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The Incursion and Spread of Highly Pathogenic Avian Influenza H5N8 Clade 2.3.4.4 Within South Africa

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SUMMARY. The report of a mass die-off of white-winged terns (*Chlidonias leucopterus*) along the shores of Lake Victoria in Uganda in January 2017 was a warning that highly pathogenic avian influenza (HPAI) H5N8 clade 2.3.4.4 had entered the avian populations of the African Rift Valley. In early June 2017, Zimbabwe reported an outbreak of the virus in commercial breeder chickens near Harare, and on June 19, 2017, the first case of HPAI H5N8 was confirmed in a broiler breeder operation near Villiers, Mpumalanga Province, South Africa, representing the first ever notifiable influenza in gallinaceous poultry in South Africa. Forty viruses were isolated from wild birds, backyard hobby fowl, zoo collections, commercial chickens, and commercial ostriches over the course of the outbreak and full genomes were sequenced and compared to determine the epidemiologic events in the introduction and spread of clade 2.3.4.4 H5N8 across the country. We found that multiple virus variants were involved in the primary outbreaks in the north-central regions of South Africa, but that a single variant affected the southernmost regions of the continent. By November 2017 only two of the nine provinces in South Africa remained unaffected, and the layer chicken industry in Western Cape Province was all but decimated. Two distinct variants, suggesting independent introductions, were responsible for the first two index cases and were not directly related to the virus involved in the Zimbabwe outbreak. The role of wild birds in the incursion and spread was demonstrated by shared recent common ancestors with H5N8 viruses from West Africa and earlier South African aquatic bird low pathogenicity avian influenza viruses. Improved wild bird surveillance will play a more critical role in the future as an early warning system.

RESUMEN. Incursión y propagación del virus de la influenza aviar altamente patógena H5N8 clado 2.3.4.4 en Sudáfrica.

El informe de una muerte masiva de fumareles aliblancos (*Chlidonias leucopterus*) a lo largo de las orillas del lago Victoria en Uganda en enero del 2017 fue una advertencia de que la influenza aviar de alta patogenicidad (HPAI) H5N8, clado 2.3.4.4 había ingresado en las poblaciones de aves del Valle del Rift Africano. A principios de junio del 2017, Zimbabwe reportó un brote del virus en pollos reproductores comerciales cerca de Harare, y el 19 de junio del 2017, el primer caso de influenza aviar de alta patogenicidad H5N8 se confirmó en una operación de pollos de engorde en la provincia de Mpumalanga cerca de Villiers, Sudáfrica, que representa el primer caso de influenza notificable en aves gallináceas en Sudáfrica. Se aislaron cuarenta virus de aves silvestres, aves de traspatio, colecciones de zoológicos, pollos comerciales y avestruces comerciales durante el transcurso del brote. Se secuenciaron los genomas completos y se compararon para determinar los eventos epidemiológicos en la introducción y propagación del subtipo H5N8 clado 2.3.4.4 a través del país. Se encontró que múltiples variantes del virus estaban involucradas en los brotes primarios en las regiones centro y norte de Sudáfrica, pero que una sola variante afectaba a las regiones más al sur del continente. En noviembre de 2017, solo dos de las nueve provincias de Sudáfrica permanecían sin afectarse y la industria de pollos en la Provincia de Cabo Occidental resultó casi diezmada. Dos variantes distintas, que sugieren introducciones independientes, fueron responsables de los dos primeros casos índices y no estuvieron directamente relacionados con el virus involucrado en el brote de Zimbabwe. El papel de las aves silvestres en la incursión y diseminación fue demostrado por los ancestros comunes compartidos con los virus H5N8 de África Occidental y los virus de la influenza aviar de baja patogenicidad de aves acuáticas de Sudáfrica detectados anteriormente. La mejora de la vigilancia de aves silvestres jugará un papel más crítico en el futuro como un sistema de alerta temprana.

Key words: clade 2.3.4.4, highly pathogenic avian influenza, H5N8, epidemiology

Abbreviations: cDNA = complementary DNA; DRC = Democratic Republic of the Congo; HA = hemagglutinin; HPAI = highly pathogenic avian influenza; LPAI = low pathogenicity avian influenza; M = matrix; NA = neuraminidase; NP = nucleoprotein; NS = nonstructural protein. PA = polymerase A; PB1 = polymerase basic 1; PB2 = polymerase basic 2; RT = reverse transcriptase; UP = University of Pretoria; WCPVL = Western Cape Provincial Veterinary Laboratory

The Gs/GD (goose/Guangdong) highly pathogenic H5 influenza virus (HPAI) lineage emerged in southern China in 1996, causing disease and deaths since then in wild birds, poultry, and humans in over 80 countries in Asia, Europe, Africa, and North America. The parental Gs/GD lineage has evolved into at least 10 genetically distinct virus clades and multiple subclades, undergoing reassortment with numerous low pathogenicity influenza viruses (LPAIs) and displaying varying degrees of virulence and host range specificity (9,11). Four main intercontinental waves are described: the first in 2005 involved clade 2.2 H5N1 viruses and their derivatives that spread in poultry throughout Asia, the Middle East, Europe, and

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West Africa, and caused human infections and deaths. The second intercontinental wave, in 2009 of clade 2.3.2.1c (H5N1), affected wild birds and poultry in Asia and Eastern Europe. The third intercontinental wave, in 2014, involved clade 2.3.4.4 (H5Nx) in wild birds and poultry in Asia and Western Europe, as well as clade 2.3.2.1c (H5N1) in Asia, Eastern Europe, the Middle East and West Africa. The fourth intercontinental wave, beginning in 2014, has been caused by clade 2.3.4.4 (H5Nx). Clade 2.3.4.4 reassortant H5N8, H5N5, H5N2, and H5N6 strains have affected Asia, North America, the Middle East, Europe, and Africa (16).

A wide range of avian species, wild and domestic, is susceptible to infection and efficient transmission of clade 2.3.4.4 H5Nx viruses. Generally, infected birds exhibit clinical disease, mortality, and pathologic features that are typical of HPAI virus infection, although with reduced virulence compared to the parental Gs/GD H5N1 virus (14). Reports of lethal as well as asymptomatic infections of wild birds with clade 2.3.4.4 viruses are accumulating, and the wide host range of clade 2.3.4.4 viruses and the ability to be carried asymptomatically might explain the successful recent globalization of this lineage (11). The involvement of wild birds in long-distance transmission of clade 2.3.4.4 Gs/Gd lineage H5 viruses during autumn migration, although uncertain with some previous intercontinental waves, is now irrefutable (16).

On the African continent, clade 2.3.4.4 H5N8 HPAI reached West and North Africa around the same time. Both geographic regions are overwintering sites for migrant wildfowl species from Europe and Asia (10). In Danbare Village, Kano State, Nigeria, on November 19, 2016, an outbreak in backyard guinea fowl, turkeys, and pigeons started after the owner had purchased birds from a local market (10). To the northwest, in the Tillabéri region of Niger, samples collected from mortalities in backyard poultry on January 23, 2017, were also confirmed as H5N8 HPAI positive (10,22), as was an outbreak on January 2, 2017, in a mixed-fowl backyard flock in the Extreme-Nord province of Cameroon. Almost simultaneously, dead Eurasian coots (*Fulica atra*) sampled on November 24, 2016, from a live bird market in the Damietta Governate, Egypt, tested positive for clade 2.3.4.4 virus (15). Mortalities among Eurasian wigeons (*Anas penelope*) and red-knobbed coots (*Fulica cristata*) in a wetland conservancy in Ghazala, Tunisia, November 24, 2016 (10), were confirmed as H5N8 HPAI positive, as were clinically healthy Eurasian teals (*Anas crecca*), sampled on December 8, 2016, in a live bird market, Port Said City, Egypt. The disease subsequently spread to Egyptian backyard poultry flocks (9).

In mid-December 2016, the deaths of 1,200 migratory white-winged terns (*Childonias leocopterus*) in an estimated population of 2,000, was reported along the shores of Lake Victoria in Lutembe Bay in Wakiso District and in Kachanga Village in Masaka District, Uganda. Clade 2.3.4.4 H5N8 HPAI was confirmed. In January 2017, a spillover of the virus from wild to domestic birds was also confirmed in Kachanga Village, and unconfirmed deaths among wild birds were reported in Kalangala District. These regions in Uganda have a high density of backyard chickens and lie on a major stopping point on the East African migratory bird flyway (5). On April 25, 2017, mass mortalities in domestic backyard ducks and chickens were first reported in the Democratic Republic of Congo's (DRC's) Ituri Territory, near Lake Albert, close to the Ugandan border (21).

Less than a month later, on May 17, 2017, clade 2.3.4.4 H5N8 HPAI broke out in a commercial broiler breeder operation in Harare, Zimbabwe. The outbreak site was located 200 m from a dam populated by a variety of wild duck species that was suspected as the source (K. Manyetu, pers. comm.). This is the farthest south that clade 2.3.4.4 had ever been recorded globally. Zimbabwe officially reported the outbreak to the World Organisation for Animal Health (OIE) on June 2, 2017 (13). Meanwhile, in South Africa contingency meetings were held, but little could prepare the country for what was to follow. On June 19, the first outbreak in South Africa was detected in a commercial broiler breeder flock near the town of Villiers, close to the Mpumalanga provincial border. The farm is situated on the banks of the Vaal River. A day later, mortalities at a commercial layer farm 35 km away near Standerton with no epidemiologic links, were diagnosed as HPAI H5N8 positive. In the following weeks and months the outbreaks spread to commercial and backyard poultry, hobby birds, exotic collections, commercial ostriches, and free-living wild birds, in most provinces of the country. In this study, we generated 40 full-genome sequences and used phylogenetic relationships and reassortment patterns to determine the epidemiologic events in the introduction of clade 2.3.4.4 H5N8 and its spread throughout the country during 2017.

MATERIALS AND METHODS

Sampling. Notifiable avian influenza serosurveillance in ostriches and backyard and commercial poultry was initiated in September 2005 throughout South Africa. Monitoring for avian influenza viruses in wild bird populations had been ongoing in some provinces, and in response to the incursion in June 2017, the number of sites for environmental sampling was increased to include high-risk water-body sites close to confirmed outbreaks in a number of provinces. Reports of increased morbidity and/or mortalities in wild bird populations were followed up and these sites were investigated.

Oropharyngeal and/or cloacal swabs or organ samples collected at necropsy by consulting poultry, clinical, and zoologic-garden veterinarians on detection of increased daily mortalities and suspect clinical symptoms in flocks were submitted to national laboratories for PCR screening and virus isolations. Samples analyzed for this study were tested at one of three laboratories, Deltamune (Pty) Ltd in Pretoria and Oudtshoorn, the Poultry Research Laboratory at the University of Pretoria (UP), or the Western Cape Provincial Veterinary Laboratory (WCPVL) in Stellenbosch. Samples submitted directly to the Agricultural Research Council–Onderstepoort Veterinary Laboratory in Pretoria are excluded from this study.

Swab samples collected from the cloaca or oropharynx were forwarded dry or in 50% phosphate-buffered saline/glycerol (v/v). Wild bird fecal and environmental swabs collected in the Gauteng region were placed into a viral transport medium supplied by UP. This transport medium consisted of brain-heart broth that contained, per liter, 100 mg of doxycycline (Mylan, Canonsburg, PA), 100 mg of enrofloxacin (Cipla, Mumbai, India), 1,000 mg of penicillin-streptomycin (Sigma-Aldrich, St. Louis, MO) and 10% glycerol (Merck KGaA, Darmstadt, Germany).

Reverse transcriptase(RT)–PCR screening. RNAs were extracted with the QIAcube HT® automated nucleic extraction system using the cador® Pathogen 96 QIAcube® HT jit (QIAGEN; at WCPVL), by TRIzol® method (Life Technologies) at UP, or by Magna Pure96 or Quick-RNA miniprep kit (Zymo Research) at Deltamune.

All laboratories used the H5 primer/probe set and method as described by Slomka *et al.* (18), in accordance with their in-house optimized standard operating procedures. At UP, the H5 primer/probe set was modified as follows (underlined) for improved detection (data not shown): modified SA H5 2.3.4.4 FOR: ACG TAT GAC TAC CCT CAG TAT TCA; modified H5 2.3.4.4 REV: AGA CCA GCC

ACC ATG ATT GC; modified H5 2.3.4.4 PRO (MGB-FAM): TCA ACA GTG GCG AGT TCC CTA GCA.

All H5 positive results were confirmed by the Agricultural Research Council–Onderstepoort Veterinary Research laboratory. All three laboratories additionally used the N8 probes and primers described by Hoffman *et al.* (8). RT-PCR results are not shown. Samples confirmed as H5N8 positive were processed for virus isolation at each of the three laboratories.

Virus isolation. All laboratories followed the OIE-prescribed method for the isolation of influenza A virus in 9- to 11-day-old embryonated specific-pathogen-free chicken eggs (12). To optimize the isolation of viruses from fecal swabs, the UP followed the method described by Tang *et al.* (20). RNA was extracted from alantoic fluids as described above and shipped to Stellenbosch University for Ion Torrent sequencing.

Ion Torrent sequencing. Total RNAs were assessed for RNA integrity scores and quantity on the BioAnalyzer 2100 using the RNA 6000 Nano Chip and reagents according to the procedure recommended by the manufacturer (Agilent Technologies, Waldbronn, Germany). The Ion Total RNA-Seq Kit v2 (Thermo Fisher Scientific, Waltham, MA) was used to convert expressed RNA transcripts into a representative complementary DNA (cDNA) library for strand-specific RNA sequencing as prescribed by the manufacturer. Briefly, 25 µl total RNA was concentrated to 10 µl at 37 C for 2 hr. The starting amount of RNA ranged from 50 to 725 ng. For library preparation, 10 µl of RNA was fragmented with RNAseIII for 2 min at 37 C. The fragmented RNA was purified, using the magnetic bead clean-up module and eluted in nuclease-free water. The yield and size of the fragmented RNA was not evaluated due to expected low concentrations. Instead, the full amount of RNA was used for subsequent hybridization and adapter ligation at 30 C for an hour. This step also incorporates the barcodes/indexes that are used to identify each sample. The adaptor-ligated RNA was reverse-transcribed to generate single-stranded cDNA. These cDNA products were amplified to prepare barcoded cDNA libraries using the Ion Xpress™ RNA-Seq Barcode Kit (Thermo Fisher Scientific). These libraries were purified and assessed for yield and fragment size distribution using the High Sensitivity DNA Kit and chips on the BioAnalyser 2100 (Agilent Technologies) according to the recommended protocol.

For template preparation and enrichment, the libraries were diluted to a target concentration of 80 pM. The diluted, barcoded cDNA libraries were combined in equimolar amounts for sequencing template preparation using the Ion PI™ HiQ™ Chef Kit (Thermo Fisher Scientific). Enriched, template positive ion sphere particles were loaded onto an Ion PI™ (v3) Chip (Thermo Fisher Scientific). Massively parallel sequencing was performed on the Ion Proton™ System using sequencing solutions, reagents, and supply kits according to the manufacturer's protocol. Flow space calibration and basecaller analysis was performed using standard analysis parameters in the Torrent Suite Version 5.4.0 Software.

Genome assembly and sequence analysis. Ion Torrent reads were imported into CLC Genomics Workbench 5.2.1 and reference genome segments KY621531-KY621538 were used as scaffolds for segment assembly. Multiple sequence alignments were prepared in BioEdit v7.2.5 (7) with reference genomes retrieved from GenBank (https://www.ncbi.nlm.nih.gov/nuccore) and the GISAID EpiFlu™ database (https://www.gisaid.org/). The latter includes sequence data generated by the National Institute of Communicable Diseases in Johannesburg, South Africa, using WCPVL RNA samples from 14 Western Cape Province cases that were RT-PCR positive but virus-isolation negative (F. Treunicht, pers. comm.; Fig 2). Phylogenies were reconstructed using the maximum likelihood statistical method in MEGA v5.5.2 (19), tested with 1000 bootstrap replicates. The Tamura-Nei nucleotide substitution model was used, specifying a uniform rate among sites. Trees were inferred with a nearest-neighbor–interchange method, with a very strong brand swap filter. MEGA v5.5 was also used to prepare pairwise distance matrices for each of the eight genome segments.

RESULTS

Fig. 1 is compiled from reports made to the OIE between June and December 2017 (13) and provides an overview of the progression of the outbreak in various provinces in South Africa, and correspondingly the various categories of birds affected. Backyard poultry and ornamental and pet birds, as well as birds in zoologic collections, have been grouped as "captive" birds here. The outbreaks peaked in August–September 2017, corresponding to late winter–early spring in the southern hemisphere. Initially, outbreaks were restricted to the northern areas of the country, in the Gauteng and Mpumalanga provinces, but shifted to the Western Cape Province in the southern region of the country at around the seventh week of the outbreak. The largest proportion of outbreaks occurred in the Western Cape Province.

Forty clade 2.3.4.4 H5N8 HPAI viruses isolated from wild birds, commercial chickens and ostriches, and captive birds during the outbreaks in South Africa in 2017 were sequenced here for phylogenetic comparison (Table 1). Ion Torrent reads with an average length of 108 bp were generated (range: 52 to 144 bp) and complete genome sequences were assembled for each of the viruses. High coverage was obtained for many of the viruses; for example, the coverage for genome Segment 8 (encoding matrix and ion channel proteins) was more than 115,000× per nucleotide position for strain A/Pekin duck/South Africa/17808481/2017. Full genome sequences were deposited in GenBank under the accession numbers listed in Table 1. Phylogenetic trees were generated for each of the eight assembled gene segments (Supplemental Figs. S1–S8). The sequence for A/chicken/Zimbabwe/810/2018 was generated at and provided by the Animal and Plant Health Agency, U.K. Genetic clusters designated a (or a_1 and a_2), b (or b_1 and b_2), c, and d, were assigned based on topology, statistical support for branches, and the percentage of nucleotide sequence identity in distance matrices (Supplemental Figs. S1–S8). In the distance matrices, classification within a cluster was qualified at $\geq$99.4% nucleotide sequence identity (data not shown). To identify genetic variants, clusters were arranged and color-coded in Fig. 2.

The eight genomic segments of influenza A virus are polymerase basic 1 (PB1), polymerase basic 2 (PB2), polymerase A (PA), hemagglutinin (HA), nucleoprotein (NP), neuraminidase protein (NA), matrix (M), and nonstructural protein (NS) here (a description of proteins encoded on each respective segment is provided in the captions to Supplemental Figs. S1–S8). Five genetic variants were identified (Fig. 2; Table 1). Based on these data, it evident that the two index events that occurred within days of each other and were situated only 35 km apart were not epidemiologically linked; the Villiers outbreak virus and the Standerton outbreak virus belong to two different variants. Interestingly, the Standerton outbreak virus had PB2 and PA genome segments that formed outgroups to the 2016/2017 clade 2.3.4.4 H5 viruses (Supplemental Figs. S1, S3). The Standerton virus PA gene shared a recent common ancestor with a broader group of viruses isolated from South African wild aquatic birds and ostriches between 2012 and 2015. This reassortment with known wild bird LPAI viruses provides evidence that wild birds introduced clade 2.3.4.4 into southern Africa.

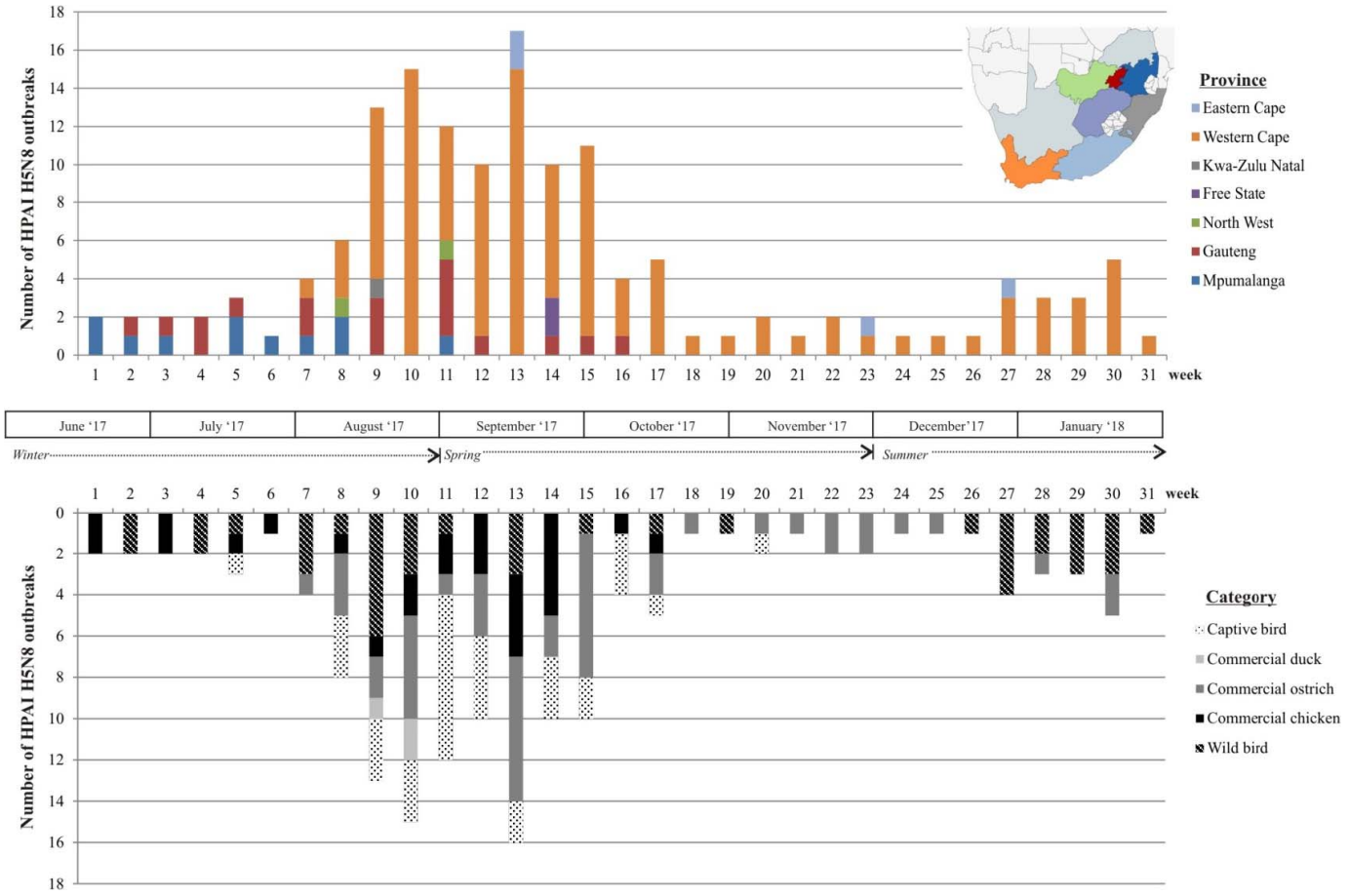

Fig. 1. Overview of laboratory-confirmed clade 2.3.4.4 HPAI H5N8 cases in South Africa during 2017, based on data reported to the OIE. The distribution of outbreaks per province is shown on the upper graph, and the distribution of species affected is on the lower graph. Captive birds comprise backyard poultry, pets, and ornamental birds as well as birds in zoologic collections.

The Villiers virus–like Variant 1 strains were isolated in the Mpumalanga and Gauteng provinces in the early stages of the epizootic (June to August 2017). A/speckled pigeon/South Africa/08-004B was the last of these in the northern region in our data set until September 23, when Variant 1 was detected again in a single outbreak in commercial chickens in the Free State Province (A/chicken/South Africa/115370/2017).

A/Egyptian goose/South Africa/001/2017 and A/sacred ibis/South Africa/009/2017 were both isolated from free-living wild birds in Pretoria, the former near Irene at the southern end of the municipal region, and the latter near Bon Accord Dam near the northern outskirts of the city. The Egyptian goose (*Alopochen aegypticus*) at the Irene site was in good body condition but moribund on the banks of a water body, unable to fly and easily caught at the time of sampling. Four sacred ibis (*Threskiornis aethiopicus*) carcasses were observed on the water body. No other moribund or dead birds were observed. Seven sacred ibis were found dead, and three living sacred ibis showed signs of disorientation and inability to fly, and were easily captured for sampling along the shoreline of Bon Accord Dam. The virus isolated from these birds was the same variant as that of the Egyptian goose at Irene (no. 3) and these two viruses were most closely related to the strain that caused the Zimbabwe outbreak 2 mo prior.

The fourth variant shares a recent common ancestor with variant 1, but is defined as a separate variant based on the statistically supported distinction between the b_1 and b_2 subclusters in the PB2, PB1, PA, NA, and M segments. There was no phylogenetic distinction in the HA, NP, and NS gene-encoding segments (Supplemental Figs. S1–S8). The first Variant 4 isolate was only obtained in August 2017, from a commercially farmed ostrich in the Western Cape Province. Variant 4 is the majority variant but remained restricted to the southern Cape region (Western and Eastern Cape provinces) in 2017.

Strain A/chicken/South Africa/443397/2017 was isolated from the only outbreak recorded in the Kwa-Zulu Natal Province, and fortunately remained restricted to the northern region (Abaquilisi District) of this highly poultry-dense province. Here, 257,568 commercial layers, a mix of Hyline, Lohmann, and Amberlink strains died or were culled, and 584,746 eggs were destroyed. This virus was phylogenetically distinct across all of its genome segments, including the HA gene (Supplemental Fig. S4), which generally provided less phylogenetic distinction compared to most of the other segments; it forms the fifth variant and wasn't detected elsewhere.

Phylogenetically, the South African strains are classified as subgroup B of clade 2.3.4.4 (11). H5N8 viruses isolated in Cameroon and Egypt in 2017 and in Korea in 2016 shared the most recent common ancestors with the South African strains, whereas viruses from the DRC and Uganda in 2017 were more distantly related. Although the genetic data are limited, they indicate that

Date	Virus	Province	PB2	PB1	PA	HA	NP	NA	M	NS	variant
15-May	A/chicken/Zimbabwe/810/2017		a	a	a	a	a_1	a	a	a	
19-Jun	**A/chicken/South Africa/Villiers/2017**	MPU	b_1	b_1	b_1	b	a_1	b_1	b_1	a	1
20-Jun	**A/chicken/South Africa/Standerton/2017**	MPU	d	a	d	a	a_1	a	a	a	2
06-Jul	**A/chicken/South Africa/436893/2017**	GAU	b_1	b_1	b_1	b	a_1	b_1	b_1	a	1
13-Jul	**A/Egyptian goose/South Africa/001/2017**	GAU	a	a	a	a	a_2	a	a	a	3
01-Aug	**A/chicken/South Africa/440638A/2017**	MPU	b_1	b_1	b_1	b	a_1	b_1	b_1	a	1
01-Aug	**A/chicken/South Africa/440638B/2017**	MPU	b_1	b_1	b_1	b	a_1	b_1	b_1	a	1
03-Aug	A/ostrich/South Africa/S2017/08 0047/2017	WC				b	b	b_2	b_2	b	4
04-Aug	**A/chicken/South Africa/441587/2017**	GAU	b_1	b_1	b_1	b	a_1	b_1	b_1	a	1
04-Aug	**A/chicken/South Africa/MC002/2017**	GAU	b_1	b_1	b_1	b	a_1	b_1	b_1	a	1
07-Aug	**A/chicken/South Africa/441839/2017**	MPU	b_1	b_1	b_1	b	a_1	b_1	b_1	a	1
09-Aug	**A/ostrich/South Africa/17080046/2017**	WC	b_2	b_2	b_2	b	b	b_2	b_2	b	4
11-Aug	A/ostrich/South Africa/S2017/08 161/2017	WC			b_2	b	b	b_2	b_2	b	4
14-Aug	A/Guinea fowl/South Africa/S2017/08 190/2017	WC	b_2	b_2	b_2	b	b	b_2	b_2	b	4
14-Aug	**A/Speckled pigeon/South Africa/08-004B/2017**	GAU	b_1	b_1	b_1	b	a_1	b_1	b_1	a	1
15-Aug	**A/Sacred ibis/South Africa/009/2017**	GAU	a	a	a	a	a_2	a	a	a	3
16-Aug	**A/Guinea fowl/South Africa/17080243/2017**	WC	b_2	b_2	b_2	b	b	b_2	b_2	b	4
16-Aug	**A/Guinea fowl/South Africa/17080274/2017**	WC	b_2	b_2	b_2	b	b	b_2	b_2	b	4
16-Aug	A/Ostrich/South Africa/S2017/08 268/2017	WC				b	b	b_2	b_2	b	4
16-Aug	A/Wild birds/South Africa/S2017/08 275/2017	WC	b_2		b_2	b	b	b_2	b_2	b	4
17-Aug	**A/chicken/South Africa/443397/2017**	KZN	c	c	c	c	c	c	c	c	5
18-Aug	A/Blue crane/South Africa/S2017/08 0322/2017	WC					b	b_2	b_2	b	4
18-Aug	**A/pigeon/South Africa/17080323/2017**	WC	b_2	b_2	b_2	b	b	b_2	b_2	b	4
18-Aug	**A/dove/South Africa/17080324/2017**	WC	b_2	b_2	b_2	b	b	b_2	b_2	b	4
21-Aug	**A/chicken/South Africa/17080336/2017**	WC	b_2	b_2	b_2	b	b	b_2	b_2	b	4
22-Aug	**A/duck/South Africa/17080340/2017**	WC	b_2	b_2	b_2	b	b	b_2	b_2	b	4
22-Aug	A/Ostrich/South Africa/S2017/08 361/2017	WC				b	b	b_2	b_2	b	4
22-Aug	A/Ostrich/South Africa/S2017/08 362/2017	WC	b_2		b_2	b	b	b_2	b_2	b	4
23-Aug	A/Chicken/South Africa/S2017/08 416/2017	WC			b_2	b	b	b_2	b_2	b	4
24-Aug	A/Ostrich/South Africa/S2017/08 484/2017	WC					b	b_2	b_2	b	4
24-Aug	**A/duck/South Africa/17080481/2017**	WC	b_2	b_2	b_2	b	b	b_2	b_2	b	4
28-Aug	**A/swan/South Africa/17080517/2017**	WC	b_2	b_2	b_2	b	b	b_2	b_2	b	4
28-Aug	A/Geese/South Africa/S2017/08 520/2017	WC				b	b	b_2	b_2	b	4
29-Aug	A/Geese/South Africa/S2017/08 558/2017	WC	b_2	b_2	b_2	b	b	b_2	b_2	b	4
29-Aug	**A/chicken/South Africa/17080561/2017**	WC	b_2	b_2	b_2	b	b	b_2	b_2	b	4
30-Aug	**A/chicken/South Africa/17080581/2017**	WC	b_2	b_2	b_2	b	b	b_2	b_2	b	4
04-Sep	A/Geese/South Africa/S2017/09 055/2017	WC	b_2	b_2	b_2	b	b	b_2	b_2	b	4
04-Sep	**A/goose/South Africa/17090065/2017**	WC	b_2	b_2	b_2	b	b	b_2	b_2	b	4
04-Sep	**A/chicken/South Africa/17090050/2017**	WC	b_2	b_2	b_2	b	b	b_2	b_2	b	4
06-Sep	**A/chicken/South Africa/17090100/2017**	WC	b_2	b_2	b_2	b	b	b_2	b_2	b	4
06-Sep	**A/chicken/South Africa/17090108/2017**	WC	b_2	b_2	b_2	b	b	b_2	b_2	b	4
08-Sep	A/Chicken/South Africa/S2017/09 184/2017	WC	b_2	b_2	b_2	b	b	b_2	b_2	b	4
11-Sep	**A/chicken/South Africa/17090202/2017**	WC	b_2	b_2	b_2	b	b	b_2	b_2	b	4
12-Sep	**A/chicken/South Africa/448475/2017**	EC	b_2	b_2	b_2	b	b	b_2	b_2	b	4
15-Sep	**A/chicken/South Africa/17090325/2017**	WC	b_2	b_2	b_2	b	b	b_2	b_2	b	4
16-Sep	**A/chicken/South Africa/449300/2017**	WC	b_2	b_2	b_2	b	b	b_2	b_2	b	4
18-Sep	**A/chicken/South Africa/17090348/2017**	WC	b_2	b_2	b_2	b	b	b_2	b_2	b	4
18-Sep	**A/chicken/South Africa/449418/2017**	WC	b_2	b_2	b_2	b	b	b_2	b_2	b	4
18-Sep	**A/chicken/South Africa/449443/2017**	WC	b_2	b_2	b_2	b	b	b_2	b_2	b	4
18-Sep	**A/chicken/South Africa/17090335/2017**	WC	b_2	b_2	b_2	b	b	b_2	b_2	b	4
19-Sep	**A/turkey/South Africa/450199/2017**	WC	b_2	b_2	b_2	b	b	b_2	b_2	b	4
23-Sep	**A/chicken/South Africa/115370/2017**	FS	b_1	b_1	b_1	b	a_1	b_1	b_1	a	1
26-Sep	**A/chicken/South Africa/450628/2017**	WC	b_2	b_2	b_2	b	b	b_2	b_2	b	4
29-Sep	**A/chicken/South Africa/451457/2017**	WC	b_2	b_2	b_2	b	b	b_2	b_2	b	4
26-Oct	**A/ostrich/South Africa/002/2017**	WC	b_2	b_2	b_2	b	b	b_2	b_2	b	4

Fig. 2. Reassortment patterns in South African 2017 HPAI H5N8 strains, arranged chronologically. Viruses sequenced in this study are in boldface. Gaps indicate partial genome sequences. Genetic clusters were assigned as indicated in the phylogenetic trees provided as supplemental figures. Provinces are abbreviated as follows: Mpumalanga (MPU), Gauteng (GAU), Western Cape (WC), Eastern Cape (EC), Free State (FS). The Zimbabwean virus is included for reference. There is no relationship horizontally between designated clusters or their color.

Table 1. Clade 2.3.4.4 HPAI H5N8 viruses isolated in South Africa in 2017

Date	Isolate	Location[D]	Accession numbers
Jun. 19	A/chicken/South Africa/Villiers/2017 (H5N8)[AB]	Villiers, MPU	MH165521–MH165528
Jun. 20	A/chicken/South Africa/Standerton/2017 (H5N8)[AB]	Standerton, MPU	MH165529–MH165536
Jul. 6	A/chicken/South Africa/436893/2017 (H5N8)[A]	Benoni, GAU	MH165537–MH165544
Jul. 13	A/Egyptian goose/South Africa/001/2017 (H5N8)[B]	Pretoria, GAU	MH165545–MH165552
Aug. 1	A/chicken/South Africa/440638A/2017 (H5N8)[A]	Standerton, MPU	MH165553–MH165560
Aug. 1	A/chicken/South Africa/440638B/2017 (H5N8)[A]	Standerton, MPU	MH165561–MH165568
Aug. 4	A/chicken/South Africa/441587/2017 (H5N8)[A]	Benoni, GAU	MH165569–MH165576
Aug. 4	A/chicken/South Africa/MC002/2017 (H5N8)[A]	Krugersdorp, GAU	MH165577–MH165584
Aug. 7	A/chicken/South Africa/441839/2017 (H5N8)[A]	Middelburg, MPU	MH165585–MH165592
Aug. 9	A/ostrich/South Africa/17080046/2017 (H5N8)[C]	Heidelberg, WC	MH165593–MH165600
Aug. 14	A/speckled pigeon/South Africa/08–004B/2017 (H5N8)[B]	Benoni, GAU	MH165601–MH165608
Aug. 15	A/sacred ibis/South Africa/009/2017 (H5N8)[B]	Pretoria, GAU	MH165609–MH165616
Aug. 16	A/guinea fowl/South Africa/17080243/2017 (H5N8)[C]	Caledon, WC	MH165617–MH165624
Aug. 16	A/guinea fowl/South Africa/17080274/2017 (H5N8)[C]	Heidelberg, WC	MH165625–MH165632
Aug. 17	A/chicken/South Africa/443397/2017 (H5N8)[A]	Newcastle, KZN	MH165633–MH165640
Aug. 18	A/pigeon/South Africa/17080323/2017 (H5N8)[C]	Heidelberg, WC	MH165641–MH165648
Aug. 18	A/dove/South Africa/17080324/2017 (H5N8)[C]	Worchester, WC	MH165649–MH165656
Aug. 21	A/chicken/South Africa/17080336/2017 (H5N8)[C]	Paarl, WC	MH165657–MH165664
Aug. 22	A/Pekin duck/South Africa/17080340/2017 (H5N8)[C]	Kraaifontein, WC	MH165665–MH165672
Aug. 24	A/Pekin duck/South Africa/17080481/2017 (H5N8)[C]	Kraaifontein, WC	MH165673–MH165680
Aug. 28	A/swan/South Africa/17080517/2017 (H5N8)[C]	Durbanville, WC	MH165681–MH165688
Aug. 29	A/chicken/South Africa/17080561/2017 (H5N8)[C]	Malmesbury, WC	MH165689–MH165696
Aug. 30	A/chicken/South Africa/17080581/2017 (H5N8)[C]	Porterville, WC	MH165697–MH165704
Sep. 4	A/domestic goose/South Africa/17090065/2017 (H5N8)[C]	Caledon, WC	MH165705–MH165712
Sep. 4	A/chicken/South Africa/17090050/2017 (H5N8)[C]	Worcester, WC	MH165713–MH165720
Sep. 6	A/chicken/South Africa/17090100/2017 (H5N8)[C]	Paarl, WC	MH165721–MH165728
Sep. 6	A/chicken/South Africa/17090108/2017 (H5N8)[C]	Paarl, WC	MH165833–MH165840
Sep. 11	A/chicken/South Africa/17090202/2017 (H5N8)[C]	Worchester, WC	MH165729–MH165736
Sep. 12	A/chicken/South Africa/448475/2017 (H5N8)[A]	Port Elizabeth, EC	MH165737–MH165744
Sep. 15	A/chicken/South Africa/17090325/2017 (H5N8)[C]	Paarl, WC	MH165745–MH165752
Sep. 16	A/chicken/South Africa/449300/2017 (H5N8)[A]	Paarl, WC	MH165753–MH165760
Sep. 18	A/chicken/South Africa/17090348/2017 (H5N8)[C]	Paarl, WC	MH165761–MH165768
Sep. 18	A/chicken/South Africa/449418/2017 (H5N8)[A]	Paarl, WC	MH165769–MH165776
Sep. 18	A/chicken/South Africa/449443/2017 (H5N8)[A]	Paarl, WC	MH165777–MH165784
Sep. 18	A/chicken/South Africa/17090335/2017 (H5N8)[C]	Stellenbosch, WC	MH165785–MH165792
Sep. 19	A/turkey/South Africa/450199/2017 (H5N8)[A]	Cape Town, WC	MH165793–MH165800
Sep. 23	A/chicken/South Africa/115370/2017 (H5N8)[A]	Welkom, FS	MH165801–MH165808
Sep. 26	A/chicken/South Africa/450628/2017 (H5N8)[A]	Paarl, WC	MH165809–MH165816
Sep. 29	A/chicken/South Africa/451457/2017 (H5N8)[A]	Paarl, WC	MH165817–MH165824
Oct. 26	A/ostrich/South Africa/002/2017 (H5N8)[B]	Oudtshoorn, WC	MH165825–MH165832

[A]Isolated at Deltamune Pty(Ltd).
[B]Isolated at the University of Pretoria.
[C]Isolated at Western Cape Provincial Veterinary Laboratory.
[D]MPU = Mpumalanga Province; GAU = Gauteng Province; WC = Western Cape Province; KZN = Kwa-Zulu Natal Province; EC = Eastern Cape Province; FS = Free State Province.

West Africa may have been the epicenter for the H5N8 spread in sub-Saharan Africa (10).

DISCUSSION

South Africa and Zimbabwe have in the past been prone to periodic outbreaks of HPAI H5 in commercial ostriches, but these unclassified H5 lineages emerged through the more traditional route of mutation of LPAI precursor strains that are naturally present in wild birds, and such outbreaks tend to have a restricted geographic range (2,11). Reports of the initial outbreaks of clade 2.3.4.4 H5N8 HPAI in northern Egypt and West Africa in late 2016 failed to raise much concern locally. Gs/GD H5 HPAI lineage viruses have reached both of these regions during previous intercontinental waves and have caused sustained outbreaks in poultry or even become endemic (16), yet did not reach southern Africa. This is in spite of an overlap in the ranges of the migratory flyways of Eurasian wildfowl that overwinter in Africa north of the equator (a range that includes West Africa, North Africa, Uganda, and eastern DRC) and the ranges of Afro-tropical wildfowl in sub-Saharan Africa (6).

Reports of clade 2.3.4.4 H5N8 HPAI in Uganda, however, were a concern. Not only do white-winged terns migrate annually to the southernmost tip of Africa, but the Ugandan territory in which the outbreaks occurred is a habitat for 240,000 wild birds and a major stopping point on the East African flyway, hosting important areas for breeding, wintering, and passage for at least 82 Palearctic and 17 Afro-tropical migrants (5). Influenza A strains isolated in South Africa in the past have contained reassorted genes of viruses isolated as far north as Zambia, linking Afro-tropical duck populations in the subregion (2,17). It was ultimately a false consolation that the outbreaks in Uganda occurred in December and January, with southbound seasonal migrations not expected to occur until September.

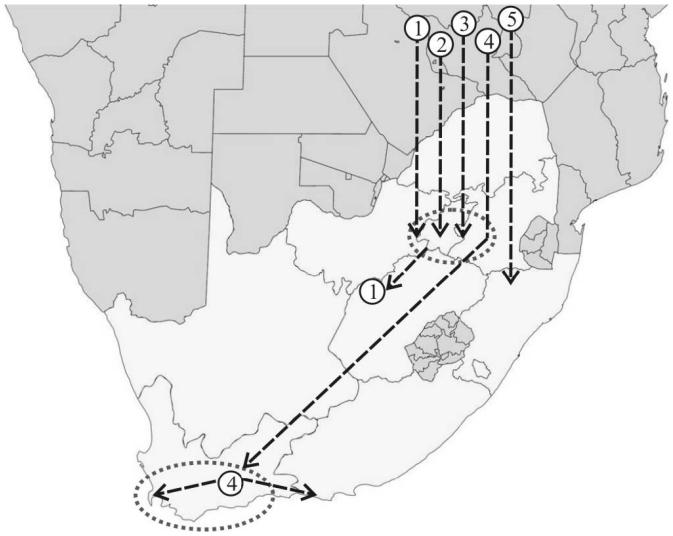

Fig. 3. Incursion and spread of clade 2.3.4.4 H5N8 HPAI in South Africa. At least five genetic variants (numbered 1 to 5) were detected in the region in mid-2017; one of these variants reached the southernmost tip of the African continent and caused devastating outbreaks in commercial chickens, ostriches, ducks, wild birds, and captive birds.

The southward movement of H5N8 to Zimbabwe by May 2017, counter to seasonal migration of Palearctic species that comprise mainly wader-type birds, suggest a role for Afro-tropical wildfowl, whose migration movements are not driven by seasonal temperatures as in northern hemisphere species, but rather by rainfall-dependent water levels of wetlands (6). When the first outbreaks occurred in the northern region of South Africa, movement of infected poultry or fomites between Harare and the index case commercial farms in South Africa were strongly suspected. However, here we determined that not only were two index cases in South Africa caused by two different variants, but that they were not directly related to the Zimbabwe virus. In fact, our data indicated that up to five primary introductions into South Africa may have occurred in 2017, as summarized by Fig. 3, with evidence for wild bird introduction supported by reassortment with southern African LPAI viruses and recent common ancestors shared with viruses from West Africa. The single variant that reached the southern Cape region caused the most devastating economic losses in the history of South African poultry production. In hindsight, a multivariate risk map published by Cumming and coworkers (3) produced a remarkably accurate fit with the incursion of the initial 2017 H5N8 HPAI outbreaks in South Africa, underscoring the usefulness of such exercises in identifying key locations for placing surveillance and monitoring points. Wild bird surveillance will play a more critical role as an early warning system for the presence of HPAI than ever before, but a lack of funding and coordinated sampling and testing efforts in most African countries cripples the capacity for early detection. Concerningly, all three laboratories that participated in the present study experienced a lack of sensitivity of the European Union–recommended H5 assay in detecting the South African variants (18), necessitating the redesign of the H5 primer and probe sequences reported here that improved detection (data not shown), but requires more extensive validation. The use of insensitive detection assays will miss infections where viruses are present in low levels.

Prior to 2017, South Africa had never had any incidence of notifiable influenza viruses in gallinaceous poultry, despite the presence of LPAI H5 and H7 in the wild duck reservoir. All epidemiologic units are, by veterinary directive, tested at least twice a year for exposure to avian influenza (2). Reasons for this could be that LPAI H5 strains typically found in wildfowl are not chicken-adapted and, under the harsh and often water-scarce environment of South Africa, the minimum viral dose required to infect chickens during a biosecurity breach is not attained. Clade 2.3.4.4 H5N8 HPAI is different. Firstly, it possibly infects chickens without prior adaptation, and secondly, clade 2.3.4.4 H5–infected species, and especially wildfowl such as ducks, have been experimentally demonstrated to shed large amounts of virus either through the cloaca or via the oropharynx, depending on the specie (14). A heavily contaminated environment would substantially increase the risk of outbreaks and viral spread in wild populations, aided by the cold temperatures in the winter months of May to July and the early spring period in South Africa.

South Africa's official method of control for HPAI is stamping out, and about 5.4 million broiler and layer chickens were culled in 2017. The effect on the layer industry was much larger than the broiler industry, with around 4.7 million birds culled in the laying sector as opposed to around 700,000 birds in the broiler sector, which was predominantly affected at the breeder level. Total losses estimated from total biologic losses, income lost from egg sales, pullet sales, day-old chick sales, and broiler meat sales is estimated at R1.66 billion rand (US$140 million) (4). Culling of infected premises and movement controls, together with suspension of all cull chickens (spent layer hens and breeder birds) for a period contributed to the success of preventing HPAI H5N8 from spreading further into the intensive poultry-producing areas in the North-West Province and southern Kwa-Zulu Natal Province. Left unchecked, a rapid epidemiologic spread of H5N8 similar to that of numerous strains of exotic Newcastle disease in South Africa (1) was certain.

The remarkable resistance of ostriches to HPAI was again evident with clade 2.3.4.4 H5N8 infections. Commercial ostriches, in which outbreaks continue, show minimal clinical signs of infection. Of all the farms infected since August 2017, only three farms recorded mortalities of any significance. Two breeder farms had 5% mortality and one slaughter farm had 10% to 12% mortality (13). Interestingly, only older birds (>6 mo) were affected; no chicks were sick (A. Olivier, pers. comm.). Isolation rates from RT-PCR–positive ostrich swabs remained low as before (2), with only two viruses recovered even though eight additional partial genome sequences were retrieved from direct sequencing on swab RNAs (Fig. 2).

Faced with the prospect that HPAI H5N8 has become established in a local reservoir, or that a new lineage could be introduced by the same route, commercial producers are lobbying government authorities for the right to vaccinate and protect their livelihoods, whist the government authorities weigh the longer-term trade and animal health implications of allowing vaccination, and the risk that this would create for an HPAI endemic status from which it will be difficult if not impossible to recover. The sale of cull chickens is at the fulcrum of this debate. The cull chicken industry is lucrative to the commercial producers as well as the depot vendors and informal traders and would be difficult to monitor and regulate. Releasing vaccinated, clinically healthy hens that may be shedding HPAI viruses into the open market cannot be permitted. A compromise that only valuable breeding stock, zoologic collections, and other valuable birds may be vaccinated against H5 HPAI, paired with very strict movement controls and testing regimes may be required.

The ultimate tragedy of the incursion of clade 2.3.4.4 H5N8 HPAI in South Africa is its effect on wild endangered birds, most of which are not found elsewhere in the world. Since December 2017, spillover from an unknown reservoir infected coastal birds along the length of the southern Cape coast, causing mass mortalities in swift terns (*Thalasseus bergii*), common terns (*Sterna hirundo*), sandwich terns (*Thalasseus sandvicensis*), Cape gannets (*Sula capensis*), Cape cormorants (*Phalacrocorax capensis*), Hartlaub's gulls (*Choroicocephalus hartlaubis*), and jackass penguins (*Spheniscus demersus*), amongst other species. By mid-February, March, and April 2018, HPAI H5N8 had been diagnosed in outbreaks in backyard poultry in the Limpopo and North West provinces, signalling that wild bird populations in the north of the country remained infected, and in June HPAI H5N8 was confirmed in commercial chickens in the southern Gauteng region, close to the locations of the 2017 index cases (13). As winter 2018 progresses in the southern hemisphere, South Africa remains braced for further outbreaks.

Supplemental figures associated with this article can be found at https://doi.org/10.1637/11869-042518-Reg.1.s1.

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