Short Communication

Outbreaks of Clade 2.3.4.4 H5N8 highly pathogenic avian influenza in 2018 in the northern regions of South Africa were unrelated to those of 2017

Running title: H5N8 HPAI viruses in northern South Africa, 2018

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SUMMARY

Asian-origin H5N8 highly pathogenic avian influenza (HPAI) viruses of the H5 Goose/Guangdong/96 lineage, clade 2.3.4.4 group B reached South Africa by June 2017. By the end of that year, 5.4 million layers and broiler chickens died or were culled, with total losses in the poultry industry estimated at US$ 140 million, and thousands of exotic birds in zoological collections, endangered endemic species and backyard poultry and pet birds also perished. The 2017 H5N8 HPAI outbreaks were characterised by two distinct spatial clusters, each associated with specific reassortant viral genotypes. Genotypes 1, 2, 3 and 5 were restricted to the northern regions, spanning the provinces of Limpopo, Gauteng, North West, Mpumalanga, KwaZulu-Natal and Free State. The second, much larger cluster of outbreaks was in the south, in the Western and Eastern Cape provinces, where in 2017 and 2018 outbreaks were caused solely by genotype 4.

The last confirmed case of H5N8 HPAI in the northern region in 2017 was in early October, and the viruses seemed to disappear over the summer. However, starting in mid-February 2018, H5N8 HPAI outbreaks resurged in the north. Viruses from two of the eight outbreaks were sequenced, one from

an outbreak in quails (*Coturnix japonica*) in the North West Province, and another from commercial pullets in the Gauteng province. Phylogenetic analysis identified the viruses as a distinct sixth genotype that was most likely a new introduction to South Africa in early 2018.

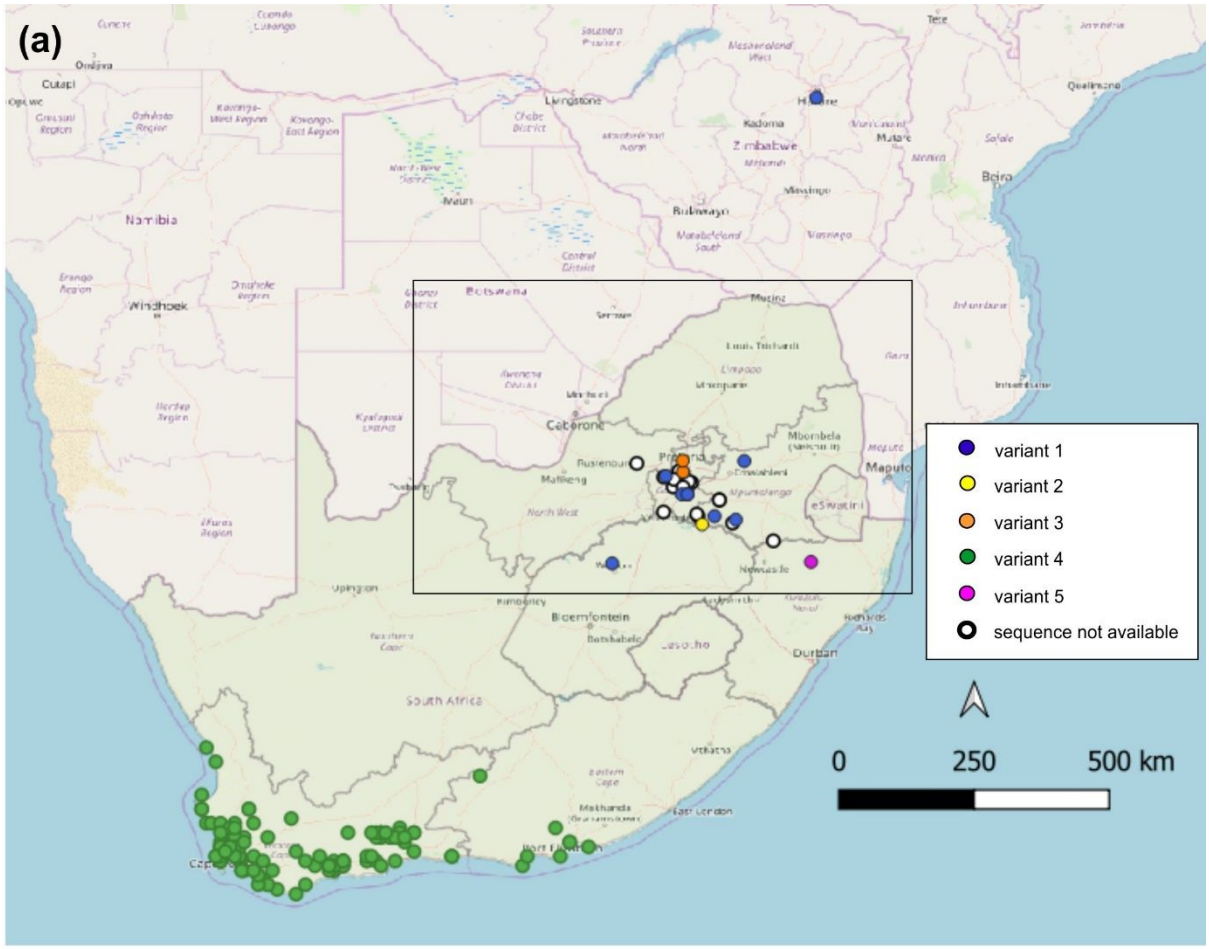
Key words: Highly pathogenic avian influenza, H5N8, quail, poultry, wild birds

1. INTRODUCTION

Asian-origin H5N8 highly pathogenic avian influenza (HPAI) viruses of the H5 Goose/Guangdong/96 (Gs/GD) lineage, clade 2.3.4.4 group B (Lee et al., 2016), reached the African continent in late 2016 with the seasonal migration of wild aquatic birds, mainly Palearctic dabbling ducks. The West African region subsequently became an important source of viruses that spread to East and Southern Africa with the regional movements of birds, likely Afrotropical ducks (Khomenko et al., 2018; Fusaro et al., 2019).

H5N8 HPAI reached Zimbabwe by May 2017, and one month later the first South African case was detected. The presence in the environment of extremely infectious Gs/GD H5N8 HPAI viruses with a wide host range was catastrophic. By the end of 2017 5.4 million layers and broiler chickens died or were culled, with total losses in the poultry industry estimated at US$ 140 million. Thousands of exotic birds in zoological collections, endangered endemic species and backyard poultry and pet birds also perished (Abolnik et al., 2018).

The 2017 South African H5N8 HPAI outbreaks were characterised by two genetically distinct spatial clusters, the first in the northern provinces of Limpopo, Gauteng, North West, Mpumalanga, northern KwaZulu-Natal and the northern Free State. The second, much larger one was in the south, in the Western and Eastern Cape provinces (Fig. 1(a)). Genomic comparison of more than 40 isolates collected across the country identified five distinct reassortant genotypes (previously referred to as "variants"). Genotypes 1, 2, 3 and 5 were only found in the northern regions of the country, whereas genotype 4 was restricted to the south (Fig. 1(b); Abolnik et al., 2018). The last confirmed case of H5N8 HPAI in the northern region in 2017 was a backyard swan sampled in the Gauteng province on 3 October (P. Geertsma, personal communication). No further outbreaks were detected in the north for the remainder of 2017, even though a new epidemic wave of genotype 4 had begun in coastal birds in the southern Cape region in late December, continuing into 2018 (Abolnik et al., 2018).



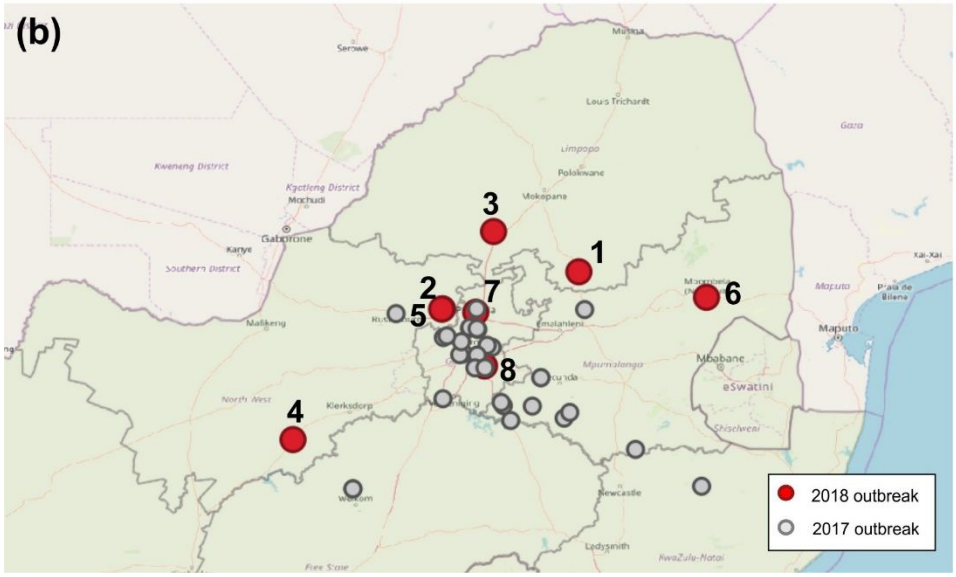


Figure 1. Clade 2.3.4.4 HPAI H5N8 outbreaks in Southern Africa in 2017 and in the southernmost regions in 2018 (a); and in the northern regions of South Africa in 2018 (b) (see Table 1).

Table 1. H5N8 highly pathogenic avian influenza outbreaks in the northern regions of South Africa in 2018

Fig. 1(b) No.	Date	Location*	Species	Epidemiological information
1	11 Feb	Groblersdal (Elias Motsoaledi, LIM)	Backyard mixed flock#	8/130 deaths; citrus farm, small number of fowls kept near farmhouse for own consumption
2	11 Mar	Brits (Madibeng, NW)	Hobby swans#	8/82 deaths
3	12 Mar	Modimolle (Modimolle, LIM)	Backyard hobby ducks#	11/85 deaths
4	12 Mar	Wolmaransstad (Maquassie Hills, NW)	Chickens	50/4500 deaths; semi-commercial farm
5	6 Apr	Brits (Madibeng, NW)	Domestic ducks# and quails (*Coturnix japonica*)	534/36,512 deaths Outbreak 1: quails hatched, grown and slaughtered on farm; A/quail/South Africa/AI5930/2018 (H5N8)† Outbreak 2: wild ducks kept domestically for ornamental purposes, less than 10 km from the quail farm
6	9 Apr	Nelspruit (City of Mbombela, MPU)	Domestic (pet) swan#	1/40 deaths
7	25 May	Pretoria (City of Tshwane, GAU)	Wild Sacred ibis (*Threskiornis aethiopicus*)	Single bird, bird weak and unable to fly
8	6 Jun	Germiston (Ekurhuleni, GAU)	Chickens	129/30,241 deaths of 15-week commercial pullets; A/chicken/South Africa/499723/2018 (H5N8)†

*Closest town/ city (district or municipality and province: LIM- Limpopo Province, NW-North West Province, MPU- Mpumalanga Province, GAU-Gauteng Province)

#Species not identified

†Full genomes sequenced in this study

Then, in mid-February 2018, the cause of sudden mortalities in a flock of mixed backyard fowl near Groblersdal in the Limpopo province was diagnosed as H5N8 HPAI. Over the following weeks, cases were confirmed in the North West, Mpumalanga and Gauteng Provinces, mainly in free-living backyard poultry, ornamental or wild birds (Table 1; Fig. 1(b)). The fifth case, in early April, involved the deaths of 534 out of a flock of 36,000 quails (*Coturnix japonica*) farmed for meat near Brits, North West Province. The eighth and final case of H5N8 HPAI in the northern region in 2018 was in early June, in a commercial pullet-rearing farm of 30,241 15-week old hens. The birds were kept in closed houses, less than one kilometre away from a site that broke in 2017. The veterinarian was alerted by the presence of indicative signs of H5N8 HPAI such as red shanks before significant mortalities could occur. The proximity of the site to a commercial layer operation where 385,000 H5N8-infected chickens were culled and disposed of a year prior raised concerns about the efficacy of the disposal method, i.e. the possibility that virus somehow leached into and survived in underground water, used for farming in the area. Alternatively, had a genotype/s detected in 2017 been maintained in unknown avian reservoir over the course of the summer? Or had southern genotype 4 finally spread northwards by bird or human movement? To answer these questions, the viral genomes from two of the cases in 2018, namely those that affected the quails and the last outbreak in commercial chickens were sequenced for phylogenetic comparison.

2. MATERIALS AND METHODS

Two total RNA extracts from tissue pools, prepared with a Quick-RNA miniprep kit (Zymo Research, Irvine, USA) were received from Deltamune Laboratories (Pty) Ltd (Pretoria) for analysis. The eight influenza A virus genome segments in each were amplified using the Superscript III One-Step RT PCR system with Platinum Taq High Fidelity (Invitrogen, Carlsbad, CA, USA) with oligonucleotide primer pair MBTuni-12/ MBTuni-13 according to the method described by Zhou et al., (2009) in a Veriti 96-well thermal cycler (Thermo Fisher Scientific, Waltham, MA, USA). PCR products were shipped to the

Central Analytical Facility at Stellenbosch University for Ion Torrent sequencing. Briefly, barcoded libraries were prepared with a NEXTflex™ DNA sequencing kit (PerkinElmer, Waltham, MA, USA). Libraries were purified and assessed for yield and fragment size distribution on the LabChip® GXII Touch (PerkinElmer, Waltham, MA, USA) according to the recommended protocol. After dilution to a target concentration of 60pM, the barcoded cDNA libraries were combined in equimolar amounts for sequencing template preparation using the Ion PI™ HiQ™ Chef Kit (Thermo Fisher Scientific). Enriched ion sphere particles loaded onto an Ion PI™ v3 Chip (Thermo Fisher Scientific) were subjected to massively parallel sequencing on the Ion Proton™ System. Flow space calibration and basecaller analysis were performed using standard analysis parameters in the Torrent Suite Version 5.12 Software.

In the CLC Genomics Workbench v5.2.1, consensus sequences for each genome segment were assembled against references KY621531-KY621538. The BLAST algorithm was used to retrieve the top 100 hits from the NCBI GenBank database (https://www.ncbi.nlm.nih.gov/nuccore) with additional African sequences retrieved from the GISAID EpiFLU database (http://www.gisaid.org). Multiple nucleotide sequence alignments were prepared in BioEdit v7.2.5 (Hall., 1999). A phylogenetic tree was constructed for each segment using the Maximum Likelihood statistical method in MEGA v5.5.2 (Tamura et al., 2011), with 1000 bootstrap replicates. The Tamura-Nei nucleotide substitution model was used, specifying a uniform rate among sites. Trees were inferred with a Nearest-Neighbour-Interchange method, with a very strong brand swap filter. Sequences generated in this study were deposited in GenBank under accession numbers MN252519-MN252534. The viral sequences for the remaining outbreaks in Table 1 had not been released by the national reference laboratory at the time of writing.

3. RESULTS

Complete genome sequences were assembled from 537,834,265 reads (mean length of 80 bp) and 795,178,218 reads (mean length of 113 bp) for A/quail/South Africa/AI5930/2018 and A/chicken/South Africa/499723/2018, respectively. BLAST analysis of the hemagglutinin (HA) and neuraminidase (NA) protein-coding segments classified both viruses within Gs/GD clade 2.3.4.4 H5N8 group B based on sequence identity to known strains, and the translated amino acid motif of PLREKRRKRGLF at the HA_0 cleavage site confirmed their highly pathogenic status.

Phylogenetic trees of the eight genomic segments were used to determine the evolutionary relationships of two viruses from 2018 outbreaks with other South African and international strains. The two viruses were 99.53 to 100 % identical at the nucleotide sequence level across all eight genome segments and clustered together in all phylograms. AI5930/18 and 499723/18 were distinct from but shared most recent common ancestors (RCAs) with strains detected in South Africa and Zimbabwe in 2017 in segment 2 (polymerase B1 (PB1) PB1 and PB1-F2 proteins; Fig. 2), segment 3 (polymerase A (PA) and PA-X proteins; Fig. 3), segment 4 (HA; Fig. 4), segment 5 (NA; Fig. 5) and segment 7 (matrix 1 (M1) + M2e; Fig. 6). Closer phylogenetic relationships were evident with the South African 2017 northern cluster viruses compared to the southern cluster viruses.

In contrast, the RCAs of AI5930/18 and 499723/18 in segment 1 (polymerase B1 (PB2) protein; Fig. 7), segment 5 (nucleoprotein (NP) gene, Fig. 8) and segment 8 (non-structural (NS) 1 and NS2 proteins; Fig. 9) were strains detected in Cameroon, Democratic Republic of Congo, Uganda and Egypt in 2017. Therefore, based on their phylogenetic characteristics, H5N8 HPAI viruses AI5930/18 and 499723/18 are reassortants possessing a gene constellation that has never been detected before, and are therefore classified as a new genotype, number six.

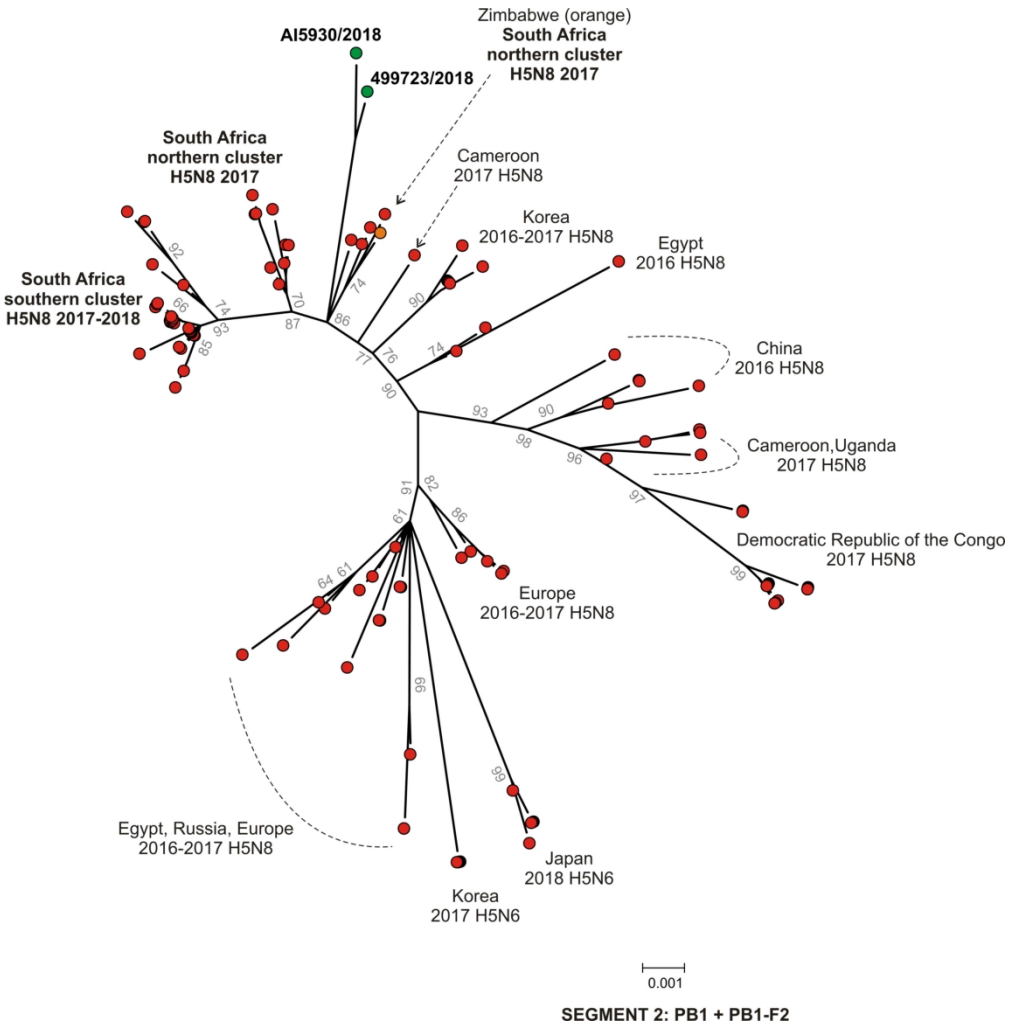


Figure 2. Radial Maximum Likelihood phylogenetic tree of segment 2 (PB1 + PB1-F2), bootstrap values >60 are shown. Viruses sequenced in this study are in green, other H5N8 HPAI viruses are in red or orange.

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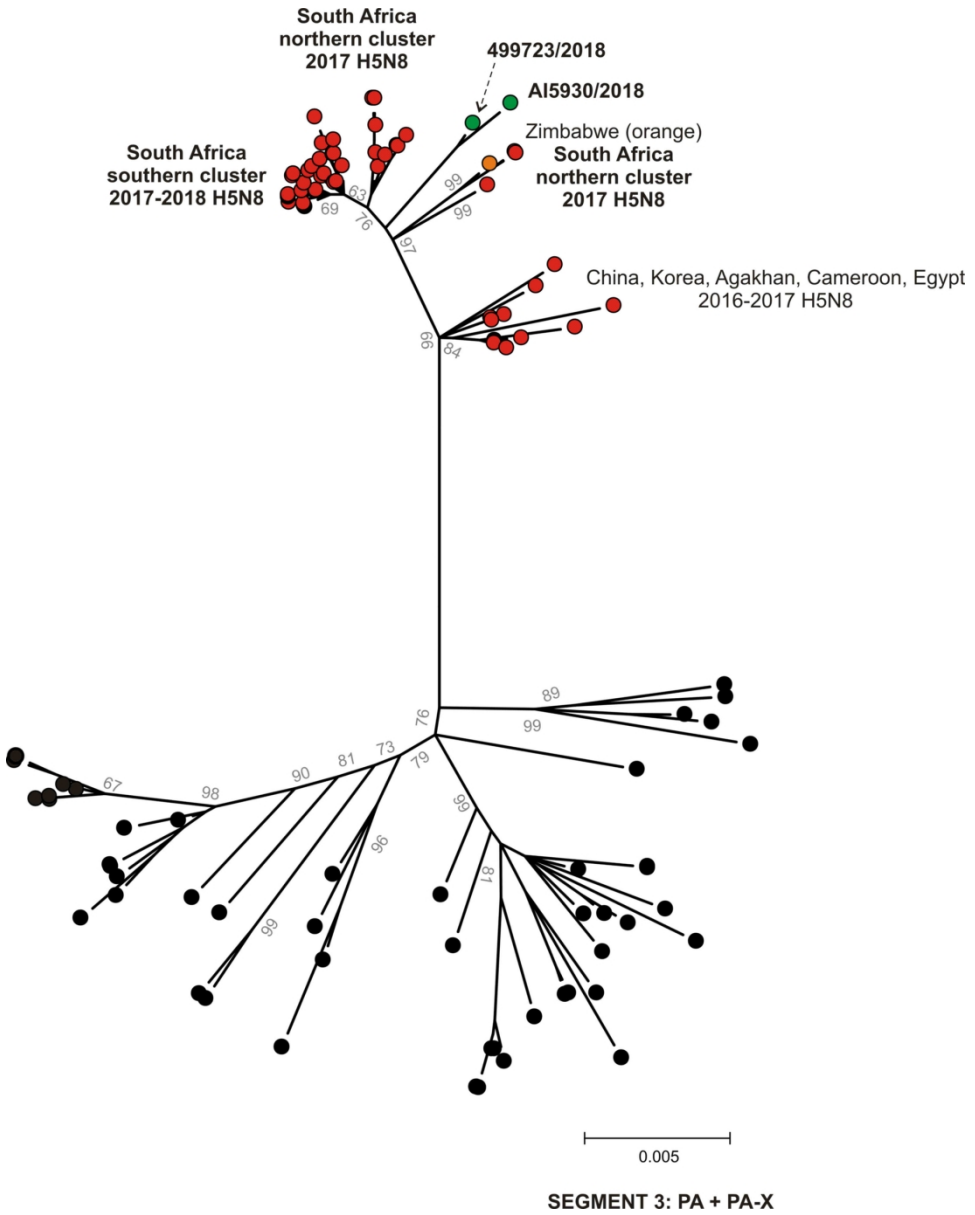

Figure 3. Radial Maximum Likelihood phylogenetic tree of segment 3 (PA + PA-X), bootstrap values >60 are shown. Viruses sequenced in this study are in green, other H5N8 HPAI viruses are in red or orange with other low pathogenic subtypes in black.

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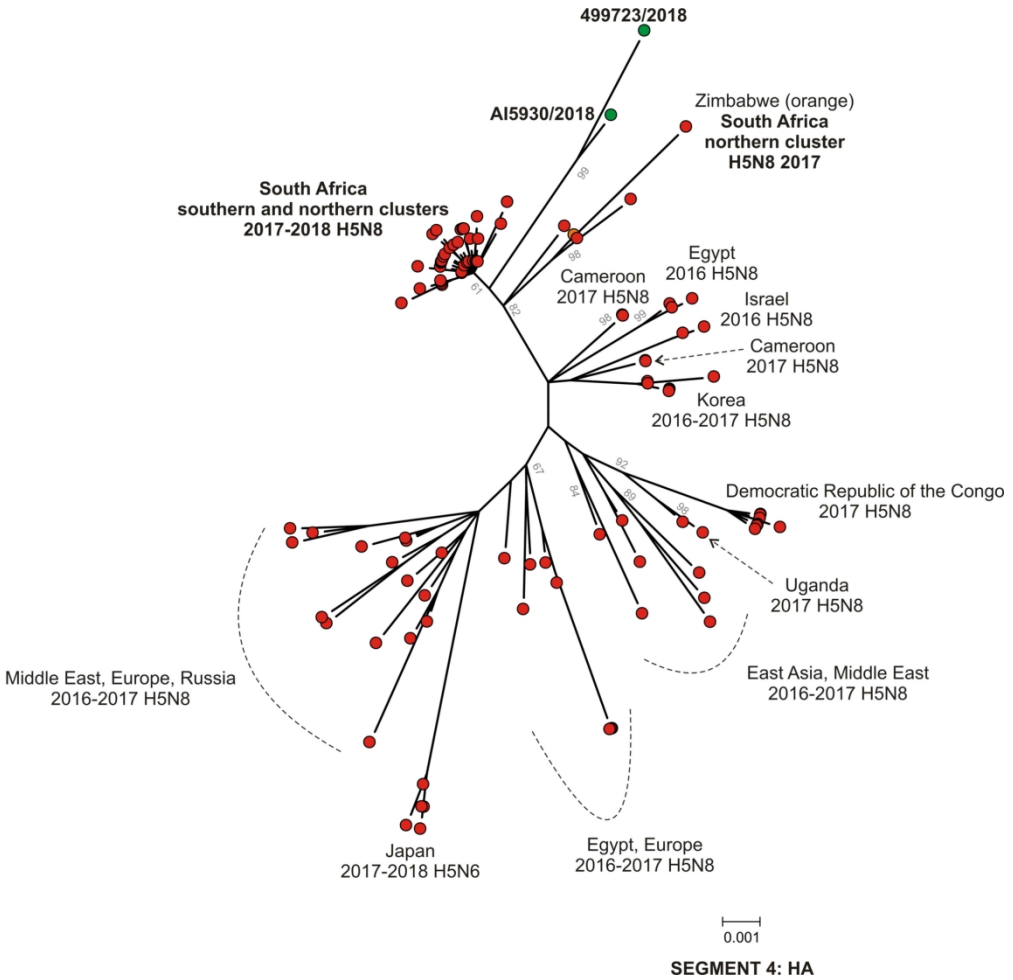

Figure 4. Radial Maximum Likelihood phylogenetic tree of segment 4 (HA), bootstrap values >60 are shown. Viruses sequenced in this study are in green, other H5N8 HPAI viruses are in red or orange.

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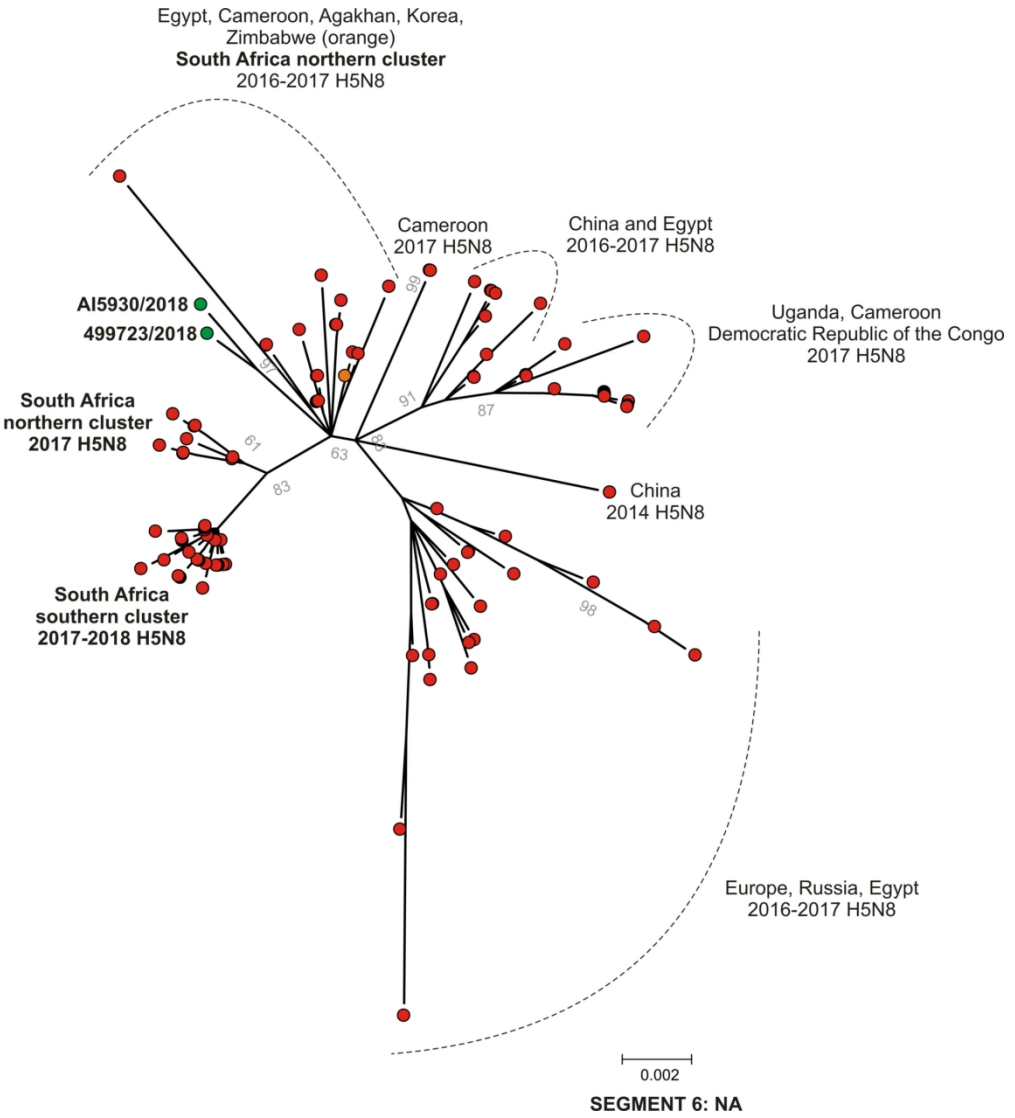

Figure 5. Radial Maximum Likelihood phylogenetic tree of segment 6 (NA), bootstrap values >60 are shown. Viruses sequenced in this study are in green, other H5N8 HPAI viruses are in red or orange.

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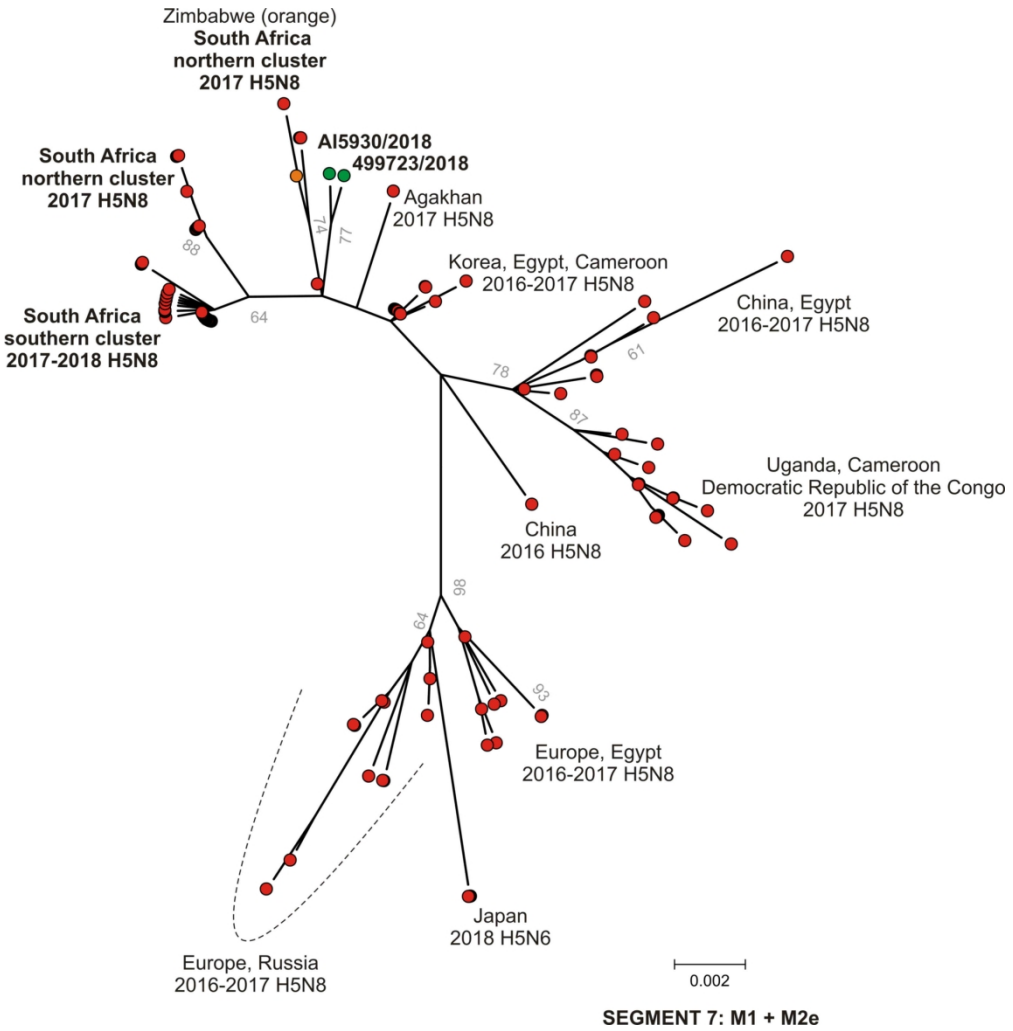

Figure 6. Radial Maximum Likelihood phylogenetic tree of segment 7 (M1 + M2e), bootstrap values >60 are shown. Viruses sequenced in this study are in green, other H5N8 HPAI viruses are in red or orange.

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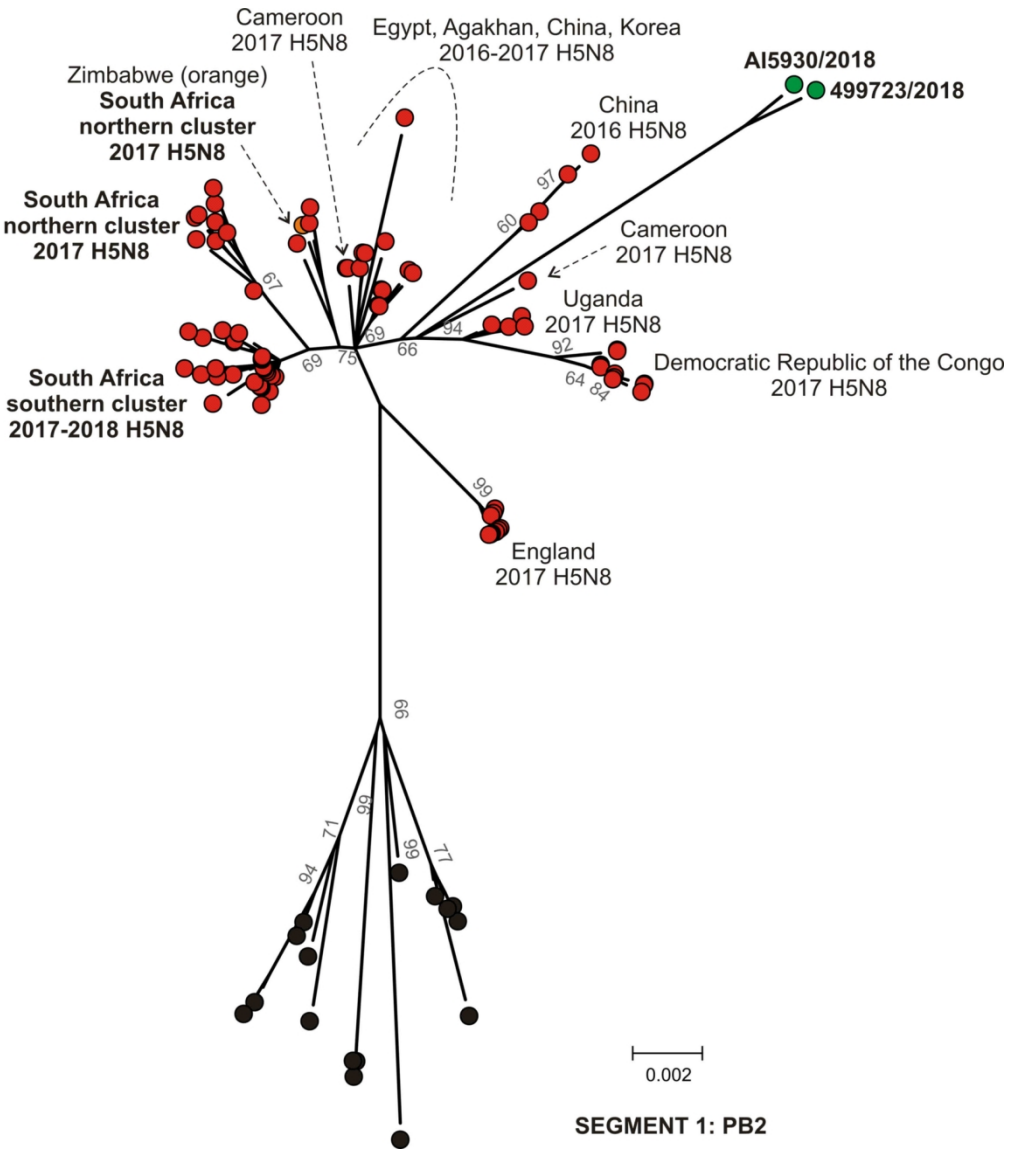

Figure 7. Radial Maximum Likelihood phylogenetic tree of segment 1 (PB2), bootstrap values >60 are shown. Viruses sequenced in this study are in green, other H5N8 HPAI viruses are in red or orange with other low pathogenic subtypes in black.

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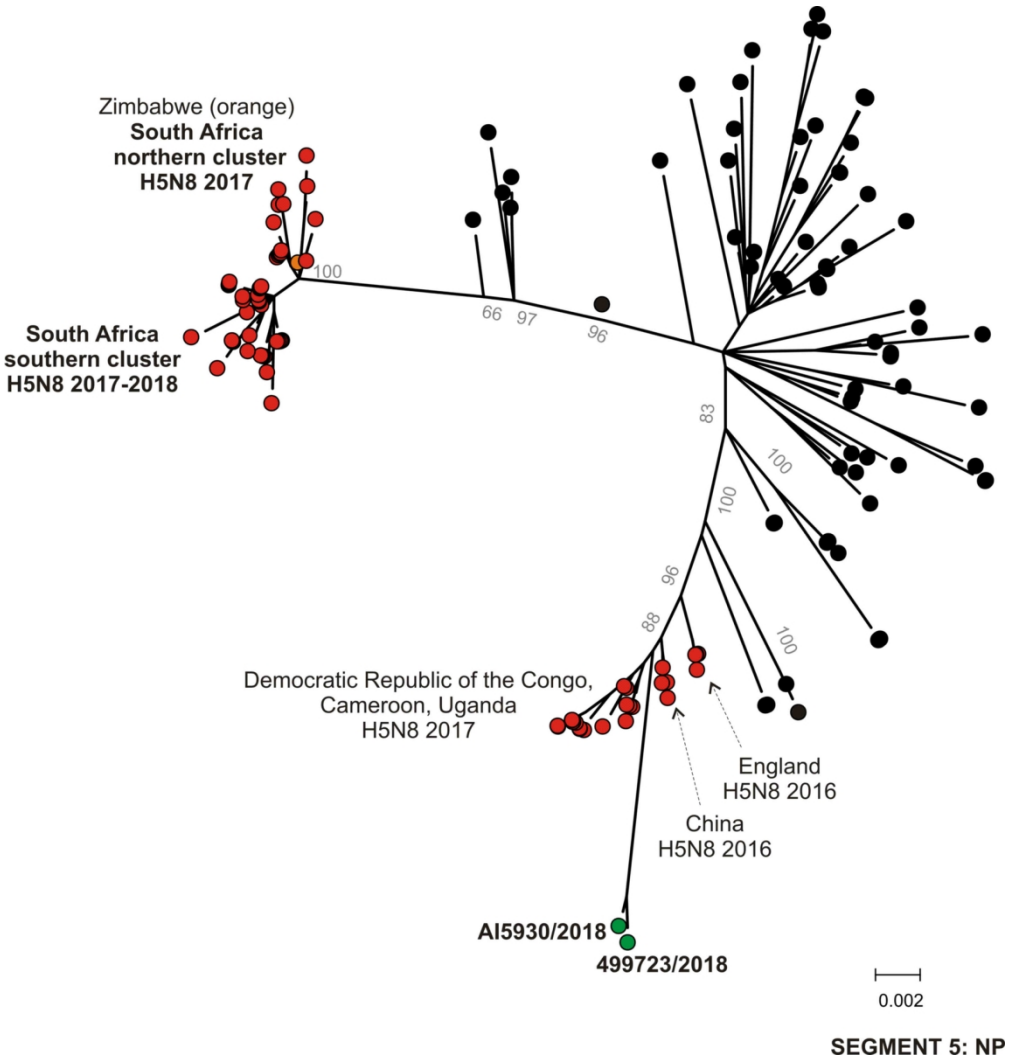

Figure 8. Radial Maximum Likelihood phylogenetic tree of segment 5 (NP), bootstrap values >60 are shown. Viruses sequenced in this study are in green, other H5N8 HPAI viruses are in red or orange with other low pathogenic subtypes in black.

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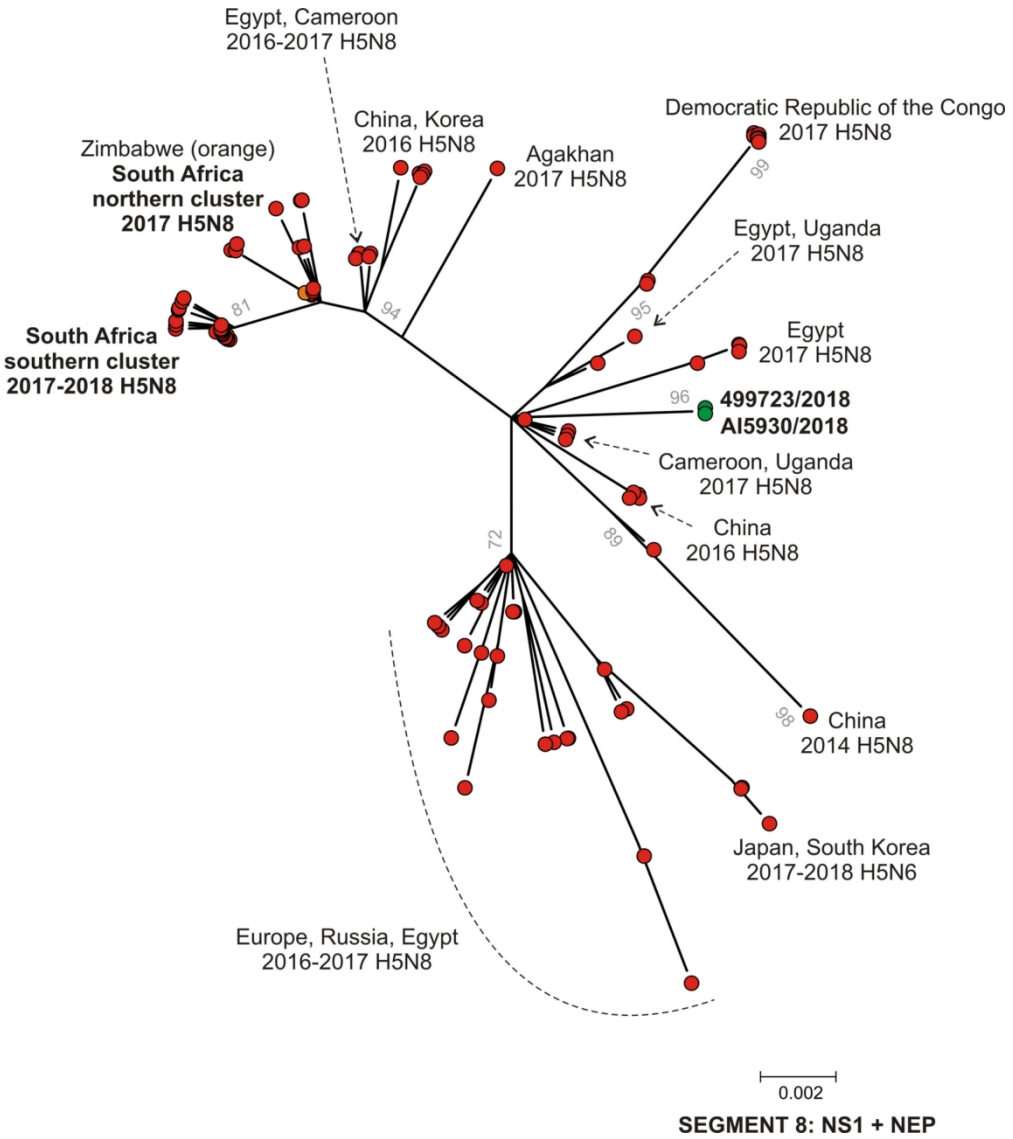

Figure 9. Radial Maximum Likelihood phylogenetic tree of segment 8 (NS1 + NS2), bootstrap values >60 are shown. Viruses sequenced in this study are in green, other H5N8 HPAI viruses are in red or orange.

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4. DISCUSSION

Two of the viruses from outbreaks in South Africa's northern regions in 2018 were identified here as a reassortant sixth genotype that was not detected before. It is clear that the reassortment event between progenitors of the prior southern African viruses and strains from West and Central Africa occurred on this continent. Moreover, the long branches, which separate the viruses from the progenitor, suggest that the strain had been circulating undetected for a while. Two possibilities for the origin of genotype 6 may be considered. Firstly, that it was introduced in 2017 around the same time as the other five, but went undetected or was present in an unknown reservoir in South Africa for almost a year. In Fig. 1(a), several viruses from 2017 were not assigned genotypes; however, most of these were sequenced by the national reference laboratory and their genomes are closely related to those reported previously (Abolnik et al., 2018; L Rotherham, personal communication). Amidst the heightened public awareness and intensified surveillance in wild birds, backyard and commercial poultry operations, with no sick or dead birds reported and no further spill overs to commercial operations in the latter half of 2017, all indications are that HPAI H5N8 disappeared from the northern regions of South Africa over the summer period. The likelihood that the virus was present in a local unknown reservoir that went undetected during 2017 is therefore low.

The second possibility is that genotype 6 was a new local introduction in early 2018. The phylogenetic relationships of genotype 6, especially in segments 1 (PB2), 5 (NP) and 8 (NS1 + NEP) that share no common ancestors with the other South African strains from 2017 (or coastal outbreaks of 2018), provide convincing evidence that this was the case. In the months prior to February 2018, H5N8 outbreaks continued to be reported in the West and Central African region, the former determined to be the key source of viruses to the southern African outbreaks in 2017 (Fusaro et al., 2019). In Niger, an outbreak in backyard birds in the Tillaberi region occurred in September 2017. Nigeria reported an outbreak in 52-week old commercial layers in Ogun State at the beginning of August, and in late January 2018 an outbreak in 5-week old broilers and cockerels in

Nasawara State, and the DRC reported an outbreak in ducklings in December 2017 the Ituri Province (OIE, 2019).

Between West-Central Africa and the northern South Africa, parts of Angola, Zambia, Tanzania, Mozambique, Malawi, Zimbabwe, Botswana and Namibia are rich in wetlands, rivers, dams and lakes that are habitats and resting sites for multitudes of Afrotropical ducks and many other intra-African migrant bird species. Except for Zimbabwe, none of these countries reported H5N8 HPAI outbreaks but it seems likely that at least some of their wild bird populations and even poultry were affected in 2017 and 2018. Sick birds and die-offs may have been unobserved, dismissed as Newcastle disease or other causes, or simply not reported. With none of the viruses from these regions available for analysis, it is difficult to pinpoint the source of the recursions of Gs/GD clade 2.3.4.4 H5N8 HPAI viruses into South Africa's northern regions in early 2018, or the species that brought them.

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ETHICS STATEMENT

The author confirms that ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No ethical approval was required as sampling of animals was not undertaken by the author.

CONFLICT OF INTEREST

The author has no conflict of interest to declare

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