Molecular and antigenic characterization of H6N2 avian influenza viruses isolated in South Africa

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List of Abbreviations

°C	degrees Celsius
%	percentage
2D	two dimensional
3D	three dimensional
A	Alanine
AIV	Avian influenza virus
AI	Avian influenza
BEI	Binary ethylenimine
bp	base pair
CDC	Centre for Disease Control
cDNA	complimentary deoxyribonucleic acid
Cp	crossing point
CRBC	chicken red blood cells
CV	coefficient of variation
D	Aspartic acid
DNA	Deoxyribonucleic acid
E	Glutamic acid
ELISA	Enzyme Linked Immunosorbent Assay
F	Phenylalanine
FAO	Food and Agriculture Organization of the United Nations
G	Glycine
GAU	Gauteng Province
H	Histidine
HA	haemagglutination

HA1	haemagglutinin protein 1
HA2	haemagglutinin protein 2
HAU	haemagglutinating units
HI	haemagglutination inhibition
HPAI	high pathogenic avian influenza
I	Isoleucine
IgG	Immunoglobulin G
K	Lysine
KZN	KwaZulu-Natal Province
L	Leucine
LPAI	low pathogenic avian influenza
M1	matrix 1 protein
M2	matrix protein 2
MDS	multiple dimensional scaling
mℓ	millilitre
N	Asparagine
NA	neuraminidase protein
NCBI	National Center for Biotechnology Information
nm	nanometre
NP	nucleoprotein
NS1	non-structural internal protein 1
NS2	non-structural internal protein 2
nt	nucleotides
OD	optical density
OIE	World Organization for Animal Health
OPD	*o*-Phenylenediamine dihydrochloride

P	Proline
PA	polymerase A protein
PB1	polymerase B1 protein
PB2	polymerase B2 protein
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RNA	ribonucleic acid
rpm	revolutions per minute
rRT-PCR	real time reverse transcription polymerase chain reaction
S	Serine
SD	standard deviation
SP-D	surfactant protein D
SPF	specific pathogen free
S/N	sample to negative
S/P	sample to positive
T	Threonine
µl	microliter
V	Valine
VLA	Veterinary Laboratory Agency
vRNA	viral ribonucleic acid
w	weeks
W	Tryptophan

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Summary

Molecular and Antigenic Characterization of H6N2 Avian Influenza Viruses Isolated in South Africa

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"Does the current H6N2 vaccine still offer sufficient protection?" is a fundamental question asked by the poultry industry in South Africa today. In this study we advanced our understanding of the genetic drift of the H6N2 virus in poultry in South Africa, by phylogenetic analyses of gene segments and antigenic characterization of the virus, and determined the efficacy of the current vaccine.

Using isolates collected between 2002 and 2013, full genome sequencing was performed to determine whether genetic re-assortment had occurred over the past decade. Vaccine batches were made from these isolates and with the use of antigenic cartography the haemagglutination inhibition (HI) data was visualized to determine antigenic similarities or differences between the viruses. From this research, information about the evolution of the H6N2 virus was revealed.

The findings show that although extensive genetic drift has occurred over the last ten years and two distinct sub-lineages have developed, the current vaccine will still offer sufficient protection for both sub-lineages of H6N2 viruses.

Keywords: avian influenza virus, poultry, H6N2, genetic drift

Chapter 1

Introduction

Avian influenza (AI) is a viral infection of birds, which infects most species of birds and can in some cases cross the species barrier and infect humans. The influenza virus is part of the Orthomyxovirus group and there are three types of influenza viruses namely A, B and C. Orthomyxoviruses are enveloped, single-stranded, negative-sense, ribonucleic acid (RNA) viruses with a segmented genome (Cheung, Poon 2007). Influenza C viruses have been isolated from humans and swine while influenza B viruses have been found in humans and seals. Influenza A viruses can infect humans, other mammals and a variety of avian species (Baigent, McCauley 2003).

Type A influenza viruses are divided into serotypes based on two surface glycoproteins: haemagglutinin – HA (H1-H17) and neuraminidase – NA (N1 – N10); all combinations of NA and HA proteins are possible and all of these can infect birds except H17N10 which has only been found in bats (CDC, 2013, Tong *et al* 2012). Type A viruses have been responsible for the human influenza pandemics in the past, where it has been shown that they resulted from the introduction of either HA or NA genes from mammalian or avian viruses (Baigent, McCauley 2003).

Influenza A type viruses that infect birds can be divided into low pathogenic (LPAI) and high pathogenic (HPAI) viruses. Whereas the highly pathogenic viruses can cause up to 100% mortality in poultry (Alexander 2000), the low pathogenic viruses do not often cause mortality.

Prior to 1990, most outbreaks of avian influenza in chickens were attributable to H5 and H7 viral serotypes and very few cases of viruses that had produced stable lineages in chickens were observed. By 2000 this had changed and H6N2 had developed stable lineages in chickens in California (Liu, Guan *et al*. 2003), while H9N2 had become endemic in China (Bi, Lu *et al* 2011). More than 94% of the H9N2 viruses

in the National Center for Biotechnology Information (NCBI) database were isolated in Asia with approximately 60% being isolated from chickens. The phylogenetic analysis of H9N2 viruses isolated from poultry in China showed that these H9N2 influenza viruses evolved quickly and new genotypes were frequently generated in the chicken flocks. It was shown that at least two stable lineages have developed and this combined with the evolution of the H9N2 viruses may represent a threat to human health (Bi, Lu *et al* 2011). The first recorded incidence of H6N2 in South Africa was in 2002 in the KwaZulu-Natal (KZN) province where it was identified by haemagglutination inhibition (HI) and NA typing as H6N2, and it was typed as a LPAI strain at the Veterinary Laboratories Agency (VLA) Weybridge laboratory. There have been sporadic incidences of H6N2 infection until the present, affecting a large number of poultry operations all over South Africa. Two distinct genetic H6N2 sub-lineages circulated in the Camperdown area of the KZN Province and these later spread to other regions of South Africa. It was shown that these viruses probably shared a common ancestor and that this virus arose from a reassortment between closely related viruses that were circulating in ostriches just prior to the emergence of the chicken strains (Abolnik 2007; Abolnik, Bisschop *et al*. 2007a, Abolnik *et al*. in press). Previous research has shown that ostriches and waterfowl can act as mixing vessels for new Avian Influenza virus (AIV) strains (Abolnik, Bisschop *et al.* 2007b, Pfitzer, Verwoerd *et al.* 2000, Zhang, Kong *et al*. 2011). With LPAI outbreaks recorded over the same period in ostriches have being attributed to H6N8, H1N8, H4N2, H10N1, H9N2 and the influenza viruses H10N7, H11N2 and H3N8 being isolated from wild ducks between 2009 and 2013, it is evident that extensive reassortment has and can still take place (Abolnik, Gerdes *et al.* 2010, Abolnik 2010, Abolnik *et al*, in press).

In April 2013 an outbreak of LPAI H7N9 influenza virus in humans in China occurred that originated from poultry (FAO, 2013; Gao, Cao *et al*. 2013). Despite the aforementioned exception, the low pathogenic viruses are not generally considered to cause severe disease and H6N2 is not thought to infect humans (Baigent, McCauley 2003). This does not mean that LPAI is not a problem as it can still spread rapidly through poultry flocks and cause either asymptomatic disease with a drop in egg production, or a mild respiratory disease with a low mortality (CDC, 2013). The poultry industry in South Africa is reliant on healthy breeding birds that can produce the

optimum amount of eggs and even a low pathogenic viral infection can have huge economic implications. The presence of a stable lineage of H6N2 within the poultry industry is of concern as this virus could provide the backbone for the reassortment of H5 or H7 should H6N2 AI infected poultry come into contact with birds infected with other AI viruses. In order to monitor the situation in South Africa there is currently compulsory surveillance for avian influenza that all poultry farmers are required to conduct every 6 months. The birds are screened for avian influenza antibodies using an Enzyme Linked Immunosorbent Assay (ELISA) screening test and any positive samples are then tested using haemagglutination inhibition (HI) tests for H5, H7 & H6 to determine the serotype (Horner and Pienaar, 2009).

The aim of this project was to achieve a better understanding of the genetic drift of the H6N2 virus in poultry in South Africa and to determine if the current LPAI vaccine used still offers sufficient protection against field strains. Full genome sequencing and phylogenetic analyses of gene segments was used to assess antigenic drift and to determine whether genetic re-assortment has occurred over the past decade. Since some of the strains are characterised by a loss of HA activity, the differences in HA activity between the viruses and their ability to produce an immune response was compared by using haemagglutination inhibition tests. The efficacy of the current H6N2 vaccine used in South Africa was examined by using immune responses to the isolates and vaccine, where these were plotted using antigenic cartography to determine the similarities or differences between the viruses.

Chapter 2

Literature Review

The genome of the Influenza A virus has eight single stranded RNA segments (vRNA) which produce at least 10 viral proteins, and are bound to the nucleoproteins (NP) and the influenza virus RNA polymerases inside the virion to form ribonucleoprotein complexes. The viral proteins can be divided into three main groups – the non-packaged protein, surface proteins and internal proteins. The surface proteins on the virus particle are the two glycoproteins mentioned before - the HA and the NA which are encoded by the fourth and sixth viral segments respectively whilst the third surface protein, the matrix protein 2 (M2) is encoded by segment seven of the genome. The three polymerase proteins – polymerase A protein (PA), polymerase B1 protein (PB1) and polymerase B2 protein (PB2), the matrix 1 protein (M1) as well as the non-structural internal protein 2 (NS2) are classified as the internal proteins. M1 is encoded by RNA segment seven, NS2 is located on segment eight, whereas PA, PB1, PB2 are encoded by the three largest segments of the genome segments three, two and one respectively (Neumann, Hatta *et al*. 2003). The only protein that is not packaged into the virus particle is the non-structural protein 1 (NS1) and this protein is coded by segment eight of the viral genome (Suarez, Schultz-Cherry 2000, Cheung, Poon 2007). The RNA segment, corresponding encoded protein and its size in nucleotides (nt) are shown in Table 1.

Table 1: RNA segments of the AI virus and the corresponding encoded proteins

RNA Segment	Encoded protein	Size (nt)
1	PB2	2341
2	PB1	2341
3	PA	2233
4	HA1, HA2	1778
5	NP	1565
6	NA	1413
7	M1, M2	1027
8	NS1, NS2	890

The HA protein is of particular importance to this study. This glycoprotein is responsible for the attachment of the virus to cellular receptors in the host which contain sialic-acid, it is then involved in the virus penetration by the fusion of the viral envelope with the cellular membranes. In order for the HA to acquire fusion activity the uncleaved form – HA_0 – has to be proteolytically cleaved by a cellular enzyme which breaks the disulphide bonds and separates the HA into the HA1 and HA2 proteins, thus cleavage is a precondition for the virus to be infectious (Figure 1). This protein is also the major viral antigen against which neutralising antibodies are formed. For Influenza A viruses, the most important determinant of pathogenicity is the cleavage site structure of the HA precursor. It has been shown that pathogenicity for chickens directly correlates with the ability of the viruses to produce cleaved HA in infected cells in culture and to form plaques on various cell types in the absence of exogenously added trypsin, while analysis of the HA1 and HA2 junctional regions confirmed that pathogenic strains invariably contain multiple basic residues at the cleavage site (Steinhauer 1999). Highly pathogenic viruses have an HA which is cleavable by furin-like proteases that are present in a variety of host tissues whereas LPAI viruses have an HA with cleavability restricted to trypsin-like enzymes, that are excreted by epithelial cells of the respiratory and gastrointestinal tracts. Consequently, LPAI replication is restricted to these tissues, whereas HPAI viruses cause systemic infections (Rott 1992). It has been shown using phylogenetic analysis that the HA genes of H5 and H7 viruses that cause severe disease in domestic birds do not form unique lineages but share common ancestors with non-pathogenic H5 and H7 viruses, this means that LPAI viruses have the potential to become HPAI and spread into mammalian hosts (Röhm, Horimoto *et al*. 1995).

Cleavage of HA produces a new amino (N)-terminal end of HA2 which contains a sequence of hydrophobic amino acids called a fusion peptide. During entry of influenza A virus into cells, the fusion peptide inserts into the endosomal membrane of the cell and causes fusion of the viral and cell membranes, this then allows the influenza viral RNA to enter the cytoplasm. Most influenza A viruses replicate efficiently in embryonated chicken eggs because of the presence of a protease in allantoic fluid that can cleave the HA protein (Rott 1992).

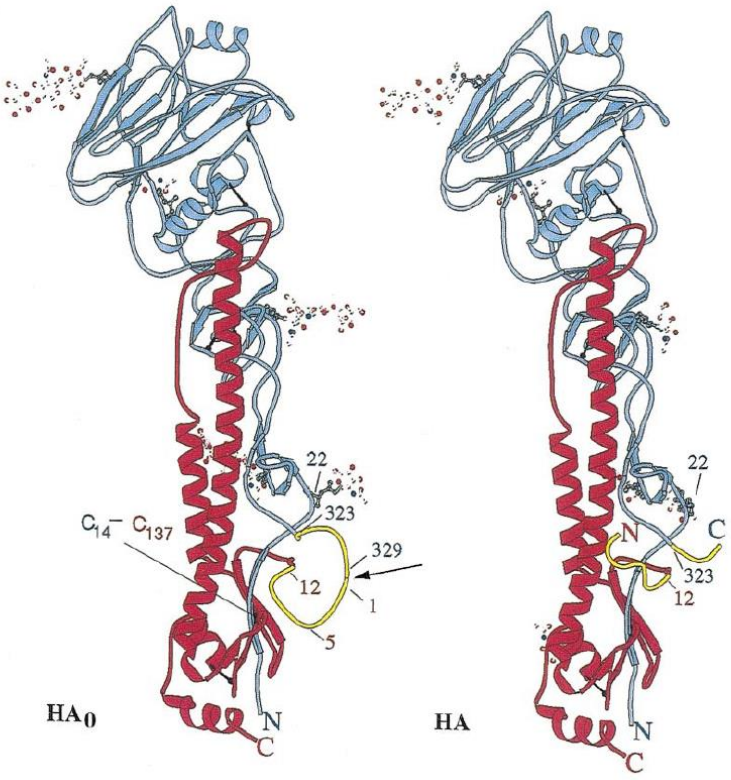

Figure 1: Ribbon diagram of uncleaved and cleaved HA (Chen, Lee et al 1998). The cleavage sites are shown, where HA1 is shown in blue, HA2 is shown in red, and the residues that are displaced after cleavage are shown in yellow. The arrow indicates the site of cleavage in the loop of uncleaved HA. The location of the disulphide bond that links HA1 and HA2 is also indicated.

Host restriction is an important factor in the transmission of avian influenza viruses to mammals, especially humans and involves multiple genetic determinants. Among the determinants of host range restriction, the genes coding for the nucleoprotein and polymerase proteins have been identified as key determinants, which together with the viral RNA segments, form the ribonucleoproteins (Naffakh, Tomoiu *et al* 2008). Of the internal proteins that can determine the host restriction, the most documented protein is the amino acid at 627 (Lysine (K) in human viruses, Glutamic acid (E) in avian viruses) on PB2. A substitution of E to K changed the host range of an avian PB2 single-gene reassortant so that it replicated in mammalian cells. However, it was also observed that multiple combinations of genetic features, involving several genomic segments can determine pathogenicity in mammals (Labadie, Dos Santos *et al*. 2007). Avian influenza viruses bind to α2,3-linked sialic acid, whereas human influenza

viruses recognise α2,6-linked sialic acid as receptors for their binding and entry into cells, and it can be argued that if a virus was to switch its binding site it could cross species and infect humans. Ha *et al* reported in 2001 that HA receptor-binding sites favouring α2,6 linkages (H9 swine, H3 human) are “wider” than the site in the avian HA (H5 avian) that favours α2,3 linkages, and where avian H5 HA hydrogen bonds through E-226, the swine H9 HA uses K-226.

Influenza A viruses bind to polysaccharides on the cell surfaces of erythrocytes of chickens causing haemagglutination. The haemagglutinating activity of AI resides in the HA1 protein (Khurana, Verma *et al.* 2011) and which is the property on which haemagglutination (HA) and haemagglutination inhibition (HI) diagnostic tests are based. The HA test indicates the presence of agglutinating viruses or bacteria in the allantoic fluid. Orthomyxoviruses, paramyxoviruses, a few strains of reovirus, egg drop syndrome 76 adenovirus, infectious bronchitis virus (if treated with neuraminidase) and some bacteria have this haemagglutinating ability. The HI test is used to detect the presence of specific circulating antibodies. For AI each H type is determined by testing the sera with a monospecific antisera to that H type. However, nonspecific inhibition of agglutination can be caused by steric inhibition when the H antigen and serum in the HI test have the same N subtype. This steric inhibition reaction results in red blood cells agglutinating in the bottom of the plate or streaming at the same rate as the control. The N type is only identified by means of a neuraminidase inhibition test (OIE 2015).

It was shown that a mutation on the HA gene at position 190 from glutamic acid (E) to aspartic acid (D) decreased the ability of the virus designated as influenza A/Aichi/51/92 (H3N2) to agglutinate chicken red blood cells and increased recognition of α2,6 (Nobusawa, Ishihara *et al*. 2000). The HA1 protein of influenza viruses has been expressed in bacterial systems and HA activity was retained (Khurana, Verma et al. 2010, Khurana, Verma *et al*. 2011).

A humoral immune response to vaccination or infection by AI in chickens is measured using ELISA and HI tests. The results of HI tests have been used to identify variants among circulating influenza strains in order to select appropriate vaccines for use in human influenza control. Human vaccine selection is aided by the use of antigenic cartography, which provides a graphical overview of the antigenic properties of isolates or vaccine candidates as revealed by haemagglutination inhibition assay data. For example, a change in a single amino acid may cause a disproportionately large change in the binding properties of a virus strain. Antigenic maps can reveal large movements in the antigenic space that may be due to minimal amino acid changes. In this process the distances between the points on an antigenic map represent the similarities or differences between viruses. Using this data, information about the antigenic evolution of a pathogen is revealed. It is thus possible, when using this technology to predict when a vaccine update is necessary, since viruses drifting away from vaccine strains will indicate the start of antigenic diversity. (Cai *et al* 2010, Foucher and Smith, 2010). This technology has to date, not been widely applied for poultry vaccines and only a few studies exist where the antigenic difference of challenge AI viruses was compared in order to select viruses for vaccine efficacy (Beato, Xu *et al* 2014).

Antigenic diversity of AIV can be divided into two phenomenon, that is, antigenic shift and antigenic drift. Antigenic shift occurs when a host is co-infected with two or more different AIVs and acts as a mixing vessel, where gene segments are interchanged during virion packing. This is termed reassortment and is defined by the exchange of intact segments between two differing influenza A viruses. Antigenic drift is normally due to the high mutational rate of the polymerase, where the lack of proofreading of the RNA polymerases contribute to a mutation rate and the selective pressure of the immune system of the host favours this substitution which leads to the development of an antigenically different virus strain (Brown, 2000, Treanor, 2004).

Vaccination for the control of AI was first used during the 1970’s in the USA where inactivated influenza A vaccines were used primarily in turkeys against H1N1, H1N2 and H3N2. Other examples of the use of inactivated AI vaccine to control LPAI are

South Africa (H6N2), Italy (H7N1, H7N3) and the Middle East (H9N2). AI vaccines have also been used to vaccinate against HPAI following outbreaks in Mexico (H5N2) and Pakistan (H7N3). In Pakistan HPAI viruses genetically close to the original HPAI virus were still being isolated in 2004, while in Mexico the HPAI virus was eradicated, but LPAI virus H5N2 continued to circulate. Vaccination was maintained as one of the control tools for these H5N2 LPAI strains, however, within a few years, multiple lineages of antigenically variant H5N2 LPAI field viruses were isolated that escaped. From the immunity induced by the original 1994 vaccine seed strain used in the inactivated vaccine, it is not clear whether the emergence of these antigenic variants was related to the use of vaccines or the improper use of vaccines. It was concluded that the use of AI vaccines to eradicate AI should not be considered as the only solution, but should be used as part of a control program. If a good quality-controlled vaccine is used as part of an integrated strategy where adequate safeguards are in place to detect low-level circulation of virus in infected flocks or the emergence of antigenic variants, vaccination may be successful in controlling the spread of AI. Without the application of monitoring systems, such as unvaccinated sentinel birds within vaccinated flocks, strict biosecurity and depopulation in the face of infection, there is the possibility that long-term circulation and the development of stable lineages of LPAI viruses in vaccinated poultry populations could result in both antigenic and genetic changes in the viruses (OIE, 2015; Swayne, Perdue *et al* 2000, Swayne, 2012).

In South Africa a H6N2 (™Avivac) vaccine is used to control the LPAI that first occurred in 2002. This whole virus AI vaccine is inactivated which reduces the risk of reassortment with other circulating AI viruses. The vaccine is prepared from infective allantoic fluid which has been inactivated by adding binary ethylenimine (BEI) and emulsified with white oil. The use of this vaccine is only allowed under strictly controlled conditions. These include the following restrictions - only serologically negative birds may be vaccinated, while unvaccinated sentinels are placed with these birds and are monitored serologically on a monthly basis for H6N2.

Chapter 3

Materials and Methods

3.1. Viral propagation in embryonated eggs

0.1mℓ of each virus (Table 2) was inoculated into the allantoic cavity of five 8 – 10 day old embryonated specific-pathogen-free (SPF) eggs obtained from Avifarms (Pty) Ltd. After incubation for 7 days at 37°C or immediately after the embryos died, the allantoic fluid was harvested by hand in a Biohazard class 2 cabinet (Airvolution Class II) and tested for haemagglutination activity.

Table 2: H6N2 viral isolates and source of viruses used in the study

Collection date	Strain	Region	Laboratory
2002, July	A/chicken/South Africa/W-04/2002	KZN*	Deltamune (Pty) Ltd
2002, July	A/chicken/South Africa/AL19/2002	Botha's Hill, KZN*	Allerton Provincial Veterinary Laboratory
2002, October	A/chicken/South Africa/AL25/2002	Verulam, KZN*	Allerton Provincial Veterinary Laboratory
2012, March	A/chicken/South Africa/BKP/2012	Eastern Pretoria, GAU*	Deltamune (Pty) Ltd
2012, March	A/chicken/South Africa/BKR2/2012	Eastern Pretoria, GAU*	Deltamune (Pty) Ltd
2012, March	A/chicken/South Africa/BKR4/2012	Eastern Pretoria, GAU*	Deltamune (Pty) Ltd
2012, October	A/chicken/South Africa/NWY/2012	Eastern Pretoria, GAU*	Deltamune (Pty) Ltd
2013, May	A/chicken/South Africa/MAS/2013	Alberton, GAU*	Deltamune (Pty) Ltd

* KZN = Zwazulu-Natal, GAU = Gauteng

3.2. Haemagglutination assay

The HA activity of the isolates was determined using the haemagglutination test which uses chicken red blood cells (CRBC) obtained from SPF blood donor birds purchased from Avifarms (Pty) Ltd and housed at the Deltamune Animal Facility. The CRBC were washed with Phosphate Buffered Saline (PBS) followed by the addition of 25µl of a 1% CRBC to serial dilutions of the viral isolates in a V-bottom 96 well plate (total volume 50µl). Agglutination was read after 40 minutes of incubation at room temperature. HA negative results were where a sharp button of red blood cells was seen at the bottom of the V-bottom well, while HA positive samples showed a diffuse film of red blood cells, no button or a very a small button of red blood cells at the bottom of the V-bottom well. One HA unit in the haemagglutinin titration is the minimum amount of virus that will cause complete agglutination of the CRBC. The last well that shows complete agglutination is the well that contains one HA unit (HAU). The HA titre was calculated as the reciprocal of the dilution that produces one HAU. For example if the dilution at 1/128 contained 1 HAU, the HA titre is the reciprocal of 1/128 = 128 = 2^7(Figure 2)

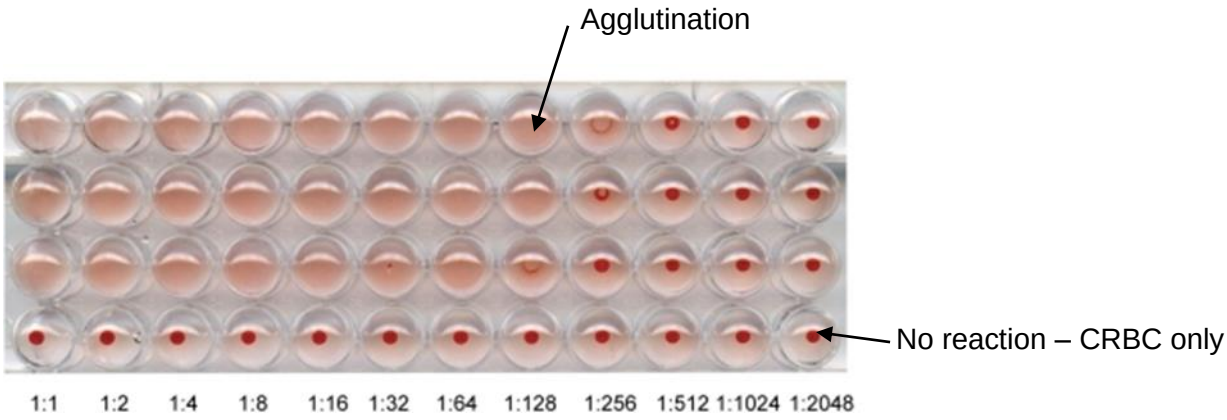

Figure 2: Haemagglutination assay showing agglutination of CRBC (Kanagarajan *et al* 2012)

3.3. Extraction of RNA

The Deltamune laboratory procedure for extracting RNA from allantoic fluid was followed whereby the avian influenza (AI) RNA was extracted from the harvested

allantoic fluid using TRIzol® reagent (Invitrogen). Briefly, 900µl of TRIzol® reagent was added to 200µl of allantoic fluid and allowed to stand for 10 minutes. 200µl of chloroform was added and the sample was vortexed for 15 seconds and allowed to stand for 3 minutes. The sample was centrifuged at 5000 rpm (Beckman Microfuge) for 30 seconds and the upper aqueous phase was transferred into a new marked microtube. 500µl Isopropyl alcohol was added to the tube and it was mixed by vortexing. The sample was allowed to stand at room temperature for 10 minutes. The tube was centrifuged at 12 000 rpm for 10 minutes and the supernatant was discarded. The RNA pellet was washed with 1mℓ 75% Ethanol and centrifuged at 12 000 rpm for 5 minutes after which the supernatant was discarded and the RNA was air-dried.

3.4. rRT-PCR

Identification of the presence of avian influenza virus in the allantoic fluid was done by performing real time reverse transcriptase polymerase chain reaction (rRT-PCR) where the primers are based on the conserved region of the Matrix gene. A master-mix was prepared where the following was pipetted in a tube: 4.3 µl H_2O PCR grade, 2.0 µl 5X concentrated mix, 0.5 µl 20x Primer/Probe Mix, 0.2 µl Enzyme Mix (Real Time Ready Roche). The reagents were mixed by carefully pipetting up and down. The tube containing the master mix was centrifuged for several seconds in a Spectrafuge mini centrifuge. 7µl of the master mix was placed into the plastic reservoir at the top the glass capillary and 3µl of the sample RNA was added. The glass capillary was centrifuged inside the centrifuge adapters at 1500 rpm for 1 minute in the Beckman Microfuge centrifuge. The glass capillary was placed into the sample carousel, and while keeping the capillaries in the upright position, the carousel was placed in the Roche 1.5 LightCycler. The run was programmed into the lightcycler and analysis of the results was performed by the software. All samples with no Crossing point (Cp) value on the graph were interpreted as not detected, while Cp values less than 40 together with a steep amplification curve were interpreted as detected.

3.5. Preparation of transcriptome libraries

The transcriptome libraries were prepared as described by Abolnik, de Castro *et al*. 2012, using a TransPlex transcriptome amplification kit (Sigma-Aldrich). Complementary deoxyribose nucleic acids (cDNAs) were generated from the influenza virus RNA and the DNA was quantified on a Thermo Scientific Nanodrop spectrophotometer. 200ng of DNA was used as an input.

3.6. Sequencing

Nextera library preparations were performed at Inqaba Biotech and analysed on an Illumina MiSeq apparatus.

3.7. Genome assembly

Illumina data was imported into CLC Genomics Workbench v6. Paired-end reads were trimmed and gene segments were assembled *de novo*.

3.8. Bioinformatic analyses

Multiple sequence alignments were prepared in BioEdit (Hall, 1999). Phylogenetic trees of full gene sequences were constructed using the Maximum likelihood interference and MEGA v5.2.2 software, with 1000 bootstrap replices to assign confidence levels to branches (Tamura, Peterson *et al*. 2011).

3.9. Antigen preparation

The harvested allantoic fluid was centrifuged at 2000 rpm for 20 minutes to remove debris and the supernatant was decanted into sterile containers. A 0.2M concentration of binary ethylenimine (BEI) was added to each of the antigen bottles to obtain a final concentration of 2% BEI in each bottle (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 so that each bottle had a final concentration of 0.4% sodium thiosulphate.

3.10. Vaccine preparation

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 was used. The white oil was added to the emulsifiers and dissolved by swirling. An ultra-turrex was inserted into the oil phase and mixing was initiated at low speed, the antigen was added gradually by means of a syringe and the speed of the ultra-turrax was gradually increased to maximum and the vaccine was mixed for 2 min and bottled.

3.11. Approval to use animals for experimental purposes

As the use of experimental animals for this study was necessary, the number of animals in the study was kept at a minimum. An application for the approval to use a total of 16 chickens aged >17w was submitted to the Deltamune Ethics committee prior to the start of the study (Appendix B). It was decided at the time of the application that four chickens per group would be an option instead of two per group as there was sufficient space to house all birds comfortably, while two birds were dedicated as control animals. In total 34 birds were approved to be used in this study (Appendix C). The chickens were housed under normal conditions for poultry at the Deltamune Animal Studies Unit.

3.12. Preparation of positive AI sera

Eight vaccines prepared from each of the 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 of each bird. A total of 34 chickens were used, where two birds were not vaccinated and were used as sentinel controls. The birds were marked with coloured tags and numbers ensuring that the study was from this point blind. Thus the identity of the vaccine group was withheld when the testing was

performed. The birds were all bled from a brachial vein prior to the first vaccination to determine a baseline antibody level. Four weeks after the initial vaccination the birds were bled from a brachial vein and 2mℓ of sera per bird was collected. Thereafter a booster injection was given to each bird. Four weeks after the booster vaccination, a terminal bled was conducted under anaesthesia where 20mℓ of sera was collected from each bird. The birds were monitored daily for clinical signs of disease or mortality over the eight week period.

3.13. Haemagglutination inhibition assay

The isolates that demonstrated a positive HA result were subjected to a haemagglutination inhibition test (HI) where 4HAU of the virus was tested against serial dilutions of the sera from all birds from the 2nd and the terminal bleeds. 25µl of phosphate buffered saline (PBS) was placed in all the wells except the first row where 50µl of the sera was placed. Two-fold serial dilutions of the sera were made in a V-bottom 96 well plate (total volume 50µl). Next 25ul of CRBC was added to all wells of the V-bottom plate (total volume 75µl). Inhibition was read after incubation at room temperature for 45 minutes. The end-point of the titration was the highest dilution of the serum in which haemagglutination was completely inhibited (where streaming of CRBCs was visible).

3.14. Enzyme Linked Immunosorbent Assay (ELISA)

3.14.1. Idexx Influenza A multispecies ELISA

Sera from all birds from the 2nd and the terminal bleeds was subjected to the Idexx Influenza A multispecies ELISA to determine the presence of antibodies to Influenza A. The ELISA was performed in a microtiter well coated with Influenza A Virus. Sera was diluted 1:10 with sample diluent and 100µl of diluted sample was dispensed into the microtiter plates. During the first incubation at room temperature for 60 minutes, Influenza A antibodies present in the sample reacted with immobilised antigens (matrix and nucleoprotein). After a wash step, an anti-influenza A monoclonal antibody enzyme conjugate was added to the microwell and the samples were further

incubated at room temperature for 30 minutes. If no anti-Influenza A antibodies were present in the sample, the enzyme-conjugated monoclonal antibodies were blocked from reacting with the antigen. Following this incubation period, the excess conjugate was removed by washing and a substrate/ chromogen solution was added. In the presence of enzyme, the substrate was converted to a product which reacted with the chromophore to generate a blue colour. The absorbance was read at 620nm using a Thermo Scientific Multiskan 355 microtitre plate spectrophotometer. Results were calculated by dividing the optical density (OD) value of the sample by the mean OD of the negative control, resulting in a sample to negative (S/N) value. The quantity of antibodies to Influenza A is inversely proportional to the OD value, and thus, to the S/N value, where for chickens a S/N value of ≥0.5 was negative.

3.14.2. H6N2 chicken specific ELISA

Sera from all birds from the 2nd and the terminal bleeds was subjected to the Deltamune H6N2 ELISA to determine the presence of antibodies to AI. The ELISA was performed in a microtiter well coated with whole inactivated AI H6N2 virus. The H6N2 ELISA has a diagnostic sensitivity of 99.47% and a diagnostic specificity of 99.05% (unpublished data). Sera was diluted 1:500 with sample diluent and 100µl of diluted sample was dispensed into the microtiter plates. During the first incubation at room temperature for 30 minutes, AI H6N2 antibodies present in the sample reacted with immobilised antigen. After a wash step, 50µl of a whole molecule anti-chicken immunoglobulin G (IgG) peroxidase conjugate developed in rabbits (Sigma Aldrich) was added to the microwells and the samples were further incubated at room temperature for 30 minutes. If no AI H6N2 antibodies were present in the sample, the conjugate was blocked from reacting with the antigen. Following this incubation period, the excess conjugate was removed by washing and 100µl of *o*-Phenylenediamine dihydrochloride (OPD) (Sigma Aldrich) was added. In the presence of peroxidase, the substrate was converted to an end product that was orange-brown in color. The OPD reaction was stopped by adding 100µl of 1.88M H_2SO_4 to each well and the absorbance was read at 492nm using a Thermo Scientific Multiskan 355 microtitre plate spectrophotometer. Results were calculated by dividing the OD value of the sample by the mean OD of the positive control, resulting in a sample to positive

(S/P) value. The titre of each sera was calculated using the formula $Log_{10}Titer = x(Log_{10}S/P) + y$, where x = 1.27 and y = 3.81. Positive results have been determined to have titre value of ≥900.

3.15. Antigenic cartography

Using AntigenMap, an online resource developed specifically for antigenic cartography construction, a low rank matrix completion algorithm was applied to fill in the entries of the HI matrix. Then a temporal multiple dimensional scaling (MDS) algorithm was utilized to map the antigens (and similarly, antibodies) into a two and three dimensional space for visualization (Barnett *et al* 2012). The scale bar on the maps represents one unit of antigenic distance, corresponding to a two-fold difference in the HI assay.

Chapter 4

Results

4.1. HA and rRT-PCR Results

After the isolates were re-passaged in the SPF eggs, AI rRT-PCRs were performed on the harvested allantoic fluid and a positive result was obtained for all isolates confirming that all the isolates did replicate in the eggs. HA activity of the isolates was tested and an HA titre was determined (Table 3). Isolates A/chicken/South Africa/BKR2/2012 and A/chicken/South Africa/NWY/2012 did not cause embryo mortality nor was there a discernible HA titre (Table 3). Isolates A/chicken/South Africa/BKR4/2012 and A/chicken/South Africa/BKP/2012 did not have any HA activity although they did cause embryo mortality. Original isolation records show that all the isolates except A/chicken/South Africa/NWY/2012 showed HA activity. The rapid replication of the AI virus in the egg could have contributed to the lack of HA activity where the spikes on the HA protein have not had sufficient time to form. It has also been suggested that interference by sialic acids on HA could also be the reason that HA activity was not observed (Nobusawa *et al* 2000).

Table 3: HA, rRT-PCR and embryo mortality results of isolate propagation

Isolate	Embryo mortality	HA (log 2) Titre	rRT-PCR result
A/chicken/South Africa/NWY/2012	0/6	Negative	Positive
A/chicken/South Africa/BKR2/2012	0/6	Negative	Positive
A/chicken/South Africa/BKR4/2012	6/6	Negative	Positive
A/chicken/South Africa/BKP/2012	4/6	Negative	Positive
A/chicken/South Africa/MAS/2013	4/6	2	Positive
A/chicken/South Africa/W-04/2002	6/6	5	Positive
A/chicken/South Africa/AL19/2002	6/6	4	Positive
A/chicken/South Africa/AL25/2002	4/6	6	Positive

4.2. HI results

Mini vaccine batches were made with each of the eight isolates and the eight groups of birds and controls were bled prior to vaccination as well as 4w and 8w post inoculation. The birds were primed with the vaccines at the start of the trial and a booster was given at 4w. Isolate - A/chicken/South Africa/W-04/2002 is the current antigen used in the H6N2 vaccines prepared for poultry in South Africa and has been in use since the start of the outbreak. The sera was tested against 4HAU of antigen of the four isolates that showed HA activity. All birds were determined to be AI negative prior to vaccination. The control group (unvaccinated birds) remained negative throughout the 8w trial. HI titres were considered positive if they had a $\log_2$ titre of four or greater (Table 4a, Table 4b). The identity of the vaccine groups was withheld while

the testing occurred. One bird (Blue 39) was euthanized midway through the trial – the necropsy report is included as Appendix D.

Table 4 a: HI log_2 titres at 4 weeks post vaccination.

	Vaccine group	Bird ID	Antigen			
			MAS	W04	AL19	AL25
Antisera	A/chicken/South Africa/BKR2/2012	Purple 4	0	0	0	0
		Purple 43	0	0	0	0
		Purple 45	0	0	0	0
		Purple 50	0	**5**	2	0
	A/chicken/South Africa/BKR4/2012	Yellow 20	0	0	0	0
		Yellow 31	0	2	1	0
		Yellow 34	0	1	1	0
		Yellow 46	0	0	0	0
	A/chicken/South Africa/MAS/2013	Orange 24	**5**	1	1	0
		Orange 41	**5**	2	2	0
		Orange 45	2	1	0	0
		Orange 46	1	0	0	0
	A/chicken/South Africa/W-04/2002	Red 2	3	**6**	**6**	1
		Red 19	**4**	**7**	**7**	1
		Red 38	**5**	**8**	**8**	1
		Red 41	**4**	**7**	**7**	1
	A/chicken/South Africa/AL19/2002	Green 13	0	**4**	3	0
		Green 17	3	**5**	**5**	0
		Green 37	0	1	1	0
		Green 38	3	**6**	**6**	3
	A/chicken/South Africa/AL25/2002	Gray 10	**6**	**8**	**8**	**6**
		Gray 34	1	3	3	1
		Gray 36	2	3	3	1
		Gray 41	**4**	**5**	**5**	**5**
	A/chicken/South Africa/NWY/2012	Blue 12	**6**	**5**	**5**	0
		Blue 19	**5**	**4**	3	0
		Blue 33	0	0	0	0
		Blue 39	1	1	0	0
	A/chicken/South Africa/BKP/2012	White 10	0	0	0	0
		White 22	0	1	2	0
		White 51	1	1	1	0
	SPF	Black 129	0	0	0	0
		Black 147	0	0	0	0

Homologous antisera-antigen reactions are shaded and positive reactions are indicated in bold.

Table 4 b: HI log_2 titres at 8 weeks post vaccination with a booster vaccination at 4 weeks.

	Vaccine group	Bird ID	Antigen			
			MAS	W04	AL19	AL25
Antisera	A/chicken/South Africa/BKR2/2012	Purple 4	1	**4**	**5**	**4**
		Purple 43	2	3	3	1
		Purple 45	3	**5**	**5**	**4**
		Purple 50	0	1	1	1
	A/chicken/South Africa/BKR4/2012	Yellow 20	**4**	**5**	**5**	3
		Yellow 31	2	**5**	**5**	**4**
		Yellow 34	0	3	**4**	1
		Yellow 46	4	**6**	**9**	0
	A/chicken/South Africa/MAS/2013	Orange 24	**8**	**5**	**9**	2
		Orange 41	**8**	**7**	**10**	**4**
		Orange 45	**7**	**7**	**9**	**4**
		Orange 46	**6**	**6**	**8**	3
	A/chicken/South Africa/W-04/2002	Red 2	**8**	**10**	**9**	**7**
		Red 19	**8**	**10**	**5**	**7**
		Red 38	**7**	**9**	**6**	**6**
		Red 41	**6**	**8**	**6**	0
	A/chicken/South Africa/AL19/2002	Green 13	**8**	**9**	**5**	**7**
		Green 17	**8**	**10**	**7**	**7**
		Green 37	**8**	**9**	**6**	**7**
		Green 38	**7**	**9**	**6**	**5**
	A/chicken/South Africa/AL25/2002	Gray 10	**6**	**8**	**9**	**7**
		Gray 34	3	**5**	**9**	**4**
		Gray 36	**5**	**6**	**8**	**5**
		Gray 41	**6**	**7**	**8**	**6**
	A/chicken/South Africa/NWY/2012	Blue 12	**7**	**7**	**7**	**4**
		Blue 19	**4**	3	3	3
		Blue 33	**7**	**7**	**7**	**4**
	A/chicken/South Africa/BKP/2012	White 10	**4**	**5**	**5**	**4**
		White 22	**5**	**7**	**6**	**6**
		White 51	**5**	**6**	**5**	**5**
	SPF	Black 129	0	0	0	0
		Black 147	0	0	0	0

Homologous antisera-antigen reactions are shaded and positive reactions are indicated in bold.

4.3. Antigenic cartography

The 2D and 3D antigenic maps were constructed using AntigenMap, with an HI titre of 1:16 as the threshold for a low reactor (Figure 3, Figure 4 and Figure 5). The antigen maps utilized low-rank matrix completion to minimise the noises in the HI data and multiple dimension scaling to generate the maps reflecting the antigenic distances embedded in the HI data. Each block on the 2D antigenic maps corresponded to a $\log_2$ unit in the HI test.

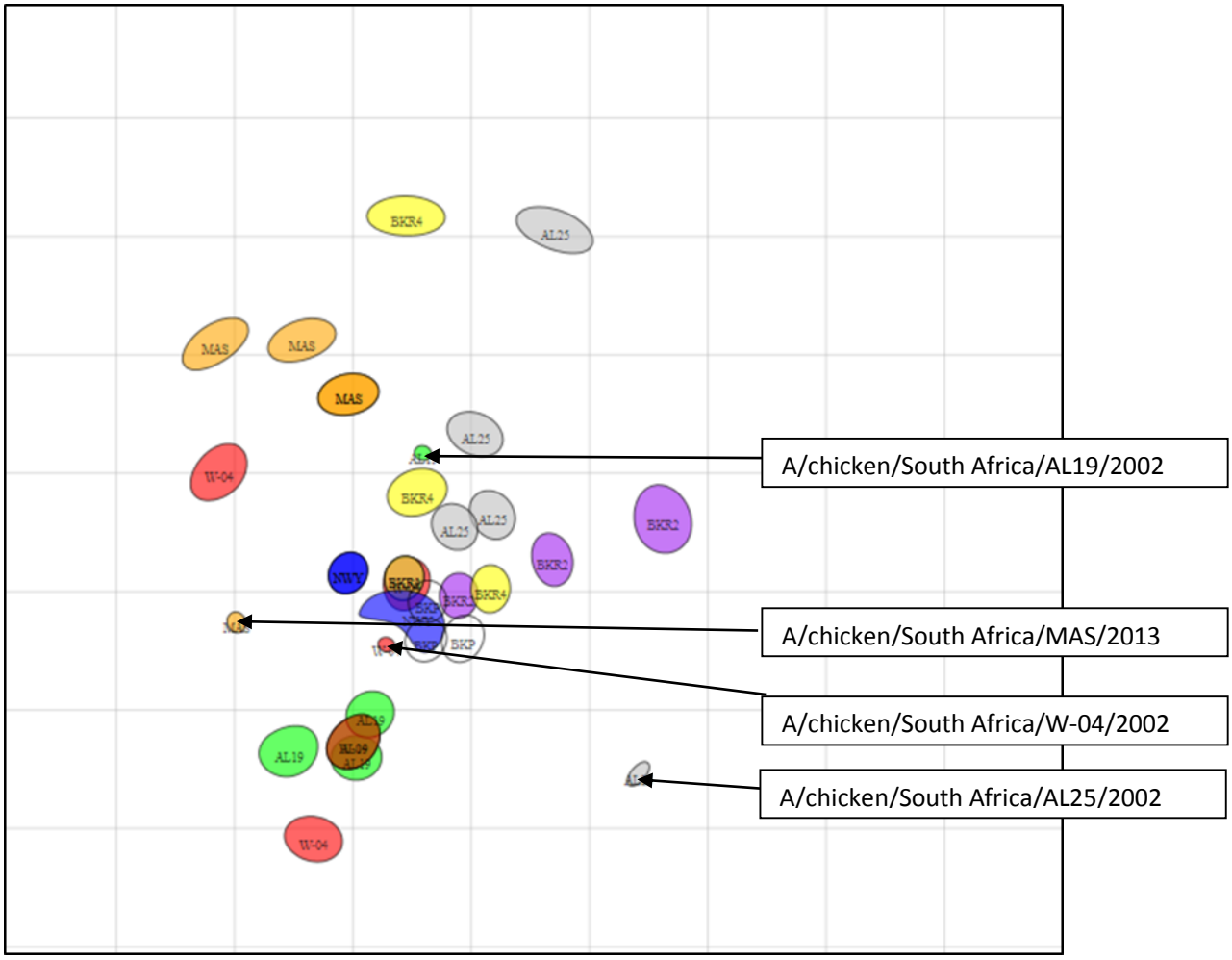

Figure 3: 2D antigenic cartography of the HI titres.

Each vaccine group's HI reactions are denoted by a colour and an amorphous shape, whereas the corresponding antigen is represented by the same colour smaller circle as indicated. A/chicken/South Africa/BKR2/2012 = purple, A/chicken/South Africa/BKR4/2012 = yellow, A/chicken/South Africa/MAS/2013 = orange, A/chicken/South Africa/W-04/2002 = red, A/chicken/South Africa/AL19/2002 = green,

A/chicken/South Africa/AL25/2002 = grey, A/chicken/South Africa/NWY/2012 = blue, A/chicken/South Africa/BKP/2012 = white.

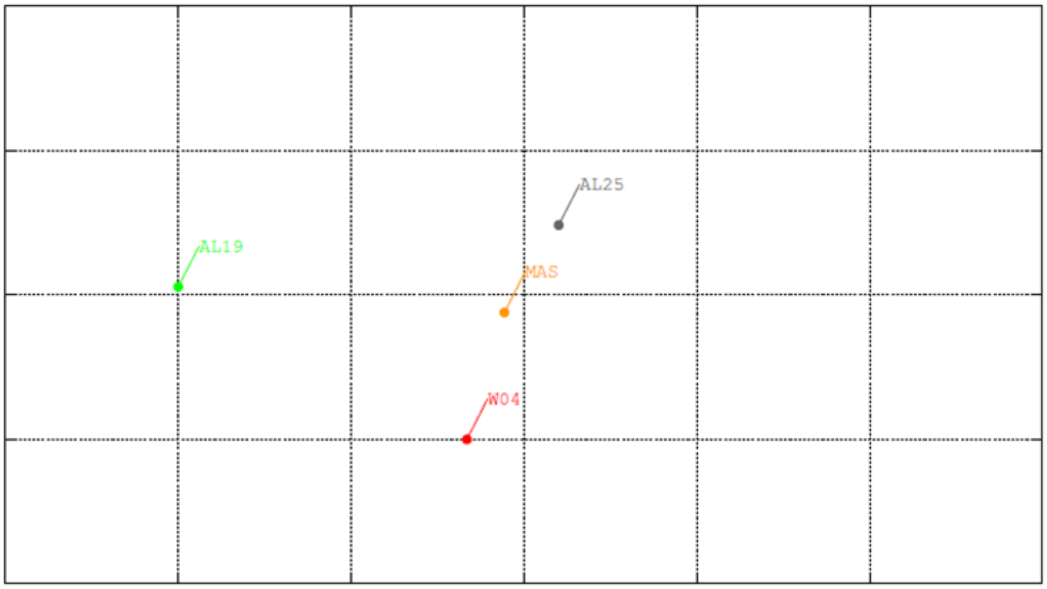

Figure 4: 2D antigenic cartography of the antigens

HA titre representation of the antigens where one block on the graph represents a $\log_2$ titre. A/chicken/South Africa/AL19/2002 = green, A/chicken/South Africa/AL25/2002 = grey, A/chicken/South Africa/W-04/2002 = red, A/chicken/South Africa/MAS/2013 = orange.

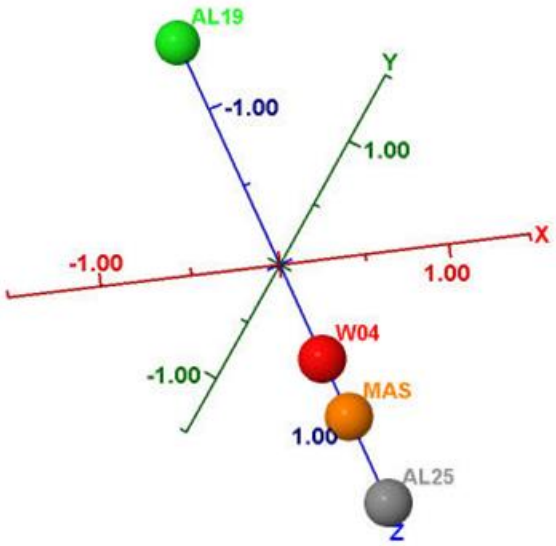

Figure 5: 3D antigenic cartography of the HI titres. HA titre representation of the antigens in 3D where one unit on the graph represents a $\log_2$ titre. A/chicken/South Africa/AL19/2002 = green, A/chicken/South Africa/AL25/2002 = grey, A/chicken/South Africa/W-04/2002 = red, A/chicken/South Africa/MAS/2013 = orange.

4.4. Influenza A multispecies ELISA

Prior to vaccination, all the birds tested negative for AI when the sera was screened using the Idexx Influenza A multispecies ELISA kit. Results were calculated by dividing the optical density (OD) value of the sample by the mean OD of the negative control, resulting in a sample to negative (S/N) value and a sample was considered as positive where a S/N ratio of <0.500 was obtained. At the first bleed 4 weeks after vaccination, 41% of the birds (13/31) already had antibodies to the vaccines while the control birds remained negative. All of the birds in the groups vaccinated with A/chicken/South Africa/AL25/2002 and A/chicken/South Africa/W-04/2002 had already seroconverted at 4 weeks post vaccination, a second booster vaccination was given at this stage. At the terminal bleed 4 weeks later, all birds except the control birds and those vaccinated with A/chicken/South Africa/BKP/2012 had developed antibodies (90%) when screened with the Idexx ELISA (Figure 6). One bird (Blue 39) was euthanized midway through the trial – the necropsy report is included as Appendix D.

Figure 7a shows the average optical densities (OD) per vaccinated group at 4w. In this figure the lower the OD the more positive the sample is, thus showing that all the birds in vaccine group A/chicken/South Africa/AL25/2002 were positive, while all the other groups still had birds that had not seroconverted. A booster vaccination was given a 4w post initial vaccination and 4w later all vaccine groups, excluding A/chicken/South Africa/BKP/2012 had seroconverted (Figure 7b).

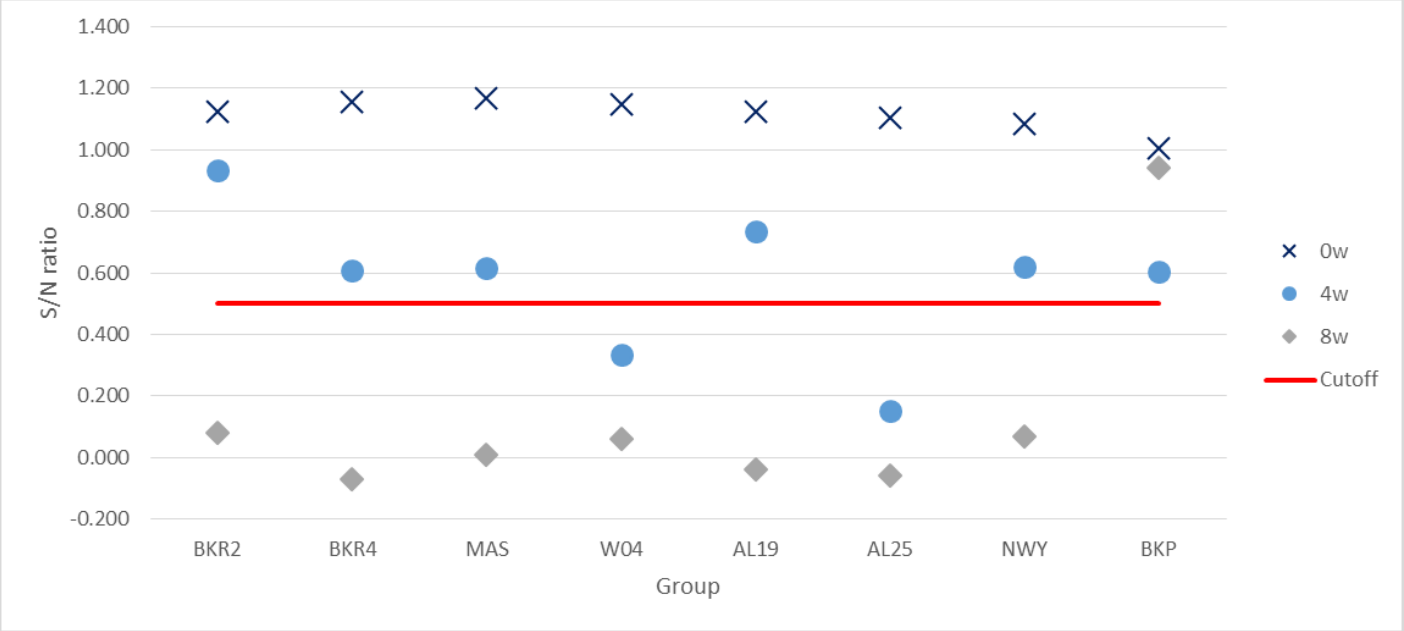

Figure 6: Idexx Influenza A ELISA S/N ratios. The average S/N values of each vaccinated group depicted at the bleeding intervals of pre-vaccination (0w), 4w and 8w post vaccination, where all values below the cut-off line on the graph are considered positive.

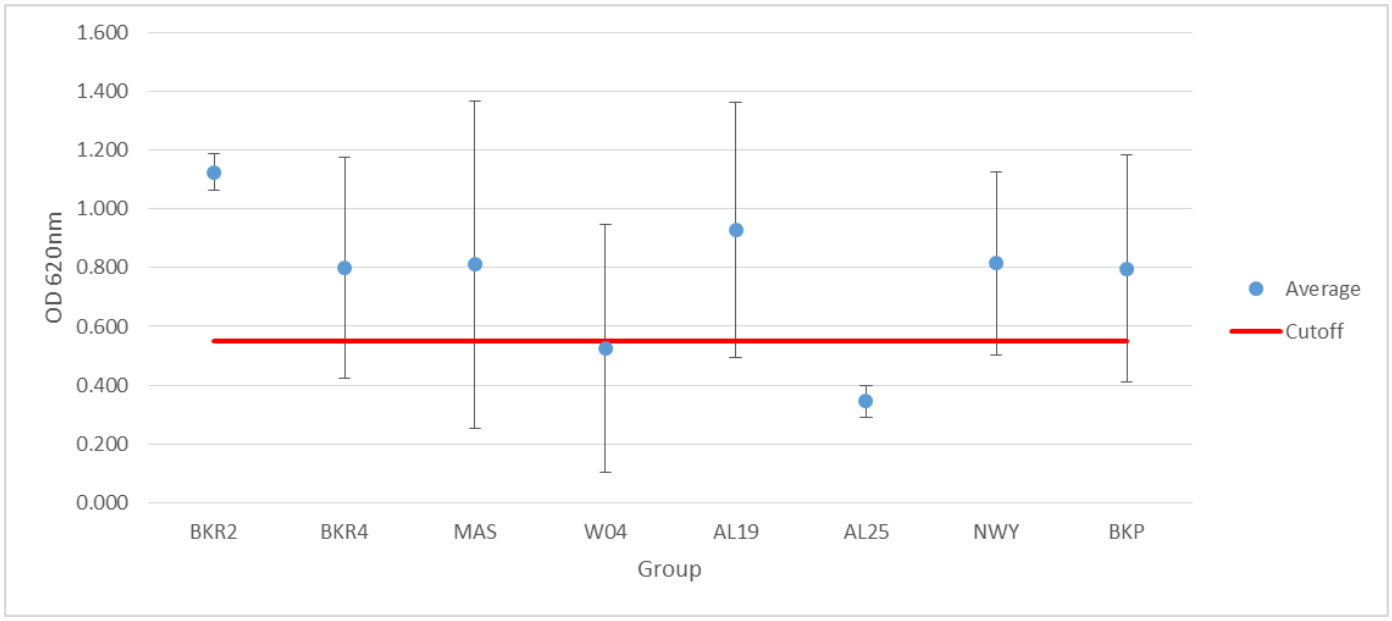

Figure 7a: Idexx influenza A ELISA - Average optical densities (OD) per vaccinated group at 4w. The bars on the graph indicate the range of values per group with the average OD within the group being indicated by ●, while the all the values below the predetermined cut-off line are considered positive.

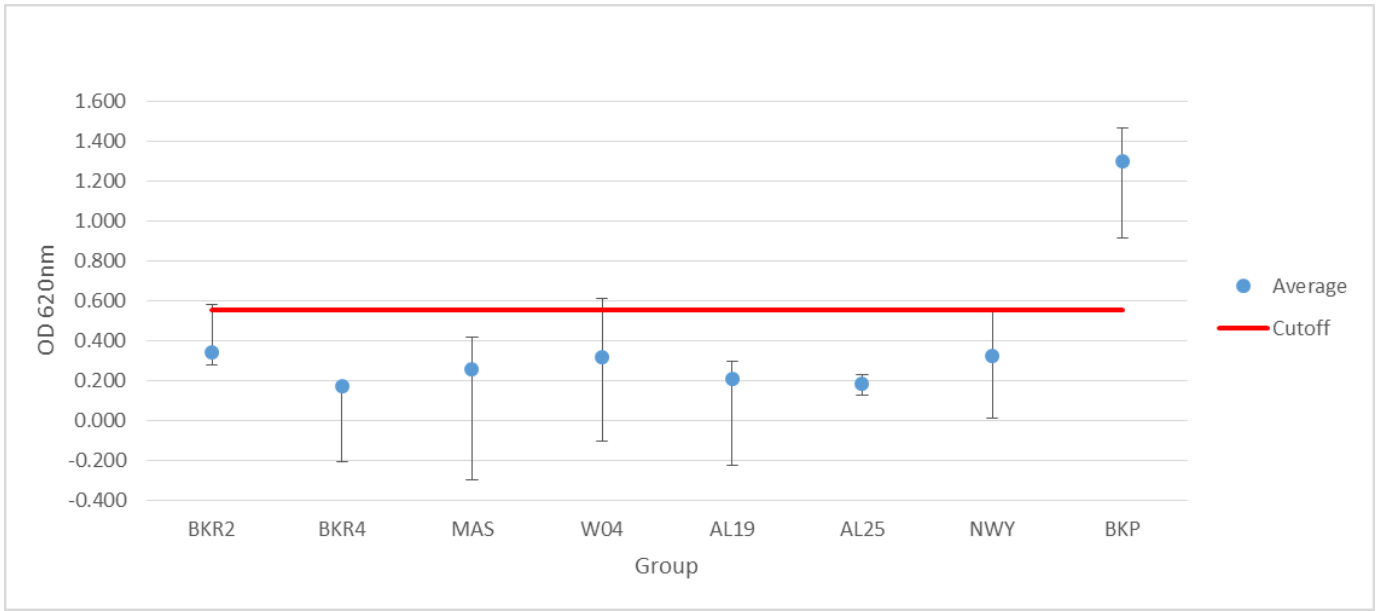

Figure 7b: Idexx influenza A ELISA - Average optical densities (OD) per vaccinated group at 8w. The bars on the graph indicate the range of values per group with the average OD within the group being indicated by ●, while the all the values below the predetermined cut-off line are considered positive.

4.5. H6N2 ELISA

As with the Idexx kit, prior to inoculation all the birds tested negative for AI when the sera was screened using the H6N2 Avian Influenza ELISA kit (Deltamune). At the first bleed 4 weeks after vaccination, 54% of the birds (17/31) had already developed antibodies to the vaccines while the control birds remained negative (Figure 8), where a titre value of >900 was considered positive. All the birds in the group vaccinated with A/chicken/South Africa/W-04/2002 had already seroconverted at 4 weeks post vaccination, while 75% of those vaccinated with A/chicken/South Africa/AL25/2002, A/chicken/South Africa/BKR2/2012 and A/chicken/South Africa/AL19/2002 had also seroconverted (Figure 9a). A booster vaccination was given at this stage and at the terminal bleed, 8 weeks after initial vaccination and 4 weeks post booster vaccination, all birds except the control birds had developed antibodies when screened with the H6N2 ELISA. One bird (Blue 39) was euthanized midway through the trial – the necropsy report is included as Appendix D. The average titres for all groups except MAS and BKP were above or at the cut-off value for positive results (Figure 8 and Table 8). When tested at 8 weeks post initial vaccination (and 4 weeks after the booster vaccination) with the H6N2 ELISA all birds had seroconverted, with the biggest standard deviation (SD) being for group A/chicken/South Africa/AL25/2002 with an SD value of 0.407 (Figure 9b).

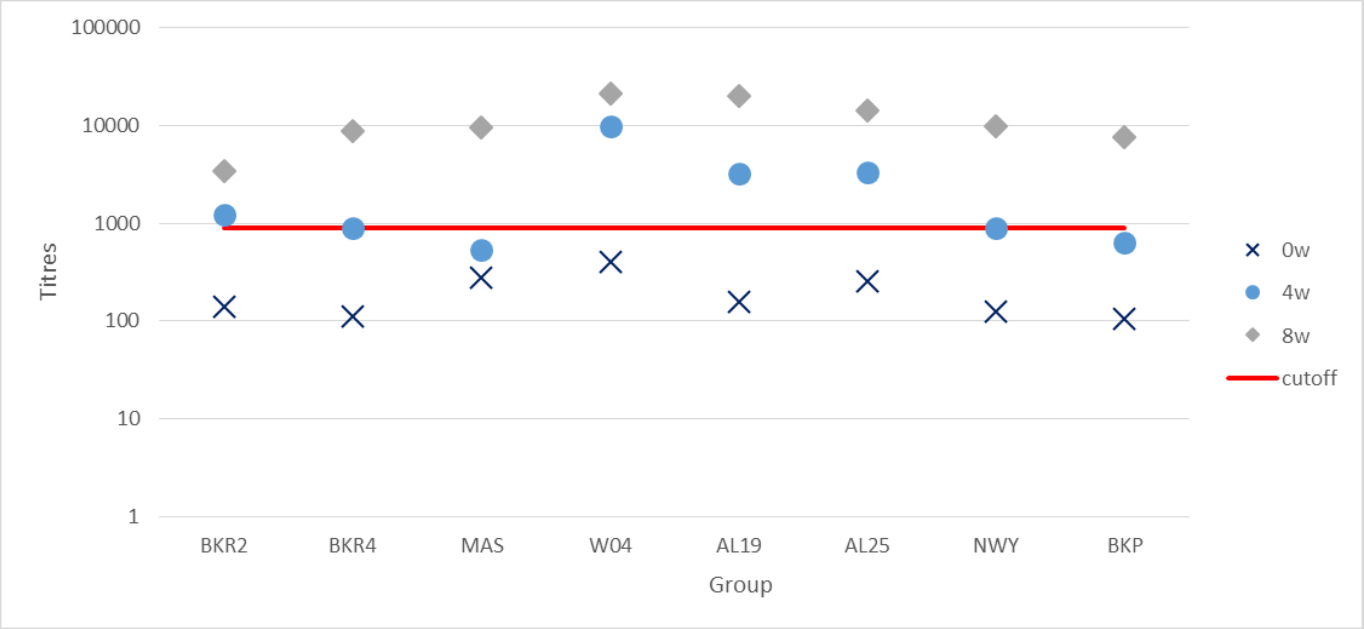

Figure 8: H6N2 ELISA Titres The average titre values of each vaccinated group depicted at the bleeding intervals of pre-vaccination (0w), 4w and 8w post vaccination, where all values above the cut-off line on the graph are considered positive.

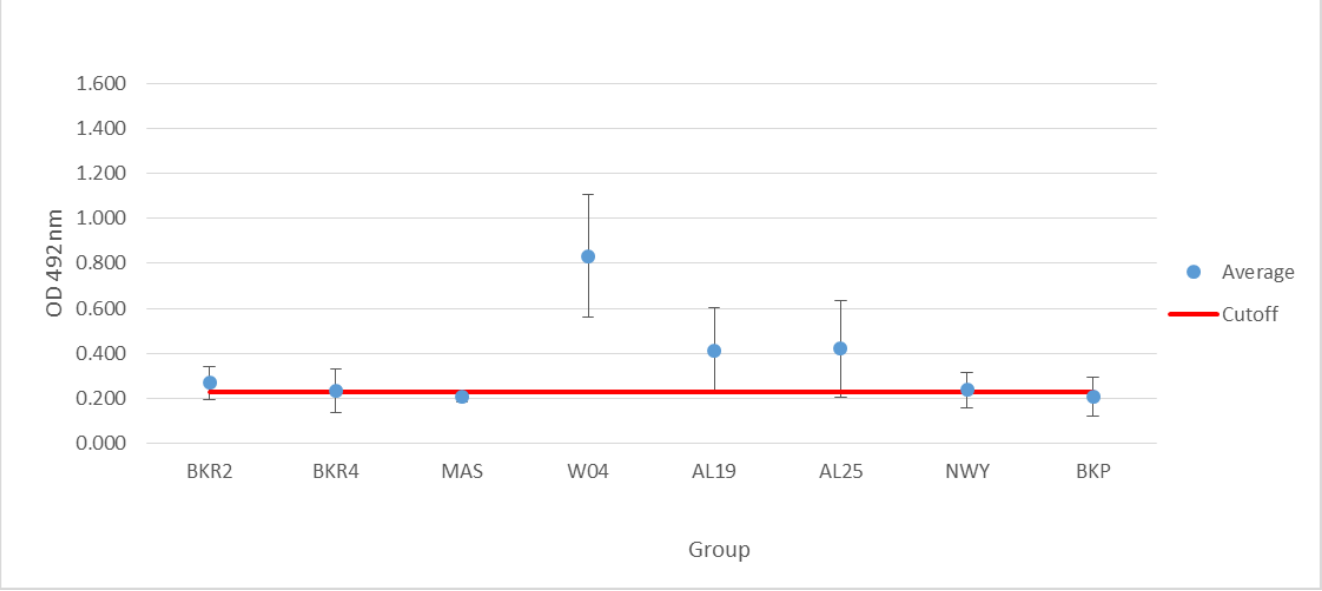

Figure 9a: Average optical densities at 4w – H6N2 ELISA. The bars on the graph indicate the range of values per group with the average OD within the group being indicated by ●, while the all the values above the predetermined cut-off line are considered positive.

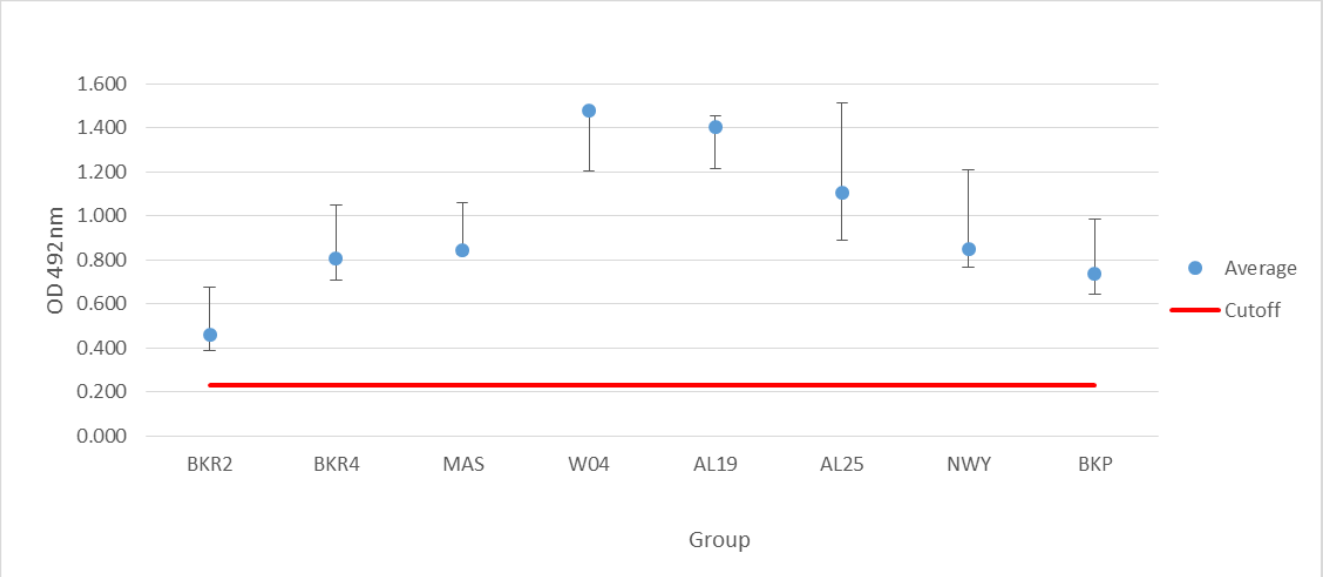

Figure 9b: Average optical densities at 8w – H6N2 ELISA. The bars on the graph indicate the range of values per group with the average OD within the group being indicated by ●, while the all the values above the predetermined cut-off line are considered positive.

4.6. Molecular characterization

Full gene sequences of the H6N2 virus strains were compared to each other and to two ostrich virus strains A/ostrich/South Africa/KK98/1998 (H6N8) and A/ostrich/South Africa/9508103/1995 (H9N2). Analysis of amino-acid sequences at HA_0 was performed to determine the cleavage site motif. Although 2 isolates have atypical sequences there is no insertion of basic amino acids thus confirming that all isolates are LPAI (Table 5).

Table 5: Amino acid sequences at HA_0

Collection date	Strain	HA_0
1998	A/ostrich/South Africa/KK98/1998 (H6N8)	PQIETR*GLF
2002, July	A/chicken/South Africa/W-04/2002	PQIETR*GLF
2002, July	A/chicken/South Africa/AL19/2002	PQIETR*GLF
2002, October	A/chicken/South Africa/AL25/2002	PQIETR*GLF
2012, March	A/chicken/South Africa/BKP/2012	PQIETR*GLF
2012, March	A/chicken/South Africa/BKR2/2012	PQIETR*GLF
2012, March	A/chicken/South Africa/BKR4/2012	PQIETR*GLF
2012, October	A/chicken/South Africa/NWY/2012	PQVETR*GLF
2013, May	A/chicken/South Africa/MAS/2013	PQVETR*GLF

The aligned nucleotide (nt) sequences for the H6 haemagglutinin genes and N2 neuraminidase genes were compared by calculating pairwise percentage identities (Table 6). The multiple sequence alignment for the HA genes is represented in Figure 18, and were examined for similarities and differences observed across the entire sequence. Once the phylogenic trees were constructed the differences were discussed (Section 4.7).

Table 6: Comparison of pairwise identities in the haemagglutinin (H6) proteins where 1720 bp sequences were aligned.

	KK98 H6N8	W-04 H6N2	AL19 H6N2	AL25 H6N2	BKP H6N2	BKR2 H6N2	BKR4 H6N2	NWY H6N2	MAS H6N2
KK98 H6N8									
W-04 H6N2	95.68								
AL19 H6N2	95.49	**99.02**							
AL25 H6N2	94.04	**94.39**	93.81						
BKP H6N2	92.74	**93.49**	92.74	93.76					
BKR2 H6N2	92.37	**95.57**	92.49	93.13	98.90				
BKR4 H6N2	92.49	**92.68**	92.61	93.24	99.02	99.88			
NWY H6N2	90.25	**93.20**	93.66	89.29	88.71	88.12	88.24		
MAS H6N2	90.36	**92.74**	93.30	88.89	88.36	87.88	87.99	98.96	

Strains that show a >90% homology with the vaccine strain are indicated in blue in the table, while the lowest homology between strains is indicated in red.

The pairwise identities of the base pairs (bp) showed the highest homology between A/chicken/South Africa/BKR2/2012 and A/chicken/South Africa/BKR4/2012 and the second highest homology between A/chicken/South Africa/W-04/2002 and A/chicken/South Africa/AL19/2002 with a pairwise identity of 99.02%. The first two isolates were not tested against each other in the HI testing, however the latter two were tested and these last two isolates showed the highest HI titres when tested against each other. A/chicken/South Africa/MAS/2013 had pairwise identities of 88.89% to A/chicken/South Africa/AL25/2002, 92.74% to A/chicken/South Africa/W-04/2002 and 93.30% to A/chicken/South Africa/AL19/2002. When comparing the pairwise identities to the average HI score for each isolate, there was a $\log_2$ HI titre of 5 or less when the pairwise identity was less than 90%.

Table 7: Comparison of pairwise identities in the neuraminidase (N2) proteins.

	9508103 H9N2	W-04 H6N2	AL19 H6N2	AL25 H6N2	BKP H6N2	BKR2 H6N2	BKR4 H6N2	NWY H6N2	MAS H6N2
9508103 H9N2									
W-04 H6N2	80.17								
AL19 H6N2	87.67	84.23							
AL25 H6N2	92.28	85.15	**90.28**						
BKP H6N2	84.87	88.42	83.37	89.92					
BKR2 H6N2	89.69	83.31	88.10	**94.92**	**94.63**				
BKR4 H6N2	88.71	83.77	87.13	**93.91**	**95.01**	**98.91**			
NWY H6N2	80.76	88.42	84.21	89.92	**74.65**	83.79	83.17		
MAS H6N2	76.69	**94.06**	80.03	81.28	85.00	79.76	80.22	**93.84**	

The lowest homology is indicated in red, while genes with homology >90% are indicated in bold.

4.7. Phylogenetic analyses

De novo assembly of trimmed, paired-end reads was applied to assemble full genomic segments for the PB2, PB1, PA, NS, NP, NA, M and HA genes. Sequences were compared in multiple sequence alignments and phylogenetically, using as references the two South Africa viruses isolated from ostriches A/ostrich/South Africa/KK98/1998 (H6N8) and A/ostrich/South Africa/9508103/1995 (H9N2). The general phylogenetic relationships of the South African H6N2 viruses to other viruses isolated from 1992 to 2014 are published elsewhere (Abolnik et al, in press). The full gene sequences of the South African H6N2 viruses in this study were compared to each other and phylogenetic trees were constructed for each of the internal genes (Figures 10 – 17).

Bootstrapping is a method for assessing confidence in a phylogenetic analysis, where bootstrap proportions in majority-rule consensus trees provide biased but highly conservative estimates of the probability of correctly inferring the corresponding clades. Bootstrap proportions of ≥70% usually correspond to a probability of ≥95% that the corresponding clade is real. When constructing the maximum likelihood tree of HA gene sequences it was rooted with A/ostrich/South Africa/KK98/1998 and two distinct sub-lineages indicated as **I** and **II** became apparent (Figure 16a). Sub-lineage **I** contained - A/chicken/South Africa/NWY/2012, A/chicken/South Africa/MAS/2013, A/chicken/South Africa/W-04/2002 and A/chicken/South Africa/AL19/2002; while sub-lineage **II** contained A/chicken/South Africa/AL25/2002, A/chicken/South Africa/BKP/2012, A/chicken/South Africa/BKR2/2012 and A/chicken/South Africa/BKR4/2012.

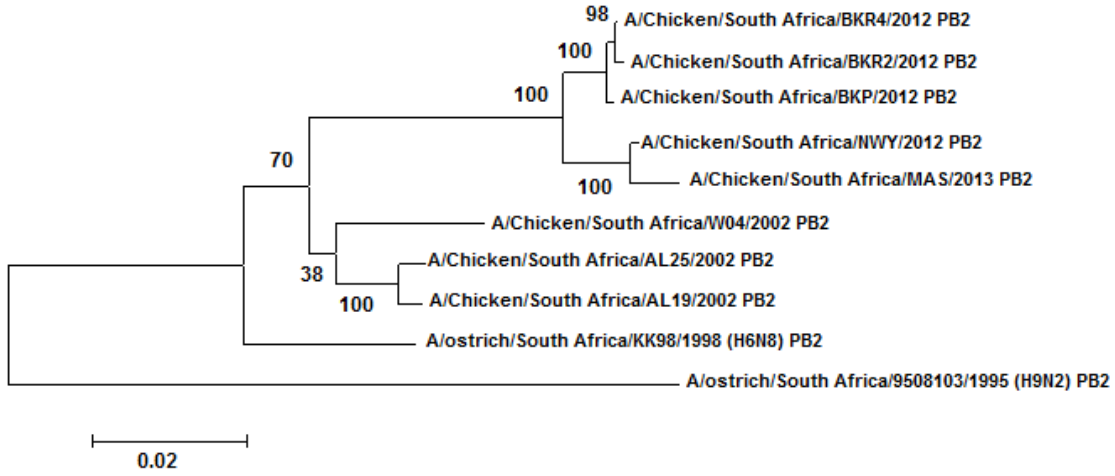

Figure 10: Maximum likelihood tree of Polymerase B2 (PB2) gene sequences (2341bp). Horizontal distances are proportional to genetic distance and the tree is rooted with A/ostrich/South Africa/9508103/1995 (H9N2).

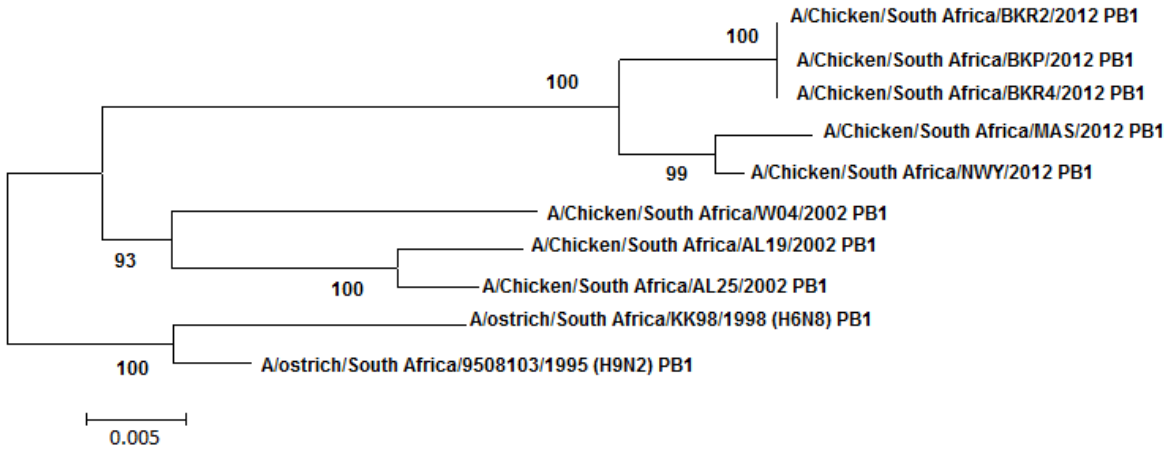

Figure 11: Maximum likelihood tree of Polymerase B1 (PB1) gene sequences (2341 bp). Horizontal distances are proportional to genetic distance and the tree is rooted with A/ostrich/South Africa/9508103/1995 (H9N2).

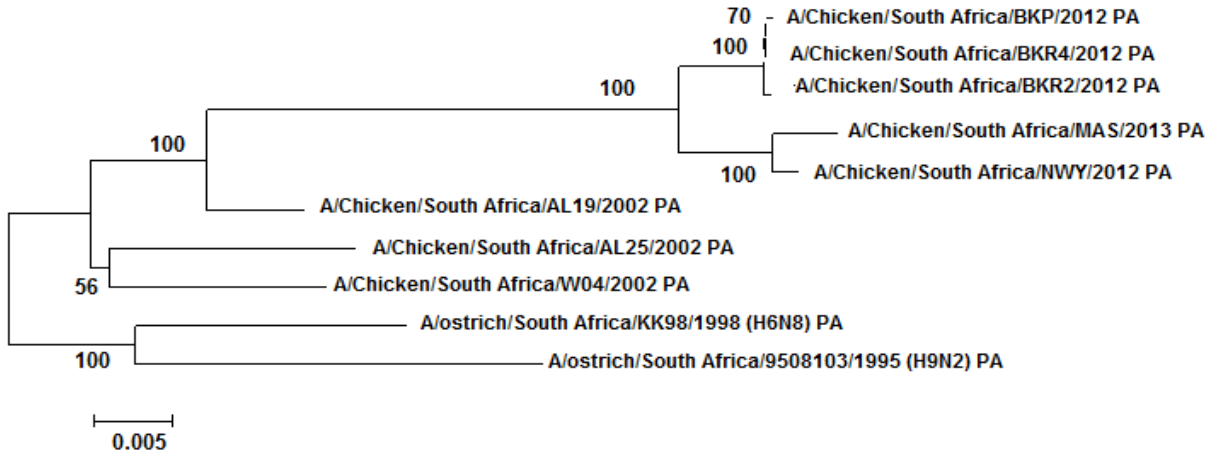

Figure 12: Maximum likelihood tree of Polymerase A (PA) gene sequences (2233 bp). Horizontal distances are proportional to genetic distance and the tree is rooted with A/ostrich/South Africa/9508103/1995 (H9N2).

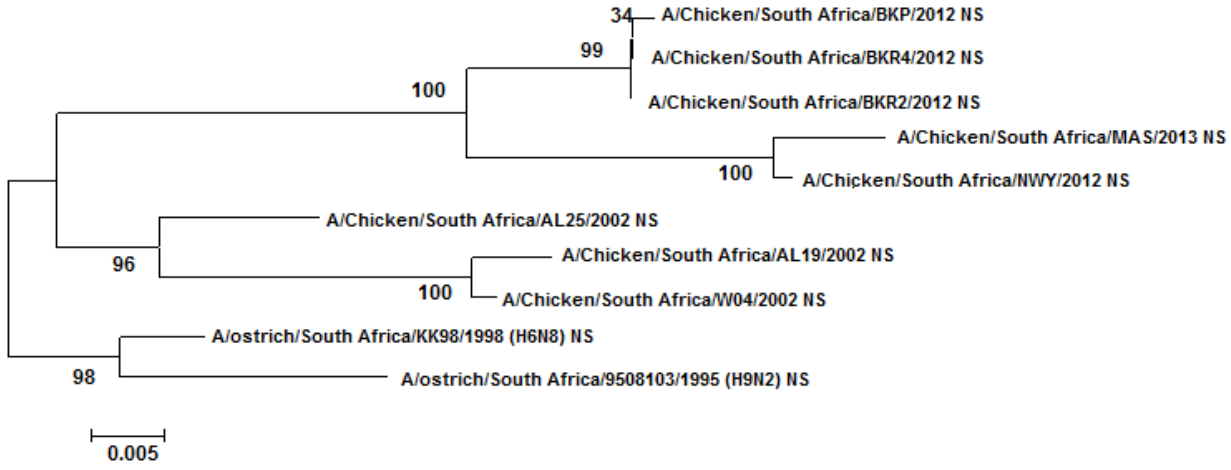

Figure 13: Maximum likelihood tree of Non-structural (NS) gene sequences (890 bp). Horizontal distances are proportional to genetic distance and the tree is rooted with A/ostrich/South Africa/9508103/1995 (H9N2).

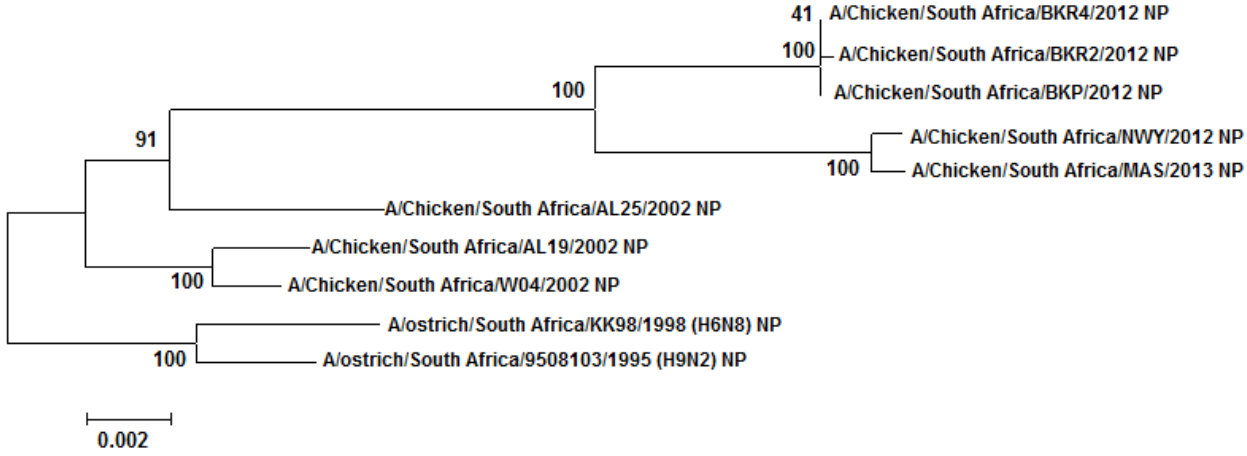

Figure 14: Maximum likelihood tree of Nucleoprotein (NP) gene sequences (1565 bp). Horizontal distances are proportional to genetic distance and the tree is rooted with A/ostrich/South Africa/9508103/1995 (H9N2).

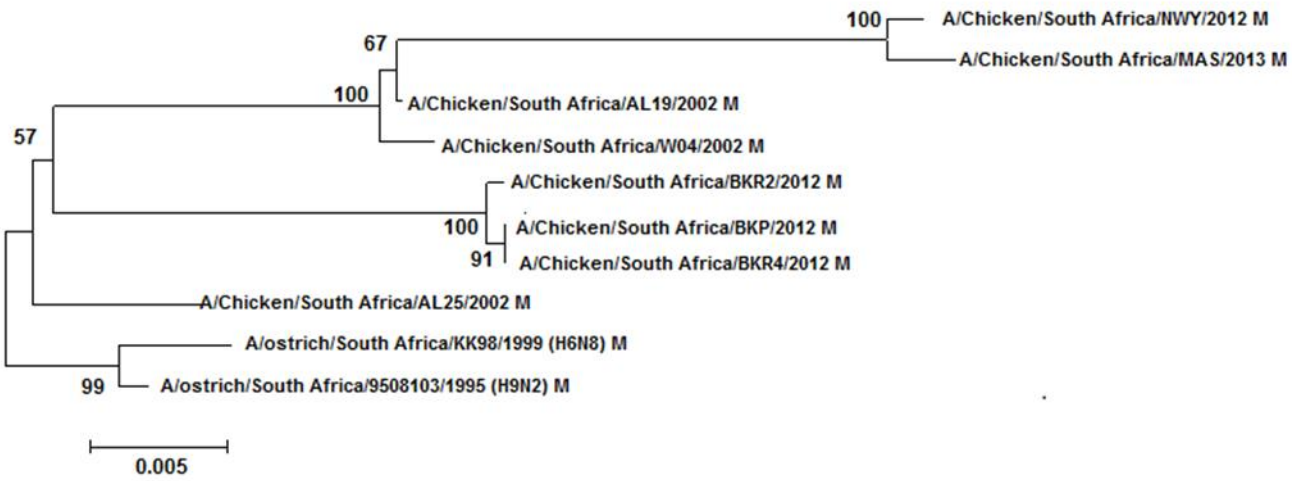

Figure 15: Maximum likelihood tree of Matrix (M) gene sequences (1027 bp). Horizontal distances are proportional to genetic distance and the tree is rooted with A/ostrich/South Africa/9508103/1995 (H9N2).

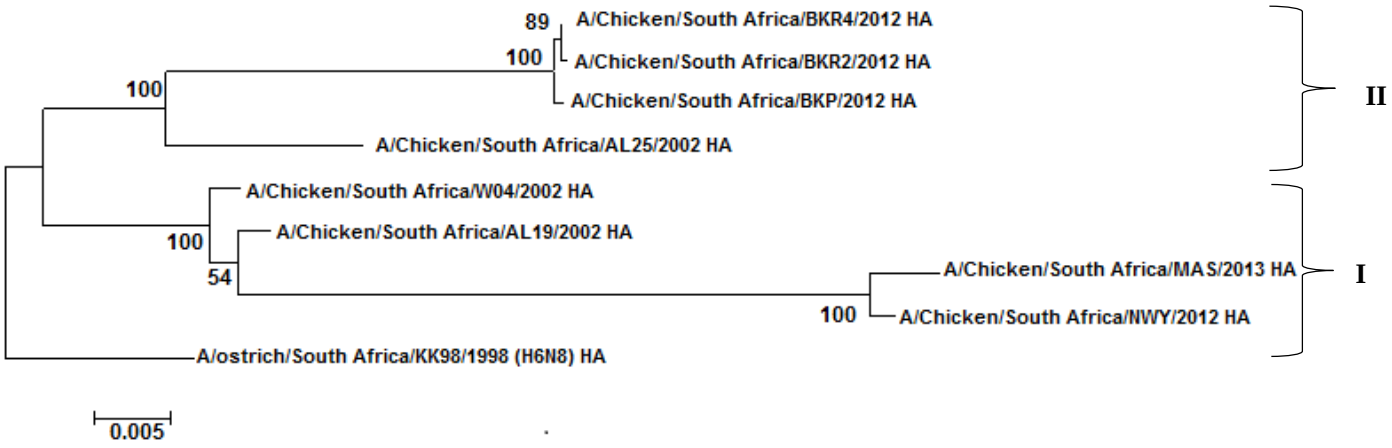

Figure 16a: Maximum likelihood tree of Haemagglutinin (HA) gene sequences (1778 bp). Horizontal distances are proportional to genetic distance and the tree was rooted with A/ostrich/South Africa/KK98/1998 and two distinct sub-lineages indicated as **I** and **II.**

When constructing the radial trees the evolutionary history was inferred using the Maximum likelihood method, which is a clustering method to group pairwise distances.

The reason that this was the favoured method when working with influenza virus was because AI viruses have different evolutionary rates in different hosts and sequences from both ostriches and poultry were compared. The trees were drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic trees. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed, while the evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. This analysis involved nine virus sequences and all positions containing gaps and missing data were eliminated. There were a total of 1717 positions in the final HA dataset and a total of 1258 positions in the final NA dataset. When the radial phylogram of the H6 HA genes (Figure 16b) was constructed it was rooted with A/ostrich/South Africa/KK98/1998 and the same two distinct sub-lineages circled I and II became apparent that were shown with the maximum likelihood tree (Figure 16a). When the maximum likelihood tree (Figure 17a) and the radial phylogram of the N2 NA genes was constructed (Figure 17b), the tree was rooted with A/ostrich/South Africa/9508103/1995 and two distinct sub-lineages indicated as I and II became apparent. Sub-lineage **I** contained - A/chicken/South Africa/NWY/2012, A/chicken/South Africa/MAS/2013, A/chicken/South Africa/W-04/2002 and A/chicken/South Africa/AL19/2002; while sub-lineage **II** contained A/chicken/South Africa/AL25/2002, A/chicken/South Africa/BKP/2012, A/chicken/South Africa/BKR2/2012 and A/chicken/South Africa/BKR4/2012.

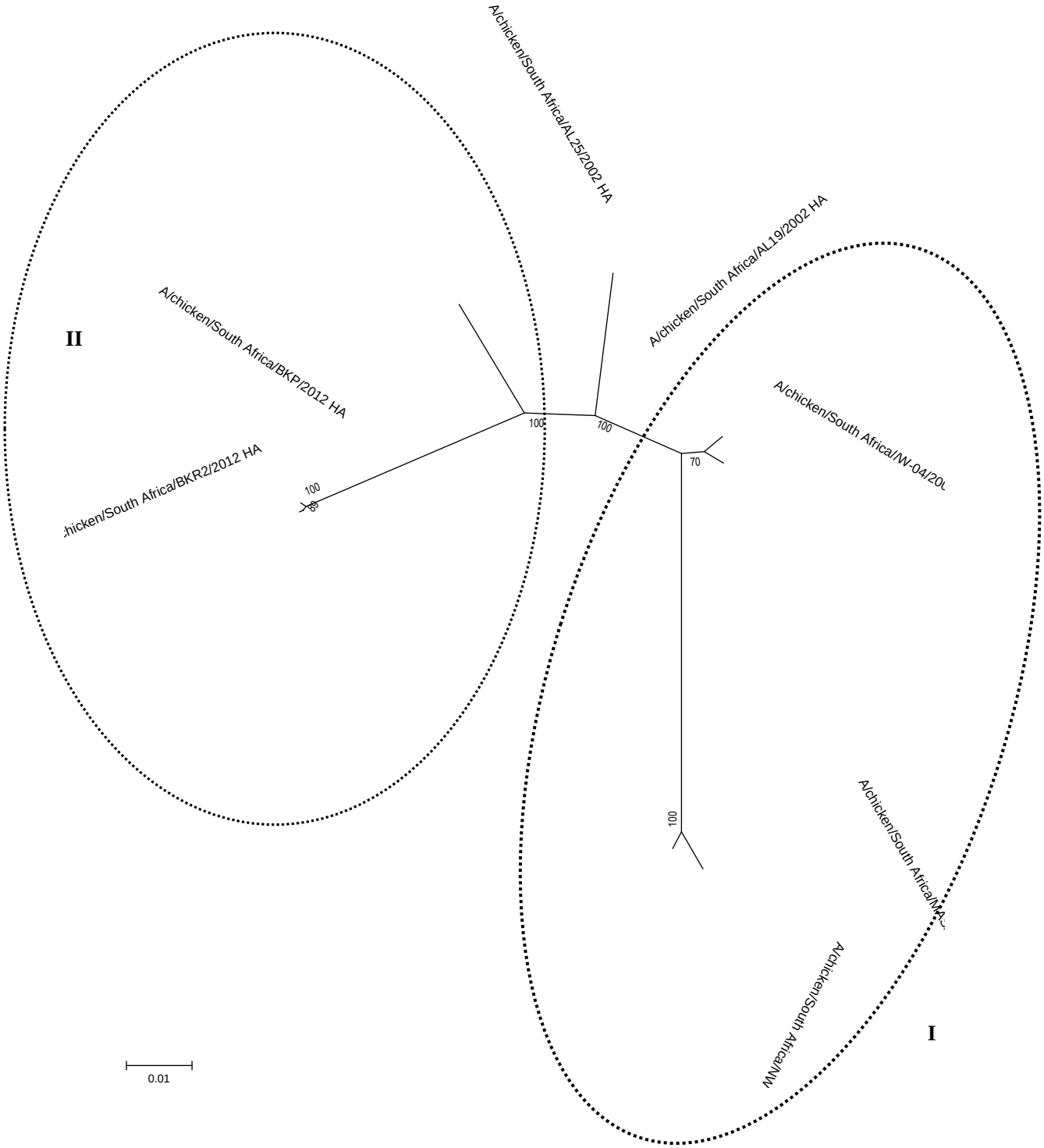

Figure 16b: Radial phylogram of H6 HA genes evolutionary relationships of H6 genes (1778 bp). The tree was rooted with A/ostrich/South Africa/KK98/1998 and two distinct sub-lineages indicated as **I** and **II.**

Examination of the alignment of the HA genes (Figure 18) revealed that sub-lineage I showed the same single point mutations at position 13 where the Alanine (A) present in the ostrich isolate and the other 4 isolates (sub-lineage II) changed to Threonine (T). This same pattern was apparent at positions 147 where Valine (V) was substituted with Isoleucine (I), 152 where Proline (P) was substituted with Serine (S), 268 where Tryptophan (W) was substituted with R, 272 where Lysine (K) was substituted with R, 274 V was substituted with Phenylalanine (F) and at 523 where Glutamic acid (E) was substituted with Aspartic acid (D).

Sub-lineage II as a group when compared to A/ostrich/South Africa/KK98/1998 (H6N8) showed single point mutations at positions 69 where R was substituted with Glycine (G), 135 where K was substituted with R, 139 where T was substituted with R, 293 where D was substituted with Histidine (H), 299 where T was substituted with A, 308 where T was substituted with R, 400 where I was substituted with V and 422 where D was substituted with G.

Both sub-lineages differs from the ostrich isolate at positions 110 and 144, where at 100 A has been substituted for P for sub-lineage I, while sub-lineage II isolates have V at this position. Position 144 is a potential glycosylation site and S in the ostrich isolate has been substituted with D in sub-lineage I and Asparagine (N) in sub-lineage II.

Position 190 showed a change for isolates A/chicken/South Africa/NWY/2012, A/chicken/South Africa/MAS/2013 in sub-lineage I, where Leucine (L) was been substituted with I.

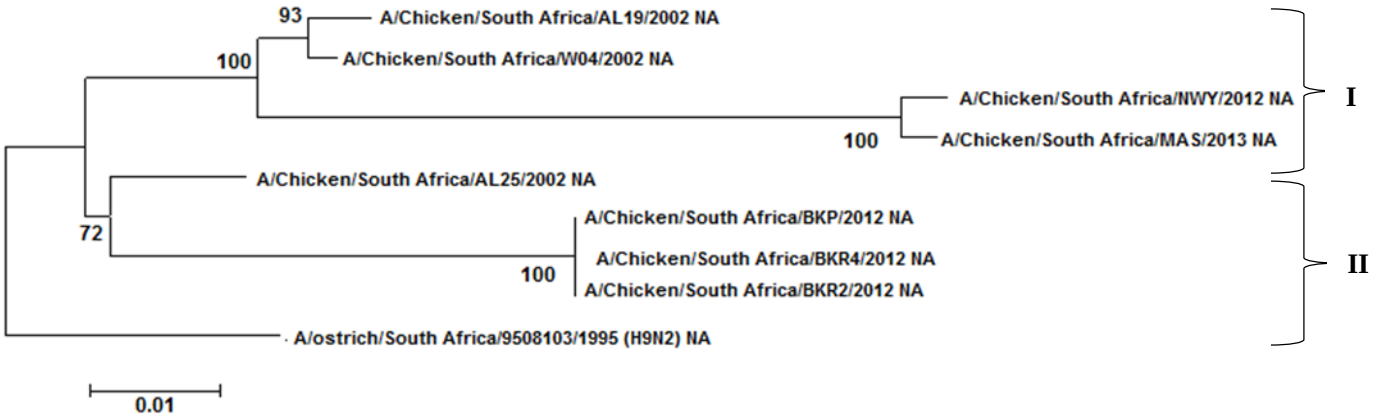

Figure 17a: Maximum likelihood tree of Neuraminidase (NA) gene sequences (1413 bp). Horizontal distances are proportional to genetic distance and the tree was rooted with A/ostrich/South Africa/9508103/1995 and two distinct sub-lineages indicated as I and II.

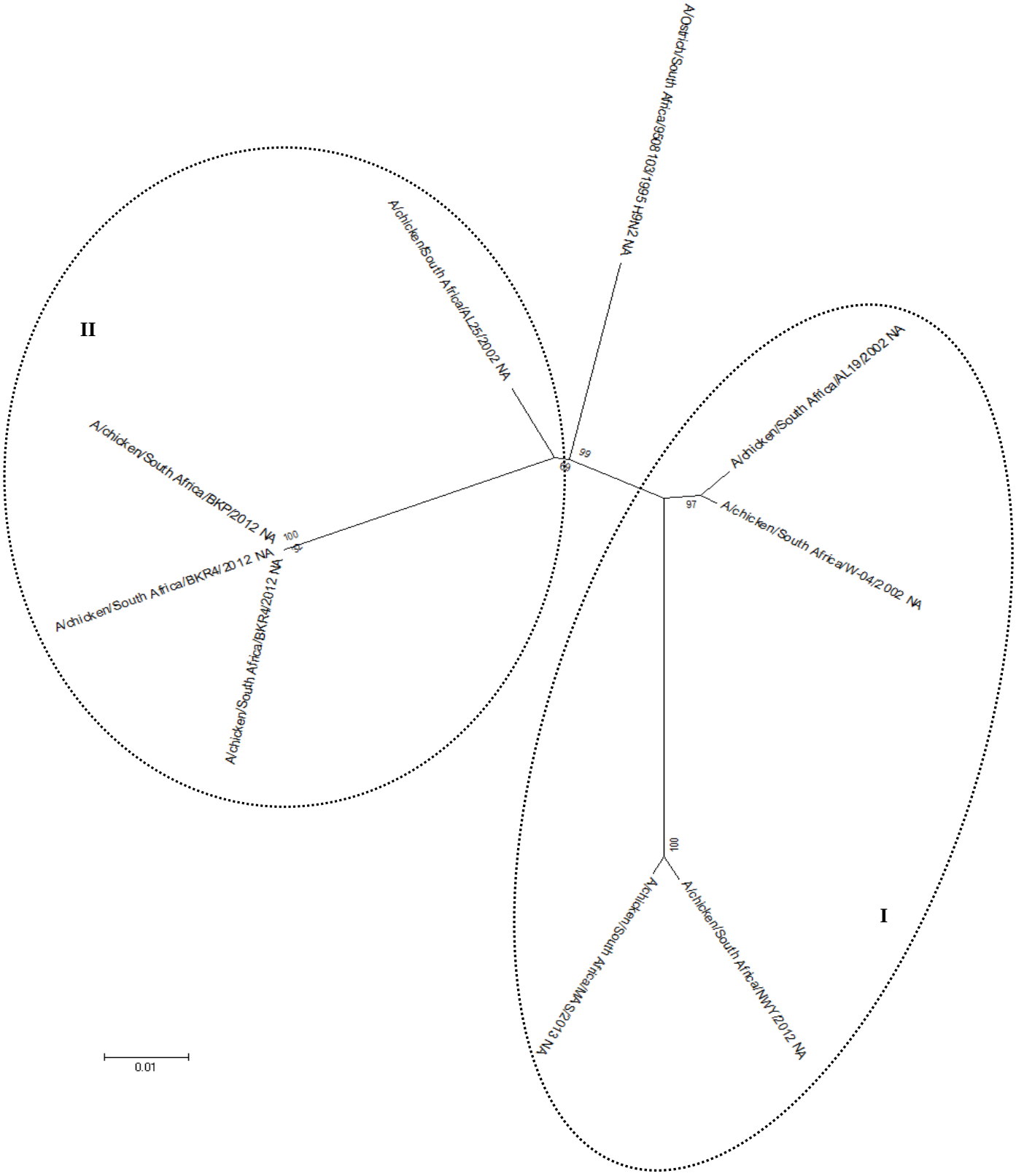

Figure 17b: Evolutionary relationship of N2 neuraminidase genes (1413 bp). The tree was rooted with A/ostrich/South Africa/9508103/1995 and two distinct sub-lineages indicated as I and II.

Chapter 5

Discussion

Initial isolation records showed that isolate A/chicken/South Africa/NWY/2012 displayed no HA activity nor did it cause embryo mortality. The presence of this isolate in the allantoic fluid was confirmed by means of electron microscopy (data not shown) and AI rRT PCR. The re-inoculation of this isolate confirmed these results. Re-inoculation of a further three isolates, namely A/chicken/South Africa/BKR2/2012, A/chicken/South Africa/BKR4/2012 and A/chicken/South Africa/BKP/2012 also showed a lack of HA activity. Although changes on the HA gene at one of the following positions - 138, 190, 103, 194 and 226 are thought to correlate with the ability of AIV to agglutinate erythrocytes, Kumari *et al*, showed that agglutination of CRBC does not correlate with altered binding to any oligosaccharide on the glycan array, and the density of HA due to replication processes may be the critical factor in the ability of viruses to agglutinate CRBC. This is confirmed by the data generated where no changes are apparent at these positions (Figure 18) except for position 190 which shows a change from leucine (L) to isoleucine (I) for isolates A/chicken/South Africa/NWY/2012 and A/chicken/South Africa/MAS/2013, however the latter isolate agglutinated CRBC.

The HI results at four weeks post vaccination (Table 4a) show that the group vaccinated with strain A/chicken/South Africa/W-04/2002 had developed the highest HI titres. As this was the current vaccine strain, and is well adapted for growth in chickens it produced a high initial HA titre (Table 3), thus most likely stimulating the immune response strongly. The negative HA values for the isolates A/chicken/South Africa/BKR2/2012, A/chicken/South Africa/BKR4/2012 and A/chicken/South Africa/BKP/2012 are most likely responsible for the low immune response at 4 weeks post inoculation. The quality of the humoral immune response could also be influenced by the way vaccine antigen uptake by dendritic cells takes place and by the more or less efficient presentation to immune effector cells. The nuclear transport of NP and PB2 genes allows AI to replicate efficiently by entering the nucleus of an infected cell (Gabriel et al 2008), thus if these 3 viruses were blocked from entering

the cell in some way, there would be a lower HA titre, due to lower numbers of virus particles present.

At 8 weeks post inoculation (Table 4b), it was shown that all vaccine groups had induced an immune response and HI titres had increased from 4 weeks prior. Although the lack of an initial HA positive result in four of the vaccine group viruses had resulted in lower titres on the HI test, there was still seroconversion, although immunity was not tested. The homologous antigens did not produce the highest titres except in the case of A/chicken/South Africa/W-04/2002, this could be as a result in the similarity of the HA gene with the lack of introduction of new genetic material. In other studies where there were antigenically distinct groups these were also determined to be separate genotypes where a re-assortment of viruses occurred (Kim et al 2010). HI titres can be linked to levels of replication of the virus and is crucial when determining the efficacy of a vaccine, as the best protection is produced by the humoral response against the HA protein (Swayne 2009). The efficacy of inactivated vaccines against H5N1 avian influenza infection in ducks and poultry were tested in challenge experiments and it was shown that lower HI titres were as a result of the lack of homology between the HA antigen and the vaccine the birds were vaccinated with; while a higher HI titre showed a close genetic relationship between vaccine and challenge viruses and in turn the antigenic match equated into to adequate protection by the vaccine (Middleton *et al* 2007, Swayne *et al* 2015).

There was a distinct grouping of the HI titres into the respective genetic sub-lineages where sub-lineage I which contained viruses - A/chicken/South Africa/NWY/2012, A/chicken/South Africa/MAS/2013, A/chicken/South Africa/W-04/2002 and A/chicken/South Africa/AL19/2002, and had a higher average HI titre; while sub-lineage II which contained A/chicken/South Africa/AL25/2002, A/chicken/South Africa/BKP/2012, A/chicken/South Africa/BKR2/2012 and A/chicken/South Africa/BKR4/2012 had lower HI titres. The HI titres of the current vaccine strain when tested against the isolates from sub-lineage I gave an average HI response of 7, while those in sub-lineage II had an HI titre of 5. The homology of the isolates as shown by their pair wise identity comparison of the HA gene (Table 6), was comparable to their

HI titres where isolates that had a homology of <90% had a lower average $\log_2$ HI titre of less than 5. Only isolates A/chicken/South Africa/W-04/2002 and A/chicken/South Africa/AL19/2002 had pairwise identities of >90% with all other isolates, thus eliminating other isolates as potential vaccine seed candidates.

When looking at the antigenic characteristics an HI titre gives information about the affinity of an antiserum for a virus strain as well as the level of antibodies produced by the virus. The HI titre value can be interpreted as a rough measure of distance between the antiserum and the virus, thus when looking at the 2D and 3D antigenic maps which are a graphic representation of the HI data (Figure 3, Figure 4, Figure 5) the antigen A/chicken/South Africa/W-04/2002 which is the basis of the current H6N2 vaccine is most centrally located and should give the most consistent immune response.

Less variation between antigen groups could be seen with the ELISAs than with the HI data. The HI test relied on a homologous binding of the surface haemagglutination antigens to the antisera, and this result was read in serially diluted wells, whereas the ELISAs produced a signal read by a spectrophotometer in a single well per sample. ELISAs produce a signal that is read in nanometres and should therefore be more sensitive, however, there could also be higher background signal thus reducing net specific signal levels.

At 4 weeks post inoculation only A/chicken/South Africa/AL25/2002 and A/chicken/South Africa/W-04/2002 had seroconverted when the Idexx influenza A ELISA was used, however at the same time when testing the sera using the H6N2 specific ELISA only two groups remained negative - A/chicken/South Africa/MAS/2013 and A/chicken/South Africa/BKP/2012. As the Idexx ELISA was prepared with conserved anti-influenza A virus nucleoproteins, the increase in detection capability of the H6N2 ELISA which was prepared using the whole virus from the homologous serotype; could have been as a result of there being more binding sites available for the H6N2 antibodies to bind.

The molecular characterization findings show that extensive genetic drift has occurred over the last ten years and the five new isolates sequenced can be divided into two distinct sub-lineages which appear to have developed independently of each other. This can be observed when examining the phylogenetic trees of each gene (Figures 10 – 17) and is especially clear in both the haemagglutinin (Figure 16b) and neuraminidase radial trees (Figure 17b). Two distinct sub-lineages (I and II) of H6 AIVs circulated during the South African outbreak in chickens in 2002, where A/chicken/South Africa/AL19/2002 was part of sub-lineage I and A/chicken/South Africa/AL25/2002 was part of sub-lineage II (Abolnik, 2007). No reassortment was observed in the isolates analysed which suggests that there was no introduction of new genetic material and these two clades seem to have developed in isolation, with sustained transmission within chicken flocks over the time period. This is supported by the pairwise identity comparison of the HA gene (Table 6), where all strains show a >90% homology with the vaccine strain, with a 92.99% homology and the lowest comparative identity being 87.88% between 2 viruses in the two sub-lineages - A/chicken/South Africa/MAS/2013 and A/chicken/South Africa/BKR2/2012. Further supporting evidence is that the recent isolates from sub-lineage II were all from one geographic area and were directly descended from A/chicken/South Africa/AL25/2002.

When comparing the amino acid differences between the two sub-lineages on the HA protein (Figure 18), there are distinct differences at positions 13, 69, 110, 135, 139, 144, 147, 152, 268, 272, 274, 293, 299, 308, 400, 422 and 523. Seven potential glycosylation sites at positions 26, 27, 39, 182, 306, 498, and 557 within the HA were conserved. Sub-lineage I has an aspartic amino acid residue (D) at position 144 and although D144 on the HA is thought to be reliable terrestrial marker sub-lineage II has asparagine (N) (Abolnik, 2007). This site is also a known glycosylation site where the HA can undergo co-translational or post-translational glycosylation modification by attaching oligosaccharides to the N side chain. The glycosylation of the HA is essential for protein folding and transport to the cell surface as well as biological functions like receptor binding activity, evasion of host immunity and HA cleavability. For the H1N1 subtype, a study showed that A/Brazil/11/78 virus containing potential

HA glycosylation sites at N144 became less sensitive to murine lung surfactant protein (SP-D) neutralization and more virulent in mice when the glycosylation site at 144 was removed. It was suggested that the glycan at 144 of the H1 HA was responsible mainly for SP-D binding and decreased susceptibility to SP-D inhibition resulting in enhanced virulence (Sun *et al* 2013). With this in mind the potential exists for the sub-lineage II viruses to be more virulent than the sub-lineage I viruses.

There is a 25-amino acid deletion in the stalk region of the NA gene (positions 56 – 80), for virus A/chicken/South Africa/AL19/2002, which is considered a typical marker of chicken adaptation. Some of the Californian H6N2 chicken viruses from 2000 to 2001 also contained a deletion in the stalk region (Webby et al 2002). Chickens that were infected with H2N2 viruses with and without the stalk deletion, and the viruses with the stalk deletion had better viral transmission and extended shedding periods. Thus it was concluded that the deletion in the stalk region of NA supports the viral replication in the respiratory tract of chickens. (Sorrell *et al* 2010). Other studies have shown that the NA stalk deletion is a major but non-essential virulence determinant which, together with an HA carrying a polybasic cleavage site, can confer high virulence (Stech, Veits *et al* 2015). This suggests that the transmission of the isolates in South Africa which do not contain the deletions could have been slower and less effective, thus explaining the low incidences of AI detected over the last 10 years.

Control programs for AI are designed to achieve at least one of the following three outcomes: prevention, management or eradication of the virus. Various essential components need to be incorporated in order to achieve these outcomes including biosecurity practices, diagnostics and surveillance, elimination of infected animals and increasing host resistance. Vaccination can be used as a single component in a control program whereby increasing host resistance to AI virus infection and decreasing environmental contamination. In Mexico after the H5N2 HPAI outbreak in 1995 an inactivated vaccine was used and has continued to be used to control H5N2 LPAI in Mexico, Guatemala and El-Salvador (Swayne, 2009). Further studies performed on this vaccine have shown that it provided protection against a variety of H5 AI viruses from other continents and conclusions were drawn that frequent

changing of the AI virus strain in the vaccine was not needed. Unfortunately in Mexico while the HPAI virus was eradicated the LPAI virus H5N2 continued to circulate and within a few years, multiple lineages of antigenically variant H5N2 LPAI field viruses were isolated that escaped from the immunity induced by the original 1994 vaccine seed strain used in the inactivated vaccine. This underlies the need for more than one control strategy, as vaccination alone is not sufficient, and that other safeguards to detect low-level circulation of virus in vaccinated flocks are needed, such as unvaccinated sentinel birds. Vaccination does not eradicate virus replication, it only decreases the shedding of the virus in the respiratory and digestive tracts of AI exposed chickens, while viruses with <90% homology at the haemagglutinin protein between vaccine and challenge virus might not result in consistent reductions in AI challenge or field virus shedding from the respiratory tract (Swayne, *et al* 2000). When considering the effectiveness of a vaccine for use in poultry, the World Organization for Animal Health (OIE) terrestrial manual suggests that a $\log_2$ HI titre response of 5 to a vaccine offers protection from mortality and that a response of 7 offers protection from virus shedding in vaccinated birds (OIE 2015).

The poultry industry in South Africa has vaccinated affected flocks with an inactivated H6N2 vaccine since 2002 when the first outbreak occurred. Monthly serological monitoring of sentinel flocks has shown that the virus is still circulating within the vaccinated flocks. In Gauteng between September 2012 and August 2013, 11 farms were quarantined for H6 infections, however, only three out of the 11 outbreaks were associated with drops in production or decreased feed consumption or respiratory symptoms and in all cases the clinical picture was relatively mild. In the majority of cases H6 was detected as an incidental finding when routine AI serology screening occurred and in most cases the virus itself was not isolated. This is most likely due to the serological monitoring occurring after shedding of the virus had stopped (Petty *et al* 2013). In challenge experiments with LPAI it was shown that the virus was detectable in infected birds 2 days post inoculation, and the virus continued to shed for 1 week, it was however only detectable in cloacal swabs until 3 days post inoculation at the same time a measurable immune response was detectable as early as 1 week after exposure. It was also found that the spread of the virus from bird to bird in adjoining cages of layers was variable and depended on the strain of virus (Lu, Castro,

2004). These findings could explain the reasons behind the few isolations made over the last 10 years even though the virus has continued to circulate.

The use of the vaccine in isolation as a control for H6 is obviously not sufficient and biosecurity practices, diagnostics and surveillance remain important. As LPAI viruses are usually shed by clinically normal chickens or birds showing minimal clinical signs, monitoring serologically for AI only every six months is not sufficient to detect the circulating virus and increased monitoring is to be recommended. The use of unvaccinated sentinel birds to detect circulating virus, remains strongly recommended; as serological tests do not distinguish between the vaccine currently used and infection with AI.

With the data gained from both the antigenic and molecular characterization of the Avian Influenza viruses isolated over the past 10 years, it can be concluded that the current vaccine should still offer sufficient protection for both groups of H6N2 viruses. This was backed up by the results of the pairwise percentage identities in the HA proteins, where the homology of HA protein and the latest isolates is greater than 90%. It is however, recommended that serological monitoring and genotypic analysis of any isolated viruses continue, and in order provide ultimate proof of vaccine efficacy the vaccine should be tested in a vaccination-challenge experiment with the latest isolated viruses.

Chapter 6

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Appendix A

Table 8: Idexx multiscreen and H6N2 ELISA data.

The raw OD values are averaged per vaccine group with positive values indicated in red. Standard deviation (SD) and Coefficient of variation (CV) of each group is also indicated. S/N values for the Idexx multiscreen ELISA and the titre values for the H6N2 ELISA are displayed.

		Idexx multiscreen				H6N2 ELISA			
Antigen Group	Bleed	Avg OD	S/N ratio	SD	CV%	Avg OD	Titre Value	SD	CV%
A/chicken/South Africa/BKR2/2012	0w	1.317	1.125	0.097	7	0.155	142.130	0.016	10
A/chicken/South Africa/BKR4/2012	0w	1.349	1.157	0.024	2	0.153	111.471	0.006	4
A/chicken/South Africa/MAS/2013	0w	1.359	1.167	0.104	8	0.172	279.267	0.026	15
A/chicken/South Africa/W-04/2002	0w	1.341	1.149	0.060	4	0.185	403.243	0.022	12
A/chicken/South Africa/AL19/2002	0w	1.318	1.126	0.058	4	0.159	159.090	0.014	9
A/chicken/South Africa/AL25/2002	0w	1.295	1.103	0.030	2	0.170	258.762	0.024	14
A/chicken/South Africa/NWY/2012	0w	1.277	1.085	0.070	5	0.155	124.191	0.011	7
A/chicken/South Africa/BKP/2012	0w	1.197	1.005	0.057	5	0.153	105.391	0.005	3
SPF	0w	1.235	1.043	0.008	1	0.155	123.520	0.012	8
A/chicken/South Africa/BKR2/2012	4w	1.126	0.934	0.062	5	0.268	1228.748	0.072	27
A/chicken/South Africa/BKR4/2012	4w	0.801	0.608	0.376	47	0.234	903.682	0.098	42
A/chicken/South Africa/MAS/2013	4w	0.810	0.618	0.559	69	0.206	530.730	0.022	11
A/chicken/South Africa/W-04/2002	4w	0.526	0.333	0.422	80	0.832	10007.305	0.272	33
A/chicken/South Africa/AL19/2002	4w	0.929	0.737	0.434	47	0.413	3233.941	0.188	46
A/chicken/South Africa/AL25/2002	4w	0.345	0.152	0.053	16	0.421	3379.630	0.215	51
A/chicken/South Africa/NWY/2012	4w	0.815	0.622	0.312	38	0.236	903.450	0.079	33
A/chicken/South Africa/BKP/2012	4w	0.796	0.604	0.388	49	0.207	636.726	0.088	43
SPF	4w	1.172	1.172	0.001	0	0.178	273.934	0.006	3
A/chicken/South Africa/BKR2/2012	8w	0.340	0.083	0.237	70	0.458	3483.440	0.217	47
A/chicken/South Africa/BKR4/2012	8w	0.169	-0.070	0.019	11	0.808	8919.770	0.243	30
A/chicken/South Africa/MAS/2013	8w	0.258	0.009	0.157	61	0.846	9538.689	0.216	25
A/chicken/South Africa/W-04/	8w	0.317	0.061	0.296	94	1.477	21562.829	0.017	1
A/chicken/South Africa/AL19/2002	8w	0.207	-0.037	0.087	42	1.403	20049.802	0.051	4
A/chicken/South Africa/AL25/2002	8w	0.181	-0.060	0.045	25	1.104	14489.743	0.407	37
A/chicken/South Africa/NWY/2012	8w	0.323	0.067	0.229	71	0.847	9820.601	0.360	42
A/chicken/South Africa/BKP/2012	8w	1.300	0.941	0.164	13	0.735	7692.881	0.253	34
SPF	8w	1.485	1.106	0.033	2	0.236	577.021	0.010	4

Positive results are indicated in red.

Figure 18: Multiple sequence alignment of the H6 haemagglutinin genes

The first sequence is used as a consensus and the similarities are plotted as a dot, the respective amino acid residue is indicated where differences occur. The HA_0 fusion site is indicated in yellow. Amino acid residues at positions 138, 190, 103, 194 and 226 are indicated in green. Sub-lineage differences in the amino acids are indicated in blue.

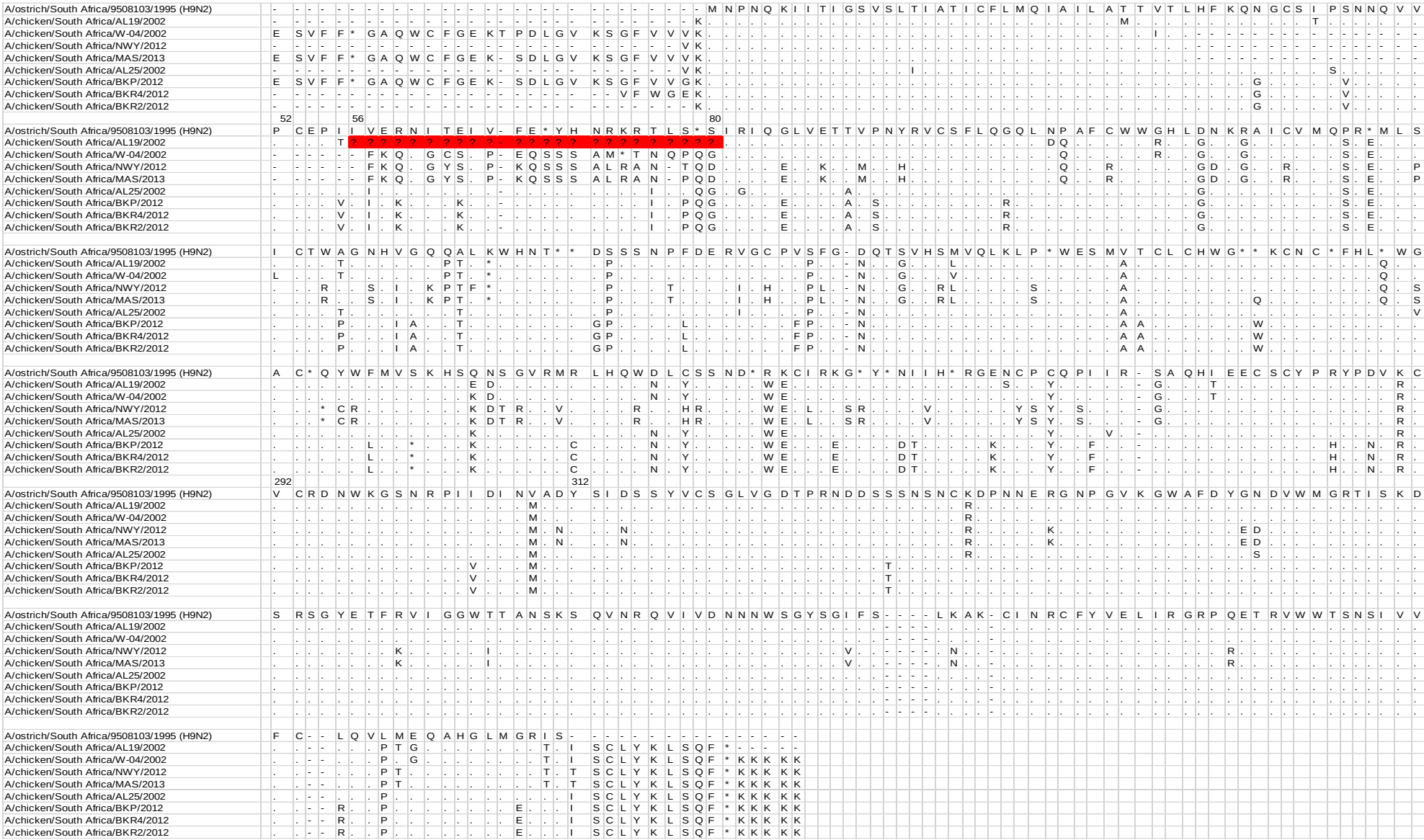

Figure 19: Multiple sequence alignment of the N2 neuraminidase genes

The first sequence is used as a consensus and the similarities are plotted as a dot, the respective amino acid residue is indicated where differences occur. The red shaded area (amino acid 56 to 80) indicates a deletion in the NA gene.

Appendix B

DELTAMUNE
a unique angle to animal health

APPLICATION FOR APPROVAL TO USE ANIMALS FOR EXPERIMENTAL PURPOSES

BY THE DELTAMUNE ETHICS COMMITTEE

- This form MUST be completed and submitted to the Deltamune Ethics Committee (DEC) for ALL animal studies.
- The word "animal" is defined as a "live, sentient non-human vertebrate, including: fish, amphibians, reptiles, birds and mammals, and encompassing domestic animals, purpose-bred animals, farm animals, wildlife and higher invertebrates such as the advanced members from the Cephalopoda and Decapoda".
- It must be signed by the Principal Investigator (the applicant) and other persons who are vouching for specialised aspects of the experimental design (e.g. statistician, safety officer, and persons responsible for supervising the use of scheduled medicinal substances) as indicated. Applications that have not been signed will not be considered.
- The application needs to be written simply, briefly and is not to exceed the limitations indicated.
- The application should be e-mailed to the co-ordinator ethics@deltamune.co.za. The application will then be distributed to the members of the DEC for review at the monthly meetings.
- Telephone enquiries on any DEC related matters may be directed to either the Chairman or one of other the members of the DEC.

A. PROJECT TITLE

Molecular Characterization of H6N2 Avian influenza viruses isolated in South African Poultry			
		PROJECT NO:	
SUBMISSION DATE	27/09/2013	ETHICS COMMITTEE No:	Co[illegible]/13/122

B. INVESTIGATOR

PRINCIPAL INVESTIGATOR	Dionne Rauff
DEPARTMENT	Test Laboratory
DEPARTMENTAL MANAGER	Stefan Swanepoel

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C. ANIMAL DATA

ANIMAL SPECIES & BREED	Chicken (Leghorn SPF)
SOURCE OF THE ANIMALS	Avifarms
NUMBER OF ANIMALS	16 Chickens
AGE	Adults and /or at least 17 weeks of age
GENDER	N/A
REPRODUCTIVE STATUS	N/A
OTHER INFORMATION	Chickens are vaccinated against pox before entering into study, chickens are allowed to acclimatise for at least 1 week before entering into the study

D. DECLARATION

1. **Moral Philosophy**
The ethical review of proposed animal experiments is predicated upon the acceptance by Deltamune (Pty) Ltd that non-human animals are organisms fully worthy of moral concern and as such their interests must be protected as far as possible in their use for advancement of biological knowledge and for the promotion of the health and welfare of animals and humans and protection of the environment.

2. **Animal Interests**
In the use of laboratory animals, animal interests obligate scientists and educators to:
- not allow animals to be used for research and/or to be killed for trivial, irrational, unjustified or inappropriate reasons;
- permit animals to live, reproduce and grow under conditions that are comfortable and reasonably natural to their species;
- keep animals free from disease, parasitism, injury and pain by prevention, rapid diagnosis and treatment;
- allow animals to be able to express normal behaviour through providing as far as possible sufficient space, proper facilities in which to live and in the company of the animal's own kind recognising the inherent social nature and hence the necessity of a social relationship for many species;
- protect animals from fear, deprivation, stress, distress and pain by ensuring that their living conditions, handling and treatment will be such that it will either minimise or eliminate the causation of these states upon those animals, that are used for research, teaching and testing;

3. **Humaneness**
The principles of humane experimental technique proposed by Russell & Burch must be followed in the planning and conduct of animal experiments. These comprise:
- **Replacement** of animals with non-sentient research systems, i.e. researchers must strive to avoid using of laboratory animals if alternative methods can yield the data they need.
- **Reduction** of the numbers of animals that are to be used to a minimum by design in order to achieve only sufficient statistical power to allow the objects of the experiment to be achieved.
- **Refinement** of the experimental methodology to be adopted by the implementation and if necessary by the improvisation of procedures which will have the least distressing or harmful effect to the animals and when this is not avoidable to counter those effects by the use of ataractics (tranquillisers), neuroleptics (dissociative agents), anaesthetics, analgesics and other

effective strategies.

4. **Animal Protection**
Animals should be protected from research designs that involve pain, illness, isolation, mutilation (whether by surgery or otherwise) and/or premature death until such research can be demonstrated to be absolutely imperative and related to health, welfare and environmental problems, which are potentially catastrophic in nature and for which alternative designs using non-sentient systems are not feasible.

5. **Relevance**
Animal-based teaching and research must address an important question relevant to Deltamune's objectives in advancing knowledge, education, science and human and animal welfare through research, be based on plausible hypotheses and have a reasonable prospect of yielding good results.

6. **Responsibility**
It is the responsibility of everyone using animals, for experimentation, testing diagnosis, teaching, sourcing of tissues or body fluids to assure that the animals that they use are afforded the highest levels of welfare and protection from abuse.

7. **Personal Declaration**

7.1 I, (full name)...Dionne Rauff....., as Principal Investigator in this application, hereby declare that I am familiar with the precepts, policies and responsibilities outlined under Section D and will personally undertake to ensure that these are upheld in the conduct of this study, should it be approved.

7.2 We undertake not to deviate from the approved application without obtaining prior approval by the DEC for any desirable or necessary significant changes that may need to be made in the methods used, which may affect the welfare of the animal subjects.

7.3 In my opinion, all persons named and working under my supervision have the appropriate training and skills needed to carry out their responsibilities for experimental procedures, care and handling of the species being used.

.....	.....27/09/2013	.....0833415045.....
Signature of Applicant	**Date**	**Cellphone number**

E. PURPOSE OF THE STUDY

PRODUCT DEVELOPMENT	☐	Name of Product
PRODUCT REGISTRATION	☐	Name of Product
QUALITY CONTROL	☐	Name of Product

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CONTRACT RESEARCH	☐	Title of Study
OTHER	x	Title of Study: **Molecular Characterization of H6N2 Avian Influenza viruses isolated in South African Poultry**

F. STUDY CATEGORY

A	☐	Animals are housed under normal (i.e. what the animals are used to) conditions. Minimal handling stress (tame animals can be handled frequently without stress). No more than two uninvasive procedures (e.g. blood collection by venipuncture) *E.g. Vaccine efficacy field trial*
B	x	Animals are housed under normal (i.e. what the animals are used to) conditions. Minimal handling stress (tame animals can be handled frequently without stress). More than two uninvasive procedures (e.g. blood collection by venipuncture) *E.g. AHS antiserum production in guinea pigs*
C	☐	Animals are housed under abnormal (or stressful) conditions (e.g. in an isolator). Minimal handling. More than two uninvasive procedures (e.g. blood collection by venipuncture) *E.g. Antibody production in an isolator*
D	☐	Animals are housed under normal (i.e. what the animals are used to) conditions. Minimal handling stress (tame animals can be handled frequently without stress). One or more invasive/painful procedures that may lead to discomfort during the procedure and/or after the procedure (e.g. challenge studies and toxin titration studies). *E.g. A challenge study in cage layers used to the cages*
E	☐	Animals are housed under abnormal (or stressful) conditions. Minimal handling stress (tame animals can be handled frequently without stress). One or more invasive/painful procedures that may lead to discomfort during the procedure and/or after the procedure (e.g. challenge studies and toxin titration studies). *E.g. Anthrax challenge in a biosafety cabinet*
F	☐	Animals are housed under abnormal (or stressful) conditions. Stressful handling (e.g. tying an animal down, put it in a neck clamp etc.) One or more invasive/painful procedures that may lead to discomfort during the procedure and/or after the procedure (e.g. challenge studies and toxin titration studies). *E.g. Testing a surgical procedure in a wild-caught primate*

G. PROJECT DETAILS

1. Proposed commencement date of study

October 2013

2. Brief justification

(Briefly introduce your project, justify the use of animals, the choice of species, the numbers to be used. If there is limited availability, or large numbers are to be used, provide additional rationale for their selection and numbers. State also what non-sentient model/s were considered and on what grounds they were rejected).

Project Introduction & why use animals?
The expected results are to have a better understanding of the genetic drift of the H6N2 virus in poultry in South Africa by phylogenetic analyses of gene segments.
Because some of the strains have a loss of HA activity the differences in HA activity between the viruses and their respective antisera need to be compared and plotted using antigenic cartography.

The positive serums are prepared by injecting vaccines containing inactivated antigens of each of the serotypes into chickens, followed by a booster vaccination, and finally the chickens are bled terminally under anaesthesia to provide a sufficient quantity of positive serum.
The positive serum is prepared in the target animal species, i.e. chickens.
What non-sentient models were considered?
None
Justify the number of animals.
Two animals per antigen are to be used, although only one bird would be sufficient, if something were to happen to that bird the experiment would have to be repeated. We expect to get ± 20ml of serum/chicken.
The following isolates are part of the trial – 197005/H4; 197005/H2; 220593; H6N2/MS; 233214/19; 233214/25; 201199; 196824
How will the experiment be refined to reduce suffering?
• Handling will only occur at inoculation and then once a month until the titres are determined to be sufficient • Euthanasia or cervical dislocation can be used if something such as broken wing occurs, provided it is done by a Veterinarian or any other trained person.
REFERENCES *(if applicable)*:
n/a

3. Aims of the study

(State these briefly and succinctly.)

To perform antigenic cartography of the various H6N2 isolates made in South Africa using positive antisera prepared in SPF chickens against the 8 isolates.

4. Experimental design and time line

(Summarise the experimental design and indicate the chronological sequence of procedures, substance administration and sample collection; and indicate the time interval between these events; *indicate the frequency of monitoring of the animals*). It can be done in text, table format or as a flow diagram.

Positive AI serums are prepared through the immune reaction of individual chickens in response to AI vaccines. For the preparation of the positive serum, vaccine prepared form each of the serotypes will be administered to chickens according to the standard vaccine schedule and route of administration, i.e. 0.5 ml injected in the pectoral muscle as described in the Standard Operating Procedure (SOP). Four weeks after the initial vaccination the birds will be bled from a brachial vein to collect 2ml of sera per bird and then a booster injection will be given. Three weeks after the booster vaccination, each bird bird will be bled from a brachial vein to collect 2ml of sera which will be tested to determine if titre is sufficient for the positive serum to be collected by a torminal bleed, performed under anaesthesia. About 20 ml blood will be collected. If the titre is not sufficient a second booster injection will be given and then the titres will be monitored 3 weeks later to determine if the terminal can bleed can occur. The birds are monitored daily for clinical signs of disease or mortality over the 7-week period. The serum will be extracted from the blood samples and submitted for laboratory evaluation as stated.

5. **Previous studies to which animals were exposed**
(If any)

None

6. **Restraint of the animals**
(Describe the methods of physical (manual procedures and use of special restraint equipment) or chemical restraint to be used on the animals)

The chickens will be gently restrained by hand on the table by competent animal handler during blood collection. Sodium pentobarbitone (200mg/ml) 0.2 – 0.5 ml/bird IV will be used for anaesthesia for the terminal blood collection

7. **Animal housing and care**

ANIMAL FACILITY	Animal Studies Unit Roodeplaat, enclosed chicken houses, the antigens are inactivated and the birds may be house in the same house
NUTRITION	Avi-farm breeder (crumbs) from Meadow Feeds (Pty) Ltd. A maximum of 40 adult hens per pan / tube feeder may be housed.
LIGHT	Natural light and intensity
VENTILATION (include temperature)	Positive pressure ventilation in the enclosed animal houses
WATER	Ad lib municipal water supply
CAGE TYPE	Chickens are housed on the floor with shavings as bedding and foraging material.
SPACE	1.45m X 3m = 4.35m^2 per cage – i.e where 16 birds per cage (maximum group size) the stocking density will be 0.27 m^2/chicken Recommended stocking density for these chickens is < 10 Chickens/m2
GROUP SIZES	2 chickens per AI serotype vaccine
ENVIRONMENTAL ENRICHMENT	Perches

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8. Experimental procedures and substance administration

PROCEDURES TO BE PERFORMED ON ANIMALS	**Procedure**			**Frequency**
	Inoculation with antigen			Initially on day 0
	Booster injection with antigen			At week 4
	Blood collection from brachial vein			At week 4
	Blood collection from brachial vein			At week 7
	Serum collection by terminal bleed if titres sufficient			At week 7
	Booster injection with antigen if titres too low			At week 7
	Blood collection from brachial vein			At week 10
	Serum collection by terminal bleed			At week 10
EXPERIMENTAL SUBSTANCES TO BE ADMINISTERED TO ANIMALS	**Substance**	**Dose**	**Route**	**Frequency**
	H6N2 antigen x 8	0.5 ml	IM	Initially, and then 4 weeks later as a booster
	H6N2 antigen x 1-8	0.5 ml	IM	3 weeks after first booster depending on titre
OTHER SUBSTANCES TO BE ADMINISTERED TO ANIMALS	**Substance**	**Dose**	**Route**	**Frequency**
	Anaesthesia (refer to point 6)			
SAMPLES TO BE COLLECTED FROM ANIMALS	**Sample**	**Volume**	**Method**	**Frequency**
	Blood	5ml	Brachial vein	At week 4 and week 7
	Blood	20ml	Terminal bleed under anaesthesia	Once at end of period

9. Severity of effects of the experimental procedures on the animals

(List the procedures that may cause deprivation, fear, distress and pain. Describe what sensations the animal may feel. Categorise these as minimal, intermediate or high.* Give their likely duration in time. Describe what specific steps will be taken to alleviate these conditions through the use of ataractics, dissociative agents, analgesics, anaesthetics or other methods. Estimate how effective these are likely to be.)

PROCEDURE	SENSATION	SEVERITY INDEX	DURATION	MEASURES TO ALLEVIATE
Blood collection	Manual restraint and	Mild	<5 minutes	Competent handler

	needle stab			and collector, needle no larger than 21G
Intramuscular vaccine inoculation	Needle stab, expansion of muscle mass	Mild mod	1-5 seconds per injection Up to days depending on absorption of product	Performed by competent personnel member, minimise volume
Blood collection via cardiocentesis	Needle stab	Mild	~1 second	Performed under anaesthesia

10. Animal welfare monitoring

Explain how the welfare of the experimental animals will be monitored during the study by completing the "clinical criteria column" in the Animal Welfare Score table.

For example:

Discomfort can be scored from 0 to 4+, where 0 represents a normal healthy status; 2+ represents the moderate discomfort (e.g. the pain associated with soft tissue surgery or the level of discomfort associated with a severe flu) and 4+ represents extreme discomfort (e.g. the pain associated with severe, widespread burn wounds or the discomfort associated with asphyxia). The criteria that would be given to the person performing the daily monitoring of the animals is a good example of the information to be included in this table. All categories are to be completed.

ANIMAL WELFARE SCORE		
Animal welfare score	**Severity of discomfort**	**Clinical criteria on which the score is based**
0	Normal	Normal behaviour
1+	Mild discomfort	Normal behaviour, slight pain reaction when inoculation site is palpated, drop in appetite, or mild drop in condition of the bird
2+	Moderate discomfort	Lethargy and swelling at the injection site
3+	Severe discomfort	Anorexia and abscess formation of tissue around injection site
4+	Unbearable discomfort	Anorexia and massive abscessation and necrosis of tissue around injection site

11. Fate of the animals

(Please note: a humane endpoint can be defined as the criteria used to determine when the animal is experiencing pain, suffering or distress and indicates the point at which corrective action should be taken to alleviate this. It does not specifically have to mean the animal undergoes euthanasia although usually does.)

FATE OF THE ANIMALS	All Birds will be euthanized after the 7 week period. And carcasses will be incinerated
HUMANE ENDPOINTS *(link the endpoints to animal welfare score)*	The nature of the procedure is such that it is unlikely that intervention based on humane endpoint will be necessary. Any chicken exhibiting signs of disease, either due to vaccine administration or due to other causes such as cannibalism, will be evaluated by an appropriated trained personnel member (veterinarian).

DELTAMUNE

APPLICATION FOR APPROVAL TO USE ANIMALS FOR EXPERIMENTAL PURPOSES

	A welfare score of 3 will necessitate withdrawal from the program and may result in euthanasia of a bird.		
EUTHANASIA, METHOD, VOLUME AND ROUTE ETC. *(incl. method for emergency euthanasia)*	**Method**	**Volume**	**Route**
	Cervical dislocation by veterinarian or trained assistant.		
	Sodium pentobarbitone 1-2ml/kg IV may also be used		

H. PERSONNEL AND RESPONSIBILITIES

	Name	Qualification	Signature
PERSON(S) RESPONSIBLE FOR DAILY CARE OF ANIMALS	Roelf Greyling	Veterinary Technologist	
	Piet Malepe	Animal Handler	
PERSON(S) RESPONSIBLE FOR CLINICAL MONITORING OF THE ANIMALS	Roelf Greyling	Veterinary Technologist	
	~~Miemie Grobler~~ Tamsyn Pulker	Veterinarian	
PERSON(S) RESPONSIBLE FOR ADMINISTRATION OF TEST SUBSTANCES	Roelf Greyling	Veterinary Technologist	
	Tamsyn Pulker	Veterinarian	
PERSON(S) RESPONSIBLE FOR COLLECTION OF SAMPLES	Solly Baloyi	Animal Handler	
	Miemie Grobler / Tamsyn Pulker	Veterinarian	
PERSON(S) RESPONSIBLE FOR GENERAL VETERINARY CARE	Miemie Grobler / Tamsyn Pulker	Veterinarian	

I. SIGNATURES

(A valid signature by Principal Investigator and the Departmental Manager is a prerequisite for conducting an animal study)

PRINCIPAL INVESTIGATOR	Dionne Rauff
DEPARTMENTAL MANAGER	Stefan Swanepoel

J. DELTAMUNE ETHICS COMMITTEE

(A valid signature by a representative of the DEC in section J is a prerequisite for conducting an animal study)

ADDITIONAL CONDITIONS FOR APPROVAL

DEC REPRESENTATIVE Type A (Chairman)		SIGNATURE	
DEC REPRESENTATIVE Type B (Deltamune Scientist)		SIGNATURE	
DEC REPRESENTATIVE Type C (NSPCA representative)		SIGNATURE	
DEC REPRESENTATIVE Type D (member of the public)		SIGNATURE	

Appendix C

CERTIFICATE OF APPROVAL
FOR THE USE OF SENTIENT ANIMALS IN AN ANIMAL STUDY

DELTAMUNE
a unique angle to animal health

APPROVAL IS HEREBY GRANTED BY THE ETHICS COMMITTEE OF DELTAMUNE (PTY) LTD FOR THE USE OF SENTIENT ANIMALS FOR:

Project title/ Study number:	Molecular characterisation of H6N2 AI viruses isolated in South Africa Poultry		
Ethics committee protocol number:	O-13-122	Study category: (A – F)	B
Project Leader/ Study Director:	Dionne Rauff		
Experimental starting date:	Oct 2013	Planned completion date:	Dec 2013
Requested by:	Dionne Rauff	Received by:	T Pulker

ANIMAL INFORMATION

SPECIES	*G.g. domesticus*						
BREED OF ANIMALS	Leghorn	AGE:	>17 wks	WEIGHT	N/A		
TOTAL NUMBER ANIMALS	16+18	MALE:	Either	FEMALE:	either		

This approval is valid for .....12..... months from the date of commencement and is give, based on the fulfilment of the following additional requirements:

Comments:
..None.....
.....

APPROVAL BY CHAIRPERSON OF DELTAMUNE ETHICS COMMITTEE

L. Santos		18/10/2013
NAME	SIGNATURE	DATE

APPROVAL BY REPRESENTATIVE FROM NSPCA

T. Pulker		18/10/2013
NAME	SIGNATURE	DATE

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Appendix C

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	Molecular characterisation of H6N2 AI viruses isolated in South Africa Poultry
PROJECT NUMBER	V059-13
RESEARCHER/PRINCIPAL INVESTIGATOR	D Rauff

STUDENT NUMBER (where applicable)	835 453 70
DISSERTATION/THESIS SUBMITTED FOR	MSc

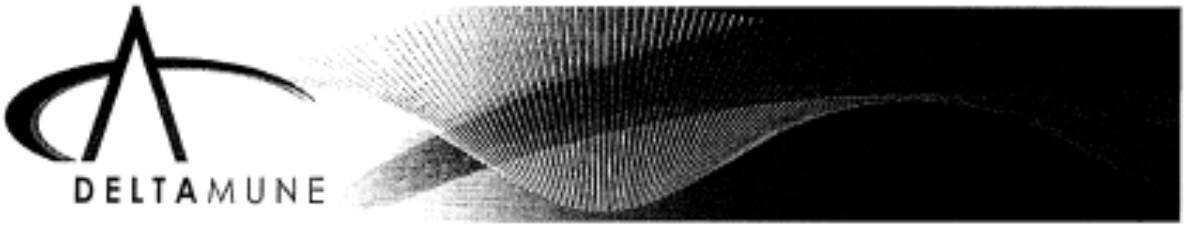
ANIMAL SPECIES	Poultry	
NUMBER OF ANIMALS	40	
Approval period to use animals for research/testing purposes		November 2013- November 2014
SUPERVISOR	Prof. C Abolnik	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date 25 November 2013
CHAIRMAN: UP Animal Ethics Committee	Signature

Appendix D



NECROPSY REPORT

Date of PM: 13/02/2014 **Project:** O-13-122 (D Rauff)

Species: *G.g.domesticus* **Breed:** Leghorn **Sex:** Male
Animal ID: Blue 39
Date of death: 13/02/2014

History
Bird euthanased on 13/02/2014 as part of the scheduled termination of the study. Primary vaccination was on 02/12/2013, 2nd vaccination on 30/12/2013. Post mortem was performed due to a purulent and ulcerated sternal blister with concomitant swelling of the feet.

Post mortem changes:
The post mortem was performed shortly after death, therefore post mortem changes were very minimal.

Specific macropathology:.
Inspissated, ulcerated, severe, encapsulated sternal blister. The soft tissue of the feet but not toes was swollen, however on cut surface no exudate was extruded. Evaluation of the joints was unrewarding, although left hock joint had some blood tinged fluid that was sampled. Wing joints were normal. The lung, liver, spleen, intestinal tract, heart and kidney were normal in appearance.

Samples collected / Laboratory tests:
Joint fluid sample was collected and cultured for growth in micro-aerophilic, anerobic and aerobic conditions with negative results.

Final Diagnosis:
Death was due to euthanasia. The severe sternal blister was likely caused by inability to perch due to bullying from conspecifics. It is difficult to establish cause and effect between the sternal blister and soft tissue swelling, however it is highly likely that the one is the result of the other.

Necropsy performed by:

Dr. Tamsyn Pulker

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Email: info@deltamune.co.za • Website: www.deltamune.co.za

Directors: Mr CM Bates (New Zealand) (Chairman) • Dr EE de Bruyn • Mr RJ Franklin
• Ms RKT Ramoupi • Dr SP Swanepoel • Dr JC Swart (CEO)

Registered name: DELTAMUNE (PTY) LTD • 1994/005061/07

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