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Evidence for exposure of asymptomatic domestic pigs to African Swine fever virus during an inter-epidemic period in Zambia

Running title: African swine fever during an inter-epidemic period

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Summary

African swine fever (ASF) causes persistent outbreaks in endemic and non-endemic regions in Zambia. However, the epidemiology of the disease is poorly understood, particularly during the inter-epidemic periods. We conducted surveillance for ASF in asymptomatic domestic pigs and soft ticks in selected Zambian provinces. Whilst serum samples (*n*=1,134) were collected from crossbred pigs from all study sites between 2014 and 2017, whole blood (*n*=300) was collected from both crossbred and indigenous pigs in Eastern Province (EP) in 2017. Soft ticks were collected from Mosi-oa-Tunya National Park in Southern Province (SP) in 2019. Sera were screened for antibodies against ASF by ELISA while genome detection in whole blood and soft ticks was conducted by PCR. Ticks were identified morphologically and by phylogenetic analysis of the 16S *rRNA* gene. Seroprevalence was highest in EP (50.9%, 95% CI [47.0 – 54.9]) compared to significantly lower rates in SP (2.9%, 95% CI [1.6 – 5.1]). No antibodies to ASFV were detected in Lusaka Province. In EP, the prevalence of ASFV genome was 11.7% (35/300), significantly higher (OR = 6.2, 95% CI [2.4 – 16.6]) in indigenous pigs compared to crossbred pigs. The pooled prevalence of ASFV genome in ticks was 11.0%, 95% CI [8.5–13.9]. Free-range husbandry system was the only factor that was significantly associated with seropositive ($p < 0.0001$, OR = 39.3) and PCR positive results ($p < 0.001$, OR = 5.7). Phylogenetically, based on the *p72* gene, ASFV from *Ornithodoros moubata* ticks detected in this study belonged to genotype I, but they separated into two distinct clusters. Besides confirming ASF endemicity in EP and the presence of ASFV-infected ticks in SP, these results provide evidence for exposure of domestic pigs to ASFV in non-endemic regions during the inter-epidemic period.

Keywords: African swine fever; *Asfarviridae*; *Ornithodoros moubata*; pigs; Seroprevalence; Zambia

Introduction

African swine fever (ASF) is a contagious, viral hemorrhagic disease of domestic and wild pigs. With mortality rates that can approach 100%, it is considered the single greatest threat to the pig industry. ASF is caused by ASF virus (ASFV), a complex linear double-stranded DNA arbovirus which is presently the sole member of the family *Asfarviridae*, genus *Asfivirus* (Dixon, Chapman, Netherton, & Upton, 2013).

Until early 2007, ASF was only considered to be endemic in sub-Saharan Africa and Sardinia, Italy (Penrith, Vosloo, Jori, & Bastos, 2013; Rolesu et al., 2007). However, by April 2007, ASFV genotype II had been introduced into Europe through Georgia (Sánchez-Cordón, Montoya, Reis, & Dixon, 2018). The disease subsequently spread through the Trans-Caucasus region and the Russian Federation where it is now established in both domestic and wild boar populations (Beltrán-Alcrudo, Lubroth, Depner, & De La Rocque, 2008; Gogin, Gerasimov, Malogolovkin, & Kolbasov, 2013). Recently, ASF was reported in Belgium in wild boars and in a number of Asian countries, including China, Vietnam, Cambodia, Mongolia, and North Korea, where it is also currently associated with high mortality in domestic pig populations (Garigliany et al., 2019; Normile, 2019). The continued, and apparently uninterrupted spread of ASF into new geographical areas raises serious concerns for both the global economy and food security.

While the European wild boar has been reported to play a role in the maintenance of ASFV in Europe (Mur et al., 2012), in sub-Saharan Africa, ASFV is primarily maintained in a sylvatic cycle involving soft ticks of the *Ornithodoros moubata* (*O. moubata*) complex and asymptomatically infected wild pigs, particularly common warthogs (*Phacochoerus africanus*) (Jori et al., 2013; Penrith et al., 2013). Additionally, ASFV can be maintained in domestic pigs through a pig-tick cycle, without involvement of warthogs and a pig-pig cycle

by direct contact with infected animals (Penrith et al., 2013; Quembo, Jori, Heath, Pérez-Sánchez, & Vosloo, 2016).

In Zambia, ASF was first reported in Eastern Province (EP) in 1912 (Wilkinson, Pegram, Perry, Lemche, & Schels, 1988). The disease was officially recognized to be endemic in indigenous free-range pigs in 1965 and as a consequence, a ban was imposed on the export of pigs and pig products from EP (Samui, Nambota, Mweene, & Onuma, 1996). Although the disease was generally considered to be restricted to EP (Wilkinson et al., 1988), ASF was reported for the first time outside this endemic zone in 1989 (Samui, Mwanaumo, & Chizyuka, 1991). By 2018, with the exception of Western Province, the disease had been reported in all provinces of Zambia (Simulundu et al., 2017, 2018a, 2018b).

Even though the epidemiology of ASFV in Zambia has not been well clarified, available evidence suggests a complex epidemiology with the possible involvement of sylvatic hosts in some areas (Simulundu et al., 2017). In non-endemic zones, there is circumstantial evidence to suggest that ASFV may be circulating in domestic pigs in these regions. Previous studies conducted in Southern Province (SP) demonstrated presence of ASFV in soft ticks and likely transmission to domestic pigs through spillover events from the sylvatic cycle (Simulundu et al., 2017; Wilkinson et al., 1988). While most previous studies have relied primarily on disease outbreaks, to date, no study has been conducted to ascertain the prevalence of ASF in domestic pigs in non-endemic and endemic zones during the inter-epidemic periods. Moreover, the latest study to demonstrate presence of infected soft ticks in non-endemic areas was conducted over three decades ago (Wilkinson et al., 1988).

Typically, diagnosis of ASF involves the detection and identification of part of the ASFV genome and/or ASFV-specific antibodies. Detection of ASFV genome by polymerase chain reaction (PCR) is useful early in infection while antibody detection by enzyme-linked immunosorbent assay (ELISA) is suitable in determining prior exposure to ASFV following

126 seroconversion. Here, we conducted serologic and virologic surveillance for ASF in three
127 provinces of Zambia during an inter-epidemic period to improve our current understanding of
128 the epidemiology of the disease.
129
130 **Materials and methods**
131 **Study sites**
132 The study was conducted in selected districts of Eastern, Southern and Lusaka
133 provinces of Zambia (Figure 1). In EP, the study was conducted in Chipata, Katete, Mambwe
134 and Vubwi districts, while in SP, Choma, Kalomo, Kazungula, Livingstone and Namwala
135 districts were investigated and finally, in Lusaka Province (LP), the study was carried out in
136 Lusaka District. The provinces were selected based on data obtained from the Ministry of
137 Fisheries and Livestock which indicated that the areas represented the four types of pig
138 husbandry systems practiced in Zambia; i.e. free-range: where pigs are allowed to roam
139 freely and scavenge for food and are occasionally supplemented with swill; semi-confined:
140 pigs are usually confined and are sometimes allowed to scavenge for food; small- to medium-
141 scale confined: pigs are always confined, but usually with poor implementation of
142 biosecurity; and large-scale confined; pigs are always confined with strict implementation of
143 biosecurity. Furthermore, while these three provinces have had repeated outbreaks of ASF in
144 the past (Simulundu et al., 2017, 2018a, 2018b), there were no reported ASF epidemics in
145 these areas during the study period based on information from the Zambian Ministry of
146 Fisheries and Livestock.
147 Although the majority of farmers (>80%) in EP rear indigenous pig breeds under the
148 free-range system (Gabriël, Mwape, Phiri, Devleesschauwer, & Dorny, 2018; Thys et al.,
149 2016), a minority of farmers rear improved crossbred pigs, i.e. crosses of Large white and
150 Landrace pigs under the small-to-medium scale confined system. In SP, pigs are

predominantly reared under a semi-confined husbandry system with supplementary feeding of swill being a common practice. Mostly, crossbred pigs are reared for commercial reasons. The largest available market for pig and pig products is in LP. While many slaughter facilities are regulated, a small number remain unregulated. Farmers in LP rear pigs under both the commercial small-to-medium scale and large-scale types of husbandry systems.

Sampling protocol

Pigs

Eastern Province

A cross-sectional study was conducted in EP in May of 2017. Although the total pig population in EP was estimated at 1,011,441 in 2016 (www.zamstats.gov.zm), there was no information available on pig populations by district or village. Therefore, the required number of samples were distributed equally among the four districts. In total, 23 villages were included in the sampling frame. Three villages selected in Mambwe District were in Msoro, a rural area bordering South Luangwa National Park. The Park is unfenced with a considerable warthog population. The remaining 20 villages were in rural areas of Chipata (n=4), Katete (n=9) and Vubwi (n=7) districts that are located far (>80 km) from the nearest National Park.

The representative sample size was obtained using an estimated ASFV antibody and genome prevalence of 50% using the formula $n = Z^2PQ/L^2$ (Martin, Meek, & Willeberg, 1987). In this study, confidence level and desired precision were set at 95% and 0.05, respectively. The resulting sample size required for the four districts was 385. Thus, 96 pigs were to be sampled per district. In addition, archived sera collected from indigenous free-range pigs in Katete and Chipata districts between January 2014 and December 2015 were

included in the study. At the time of sample collection, there were no reported cases of ASF in these districts.

Lusaka and Southern provinces

Archived serum samples collected from pigs in Lusaka and Southern provinces between January 2014 and December 2016 were included in the study. Notably, at the time of sampling, there were no reported cases of ASF in these areas. In Lusaka District, unregulated and regulated slaughter facilities were selected. At an unregulated slaughter facility in Chibolya market, the majority of slaughtered pigs are those reared under the semi-confined husbandry system by resource-limited farmers in SP. Usually, pigs are slaughtered with no or minimal veterinary inspection (Siamupa, Saasa, & Phiri, 2018). At regulated slaughter facilities, prior to slaughter, pigs are routinely tested for ASF at the Central Veterinary Research Institute. Most pigs supplied to these facilities are reared under the large-scale type of husbandry system. At both regulated and unregulated slaughter facilities, predominantly crossbred pigs are slaughtered.

In SP, the districts were selected according to their proximity to national parks and past reports of ASF outbreaks. Kalomo and Namwala districts border the Kafue National Park while Kazungula District borders the Mosi-oa-Tunya and Kafue National parks. Choma District is far (>80 km) from the nearest National park and acts as a transit point for animals that come from different parts of SP, which are usually transported to Lusaka's Chibolya market for slaughter (Figure 2).

Soft ticks

Soft ticks were collected from warthog burrows and culverts within Mosi-oa-Tunya National Park in Livingstone District. The desired sample size was calculated as previously

200 described (Quembo et al., 2018) using an estimated warthog population of 300 and 50
201 burrows. Thus, a minimum of three burrows needed to be sampled to detect at least one
202 infected burrow at a 95% level of confidence, assuming 40% prevalence of warthog burrows
203 infested with soft ticks. In total, seven burrows located on anthills, four culverts and one
204 dwelling under a house were examined for the presence of soft ticks.
205
206 **Sample and data collection**
207 **Pigs**
208 A total of 300 crossbred confined and indigenous free-range pigs were sampled from
209 60 small-scale farmers clustered in 23 villages in EP in May 2017. Of the 300 samples, 34
210 were collected in Mambwe District from seven households in three villages while 78 samples
211 were obtained from 17 households in nine villages in Katete District. In Chipata District, 130
212 samples were collected from 21 households in four villages, and 58 samples from 16
213 households were obtained from seven villages in Vubwi District. Blood was collected from
214 the cranial vena cava vein from each animal using vacutainer needles into plain and EDTA
215 tubes. Blood in plain tubes was allowed to clot and serum was separated by centrifugation at
216 1500 x *g* for 5 minutes and stored at -30°C until analysis. Blood in EDTA tubes was
217 aliquoted and stored at -80°C until analysis. Additionally, a semi-structured questionnaire
218 was administered to all farmers whose pigs were sampled. Information such as knowledge of
219 ASF, clinical signs associated with ASF, local names of ASF, history of ASF outbreaks,
220 numbers of animals lost during an outbreak, presence of ectoparasites and presence or
221 interaction of domestic pigs with warthogs and bush pigs was noted. Farmers were also asked
222 to confirm if they recognized seeing soft ticks in pig pens after being shown an image of the
223 *O. moubata* tick.

In addition, archived serum samples collected from indigenous free-range pigs in EP (*n*=323) and crossbred pigs in LP (*n*=128) and SP (*n*=383) were included in the study. Of the 323 archived samples from EP, 303 samples were collected from Katete District while 20 samples were collected from Chipata District. In SP, out of the 383 samples collected, 249 were collected from Choma District, while 92 were collected from Namwala District. Eighteen and 24 serum samples were collected from Kalomo and Kazungula districts, respectively. In LP, 81 samples were obtained from an unregulated abattoir while 47 samples were obtained from a regulated abattoir. The mean number of blood and serum samples collected per district in EP was 75 and 155, respectively. In SP, the mean number of serum samples collected per district was 96. In total, 1,134 serum and 300 blood samples collected from pigs between the ages of three months to approximately four years were analyzed in the present study. The number of collected samples by area and breed is summarized in Table 1.

Soft ticks

Soft ticks (*n*=724) were collected from Mosi-oa-Tunya National Park using a previously described method (Jori et al., 2013). Briefly, loose soil and litter were manually collected from warthog burrows using a shovel and placed on black polyethylene bags and exposed to sunlight to elicit tick movement. The excited ticks were collected using entomological forceps. The ticks were kept alive and transported to the laboratory in 50 mL centrifuge tubes supplied with fresh leaves to provide humidity. Ticks were identified morphologically and by molecular methods as previously described (Black & Piesmant, 1994; Walton, 1979, 1962). The ticks were then stored at -80°C until further processing.

DNA extraction, virus genome, sequencing and antibody detection

Pigs

DNA was extracted from 200 µL of whole blood in EDTA using the Quick-gDNA Miniprep Kit (Zymo Research, Orange, CA, USA), according to the manufacturer's protocol. Screening for ASFV genome was conducted essentially as described previously (Thoromo et al., 2016; Yabe et al., 2014) using the OIE recommended diagnostic primer pairs: PPA1 (5'-AGTTATGGGAAACCCGACCC-3') and PPA2 (5'-CCCTGAATCGGAGCATCCT-3') (Agüero et al., 2003). PCR was carried out on a 96-Well Thermal Cycler (Applied Biosystems, CA, USA) using the OneTaq Quick-Load 2X Master Mix PCR kit (New England Biolabs, Beverly, MA, USA). Serologic analysis for ASFV-specific antibodies was performed using the Ingezim PPA COMPAC double sandwich ELISA (Ingenesa, Madrid, Spain) according to the manufacturer's instructions. Data entry and analyses were carried out using Microsoft Excel software. Samples which gave inconclusive results were re-tested.

Soft ticks

Ticks were tested in pools, with each pool containing two or three adult ticks. For immature ticks, each pool had six or eight nymphs. In total, 124 pools were generated and homogenized as previously described (Quembo et al., 2018). DNA was extracted from 200 uL of clarified homogenate using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to manufacturer's guidelines. Initial analysis for ASFV genome was conducted using the OIE prescribed real-time PCR assay (King et al., 2003). Threshold cycle values below 35 were considered positive for ASFV. In addition, a nested PCR assay reported by Basto et al., (2006) was employed to screen for ASFV genome. Amplification and sequencing of the *p72* gene was condcucted as previously described (Simulundu et al., 2018b). Phylogenetic analysis was performed in MEGA V7.0 (http://www.megasoftware.net). Sequences were deposited in GenBank under accession numbers LC528860 – LC528881.

274

275 **Molecular identification of soft ticks**

276 DNA from 10 pools of soft ticks obtained from each burrow were analyzed using
277 primers targeting the mitochondrial 16S *rRNA* gene as previously reported (Black &
278 Piesmant, 1994). Amplicons of the expected size were purified from agarose gels using the
279 QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) according to manufacturer's
280 protocol. Purified PCR products were sequenced directly using BigDye terminator cycle
281 sequencing ready reaction kit version 3.1 (Applied Biosystems, Foster City, CA, USA) and
282 analyzed on a 3500 Genetic Analyzer (Applied Biosystems). Sequences were assembled and
283 edited using Geneious software (Biomatters Ltd., Auckland, New Zealand) and phylogenetic
284 analysis was implemented using MEGA V7.0 (http://www.megasoftware.net/). The
285 sequences were deposited in GenBank under accession numbers LC492098-LC492107.

286

287 **Statistical analysis**

288 The prevalence of ASFV antibody and viral genome were calculated using EpiTools
289 epidemiological calculators (http://epitools.ausvet.com.au). The pooled prevalence for ASFV
290 genome in soft ticks was estimated as previously described (Williams & Moffitt, 2010). The
291 dependency of the outcome variables (ELISA and PCR results) on potential risk factors i.e.
292 pig breed, proximity to National Park, history of ASF outbreaks, and management system
293 was modelled using multiple logistic regression in R version 3.5.1 (https://www.rstudio.com).

294

295 **Results**

296 **Questionnaire survey**

297 A total of 60 pig farmers from Chipata (*n*=21), Katete (*n*=16), Vubwi (*n*=16) and
298 Mambwe (*n*=7) districts participated in the questionnaire survey. The average litter size per

299 household was seven pigs (95% CI [6.5 – 7.6]) with a mean farrowing per year of 2.0 (95%
300 CI [1.9 – 2.2]). All respondents positively acknowledged knowledge of ASF. They also
301 reported to have lost pigs in the last 12 months to a disease they described as having clinical
302 signs similar to ASF. The most common signs described were diarrhea, depression,
303 staggering, inappetence, purple skin in light skinned pigs, dyspnea, recumbency and sudden
304 death. Two local names (Chimpupu and Chigodola) in the Nyanja language were frequently
305 used to describe the disease, both of which have connotations suggestive of mass mortalities.
306 Reported mortality rates ranged from 10% to as high as 80%. The periods of most recent
307 experience with ASF ranged from less than three months in Chipata and Mambwe districts to
308 between six and 12 months in Katete and Vubwi districts. The most common ectoparasite
309 reported (100%) was lice (*Haematopinus suis*), however, none of the respondents
310 acknowledged seeing *O. moubata* soft ticks in pigsties. In Mambwe District, all respondents
311 acknowledged seeing warthogs and/or bush pigs near the villages.
312
313 **Antibody detection**
314 Antibodies to ASFV were detected in 325 of the 1,134 (28.7%) pigs tested by ELISA
315 (Table 2). The overall seroprevalence was 29.0%, 95% CI [26.4–31.7]. In EP, 50.9%
316 (314/623) of the pigs were seropositive for antibodies to ASFV (95% CI [47.0–54.9])
317 compared to 2.9%, 95% CI [1.6–5.1] of the pigs (11/128) tested in SP. Antibodies to ASFV
318 were detected in all study sites in EP; i.e. Chipata (4.0%; 95% CI [1.9 – 8.5]), Katete (79.0%;
319 95% CI [74.6 – 82.9]), Mambwe (23.8%; 95% CI [12.6 – 40.4]) and Vubwi districts (3.5%;
320 95% CI [1.0 – 11.9]). In SP, antibodies were detected in Choma (2.8%; 95% CI [1.4 – 5.8]),
321 Kalomo (5.6%; 95% CI [0.3 – 26.0]) and Kazungula districts (12.6%; 95% CI [4.4 – 31.3]),
322 but not in Namwala District. Also, no antibodies to ASFV were detected from pigs in
323 Chibolya market and at a regulated slaughter facility in Lusaka District. Although no

antibodies to ASFV were detected in crossbred pigs in EP, the seroprevalence in indigenous free-range pigs (65.7%; 95% CI [61.3 – 69.8]) was significantly higher compared to crossbred pigs (2.9%; 95% CI [1.6 – 5.1]) in SP. Katete District in EP had the highest seroprevalence (79.0%; 95% CI [74.6 – 82.9]) among all study sites.

Genome detection and phylogenetic analysis

Pigs

Of the 300 blood samples analyzed, 35 (11.7%) yielded amplicons of the expected size (257 bp), whereas 265 (88.3%) were classified as negative (Table 2). While no ASFV genome was detected in pigs from Vubwi and Katete districts, there was a significant difference (OR = 5.2, 95% CI [1.9 – 5.6]) in the proportion of PCR-positive pigs between Mambwe and Chipata District (47.1% [16/34] versus 14.6% [19/130], respectively). The prevalence of ASFV genome was significantly higher in indigenous free-range pigs compared to confined crossbred pigs (OR = 6.2, 95% CI [2.4 – 16.6]). Also, the prevalence of ASFV genome in indigenous free-range pigs was higher in Mambwe District (94.1% [16/17]) when compared to Chipata District (21.9% [14/64]). Only 4% (12/300) of the pigs tested positive by both ELISA and PCR. Notably, all the pigs with positive ASFV genome were asymptomatic at the time of sampling and no ASF outbreaks were reported from these areas during subsequent follow-ups through the District Veterinary office three months post-sampling, which was suggestive of sub-clinical circulation of ASFV.

Soft ticks

Of the 724 ticks collected, 39.9% (289) were collected from warthog burrows, 13.4% (97) from culverts while 46.7% (338) were collected from a warthog dwelling under a house. From the 124 pools analyzed, 17.7% (22/124) were positive for ASFV genome by real-time

PCR assay while 47% (59/124) yielded amplicons of expected size (243 bp) on nested PCR assay. The positive rate in nymphs and adult ticks was 54.2% and 45.8%, respectively. The overall pooled prevalence of ASFV genome by the nested PCR assay was 11.0%, 95% CI [8.5–13.9]. Also, soft ticks were found in 100% (7/7) of the inspected warthog burrows while only 50% (2/4) of the calverts yielded soft ticks. Overall, soft ticks were recovered in 83.3% (10/12) of the inspected warthog dwellings.

ASFV detected in ticks in this study belonged to genotype I (Figure 3). Topologically, genotype I isolates analysed formed three clusters. Cluster I was mostly composed of tick isolates from Mosi-oa-Tunya National Park with the exception of an isolate (47/Ss/2008; accession no. KX354450) from domestic pigs in Sardinia, Italy. By contrast, cluster II had isolates from ticks along with viruses that were responsible for the 2013/2015 ASF outbreaks in Zambia. Cluster III was composed of only two isolates that were detected from domestic pigs during the 2001-2002 ASF outbreak in Zambia.

Soft tick identification

Soft ticks were morphologically identified as belonging to the *Ornithodoros* genus. On phylogenetic analysis of the 16S *rRNA* gene, the soft ticks clustered within the *O. moubata* complex (Figure 4). While nucleotide sequences of nine of the 10 pools were 100% identical, one pool, Zmq0519_UH, showed a single nucleotide difference.

Regression analysis

Four variables i.e. pig breed, proximity to a National Park, history of an ASF outbreak and management system were included in the multivariate analysis. Free-range management system was the only factor that was significantly associated with seropositivity ($p < 0.0001$, OR = 39.3) and positive PCR results ($p < 0.001$, OR = 5.7).

374

375 **Discussion**

376 In this study, we have determined the prevalence of ASFV antibodies and viral
377 genome in domestic pigs and soft ticks. Whilst there would be a potential for sampling bias in
378 SP, considerable differences in seroprevalence were noted among each of the four study
379 districts. For example, Kazungula District, which shares boundaries with Mosi-oa-Tunya and
380 Kafue National Parks, had the highest seroprevalence (12.6%). This is potentially attributable
381 to the semi-confinement husbandry practice and proximity to national parks. Moreover, this
382 study corroborates earlier findings by Simulundu et al., (2018a, 2017) that associated Mosi-
383 oa-Tunya National Park with past ASF outbreaks. While the presence of infected soft ticks in
384 Mosi-oa-Tunya National Park presents a continued risk for ASFV spill-over to
385 immunologically naïve pigs, tick-pig or pig-pig cycle of maintenance and transmission may
386 present a greater risk and deserves further investigation to inform preventive and control
387 strategies.

388 The seroprevalence in Kalomo District (5.6%) in SP was similar to what has been
389 reported in Mbeya (3.0 – 5.0%) in Tanzania (Uttenthal et al., 2013), an area known to be
390 endemic for ASF. Similarly, this is possibly attributable to semi-confinement husbandry
391 system and proximity to Kafue National Park. Previous studies reported detection of ASFV
392 from soft ticks captured in Kafue National Park (Dixon & Wilkinson, 1988; Lubisi et al.,
393 2005; Wilkinson et al., 1988). In contrast, no antibodies were detected in pigs from Namwala
394 District, perhaps due to a small sample size. Nevertheless, evidence suggests possible
395 involvement of a sylvatic cycle in this area (Simulundu et al., 2017; Wilkinson 1988).
396 Interestingly, Choma District in SP is not associated with a National Park but had ASFV
397 seropositive pigs. Notably, farmers transport pigs to Choma District from different parts of
398 SP, from where they are mainly transported to be sold at Chibolya market in Lusaka. This

poses a continued threat of ASF outbreaks in Choma as well as other parts of the country. Some genotype I ASFV detected in soft ticks were 100% identical in the *p72* gene to ASFV that caused the 2013-2015 outbreaks in Choma and other distrcts, which supports the idea of possible sylvatic spillover of the pathogen into domestic pigs.

Despite the seronegative findings for ASFV in pigs from LP, the potential spread of ASF from infected pigs in SP to other parts of the country remains high due to free movement of pigs and pig products. Our findings suggest that some pigs in Kazungula and Kalomo districts in SP were exposed to ASFV. Moreover, detection of infected soft ticks in Mosi-oa-Tunya National Park confirms earlier reports (Dixon & Wilkinson, 1988; Wilkinson et al., 1988) of the potential presence of a sylvatic cycle. Taken together, our data support the idea that ASF may be endemic in domestic pigs in some parts of SP. To further clarify the transmission dynamics of ASFV at the wildlife-livestock interface areas in SP, more studies on the interaction between domestic pigs and sylvatic hosts, coupled with comprehensive genomic analyses are required.

The seroprevalence in EP (50.9%) was significantly higher compared to SP (2.9%). This was expected since it has been well-established that ASF is endemic in EP. In addition, the free-range type of husbandry practices in EP presents more opportunities for disease transmission. While seroprevalence was high in indigenous free-range pigs (65.7%), we did not detect antibodies to ASFV in confined crossbred pigs. Katete District had the highest seroprevalence (79.0%) among all study sites, representing the highest rate reported to date when compared to findings of up to 52.96% from other endemic areas (Atuhaire et al., 2013; Haresnape et al., 1987; Penrith et al., 2004; Quembo et al., 2016; Uttenthal et al., 2013). The reasons for high seroprevalence rate in Katete District are unclear, considering that the area is not near a National Park. However, in the absence of spill-over events from sylvatic cycle, we speculate possible involvement of pig-pig cycle of maintenance and transmission of

ASFV. The presence of serologically naïve pigs during restocking activities in Chipata District might account for the observed low prevalence rate (4.0%). In Vubwi District, farmers were reluctant to restock, thus, fewer indigenous pigs could be sampled and this may have contributed to the low seroprevalence (3.5%).

ASFV DNA was detected in asymptomatic pigs from Mambwe and Chipata districts in EP. Similarly, Abworo et al., (2017) reported detection of ASFV from asymptomatic pigs along the Kenya-Uganda border (Abworo et al., 2017). The high prevalence of ASFV genome (94.1%) and antibodies (46.9%) in indigenous free-range pigs in Mambwe District may be attributable to the proximity to South Luangwa National Park. During sampling, farmers acknowledged seeing warthogs near villages. Furthermore, earlier studies showed evidence of infected soft ticks in warthog burrows in South Luangwa National Park (Dixon & Wilkinson, 1988; Lubisi et al., 2005; Wilkinson et al., 1988). In Chipata District, we unexpectedly detected ASFV genome in asymptomatic crossbred confined pigs. While this finding was surprising since crossbred pigs are highly susceptible to ASF, it was determined that the pigs had been sourced locally within Chipata District, unlike other farmers who opted to purchase crossbred pigs from Lusaka, which was free of ASF. This finding might imply that pigs in EP, regardless of the breed, are likely to develop some level of resistance or tolerance to ASF.

Failure to detect ASFV genome by PCR in Katete and Vubwi districts in EP is indicative of a lack of recent exposure to ASFV. Fully recovered pigs do not remain long-term carriers (Penrith et al., 2004; Petrov, Forth, Zani, Beer, & Blome, 2018; Muhangi et al., 2015). In addition, we detected ASFV antibodies and genome in only 4.0% (12/300) of the pigs tested. At the time of sampling, all pigs were asymptomatic. Occasional ASF outbreaks do occur in EP (Simulundu et al., 2018a, 2018b), however, most remain unreported, as evidenced by farmer interviews and lack of veterinary records. In contrast to SP, ASF

outbreaks in EP are usually characterized by low mortality rates. This was determined both from the number of surviving seropositive pigs and farmer interviews. Similarly, lower-than-usual mortality rates in other endemic areas have been reported (Haresnape & Wilkinson, 1989; Penrith et al., 2004; Uttenthal et al., 2013). The mechanism(s) underlying ASFV endemicity in EP remains to be fully elucidated. Probably, depending on the area, there may be involvement of either the tick-pig or pig-pig cycle of ASFV maintenance and transmission.

In conclusion, through serological and molecular analyses, this study has revealed a relatively high prevalence of ASFV in asymptomatic domestic pigs during an interepidemic period in Eastern and Southern provinces of Zambia. The findings highlight the need to consider revising the policy on ASF prevention and control strategy, particularly relating to movement of pigs and pig products from Southern Province, which is currently considered to be an ASF nonendemic region. Indeed, further studies are warranted in endemic and non-endemic areas in Zambia in order to provide empirical evidence on the possible endemicity of ASF in areas thought to be non-endemic as well as to better clarify the maintenance and transmission dynamics of ASFV in these regions**.** It is anticipated that this will help in the formulation of evidence-based control strategies to mitigate disease outbreaks and the associated socioeconomic impact.

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Conflicts of interest

The authors declare no conflicts of interest.

Ethical considerations

This study was commissioned and approved by the Ministry of Fisheries and Livestock of the Government of the Republic of Zambia with support from various organisations.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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640

641 **Figure legends**

642 **Figure 1.** Map showing the locations of sample collection points by district and province.

643

644 **Figure 2.** Map of Southern and Lusaka provinces of Zambia showing the trade routes used
645 by farmers to move pigs across Southern Province into Lusaka District.

646

647 **Figure 3.** Phylogenetic tree of *p72* gene of ASFV detected in soft ticks from Mosi-oa-Tunya
648 National Park in Zambia. The tree was generated using the Neighbor-Joining method based
649 on the Kimura 2-parameter model. Analysis was based on 448 bp. Numbers at the branch
650 nodes indicate bootstrap values (>60%). Viruses characterized in the present study are in red
651 text. All genotype I ASFV isolates detected in domestic pigs in Zambia are Italicized. Right
652 brackets denote cluster. Dotted lines reprersent genotype. Bar, number of substitutions per
653 site.

654

655 **Figure 4.** Phylogenetic tree of 16S *rRNA* gene of soft ticks detected in Mosi-oa-Tunya
656 National Park in Zambia. The tree was generated using the Neighbor-Joining method based
657 on the Tamura-3 parameter model. Analysis was based on 430 bp. Numbers at the branch
658 nodes indicate bootstrap values (>60%). The tree is rooted to *Argas monachus* (Accession
659 number EU283344). Soft ticks characterized in the present study are in red text. Bar, number
660 of substitutions per site.

Table 1. Summary of ASF blood and serum samples collected from crossbred and indigenous pig breeds in different provinces of Zambia.

Province	District	Sampled	Serum			Blood			Total
			Total	Crossbred	Indigenous	Total	Crossbred	Indigenous	
Eastern	Chipata	2014; 2017	150	66	84	130	66	64	280
	Katete	2015; 2017	381	11	370	78	11	67	459
	Mambwe	2017	34	17	17	34	17	17	68
	Vubwi	2017	58	46	12	58	46	12	116
Sub-total[†]			623	140	483	300	140	160	923
Southern	Choma	2014; 2015	249	249	N/S	N/S	N/S	N/S	249
	Kalomo	2014	18	18	N/S	N/S	N/S	N/S	18
	Namwala	2014	92	92	N/S	N/S	N/S	N/S	92
	Kazungula	2014	24	24	N/S	N/S	N/S	N/S	24
Sub-total[†]			383	383	N/S	N/S	N/S	N/S	383
Lusaka	CHSLS	2016	81	81	N/S	N/S	N/S	N/S	81
	RSLH	2016	47	47	N/S	N/S	N/S	N/S	47

Sub-total[†]	128	128	N/S	N/S	N/S	N/S	128
Total	1,134	651	483	300	140	160	1434

Key: [†] Provincial sub-total. N/S, Not sampled. CHSLS, Chibolya slaughter slab. RSLH, Regulated slaughter facility.

Table 2. Prevalence of ASFV-specific antibodies and ASFV genome in Eastern, Southern, and Lusaka provinces of Zambia.

Province	**District**	**ELISA[†]**			**PCR[‡]**		
		Total	**Crossbred**	**Indigenous**	**Total**	**Crossbred**	**Indigenous**
Eastern	Chipata	6 (4.0, 1.9 – 8.5)	0	6 (7.2, 3.4 – 14.9)	19 (14.6)	5 (7.6)	14 (21.9)
	Katete	298 (79.0, 74.6 – 82.9)	0	298 (81.4, 77.0 – 85.1)	0	0	0
	Mambwe	8 (23.8, 12.6 – 40.4)	0	8 (47.5, 26.4 – 69.7)	16 (47.1)	0	16 (94.1)
	Vubwi	2 (3.5, 1.0 – 11.9)	0	2 (16.8, 4.7 – 45.3)	0	0	0
Sub-total[§]		314 (50.9, 47.0 – 54.9)	0	314 (65.7, 61.3 – 69.8)	35 (11.7)	5 (7.6)	30 (18.5)
Southern	Choma	7 (2.8, 1.4 – 5.8)	7 (2.8, 1.4 – 5.8)	-		-	
	Kalomo	1 (5.6, 0.3 – 26.0)	1 (5.6, 0.3 – 26.0)	-		-	
	Namwala	0	0	-		-	

	Kazungula	3 (12.6, 4.4 – 31.3)	3 (12.6, 4.4 – 31.3)	-		-	
Sub-total[§]		11 (2.9, 1.6 – 5.1)	11 (2.9, 1.6 – 5.1)	-		-	
Lusaka	Lusaka	0	0	-		-	
Sub-total[§]		0	0	-		-	
Total		**325 (29.0, 26.4 – 31.7)**	**11 (2.9, 1.6 – 5.1)**	**314 (65.7, 61.3 – 69.8)**	**35 (11.7)**	**5 (7.6)**	**30 (18.5)**

Key: [†]positive (%, 95% CI); %, Percent; CI, Confidence interval; [‡]positive (%); [§] Provincial sub-total; -, Not done