Original Article

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

Running title: Influenza A viruses in pigs in Zambia

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Summary (300 words)

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

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

1. Introduction

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

Three predominant subtypes of swine IAVs (H1N1, H1N2, and H3N2) have been found in pig populations worldwide (Brown, 2000; Vincent et al., 2014). Classical swine H1N1 IAV has the same origin as the Spanish influenza virus, which caused a pandemic in humans in 1918, and has been circulating in pig populations worldwide (Webster, Bean, Gorman, Chambers, & 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

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

2. Materials and Methods

2.1 Ethics statement

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

2.2 Sample collection

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

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

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

2.3 Cells and Viruses

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

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

four wells per dilution, confluent monolayers of MDCK cells on 96-well plates were infected with 10-fold serially diluted viruses. After adsorption for 1 hour, the inoculum was removed and the cell monolayers were maintained in MEM containing 5 μg/ml trypsin (Gibco, Thermo Fisher Scientific, Waltham, MA, USA), 0.3% BSA, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 1.0 mg/ml $NaHCO_3$ at 35°C in 5% CO_2. A cytopathic effect (CPE) was observed three days post-infection and viral titers were calculated by the Reed-Muench method (Reed & Muench, 1938).

2.4 Serological assays

HI and neutralizing activities of serum samples were evaluated according to the standard method (WHO, 2002). Pig sera were treated with receptor-destroying enzyme (RDE II; Denka Seiken Co., Ltd., Tokyo, Japan) to remove nonspecific inhibitors of IAV replication. For HI tests, twofold serially diluted sera were mixed with an equal volume of diluted virus solutions (4 × HA units) and incubated for 1 hour at room temperature followed by adding an equal volume of 0.5% chicken erythrocytes. HI titers were defined as reciprocals of the highest serum dilutions completely protecting hemagglutination. HI tests were carried out twice for each reference IAV strain, and the mean values were adopted as HI titers of individual sera. For neutralization tests, twofold serially diluted sera were mixed with an equal volume of diluted virus solutions (200 × $TCID_{50}$) and incubated for 1 hour at room temperature. Then the serum-virus mixtures were inoculated onto a monolayer of MDCK cells on 96-well tissue culture plates (4 well per dilution). After 1-hour incubation at 35°C, the inoculum was removed, and the cells were washed once with PBS and maintained with MEM containing 5 μg/ml trypsin and 0.3% BSA. CPE was observed three days post-infection and neutralization titers were defined as the reciprocal of the highest serum dilution completely protecting the cells from CPE in at least two wells of each dilution. As initial screening for neutralizing activities, 40-fold and 80-fold diluted pig sera were subjected to neutralization tests with each reference IAV. For the sera showing neutralization titers of ≥ 80, the assay was performed twice, and the mean values were used as neutralization titers of individual

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

2.5 Detection of IAV genome by RT-PCR

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

2.6 Virus isolation

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

2.7 Genomic sequencing

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

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

2.8 Phylogenetic analysis

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

3. Results

3.1 Seroprevalence of IAV infection in pigs in Zambia

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

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

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

3.2 Isolation of IAV strains from nasal swab samples

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

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

3.3 Phylogenetic characterization of the isolated IAVs

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

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

4. Discussion

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

The IAV strains isolated from pigs in this study were genetically close to IAVs causing seasonal influenza in humans, as well as IAVs found in Nigerian pigs (Meseko et al., 2019). These swine IAV isolates shared 99–100% nucleotide identities with human IAVs, suggesting reverse zoonotic transmission from humans to pigs. It is noteworthy that all of the Zambian swine IAV isolates were phylogenetically classified into the well-known lineages consisting of recent human IAV isolates. Since sustained circulation of such human IAVs in pig populations for a long term leads to the establishment of novel IAV lineages in the swine host (Kyriakis et al., 2011; Van Reeth, 2007), our data suggest that the IAVs isolated in this study were sporadically introduced into the pig population from humans in Zambia. In fact, the IAV isolates were obtained from each farm only at a single time point in the multiple sampling dates (Table 2). It is conceivable that continuous IAV circulation in pigs is associated with farming systems such as the farrow-to-finish 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 = 700–7700) 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

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

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

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Conflict of interest statement

The authors have no conflict of interest to declare.

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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)

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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)	99.5
		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).

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

Figure legends

Fig. 1

HI antibodies detected in the sera collected from pigs in Zambia during the years 2011-2018. Serum samples were tested for HI antibodies to A(H1N1)pdm09, classical swine H1N1, classical swine H1N2, and swine H3N2 viruses. HI titers of 80 or higher were considered positive and are shown in the figure. Each value represents the mean of HI titers of two independent experiments. All HI titers are represented in Supplementary Table S2.

Fig. 2

Neutralizing antibodies detected in the pig sera during the years 2011-2018. Serum samples were tested for neutralizing antibodies to A(H1N1)pdm09, classical swine H1N1, classical swine H1N2, and swine H3N2 viruses. Neutralizing titers of 80 or higher were considered positive and are shown in the figure. Each value represents the mean of neutralizing titers of two independent experiments. All neutralizing titers are represented in Supplementary Table S2.

Fig. 3

Phylogenetic trees of the HA gene of H1 and H3 subtypes. The complete ORF nucleotide sequences of HA genes of 9 Zambian isolates were phylogenetically analyzed with corresponding genes from the representative swine, avian, and human 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 IAVs isolated in this study, respectively.

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.

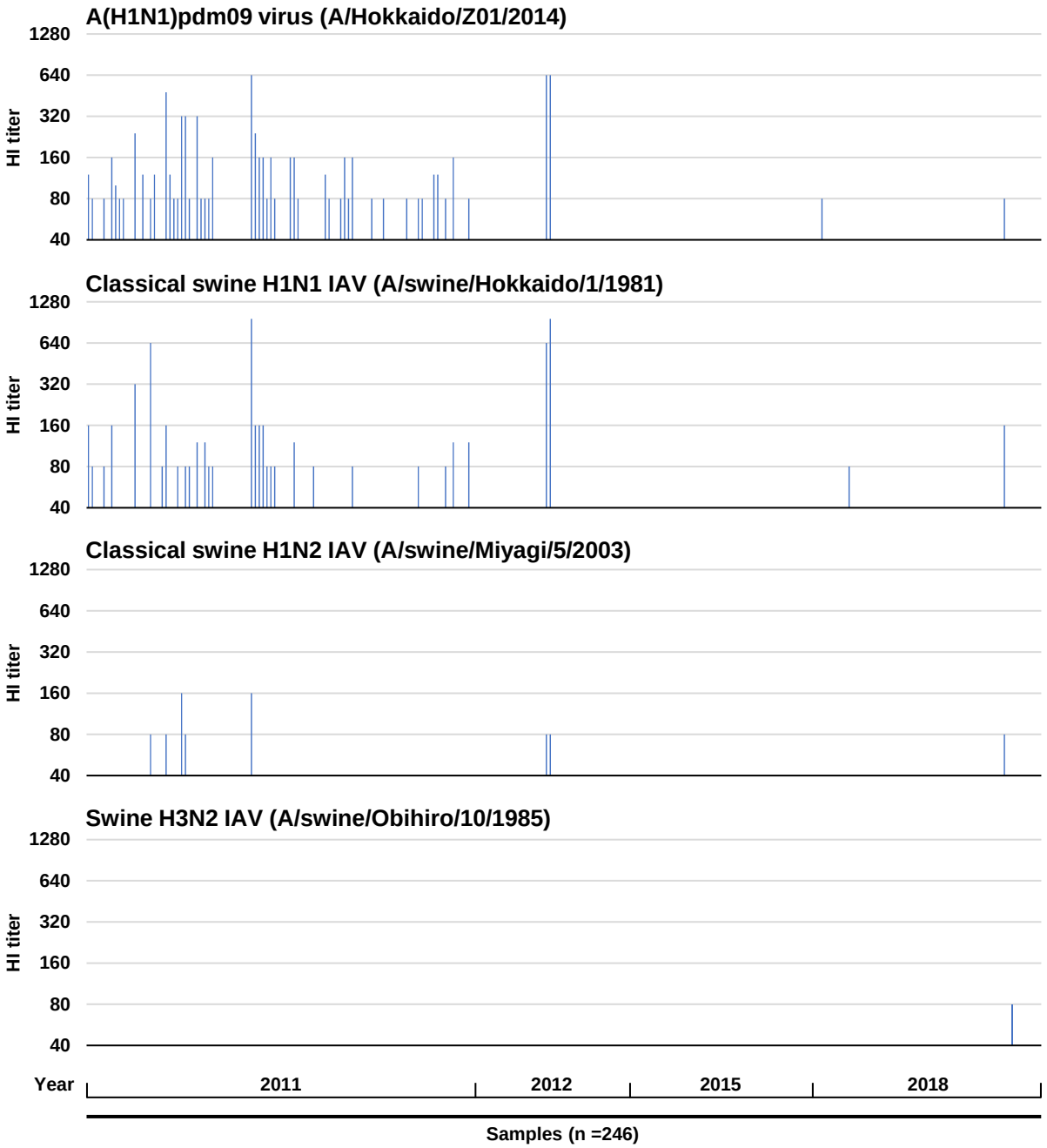
A(H1N1)pdm09 virus (A/Hokkaido/Z01/2014)
1280
640
320
160
80
40
HI titer
Classical swine H1N1 IAV (A/swine/Hokkaido/1/1981)
1280
640
320
160
80
40
HI titer
Classical swine H1N2 IAV (A/swine/Miyagi/5/2003)
1280
640
320
160
80
40
HI titer
Swine H3N2 IAV (A/swine/Obihiro/10/1985)
1280
640
320
160
80
40
HI titer
Year
2011
2012
2015
2018
Samples (n =246)

Fig. 1

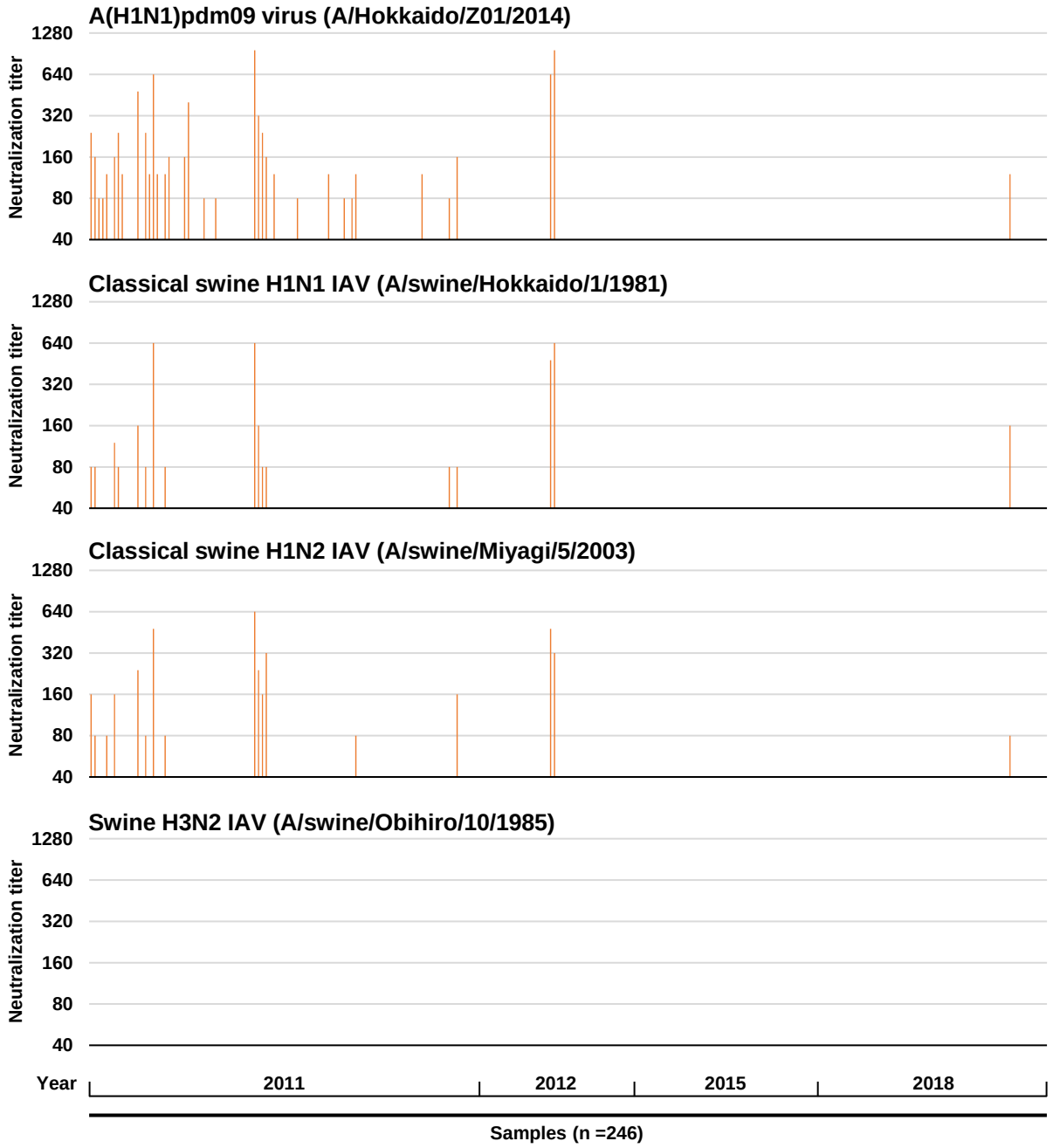
A(H1N1)pdm09 virus (A/Hokkaido/Z01/2014)
Neutralization titer
1280
640
320
160
80
40
Classical swine H1N1 IAV (A/swine/Hokkaido/1/1981)
Neutralization titer
1280
640
320
160
80
40
Classical swine H1N2 IAV (A/swine/Miyagi/5/2003)
Neutralization titer
1280
640
320
160
80
40
Swine H3N2 IAV (A/swine/Obihiro/10/1985)
Neutralization titer
1280
640
320
160
80
40
Year
2011
2012
2015
2018
Samples (n =246)

Fig. 2

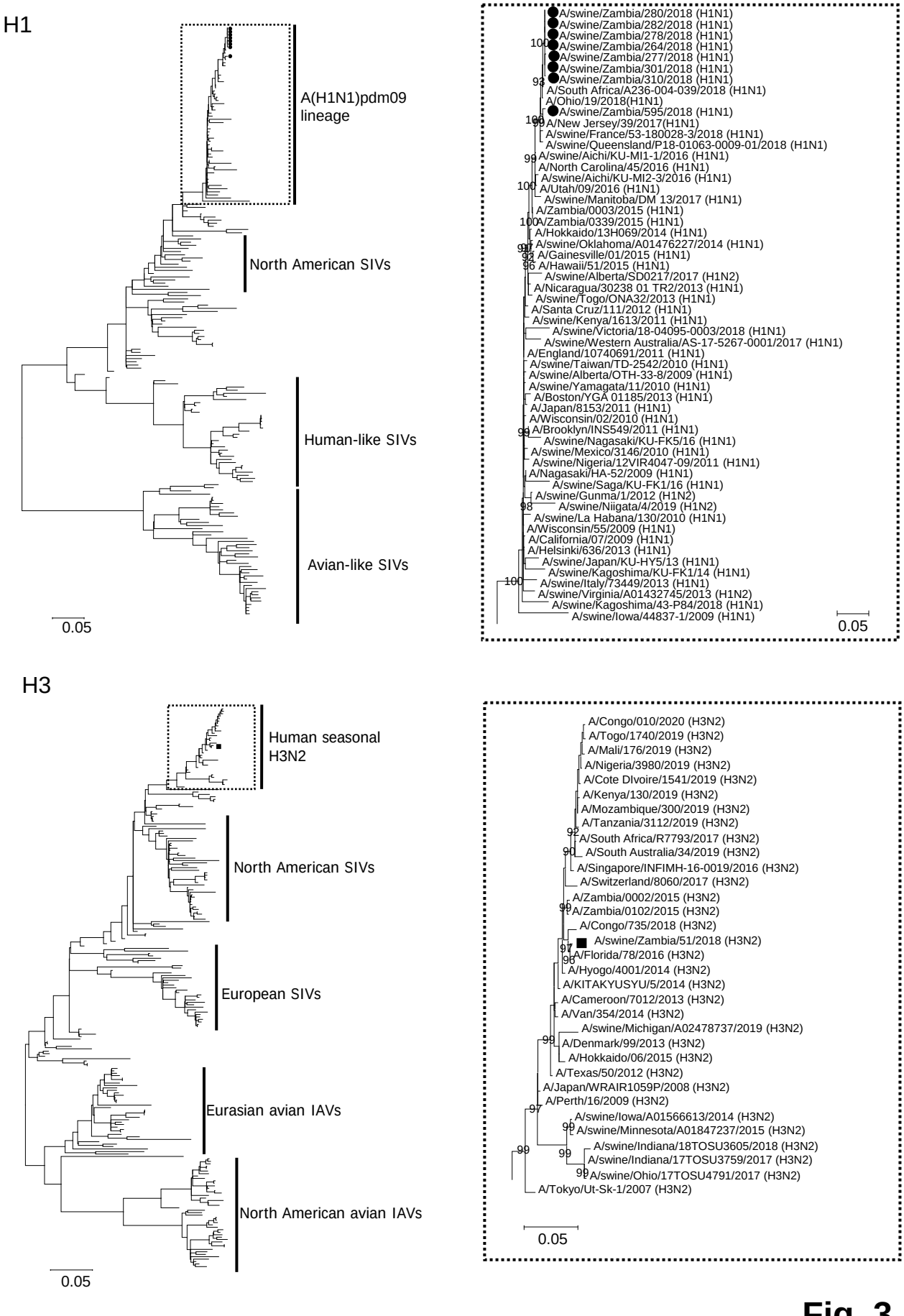
H1
A(H1N1)pdm09 lineage
North American SIVs
Human-like SIVs
Avian-like SIVs
0.05
A/swine/Zambia/280/2018 (H1N1)
A/swine/Zambia/282/2018 (H1N1)
A/swine/Zambia/278/2018 (H1N1)
A/swine/Zambia/264/2018 (H1N1)
A/swine/Zambia/277/2018 (H1N1)
A/swine/Zambia/301/2018 (H1N1)
A/swine/Zambia/310/2018 (H1N1)
A/South Africa/A236-004-039/2018 (H1N1)
A/Ohio/19/2018(H1N1)
A/swine/Zambia/595/2018 (H1N1)
A/New Jersey/39/2017(H1N1)
A/swine/France/53-180028-3/2018 (H1N1)
A/swine/Queensland/P18-01063-0009-01/2018 (H1N1)
A/swine/Aichi/KU-MI1-1/2016 (H1N1)
A/North Carolina/45/2016 (H1N1)
A/swine/Aichi/KU-MI2-3/2016 (H1N1)
A/Utah/09/2016 (H1N1)
A/swine/Manitoba/DM 13/2017 (H1N1)
A/Zambia/0003/2015 (H1N1)
A/Zambia/0339/2015 (H1N1)
A/Hokkaido/13H069/2014 (H1N1)
A/swine/Oklahoma/A01476227/2014 (H1N1)
A/Gainesville/01/2015 (H1N1)
A/Hawaii/51/2015 (H1N1)
A/swine/Alberta/SD0217/2017 (H1N2)
A/Nicaragua/30238 01 TR2/2013 (H1N1)
A/swine/Togo/ONA32/2013 (H1N1)
A/Santa Cruz/111/2012 (H1N1)
A/swine/Kenya/1613/2011 (H1N1)
A/swine/Victoria/18-04095-0003/2018 (H1N1)
A/swine/Western Australia/AS-17-5267-0001/2017 (H1N1)
A/England/10740691/2011 (H1N1)
A/swine/Taiwan/TD-2542/2010 (H1N1)
A/swine/Alberta/OTH-33-8/2009 (H1N1)
A/swine/Yamagata/11/2010 (H1N1)
A/Boston/YGA 01185/2013 (H1N1)
A/Japan/8153/2011 (H1N1)
A/Wisconsin/02/2010 (H1N1)
A/Brooklyn/INS549/2011 (H1N1)
A/swine/Nagasaki/KU-FK5/16 (H1N1)
A/swine/Mexico/3146/2010 (H1N1)
A/swine/Nigeria/12VIR4047-09/2011 (H1N1)
A/Nagasaki/HA-52/2009 (H1N1)
A/swine/Saga/KU-FK1/16 (H1N1)
A/swine/Gunma/1/2012 (H1N2)
A/swine/Niigata/4/2019 (H1N2)
A/swine/La Habana/130/2010 (H1N1)
A/Wisconsin/55/2009 (H1N1)
A/California/07/2009 (H1N1)
A/Helsinki/636/2013 (H1N1)
A/swine/Japan/KU-HY5/13 (H1N1)
A/swine/Kagoshima/KU-FK1/14 (H1N1)
A/swine/Italy/73449/2013 (H1N1)
A/swine/Virginia/A01432745/2013 (H1N2)
A/swine/Kagoshima/43-P84/2018 (H1N1)
A/swine/Iowa/44837-1/2009 (H1N1)
0.05
H3
Human seasonal H3N2
North American SIVs
European SIVs
Eurasian avian IAVs
North American avian IAVs
0.05
A/Congo/010/2020 (H3N2)
A/Togo/1740/2019 (H3N2)
A/Mali/176/2019 (H3N2)
A/Nigeria/3980/2019 (H3N2)
A/Cote DIvoire/1541/2019 (H3N2)
A/Kenya/130/2019 (H3N2)
A/Mozambique/300/2019 (H3N2)
A/Tanzania/3112/2019 (H3N2)
A/South Africa/R7793/2017 (H3N2)
A/South Australia/34/2019 (H3N2)
A/Singapore/INFIMH-16-0019/2016 (H3N2)
A/Switzerland/8060/2017 (H3N2)
A/Zambia/0002/2015 (H3N2)
A/Zambia/0102/2015 (H3N2)
A/Congo/735/2018 (H3N2)
A/swine/Zambia/51/2018 (H3N2)
A/Florida/78/2016 (H3N2)
A/Hyogo/4001/2014 (H3N2)
A/KITAKYUSYU/5/2014 (H3N2)
A/Cameroon/7012/2013 (H3N2)
A/Van/354/2014 (H3N2)
A/swine/Michigan/A02478737/2019 (H3N2)
A/Denmark/99/2013 (H3N2)
A/Hokkaido/06/2015 (H3N2)
A/Texas/50/2012 (H3N2)
A/Japan/WRAIR1059P/2008 (H3N2)
A/Perth/16/2009 (H3N2)
A/swine/Iowa/A01566613/2014 (H3N2)
A/swine/Minnesota/A01847237/2015 (H3N2)
A/swine/Indiana/18TOSU3605/2018 (H3N2)
A/swine/Indiana/17TOSU3759/2017 (H3N2)
A/swine/Ohio/17TOSU4791/2017 (H3N2)
A/Tokyo/Ut-Sk-1/2007 (H3N2)
0.05

Fig. 3

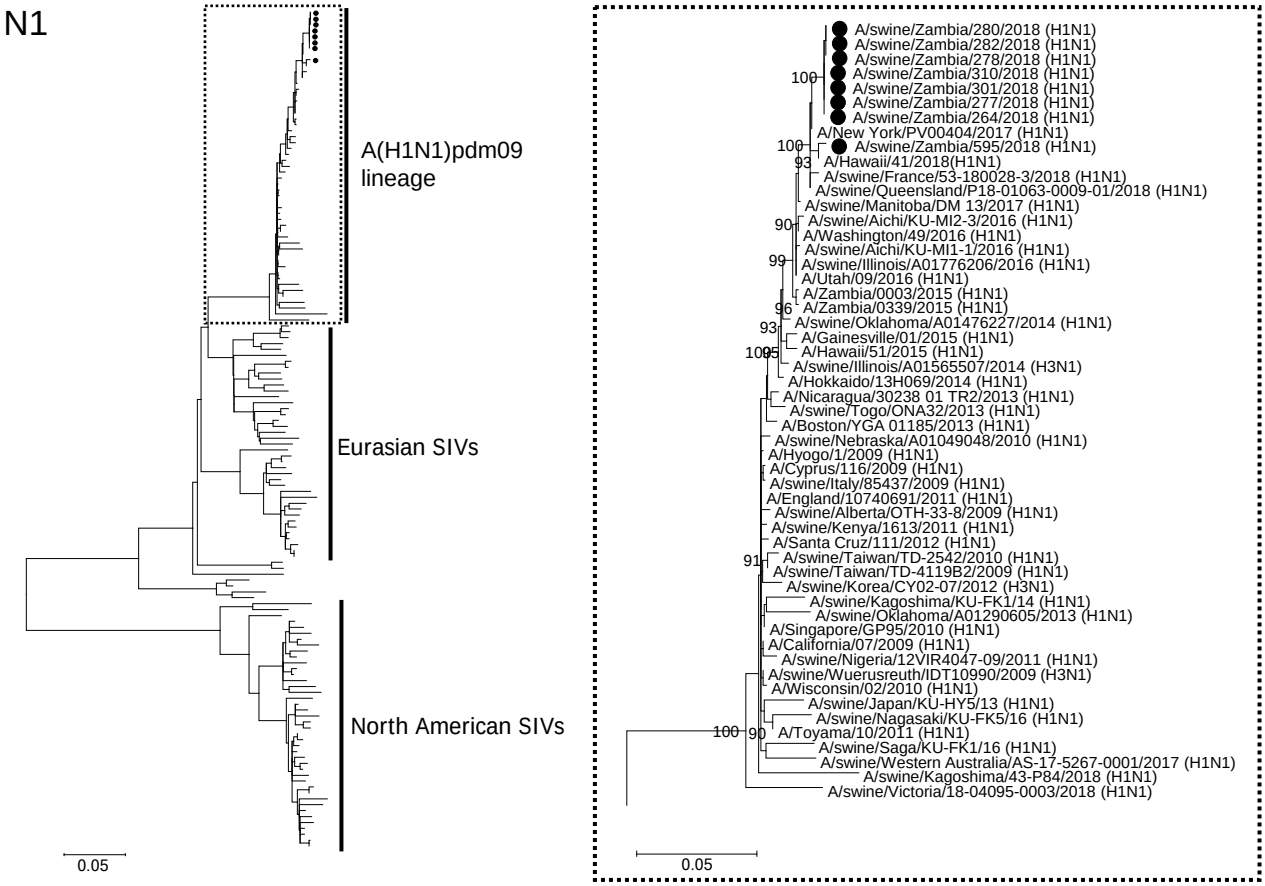
N1
A(H1N1)pdm09 lineage
Eurasian SIVs
North American SIVs
0.05
A/swine/Zambia/280/2018 (H1N1)
A/swine/Zambia/282/2018 (H1N1)
A/swine/Zambia/278/2018 (H1N1)
A/swine/Zambia/310/2018 (H1N1)
A/swine/Zambia/301/2018 (H1N1)
A/swine/Zambia/277/2018 (H1N1)
A/swine/Zambia/264/2018 (H1N1)
A/New York/PV00404/2017 (H1N1)
A/swine/Zambia/595/2018 (H1N1)
A/Hawaii/41/2018(H1N1)
A/swine/France/53-180028-3/2018 (H1N1)
A/swine/Queensland/P18-01063-0009-01/2018 (H1N1)
A/swine/Manitoba/DM 13/2017 (H1N1)
A/swine/Aichi/KU-MI2-3/2016 (H1N1)
A/Washington/49/2016 (H1N1)
A/swine/Aichi/KU-MI1-1/2016 (H1N1)
A/swine/Illinois/A01776206/2016 (H1N1)
A/Utah/09/2016 (H1N1)
A/Zambia/0003/2015 (H1N1)
A/Zambia/0339/2015 (H1N1)
A/swine/Oklahoma/A01476227/2014 (H1N1)
A/Gainesville/01/2015 (H1N1)
A/Hawaii/51/2015 (H1N1)
A/swine/Illinois/A01565507/2014 (H3N1)
A/Hokkaido/13H069/2014 (H1N1)
A/Nicaragua/30238 01 TR2/2013 (H1N1)
A/swine/Togo/ONA32/2013 (H1N1)
A/Boston/YGA 01185/2013 (H1N1)
A/swine/Nebraska/A01049048/2010 (H1N1)
A/Hyogo/1/2009 (H1N1)
A/Cyprus/116/2009 (H1N1)
A/swine/Italy/85437/2009 (H1N1)
A/England/10740691/2011 (H1N1)
A/swine/Alberta/OTH-33-8/2009 (H1N1)
A/swine/Kenya/1613/2011 (H1N1)
A/Santa Cruz/111/2012 (H1N1)
A/swine/Taiwan/TD-2542/2010 (H1N1)
A/swine/Taiwan/TD-4119B2/2009 (H1N1)
A/swine/Korea/CY02-07/2012 (H3N1)
A/swine/Kagoshima/KU-FK1/14 (H1N1)
A/swine/Oklahoma/A01290605/2013 (H1N1)
A/Singapore/GP95/2010 (H1N1)
A/California/07/2009 (H1N1)
A/swine/Nigeria/12VIR4047-09/2011 (H1N1)
A/swine/Wuerusreuth/IDT10990/2009 (H3N1)
A/Wisconsin/02/2010 (H1N1)
A/swine/Japan/KU-HY5/13 (H1N1)
A/swine/Nagasaki/KU-FK5/16 (H1N1)
A/Toyama/10/2011 (H1N1)
A/swine/Saga/KU-FK1/16 (H1N1)
A/swine/Western Australia/AS-17-5267-0001/2017 (H1N1)
A/swine/Kagoshima/43-P84/2018 (H1N1)
A/swine/Victoria/18-04095-0003/2018 (H1N1)
0.05
N2
Human seasonal H3N2
North American SIVs
European SIVs
Avian IAVs
0.05
A/Congo/010/2020 (H3N2)
A/Togo/1740/2019 (H3N2)
A/Nigeria/3980/2019 (H3N2)
A/Mali/176/2019 (H3N2)
A/Cote DIvoire/1541/2019 (H3N2)
A/Kenya/130/2019 (H3N2)
A/Mozambique/300/2019 (H3N2)
A/Tanzania/3112/2019 (H3N2)
A/South Australia/34/2019 (H3N2)
A/South Africa/R7793/2017 (H3N2)
A/Congo/735/2018 (H3N2)
A/Switzerland/8060/2017 (H3N2)
A/swine/Michigan/A02478737/2019 (H3N2)
A/Singapore/INFIMH-16-0019/2016 (H3N2)
A/swine/Zambia/51/2018 (H3N2)
A/Victoria/146/2016 (H3N2)
A/Zambia/0102/2015 (H3N2)
A/Zambia/0002/2015 (H3N2)
A/Hong Kong/4801/2014 (H3N2)
A/Switzerland/9715293/2013 (H3N2)
A/Victoria/361/2011(H3N2)
A/Texas/50/2012 (H3N2)
A/Perth/16/2009(H3N2)
A/Brisbane/10/2007(H3N2)
A/California/7/2004(H3N2)
A/Wisconsin/67/2005(H3N2)
A/Fujian/411/2002(H3N2)
A/swine/Irkutsk/85/2018 (H3N2)
0.05

Fig. 4

Transboundary and Emerging Diseases
Original Article

Serological and molecular epidemiological study on swine influenza in Zambia

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Table S1. Summary of pig sera used for serosurveillance of IAV infection in Zambia

District	Year	Place	Stage or age of pigs	No. of sera	Sample ID
Lusaka	2011	Abattoir 1	Slaughtering	20	ZP11-1~20
		Abattoir 2		13	ZP11-21~33
		Abattoir 3		36	ZP11-34~69
		Abattoir 4		31	ZP11-70~100
	2012	Abattoir 2		19	ZP12-25~43
		Abattoir 1		21	ZP12-44~64
	2015	Abattoir 2		47	ZP15-1~47
	2018	Farm A	4-12 weeks old	40	ZP18-1~40
			> 20 weeks old	19	ZP18-41~59

Table S2. Antibody titers of pig sera collected in Zambia in hemagglutination-inhibition and neutralization tests

	Hemagglutination-inhibiting antibody titers				Neutralizing antibody titer			
Sample ID	A(H1N1)pdm09	SwH1N1	SwH1N2	SwH3N2	A(H1N1)pdm09	SwH1N1	SwH1N2	SwH3N2
ZP11-1	120	160	-	-	240	80	160	-
ZP11-2	80	80	-	-	160	80	80	-
ZP11-3	-	-	-	-	80	-	-	-
ZP11-4	-	-	-	-	80	-	-	-
ZP11-5	80	80	-	-	120	-	80	-
ZP11-6	-	-	-	-	-	-	-	-
ZP11-7	160	160	-	-	160	120	160	-
ZP11-8	100	-	-	-	240	80	-	-
ZP11-9	80	-	-	-	120	-	-	-
ZP11-10	80	-	-	-	-	-	-	-
ZP11-11	-	-	-	-	-	-	-	-
ZP11-12	-	-	-	-	-	-	-	-
ZP11-13	240	320	-	-	480	160	240	-
ZP11-14	-	-	-	-	-	-	-	-
ZP11-15	120	-	-	-	240	80	80	-
ZP11-16	-	-	-	-	120	-	-	-
ZP11-17	80	640	80	-	640	640	480	-
ZP11-18	120	-	-	-	120	-	-	-
ZP11-19	-	-	-	-	-	-	-	-
ZP11-20	-	80	-	-	120	80	80	-
ZP11-21	480	160	80	-	160	-	-	-
ZP11-22	120	-	-	-	-	-	-	-
ZP11-23	80	-	-	-	-	-	-	-
ZP11-24	80	80	-	-	-	-	-	-
ZP11-25	320	-	160	-	160	-	-	-
ZP11-26	320	80	80	-	400	-	-	-
ZP11-27	80	80	-	-	-	-	-	-
ZP11-28	-	-	-	-	-	-	-	-
ZP11-29	320	120	-	-	-	-	-	-
ZP11-30	80	-	-	-	80	-	-	-
ZP11-31	80	120	-	-	-	-	-	-
ZP11-32	80	80	-	-	-	-	-	-
ZP11-33	160	80	-	-	80	-	-	-
ZP11-34	-	-	-	-	-	-	-	-
ZP11-35	-	-	-	-	-	-	-	-
ZP11-36	-	-	-	-	-	-	-	-
ZP11-37	-	-	-	-	-	-	-	-
ZP11-38	-	-	-	-	-	-	-	-
ZP11-39	-	-	-	-	-	-	-	-
ZP11-40	-	-	-	-	-	-	-	-

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	-	-	-	-	-	-	-	-
ZP11-51	-	-	-	-	-	-	-	-
ZP11-52	-	-	-	-	-	-	-	-
ZP11-53	160	-	-	-	-	-	-	-
ZP11-54	160	120	-	-	80	-	-	-
ZP11-55	80	-	-	-	-	-	-	-
ZP11-56	-	-	-	-	-	-	-	-
ZP11-57	-	-	-	-	-	-	-	-
ZP11-58	-	-	-	-	-	-	-	-
ZP11-59	-	80	-	-	-	-	-	-
ZP11-60	-	-	-	-	-	-	-	-
ZP11-61	-	-	-	-	-	-	-	-
ZP11-62	120	-	-	-	120	-	-	-
ZP11-63	80	-	-	-	-	-	-	-
ZP11-64	-	-	-	-	-	-	-	-
ZP11-65	-	-	-	-	-	-	-	-
ZP11-66	80	-	-	-	80	-	-	-
ZP11-67	160	-	-	-	-	-	-	-
ZP11-68	80	-	-	-	80	-	-	-
ZP11-69	160	80	-	-	120	-	80	-
ZP11-70	-	-	-	-	-	-	-	-
ZP11-71	-	-	-	-	-	-	-	-
ZP11-72	-	-	-	-	-	-	-	-
ZP11-73	-	-	-	-	-	-	-	-
ZP11-74	80	-	-	-	-	-	-	-
ZP11-75	-	-	-	-	-	-	-	-
ZP11-76	-	-	-	-	-	-	-	-
ZP11-77	80	-	-	-	-	-	-	-
ZP11-78	-	-	-	-	-	-	-	-
ZP11-79	-	-	-	-	-	-	-	-
ZP11-80	-	-	-	-	-	-	-	-
ZP11-81	-	-	-	-	-	-	-	-
ZP11-82	-	-	-	-	-	-	-	-
ZP11-83	80	-	-	-	-	-	-	-
ZP11-84	-	-	-	-	-	-	-	-
ZP11-85	-	-	-	-	-	-	-	-
ZP11-86	80	80	-	-	120	-	-	-
ZP11-87	80	-	-	-	-	-	-	-
ZP11-88	-	-	-	-	-	-	-	-
ZP11-89	-	-	-	-	-	-	-	-
ZP11-90	120	-	-	-	-	-	-	-
ZP11-91	120	-	-	-	-	-	-	-
ZP11-92	-	-	-	-	-	-	-	-
ZP11-93	80	80	-	-	80	80	-	-
ZP11-94	-	-	-	-	-	-	-	-
ZP11-95	160	120	-	-	160	80	160	-
ZP11-96	-	-	-	-	-	-	-	-
ZP11-97	-	-	-	-	-	-	-	-
ZP11-98	-	-	-	-	-	-	-	-

ZP11-99	80	120	-	-	-	-	-	-
ZP11-100	-	-	-	-	-	-	-	-
ZP12-25	-	-	-	-	-	-	-	-
ZP12-26	-	-	-	-	-	-	-	-
ZP12-27	-	-	-	-	-	-	-	-
ZP12-28	-	-	-	-	-	-	-	-
ZP12-29	-	-	-	-	-	-	-	-
ZP12-30	-	-	-	-	-	-	-	-
ZP12-31	-	-	-	-	-	-	-	-
ZP12-32	-	-	-	-	-	-	-	-
ZP12-33	-	-	-	-	-	-	-	-
ZP12-34	-	-	-	-	-	-	-	-
ZP12-35	-	-	-	-	-	-	-	-
ZP12-36	-	-	-	-	-	-	-	-
ZP12-37	-	-	-	-	-	-	-	-
ZP12-38	-	-	-	-	-	-	-	-
ZP12-39	-	-	-	-	-	-	-	-
ZP12-40	-	-	-	-	-	-	-	-
ZP12-41	-	-	-	-	-	-	-	-
ZP12-42	-	-	-	-	-	-	-	-
ZP12-43	640	640	80	-	640	480	480	-
ZP12-44	640	960	80	-	960	640	320	-
ZP12-45	-	-	-	-	-	-	-	-
ZP12-46	-	-	-	-	-	-	-	-
ZP12-47	-	-	-	-	-	-	-	-
ZP12-48	-	-	-	-	-	-	-	-
ZP12-49	-	-	-	-	-	-	-	-
ZP12-50	-	-	-	-	-	-	-	-
ZP12-51	-	-	-	-	-	-	-	-
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ZP12-57	-	-	-	-	-	-	-	-
ZP12-58	-	-	-	-	-	-	-	-
ZP12-59	-	-	-	-	-	-	-	-
ZP12-60	-	-	-	-	-	-	-	-
ZP12-61	-	-	-	-	-	-	-	-
ZP12-62	-	-	-	-	-	-	-	-
ZP12-63	-	-	-	-	-	-	-	-
ZP12-64	-	-	-	-	-	-	-	-
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ZP15-2	-	-	-	-	-	-	-	-
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ZP15-4	-	-	-	-	-	-	-	-
ZP15-5	-	-	-	-	-	-	-	-
ZP15-6	-	-	-	-	-	-	-	-
ZP15-7	-	-	-	-	-	-	-	-
ZP15-8	-	-	-	-	-	-	-	-
ZP15-9	-	-	-	-	-	-	-	-
ZP15-10	-	-	-	-	-	-	-	-
ZP15-11	-	-	-	-	-	-	-	-
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ZP15-13	-	-	-	-	-	-	-	-
ZP15-14	-	-	-	-	-	-	-	-
ZP15-15	-	-	-	-	-	-	-	-
ZP15-16	-	-	-	-	-	-	-	-

ZP15-17	-	-	-	-	-	-	-	-
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ZP15-21	-	-	-	-	-	-	-	-
ZP15-22	-	-	-	-	-	-	-	-
ZP15-23	-	-	-	-	-	-	-	-
ZP15-24	-	-	-	-	-	-	-	-
ZP15-25	-	-	-	-	-	-	-	-
ZP15-26	-	-	-	-	-	-	-	-
ZP15-27	-	-	-	-	-	-	-	-
ZP15-28	-	-	-	-	-	-	-	-
ZP15-29	-	-	-	-	-	-	-	-
ZP15-30	-	-	-	-	-	-	-	-
ZP15-31	-	-	-	-	-	-	-	-
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ZP15-36	-	-	-	-	-	-	-	-
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ZP15-38	-	-	-	-	-	-	-	-
ZP15-39	-	-	-	-	-	-	-	-
ZP15-40	-	-	-	-	-	-	-	-
ZP15-41	-	-	-	-	-	-	-	-
ZP15-42	-	-	-	-	-	-	-	-
ZP15-43	-	-	-	-	-	-	-	-
ZP15-44	-	-	-	-	-	-	-	-
ZP15-45	-	-	-	-	-	-	-	-
ZP15-46	-	-	-	-	-	-	-	-
ZP15-47	-	-	-	-	-	-	-	-
ZP18-1	-	-	-	-	-	-	-	-
ZP18-2	-	-	-	-	-	-	-	-
ZP18-3	80	-	-	-	-	-	-	-
ZP18-4	-	-	-	-	-	-	-	-
ZP18-5	-	-	-	-	-	-	-	-
ZP18-6	-	-	-	-	-	-	-	-
ZP18-7	-	-	-	-	-	-	-	-
ZP18-8	-	-	-	-	-	-	-	-
ZP18-9	-	-	-	-	-	-	-	-
ZP18-10	-	80	-	-	-	-	-	-
ZP18-11	-	-	-	-	-	-	-	-
ZP18-12	-	-	-	-	-	-	-	-
ZP18-13	-	-	-	-	-	-	-	-
ZP18-14	-	-	-	-	-	-	-	-
ZP18-15	-	-	-	-	-	-	-	-
ZP18-16	-	-	-	-	-	-	-	-
ZP18-17	-	-	-	-	-	-	-	-
ZP18-18	-	-	-	-	-	-	-	-
ZP18-19	-	-	-	-	-	-	-	-
ZP18-20	-	-	-	-	-	-	-	-
ZP18-21	-	-	-	-	-	-	-	-
ZP18-22	-	-	-	-	-	-	-	-
ZP18-23	-	-	-	-	-	-	-	-
ZP18-24	-	-	-	-	-	-	-	-
ZP18-25	-	-	-	-	-	-	-	-
ZP18-26	-	-	-	-	-	-	-	-
ZP18-27	-	-	-	-	-	-	-	-

ZP18-28	-	-	-	-	-	-	-	-
ZP18-29	-	-	-	-	-	-	-	-
ZP18-30	-	-	-	-	-	-	-	-
ZP18-31	-	-	-	-	-	-	-	-
ZP18-32	-	-	-	-	-	-	-	-
ZP18-33	-	-	-	-	-	-	-	-
ZP18-34	-	-	-	-	-	-	-	-
ZP18-35	-	-	-	-	-	-	-	-
ZP18-36	-	-	-	-	-	-	-	-
ZP18-37	-	-	-	-	-	-	-	-
ZP18-38	-	-	-	-	-	-	-	-
ZP18-39	-	-	-	-	-	-	-	-
ZP18-40	-	-	-	-	-	-	-	-
ZP18-41	-	-	-	-	-	-	-	-
ZP18-42	-	-	-	-	-	-	-	-
ZP18-43	-	-	-	-	-	-	-	-
ZP18-44	-	-	-	-	-	-	-	-
ZP18-45	-	-	-	-	-	-	-	-
ZP18-46	-	-	-	-	-	-	-	-
ZP18-47	-	-	-	-	-	-	-	-
ZP18-48	-	-	-	-	-	-	-	-
ZP18-49	-	-	-	-	-	-	-	-
ZP18-50	80	160	80	-	120	160	80	-
ZP18-51	-	-	-	-	-	-	-	-
ZP18-52	-	-	-	80	-	-	-	-
ZP18-53	-	-	-	-	-	-	-	-
ZP18-54	-	-	-	-	-	-	-	-
ZP18-55	-	-	-	-	-	-	-	-
ZP18-56	-	-	-	-	-	-	-	-
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

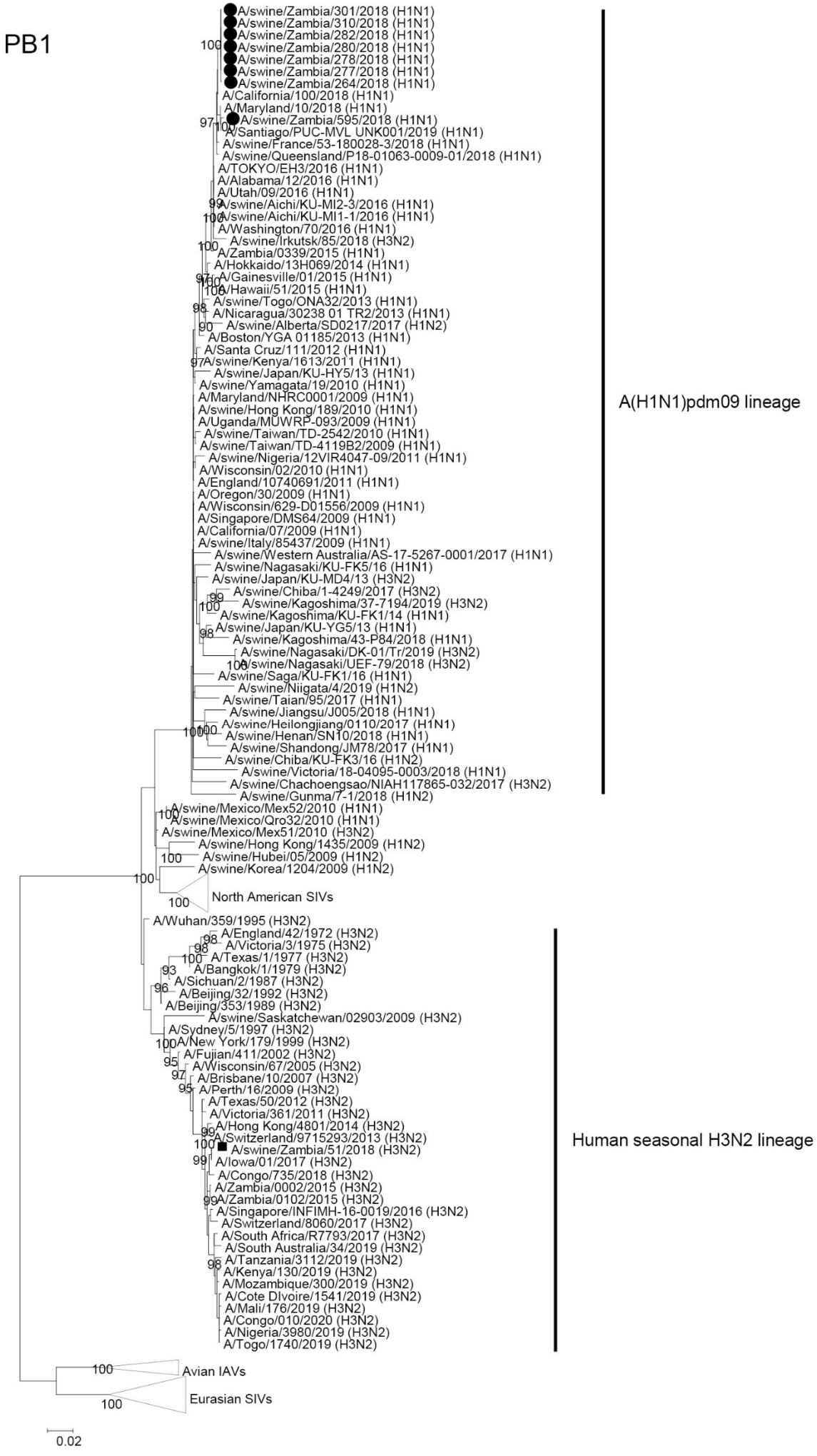
-: Not pooled, N.A.: Not analyzed

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/swine/Zambia/282/2018 (H1N1)
A/swine/Zambia/310/2018 (H1N1)
A/swine/Zambia/280/2018 (H1N1)
A/swine/Zambia/278/2018 (H1N1)
A/swine/Zambia/264/2018 (H1N1)
A/swine/Zambia/277/2018 (H1N1)
A/swine/Zambia/301/2018 (H1N1)
A/swine/Zambia/595/2018 (H1N1)
A/Minnesota/20/2018 (H1N1)
A/Mississippi/01/2018 (H1N1)
A/swine/Queensland/P18-01063-0009-01/2018 (H1N1)
A/swine/France/53-180028-3/2018 (H1N1)
A/New Jersey/37/2017 (H1N1)
A/swine/Irkutsk/85/2018 (H3N2)
A/Utah/09/2016 (H1N1)
A/swine/Aichi/KU-MI1-1/2016 (H1N1)
A/Texas/69/2016 (H1N1)
A/swine/Aichi/KU-MI2-3/2016 (H1N1)
A/Colorado/20/2016 (H1N1)
A/TOKYO/EH3/2016 (H1N1)
A/Zambia/0339/2015 (H1N1)
A/Gainesville/01/2015 (H1N1)
A/Hawaii/51/2015 (H1N1)
A/Hokkaido/13H069/2014 (H1N1)
A/swine/Alberta/SD0217/2017 (H1N2)
A/Boston/YGA 01185/2013 (H1N1)
A/swine/Nebraska/A01049048/2010 (H1N1)
A/Viet Nam/001-1716/2009(H1N1)
A/California/07/2009 (H1N1)
A/England/10740691/2011 (H1N1)
A/Mexico/24024/2009 (H1N1)
A/Wisconsin/629-S0252/2009 (H1N1)
A/Singapore/GP1120/2009 (H1N1)
A/swine/Korea/CY02-08/2012 (H3N1)
A/swine/Italy/85437/2009 (H1N1)
A/swine/Nigeria/12VIR4047-09/2011 (H1N1)
A/Wisconsin/02/2010 (H1N1)
A/Santa Cruz/111/2012 (H1N1)
A/swine/Kenya/1613/2011 (H1N1)
A/Mexico City/INERPHAC3/2010 (H1N1)
A/Singapore/DMS623/2009 (H1N1)
A/swine/Taiwan/TD-2542/2010 (H1N1)
A/swine/Chiba/KU-FK3/16 (H1N2)
A/swine/Japan/KU-HY5/13 (H1N1)
A/swine/Nagasaki/KU-FK5/16 (H1N1)
A/swine/Japan/KU-YG5/13 (H1N1)
A/swine/Kagoshima/43-P84/2018 (H1N1)
A/swine/Japan/KU-MD4/13 (H3N2)
A/swine/Kagoshima/KU-FK1/14 (H1N1)
A/swine/Chiba/1-4249/2017 (H3N2)
A/swine/Kagoshima/37-7194/2019 (H3N2)
A/swine/Nagasaki/DK-01/Tr/2019 (H3N2)
A/swine/Nagasaki/UEF-79/2018 (H3N2)
A/swine/Taian/95/2017 (H1N1)
A/swine/Gunma/7-1/2018 (H1N2)
A/swine/Victoria/18-04095-0003/2018 (H1N1)
A/swine/Chachoengsao/NIAH117865-032/2017 (H3N2)
A/swine/Chachoengsao/NIAH117865-082/2017 (H3N2)
A/swine/Henan/SN10/2018 (H1N1)
A/swine/Heilongjiang/0110/2017 (H1N1)
A/swine/Shandong/JM78/2017 (H1N1)
A/swine/Niigata/4/2019 (H1N2)
A(H1N1)pdm09 lineage
North American SIVs
A/Bangkok/1/1979 (H3N2)
A/Texas/1/1977 (H3N2)
A/England/42/1972 (H3N2)
A/Victoria/3/1975 (H3N2)
A/Beijing/32/1992 (H3N2)
A/Wuhan/359/1995 (H3N2)
A/Beijing/353/1989 (H3N2)
A/Sichuan/2/1987 (H3N2)
A/New York/179/1999 (H3N2)
A/Sydney/5/1997 (H3N2)
A/Fujian/411/2002 (H3N2)
A/Wisconsin/67/2005 (H3N2)
A/swine/Guatemala/CIP049-IP040078/2010 (H3N2)
A/Brisbane/10/2007 (H3N2)
A/Perth/16/2009 (H3N2)
A/Congo/010/2020 (H3N2)
A/Mali/176/2019 (H3N2)
A/Togo/1740/2019 (H3N2)
A/Nigeria/3980/2019 (H3N2)
A/Cote DIvoire/1541/2019 (H3N2)
A/Kenya/130/2019 (H3N2)
A/Mozambique/300/2019 (H3N2)
A/Tanzania/3112/2019 (H3N2)
A/South Australia/34/2019 (H3N2)
A/Victoria/361/2011 (H3N2)
A/Texas/50/2012 (H3N2)
A/Hong Kong/4801/2014 (H3N2)
A/Switzerland/9715293/2013 (H3N2)
A/swine/Zambia/51/2018 (H3N2)
A/Michigan/104/2016 (H3N2)
A/Congo/735/2018 (H3N2)
A/Singapore/INFIMH-16-0019/2016 (H3N2)
A/Switzerland/8060/2017 (H3N2)
A/Zambia/0002/2015 (H3N2)
A/Zambia/0102/2015 (H3N2)
Human seasonal H3N2 lineage
Avian IAVs
A/goose/Zambia/06/2008 (H3N8)
A/ostrich/South Africa/AI2887/2011 (H1N2)
A/duck /Zambia/03/2008 (H6N2)
Eurasian SIVs
0.05


PB1
A/swine/Zambia/301/2018 (H1N1)
A/swine/Zambia/310/2018 (H1N1)
A/swine/Zambia/282/2018 (H1N1)
A/swine/Zambia/280/2018 (H1N1)
A/swine/Zambia/278/2018 (H1N1)
A/swine/Zambia/277/2018 (H1N1)
A/swine/Zambia/264/2018 (H1N1)
A/California/100/2018 (H1N1)
A/Maryland/10/2018 (H1N1)
A/swine/Zambia/595/2018 (H1N1)
A/Santiago/PUC-MVL UNK001/2019 (H1N1)
A/swine/France/53-180028-3/2018 (H1N1)
A/swine/Queensland/P18-01063-0009-01/2018 (H1N1)
A/TOKYO/EH3/2016 (H1N1)
A/Alabama/12/2016 (H1N1)
A/Utah/09/2016 (H1N1)
A/swine/Aichi/KU-MI2-3/2016 (H1N1)
A/swine/Aichi/KU-MI1-1/2016 (H1N1)
A/Washington/70/2016 (H1N1)
A/swine/Irkutsk/85/2018 (H3N2)
A/Zambia/0339/2015 (H1N1)
A/Hokkaido/13H069/2014 (H1N1)
A/Gainesville/01/2015 (H1N1)
A/Hawaii/51/2015 (H1N1)
A/swine/Togo/ONA32/2013 (H1N1)
A/Nicaragua/30238 01 TR2/2013 (H1N1)
A/swine/Alberta/SD0217/2017 (H1N2)
A/Boston/YGA 01185/2013 (H1N1)
A/Santa Cruz/111/2012 (H1N1)
A/swine/Kenya/1613/2011 (H1N1)
A/swine/Japan/KU-HY5/13 (H1N1)
A/swine/Yamagata/19/2010 (H1N1)
A/Maryland/NHRC0001/2009 (H1N1)
A/swine/Hong Kong/189/2010 (H1N1)
A/Uganda/MUWRP-093/2009 (H1N1)
A/swine/Taiwan/TD-2542/2010 (H1N1)
A/swine/Taiwan/TD-4119B2/2009 (H1N1)
A/swine/Nigeria/12VIR4047-09/2011 (H1N1)
A/Wisconsin/02/2010 (H1N1)
A/England/10740691/2011 (H1N1)
A/Oregon/30/2009 (H1N1)
A/Wisconsin/629-D01556/2009 (H1N1)
A/Singapore/DMS64/2009 (H1N1)
A/California/07/2009 (H1N1)
A/swine/Italy/85437/2009 (H1N1)
A/swine/Western Australia/AS-17-5267-0001/2017 (H1N1)
A/swine/Nagasaki/KU-FK5/16 (H1N1)
A/swine/Japan/KU-MD4/13 (H3N2)
A/swine/Chiba/1-4249/2017 (H3N2)
A/swine/Kagoshima/37-7194/2019 (H3N2)
A/swine/Kagoshima/KU-FK1/14 (H1N1)
A/swine/Japan/KU-YG5/13 (H1N1)
A/swine/Kagoshima/43-P84/2018 (H1N1)
A/swine/Nagasaki/DK-01/Tr/2019 (H3N2)
A/swine/Nagasaki/UEF-79/2018 (H3N2)
A/swine/Saga/KU-FK1/16 (H1N1)
A/swine/Niigata/4/2019 (H1N2)
A/swine/Taian/95/2017 (H1N1)
A/swine/Jiangsu/J005/2018 (H1N1)
A/swine/Heilongjiang/0110/2017 (H1N1)
A/swine/Henan/SN10/2018 (H1N1)
A/swine/Shandong/JM78/2017 (H1N1)
A/swine/Chiba/KU-FK3/16 (H1N2)
A/swine/Victoria/18-04095-0003/2018 (H1N1)
A/swine/Chachoengsao/NIAH117865-032/2017 (H3N2)
A/swine/Gunma/7-1/2018 (H1N2)
A(H1N1)pdm09 lineage
A/swine/Mexico/Mex52/2010 (H1N1)
A/swine/Mexico/Qro32/2010 (H1N1)
A/swine/Mexico/Mex51/2010 (H3N2)
A/swine/Hong Kong/1435/2009 (H1N2)
A/swine/Hubei/05/2009 (H1N2)
A/swine/Korea/1204/2009 (H1N2)
North American SIVs
A/Wuhan/359/1995 (H3N2)
A/England/42/1972 (H3N2)
A/Victoria/3/1975 (H3N2)
A/Texas/1/1977 (H3N2)
A/Bangkok/1/1979 (H3N2)
A/Sichuan/2/1987 (H3N2)
A/Beijing/32/1992 (H3N2)
A/Beijing/353/1989 (H3N2)
A/swine/Saskatchewan/02903/2009 (H3N2)
A/Sydney/5/1997 (H3N2)
A/New York/179/1999 (H3N2)
A/Fujian/411/2002 (H3N2)
A/Wisconsin/67/2005 (H3N2)
A/Brisbane/10/2007 (H3N2)
A/Perth/16/2009 (H3N2)
A/Texas/50/2012 (H3N2)
A/Victoria/361/2011 (H3N2)
A/Hong Kong/4801/2014 (H3N2)
A/Switzerland/9715293/2013 (H3N2)
A/swine/Zambia/51/2018 (H3N2)
A/Iowa/01/2017 (H3N2)
A/Congo/735/2018 (H3N2)
A/Zambia/0002/2015 (H3N2)
A/Zambia/0102/2015 (H3N2)
A/Singapore/INFIMH-16-0019/2016 (H3N2)
A/Switzerland/8060/2017 (H3N2)
A/South Africa/R7793/2017 (H3N2)
A/South Australia/34/2019 (H3N2)
A/Tanzania/3112/2019 (H3N2)
A/Kenya/130/2019 (H3N2)
A/Mozambique/300/2019 (H3N2)
A/Cote DIvoire/1541/2019 (H3N2)
A/Mali/176/2019 (H3N2)
A/Congo/010/2020 (H3N2)
A/Nigeria/3980/2019 (H3N2)
A/Togo/1740/2019 (H3N2)
Human seasonal H3N2 lineage
Avian IAVs
Eurasian SIVs
0.02


PA
A/swine/Zambia/301/2018 (H1N1)
A/swine/Zambia/310/2018 (H1N1)
A/swine/Zambia/282/2018 (H1N1)
A/swine/Zambia/280/2018 (H1N1)
A/swine/Zambia/278/2018 (H1N1)
A/swine/Zambia/277/2018 (H1N1)
A/swine/Zambia/264/2018 (H1N1)
A/New Jersey/11/2018 (H1N1)
A/swine/Zambia/595/2018 (H1N1)
A/Texas/93/2018 (H1N1)
A/swine/France/53-180028-3/2018 (H1N1)
A/swine/Queensland/P18-01063-0009-01/2018 (H1N1)
A/Idaho/37/2015 (H1N1)
A/Alabama/12/2016 (H1N1)
A/TOKYO/EH3/2016 (H1N1)
A/Utah/09/2016 (H1N1)
A/Zambia/0003/2015 (H1N1)
A/swine/Aichi/KU-MI1-1/2016 (H1N1)
A/Hokkaido/13H069/2014 (H1N1)
A/Gainesville/01/2015 (H1N1)
A/Hawaii/51/2015 (H1N1)
A/swine/Kenya/1613/2011 (H1N1)
A/Santa Cruz/111/2012 (H1N1)
A/Boston/YGA 01185/2013 (H1N1)
A/swine/Togo/ONA32/2013 (H1N1)
A/Nicaragua/30238 01 TR2/2013 (H1N1)
A/swine/Alberta/SD0217/2017 (H1N2)
A/England/10740691/2011 (H1N1)
A/swine/Italy/85437/2009 (H1N1)
A/Cambodia/NHRCC00006/2009 (H1N1)
A/Scotland/Glasgow 413608/2009 (H1N1)
A/swine/Wuerusreuth/IDT10990/2009 (H3N1)
A/Montana/22/2009 (H1N1)
A/California/07/2009 (H1N1)
A/swine/Taiwan/TD-2542/2010 (H1N1)
A/swine/Taiwan/TD-4119B2/2009 (H1N1)
A/swine/Mexico/SG1444/2011 (H3N2)
A/Tallinn/INS373/2010 (H1N1)
A/swine/Nigeria/12VIR4047-09/2011 (H1N1)
A/Wisconsin/02/2010 (H1N1)
A/Hong Kong/H090-770-V22/2009 (H1N1)
A/swine/Chiba/KU-FK3/16 (H1N2)
A/Singapore/DMS78/2010 (H1N1)
A/swine/Japan/KU-YG5/13 (H1N1)
A/swine/Japan/KU-HY5/13(H1N1)
A/swine/Manitoba/SG1438/2011 (H3N2)
A/Minnesota/24/2009 (H1N1)
A/swine/Iowa/A01202443/2011 (H1N2)
A/swine/California/A00968949/2014 (H3N2)
A/swine/Iowa/17TOSU3113/2017 (H1N1)
A/swine/Nagasaki/KU-FK5/16 (H1N1)
A/swine/Japan/KU-MD4/13 (H3N2)
A/swine/Kagoshima/43-P84/2018 (H1N1)
A/swine/Kagoshima/KU-FK1/14 (H1N1)
A/swine/Chiba/1-4249/2017 (H3N2)
A/swine/Kagoshima/37-7194/2019 (H3N2)
A/swine/Nagasaki/DK-01/Tr/2019 (H3N2)
A/swine/Nagasaki/UEF-79/2018 (H3N2)
A/swine/Manitoba/DM 13/2017 (H1N1)
A/swine/Irkutsk/85/2018 (H3N2)
A/swine/Taian/95/2017 (H1N1)
A/swine/Chachoengsao/NIAH117865-032/2017 (H3N2)
A/swine/Western Australia/AS-17-5267-0001/2017 (H1N1)
A/swine/Jiangsu/J005/2018 (H1N1)
A/swine/Heilongjiang/0110/2017 (H1N1)
A/swine/Shandong/JM78/2017 (H1N1)
A/swine/Saga/KU-FK1/16 (H1N1)
A/swine/Gunma/7-1/2018 (H1N2)
A/swine/Niigata/4/2019 (H1N2)
A(H1N1)pdm09 lineage
North American SIVs
A/swine/Henan/Y1/2009 (H9N2)
Avian IAVs
Eurasian SIVs
A/Bangkok/1/1979 (H3N2)
A/Texas/1/1977 (H3N2)
A/England/42/1972 (H3N2)
A/Victoria/3/1975 (H3N2)
A/Beijing/32/1992 (H3N2)
A/Wuhan/359/1995 (H3N2)
A/Beijing/353/1989 (H3N2)
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A/Congo/735/2018 (H3N2)
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A/Nigeria/3980/2019 (H3N2)
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A/Mozambique/300/2019 (H3N2)
A/Tanzania/3112/2019 (H3N2)
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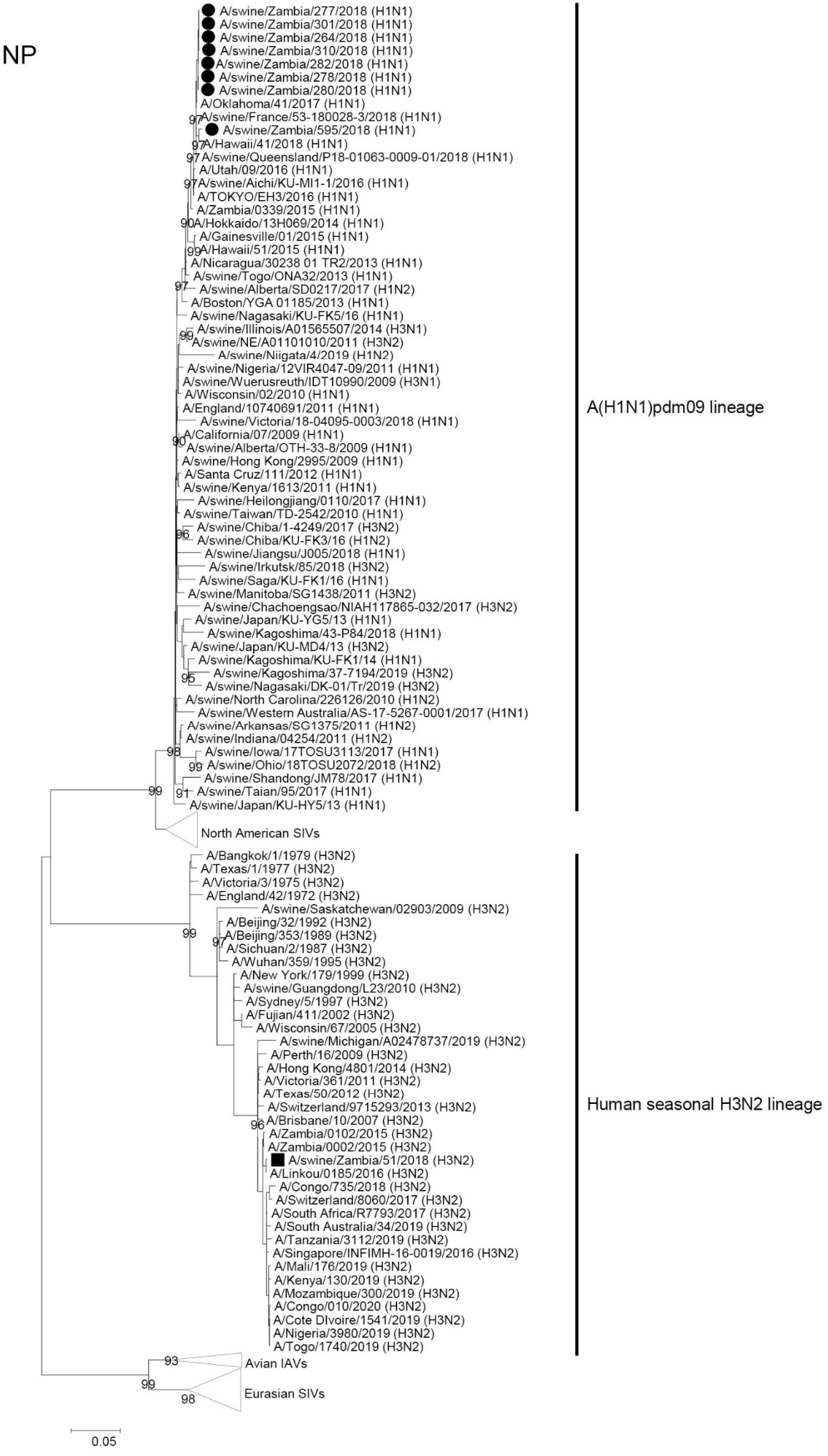
A/South Australia/34/2019 (H3N2)
Human seasonal H3N2 lineage
0.05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A/Gainesville/01/2015 (H1N1)
A/Hawaii/51/2015 (H1N1)
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A/swine/Saga/KU-FK1/16 (H1N1)
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A/Wuhan/359/1995 (H3N2)
A/New York/179/1999 (H3N2)
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A/Sydney/5/1997 (H3N2)
A/Fujian/411/2002 (H3N2)
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A/Brisbane/10/2007 (H3N2)
A/Zambia/0102/2015 (H3N2)
A/Zambia/0002/2015 (H3N2)
A/swine/Zambia/51/2018 (H3N2)
A/Linkou/0185/2016 (H3N2)
A/Congo/735/2018 (H3N2)
A/Switzerland/8060/2017 (H3N2)
A/South Africa/R7793/2017 (H3N2)
A/South Australia/34/2019 (H3N2)
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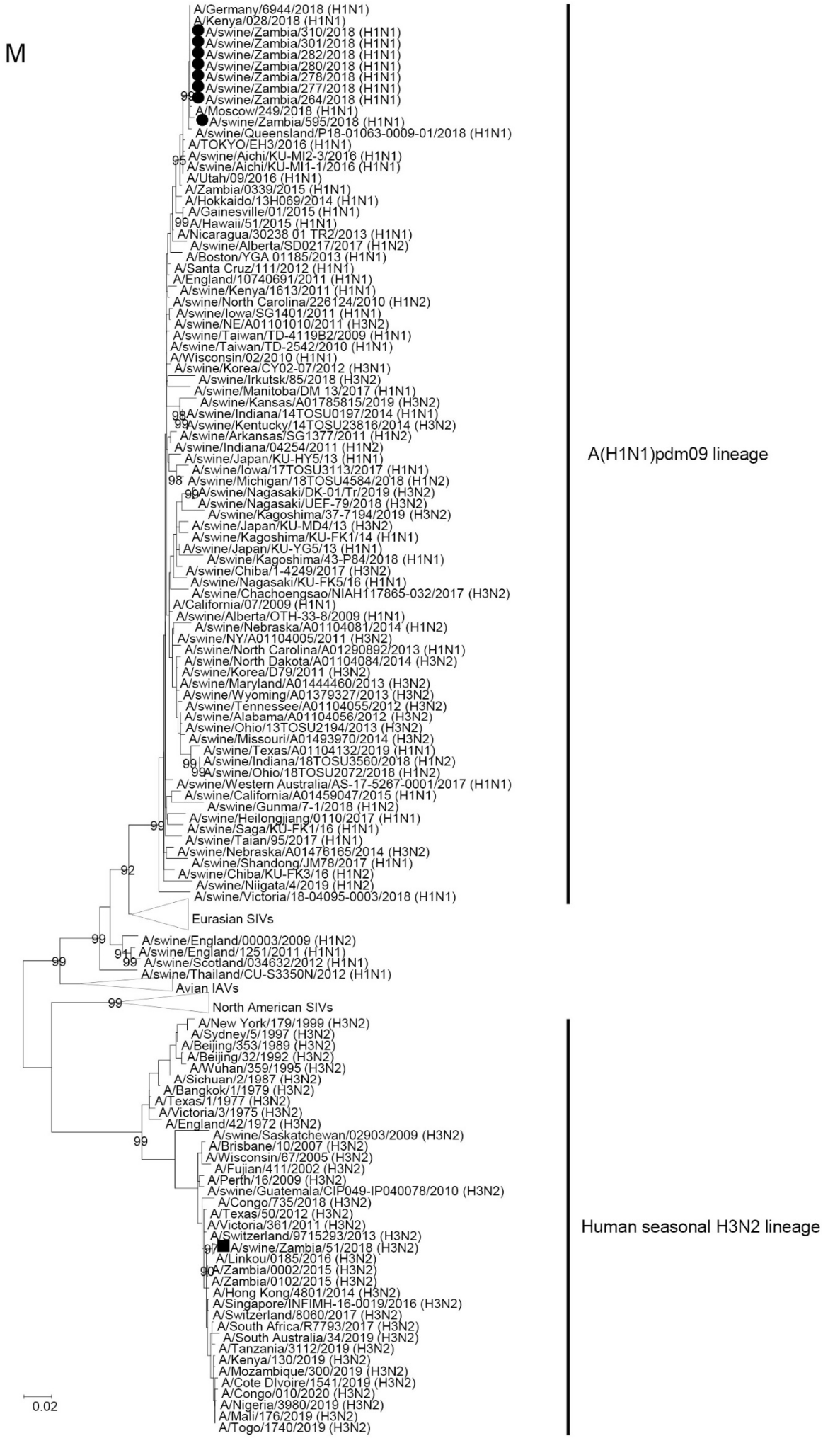
A/Mali/176/2019 (H3N2)
A/Kenya/130/2019 (H3N2)
A/Mozambique/300/2019 (H3N2)
A/Congo/010/2020 (H3N2)
A/Cote DIvoire/1541/2019 (H3N2)
A/Nigeria/3980/2019 (H3N2)
A/Togo/1740/2019 (H3N2)
Avian IAVs
Eurasian SIVs
0.05
A(H1N1)pdm09 lineage
Human seasonal H3N2 lineage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A/swine/Zambia/277/2018 (H1N1)
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A/Zambia/0339/2015 (H1N1)
A/Hokkaido/13H069/2014 (H1N1)
A/Gainesville/01/2015 (H1N1)
A/Hawaii/51/2015 (H1N1)
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A/Santa Cruz/111/2012 (H1N1)
A/England/10740691/2011 (H1N1)
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A/swine/Japan/KU-YG5/13 (H1N1)
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A/swine/Taian/95/2017 (H1N1)
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A/swine/Shandong/JM78/2017 (H1N1)
A/swine/Chiba/KU-FK3/16 (H1N2)
A/swine/Niigata/4/2019 (H1N2)
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Eurasian SIVs
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A/swine/Thailand/CU-S3350N/2012 (H1N1)
Avian IAVs
North American SIVs
A/New York/179/1999 (H3N2)
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A/Zambia/0002/2015 (H3N2)
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A/Cote DIvoire/1541/2019 (H3N2)
A/Congo/010/2020 (H3N2)
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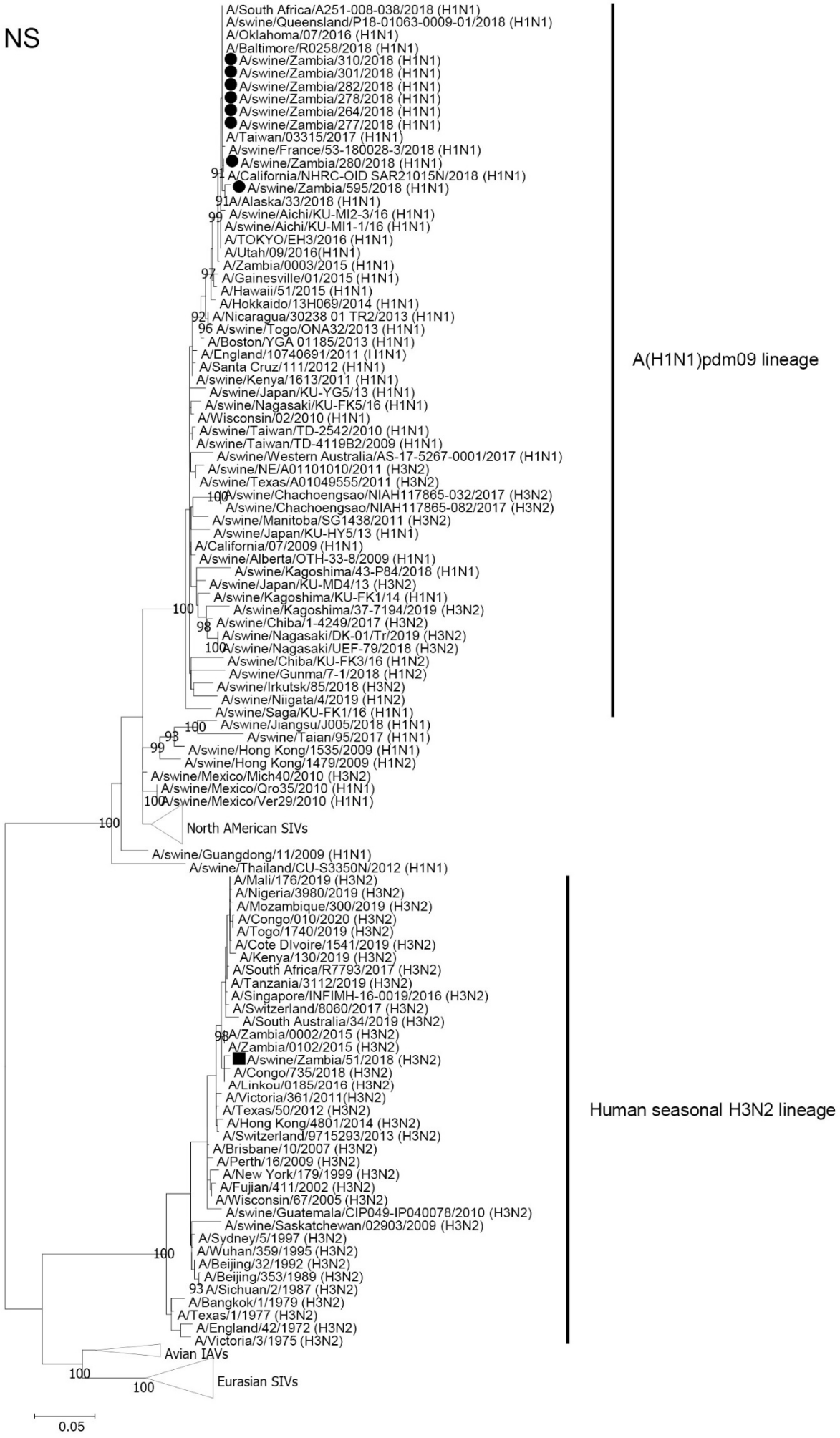
A(H1N1)pdm09 lineage
Human seasonal H3N2 lineage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91
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97
A/Gainesville/01/2015 (H1N1)
A/Hawaii/51/2015 (H1N1)
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92
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96
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A/Santa Cruz/111/2012 (H1N1)
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A/swine/Japan/KU-YG5/13 (H1N1)
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A/Wisconsin/02/2010 (H1N1)
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93
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99
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A/Mozambique/300/2019 (H3N2)
A/Congo/010/2020 (H3N2)
A/Togo/1740/2019 (H3N2)
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A/South Australia/34/2019 (H3N2)
98
A/Zambia/0002/2015 (H3N2)
A/Zambia/0102/2015 (H3N2)
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A/Linkou/0185/2016 (H3N2)
A/Victoria/361/2011(H3N2)
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93
A/Sichuan/2/1987 (H3N2)
A/Bangkok/1/1979 (H3N2)
A/Texas/1/1977 (H3N2)
A/England/42/1972 (H3N2)
A/Victoria/3/1975 (H3N2)
Avian IAVs
100
100
Eurasian SIVs
0.05
A(H1N1)pdm09 lineage
Human seasonal H3N2 lineage

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.