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Susceptibility and Status of Avian Influenza in Ostriches

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SUMMARY. The extensive nature of ostrich farming production systems bears the continual risk of point introductions of avian influenza virus (AIV) from wild birds, but immune status, management, population density, and other causes of stress in ostriches are the ultimate determinants of the severity of the disease in this species. From January 2012 to December 2014, more than 70 incidents of AIV in ostriches were reported in South Africa. These included H5N2 and H7N1 low pathogenicity avian influenza (LPAI) in 2012, H7N7 LPAI in 2013, and H5N2 LPAI in 2014. To resolve the molecular epidemiology in South Africa, the entire South African viral repository from ostriches and wild birds from 1991 to 2013 ($n = 42$) was resequenced by next-generation sequencing technology to obtain complete genomes for comparison. The phylogenetic results were supplemented with serological data for ostriches from 2012 to 2014, and AIV-detection data from surveillance of 17 762 wild birds sampled over the same period. Phylogenetic evidence pointed to wild birds, e.g., African sacred ibis (*Threskiornis aethiopicus*), in the dissemination of H7N1 LPAI to ostriches in the Eastern and Western Cape provinces during 2012, in separate incidents that could not be epidemiologically linked. In contrast, the H7N7 LPAI outbreaks in 2013 that were restricted to the Western Cape Province appear to have originated from a single-point introduction from wild birds. Two H5N2 viruses detected in ostriches in 2012 were determined to be LPAI strains that were new introductions, epidemiologically unrelated to the 2011 highly pathogenic avian influenza (HPAI) outbreaks. Seventeen of 27 (63%) ostrich viruses contained the polymerase basic 2 (PB2) E627K marker, and 2 of the ostrich isolates that lacked E627K contained the compensatory Q591K mutation, whereas a third virus had a D701N mutation. Ostriches maintain a low upper- to midtracheal temperature as part of their adaptive physiology for desert survival, which may explain the selection in ratites for E627K or its compensatory mutations—markers that facilitate AIV replication at lower temperatures. An AIV prevalence of 5.6% in wild birds was recorded between 2012 and 2014, considerably higher than AIV prevalence for the southern African region of 2.5%–3.6% reported in the period 2007–2009. Serological prevalence of AI in ostriches was 3.7%, 3.6%, and 6.1% for 2012, 2013, and 2014, respectively. An annual seasonal dip in incidence was evident around March/April (late summer/autumn), with peaks around July/August (mid to late winter). H5, H6, H7, and unidentified serotypes were present at varying levels over the 3-yr period.

RESUMEN. Susceptibilidad y situación de la influenza aviar en avestruces.

La extensa naturaleza de los sistemas de producción de avestruz enfrenta el riesgo continuo de la presentación del virus de la influenza aviar (AIV) originado de aves silvestres, pero el estado inmunológico, el manejo, la densidad de población, y otras causas de estrés en avestruces son determinantes importantes en la severidad de esta enfermedad en esta especie. De enero del 2012 a diciembre del 2014, se registraron más de 70 casos de virus de influenza aviar en avestruces en Sudáfrica. Estos virus incluyeron virus de baja patogenicidad H5N2 y H7N1 en el año 2012, virus de baja patogenicidad H7N7 en el año 2013 y virus de baja patogenicidad H5N2 en 2014. Para resolver la epidemiología molecular en Sudáfrica, todo el repositorio de muestras virales de avestruces y aves silvestres en Sudáfrica del año 1991 al 2013 ($n = 42$) fue re-analizado por análisis de secuencias de próxima generación para obtener genomas completos para su comparación. Los resultados filogenéticos se complementaron con datos serológicos para avestruces del año 2012 al 2014, y con los datos de detección en la vigilancia del virus de influenza aviar de 17 762 aves silvestres muestreadas durante el mismo período. La evidencia filogenética señaló el papel de aves silvestres como los, ibis sagrados africanos (*Threskiornis aethiopicus*), en la difusión de virus de influenza de baja patogenicidad H7N1 a las avestruces en las provincias del Este y del Cabo Occidental durante el año 2012, en incidentes separados que no pudieron ser relacionados epidemiológicamente. Por el contrario, los brotes con virus H7N7 de baja patogenicidad en el año 2013, que se limitaban a la Provincia Occidental del Cabo parecen haberse originado a partir de una introducción de un solo punto de aves silvestres. Se determinó que dos virus H5N2 detectados en avestruces en el año 2012 de baja patogenicidad eran introducciones nuevas, que no estaban relacionadas epidemiológicamente con los brotes de influenza aviar en el año 2011. Diecisiete de 27 (63%) virus de avestruces contenían el marcador PB2 E627K, y dos de los aislados de avestruz que carecían del marcador E627K contenían la mutación compensatoria Q591K, mientras que un tercer virus tenía una mutación D701N. Las avestruces mantienen una temperatura baja en la parte media y baja de la tráquea como parte de su fisiología de adaptación para sobrevivir en el desierto, lo que puede explicar la selección de las ratites para la mutación E627K o sus mutaciones compensatorias que son marcadores que facilitan la replicación del virus de influenza aviar a temperaturas más bajas. Se registró una prevalencia del virus de influenza aviar de 5.6% en las aves silvestres, entre 2012 y 2014, considerablemente más alta que la prevalencia del virus de influenza aviar de la región de África meridional de 2.5% −3.6% reportada en el periodo entre los años 2007-2009. La prevalencia serológica de la influenza aviar en avestruces fue del 3.7%, 3.6% y 6.1% para los años 2012, 2013 y 2014, respectivamente. Fue evidente una

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caída estacional en la incidencia anual alrededor de Marzo y Abril (finales de verano/otoño), con picos alrededor de Julio y Agosto (mediados a finales de invierno). Los subtipos H5, H6, H7, y serotipos no identificados estuvieron presentes en diferentes niveles durante el período de tres años.

Key words: avian influenza, ostrich, PB2, phylogenetic, serology, wild birds

Abbreviations: AI = avian influenza; AIV = avian influenza A virus; HA = hemagglutinin; HA_0 = hemagglutinin cleavage site; HI = hemagglutinin inhibition; HPAI = highly pathogenic avian influenza; IFN = interferon; LPAI = low pathogenicity avian influenza; M = matrix; NA = neuraminidase; NS1 = nonstructural protein 1; NP = nucleocapsid protein; OIE = World Organization for Animal Health; PA = polymerase A; PB1 = polymerase basic 1; PB2 = polymerase basic 2; PCR = polymerase chain reaction; rRT-PCR = real-time reverse transcription PCR; RCA = recent common ancestor; RIG-1 = retinoic acid-inducible gene I

Avian influenza (AI), especially when caused by the highly pathogenic (HPAI) H5 or H7 viral serotypes, is a serious disease of poultry with zoonotic potential (23). Three epidemiologically unrelated outbreaks of H5N2 HPAI in 2004, 2006, and 2011 had devastating economic impacts on the South African ostrich industry through control measures, restrictions on meat exports, and other socioeconomic factors (7,22). South Africa is the largest global producer of ostriches, and farming operations are concentrated in the Klein Karoo, a semi-desert region that spans the Eastern and Western Cape provinces. Ostriches (*Struthio camelus*) are valued for their lean meat and their skins, which are used to produce luxury leather goods. Farmed ostriches are classified as poultry by the World Organisation for Animal Health (OIE); all guidelines for poultry surveillance and diagnosis, and regulations for control of AI, therefore apply (23). The extensive nature of ostrich farming production systems bears the continual risk of point introductions of avian influenza virus (AIV) from wild birds, the natural reservoirs (29), but immune status, management, population density, and other causes of stress in ostriches are the ultimate determinants of the severity of the disease in this species.

AIVs are members of the *Orthomyxoviridae* family, typified by single-stranded RNA genomes of negative coding sense. The genome is distributed across eight segments, encoding at least 11 protein genes: polymerase basic 2 (PB2) on segment 1, polymerase basic 1 (PB1) plus mitochondria-associated protein (PB1-F2) on segment 2, polymerase A (PA) plus PA-X fusion protein on segment 3, hemagglutinin (HA) glycoprotein on segment 4, nucleocapsid protein (NP) on segment 5, neuraminidase (NA) glycoprotein on segment 6, matrix protein (M1) plus the ionic channel protein (M2) on segment 7, and nonstructural protein 1 (NS1) plus nuclear export protein on segment 8 (21,30). Phylogenetically, NS1 is further divided into alleles A and B that differ by more than 30% sequence identity. Allele A is found in viruses of both avian and mammalian origin, and is the more common of the two, whereas allele B occurs mainly in avian-origin viruses (1). The HA and NA glycoproteins are arranged in combination on the virus surface to form the H/N serotype; 16 X H and 9 X N glycoprotein serotypes have been discovered in birds, and these may theoretically occur in any combination. Because the AIV genome is segmented, co-infecting viruses are capable of producing hybrid progeny by reassortment (30). The HA cleavage site, HA_0, is an important virulence determinant required for the proteolytic cleavage of the HA protein into a functional structure for the entry of virus into cells. Low pathogenic avian influenza viruses (LPAI) are restricted to replicating in the epithelial cells lining the respiratory and intestinal tract; their HA_0 motif is cleaved by trypsin-like proteases secreted in these tissues. HPAI HA_0 is cleavable by furin-like proteases, present in a much wider variety of tissues, leading to multiorgan replication of the virus and hence greater pathogenicity. Only the H5 and H7 subtypes have been known to acquire the multibasic cleavage sites at HA_0 that define HPAI naturally, and the amino acid sequence at HA_0 is routinely determined during the identification of the viral subtype for pathotyping purposes and reporting (23).

The 2011 South African H5N2 HPAI outbreak in ostriches was brought under control and ceased at the end of 2011. Routine post-movement and biannual serological testing continued, with supplementary polymerase chain reaction (PCR) testing on serologically positive farms. From January 2012 to December 2014, more than 70 incidents of AIV in ostriches were reported. These included H5N2 and H7N1 LPAI in 2012, H7N7 LPAI in 2013, and H5N2 LPAI in 2014.

To resolve the molecular epidemiology in South Africa, the entire South African viral repository from ostriches and wild birds dating from 1991 to 2013 (42 viruses plus four H6N2 chicken strains) was resequenced with Illumina technology to obtain complete genomes for comparison. Protein sequences were analyzed for important genetic markers. To summarize the current status and better understand the factors affecting the susceptibility of ostriches to avian influenza, the phylogenetic results were supplemented with serological data for ostriches and AIV-detection data from surveillance of wild birds over the past 3 yr.

MATERIALS AND METHODS

Serological analysis. Ostrich sera submitted to Deltamune (Pty.) Ltd., Oudtshoorn, South Africa from 2012 to 2014 were tested with the use of the IDEXX Influenza A Ab Test Kit (IDEXX Laboratories [Pty.] Ltd., Johannesburg, South Africa) according to the recommended procedure. Positive ostrich sera were further tested by hemagglutination inhibition (HI) assays for H5-, H6-, and H7-specific reactions in accordance with the OIE-recommended procedure (23).

Viruses and RNA. Pooled tracheal and cloacal swabs in viral transport medium were collected from wild birds across South Africa (n = 1762) between April 2012 and December 2014. Stool Transport and Recovery (S.T.A.R.) buffer (Roche) was added to 200 µl of the sample and MagNAPure 96 External Lysis Buffer (Roche) prior to high-throughput extraction with the use of the DNA and Viral Nucleic Acid Small Volume kit on a MagNaPure (Roche). Species included Egyptian goose (*Alopochen aegyptiaca*), spur-winged goose (*Plectropterus gambensis*), yellow-billed duck (*Anas undulata*), red-billed teal (*Anas erythrorhyncha*), Cape teal (*Anas capensis*), white-faced whistling duck (*Dendrocygna viduata*), red-knobbed coot (*Fulica cristata*), common moorhen (*Gallinula chloropus*), and African sacred ibis (*Threskiornis aethiopicus*). Other species associated with farmed ostriches sampled in the Klein Karoo and Southern Cape Coastal region included cattle egrets (*Bubulcus ibis*) and feral pigeons (*Columba livia*). Several raptor and gull species from a regional rehabilitation centre were also tested.

Viral isolates in infective allantoic fluid, extracted RNA and AIV-infected tissues used in the study (Table 1) were provided by Deltamune (Pty.) Ltd., Stellenbosch Provincial Veterinary Laboratory, Klein Karoo International Research Laboratory, the University of Pretoria, and the Agricultural Research Council–Onderstepoort Veterinary Institute

Table 1. South African avian influenza strains in poultry and wild birds, 1991–2013.

Collection date	Strain	Serotype	Host	Region[A]	HA0	Full genome	Reference or source
1991	A/ostrich/South Africa/1991	H7N1	Ostrich	De Rust, WC and EC	PEIPKGR*GLF	Yes	GU052954-GU052960
August 1995	A/ostrich/South Africa/9508103/1995	H9N2	Ostrich	Klein Karoo district, WC	PAASYR*GLF	Yes	AF508705, AF508684, AF508662, AF508640, AF508618, AF508569, AF508596; this paper
May 1998 to July 2001	A/ostrich/South Africa/KK98/1998	H6N8	Ostrich	Leeu Gamka, WC	PIEPR*GLR	Yes	(3)
	A/ostrich/South Africa/2001	H10N1	Ostrich	Oudtshoorn, WC	PEIMQGR*GLF	Yes	This paper
July 2002	A/chicken/South Africa/AL19/2002	H6N2	Chickens	Botha's Hill, KZN	PQIEPR*GLF	Yes	This paper
October 2002	A/chicken/South Africa/AL25/2002	H6N2	Chickens	Verulam, KZN	PQIEPR*GLF	Yes	This paper
June 2004	A/Egyptian goose/South Africa/AI23/2004	H5N2	Egyptian goose	Oudtshoorn, WC	PQRETR*GLF	No	(6)
July 2004	A/ostrich/South Africa/N227/2004	H5N2	Ostrich	Middleton, EC	PQREKRRKKR*GLF	Yes	(6); this paper
April–July 2004	A/Cape teal/South Africa/1108/2004	H3N8	Cape teal/Cape shoveller	Blesbokspruit, GAU	PEKQTR*GLF	Yes	(4); this paper
April–July 2004	A/Red-billed teal/South Africa/1233A/2004	H4N8	Red-billed teal	Blesbokspruit, GAU	PEKASR*GLF	Yes	(4); this paper
April–July 2004	A/Yellow-billed duck/South Africa/811/2004	H5N1	Yellow-billed duck	Blesbokspruit, GAU	PQRETR*GLF	Yes	(4); this paper
June 2006	A/ostrich/South Africa/AI1091/2006	H5N2	Ostrich	Albertinia/Riversdale district, WC	PQRRKKR*GLF	Yes	(3); this paper
August 2006	A/ostrich/South Africa/AI1160/2006	H5N2	Ostrich	Oudtshoorn, WC	PQRETR*GLF	Yes	(3); this paper
August 2007	A/ostrich/South Africa/AI1447/2007	H6N8	Ostrich	Oudtshoorn, WC	PIETR*GLF	Yes	(5); this paper
June 2007	A/Egyptian goose/South Africa/AI1448/2007	H1N8	Egyptian goose	Baberspan, NWP	PSIQSR*GLF	Yes	(5); this paper
July 2008	A/Egyptian goose/South Africa/AI1556/2008	H4N2	Egyptian goose	Oudtshoorn, WC	PEKASR*GLF	Yes	(5); this paper
August 2008	A/ostrich/South Africa/AI1586/2008	H9N2	Ostrich	Oudtshoorn, WC	PAVSDR*GLF	Yes	(5); this paper
February 2009	A/Pekin duck/South Africa/AI1642/2009	H10N7	Pekin duck	Joostenburgvlakte, WC	PEIMQGR*GLF	Yes	(5); this paper
July 2009	A/ostrich/South Africa/AI1713/2009	H6N1	Ostrich	Worchester, WC	PQIETR*GLF	Yes	This paper
December 2010	A/ostrich/South Africa/AI1915/2010	H1N2	Ostrich	Oudtshoorn, WC	Unknown	No	This paper; (7)
March 2011	A/ostrich/South Africa/AI2114/2011	H5N2	Ostrich	Oudtshoorn, WC	PQRRKKR*GLF	Yes	(7)
June 2011	A/ostrich/South Africa/AI2214/2011	H5N2	Ostrich	Oudtshoorn, WC	PQRRRKKR*GLF	Yes	(7)
July 2011	A/ostrich/South Africa/AI2512/2011	H5N2	Ostrich	Oudtshoorn, WC	PQRRKKR*GLF	Yes	(7)
September 2011	A/ostrich/South Africa/AI2887/2011	H1N2	Ostrich	Stellenbosch, WC	PSIQSR*GLF	Yes	(7)
December 2011	A/ostrich/South Africa/AI3246/2011	H6N2	Ostrich	Oudtshoorn, WC	Unknown	No	(7)
February 2012	A/ostrich/South Africa/ C42 DKF/2012	H5N2	Ostrich	Uniondale, WC	PQRETR*GLF	No	This paper
March 2012	A/ostrich/South Africa/ORD/2012	H7N1	Ostrich	Graaf-Reinet, EC	PELPKGR*GLF	Yes	This paper
April 2012	A/Red-billed teal/South Africa/KZN002/2012	H11N2	Red-billed teal	Pongola Nature Reserve, KZN	PAIASR*GLF	Yes	This paper
May 2012	A/ostrich/South Africa/KVL/2012	H7N1	Ostrich	Mosselbay, WC	PELPKGR*GLF	Yes	This paper
June 2012	A/ostrich/South Africa/GWA/2012	H5N2	Ostrich	Heidelberg, WC	PQRETR*GLF	No	This paper
June 2012	A/ostrich/South Africa/MKT/2012	H7N1	Ostrich	Aberdeen, EC	PELPKGR*GLF	Yes	This paper
June 2012	A/ostrich/South Africa/FWS/2012	H7N1	Ostrich	Aberdeen, EC	PELPKGR*GLF	Yes	This paper
July 2012	A/ostrich/South Africa/OUD/2012	H7N1	Ostrich	De Rust, WC	PELPKGR*GLF	Yes	This paper
July 2012	A/Sacred ibis/South Africa/120701/2012	H7N1	Sacred ibis	Leeu Gamka, WC	PELPKGR*GLF	Yes	This paper
July 2012	A/Shellduck/South Africa/DLH/2012	H7N8	Shellduck	Graaf-Reinet, EC	PELPKGR*GLF	Yes	This paper
March 2012	A/chicken/South Africa/BKP/2012	H6N2	Chickens	Eastern Pretoria, GAU	PQIETR*GLF	Yes	This paper
October 2012	A/chicken/South Africa/NWY/2012	H6N2	Chickens	Eastern Pretoria, GAU	PQVETR*GLF	Yes	This paper

Table 1. Continued.

Collection date	Strain	Serotype	Host	Region[A]	HA0	Full genome	Reference or source
October 2012	A/Sacred ibis/South Africa/IZN/2012	H7N1	Sacred ibis	Pretoria, GAU	PELPKGR*GLF	No	This paper
February 2013	A/Yellow billed duck/South Africa STR0963/2013	H4N8	Yellow-billed duck	Strandfontein, WC	PERASR*GLF	Yes	This paper
February 2013	A/Cape shoveller/South Africa/STR0982/2013	H4N8	Cape shoveller	Strandfontein, WC	PERASR*GLF	Yes	This paper
April 2013	A/ostrich/South Africa/KRB/2013	H7N7	Ostrich	Oudtshoorn, WC	PELPKGR*GLF	Yes	This paper
April 2013	A/ostrich/South Africa/STV/2013	H7N7	Ostrich	Oudtshoorn, WC	PELPKGR*GLF	Yes	This paper
May 2013	A/ostrich/South Africa/AI3246 DWG/2013	H6N1	Ostrich	George, WC	Unknown	No	This paper
May 2013	A/ostrich/South Africa/DWG/2013	H7N7	Ostrich	George, WC	PELPKGR*GLF	Yes	This paper
May 2013	A/ostrich/South Africa/BDR/2013	H7N7	Ostrich	Oudtshoorn, WC	PELPKGR*GLF	Yes	This paper
June 2013	A/ostrich/South Africa/IMP/2013	H7N7	Ostrich	George, WC	PELPKGR*GLF	Yes	This paper

[A]Provinces: WC = Western Cape, EC = Eastern Cape, GAU = Gauteng, KZN = KwaZulu-Natal.

(ARC-OVI). RNA extraction and cDNA preparation for Illumina sequencing was performed as previously described (7).

In October 2012, about a dozen African sacred ibises with signs of progressive paresis and death were observed on a crocodile farm near Pretoria, Gauteng Province. Botulism was an important differential diagnosis, but RNA extracted from one spleen and two brain necropsy samples tested positive by real-time reverse transcription PCR (rRT-PCR) for the AIV group (M gene assay) and subsequently the H7 subtype. RNA was provided for this study.

In April 2013, 10 hunter-harvested pigeons were obtained from an ostrich farm at the same time that A/ostrich/South Africa/KRB/2013 (H7N7) was isolated. Trachea, spleen, liver, and kidney were collected aseptically into pools for each bird, and the pairs of feet were stored separately. Pigeon feet were swabbed and swabs were resuspended in 1 ml of phosphate-buffered saline for extraction of the RNA.

rRT-PCR. RNAs extracted from tracheal and oropharyngeal swabs, organ pools, and foot swabs were tested by rRT-PCR assay targeting the matrix protein gene. Probes and primers (27) were used in conjunction with a VetMAX^{TM}-Plus One-Step RT-PCR kit (Applied Biosystems, Austin, TX) and reactions were cycled on an Applied Biosystems Step One Plus machine (Life Technologies, Johannesburg, South Africa). PCR-positive samples were prepared and inoculated into embryonated chicken eggs, and tested for hemagglutinating activity in accordance with standard protocols (23).

Genomic and phylogenetic analysis. Genomic assembly and analysis was performed in CLC Genomics Workbench v 5.1.2. Paired-end reads were trimmed and gene segments were assembled by either an assemble-to-reference strategy using reference segments retrieved from GenBank, or alternatively by *de novo* assembly. Viral sequences were deposited in GenBank under the accession numbers DQ408509-DQ408516, EF041479-EF041502, EF591746-EF591761, FJ5919978-FJ519989, and GQ404695-GQ404732.

Multiple sequence alignments were prepared in BioEdit (18). Maximum-likelihood trees using full gene sequences were constructed with the use of MEGA v5.2.2 (28). Preliminary trees were initially constructed with South African sequences only. A representative from each clade was then used in a BLAST analysis to identify the closest relatives in GenBank. All reference sequences above a cutoff of 96% nucleotide sequence identity were incorporated into the multiple sequence alignment, trimmed, and maximum-likelihood trees inferred with the use of 1000 bootstrap replicates. Where full sequences were not available for South African AIVs (e.g., where sequences were obtained directly from sequencing of tissues; Supplemental Table S1), multiple sequence alignments were trimmed to remove gaps prior to phylogenetic construction.

RESULTS

Serological detection of AIV detection in ostriches, 2011–2014. Premovement, preslaughter, and compulsory biannual serological surveillance of ostriches was performed, to determine the exposure of ostriches to H5, H6, and H7 subtypes at $>10\%$ prevalence with 95% confidence in every epidemiological group. Fig. 1 provides a summary of total ELISA tests over the 3-yr period, with HI results for H5, H6, and H7. The decline in total ELISA tests from 2012 to 2014 was attributed to a significant reduction in slaughter numbers over the period. Serological prevalence (percentage positive ELISAs) of AI in ostriches was 3.7%, 3.6%, and 6.1% for 2012, 2013, and 2014, respectively. The increase in prevalence in 2014 is in line with a global trend in the emergence and spread of H5 and H7 AIV (unpublished data).

In Fig. 2, only the epidemiological groups containing positive sera were analyzed, providing firstly an indication of seasonality of AIV in ostriches and secondly, the relative frequencies of specific serotypes circulating in the country for certain years. An annual seasonal dip in incidence was evident around March/April (late summer/autumn) of each year, with peaks around July/August (mid to late winter). H7

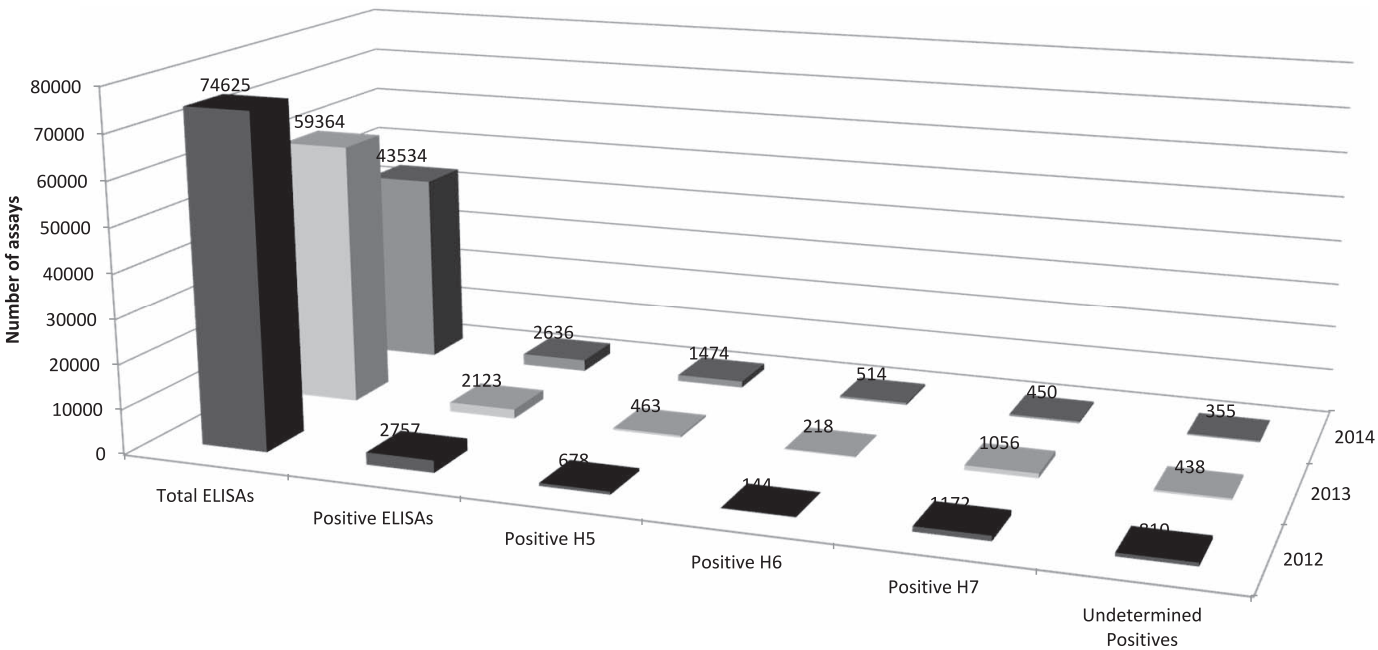

Fig. 1. Cumulative ostrich ELISA tests for 2012–2014 in South Africa. H5-, H6-, and H7-positive results were determined by HI assay. "Undetermined positives" refer to sera that were either negative for the H5/H7/H7 HI assays or below the threshold of a value considered to be positive for the HI assay.

showed some significant activity during 2013, but H5 became dominant in 2014. H6 and other serotypes were present at lower levels for most of the time, apart from a spike in H6 incidence on just a few farms around July 2013.

H7N1 and H7N7 viruses in Cape ostriches, 2012–2013. H7-specific serological reactions had been detected in the Western Cape Province since January 2012, but the first isolation of an H7N1 virus was made in March 2012 from ostriches on a farm near Graaff-Reinet, Eastern Cape Province. The HA_0 sequence of this virus, and all H7 ostrich strains isolated subsequently, was PELPKGRF, consistent with LPAI (Table 1). Two months later, H7N1 LPAI was identified in the Albertinia District of the Western Cape Province, and a virus was isolated from ostriches on this farm which is located near Mosselbay. Isolations of H7N1 LPAI continued in both provinces until July 2012. At the end of March 2013, a chick-raising farm near Oudtshoorn tested serologically positive for H7 during routine 28-day postmovement surveillance. In April 2013 an isolate was obtained from tracheal swab pools collected from these chicks, and was subsequently identified as an H7N7 LPAI virus by sequence analysis. The H7N7 incidents were restricted to the Western Cape Province, clustered around the Oudtshoorn region.

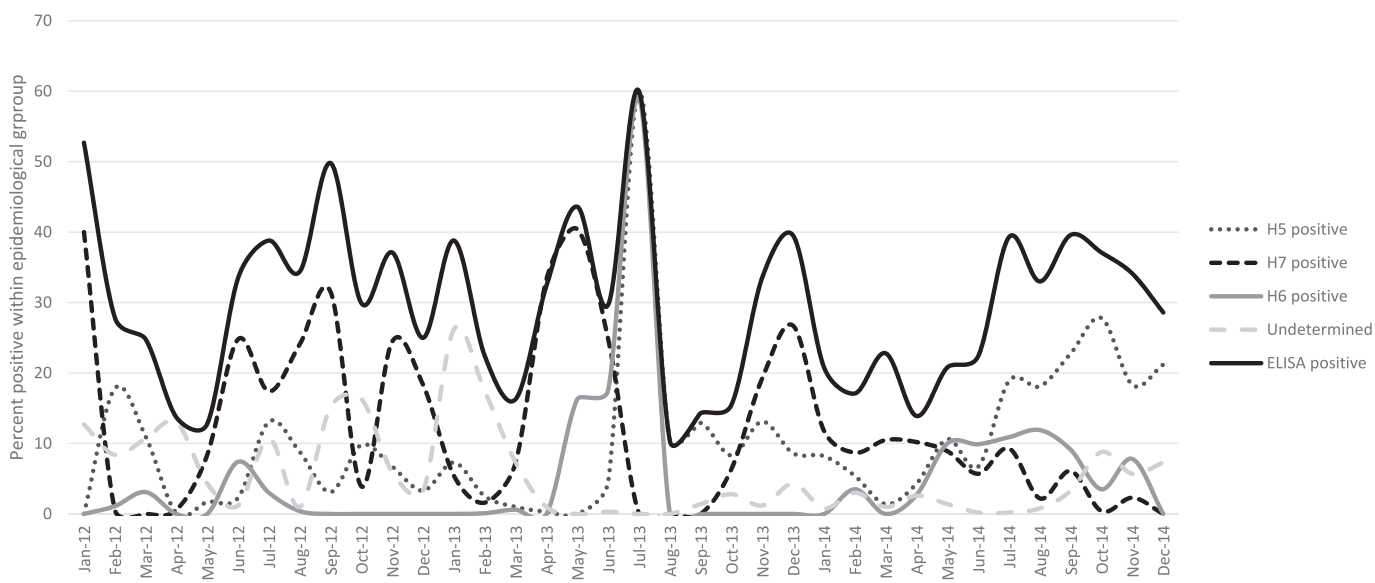

Fig. 2. Temporal distribution of H5-, H6-, and H7-specific antibodies in ostriches, as a percentage of the positive ELISA results obtained per positive epidemiological unit. "Undetermined positives" refer to sera that were either negative for the H5/H6/H7 HI assays or below the threshold of a value considered to be positive for the HI assay.

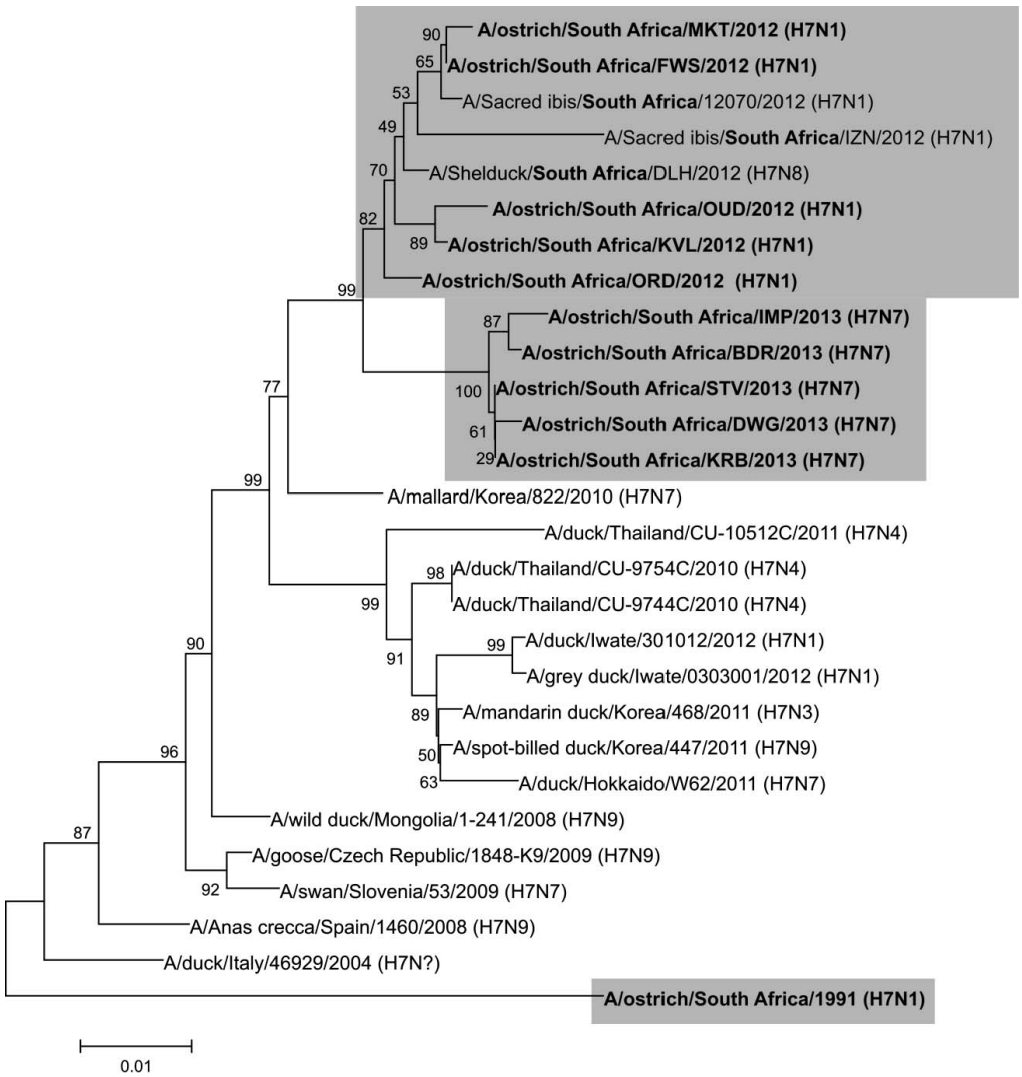

Fig. 3. Maximum-likelihood tree of full H7 HA sequences. South African strains are shaded.

The most prominent clinical observation on the H7-affected ostrich farms was poor appetite resulting in emaciation that was more pronounced in the 3–4-mo age group with weights below 40 kg. Older chicks and breeders exhibited signs of unthriftiness, poor appetite, and mild bilirubinuria. Postmortem examinations revealed mild to moderate bile stasis in the liver, severe serous fat atrophy, muscle atrophy, and dry intestinal content with severe bile stasis and staining in the gizzard and proventriculus. The most severely affected farms were those that received chicks towards the end of the chick production season (January–April). These chicks were poor growers with slow weight gain. Another prominent factor was a typically high population density of this age group of chicks. Mortalities were ascribed to a catabolic state with protein and energy deficiency rather than a viremia. Sporadic individual postmortem investigations showed lesion of secondary, opportunistic bacterial infections ranging from severe airsacculitis and tracheitis to fibrinous serositis and peritonitis. Mortality rates varied according to age, condition, management practices, and climatic conditions. Secondary infections contributed to increased mortality up to 30%. Older chicks and breeders showed low mortality of around 1%–3%. In contrast to H5N2, isolations of H7N1 and H7N7 viruses were easily made from ostrich swabs and tissues (Table 1).

Phylogenetically, all South African H7 viruses isolated since 2012 were rooted by a recent common ancestor (RCA), to which A/mallard/Korea/822/2010 (H7N7) was the closest relative (Fig. 3). The South African H7 viruses were further split into two clades: the H7N7 viruses from 2013, comprised solely of ostrich isolates from the Western Cape Province, and the H7N1 isolates from 2012. The index H7N1 virus isolated in March 2012 in the Eastern Cape Province, A/ostrich/South Africa/ORD/2012, and was basal to the H7N1 clade; within this clade, the Western Cape isolates A/ostrich/South Africa/OUD/2012 and A/ostrich/South Africa/KVL 2012 clustered together. Similarly, the other two Eastern Cape isolates from June 2012, A/ostrich/South Africa/MKT/2012 and A/ostrich/South Africa/FWS/2012 clustered together, but basal to this subclade were two sacred ibis viruses, discussed below. Similar topology was observed in the N1 gene tree (Supplemental Fig. S1), and in the PB2 genes, where the index H7N1 virus shared 98.6% sequence identity with another wild bird isolate, A/Shelduck/South Africa/DLH/2012 (H7N8). Phylogenetically, it seems likely that wild birds were responsible for the point introduction of H7N1 to ostriches in both provinces.

The H7N7 LPAI strains from 2013 contained internal genes that were phylogenetically distinct from those of the H7N1 viruses (Supplemental Figs. S2–S8), and all internal genes of the H7N7 strains shared RCAs with wild duck viruses isolated in 2012 and 2013, namely, A/Red-billed teal/South Africa/KZN002/2012 (H11N2),

and/or A/Yellow-billed duck/South Africa/STR0963/2013 (H3N8) plus A/Cape Shoveler/South Africa/STR0982/2013 (H3N8). Furthermore, the ostrich H7N7 strains' NS1 genes fell within the Allele-A clade, and the H7N1 NS1 genes were classified as Allele B, evidence that the H7N7 outbreak was as a result of an independent point introduction from wild birds to ostriches (Table 1; Supplemental Figs. S7, S8). Feral pigeons observed to be associating with ostriches on an H7N7-positive ostrich farm during the outbreak in 2013 were sampled and tested. One of 10 paired pigeon foot swabs was H7 positive on rRT-PCR, but none of the tissues tested positive. Free-flying wild birds may thus have aided in fomite transmission of the H7N7 infection between closely situated ostrich farms, but pigeons and doves on the farm are not expected to have been carriers and multipliers of the virus (2).

LPAI H7N1 in African sacred ibises. Two cases of H7N1 LPAI in sacred ibises were recorded for the first time**.** A/Sacred Ibis/South Africa/120701/2012 was isolated in July 2012 on an ostrich farm in the Leeu Gamka district of the Western Cape. The bird, culled for disease surveillance purposes, appeared to be healthy, with no signs of neurological disease. Phylogenetically, this virus grouped closely with the ostrich H7N1 viruses isolated from Eastern and Western Cape provinces ostriches in 2012 (Fig. 3; Supplemental Figs. S1–S8). An interesting feature of this isolate was the presence of the E627K mutation in the PB2 gene (Fig. 3). PB2 E627K is a well-recognized mammalian adaptation marker found in human isolates of the H5N1 and H7N7 subtypes. PB2 E627K was associated with increased transmission of HPAI H5N1 in humans, essentially by the increased viral polymerase activity at lower temperatures, driving faster transcription of viral genes and hence production of progeny at a higher rate. Other amino acid substitutions in PB2, e.g., T271I, Q591K, and D701N, can partially substitute for the function of E627K (15). E627K is known to be generated in ratites (26) but not waterfowl. The presence of E627K in an African sacred ibis on an ostrich farm provides circumstantial evidence for ostriches reinfecting ibises, and a possible bridge species role for ibises during outbreaks.

In October 2012, the second case of H7N1 LPAI in an African sacred ibis was detected in the Gauteng Province, about 1400 km northwest of the Leeu Gamka district. Virus isolation of A/Sacred Ibis/South Africa/IZN/2012 was unsuccessful, but direct deep sequencing on brain tissue produced 23.08% of the total genome sequence for comparison (Supplemental Table S1). A/Sacred Ibis/South Africa/IZN/2012 too was interspersed with wild bird and ostrich H7N1 strains from 2012, suggesting that LPAI H7N1 was widespread in wild birds throughout Southern Africa in 2012.

Phylogenetic relationships and origins of HPAI H5N2 and LPAI H5N2 viruses. An in-depth study of the phylogeny of H5N2 viruses was carried out by analyzing the complete genome sequences. The reassortment patterns and RCAs with Eurasian strains reaffirmed that the 2004, 2006, and 2011 HPAI H5N2 outbreaks in ostriches were unrelated (Fig. 4; Supplemental Figs. S2–S9). Detection of H5N2 by PCR and serology continued throughout 2012 and into 2014, but no isolates could be made. Direct deep sequencing on A/ostrich/South Africa/C42 DKF/2012 (March) and A/ostrich/South Africa/GWA/2012 (June) yielded 88.23% and 55.14% of the total genome sequences, respectively (Supplemental Table S1). Both were confirmed to be LPAI according to the PQRETRGLF motif at HA0. Evidence that these two H5N2 LPAI viruses were new point introductions to ostriches was provided in the phylogenetic analyses. In Fig. 4, the HA genes of the two H5N2 LPAI strains from 2012 shared an RCA with viruses isolated in Nigeria, the Czech Republic and Italy (2008–2012). In the PB2 and PA gene trees, for example,

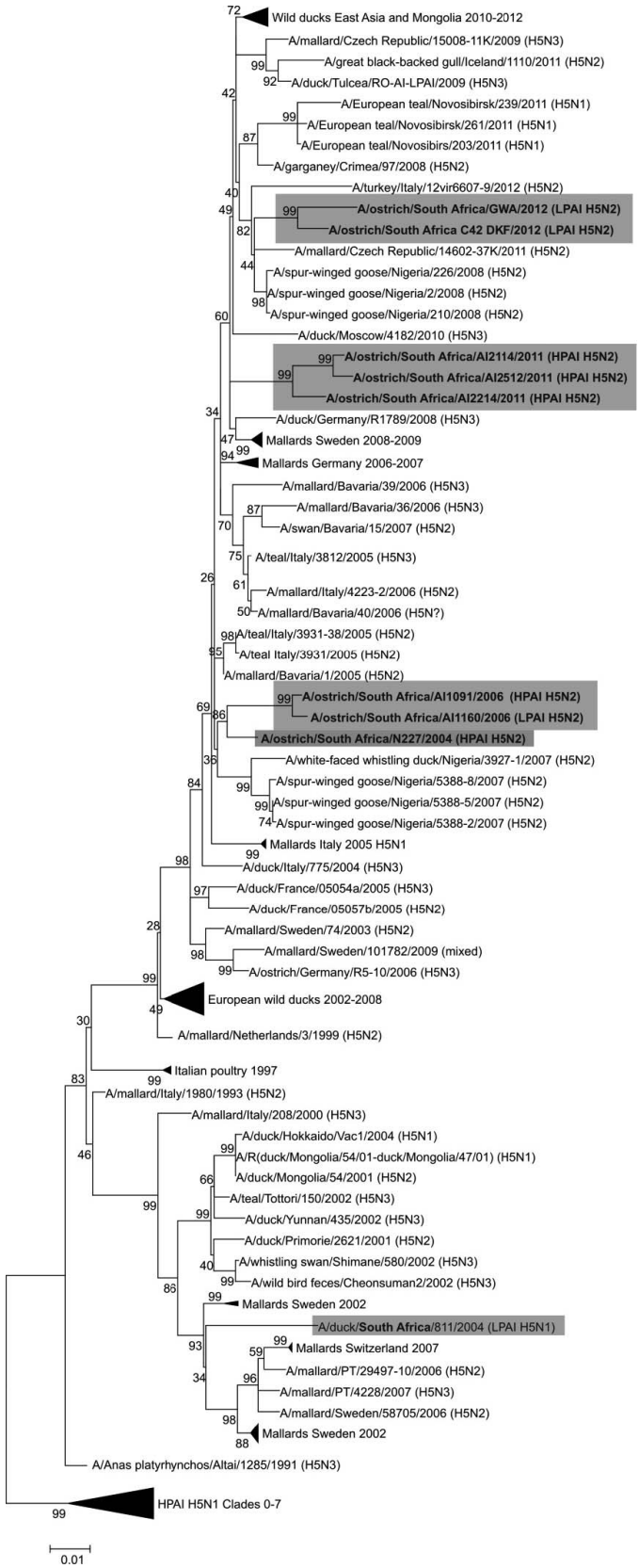

Fig. 4. Maximum-likelihood tree of H5 HA genes. South African HPAI H5 strains are shaded in dark grey, and LPAI H5 strains in light grey.

A/ostrich/South Africa/GWA/2012 grouped most closely with the H7N1 LPAI ostrich strains from 2012 (Supplemental Figs. S2, S4).

Evolutionary relationships of southern African viruses. The increase in international avian influenza sequences in public sequence databanks enabled a comprehensive analysis of the evolutionary relationships of southern African viruses. Twenty-two new isolates from ostriches and wild birds were sequenced, and genomes were completed for strains of which only partial sequences were previously available (Supplemental Figs. S1–S9). A common feature of all the phylogenetic trees was that southern African viruses did not form a monophyletic clade for each gene segment; rather, the southern African viruses were interspersed in a temporal distribution with Eurasian viruses. Although the genetic database still reflects a bias toward those countries that undertake active surveillance in wild ducks, many of the strains with which southern African viruses shared RCAs were sampled in northern and eastern Europe and central Russia. Although there is little evidence for introductions of AIVs by Eurasian migrants into southern Africa, the first recorded case of an AIV in South Africa was from a migratory species, the swift tern (*Sterna hirundo*) (8). Intensive sampling at the Banc d'Arguin in Mauritania indicated that even in West African wetlands, where mixing with European waterfowl would be common, the prevalence of LPAIV strains is relatively low; only red knot (*Calidris canutus*), some tern species (Caspian tern [*Sterna caspia*], royal tern [*Sterna maxima*], and Sandwich tern [*Sterna sandvicensis*]), and a small group of waders/shorebirds (curlew sandpiper [*Calidris ferruginea*], little stint [*Calidris minuta*], common ringed plover [*Charadrius hiaticula*], Eurasian oystercatcher [*Haematopus ostralegus*], bar-tailed godwit [*Limosa lapponica*], grey plover [*Pluvialis squatarola*], common redshank [*Tringa tetanus*], grey-headed gull [*Chroicocephalus cirrocephalus*], black-headed gull [*Chroicocephalus ridibundus*]) had AIV prevalences >1% and no species had prevalence >1.7% (17). Many of these species are migrants that might enter southern Africa after a stopover in West Africa; although documented prevalences of AIVs are low, they probably represent the most likely transmission route for AIVs from Europe to southern Africa. Alternatively, a small number of ringing recoveries suggest that knob-billed ducks (*Sarkidiornis melanotos*) may move between southern and western Africa. Such a transmission pathway would, however, require a multiple-step transmission process, because knob-billed ducks do not migrate to Europe. It is evident from the randomization of gene segments between different clades of southern African viruses that extensive reassortment had taken place. Reassortment of the internal gene segments is an indication of the mixing of viral populations, and by extension, mixing of wild bird populations. Southern African waterfowl breed in isolated ephemeral wetlands during the wet season, following which they undertake long-distance annual movements to form large molting aggregations at permanent water bodies (14,20). Moult sites, where birds from different populations share resources in close proximity, provide areas with high potential for virus reassortment. Between January and April 2012 and December 2014, 1762 pooled cloacal and oropharyngeal swabs from wild birds, sampled across South Africa, were tested for AIV. Ninety-eight (5.6%) of these were PCR positive for the AIV group, and two H3N8 strains were isolated from wild ducks at Strandfontein sewage treatment facility in the Western Cape Province. H5/H7 subtyping was not performed.

The sources of the periodic infections in ostriches were incontrovertibly the wild bird population, as evidenced by nodes with RCAs and high percentage sequence identity between genes. Contact transmission of AIV from wild birds in the ostrich-farming districts have been limited as far as possible by chlorination of drinking water and fencing off canals and rivers, but the availability of open feed in the camps and alfalfa pastures favored by grazing ducks such as Egyptian geese still allows contact between the species. In July 2012, 6 out of 11 sera samples from wild Egyptian geese and one sample from a South African shelduck on an ostrich farm in Aberdeen, Eastern Cape Province, tested positive on AI ELISA. Subsequently, HI tests were positive for H5-specific reactions. Although the organ samples from the wild ducks tested negative on PCR for AIV, detection of H5-specific antibodies indicated a prior infection of these wild ducks with H5 virus and a circumstantial link between wild ducks and ostriches at the time of the outbreak on the farm. Other serological evidence of H5 infection in wild ducks on ostrich farms was detected in March in 3 out of 10 Egyptian goose sera in Graaff-Reinet, Eastern Cape Province, and towards the end of July in 2 out of 12 Egyptian goose samples in Oudtshoorn.

Supporting data for the source of endemic H6N2 strain in chickens. The geophysical separation of ostrich- and chicken-producing regions and a lack of opportunities for contact between chickens and wild or domestic waterfowl (reviewed in 12) has meant that the South African chicken population has, to date, only been infected with H6N2. The earliest H6N2 chicken strain was traced back to June 2001 (3). The outbreak in chickens is thought to have started when a flock of infected spent layer hens was transported from the Western Cape Province to the KwaZulu-Natal Province, where the infection established in backyard/village chickens and was introduced via poor biosecurity by poultry workers onto a commercial farm, resulting in an epidemic that swept the country (3) and continues to date. The hypothesis is that the chickens had contracted the H6N2 strain in the Western Cape Province, through contact (a fomite source is a strong possibility) with a strain that originated in ostriches in the region. Strong phylogenetic evidence of the genetic relatedness of all eight chicken H6N2 virus gene segments pointed to RCAs of two viruses isolated from ostriches in the 1990s (H6N8 and H9N2; Table 1) as the donors. The phylogenetic evidence was contested by the ostrich industry, but here, analyzing longer gene lengths of the full genes of H6N2 chicken-origin strains and its hypothetical progenitors enabled more robust phylogenetic analysis. A/ostrich/South Africa/2001 (H10N1) was included in the analysis for the first time. The H10N1 ostrich virus is used in many international laboratories, but only the HA and NA sequences had been available in GenBank. The PB1 and NP gene sequences of A/ostrich/South Africa/2001 (H10N1) determined in the present study shared a more recent common ancestor with the 2002 H6N2 chicken outbreak strains than the H6N8 and H9N2 ostrich virus progenitors reported previously (Supplemental Figs. S3, S6). This H10N1 ostrich strain, isolated in the Oudtshoorn region around the same time that the H6N2 chicken strain is thought to have emerged by reassortment in the Western Cape Province, is the third ostrich virus with high sequence homology to the original chicken outbreak strain. This revised phylogeny supports the previous finding that the progenitor of the chicken H6N2 outbreak strains was a product of reassorted ostrich-origin viruses. Today, the H6N2 infection in chickens is a monophyletic clade, with long branch lengths typical of sustained circulation over a long period (Supplemental Figs. S2–S9).

Analysis of molecular virulence and host-specific markers. The amino acid sequences of the H7N7 and H7N1 HA and NA genes, and the internal protein-encoding genes of the total strains in Table 1, were examined for known virus-specific factors that are associated with zoonotic and pandemic potential (data not shown). One H7N1 virus, A/ostrich/South Africa/ORD/2012, contained the A143T marker in the H7 protein gene, which was demonstrated to increase replication efficiency of an H7N7 strain in human respiratory tissues by enhancing the binding capability of the virus (15). An H7N7 virus, A/ostrich/South Africa/DWG/2013, contained the

T314I mutation that was implicated in enhanced receptor binding, one of three mutations in a genetically modified H5N1 strain that facilitated the airborne transmission in ferrets (19).

Seventeen of 27 (63%) ostrich viruses in Table 1 contained the PB2 E627K marker (Supplemental Fig. S10). Two of the ostrich isolates that did not contain E627K, A/ostrich/South Africa/OUD/2012 (H7N1) and A/ostrich/South Africa/AI2887/2011 (H1N2), contained the compensatory Q591K mutation, and the 2004 HPAI H5N2 strain lacked E627K but contained the D701N mutation. The only nonostrich viruses that contained E627K or its compensatory mutations were A/Sacred ibis/South Africa/120701/2012 (H7N1) (E627K) as discussed above and the H6N2 chicken outbreak strain A/chicken/South Africa/AL19 (2002) (D701N), although the latter was previously demonstrated to have originated from ostriches through phylogenetic analysis (3).

In the PA protein sequences (data not shown), A/ostrich/South Africa/1991 (H7N1) contained a T85I mutation, previously associated with the enhancement of avian polymerase viral activity of H1N1 in mammalian cells (9). Finally, in the NP protein, A/ostrich/South Africa/AI2887/2011 (H1N2) contained a N319K mutation linked to the increased binding of mammalian α-1 proteins and polymerase activity of H7N7 viruses (16).

DISCUSSION

The phylogenetic analysis confirmed several general findings from previous studies: 1) South Africa is at the end of a transmission pathway, through which avian influenza viruses of Eurasian origin are periodically introduced to the region by wild birds; 2) populations of avian influenza viruses circulated in southern African waterfowl populations for periods of up to 6 yr; 3) all ostrich outbreaks or point introductions could be linked to wild bird viruses circulating in the region; and 4) ostriches were the likely original source of the H6N2 strain, introduced to chickens in the early 2000s, which is still endemic in the chicken population.

An AIV prevalence of 5.6% in wild birds was recorded between 2012 and 2014, which is considerably higher than AIV prevalence for the southern African region of 2.5% to 3.6% reported in the period 2007–2009 (10,13), although sampling, storage, and testing procedures remained similar. A higher viral load in the local waterfowl population may explain the frequency of point introductions to ostriches since 2012, but the factors that might be driving this trend are unknown. Further to the detection of H7N1 LPAI in sacred ibises, numerous healthy endemic and exotic ibises in open aviaries in the National Zoological Gardens in Pretoria, Gauteng Province, tested positive for AIV on rRT-PCR, where other orders in similar housing remained negative (data will be published elsewhere). The role of the *Ciconiiformes* as hosts permissive for AIV replication in subclinical infection, like the *Anseriformes* and *Charadriiformes,* is emerging. This is of concern, because sacred ibises, unlike ducks and waders, might feed on AIV-infected carrion, yet still mingle with other waterfowl at open water sources, and they are often observed on poultry farms. Populations of migratory European white storks (*Ciconia ciconia*) also appear to be growing, and these birds are increasingly observed on ostrich farms and landfills across the country.

It is thought that wild birds played a key role in the spread of H7N1 LPAI in ostriches in two provinces during 2012, and phylogenetic evidence supported this. The H7N7 LPAI outbreaks in 2013, however, appear to have originated from a single introduction from wild birds with subsequent spread between ostriches and farms. Other wild bird species may have acted as carriers of the H7N1 LPAI virus (African sacred ibis) or as short-range fomite vectors between farms (feral pigeons). The H7N1 and H7N7 LPAI outbreaks generally presented with more severe clinical signs than H5N2 HPAI, but no evidence of conversion to H7 HPAI at HA_0 was evident at the subgenomic level (data not shown). Extended circulation of H7 LPAI in ostriches (e.g., 1991) has never resulted in H7 HPAI in South Africa or internationally, raising an interesting question of whether H7 is capable of conversion to HPAI in the ostrich—this warrants further scientific investigation.

Innate susceptibility of ostriches to infection with waterfowl-origin AIV, and the ways in which their unique immune system and physiology affect the virus, will be future key research areas for ostriches. Little is known about the replication of AIV in ostriches under natural conditions. The innate immune system acts as the first line of defense against invading viruses and plays an important role in the subsequent activation of antiviral responses, of which the potent antiviral molecule type-I interferon (IFN) is a prime example. Chickens, unlike ducks, geese and finches, lack a retinoic acid-inducible gene I (RIG-I)-like receptor. The expression of IFN-β following avian influenza infection is mainly dependent on the expression of RIG-I, and the lack of this receptor may be a contributing factor to the higher resistance of ducks than chickens to HPAI infections (11). It would be interesting to know whether ostriches and other ratites express RIG-I–like receptors and whether this accounts for their apparent lack of clinical symptoms during infection.

The propensity of ratites to select for the E627K marker was experimentally demonstrated *in vitro* and *in vivo* (26); viruses possessing the mammalian-type E627K mutation were positively selected following passage in ostrich embryo fibroblast cells, but not chicken embryo fibroblasts, and after 3 days in 3-wk-old ostrich chicks infected experimentally. AIVs are adapted for replication in the avian enteric tract at 40–41 C, and both human and avian influenza viruses are able to infect efficiently at 37 C (the temperature of the human distal airways). AIVs are, however, restricted for infection at the cooler temperatures of the human proximal airways at 32 C, which can be overcome by the mutation of E627 to lysine, demonstrated with the use of a ferret model (24). The core body temperature of ostriches, unlike most other avian species, is slightly below 40 C. As birds do not have sweat glands, they rely on increased evaporation from the respiratory system by panting for heat dissipation. Ostriches are able to maintain body temperatures below 40 C during 8 hr at ambient temperatures as high as 50 C (25). The surface temperatures of the respiratory tract of an adult ostrich kept at an air temperature of 40 C was measured at 34 C 10 cm from the glottis (upper trachea), 35 C 40 cm from the glottis (mid trachea) and 36 C 60 cm from the glottis (lower trachea) (25). Ostriches therefore maintain an unusually low mid- to upper tracheal temperature as part of their adaptive physiology, which may explain why the E627K mutation or its compensatory mutations, which facilitate AIV replication at lower temperatures, are selected for in ostriches.

As far as susceptibility to AI is concerned, ostriches are frequently exposed to AIVs in their environment, based on the prevalence of AIV detected in wild birds, and ostriches frequently become infected based on continuous serological positive results detected during routine surveillance of ostrich flocks. Ostriches are also able to manifest infections (both HPAI and LPAI) with minimal clinical signs, precluding factors such as inclement weather and poor management practices. Significant improvements by the industry have helped to mitigate point introductions, and the use of the more sensitive high-throughput ELISA has aided in detecting point introductions more quickly. At the time of writing, the ban of export on fresh ostrich meat by the European Union was still in force, but the industry is adapting to the *status quo* through the use of innovative heat

treatment for ostrich meat products. For the time being, it seems that avian influenza in extensively farmed ostriches is a reality that producing countries must cope with through constant and rigorous management.

Supplementary data associated with this article can be found at http://dx.doi.org/10.1637/11110-042815-Reg.s1.

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