Outbreaks of avian influenza H6N2 viruses in chickens arose by a reassortment of H6N8 and H9N2 ostrich viruses

C. Abolnik
ARC-Onderstepoort Veterinary Institute, Private Bag X5, Pretoria 0110, South Africa / Department of Zoology, University of Pretoria, Pretoria 0002, South Africa. e-mail: abolnikc@arc.agric.za
T. Gerdes
ARC-Onderstepoort Veterinary Institute, Private Bag X5, Pretoria 0110, South Africa
S. Bisschop
Poultry Reference Laboratory, University of Pretoria, Pretoria 0002, South Africa
A. Olivier
Klein Karoo Group, Ostrich Laboratory, PO Box 241, Oudtshoorn 6620, South Africa
R. Horner
Allerton Provincial Veterinary Laboratory, 458 Town Bush Road, Montrose, Pietermaritzburg 3201, South Africa / Vetdiagnostix Laboratory, PO Box 13624, Cascades, Pietermaritzburg 3202, South Africa

The nucleotide sequence data reported in this paper have been submitted to the GenBank nucleotide sequence database andhave been assigned the accession numbers DQ408506-DQ408529.

Abstract

The first recorded outbreak of avian influenza (AI) in South African chickens (low pathogenicity H6N2) occurred at Camperdown, KwaZulu/Natal Province (KZN) in June 2002. To determine the source of the outbreak, we defined the phylogenetic relationships between various H6N2 isolates, and the previously unpublished gene sequences of an H6N8 virus isolated in 1998 from ostriches in the Leeu Gamka region (A/Ostrich/South Africa/KK98/98). We demonstrated that two distinct genetic H6N2 lineages (sub-lineages I and II) circulated in the Camperdown area, which later spread to other regions. Sub-lineages I and II shared a recent common H6N2 ancestor, which arose from a reassortment event between two South African ostrich isolates A/Ostrich/South Africa/9508103/95 and (H9N2) /Ostrich/South Africa/KK98/98 (H6N8). Furthermore, the H6N2 sub-lineage I viruses had several molecular genetic markers including a 22-amino acid stalk deletion in the neuraminidase (NA) protein gene, a predicted increased N-glycosylation, and a D_{144} mutation of the HA protein gene, all of which are associated with the adaptation of AI viruses to chickens. The H6N2 NS1 and PB1 genes shared recent common ancestors with those of contemporary Asian HPAI H5N1 viruses. Our results suggest that ostriches are potential mixing vessels for avian influenza viruses (AIV) outbreak strains and support other reports that H6 viruses are capable of forming stable lineages in chickens.

Introduction

In June 2002, avian influenza (AI) was detected for the first time in commercial chickens at Camperdown in the KwaZulu/Natal (KZN) Province of South Africa. The virus was initially typed as a low pathogenic avian influenza (LPAI) H6N2 strain at the VLA Weybridge laboratory, UK. In South Africa, testing of archival material from the same region traced the infection back to June 2001, but the exact source of the H6N2 outbreak was never determined.Most outbreaks ofAI in domestic poultry are thought to have originated by the transfer of viruses from feral birds [1, 2]. Chickens are generally not considered to be natural hosts for AI viruses and H6 viruses have never been associated with serious disease in poultry. It has been suggested that H6 viruses may be capable of developing stable lineages in chickens [3]. Several H6N1 and H6N2 outbreaks have been recorded in recent years in South-Eastern Asia and California [4,5]. Outbreaks of LPAI in ostrich-producing areas of the Western, Northern and Eastern Cape Provinces, have been reported sporadically in the winter months. H7N1 was isolated from ostriches in South Africa in 1991, H5N9 in 1994, H9N2 in 1995, H6N8 in 1998, and H10N1 in 2001. The first outbreak of a highly pathogenic (HPAI) H5N2 virus in South Africa was recorded in ostriches in 2004.

The objective of this study was to elucidate the phylogenetic relationships of H6N2 chicken virus isolates and to determine the origins of the outbreak, by phylogenetically comparing them to viruses isolated in other countries. The previously unpublished gene sequences of the 1998 ostrich virus that was isolated at the Klein Karoo Laboratory (South Africa) and typed at the VLA laboratory, Weybridge (UK) as H6N8, were also analyzed. The results showed that two separate sub-lineages of H6N2 chicken viruses circulated during the outbreak period, and that the common ancestor of these viruses was produced by a reassortment between the 1995 South African H9N2 ostrich virus and the 1998 South African H6N8 ostrich virus.

Materials and methods

Viruses

Homogenates of organs from infected birds were inoculated into the allantoic cavity of 10-day-old embryonated chicken eggs. Haemagglutinating agents were tested for and avian influenza viruses (AIV) were identified by specific haemagglutination inhibition with known avian influenza anti-sera and by RT-PCR. Thirty-two of the H6N2 isolates collected from June 2001 to March 2005 were analyzed in this study.
RNA extraction and RT-PCR Viral RNA was extracted from allantoic fluid using TRIzol_ reagent (Gibco, Invitrogen), or with the QIAamp Viral RNA mini kit (Qiagen), according to the manufacturer's instructions. Reverse transcription was performed with M-MLV reverse transcriptase (Promega) at 42_C for 90 min on 5 /l of extracted viral RNA, using gene-specific oligonucleotides (6, 7; D.Suarez, personal communication). The same genespecific oligonucleotides and a GeneAmp 2400 PCR System (Perkin Elmer) were used for PCR according to the published thermo-cycling parameters [6, 7].

Gene sequencing

PCR products were purified with the QIAquick_ Gel Extraction Kit (Qiagen), and quantified with a NanoDrop_ ND-1000 Spectrophotometer (NanoDrop Technologies, Inc, USA). BigDye_ Terminator V3.1 chemistry (Perkin Elmer/Applied Biosystems) and gene-specific primers were used for cycle sequencing, according to the manufacturer's instructions. Reactions were run on a 3130 Genetic Analyzer (Applied Biosystems).

Phylogenetic analysis

Sequences were visualized with Chromas Lite 1.0 software (http://www.technelysium.com.au) and edited with BioEdit V.7.5.0.2 [8]. BLAST homology searches (www.ncbi.nlm.nih.gov/blast) were used to retrieve the top 50 homologous sequences for each gene from the GenBank database. For the South African H6N2 HA genes, the region analyzed corresponds to nucleotides 838–954 (116 nt) of the complete 1744 nucleotide coding region for H6 genes [6]. The phylogenetic topology for the 116 nt region is similar to that found in the full-length HA sequences of some of the South African H6N2 H6 genes and was thus deemed to be a suitable size for phylogenetic comparison (data not shown).
Multiple sequence alignments were prepared with ClustalW (http://www.ebi.ac.uk/clustalw/index.html) and phylogenies were reconstructed with MEGA 3.1 software [9] using the Neighbor-Joining tree inference method, with 1000 bootstrap replicates to assign confidence levels to branches.
Potential N-glycosylated sites were predicted using the NetNGlyc 1.0 Server ttp://www.cbs.dtu.dk/services/NetNGlyc/).

Results

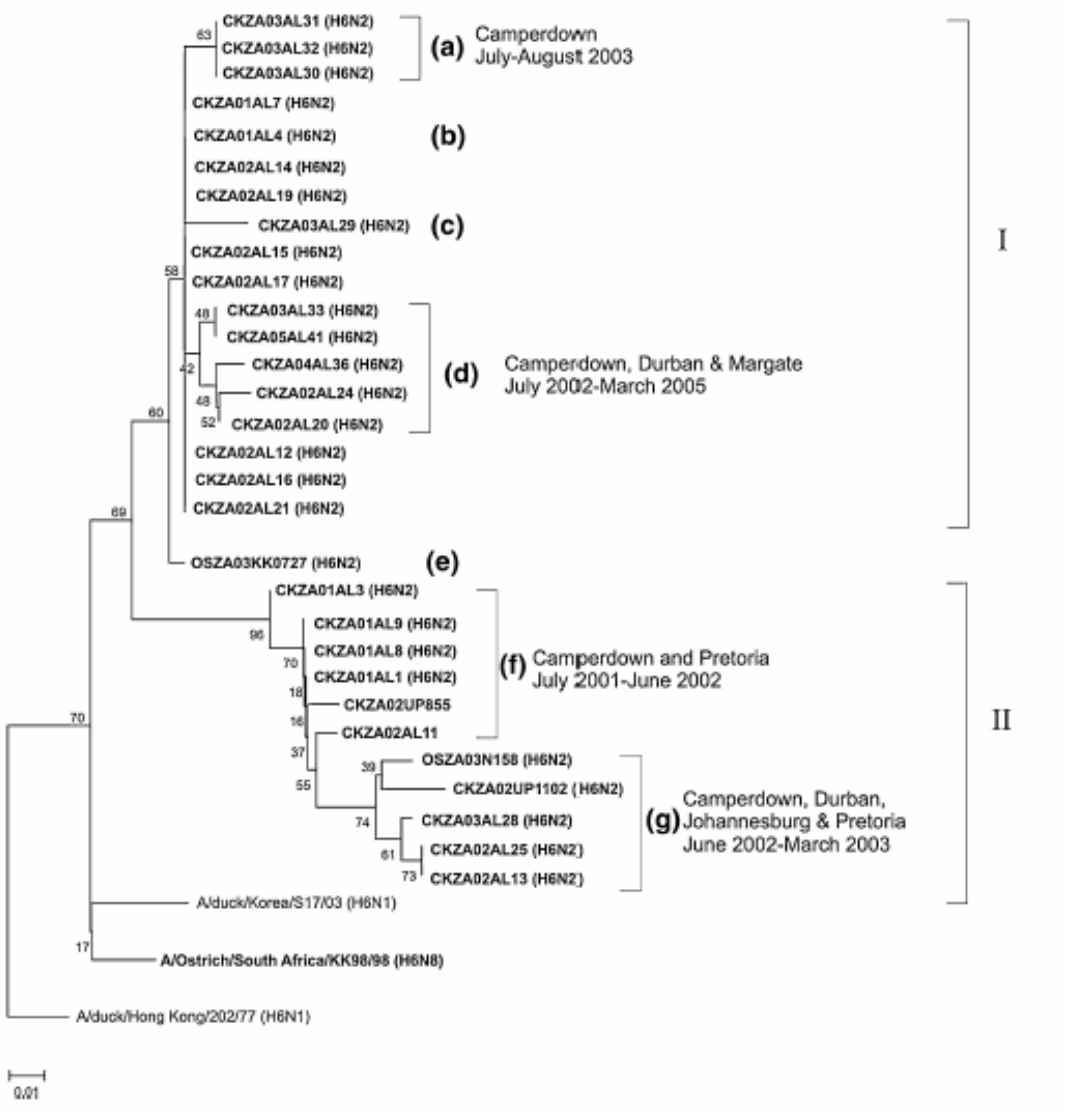
Molecular epidemiology of the South African H6N2 chicken outbreak strains

Two distinct sub-lineages (I and II) of H6 AIVs (Fig. 1) circulated during the South African outbreak. Sub-lineage I viruses were isolated only at commercial operations in the KZN Province. The isolates of I(a), CKZA03AL30 and –31 were isolated within a week of each other from the same Camperdown farm, and CKZA03AL32 almost 5 weeks later from a farm near the neighboring town of Hammarsdale. The isolates of I(b) were isolated from 2001 to 2002, mostly from the original sites in Camperdown, but also from the Botha's Hill near Durban (CKZA02AL14 and CKZA02AL19). CKZA02AL14 was isolated from a farm in Empangeni situated about 170 km up the North Coast, but the farmer had visited a poultry farm in the Camperdown area a week previously. The isolates of I(d) originated in Camperdown (CKZA02AL16; July 2002), then appeared in Durban a month later (CKZA02AL24), before being detected in Margate about 150 km away on the South Coast at the end of September 2003 (CKZA03AL33). There it persisted throughout 2004 (CKZA04AL36) until the last known isolate of the H6N2 outbreak was collected in March 2005 (CKZA05AL41). The ostrich virus OSZA03KK0727 (I(e)) is an outlier to sub-lineage I and was isolated in the Oudtshoorn region in September 2004.
Sub-lineage II circulated over the same time period as sub-lineage I viruses but contains viruses isolated from both the KZN Province and the Gauteng Province. CKZA02UP855 (II(f)) was isolated in Pretoria in September 2002, and OSZA03N158 (II(g)) from ostriches near Johannesburg in March 2003.
Analysis of the peptide sequences surrounding the neuraminidase (NA) stalk region (data not shown) indicated that sub-lineage I viruses all contained a 22-amino acid deletion in the stalk region, which was absent from sub-lineage II viruses. A representative from sub-lineage I, A/Chicken/South Africa/AL19/02 (H6N2) and one from sub-lineage II A/Chicken/SouthAfrica/UP1102/02 (H6N2) were selected for further comparison.

The origins of the H6N2 chicken virus genes

Phylogenetic analyses of the surface glycoprotein and internal genes of two chicken H6N2 and one ostrich H6N8 viruses were conducted (Figs. 2–4) and the proposed origins of these genes are summarized in Fig. 6. In all genes the H6N2 chicken viruses shared a recent common ancestor. The closest relative to the H6N2 HA was the H6N8 ostrich virus HA gene (Fig. 2), and the closest N2 ancestor was the H9N2 N2 gene (Fig. 4). The H6N8 NA gene (Fig. 3) shared recent common ancestors with contemporary northern European H3N8 viruses, but the virus probably derived its M, NP, PB1 and PA genes from the H9N2 1995 ostrich virus (Fig. 5).

Fig. 1 Phylogenetic tree of the H6 HA nucleotide sequences (116 nt). The tree is rooted with A/duck/Hong Kong/202/77 (H6N1). South African viruses isolated and sequenced in the present study are in boldface, and sub-lineages I and II and (a)–(g) are indicated. The names contain the following information: CK-ZA-03-AL28 = host-country-yearlab number; where CK = chicken, OS = ostrich, and ZA = South Africa



The internal genes of only two other virus isolates had closer phylogenetic relationships to the H6N2 chicken viruses. The A/chicken/Taiwan/7-5/99 (H6N1) NS1 gene appears to be most closely-related to those of the chicken H6N2 viruses, but at the nucleotide level, higher nucleotide sequence identities were observed with the ostrich NS1 gene (95–97%) than with the Taiwanese chicken NS1 gene (93–94%). A/Finch/Canada/NS13011/01 (H3N8) was isolated from caged quarantined finches from the Netherlands [10], but the observed similarities between the PB1 genes may be due to convergent evolutionary events. Fig. 6

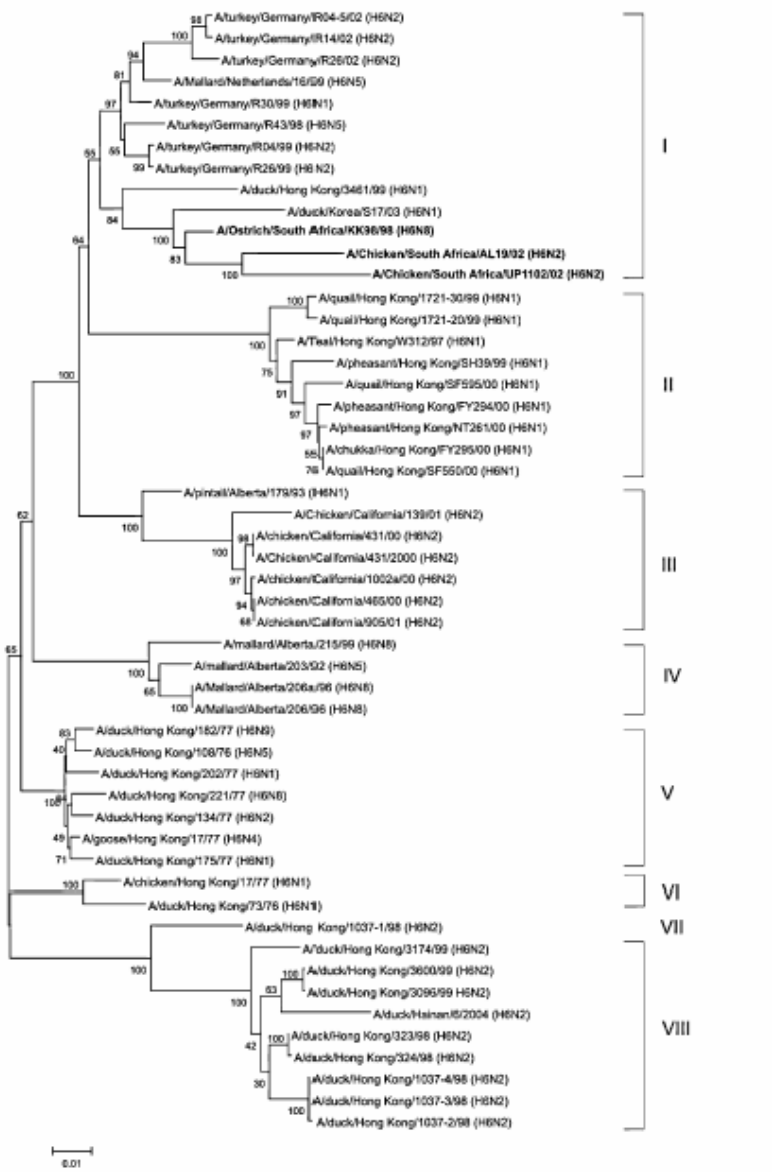
Molecular determinants

The peptide cleavage sites at H_0 were determined to be $_{339}$PQIEPRGLR$_{347}$ for both A/Chicken/South Africa/AL19 (H6N2) and A/Ostrich/South Africa/KK98/98(H6N8), but $_{339}$PQIETRGLR$_{347}$ for A/chicken/South Africa/UP1102/02 (H6N8). Five potential N-glycosylation sites were predicted in A/chicken/South Africa/AL19/02 (H6N2), three in A/Chicken/South Africa/UP1102/02 (H6N2) and four in A/Ostrich/South Africa/KK98/98 (H6N8) HA proteins. In the HA protein, A/Chicken/South Africa/AL19/02 (H6N2) contained the aspartic acid residue D_{144}, whereas A/Chicken/South Africa/UP1102/02 (H6N2) had an asparagine residue, shared by duck isolates and A/Ostrich/South Africa/KK98/98 (H6N8) had a serine residue at this position, shared by isolates from ducks, turkeys chickens and quails (data not shown).

Discussion

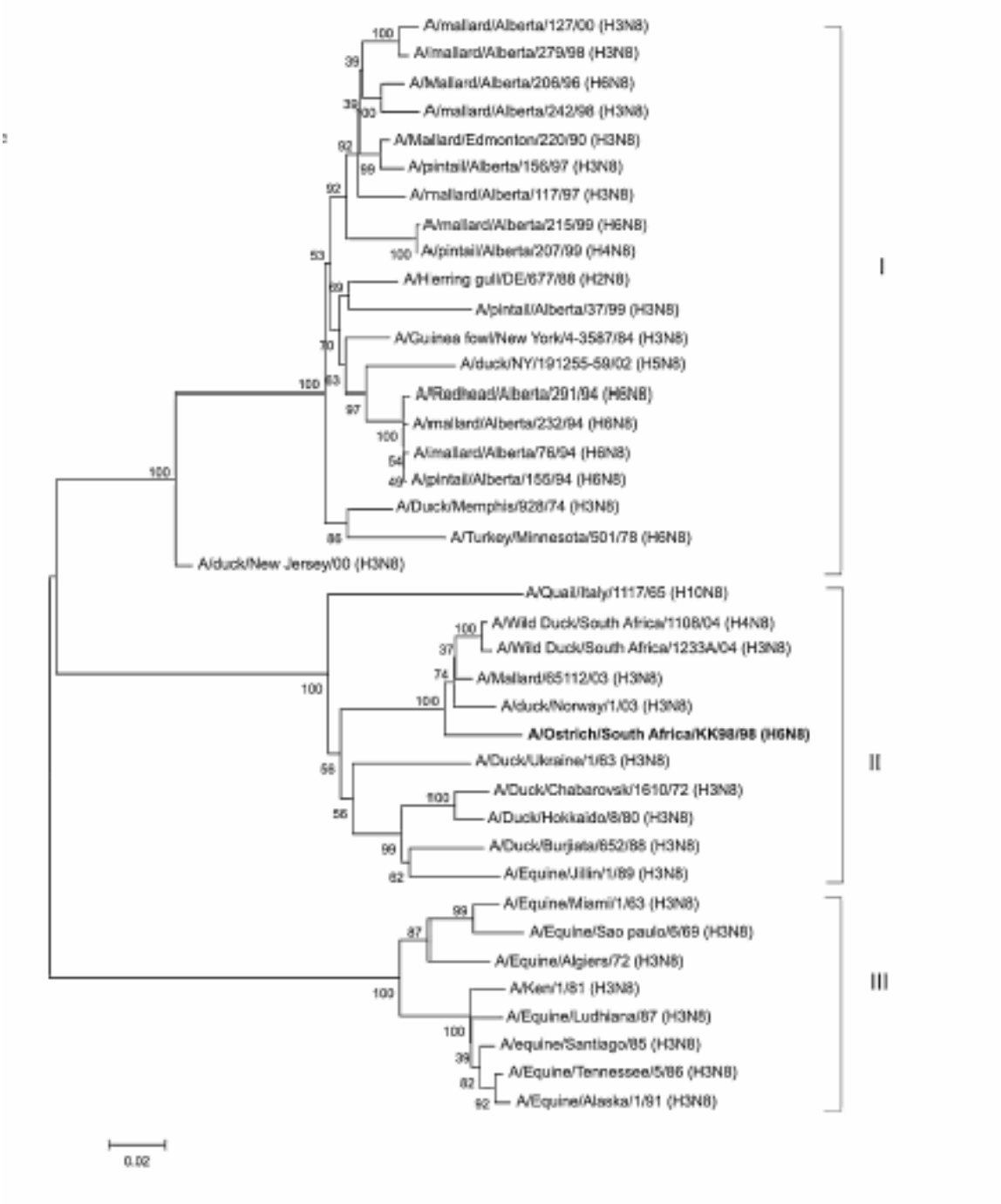
We demonstrated that the H6N2 viruses, although divided between two distinct genotypes (sub-lineages I and sub-lineage II), probably shared a recent common ancestor and that this ancestral virus arose from a reassortment event between the 1995 H9N2 virus, A/Ostrich/South Africa/9508103, and the H6N8 virus, (A/Ostrich/South Africa/KK98/98) isolated in 1998. Both H6N2 sub-lineages I and II were isolated at Camperdown early on, sometimes even from the same farm. Although sub-lineages I and II became widespread in the KZN Province, only sub-lineage II was detected in the northern Gauteng Province.

Fig. 2 Unrooted phylogenetic tree of the H6 HA nucleotide sequences (1367 nt). Deletions and insertions in the nucleotide sequence were removed to facilitate comparison. South African viruses isolated and sequenced in the present study are in boldface, and sub-lineages I–VIII are indicated



The outbreak was restricted to commercial chickens, and the probable mode of transmission was via vendors of spent hens (a.k.a. cull buyers) whilst moving between farms and their depots. A case where a breeder company in the North-West Province brought H6N2-infected male breeders to replace existing males was also reported. The most likely vectors for the introduction of AIV into the Western Cape ostrich population are the wild waterfowl with which they are in contact with through their attraction to water and feed troughs, or who graze on ostrich pastures each winter. In fact, H6 antibodies were detected in the sera of wild ducks in the Oudtshoorn region during the winter of 1998 [11], about the same time that the H6N8 virus was isolated from the ostriches.

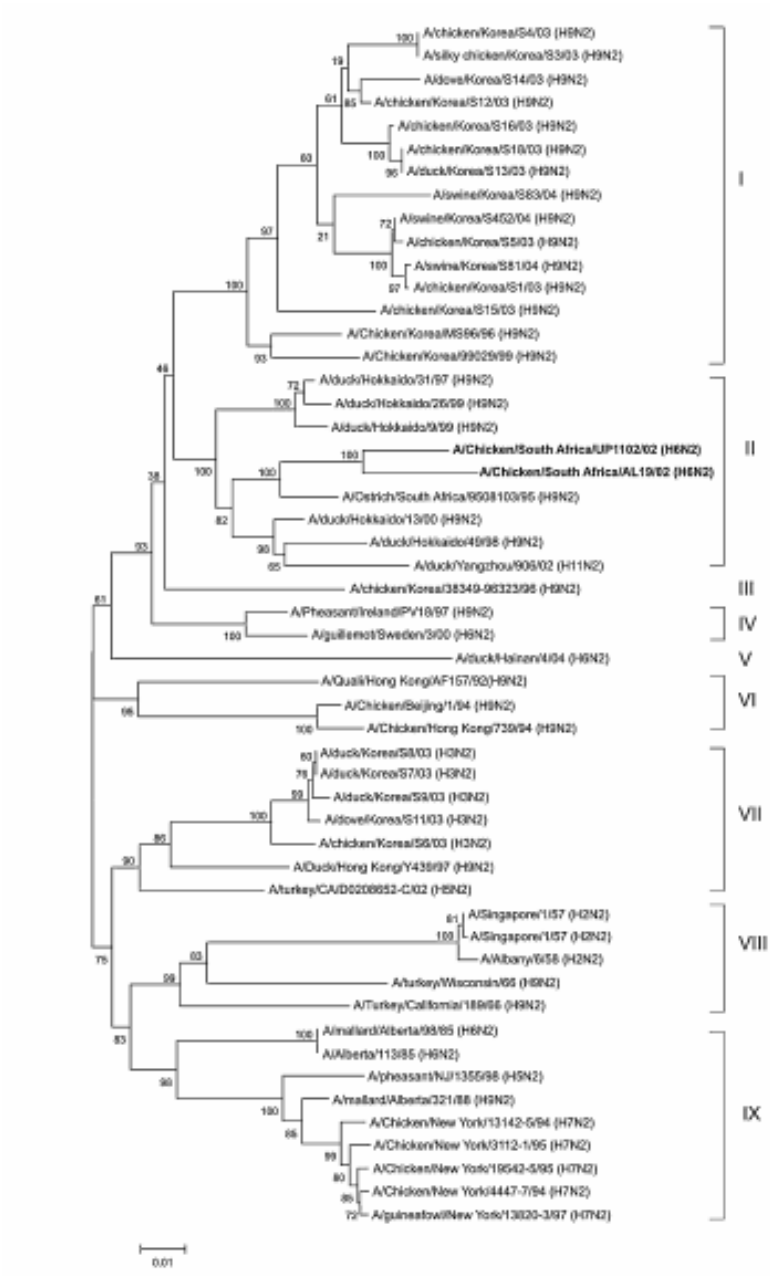
Fig. 3 Unrooted phylogenetic tree of the N8 neuraminidase nucleotide sequences (1314 nt). Deletions and insertions in the nucleotide sequence were removed to facilitate comparison. South African viruses isolated and sequenced in the present study are in boldface, and sub-lineages I–III are indicated



There is mounting evidence that ostriches exhibit atypical (for poultry) and often sub-clinical responses to infections with AI viruses, even with highly pathogenic strains [12, 13]. Therefore, it is possible that mature ostriches could act as

mixing vessels for strains of AIV without showing clinical disease, and that the viruses could be shed for up to 2 weeks [13]. How the infection is spread from ostriches to chickens in the area is unclear as ostrich and commercial poultry production areas are separate. During 2001, however, a commercial chicken flock in the Worchester area was suspected to be infected with AIV, but before control measures could be undertaken, infected birds were sold to cull-buyers from KZN (Carine Pienaar, Chief State Veterinarian, personal communication). Chicken H6N2 sub-lineages I and II circulated for roughly the same time periods, but sub-lineage I viruses contained multiple genetic markers associated with the adaptation of AIV to chickens. Firstly, a 22-amino acid deletion in the stalk region of the NA gene was observed. NA stalk deletions have been shown to reduce the enzymatic activity of the protein [14] and, presumably, adversely affect the spread of the virus to uninfected cells. Secondly, changes in the HA gene that

Fig. 4 Unrooted phylogenetic tree of the N2 neuraminidase nucleotide sequences (1104 nt). Deletions and insertions in the nucleotide sequence were removed to facilitate comparison. South African viruses isolated and sequenced in the present study are in boldface, and sub-lineages I–IX are indicated



compensate for a shortened N stalk have been described, including increased glycosylation near the receptor binding site thereby decreasing receptor binding affinity [15, 16]. Sub-lineage I displayed this hyperglycosylation, with five predicted N-glycosylations sites in the HA gene compared to only three in sub-lineage II, which did not contain the N-

stalk deletion. Thirdly, the presence of D_{144}, a proposed genetic marker that distinguishes terrestrial bird isolates from aquatic ones [4], was observed in the sublineage I HA gene. These adaptations combined with the example of a particular strain (Fig. 1, sub-lineage I(d)) persisting in a small geographical region (KZN South Coast) isolated for 3 years, supports the findings of other investigators [4, 17, 18] that H6 viruses are capable of forming stable lineages in chickens.

Fig. 5 Unrooted neighborjoining trees of the internal protein genes. A = A/Chicken/South Africa/UP1102/02(H6N2); B = A/chicken/South Africa/AL19/02 (H6N2); C = A/Ostrich/South Africa/KK98/98(H6N8); D = A/Ostrich/South Africa/9508103(H9N2); E = A/Finch/Canada/NS13011/01(H3N8); F = A/chicken/Taiwan/7-5/99(H6N1)

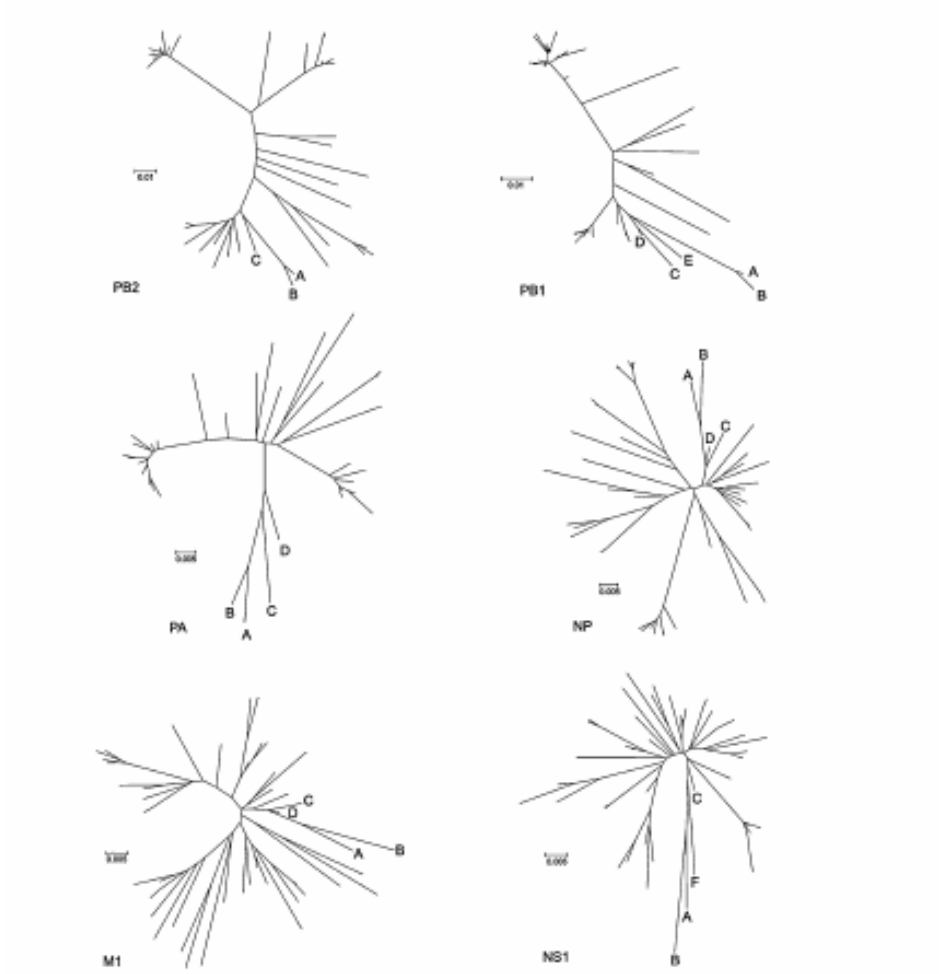
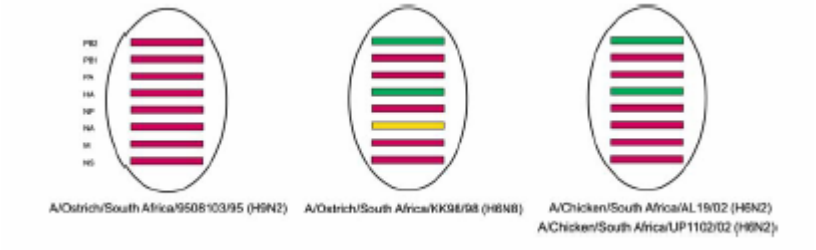


Fig. 6 Schematic diagram representing the origins of genes of the South African viruses



The results of this study suggest that ostriches can act as mixing vessels for AIV subtypes. These viruses in turn potentially pose a threat to the poultry industry if there is a breakdown in the implementation of biosecurity measures. In the case of the H6N2 chicken viruses, the infection seems to have spread by the movement of infected chickens and

not by wild waterfowl, as the outbreak was limited to commercial chickens. The presence of two internal genes, viz. NS1 and PB1, with recent common ancestors to those of current Asian HPAI H5N1 strains in the South African AIV gene pool is a cause for concern, particularly since HPAI H5 and LPAI H7 viruses have been isolated from South African ostriches in the past. Monitoring of the gene pool of AI viruses in South Africa should remain a research priority as part of an international surveillance program.

Ostriches can be used as sentinels for the surveillance and monitoring of AI in South Africa. In addition, the potential for long-term maintenance of AIV in ostriches and local waterfowl is an area that requiresmore research.

Acknowledgements

We wish to thank Ruth Manvell for resupplying stock of the H6N8 virus, Margaret Parker, Raina Maharaj, Boto Ganzevoort, C Mutlow, S Abrahams and Elsa Cornelius for excellent technical assistance and Marco Romito for critical review of the manuscript.

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