates.

Key words: African swine fever virus, genotyping, genomic similarity, DRC, West Africa

Introduction

African swine fever (ASF) is a complex and devastating disease of domestic swine, occurring in forms ranging from peracute to acute, subacute, chronic and inapparent; with mortality rate ranging from 100% to as little as 3% (1). The most specific signs during outbreaks are pyrexia (40.5-42.2°C), hyperaemia of the skin, abortions, oedema, and haemorrhage in many internal organs, especially lymph nodes (2).

ASF symptoms may also vary geographically (3). It has been established that all the field strains do not behave in a similar manner (4) and, depending on the hosts, can produce diseases with different clinical pictures (5). Clinically, ASF is similar to other heamorrhagic diseases of swine (6, 7), so-called ASF-like diseases, in Central Africa: Erysipelas, Pasterollosis and Salmonellosis; laboratory tests are required to specify a correct diagnosis (6, 8). The causative agent of ASF is an enveloped, icosahedral, cytoplasmic, deoxyvirus with a double-stranded DNA genome of 170 to 190 kbp (6, 9, 10, 12). At the ends of this genome, the two strands are linked by hairpin loops incompletely base-paired, with terminal inverted repeat tandems (3, 9, 11). The two extreme and variable regions of the virus genome flank a central region of 125 kbp, which is conserved in length (9, 11, 12). Morphologically ASFV is similar to the Iridoviridae family in which it was classified, but as only some *molecular* similarities were established with the Poxviridae family, it was recently removed from this family (9, 10, 11, 12), and placed in the Asfarviridae family, where it is the only member (12, 14). It has not been possible to produce a conventional vaccine (3, 9, 11) against ASF. This disease was described for the first time in 1921, by Montgomery in overseas pigs introduced for the first time in Kenya (9, 14) since 1910, but prior to this time, similar outbreaks were observed in South Africa (15, 16). ASF escaped from Africa in 1957, and spread to Europe through Iberian Peninsula; notably to Portugal (2, 15), very probably from Angola (9, 19). Since then, several epizootics causing heavy losses in domestic pig's populations were claimed elsewhere in the world, chiefly in the Caribbean, and Latin America (2, 7-15, 17-19). Apart from Sardinia, the disease was eradicated in all these outbreaks by compulsory slaughter (9, 12, 19) as well as restriction of pigs and pork products movement (2, 7). Until the period of time concerned by this retrospective study, ASF was confined to sub-Saharan Africa (6, 19), its enzootic area (9, 15, 19), and Sardinia. ASF is a threat to the world pig industry (2, 7-9, 12, 15, 17-19), and a major concern for all swine-producing countries (15). In Africa, ASF is a great constraint to the rural economy, and the rural poor populations are therefore the main victims as in villages domestic pigs form valuable source of income (19). In order to allow a proper control of the disease, some paths have been recommended, i.e. -further global epidemiological studies including characterisation of isolates circulating (2) and, better knowledge of virus genes involved in infections (18). Little is known about ASF in Central Africa. This region has never been systematically surveyed, and most of the field staff is not of veterinary profession. Consequently, ASF occurrences are confused with other heamorrhagic diseases of swine and, the socioeconomic impact is still underestimated (8). Furthermore, most of ASFV field isolates from Africa, used in a broad range of studies, are all from Eastern and Southern Africa (13). The 2 or 3 DRC ASFV strains, which have been studied, are all from Katanga; the DRC area very linked on the Southern Africa in terms of livestock business. The purpose of this study was to monitor the occurrence of ASF in DRC and to characterise the strains circulating, to establish their relationship with other African and European isolates. In order to get more realistic data of novel isolates introduction within the surveyed area, temporally, the period of time marked by severe outbreaks was selected, which makes this study very retrospective. As a matter of fact, high apparent prevalence was observed; which is an indication of the disease endemicity. Interestingly, a genetic similarity was established between ASFV strains circulating in DRC and in the Western Africa.

Material and methods

Samples collection

Pig's specimens used in analyses were both randomly collected in 1997 and in 2001, the years during which unidentified outbreaks with higher mortality rate, about 100%, were recorded in pig farms within the study area, incriminating new epizootics. The study was conducted in two parts. The first part was more explorative and was carried out most

randomly, focusing on provincial origins of pigs.

Samples and locations

For the explorative step, 534 plasma specimens from 534 pigs of which *469* were European breeds of pigs and *65* local-breed (indigenous) pigs were tested using enzyme linked immunosorbent assay (ELISA), in order to trace areas with sero-positive pigs. From the same pool of pigs, organs from *72* animals were tested using Porcine Bone Marrow cells culture (BMCC) in order to try the virus isolation. 40 congestivo-haemorrhagic tissues of 40 pigs from the same pool were tested by Polymerase Chain Reaction (PCR) for viral deoxynucleic acid (DNA) detection. All the tested pigs originated from 4 provinces (Figure 1) within the western part of the country, they were all ASF symptomless. The second part of the study targeted the same two types of breeds. 350 pigs were tested. Apart from 47 indigenous, 303 pigs originated from commercial farms, selected on the basis of current or past history of mortality associated with haemorrhagic syndromes, PCR was the main test used to detect ASFV.

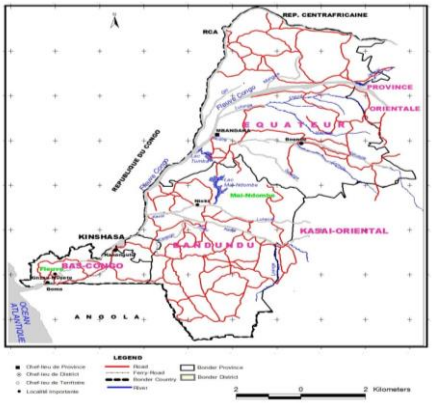

Figure 1. Study area including the target locations

In this grouping, DNAs were extracted from blood clots of 350 pigs, and from solid tissues including lymph nodes, spleens, kidneys, lungs, and livers of 268 pigs. The 350 plasma specimens were also screened by ELISA in order to obtain more structured information about disease prevalence. From the solid tissues, 20 blocks of formalin fixed - paraffin embedded tissues from 5 carcasses with critical post-mortem gross changes were prepared for confirmatory diagnostic by Immunohistochemistry (IHC). VP72 gene fragments from extracted DNAs were sequenced and compared with other isolates. The molecular epidemiology survey covered 10 locations of which 9 were already suspected to have ASF and the majority of the tested pigs originated from peri-urban areas (Figure 2).

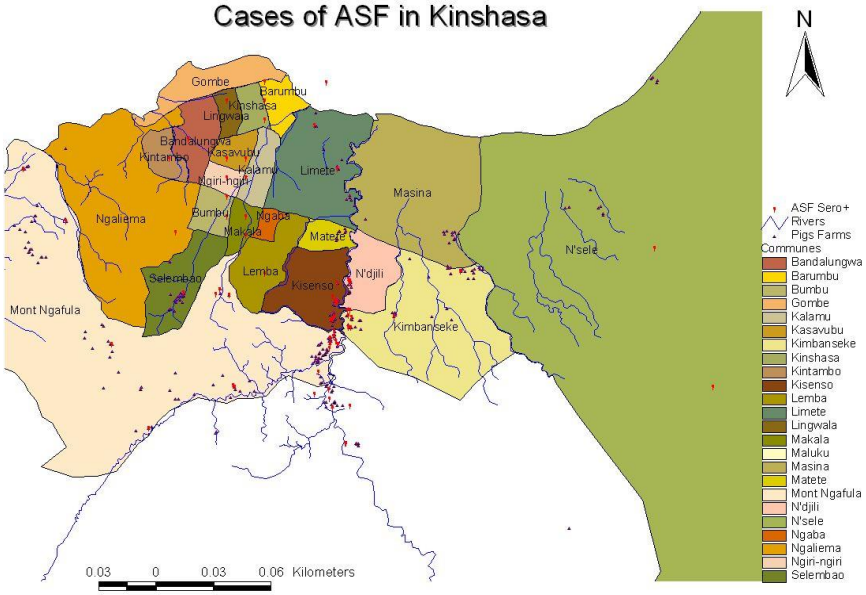

Figure 2. Urban and peri-urban surveyed area involved in 2001 severe outbreak

DNAs extraction

DNAs were purified using QIAGEN protocol as described in their September 2001 handbook, for blood and body fluids. Organs were initially prepared in order to provide supernatants. 2 grs of the tissue was ground with sand, suspended in PBS and the supernatant collected after a 10 minutes centrifugation at 1500 rpm. Liquid samples were then used to provide DNAs after lysis by Protease K, maximisation by Buffer AL, Ethanol and detergent, ionic capture by QIAamp column, and elution with distilled water.

Primers selection

Three ASFV genomic regions were specifically targeted and primers were selected accordingly. Concerned regions were the highly conserved gene encoding the viral protein 72 (VP72) and variable genes encoding the viral protein 54 (j13L) and the CD2 like protein (Figure 3).

	Primer name	*Primer sequence (5' to 3')*	***Init. Denat** Temp (°C)*	*Ann. Temp (°C)*	*Taq Poly*	*Frag. size*	*+ve location*
VP72	*VP72 3 F*	CTGTAACGCAGCACAGCTGAACCGTTCTG			*Pcia*		5/5
	VP72 4 R	ATAGGATTAAAACCTACCTGGAACATCTCCG	94	55	*Mast*	500 bp	100%
j13L	*J13L 3 F*	GGTTGGTTTTCAAATGTTGGCGAAAGTAG		55	*Pcia*		3/5
	J13L 4 R	CCATAAATTCTGTAATTTCATTGCGCCACAAC	94	50	*Trip*	700 bp	60%
CD2	*CD2v 1 F*	GGGTTAACAATACGGTAATATTAC GTGGTG					0/5
	CD2v 2 R	GATTCAGAGGGTGGTGGAATTTCTATTTC	94	50	Pcia	500 p*	0%
EP	*ep152 F 5'*	CCAAAGCAGTTTAACTTTCTGTTAGACAGCGCAGAAGG			*Trip*		2/5
	ep364R 5'2	CATTGGATTATGTTGCATATCATGAAAATCGG	94	50	*Mast*	1700 p	40%

Figure 3. Primers (oligonucleotids used for this study)

Primers were supplied by Life Technologies, namely: -VP72 $_3$ (f) CTG TAA CGC AGC ACA GCT GAA CCG TTC TG (*50.9 nmol/ml*) and VP2 $_4$ (r) ATA GGA TTA AAA CCT ACC TGG AAC ATC TCC G (*50.9 nmol/ml*) ; j13L $_3$ (f) GGT TGG TTT TCA AAT GTT GGC GAA AGT AG (*54.8 nmol*)and j13L $_4$ (r) CCA TAA ATT CTG TAA TTT CAT TGC GCC ACA AC (*51.1 nmol/ml*); CD2v $_1$ (f) GGG TTA ACA ATA CGG TAA TAT GTG GTG (*61.3 nmol/ml*) and CD2v $_2$ (r) GAT TCA GAG GGT GGA CTT TCT ATT TC (55.*4 nmol/ml*). The genomic region encoding CD2 was not easy to recover such that a set of primers dealing with its flanking regions was used, i.e. ep152 (*f*)$_{5'}$ CCA AAG CAG TTT AAC TTT CTG TTA GAC AGC GCA GAA GG (30.1 *nmol/ml*) and ep364 *(r)* $_{5'2}$ CAT TGG ATT ATG TTG CAT ATC ATG AAA ATC GG (*31.6 nmol/ml*).

Polymerase chain reaction (PCR)

For DNAs manipulations, standard methods as described by Sambrook et *al*. (1989) were referred to. *PCRs reactions were carried out in 25µl volume* in which 0.5µl of each primer, 10µl of template DNA (empirically suggested), 10µl (1 unit) of Taq DNA polymerase, and 4µl of double distilled water. For positive control, the *25µl* were composed of 0.5µl of each primer, 1µl of positive control DNA, 13µl of double distilled water and 10µl of Taq DNA polymerase and; for negative control, 0.5µl of each primer, 14µl of double distilled water and 10µl of Taq DNA polymerase. The MJ Research Inc. programmable thermal cycler was used with the following series of cycles: *cycle 1*, 94˚C for 5 min, 50-55˚C for 1 min, 72˚C for 2 min; *cycles 2 to 29*: 94˚C for 1 min, 50-55˚C for 1 min, 72˚C for 2 min; *cycle 30*: 94˚C for 1 min, 50-55˚C for 1 min, and 72˚C for 10 min. Owing to the reagents shortage, 3 types of Taq DNA polymerase were used, i.e. Ready.To. Go PCR Beads (Amersham Pharmacia), Eppendorf Mastermix (2.5×) and Eppendorf Triple Master (for the CD2 like protein gene). *PCR* products were separated and sized by *Electrophoresis* against a 1Kb DNA Ladder scale, in 0.5 x Tris acetate Edta (TAE) buffer including 0.6% Agarose gel and

Ethidium Bromide. The viral DNAs visualization was permitted by a BIO-RAD computer facility. Prior to proceed to the sequencing and relationship steps, confirmatory diagnoses were performed.

Enzyme-linked immunosorbent assay (ELISA)

As antigens, two types of VP72 were used: crude VP72 (21, 22) for the explorative step and, cytosoluble (21, 23) in the second step. Apart from the antigens formats, other steps of this test were similar, i.e. Raifort peroxydase as enzyme; Ortho-phenylene diamine (OPD) as substrate; precoated immuno-microplates at 4^{o} C overnight; required buffers for dilutions, washings, and non specific antibodies blocking; 37˚C shaking incubation; sulphuric acid for reaction stopping; and automatic reader for optical density (OD) measurement.

Immunohistochemistry (IHC)

According to previous studies, the ASFV immunodominant protein (p72) (3, 14, 24) is histologically significant (25). Some paraffin-embedded organs from a PCR positive pig, were tested by IHC for ASF confirmation using antibodies against VP72 as DNAs from the same tissues were selected for sequencing and relationship steps. Spleen, and kidney sections were dewaxed in Xylene, rehydrated in decreasing degrees of alcohol, pre-treated with 0.5 Trypsin (Sigma UK) and allowed to incubate during 15 min at 37°C. Antibodies nonspecific bindings were blocked with Horse serum (1% in PBS) and, using ImmunoPure Peroxydase Suppressor (Pierce, UK) tissues endogenous Peroxydase was quenched. ASFV antigen sites were detected by the use of a Primary antibody that bound to a Secondary one (anti-mouse), biotinylated, 1% dilution in normal swine serum, and the mixture was localised thanks to Avidin-Biotin Complex (Vectastain ABC Kit). The other steps such as PBS washing and incubations in humidified chambers were respected according to manufacturer's SOP. The revelation of reaction was enabled by 3,3 diaminobenzidine tetrahydrochloride (DAB) (Vector UK), the substrate which hydrolysed the enzyme, Peroxydase and, finally ASFV Ag sites were smartly overlooked as dark brown sites in tissues sections, after counterstaining with Hematoxylin.

Sequencing and sequences comparison

DNA bands were cut out from agarose gel under the UV light. Purification procedures were completed according to QIAGEN kits instructions. Plasmid *pGEMT* was selected as vector and, associated steps such as insertion and ligation, transformation and analysis of plasmid DNA were carried out by standard procedures as described by Sambrook et *al*. (1989). Nucleotide sequences were determined according to Promega Corporation protocol (revised 4 / 00) / Part # TMO24 / page 8 and, analysed automatically using the Genetic Computer Group (GCG) programme. DNAs for the VP72 gene, detected in lymphoid tissues of a pig which died in a commercial farm ASF outbreak, as revealed at PCR and confirmed by IHC, was selected for these operations.

Results

Explorative survey

Explorative tests using PCR and ELISA were carried out on a random basis, using 534 plasmas, and 72 tissues as samples from 534 clinically healthy pigs, originating from 4 provinces supposed to be ASF-free. This revealed that these 4 provinces were already challenged by ASFV (Table 1). According to these tests, 6 pigs out of 534 were positive, of which 5 were identified by ELISA: 3 from Equator Province, 1 from Bas - Congo and 1 from Bandundu Provinces; and 1 by PCR from Kinshasa. With respect to breeds as well as locations, the 5 ELISA positive pigs were all of indigenous origin, free ranging, and were all sampled in the market, where healthy pigs are brought from the country side for trade. The only PCR positive pig was a Large White pig, and viral DNA was detected from all of its tested tissues (kidney, spleen, and lymph node). The ODs values of the ELISA positive pigs, i.e. the free ranging pigs were higher than those of the positive controls used in the assay.

Table 1. Explorative study data. The BMCC analysis was negative for all the tested tissues given the high level of degradation

Nº	Year	Location	Breed	Sample	Positive Samples / Tested Samples				Specific Details			
					ELISA	BMCC	PCR	IMP	+ ve/Age	+ ve Organs	StorageTemp.	+ ve Provinces
1	1997	Cataractes ·	European	Plasma	0 / 445	-	-	-	-	-	-25º C	-
2	1997	Mket Kbu ·	Local	Plasma	5 / 65	-	-	-	Ad.	-	-25º C	Eq, Bc, Bdd
				Organs	-	0 / 72	-	-	-	-	-25º C	-
3	1997	Boly ·	European	Organs	-	0 / 6	3 / 3	-	Yg.	Lg,Kn,Ln	-25º C	Kin.
4	1997	Kalenga ·	European	Plasma	0 / 13	-	-	-	-	-	-25º C	-
5	1997	Monastere ·	European	Plasma	0 / 5	-	-	-	-	-	-25º C	-
6	1997	Mona ·	European	Plasma	0 / 6	-	-	-	-	-	-25º C	-
				Organs	-	0 / 5	0 / 2	-	-	-	-25º C	-
OVERALL SITUATION					**5 / 534**	**0 / 83**	**3 / 5**	-	**Ad., Yg.**	Lg,Kn,ln	**-25º C**	**Eq, Bc, Bdd, Kin.**

Molecular analsysis

Five (5) locations out of the 10 surveyed using the samples including organs, blood clots, EDTA blood and serums were positive at PCR. These samples were collected on 268 pigs originating from two neighbour provinces Kinshasa and Bas-Congo (Table 2). In regard to characterisation (Figure 4), VP72 primers allowed the detection of virus DNA in all the positive samples, i.e. from all the 5 positive locations (Figure 4a). Primers of the gene encoding virus protein 54 (j13L) amplified also ASFV DNA, but only in samples from 4 locations out of the 5 positive (Figure 4c). The CD_2 like protein encoding gene primers failed to reveal virus DNA, but the CD_2 positive control DNA used in the same reaction was amplified. Primers from genomic regions flanking the CD_2-gene amplified the relevant regions and the size of the detected regions was 1700 bp (Figure 4b).

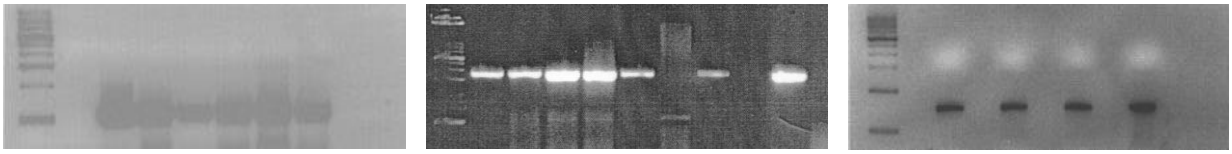

Figure 4. PCR results in relation with genes' primers and locations
a) VP72 gene amplicons: 500 bp *b)* CD_2 gene amplicons: 1700 bp *c)* j13L gene amplicons: 700 bp

The sizes of the two former genomic regions i.e. the VP72 and VP54 (j13L) genes were respectively 500 bp and 700 bp. It is important to point out that for these results; the primers used were 30 nucleotides long and with 50% of GC rate. The partial sequence of the VP72 gene of the selected field isolate was determined (Figure 5) and sequences comparison with other published using the GCG package revealed close similarities with isolates circulating in West Africa, i.e. from Nigeria and Ghana (Figure 6). With respect to the clinical picture, ASFV DNA was detected from a spleen of a healthy indigenous sow, a free-ranging pig, from the countryside, sampled in the market. This specimen was positive with VP72 and j13L primers.

TTATAGGATTAAAACCTACCTGGAACATCTCCGATCAAAATCCTCATCAGCA (52)
CCGAGATTGGCACAAGTTCGGACATGTTGTTAACGCCATTATGCAGCCTACT (104)
CACCACGCAGAGATAAGCTTTCAGGATAGAGATACAGCTCTTCCAGACGCAT (156)
GTTCATCTATATCGGATATTAGCCCCGTTACGTATCCGATCACATTACCTAT (208)
TATTAAAAATATTTCCGTAACTGCTCATGGTATCAATCTTATCGATAAGTTT (260)
CCATCAAAGTTCTGCAGCTCTTACATACCCTTCCACTACGGAGGCAATGCAA (312)
TTAAAACCCCCGATGATCCGGGTGCGATGATGATTACCTTTGCTTTGAAGCC (364)
ACGGGAGGAATACCAACCCAGTGGTCATATTAACGTATCCAGAGCAAGAGAA (416)
TTTTATATTAGTTGGGACACGGATTACGTGGGGTCTATCACTACGGCTGATC (468)
TTGTGGTATCGGCATCTGCTATTAACTTTCTTCTTCTTCAGAACGGTTCAGC (520)
TGTGCTGCGTTACAG (535)

Figure 5. ASFV isolate: VP72 gene sequence (5'-3'), 2001 outbreak / Kinshasa

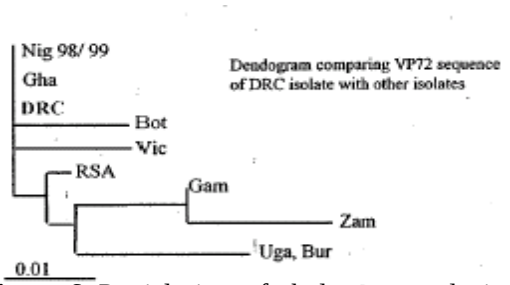

Figure 6. Partial view of phylogramme depicting genetic relationship between ASFV isolates including Kinshasa 2001 outbreak' strain indicating that West African and DRC isolates belong to the same genotype

ASFV DNA was also detected in gastro-hepatic lymph node of a young pig with only growth delay and exudative epidermitis as apparent clinical signs. This pig was from a commercial farm. From the same farm which has never been depopulated after the outbreak, ASFV DNA was also detected from lung and gastro-hepatic specimens of a freshly died sow with severe haemorrhage in internal organs. Lymphoid tissues, kidneys, and liver were the best infectious tissues. Only 1 blood sample enabled viral DNA detection, and only with j13L (p 54) primers.

ELISA (Enzyme linked immunosorbent assay)

ELISA tests to detect sero-positive pigs were carried out on samples from the same 10 locations as for the molecular epidemiology survey. ASFV antibodies were detected in the sera of pigs from 8 locations out of the 10 tested (Table 2).

Table 2. Overall results with respect to origins, nature and amount of tested samples

			Breed	**Sample**	**Positive Samples / Tested Samples**							
Nº	Year	Location							Specific Details			
					PCR	**ELISA**	**IMP**	**H.- PATH**	**+ve Age**	**+ ve Organs**	**Storage Temp. ***	**+ ve Provinces**
1	2000	MaTombe ✖	European	Organs	**9 / 12**	-	2 / 3	3 / 3	Ad	Kn,Lg,Spl	-25º C	Kin.-kinsh.
				Plasma	-	28 / 48	-	-	Ad	Kn,Lg,Spl Ln:gh+Pcr	-25º C	Kin.-kinsh.
2	2000	Banga ♦	European	Bl.clots	0 / 4	-	-	-	Ad	-		Kin.-mgfl
				Plasma	0 / 4	4 / 4	-	-	-	-	-25º C	-
3	2000	Mona ♦	European	Organs	**9 / 14**	-	-	-	Ad,Yg	-	-25º C	Kin-ndjili
				Bl.clots	0 / 19	-	-	-	Ad,Yg	Kn,Lg,Spl .gh Ln, Liv	-25º C	"
				Plasma	-	18 / 21	-	-	-	-	-25º C	-
4	2000	Mongul ·	European	Bl.clots	0 / 10	-	-	-	-	-	-25º C	-
				Plasma	-	0 / 17	-	-	-	-	-25º C	-
5	2000	Musube ♦	European	Bl.clots	0 / 7	-	-	-	Ad,Yg	-	-25º C	Kin.-kibwa
				Plasma	-	10 / 10	-	-	-	-	-25º C	-
6	2000	Kibuila ·	Local	Bl.clots	0 / 15	-	-	-	Ad	-	-25º C	Bas-Congo
				Plasma	-	1 / 21	-	-	-	-	-25º C	-
7	2000	Mket-Mte ·	Local	Organs	**1 / 101**	-	-	-	Ad,Yg	-	-25º C	Bas C, Kin:kbwa Lba,mgfl,Mte,sle
				Edta Bl	0 / 45	-	-	-	-	-	-25º C	-
				Plasma	-	7 / 47	-	-	Ad	Spl.	-25º C	Bas-Congo
8	2000	Banza-Lba ♦	European	Edta Bl	**1 / 6**	-	-	-	Ad	-	-25º C	Kin.-lba
				Plasma	-	1 / 5	-	-	Ad	Blood	-25º C	Kin.-lba
9	2000	Cataractes ♦	European	Organs	0 / 6	-	-	-	Ad,Yg	-	-25º C	Bas-Congo
				Plasma	-	4 / 177	-	-	-	-	-25º C	-
				Edta Bl	0 / 22							
10	2000	Kasavubu ✖	European	Organs	**3 / 3**	-	1 / 3	-	Ad	Kn,Spl,Ln	-25º C	Kin.-kbu
OVERALL SITUATION					**23/268 (5 farms)**	**73/350**	**3/6**	**3/3**	Ad, Yg	**Ln,Bl,Liv Kn,Lg,Spl**	**-25º C***	**Bas-Congo,Kinshasa**

Legend: Bl, blood; Ln, lymph node; Lg, lung; Liv, liver; Kn, kidney; Spl, spleen; gh, gastro-hepatic; pcr, precrural; Ad, adults; Yg, young; ✖-mortality cases associated with haemorrhagic syndrome during the sampling period; ♦ -mortalities in relation with haemorrhagic syndrome according to ancient history; ·-samples collected randomly, as from healthy animals, * -samples held accidently 9 days without refrigeration.
Bone Marrow Cells Culture never worked due to organs temperature-based degradation

Four PCR positive locations were included within the 8 ELISA positive. 2 of the 8 positive locations, were the markets where indigenous pigs, clinically healthy were supplied for commercial purpose. 1 of these 2 markets was also positive at PCR with the

same animal. 6 of the 8 positive locations were commercial farms, suspected for ASF, due to haemorrhagic syndrome occurrences, which were furthermore confirmed as ASF outbreaks according to PCR results (Table 2). For all the tested *serums*, the best positive OD values were these observed in the market-pigs, i.e. indigenous or free ranging with high antibodies titers. Within each positive location, the rate of positive cases were of 100% in 2 locations and successively 86%, 58%, 20%, 15%, 5% and 2% in the following locations (Figure 7).

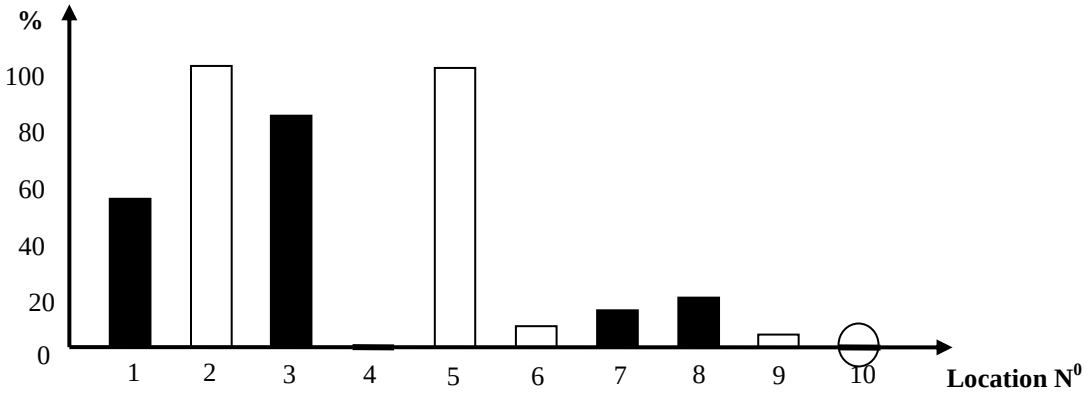

Figure 7. ASF: apparent prevalence estimate per location within the surveyed area
■ Locations with positive results at PCR and ELISA tests
▢ Locations with positive results only at the ELISA tests
O Locations with positive results at PCR tests
▪ Locations with negative results

The ASF positiveness for the two symptomless herds i.e. the 2 market-pigs populations, ranges from 5 to 15%. Concerning the overall number of the tested pigs, 73 animals out of 350 were positive, revealing 21% of of presumable apparent prevalence (Table 2). When both PCR and ELISA tests results are considered, 9 locations out of the 10, i.e 90% of tested locations were affected.

Immunohistochemistry (IHC)
ASFV protein 72 was localised in kidney, and lymph node and tonsils tissues sections from the selected carcass (Figure 8). With this investigation PCR result for the same pig was confirmed; the ELISA test for the same location was also positive. It is also interesting to point out that routinely microscopic changes in the same lymphoid tissues were very suggestive for ASF as lymphocytes karyorrhexis and depletion were very visible.

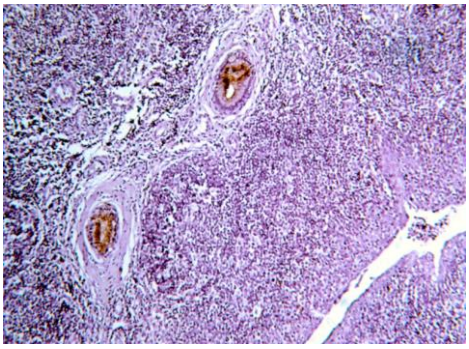

Figure 8. Pig tonsil with immunoperoxydase (IMP) staining positive to VP72 for both blood and lymphatic vessels

Discussion

The above described results confirm the existence of ASF at least within the surveyed regions. Advanced laboratory techniques as well as a broad range of specimens have been exploited and some specific circumstances under which this partial study has been carried out should be pointed out; 3 of provinces from which samples were collected contained two main forest parks. When scavenging for food (26), free ranging pigs within the villages neighbouring the forests may get in contact with wild suids. According

to final investigations, i.e. *4* positive provinces out of the *4* tested, and *9* positive locations out of the *10* tested, and according to criteria used by previous workers (27), it should be concluded that ASF is endemic within the surveyed areas. The study area is entirely located within the Sub-Saharan Africa, which is globally recognised as the ASF most endemic region (18). ELISA tests for the two study steps revealed that the best positive cases, i.e. serums with the highest antibodies titers (highest OD values) were observed in the free ranging pigs group, suggesting that these animals had been in contact with ASF virus possibly for long exposure (14). In the second step of the survey, 1 pig out of the 47 healthy pigs tested, was positive at PCR (with VP72 and j13L primers). Such investigation provides an indication of ASFV maintenance (28) in healthy pigs within this endemic region. ELISA test for this same batch of pigs, revealed 7 positive pigs out the 47 tested, and the PCR positive pig was also positive at this test. This double positive pig condition recalls two possible situations, either an outbreak survivor or subclinical ASF status but in both conditions, the carrier status is established *(*15, 29). According to some authors, the subclinical ASF is not frequent among African domestic pigs (29). Six (*6*) other pigs, i.e. ELISA positive and PCR negative, could be considered as temporally protected as ASFV does not induce neutralising antibodies (3, 9, 11). A breed-based resistance could also be suggested especially in enzootic regions were pigs have been in contact with ASFV for many generations and, according to Gusmao Vasco (1984) experiments; it was also demonstrated that some resistant pigs are not carriers (6). Haemorrhagic syndrome occurrences, the most suspected criterion in many surveyed commercial farms could be finally confirmed as a suggestive indication of ASF occurrence even in post-mortem exploration. The persistence of such syndrome is also evidence that within the study area ASF was really a long term infection, even in well fenced commercial farms. The reduction of the classic clinical signs of ASF in ASF endemic areas, owing chiefly to the decrease of isolates virulence has been reported by many workers (15, 19, 27). ASFV DNA was detected from a spleen of a young pig showing only growth delay and exudative epidermitis as symptoms. Apart from the growth delay (19), exudative epidermitis might as well be associated to skin lesions suggesting ASF in long term infection. The same commercial farm lost about 700 pigs out of 1500 in relation with haemorrhagic disease history 8 months before the current sampling; since this occurrence, no depopulation was applied. It may be concluded that chronic ASF ending in low, but constant mortality rate was superficially created in this farm within the remaining survivors. This type of condition has been already described as source of endemicity by the past (19, 30). From the same piggery, ASFV DNA was detected from a sow which died with severe heamorrhagic lesions in lung and spleen, suggesting acute form of ASF. Reactivation of ASF mild infection with low virulence strains because of stress has been experimentally also produced (19), and deserves to be considered as source of disease recrudescence (30). In terms of virus tropism, beside lymphoid tissues (31), ASFV DNA was easily detected from kidneys, lungs, and livers tissues. Apart from one sample tested with j13L primers, ASFV DNA was not easily detected from blood specimens. Furthermore, the unique positive blood sample showed two bands on the gel, indicating two sizes which were repeated after a couple of tests. This might be the effect of interference between haemoglobin and Taq DNA polymerase already reported by some workers (32). Plasmas samples used for PCR were all negative despite positive ELISA results for the same animals. This might be due to the fact that within the circulating blood, virus particles are more attached to erythrocytes membranes (12) such that after decantation manipulations, blood plasma become poor and may be free of viral material (32). Epidemiologically, the 3 indicated genes were investigated at least in 2 locations of our surveyed area, suggesting that relevant proteins, i.e. *p72*, *p54* (*j13L*), and the CD_2 like could be successfully exploited for further studies within the surveyed areas. The ASFV protein 72 has

been easily detected in all the PCR positive samples, confirming that it is highly conserved in most of field isolates (22). As ASFV field isolates from Africa are antigenically the most variable (33), it was very important to understand the relationship existing between these isolates and others from African and other continents. VP72 gene nucleotides sequences were compared and genetic similarities have been revealed between the surveyed area isolates and the others, from West Africa. Some DRC ASFV strains, isolated by the past in the Southern African laboratories, through routine diagnostics requests, have been the most exploited in many relationship studies. These strains originated from the Southern region of DRC the Southern Africa in terms of trade. Also, the majority of African field isolates used in relationship studies have been supplied from the Southern and Eastern Africa (13). The following genetic similarities have been revealed through the above mentioned studies: - *Katanga / 67* isolate is close to *Angola 70 and 72*, and to *Lisbon / 57*, according to restriction enzyme mapping-based study (13), -*Zaire 77* is close to isolates from South Africa on the basis of VP72 portion comparison study (34). A net separation between virus isolates could not be established on the basis of antigenic homology, isolates from Africa, Europe and Latin America belong to the Genotype I group (11). The Genotype I group is mainly made ASFV isolates circulating in the Eastern and Southern Africa, Carribean, America and West Africa (ESACAW) and according to the same reports, the DRC is shared between the two ecosystems, due to co-circulation of Genotype I and Genotype II isolates (34). Genotype II is the most dominant in the Southern and the Eastern Africa (34).

Conclusion

Partial sequence of the DRC ASFV isolate based on the highly conserved gene is determined and the sequences comparison exercise indicate strong genetic similarity with Western Africa isolates, with only 0,01% of nucleotids substitution.

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Chapter 7 - Estimation of the prevalence of African swine fever in the Democratic Republic of Congo and assessment of sensibility and specificity of three ELISA tests in field conditions

Chapter 7 – Estimation of prevalence of African swine fever in the Democratic Republic of Congo and assessment of sensitivity and specificity of three ELISA tests in field conditions.

7.1. – Preamble

Good knowledge of ASF epidemiology including prevalence and spatial distribution is very crucial for instigation of targeted control measures (Costard et al., 2009). In endemic ASF areas, early and accurate detection followed by strict control measures, even if difficult and costly are the only way for prevention and control of the disease, given the lack of vaccine and treatment (Cubillos et al., 2013). Good screening technique, with good sensitivity and specificity should allow fruitful control programme, due to the lack of false results (Toma et al., 1999).

7. 2 – The following section is devoted to the estimation of prevalence rate and characteristics of three screening tests.

ARTICLE 4:

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Estimation of the prevalence of African swine fever in the Democratic Republic of Congo and assessment of sensibility and specificity of three ELISA tests in field conditions

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ABSTRACT

A study was conducted on 495 apparently healthy domestic pigs from the Democratic Republic of Congo in order to determine the exposure of African swine fever virus (ASFV) based on three ELISA tests using crude (cp72) and recombinant (rp72) VP72, and recombinant p30 (rp30) antigens respectively. In addition, PCR targeting the conserved gene B646L encoding VP72 capsid protein was used in order to determine the exposure in function of the stage of ASFV infection.

The apparent prevalence (AP) calculated for the ELISA tests ranged from 10% (48/482) for rp30 to 22.3% (79/354) for rp72 based-antigen ELISA test, respectively. Based on positive results reproduced at least by two diagnostic tests, the overall AP was estimated at 17 % (55/328). Among 495 animals tested with PCR, ASFV DNA was detected in 7 animals (1.4%), including 5 pigs also positive for ASFV antibodies. In addition, assuming the cp72 ELISA as reference, relative sensitivity and specificity of the rp72 and rp30 ELISA tests were estimated on a subset of 328 pigs for which results to all tests were available. The rp72 test

had a relative sensitivity of 93.5% (95% CI: 82.1-98.6) and a relative specificity of 88.0 % (95% CI: 83.6-91.5). The rp30 test had a relative sensitivity of 34.8% (95% CI: 21.3-50.2) and a relative specificity of 93.7% (95% CI: 89.7-95.9). The overall combination of PCR and the three ELISA tests revealed that 62 % of the positive cases had at least one of their result confirmed by another test.
The rp72 ELISA test can be recommended in large scale screening of the disease in ASF endemic areas of Africa as an alternative to the cp72 ELISA test. In addition, the rp30 ELISA test can be recommended for confirmation at any stage of infection, including in the early ones in parallel with PCR.

Keywords: African swine fever (ASF), African swine fever virus (ASFV), Africa, Epidemiology, ELISA test, Sensitivity, Specificity, Field conditions.

Running title: ASFV prevalence based on three ELISA tests in DRC

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INTRODUCTION

African swine fever (ASF) is caused by ASF virus (ASFV), a large icosahedral arbovirus which is the only member of the *Asfivirus* genus and the *Asfarviridae* family (Dixon et al., 2004). ASFV contains a long linear double-stranded DNA varying in length from 170 to 193 Kbp depending on the viral isolate and encodes up to 165 proteins.
ASF is a complex and devastating disease of domestic pigs, occurring in different clinical forms ranging from peracute, acute, subacute, chronic and subclinical. The mortality rate is quite variable, from close to 100% to as little as 3% (Hess, 1987). ASF poses a major

constraint to poverty alleviation and food security especially in developing countries where the rural populations are the most affected. It also threatens the world pig industry due to international trade of live pigs and pig products (EFSA, 2010; FAO, 1998). As a devastating transboundary animal disease, ASF is a reportable disease to the OIE (Cubillos et al., 2013). ASF has already been demonstrated as endemic in many countries of Sub-Saharan Africa (Haresnape, 1985 and 1987; Plowright et al., 1994; Wilkinson, 1989) and in Sardinia (Costard et al., 2009; Wilkinson, 1989). In addition, since 2007 it has become endemic in parts of the Trans Caucasian Countries (TTC) and the Russian Federation (RF) (EFSA, 2010; Rowlands et al., 2007). The wide dispersal of ASF in the Russian Federation (EFSA, 2010; FAO, 2012) and more recently, the occurrence of sporadic ASF cases in Lithuania, Poland, Estonia and Latvia (WAHID, 2014), give an indication of the capacity for rapid disease spread.

Geographically, DRC is the largest country of Sub-Saharan Africa, with possible co-existence of both domestic and sylvatic cycles (Costard et al., 2009; Plowright et al., 1994) which might make the ASF epidemiological situation complex. Historically, ASFV has been circulating over many years in DRC, with the first signs of disease reported as early as in 1939 (Saliki et al., 1985). The most dominant risk factors that contribute to ASF spread in DRC include farming system, restocking of backyard and commercial farms without depopulation after outbreaks, poor management of waste disposal after outbreaks, movements of both pigs and pig products from inside and outside the country across a long and porous border shared with nine countries. The high proportion of pigs raised in the DRC is located in rural and peri-urban areas where animals are mostly free-ranging. Apart from quarantine in infected and suspected areas and drastic slaughter without compensation, no other important control measures are applied. These factors were suggested to be important for the disease spread in other African countries (Penrith et al., 2007). Generally alerts of ASF outbreaks are reported during the period of time starting from the dry season to the beginning of the following raining season. Low virulence isolates of ASFV have already been identified in DRC since the 1980s (Haresnape et al., 1985).

In the absence of vaccines, the only way to prevent ASFV maintenance and spread is a rapid and accurate detection and an appropriate control strategy timely implemented (Sanchez-Vizcaino and Mur, 2013). For diagnosis, several target proteins have been identified as antigens for serological and molecular assays (Cubillos et al., 2013). For most large scale investigations, the major structural capsid protein 72 (VP72) which is coded by the conserved ASFV gene B646L was used (Fernandez-Pinero et al., 2013; Freidje et al., 1992; Oura et al., 2013; Oura et al., 1998; Tabares, 1987). Indeed the conventional ELISA test approved by

FAO and OIE is an indirect ELISA based on the cytosoluble form of the VP72 protein (Cubillos et al., 2013). The ASFV protein 30 (p30), coded by the CP204L gene, is also used to detect anti-ASFV antibodies in pigs at any stage post infection (Cubillos et al., 2013; Gallardo et al., 2009). With acute ASF, pigs usually die before development of ASFV specific antibodies, thus real time or conventional PCR tests are advised to confirm the infection (Oura et al., 2013).

The goal of this study was to determine the prevalence of ASFV in domestic pigs in selected regions of the DRC, and to assess the sensitivity and specificity of some diagnostic assays. To achieve this goal, PCR and three antigen-based ELISA tests were used for the first time in the DRC during a large scale screening in apparently healthy pigs.

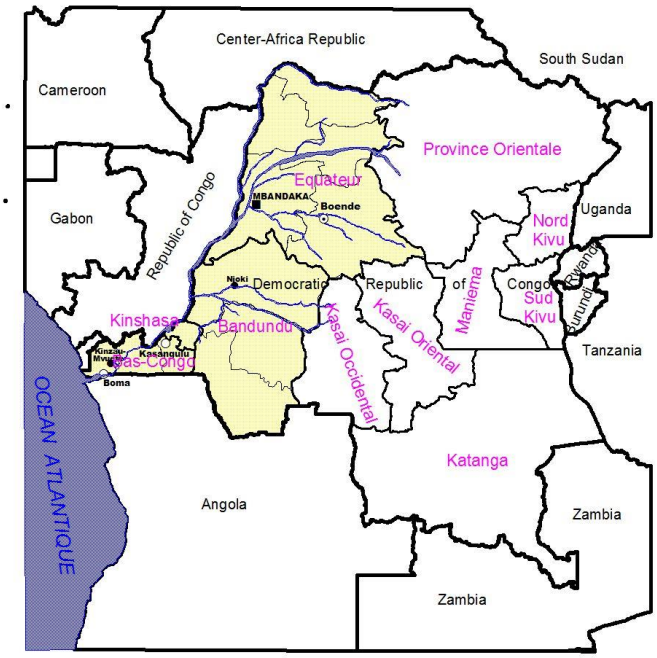
MATERIALS AND METHODS

Study area and time period

The study was conducted in the West of the country and covered four provinces (Equateur, Bandundu, City province of Kinshasa and Bas Congo) (**Figure 1**). Geographically the surveyed areas were located between 0.05’ and 5.12’ South and between 15.12’ and 24.25’ East. Apart from the peri-urban areas, the region is characterized by two major ecosystems dominated by dense forests and savannahs. Those ecosystems have an impact on the habitats of wild pigs and ticks (Wilkinson et al., 1988).

Active surveillance was conducted from April to December 2008. This period corresponds to the second half of the first raining season, the dry season and the first half of the following raining season. The choice of the period was based on alerts indicating that ASF outbreaks were more prevalent during the dry seasons and the beginning the rainy seasons.

Figure 1. Geographical location of the survey area within the DRC



Sample collection

The study involved collection of samples in three selected markets of Kinshasa City Province where pigs were apparently healthy. Those markets were selected because of their importance for the trade of live animals including pigs, originating from the peri-urban area and other provinces. The animals sent to these markets are sold for both butchery and breeding purposes. The overall population of surveyed pigs was 495 in total with 354 females and 141 males including castrate and intact. These pigs were of mixed ages, 36 young animals ranging from 1 to 4 month-old, and 459 adults between 5 and 36 month-old. Body weight of sampled pigs varied from 15 to 120 kg. Different breeds of animals were also included as follows: 358 Large White animals, 109 indigenous pigs, 18 crossed breed animals, 4 Landrace, 4 Pietrain, 1 Chinese, and 1 Duroc. All the surveyed animals were in good nutritional condition.

Blood samples were collected within auricular vein, directly in both, EDTA and dry vacutainer tubes per sampled animal. After slaughter, several tissues were sampled including spleen and gastro-hepatic lymph nodes. If hemorrhages were observed, kidney, lung, and liver samples were also collected. Tissues samples were kept in sealed plastic bags placed in cooler boxes and directly brought to the laboratory where they were stored at -20°C. Blood samples in dry tubes were left at +4^{o}C overnight in order to allow sera and clots to separate. The sera were recovered 24 hours later and stored in cryotubes at -20°C until analysis.

ASFV antibody detection

Three ELISA tests were used to detect antibodies against ASFV. Two were based on the ASFV VP72 protein as coating antigen (Ag) and one on the ASFV recombinant protein 30 (rp30).

The differences between the two ELISAs based on the VP72 proteins were on the purity level of the coating Ag and on the detection format of the ELISA tests. Indeed, one of the two tests was based on the crude, cytosoluble form of the ASFV VP72 (cp72), prepared from Monkey stable (MS) cells, infected with ASFV (OIE, 2012). This test has been approved by FAO/OIE as indirect ELISA and was supplied by CISA-INIA, Madrid, Spain. The second VP72 antigen was a recombinant form expressed in *E. coli* and used as coating antigen in a blocking ELISA, supplied by INGENASA, Madrid, Spain.

The rp30 expressed using a baculovirus vector in insect larvae was used as antigen in a third indirect ELISA test (ALGENEX, Madrid, Spain). All ELISA tests were used according to different manufacturer's instructions.

The first indirect ELISA approved by the FAO/OIE with a sensitivity of 85% and a specificity of 93%, was referred to as the gold standard in this study.

ASFV DNA detection

DNA extraction

Depending on the nature of the collected tissues, ASFV DNA detection was performed using templates from both blood and animal organs.

ASFV DNA was extracted using DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) (provided by Dr L. Dixon, The Pirbright Institute and Dr I. Damon, CDC Atlanta). Two grams of solid tissue were crushed with mortar and pestle using sterile sand, and suspended in 8 ml of PBS; the mixture was centrifuged at 1500 rpm for 10 minutes at 4^{o}C and DNA was prepared using the supernatants. A total of 200µl of plasma was centrifuged at 1400rpm for 5 minutes, and then frozen at -20°C, thawed during 24 hours and DNA was prepared from 20µl of the supernatant. DNA extraction from the supernatants was performed according to the manufacturer's recommendations (QIAGEN, Hilden, Germany).

DNA amplification, separation and sizing

Partial amplification of the p72 gene was realized to detect ASFV DNA (Bastos et al., 2003; Agüero et al., 2003). The PCR targeted the conserved gene B646L (GeneBank access numbers FJ174377.1 and AY578708.1). Primers' pair used were $VP72_3$-F (Primer sequence 5' to 3': CTG TAA CGC AGC ACA GCT GAA CCG TTC TG) and $VP72_4$-R (Primer sequence 5' to 3': ATA GGA TTA AAA CCT ACC TGG AAC ATC TCC G). These primers were supplied by Life Technology (Carlsbad, California, U.S.) or by MWG-Biotech AG (Ebersberg, Deutshland). All the PCRs reactions were performed in 25µl volume including 1µl of primers mix (0.5µM of forward and reverse primers), 10µl of DNA template, 10µl of Taq DNA polymerase, and 4µl of double distilled water. A positive and a negative control of amplification were always used and amplified in parallel with the DNA samples. The Taq DNA polymerase consisted of a mastermix (2.5x) supplied by Eppendorf (Hamburg, Germany), containing: Taq DNA polymerase (62.5 U/ml), 125mM KCl, 75mM Tris-HCl pH 8.3, 3.75mM Mg $(OAc)_2$, 0.25% Igepal-CA630 and 500µM of each dNTP and stabilizers. PCRs were performed using the following cycling conditions: cycle 1, 94˚C for 5 min, 50-55˚C for 1 min, 72˚C for 2 min; cycles 2 to 29: 94˚C for 1 min, 50-55˚C for 1 min, 72˚C for 2

min; cycle 30: 94˚C for 1 min, 50-55 ˚C for 1 min, and 72˚C for 10 min. Amplicons were loaded on a 1% agarose gel with 10% bromophenol blue dye (Gerrit et al., 2005) and, separated in 0.5 x Tris acetate EDTA (TAE) buffer mixed with 1µl of Ethidium Bromide. Sizes were determined by comparison with a 1 Kb DNA molecular weight marker. ASFV's DNA was visualised using a UV transluminator and, the expected size of the VP72 amplicon was 500 bp.

Statistical analysis

The 95% confidence interval of the diagnostic sensitivity (Se) and the specificity (Sp) of ELISA tests were estimated using a binomial exact distribution. Only samples in all of the screening tests (N = 328) were taken into consideration for the Se and Sp calculation, in order to increase the objectivity of characteristics assessment.

The kernel density estimation of the distribution of final results obtained in each ELISA test was used to evaluate the potential of separation between infected and uninfected sub-populations of animals (Chen and Wang, 2013). The Receiver-Operating-Characteristic (ROC) curve was used to test the diagnostic usefulness of rp72 and rp30-based ELISA tests in comparison with the cp72 ELISA test as FAO/OIE reference test.

The level of agreement between ELISA tests was assessed using the Kappa coefficient (Kenny et al., 2006).

The apparent prevalence was the number of animals testing positive by a diagnostic test divided by the total number of animals tested. The 95% confidence interval of the apparent prevalence was estimated using a binomial exact distribution.

The estimation of true prevalence was estimated using the following formula (Rogan and Gladen, 1978):

$$TP = \frac{AP + Sp - 1}{Se + Sp - 1}$$

With: TP, the true prevalence; AP, the apparent prevalence; Sp, the specificity and Se, the sensitivity.

RESULTS

ELISA tests

Using the cp72-based antigen ELISA as gold standard, the relative Sensitivity (Se) and Specificity (Sp) of the two other ELISA tests were assessed. The rp72-based test had a

relative Se of 93.5% (95% CI: 82.1-98.6) and a relative Sp of 88.0 % (95% CI: 83.6-91.5). The rp30-based test had a relative Se of 34.8% (95% CI: 21.4-50.2) and a relative Sp of 93.7% (95% CI: 89.7-95.9). The general tendency of the separation of infected and uninfected animals as detected by each ELISA test was determined by the frequency histogram (**Figure 2A, B and C**) and the correspondent kernel density (**Figure 2D, E and F**). Therefore it was observed that the two sub-populations of infected and uninfected pigs were more separated in the rp72 and rp30-based tests.

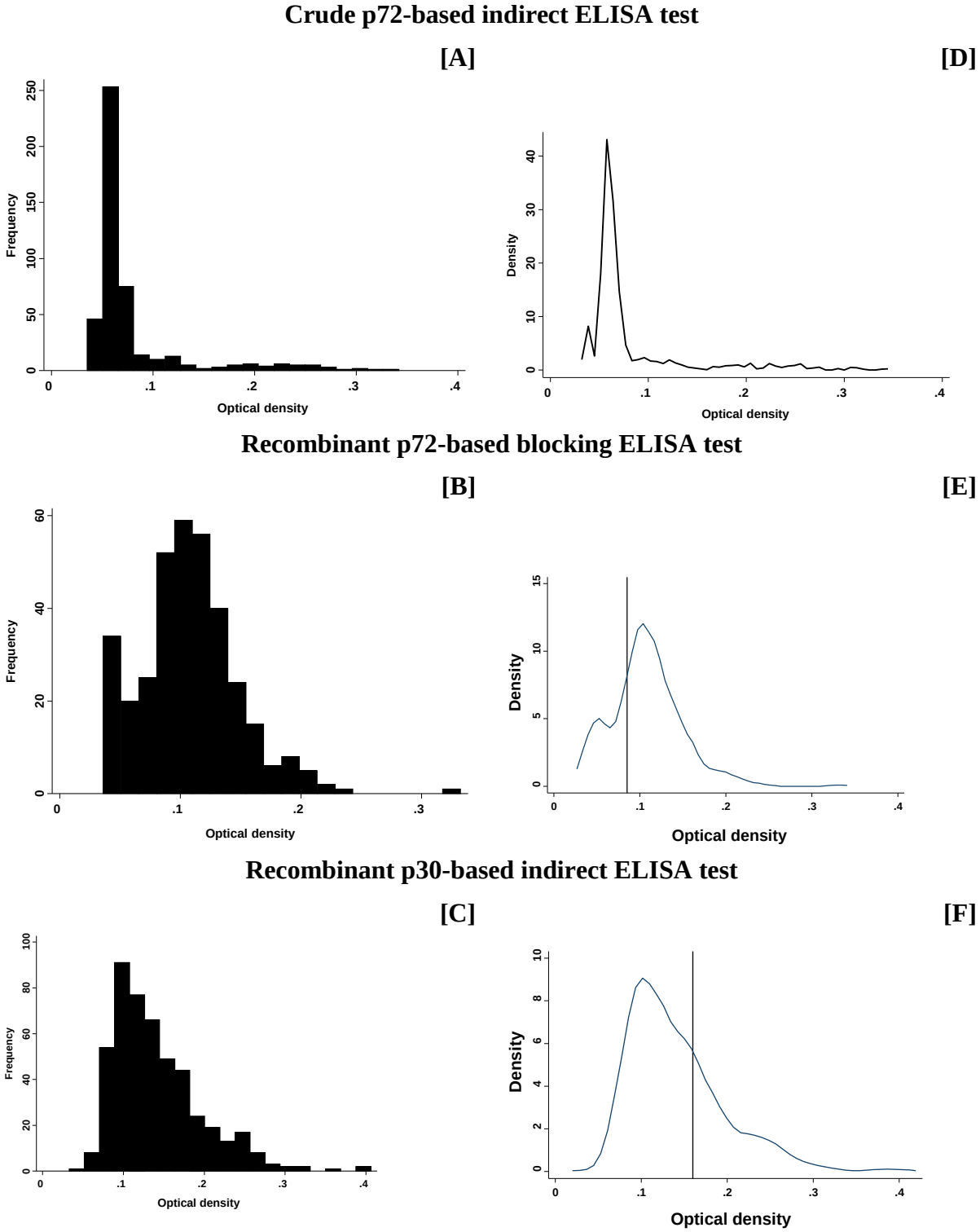
According to ROC curve calculation using cp72 ELISA as gold standard, the diagnostic value of both rp72 ELISA (area under the curve [AUC] = 0.86) and rp30 ELISA (AUC = 0.76) appeared to be good (**Figure 3**). Furthermore, the two AUC were not significantly different (Chi2 = 3.41; 1 df; $P = 0.06$).

The cut-off point (CO) for the rp72 ELISA corresponds to an optical density (OD) of 0.16. With this CO, the sensitivity and the specificity of the test are 76.5% and 83.5 %, respectively. The CO for the rp30 ELISA corresponds to an OD of 0.085. With this CO, the sensitivity and the specificity are 65 % and 75%, respectively.

The concordance agreement between rp30 and cp72 antigens-based tests (Kappa coefficient = 0.38 with 95% CI: 0.29-0.47) as well as the concordance agreement between rp30 and rp72 antigens-based screening tests (Kappa coefficient = 0.40 with 95% CI: 0.31-0.50) were poor whereas the concordance between the cp72 and rp72 antigens tests was good (Kappa coefficient = 0.64 with 95% CI: 0.53-0.74).

Based on the number of samples tested and positive results recorded, the apparent prevalence (AP) was estimated for each ELISA test as follows: for the rp30 ELISA, 48 out of 482 were positive (AP = 10.0%; 95% CI: 7.4-13.0); for the cp72 ELISA, 64 out of 460 were positive (AP = 13.9%; 95% CI: 10.9-17.4); and for the rp72 ELISA, 79 out of 354 were positive (AP = 22.3; 95% CI: 18.1-27.0). According to Se and Sp of the ELISA tests previously published (Perez-Filgueira et al., 2006) for rp30 (Se = 98.2%; Sp = 97.4%) and cp72-based (Se = 98.8%; Sp = 87.8%) screening tests and, the use the formula of Rogan and Galden (Rogan and Gladen., 1978), the true prevalence was estimated between 2.0% with cp72 ELISA and 7.7% with rp30 based-ELISA. Regarding the rp72 ELISA test, no sufficient data on Se and Sp were so far published (Oura et al., 2013), then the true prevalence could not be estimated on this base.

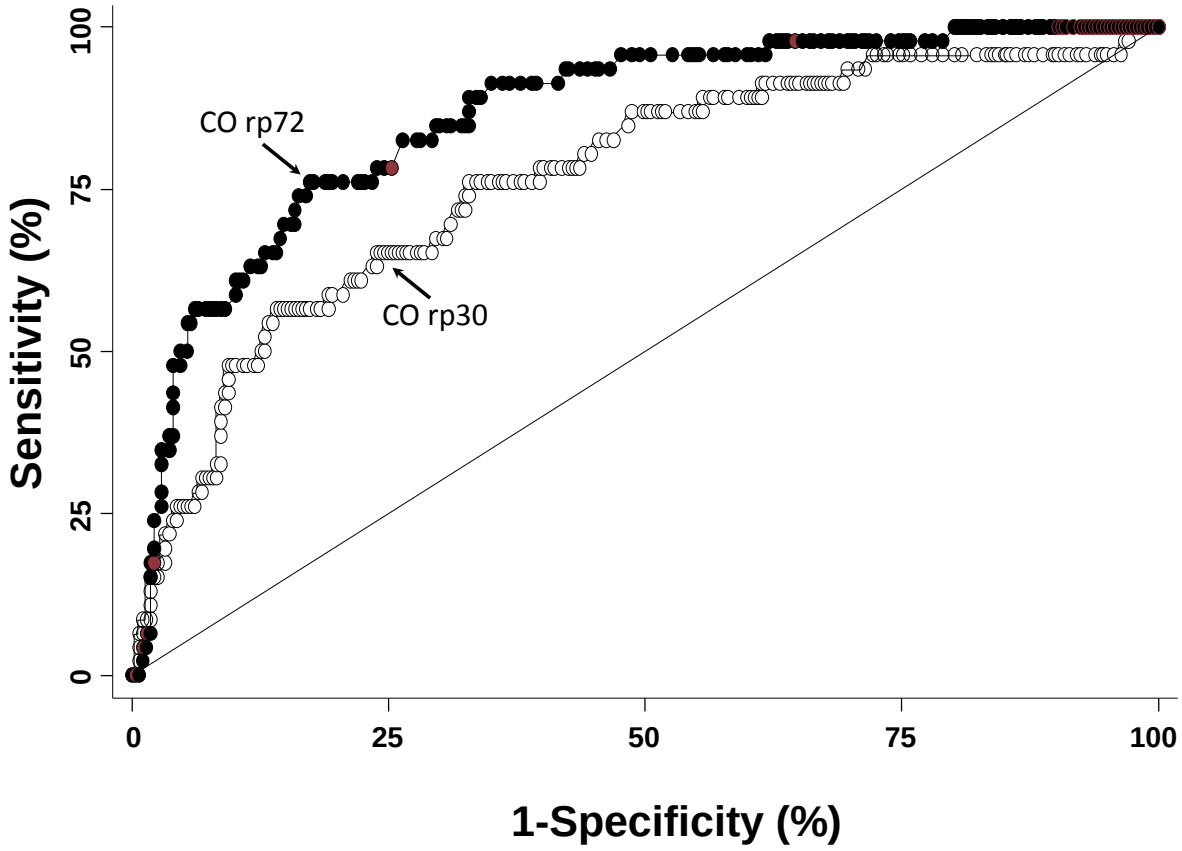
Figure 2: Frequency histogram of optical density for each ELISA test and corresponding Kernel density estimation



Legend: [A], [B] and [C], frequency histograms of optical density for protein 72, recombinant protein 72 and recombinant protein 30-based antigen ELISA tests, respectively ; [D], [E] and [F], kernel density estimation for protein 72, recombinant protein 72 and recombinant protein

30-based antigen ELISA tests, respectively. Optical density values on the X-axis line should be read as 0.01 to 0.4.

Figure 3: The Receiver-Operating-Characteristic (ROC) curve for the recombinant protein 72 (●) and the recombinant protein 30 (○) - based antigen ELISA tests.



Legend: For the recombinant protein 72, more higher is the optical density (OD), more negative is the result. For the recombinant protein 30 it is the reverse. In order to compare the two tests, the representation of ROC curve for recombinant protein 72-based test considers the value 1 minus the OD. For the recombinant protein 72-based antigen ELISA test, the area under the curve is 0.86 and for the recombinant protein 30-based ELISA test, the area under the curve is 0.76.

ASFV DNA detection by PCR

Out of the 495 specimens tested with PCR, ASFV DNA was detected in 7 animals. The size of the amplicon corresponded to the expected one (500 bp). Among these 7 cases, anti-ASFV antibodies were detected in 5 cases (4 detected by the rp30 ELISA including 1 jointly detected by cp72, and 1 detected both by the cp72 and rp72 ELISA tests).

Similarly to what previously described for the three ELISA tests, the apparent prevalence for the PCR assay was calculated using the 7 positive samples out of 495 tested (AP = 1.4%; 95% CI: 0.6-2.9).

Combination of ELISA and PCR results

The combined results obtained by each animal to one or more tests were assessed in order to more accurately understand the behavior of each test with regard to the moment of infection. Within the parallel combination of ELISA and PCR test results, a pig was considered negative (0), with a negative result in each of the 4 tests and it was considered positive (1) in the presence of at least in one positive result among the 4 tests. Moreover, the combination of two or more positive results was considered as a confirmation of the positive condition of the animal.

Out of 328 pigs for which the results were available for the three ELISAs and the PCR tests, 239 animals (72.9 %) were negative to all tests, whereas 89 (27.1 %) were positive in at least one of the four screening tests (**Table 1**). Within the positive pigs, 9 different combinations of test results were observed. To express this combination, a number with 4 positions was created (with the following order: PCR, rp30, cp72 and rp72-based ELISA tests) and in function of the result for each test, a binary codification was used per position (1 as positive result and 0 as negative result). The different combinations are presented in **Table 1.**

The 9 positive combinations could be grouped in 2 categories: category A with one single screening test having a positive result (combinations 1000, 0100, 0010 or 0001) and category B including cases in which one positive result was confirmed by one or more different positive results (inter-observation between different screening tests, combinations 1100, 0011, 1011, or 0111). Category A included 34 cases out of 89 positive animals (38%) and category B included the remaining 55 cases (62%). This data analysis showed that 62% of positive results were confirmed by at least one other positive test.

Within the category A, the highest value (7.0%) was corresponding to solitary rp72 positive result, suggesting that this was the best screening test when considered separately. Within category B, the highest proportion (7.9%) was represented by the combination 0011 composed by both cp72 and rp72 positive test results. Within the positive animal's categories, three different small groups emerged in relation with infection stages. The first one (0.3 %) was composed by the animals with positive results in PCR but negative in all the ELISA tests. The second group (0.6 %) included animals with positive results in PCR as well as in ELISA, and the third one, (26.2%) was the group including the animals with positive results at ELISA (one or more), but negative in PCR.

Table 1: Different combinations of results obtained using three ELISA tests and a VP72 gene-based PCR (N = 328 apparently healthy pigs).

Combinations of results	PCR	ELISA rp30	ELISA p72	ELISA rp72	Number of pigs	% of total
1	0	0	0	0	239	72.9
2	1	0	0	0	1	0.3
3	0	1	0	0	7	2.1
4	0	0	1	0	3	0.9
5	0	0	0	1	23	7.0
6	1	1	0	0	1	0.3
7	0	0	1	1	26	7.9
8	0	1	0	1	11	3.4
9	1	0	1	1	1	0.3
10	0	1	1	1	16	4.9

Legend: A binary codification was used to express the result of each test (1 as positive result and 0 as negative result).

DISCUSSION

The study was undertaken to determine which diagnostic tests were most appropriate to use in large scale screening to identify ASFV exposure of pigs in the epidemiological context of DRC but also to estimate the prevalence of ASFV in apparently healthy pigs.

The results of the study indicate that, the rp72-based ELISA test is useful in detecting exposure to ASFV in DRC and possibly in other endemic areas of Sub-Saharan Africa. To detect virus infection at an early stage, i.e. before the development of a detectable antibody response, the PCR is the most reliable test. Several real time and conventional PCR assays have been developed and confirmed this finding (Steiger et al., 1992; Agüero et al., 2003). The rp30 ELISA test may have better potential to detect antibodies at any stage of infection including the early one compared to the rp72 or cp72 ELISA test, since detection by PCR correlated well with positive results obtained using rp30 ELISA test. The early detection capacity of ASFV rp30 antigen-based ELISA would be probably based on the involvement of p30 in the cellular internalization of ASFV at the earlier steps of resorbative endocytosis. However, further evaluation of this is required.

Using a binomial exact distribution and the cp72-based antigen ELISA as gold standard, the relative Se and the relative Sp of the rp72-based antigen ELISA were 93.5% and 88.0 %, respectively. In contrast, the rp30-based test had a relative Se of 34.8% and a relative Sp of 93.7%. Surprisingly, the Se and Sp of rp30-based ELISA were lower than the ones reported from a previous study (i.e. Se of 97.8% and Sp of 98.8%) performed in the same geographical area (Cubillos et al., 2013). However, Cubillos and collaborators used samples collected in 2007 while in the present study the sampling was realized in 2008. It should be considered that several factors could affect diagnostic test performances (Costa, 1988; Plowright et al., 1994; Wilkinson, 1989; Freidje et al., 1992; Tabares, 1987; Castello et al., 2009; Cubillos et al., 2013). Among them, the ability in detecting antibodies from symptomless or healthy carriers could be a determining factor (Costa, 1988), together with the use of different antigens for coating the plate (Morara/Georgia ASFV strain for the study of Cubillos and collaborators *versus* Spanish ASFV isolate 120VR15 in this study). Nonetheless, within the current study, a better correspondence was observed between rp30 ELISA results and PCR, as 4/7 PCR positive results were reproduced by rp30 ELISA. This suggests that the rp30 ELISA test may detect antibodies at an earlier stage of infection, when the virus is still

circulating in apparently healthy pigs, compared to the tests based on rp72 or cp72. Furthermore, our results suggested the potential to improve the sensitivity of the rp30 ELISA, since 9 sera among 482 (19 %) had an optical density value significantly below the manufacturer cut-off but were positive according to the color intensity criteria and were subsequently confirmed by p30 immunoblotting test (data not shown).

The Kappa coefficient calculation indicated a good agreement between the cp72 and rp72-based ELISA tests. This was expected since both tests included the same VP72 antigen. However, the Kappa coefficient showed a poor agreement between rp30 and both cp72 and rp72-based ELISA tests. In addition, no significant difference of the area under the ROC curve was observed between the rp30 and the cp72 tests, although the *P* value was close to the threshold value 0.05. It should be interesting to test more samples in the future to consolidate this preliminary finding.

The PCR technique is an expensive diagnostic assay but given the objectives of this survey, conventional PCR was also selected as one of the screening tests to assess the infection status of apparent healthy pigs. Although the number of animals from which ASFV DNA was detected was very low, a proportion of these pigs were negative in ELISA assays. This suggests that the pigs were infected with ASFV and were detected at early stage of infection before seroconversion. In other words, a preclinical ASF situation was presumably present, which is confirming the PCR assay value in the prediction of ASF outbreak (Zsak et al., 2005). Another proportion of PCR positive animals were also positive by ELISA.

As highly virulent ASFV isolates usually cause death of most if not all the infected pigs before the detection of antibody response, these results indicate that at least a proportion of pigs survive long enough to develop a detectable antibody response and suggests that the circulating ASFV isolates have a reduced virulence in DRC. Circulation of low virulence strains, outbreaks survivors, long-term and old infection were already reported in ASF endemic areas by many investigators in the past (DeTray, 1963; Haresnape, 1987; Anderson *et al*., 1998).

Assuming that positive results in at least two screening tests confirmed ASFV exposure of pigs, the minimum apparent prevalence (AP) estimation is 17%. Including the pigs which were positive in just one test resulted in an increase of AP up to 27.1% but significantly higher than that estimated in Senegal (16.9%) (Etter et al., 2011). This estimation is quite different from the estimation of the true prevalence using the Rogan and Gladen formula (between 2.0% or 7.7% in function of the ELISA test considered and using available published priors for its Se and Sp).

The group of positive pigs that was positive by ELISA but negative by PCR analysis represented 26.6 % of the positive pigs tested. These pigs have presumably recovered from infection. This relatively high percentage suggests that ASFV isolates of reduced virulence may be circulating in the region. These pigs may be important in disease spread if they remain persistently infected (Alvarez Ordas, 1987) because they may represent a potential source of infection. In addition these pigs are likely to be immune to challenge with related virulent viruses and may thus survive after ASFV outbreak. It would be interesting to further characterize the ASFV strains circulating by complete genome sequencing and pathogenesis studies. Further large scale and more structured studies are required to obtain a better informed assessment of the prevalence of ASF in DRC. Given the size of the country and its central location within Sub-Saharan Africa, a study of ASF true prevalence and the genotyping of circulating ASFV isolates combined with risk factor analysis would be a prerequisite for introduction of appropriate control strategies.

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Chapter 8 - Genetic assessment of African swine fever isolates involved in outbreaks in the Democratic Republic of Congo between 2005 and 2012 reveals co-circulation of p72 genotypes I, IX and XIV and 19 variants

Chapter 8 - Genetic assessment of African swine fever isolates involved in outbreaks in the Democratic Republic of Congo between 2005 and 2012 reveals co-circulation of p72 genotypes I, IX and XIV and 19 variants

81. – Preamble

Molecular characterisation has been demonstrated by several workers as a worthy tool for implementation of prevention and control measures against ASF (Atuhaire et al., 2013), due to the fact that links and source of outbreaks have become traceable (Nix et al, 2006; Boshoff et al., 2007; Rowlands et al., 2008, Gallardo et al., 2011), including the period and the number of introductions (Giammarioli et al., 2011; Lubisi et al., 2005).

8.2. – The following section is devoted to the genetic assessment of African swine fever isolates involved in outbreaks in the Democratic Republic of Congo between 2005 and 2012.

ARTICLE 5:

Mulumba-Mfumu L. K., Achenbach, J. E, Mauldin, M. R., Dixon, L. K., Tshilenge, M. G., Thiry, E., Moreno, N., Blanco, E. Saegerman, C., Lamien, C. E., and Diallo, A.

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Genetic assessment of African swine fever isolates involved in outbreaks in the Democratic Republic of Congo between 2005 and 2012 reveals co-circulation of p72 genotypes I, IX and XIV and 19 variants.

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ABSTRACT

African swine fever (ASF) is a devastating disease of domestic pigs. It is a socioeconomically important disease, initially described from Kenya, but subsequently reported in most countries in Sub-Saharan Africa where it is endemic. ASF spread to Europe, South America and Caribbean where it has been eradicated apart from Sardinia. In 2007, Eurasia was affected from East Africa. In this study of ASF within the Democratic Republic of Congo, 62 domestic pig samples collected from 2005-2012, were examined for viral DNA and sequencing at multiple loci: C-terminus of B646L gene (p72 protein), central hypervariable region (CVR) of the B602L gene (9RLORF protein), and the K183L gene (p54 protein). Phylogenetic analyses identified three circulating genotypes: I (64.5% of sampled isolates), IX (32.3%), and XIV (3.2%). This is the first evidence of genotypes IX and XIV within this country. Examination of the CVR revealed high levels of intragenotypic variation, with 19 variants.

Key words: African swine fever virus; Outbreaks; Democratic Republic of Congo, Suids; Genotypes; Molecular epidemiology; p72 gene; p54 gene; CVR

INTRODUCTION

African swine fever (ASF) is a complex and highly lethal disease of domestic pigs with mortality rates ranging from 100% to as little as 3% (Hess, 1987). Presentation varies from peracute, acute, subacute, to chronic and unapparent. Domestic pigs, wild suids and eyeless, burrow dwelling soft ticks of the genus *Ornithodoros* are susceptible to ASF, although wild suids are symptomless. *Ornithodoros* ticks are both reservoirs and vectors. Depending on involved strains, exposure intensity, disease course, infection route and infected host, ASF clinical and pathological signs differ (Guinat et al., 2014; Saliki, 1985; Wilkinson, 1989). ASF is a threat to the world pig industry, as well as a major constraint to poverty reduction and to food security in rural areas of sub-Saharan Africa (SSA), where pigs are one of the few domestic livestock species that form an affordable source of income and animal proteins (Penrith et al., 2013; FAO, 1998a). ASF is on the World Organization for Animal Health's (OIE) list of notifiable animal diseases (Sanchez-Vizcaino et al., 2014).

ASF was first documented in Kenya in 1910, as a result of unusual clinical signs observed in domestic pigs (Montgomery 1921). Since the recognition of the virus, ASF has been documented in most sub-Saharan countries (Costard et al., 2009). The impact of ASF has been substantial across Africa, in both the commercial and subsistence pig sectors (Edelsten & Chinombo 1995; Costard et al., 2009). Some countries, such as Cote D'Ivoire and Madagascar have lost an estimated 30-50% of their pig populations following the first ASF outbreaks they experienced in the 1990s (El Hicheri et al. 1998; Roger et al., 2001). However, the economic impact of ASF is not limited to Africa. The first recorded exportation of ASF case outside Africa occurred in 1957 in Portugal (De Tray, 1963). The second and third exportations to Europe occurred in 1960 and 2007, respectively (Sanchez-Vizcaino, 2012; Rowlands et al., 2008). Based on genetic similarities, the first two exportation of ASF to Europe were thought to have originated from western Africa, more specifically Angola (1957; De Tray, 1963) and Senegal (1960; Sanchez-Vizcaino, 2012), whereas the 2007 release were determined to have originated from eastern Africa (Rowlands et al., 2008). Introduction of ASF was not limited to Europe, as outbreaks with putative causal links to Spain have occurred in the Caribbean and South America in the 1970s (Plowright, 1994). The high mortality rate in domestic pig outbreaks has resulted in major economic implications. The introduction of ASF to Cuba cost an estimated $9.4 million USD (Simeon-Negrin &

Frias-Lepoureau 2002), whereas the last five years of the eradication program in Spain reportedly cost $92 million USD (Arias & Sanchez-Vizcaino 2002).

ASF is caused by African Swine Fever Virus (ASFV), which is transmitted to susceptible suids through three main routes: 1) an ancient sylvatic cycle involving wild suids and *Ornithodoros* ticks, 2) from the sylvatic cycle to domestic pigs, and 3) the domestic pig cycle involving domesticated pig to pig transmission (Lubisi, 2005). ASFV is a large icosahedral arbovirus which belongs to the monotypic genus *Asfivirus* of the family *Asfarviridae* (Dixon et al., 2004). The genome of ASFV is comprised of a long linear double stranded DNA molecule varying between 170-193 Kbp, containing 150-167 open reading frames (ORFs) depending on the isolate (Dixon et al., 2013). The ASFV genome is close-ended by imperfectly base-paired and covalent terminal cross links hair-pin loops. The mapping of ASFV genome using restriction enzymes revealed the presence of a large, highly conserved 125 kbp central region flanked by long and short variable termini at the left and right ends, respectively. These variable termini include inverted tandem repeats where indels have been described by several workers (Vinuela, 1987; Blasco et al., 1989; Costa, 1990). Antigenic and pathogenic divergences between ASFV isolates are associated with genomic variability within those terminal flanking regions, especially in the multigene families (MGFs; Chapman et al., 2008; Zack et al., 2001). Five different multigene families have been identified: MGF 360, MGF 300, MGF 530/505, MGF 110, and MGF 100 (Dixon et al., 2004). Indels within these regions can include entire ORFs or variation in numbers of short tandem repeats, either within coding regions or intergenic regions (Dixon et al., 1990; Lubisi et al., 2007).

Due to the lack of treatment and vaccine, as well as the inability to discern serotypes of ASFV isolates (Cubillos et al., 2013), rapid and accurate diagnosis completed by the genotyping of circulating isolates may contribute to timely improvement of prevention and control strategies (Owolodun et al., 2010). In order to identify and determine the heterogeneity of circulating ASFV isolates, genetic characterization was initially achieved through use of restriction fragment length polymorphisms (RFLPs; Wesley and Tuthill 1984; Dixon and Wilkinson 1988; Blasco et al., 1989), and more recently by PCR-based sequencing of a 179 bp fragment at the C-terminal end of the p72 gene (Bastos et al., 2003). Although both RFLP and nucleotide sequence data achieved similar classification results, the latter method identified greater levels of genetic variation, and provided a faster, more accurate means of genotyping ASFV isolates circulating in the field (Bastos et al., 2003).

Given the low level of genetic variation detected at the p72 locus among ASFV isolates recovered in domestic pig outbreaks, examination of more variable genomic regions as well as a wide range of multi-locus approaches were tried to discern between isolates within a single outbreak. The central hypervariable region (CVR) of the 9RL ORF encoding the pB602L protein (Phologane, 2005) has been used as a single locus marker and in combination with other loci. Multilocus datasets have varied from two markers (Boshoff et al., 2007), to three (Atuhaire et al., 2014; Gallardo et al., 2009; Giammarioli et al., 2011; Misinzo et al., 2011; Owolodun, 2010b; Goller, 2015), and even four (Nix et al., 2006). Continued research highlighting useful genomic regions for further discrimination of ASFV isolates is required to increase our understanding of the natural history, evolution, and molecular epidemiology of this socio-economically important virus.

DRC, located between 12^{o} and 33^{o} E, 5^{o} N and 14^{o} S, is the second largest country in Africa, and the largest of Central Africa, with an area of 2,345,000 km^2. The central and western portions of the country are dominated by the second largest block of rainforest in the world, whereas the southern and eastern regions are characterized by savannas. DRC is surrounded by nine countries along its 9,000 km border. This lengthy border is somewhat porous due to many trans-frontier tribes that inhabit the DRC and surrounding countries. A portion of this border is comprised of the Congo River, one of the longest and deepest in the world. This river is a major thoroughfare of the country (Siradou, 2012) that links 7 of the 11 provinces. Kinshasa, the capital, is home to more than 10 million inhabitants, and is the greatest consumption center in the country, with increasing demand for pigs and pork products.

In 2011, DRC reported 84 outbreaks and a loss of 105,614 suids, leading African countries in both statistics (El-Sawalhy et al., 2011). Despite the prevalence of ASF in the DRC, the variety of genotypes reported in surrounding countries (Gallardo et al., 2011; Lubisi et al, 2005), and the large number of studies that have examined this disease, no large scale study has focused on understanding the genetic diversity of ASFV within this country. The goal of this study was to improve the scientific community's understanding of ASFV strains circulating in the DRC. To achieve this goal the following objectives were developed: 1) collect samples from suids that exhibited symptoms attributable to ASF over a broad geographic and temporal range, 2) utilize a multi-locus genotyping approach to categorize isolates into genotypes, and 3) utilize these data to improve the understanding of the natural history and ecology of ASF in the DRC.

MATERIALS AND METHODS

Study areas and samples

Based on disease occurrence history and recognition, 62 tissue samples (spleen, lymph node, kidney, lung, liver, heart and stomach) were collected from 57 domestic pig carcasses from 25 sampling locations. Of these carcasses, 54 were collected in outbreaks and 3 were slaughtered in urban markets. Sampling localities were located in six provinces (Kinshasa, Equateur, Katanga, Orientale, Bas-Congo and Maniema) that contain the majority of the country's domestic pig population. Samples were collected over seven years (2005-2012). During the sampling operations, the collected specimens were directly placed in plastic bags, sealed, and transported to the laboratory at ambiant temperature. Once in the laboratory, samples were registered, labelled, and stored at -20^{o} C. The following day the tissues were thawed and homogenized by grinding 2 grams of tissue with sterile sand using a pestle and mortar. The ground tissue was then suspended in 8 mL of PBS before being transferred into 50 mL Falcon conical bottles and centrifuged at 2500 rpm for 30 minutes. The supernatant for each sample was labelled and stored at -90 ^{o} C. Samples, collection localities, and other details are provided in **Table 1**.

PCR-based ASFV DNA detection assay

ASFV DNA detection for each tissue sample was performed in two independent reactions: 1) for confirmation of outbreaks, and 2) for sequencing. For ASF outbreak confirmation, ASFV DNA was extracted using QIAGEN blood and tissue extraction kit (Qiagen) according the manufacturers' protocol. The amplification process was achieved using primers $VP72_3$ (F)-CTGTAACGCAGCACAGCTGAACCGTTCTG , and $VP72_4$ (R)-ATAGGATTAAAACCTACCTGGAACATCTCCG, corresponding to the p72 gene as described in a previous study (Mulumba-Mfumu et al., 2013). In a 50µl volume reaction, Taq DNA Polymerase (1.25 U) was added to a mastermix containing 50 mM KCl, 30 mM Tris-HCl, 1.5 mM Mg^{2+}, 0.1 % Igepal-CA630, and 200 µM of each dNTP (Eppendorf AG, Hamburg, Germany). PCR thermal profiles were as follows: cycle 1, 94 °C for 5 min; cycles 2 to 29, 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min; cycle 30, 72 °C for 10 min. PCR products were separated in a 1% agarose gel using Tris acetate EDTA (TAE) buffer and ethidium bromide for 1 hour at 100 V, followed by visualization under UV light. Amplicons were sized using 1 Kbp DNA ladder (Bioline, London, UK). Confirmation of ASFV DNA was dependent upon the presence of a 500bp amplicon.

Table 1. Samples, isolates details and genotypes

GENOTYPE I

Virus.	Date	Location	GPS	Province	Ecological profile	Tissues	Species	Farming System	CVR TRs	Reference
drc49/05/1a	May 2005	Limete	4^{O}18S /15^{o}22E	Kinshasa	City	Spl	DP	Commercial	**9**	This study
drc49/05P1b	May 2005	Limete	4^{O}18S /15^{o}22E	Kinshasa	City	Ln	DP	Commercial	9	This study
drc49/05/P2	May 2005	Limete	4^{O}18S /15^{o}22E	Kinshasa	City	Spl	DP	Commercial	10	This study
drc70/05	2005	Limete	4^{O}18S /15^{o}22E	Kinshasa	City	Spl	DP	Commercial	6	This study
drc75/05	2005	Maniema	2^{O}93S /25^{o}86E	Kindu	City	Spl	DP	Commercial	9	This study
drc99/05/a	2005	Ngafula	4^{O}21S /15^{o}14E	Kinshasa	Peri-urban	Spl	DP	Commercial	14	This study
drcKG28110805	Nov 2008	Ngaliema	4^{O}21S/15^{o} 21E	Kinshasa	City	Spl	DP	Backyard	45	This study
drcKG28040802	Apr 2008	Kasavubu	4^{O}20S/15^{o} 20E	Kinshasa	City	Spl	DP	Backyard	16	This study
drc74/09/2	2009	Nsele	4^{O}24S/15^{o} 30E	Kinshasa	Peri-urban	Kd	DP	Commercial	23	This study
drc74/09/3	2009	Nsele	4^{O}24S/15^{o} 30E	Kinshasa	Peri-urban	Lg	DP	Commercial	23	This study
drc74/09/4	2009	Nsele	4^{O}24S/15^{o} 30E	Kinshasa	Peri-urban	Lv	DP	Commercial	23	This study
drc74/09/6	2009	Nsele	4^{O}24S/15^{o} 30E	Kinshasa	Peri-urban	Hrt	DP	Commercial	23	This study

drc94/09/2	2009	Kintambo	4^{O}20S/15^{o} 18E	Kinshasa	City	Ln	DP	Commercial	23	This study
drc35/10/1	2010	Ngaliema	4^{O}21S/15^{o} 05E	Kinshasa	City	Spl	DP	Backyard	51	This study
drc35/10/5	2010	Ngaliema	4^{O}21S/15^{o} 05E	Kinshasa	City	Ln	DP	Backyard	Sng	This study
drc35/10/4	2010	Ngaliema	4^{O}21S/15^{o} 05E	Kinshasa	City	Ln	DP	Backyard	Sng	This study
drc51/10/23	2010	Ndjili	4^{O}24S/15^{o} 21E	Kinshasa	City	Spl	DP	Commercial	12	This study
drc73/10/2	2010	Ngaliema	4^{O}21S/15^{o} 05E	Kinshasa	City	Kn	DP	Backyard	11	This study
drc73/10/3	2010	Ngaliema	4^{O}21S/15^{o} 05E	Kinshasa	City	Lv	DP	Backyard	11	This study
drc73/10/4	2010	Ngaliema	4^{O}21S/15^{o} 05E	Kinshasa	City	Ln	DP	Backyard	11	This study
drc85/10/1/s	2010	Ngafula	4^{O}21S /15^{o}14E	Kinshasa	Peri-urban	Spl	DP	Commercial	9	This study
drc85/10/1/h	2010	Ngafula	4^{O}21S /15^{o}14E	Kinshasa	Peri-urban	Hrt	DP	Commercial	9	This study
drc85/10/1/lg	2010	Ngafula	4^{O}21S /15^{o}14E	Kinshasa	Peri-urban	Lg	DP	Commercial	12	This study
drc85/10/2/h	2010	Ngafula	4^{O}21S /15^{o}14E	Kinshasa	Peri-urban	Hrt	DP	Commercial	9	This study
drc85/10/2/lv	2010	Ngafula	4^{O}21S /15^{o}14E	Kinshasa	Peri-urban	Lv	DP	Commercial	12	This study
drc86/10/kd	2010	Ngafula	4^{O}21S /15^{o}14E	Kinshasa	Peri-urban	Kd	DP	Commercial	12	This study
drc86/10/3	2010	Ngafula	4^{O}21S /15^{o}14E	Kinshasa	Peri-urban	Lv	DP	Commercial	12	This study
drc108/10/3	2010	Ngafula	4^{O}21S /15^{o}14E	Kinshasa	Peri-urban	Lv	DP	Commercial	12	This study

drc108/10/5	2010	Ngafula	4^{O}21S /15^{o}14E	Kinshasa	Peri-urban	Spl	DP	Commercial	12	This study
drc27/11/1	2011	Ngafula	4^{O}21S /15^{o}14E	Kinshasa	Peri-urban	Spl	DP	Commercial	sng	This study
drc27/11/2	2011	Ngafula	4^{O}21S /15^{o}14E	Kinshasa	Peri-urban	Ln	DP	Commercial	sng	This study
drc27/11/3	2011	Ngafula	4^{O}21S /15^{o}14E	Kinshasa	Peri-urban	Stm	DP	Commercial	23	This study
drc27/11/5	2011	Ngafula	4^{O}21S /15^{o}14E	Kinshasa	Peri-urban	Lv	DP	Commercial	23	This study
drc65/11/2	2011	Nsele	4^{O}24S/15^{o} 30E	Kinshasa	Peri-urban	Kd	DP	Commercial	sng	This study
drc65/11/3	2011	Nsele	4^{O}24S/15^{o} 30E	Kinshasa	Peri-urban	Spl	DP	Commercial	8	This study
drc65/11/4	2011	Nsele	4^{O}24S/15^{o} 30E	Kinshasa	Peri-urban	Lg	DP	Commercial	24	This study
drc96/12/1	2012	Mayanda	5^{o}12S /15^{o}12E	BasCongo	Rural	Lg	DP	village	16	This study
drc96/12/2	2012	Mayanda	5^{o}12S /15^{o}12E	BasCongo	Rural	Spl	DP	village	16	This study
drc96/12/3	2012	Mayanda	5^{o}12S /15^{o}12E	BasCongo	Rural	Hrt	DP	village	16	This study
drc108/10/1	Dec 2010	Ngafula	4^{o}21 S/15^{o} 14E	Kinshasa	Peri-urban	Lg	DP	Commercial	12	This study
drc46/11/2	Jun 2011	Kinshasa	4^{o}20 S/15^{o}18E	Kinshasa	City	Ht	DP	Backyard	sng	This study

p72 GENOTYPE IX

Virus	Outbreak	Location	GPS	Province	Ecological Profile	Tissues	Species	Farming System	CVR TRs	Reference
drc20/07/19	Apr 2007	Mahagi	2°S / 31°E	P. Orientale	Rift Valley	Kd	DP	Backyard	23	This study
drc20/07/20	Apr 2007	Mahagi▪	2°S / 31°E	P. Orientale	Rift Valley	Ln	DP	Backyard	23	This study
drc25/08/3/k	Mar 2008	Boende	0°15 S/ 21° 01 E	Equateur	Forest	Kd	DP	Free ranging	24	This study
drc25/08/s	Mar 2008	Boende	0°15 S/ 21° 01 E	Equateur	Forest	Spl	DP	Free ranging	24	This study
drc25/08/4/k	Mar 2008	Boende	0°15 S/ 21° 01 E	Equateur	Forest	Kd	DP	Free ranging	24	This study
drc25/08/9	Mar 2008	Boende	0°15 S/ 21° 01 E	Equateur	Forest	Spl	DP	Free ranging	24	This study
drc35/08/1	Apr 2008	Boende	0°15 S/ 21° 01 E	Equateur	Forest	Spl	DP	Free ranging	24	This study
drc35/08/13	Apr 2008	Boende	0°15 S/ 21° 01 E	Equateur	Forest	Spl	DP	Free ranging	24	This study
drc35/08/P4$_2$	Apr 2008	Boende	0°15 S/ 21° 01 E	Equateur	Forest	Spl	DP	Free ranging	24	This study
drc35/08/15	Apr 2008	Boende	0°15 S/ 21° 01 E	Equateur	Forest	Spl	DP	Free ranging	24	This study
drc35/8/18/k	Apr 2008	Boende	0°15 S/ 21° 01 E	Equateur	Forest	Kd	DP	Free ranging	24	This study
drc35/8/20	Apr 2008	Boende	0°15 S/ 21° 01 E	Equateur	Forest	Spl	DP	Free ranging	24	This study

drc35/08/3	Apr 2008	Boende	0°15 S/ 21° 01 E	Equateur	Forest	Spl	DP	Free ranging	24	This study
drc66/7/43	Nov 2007	Yakoma	4° S / 22° E	Equateur	Forest	Lg	DP	Free ranging	24	This study
drc66/7/48	Nov 2007	Yakoma	4° S / 22° E	Equateur	Forest	Spl	DP	Free ranging	24	This study
drc66/7/49$_1$	Nov 2007	Yakoma	4° S / 22° E	Equateur	Forest	Spl	DP	Free ranging	24	This study
drc66/7/49$_2$	Nov 2007	Yakoma	4° S / 22° E	Equateur	Forest	Ln	DP	Free ranging	24	This study
drc66/7/50	Nov 2007	Yakoma	4° S / 22° E	Equateur	Forest	Spl	DP	Free ranging	24	This study
drcKG31208/3	Dec 2008	Lingwala	4°20 S/ 15° 19 E	Kinshasa	City	Spl	DP	Backyard	24	This study

p72 GENOTYPE XIV

Virus	**Outbreak**	**Location**	**GPS**	**Province**	**Ecological profile**	**Tissues**	**Species**	**Farming system**	**CVR p602L gene**	**Reference**
drc21/07/22	2007	Kipushi●	12 ° S / 28 ° E	Katanga	City	Spl	DP	Backyard	13	This study
drc35/10/3	Apr 2010	Ngaliema	4°21S/15° 05E	Kinshasa	City	Kd	DP	Backyard	51	This study

Legend: **sng**-sequence not generated; ▪ - Uganda border; ●-Zambia border; **Ln**-lymph node; **Hrt**– Heart; **Spl**– Spleen; **Kd**-Kidney; **Lg**-Lung; **Lv**-Liver; **Stm**-Stomach; **GPS**-Global Positioning System; **CVR TRs**-Central hypervariable region-Tetrameric tet-type; **DP**-Domestic pig.

Generation of ASFV DNA sequence data

DNA was extracted as detailed above and target gene fragments were amplified separately using PCR protocols outlined below. The C terminal end of the B646L (p72) gene was amplified using primers p72D and p72U as previously described (Bastos et al., 2003), using the Taq DNA polymerase kit (Qiagen) and a 50µl volume in the presence of 0.25mM dNTPs, 0.5µM of each primer, 1X buffer, 2.5U Taq DNA polymerase and 5µl of DNA extract. The thermal profile included an initial denaturation at 95° C for 5 min, followed by 35 cycles of 95° C, 30 sec, 54° C, 30 sec, 72° C, 1 min, and a final elongation step at 72° C for 5 min. The CVR locus was amplified using primers, ORF9RLW_F and ORF9RLW_R (Nix, 2006), using the protocol above with a change in annealing temperature to 55° C. The full E183L (p54) gene was amplified using primers ASFlongP54F, and ASFlongP54R using a touchdown PCR protocol as follows, 95° C for 5 min, followed by 15 cycles of 95° C, 30 sec, 60° C, 30 sec, 72° C, 1 min, then 25 cycles of 95° C, 30 sec, 58° C, 30 sec, 72° C, 1 min, with a final extension or 72° C for 5 min. PCR products were purified using Wizard ® SV Gel and PCR Clean Up System kit, according to manufacturers' protocol (Promega Corporation, USA). Purified PCR products were submitted to LGC Genomics (Berlin, Germany) with amplification primers, for Sanger sequencing. Raw sequences were assembled and edited using Vector NTI 11.5 Software (Life Technologies). Sequences were then aligned with GenBank reference sequences using MEGA (Version 5.2) or BioEdit (Version 7.2.3; Hall 1999) using the Clustal W method.

Phylogenetic inference of ASFV sequence data

Separate multiple sequence alignments of both the p54 and p72 loci were generated in MEGA6 (Tamura et al., 2013) using default values of the 'by codon' option with the Clustal W algorithm. The p54 alignment required additional manual alignment as a result of indels. The p72 alignment was 404 bp in length and contained 118 sequences. Of these 118 sequences, 62 were generated for this study and 56 were reference sequences representing at least one isolate for each of the 22 previously described genotypes. The p54 alignment was 657 bp in length and included 83 sequences, 34 were generated for this study, and 49 were reference sequences of previously reported genotypes. Of the 22 reported genotypes, all but three were represented (XI, XII, and XVIII). The most appropriate model of molecular evolution was determined by the Akaike Information Criterion (AIC) using MEGA6 (Tamura et al., 2013). Maximum likelihood (ML) analyses with 1000 bootstrap replicates were

performed using the program MEGA6 with the predetermined model of molecular evolution (GTR+I+G for both datasets) using all sites.

CVR locus typing

Grouping of amino acids into tetramers at this locus has been utilized by other researchers, therefore the coding of tetramers followed methods outlined previously (Boshoff et al., 2007; Lubisi et al., 2007; Misinzo et al., 2011; Misinzo et al., 2014; Nix, 2006). Labelling of length polymorphisms also followed the previous designation (Owolodun, 2010). CVR sequences were first translated into amino acids then converted to the tetramer code using a Microsoft excel based analysis. The amino acid tetramer code is provided in **Table 2**.

RESULTS

Clinical findings and ASF diagnosis

Field identification and description of collecting localities. Fifty-four out of the 57 sampled pigs exhibited many of the following symptoms: hemorrhagic edema, enlargement of spleen and some internal lymph nodes, hydropericarditis, hydrothorax, ascites, as well as skin redness and petechiae. The 3 remaining pigs sampled in the markets appeared superficially healthy, but presented with enlarged, congestive or hemorrhagic spleens and/or gastrohepatic lymph nodes. Massive mortality, including a minimum weekly loss of 5 pigs were documented in the 25 sampled localities. Four of the 25 sampling sites, including the cities of Yakoma, Boende, Mahagi and Kipushi, recorded indigenous pigs of local breeds, primarily free ranging to be the most commonly lost. The remaining 21 locations were commercial farms raising improved breeds of pigs in backyards or securely fenced areas, minimizing intermingling of wildlife and domestic suids.

Laboratory diagnostics. Extraction, amplification, and subsequent electrophoresis of genomic DNA extracted from various suid tissues identified 62 samples (100 %) as positive for ASFV DNA. All 62 isolates were sequenced for locus 1 (c-terminus of the p72 gene), 56 were additionally sequenced at both locus 2 (p54 full genome) and locus 3 (CVR) (**Table 1**).

Table 2. CVR locus-based intra-genotype resolution (Amino-acid tetramer codes as labeled in previous studies: Boshoff et al., 2007; Lubisi et al., 2007; Nix et al., 2006; Misinzo et al., 2011; Misinzo et al., 2014)

Isolate	Location	Province	Year	Genotype	Tetrameric repeats	TRs
drc35/10/p1	Ngaliema	Kinshasa	2010	I	AAAAAAAAAAAAAAAAAAAAAAAAAAAAABNABNBTDBNAAAAAAAAAAAF	51
drc281108p5	Ngaliema	Kinshasa	2010	I	AAAAAAAAABNABNBTABNAAAAAAAAAAAAAAAAAAAAAAAAAF	45
drc65/11/p4	Nsele	Kinshasa	2011	I	AAAAAABNABNBTDBNAAAAAAAAF	25
drc74/09/p2	Nsele	Kinshasa	2009	I	AAAAAAAAAAAAAAAAAAAAAF	23
drc74/09/p3	Nsele	Kinshasa	2009	I	AAAAAAAAAAAAAAAAAAAAAF	23
drc74/09/p4	Nsele	Kinshasa	2009	I	AAAAAAAAAAAAAAAAAAAAAF	23
drc74/09/p6	Nsele	Kinshasa	2009	I	AAAAAAAAAAAAAAAAAAAAAF	23
drc94/09/p2	Kintambo	Kinshasa	2009	I	AAAAAAAAAAAAAAAAAAAAAF	23
drc27/11/p3	Ngafula	Kinshasa	2011	I	AAAAAAAAAAAAAAAAAAAAAF	23
drc27/11/p5	Ngafula	Kinshasa	2011	I	AAAAAAAAAAAAAAAAAAAAAF	23
drccokg28408p2	Kasavubu	Kinshasa	2008	I	AAAAAAAAAAAAAAF	16
drc96/12/p1	Mayanda	Bas Congo	2012	I	AAAAAAAAAAAAAAF	16
drc96/12/p2	Mayanda	Bas Congo	2012	I	AAAAAAAAAAAAAAF	16
drc96/12/p3	Mayanda	Bas Congo	2012	I	AAAAAAAAAAAAAAF	16
drc99/05	Ngafula	Kinshasa	2005	I	AAAAAAAAAAAAF	14
drc51/10/p23	Ndjili	Kinshasa	2010	I	AAAAAAAAAAF	12
drc85/10/p13	Ngafula	Kinshasa	2010	I	AAAAAAAAAAF	12
drc85/10/p27	Ngafula	Kinshasa	2010	I	AAAAAAAAAAF	12
drc86/10/p3	Ngafula	Kinshasa	2010	I	AAAAAAAAAAF	12
drc86/10/p1	Ngafula	Kinshasa	2010	I	AAAAAAAAAAF	12
drc108/10/p1	Ngafula	Kinshasa	2010	I	AAAAAAAAAAF	12
drc108/10/p3	Ngafula	Kinshasa	2010	I	AAAAAAAAAAF	12
drc108/10/p5	Ngafula	Kinshasa	2010	I	AAAAAAAAAAF	12
drc85/10/p12	Ngafula	Kinshasa	2010	I	AAAAAAAAAF	11
drc73/10/p2	Ngaliema	Kinshasa	2010	I	AAAAAAAAAF	11
drc73/10/p3	Ngaliema	Kinshasa	2010	I	AAAAAAAAAF	11
drc73/10/p4	Ngaliema	Kinshasa	2010	I	AAAAAAAAAF	11
drc49/05/p2	Limete	Kinshasa	2005	I	AAAAAAAAAF	10
085/10/p25	Ngafula	Kinshasa	2010	I	AAAAAAAAF	9
drc75/05	Maniema	Kindu	2005	I	AAAAAAAAF	9
drc49/05/p1b	Limete	Kinshasa	2005	I	AAAAAAAAF	9
drc65/11/p3	Nsele	Kinshasa	2011	I	AAAAAAAF	8
070/05	Limete	Kinshasa	2005	I	AAAAAF	6
drc49/05/p1a	Limete	Kinshasa	2005	I	AAAAF	5
Isolate	**Location**	**Province**	**Year**	**Genotype**	**Tetrameric repeats**	**TRs**
drc66/07/49$_1$	Yakoma	Equateur	2007	IX	AAA-BB AABBN ABBAABBN ABN ABA	24a
drc66/07/p43	Yakoma	Equateur	2007	IX	AAA-BN ABBBN ABBAABBN ABN ABA	24b
drc66/07/p50	Yakoma	Equateur	2007	IX	AAA-BN ABBBN ABBAABBN ABN ABA	24b
drc66/07/49$_2$	Yakoma	Equateur	2007	IX	AAA-BN ABBBN ABBAABBN ABN ABA	24b
drc66/07/p48	Yakoma	Equateur	2007	IX	AAAABN ABB-N ABBAABBN ABN ABA	24c
drc35/08/p4$_2$	Boende	Equateur	2008	IX	AAAABN ABB-N ABBAABBN ABN ABA	24c
drc35/08/p18	Boende	Equateur	2008	IX	AAAABN ABB-N ABBAABBN ABN ABA	24c
drc35/08/p13	Boende	Equateur	2008	IX	AAAABN ABB-N ABBAABBN ABN ABA	24c
drc35/08/p3	Boende	Equateur	2008	IX	AAAABN ABB-N ABBAABBN ABN ABA	24c
drc35/08/p20	Boende	Equateur	2008	IX	AAAABN ABB-N ABBAABBN ABN ABA	24c
drc35/08/p1	Boende	Equateur	2008	IX	AAAABN ABB-N ABBAABBN ABN ABA	24c
drc35/08/p15	Boende	Equateur	2008	IX	AAAABN ABB-N ABBAABBN ABN ABA	24c
drc25/08/p3a	Boende	Equateur	2008	IX	AAAABN ABB-N ABBAABBN ABN ABA	24c
drc25/08/p9	Boende	Equateur	2008	IX	AAAABN ABB-N ABBAABBN ABN ABA	24c
drc25/08/p4$_2$	Boende	Equateur	2008	IX	AAAABN ABB-N ABBAABBN ABN ABA	24c
drc25/08/p3b	Boende	Equateur	2008	IX	AAAABN ABB-N ABBAABBN ABN ABA	24c
cokg031208p3	Lingwala	Kinshasa	2008	IX	AAAABN ABB-N ABBAAABN ABN ABA	24d
drc20/07/p19	Mahagi	P.Orientale	2007	IX	AAA-BN ABB-N ABBAABBN ABN ABA	23a
drc20/07/p20	Mahagi	P.Orientale	2007	IX	AAA-BN ABB-N ABBAABBN ABN ABA	23a
drc86/10/p2	Ngafula	Kinshasa	2010	IX	AAAAAAAAAAAF	12
drc35/10/p3	Ngaliema	Kinshasa	2010	XIV	AAAAAAAAAAAAAAAAAAAAAAAAAAAAABNABNBTDBNAAAAAAAAAAAF	51
drc21/07/p22	Kipushi	Katanga	2007	XIV	AVVOVAVVNBVOV	13

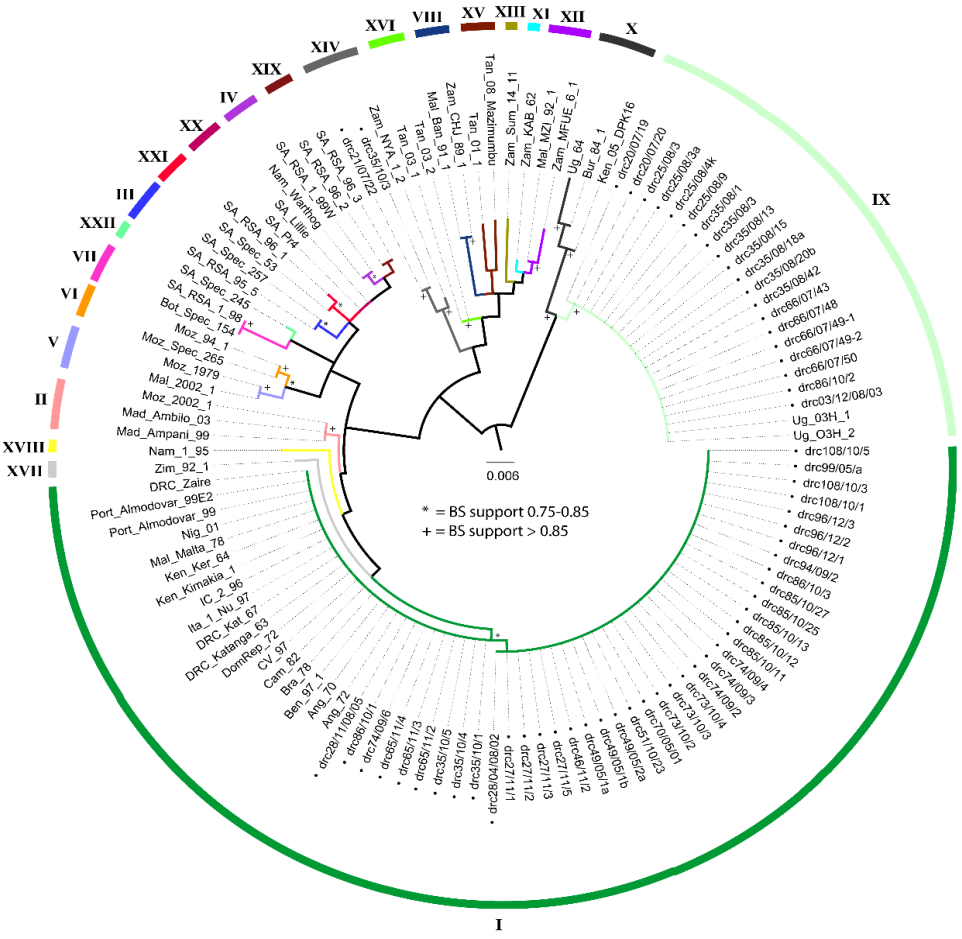
Legend: The tetramer code is the sequence of four amino acids: A=CAST, CVST, CTST, or CASI; B=CADT, CADI, CTDT, or CAGT; C=GAST or GANT; F=CANT or CAAT; N=NVDT, NVGT, NVDI, or NCDT; T=NVNT; H=RAST; S=SAST; O=NANI, NADI, or NASI; V=NAST, NAVT, NADT, or NANT; D=CASM; G=CTNT; M=NEDT; W=SADT or SVDT; U=NIDT or NTDT.

Phylogenetic inference of ASFV sequence data

Phylogenetic analyses of the C-terminal end of the p72 gene revealed that the newly sequenced ASFV isolates which circulated in the DRC from 2005 to 2012 clustered into three p72 genotypes, I, IX and XIV (**Figure 1**). Of the 62 newly sequenced isolates, 40 (64.5%) grouped with isolates previously identified as belonging to genotype I, including three published isolates from DRC (Katanga_63, Kat_67, and Zaire_77); 20 isolates (32.3 %) formed a monophyletic group with recognized genotype IX isolates , and 2 (3.2 %) were determined to belong to genotype XIV. This is the first report of genotypes IX and XIV circulating in the DRC. Although isolates generated herein grouped with high bootstrap support (>75%) with reference samples of the respective genotype, support for inter-genotypic relationships and intra-genotypic resolution varied. Isolates within genotype I grouped into two polytomies; however, the bootstrap support separating the two polytomies was low. Genotype IX samples all formed a single highly supported polytomy, and topology of genotype XIV samples was highly resolved with the two new isolates grouping sisters with high support, followed by the genotype XIV reference sequence.

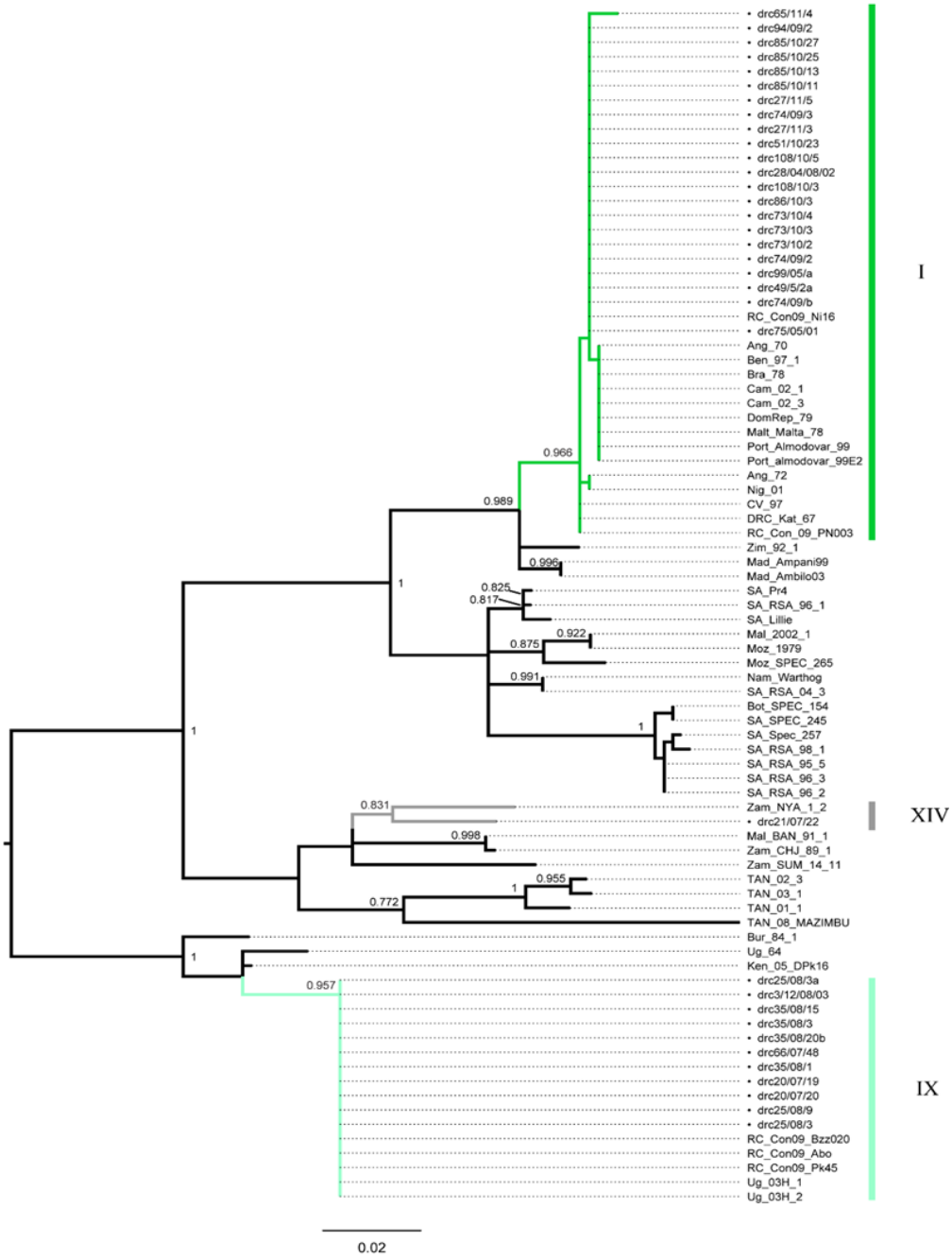
Phylogenetic analyses of the p54 gene sequences recovered the same groupings of new sequences with respective genotypes as the p72 analyses (**Figure 2**). New samples identified by the p72 analysis as genotypes I, IX, and XIV grouped with reference sequence of the respective genotype with high bootstrap support ($\geq$83%). Although monophyletic groupings of newly sequenced isolates corroborated the p72 analyses, the p54 analysis failed to recover monophyletic assemblages for reference isolates belonging to the following genotypes: III, VII, X, XV, and XX. Discrepancies between markers is not uncommon, and monophyly of genotypes XV and XX was not highly supported in the p72 analysis; however, high bootstrap support was recovered for monophyletic clades of genotypes III, VII, and X in the p72 analyses. The intergenotypic relationships were poorly resolved for both the p54 and p72 datasets, as was the intragenotypic resolution of isolates given the low level of genetic variability at these loci; however, more genetic variability was detected at the p54 locus, as is evidenced by the increased structure within the genotype I clade.

Figure 1. p72 Genotype phylogenetic tree



Legend: Maximum likelihood tree depicting genetic relationships of isolates examined in this study utilizing the p72 locus. Bootstrap support (BS) is indicated by * for values between 75-85%, and by + for values greater than 85% support. The scale bar indicates the number of nucleotide substitutions per site. Each color is a different genotype, indicated by the corresponding roman numerals. Labels for all isolates generated by this study begin with •drc. Isolates are named in the following manner: Country Strain Name. Acronyms used for countries of origin are as follows: Ang=Angola, Ben=Benin, Bot=Botswana, Bra=Brazil, Bur=Burundi, Cam=Cameroon, CV=Cape Verde, DomRep=Dominican Republic, DRC=Democratic Republic of Congo, IC=Ivory Coast, Ita=Italy, Ken=Kenya, Mad=Madagascar, Mal=Malawi, Malt=Malta, Moz=Mozambique, Nam=Namibia, Nig=Nigeria, Port=Portugal, SA=South Africa, Tan=Tanzania, Ug=Uganda, Zam=Zambia, Zimb=Zimbabwe.

Figure 2. p54 gene Genotypes Tree



Legend: Maximum likelihood tree depicting genetic relationships of examined isolates using the p54 locus (*E183L* gene). The scale bar is indicating the of nucleotide substitutions per site. The colored clades indicate genotypes represented by isolates sequenced in this study. Labels for all isolates generated by this study begin with •drc. Isolates are named in the following manner: Country_StrainName. Acronyms used for countries of origin are as follows: Ang=Angola, Ben=Benin, Bot=Botswana, Bra=Brazil, Bur=Burundi, Cam=Cameroon, CV=Cape Verde, DomRep=Dominican Republic, DRC=Democratic Republic of Congo, IC=Ivory Coast, Ita=Italy, Ken=Kenya, Mad=Madagascar, Mal=Malawi, Malt=Malta, Moz=Mozambique, Nam=Namibia, Nig=Nigeria, Port=Portugal, RC=Republic of Congo, SA=South Africa, Tan=Tanzania, Ug=Uganda, Zam=Zambia, Zimb=Zimbabwe.

CVR locus typing

CVR locus variability within this dataset was first demonstrated by the 15 discernable amplicon lengths. Sequence analysis of the same locus allowed further discrimination, and recognized 19 unique nucleotide sequences, which were translated into amino acid sequences, and subsequently coded as amino acid tetramers (tet-types), 13 tet-types were detected within isolates identified as belonging to p72 genotype I, six from isolates belonging to genotype IX, and two within genotype XIV isolates (**Table 2**). All isolates grouped within a tet-type contained identical CVR nucleotide sequences, therefore no resolution was lost by converting nucleotide sequences to tetrameric repeat sequences. Tet-types 12 and 51 were both detected in p72 genotype I isolates, but tet-12 was also found in a p72-IX isolate (drc86/10/p2), and tet-51 was also found in p72-XIV isolate (drc35/10/p3).

DRC ASFV genotypes geographical distribution

ASF isolates were identified in the provinces of Bas-Congo, Equateur, Katanga, Kinshasa, Maniema, and Orientale (**Figure 5**). Genotype I isolates were detected in Bas-Congo, Kinshasa and Maniema, whereas genotype IX isolates recovered from Equateur, Kinshasa, and Oriental provinces. Genotype XIV was detected from Katanga and Kinshasa. All the three genotypes were identified in Kinshasa, the capital, which is the most populous city in the country (**Figure 4**). Kinshasa contained the largest number of genotypes (3) as well as tet-types (16; 13, 2, and 1 for tet-types I, IX, and XIV, respectively) (**Table 2**; **Figures 3 and 4**). Regarding the five remaining provinces: Equateur presented four tet-types (genotype IX); Bas-Congo and Maniema each had single tet-types (genotype I); and Katanga contained a single tet-type (genotype XIV); Oriental contained a single tet-type (genotype IX).

Figure 3. Spatial distribution of p72 genotypes and variants of the DRC ASFV isolates recovered in this study: Kinshasa City Province and country

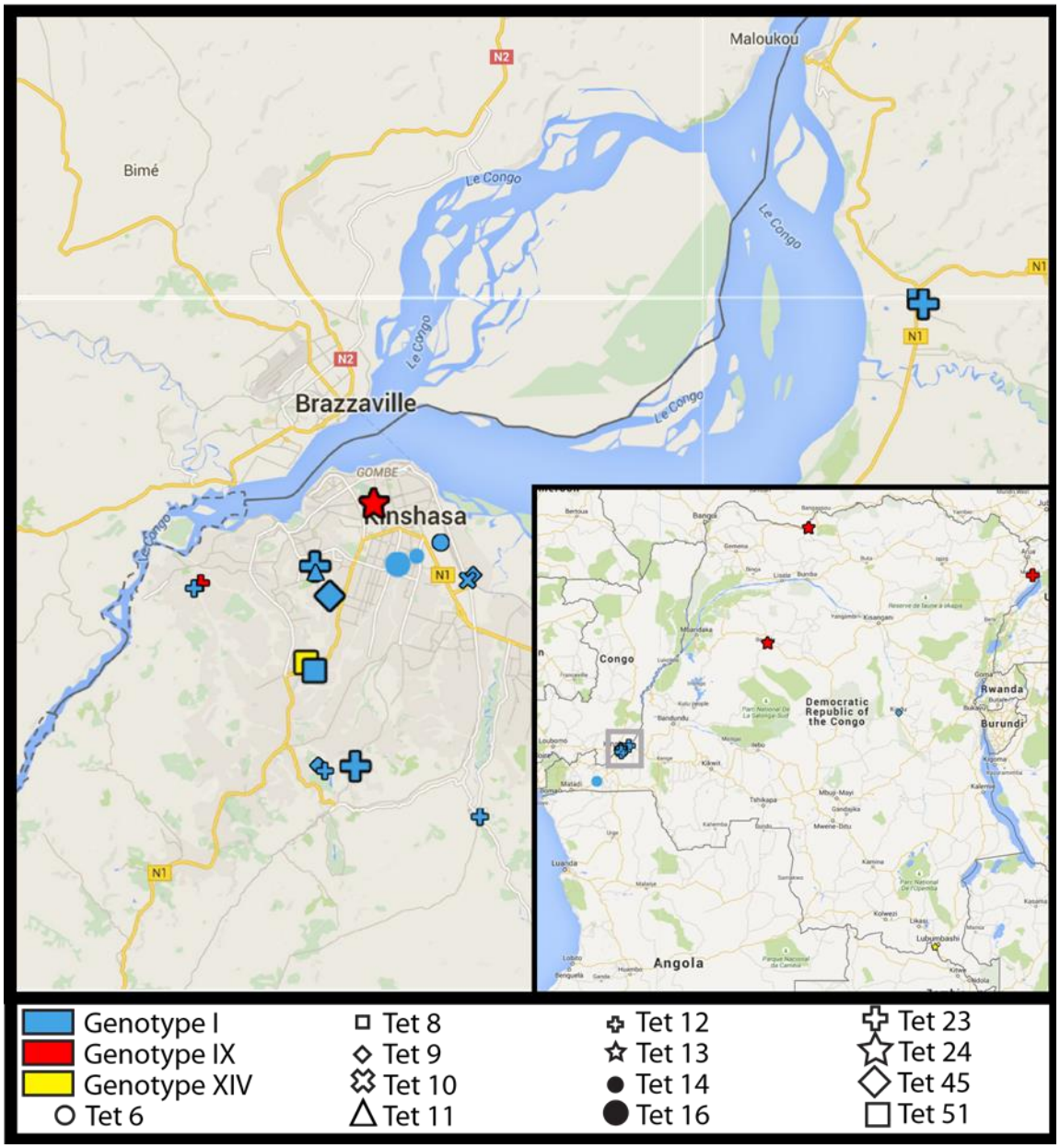


Figure 4. Localization of ASFV p72 genotypes revealed in this study within the Kinshasa City Province

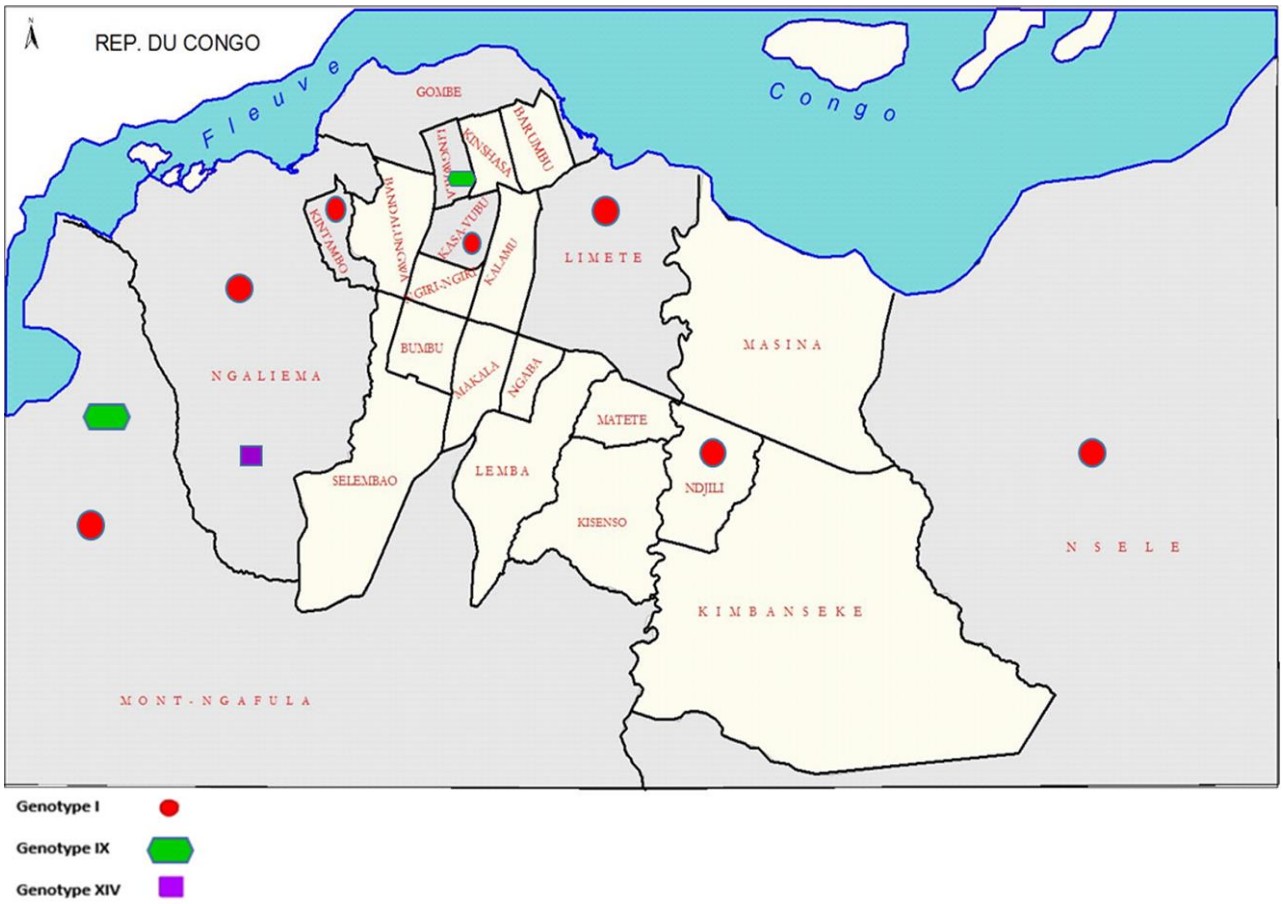


Figure 5. Provincial localization of revealed p72 Genotypes and presumable links with some ASFV historical isolates in the neighboring countries

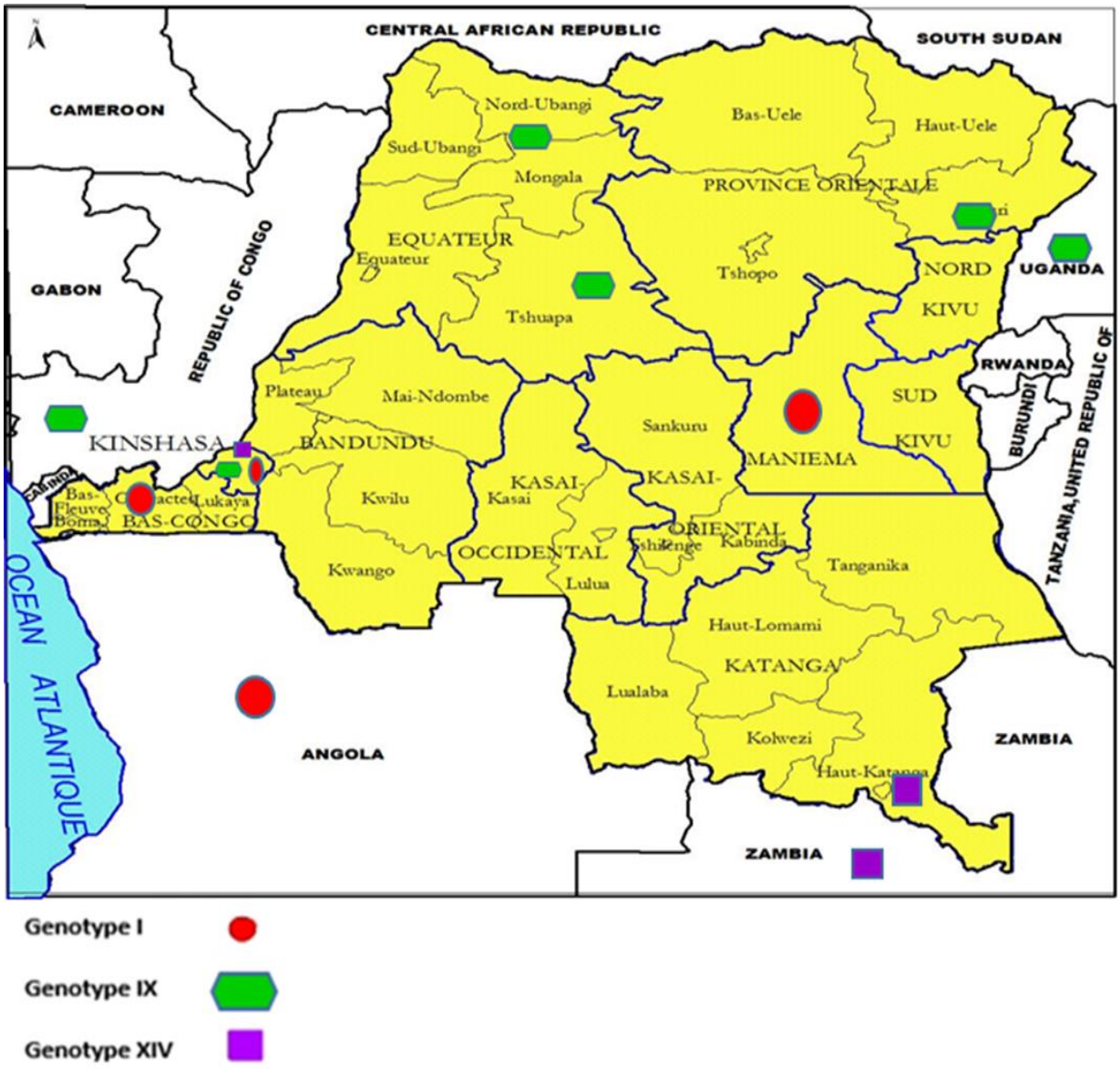
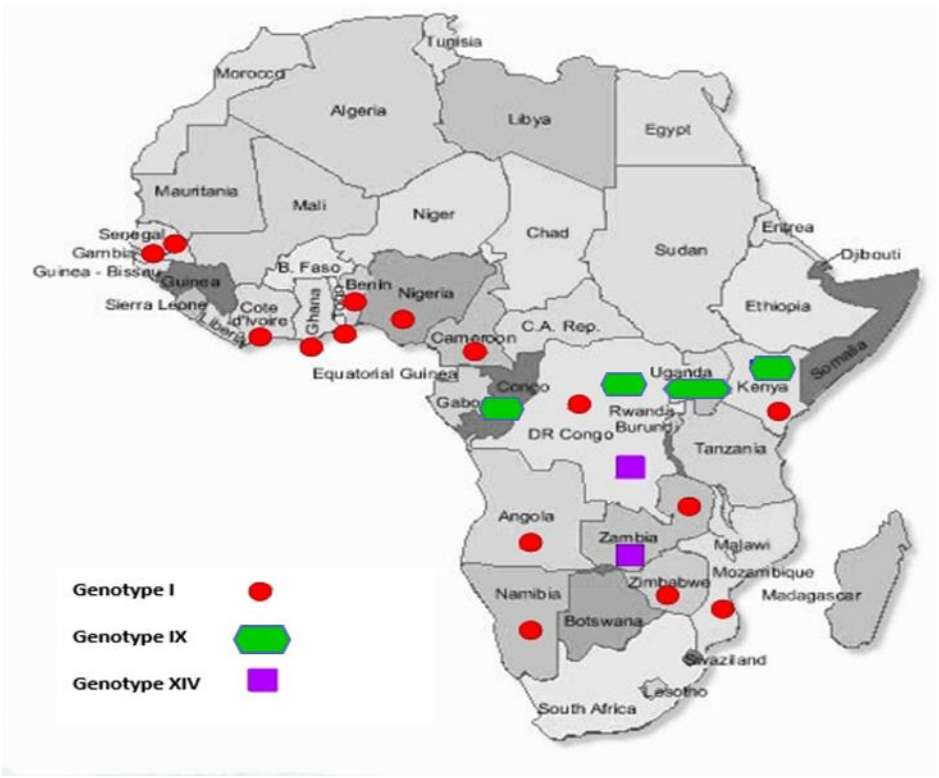


Figure 6. Continental distribution of the three p72 genotypes revealed by this study in the DRC. Position of symbols represents presence of genotype within the country, and are not indicative of any specific geographic localities (modified from Lubisi et al., 2005)



DISCUSSION

Molecular Characterization

This was the first extensive molecular evaluation of circulating ASFV genotypes in the DRC. Previous knowledge was based on publication of data from single samples submitted for ASF diagnosis (Bastos et al., 2003, Boshoff et al., 2007, Ekwe and Wilkinson 2000, Lubisi, 2005, 2007, Mulumba-Mfumu, 2015, 2013; Nix, 2006). Little was known about the dynamics of ASFV genotypes and strains circulating in the largest country of SSA. Analysis of the p72 locus identified three genotypes (I, IX and XIV) circulating in DRC. Forty of 62 (64.5 %) clustered with DRC historical isolates (DRC_Kat63 and DRC_Kat67), as well as other isolates from the often named ESAC-WA genotype, which has been involved in ASF outbreaks in Europe, South America, Caribbean, as well as Central and West Africa (often referred to as the ESAC-WA genotype), confirming that genotype I is the most prevalent in the country, as previously reported (Bastos et al., 2003, Lubisi 2005). Additionally, DRC isolates of this genotype exhibited limited genetic variability, which has been repeatedly documented within the ESAC-WA clade, as it was hypothesized to be maintained predominantly by the domestic pig cycle (Lubisi, 2005). The p72 genotype IX was represented by 20 isolates (32.3 %) with little or no genetic variation detected at this locus. Previously undocumented within the DRC, Genotype IX is much more geographically restricted, as it is endemic to East and Central Africa, having been reported previously in Republic of Congo, Uganda, and Kenya where it is involved in sylvatic and domestic cycles (Lubisi et al., 2005; Gallardo et al., 2011). The third genotype detected herein, genotype XIV, was also not previously reported in the country prior to the two (3.2%) isolates reported herein. Genotype XIV, similarly to genotype IX has a much more geographically restricted distribution, and was previously only reported from Zambia, where it was originally isolated from a tick of the genus *Ornithodoros* in 1986 (Lubisi et al., 2005). The presence of the same genotype in domestic pig in DRC suggests transmission of Genotype XIV ASFV isolates between sylvatic and domestic cycles; however it cannot be determined given the available data, whether this transition between cycles occurred in Zambia or DRC, and which reservoir (ornithodoros tick, domestic or feral suids) was responsible for importing this strain into DRC. In regards to distribution of ASFV isolates within DRC recovered in this study, isolates of all genotypes were found near the eastern and western borders, and confirmed localities for all the three genotypes occurred in the southern half of the country; however,

only isolates of genotype IX were collected from localities in the northern portion of the DRC (**Figures 3 and 5**). The presence of all three genotypes within the Kinshasa province is likely a result of the pig shipments, as pork sold at markets in Kinshasa have been documented to originate from multiple regions of the country, including the provinces of Equateur, Bandundu, and Bas-Congo (Praet et al., 2010). Once again, at the regional level, as reported in previous studies based on geographical distribution of historical isolates, genotype I is likely the most dominant in West and Central Africa (**Figure 4**) **(**Lubisi et al., 2005; Bastos et al., 2003; Ekwe and Wilkinson, 2000).

Although the p54 gene was previously determined to be a valuable locus for fine levels of discrimination (Atuhaire et al., 2014; Gallardo et al., 2011; Giammarioli et al., 2011), no additional resolution was gained using the p54 gene in this study; however, the p54 locus did corroborate the topology generated by the p72 analysis. Given the sampling scheme of this study (domestic suids only), failure to achieve higher resolution may be due to the low genetic variation consistently detected within the domestic pig cycle, when compared to the higher levels of variation previously detected within the sylvatic cycle (Lubisi et al., 2005).

The CVR locus was capable of providing further resolution of isolates than either the p72 or p54 locus. The pattern of genetic variability at this locus varied dependent upon the p72 genotype examined. Within p72 genotype I isolates, genetic variation was primarily a result of variability in the number of tetramers as previously reported (Goller 2015), specifically the tetrameric amino acid repeats CAST and CVST coded as ‘A’. Also of interest within genotype I isolates, is that tet-types 25, 45, and 51 (also found in one genotype XIV isolate) contained a conserved sequence of 10 tetramers 'BNABNDT[D/A]BN’ not found in other tetrameric sequences. This sequence was flanked by a variable number of ‘A’ coded tetramers. CVR tet-types of p72 genotype IX consisted primarily of a more complex sequence than genotype I isolates, with the exception of tet-type 12 (common to genotype I isolates) being documented in one genotype IX isolate (drc86/10/p2). Of the two tet-types detected in genotype XIV isolates, the first (tet-type 51) was identical to a tetrameric repeat sequence found in a genotype I isolate, and the second was extremely different than any other tet-type reported herein (**Table 2**). Several CVR tet-types are discussed below in reference to detection of multiple strains within outbreaks, and potential links between outbreaks based on this locus.

Disease Ecology, Molecular Epidemiology and Case Investigations

The higher resolution offered by the CVR locus allowed for the three genotypes to be further broken down into 19 tet-types. The high level of variability previously noted at this locus (Irusta et al., 1996; Lubisi et al., 2005; Bastos et al., 2004) would suggest that highly similar/identical tet-types are more closely related than more divergent tet-types; however, given the nature of nucleotide repeat regions, such as the CVR locus, identical sequences can occur due to homoplasy. With these possibilities in mind, a number of putative outbreak connections are proposed and discussed below, as are details of co-circulation of multiple variants (genotypes and tet-types).

The detection of genotype I, tet-type 23 isolates during the 2009 outbreaks at Nsele and Kintambo, as well as the 2011 outbreak in Ngafula suggests that these geographically proximal outbreaks were caused by closely related strains, even though they occurred over a two year time span. Continued, undetected circulation of a single strain during the nearly two year lapse between these outbreaks could be facilitated by the asymptomatic infection of domestic pigs in the area. A second putative association between outbreaks was made after examination of samples from March to April (drc25/08 & drc35/08 isolates) in the 2008 outbreak in Boende, as genotype IX tet-type 24c isolates were detected in both outbreaks, suggesting these isolates share a recent common ancestor. A third potential connection is suggested by analysis of isolates from the Mahagi outbreak in April 2007 (near the Ugandan border), as they were of identical genotype (IX) and tet-type (23a) to isolates Uganda03H1, Kenya 06B1, and Kenya06Bus examined in a previous study (Gallardo et al., 2009).

A fourth connection stems from an isolate of a symptomless pig sampled at the Kinshasa market (drccokg28408p2) in 2008. This isolate belonged to the same genotype (I) and tet-type (16) as samples isolated from the 2012 outbreak in Mayanda, Bas-Congo province. The geographic proximity of these 2 provinces, in combination with the high level of commercial traffic between them could easily result in transfer of ASF from one province to the other (Sanchez-Vizcaino, 2014; Misinzo et al., 2011). Again, either the ability of ASF to persist in asymptomatic pigs (domestic or feral) or the sylvatic cycle (including ticks) could explain how closely related isolates were responsible for outbreaks separated by four years, as Mayanda is rural locality, where there is a high potential for interaction between domestic and feral suids. A fifth potential outbreak link was made apparent upon examination of genotype IX isolates collected from outbreaks in Yakoma (April 2007) and Boende (March and April 2008; see Table 2). Tet-type 24c isolates were detected in both outbreaks, which severely affected both feral and domestic pigs (Plowright et al., 1994; OIE, 2014). The Boende

outbreak was estimated to have caused more than 4500 suid fatalities (Central Veterinary Laboratory, 2010). Both Yakoma and Boende are forested sites, located in North Ubangi and Tshuapa Districts of the Equateur Province, respectively. The forest environment and free ranging animal husbandry practices enable interaction between domestic and wild suids, potentially allowing for crossover between infection cycles and occurrence of multiple strains. The presence of tet-type 24c at both outbreaks could represent a link between these geographically distant outbreaks.

Despite the large number of geographically and/or temporally separated outbreaks for which genetic evidence suggests potential recent common ancestry, other temporally separated outbreaks from a single locality, such as the 2009 and 2011 outbreaks at Nsele display evidence of infection by divergent tet-types (tet-type 23 in 2009 and tet-type 25 in 2011). Co-circulation of two ASFV tet-types (drc49/05/p2 [Tet-10] and 49/05/p1a [Tet-5]) was observed in the 2005 outbreak in the Interchix commercial Farm in Limete, and three tet-types (24a-c) of genotype IX were isolated from the outbreak in Yakoma in April of 2007. Additionally, co-circulation of genotype I isolates drc85/10/p25 (tet-9), drc85/10/p12 (Tet-11), drc85/10/13 (Tet-12), and drc85/10/27 (Tet-12), representing three tet-types, was detected in Sebo farm, during the 2010 outbreak in Ngafula. Further, evidence of co-circulation of both tet-types and genotypes was detected at multiple localities. Isolates from the Ngafula outbreak in 2010 included genotypes I (tet-types 9, 11, 12) and IX (tet-type 12), indicating that multiple variants can occur within a single outbreak, and that divergent genotypes can possess identical tet-types. Another instance of multiple genotypes with identical tet-types occurred in the Ngaliema 2010 outbreak, where tet-type 51 was found in two isolates: drc35/10/p1 (genotype I) & drc/35/10/p3 (genotype XIV). Co-infection of different tet-types was even documented within the same pig, when two genotype I isolates, drc49/05/p1a (tet-type 5) and drc49/05/p1b (tet-type 9), were extracted from the spleen and lymph node respectively, of a pig in 2005 from Kinshasa. In a similar study conducted in Nigeria, individual co-infection was not observed (Bastos et al., 2004; Owolodun et al., 2010).

Understanding the details of ASFV circulation across DRC is clearly complex. The co-circulation of genotypes and tet-types, as well as co-infection of a pig by different tet-types suggests a high level of genetic variation; however, detection of conserved tet-types across large geographic areas, and over multiple years suggests current markers can provide insight into the movement of ASFV isolates. Unfortunately, connecting cases is not straight forward, as anthropogenic factors, such as trade of pigs and animal husbandry practices (Eldelsten and

Chinombo, 1995), i.e., intermingling of feral and domesticated suids, likely influence the phylogeographic signal of ASF. Further, presence of identical tet-types, as identical amino acid tetramers could be caused by homoplasy, retention of ancestral polymorphisms, or recombination. Retention of ancestral alleles is perhaps the least likely of the three scenarios, given the highly variable nature of the CVR locus. Homoplasy is plausible given the nature of tandemly repeated sequences, and recombination has previously been hypothesized to occur in vivo, potentially generating indels (Smith 1976; Blasco et al., 1989). The two examples presented herein (Ngaliema 2010 & Ngafula 2010 outbreaks in Kinshasa province), in which identical tet-types were found within divergent (more conservative) genotypes may be explained by homoplasy at the CVR locus; however, the co-circulation of multiple genotypes within an outbreak and co-infection of multiple tet-types within a single pig lend credibility to the hypothesis that recombination could explain highly similar CVR sequences (at a more variable locus) between highly divergent p72 genotypes (a more conserved locus). Whole genome examination of these isolates may provide a more definitive understanding of relationships.

As the DRC is the largest country in SSA, and borders nine countries, understanding the prevalence and distribution of ASFV genotypes within the DRC is an important step in better understanding large scale patterns of ASF. Given the high degree of similarity, at all examined loci, between genotype IX isolates collected in Mahagi, DRC (presented herein), and Uganda isolates of a previous study (Gallardo et al., 2009), it appears the distribution of this strain spans the border between DRC and Uganda, suggesting this strain has been transmitted across political boundaries by movement of either feral or domesticated suids.

Two more potential cross-boundary transmissions of ASF into/out of DRC are worth mentioning; however, their molecular evidence is less direct. The first possibility is that genotype XIV may have been transferred, between Zambia (Zam_NYA_1_2-isolated in 1986) and DRC (drc21/07/22-isolated in 2007) through Kipushi (**Figure 3**), as p72 sequences between these isolates share 99.26% nucleotide sequence identity. Unfortunately, no CVR sequence for isolate Zam_NYA_1_2 was available for a more detailed comparison. The next putative trans-boundary migration of ASF could have occurred between Brazzaville, Republic of Congo (Con09/P003 - isolated 2009; Gallardo et al., 2011) and the DRC (drc86/10/1-isolated in 2010). The p72 sequences were identical, and the CVR locus differed by the insertion of two amino acid tetramers coded as A (AAAAAAAAAF from the Brazzaville isolate and AAAAAAAAAAAF in the DRC isolate). Although the CVR tet-types are not identical, they are not highly divergent, and based on nucleotide analyses, a single 24bp

insertion could explain the difference between these isolates. Rapid mutation rates have been shown in vitro (Garcia-Barreno et al., 1986), therefore, given the highly variable nature of the CVR locus, it is difficult to omit the possible link between these outbreaks, especially as the boundary is a narrow aquatic border with high levels of traffic (both commercial and civilian).

This first, in depth examination of ASF in the DRC has provided evidence of 1) multiple genotypes previously not known to circulate within the DRC, 2) putative links between both geographically and temporally separated outbreaks, 3) potential movement of isolates across political borders between Uganda, Zambia, Congo, and DRC, respectively, 4) co-circulation of multiple genotypes within outbreaks and, 5) a pig co-infected with two tet-types. This provides a solid first step at understanding patterns of ASF circulation within the DRC. These data, in combination with examination of genotype relationships (Maloglovkin et al., 2015) may be useful for optimization of current prevention and control strategies at the regional level given the location and size of this country. Increased geographic sampling and genomic analysis may provide better fine scale resolution of ASFV circulation within the DRC.

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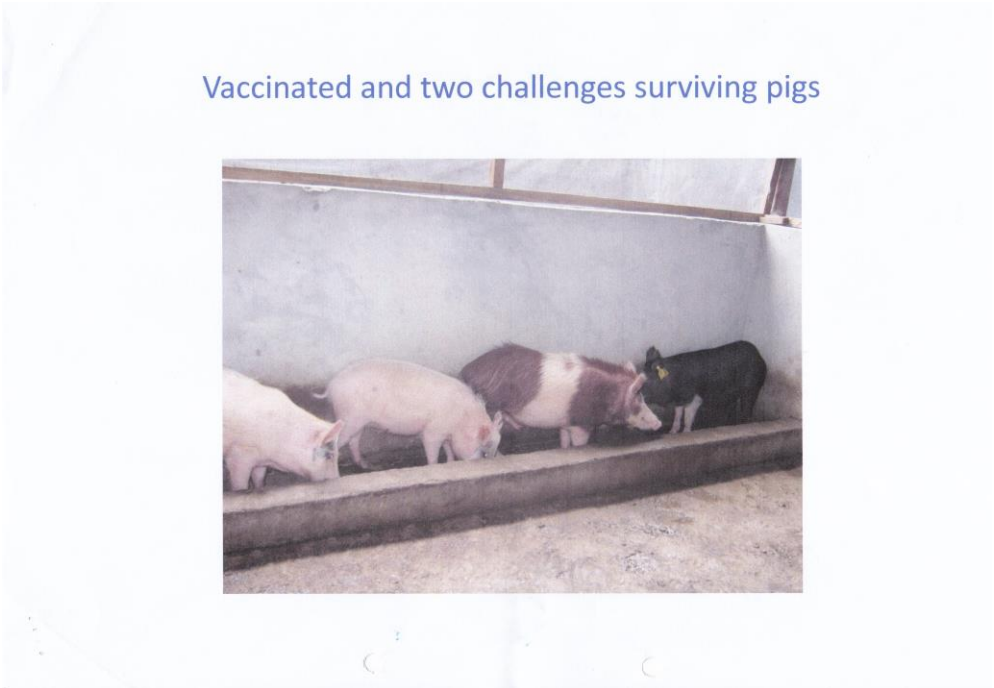
Chapter 9 - Immunization of African indigenous pigs with attenuated Genotype I African swine fever virus OURT88/3 induces protection against challenge with virulent strains of the same Genotype



Chapter 9 - Immunization of African indigenous pigs with attenuated Genotype I African swine fever virus OURT88/3 induces protection against challenge with virulent strains of the same Genotype

9.1. – Preamble

Neither protective vaccine nor treatment have been developed against ASF (PigTrop, 2015) but, to date, naturally attenuated vaccines have provided promising results (King et al., 2011; Sanchez-Vizcaino et al., 2014).

9.2. – The following section is devoted to the vaccination of indigenous pigs with attenuated Genotype 1 ASFV.

ARTICLE 6:

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ORIGINAL ARTICLE

Immunization of African Indigenous Pigs with Attenuated Genotype I African Swine Fever Virus OURT88/3 Induces Protection Against Challenge with Virulent Strains of Genotype I

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Keywords:
African swine fever virus; vaccine; Africa

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Summary

The attenuated African swine fever virus genotype I strain OURT88/3 has previously been shown to induce protection of European breeds of domestic pigs against challenge with virulent isolates. To determine whether protective immune responses could also be induced in indigenous breeds of pigs from the Kinshassa region in Democratic Republic of Congo, we immunized a group of eight pigs with OURT88/3 strain and challenged the pigs 3 weeks later with virulent genotype I strain OURT88/1. Four of the pigs were protected against challenge. Three of the eight pigs died from African swine fever virus and a fourth from an unknown cause. The remaining four pigs all survived challenge with a recent virulent genotype I strain from the Democratic Republic of Congo, DRC 085/10. Control groups of non-immune pigs challenged with OURT88/1 or DRC 085/10 developed signs of acute ASFV as expected and had high levels of virus genome in blood.

Introduction

African swine fever virus (ASFV) causes an acute haemorrhagic fever, African swine fever (ASF), in domestic pigs. This can result in very high mortality and has a severe socio-economic impact in affected countries. Currently, no vaccine is available to protect pigs against ASF, and this limits options for disease control. African swine fever is endemic in many sub-Saharan African countries, Sardinia and parts of the Trans-Caucasus region and Russian Federation (Wilkinson, 1999; Arias and Sanchez-Vizcaino, 2002; Beltran-Alcrudo et al., 2008; Penrith et al., 2013; OIE-WAHID).

Knowledge of the genotypes of ASFV isolates circulating in different regions could provide information relevant for design of vaccination campaigns as these would be expected to be more successful in regions with single or few genotypes circulating. African swine fever virus isolates have been classified into 22 different genotypes, by partial sequencing of the gene, which encodes the VP72 major capsid protein. All of these genotypes have been detected in eastern and southern Africa. In Central and West Africa and Sardinia (Wilkinson, 1999), only genotype I was described until recently although genotype IX has now been reported in the Republic of Congo and Democratic Republic of Congo (Boshoff et al., 2007; Gallardo et al., 2011a,b). In the Trans-Caucasus and Russian Federation, the virus strains described are from genotype II (Rowlands et al., 2008; Chapman et al., 2011). Genotype I is considered the predominant genotype circulating in Central and West Africa. Therefore in the current study, we tested the ability of an attenuated genotype I virus isolate, OURT88/3, to protect pigs against virulent genotype I isolates included a recent strain from DRC.

European pigs immunized with attenuated genotype I ASFV strain OURT88/3 can be protected against challenge

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with related virulent strains including from genotype X (Boinas et al., 2004; King et al., 2011). The protection levels varied from 66% to 100% dependent on the pigs and the challenge virus (Boinas et al., 2004; King et al., 2011). Complete genome sequencing showed that the OURT88/3 isolate has a large deletion near the left genome end compared to virulent isolates and interruptions in three other genes involved in immune evasion. These gene deletions and interruptions are likely to be important for attenuation of the virus and induction of a protective immune response (Chapman et al., 2008). The protective response induced by immunization of pigs with this strain is dependent on CD8+ T cells as protection is abrogated if this cell subset is depleted (Oura et al., 2005).

In this study, we have determined the protection induced by OURT88/3 in indigenous African pigs from the Kinshasa Region of the Democratic Republic of Congo (DRC) against consecutive challenge with virulent genotype I strains from Europe and from DRC.

Materials and Methods

Virus isolates and cell culture

The OURT88/3 and OURT88/1 isolates have been described previously (Chapman et al., 2011). The DRC 085/10 isolate was obtained from an ASF outbreak in domestic pigs in Kimwenza/Democratic Republic of Congo in October 2010 and confirmed to be genotype I by partial sequencing of the B646L gene.

All virus isolates were passaged at maximum five times in primary pig macrophage cultures, and virus titres were obtained by limiting dilution in primary macrophage cultures and detection of virus infection by haemadsorption (HAD) assay or by immunofluorescence using an antibody against ASFV protein p30.

Pig immunization and challenge

Indigenous pigs, at least 3 months old, were bought from farms near Kinshasa and tested negative for ASFV by antibody detection ELISA (Ingenasa Spain) and PCR. To confirm their non-viraemic status, the animals were kept for more than thirty days before the beginning of the experiments. The pigs were also negative for cysticercosis and trypanosomosis. Those in which coccidiosis was detected were treated with amprolium. The animals were kept in four buildings of level three vivarium standard, within a double-fenced restricted area. The experiments were approved by the Provincial Veterinary Office of the Ministry of Agriculture and Rural Development DRC. Pigs were randomly attributed in each group. The weight of pigs by group at arrival was compared and tested. The average weight in each group was not significantly different (linear regression with group A as reference group; P value ≥ 0.28). The average and SD for each group were, respectively, group A (32.4; 13.0), group B (24.2; 8.7) and group C (24.0; 19.3). A photograph of the pigs which survived challenge is shown (Figure S1). These were representative of the groups used.

In group A, eight pigs were immunized intramuscularly (IM) with 10^4 $TCID_{50}$ attenuated OURT88/3 strain and after 21 days challenged IM with 10^4 HAD_{50} OURT88/1 virulent strain. After a further 21 days, remaining pigs were challenged IM with 10^4 HAD_{50} DRC strain 085/10.

In group B, six pigs were challenged IM with 10^4 HAD_{50} virulent strain OURT88/1 21 days after the start of the experiment, at the same time as those in group A. In group C, seven pigs were challenged IM with 10^4 HAD_{50} DRC strain 085/10 at 42 days at the same time that group A pigs were challenged with this strain.

Pigs were immunized and challenged by the intramuscular (IM) route with 10^4 HAD_{50} or $TCID_{50}$ (tissue culture infective dose 50%) of the virus isolate indicated. Pigs were monitored daily for clinical signs, and these were scored using a clinical scoring system previously reported (King et al., 2011). Blood samples were collected at different days post-immunization and challenge. At termination, tissue samples were collected from spleen, lymph nodes and kidney. The ASFV genome copy numbers in blood and tissue samples were estimated by quantitative PCR (King et al., 2003).

Results

Immunization with OURT88/3

All eight pigs in group A developed a slightly elevated temperature between 39.1 and 40°C over the first 9 or 10 days after immunization with OURT88/3. Few other clinical signs were observed. Blood samples were collected from all pigs at days 7, 14 and 21 days post-immunization. No virus DNA was detected in any of the samples.

After challenge of pigs in group A with virulent OURT88/1 strain, four pigs, 1, 3, 7 and 8, survived. Two of these pigs showed no clinical signs, one had an elevated temperature from day 1 to 6 post-challenge, and the fourth pig had a reduced food intake on days 3 and 4 (Fig. 1). Two pigs (5 and 6) were terminated at day 6 post-challenge with virulent strain OURT88/1 showing typical signs of acute ASF (high body temperature, redness, ecchymosis and cyanosis of legs and extremities, apathy and icterus of conjunctiva). Two pigs (Arias and Sanchez-Vizcaino, 2002; Penrith et al., 2013) died at days 7 or 5, respectively, without showing typical clinical signs of ASF.

Quantitative PCR was carried out to determine the level of viraemia by estimating virus genome copy numbers in blood and tissues (Tables 1 and 2). Of the pigs which survived (Wilkinson, 1999; Beltran-Alcrudo et al., 2008; Chap-

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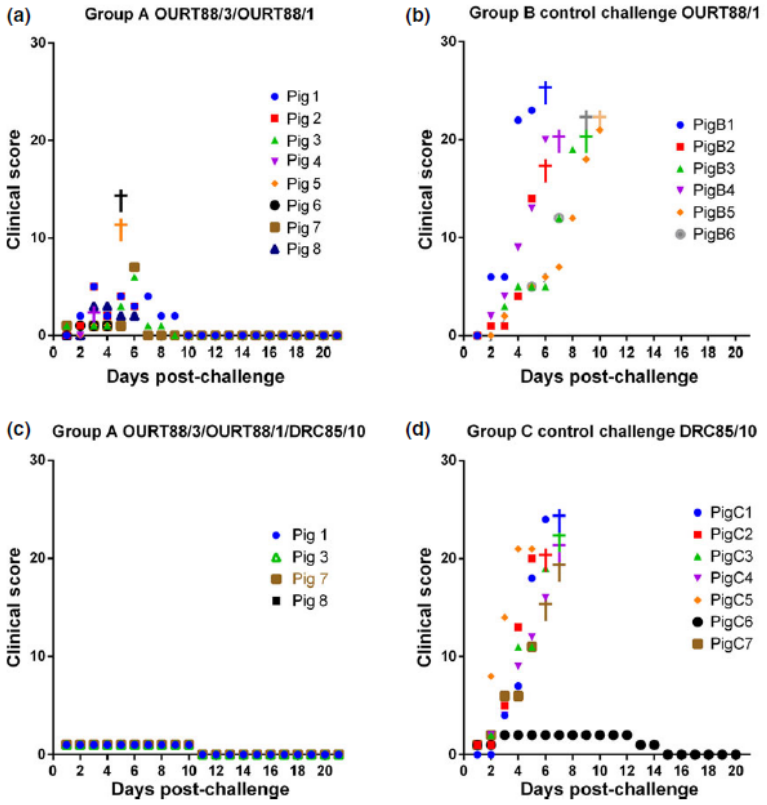

Fig. 1. Clinical scores of pigs post-immunization with OURT88/3 and challenged with OURT88/1 and DRC85/10. Clinical scores were recorded daily. Panels a and c show values recorded for pigs in Group A, which were immunized with OURT88/3 virus, at different days post-challenge with virulent virus OURT88/1 (Panel a) and DRC85/10 (Panel c). Values for control pigs in Group B, not immunized with OURT88/3, challenged with OURT88/1 are shown in Panel b and for control pigs in Group C challenged with DRC85/10 are shown in Panel d. The clinical scoring was as described previously. Values for different pigs are shown in different colours and shapes. Crosses show termination days, the colour indicates which pigs were terminated.

Table 1. Genome copy numbers of ASFV DNA in blood from pigs in Group A at different days post-challenge with OURT88/1

Pig	3	6	SD	14	21
1	2.54E+01	3.41E+04	2.84E+04	0.00	0.00
2	0.00	1.27E+06	9.53E+04	N/A	N/A
3	0.00	9.05E+00		0.00	0.00
4	0.00	N/A		N/A	N/A
5	0.00	N/A		N/A	N/A
6	0.00	N/A		N/A	N/A
7	0.00	1.04E+01		0.00	0.00
8	0.00	0.00		0.00	0.00

Pigs were immunized with OURT88/3 and at 21 days post-immunization were challenged with OURT88/1. DNA was extracted from blood samples and genome copy numbers per ml were estimated by qPCR. Two separate DNA extractions were made, and each of these samples was analysed in duplicate. Standard deviations (SD) are shown for those samples giving values >1.0E+02.

man et al., 2011; Gallardo et al., 2011b) virus DNA at 3.4×10^4 genome copies per ml of blood was detected only from pig 1 at day 6 post-challenge. Of those pigs which did not survive (Arias and Sanchez-Vizcaino, 2002; Boshoff et al., 2007; Gallardo et al., 2011a; Penrith et al., 2013), pigs 5 and 6 had high levels of ASFV DNA in tissues collected at post-mortem. Pig 5 had $>1.0 \times 10^4$ genome copy numbers per mg of tissue in lymph and spleen and pig 6 had 2.3 E+04 in lymph and 1.09E+05 per mg spleen, similar to virus levels detected in pigs from control Group B. Pig 2 had high levels of virus DNA in blood at day 6 post-challenge and high levels in tissues at termination (1E+06 genome copies per ml blood and 8.1E+03 per mg in spleen and 1.2×10^3 in lymph tissue). Pig 4 had no detectable ASFV DNA in blood or tissues. The six control pigs in Group B, which were challenged with OURT88/1 without prior immunization with OURT88/3, all exhibited high clinical scores typical of acute ASFV infection and were terminated between days 7 and 11 post-challenge. These pigs had as expected high genome copy numbers of ASFV DNA in tissues at termination (Tables 1 and 2). Therefore, we conclude that of the pigs immunized with OURT88/3, three of the eight pigs died from ASFV post-challenge with OURT88/1 and one pig died from unknown causes not related to ASFV.

Challenge with DRC085/10

The four pigs from group A (Wilkinson, 1999; Beltran-Alcrudo et al., 2008; Chapman et al., 2011; Gallardo et al.,

Table 2. Genome copy numbers of ASFV DNA from tissues collected at termination from pigs in groups A, B and C

Pig	Kidney	SD	Spleen	SD	Lymph	SD	Gang hep	SD
Group A								
1			0.00E+00		9.41E-01	1.14E+00		
2	5.43E+02	6.99E+01	8.16E+03	5.14E+02	1.23E+03	8.67E+01		
3			0.00E+00		1.05E+00	1.37E+00		
4	0.00E+00				3.94E+00	7.89E+00		
5	1.07E+04	1.15E+03	1.17E+05	1.61E+04	1.09E+05	6.97E+03		
6	6.91E+03	1.20E+03	1.09E+05	7.55E+03	2.34E+04	5.20E+03		
7			0.00E+00		0.00E+00			
8			0.00E+00		1.85E-01	1.01E+00		
Group B								
B1	5.79E+04	6.28E+03	1.50E+05	2.50E+04	1.71E+04	1.79E+03		
B2	1.18E+04	1.51E+03	4.59E+04	4.90E+03	4.29E+03	1.30E+03		
B3	1.43E+04	1.19E+03	3.88E+05	2.53E+04	3.75E+04	1.71E+03		
B4	2.29E+03	3.73E+02	1.12E+04	4.77E+02	3.42E+03	2.05E+02		
B5	2.27E+03	4.72E+01	2.97E+04	5.93E+03			8.50E+02	9.37E+0 1
B6	2.86E+02	6.19E+01	4.46E+03	7.09E+02	1.51E+01	1.64E+01		
Group C								
C1	1.51E+04	4.99E+02	3.19E+05	2.16E+04	7.83E+04	4.27E+03		
Control C3	1.37E+04	5.17E+02	7.74E+05	4.61E+04	2.79E+05	3.54E+04		
C6	6.37E+00	1.98E+00		2.74E+00	3.60E+02	1.70E+02		

Genome copy numbers of ASFV DNA detected in tissues at termination from pigs in Groups A, B and C. Values given are per mg tissue. Two separate DNA extractions were carried out, and DNA from each analysed in duplicate. The values shown are the means and standard deviation is indicated (SD). Standard deviations (SD) are shown for those samples giving values >1.0E+02.

2011b), which had survived the immunization with OURT88/3 and challenge with OURT88/1, were challenged with DRC strain 085/10 at 21 days post-challenge with OURT 88/1. All pigs survived the challenge (see Figure S1). All four pigs showed a slight temperature increase to a maximum of 39.4°C, until the 11th day post-challenge but no other clinical signs. There was a substantial increase of body weight in all of the pigs compared to when they were introduced in the experiment. No or very low levels (9.4 E+01 per mg in lymph from pig 1) ASFV DNA was detected in any tissue collected from those four pigs at termination.

A control group (C) of seven non-immunized pigs were challenged with DRC strain 085/10. Six of the pigs died between days 5 and 7 with typical clinical and post-mortem signs of acute ASF. The presence of ASFV DNA was confirmed in blood as well as other tissues by conventional PCR and for two of the pigs by qPCR. One pig from this control group (3 months of age and 10 kg) survived the challenge and was euthanized at day 21 post-challenge. In this surviving pig, a slight increase in temperature was observed between days 2 and 16 but no other clinical signs were observed. At post-mortem, this pig showed some typical lesions of ASF, consisting of ecchymosis, petechiae, small red and white infarctions on kidneys surface, fibrinous hydro-pericarditis, fibrinous hydrothorax and fibrinous adhesions to adjoining tissues/organs, but no ASFV DNA was detected in spleen and kidney and low levels (3.6×10^2) in lymph nodes.

The copy numbers of ASFV DNA in kidney, spleen and lymph nodes were compared with group C and kidney as references. This showed significantly more copy numbers of ASFV DNA in spleen (negative binomial regression; $P = 0.03$) and less for group A (negative binomial regression; $P = 0.06$) but for the later, P was close to the signification level.

Discussion

In previous experiments up to 100% of pigs immunized with OURT88/3 survived challenge with OURT88/1. The reduced survival observed in the experiment reported here may be due to differences in genetics, size, husbandry system or environment of pigs. In future experiments, the immunization and challenge regimen could be modified to improve the efficacy.

In the control non-immune group all pigs challenged with OURT88/1 developed acute ASF signs and had high levels of virus DNA in blood and tissues. Six of the seven non-immune pigs challenged with DRC085/10, died between days 5 and 7 post-challenge with typical clinical and post-mortem signs of acute ASF and presence of ASFV DNA in blood. This confirms that this is a highly virulent strain supporting field observations.

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The results indicate that the OURT88/3 strain may have potential for use as a vaccine in certain regions including DRC. However, extensive further safety testing would be required to evaluate the level and duration of adverse reactions observed in immunized pigs over longer periods and the potential for the strain to persist and be transmitted between pigs. The stability of the virus during passage in pigs should also be confirmed. The high levels of mortality and socio-economic impact of ASF in DRC and other countries where disease is endemic and difficulty associated with control of ASF mean that the deployment of an effective vaccine could have a high impact in both backyard and commercial pig farming.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Photo of the 4 pigs which survived immunisation with OURT88/3 and challenge with OURT88/1 and DRC85/10 virulent isolates.

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PART IV - GENERAL DISCUSSION, CONCLUSIONS, RECOMMENDATIONS AND PERSPECTIVES

Chapter 10 – General discussion

Chapter 10 - General Discussion

As explained for justification of this work, confusion about a precise diagnosis between the so-called "red diseases of pig" (Mulumba-Mfumu et al., 2013), lack of specific diagnostic facility and heavy losses related to a killing syndrome in pig populations that occurred in the 1990s within the city province of Kinshasa and surrounding areas formed the starting point of this research.

As a matter of fact, misunderstanding amongst field professionals contributed to delay in the implementation of prevention and control strategies, which definitely contributed to persistence of ASF and therefore, to establishment of its endemicity (Thomson, 1998). For instance, public awareness, compulsory slaughtering and other preventive actions could not timely be initiated. Devastated pens were restocked with healthy pigs, without removing outbreaks' survivors, which unfortunately ended up in the establishment of healthy carrier's status.

Within the DRC, and possibly within its surrounding countries, common erythematous diseases of swine that share similar clinical signs with acute course of ASF comprise Erysipelas, Pasteurellosis and Salmonellosis as stipulated in several text books in terms of differential diagnosis (OIE, 2013). Based on field observations, especially with reference to post-mortem lesions like bronchopneumonia, polyarthritis and hydropic changes such as ascites, hydropericarditis, hydrothorax usually observed in subacute course of ASF are also typical for Enzootic Pneumonia (Martineau and Morvan, 2020). Enzootic pneumonia (EP) is a swine mycoplasmosis caused by *Mycoplasma hyopneumonia*, in which the respiratory syndrome is the most dominant. As insidious disease, EP kills few numbers of pigs but, permanently until the piggery herd is terminated if specific treatment or control measure is not timely implemented. Clinically suspected in a commercial farm within this research area, EP has been confirmed and stabilised in 1999 using Long Acting Oxytetracyclin and limitations of movements (Mulumba-Mfumu, data shown). Apart from EP, another swine disease not present in the DRC but, which needs to be differentiated from ASF is Classical Swine Fever (CSF) or Hog Cholera suspected in the region at the beginning of the last century, before description of ASF by Montgomery (Plowright et al., 1994), and recently confirmed in South Africa (OIE, 2012). CSF is as well as haemorrhagic fever of domestic pigs, caused by CSF virus (CSFV) a pestivirus of *Flaviviridae* family. CSF clinical lesions are similar to those of ASF but, some clinical particularities like vertical transmission, predominance in piglets, predominance abortion and stillbirth syndrome and, quite long acute course which may last

more than ten days makes it from ASF. CSF is as well an OIE old list A disease. Epidemiologically, for differentiation with ASF, Erysipelas, Pasteurellosis, Salmonellosis and Enzootic Pneumonia are easily treated using appropriated antibiotics, while CSF may be easily controlled using appropriated vaccines (OIE, 2012). International trade of live pigs (Kleiboeker, 2002), currently frequent in the DRC, specifically with importation of improved races boars from Europe and South Africa, forms a major risk for CSF introduction in this country and others of the sub-Saharan Africa.

Due to the lack of reliable diagnostic facility in that period of time, that could enable differentiation between a ASF and the aforementioned range of pig diseases, the most speculated disease was Erysipelas. ASF was ignored, in spite of resistance to large spectrum antibiotics that were unsuccessfully used for eradication. This confusion lasted until the end of 1987, when a severe red disease of pig broke out within a commercial farm in Kingantoko, 50 km far from Kinshasa City in the neighbouring Bas-Congo Province.

Syndromically, and based on haemorrhages level in internal organs involving kidney, spleen and lymph nodes, as watched directly at the laboratory level, strong presumption was made in connection with ASF occurrence, presumably in a naïve population of susceptible animals. Unfortunately and once again, we ran in a lack of appropriate diagnostic facility. We came up to a need of implementation of such a diagnostic unit, justified by a specific field and laboratory research study entirely devoted to ASF in the DRC.

Our scientific question was then formulated as follows, "What is the epidemiological situation of ASF in the DRC?", definitely this implied confirmation of suspected outbreaks, study of transmission cycle, assessment of disease prevalence and endemicity, assessment of disease ecology including virus isolates dynamics and, suggestion of control strategies in terms of protection of African breeds of pigs. Overall and specific objectives of such a research study were determined and, accordingly, a frame work dividing the main study in 6 components or sub-studies was set up.

ASF is endemic in the majority of SSA countries, with forms ranging from peracute, acute, subacute, chronic to unapparent and, with mortality rate ranging from as higher as 100 % to as little as 3 % (Hess, 1987). Mild forms of ASF were described in the SSA by other researchers (Plowright et al., 1994). Long term infection were already described in Malawi (Haresnape, 1985 and 1987). Unapparent forms of the disease were described even in severe outbreaks (Gallardo et al., 2015). Low rate of mortality was also observed by other authors (Kleiboeker et al., 2001). Depending on geographical areas, some ASF-like diseases have also been observed. Unfortunately, only the dramatic picture of the disease consisting in 100 %

hemorrhagic disease and killing 100 % of infected pigs is known by field professionals. This empiric knowledge of ASF has largely contributed to persistence and endemicity of the disease in the SSA. Because of ignorance of exact diagnosis, drastic measures are either omitted, or applied with delays.

As a matter of fact, the classical picture of ASF newly introduced in naïve population is the most taught and the most presented in literature, which is misguiding for some technicians working in the areas where the disease is endemic, with pig populations being repeatedly exposed.

Our general discussion will be developed following different components of our study, as follows:

i. Literature review;

ii. Initial and prospective study entitled " Molecular characterisation of African swine fever virus involved in infection persistence in Central Africa: Democratic Republic of Congo", published in *Annales Africaines de Medecine*;

iii. Field investigation aimed on ASF transmission cycle in our research area, specifically in wild suids and ticks, which enable us to find new ticks never described in existing literature. A paper entitled "A new species of *Rhipicephalus* (Acari: *Ixodidae*), a parasite of Red River Hogs and domestic pigs in the Democratic Republic of Congo" was devoted to this finding published in the *Journal of Medical Entomology*;

iv. A study combining both, field and laboratory approaches, consisting in prevalence studies and assessment of screening tests used in the field. Two papers temporally separated, with the first being partial and the second being more global, entitled "The contribution of molecular epidemiology to informing control measures for African swine fever", and "Estimation of the prevalence of African swine fever in the Democratic Republic of Congo and assessment of sensibility and specificity of three ELISA tests in field conditions" respectively, were written; with the first published in the international congress proceedings and the second submitte in *Transboundary and Emerging Disease*.

v. A study on ASF ecology titled "Genetic characterisation of African swine fever isolates involved in outbreaks in the Democratic Republic of Congo between 2005 and 2012 reveals co-circulation of p72 Genotypes I, IX, and XIV and, 19 variants";

vi. Pig protection study, or Anti-ASFV vaccine trial of which the final report entitled "Immunisation of African indigenous pigs with attenuated Genotype I African swine fever virus OURT88/3 induces protection against challenge with virulent strains of Genotype I" was published in *Transboundary and Emerging Disease*.

Three main approaches will be addressed within this section, that is to say, results, research strategies exploited during investigations, both in the field and at the laboratory level and, risk factors or drivers. The reason why our results will be debated is to appreciate whether the gap missing before the beginning of the studies has been filled in terms of research outcome. Discussing about research strategies will enable the scientific community as well as the reader to understand the strengths and weakness of our studies and, debating the risk factors or drivers will allow to determine which of them plaid a key roles in occurrence and spread of the disease within the surveyed area.

Regarding laboratory methods, only the basic strategies exploited in the used tests will be discussed in this section as the standard operating procedures (SOPs) have been already provided with details within the relevant articles.

1. Literature review

Essential old and newly published papers related to our topic have been carefully reviewed while before redaction of this thesis. When comparing basic scientific information delivered in published papers or text books about ASF, some observations need to be highlighted.

In respect with the escape and spread of ASF from Africa to overseas, it should be noted that two distinct ecosystems or watersheds have been naturally created. One ecologically related to Atlantic Ocean comprising a pool of ASF viruses that spread from the western coast of Africa, moving to Europe, South America and Caribbean. The second ecosystem is linked to Indian Ocean, comprising ASF viruses that recently moved to Eurasia through the same ocean's islands, apparently from the South-eastern coast of Africa.

Other observations that were less written in existing literature and that need to be recalled in endemic ASF areas concern the clinical profile of disease as it is the case for skin vesicles mainly localised on affected animals 'abdomens, growth delays in young animals, exudative epidermitis, stomach and heart as most associated target tissues, jaundice associated with anorexia in acute phase in some breeds. Skin vesicles, growth delay and exudative epidermitis

and joints swelling are usually associated with subacute or chronic course of ASF. Furthermore, as recently noted in Poland and Belarus for European boars, African feral pigs are as well most susceptible to ASF during outbreaks.

No specific reports are found in existing literature about surviving phenomenon observed after disease occurrences. No specific studies have been conducted about race-based resistance to ASF, in order to better understand whether this is related to circulation of avirulent strains of virus, environmental or genetic factors. Without explanation, it has been observed during our investigations that the few cases of surviving animals observed upon ASF outbreaks even for naïve populations were associated with some specific breeds of pigs. Amongst these breeds, Nganda (Nsalambi, 1987) which is the indigenous Large Black race of pigs has been observed as more resistant than the European Large White (LW) and the South African Duroc.

When compared newly introduced exotic breeds of pigs among themselves, the European pure race stress-negative Pietrain was found more resistant than LW, Duroc, and even with its own cross-bred offspring, given the higher survival rate roughly observed upon outbreaks.

2. Molecular characterisation of African swine fever virus involved in infection persistence in Central Africa: Democratic Republic of Congo.

This was the first prospective study. This was a kind of survey conducted in two main steps. The first step was a randomly conducted survey, more explorative and qualitative as aimed on checking of exposure to ASF in terms of assessment of infection within the suspected locations during the concerned period of time. A total of 534 sera samples from 534 symptomless pigs and haemorrhagic tissues from 40 pigs out of 534, collected before and during 1998 were tested for confirmation of ASF in the second half of 1998 through a specific trip at the regional reference laboratory in South Africa. The second step, quantitative and more structured, was carried on 3 years later, due to similar alerts from the previous municipalities and others. A second pool of 350 sera samples from 350 pigs and solid tissues from 268 out of 350 collected between 1999 and 2001 were tested for ASF diagnosis another specific trip at the world reference laboratory in Pirbright, UK.

Amongst the exploited laboratory techniques, indirect ELISA using cp72 antigen was selected for assessment of the serological status of animals though detection of anti-ASFV Ig G, PCR for detection of ASFV DNA targeting amplification of the highly conserved genomic region (p72 gene), cell culture for identification and collection of live viruses, IHC as confirmatory diagnostic and, sequencing for molecular characterisation of detected viruses. Apart from

sequencing and IHC, the remaining methods were used in the first step and, all the mentioned methods were used during the analysis of the second step samples. The samples originated from syndromically suspected farms in peri-urban areas and villages of 4 provinces of the country (Bas-Congo, Bandundu, Equateur and Kinshasa) between 1997 and 2001.

Roughly, the first step results confirmed exposure of tested animals to ASF by detection of Anti-ASFV Ig G in sera samples, in four free ranging pigs, and detection of ASFV DNA in kidney and spleen of one pig from a suspected commercial farm in Ndjili, in the West of Kinshasa. These findings confirmed that the heavy losses that were undergone by many farms and pig owners in these areas were due to ASF occurrence (probably with involvement of low virulent strains), rather than other so-called "red diseases of pigs" like Erysipelas which was mostly cited.

Interestingly ASF diagnosis was also reproduced when analysing the second pool of samples upon the second step of the study, both by detection of anti-ASFV IgG and ASF DNA. It is important to point out that, based on those initial findings, we were already aware about the circulation of the disease in these areas before starting the second step of our study. That is the reason why it was more selective, structured, and more detailed, with extension to molecular characterisation. These results were confirmed by IHC, with antibodies-antigen complexes revealed in tonsils tissues (see picture in relevant article).

All the five diagnostic techniques were applied during the analyses of the second pool of samples, unfortunately, no live virus could be identified by cell culture apparently due to degradation of samples because of poor cold chain during the storage but, that was as well experienced by previous investigators who incriminated long-term infection status of sampled animals (Carrillo et al., 1993).

When trying to interpret these results for the Kinshasa's province which was the most challenged, and assuming each concerned municipality as an epidemiological unit (Toma et al., 1999), we found that Anti-ASFV IgG were detected in 8 units out 10 (80%) that were surveyed during the second step on the study.

When considering the molecular biology results using PCR (Gerrit, 2005) also exploited as screening tool during the second step survey, assuming each municipality as an epidemiological unit, we found that ASFV DNA was amplified in tissues collected from 6 pigs representing 4 municipalities belonging separately to of each of the four surveyed provinces. This means that 4 provinces out the 4 surveyed (100 %) were not ASF free, which is reflecting the picture of the epidemiological profile of all the country with respect to the spread of this epizootic.

Globally, combination of the ELISA and PCR results for the second step of the study revealed that 9 locations taken as epidemiological units out of 10 (90%) were affected. Finally in terms of diseases frequency and based on serological status of tested animals, we found that 73 pigs out 350 anti-ASFV antibodies, indicating an apparent prevalence of 21%. Two animals, among the 73 were asymptomatic pigs sampled in urban markets; these animals were found positive with ELISA test and one of them was also positive by PCR, which was suggesting the presence of healthy carrier's status, maintenance of source of infection, and confirmation of domestic pig to domestic pig cycle.

All the 3 ASFV target genomic regions, the pB646L gene encoding the most immunodominant, major capsid protein 72 (Costa, 1990; Tabares, 1987), the pK83L gene encoding the structural protein 54 and the CD2v gene were amplified. The p54 and CD2v proteins play a critical role in cytolysis and haemadsorption of infected cells respectively (Dixon, 2004), which is an indication of potential virulence of circulating strains of ASFV.

The analysis of the phylogenic tree involving the p72 gene sequence of one of ASFV strains collected in the second step of this study delineated it as genotype I, clustering within the same taxa with other historical viruses from DRC (Katanga 67, Zaire 77), Lisbon 57 and Angola 70 and 72, which is indication of more than 30 years presence of this virus strain in the DRC. This is also evidence that this ASFV strain shares the same ancestor with the aforementioned historical isolates.

3. A new species of *Rhipicephalus* (Acari: *Ixodidae*), a parasite of Red River Hogs and domestic pigs in the Democratic Republic of Congo

The specific field investigation conducted for determination of ASFV infection in wild and domestic pigs and ticks in interfaces with interactions between these susceptible species involved 4 Provinces: Equateur (0º56'S, 20º33'E), Bas-Congo (-5º23'S, 13º91'E), Kasaï Oriental (-3º00'S, 23º41'E) and Kinshasa (- 4º38'S, 15º, 96'E). This field study allowed us to recover only hard ticks instead of Ornithodoros ticks. Among the recovered hard ticks, the following were identified: *Ixodes muniensis*, *Rhipicephalus compositus*, *Rhipicephalus complanatus*, *Rhipicephalus planus*, *Rhipicephalus ziemani*, *Amblyoma paulopunctatum* and 26 ticks of unknown species which microscopically belonged to the Rhipicephalus genus but, for which the species has never been described in the existing literature. Based on morphologic features which have been revealed by the SEM, as they were all similar, we named this new species as "*Rhipicephalus congolensis*" (Apanaskevich et al., 2013). Both, female and males of this new species were collected on Red River Hogs and on domestic pigs in the Equateur Province which is fully located in forest ecosystem.

All the 330 hard ticks were collected on the bodies of 45 animals comprising 38 wild suids of which 36 Red River Hogs, all from Equateur province, and one *Potamocherus larvatus* from Bas-Congo on the border with Angola, and one *Potamocherus larvatus* from Sankuru within the Oriental Kasaï province; and, 7 domestic pigs. No *Ornithodoros* ticks was found among those 330 collected ticks and, all the new ticks, identified as *Rhipicephalus congolensis* were originating from the Equateur province, namely from Boende in the Tshuapa district and from Mbandaka district. We homogenised a significant number of ticks collected in Boende and Mbandaka, as anti-ASFV IgG were detected in some wild suids from these two locations, we extracted DNA from the obtained mixture, and we ran PCR using the primers VP723 F and VP724 R, as previously described (Mulumba-Mfumu et al., 2013) but, we failed to amplify ASFV DNA.

These findings correlated with the ones previously published by other authors (Plowright et al., 1994; Owolodun, 2010). Although Ornithodoros ticks of *O. moubata* complex (sensu Walton) are the only recognised as ASFV natural host and vector of ASFV (Kleiboeker, 2001), mechanic transmission by *Rhipicephalus* genus has been experimentally reported by other authors, in endemic ASF countries of SSA like the Republic of Congo Brazzaville (Badaev et al., 1992). Basically and as for other blood sucking arthropods, mechanical transmission of ASFV from infected to uninfected pigs requires freshness of blood meal, which means that the time between feeding on the two animals should be significantly short (Plowright et al., 1994). This is not or only exceptional the case for *Rhipicephalus*.

Of important significance are some biological and ecological properties of Ornithodoros ticks, naturally they are known to be burrows dwelling as colonising wild suids nets such as warthog's habitats, beside the fact they do not stay longer on suids' bodies, staying just for the feeding time (Jori et al., 2013; Plowright, 1994). This means that rather than collecting them on suids 'bodies, habitats and nets would be the correct places, unfortunately there are not easy to find within a rainy forest which is permanently flooded. Further field studies using appropriated means are required.

4. Prevalence studies

4.1 - Estimation of the prevalence of African swine fever in the Democratic Republic of Congo and assessment of sensibility and specificity of three ELISA tests in field conditions

Given the fact that ASF, which is as a highly lethal and destructive disease of pigs with major negative impact on rural economy in the SSA countries, is characterised by the lack of vaccine and treatment. The only good available mean that could efficiently contribute to implementation of prevention and control strategies would be early and accurate diagnostic of this disease (Cubillos, C., et al., 2013). The aim of this study was to estimate the prevalence of this epizootic in the DRC and, during the same time to assess the sensitivity and specificity of two serological tests that currently are the most used in large scale screening operations in the aforementioned country.

The two assessed tests were the rp72-based competitive ELISA, targeting the highly conserved and immunodominant ASFV capsid protein 72 encoded by the B646L gene, and the rp30-based indirect ELISA targeting another immunocompetent ASFV structural protein 30 encoded by the CP204L gene. The 2 techniques were set up and supplied by INGENASA, Madrid, Spain and, ALGENEX, Madrid, Spain, respectively. The two format of ELISA are based on recombinant proteins, which is making them safer than the ones initially in use and that were presenting contamination risk in terms of biosafety in that they were mounted with infective coating proteins and thus quite dangerous for laboratory and surrounding environment. The 2 recombinant proteins were expressed in *E. coli* and baculovirus respectively. In order to facilitate a large-scale production of rp30, it was furthermore cloned in *Trichoplusia ni* larvae, which is a cabbage looper, given the lack of fermentation procedures, not existing for this virus (Perez-Filgueira et al., 2007).

For estimation of disease prevalence and assessment of Se and Sp of the two aforementioned tests, 495 samples were used. These two tests to be assessed were simultaneously run with two others comprising the crude and cytosoluble ASFV protein 72 (cp72)-based ELISA, recommended by OIE and FAO as serological gold standard test and, the PCR using primers binding the C terminus end of the p72 gene.

Of the 495 involved samples, only 328 samples that were analysed by each of the 4 assays out were taken in consideration for calculations. When taking in consideration combined results of the 4 exploited tests, it has been found that comparatively to single positive results per test, combined positive results represented 55 cases out 89, suggesting that 62 % of positive samples were reproduced at least by one of other tests. This was a good parameter in terms of internal validation of our study's results.

For assessment of sensitivity and specificity of our two field screening tests, the comparison of ELISA results as revealed by the ODs values, with cp72-based ELISA as gold standard demonstrated that the rp72-based ELISA had 93. 48 % (95% CI: 82.10-98.63) as the

relative Sensitivity (Se) and 87. 98 % (95 % CI: 83.62-91.53) as the relative Specificity (Sp). And, the rp30-based ELISA had 34. 78 % (95 % CI: 21.35-50.24) as the relative sensitivity (Se) and 93. 68 % (95 % CI: 89.68-95. 90) as the relative specificity (Sp). This observation was clarified by some statistical tools. The general tendency separating infected and non-infected animals, was illustrated by frequency histograms and correspondent kernel density which showed a clear cleavage between the two sub-populations. The concordance agreement involving each of the compared test against the gold standard (the crude p72 test), was illustrated by the Kappa coefficient which was demonstrated as good between the gold standard and the rp72-based test (0.64) and poor between the gold standard and the p30 based-test (0.50). On the other hand, two other parameters, comprising the Receiver-Operating Characteristics (ROC) curve and the Chi-square test, demonstrated that the difference between both, the rp72-based (Ingenasa) test and the rp30 (Algenex test) was not too much significant.

According to ROC, the area under the curve (AUC) for Ingenasa was 0.86 while for Algenex, it was 0.76 and consequently the Chi-square test was 3.41 (1 df, and p-value = 0.06), even if the p-value was closed to the threshold of 0.05. According the same ROC, the optimal cut-off (CO) point for the rp72-based test is 0.16 and with this cut-off, the Se is 76.5 % and the Sp is 83.5 %, and the optimal CO for the rp30-based test is 0.08, the Se for p30-based test is 65 % and the Sp is 75 %, which is an indication that the characteristics values of the two tests were in closed limits.

In terms of interpretation, a huge divergence appeared between these results and those of previous studies concerning the same rp30-based test, where many sera (samples) from Africa including those from our research area were used; according the mentioned studies, the Se of the same test reached 97.78 % and the Sp 98.81 % (Cubillos, 2013). Many reasons may be referred to for explanation of the observed discrepancy among which: exploited coating antigens, ability of each test to detect antibodies in symptomless positive animals, poor preservation of sera-samples.

On the basis of these data and chiefly on reproduced results by at least another test, the apparent prevalence appeared to be minimal, i.e. 17 %, close to the one found in Senegal (16.9%) (Etter et al., 2011) and when taking in consideration the results per each used test, the maximum was 27.13 % which was largely higher than that revealed in Senegal.

The rp30-based ELISA results correlated with the PCR ones, suggested the former as alternative test of the latter for detection of ASFV in the early stage of infection.

4.2 - The contribution of molecular epidemiology to informing control measures for African swine fever.

Between 2005 and 2006, a field study was conducted in three provinces of DRC (Bas-Congo, Equateur, and Kinshasa), in order to investigate the ASF prevalence in relation with the farming system, outbreaks alerts and sub-grouping of ASFV viruses. Alerts about ASF were collected from local populations through participative approaches when collecting the samples. This partial study was therefore aimed on apparently healthy pigs of rural, peri-urban and urban premises of the study area during the indicated period of time. Pig exploitation in this territory is characterised by three farming systems, i.e. traditional free-ranging, traditional backyard and, fenced commercial farms.

In terms of pigs' breeds, three types of races are dominantly raised, comprising African indigenous pigs, exotic improved breeds also called race-pigs and, cross-bred pigs which, depending on the level of phenotype are assimilated either to local breeds or to exotic ones. Generally, the most encountered exotic breeds of pigs in the DRC are: European pure LW, Landrace, and Pietrain (recently introduced); South-African Duroc and the Chinese pig (small size format, pure or cross-bred). A total of 520 sera samples, completed with organs from some identified outbreaks were collected. The sera samples were selectively collected with, one part collected within villages with alerts about ASF and, another part originating from the villages areas without ASF alerts.

The purpose of the study was to assess the circulation of disease in terms of serological status of domestic pigs with respect to the farming system and of ASF occurrence. Blood samples were therefore collected for serological screening and, solid tissues from some outbreaks were made available for assessment of ASFV sub-grouping. This was the pilot survey touching the differentiation of ASFV strains circulating in the country and using p72 and 9RLORF genes as markers. The surprising findings that resulted from this survey allowed the initiation of a more extensive study that was finally carried out at the level of the country.

At the end of the survey, the overall apparent prevalence revealed was 26.9 % (CI 95 %: 23.1-30.7 %), which is close to 27.13 % revealed in similar study conducted between 2007 and 2008 by the rp72 ELISA which performed better (Mulumba-Mfumu et al., article in press), and which approximatively seems to be in the same limits with 21% revealed in another previous study conducted between 1997 and 2001 (Mulumba-Mfumu et al., 2013). When compared per farming system versus outbreak alerts, apparent prevalence was 23.8 % (CI 95 %: 18.6-29%) for free-ranging pigs, while for pigs from fenced and clustered

commercial farms it was 30.1 % (CI 95 %: 24.5-35.7 %), presumably due the fact that the majority of commercial farms, at least in the covered area, do not totally depopulate the farms after outbreaks, before introduction of new animals. This husbandry mistake forms the basis of next outbreaks due to presence of survivors among the new pigs, which is a best way for persistence of the disease in the pigsties and, a major critical risk factor the neighbouring disease-free areas.

When free-ranging pigs were taken separately in consideration, the apparent prevalence was higher in free-ranging pigs from rural areas where no outbreak was reported, which suggested high level of disease circulation in both, indigenous pigs and the villages.

PCR characterisation using 9RLORF primers revealed co-circulation of different sub-groups even in the same farms during the same outbreaks. These findings correlate with those of the extensive study conducted later at the country level, which has been initiated on the same basis (data not shown). On the basis of these findings it should be noted that, variation of ASF prevalence depends also on the sampling area, the husbandry method in practice, the epidemiological situation of the disease within the concerned area as previously reported for other diseases (Toma et al., 1999). It was also understood that infection pathways of diseases vary in terms of countries and, within the same country they differ between regions and, within the region they will differ according to pig production characteristics (Costard, 2007).

Other lessons to be learned from this study are the need of risk assessment framework which could be referred to for efficient implementation of prevention and control strategies against ASF. Some critical points like genotypes of circulating isolates, husbandry system and environmental scenario should be integrated within the risk assessment frame work. Risk assessment is a valuable approach for differentiation of levels of pig exposure to ASF, as already reported in terms of ASF transmission models (Costard, 2007).

In other words, molecular epidemiology tools combined with risk assessment could inform on targeted surveillance, and control or even eradication strategies at any level.

5. Genetic assessment of African swine fever isolates involved in outbreaks in the Democratic Republic of Congo between 2005 and 2012 reveals co-circulation of p72 genotypes I, IX and XIV and 19 variants

Due to the size of the DRC, in the sub-Saharan Africa in general and particularly in the Central Africa, the knowledge of ASFV circulating viruses which was missing at the level of the country was a real need. According to existing and published data, only 3 isolates, i.e.

Kat_63, Kat_67, and Zaire_77 recovered from the samples that were submitted by the past for laboratory diagnostic in some regional reference centres like the Onderstepoort Veterinary Institute laboratories were the only existing. These isolates are still largely exploited in phylogenetic studies conducted by several authors. These ASFV isolates were all assigned as genotype I.

The worldwide spread of ASF started with Europe, affected three times in 1957, 1960 and 2007 when the disease escaped from Africa, probably from Angola for the first time (DeTray, 1963), from Senegal for the second time (Sanchez-Vizcaino et al., 2012) and from south-eastern Africa through the Caucasus (Georgia) for the third time (Rowlands et al., 2008). Associated with the two first escapes was the spread to Caribbean and South America from Europe with involvement of what was designated as the Iberian ASF isolates (Plowright et al., 1994). Based on genetic similarity, the ASF viruses involved that dynamics were assigned to genotype I, sometimes named 'ESACWA genotype' by some authors (Bastos et al., 2003; Lubisi et al., 2005).

The maintenance of genetic similarity across the world by these viruses was attributed to internal propagation in terms of transmission cycle which, in this case, was supposed to be completely domestic cycle, i.e. domestic pig to domestic pig, without involvement of sylvatic cycle's components. On the other hand, that is not the case same with ASFV isolates circulating within the Eastern and Southern Africa which genetically differ among themselves temporo-spatially. The heterogeneity of ASF viruses that are circulating in these latter regions has been already reported in details (Lubisi, 2005; Boshoff et al., 2007) and, apparently it has been attributed to quasi-chronic involvement of sylvatic cycle components's viruses, i.e. wild suids and invertebrate hosts, in the propagation cycle (Lubisi 2005; Bastos et al., 2009; Gallardo, 2009). Also, based on the fact that conventional serological diagnostic tests prescribed by OIE, including recombinant antigens, do not easily detect ASFV response in susceptible animals from the Eastern Africa (Perez-Filgueira et al., 2006), it was suggested that the possible reason might be the immune-genetic status of the indigenous pigs of this region, rather than polymorphism in immunodominant viral antigens of ASFV isolates (Gallardo, 2011).

On the other hand, genotype I isolates (ESACWA), are likely not easy to be genetically differentiated due to their genetic similarity, especially when using p72 gene's loci reputed to be genetically stable, i.e. with low resolution level when compared to some other genes (Giammarioli et al., 2011).

In order to have a better understanding of heterogeneity of circulating ASFV isolates in the DRC, a discrimination strategy based on three approaches, i.e. partial (C-terminal end) B646L gene encoding p72 protein, full K183L gene encoding p54 protein and B602L CVR gene encoding 9RLORF protein has been exploited. This strategy revealed the presence of three genotypes: I, IX and XIV; and 19 variants.

Phylogenetic analysis of partial p72 gene revealed that all the newly recovered ASFV isolates clustered into three p72 genotypes, I (64.5%), IX (32.3%) and XIV (3.2%). These newly recovered isolates grouped with reference isolates with bootstrap value of more than 75%, but when compared among themselves inter and intra-genotypes variation of genetic relationship was observed. Within genotype I, isolates grouped in two polytomies with low bootstrap value; within genotype IX these new isolates grouped in one strongly supported polytomy and with topology and, genotype XIV with two sister groups highly supported, including reference isolate reference.

With 64.5 % of recovered isolates, apart from previous assignment of DRC historical isolates such as DRC_Zaire, Kat-63, and 67 to genotype I, it is clearly demonstrated, when compared to other genotypes, that this genotype is the most dominant and even the oldest in this country.

Phylogenetic analysis of p54 gene tree, corroborated the results of p72 gene tree with the same grouping of new sequences in two taxa, apart from some differences, i.e. the bootstrap value of the grouping of the two taxa was low with the p72 marker or more discriminating with the p54 marker. Furthermore, the grouping with reference sequences was well supported with more than 83% of bootstrap value using this latter marker. On the other hand, the p54 marker failed to recover monophyletic assemblage of reference isolates (available and included in the dataset) belonging to genotypes III, VII, X, XV and XX; even if the bootstrap support was low with the p72 marker. Surprisingly, the bootstrap support of monophyletic clades of genotypes III, VII and X was strongly supported with the latter marker. Finally and on the basis the current phylogenetic analyses, p72 and p54 genes approaches showed low level of variability and thus poor discrimination capacity in terms of inter and intra-genotypes resolution of circulating variants. In other previous studies, the p54 gene approach has been presented as a good complementary tool for discrimination of ASFV isolates (Nix, 2006; Boshoff, 2007; Giammarioli et al., 2011).

On the other hand, the use of the CVR locus as discrimination marker, demonstrated a good resolution level by revealing 15 discernible amplicons lengths, just at the beginning when sizing the PCR products which have been increased to 19 variants upon sequences

analysis. Recovered nucleotides sequences were translated in amino acids sequences and, finally coded as amino acids tetramers (tet-types). The final analysis process revealed that, genotype I presented 13 tet-types; genotype IX, 6 tet-types and genotype XIV, 2 tet-types. All the isolates grouping within one tet-type contained an identical nucleotides sequence, which means that no resolution was lost by converting them to tet-types. Of critical importance, was the fact tet-types is the fact that tet-type 12 and tet-type 51 were detected in more than one genotype, i.e. tet-type 12 and tet-type 51 were detected in genotype I, tet-type 12 was as well detected in genotype IX and tet-type 51 detected as well in genotype XIV.

Phylo-geographically, spatial distribution of DRC ASFV isolates recovered in this study is presented as follows: genotype I in Bas-Congo, Kinshasa and Maniema; genotype IX from Equateur, Kinshasa, and Oriental provinces; Genotype XIV from Katanga and Kinshasa. Details dealing with Tet-types locations are provided in Table 2, Figures 3 and 4 of the dedicated chapter.

This is the first extensive genotyping study about ASFV isolates circulating in the DRC; previously, only single samples submitted at the regional reference laboratories were unsystematically analysed and, of which some sequences have been largely exploited (Ekwe and Wilkinson, 2000, Bastos et al., 2003; Lubisi et al., 2005; Nix et al., 2006; Boshoff et al., 2007; Gallardo et al., 2009, Gallardo et al., 2011). Little was known about the dynamics of ASFV strains in this largest country of the sub-Saharan Africa.

Ultimately, three genotypes, i.e. I, IX, and XIV were delineated by our genetic assessment which revealed the genotype I, with 64.5%, as the most prevalent as previously reported by the above mentioned workers. Limited genetic variability was revealed with this genotype, probably due to its restriction to domestic cycle. Genotype IX with 32.3%, reported in Eastern and Central Africa (recently), circulating both in domestic and sylvatic cycles exhibited little or no genetic variation. The genotype XIV previously reported in Zambia where it was detected from Ornithodoros tick suggested transmission from sylvatic cycle; its introduction in the DRC might have been due to cross-boundary pathway or demonstrated; it could also be due involvement O. ticks, wild suids or even domestic pig.

The three genotypes were detected in the southern part of the country; the genotype IX was mainly dominant in the northern part. Furthermore all the three genotypes were present in Kinshasa, the capital and the main market on live animals trade (Praet et al., 2010).

The sequencing of the p54 gene reported by some workers as valuable discrimination tool showed limited resolution capacity, apart from revelation of slight differences between the two genotype I taxa when corroborating with p72 gene. Our sampling frame, totally aimed on

domestic pigs, might have plaid an important role in low resolution level; as a matter of fact, low genetic variation has been consistently detected in domestic cycle when compared to sylvatic cycle (Lubisi et al., 2005).

Sequencing of CVR locus allowed fragmentation of the three genotypes in 19 variants and revealed some critical data such as, co-circulation of isolates within the same outbreaks and same locations, co-circulation of more than one genotype within the same farm and during the same outbreak, exchange of amino acids tetramers (tet-types) within and between genotypes, and co-infection of the same pig by different isolates.

Identical tet-types is more likely due to homoplasy and with this background, putative connections between outbreaks are being discussed as follows:

i. Genotype I, tet-type 23 isolates were detected in the 2009 outbreak in Kintambo and Nsele and, in 2011 in Ngafula, involvement of asymptomatic might be suspected;
ii. Genotype IX, tet-type 24c, was detected in both, March and April 2008 outbreaks, common ancestor might be suspected;
iii. Genotype IX, tet-type 23a detected in the 2007 outbreak in Mahagi located not far from the border with Uganda was also involved in outbreak in Uganda (Ug_0H31), cross-boundary transmission is confirmed;
iv. Genotype I, tet-type 16, detected in asymptomatic pig from a market in Kinshasa in 2008, was also detected in Mayanda/Bas-Congo in 2012, involvement of live pigs trade and healthy carrier status might be involved;
v. Genotype IX, tet-type 24c, was detected in the 2007 outbreak in Yakoma and, the March and April 2008 outbreaks in Boende, feral pigs were killed during the 2007 outbreak, geographic proximity, forest environment, free ranging pigs and interaction between domestic and feral pigs might be suspected as risk factors;
vi. Genotype I, tet-type 10 and tet-type 5 co-circulated within the 2005 outbreak in the same farm (Interchix) in Limete/Kinshasa;
vii. Genotype XI, tet-type 24a,b and c co-circulated in the 2007 outbreak in Yakoma;
viii. Genotype I, tet-types 9, 11, and 12 co-circulated in the 2010 outbreak in the same farm (SEBO) in Ngafula, more than 3000 pigs were lost in this industrial farm;
ix. Genotype I (tet-types 9, 11, and 12) and genotype IX (tet-type 12) in Ngafula in 2010, with the genotypes sharing identical tet-type (12), genetic recombination or homoplasy might be suspected;
x. Genotype I (drc35/10/p1) and genotype XIV (drc/35/10/p3) both co-circulating in the 2010 outbreak in the same farm (Kuswamina) shared a same tet-type (51) and

xi. Co-infection of the same pig by two genotype I isolates tet-type 5 (drc49/05/p1a) and tet-type 9 (drc49/05/p1b) detected in the spleen and lymph node respectively was also revealed by this study. This was not found in similar studies conducted in Madagascar and Nigeria (Bastos et al., 2004; Owolodun et al., 2010).

In accordance with this dataset, a couple of speculations have been raised:

i. Co-circulation of genotypes and tet-types as well as co-infection of a pig by different isolates suggest high level of genetic variation and then, high value of the exploited marker, i.e. the CVR locus; and genetic recombination;
ii. Trade of live pigs and interactions between domestic and feral pigs may influence phylo-geographical signals of ASF;
iii. Identical tet-types within variants and genotypes could be caused by homoplasy but, suspicion of the same ancestor can also be made and
iv. Cross-boundary transmission would be an important pathway of ASF introduction within the DRC.

6. Immunisation of African indigenous pigs with attenuated Genotype I African swine fever virus OURT88/3 induces protection against challenge with virulent strains of Genotype I.,

The overall outcome of this vaccine trial was that 50 % of vaccinated pigs or 4 animals out of 8 were protected against the two challenge virulent isolates, OURT88/1 and DRC85/10, each time at 21 days of interval between inoculations. Basically, in addition with one pig not killed by any of the challenge viruses during this trial, the overall number of protected animals would be 6 pigs out 8 (75 %) as previously reached and, even overcome in similar study (King et al, 2011). Pigs selected for this trial were all of indigenous origin, purchased in rural areas in Bas-Congo and Bandundu, the neighbouring provinces of Kinshasa, ASF free as revealed by initial analyses, first with chromogenic p72-based rapid test (Ingenasa) at the pre-selection step, before purchasing, then with rp72-based competitive ELISA (Ingenasa) and after, rtPCR (using blood clot) during pre-quarantine step before introduction in the vivarium facility for vaccination and challenge operations. Within the vivarium facility, all the selected pigs were gathered in 3 groups representing vaccinated pigs (Group A), control pigs for the first challenge using OURT88/1 (Group B) and control pigs for the second challenge using DRC85/10 (Group C). All the challenge viruses were p72 Genotype I. Clinical score aiming on key ASF symptoms such as body temperature, skin hyperaemia and haemorrhage,

anorexia, apathy and moribundity, were collected along with blood samples after each inoculation. Step by step results revealed worst parameters for all terminating pigs than for the 4 vaccinated pigs for which only transient temperature elevation and slight clinical signs like reduced food intake were observed, which was an indication of protective response being induced (Wilkinson, 1989, Gomez-Puertas and Escribano, 1997). After challenge with DRC85/10, all the four vaccinated pigs showed a slight elevation of body temperature increased to a maximum of 39.4 º C without other clinical signs. And, the qPCR analysis at the end of trial, at day 21 post the second challenge (pc) revealed that apart from a very low number of ASFV DNA copies (9.4 E+0 per mg of lymph node) observed in pig # 1, no one was detected in any tissue collected from the four vaccinated pigs, which suggested a good level of immunity for tested pigs and, lack of side effects in terms of inocuity. On the other hand, within the non-vaccinated pigs, in Group C, 6 pig out of 7 died between days 5 and 7 pc, which confirmed the high level of virulence of DRC85/10 isolate which was observed even during the outbreak. Within this non vaccinated control Group C animals, one three months old pig of 10 Kgs, survived the challenge but, with typical clinical lesions of ASF, progressing between subacute and chronic course (Maurer, 1985; Plowright, 1994). Fibrineous hydro-pericarditis, hydrothorax and fibrineous adhesions to adjoining tissues associated to ecchymosis, petechiae, red and white infarction micro-spots in kidneys were observed in that pig. Caseous type of lesions was described as one of the most encountered gross changes in subacute and chronic forms of ASF (Mc Daniel, 1986). But, no ASFV DNA was detected in spleen and kidney of this pig apart from low level of nucleic acid copies (3.6 x 10^2) revealed in lymph nodes. This resistance might be related to maternal immunity, suggesting acquired neutralising antibodies but, not inducing sustainable protection. In fact, piglets receiving clolostral antibodies have been reported as showing delayed onset of clinical signs, brief period of fever, low level of viraemia and high survival rate than those receiving normal colostrum (Wilkinson, 1989). Production of diverse clinical forms of ASF including healthy carriers pigs through large scale immunisation with artificially attenuated vaccines largely contributed to endemicity of the disease in some countries like Portugal and Spain where protection attempts using this strategy were tried (Wilkinson, 1989). Among other strategies exploited for production of ASF safe vaccines, alternative formats of vaccine such as the ones based on the use of virus proteins, genomic deletions, and DNA were either ineffective or provided only very limited protection (Sanchez-Vizcaino, 2014). Without effective and reliable vaccine against ASF, this epizootics will permanently remain a major constraint to pig industry and rural economy.To date, promising results were obtained with a

naturally attenuated and non-virulent strain OURT 88 / 3, assigned to the VP72 genotype I, isolated from Ornithodoros tick in Portugal, and intramuscularly (IM) inoculated (King, K., et al, 2011; Mulumba-Mfumu et al., 2015). Compared to ASFV virulent isolates, the molecular basis of natural attenuation and protective power of OURT88/3 is presumably based on deletion of a large region near the left end of the virus genome, in addition to interruptions in 3 genes recognised as playing a key role in the immune response evasion (Chapman et al., 2008). Proteomically, it has also been demonstrated that the protective response induced by this strain is due to CD8+ T cells given the fact that when these cell subset are depleted, the aforementioned protection was abrogated (Oura et al., 2005). In order to determine the type of the viral nature of ASFV DNA detected in one of the 4 vaccinated pigs, by confirming whether or not it is from live or dead virus, further assortment tests usimg competent cells such as porcine bone marrow, alveolar macrophages, peripheral blood leucocytes (Pan, 1987) or Vero cells are required. Before fruitful use of this vaccine candidate, specific studies aimed on the abovementioned determination of remaining ASFV DNA copies in target tissues, and pre-assessment of virus genotypes involved in concerned areas should be a crucial pre-requisite. Also at the factory level, this study's workers suggest to increase the number of passages when achieving limiting dilutions through porcine bone marrow macrophages. Another hypothesis that need to be sorted out is to know whether, the remaining materials of ASFV detected in target cells upon pigs vaccination would play any role in terms of acting protective premunition as previously reported in some protozoal diseases like equine piroplasmosis (Tamzali et al., 2013). Basically, the lack of vaccine against ASF is directly linked on couple of factors including: 1) lack of neutralising antibodies (Vinuela, 1987) which is apparently justified by the lack of surface gylcoproteins in ASFV virion (Costa, 1988); in fact, the ASFV virion is enveloped by a outer lipid envelop (Vinuela, 1987), 2) presence of proteins involved in host immune response evasion in MGFs (Linda, 2004, Chapman, 2008), 3) genetic variability due to the fact the double stranded DNA linear molecule of ASFV, of 170-193 Kbp (depending on isolate) contains two terminal variable regions, apart from its central region of about 125 Kbp which is higly conserved and, the two terminal variable regions which are convalently cross-linked by hair-loops, are characterised by inverted tandem repeats of nucleotide sequences (Vinuela, 1987; Plowright, 1994), 4) antigenic variability due to the fact the 170-193 Kbp (depending on isolate) encode for more than 163 Open Reading Frames (ORFs) (Dixon, 2005). In regard to the level of variability within the ASFV DNA molecule, apart from the two terminal variable regions, other variable loci have been reported within the encoding genes, within the intergenic regions, and within the

multigene families (MGFs). Five MGFs were so far discribed comprising MGF100, MGF110, MGF 350, MGF 500 and MGF 510 (Dixon, 2004). The majority of ASFV proteins involved in host immune response are located in MGFs. Another major genetic mechanism is that ASFV has been so far delineated in 22 Genotypes, the majority of which were identified in East and Southern Africa, the 22 Genotypes have been divided in sub-components by some workers, i.e. sub-groups based on amplicons size (Phologane et al., 2005). That means that when corroborating together these actors form the basis of the lack of effective and sustainable vaccine against ASF. Consenquently, further studies are still required.

7. Methodology

a. Field operations

Our field operations were conducted in the locatins selected on the basis of some specific conditions, i.e., accessibility to the site, ecological scenario, availability of some critical risk factors like free ranging pigs and interfaces with interactions between domestic and wild suids, availability of wild pigs samples for determination of sylvatic cycle. The main risk factors recognised as most involved in determination of exposure and spread of ASF, have been characterised (Costard et al., 2007). As initially explained at the beginning of this field study a couple of risk factors have been roughly envisaged and referred to according to local observations. These comprised: sampling location, animal origin location, with GPS details, temperature during transportation of samples to the laboratory, distance between the origin location and with the nearest pig farm, skin parasites, pig race, age, sex, weight, health status, presence of haemorrhagic clinical signs or lesions, farming practice, alert about ASF within surrounding areas. Some difficulties were faced with some of these data, among which: a) data about sylvatic cycle, b) difference between traditional and free ranging farming systems in rural areas, c) GPS data for spatially closed farms or locations. Regarding exploration of sylvatic cycle data, given the short time of our stay in the forest environment, we fully relied on our collaboration with the hunters as sometimes advised (Costard, et al., 2009). Some tissues and blood including ticks from both Red Rivers Hogs and domestic pigs were collected directly on the animals from the markets. In order to avoid confusion about pigs categories as exploited in the villages, which negatively impact on the estimation of prevalence or other calculation, both traditional and free ranging pigs were clustered together as indigenous pigs. As a matter of fact, the traditional system consist of raising pigs in backyard of fenced houses or gardens which is different with but it was sometimes observed

that pigs were clustered in backyard only for the night and, released as free-ranging during the day for feeding. No strict limit could be made between the two types of exploitation. Regarding the GPS data, spatially closed farms or locations with opposite results were difficult to be segregated on the map especially when dealing with calculation of prevalence; luckily, GPS data detailed in decimals were made available thanks to Google earth.

b. **Laboratory analysis**

At the laboratory level, some critical observations about diagnostic techniques need to be pointed out: a) use of crude ASFV cytosoluble antigen-based indirect ELISA (iELISA); b) use of PCR; c) confirmatory diagnostic using rtPCR instead of immunoblotting; d) IMP as appropriated diagnostic tool for wild suids (red rivers hogs) tissues; e) B602L CVR gene sequencing as a powerful approach for discrimination of genetically closed ASFV isolates. When comparing other screening tests used in the field in DRC, the crude ASFV cytosoluble antigen-based indirect ELISA (iELISA), which is so far, conventionally recommended by the OIE and adopted by FAO (OIE, 2012), was used either alone for diagnostic through detection of anti-ASFV IgG during the 1997-2006 prospective step or, as a reference test for comparison of other two field screening tests. According to some published literature data (Cubillos et al., 2013), this serological test which is still presented as the gold standard has been incriminated as being of less specific. This weakness might have impacted on the results of the assessment of the screening tests ‘characteristics when comparing them and, the same calculation of the overall prevalence. Regarding PCR, during the amplification step, two types of DNA templates were assayed, i.e., consisting of the direct use of the crude organ, empirically estimated to 50 ng and, the other consisting in the well extracted DNA with QIAGEN kit following specific protocols as recommended by the supplier. In terms of learned lessons, some advantage could be taken from the former strategy especially in the African laboratories due to the costly price of molecular biology reagents. In order to fill the gap left by the low relative specificity of the OIE/FAO cytosoluble antigen-based iELISA kit, Immunoblotting (IB) using p72 or p30 proteins were suggested by the OIE (Cubillos et al., 2013, OIE, 2012). Apart from the fact of not being available because of the price, two kits containing the two western blot reagents supplied by INIA, Madrid, Spain and INGENASA, Madrid Spain, respectively behaved very well as confirmatory diagnostic but, for this type of confirmation , rtPCR has been suggested as the gold standard test for ASFV diagnosis (Oura et al., 2013). For frequent availability of bush pigs specimens our collaboration with the hunters and the rural traders was very helpful, even if very costly. Given the long distance

between villages and hunting areas and the lack of gold chain, ethanol and 10% formalin fixed tissues was the only way to be exploited in our field conditions for preservation of wild suids specimens during both the hunting season and transportation to laboratory. With this alternative sampling strategy, only Immunohistochemistry (IHC) and or PCR could be performed at the level of laboratory, as diagnostic tests. We furthermore observed that, Immunohistochemistry as a more sensitive and more specific diagnostic test was more appropriated for the analysis of feral pigs specimens collected during hunting operations (Oura, et al., 2013, 1998a and 1998b). We also discovered that, for a good resolution in terms discrete discrimination of closely related viruses, sequencing of B602L CVR gene, encoding the 9rl ORF protein was more powerful in terms of genetic marker than the p72 and p54 genes, in terms of differentiation of genetically closed ASFV isolates. This finding has been also confirmed reported by several previous authors (Boshoff et al., 2007; Gallardo et al., 2009, Owolodun, et al., 2010).

8. Research strengths and difficulties

In terms of strengths, we were able to confirm that this is the first extensive study devoted to ASF molecular epidemiology in the DRC, in regard to all the approaches developed in all the chapters of this research work. A wide range of novel findings have been yielded by this research work which, hopefully will benefit to animal health officials of the DRC government and those of other Central Africa‘ s countries. As first instance, we may mention the revealed promising pathway in the direction of vaccine production prospects appropriated to endemic ASF areas of the SSA (however the safety of the vaccine was still not tested). Secondly the knowledge of circulating isolates in terms of genotypes and virus evolution should be improved. Thirdly, the knowledge of the prevalence of ASF should be improved, especially in the Western part of the country which was targeted different steps of this research work. Finally, a wide of range of risk factors and risk drivers were briefly described or discussed, which importantly will be a strong epidemiological contribution to prevention and control strategies. Indirectly, several stakeholders including public and private sectors researchers, diagnosticians, teachers, students, breeders and other national and international experts operating in the field of animal health will hopefully take advantage of these few inputs.

On the other hand, the large surface and size of the DRC made it quite difficult to explore. The cornucopia of natural reserves including variety of ticks and huge amount of wild suids will still need profound exploration. The low number of published materials about the disease,

not only the DRC, but also the sub-Saharan Africa region and even for the world was real bottle neck for the achievement of this research work. More than 150 published papers were found and, the majority of these were written in the 1990s and before, by non-African scientists and by researchers of the same scientific reference institutes.

Thus, little is still known about ASF in the sub-Saharan Africa, further and diverse studies are still needed.

9. Risk Factors and control measures

Due to complexity of the disease in the large majority of Sub-Saharan countries, control and eradication of the disease will be difficult but, even if strict zoo-sanitary measures are difficult, they are the only existing way if any prevention and eradication of this epizootic disease is an option (Sanchez-Vizcaino, 2012). The transmission chain between infected and non-infected susceptible species must be interrupted, the same with infected areas and non-infected ones. As demonstrated above, the important reservoir of ASFV are wild suids and *Ornithodoros* ticks, beside healthy carriers among domestic pigs, presented in our results as apparently healthy pigs. The first action should be the protection of disease-free areas which are existing in endemic ASF countries. This should be the starting point, as successfully implemented in South Africa (Costard et al., 2009). The second bottle neck to be addressed is contact between wild and domestic pigs including classic vectors, i.e. *Ornithodoros* ticks chiefly, using double fences (including electric ones if possible) as successfully implemented in South Africa (Perinth et al., 1998), around the Paul Kruger National Park. Housekeeping of free-ranging pigs within strongly fenced backyard premises as currently being tried in Cote d'Ivoire where the disease was drastically eradicated (FAO, 2001), and finally starting with progressive elimination of symptomless pigs, progressively detected through programmed surveillance and automatically separated from uninfected ones, followed by compulsory slaughter. These operations will be associated with others such as observation of biosafety rules, public awareness, vulgarisation involving all the stakeholders, indeminisation, observation of classic strict measures and national contingency plans. This strategy could be achieved as a pilote project, which will progressively be implemented in other districts with the support of local administrative chiefs and other social structures.

Chapter 11 – Conclusions, recommendations and perspectives

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1. Conclusions

This is the first study to be fully devoted to African swine fever in the Democratic Republic of Congo (DRC). Some of generated results are not only crucial, but also novel due to the fact of being revealed as a first hand outcome. By the time being, it may be considered that:

1. More than one Genotype of the Africa swine fever virus (ASFV) are circulating within the DRC territory, comprising one (the Genotype I) historically known as restricted to domestic pigs and as ESACAWA, and two previously described as specific to wildlife (the Genotypes XIV and XI) in southern and eastern Africa;
2. During one outbreak, in the same farm (commercial or backyard) more than one Genotype may co-circulate;
3. During just one outbreak, in the same farm, more than ASFV variants may co-circulate;
4. Co-infection of one animal by two ASFV variants is possible;
5. Different ASFV genotypes, in domestic pigs, during outbreaks occurring in the same areas may have identical nucleotides sequences which need to be clarified between homoplasy or genetic recombination through further studies and
6. Estimated prevalence of ASF in the DRC is between 20 to 30 %, depending on the area, farming system, ecological profile, and epidemiological situation of the area in regard to ASF, genotypes, virus variants or other critical risk factors.

Other confirmed information revealed by this research are:

1. Complexity of ASF epidemiology in the DRC, given presumable introduction from sylvatic cycle to domestic pig cycle directly from sylvatic cycle (Genotype XIV in Katanga and Genotype IX in Orientale and Equateur provinces);
2. Endemicity of ASF in the DRC, based not only on serological evidence, but also on detection of the virus in healthy pigs and wild suids and
3. Cross-boundary introduction through the long and porous national border.

2. Recommendations

In terms of recommendations, due to the fact that ASF has been for long time, a major constraint to pig sector within the country, with only 961,000 heads against more than 75,000,000 of population of which 75 % living in rural areas, our master recommendation is addressed not only to the Congolese government, but also to other international partners, from which active engagement is still expected. As a matter of fact, 193 UN member states including 23 international organisations agreed to achieve by the end of 2015, the eight WHO millennium development goals. The number one of these goals is "eradication of extreme poverty and hunger". ASF is a major constraint to food security and poverty alleviation in poor rural areas in Africa, beside the fact of being a threat to pig industry in developing countries. More research funds are needed for research, prevention and control of ASF in the world.

The key recommendation is "breaking the transmission chain between infected and uninfected pigs" through careful control of major risk factors and depending on whether or not we are at the international or national level and; if on the national depending on the type of farming whether it is traditional (dominated by free-ranging or rear raising exploitation) or modern (characterised by commercial farms with house kept pigs).

At the international level:

1. Control of pig and pork movement across the national borders which is comprising land borders, airports and sea ports. In these circumstances import or circulation permits should be required, and quarantine facilities for long term observation of live animals should be implemented and for that,
2. Qualified and competent veterinary staff should be appointed and requested to be permanent at their posts,
3. Trans-frontier parks need to be either double fenced or closely supervised,

At the national level:

4. With respect to the traditional system (free ranging exploitation):
    a. Clustering of pigs in well fenced backyard premises is required,
    b. Feeding supplementation in order to avoid scavenging of animals across the villages should be associated with,

c. Depending of the level, whether municipalities, districts and provinces, circulation permits must be pre-requisites for pigs' movement across relevant borders,
d. Public awareness, and mobilisation of stakeholders, involving pigs' keepers, traders, carriers, butchers, hunters, conservators, administrative officials, and public media should be done on regular basis.

5. With regard to commercial and modern system:
    a. Implementation and careful application of Biosafety measures should be due,
    b. Cleaning and disinfection of farms,
    c. Observation of legal measurements dealing with restocking.

These general measures should be completed with others depending on specific circumstances such as:

1. During outbreaks:
    a. Strict observation of biosafety measures, e.g. good disposal of dead pigs carcasses and other related waste using incineration,
    b. Compulsory slaughtering of infected and in contact pigs should applied,
    c. Prohibition of selling neither live pigs nor pork from infected premises;
    d. Early detection and reporting of disease occurrence etc.
2. Within interfaces where interactions between domestic and wild suids are existing, the hunting activities should be strongly regulated beside clustering of pigs in strongly fenced backyard as advised above and,
3. Availability of well-equipped diagnostic facilities is strongly recommended.

Holistically, those should be the most observed recommendations for our endemic ASF ground, still needing government's engagements and hopefully with indeminisation of pig keepers, if successful outcome is needed.

3. Perspectives

Given the lack of vaccine and drugs against ASF and due to the persistence of the disease in the large majority of the sub-Saharan Africa and, the threat to the world pig industry, including its causal agent is strongly needed.

In terms of perspectives and, depending on available logistic and financial support, the following activities are expected:

- Short Term:
    i. To determine the virological nature of ASFV DNA detected the group A pigs 'tissues (lymph nodes), i.e., to verify whether or not they were alive or dead viruses, using PMMC cell culture technique;
    ii. To propose to the DRC' government through the Ministry of Agriculture, Fishery and Husbandry, the specific contingency plan draft requested at the Ouagadougou – ASF – Regional Meeting (November, 2015) for each concerned country;
- Mid Term:
    i. To go ahead with field investigation about determination of sylvatic cycle, i.e. Ornithodoros ticks and wild suids, in terms of both, existence and infection;
    ii. To conduct large scale genotyping studies, at the level of the country involving all the ASF susceptible species, i.e. with domestic, wild suids, and ticks samples from all the provinces;
- Long Term:
    i. To conduct a large scale vaccine trial using OURT88/3 involving not only genotype I, but also genotypes X, XIV and others to be delineated from the abovementioned samples. During this next trial, some critical steps of the protocol such as number of passages of our vaccine candidate in primary PBMM, number of days between day 21 post last challenge and the humane end point will be increased. And, at this stage of assay, target tissues containing ASF DNA copies will be analysed for determination of virological nature of relevant strains.

PART V – BIBLIOGRAPHICAL REFERENCES

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PART VI – APPENDIXES

Appendix - 1

A New Species of *Rhipicephalus* (Acari: Ixodidae), a Parasite of Red River Hogs and Domestic Pigs in the Democratic Republic of Congo

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ABSTRACT A new tick species belonging to the genus *Rhipicephalus* Koch, 1844 (Acari: Ixodidae), namely, *Rhipicephalus congolensis* n. sp., is described. Males and females of this species are similar to those of *Rhipicephalus complanatus* Neumann, 1911 and *Rhipicephalus planus* Neumann, 1907, but it can be distinguished from them by a pattern of dense medium-sized punctations on the conscutum and scutum. Males of *R. congolensis* may be distinguished by the following characters: posterior half of the marginal groove deep with a sharp outer edge; anterior portion of the groove shallow with rounded edges; posteromedian groove distinct, long, and deep; adanal plates broadly sickle-shaped; bluntly pointed posteromedian spur on coxa I; and posterolateral spur on coxa I slightly longer or subequal to posteromedian spur. Females of *R. congolensis* may be distinguished by the following characters: outer edge of cervical grooves smooth and not clearly defined either by slope or punctations; genital aperture broad, bowl-shaped, and tripartite in appearance, with central flap flanked on either side by an oval depression; and posteromedian spur on coxa I tapering to its apex. *R. congolensis* is known only from the Democratic Republic of Congo, where the adults were collected from red river hogs, *Potamochoerus porcus* (L.), and domestic pigs, *Sus scrofa* (L.), within the dense equatorial forest in the districts of Équateur and Tshuapa, in the province of Équateur.

KEY WORDS *Rhipicephalus*, new species, Democratic Republic of Congo, Suidae

The genus *Rhipicephalus* Koch, 1844 (Acari: Ixodidae) currently comprises 82 valid species, including the five species previously belonging to the genus *Boophilus* (Curtice 1891 and Guglielmone et al. 2010). With the exception of the four ornate types and a few other species with unique taxonomic characters, species of this genus are generally considered difficult to identify. This difficulty stems from their overall morphological similarity, including their brown color, but marked geographical and individual variation in taxonomic characters. The last major taxonomic revision of the genus was that by Walker et al. (2000), who provided detailed descriptions and illustrations of the adults of all species they considered valid at that time. They also described and illustrated the nymphs and larvae of those species for which they were known.

Based on line drawings of the morphological characters of the dorsal aspect of the gnathosoma of the nymph and larva, Walker et al. (2000) proposed species groups within the genus. These proposals proved to be extremely accurate in predicting which adult ticks belonged to a species within a particular grouping. Unfortunately, in those instances for which the immature stages were not known, Walker et al. (2000) did not assign the adults to a specific group. However, males and females of species within a group usually possess a common or similar set of characters that can be used for designating them to that specific group.

A new species of *Rhipicephalus* seemingly closely related to *Rhipicephalus complanatus* Neumann, 1911 and *Rhipicephalus planus* Neumann, 1907 has recently been discovered in Democratic Republic of Congo parasitizing both red river hogs, *Potamochoerus porcus* (L.), and domestic pigs, *Sus scrofa* (L.); the adults are described here.

Materials and Methods

The material examined for the description is summarized in Table 1. Only field-collected ticks were available for study. The specimens that we examined are deposited in the U.S. National Tick Collection (USNTC), The James H. Oliver, Jr. Institute of Arthropodology and Parasitology, Georgia Southern University (Statesboro, GA) and the I. G. Horak tick collection (IGHTC), Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria (Onderstepoort, South Africa). Some of the paratypes will be deposited in the collections of

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Table 1. ***Rhipicephalus congolensis*** **n. sp., material studied**

No. of ticks ♂	No. of ticks ♀	Host	Locality	Date	Collector	Accession. no.
24	15	*S. scrofa*	Boende	21-VII-2007	L.K.M.-M.	USNMENT 00714032 USNMENT 00714120 USNMENT 00714223 USNMENT 00714126
2		*P. porcus*	Boende	21-VII-2007	L.K.M.-M.	IGHTC
5	3	*S. scrofa*	Boende	24-VII-2007	L.K.M.-M.	USNMENT 00714410
3		*S. scrofa*	Boende	9-IX-2007	L.K.M.-M.	IGHTC
4		*P. porcus*	Mbandaka	14-VII-2007	L.K.M.-M.	USNMENT 00714306
2	2	*P. porcus*	Mbandaka	20-VII-2007	L.K.M.-M.	IGHTC
2			Mbandaka	29-VII-2007	L.K.M.-M.	IGHTC
42	20	Total				

All specimens were collected in Équateur Province, Democratic Republic of Congo.

the Zoological Institute, Russian Academy of Sciences (St. Petersburg, Russia), and the Gertrud Theiler Tick Museum of the ARC-Onderstepoort Veterinary Institute (Onderstepoort, South Africa).

The finer structures of the adults were examined with scanning electron microscopy; the macrostructures of males and females were examined under a stereoscopic microscope (Olympus SZX16, Olympus Corporation, Tokyo, Japan). Measurements are given in millimeters. Measurements are arranged as follows: minimum–maximum (average; n = number of specimens measured). All illustrations were drawn by D.A.A.

Rhipicephalus congolensis n. sp. (Figs. 1–3)

Male. Conscutum (Fig. 1A): length 3.12–3.78 mm (3.48 mm; $n = 5$), width 2.07–2.73 mm (2.41 mm; $n = 5$), ratio length to width 1.39–1.51 (1.45; $n = 5$). Broadly oval, widest slightly posterior to mid-length; reddish brown; central and posterior surfaces depressed, giving conscutum a concave appearance; cervical grooves indistinct, very short and shallow; marginal grooves long, almost reaching eyes, shallow anteriorly with rounded edges and deeper posteriorly with sharp outer edges, demarcated by medium-sized punctations, enclosing the first festoons; posteromedian groove long, distinct; paramedian grooves indistinct, shallow depressions; medium-sized punctations densely aggregated on central part of conscutum; smaller punctations densely spaced on scapulae and sparsely distributed along lateral margins of conscutum; 11 distinct festoons posteriorly. Eyes oval, almost flat. In engorged males, body wall is expanded posterolaterally and forming a short, blunt caudal process posteromedially. Anal plates (Fig. 2A): two pairs; adanal plates long, fairly broad, sickle-shaped, mildly convex, with a long lateral margin, broadly rounded posterior margin, and inner margin distinctly concave posterior to the anus; accessory plates small, pointed. Spiracular plates (Fig. 2B): oval with short, broad dorsal prolongation. Circumspiracular setae sparse.

Gnathosoma (Fig. 2C and D): slightly longer than broad dorsally; length 0.79–0.92 mm (0.86 mm; $n = 5$), width 0.73–0.89 mm (0.81 mm; $n = 5$), ratio length to width 1.04–1.10 (1.07; $n = 5$). Basis capituli (Fig. 2C and D): hexagonal, with short lateral angles at about the anterior quarter of its length; dorsal cornua large, triangular, with bluntly pointed tips, equal to between one fifth and one sixth of the length of the basis capituli; ventral auriculae short, broadly rounded. Palpi (Fig. 2C and D): short, 1.5× or less as long as broad dorsally; palpal segment II subrectangular, palpal segment III broadly rounded apically; segment II slightly longer than segment III dorsally. Hypostome (Fig. 2D): club-shaped, dental formula 3/3.

Legs (Fig. 1A) increase slightly in size from I to IV. Coxae (Fig. 2E and F): anterior process on coxa I inconspicuous from above; posteromedian and posterolateral spurs of coxa I are juxtaposed, long, subequal in length or posterolateral spur slightly longer than posteromedian spur, both spurs tapering to apex; coxae II–IV each with distinct, triangular posterolateral spur; coxae II and III each with modest, broadly arcuate posteromedian spur; coxa IV with distinct, triangular posteromedian spur.

Female. Scutum (Fig. 1B): broader than long; length 1.71–2.10 mm (1.92 mm; $n = 4$), width 1.93–2.27 mm (2.12 mm; $n = 4$), ratio length to width 0.88–0.93 (0.91; $n = 4$). Reddish brown; posterior margin sinuous; cervical pits deep; cervical grooves broad, shallow, diverging, outer edge of cervical grooves smooth; dense pattern of medium-sized punctations on central and cervical fields; smaller punctations less densely spaced on lateral fields. Eyes at about mid-length of scutum, oval, almost flat. Genital aperture (Fig. 3A): broad, bowl-shaped, tripartite in appearance, with the central pillar flanked on either side by an oval depression. Spiracular plate (Fig. 3B): broadly oval, with short, broad dorsal prolongation.

Gnathosoma (Fig. 3C and D): slightly longer than broad dorsally; length 0.92–1.13 mm (1.04 mm; $n = 4$), width 0.91–1.08 mm (1.00 mm; $n = 4$), ratio length to width 1.02–1.05 (1.03; $n = 4$). Basis capituli (Fig. 3C and D): hexagonal; with short lateral angles slightly anterior to its mid-length; dorsal cornua large, triangular, with rounded tips, comprising approximately one seventh of length of basis capituli; porose areas oval, ≈1.5× their own diameter apart; ventral auriculae short, broadly rounded. Palpi (Fig. 3C and D): short, 2× or slightly less as long as broad dorsally; palpal segment II subrectangular, palpal segment III

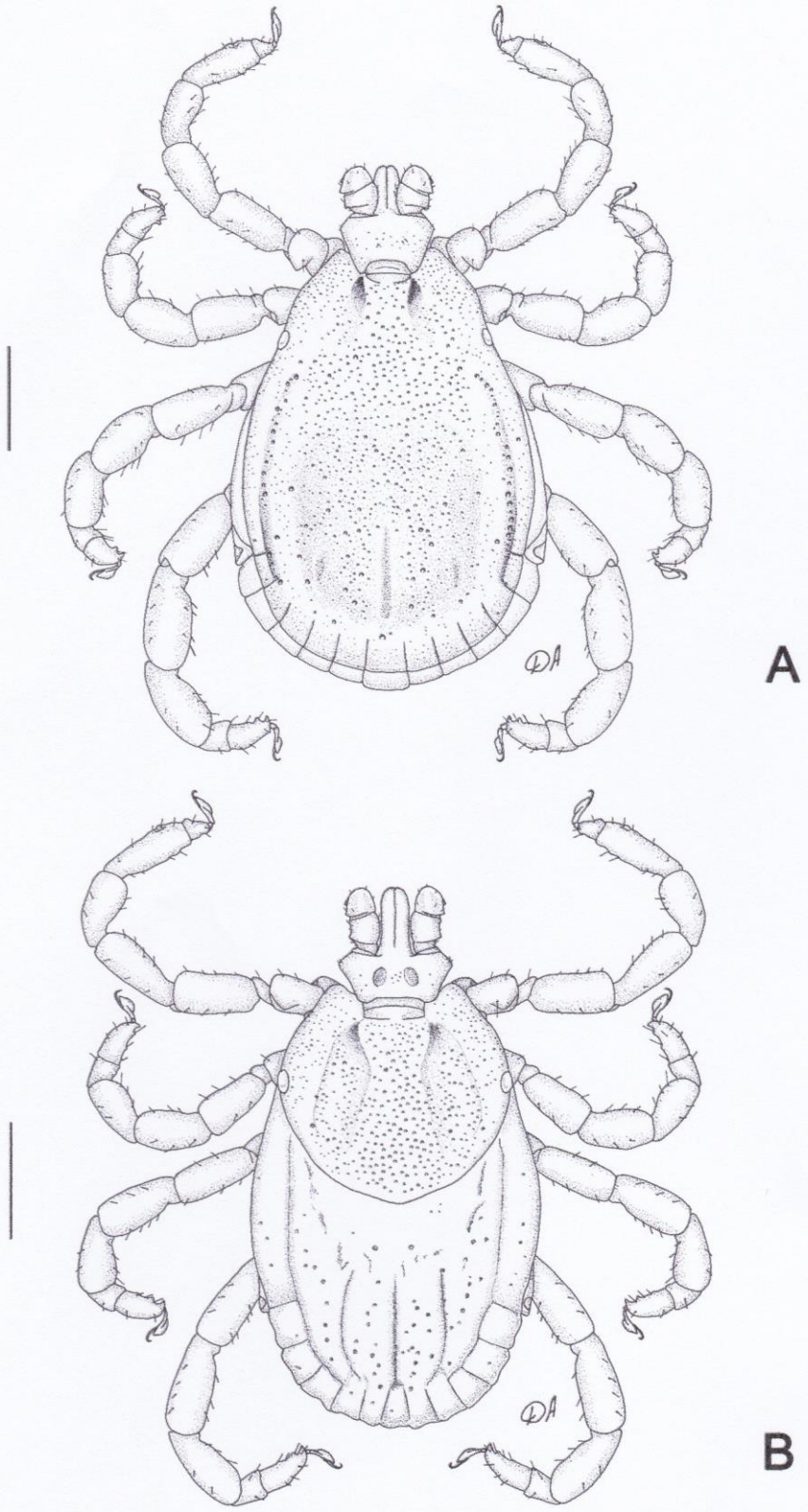

Fig. 1. *Rhipicephalus congolensis* n. sp. (A) Male dorsally. Bar = 1 mm. (B) Female dorsally. Bar = 1 mm.

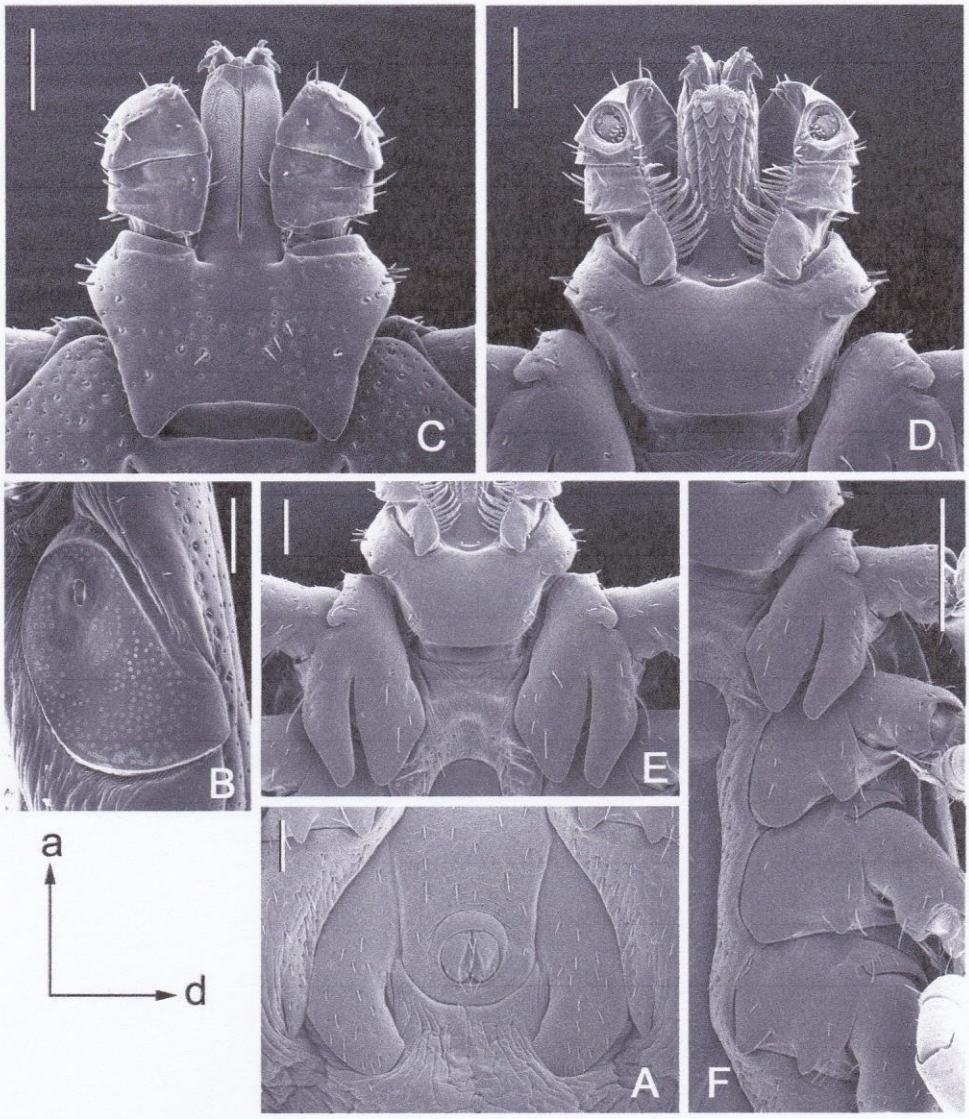

Fig. 2. *Rhipicephalus congolensis* n. sp., male. (A) Anal plates. Bar = 0.2 mm. (B) Spiracular plate. Bar = 0.2 mm. Arrows indicate orientation of spiracular plate (a = anterior; d = dorsal). (C) Gnathosoma dorsally. Bar = 0.2 mm. (D) Gnathosoma ventrally. Bar = 0.2 mm. (E) Coxae I. Bar = 0.2 mm. (F) Coxae. Bar = 0.5 mm.

broadly rounded apically; segment II slightly longer than segment III dorsally. Hypostome (Fig. 3D): club-shaped; dental formula 3/3.

Coxae (Fig. 3E and F): anterior process on coxa I inconspicuous from above; posteromedian and posterolateral spurs of coxa I juxtaposed, long, subequal in length or posterolateral spur slightly longer than posteromedian spur, posteromedian spur tapering to apex; coxae II–IV each with distinct, triangular posterolateral spur; coxae II and III each with modest, broadly arcuate, posteromedian spur; posteromedian spur on coxae IV distinct, broadly triangular.

Nymph and Larva. Unknown.

HOLOTYPE. Male, from *S. scrofa*, Boende, Équateur Province, Democratic Republic of Congo (0° 15′ S; 20° 45′ E), 21-VII-2007, L. K. Mulumba-Mfumu; deposited in the USNTC (USNMENT 00714032; CEN 126545).

ALLOTYPE. Female (USNMENT 00714120; CEN 126545), with the same collection data as for holotype.

PARATYPES. Total: 41 males and 19 females. Collection data are listed in Table 1. Seven males and four females are deposited in the USNTC (USNMENT 00714223, CEN 126545; USNMENT 00714126, CEN 126546; USNMENT 00714306, CEN 126547; USNMENT 00714410, CEN 126548); the remaining specimens are deposited in the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria. Some of the paratypes will be deposited in the collections of the Zoological Institute, Rus-

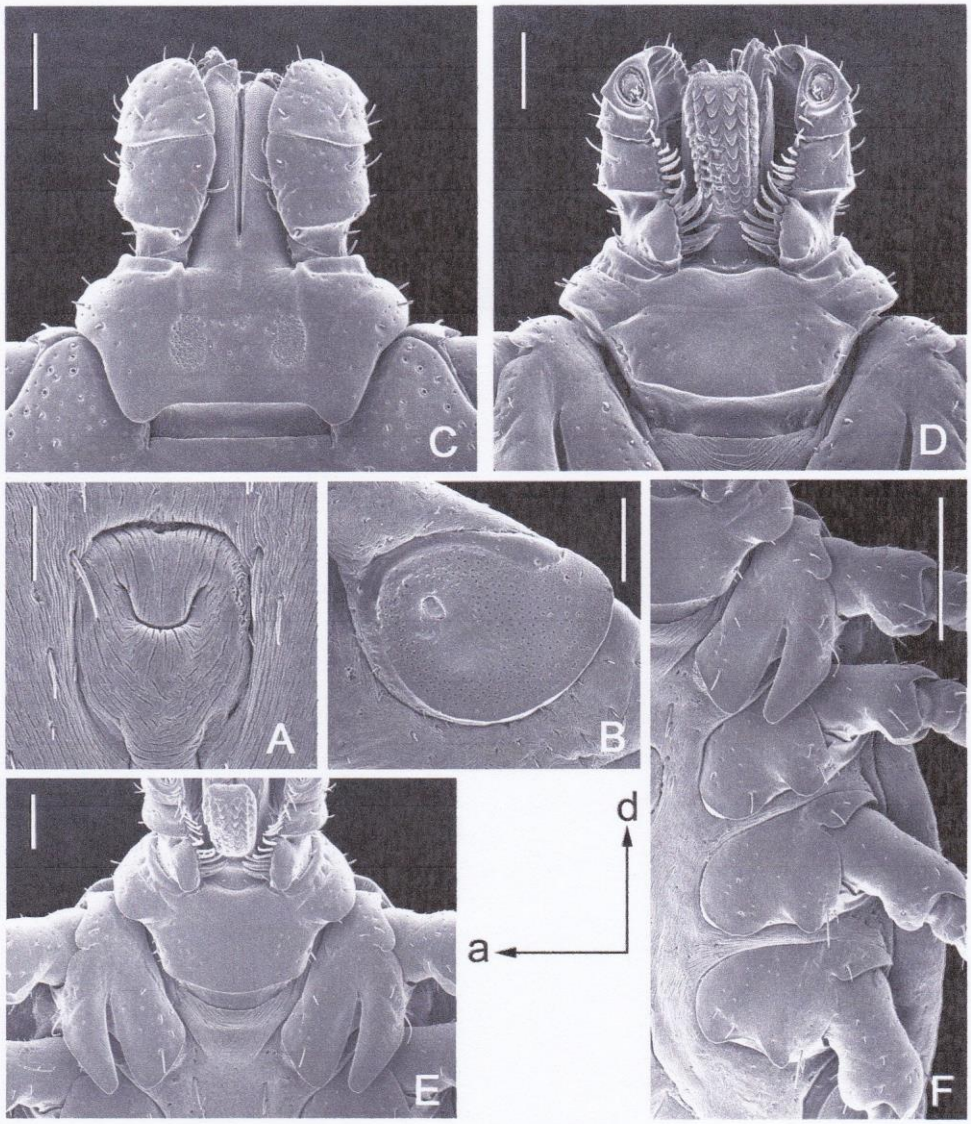

Fig. 3. *Rhipicephalus congolensis* n. sp., female. (A) Genital aperture. Bar = 0.1 mm. (B) Spiracular plate. Bar = 0.2 mm. Arrows indicate orientation of spiracular plate (a = anterior; d = dorsal). (C) Gnathosoma dorsally. Bar = 0.2 mm. (D) Gnathosoma ventrally. Bar = 0.2 mm. (E) Coxae I. Bar = 0.2 mm. (F) Coxae. Bar = 0.5 mm.

sian Academy of Sciences, and the Gertrud Theiler Tick Museum of the ARC-Onderstepoort Veterinary Institute.

Distribution and Hosts. The collection data for *R. congolensis* is that of the type series (Table 1). This species is confined to the Democratic Republic of Congo, Équateur Province (Fig. 4). All adult specimens have been collected from red river hogs and domestic pigs.

Etymology. The species is named after the Democratic Republic of Congo, in which it was discovered.

Related Species. Morphologically the adults of *R. congolensis* are most similar to those of *R. complanataus* and *R. planus*. The surface of the central and posterior regions of the conscutum of the males of these species is depressed, giving the conscutum a concave appearance. Based on the shape of the adanal plates of the male and tripartite genital structures of the female, the new species is closer in appearance to *R. planus* than to *R. complanatus*.

The males of *R. congolensis* can be distinguished from those of *R. complanatus* and *R. planus* by the following characters: central part of conscutum densely covered with medium-sized punctations (smooth, sparsely covered with larger punctations in *R. complanatus* and *R. planus*); posterior half of marginal grooves deep with sharp outer edge, anterior portion of the groove shallow with rounded edges (marginal grooves deep throughout their length, with sharp lateral edges, in *R. complanatus* and *R.*

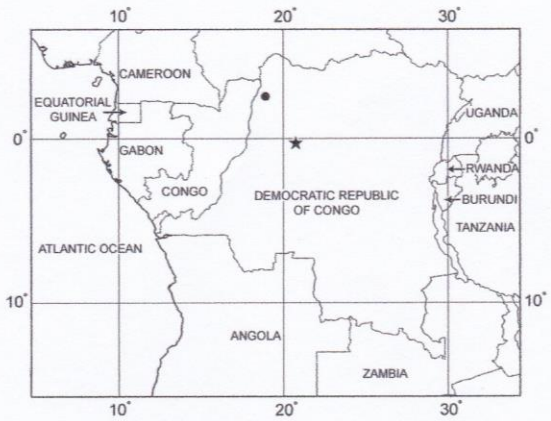

Fig. 4. *Rhipicephalus congolensis* n. sp., distribution. Star shows type locality, and filled circle shows confirmed locality.

planus); posteromedian groove distinct, long and deep (usually appears indistinct or sometimes as a short and shallow groove in *R. complanatus* and *R. planus*); adanal plates fairly broad, sickle-shaped (*R. complanatus* with broad adanal plates, particularly posteriorly, with concave posterior margin); posteromedian spur on coxa I pointed (rounded in *R. planus*); posterolateral spur on coxa I slightly longer or subequal to posteromedian spur (considerably longer in *R. complanatus*).

The females of *R. congolensis* can be distinguished from those of *R. complanatus* and *R. planus* by the following characters: central field of scutum densely covered with medium-sized punctations (smooth, sparsely punctate in *R. complanatus* and *R. planus;* rarely moderately covered with shallow punctations or ones with sloping walls in *R. planus*, giving an irregular appearance to the scutal surface); outer edge of cervical grooves smooth, not clearly defined by slope or punctations (sharp, clear outer edge defined by large punctations in *R. planus*); genital aperture tripartite in appearance, with moderately broad central pillar with straight posterior margin, flanked on each side by a rounded depression (not tripartite in *R. complanatus;* central pillar very narrow and tapering posteriorly in *R. planus*); posteromedian spur on coxa I tapering to apex (rounded in *R. planus*).

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Appendix - 2

THE CONTRIBUTION OF MOLECULAR EPIDEMIOLOGY TO INFORMING CONTROL MEASURES FOR AFRICAN SWINE FEVER

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ABSTRACT

Genotyping methods have been shown to be a useful tool for outbreak investigation and for improving our understanding of the epidemiology of various animal diseases at local and global level. For African swine fever (ASF) a large genetic variability among strains was found in East Africa, reflecting introductions into domestic pigs through the sylvatic cycle. However, for Western and Central Africa little is known about the sub-grouping of circulating genotypes. During a prevalence study in the Democratic Republic of Congo, regional differences in prevalence and virulence of circulating strains were observed in free-range pigs and pigs kept in closed housing systems. To identify the circulating genotypes in these different environmental settings, ASF isolates from apparently healthy pigs were compared with strains isolated during outbreaks in urban areas. This study aimed at investigating if certain ASF genotypes can be linked with specific exposure pathways, which might also result in different incidence and prevalence in the respective region. The results of this study not only provide important insights into the epidemiology of ASF, but will also allow the formulation of targeted preventive measures suitable for the respective husbandry system.

INTRODUCTION

African swine fever (ASF) has become endemic in most sub-Saharan countries over the last twenty years. The main infection pathways for African swine fever have been identified using cross-sectional, case-control studies and outbreak investigations. Direct contact between pigs is regarded as the main factor for spreading of the disease within the domestic pig population. Indirect contact through fomites and people involved in the pig production chain has also been associated with infection of domestic pigs. The virus has been found in warthogs and bush pigs, in which infection is sub-clinical most of the time. With

these hosts and the soft tick species *Ornithodoros moubata* (in East Africa) and *Ornithodoros erraticus* (in Europe) which can remain persistently infected over a long time, an endemic cycle can be maintained in a region. Indirect transmission and the presence of a wildlife cycle especially compromise the effectiveness of control measures. So far, no vaccine is available and disease control largely depends on stamping out and pig movement restrictions. The importance of the various infection pathways differs between affected countries depending on potential contact with wildlife, characteristics of the pig production chain and husbandry methods in place. Even within a country the importance of the various known infection pathways differs from region to region due to the differences in the aforementioned factors. This is also the case in the Democratic Republic of Congo (DR Congo) where regular outbreaks have been reported across the country. Little is known in relation to the genetic variability of circulating strains in DR Congo. In a study assessing the genetic heterogeneity of circulating ASF isolates from across Africa (Lubisi *et al.,* 2005), sequencing of the conserved region of the p72-gene placed the only previously characterized strain from DR Congo in the ESACWA genotype group which comprises European, South American, Caribbean and West African isolates. Recently described methods for subtyping of ASF isolates have been useful for understanding epidemiological complexities at the country, genotype and regional level (Bastos *et al.,* 2004; Nix *et al.,* 2006; Lubisi *et al.,* 2007). The current understanding is that higher genetic variability is present in the wildlife cycle in E. and S. Africa compared to the domestic cycle. Various investigations have been carried out to quantify exposure of domestic pigs to specific infection pathways, and transmission models for ASF have been developed (Costard *et al.,* 2007). Risk assessment offers a structured approach to dealing with local and regional differences in exposure of domestic pigs to potential infection sources and the resulting consequences, which in this case would be an ASF outbreak. However, poor data quality or missing data has often been an important constraint for many risk assessments carried out in Africa. This paper aims at demonstrating the potential for including molecular epidemiology tools into a risk assessment approach, focusing on exposure assessment, to investigate the importance of infection pathways for African swine fever for domestic pig herds. Knowing which genotypes circulate in a geographical area with known prevalence and what the genetic variability among these strains is, can be a helpful indicator for assessing the relative importance of exposure pathways. The outcomes of such a combined approach will not only corroborate findings of a risk assessment, but can also inform the formulation of targeted control and surveillance strategies at regional level.

MATERIALS AND METHODS

The study is based on data collected during a cross-sectional study carried out in 2005/2006, where 520 serum samples of apparently healthy pigs were collected in three markets in the Democratic Republic of Congo, representing 37 villages of three provinces Bas Congo, Equateur, and Kinshasa. Different environmental settings, including rural, peri-urban and urban settings, free range and closed husbandry systems are represented with this sample. The sample sites also differed in possible contact rates of domestic pigs with wild suids and the presence of soft ticks. Samples were tested for ASF antibodies using a VP72-based indirect ELISA and sero-positive samples were confirmed by PCR. In 2007, samples from bush pigs and warthogs were collected in areas where contact with domestic pigs can occur.

Genotyping of ASF isolates was carried out by sequencing the C terminal end of the *p72* gene (Bastos *et al.,* 2003). To increase the discriminatory power at local and regional level, subtyping of strains was carried out by comparing the size of various amplicons of the central variable region of the 9RL open reading frame ORF (Nix *et al.,* 2006) and sequencing of its central variable region of (Lubisi *et al.,* 2007).

Following a risk assessment framework, the exposure pathways for domestic pigs were defined and the likelihood of exposure of a domestic pig herd was estimated qualitatively for the different study areas and the respective sub-areas. Relevant for the exposure assessment were data on the local structure of the pig industry, including herd management characteristics such as husbandry system, herd size, density of pigs and farms, movements within and between farms, and biosecurity measures. Further characteristics included were direct and indirect contact patterns of different stakeholders of the pig production chain with pig farms. For the exposure assessment, the presence of wildlife reservoirs, in particular the density of *Ornithodoros* soft ticks and wild boars were also included and their potential contacts with domestic pigs or elements of the pig production chain were estimated.

RESULTS

Analysis of the 520 samples collected revealed an overall seroprevalence of 26.9% (confidence interval [CI] 95%: 23.1-30.7%). The prevalence found in free-ranging pigs was 23.8% (95% CI: 18.6-29%) and in pigs from farms with a closed husbandry system 30.1%

(95% CI: 24.5-35.7%), with considerable variation between villages. The ASF prevalence in free-ranging pigs originating from the rural areas where no outbreaks were reported was higher than in free-ranging pigs in peri-urban areas where ASF outbreaks had been observed recently. Preliminary analysis using p72 sequencing placed all the isolates in the ESACWA genotype, however, with subtyping of the 9RL ORF different circulating strains were identified.

DISCUSSION

The preliminary results of this study indicate that different African swine fever virus strains are circulating in the DR Congo and that prevalence in domestic pigs differs between sampling areas and husbandry systems. This suggests the possibility of important differences in sources of exposure to infection. Preliminary results also suggest that the level of prevalence depends on the genotypes present in an area. To further investigate if these observations can be linked with a higher exposure of pigs to certain infection pathways, more isolates need to be included in the analysis. Included ASF isolates will be from apparently healthy pigs in rural areas, from outbreaks in urban areas and from wild suids.

Insights gained through genotyping of isolates collected in the different husbandry and environmental settings, including wildlife isolates, will be combined with estimates of the relative importance of different infection pathways, which will improve the understanding of the epidemiology of ASF in the DR Congo. The results will also provide a basis for the formulation of targeted preventive and control measures at village or regional level. This study demonstrates that tools applied in molecular epidemiology can be successfully integrated into a risk assessment framework.

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