

Evolutionary consequences of a decade of vaccination against subtype H6N2 influenza

Dionne Rauff^{a,b}, Christine Strydom^a, Celia Abolnik^{c,*}

^aDeltamune (Pty) Ltd, 248 Jean Avenue, Centurion, Pretoria, 0140, South Africa

^bDepartment of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, Onderstepoort, 0110, South Africa

^cPoultry Section, Department of Production Animal Studies, Faculty of Veterinary Science, University of Pretoria, Onderstepoort, 0110, South Africa

*corresponding author

Email: celia.abolnik@up.ac.za

Abstract

The evolutionary dynamics of chicken-origin H6N2 viruses isolated in South Africa between 2002 and 2013 were investigated. Sub-lineages I and II continued to co-circulate under vaccination pressure, but sub-lineage I, from which the inactivated vaccine was derived, displayed a markedly higher mutation rate and a three-fold increase in the emergence of potential antigenic sites on the globular head of HA compared to sub-lineage II. Immunological pressure culminated in a critical phenotypic change as four of the five isolates from 2012-2013 had lost the ability to haemagglutinate chicken erythrocytes, correlating with a pattern of predicted O-glycosylation sites at residues 134, 137 and 141 within the critical 130 loop of the receptor binding domain site. Coassortment of the HA, NA and M genes in the respective sub-lineages contrasted reassortment of the other internal protein genes, and the vaccine seed strain itself was the probable donor of segments to sub-lineage II field strains.

Key words

Avian influenza, H6N2, glycosylation, reassortment, coassortment, genetic drift, vaccine

Highlights

- Prolonged vaccination pressure produced increased antigenic drift in the homologous field strains
- Four of out five recent isolates had lost the ability to haemagglutinate erythrocytes, correlating with predicted O-glycosylation sites in the 130 loop of the receptor binding domain
- Coassortment and reassortment occurred

1. Introduction

Avian influenza outbreaks in poultry are associated with a restricted number of subtypes, suggesting that the haemagglutinin (HA; H) and neuraminidase (NA; N) glycoprotein combination poses a major adaptive barrier within terrestrial birds. The highly pathogenic (HPAI) forms of the disease in poultry, caused by the H5 and H7 viral subtypes are notorious zoonotic pathogens, but until recently the low pathogenic (LPAI) subtypes of AIV in poultry were considered a negligible risk to humans. This status has changed. Since 2013, an increasing number of often fatal human infections have been reported in Asia, and influenza-like illnesses contracted through the handling of infected poultry have been attributed to H7N9 LPAI, H10N8, and H9N2 subtypes (Gao et al 2013; Li et al 2014; Ni et al 2015; WHO 2016). The H6N1 subtype has also infected humans causing influenza-like illness (Wei et al 2013). Alarmingly, more than 30% of the H6 subtype influenza viruses circulating in the live poultry markets in China have acquired the ability to recognise human type receptors- the sialic acid (SA) α 2,6-Gal moiety instead of the SA α 2,3-Gal variety that avian influenza viruses usually target (Lamb and Krug 1996; Wang et al 2014; Wang et al 2015). H6N1 is also able to replicate without prior adaptation in a variety of mammalian hosts and transmit efficiently in some, thus the H6 subtype is considered a potential human pandemic threat (Ni et al 2015).

H6 and H9N2 are the most prevalent subtypes in poultry, have formed stable lineages, and are endemic in the poultry of many regions (Bi et al 2011, Liu et al 2003, Chin et al 2002, Huang et al 2015, Wang et al 2014, Wu et al 2015, Lee et al 2016). LPAI infections don't typically cause severe disease or high mortalities on their own, but co-infections with other pathogens can exacerbate disease, making these viruses of great economic importance. A significant risk of endemic poultry-adapted LPAI strains is that they contribute their replication-competent genes to notifiable strains having pathogenic potential, by reassortment. For example, H6 and H9 strains were the original source of internal protein genes for the pandemic H5N1 HPAI virus (Cheung et al 2007). Many countries therefore attempt to control LPAI by vaccination. Vaccination does not eradicate virus replication, but it decreases the shedding of the virus in the respiratory and digestive tracts of exposed chickens (Swayne et al 2014). The caveat of vaccination is the occurrence of accelerated antigenic drift in field strains. Antigenic drift and reassortment are the primary mechanisms exploited by AIV to escape host defences, especially under vaccination pressure.

Reassortment is facilitated by the segmented nature of the genome. In addition to genes for the HA and NA glycoproteins, located on genome segments 4 and 6 respectively, at least nine other proteins are encoded as follows: 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,

nucleocapsid protein (NP) on segment 5, neuraminidase glycoprotein (NA) on segment 6, non-structural protein 1 (NS1) plus nuclear export protein (NEP) on segment 7, and matrix protein (M1) plus the ionic channel protein (M2) on segment 8 (Wright et al., 2007; Jagger et al., 2012). HA and NA are the major antigens in the viral outer membrane, but vaccination using inactivated virus also elicits good levels of antibodies directed against the NP and M proteins which are packaged into the virion, although these are not protective (Lamb and Krug 1996). Antigenic drift ensues during the error-prone replication of the negative sense single stranded RNA genome, coupled with host selection pressures. Matching the challenge virus and vaccine is one of the critical factors affecting vaccine efficacy, and in countries that used the same vaccines for an extended period of time, the field viruses evolved to evade the protective immune response of the vaccine (Spackman et al 2014). Consequently, the World Organization for Animal Health (OIE) recommends that vaccine strains be re-evaluated for efficacy against circulating viruses every two to three years and updated if needed (OIE 2015).

An H6N2 outbreak in chickens started in South Africa around 2001 and rapidly spread throughout the country by movement of infected chickens. Two distinct genetic H6N2 sub-lineages, I and II, were detected from the outset and it was determined through phylogenetic studies that the sub-lineages shared a common ancestor. Phylogenetic analysis also demonstrated that the common ancestor most likely originated from a reassortment of viruses that were circulating in ostriches in the late 1990s (Abolnik et al 2007). An inactivated H6N2 vaccine, produced from a strain isolated in 2002 has been in use ever since to control sporadic outbreaks in commercial flocks. In this study, we assessed the evolutionary dynamics of H6N2 in chickens under vaccination pressure over a period of ten years.

2. Materials and Methods

2.1 Ethical considerations

The use of sentient animals and the experimental procedures were approved by both the Deltamune and University of Pretoria Animal Ethics Committees.

2.2 Viral propagation and sequencing

Viruses were isolated from chickens displaying typical signs of LPAI infection such as drops in egg production or respiratory symptoms (Table 1). Isolate A/chicken/South Africa/W-04/2002, abbreviated as CKW04VACC02 here was isolated in 2002 and is the current antigen used in the H6N2 vaccine prepared for poultry in South Africa. For further propagation, 0.2m^l of each isolate was inoculated into the allantoic cavity of five 8 – 10 day old embryonated specific-pathogen-free (SPF) eggs followed by incubation at 37°C. Allantoic fluids were tested for haemagglutination activity (HA) after embryo deaths, or after 7 days according to the standard method (WHO 2015). We extracted viral RNAs using TRIzol® reagent (Thermo Fischer Scientific, Waltham Massachusetts, USA) and confirmed the presence of AIV by real-time reverse transcriptase polymerase chain reaction (rRT-PCR) (Spackman et al 2002). Transcriptomic libraries were generated as previously described (Abolnik et al 2012) and Nextera libraries were prepared and analysed on an Illumina MiSeq apparatus by the sequencing service provider, Inqaba Biotech (Pretoria). Electron microscopy was performed at the Agricultural Research Council- Onderstepoort Veterinary Institute.

2.3 Genome assembly and molecular characterization

The CLC Genomics Workbench v6 software package was used for processing and analysing Illumina sequence data. Paired-end reads were trimmed and gene segments were assembled *de novo*. Multiple sequence alignments were prepared in BioEdit v7.1.3.0 (Hall, 1999), with reference sequences yielding the highest percentage nucleotide sequence identity as retrieved by BLAST searches (www.ncbi.nlm.nih/blast). Phylogenetic trees of full gene sequences were constructed using the Maximum Likelihood interference in MEGA v5.2.2 software, with 1000 bootstrap replications to assign confidence levels to branches (Tamura et al 2011). Sequences were deposited in Genbank under the accession numbers DQ408509-DQ408515, KT777818-KT777824, KT777899, DQ408517-DQ408529 and KX595237-KX595268.

Table 1. Propagation and haemagglutinating activity (HA) of H6N2 chicken viruses used in the study

Collection date	Isolate	Abbreviation	Embryo mortality	HA titre	AIV rRT-PCR result	Phylogenetic grouping
2002, July	A/chicken/South Africa/W-04/2002	CKW04VACC02	6/6	32	Positive	Sub-lineage I
2002, July	A/chicken/South Africa/AL19/2002	CKAL1902	6/6	16	Positive	Sub-lineage I
2002, October	A/chicken/South Africa/AL25/2002	CKAL2502	4/6	64	Positive	Sub-lineage II
2012, March	A/chicken/South Africa/BKP/2012	CKBKP12	4/6	0 (Negative)	Positive	Sub-lineage II
2012, March	A/chicken/South Africa/BKR2/2012	CKBKR212	0/6	0 (Negative)	Positive	Sub-lineage II
2012, March	A/chicken/South Africa/BKR4/2012	CKBKR412	6/6	0 (Negative)	Positive	Sub-lineage II
2012, October	A/chicken/South Africa/NWY/2012	CKNWY12	0/6	0 (Negative)	Positive	Sub-lineage I
2013, May	A/chicken/South Africa/MAS/2013	CKMAS13	4/6	4 (Negative)	Positive	Sub-lineage I

Fifty seven Eurasian-lineage H6 HA sequences isolated from chickens were retrieved from the Global Initiative on Sharing All Influenza Data (GISAID) EpiFlu™ database (<http://platform.gisaid.org/>). We used these reference sequences together with nine H6N2 strains from South Africa to assess evolutionary dynamics. Molecular dating, calculation of the mean nucleotide substitution rate and time to the most recent common ancestor (tMCRA) were performed using the Markov chain Monte Carlo (MCMC) method available in BEAST version 2.3.2 (Bouckaert et al 2014) with the BEAGLE library (Suchard et al 2009). A HKY nucleotide substitution model, Bayesian skyline coalescent tree prior, and uncorrelated relaxed lognormal clock were applied. The MCMC chain was run for 80 million iterations with sampling every 10 000 steps to achieve convergence as assessed using the Tracer v1.6 program (<http://tree.bio.ed.ac.uk/software/tracer>), thereby ensuring that the effective sample size (ESS) was >200. A maximum clade credibility phylogenetic tree was summarized from the posterior probability of trees after removal of 10% burn-in. The summarized tree was visualised using FigTree version 1.4.2.

The HA gene sequences were translated to amino acids in BioEdit, and scanned for potential N-glycosylation sites using the NetNGlyc1.0 Server. (<http://www.cbs.dtu.dk/services/NetNGlyc/>). O-glycosylation sites were similarly predicted with the NetOGlyc4.0 algorithm (Steentoft et al 2013). RaptorX (Kallberg et al 2012) and CCP4MG v2.9.0 were used for secondary structure prediction and visualisation, respectively, of the HA protein of CKNWY12.

In order to assess reassortment between sub-lineage I and II viruses we concatenated the eight genome segments to form a single chromosome for each H6N2 chicken virus. Genes segments (trimmed for length and gaps) were concatenated in the order largest to smallest, each separated by five N's as follows: PB2: 1-2288; PB1:2294-4572; PA: 4578-6687; HA: 6693-9396; NP: 8402-9884; NA: 9890-11175; M: 11181-12162; NS: 12168-12849. The multiple sequence alignment was then analysed for reassortment as well as recombination events in the Recombination Detection Programme (RPD) v4.69 (Martin et al 2010). A full exploratory scan using seven of the different recombination detection methods implemented in RDP4 was applied (RDP, Bootscan, GENECONV, MaxChi, Chimaera, SiScan, and 3Seq), retaining the default settings. Reassortment events were considered credible when an event was identified by at least two detection methods with an associated P value <0.05 and with at least one method having an associated P value <0.001 (Stainton et al 2015).

2.4 Vaccine preparation and sera production

Harvested allantoic fluids were clarified by centrifugation at 2000 rpm for 20 minutes. A 0.2M concentration of binary ethylenimine (BEI) was added to each of the antigen bottles to obtain a final concentration of 2% BEI (20mℓ BEI / 1ℓ antigen). The bottles were mixed well and incubated for 26 hours (± 2 h) at 37 (± 1) °C, to inactivate the virus. The inactivation was stopped by aseptically adding the required volume of 20% sodium thiosulphate to a final concentration of 0.4%. The inactivated antigens were used to make small batches of oil-based AI vaccines, where 20% antigen, 72% white oil, 6% Arlacel and 2% Tween 80 were emulsified and bottled. Thirty four specific pathogen free (SPF) white leghorn chickens were used in the study.

Vaccines prepared from each of the eight isolates were administered to eight individual groups of chickens with four chickens per group, where 0.5 mℓ of vaccine was injected into the pectoral muscle. Two unvaccinated chickens served as negative controls. Chickens were bled from a brachial vein prior to the first vaccination to determine a baseline antibody level. Four weeks after the initial vaccination, another blood sample was collected and a booster injection was administered. Four weeks after the booster vaccination, a terminal bled was conducted under anaesthesia where 20mℓ of serum was collected from each bird.

2.5 Serological tests

The haemagglutination inhibition test (HI) was performed according to the standard procedure (WHO 2015) on HA-positive sera from all birds from the second and the terminal bleeds. Titres were calculated as the reciprocal of the last HI positive serum dilution. All sera were tested using the Influenza A multispecies ELISA (IDEXX) according to the manufacturer's recommended protocol. Absorbance was read at 620nm using a Thermo Scientific Multiskan 355 microtitre plate spectrophotometer. The sample to negative (S/N) was calculated. We also tested the sera using an indirect H6N2-specific ELISA developed for in-house use by Deltamune (Pty) Ltd. Microtiter plates (Nunc Polysorp) were coated with whole inactivated AI H6N2 virus and incubated with test sera at a dilution of 1:500. After washing, rabbit anti-chicken immunoglobulin G (IgG) peroxidase conjugate (Sigma-Aldrich, St Louis, Missouri, U.S.A) was added. After a final wash step, o-Phenylenediamine dihydrochloride substrate (OPD) (Sigma-Aldrich, St Louis, Missouri, U.S.A) was added. The OPD reaction was stopped by adding 1.88M H₂SO₄ to each well and the absorbance was read

at 492nm using a Thermo Scientific Multiskan 355 microtitre plate spectrophotometer. The sample to positive (S/P) value was calculated. The titre of each serum was calculated using the formula

$\text{Log}_{10}\text{Titer} = x(\text{Log}_{10}\text{S/P}) + y$, where $x = 1.27$ and $y = 3.81$.

After further validation (data not presented), the cut-off for a positive result was determined to be a titre value of ≥ 900 . This assay had a diagnostic sensitivity of 99.47% and a diagnostic specificity of 99.05%.

3. Results and discussion

3.1 Antigen propagation and immunogenicity

The replication of eight H6N2 viruses investigated in this study (Table 1) in embryonated chicken eggs (ECE) was confirmed by AIV-specific rRT-PCR performed on RNAs extracted from the allantoic fluids (data not shown). Viruses CKBKR212 and CKNWY12 did not kill the embryos and four of the viruses, namely CKBKP12, CKBRK212, CKBKR412 and CKNWY12 did not agglutinate chicken red blood cells (CRBCs). All non-embryo lethal/ non-haemagglutinating viruses were isolated in 2012, and represented both sub-lineage I and sub-lineage II.

After the primary vaccination, only CKW04VACC02 and CKAL2502 had induced antibody titres that were positive on the IDEXX ELISA (Fig 1), an assay that detects NP-specific antibodies. After the booster vaccines had been administered, all vaccinated groups had seroconverted, except for the group vaccinated with CKBKP12, which remained below the positive cut-off threshold. When the same sera were tested by H6N2 ELISA (Fig 2), the CKW04VACC02 vaccine had induced the strongest antibody response at four weeks, and positive values were also recorded for CKAL1902 and CKAL2502. The early induction of a strong humoral response is an important attribute for a vaccine, and the high serum antibody response reflects that the strain replicates well in chickens. After 8 weeks, all groups, including the CKBKP12-vaccinated group had seroconverted with H6 and N2-specific antibodies. We surmise that the internal NP proteins were somehow damaged during vaccine preparation for CKBKP12, but the membrane-embedded H6 and N2 glycoproteins remained intact, resulting in antibody responses to the external but not the internal viral proteins.

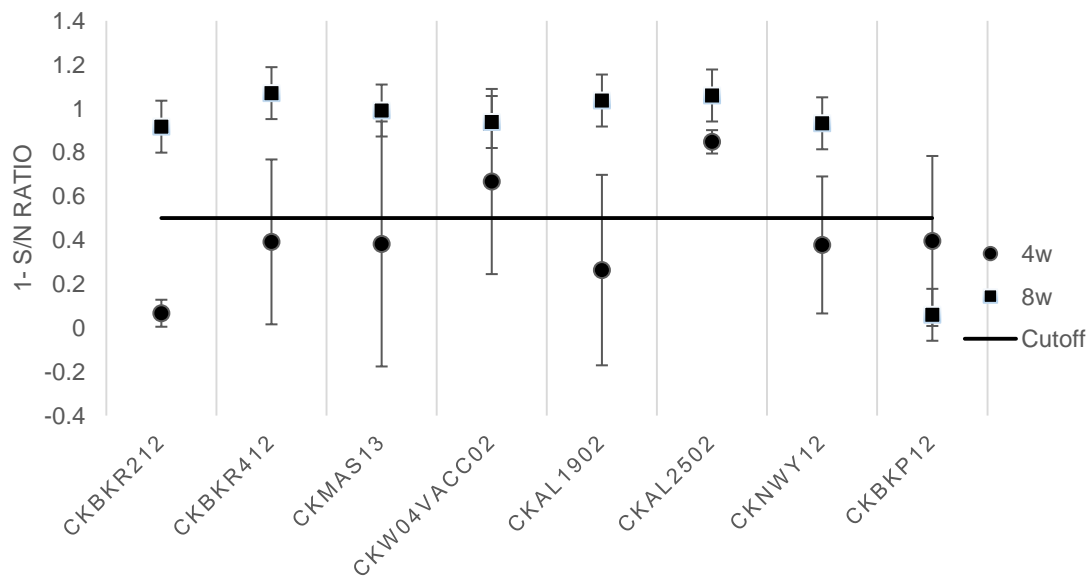


Figure 1: IDEXX ELISA NP-specific antibody responses of chickens immunised with inactivated H6N2 viruses after primary exposure (4 weeks) and a booster (8 weeks). The positive threshold is indicated.

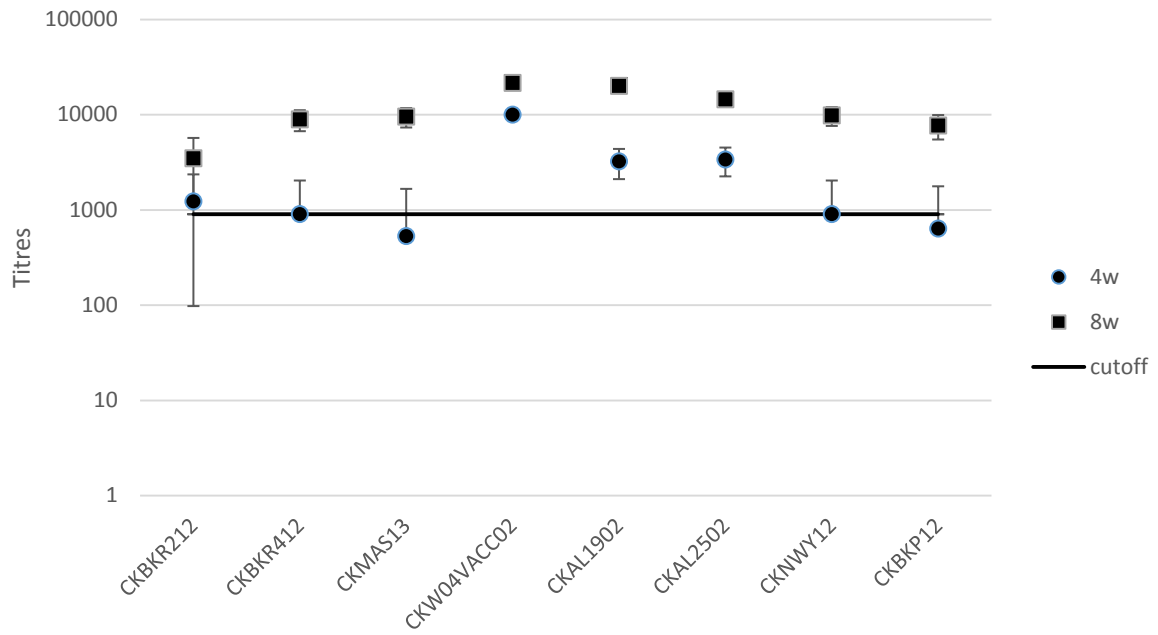


Figure 2: H6N2 indirect ELISA antibody responses of chickens immunised with inactivated H6N2 viruses after primary exposure (4 weeks) and a booster (8 weeks). The positive threshold is indicated.

Table 2. Geometric mean HI titers of sera collected from vaccinated chickens at 8 weeks

	Vaccine group	Antigen			
		CKMAS13 (I)	CKW04VACC02 (I)	CKAL1902 (I)	CKAL2502 (II)
Antisera	CKBKR212 (II)	4 [86.60] ^a	15 [92.94]	19 [92.94]	9 [95.59]
	CKBKR412 (II)	9 [86.60]	34 [92.94]	148 [92.94]	7 [95.59]
	CKMAS13 (I)	176 [100]	88 [92.59]	576 [92.59]	11 [88.54]
	CKW04VACC02 (I)	176 [92.59]	704 [100]	168 [99.64]	80 [94.88]
	CKAL1902 (I)	224 [92.59]	640 [99.64]	288 [100]	104 [94.53]
	CKAL2502 (II)	42 [88.54]	120 [94.88]	384 [94.53]	60 [100]
	CKNWY12 (I)	91 [98.94]	88 [93.12]	91 [93.12]	13 [89.42]
	CKBKP12 (II)	27 [88.95]	75 [93.30]	43 [92.94]	37 [95.94]

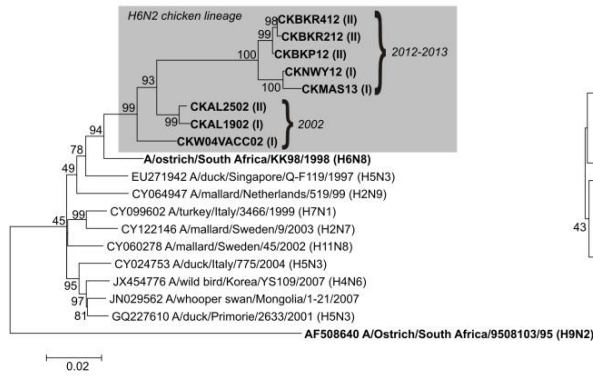
^aPercent amino acid sequence identity in the H6 protein gene, from supplemental table 1

HI titres of the antisera raised against the eight vaccine antigens could only be assessed using the four viruses that retained hemagglutinating activity, namely CKMAS13, CKW04VACC02, CKAL1902 and CKAL2502 (Table 2). HI results were considered positive if they had a titre of 16 or greater. Antisera against the strains that were haemagglutination negative (CKBKR212, CKBKR412, CKNWY12 and CKBKP12) produced lower geometric mean titres (GMT) against the test antigens, regardless of whether the sub-lineages were matched. Nonetheless, the ELISAs demonstrated that these non-haemagglutinating vaccines elicited comparable levels of H6/N2-specific antibodies to the haemagglutination-competent strains after the booster had been administered. Interestingly, the homologous antigen did not produce the highest titre in each case, for example, CKMAS13 HI titres were highest against the CKAL1902 antigen, and similarly the CKAL1902 antisera reacted more strongly with the CKW04VACC02 vaccine antigen than to the homologous antigen. A general correlation was evident between HI titre and percentage amino acid sequence identity in the HA protein (Table 2; Supplemental table 1, Supplemental figure 1). The CKW04VACC02 vaccine strain produced GMTs in the range of 75 to 640 with other sub-lineage I strains and GMTs in the range of 15 to 120 with the sub-lineage II strains. Some studies have reported that titres greater than 120 prevent replication (Swayne et al 2006), but others have found that antibody titres to vaccines are neither predictive of survival (in the case of pathogenic strains), nor shedding (Spackman et al 2014). The OIE terrestrial manual guideline is that a HI titre response 32 to a vaccine offers protection from mortality and that a response of 128 offers protection from virus shedding in vaccinated birds (WHO 2015). Neither cross-protection offered by the vaccine strain against clinical symptoms nor its ability to reduce shedding against either of the sub-lineages were assessed here, but the HI test does provide a practical alternative to *in vivo* assessment of vaccine efficacy and minimum protection level (Swayne et al 2014).

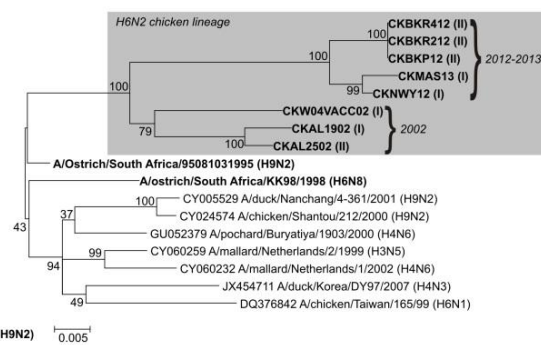
3.2 Phylogenetic and reassortment analysis

We analysed the eight full genomic segments for each virus to demonstrate that the South African H6N2 chicken viruses formed a well-supported monophyletic group for each segment (Fig 3), to which the hypothetical progenitor strains *A/ostrich/South Africa/KK98/1998* (H6N8) (OSKK98) and *A/Ostrich/South Africa/9508103/05* (H9N2) remained basal. BLAST searches did not identify related sequences from external sources. Extensive genetic drift in the H6N2 viruses over the ten-year period was evident from the long branch lengths. The sub-lineage I strains isolated in 2012/ 2013 had decreased to 92.59 and 93.12% amino acid identity in the HA protein to the chicken viruses isolated in 2002 (supplemental table 1).

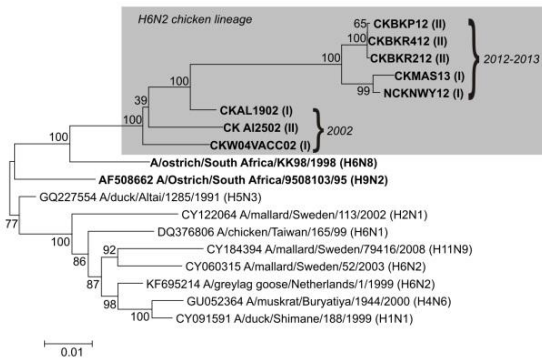
Segment 1- Polymerase B2 (PB2)



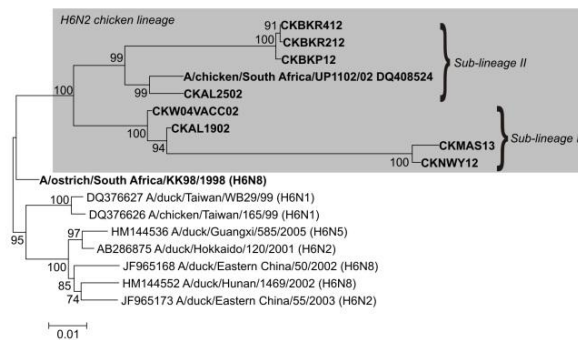
Segment 2- Polymerase B1 (PB1)



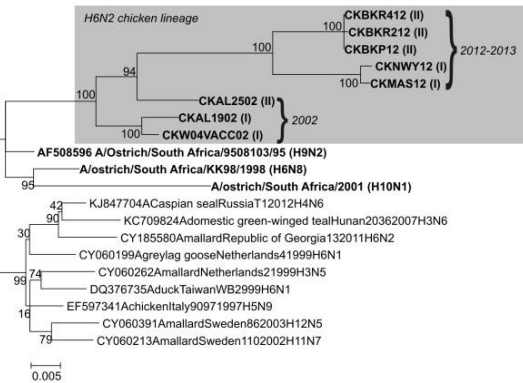
Segment 3- polymerase A (PA, PA-X)



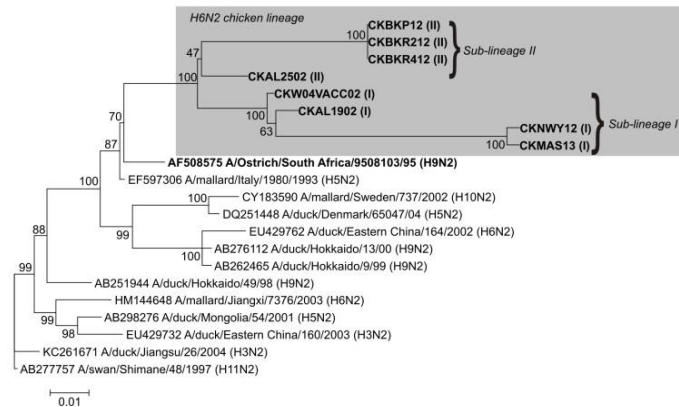
Segment 4- Haemagglutinin (H6)



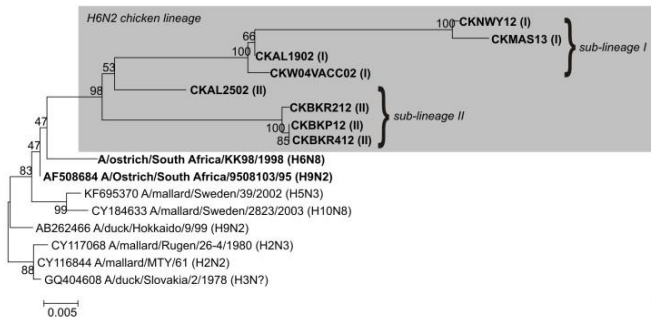
Segment 5- Nucleoprotein (NP)



Segment 6- Neuraminidase (N2)



Segment 7- Matrix proteins (M1, M2)



Segment 8- Non-structural proteins (NS1, NEP)

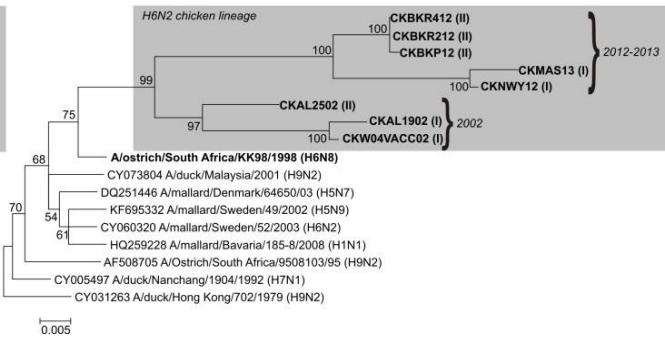


Figure 3. Phylogenetic analysis of the eight genomic segments of H6N2 viruses and closest relatives retrieved from sequence databases.

Similarly, in sub-lineage II the viruses isolated in 2012 were only 92.94 to 93.3% identical to strains from a decade earlier. Between sub-lineages, the amino acid sequence identity between sub-lineage I and II in 2002 was 94.53-94.88%, but in 2012 this had decreased to 86.60-88.54%, indicating that the sub-lineages are diverging.

To further investigate the evolutionary dynamics of the H6N2 viruses, we applied molecular dating to the HA genes. Nucleotide substitution rates for influenza viruses are in the order of 10^{-3} substitution per site per year (subs/site/year), and genes coding for the external H and N proteins are reported to have higher evolutionary rates than internal genes (Fournment and Holmes, 2015). Fournment and Holmes (2015) investigated the evolutionary rates of heterogeneity to determine whether there were differences in rates between wild birds and poultry, and found that estimates of substitution rates in poultry for H6 viruses were similar to those of H5N1 in poultry and were significantly higher than those of H6 from wild birds. More frequent contact transmission in poultry leading to more replications and hence a greater number of mutations per unit time was proposed to explain this phenomenon, but they conceded that host-specific selection pressures and ecological differences are also possibilities. In view of these findings and the implication that a single substitution rate cannot be applied to wild birds and poultry equally, in estimating a molecular clock for the South African H6N2 HA genes, only Eurasian lineage H6 viral sequences derived from chickens were compared (Fig 4). The evolutionary rate between 2002 and 2012 for sub-lineage I was calculated as 7.7×10^{-3} nucleotide subs/ site/year and for sub-lineage II the rate was 4.05×10^{-3} subs/ site/year revealing that sub-lineage I is the faster evolving sub-lineage. The time-scaled phylogeny of Eurasian lineage H6 chicken HA genes placed the tCMRA for sub-lineage I in early 2001, with the tCMRA for sub-lineage II set some months earlier prior to 2000, which is fairly close to the detection of the index case in June 2001 (Abolnik et al 2007). The tCMRA of both sub-lineages was calculated to be mid-1997. This molecular dating remains consistent with the period of circulation of the hypothetical progenitors, field strains OSKK98 (H6N8) and H9N2 from which all segments were derived (Abolnik et al 2007). OSKK98 was isolated in July 1998 from extensively-reared four-month old ostrich chicks. A recent study showed that the H6 serotype is periodically introduced to farmed ostriches from the wild duck reservoir and can circulate for extended time periods, for example, H6 infection was detected serologically in ostrich flocks from January to August 2012, March to August 2013 and January to December 2014. These ostrich and wild duck H6 strains are phylogenetically unrelated to the H6 chicken lineage (Abolnik et al 2016).

Phylogenetic groupings of H6N2 strains in the eight genome segment trees did not correlate. Sub-lineages I and II were clearly distinguishable with the HA, NA and M (M1, M2) gene-encoding segments. However,

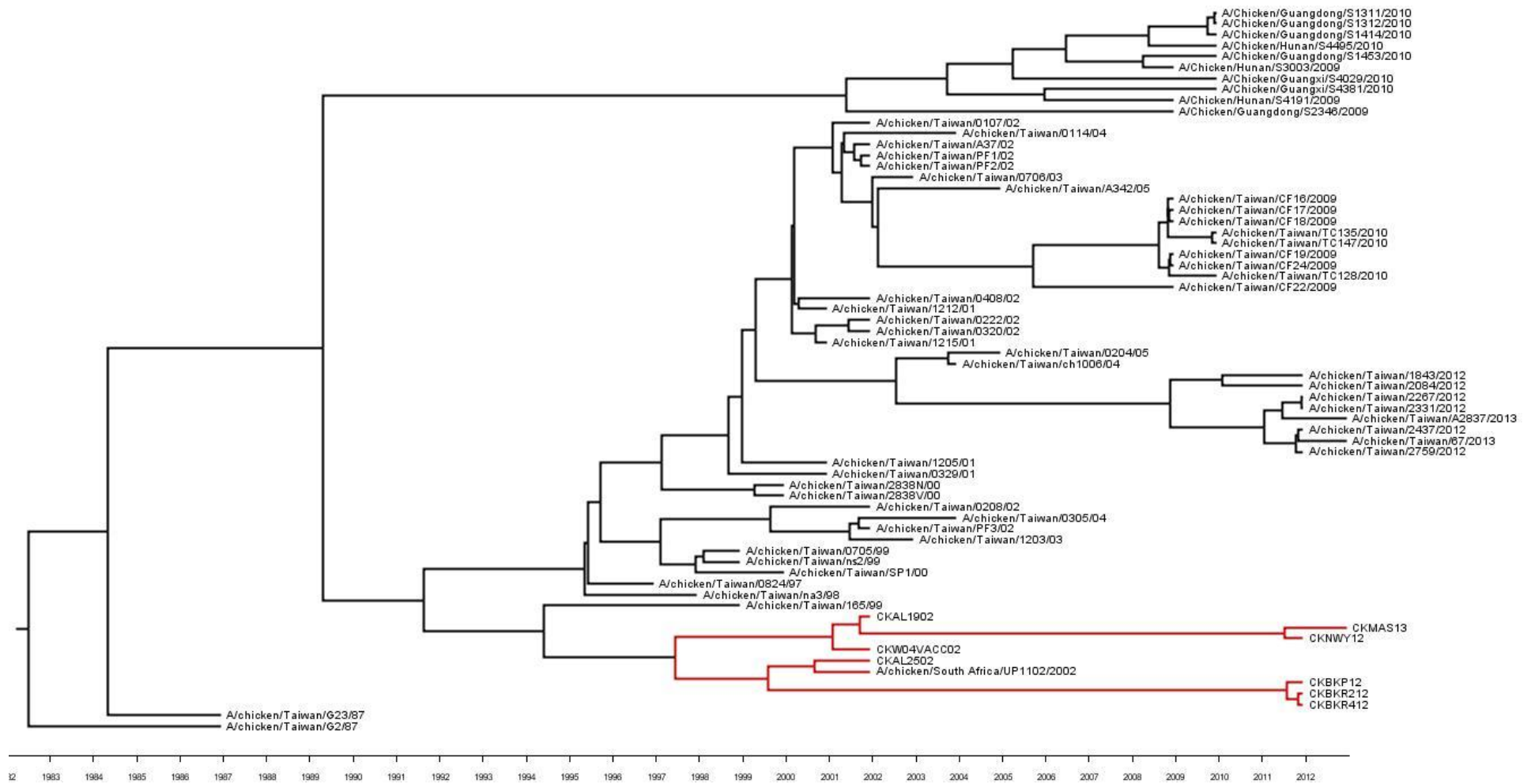


Figure 4. Time-scaled phylogeny of Eurasian lineage HA genes of H6 subtype viruses isolated from chickens only. The South African H6N2 viruses are highlighted by the red branches.

Table 3: Reassortment detection in concatenated H6N2 genome segments

Breakpoint positions		Recombination detection			Detection method P-value						
Begin	End	Recombinant Sequence	Minor Parental Sequence	Major Parental Sequence	RDP	GENECONV	Bootscan	MaxChi	Chimaera	SiScan	3Seq
6715	12154	CKBKP12 (II) CKBKR212 (II) ^a CKBKR412 (II) ^a	CKNWY12 (I)	CKW04VACC02 (I)	1.30x10 ⁻³⁸	6.95x10 ⁻³³	2.35x10 ⁻²⁴	1.07x10 ⁻²⁵	9.33x10 ⁻²³	7.27x10 ⁻³³	8.66x10 ⁻²⁶
8431	10013	CKBKP12 (II) CKBKR212 (II) ^a CKBKR412 (II) ^a	CKNWY12 (I)	CKW04VACC02 (I)	1.31x10 ⁻¹⁶	-	2.51x10 ⁻¹⁴	2.47x10 ⁻⁰⁹	6.70x10 ⁻⁰⁹	5.99x10 ⁻¹⁵	1.20x10 ⁻²

^aSequences with the same recombination event

in the other internal gene segment trees, lineages for segments 1 (PB2), 2 (PB1; PB1-F2), 3 (PA; PA-X), 5 (NP) and 8 (NS1; NEP) segregated chronologically, i.e. the 2012/2013 strains formed a discrete cluster, separate from the strains isolated in 2002. To investigate whether the topology of the internal genome segment trees could be explained by recombination or reassortment events, we concatenated genomes and performed RDP4 analysis. Two main recombination events were identified in strain CKBKP12 (II), with similar events detected in CKBKR212 (II) and CKBKR412 (II) (Table 3). The first event predicted breakpoints at positions 6715 and 12154, and the second recombination event occurred internally to this, at breakpoint positions 8431 and 10013. Mapped against the concatenated genes (data not shown), the recombination breakpoints generally correlated with gene segments, thereby confirming that reassortment had occurred. Essentially, this indicates that the PB2, PB1, PA, NP and NS genes for CKBKP12 (II) were derived from a lineage I strain. Interestingly the major parental sequence was identified as the vaccine CKW04VACC02 (I) and the minor parental sequence as CKNWY12 (I) for both reassortment events, with strong statistical support with $P < 9.33 \times 10^{-23}$ for 7/7 methods for the first event and $P < 2.51 \times 10^{-14}$ for 3/7 methods in the second. Lack of reassortment in the HA, NA and M gene segments for CKBKP12 (II) (as well as CKBKR212 (II) and CKBKR412 (II)) concurred with phylogenetic tree topologies.

3.3. Glycosylation patterns in the HA protein correlate with the haemagglutination phenotype

Original laboratory isolation records as well as re-inoculation in our study showed that isolate CKNWY12 displayed no HA activity nor did it cause embryo mortality. Another three late isolates, namely CKBKR212, CKBKR412 and CKBKP12 also showed a complete lack of HA activity, and CKMAS13 yielded a weak titre of 4, below the threshold of what is considered positive. Changes on the HA gene at positions - 138, 190, 193, 194 or 226 (H3 numbering) are thought to correlate with the ability of AIV to agglutinate erythrocytes but Kumari et al, (2007) reported that agglutination of CRBCs did not correlate with altered binding to any oligosaccharide a glycan array, and suggested that the density of HA due to replication processes may be the critical factor in the ability of viruses to agglutinate CRBC. However, under the electron microscope, the density of the glycoprotein spikes of CKNWY12 appeared typical (supplemental figure 2). Therefore, we considered that another factor influences the failure of some H6 viruses to agglutinate CRBCs, and it is already well-established that oligosaccharides in close proximity to the receptor-binding site of HA can alter its binding avidity and/or specificity for sialylated receptors (Tate et al 2014).

Viral proteins destined for the virion surface travel through the host's secretory pathway where hundreds of glycosyltransferases affix a diverse array of glycan structures to them. Protein glycosylation is one of the most abundant and diverse forms of modification, and glycosylation of the HA is essential for protein folding and transport to the cell surface as well as biological functions such as receptor binding activity, evasion of host immunity and HA cleavability. A number of different types of protein glycosylation exist, including N-linked, several types of O-linked to S, T, hydroxyline and Y residues, and C-mannosylation to W (Steentoft et al 2013). N-linked glycosylation is initiated by a single oligosaccharyltransferase complex, and the consensus sequence motif NXS/T (where X represents all amino acids except P) enables reliable prediction of N-glycosylation sites (Tate et al 2014). On the other hand, O-glycosylation is highly complex and regulated by up to 20 distinct oligosaccharyltransferase complexes with different but partially overlapping peptide specificities, and no straightforward consensus sequence motifs for prediction and identification of O-glycoproteins. Steentoft et al (2013) recently mapped the first-generation human O-glycoproteome using a dozen human cancer cell lines and subsequently applied the data to improve the NetOGlyc O-glycosylation prediction algorithm. Using the respective servers, we screened the HA amino acid sequences of the H6N2 subtype viruses for N-glycosylation and O-glycosylation site motifs, specifically for any glycosylation sites that may cause steric hindrance in or around the receptor binding site. The region of HA responsible for receptor binding is located at the tip of each monomer of the HA trimer, and it has four main structural features. The binding site is flanked by the 220 and 130 loops, which contain amino acids that interact with sialic acid or internal sugars of the glycan chain on the host cell receptor. The 190 helix also includes residues with the potential to contact the receptor at either the sialic acid residue (residue 194) or internal glycans on the receptor (residues 190 and 193), and the base of the site contains several highly conserved residues that form an extensive hydrogen bond network (Bradley et al 2011)

The number of potential N-glycosylation sites identified in the HA sequences (Figure 5) was conserved at positions 27, 44, 183, 499 and 559 between sub-lineage I and sub-lineage II strains, but the sub-lineage I strains contained an additional N-glycosylation site at position 307. There was no correlation between viruses that haemagglutinate CRBCs and those that do not based on predicted N-glycosylation patterns, and no N-glycosylation was predicted in the 130 loop, 190 helix or 220 loop regions.

In contrast, we observed notable differences in the predicted O-glycosylation sites, furthermore these differences predominantly occurred in the 130 loop (Fig 5, Table 4) and a pattern is discernible that corresponds to the CRBC agglutination phenotype. The combination of three amino acids at H6 positions

Table 4. O-glycosylation in the H6 haemagglutinin protein 130 loop

Virus	Amino acid			Haemagglutination
	145 (134 ^a)	148 (137 ^a)	152 (141 ^a)	
CKAL1902 (I)	S	T	S*	Yes
CKW04VACC02 (I)	S	T	S*	Yes
CKMAS13 (I)	N	T*	S*	No^b
CKNWY12 (I)	N	T*	S*	No
CKBKP12 (II)	S*	T	P	No
CKBKR412 (II)	S*	T	P	No
CKBKR212 (II)	S*	T	P	No
CKAL2502 (II)	S*	T*	P	Yes

^a H3 numbering

^b An HA titre of 4 was obtained (Table 1)

*denotes O-glycosylation

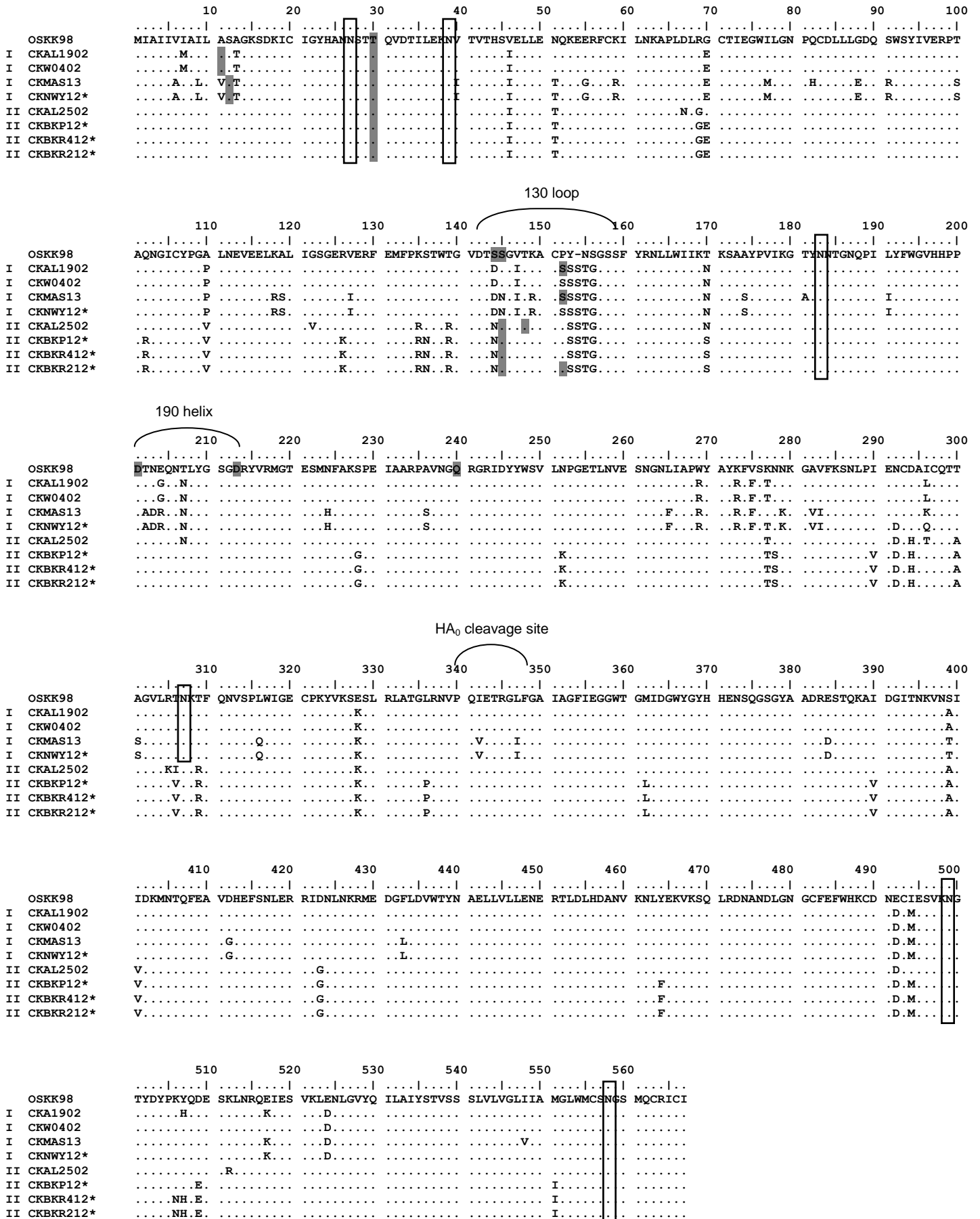


Figure 5. Amino acid alignment of H6 HA proteins. Potential N-glycosylation sites are boxed, and O-glycosylation sites are shaded. Viruses that lack haemagglutination activity are indicated by asterisks.



Figure 6. Predicted secondary structure of CKNWY12 showing the locations of potential O-glycosylated sites in the 130 loop of the receptor-binding site that regulates the haemagglutination phenotype in the H6N2 viruses (residues 145, 148 and 152) as well as the non-glycosylated residue at position 82 in close proximity

145, 148 and 152 (H3 numbering 134, 137 and 141 respectively) could thus regulate the haemagglutination phenotype in these H6 viruses. Their relative positions in the RBS on the globular head of the H6 monomer are shown in Fig. 6. O-glycosylation at position 152 only permits haemagglutination (CKAL1092, CKW04VACC02, CKMAS13), but if the amino acid at position 145 alone is glycosylated, haemagglutination is abolished (CKBKP12, CKBKR412, CKBKR414). Haemagglutination is unaffected if both residues 145 and 148 are O-glycosylated (CKAL2502). CKMAS13 and CKNWY12 are both late sub-lineage I strains with identical O-glycosylation patterns in the 130 loop, at residues 148 and 152. CKNWY12 was completely HA negative, but CKMAS13 produced a very low HA titre of 4, which remains below the positive threshold. The only amino acid differences in the HA protein between these two strains occur at positions ⁸²Q→H, ¹⁸¹A→T, ²⁸⁷K→T, ²⁹⁶K→Q and ⁵⁴⁸V→I. The ⁸²H→Q mutation is situated in close proximity to the RBS on the globular head of the HA monomer (Fig. 6) and we consider that this mutation potentially affects secondary structure around the glycosylated residues ¹⁴⁸T and ¹⁵²S, partially restoring the HA function in CKMAS13.

3.4 Selection of antigenic epitopes on the HA protein under immune pressure

Strong humoral immunity induced by vaccination can be an important factor promoting the selection of escape mutants in vaccinated birds (Lee et al 2016), and our ELISA and HI results demonstrated that the vaccine strain indeed induces strong humoral immunity in chickens, even after a single exposure. We identified antigenic sites under immunological selection in the amino acid sequences (Fig 5), but a limitation of our study was the small sample size that did not permit the analysis of positive selection pressure in a statistically meaningful manner. Nevertheless, in sub-lineage I, between the viruses isolated in 2002 (CKAL1902 and vaccine strain CKW04VACC02), and the field strains from 2012-2013, (CKMAS13 and CKNWY12), 32 amino acid changes occurred in the HA protein (Fig. 5). In sub-lineage II, 16 amino acid changes occurred between 2002 (CKAL2502) and 2012 (CKBKP12, CKBKP412 and CKBKR212). The locations of the mutations that emerged in the two sub-lineages were mapped to the predicted three-dimensional structure for the CKNW12 HA monomer (Figs 7a and b). Sub-lineage I viruses acquired 27 unique mutations in the globular head domain over a ten-year period compared to 9 new mutations in sub-lineage II strains. Mutations in the stalk region numbered four in sub-lineage I, one of which occurred in the hemagglutinin cleavage site (HA0), and five in sub-lineage II. Sub-lineage I had three mutations in the cytoplasmic tail region compared to two in sub-lineage II.

3.5 Additional molecular markers for host adaptation

Thirty four percent of H6 subtype viruses isolated from Chinese poultry sampled in 2014 had acquired the ability to bind to α 2,6-linked sialic acid, which is a prerequisite for human to human infection, and were able to replicate in mice without preadaptation (Wang et al 2014). The change in receptor preference for these H6 strains was mapped to amino acid changes $^{138}\text{A}\rightarrow\text{S}$, $^{226}\text{Q}\rightarrow\text{L}$, and $^{228}\text{G}\rightarrow\text{S}$ coupled with the absence of glycosylation at positions 158 to 160 in HA that was caused by an ^{160}A mutation in the HA. The aforementioned residues are H3 coordinates, and in our amino acid alignment (Fig. 5) these correspond to positions 150 (A), 240 (Q), 242 (G). Neither N- nor O-glycosylation signals were detected at or around position 174 that corresponds to ^{160}A in the Chinese strains. The H6N2 strains isolated from South African chickens have therefore not evolved a receptor binding preference for mammalian cells.

The HA and NA glycoproteins work in conjunction to facilitate the attachment and release, respectively, of the virus to its sialic acid receptor on the host cell (Mitnaul et al 2000). Numerous studies have pointed to the critical importance of the balance between the HA and NA activities for viral fitness, that is determined by NA stalk length and levels of glycosylation on the HA. A shortened NA stalk is an evolutionary adaptation of AIVs to terrestrial poultry, and is accompanied by the selection of compensatory mutations in HA that increase glycosylation levels (Luo *et al.*, 1993, Wagner *et al.*, 2000, Mitnaul et al 2000). A 22 amino acid deletion in the NA stalk in sub-lineage I viruses, accompanied by increased N-glycosylation in HA was previously reported (Abolnik et al 2007). In this study, we confirm that the 22 amino acid deletion in late sub-lineage I strains CKMAS13 and CKNWY12 has been retained (data not shown), and that sub-lineage I strains contain comparatively more N-glycosylated as well as O-glycosylated sites in the HA protein compared to sub-lineage II viruses (Fig 5).

The structural complementation between the specific HA and NA proteins in sub-lineage I and II was reflected in the phylogenetic and reassortment analyses, since no reassortment of the glycoproteins was detected, even though inter-sub-lineage reassortment occurred with most of the internal protein segments. The notable exception was the matrix proteins that followed a similar evolutionary path as the surface glycoproteins. The partially overlapping genes on segment 7 encode a highly conserved 252-amino acid M1 protein and a 97-amino acid M2 protein, both of which play essential roles in the viral life cycle. During viral assembly, the M1 protein binds to the cytoplasmic tails of HA and NA, and bridges interactions between the viral lipid membrane and the RNP core (Ali et al 2000; Nayak et al 2004). In the H6N2 chicken viruses, the M1 protein sequence was largely conserved but lineage-specific mutations were

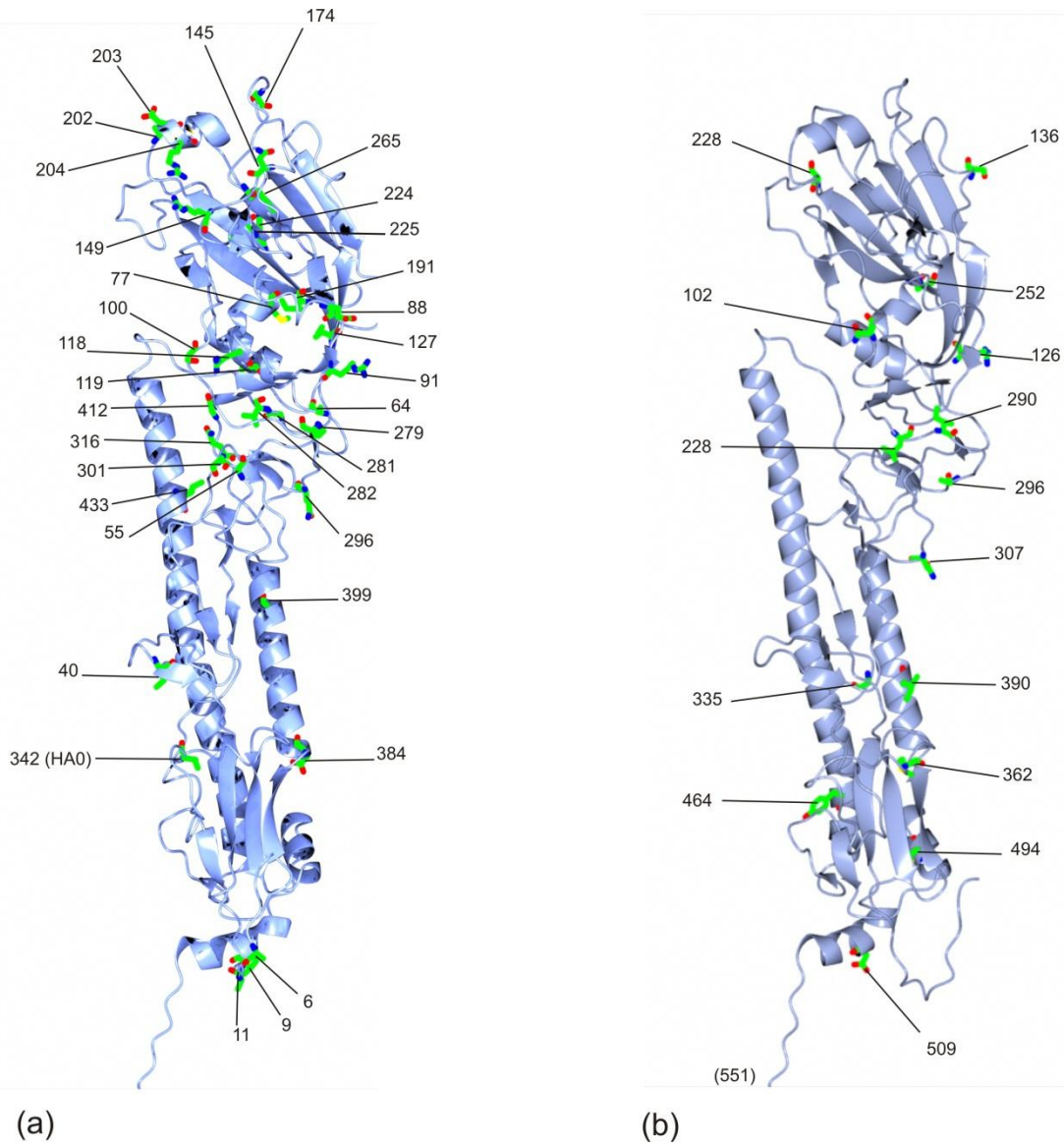


Figure 7. Secondary structure of CKNWY12 showing the potential antigenic sites selected under vaccination pressure over a period of ten years in sub-lineage I (a) and sub-lineage II (b). The vaccine strain CKW04VACC02 is a sub-lineage I strain.

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      10      20      30      40      50      60      70      80
OSKK98      MSLLTVEVETV VLSIVPSGPL KAEIAQRLED VFAGKNTDLE ALMEWLKTRP ILSPLTKGIL GFVFTLTVPS ERGLQRRREV
CKW04VACC02 (I) .....
CKAL1902 (I) .....
CKNWX12 (I) .....
CKMAS13 (I) .....
CKAL2502 (II) .....
CKBKP12 (II) .....
CKBKR412 (II) .....
CKBKR212 (II) .....

      90      100     110     120     130     140     150     160
OSKK98      QNALNGNGDP NNMDRAVKLY RKLKREITFH GAKEVALSYS TGALASCMGL IYNRMGTVTT EVAFGLVCAT CEQIADSQHR
CKW04VACC02 (I) .....
CKAL1902 (I) .....
CKNWX12 (I) .....
CKMAS13 (I) .....
CKAL2502 (II) .....
CKBKP12 (II) .....
CKBKR412 (II) .....
CKBKR212 (II) .....

      170     180     190     200     210     220     230     240
OSKK98      SHRQMVTTTN PLIRHENRMV LAAITAKAME QMAGSSEQAA EAMEVASQAR QMVQAMRTIG THPSSSAGLK DDLENLQAY
CKW04VACC02 (I) .....
CKAL1902 (I) .....
CKNWX12 (I) .....
CKMAS13 (I) .....
CKAL2502 (II) .....
CKBKP12 (II) .....
CKBKR412 (II) .....
CKBKR212 (II) .....

      250     260
OSKK98      QKRMGVQMQR FKVYEGRISA GTAECCGC
CKW04VACC02 (I) .....
CKAL1902 (I) .....
CKNWX12 (I) .....
CKMAS13 (I) .....
CKAL2502 (II) .....
CKBKP12 (II) .....
CKBKR412 (II) .....
CKBKR212 (II) .....

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(a)

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      ectodomain (M2e)      transmembrane region      cytoplasmic tail
      ┌──────────────────┬──────────────────┬──────────────────┐
      │ 10 20 30 40 50 60 70 80 │
      │.....│.....│.....│.....│.....│.....│.....│.....│
OSKK98      MIFLKICRPT RNgWECKCSd SSdPLVIAAS IIGILHLILW ILDRlFFKCI YRRLKYGLKR GPSTEGVPES MR EEYRQEQQ
CKW04VACC02 (I) .....
CKAL1902 (I) .....
CKNWX12 (I) .....
CKMAS13 (I) .....
CKAL2502 (II) .....
CKBKP12 (II) .....
CKBKR412 (II) .....
CKBKR212 (II) .....

      90
OSKK98      SAVDvDDGHF VNIELE
CKW04VACC02 (I) .....
CKAL1902 (I) .....
CKNWX12 (I) .....
CKMAS13 (I) .....
CKAL2502 (II) .....
CKBKP12 (II) .....
CKBKR412 (II) .....
CKBKR212 (II) .....

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(b)

Figure 8. Amino acid alignment of M1 proteins (a) and the M2 proteins (b) encoded on segment 7.

evident at residues 15, 167, 227 and 230 (Fig 8a). The M2 protein is a type III integral membrane protein present on the surface of the influenza A virus particle where it functions as a pH-activated ion channel. M2 consists of three domains, an extracellular domain (M2e) at the N-terminus, a transmembrane domain and the cytoplasmic tail. It was recently reported that selective pressure is stronger on M2 than that on M1 as more sites under positive selection were identified in M2 than in M1. Among them, most of the sites (5 out of 8) were located in the extracellular domain (Xiarong et al 2015). Our results were similar, with relatively more mutations in M2 compared to M1 (Fig 8b). The M2e region contained amino acid mutations that emerged over time in sub-lineage II, namely ³F→S, ⁹P→H and ²³D→N, suggestive of immune selection pressure similar to that of the surface glycoproteins. The M2 cytoplasmic domain, which is important for interaction with M1, genome packaging and formation of viral particle (Nayak et al 2004), contained lineage-specific mutations at residues 43, 60 and 88. The precise domains in M1 that interact with the cytoplasmic tails of HA and NA, and in turn, of M1 with the cytoplasmic tail of M2, remain undetermined or speculative (Rossman and Lamb, 2011).

4. Conclusions

Vaccination is but one of the control tools applied globally to reduce or eradicate avian influenza infections in poultry, and where applied properly, is not only able to prevent illness and death, but also significantly reduce the amount of virus replicating in respiratory and gastrointestinal tracts (Swayne et al 2014). Antigenic matching between the vaccine and field virus is a critical factor in achieving optimal vaccine efficacy, emphasized in numerous cases where circulating field strains have evolved to escape immunity provided by inactivated vaccines applied long-term (Lee et al 2004; Swayne et al 2014; Spackman et al 2014, Wei et al 2016; Lee et al 2016). In continuing South African H6N2 outbreaks in chickens that started in 2001, it was established early on that two H6N2 sub-lineages (I and II) with a common progenitor were involved. The veterinary authority permitted the use of a registered inactivated H6N2 vaccine to control the infection and limit production losses in commercial broilers and layers, but only a sub-lineage I strain was included in the vaccine. This vaccine, although demonstrated here to elicit good antibody responses in vaccinated chickens, as well as cross-reactivity against both sub-lineages as assessed by HI, has not been updated since, and the evolutionary effects on the field strains after a ten-year period are dramatic.

Firstly, both sub-lineages are still co-circulating and substantial genetic and antigenic drift has occurred. Vaccination pressure on sub-lineage I, from which the vaccine strain is derived, is reflected in the higher mutation rate of sub-lineage I (7.7×10^{-3} subs/site/year) compared to sub-lineage II (4.05×10^{-3} subs/site/year). Furthermore, three-fold more potential antigenic epitopes have emerged on the globular head domain of sub-lineage I strains compared to sub-lineage II. A remarkably similar situation with H9N2 was reported in Korea, where two clades (A and B) arose around 2002, also originally derived from a single introduction into poultry (Lee et al 2016). However, implementation of an H9N2 vaccine prepared from an early clade A strain resulted in the extinction of clade A around 2006, whereas clade B persisted and diversified into multiple sub-lineages.

It was also evident that despite prolonged circulation of the field strains no unrelated viruses have contributed internal genes by reassortment to the chicken H6N2 lineage in South Africa. This is atypical of a prolonged endemic LPAI situation, and the homogeneity of H6N2 in South African chickens is probably linked with the lack of opportunities for contact with wild waterfowl. Live bird markets are largely absent and the region is water-scarce (reviewed in Cumming et al, 2015). Other regions, including the European member states and North America but East Asia in particular occasionally report new transmissions of into commercial and backyard gallinaceous poultry of various LPAI strains, including the potentially pathogenic

H5 and H7 subtypes (Cheung et al 2007; Gonzales et al 2010; Corrand et al 2012; Krauss et al 2015), but countries have varying levels of competence in preventing these events from becoming established in poultry.

Throughout their prolonged circulation the sub-lineages retained unique features that are typical evolutionary adaptations of AIVs to terrestrial poultry. Early in their emergence in chickens, sub-lineage I acquired a 22-amino acid deletion in the NA stalk, which was accompanied by compensatory additional N-linked and O-linked glycosylation in the HA protein. Sub-lineage II retained a full-length stalk with less glycosylation. Given the low isolation rates of H6N2 (reasons are discussed below) our study was limited in the number of viruses available for comparison, but we do show that that both sub-lineages have persistently co-circulated under vaccination pressure, unlike other cases where the sub-lineage or clade homologous with the vaccine strain had become extinct and another sub-lineage became dominant (Lee et al 2016).

The functional requirement and balance between the specific HA and N-stalk length was demonstrated in the lack of reassortment between the HA and NA proteins between the two lineages, however, this was not the case with most of the other internal genes. Lindstrom et al (1998) found that H3N2 human influenza viruses isolated over a five-year period displayed a similar pattern. HA and NA proteins evolved in a single lineage, with amino acid changes that accumulated sequentially with respect to time, but in contrast the evolutionary pathways of the six internal genes were not linked to the surface glycoproteins. They concluded that the lack of correlation between the topologies of the phylogenetic trees of the genes coding for the surface glycoproteins and internal proteins was a reflection of genetic reassortment among human H3N2 viruses. Similarly, the phylogenetic topology of the H6N2 viruses showed little correlation with the chronology of the virus isolates. We detected reassortment between lineage I and lineage II internal genes by concatenating the genomes and conducting recombination analysis, but more interesting was the probability that the vaccine seed strain itself was the donor of segments to sub-lineage II field strains. Production, sale and use of the H6N2 inactivated vaccine is strictly regulated by the veterinary authority in South Africa, but there has been at least one other case where use of illegal unregistered AIV vaccines was suspected in other poultry species (Abolnik et al 2013). Facilities with insufficient quality control risk producing vaccines that are inefficiently inactivated. Although many chemicals and procedures effectively render AIV replication deficient, intact genomic nucleic acid may still remain. For example, BEI renders AIV replication deficient even at concentrations as low as 0.001M, but intact AIV genomic nucleic acid is still present at incubation times shorter than 8 hours (Sarachai et al 2010). The presence of nucleic acids in

inefficiently-inactivated vaccines may thus be complicit in reassortment of segments, or even recombination. Cases of intra-segment homologous recombination in AIVs have been reported in the PB1, PA, HA, NP and NA genes (He et al 2009, Chen et al 2016), and the “rescue” of portions of vaccinal genome by recombination, although not yet reported, remains theoretically possible.

The coassortment of HA and NA within the H6N2 sub-lineages due to functional complementation extended to the matrix proteins of the H6N2 viruses. Selective pressure is stronger on M2 than M1, and the M2e domain although small and represented in low amounts on the viral surface, is evidently under immunological pressure (Feng et al 2006; Xiaorong et al 2015; Swayne and Kapczynski, 2008). Specific amino acids in the cytoplasmic domain of M2, or any of the domains in M1 that interact with the specific amino acid residues in cytoplasmic tails of HA and NA might be involved in structural complementation, but this remains to be explored. Since both M1 and M2 are encoded on the same segment, the functional requirement for one would result in the selection and retention of both. Our results are supported by those of a phylogenetic study that analysed >600 matrix protein sequences from multiple virus subtypes (H3 to H11) in Chinese poultry and other avian species, where only the matrix protein genes from the H6N2 subtype formed a monophyletic group (Xiaorong et al 2015). Coassortment of the internal protein segments has not been extensively studied in AIV, but as the tendency shifts towards full AIV genome sequencing thanks to next generation sequencing technologies, more insight into this phenomenon will be gained.

Immunological pressure caused by long-term vaccination culminated in a drastic phenotypic change in the late H6N2 strains. Four of the five viruses isolated in 2012 and 2013 had lost the ability to haemagglutinate CRBCs, and both sub-lineages were implicated. The portion of HA responsible for haemagglutination activity reside in residues 123 to 239 in the HA1 region (Morishita et al 1996), and it is also known that glycosylation of oligosaccharides on viral envelope proteins is a ploy by viruses to escape host immune pressure. Oligosaccharides mask or modify antigenic sites on HA and their presence focuses variation on uncovered antigenic sites. Numerous studies have addressed the structures and functions of N-linked glycans on membrane glycoproteins from different viruses, and N-glycosylation has attracted particular attention for the human immunodeficiency virus (HIV), where a cluster of N-glycans constitute the epitope for the 2G1G and other antibodies with broadly neutralizing function epitopes (Bagdonaite et al, Tate et al 2014).

Oligosaccharides attached to the stalk region of the viral HA tend to be conserved across different viral strains, whereas those attached to the globular head display considerable variation in both number and

location (Ohuchi et al 1997, Tate et al.,2014). Evolutionary studies of pandemic and seasonal H1N1 and H3N2 viruses indicate that the number of N-linked glycosylation sites on the head of HA increased after their emergence in the human population, suggesting that the addition of glycans conferred a selective advantage, likely by preventing the binding of neutralising antibody to antigenic epitopes (Tate et al., 2014). HIV-1 gp160 is another example where hyper-glycosylation is employed as an effective strategy for deflecting neutralizing Abs (Tate et al 2014). For example, Eggink et al (2014) successfully hyper-glycosylated recombinant HA in an attempt to shield immunodominant epitopes on the globular head and redirect antibody responses towards the conserved stalk domain of HA.

Thus, the shielding function is well-documented for N-glycans, but presumably O-glycans serve similar functions (Machiels et al 2011). Substantial evidence suggests that O-glycosylation is important for viral infectivity and virus-induced immunomodulation for several viruses, for example, the HSV-1 envelope proteins were demonstrated *in vivo* to be heavily O-glycosylated (Bagdonaite et al 2015). The lack of a reliable O-glycosylation prediction tool has hampered analysis of these sites and their effects in viruses up until now, and to our knowledge, this study represents the first where O-glycosylation of AIV proteins is investigated. We found that combinations of O-glycosylation site at residues 134, 137 and 141 (H3 numbering) within the critical 130 loop of the RBS correlated with the haemagglutination phenotype in the H6 viruses.

The evolution to loss of the haemagglutinating phenotype of recent H6 isolates presents a major diagnostic dilemma and that this has occurred in both sub-lineages is also very interesting. At the start of the outbreak H6N2 viruses were relatively easily and frequently isolated from infected flocks, but over time isolates have become increasingly difficult to obtain, even though samples from sentinels have been routinely inoculated into ECE. The preferred method for influenza A virus identification is virus isolation in SPF embryonated chicken eggs (OIE 2015). Lack of HA activity in isolates affects the isolation rate of these viruses, and because HA activity is used as a first step to indicate the growth of virus in the allantoic fluid, many laboratories would discard HA-negative eggs if they are not including a PCR as a confirmation step. Coupled with the failure to isolate the strains for sequencing is the inability to update oligonucleotide design for assays that detect and differentiate between specific subtypes, and as we demonstrate, both sub-lineages have undergone significant genetic drift. As veterinary authorities contemplate whether to discontinue H6N2 vaccination completely or whether vaccination should be continued to be used in conjunction with other control strategies, it is clear from our results that a new bivalent vaccine containing late strains of both sub-lineages would be prudent, and that diagnostic procedures must be revised. For the

time being, the H6N2 strains that continue to circulate have not evolved human virulence markers, but this must be closely monitored.

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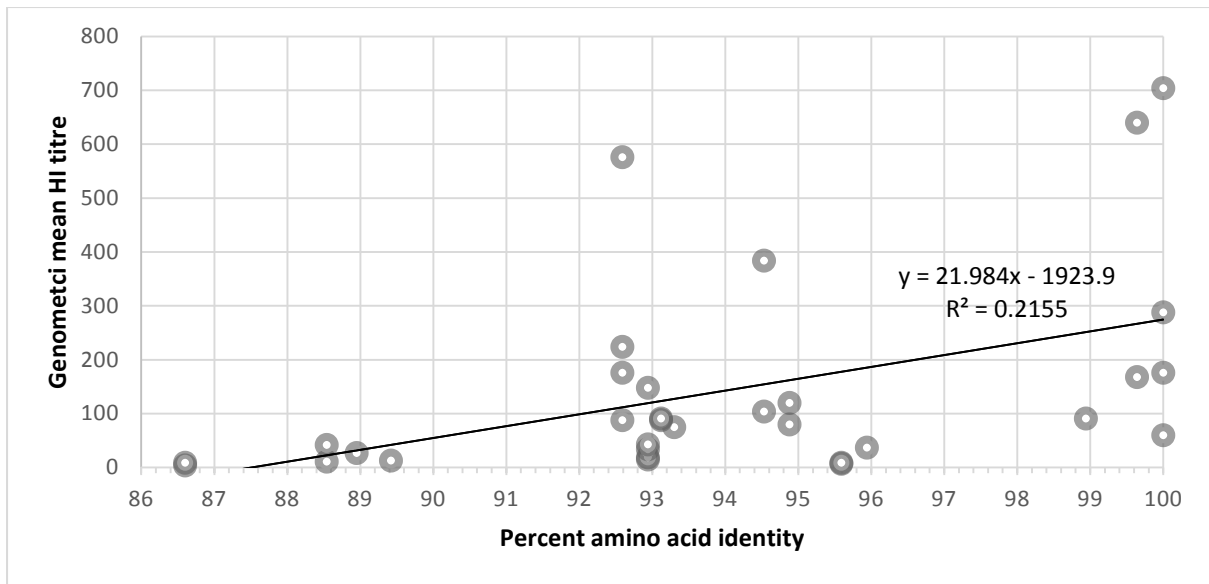
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Supplemental figure 1. Correlation between the HI titre and percent amino acid identity in the HA protein of H6N2 viruses



Supplemental figure 2: Electron micrograph of CKNWY12

Supplemental table 1. Percent amino acid sequence identity in the haemagglutinin (H6) protein gene, with nucleotide sequence identity in square brackets

	CKW04VACC02(I)	CKAL1902(I)	CKAL2502(II)	CKBKP12(II)	CKBKR212(II)	CKBKR412(II)	CKNWY12(I)
CKA1902(I)	99.64 [99.3]	-					
CKAL2502(II)	94.88 [95.0]	94.53 [94.50]	-				
CKBKP12(II)	93.3 [93.12]	92.94 [92.60]	95.94 [94.33]	-			
CKBKR212(II)	92.94 [93.05]	92.94 [92.67]	95.59 [94.27]	99.65 [99.77]	-		
CKBKR412(II)	92.94 [93.12]	92.94 [92.73]	95.59 [94.33]	99.65 [99.83]	100 [99.94]	-	
CKNWY12(I)	93.12 [93.04]	93.12 [93.49]	89.42 [89.29]	87.83 [87.88]	87.48 [87.81]	87.48 [87.88]	-
CKMAS13(I)	92.59 [93.04]	92.59 [92.98]	88.54 [88.88]	86.95 [87.47]	86.60 [87.40]	86.60 [87.40]	98.94 [99.06]