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1 Original Article

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3 **Title: Serological and molecular epidemiological study on swine influenza in Zambia**

4 Running title: Influenza A viruses in pigs in Zambia

5

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43 **Summary (300 words)**

44 Influenza A viruses (IAVs) cause highly contagious respiratory diseases in humans and
45 animals. In 2009, a swine-origin pandemic H1N1 IAV, designated A(H1N1)pdm09 virus, spread
46 worldwide, and has since frequently been introduced into pig populations. Since novel reassortant
47 IAVs with pandemic potential may emerge in pigs, surveillance for IAV in pigs is therefore
48 necessary not only for the pig industry but also for public health. However, epidemiological
49 information on IAV infection of pigs in Africa remains sparse. In this study, we collected 246
50 serum and 605 nasal swab samples from pigs in Zambia during the years 2011-2018. Serological
51 analyses revealed that 49% and 32% of the sera collected in 2011 were positive for
52 hemagglutination-inhibition (HI) and neutralizing antibodies against A(H1N1)pdm09 virus,
53 respectively, whereas less than 5.3% of sera collected during the following period (2012–2018)
54 were positive in both serological tests. The positive rate and the neutralization titers to
55 A(H1N1)pdm09 virus were higher than those to classical swine H1N1 and H1N2 IAVs. On the
56 other hand, the positive rate for swine H3N2 IAV was very low in the pig population in Zambia
57 in 2011–2018 (5.3% and 0% in HI and neutralization tests, respectively). From nasal swab
58 samples, we isolated one H3N2 and eight H1N1 IAV strains with an isolation rate of 1.5%.
59 Phylogenetic analyses of all eight gene segments revealed that the isolated IAVs were closely
60 related to human IAV strains belonging to A(H1N1)pdm09 and seasonal H3N2 lineages. Our
61 findings indicate that reverse zoonotic transmission from humans to pigs occurred during the
62 study period in Zambia and highlight the need for continued surveillance to monitor the status of
63 IAVs circulating in swine populations in Africa.

64

65 **Keywords:** influenza A virus; pig; surveillance; complete genome; Zambia

66

67 1. Introduction

68 Influenza A virus (IAV), belonging to the family *Orthomyxoviridae*, is a major pathogen that
69 causes highly contagious diseases in vertebrates, including mammals and birds (Wright, Neumann,
70 & Kawaoka, 2013). Similar to human IAVs responsible for seasonal influenza, swine IAVs are
71 also broadly distributed in pig populations worldwide (Chauhan & Gordon, 2020). Although
72 swine IAVs generally cause asymptomatic or mild respiratory symptoms in pigs, coinfections
73 with other pathogens such as porcine reproductive and respiratory syndrome virus or *Mycoplasma*
74 *hyopneumoniae* have been demonstrated to aggravate clinical outcomes of IAV-infected pigs
75 (Kitikoon et al., 2009; Yazawa et al., 2004). Therefore, a high prevalence of swine influenza
76 negatively impacts animal health and the economic performance of the pig industry. Additionally,
77 it is well known that pigs play a pivotal role in the emergence of novel reassortant IAVs with
78 pandemic potential (Ma, Kahn, & Richt, 2008). Since pigs are susceptible to both human and
79 avian IAVs, genetic reassortment among human, swine, and avian IAVs can occur upon
80 coinfection (Castrucci et al., 1993; Ito et al., 1998). In 2009, a novel swine-origin pandemic H1N1
81 virus, which was designated A(H1N1)pdm09 virus, emerged most likely in North America, and
82 rapidly spread among humans (Dawood et al., 2009). A(H1N1)pdm09 virus was a reassortant
83 between two swine IAVs; Eurasian avian-like swine IAV and North American triple reassortant
84 swine IAV generated by genetic reassortment among human, swine, and avian IAVs (Dawood et
85 al., 2009). Therefore, control of swine influenza is important for minimizing economic losses in
86 the pig industry and also for monitoring the emergence of novel IAV strains with pandemic
87 potential.

88 Three predominant subtypes of swine IAVs (H1N1, H1N2, and H3N2) have been found in
89 pig populations worldwide (Brown, 2000; Vincent et al., 2014). Classical swine H1N1 IAV has
90 the same origin as the Spanish influenza virus, which caused a pandemic in humans in 1918, and
91 has been circulating in pig populations worldwide (Webster, Bean, Gorman, Chambers, &
92 Kawaoka, 1992). In Europe, an avian H1N1 IAV was introduced into pigs in 1979 and replaced

93 the classical swine H1N1 IAV (Ludwig et al., 1995; Pensaert, Ottis, Vandeputte, Kaplan, &
94 Bachmann, 1981). In Asia, a human H3N2 IAV was transmitted to pigs and is co-circulating with
95 classical swine H1N1 IAV (Shortridge, Webster, Butterfield, & Campbell, 1977; Yu et al., 2008).
96 Meanwhile, genetic reassortment between the classical swine H1N1 IAV and the human H3N2
97 IAV generated a classical swine H1N2 IAV possessing a human H3N2-derived NA gene and the
98 other genes from the classical swine H1N1 IAV (Nerome et al., 1983). Other swine H1N2 and
99 H3N2 IAVs generated by reassortments among multiple human, swine, and avian IAVs have been
100 reported in North America and Europe since the 1980s and have circulated in pig populations
101 (Brown, Harris, McCauley, & Alexander, 1998; Kyriakis et al., 2011; Olsen, 2002). These
102 endemic swine IAVs have individually established genetically and antigenically distinct lineages
103 in each region due to limited intercontinental transport of pigs (Kyriakis et al., 2011; Van Reeth,
104 2007). However, these situations were dramatically changed by the emergence of A(H1N1)pdm09
105 virus in 2009. Since the 2009 pandemic, A(H1N1)pdm09 virus has frequently been transmitted back
106 to pig populations and reassorted with the endemic swine IAVs, further increasing genetic diversity
107 among swine IAVs worldwide (Hiromoto et al., 2012; Mine, Uchida, Takemae, & Saito, 2020).

108 IAVs have been found in pig populations from eight African countries; Egypt, Cameroon,
109 Nigeria, Togo, Ghana, Kenya, Uganda, and Burkina Faso (Chauhan & Gordon, 2020; Tialla et al.,
110 2020). In 1990, hemagglutination-inhibition (HI) antibodies against H1 and H3 IAVs were
111 detected in pigs in Nigeria, which was the first evidence of IAV infection in African pigs (Olaleye,
112 Omilabu, Baba, & Fagbami, 1990). During 2009-2017, A(H1N1)pdm09 viruses were recognized
113 in pigs in the aforementioned countries except Uganda and Burkina Faso (Chauhan & Gordon,
114 2020; Tialla et al., 2020). Human H3N2 IAVs have also been detected in pigs in Nigeria and
115 Ghana (Adeola, Olugasa, & Emikpe, 2016). However, epidemiological data on swine influenza
116 in Africa still remain sparse and serological and genetic information on IAVs in pig populations
117 is limited.

118 In a previous study, HI antibodies against H1 and H3 IAVs were not detected in pig sera

119 collected in Zambia in 1989 (Stafford, Stafford, Paton, & Gamble, 1992). To understand the
120 prevalence of IAV infection of pigs in Zambia after the 2009 pandemic, we tested pig sera
121 collected during the years 2011 to 2018 for IAV-specific antibodies. Furthermore, we genetically
122 analyzed IAV strains isolated from nasal swab samples of pigs.

123

124 **2. Materials and Methods**

125 *2.1 Ethics statement*

126 Influenza surveillance in pigs in Zambia was carried out with approval from the Ministry of
127 Fisheries and Livestock of the Republic of Zambia (permit number: DVS/9/7/2; The Animal
128 Health Act, No. 27 of 2010). For sample collection, permission was obtained from abattoir and
129 farm owners through local veterinary officers of the Ministry of Fisheries and Livestock of the
130 Republic of Zambia. For the purpose of sample collection at pig farms, verbal consent was
131 obtained from the pig farmers. For humane animal treatment, all experimental procedures
132 complied with the guidelines of the Animal Care and Use Committee of Hokkaido University
133 following the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related
134 Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education,
135 Culture, Sports, Science and Technology in Japan.

136 *2.2 Sample collection*

137 In 2011–2015, 187 blood samples were collected from pigs taken for slaughter at four
138 abattoirs in Lusaka in Zambia (Table S1). In January 2018, 40 and 19 blood samples were taken
139 from clinically healthy 4–12-week-old pigs and >20-week-old symptomatic pigs (with cough),
140 respectively, at Farm A in Lusaka in Zambia. Collected blood samples were processed for serum
141 separation by a standard method, and sera were stored at –80°C until use for serological analyses.

142 We designed the influenza surveillance in pigs for IAV isolation according to the sampling
143 strategy as previously described (Takemae et al., 2011; Takemae et al., 2016). We focused on
144 relatively large pig farms in Zambia and selected six farms (herd sizes: 700–7700) where the

145 owners agreed to have their pigs sampled (Farms A and F in Lusaka, Farm B in Chilanga, Farm
146 C in Kafue, and Farms D and E in Chibombo District). Nasal swabs were collected from clinically
147 healthy 4–12-week-old pigs (40–50 specimens for each visit). This age range yielded IAV
148 isolation rates ranging from 0.5 to 10.9% in previous studies (Mine et al., 2019; Takemae et al.,
149 2011). We also collected nasal swabs from coughing pigs (> 20 weeks old) at Farm A in Lusaka
150 in Zambia. A total of 605 nasal swabs were obtained through 13 visits during the period from
151 January through December in 2018 (three times at Farm A; three times at Farm B; three times at
152 Farm C; once at Farm D; once at Farm E; twice at Farm F). Nasal swabs were promptly placed in
153 Eagle’s minimum essential medium (MEM) supplemented with 1,000 units/ml penicillin, 1,000
154 $\mu\text{g/ml}$ streptomycin, 25 $\mu\text{g/ml}$ amphotericin B, 0.01 M HEPES, and 0.5% bovine serum albumin
155 (BSA) and were kept at 4°C during transportation to our laboratory. After centrifugation at 1,750
156 $\times g$ for 5 min, the supernatants were stored at -80°C .

157 *2.3 Cells and Viruses*

158 Madin–Darby canine kidney (MDCK) cells were maintained in Dulbecco’s Modified Eagle’s
159 Medium (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% fetal bovine serum
160 (FBS), 2 mM L-glutamine, 100 units/ml penicillin, 100 $\mu\text{g/ml}$ streptomycin, 3.5 mg/ml D-glucose,
161 and 1.0 mg/ml NaHCO_3 at 37°C in an atmosphere of 5% CO_2 .

162 Four IAV strains, A/Hokkaido/Z01/2014 (H1N1), A/swine/Hokkaido/1/1981 (H1N1)
163 (SwH1N1), A/swine/Miyagi/5/2003 (H1N2) (SwH1N2), and A/swine/Obihiro/10/1985 (H3N2)
164 (SwH3N2), were used as reference strains for A(H1N1)pdm09 virus, classical swine H1N1 IAV,
165 classical swine H1N2 IAV, and swine H3N2 IAV, respectively, in HI and neutralization tests. The
166 hemagglutinin (HA) antigenicities of these H1 IAVs are slightly different from each other
167 (Okamatsu, Sakoda, Hiono, Yamamoto, & Kida, 2013). These reference strains were propagated
168 in embryonated chicken eggs to be used for serological assays. HA titers of the reference IAVs
169 were determined using 0.5% chicken erythrocytes. Fifty-percent tissue culture infectious doses
170 (TCID_{50}) of the reference IAVs were determined using MDCK cells. For the TCID_{50} assay using

171 four wells per dilution, confluent monolayers of MDCK cells on 96-well plates were infected with
172 10-fold serially diluted viruses. After adsorption for 1 hour, the inoculum was removed and the
173 cell monolayers were maintained in MEM containing 5 µg/ml trypsin (Gibco, Thermo Fisher
174 Scientific, Waltham, MA, USA), 0.3% BSA, 2 mM L-glutamine, 100 units/ml penicillin, 100
175 µg/ml streptomycin, and 1.0 mg/ml NaHCO₃ at 35°C in 5% CO₂. A cytopathic effect (CPE) was
176 observed three days post-infection and viral titers were calculated by the Reed-Muench method
177 (Reed & Muench, 1938).

178 2.4 Serological assays

179 HI and neutralizing activities of serum samples were evaluated according to the standard
180 method (WHO, 2002). Pig sera were treated with receptor-destroying enzyme (RDE II; Denka
181 Seiken Co., Ltd., Tokyo, Japan) to remove nonspecific inhibitors of IAV replication. For HI tests,
182 twofold serially diluted sera were mixed with an equal volume of diluted virus solutions (4 × HA
183 units) and incubated for 1 hour at room temperature followed by adding an equal volume of 0.5%
184 chicken erythrocytes. HI titers were defined as reciprocals of the highest serum dilutions
185 completely protecting hemagglutination. HI tests were carried out twice for each reference IAV
186 strain, and the mean values were adopted as HI titers of individual sera. For neutralization tests,
187 twofold serially diluted sera were mixed with an equal volume of diluted virus solutions (200 ×
188 TCID₅₀) and incubated for 1 hour at room temperature. Then the serum-virus mixtures were
189 inoculated onto a monolayer of MDCK cells on 96-well tissue culture plates (4 well per dilution).
190 After 1-hour incubation at 35°C, the inoculum was removed, and the cells were washed once with
191 PBS and maintained with MEM containing 5 µg/ml trypsin and 0.3% BSA. CPE was observed
192 three days post-infection and neutralization titers were defined as the reciprocal of the highest
193 serum dilution completely protecting the cells from CPE in at least two wells of each dilution. As
194 initial screening for neutralizing activities, 40-fold and 80-fold diluted pig sera were subjected to
195 neutralization tests with each reference IAV. For the sera showing neutralization titers of ≥ 80, the
196 assay was performed twice, and the mean values were used as neutralization titers of individual

197 sera. A cutoff value of 80 was used for HI and neutralization tests as previously described (Cao et
198 al., 2013).

199 *2.5 Detection of IAV genome by RT-PCR*

200 Initial screening for the detection of the IAV RNA genome was conducted using pooled swab
201 specimens (4–5 nasal swabs per pool). Total RNA was extracted from the pools using the QIAamp
202 Viral RNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer’s instructions.
203 The IAV RNA genome was detected by reverse transcription-PCR (RT-PCR) using the QIAGEN
204 OneStep RT-PCR kit (QIAGEN) with primer sets specific to either viral nucleoprotein (NP) (Lee,
205 Chang, Shien, Cheng, & Shieh, 2001) or matrix (M) protein (Ngo et al., 2012) genes of IAV. For
206 a positive pool in initial screening, RNA was reextracted from the individual specimens
207 constituting the positive pool and retested by RT-PCR to identify individual positive specimens.

208 *2.6 Virus isolation*

209 IAV genome (both NP and M genes)-positive swab specimens were subjected to virus
210 isolation as previously described (Takemae et al., 2016). Briefly, media from nasal swabs were
211 filtered with 0.45 µm-pore size filters and inoculated into cultures of floating MDCK cells in
212 MEM containing 100 units/ml penicillin, 100 µg/ml streptomycin, 2.5 µg/ml amphotericin B, 25
213 µg/ml gentamicin, and 0.4 % BSA, and 4.0 µg/ml TPCK-trypsin (Thermo Fisher Scientific,
214 Waltham, MA, USA). After 2- to 4-day incubation, the supernatants of these cells showing CPE
215 were harvested and stored at –80°C. Isolation of IAV was confirmed by reinoculation into
216 MDCK cells and RT-PCR.

217 *2.7 Genomic sequencing*

218 All gene segments of isolated viruses were sequenced by next generation sequencing as
219 previously described (Harima et al., 2020). Briefly, the cDNA library was synthesized from total
220 RNAs extracted from the supernatants of infected cells and subjected to whole-genome
221 sequencing on a MiSeq instrument (Illumina, San Diego, CA, United States). Obtained sequence
222 data were analyzed using CLC Genomics Workbench software (CLC bio, Hilden, Germany).

223 Sequence reads were analyzed by *de novo* assembly, and the obtained contigs were subjected to
224 a BLAST search (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) to identify the sequence most similar
225 to each segment of the isolated viruses. Then sequence reads were mapped to the selected
226 reference sequences of IAV, and consensus sequences with coverage of over 20 reads were
227 obtained. Additionally, full-length sequences of open reading frames (ORFs) of each viral
228 segment were determined by conventional RT-PCR and Sanger sequencing with the segment-
229 specific primer sets (Hoffmann, Stech, Guan, Webster, & Perez, 2001). The determined nucleotide
230 sequences of the isolated viruses were deposited in the DNA Data Bank of Japan (DDBJ) under
231 the accession numbers shown in Table S4.

232 2.8 Phylogenetic analysis

233 The nucleotide sequences of human, swine, and avian IAVs that represent a wide temporal
234 and geographical range were extracted from the Global Initiative on Sharing Avian Influenza Data
235 (GISAID) EpiFlu database (<http://platform.gisaid.org/>). Phylogenetic analyses based on the
236 nucleotide sequences of each IAV gene were performed using MEGA7 software (Kumar, Stecher,
237 & Tamura, 2016). The MUSCLE protocol was used to align the sequences. A phylogenetic tree
238 of each viral gene was constructed using the maximum likelihood method based on the general
239 time-reversible and gamma distributed nucleotide substitutions with invariant sites (GTR+G+I)
240 model. The robustness of each node was assessed by the bootstrap method (200 replicates).

241

242 3. Results

243 3.1 Seroprevalence of IAV infection in pigs in Zambia

244 To investigate the prevalence of IAV infection in the tested pigs, HI tests were performed
245 using IAVs of H1 and H3 subtypes, which have been globally and constantly found in pigs. We
246 found that 57 of the 246 pig sera (23.1%) showed HI activities against H1 or H3 IAVs, and that
247 53, 33, and 8 pig sera were positive for HI antibodies against A(H1N1)pdm09 virus, SwH1N1,
248 and SwH1N2, respectively (Table 1 and Fig. 1). Most of the positive sera were those collected in

249 2011, and their seroprevalence against A(H1N1)pdm09 virus and SwH1N1 were particularly high
250 (49% and 29%, respectively). Of note, the positive rate for A(H1N1)pdm09 virus was higher than
251 those for SwH1N1 and SwH1N2. HI titers against SwH1N2 were lower than those against the
252 other H1 viruses tested. On the other hand, HI antibodies against SwH3N2 were not detected in
253 the pig sera except for one collected in 2018. None of the sera collected in 2015 showed HI
254 activity against the reference IAV strains.

255 Neutralizing activity of the pig sera against IAVs of H1 and H3 subtypes was also evaluated
256 using the same reference strains (Table 1 and Fig. 2). We found that 35 of the 246 pig sera showed
257 neutralizing activities against H1 IAVs, and that 35, 17, and 17 of the pig sera showed neutralizing
258 activities against A(H1N1)pdm09 virus, SwH1N1, and SwH1N2, respectively. Note that 19 of the
259 35 A(H1N1)pdm09-positive sera showed cross-neutralizing activities against SwH1N1 and/or
260 SwH1N2 (Table S2). Consistent with the HI antibodies, the positive rate of the sera collected in
261 2011 was higher against A(H1N1)pdm09 virus than against SwH1N1 and SwH1N2. The
262 neutralization test showed lower sensitivity compared to the HI test except for the assay using
263 SwH1N2. The titers against SwH1N2 in the neutralization test were higher than those in the HI
264 test, and the positive rate in the neutralization test against SwH1N2 was the same as that against
265 SwH1N1. Although one of the pig sera was positive for HI antibodies against SwH3N2, none of
266 the pig sera showed neutralizing activity against the H3 virus. As was the case with HI antibodies,
267 the sera collected in 2015 were not positive for neutralizing antibodies against any of the reference
268 strains.

269 *3.2 Isolation of IAV strains from nasal swab samples*

270 To isolate IAVs circulating in pigs in Zambia, RT-PCR-positive nasal swab samples were
271 inoculated into MDCK cells (Table S3). IAVs were isolated from a total of nine specimens from
272 three farms; one specimen collected at Farm A on January 7 in 2018 (isolation rate, 1.8%), seven
273 specimens collected at Farm C on June 8 in 2018 (isolation rate, 14%), and one specimen collected
274 at Farm F on December 20 in 2018 (isolation rate, 2.1%) (Table 2). Of note, we obtained IAV

275 isolates from pigs at Farm C only at the second sampling (June 8, 2018) although nasal swab
276 samples were collected on three different dates at this farm. Similarly, Farms A and F had IAV-
277 positive samples only at a single time point (January 7 and December 20, respectively). The
278 overall isolation rate during the study period reached 1.5% (9/605). One of the IAV isolates,
279 designated A/swine/Zambia/51/2018 (H3N2) (Z51), was obtained from pigs aged 32 weeks that
280 were coughing at Farm A, while the others, designated A/swine/Zambia/264/2018 (H1N1) (Z264),
281 A/swine/Zambia/277/2018 (H1N1) (Z277), A/swine/Zambia/278/2018 (H1N1) (Z278),
282 A/swine/Zambia/280/2018 (H1N1) (Z280), A/swine/Zambia/282/2018 (H1N1) (Z282),
283 A/swine/Zambia/301/2018 (H1N1) (Z301), A/swine/Zambia/310/2018 (H1N1) (Z310), and
284 A/swine/Zambia/595/2018 (H1N1) (Z595), were obtained from clinically healthy (asymptomatic)
285 pigs aged 8–12 weeks at Farms C and F (Table 3).

286 3.3 *Phylogenetic characterization of the isolated IAVs*

287 To genetically characterize the IAV isolates obtained from pigs in Zambia, we determined
288 their full genome sequences. BLAST analyses of the nucleotide sequences revealed that all eight
289 gene segments of the isolated H1N1 and H3N2 IAVs shared more than 99% nucleotide identities
290 with those of human H1N1 and H3N2 IAVs, respectively (Table 4). The determined complete
291 ORF sequences of all eight gene segments (PB2, PB1, PA, HA, NP, NA, M, and NS) were
292 phylogenetically compared with the representative swine, avian, and human IAV strains. The
293 phylogenetic analysis of H1 HA and N1 NA sequences demonstrated that all of the isolated H1N1
294 IAVs (Z264, Z277, Z278, Z280, Z282, Z301, Z310, and Z595) were classified into the
295 A(H1N1)pdm09 lineage consisting of IAVs globally circulating in humans and pigs (Figs. 3 and
296 4). Similar results were obtained in the phylogenetic tree of the other gene segments (PB2, PB1,
297 PA, NP, NS, and M) (Fig. S1). The phylogenetic tree of the H3 HA and N2 NA sequences also
298 revealed that Z51 was not genetically close to IAVs in the swine H3 lineage, including European
299 and North American swine H3N2 viruses, but clustered together with human seasonal H3N2
300 IAVs. It was noteworthy that Z51 was closely related to human H3N2 strains isolated in Zambia,

301 A/Zambia/0002/2015 (H3N2) and A/Zambia/0102/2015 (H3N2). The phylogenetic analyses of
302 the other gene segments showed similar topologies.

303

304 **4. Discussion**

305 In this study, we demonstrated the detection of IAV-specific antibodies and isolation of IAVs
306 from pigs in Zambia. To the best of our knowledge, this is the first report of IAV infection in pigs
307 in this country and we further characterized the isolated strains genetically. Although swine IAVs
308 have been detected in some African countries, the genetic information is still limited. Prior to this
309 study, only seven full genome sequences of IAVs from swine in Africa have been deposited in the
310 Genbank and GISAID EpiFlu databases: four strains from Kenya, two strains from Nigeria, and
311 one strain from Togo (Ducatez, Awoume, & Webby, 2015; Meseko, Heidari, Odaibo, & Olaleye,
312 2019; Munyua et al., 2018). The present study provides new genetic information on swine IAVs
313 in Africa and may help to improve our understanding of the molecular epidemiology of swine
314 IAVs circulating on the continent.

315 The IAV strains isolated from pigs in this study were genetically close to IAVs causing
316 seasonal influenza in humans, as well as IAVs found in Nigerian pigs (Meseko et al., 2019). These
317 swine IAV isolates shared 99–100% nucleotide identities with human IAVs, suggesting reverse
318 zoonotic transmission from humans to pigs. It is noteworthy that all of the Zambian swine IAV
319 isolates were phylogenetically classified into the well-known lineages consisting of recent human
320 IAV isolates. Since sustained circulation of such human IAVs in pig populations for a long term
321 leads to the establishment of novel IAV lineages in the swine host (Kyriakis et al., 2011; Van
322 Reeth, 2007), our data suggest that the IAVs isolated in this study were sporadically introduced
323 into the pig population from humans in Zambia. In fact, the IAV isolates were obtained from each
324 farm only at a single time point in the multiple sampling dates (Table 2). It is conceivable that
325 continuous IAV circulation in pigs is associated with farming systems such as the farrow-to-finish
326 system, which constantly supplies young naive piglets and thus provides opportunities for IAVs

327 to circulate among pig populations (Takemae et al., 2011). Although this system was implemented
328 in the pig farms selected in this study, their herd sizes ($n = 700\text{--}7700$) might be too small to
329 maintain IAVs on the respective farms. Therefore, IAV surveillance including larger farms will
330 be required for more comprehensive monitoring of IAVs in the pig population in Zambia.

331 Our serological investigation revealed the high seroprevalence of H1 IAV infection in pigs
332 in 2011. The positive rate and neutralization titers for A(H1N1)pdm09 virus were higher than
333 those for the other H1 IAVs tested, suggesting that Zambian pigs in 2011 were predominantly
334 infected with A(H1N1)pdm09 or other H1 viruses antigenically similar to A(H1N1)pdm09 virus.
335 This seroprevalence pattern was also observed in pigs in Nigeria in 2012 (Snoeck et al., 2015).
336 These results may suggest that IAVs of the H1 subtype, most likely A(H1N1)pdm09, were
337 introduced into pig populations in Africa just after the 2009 pandemic and circulated in the
338 early 2010s. On the other hand, anti-H3 antibodies were detected only in one pig during the
339 study period and its antibody titer was very low. Although one human H3N2 strain was isolated
340 from a pig, our serological data suggest that human-to-swine transmission of human H3N2 IAVs
341 might have occurred rarely in Zambia during 2011–2018. Although human seasonal influenza
342 H3N2 viruses were also isolated from pigs in Japan, no descendant strains were subsequently
343 isolated, suggesting that these viruses were sporadically introduced into pigs from humans
344 without establishment of novel IAV lineages (Takemae et al., 2013). On the other hand, H3N2
345 IAV variants recently generated by the reassortment between A(H1N1)pdm09 and human
346 seasonal H3N2 viruses might be well adapted to swine and have established a novel IAV lineage
347 in pig populations in Asia, including Japan and Thailand (Hiromoto et al., 2012; Mine et al., 2020;
348 Ozawa et al., 2015). Since both A(H1N1)pdm09 and human H3N2 viruses were isolated from
349 pigs in Zambia, it might be possible for such a reassortant IAV to emerge in pigs in Zambia. A
350 regular surveillance program is required to detect such an event as well as to understand the status
351 of swine influenza in Africa.

352 It is of interest that none of the samples collected in 2015 were IAV-antibody positive. This

353 result might be associated with the outbreak of African swine fever (ASF). During 2013–2015,
354 Zambia experienced widespread outbreaks of ASF in domestic pigs (Simulundu et al., 2018).
355 Many pigs died from the disease and pigs affected with ASF were culled to stop the spread of
356 ASF. These facts suggest that A(H1N1)pdm09 viruses, which might have been highly prevalent
357 in pigs in Zambia before 2012, could have been almost undetectable as a result of the massive
358 death of ASF virus-infected pigs and the compulsive all-in/all-out management practice by culling.
359 ASF is broadly endemic in sub-Saharan Africa, and repeated outbreaks of ASF have been reported
360 in various African countries since 1950 (Penrith, Vosloo, Jori, & Bastos, 2013). Under such
361 particular circumstances in Africa, swine IAVs may be unlikely to be maintained in pig
362 populations for a long term.

363 In this study, one IAV strain was isolated from a symptomatic pig in Farm A. On this farm,
364 two pigs were reported to die from respiratory diseases after our sampling. Although swine
365 influenza is generally considered to be a mild disease without significant clinical impact, co-
366 infection with other pathogens may cause severe respiratory disease in these pigs. Control of
367 swine influenza is important to improve animal health and productivity in the pig industry as well
368 as to prevent zoonotic transmission of IAVs to humans.

369

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379

380 **Conflict of interest statement**

381 The authors have no conflict of interest to declare.

382

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516

517

518 **Tables**

519 Table 1. Seropositivity for IAV infection in pigs in Zambia

Year	Stage or age of pigs	Positive rates (%) for the respective viruses (Positive/Total) †									
		Hemagglutination-inhibition test					Neutralization test				
		A(H1N1)pdm09	SwH1N1	SwH1N2	SwH3N2	Subtotal	A(H1N1)pdm09	SwH1N1	SwH1N2	SwH3N2	Subtotal
2011	Slaughtering	49.0 (49/100)	29.0 (29/100)	5.0 (5/100)	0 (0/100)	51.0 (51/100)	32.0 (32/100)	14.0 (14/100)	14.0 (14/100)	0 (0/100)	32.0 (32/100)
2012	Slaughtering	5.0 (2/40)	5.0 (2/40)	5.0 (2/40)	0 (0/40)	5.0 (2/40)	5.0 (2/40)	5.0 (2/40)	5.0 (2/40)	0 (0/40)	5.0 (2/40)
2015	Slaughtering	0 (0/47)	0 (0/47)	0 (0/47)	0 (0/47)	0 (0/47)	0 (0/47)	0 (0/47)	0 (0/47)	0 (0/47)	0 (0/47)
2018	4-12 weeks old	2.5 (1/40)	2.5 (1/40)	0 (0/40)	0 (0/40)	5.0 (2/40)	0 (0/40)	0 (0/40)	0 (0/40)	0 (0/40)	0 (0/40)
	>20 weeks old	5.3 (1/19)	5.3 (1/19)	5.3 (1/19)	5.3 (1/19)	10.0 (2/19)	5.3 (1/19)	5.3 (1/19)	5.3 (1/19)	0 (0/19)	5.3 (1/19)
Total		21.5 (53/246)	13.4 (33/246)	3.3 (8/246)	0.4 (1/246)	23.1 (57/246)	14.2 (35/246)	6.9 (17/246)	6.9 (17/246)	0 (0/246)	14.2 (35/246)

520 Abbreviations: A(H1N1)pdm09 (A/Hokkaido/Z01/2014); SwH1N1, classical swine H1N1 (A/swine/Hokkaido/1/1981); SwH1N2, classical swine H1N2
 521 (A/swine/Miyagi/5/2003); SwH3N2, swine H3N2 (A/swine/Obihiro/10/1985).

522 † HI and neutralization titers of 80 or higher were considered positive.

523 Table 2. Summary of nasal swab sampling and IAV isolation from pigs in 2018

Farm	District	Sampling date	No. of isolates/No. of collected samples	
			4-12 weeks old	> 20 weeks old
A	Lusaka	January 7	0/50	1/6
		January 14	-	0/13
		June 14	0/50	-
B	Chilanga	January 25	0/40	-
		July 10	0/50	-
		December 4	0/48	-
C	Kafue	February 13	0/50	-
		June 8	7/50	-
		August 16	0/50	-
D	Chibombo	March 2	0/50	-
E	Chibombo	March 15	0/50	-
F	Lusaka	July 17	0/50	-
		December 20	1/48	-

524 -; Not collected

525

526 Table 3. IAV strains isolated from pigs throughout the study period

Strain name	Farm	Sampling date	Isolated from (age)
A/swine/Zambia/51/2018 (H3N2)	A	2018/1/7	Coughing pig (32 wks)
A/swine/Zambia/264/2018 (H1N1)	C	2018/6/8	Healthy pig (8 wks)
A/swine/Zambia/277/2018 (H1N1)			Healthy pig (10 wks)
A/swine/Zambia/278/2018 (H1N1)			Healthy pig (12 wks)
A/swine/Zambia/280/2018 (H1N1)			Healthy pig (12 wks)
A/swine/Zambia/282/2018 (H1N1)			Healthy pig (12 wks)
A/swine/Zambia/301/2018 (H1N1)			Healthy pig (10 wks)
A/swine/Zambia/310/2018 (H1N1)			Healthy pig (11 wks)
A/swine/Zambia/595/2018 (H1N1)	F	2018/12/20	Healthy pig (8–12 wks)

527

528

529 Table 4. Highest nucleotide identities for each genome segment of the isolated IAVs

Farm	Strain	Gene	Closest virus (subtype)†	Identity (%)
A	Z51	HA	A/Florida/78/2016 (H3N2)	99.8
		NA	A/Michigan/104/2016 (H3N2)	99.4
		PB2	A/Michigan/104/2016 (H3N2)	99.6
		PB1	A/Iowa/01/2017 (H3N2)	99.8
		PA	A/Linkou/0185/2016 (H3N2)	99.6
		NP	A/Linkou/0185/2016 (H3N2)	99.8
		M	A/Linkou/0185/2016 (H3N2)	99.7
		NS	A/Linkou/0185/2016 (H3N2)	99.4
C	Z264, Z277, Z278, Z280, Z282, Z301, Z310	HA	A/California/69/2017 (H1N1)	99.4-99.5
		NA	A/California/69/2017 (H1N1)	99.4-99.5
		PB2	A/Mississippi/01/2018 (H1N1)	99.6-99.7
		PB1	A/California/100/2018 (H1N1)	99.7
		PA	A/New Jersey/11/2018 (H1N1)	99.7-99.9
		NP	A/Oklahoma/41/2017 (H1N1)	99.7-99.9
		M	A/Kenya/028/2018 (H1N1)	99.8-100
	Z264, Z277, Z278, Z282, Z301, Z310	NS	A/Oklahoma/07/2016 (H1N1)	100
	Z280		A/California/NHRC- OID_SAR21015N/2018 (H1N1)	100
	F	Z595	HA	A/New Jersey/39/2017 (H1N1)
NA			A/Hawaii/41/2018 (H1N1)	99.7
PB2			A/New Jersey/39/2017 (H1N1)	99.6
PB1			A/Maryland/10/2018 (H1N1)	99.7
PA			A/Texas/93/2018 (H1N1)	99.8
NP			A/Hawaii/41/2018 (H1N1)	99.6
M			A/Kenya/028/2018 (H1N1)	99.6
NS			A/Alaska/33/2018 (H1N1)	99.5

530 Abbreviations: Z51, A/swine/Zambia/51/2018 (H3N2); Z264, A/swine/Zambia/264/2018
531 (H1N1); Z277, A/swine/Zambia/277/2018 (H1N1); Z278 A/swine/Zambia/278/2018 (H1N1);
532 Z280, A/swine/Zambia/280/2018 (H1N1); Z282, A/swine/Zambia/282/2018 (H1N1); Z301,
533 A/swine/Zambia/301/2018 (H1N1); Z310, A/swine/Zambia/310/2018 (H1N1); Z595,
534 A/swine/Zambia/595/2018 (H1N1).

535 † Representative viruses with the highest nucleotide identity found by BLAST analyses are listed.

536

537 **Figure legends**

538 Fig. 1

539 HI antibodies detected in the sera collected from pigs in Zambia during the years 2011-2018.

540 Serum samples were tested for HI antibodies to A(H1N1)pdm09, classical swine H1N1, classical

541 swine H1N2, and swine H3N2 viruses. HI titers of 80 or higher were considered positive and are

542 shown in the figure. Each value represents the mean of HI titers of two independent experiments.

543 All HI titers are represented in Supplementary Table S2.

544

545 Fig. 2

546 Neutralizing antibodies detected in the pig sera during the years 2011-2018. Serum samples were

547 tested for neutralizing antibodies to A(H1N1)pdm09, classical swine H1N1, classical swine H1N2,

548 and swine H3N2 viruses. Neutralizing titers of 80 or higher were considered positive and are

549 shown in the figure. Each value represents the mean of neutralizing titers of two independent

550 experiments. All neutralizing titers are represented in Supplementary Table S2.

551

552 Fig. 3

553 Phylogenetic trees of the HA gene of H1 and H3 subtypes. The complete ORF nucleotide

554 sequences of HA genes of 9 Zambian isolates were phylogenetically analyzed with corresponding

555 genes from the representative swine, avian, and human IAVs. Bootstrap values greater than 90%

556 are shown on the interior branch nodes, and scale bars indicate the number of substitutions per

557 site. The black circles and square represent the H1N1 and H3N2 IAVs isolated in this study,

558 respectively.

559

560 Fig. 4

561 Phylogenetic trees of the NA gene of N1 and N2 subtypes. The complete ORF nucleotide
562 sequences of NA genes of 9 Zambian isolates were phylogenetically analyzed with corresponding
563 genes from the representative swine, avian, and human IAVs. Bootstrap values greater than 90%
564 are shown on the interior branch nodes, and scale bars indicate the number of substitutions per
565 site. The black circles and square represent the H1N1 and H3N2 IAVs isolated in this study,
566 respectively.

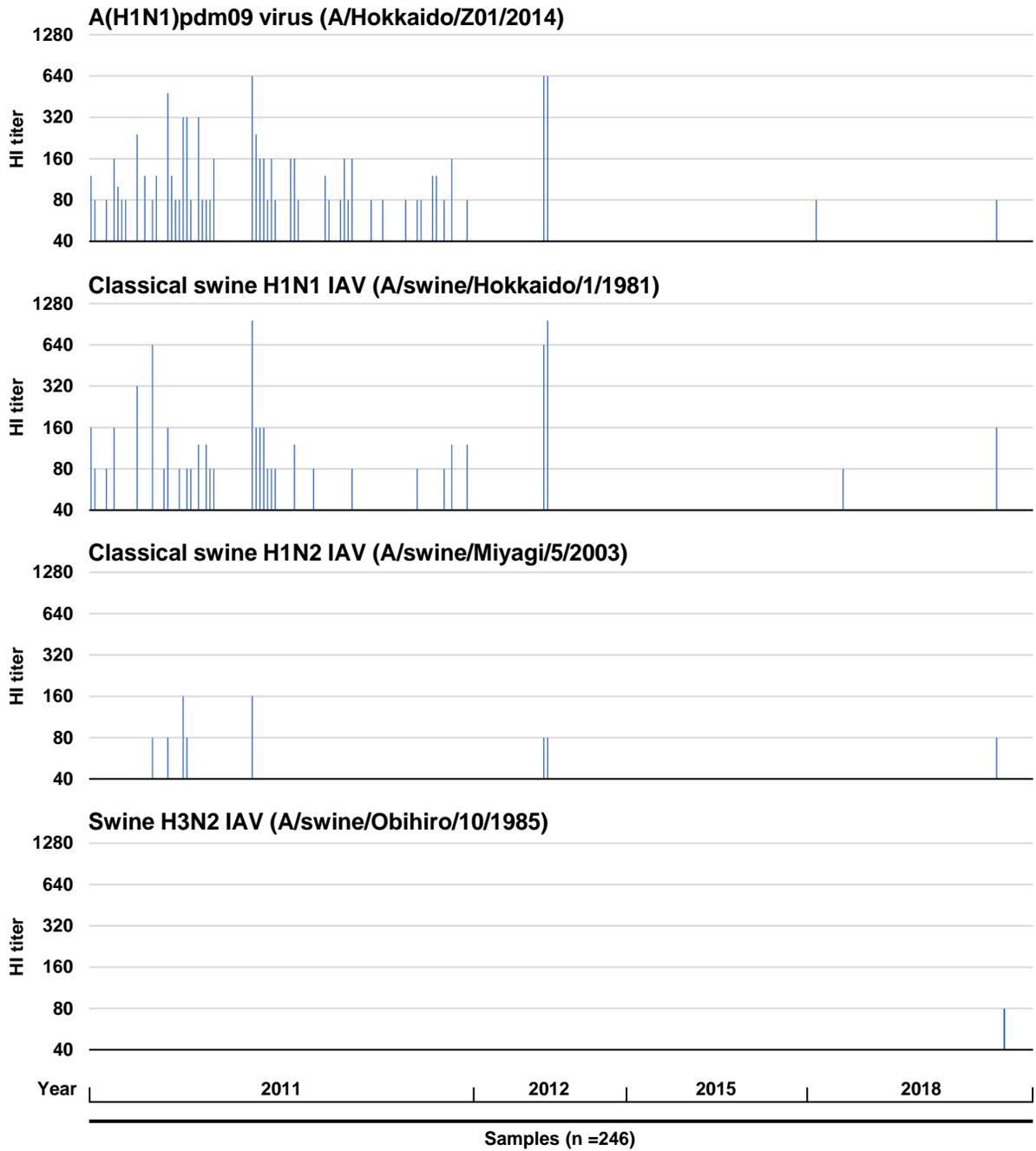


Fig. 1

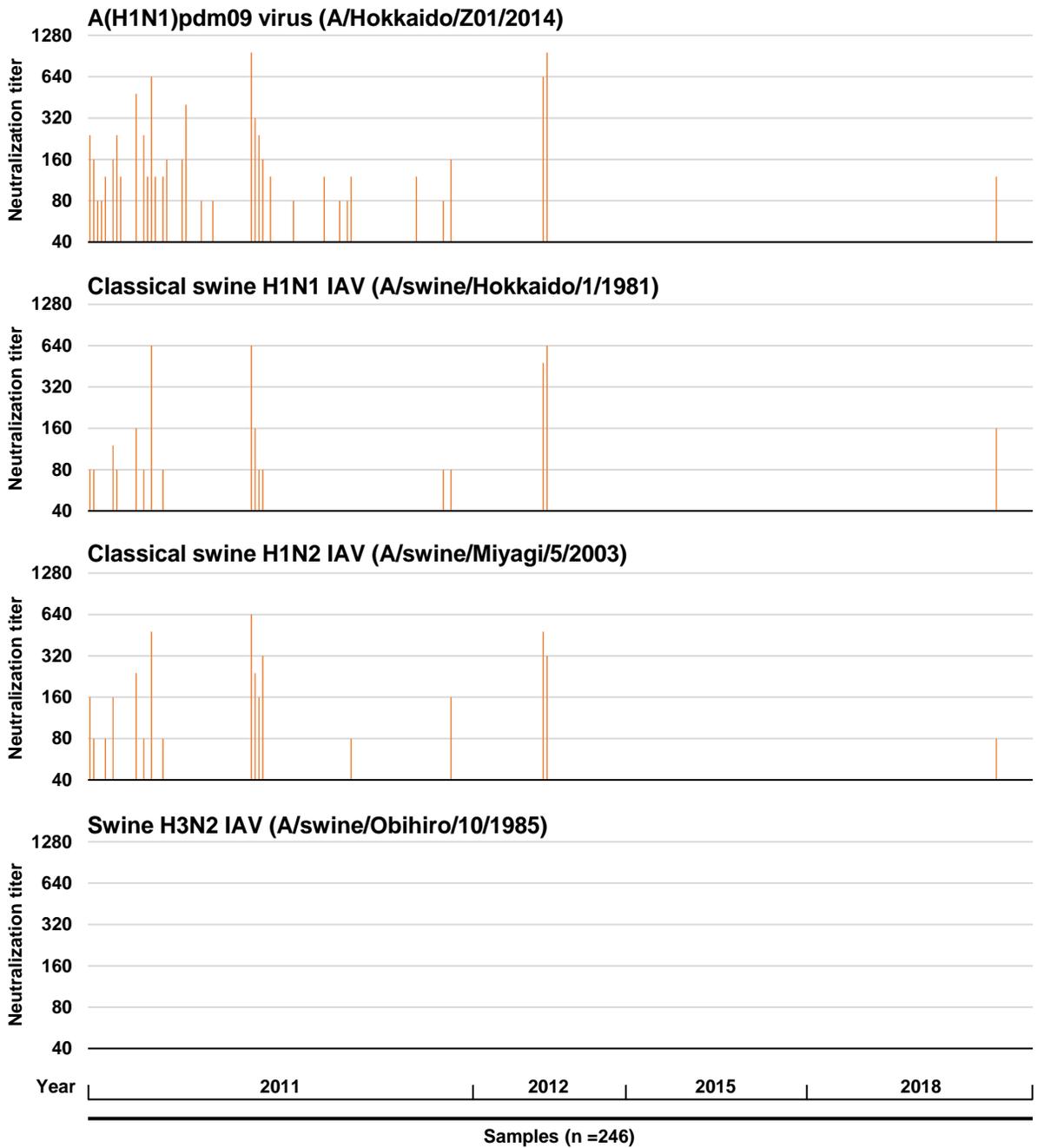
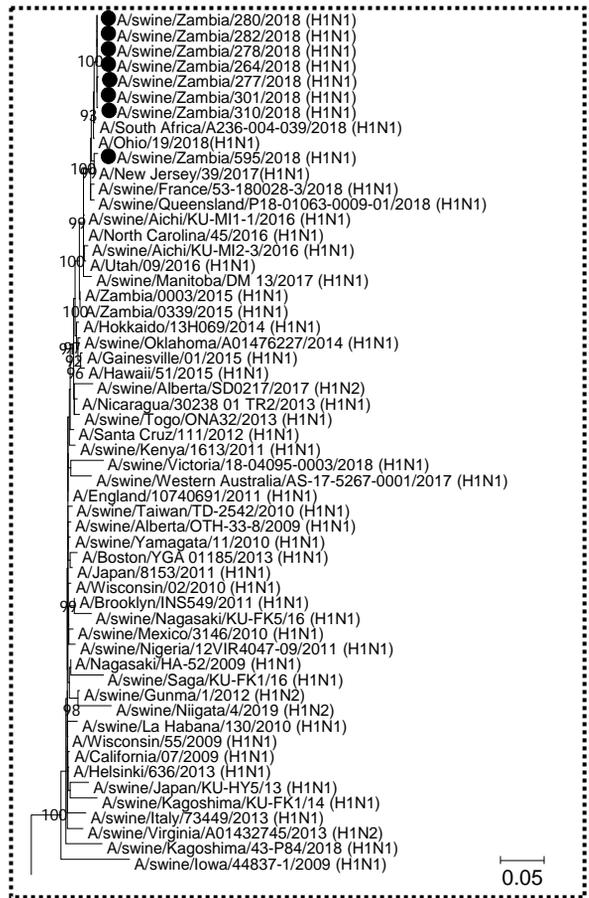
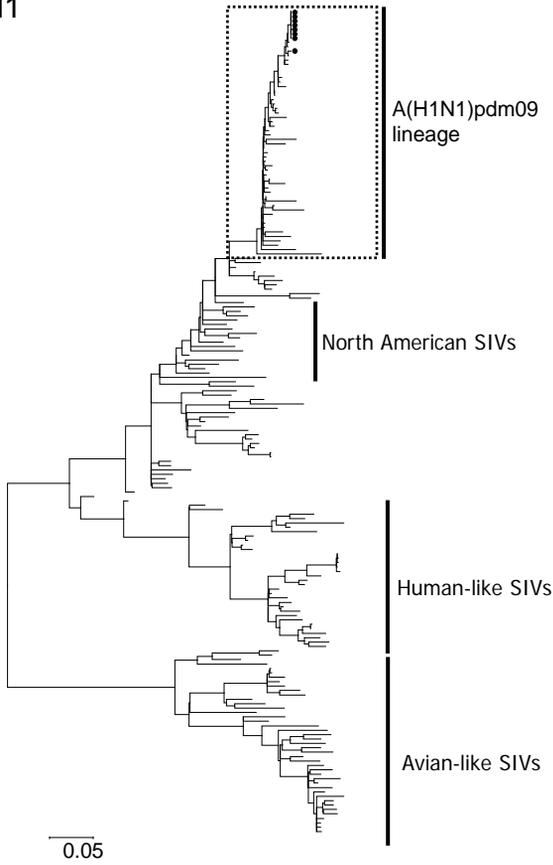


Fig. 2

H1



H3

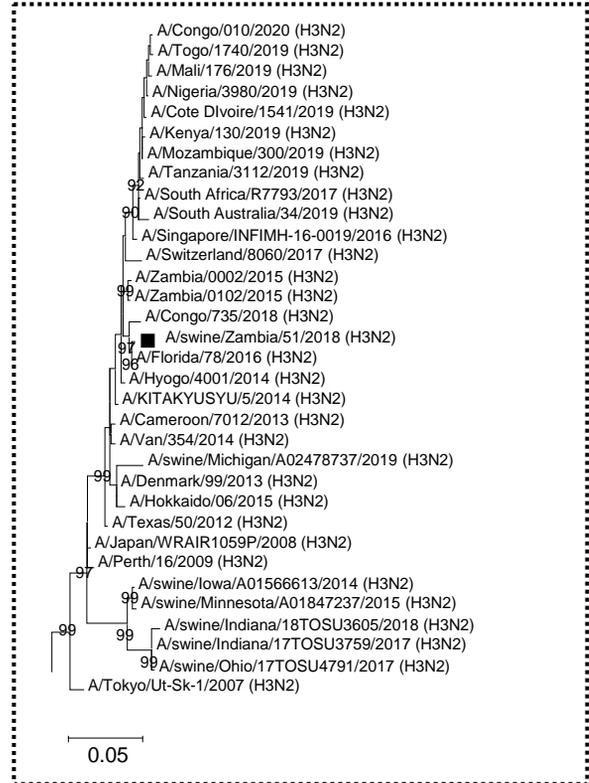
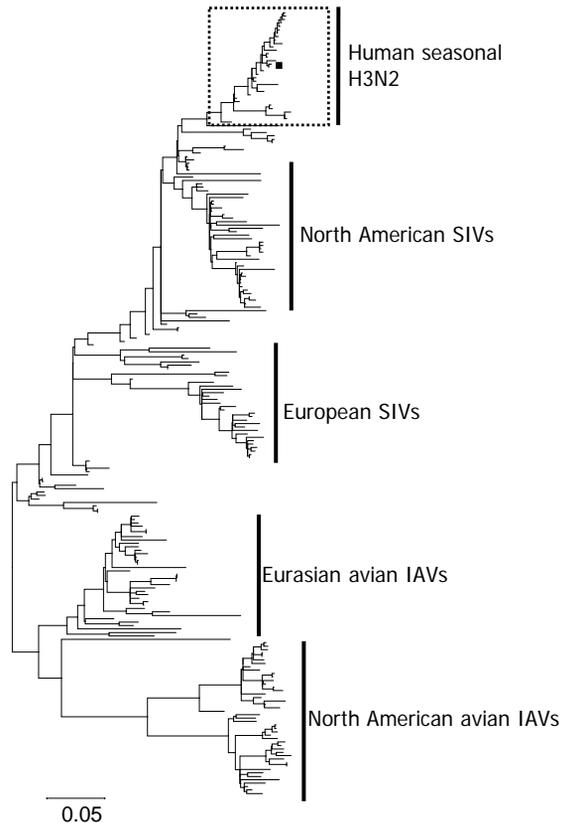


Fig. 3

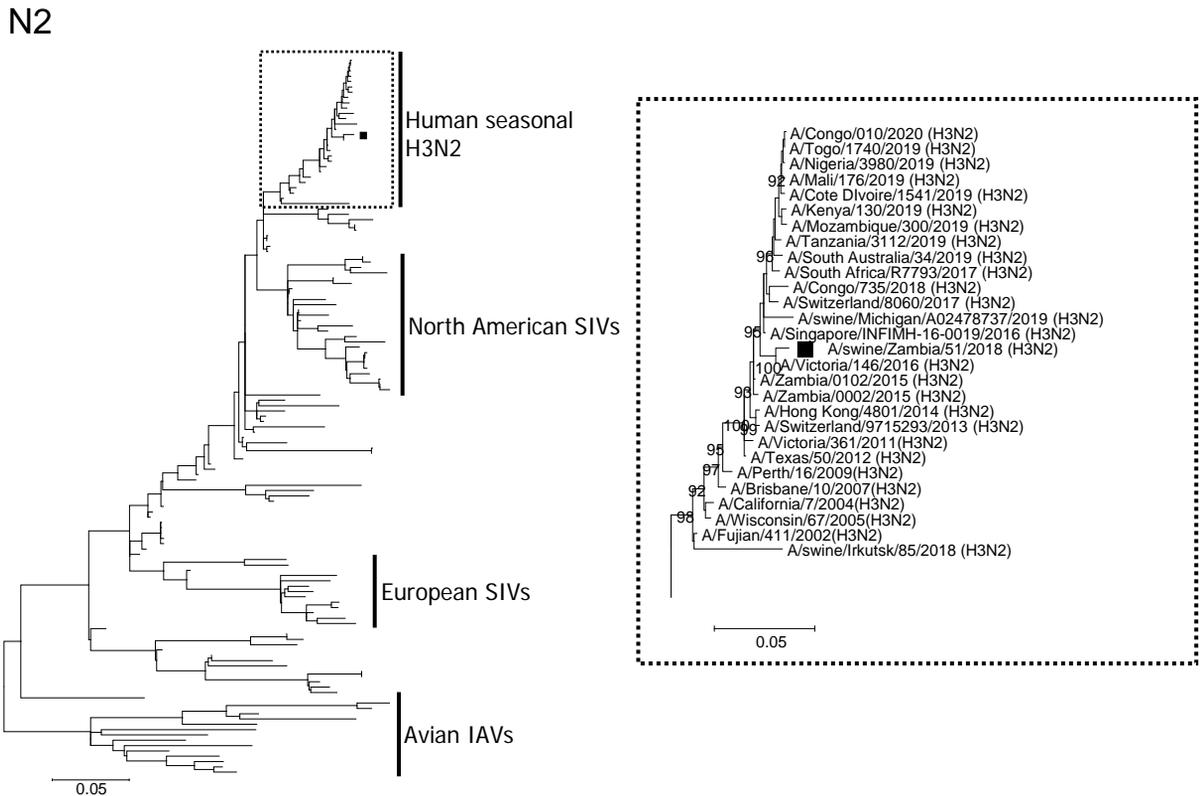
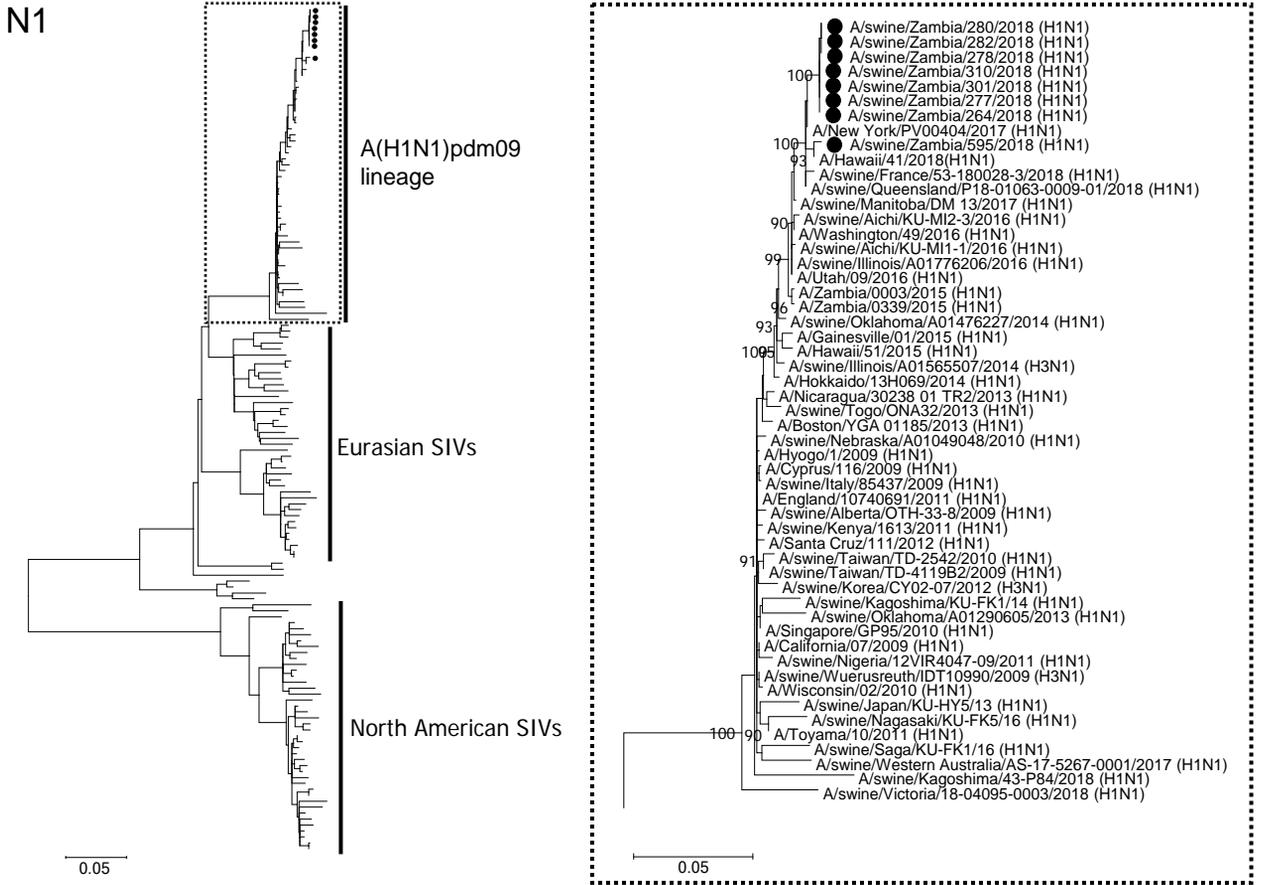


Fig. 4

Serological and molecular epidemiological study on swine influenza in Zambia

Hayato Harima, Kosuke Okuya, Masahiro Kajihara, Hirohito Ogawa, Edgar Simulundu, Eugene Bwalya, Yongjin Qiu, Akina Mori-Kajihara, Musso Munyeme, Yoshihiro Sakoda, Takehiko Saito, Bernard M. Hang'ombe, Hirofumi Sawa, Aaron S Mweene, Ayato Takada*

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ZP11-41	-	-	-	-	-	-	-	-
ZP11-42	-	-	-	-	-	-	-	-
ZP11-43	640	960	160	-	960	640	640	-
ZP11-44	240	160	-	-	320	160	240	-
ZP11-45	160	160	-	-	240	80	160	-
ZP11-46	160	160	-	-	160	80	320	-
ZP11-47	80	80	-	-	-	-	-	-
ZP11-48	160	80	-	-	120	-	-	-
ZP11-49	80	80	-	-	-	-	-	-
ZP11-50	-	-	-	-	-	-	-	-
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ZP18-57	-	-	-	-	-	-	-	-
ZP18-58	-	-	-	-	-	-	-	-
ZP18-59	-	-	-	-	-	-	-	-

Abbreviations: A(H1N1)pdm09 (A/Hokkaido/Z01/2014); SwH1N1, classical swine H1N1 (A/swine/Hokkaido/1/1981); SwH1N2, classical swine H1N2 (A/swine/Miyagi/5/2003); SwH3N2, swine H3N2 (A/swine/Obihiro/10/1985).

-: Negative, < 80

HI and neutralization titers of 80 or higher were considered positive and shown. Each value represents the mean of HI and neutralization titers of two independent experiments.

Table S3. Summary of RT-PCR screening of IAV detection from pigs tested in 2018

Farm	District	Sampling date	age	No. of collected samples	No. of pools	No. of positive pools	No. of positive individuals
A	Lusaka	January 7	4–12 weeks old	50	10	0	N.A.
			> 20 weeks old	6	-	-	1
		January 14	> 20 weeks old	13	4	0	N.A.
		June 14	4–12 weeks old	50	12	0	N.A.
B	Chilanga	January 25	4–12 weeks old	40	10	1	2
		July 10	4–12 weeks old	50	12	0	N.A.
		December 4	4–12 weeks old	48	12	0	N.A.
C	Kafue	February 13	4–12 weeks old	50	12	0	N.A.
		June 8	4–12 weeks old	50	12	6	8
		August 16	4–12 weeks old	50	12	0	N.A.
D	Chibombo	March 2	4–12 weeks old	50	12	0	N.A.
E	Chibombo	March 15	4–12 weeks old	50	12	0	N.A.
F	Lusaka	July 17	4–12 weeks old	50	12	0	N.A.
		December 20	4–12 weeks old	48	12	1	2

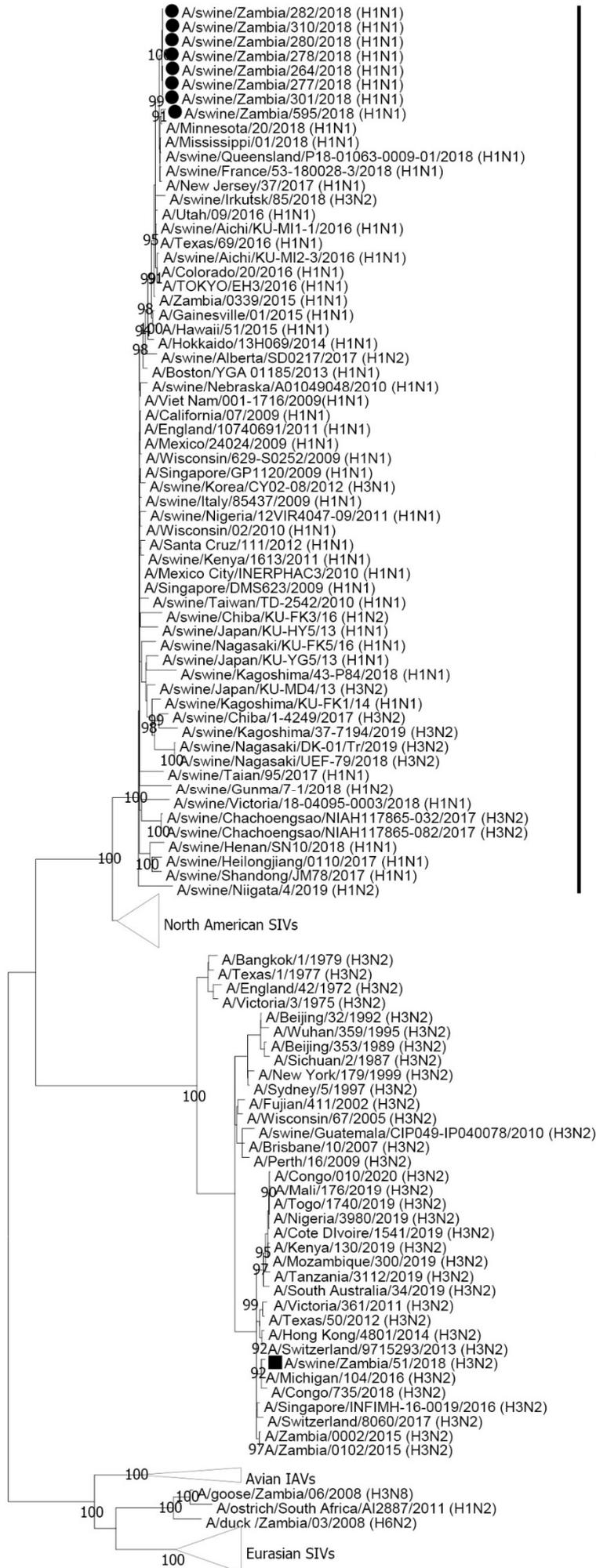
-.: Not pooled, N.A.: Not analyzed

Nasal swabs, which both M and NP genes were detected by RT-PCR, were considered as IAV-positive.

Table S4. Accession numbers of IAV strains isolated in this study

Isolate	Accession number							
	PB2	PB1	PA	HA	NP	NA	M	NS
A/swine/Zambia/51/2018 (H3N2)	LC644995	LC644996	LC644997	LC644998	LC644999	LC645000	LC645001	LC645002
A/swine/Zambia/264/2018 (H1N1)	LC645003	LC645004	LC645005	LC645006	LC645007	LC645008	LC645009	LC645010
A/swine/Zambia/277/2018 (H1N1)	LC645011	LC645012	LC645013	LC645014	LC645015	LC645016	LC645017	LC645018
A/swine/Zambia/278/2018 (H1N1)	LC645019	LC645020	LC645021	LC645022	LC645023	LC645024	LC645025	LC645026
A/swine/Zambia/280/2018 (H1N1)	LC645027	LC645028	LC645029	LC645030	LC645031	LC645032	LC645033	LC645034
A/swine/Zambia/282/2018 (H1N1)	LC645035	LC645036	LC645037	LC645038	LC645039	LC645040	LC645041	LC645042
A/swine/Zambia/301/2018 (H1N1)	LC645043	LC645044	LC645045	LC645046	LC645047	LC645048	LC645049	LC645050
A/swine/Zambia/310/2018 (H1N1)	LC645051	LC645052	LC645053	LC645054	LC645055	LC645056	LC645057	LC645058
A/swine/Zambia/595/2018 (H1N1)	LC645059	LC645060	LC645061	LC645062	LC645063	LC645064	LC645065	LC645066

PB2

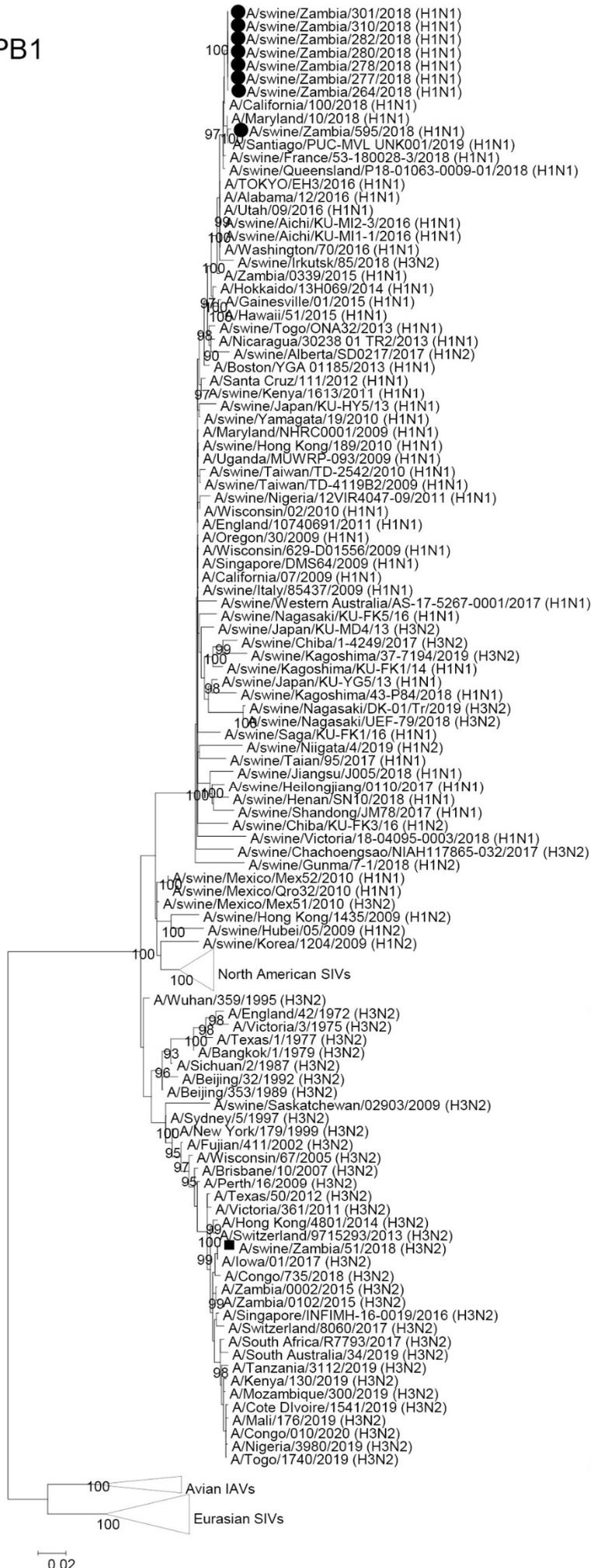


A(H1N1)pdm09 lineage

Human seasonal H3N2 lineage

0.05

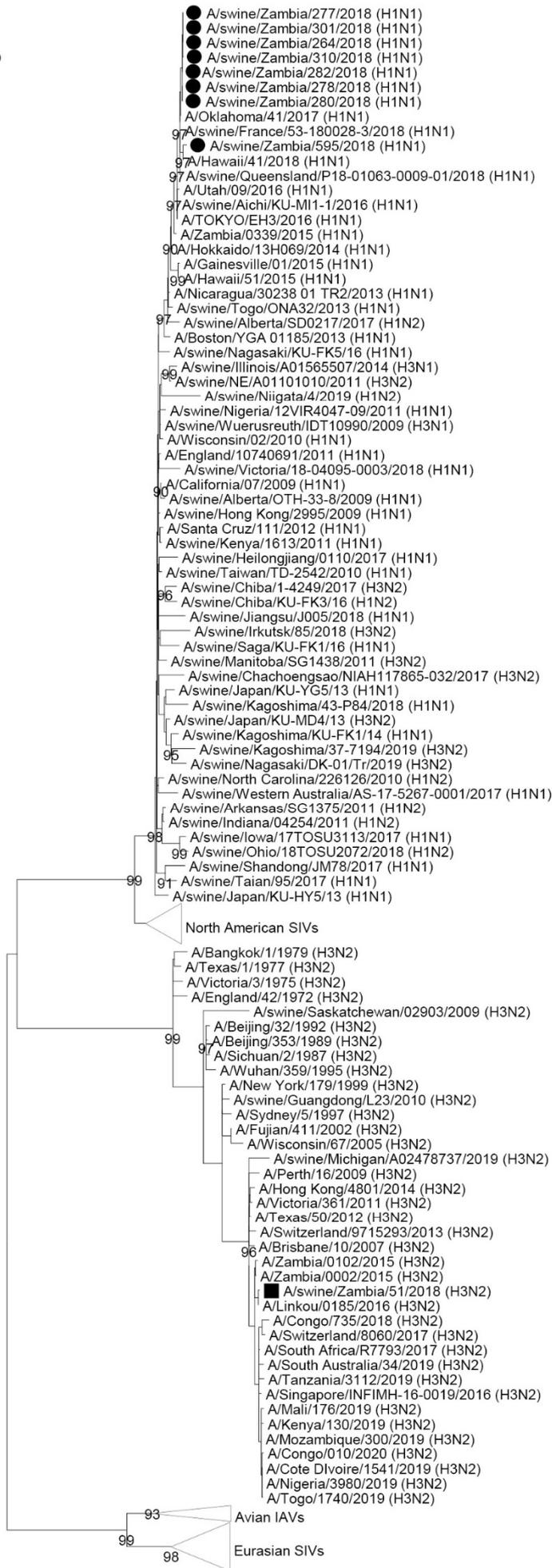
PB1



A(H1N1)pdm09 lineage

Human seasonal H3N2 lineage

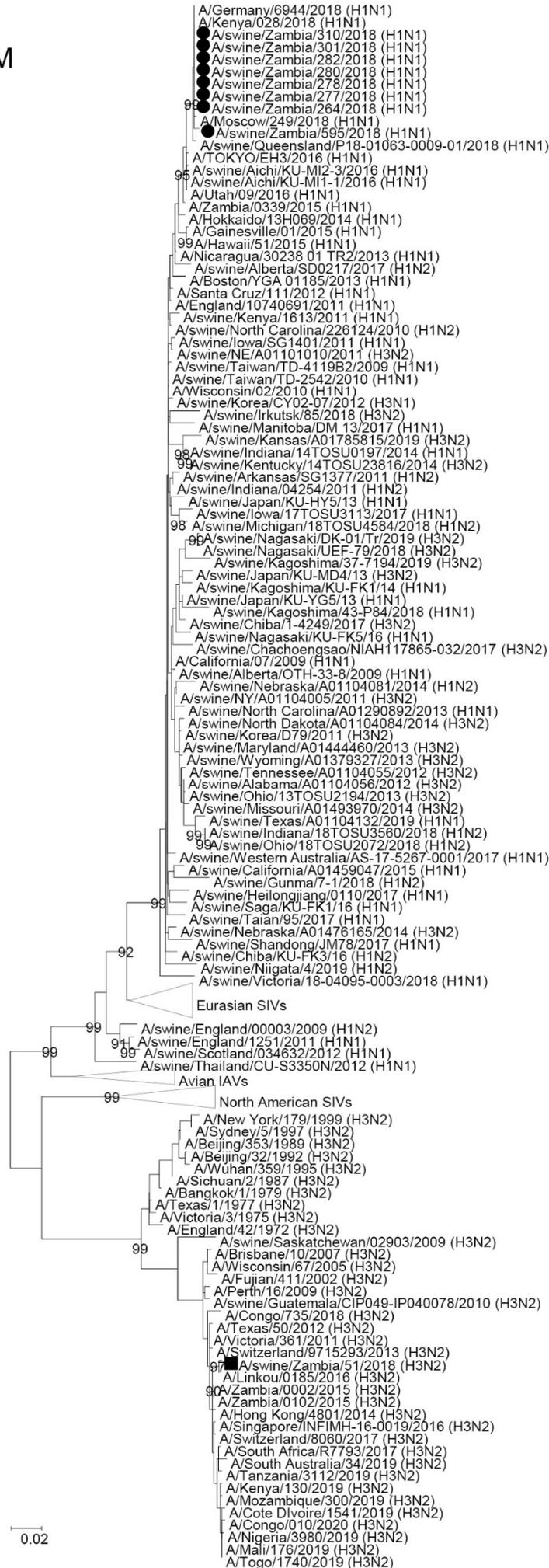
NP



A(H1N1)pdm09 lineage

Human seasonal H3N2 lineage

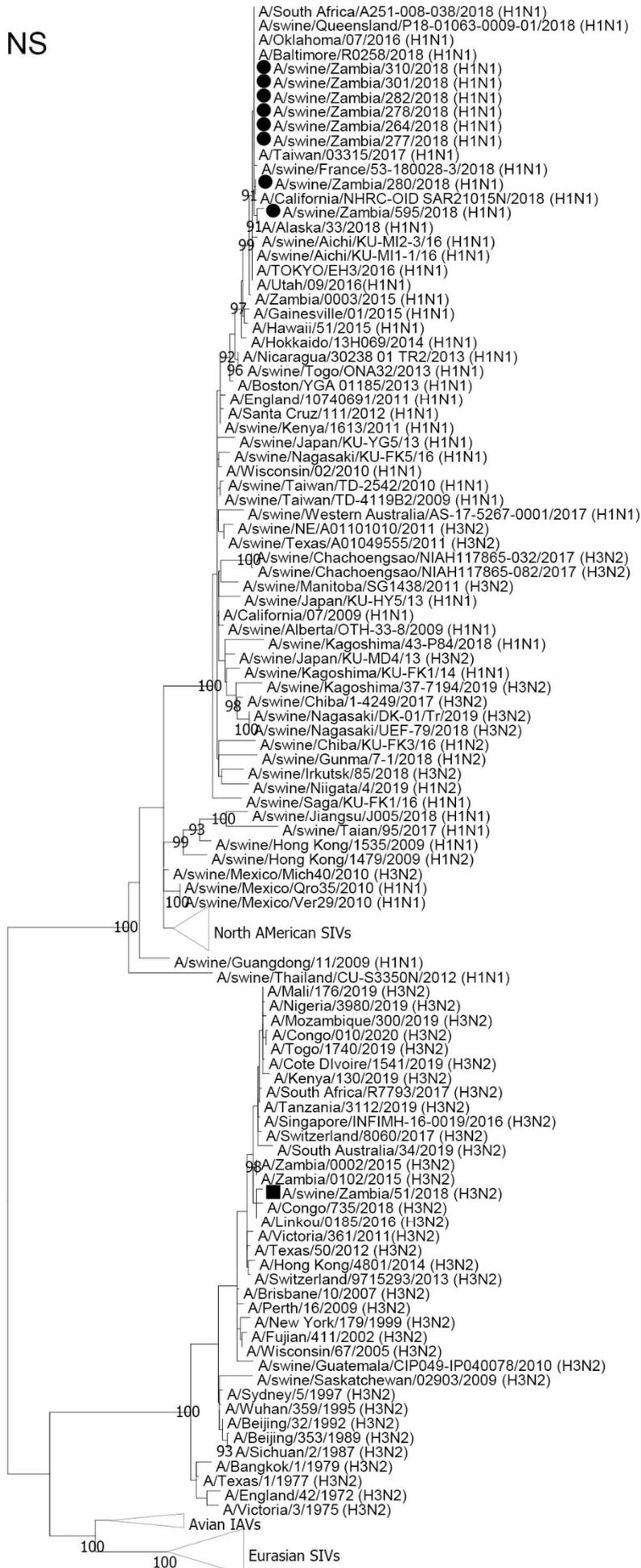
M



A(H1N1)pdm09 lineage

Human seasonal H3N2 lineage

NS



A(H1N1)pdm09 lineage

Human seasonal H3N2 lineage

0.05

Fig S1

Phylogenetic trees of the PB2, PB1, PA, NP, M, and NS genes. Phylogenetic analyses based on the complete ORF nucleotide sequences of the PB2, PB1, PA, NP, M, and NS genes were conducted with corresponding genes from our 9 isolates and the representative swine, human, and avian IAVs. Bootstrap values greater than 90% are shown on the interior branch nodes, and scale bars indicate the number of substitutions per site. The black circles and square represent the H1N1 and H3N2 IAV strains isolated in this study, respectively.