Evaluation of swab pooling and transport medium to improve the identification of avian influenza viruses in farmed ostriches in South Africa

By

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Mini-Dissertation submitted in partial fulfilment of the requirements for the degree Magister Scientiae (Tropical Animal Health) in the Department of Veterinary Tropical Disease, Faculty of Veterinary Science, University of Pretoria

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Declaration

I, Reneé Pieterse declare that this mini-dissertation submitted to the University of Pretoria for the degree of Magister Scientiae (Tropical Animal Health) in the Department of Veterinary Tropical Disease, Faculty of Veterinary Science, has not been previously submitted by me for the degree at this or any other university, that it is my own work, and that all material contained therein has been duly acknowledged.

Signed: 

Date: 2019-12-13

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Summary

Evaluation of swab pooling and transport medium to improve the identification of avian influenza viruses in farmed ostriches in South Africa

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Avian influenza (AI) control in farmed ostriches relies on rapid and accurate diagnostic testing to detect the presence and spread of disease in avian populations, as flocks are in unavoidable contact with wild bird reservoirs, increasing the possibility of infection. Increasing swab pools from five to ten would significantly decrease the cost of testing by reducing the number of tests performed; however detection of highly pathogenic avian influenza (HPAI) at low virus titres by RT-qPCR and virus isolation is critical to ensure that the disease is detected and accurately characterised. The present study found that pooling a single HPAI positive ostrich swab with four or nine AI negative ostrich tracheal swabs did not significantly affect the detection of influenza A virus by RT-qPCR ($p < 0.05$) over the dilution range. RT-qPCR had a high sensitivity detecting up to $10^{2.5}$ EID_{50}/ ml (~ RT-qPCR Ct value = 35) virus titre compared to virus isolation, that detected only up $10^{4.8}$ EID_{50}/ml (~ RT-qPCR Ct value = 29) virus titre, confirming that weak positive swab pools with a low virus titre detected by RT-qPCR (Ct value ≥ 35) may not be detected by standard virus isolation techniques. Both virus transport media evaluated (10% v/v glycerol in brain heart infusion (BHI) broth supplemented with antibiotics (VTM) and 50% v/v glycerol PBS transport medium (pH 7.2)) facilitated the isolation of influenza A virus at ~ $10^{4.5}$ EID_{50}/ml (RT-qPCR Ct value ~ 27); however VTM improved the efficiency of the method, potentially reducing the reporting time. Sampling costs can be reduced by increasing swabs pools to ten and efficacy of virus isolation can be improved by using antibiotic supplemented VTM, without compromising the detection of influenza A virus by RT-qPCR. It is recommended that costs and efforts are focused at isolating virus from pooled swab samples, with a virus titre of ≥ 10^4 EID_{50}/ ml (RT-qPCR Ct value ≤ 29), followed by full molecular sequencing and characterisation of the viral isolate. Samples with a low virus titre < 10^4 EID_{50}/ ml (RT-qPCR Ct value > 29) should be characterised by direct sequencing of PCR amplicons or type-specific RT-qPCR assays.

Abbreviations

AI:	Avian influenza
Ct:	Crossing threshold
DAFF:	Department of Agriculture, Forestry and Fisheries
DNA:	Deoxyribose nucleic acid
EID_{50}:	Median egg infectious dose
ELISA:	Enzyme-linked immuno-absorbent assay
HA:	Haemagglutinin
HAT:	Haemagglutination test
HI:	Haemagglutination inhibition
HPAI:	Highly pathogenic avian influenza or high pathogenic AI
IPC:	Internal positive control
LPAI:	Low pathogenic avian influenza
ml:	Millilitre
NA:	Neuraminidase
OFFLU:	OIE (Office International des Epizooties; World Organisation for Animal Health) - FAO (Food and agricultural Organization of the United Nations) global network of expertise on animal influenza
OIE:	Office International des Epizooties; World Organisation for Animal Health
PBS:	Phosphate buffered saline
PCR:	Polymerase chain reaction
RNA:	Ribose nucleic acid
RT-qPCR:	Real-time reverse transcription polymerase chain reaction
SAOBC:	South African Ostrich Business Chamber
TCID:	Tissue culture infectious dose
v/v:	Volume per volume
VTM:	Virus transport medium
VAT:	Value added tax
USDA:	The United States Department of Agriculture
WC:	Western Cape province, South Africa
WHO:	World Health Organization

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1. Introduction

South Africa suffered a severe outbreak of highly pathogenic avian influenza (HPAI) H5N8 Clade 2.3.4.4. affecting multiple avian species including domestic poultry and wild bird species in 2017 and 2018 (Abolnik et al., 2018). Rapid and accurate diagnostic testing is essential to detect and monitor the presence and spread of disease in avian populations. HPAI was first detected in chickens in June 2017 on a commercial breeder flock in Mpumalanga. There was little warning and no clinical symptoms were reported prior to the deaths. Serology testing did not detect antibodies for influenza A virus; however molecular tests confirmed the presence of HPAI virus H5N8. Six weeks later, in August 2017 the virus was detected in two tracheal swab pools taken for routine surveillance testing from an ostrich farm in Heidelberg, Western Cape province. No clinical symptoms or mortalities occurred and swift quarantine action was taken to restrict movement and reduce the risk of spread of infection (van Helden and Roberts, 2017).

Farmed ostriches in South Africa are used for the production of meat and other products such as leather, feathers and ornaments and account for 75% of the world's market share. Products are exported primarily to European markets (98%) that consume ostrich meat valued for the low cholesterol and fat content (Department of Agriculture, 2012a). Since 2004, the industry has experienced instability partly due to HPAI outbreaks that occurred in 2004, 2006 and 2011 (Abolnik, 2007, Abolnik et al., 2012, Manvell et al., 2005), resulting in export bans to countries in the European Union (EU). During this period at its peak in 2009, exports of 7.4 million kg valued at almost 450 million rand (53.2 million US dollars)[1] were reported, but this declined rapidly in 2011 to less than 2 million kg valued at approximately 140 million rand (19.2 million US dollars)[2] (Department of Agriculture, 2012a).

Since the HPAI H5N2 outbreak in ostriches in 2004, the surveillance, registration, movement and official control of ostriches in South Africa has prescribed compulsory diagnostic testing, resulting in increased high financial demands on industry and government resources (Department of Agriculture, 2006, Department of Agriculture, 2012b). In order to achieve animal health objectives, the surveillance of avian influenza should result in the early detection of the virus which will allow for follow up testing and control strategies to be implemented.

To reduce costs of laboratory testing for surveillance, five swabs are pooled to minimise the number of RT-qPCR tests performed. The number of ostriches from each epidemiological group are sampled for detection of H5, H6 and H7 influenza A virus subtypes at a ">5% prevalence with 95% confidence in every epidemiological group". Where the number of ostriches exceeds 4500 per epidemiological unit, a

[1] https://www.poundsterlinglive.com/bank-of-england-spot/historical-spot-exchange-rates/usd/USD-to-ZAR-2009
[2] https://www.poundsterlinglive.com/bank-of-england-spot/historical-spot-exchange-rates/usd/USD-to-ZAR-2011

minimum number of 59 ostriches must be sampled (Department of Agriculture, 2012b). To meet these requirements in South Africa, where ostrich flocks range from 150 to > 1000, at least 60 ostriches from each epidemiological group are routinely sampled, thus resulting in 12 sample pools of five swabs each that are tested by RT-qPCR. Routine testing costs are borne by farmers and industry players such as the South African Ostrich Business Chamber (SAOBC), where accredited diagnostic laboratories charge between R316 and R438 (Table 6.) per sample (swab pool) for RT-qPCR tests. For epidemiological investigations during HPAI outbreaks, test costs are covered by national and provincial government.

Identifying the subtype and genetic sequencing of the virus is essential in order to show mutations, genetic evolution and regional patterns of transmission (OFFLU, 2013). When sampled ostriches are tested using molecular tests for influenza A virus, typically only a few of the swab pools test positive. Where seroconversion occurs for AI, reactions against multiple antigen subtypes are reported (Tables 1 and 2), making definitive diagnosis decisions difficult for state veterinary services. Ostrich flocks are in unavoidable contact with wild bird reservoirs, increasing the possibility of infection occurring, thus it is essential to evaluate sampling protocols including swab pooling and transport medium to improve the detection, characterisation and diagnosis of AI in farmed ostriches. This is only possible if the viruses themselves are detected in swab samples and successfully isolated for further characterisation.

It has been demonstrated that low pathogenic AI (LPAI) virus is readily detected by RT-qPCR in chickens and turkeys when the number of swabs per pool is increased to 11 (Ladman et al., 2012, Spackman et al., 2013). If increasing the number of swabs per pool could be similarly applied to ostriches, the number of tests performed from birds sampled at each epidemiological site could be decreased, thereby reducing the costs incurred by government and other stakeholders. Samples taken from the trachea of ostriches may result in lower virus titres on the swabs as the secretion of virus occurs over a larger surface area when compared to swabs taken from chickens and turkeys. Ostriches may therefore not be directly comparable to chickens and turkeys if the number of swabs per pool is increased and it is important to ensure that the sensitivity of the assay is not reduced.

During the 2011 HP H5N2 outbreak, only three viruses were isolated from pooled swabs originating from twenty PCR positive farms (Abolnik et al., 2012). Similarly, in the recent HPAI (H5N8) outbreak in 2017, there was limited success in isolating virus from the thirty-eight confirmed PCR positive swab pools (Table 2) in 50% v/v glycerol PBS transport medium from ostrich flocks at accredited testing laboratories, where only two viruses were isolated. In contrast thirty-nine viruses were successfully isolated during this period from other avian species including chickens and wild birds following increased mortalities from oropharyngeal and cloacal swabs or tissue samples (Abolnik et al., 2018). Virus transport medium (VTM) containing brain heart infusion (BHI) broth with antibiotics has been shown to be optimal when compared to using PBS in chickens (Spackman et al., 2013), but was comparable to a medium using 10% v/v

glycerol PBS medium supplemented with antibiotics in a another study (Zhang et al., 2015). Transport media supplemented with antibiotics has not been adopted for ostrich testing in South Africa, presumably due the limited shelf life of the medium and higher costs of supply.

This study evaluated sampling protocols that could potentially reduce the number of molecular tests performed by increasing the number of swabs pooled per sample. Virus isolation from tracheal swab pools was performed and in a parallel study an alternative protein-based virus transport medium, supplemented with antibiotics was compared to the standard 50 %v/v glycerol PBS transport media to evaluate improved virus isolation from ostrich swab samples.

2. Literature study

2.1 Influenza A virus

AI is caused by the influenza A virus (*Orthomyxoviridae* family, genus *Alphainfluenza virus A*). Influenza A virus is made up of eight negative sense RNA segments, the fourth and sixth segment encode the haemagglutinin (HA) and neuraminidase (NA) proteins, respectively. Antigenic variation in the HA and NA proteins classifies the influenza A virus into subtypes. There are currently 16 HA and 9 NA subtypes in avian species, resulting in many H- and N- subtype combinations. Regulatory or structural functions are directed by matrix proteins (M1 and M2), non-structural protein and nucleoprotein (NP) (Neumann et al., 2003). Direct RNA detection of influenza A viruses by real time reverse transcriptase polymerase chain reaction typically targets the highly conserved M gene sequence (Spackman et al., 2002, Fouchier et al., 2000).

The HA protein attaches to host cell receptors to initiate infection and is also recognized as a primary virulence determinant. Cellular protease enzymes cleave the HA_0 protein into the functional HA_1 and HA_2 proteins. The amino acid sequence at the cleavage site of the HA_0 protein therefore determines if protease enzymes are able to cleave the HA protein and cause either localized or systemic infection in the host (Office International des Epizooties; World Organisation for Animal Health (OIE, 2015).

RNA viruses lack mechanisms in replication to correct transcriptional mistakes making their genomes prone to mutations. In the case of influenza virus consisting of a segmented RNA genome, mutations (i.e. antigenic drift) and reassortment of segments of the genome where a coinfection may occur results in new virus particles (i.e. antigenic shift) that allow it to adapt to and better evade the host's immune system (Perdue and Suarez, 2000, Perdue, 2008). LPAI infection is caused by influenza viruses that have a single-basic amino acid cleavage site at the critical position between the arginine (R) and glycine (G) amino acids (PEKQTR/GLF). The proteases capable of cleavage at the site are restricted to respiratory and gastrointestinal

tract tissues, limiting the area of infection in the host. The HPAI causes multiple tissue and organ infection as the basic amino acid present at the HA cleavage site is substituted by multiple basic amino acids including arginine (R) and lysine (K) (e.g. PQRESRRKK/GLF), or by insertions of multiple basic amino acids or recombination with other gene segments leading to a lengthening of the cleavage site and facilitating the cleavage of the HA gene by many cellular proteases enzymes that are present in a wider range of host tissues (OIE, 2015, OFFLU, 2018). HPAI infection is therefore more widely spread as the virus is able to replicate in multiple host cell tissues or organs.

AI subtypes H5 and H7 are reported to be the only subtypes where multiple-basic amino acids are inserted at the HA_0 cleavage sites. Antigenic mutation and the reassortment of virulence determinants could result in LPAI becoming HPAI. As such LPAI and HPAI, including H5 and H7 subtypes are notifiable in South Africa and also to the OIE (OIE, 2015, Department of Agriculture, 2012b).

2.2 History of avian influenza outbreaks in ostriches in South Africa (1991 to May 2017)

AI outbreaks in South Africa have long been associated with ostriches farmed in the Western Cape province. The first reported outbreak of LPAI H7N1 occurred in 1991 in ostrich chicks in De Rust, Oudsthoorn Western Cape province. High mortality rates (80%) were reported in birds under a month old, usually dying within two days of showing clinical symptoms (poor appetite, depression, ocular discharge and green discoloration in the urine). Birds between eight and 14 months developed milder symptoms with a mortality rate of 20%, while adult birds were largely unaffected (Allwright et al., 1993). AI was detected in ostriches in 1995 (H9N2), 1998 (H6N8), July 2001 (H10N1) and in 2002, the first reported case of AI (H6N2) in chickens occurred in Camperdown, Kwa-Zulu Natal province. Molecular characterization of the H6N2 strain from the chicken outbreak and subsequent phylogenetic analysis suggested that the H9N2, H6N8 and other strains circulating in the ostrich populations (1995 to 1998) may have played a role in the reassortment of the virus and subsequent spillover to chickens (Abolnik et al., 2007).

In July 2004, deaths were reported in young ostriches in Middleton (Eastern Cape province) exhibiting similar symptoms to the LPAI H7N1 outbreak in 1991. HPAI H5N2 was isolated from tissue samples following postmortem examination (Abolnik et al., 2009). The OIE Terrestrial Animal Health Code defines poultry as “all domesticated birds, including backyard poultry, used for the production of meat or eggs for consumption, for the production of other commercial products, for restocking supplies of game, or for breeding these categories of birds, as well as fighting cocks used for any purpose” (OIE, 2019c). The OIE recommends stamping out by culling exposed and infected birds, strict quarantine and movement controls if infection is detected (OIE, 2019b). Subsequently, 26 000 infected and exposed ostriches were culled in the Eastern Cape province. A ban on trade for all fresh ostrich products was imposed, resulting in a net loss of

approximately five million rand[3] (750 000 US dollars) from 2004 to October 2005 when the suspension was lifted (Department of Agriculture, 2012a).

Outbreaks of HPAI H5N2 occurred in 2006 and again in 2011 in the Oudtshoorn area (Abolnik, 2007, Abolnik et al., 2012). The HPAI virus isolated in 2006 was unrelated to the 2004 HPAI H5N2 outbreak. In addition a LPAI H5N2 isolate was also detected. Phylogenetic analysis revealed two basic amino acid deletions at the HA_0 cleavage site and introduction events were most likely attributed to wild duck populations. Clinical symptoms, although similar to the 2004 outbreak event were less severe and very few mortalities were reported (Abolnik, 2007).

An increasing trend in the value of export was evident after the country resumed exports and during this period at its peak in 2009, exports of 7.4 million kg valued at almost 450 million rand (53.2 million US dollars)[4] were reported, but this declined rapidly in 2011 to less than 2 million kg valued at approximately 140 million rand (19.2 million US dollars)[5] (Department of Agriculture, 2012a).

The first indications of the impending outbreak in 2011 were through routine serological detection of antibodies against H5 in May 2010. Seropositive results persisted, but no PCR positive farms were detected until March 2011 (Abolnik et al., 2012, van Helden et al., 2016). Authorities responded by suspending exports of fresh meat products (Barroso, 2011a). Farms outside the containment zone were very briefly permitted to resume exports in October 2011, but this was quickly revoked when further samples from farms tested positive for influenza A virus (Barroso, 2011b). As a direct result of the outbreak, there was a severe economic impact for both the industry as well government stakeholders where 53 million rand was spent on compensation alone (excluding surveillance and laboratory testing). It was estimated that at least 10% of the total ostrich population in South Africa was lost due to disease control measures implemented by authorities by culling approximately 37 000 ostriches (van Helden et al., 2016).

The presence of LPAI (H7N1, H7N7 and H5N2) was detected through routine serology and PCR surveillance testing of registered ostrich farms in 2012, 2013 and 2014. Influenza A virus was isolated from a farm in the Eastern Cape province in March 2012 (H7N1) and three LPAI H7N1 isolates and one LPAI H5N2 virus were isolated from farms located in the Western Cape province (Mossel Bay, Aberdeen, Heidelberg and De Rust) from May to June 2012. In May 2013, LPAI H7N7 was also isolated from one farm in Oudtshoorn. Ostriches infected with LPAI were reported to have a suppressed appetite, primarily in younger birds (three to four months old) and low mortalities were reported (30%), but deaths were not attributed to influenza infection but rather due other factors including poor management practices and

[3] https://www.poundsterlinglive.com/bank-of-england-spot/historical-spot-exchange-rates/usd/USD-to-ZAR-2004
[4] https://www.poundsterlinglive.com/bank-of-england-spot/historical-spot-exchange-rates/usd/USD-to-ZAR-2009
[5] https://www.poundsterlinglive.com/bank-of-england-spot/historical-spot-exchange-rates/usd/USD-to-ZAR-2011

secondary bacterial infections (Abolnik et al., 2016). Phylogenetic analysis of isolates from 2012 and 2013 attributed the origin of the outbreak to introduction events by wild bird species and specifically in 2013, a LPAI H7N1virus isolated from a sacred ibis (*Threskiornis aethiopicus*) suggested that this species may have been a carrier of the virus between ostrich farms (Abolnik et al., 2016). Limited export to the EU market was permitted in February 2014 from farms located in 'super compartments' or closed holdings subjected to strict biosecurity and official monitoring of influenza (Barroso, 2014). The country was officially declared free of influenza infection and the ban on exports to the EU was lifted in August 2015 (Juncker, 2015). Sporadic cases of influenza were reported by state veterinary services in the Western Cape province in ostriches and other birds from January 2016 to May 2017 (Table 1) (van Helden, 2016, van Helden and Roberts, 2017). Where seroconversion occurs for influenza A virus, reactions against multiple antigen subtypes are reported (Table 1), making definitive diagnosis decisions difficult for state veterinary services. In addition, where AI PCR positive swabs sample were reported, H5 and H7 subtype PCR results were negative and virus isolation was unsuccessful, therefore full charaterisation of the AI H- and N-type was not established.

Table 1. Avian influenza reported in the Western Cape province in ostriches and other birds (January 2016 to May 2017).

Start date	Host	No. of farms / locations	Region	Diagnostic method		Serotype(s)
				Serology (NP ELISA + H5/H6/H7 HI)	PCR (AIV M gene + H5/H7)	
Jan 2016	Ostrich	1	Oudtshoorn	ELISA positive H6 positive	Negative	H6 N2/N8
Feb 2016	Ostrich	1	Tulbagh	ELISA positive	Negative	Unidentified subtype
Feb 2016	Duck breeder farm	1	Joostenbergvlakte	ELISA positive H5 positive	Negative	HI titres: H5N2 H5N1 H6N2 Attributed to H5N2
Mar 2016	Ostrich	2	Mossel Bay Oudtshoorn	ELISA positive H5 positive	Negative	Attributed to H5N2
Apr 2016	Ostrich	1	Oudtshoorn	H5N2	Negative	Attributed to H5N2
Apr 2016	Ostrich	2	Oudtshoorn	ELISA positive	Negative	Unidentified subtype
May 2016	Ostrich	1	Heidelberg	ELISA positive H6 positive	Negative	
Aug 2016	Ostrich	2	George	-	AIV positive	H5/H7 negative Unidentified subtype
Sep 2016	Ostrich	2	Oudtshoorn	-	AIV positive	H5/H7 negative Unidentified subtype
Oct 2016	Ostrich	1	Oudtshoorn	-	AIV positive H5N2 positive	PCR positive for H5 and N2
Oct 2016	Ostrich	1	Heidelberg	ELISA positive H5 positive	negative	Attributed to H5N2
Nov 2016	Chicken (layers)	1	Unknown	ELISA positive H6 positive	negative	
Nov 2016	Ostrich	1	Unknown	ELISA positive H5 positive	negative	Attributed to H5N2
Nov 2016	Ostrich	3	Unknown	ELISA positive H6 positive	negative	
Jan 2017	Pied Starling (*Lamprotornis bicolor)*	-	Oudtshoorn	-	AIV positive	Shot for surveillance purposes Unidentified subtype
Jan 2017	Ostrich	1	Worcester	ELISA positive		Unidentified subtype
Feb 2017	Chicken (layers)	1	Cape Town	ELISA positive H6 positive		
Feb 2017	Ostrich	2	Oudtshoorn	ELISA positive	negative	Unidentified subtype
Feb 2017	Wild bird faecal samples	1	Oudtshoorn		AIV positive	Collected surveillance purposes Unidentified subtype
April 2017	Ostrich	1	Heidelberg	ELISA positive H6 positive		
April 2017	Ostrich	1	Heidelberg	ELISA positive		Unidentified subtype
April 2017	Ostrich	2	Oudtshoorn	Negative	AIV positive	H5/H7 negative Unidentified subtype

WC: Western Cape Province, South Africa; HI: Haemagglutination inhibition; ELISA: Enzyme-linked immuno-absorbent assay; PCR: Polymerase chain reaction

2.3 High pathogenic avian influenza Clade 2.3.4.4 H5N8 outbreak in poultry in South Africa

Since 2004 the OIE has characterized outbreaks of Clade 2.3.4.4 HPAI H5Nx influenza A virus into panzootic events; the first event was from 2004 until 2012 and the second from 2013 (ongoing event), and the most severe outbreaks were recorded between 2015 and 2017 (OIE, 2018d). A unique strain of H5N8 was detected in wild birds in the Republic of Tyva, (southern Russian Federation) in May 2016, followed by outbreaks in India and Europe in what has become known as the fourth intercontinental wave, resulting in the spread and deaths in poultry and wild birds globally (Lee et al., 2017). Clade 2.3.4.4 HPAI H5N8 originated from the A/Goose/Guangdong/1/1996 H5N1 lineage (Gs/GD) first detected in China in 1996 and has evolved into ten genetically distinct clades (0-9) and subclades (Donis et al., 2008). Since 2012, four distinct genetic groups (2.3.4.4 A-D) have been identified within this clade (Lee et al., 2017). In 2016 and 2017 as clade 2.3.4.4 H5 viruses spread, multiple reassortment of genes from wild bird populations resulted in the emergence of H5N8, H5N2, H5N5 and H5N6 viruses (Sims et al., 2017).

In Africa, only 3 subtypes of HPAI have predominated namely H5N1, H5N2, and H5N8 (OIE, 2018d). H5N1 clade 2.2.1.2 has been present in Egypt since the first intercontinental wave in 2006, where the first warning signs of the impending HPAI H5N1 outbreak in poultry were found through surveillance of migratory common teal (*Anas crecca*) (Saad et al., 2007). Clade 2.3.4.4 H5N8 was detected in green-winged teals (*Anas carolinensis*) in December 2016 (Kandeil et al., 2017). Outbreaks were also reported in Nigeria in May 2017 (Sims et al., 2017, OIE, 2018b) affecting free range birds including Guinea fowl (*Numididae*), turkey (*Meleagris*) and pigeon (*Columbidae*). The virus spread south to Zimbabwe in May 2017, presumably with migratory birds (OIE, 2018c).

In South Africa the HPAI subtype H5N8 was first detected in chickens in June 2017 in a commercial breeder flock in Mpumalanga province. Serology testing did not detect antibodies for influenza A virus; however molecular tests confirmed the presence of HPAI H5N8. Six weeks later, in August 2017 the virus was detected in two tracheal swab pools taken for routine surveillance testing from an ostrich farm in Heidelberg, Western Cape province. No clinical symptoms or mortalities occurred and swift quarantine action was taken to restrict movement and reduce the risk of spread of infection (van Helden and Roberts, 2017). The outbreak continued to spread to chickens farms, backyard operations, hobby bird species, ostriches and wild bird populations. One hundred and thirty-nine HPAI H5N8 outbreaks were reported to the OIE by February 2018.

Outbreak events decreased significantly after March 2018 due to increased biosecurity and stamping out measures for poultry species. Two outbreaks were reported in the North West province within ten kilometers of each other affecting quail (*Coturnix coturnix*) and pet wild ducks in the Madibeng district and almost 250 km North-East, a commercial chicken farm was affected in Maquassie Hills.

Fifty-eight outbreaks on commercial ostrich farms were reported to the OIE between August 2017 and February 2019. The majority of these outbreaks occurred in the Western Cape province (55) (Table 2). Three districts in the southern Cape were primarily affected, namely, Hessqua district with farms located closest to Heidelberg, Riversdal and Slagrivier; the Oudsthoorn district and farms near Calitzdorp (Kannaland district) (OIE, 2019a). Twelve of the 55 positive ostrich farms reported mortalities. The low mortality rate suggested that ostriches had innate resistance that rarely resulted in mortality unless other factors such as poor management and nutrition caused undue stress to the birds (Abolnik et al., 2016). At the time of writing, a ban on exports of fresh ostrich meat remains in effect.

Table 2. Avian influenza reported in the Western Cape province in ostriches (August 2017 to February 2019) (OIE, 2019a).

		Diagnostic method[1]			
Start date	**Region**	**Serology (NP ELISA + H5/H6/H7 HI)**	**PCR (AIV M gene + H5/H7)**	**Serotype(s)**	**% Mortality**
2017/08/02	Oudsthoorn	AI ELISA positive H6N2, H6N8, H7N1, H5N8 titres detected	Matrix gene positive only	Suspect H6 or suspect H5N8 §	0.0
2017/08/09	Hessequa	AI ELISA positive H6N2, H6N8, H5N1, H5N2, H5N8 titres detected	HP H5N8 positive	HPAI H5N8 *	0.0
2017/08/09	Hessequa	AI ELISA positive only	HP H5 positive	HPAI H5N8 *	0.0
2017/08/15	Hessequa	AI ELISA positive only	N8 positive, suspect HP H5	HPAI H5N8 *	0.0
2017/08/18	Hessequa	AI ELISA only (H5N8 not tested)	HP H5N8 positive	HPAI H5N8 *	0.0
2017/08/21	Hessequa	AI ELISA positive H5N1, H5N2, H5N8, H6N8 titres detected	HP H5N8 positive	HPAI H5N8 *	0.0
2017/08/21	Hessequa	AI ELISA positive H6N8 titres detected (H5N8 not tested)	HP H5N8 positive	HPAI H5N8 *	0.0
2017/08/23	Hessequa	AI ELISA positive H6N8, H5N1, low H5N8 titres detected	HP H5N8 positive	HPAI H5N8 *	0.0
2017/08/24	Hessequa	AI ELISA positive only	HP H5N8 positive	HPAI H5N8 *	**2.1**
2017/08/25	Hessequa	AI ELISA positive H5N1, H5N2, H6N8 titres detected (H5N8 not tested)	HP H5N8 positive	HPAI H5N8 *	0.0
2017/08/28	Hessequa	AI ELISA positive H6N2, H6N8, H5N8 titres detected	H6N8 positive	LPAI H6N8 *	0.0
2017/09/05	Hessequa	AI ELISA positive H6N2, H6N8, H5N2, H5N8 titres detected	Negative	Suspect H5N8 §	0.0
2017/09/08	Oudsthoorn	AI ELISA positive H6N2, H6N8, H5N1, H5N2, H5N8 titres detected	Matrix gene positive only	Suspect H5N8 and suspect H6 §	0.0
2017/09/11	Kannaland	Serology not done	HP H5N8 positive	HPAI H5N8 *	0.0
2017/09/11	Kannaland	AI ELISA positive H5N2, H6N8, H5N8 titres detected	Negative	Suspect H5N8 §	0.0
2017/09/12	Kannaland	AI ELISA positive H6N2, H6N8, H5N1, H5N2, H5N8 titres detected	HP H5N8 positive	HPAI H5N8 *	0.0
2017/09/13	Kannaland	Serology not done	HP H5N8 positive	HPAI H5N8 *	0.0
2017/09/13	Oudsthoorn	AI ELISA positive H6N8, H5N8 titres detected	H6 positive	Suspect H6 or suspect H5N8 §	0.0
2017/09/15	Kannaland	Negative	HP H5N8 positive	HPAI H5N8 *	0.0
2017/09/15	Oudsthoorn	AI ELISA positive	HP H5N8 positive	HPAI H5N8 *	0.0

		Diagnostic method[1]			
Start date	**Region**	**Serology (NP ELISA + H5/H6/H7 HI)**	**PCR (AIV M gene + H5/H7)**	**Serotype(s)**	**% Mortality**
		H6N2, H6N8, H5N1, H5N2, H5N8 titres detected			
2017/09/19	Kannaland	AI ELISA positive H6N2, H6N8, H5N8 titres detected	N8 positive only	Suspect H5N8 and H6 *	0.0
2017/09/19	Oudsthoorn	AI ELISA positive H6N2, H6N8, H5N8 titres detected	Matrix gene positive only	Suspect H6 or suspect H5N8 §	0.0
2017/09/26	Oudsthoorn	AI ELISA positive only	H5N8 positive	HPAI H5N8 *	0.0
2017/09/26	Mossel Bay	Negative	HP H5N8 positive	HPAI H5N8 *	0.0
2017/09/27	Oudsthoorn	Serology not done	HP H5N8 positive	HPAI H5N8 *	0.0
2017/09/27	Hessequa	AI ELISA positive H6N8, H5N8, H5N2 titres detected	Negative	Suspect H5N8 §	0.0
2017/10/11	Hessequa	AI ELISA positive H5N1, H5N2, H6N8 titres detected	Negative	Suspect HPAI H5N8 §	0.0
2017/10/11	Oudsthoorn	AI ELISA positive H6N8, H5N8 titres detected	Matrix gene positive only	Suspect HPAI H5N8 §	**0.4**
2017/10/16	Oudsthoorn	AI ELISA positive H6N8, H5N8 titres detected	H6N8 positive	LPAI H6N8 *	0.0
2017/10/20	Oudsthoorn	AI ELISA positive H6N2, H6N8, H5N8 titres detected	Matrix gene positive only	Suspect H6 or suspect H5N8 §	0.0
2017/10/25	Oudsthoorn	AI ELISA positive H5N8, H6N2, H6N8 titres detected	Negative	Suspect H5N8 §	0.0
2017/10/26	Oudsthoorn	AI ELISA positive H6N2, H6N1, H5N8 titres detected	H6N8 positive	LPAI H6 *	0.0
2017/10/31	Oudsthoorn	AI ELISA positive H6N2, H6N8, H5N1, H5N2, H5N8 titres detected	HP H5N8	HPAI H5N8 *	0.0
2017/11/07	Hessequa	AI ELISA positive H6N2, H5N8, H6N8 titres detected	Negative	Suspect H5N8 §	0.0
2017/11/13	Witzenberg	AI ELISA positive H5N2, H5N8, H6N8 titres detected	Negative	Suspect HPAI H5N8 §	0.0
2017/11/17	Swartland	AI ELISA positive H6N8, H5N8 titres detected	Negative	Suspect HPAI H5N8 §	0.0
2017/11/23	Hessequa	AI ELISA positive H6N2, H5N1, H5N2, H5N9 titres detected	Matrix gene positive only	Suspect H5N8 or Undefined AI §	0.0
2017/11/28	Oudsthoorn	AI ELISA positive H6N2, H6N8, H5N1, H5N2, H5N8 titres detected	H6N8 positive	LPAI H6N8 *	0.0
2017/12/04	Witzenberg	AI ELISA positive H5N1, H5N2, H5N8, H6N8 titres detected	Negative	Suspect HPAI H5N8 §	0.0
2018/01/03	Kannaland	AI ELISA positive H5N1, H5N2, H6N8, H5N8 titres detected	Negative	Suspect HPAI H5N8 §	0.0
2018/01/15	Hessequa	AI ELISA positive H5N8, H5, H6N8 titres detected	Negative	Suspect HPAI H5N8 §	0.0
2018/01/16	Swellendam	AI ELISA positive H5N8 titres detected	Negative	Suspect HPAI H5N8 §	0.0
2018/01/16	Witzenberg	AI ELISA positive H5N8, H6N8	Negative	Suspect HPAI H5N8 §	0.0
2018/02/15	Witzenberg	AI ELISA positive H6N2, H6N8, H5N1, H5N2, H5N8 titres detected	Negative	Suspect HPAI H5N8 §	**0.3**
2018/03/13	Witzenberg	AI ELISA positive H5N1, H5N8, H7N1, H6N8, H6N2 titres detected	H6N8 positive	LPAI H6N8 *	**0.2**
2018/04/03	Mossel Bay	AI ELISA positive H5N1 H5N8 titres detected	Negative (April 2017: LPAI, H12 confirmed by sequencing	Suspect H5 (April 2017: AI H12*)	**28.9**

		Diagnostic method[1]			
Start date	**Region**	**Serology (NP ELISA + H5/H6/H7 HI)**	**PCR (AIV M gene + H5/H7)**	**Serotype(s)**	**% Mortality**
2018/04/05	Oudsthoorn	AI ELISA positive Serology not done	H5N8 positive	HPAI H5N8 *	0.0
2018/09/15	Hessequa	AI ELISA positive H5N1, H5N8, H7N1, H6N8 titres detected	Negative	Suspect AI - undefined §	**0.2**
2018/09/26	Oudsthoorn	AI ELISA positive H5N2, H5N8 titres detected	Negative	Suspect AI - undefined §	**5.9**
2018/09/27	Hessequa	AI ELISA positive H6N8, H5N8 titres detected	Negative	Suspect AI - undefined §	**2.0**
2018/10/29	Swellendam	AI ELISA positive H5N1, H5N8 titres detected	Negative	Suspect H5 §	**4.3**
2018/11/19	Hessequa	AI ELISA positive H5N8, H6N8 titres detected	Negative	Suspect AI - undefined §	**1.3**
2018/11/20	City of Cape Town	AI ELISA positive H6N2, H5N8, H6N8 titres detected	Negative	Suspect AI - undefined §	0.0
2018/12/07	Kannaland	AI ELISA positive H5N2, H5N8, H6N8 titres detected	Negative	Suspect AI - undefined §	**5.0**
2019/01/08	Hessequa	AI ELISA positive H5N1, H5N8 titres detected	Negative	Suspect H5 §	**0.2**
					0.7 ^

[1] Refer to Avian influenza control for ostriches in South Africa (page 15) for diagnostic testing requirements for registered ostrich farms following the detection of avian influenza.

* Confirmed status

§ Suspected or unconfirmed status due to multiple seropositive reactors.

^ Average mortality % reported

2.4 High pathogenic avian influenza Clade 2.3.4.4 H5N8 outbreak in wild birds in South Africa

Sporadic outbreaks of HPAI H5N8 occurred from June 2017 in the Gauteng province in wild bird species. Six months later, in December 2017, the public began reporting deaths of tern species in the coastal regions of Gansbaai and Hermanus. Clinical symptoms reported included weakness, corneal opacity, neurological signs such as tremors and seizures, disorientation and the inability to fly. Death occurred within days of the first clinical symptoms. The first report of deaths as result of HPAI H5N8 in African penguins (*Spheniscus demersus*) on Dyer Island, was in January 2018. As this is listed as an endangered species in South Africa, the ecological impact of HPAI was potentially devastating. Swift terns (*Thalasseus bergii*) are the coastal bird species that have been most affected by the disease in South Africa to date; authorities managing wild life species reported 5929 deaths for this species followed by Cape gannet (*Morus capensis*), where 30 deaths were reported. Other wild bird species affected included common terns (*Sterna hirundo*), Hartlaub's gull (*Chroicocephalus hartlaubii*) and African black oystercatchers (*Haematopus moquini*) (OIE, 2018a).

The cases reported to the OIE are likely a gross underestimation of the true number of wild birds affected during this period. Inadequate resources prevented the wider and more thorough collection and testing of samples due to the large number of mortalities occurring over multiple locations on western and southern coastal areas. In general, AI control measures for wild birds are limited as mass culling is not supported as an effective in the control of influenza and furthermore the presence of influenza A virus in wild birds does not result in trade restrictions for poultry by the OIE (OIE, 2018d).

2.5 Drivers of avian influenza infection in ostriches in South Africa

Ostrich farming in South Africa is located primarily in the Western Cape province in the semi-arid Karoo region close to Oudtshoorn and near Heidelberg in the Southern Cape, that fall within a winter rainfall region. A free-range system is adopted where ostriches are likely to come into contact with waterfowl and other wild bird species close to feed points, irrigated pastures and water sources such as dams, troughs and streams. One of the key drivers for influenza infection in ostriches is this interaction between ostriches and wild bird species (Abolnik et al., 2016).

In all the reported outbreaks of HPAI and LPAI in ostriches in South Africa, low mortality occurred (Manvell et al., 1998, Manvell et al., 2005, Sinclair et al., 2006, van Helden et al., 2016, Abolnik, 2007, Abolnik et al., 2009, Abolnik et al., 2016) and clinical symptoms may be undetected in adult birds, increasing the risk of the spread of the virus between ostriches and wild bird species. Sacred ibis (*Threskiornis aethiopicus*) and waterfowl species such as ducks and geese (*Anseriformes*) can be infected subclinically with AI and therefore also act as reservoirs and fomite carriers between farms (Abolnik et al., 2016). Surveillance of wild birds, specifically migratory waterfowl (*Anseriformes*) species, would alert authorities of circulating influenza

strains and the potential for mutation of LPAI to HPAI outbreaks in poultry, including ostriches. The transfer of influenza A viruses has been inferred using phylogentic analysis of viruses isolated from domestic avian species and wild life species further demonstrating the role of migratory birds in the global spread of AI (Lycett et al., 2016, Olsen et al., 2006, Ramey et al., 2018).

2.6 Avian influenza control for ostriches in South Africa

Following the HP H5N2 outbreak in 2011, the National Directorate of Animal Health in South Africa revised the standard for the requirement for the registration and official control of registered ostriches. The standard recognised the risk factors and drivers of AI infection in ostriches and wild bird species and therefore aimed to implement measures intended at controlling and managing the spread of AI in ostrich populations, in turn preventing the spread of infection to other commercial poultry. The ultimate aim is to ensure food security and facilitate trade exports to major trading partners such as the EU for this industry (Department of Agriculture, 2012b).

In line with OIE requirements, ostrich farms must be registered and comply with stipulated biosecurity measures. In addition traceability of birds and record keeping must be practiced to ensure that all ostriches are individually identifiable. Serological surveillance for influenza A virus infection of all registered ostrich farms is compulsory every six months. Diagnostic testing firstly involves screening using a validated commercially available AI MultiS-Screen antibody (Ab) test that has a high diagnostic sensitivity (≥ 99%) (IDEXX, Maine, USA). Any ELISA positive samples must be tested to detect exposure to H5, H6 and H7 subtypes using haemagglutination inhibition (HI) tests. A panel of antigens is used to test each ELISA positive serum; at the time of writing this panel consisted of seven antigens, namely H5N1, H5N2, H5N8, H6N2, H6N8, H7N1 and H7N7. As per the case definition of notifiable AI by the OIE, H5 and H7 subtypes are reportable (OIE, 2015), however screening for H6 subtypes is also required in South Africa due to the presence of LPAI H6N2 in chicken populations in the region (Abolnik et al., 2016, Rauff et al., 2016). The epidemiological unit is tested to detect infection at >10% prevalence with 95% confidence in each unit within each ostrich compartment (Department of Agriculture, 2012b).

When H5 or H7 antibodies are detected, immediate disease investigation and control measures are implemented. This will include intensifying sampling for serological testing and RT-qPCR for the detection of the AI matrix gene. Any PCR positive results must be tested further to characterize the HA subtype using sensitive and specific H5 and H7 subtype RT-qPCR tests, followed by amplicon sequencing to determine the HA cleavage site and characterization of positive samples as either low or high pathogenic AI when reporting the outbreak to the OIE. Low RNA concentrations extracted from avian influenza PCR positive swab samples present a challenge for further characterization using amplicon sequencing, including next generation sequencing as PCR assays used to generate the PCR products have limited sensitivity. Virus isolation remains

a “gold standard” diagnostic assay for avian influenza, although it is time consuming and laborious (OIE, 2015). However successful virus isolation amplifies virus titres that can be used for further laboratory testing with ease making it possible to sequence all eight gene segments of isolates for phylogenetic comparison of influenza A viruses in the region and elsewhere, and to identify genetic shift and reassortment (Zhou, et al., 2009). In addition, further advantages of successful virus isolation include the possible determination of the intravenous pathogenicity index (IVPI) for chickens and the virus isolates can also be used to produce geographically relevant antigens that are required for serology HI tests (OIE, 2015). Thus, efficient and successful virus isolation techniques from routine surveillance samples (tracheal swab pools) will be of great diagnostic value when outbreaks are detected in ostrich flocks (Department of Agriculture, 2012b).

During an outbreak, intensified sampling and testing inevitably places additional pressure on already strained veterinary service resources in the country. Repeated sampling events must be done in order to detect the presence or absence of AI antibodies and the virus at > 5% prevalence with 95% confidence in each epidemiological group. A registered ostrich farm may include different epidemiologic units, which are based on relative distance between groups of ostriches. If the distance exceeds more than 500 meters, then it would be considered to be a separate epidemiologic unit. Epidemiologic units are also separated based on age of the ostrich. Young birds are classified as those from 6 weeks to 5 months of age and older birds are those greater than 5 months of age to slaughter age (10- to 14-months old). The number of samples taken depends on the size of the epidemiologic unit. For example, if the epidemiologic unit is 51 to 100, then at least 45 ostriches must be sampled; 201-300, then at least 54 ostriches must be sampled; if the epidemiological unit >4500, then at least 59 birds must be sampled (Department of Agriculture, 2012b). However, routine sampling done by the Western Cape Animal Health Services usually exceeds the prescribed number of samples, where 60 to 120 ostriches are sampled from each epidemiological unit (Dr L. Roberts and Mr D. Visser [Animal Health, Department of Agriculture, Western Cape Province] pers. comm., 14 February 2018).

Where the presence of HPAI infection is confirmed, strict quarantine and movement restrictions come into effect as well as the suspension of exports from the infected zones, province and or the country. Increased sampling must be done and only when the infected zone or compartment is free from infection for at least 6 months from the date of the last infected farm or compartment is movement of ostriches permitted. Vaccination of ostriches is strictly prohibited in South Africa as a control measure (Department of Agriculture, 2012b).

2.7 Sampling and collection protocols for the detection of influenza A virus

The type of specimen collected is determined by the type of test to be conducted and the target species. Serum is collected for serological testing, whilst oropharyngeal (or tracheal) and cloacal swabs are commonly collected for RT-qPCR testing and virus isolation for avian species. Cloacal, oropharyngeal and faecal swabs

are sampled for wild waterfowl where the site of shedding of LPAI virus may vary, but oropharyngeal (or tracheal) swabs are optimal for gallinaceous poultry (Munster et al., 2009, Latorre-Margalef et al., 2016, USDA, 2016). Tissues such as lung, brain, kidney and spleen are also collected for the detection of influenza A virus by RT-qPCR and virus isolation (Spackman et al., 2017).

Aforementioned sampling for surveillance of AI in ostriches includes tracheal swabs (Department of Agriculture, 2012b). LPAI and HPAI virus is known to attach to and infect epithelial cells in the respiratory and gastrointestinal tracts (OIE, 2015). Sampling is therefore directed to detect influenza A virus that may be collected from the surface of infected tissues, where virus is excreted from epithelial cells with tracheal swabs (Spackman et al., 2017).

Swab samples should be collected and placed into tubes in a virus transport medium as soon as possible after collection, as transporting swabs dry decreases the sensitivity of RT-qPCR and virus isolation (Spackman et al., 2013, USDA, 2016, Latorre-Margalef et al., 2016). Swab pooling has been practiced to reduce costs of testing. Pooling of up to 11 swabs per pool in experimentally infected chickens has been evaluated for low virus titres and has not decreased the sensitivity of AI detection using RT-qPCR and virus isolation when compared to one swab and five swabs (Spackman et al., 2013, Ladman et al., 2012, Arnold et al., 2013). Swab pooling of more than five swabs has however not been adopted for ostriches in South Africa in surveillance testing because the Department of Agriculture, Forestry and Fisheries (DAFF) maintains that methods optimized for chickens cannot be applied directly to ostriches, and requires empirical proof that pooling higher numbers of ostrich swabs will not dilute influenza A virus where the titre is low.

In South Africa, within each epidemiologic unit, tracheal swabs are collected from individual ostriches and five swabs are pooled together in a single tube containing 50% v/v glycerol PBS medium (pH 7.2) for AI surveillance for RT-qPCR; virus isolation is performed on all positive samples as this is still considered to be the "gold standard" for the detection of Influenza A virus (OIE, 2015). In order to successfully characterize Influenza A virus in an outbreak, the virus should be isolated so that full genetic sequencing can be done. Historically, in all influenza outbreaks in ostriches in South Africa, the recovery of Influenza A virus has had limited success. One of the reasons could be degradation of the virus in the standard transport medium. Another reason is the possible existence of a species barrier between ostrich-adapted viruses and the embryonated chicken egg propagation system (Spackman and Killian, 2014). Ostrich farms are typically located far from diagnostic test laboratories. Without the addition antibiotics in a transport medium, and especially if the cold chain is not maintained, the growth of bacteria and fungi in tracheal swab pools could produce proteases that destroy the viral envelope. During the 2011 HP H5N2 outbreak, only three viruses were isolated from pooled swabs originating from twenty farms that tested positive by PCR (Abolnik et al., 2012). Similarly, in the 2017 HPAI (H5N8) outbreak, there was been limited success in isolating virus from PCR positive swab pools in 50% v/v glycerol PBS transport medium from ostrich flocks at accredited testing

laboratories (Abolnik et al., 2018). Spackman et al. (2013) evaluated PBS (pH 7.2) and BHI broth (pH 7.3) medium used to transport swabs for AI detection by virus isolation and RT-qPCR. A buffered medium containing protein such as BHI was found to be superior for the isolation of Influenza A virus. No significant difference was found in the detection of influenza RNA by RT-qPCR. Influenza A virus has been however, successfully isolated from faecal swabs sampled from chickens, ducks and pigeons in antibiotic supplemented glycerol PBS medium in China (Zhang et al., 2015).

For the ostrich production industry to be sustainable, the demands of the European export market and local South African animal health services must be met. Rapid and accurate diagnostic testing is essential in ensuring early detection and monitoring the presence and spread of disease in ostriches and other poultry populations. Increased swab pooling from five to ten swabs, without comprising the epidemiology requirements for the detection of AI in farmed ostriches for surveillance in an outbreak period and routine surveillance for movement and preslaughter, would reduce the number of tests performed at accredited laboratories. This would result in cost benefits for farmers as well as government stakeholders. Accredited laboratories charge between R316 and R 438 (ZAR, South African rand) per sample (swab pool) for RT-qPCR tests (Table 6). This is equivalent to approximately $22 to $30 US dollars[6]. Where at least 60 ostriches from each epidemiological group are routinely sampled, this results in a minimum of 12 sample pools of five swabs each that are tested by RT-qPCR, costing between R3792 to R5256 per unit. Increasing the number of swabs pooled to ten would reduce the costs of RT-qPCR testing for the epidemiological unit by half (i.e. R1896 to R2628).

Virus transport medium (VTM) containing BHI broth with antibiotics has been shown to be optimal when compared to PBS in chickens (Spackman et al., 2013), but this has not been adopted for ostrich testing in South Africa, presumably due to supply and cost of VTM. The cost benefit of continued use of 50% v/v PBS glycerol transport medium for tracheal swabs can be compared to the benefit of increasing the success of virus isolation using a more expensive transport medium (BHI broth, 10% glycerol with broad-spectrum antibiotics). Successful and efficient virus isolation from routine tracheal swab surveillance samples would facilitate accurate identification and characterisation of the virus at every epidemiological site and will improve the accuracy of reporting in outbreak situations and management practices for the ostrich industry.

[6] https://www.poundsterlinglive.com/bank-of-england-spot/historical-spot-exchange-rates/usd/USD-to-ZAR-2019

3. Aims and objectives

The proposed study sought to evaluate sampling protocols currently used for surveillance of avian influenza in farmed ostriches in South Africa. The first objective of the study was to evaluate if the detection of influenza A virus by RT-qPCR and virus isolation was reduced by increasing the number of tracheal swab pools from five to ten swabs in the presence of low titres of influenza A virus (null hypothesis). The second objective was to evaluate the effect of the virus transport media on the detection of Influenza A virus by RT-qPCR and virus isolation. The virus transport medium that is currently and widely used in South Africa to transport ostrich swabs contains 50% v/v glycerol PBS, without the addition of antibiotics or antifungals. This virus transport medium was compared to an alternative transport medium, namely BHI broth (VTM), which contains 10% glycerol and broad-spectrum antibiotics. The third objective was to conduct a cost benefit analysis for increasing ostrich swab pools from five to ten with VTM.

Previous studies that evaluated the pooling of swabs in chickens and turkeys were conducted by experimental infection of birds (Spackman et al., 2013, Ladman et al., 2012, Arnold et al., 2013); however housing large ostriches within Biosafety Level 3 facilities presents unique challenges. Therefore, instead of experimental infection to generate the samples required, i.e. swabs that contain a natural matrix of ostrich tracheal mucous, we targeted sampling of a flock with an active field infection of AI. All the tracheal swabs sampled from the selected flock would be screened for Influenza A virus by a commercial rapid antigen lateral flow immunoassay and by RT-qPCR to identify the positive and negative swabs in the sampling unit. The tracheal swabs identified as negative during the initial screening assays were to be used for the preparation of the required number of swab pools with a single influenza A virus positive swab.

4. Materials and Methods

4.1 Selection of the sampling site

The strategy used to select a suitable sampling site was to identify a farm where ostriches could be sampled from a flock with a high likelihood of active infection based on the history of surveillance and diagnostic testing, including RT-qPCR and serology testing. A conservative approach with 50% prevalence (P) and 5% precision (e) in a finite population size (N) of 1000 (95% confidence interval; $Z = 1.96$), was used to estimate the required sampling size ($Z^2 * P(1-P)/e^2$). The sample size, $n = 382.2$ was adjusted to take the finite population into account to give a sample size of 278 birds ($N_{adj} = (N*n)/(N+n)$ (Thrusfield, 2007). This was done to ensure that sufficient positive and negative field samples were available to perform the swab pooling experiments. For contingency purposes, the option of spiking ostrich field swabs with

cultured influenza A virus was also considered if the expected number of PCR positive swabs was not available for swab pooling. In addition, if the expected number of PCR negative swabs was not available, additional sampling of a known negative site was also proposed.

On the 5th of September 2018, tissue samples from two wild Egyptian geese (*Alopochen aegyptiaca*) that had been shot on an ostrich farm located in the Oudsthoorn area, Western Cape Province were submitted for wild bird AI surveillance to the regional AI reference laboratory in South Africa (Agricultural Research Council-Onderstepoort Veterinary Research (ARC-OVR), Onderstepoort, Pretoria, South Africa). At the time of sampling, approximately 3600, ten- to fourteen-month old (slaughter age) ostriches were present on the farm. A variety of wild birds were frequently sighted on the 5400 hectare property, including Egyptian geese (*Alopochen aegypti*), blue cranes (*Anthropoides paradiseus*) (approximately 150), spurwing geese (*Plectropterus gambensis*), common house crows (*Corvus splendens*) and pigeons (*Columbidae*), where some water points are shared between ostriches and wild birds on the farm (Mr Eddie Lottering, [Animal Health, Department of Agriculture, Western Cape Province] pers. comm., 26 April 2019]). The presence of AI matrix gene was confirmed in the Egyptian Geese samples by RT-qPCR testing on the 10th of September, but tested negative for H5 and H7 subtype RT-qPCR.

As a precaution, control measures were initiated by veterinary services and the farm was placed under quarantine. On the 15th of September 2018, four ostriches died on the farm, but the mortality was attributed to the sudden drop in temperatures. Tissue samples were submitted for AI testing to the Provincial Veterinary Laboratory in Stellenbosch, Western Cape Province. Tissue samples were tested for the influenza A virus using RT-qPCR (VetMAX™-Gold AIV Detection Kit (Applied Biosystems Thermo Fisher Scientific, Waltham, US) by the author and also processed in virus transport medium and inoculated into 9- to 11-day old embyronated chicken eggs according to standard virus isolation methods (OIE, 2015) by Ms Belinda Peyrot. An agglutinating virus was isolated from the tissue samples; however the hemaglutination inhibition (HI) test results using H5 and H7 antisera were negative. Both the original tissue and virus isolate were confirmed to be Influenza A virus by RT-qPCR testing; samples tested negative for H5, H7 and N8 subtypes. Purified RNA from the virus isolate was submitted for Sequencing (Ion Torrent) of the HA and NA genes described elsewhere (Abolnik et al., 2018) and were confirmed as LPAI H11N1 subtype. No further ostrich mortalities were reported on this farm, but some ostriches showed respiratory distress and increased mucous was observed in the tracheal swabs at sampling (Dr Edwin Dyason, [Animal Health, Department of Agriculture, Western Cape Province] pers. comm., 1 November 2018]).

Surveillance testing by state veterinary services followed and samples taken on the 17th of September (serum only) were sent to Deltamune (Pty) Ltd, Oudtshoorn, Western Cape Province for AI ELISA and HI testing. Samples taken on the 25th of September and the 3rd of October (tracheal swabs and serum) were sent to ARC-OVR for ELISA, HI, RT-qPCR testing. Six (out of 24) and four (out of 24) influenza A virus PCR positive

tracheal swab pools were reported for sampling done on the 25th September and the 3rd of October, respectively. The RT-qPCR crossing threshold values for six of the 10 positive samples were characterized as "weak positives", with Ct values > 35 cycles. Serology testing included ELISA tests, followed by HI testing; positive HI reactions were reported against H5N1, H5N8 and H7N1 (sampling date: 17th September), H5N1 and H5N8 (sampling date: 25th September), H5N1, H5N2, H5N8, H6N2, H6N8 (sampling date: 3rd October), indicating that the ostriches had been exposed to various serotypes that could not be conclusively identified using the available serological reagents. The multiple antigen reactions reported illustrated the limitations of serology HI diagnostic tests available to accurately diagnose AI subtypes. Due to the epidemiological history and the likelihood of continuing or reintroduced virus, the site was selected as a suitable sampling site for the swab pooling study. Ethics approvals for sampling and research was obtained, including: University of Pretoria, Animal Ethics V015-18, 26 March 2018; University of Pretoria, Research Ethics REC025-18, 5 June 2018; and Department of Agriculture Forestry and Fisheries (DAFF) permission was granted under Section 20 of the Animal Diseases Act, 1984 (Act No 35 of 1984) Reference 12/1/9, 25 June 2018 (Appendix A – D).

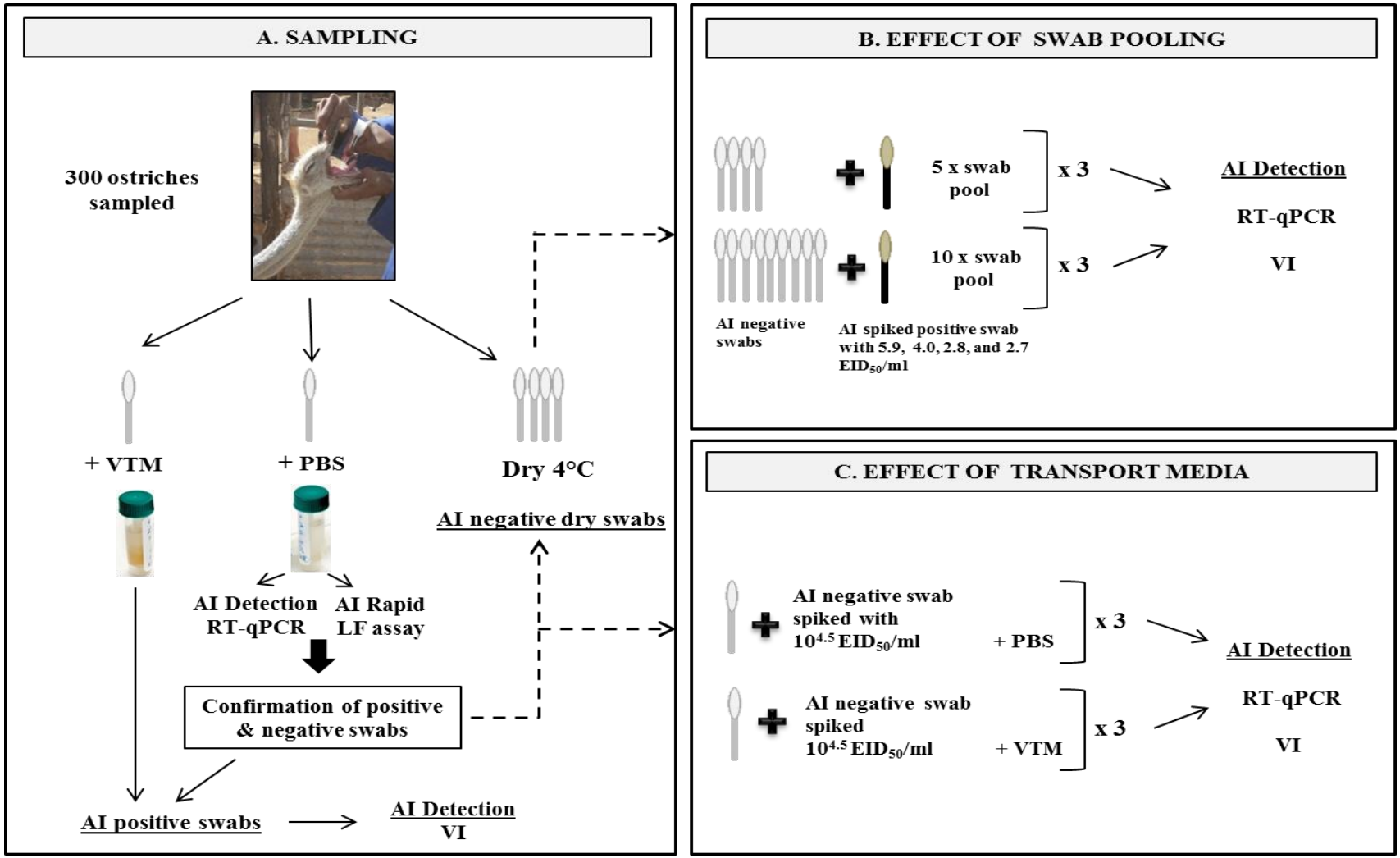

Figure 1. Schematic diagram illustrating the sampling and testing protocol, including sampling and screening of 300 ostriches for influenza A virus (A), followed by spiking negative tracheal swabs to evaluate the effect of swab pooling (B) and transport medium (C) on the detection of influenza A virus. (AI Rapid LF assay: Avian influenza Rapid lateral flow immune assay).

4.2 Sample collection

On 1 November 2018, 52 days after the initial AI positive samples were confirmed in wild Egyptian geese (*Alopochen aegypti*) three hundred 10- to 14-month ostrich chicks that had not been sampled previously were selected from two camps on the farm. Each ostrich was captured by four experienced farm handlers and sampled by Western Cape Provincial State Veterinary officials (State Vet Office George, Western Cape) including State Veterinarian Dr Edwin Dyason and Animal Health Technicians, Mr Eddie Lottering and Ms Rebecca Mabula, shown in Fig. 2. Six replicate tracheal swabs (rayon tipped, Copan Italia SPA, Brescia, Italy) were sampled from each ostrich, packed on ice and transported to the State Veterinary office in George, Western Cape for initial sample processing. Two replicate swabs were each immersed into either 2 ml virus transport medium (VTM) containing 10% v/v glycerol in BHI broth (pH 7.2) containing the following antibiotics per litre: 100mg of doxycycline (Mylan), 100mg of enrofloxacin (Cipla), 1000mg of pencillin-streptomycin (Sigma-Aldrich), or standard 50% v/v glycerol PBS transport medium (pH 7.2). Swabs in transport medium and the remaining four replicate swabs were kept at 4 – 8°C and transported to the Western Cape Provincial Veterinary Laboratory in Stellenbosch, Western Cape for testing on 2 November 2018.

Figure 2. Sampling of ostrich farm on 1 November 2018; (a) Ostriches were kept in camps before sampling, (b) Ostriches were captured and held securely by four experience farm handlers, (c) and (d) tracheal swab sampling was performed by State Veterinary officials, (d) ostriches were released after sampling (e).

4.3 Detection of influenza A virus

In the laboratory, the tracheal swabs in 50% v/v glycerol PBS transport medium were screened for the presence of influenza A virus using the ANIGEN Rapid AIV Ag lateral flow immune assay test kit (Animal Genetics, Inc, South Korea) as per the manufacturer's instructions. The sole purpose of using the lateral flow immunoassay was to identify positive samples for the swab pooling experiments. Viral RNA was purified from 200µl transport fluid for RT-qPCR using the *cador*® Pathogen HT 96 QIAcube® HT Kit and QIAcube HT system (QIAGEN, Germany). Two microliters of a synthetic RNA internal positive control (IPC) VetMax™ Xeno™ RNA was included in nucleic acid extraction to detect for PCR inhibition at a concentration of 10 000 copies/µl (Applied Biosystems Thermo Fisher Scientific, Waltham, US). The VetMAX™-Gold AIV Detection Kit (Applied Biosystems Thermo Fisher Scientific, Waltham, US) was used to perform RT-qPCR targeting the matrix and the nucleoprotein genes of influenza A virus and the Xeno™ RNA IPC target using a fluorescent TaqMan® hydrolysis probe. Four microliters of the eluted fluid and 8.5 µl of master mix were used per RT-qPCR reaction as per validated and accredited protocol in the laboratory.

A Roche LightCycler® 480 Instrument II (Roche Molecular Diagnostics, Rotkreuz, Switzerland) was used for the RT-qPCR reaction with the following thermal cycle profile: reverse transcription, 48 °C for 10 minutes, inactivation and initial denaturation step, 95°C for 10 minutes, followed by 45 amplifications cycles of 95 °C for 15 seconds and 60 °C for 45 seconds and a final cooling step of 40 °C for 30 seconds. Results were analysed using the system software for absolute quantification using the second derivative maximum method, where the Ct value of the sample was recorded at the point where the fluorescence curve turned sharply upward above the background signal in the exponential phase of the PCR amplification reaction. Samples with a Ct value of ≤ 36 were interpreted as "positive", samples with a Ct value of 37 to 39 were considered to be "weak positive", and samples with a Ct values greater than 39 or where no Ct value was recorded were considered to be negative.

4.4 Virus isolation

After screening for influenza A virus, positive RT-qPCR tracheal swab samples (in both VTM and 50% v/v glycerol PBS transport media) were sent to an accredited testing laboratory (Ms Christine Strydom, Deltamune (Pty) Ltd, Centurion, Pretoria) for virus isolation by inoculating 9- to 11-day old embryonated eggs using standard methods (OIE, 2015).

4.5 Spiking experiments

The high pathogenic influenza A virus A/Speckled pigeon/South Africa/08-004B/2017(H5N8) with an egg infectious dose (EID) of $10^{8.5}$ EID_{50}/ml was provided by the University of Pretoria and this was used to spike negative tracheal swabs pools as described below. In order to determine the viral stock titres and

the corresponding Ct value of RT-qPCR, the viral stock was diluted 10-fold in 50% v/v glycerol PBS transport medium and the Ct values were plotted in a standard curve against the EID_{50}/ml after RNA extraction and RT-qPCR testing in triplicate as described in section 4.3.

To model the detection of influenza A virus by RT-qPCR and virus isolation at low viral titres in pooled ostrich swabs, where RT-qPCR Ct values greater than 36 are typically reported as "weak positive" at the cut-off for detection, the virus stock was diluted 10-fold (10^{-1} to 10^{-4}), followed by seven two-fold serial dilutions ($10^{-4.3}$ to $10^{-6.4}$). Transport medium (50% v/v glycerol PBS) pooled from surplus PCR negative swab samples used as virus stock diluent to ensure that the matrix of subsequent swab pooling matched tracheal swabs sampled from ostriches in routine surveillance.

Six sterile swabs were spiked with virus by cutting and immersing the swabs tips into each dilution tube. To ensure sufficient adsorption of all 6 swabs, the tubes were left at room temperature for approximately 30 minutes. Each spiked swab tip was removed aseptically and added to 3 ml sterile 50% v/v glycerol PBS transport medium containing either 4 or 9 negative tracheal swab tips, each prepared in triplicate. The pooled samples were refrigerated for four days before RT-qPCR and virus isolation was performed, as ostrich swabs sampled during routine surveillance can typically take 72 to 96 hours before they reach the testing laboratory.

Each swab pool was tested for influenza A virus by RT-qPCR as described in section 4.3 however for the purposes of data analysis sample pools that were negative where no Ct value was recorded, these results were assigned a Ct value of 40 as this is highest Ct value that is reported by the system software for absolute quantification using the second derivative maximum method when 45 amplification cycles are selected. The significance of the mean RT-qPCR Ct values recorded for the five and ten swab pools was tested using Welch's paired t-test. Calculations were made using R-studio (Boston, MA, USA) and Excel (Microsoft, Redmond, WA, USA).

The fluid from the triplicate five- and ten-swab pooled samples at low (10^{-1}), medium (10^{-3}) and high ($10^{-4.6}$ and $10^{-5.6}$) dilutions were each pooled into a single sample and aliquots were used for virus isolation at two accredited testing laboratories (denoted as "Laboratory A" and "Laboratory B" in this study to maintain confidentiality), using standard methods (OIE, 2015).

4.6 Comparison of virus transport media with PBS glycerol media

To evaluate whether there was a significant difference in virus isolation between standard 50% v/v glycerol PBS and VTM transport medium, one negative tracheal swab was immersed in 2ml of transport medium spiked with HPAI (A/Speckled pigeon/South Africa/08-004B/2017(H5N8)) at ~$10^{4.5}$ EID_{50}/ml

concentration. Each tube was prepared in triplicate and refrigerated for 4 days before sending the sample to an accredited laboratory for virus isolation.

5. Results and discussion

5.1 Screening of the ostrich flock for influenza A virus

Three hundred ostriches were sampled to collect sufficient positive and negative tracheal swabs in order to achieve the research objectives. RT-qPCR and a lateral flow immunoassay test were used to screen for influenza A virus. Only two weak positive samples with Ct values of 36.4 and 36.7 out of 300 birds sampled were detected using RT-qPCR, these samples however tested negative using the Rapid AIV Ag test kit. Three samples tested positive using the Rapid AIV Ag test kits (Figure 3), but negative using RT-qPCR. Lateral flow test kits have been evaluated in poultry, but their use in the field as a pen side diagnostic tool has been variable with low detection sensitivity reported from cloacal or oropharyngeal swab samples. A 61% (56-80%, 95% CI) sensitivity was reported for the Rapid AIV Ag test kit (ANIGEN) when evaluated against RT-qPCR testing under field conditions in chickens in Indonesia (Loth et al., 2008). Woolcock and Cardona (2005) evaluated five immunoassay kits reporting poor sensitivity of detection in cloacal and oropharyngeal swabs in experimentally infected chickens, where single and pooled oropharyngeal swab samples from experimentally infected chickens were compared with virus isolation and the best performing rapid test kit (Directigen FluA, Becton Dickinson, Franklin Lakes, NJ, USA) detected only 2 of 39 single swab samples (5%), 5 of 29 pool swab samples (7%), and 1 of 5 pooled cloacal swabs. A detection limit of $10^{4.7}$ EID_{50}/ml LPAI H6N2 was reported the Directigen FluA kit (Becton Dickinson, Franklin Lakes, NJ, USA). The sole purpose of using the Rapid AIV Ag test kit for screening in the present study was to identify strong positive samples to be used in the swab pooling experiments, but inconsistencies in the results between RT-qPCR testing and AI Rapid LF immune assay in the current study reflect that low levels of virus in ostrich tracheal swabs may limit their use in field surveillance. Virus isolation was attempted on the five field samples that tested positive using either RT-qPCR or Rapid AIV Ag test, but no virus was isolated.

Figure 3. ANIGEN Rapid AIV Ag lateral flow immune assay results. Three out of the 300 samples screened for influenza A virus using the ANIGEN Rapid AIV Ag lateral flow immune assay were positive, indicated by two visible red lines on the test strip: (a) 91 (b) 111 and (c) 247; (a) 92 where only a single red line is visible is indicated as an example of a negative result.

At the time of sampling on 1 November 2018, the presence of active AI infection in the 300 ostriches sampled was low as only two birds tested positive by RT-qPCR positive, indicating that despite the associated risk factors, neither prolonged circulation of the H11N1 virus in mid-September 2018, nor reintroduction of other virus serotypes into the population had occurred. The short period of H11N1 circulation in the flock was possibly due to biosecurity measures imposed during quarantine of the property along with the innate resistance of ostriches to AI infection (Abolnik et al., 2016). In addition, planning and consultation delayed sampling by a few weeks. The sampling exercise was labour intensive, requiring additional farm personnel and veterinary officials than what would usually be required for routine surveillance sampling, where a maximum of 120 ostriches are sampled and only a single tracheal swab and blood tube is required for testing. The logistics in arranging the sampling event potentially reduced the possibility of obtaining additional positive samples from active infection in the ostriches for the study.

Since insufficient influenza A virus positive field swabs were identified, it was necessary to revert to the contingency plan of spiking the AIV-negative swabs collected from ostriches during the field sampling with H5N8 virus, for the swab pooling, transport medium and isolation experiments.

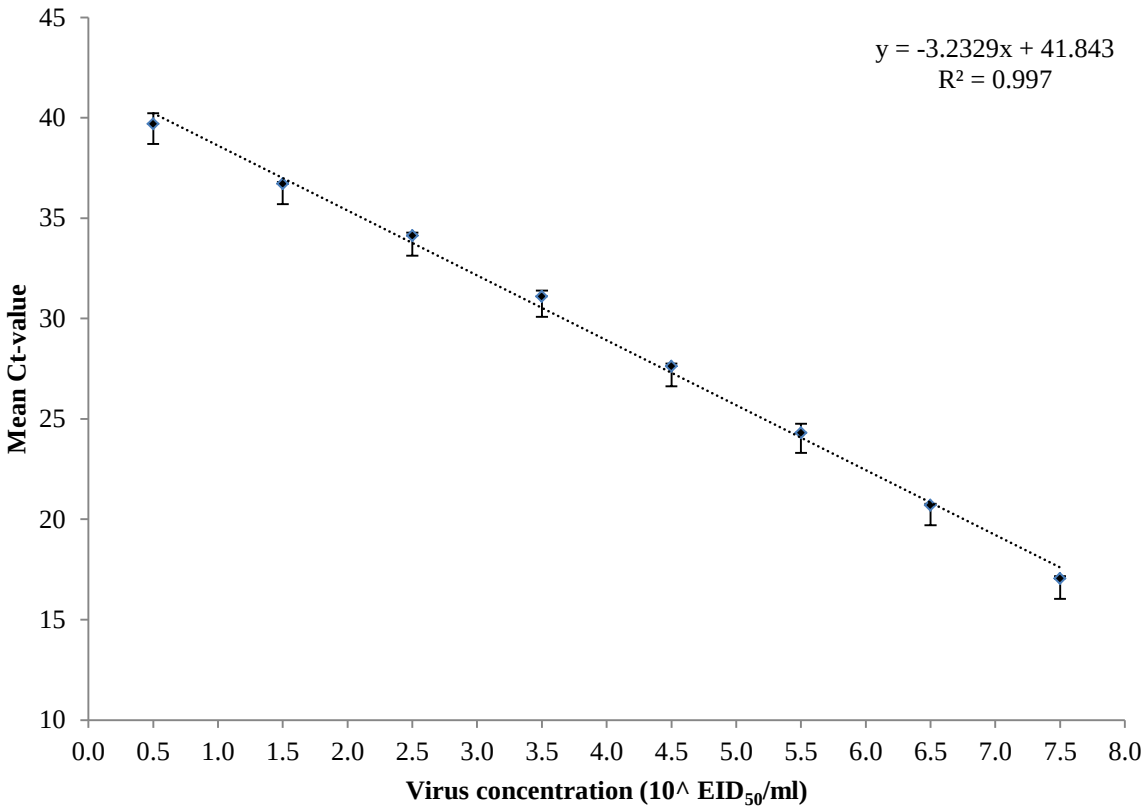

Figure 4. EID_{50}/ml viral stock titres of HPAI virus (A/Speckled pigeon/South Africa/08-004B/2017(H5N8)) plotted against the corresponding RT-qPCR crossing threshold (Ct) value for influenza A virus detection.

5.2 Comparison of pooling five versus ten ostrich swab pools for influenza A virus detection by RT-qPCR

RT-qPCR results for the detection of influenza A virus in five versus ten tracheal swab pools is presented in Table 3. The present study found that pooling a single HPAI positive swab with four or nine AI negative tracheal swabs did not significantly affect the detection of influenza A virus by RT-qPCR ($p < 0.05$) over the dilution range. The cycle threshold increased with a decreasing viral load, where the lowest virus concentration detected for all three replicates of five and ten swab pools was $10^{2.5}$ EID_{50}/ ml. In a similar study where faecal samples were spiked with LPAI using a single positive swab to prepare 5, 10 and 15 pools with negative swabs at high, medium and low virus loads (10^5, 10^4 and 10^3 $TCID_{50}$/ml, respectively), no difference was reported in swab pools at high and medium virus loads, but at lower virus titre (10^3 $TCID_{50}$/ml), the 10 and 15 swab pools were not generally detected by RT-qPCR (Fereidouni et al., 2012). Ladman et al. (2012) reported that the mean Ct value of the RT-qPCR for swab pools of 11 swabs were significantly higher than five swabs pools at three and five days post inoculation of a LPAI H7N2, but Ct values were below 35 and therefore easily detected as positive samples. The pathogenicity of the virus and pooling has also been evaluated where experimentally infected broiler chickens were inoculated with a either HPAI H5N2 or LPAI H7N2 virus. The proportion of positive samples was lower for HPAI than for LPAI swab pool groups (60.9% and 95%, respectively), but within each group there was very little difference in the detection of influenza A virus by RT-qPCR between 5, 6 and 11 swab pools (Ssematimba et al., 2018). This was also comparable to a study performed on experimentally infected chickens where comparisons were made between 1, 5 and 11 swab pools (Spackman et al., 2013). The United States Department of Agriculture (USDA) AI sampling protocol allows for the collection of 5 or 6 swabs in 3 ml virus transport medium for gallinaceous poultry, which can subsequently be pooled as an 11-swab pool at the testing laboratory before testing (USDA, 2016).

Table 3. Detection of influenza A virus by RT-qPCR in five and ten tracheal swab pools.

Virus concentration (EID_{50}/ml)[1]	5 swab pool (1 positive + 4 negative)[3]			10 swab pool (1 positive + 9 negative)[3]			P- value
	Mean Ct value[2]	Standard deviation	Number of AI positive pools/total replicates	Mean Ct value[2]	Standard deviation	Number of AI positive pools/total replicates	
5.9	22.80	0.27	3/3	22.46	0.51	3/3	0.374
4.9	25.99	0.29	3/3	25.80	0.10	3/3	0.376
4.0	29.45	0.53	3/3	28.42	0.33	3/3	0.056
3.4	30.48	1.56	3/3	31.29	0.14	3/3	0.463
2.9	32.73	0.13	3/3	32.00	0.55	3/3	0.142
2.8	32.92	0.06	3/3	32.44	0.38	3/3	0.155
2.6	33.08	0.40	3/3	33.58	1.24	3/3	0.558
2.7	33.45	0.91	3/3	32.89	1.53	3/3	0.619
2.5	34.77	0.63	3/3	32.99	0.89	3/3	0.054
1.9	35.34	4.04	2/3	36.30	3.30	2/3	0.990
1.5	35.53	3.91	2/3	38.17	3.16	0/3	0.416
0.6	40.00	0.00	0/3	40.00	0.00	0/3	0.374

[1] Virus concentration was calculated using the standard linear regression formula y = -3.2329x+41.843 (Figure 3)

[2] Ct values ≤ 36 were considered to be RT-qPCR positive; where no Ct values were recorded, a Ct value of 40.00 was assigned for data analysis

[3] The number of influenza A virus RT-qPCR positive results for pools of five and ten swabs at each virus concentration were analysed using Welch Two Sample t-test. No statistical differences were found ($p < 0.05$).

5.3 Effect of swab pooling and transport medium on virus isolation

In previous outbreaks of AI in ostriches in South Africa, the isolation of virus from pooled RT-qPCR samples in 50% v/v glycerol PBS transport medium has had limited success (Abolnik et al., 2012). It has been suggested that the virus transport medium lacks stabilization proteins and antibiotics and fungicides that could reduce bacterial and fungal growth, and thus the degradative action of proteolytic enzymes (Munster et al., 2009, Spackman et al., 2013). Spackman et al. (2013) evaluated PBS (pH 7.2) and BHI broth (pH 7.3) medium used to transport swabs for influenza A virus detection by virus isolation and RT-qPCR. A buffered medium containing protein such as BHI was found to be superior for the isolation of Influenza A virus. No significant difference was found in the detection of influenza A virus RNA by RT-qPCR; however Influenza A virus was isolated successfully from faecal swabs in 50% v/v glycerol PBS medium from chickens, ducks and pigeons (Zhang et al., 2015).

In the present study, two accredited laboratories attempted virus isolation from five and ten swab pools at a high ($10^{5.9}$ EID_{50}/ml), medium ($10^{4.0}$ EID_{50}/ml) and low virus load ($10^{2.8}$ and $10^{2.7}$ EID_{50}/ml) near to the cut-off for a positive RT-qPCR (Ct value ~ 33) to evaluate firstly whether the type of transport medium affected the success rate of virus isolation and secondly to evaluate swab pooling and virus load effect on virus isolation efficiency. The results, presented in Table 4, show that influenza A virus was isolated after the 1st passage from both the 5 and 10 swab pools at the high and medium virus load by Laboratory A. This corresponded to RT-qPCR Ct values of ~ 23 and ~ 28, respectively. Laboratory B isolated virus after the 1st passage from both the 5 and 10 swab pools at the high concentration only, and only after the 3rd passage from the sample with the medium virus load ($10^{4.0}$ EID_{50}/ml) where ten swabs were present in the pool, but not where five swabs were present in the pool. No virus was isolated at the low virus load concentration ($10^{2.7\text{-}2.8}$ EID_{50}/ml), where the RT-qPCR Ct values corresponded to ~ 32 to 33. Both laboratories reported bacterial and or fungal contamination in the samples, necessitating the addition of antibiotics, fungicides and filtration of the fluid (using 0.2µm filters) before inoculation in subsequent passages. Additional sample processing steps such as filtration would remove contaminating bacteria, yeast and fungal cells, but may also result in reducing the amount of viable virus particles as these may be bound closely to contaminates in the inoculum, which may be significant if a low initial viral load was present in the swab fluid reducing the sensitivity of the test method.

A separate experiment was conducted to evaluate whether there was a difference in the efficiency of virus isolation between swabs stored in standard 50% v/v glycerol PBS compared to VTM at a selected virus concentration of approximately $10^{4.5}$ EID_{50}/ml (RT-qPCR Ct value ~ 27) (Table 5). Virus was isolated from VTM in 2 of the 3 replicate samples after the first passage and only from 1 of the 3 replicates from standard 50% v/v glycerol PBS medium. Virus was isolated in the remaining negative samples in the second passage. The study suggested that virus transport media containing antibiotics

could reduce the presence and growth of bacteria in swab fluid during transport thereby eliminating the need for filtration. This would minimise the likelihood of reducing the viral load in subsequent passages and the total time required for the test to be completed would be reduced if the virus is detected in the 1st passage.

The present study showed that samples with low virus titres ($10^{2.7}$ EID_{50}/ml), although detected by RT-qPCR, were not isolated from 50% v/v PBS glycerol transport medium. This transport medium is cost efficient and if refrigerated can be utilized by field personnel for up to 12 months for intermittent sampling events. The USDA recommends a number of protein based media, including BHI broth and tris-buffered tryptose broth (TBTB) (USDA, 2016). Commercial universal virus transport media in three ml tubes contain antibiotics that stabilize the virus and reduce the growth of contaminants. At a cost of approximately R27 per 3ml tube, equivalent to $1.90 US dollars[7], imported commercial universal transport medium in tubes is cost prohibitive. In addition, imported commercial universal virus transport media in tubes are designed to accommodate a single swab in the tube, therefore cannot be used for the routine collection of pooled tracheal swabs for surveillance of ostriches in South Africa. Preparing VTM or 50% glycerol PBS, supplemented with anitbiotics using individual components could reduce the costs compared to purchasing imported commercial universal transport medium (Table 7) but would require stricter quality control protocols to ensure that the media is handled and stored appropriately by field personnel to prevent contamination of unused media or the use of media that has expired. Fifty percent v/v glycerol PBS transport medium with added antifungals and antibiotics has also been recommended as one of the transport mediums that can be used for the collection and storage of swab samples for AI testing by the World Health Organisation (WHO, 2006). The results of our study were comparable to another study, where virus was successfully isolated from faecal samples prepared in 10% v/v glycerol PBS transport medium spiked with LPAI H6N2 (Zhang et al., 2015), up to a virus titre of $10^{4.6}$ EID_{50}/ml, even after repeated freeze thaw after storage at -20°C for 7 days, but was not isolated from samples stored in BHI. Virus was isolated from samples stored at 4°C for 7 days in glycerol PBS and BHI transport media under the same test conditions, suggesting that the addition of glycerol provides protection of the virus against the formation of ice crystals in samples stored at -20°C.

Transporting dry swabs should be avoided as heat and desiccation can inactivate the virus in less than 24 hours (USDA, 2016). This study showed that increasing the swab pool from five to ten swab tips, in three ml of transport media in five ml transport tubes with a diameter of at least 20 mm was prepared with ease and also ensured that all swabs are wet and immersed in transport fluid (Figure 5). A three ml sample volume was sufficient for the detection of influenza A virus using RT-qPCR and subsequent virus isolation tests.

[7] https://www.poundsterlinglive.com/bank-of-england-spot/historical-spot-exchange-rates/usd/USD-to-ZAR-2019

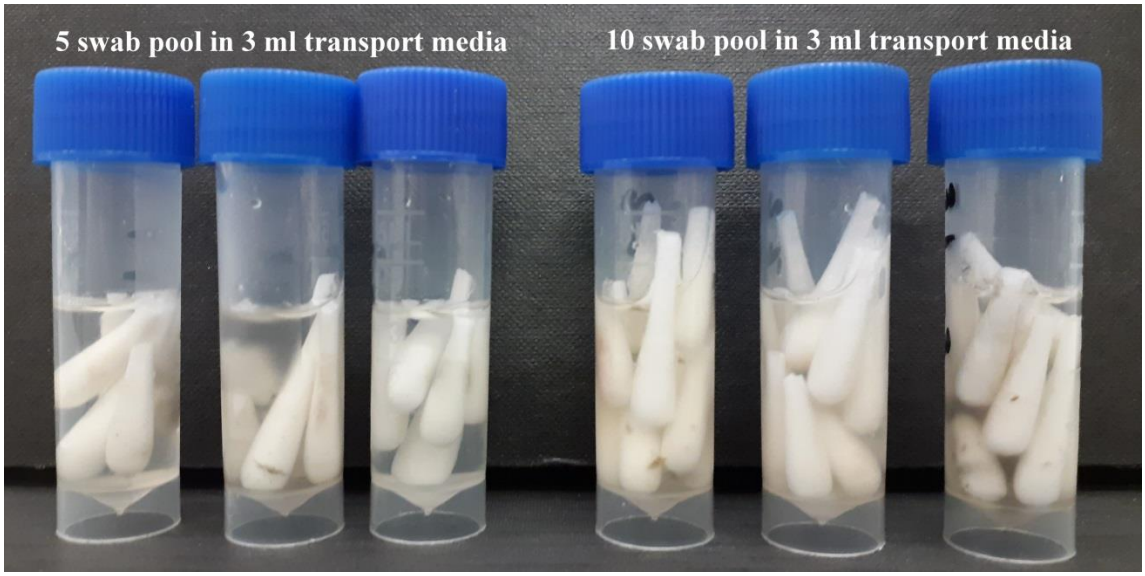

Figure 5. Five and ten swabs pooled in three milliliters 50 % v/v PBS glycerol transport media.

Table 4. Detection of influenza A virus by virus isolation in RT-qPCR positive pools.

Virus concentration (EID_{50}/ml)[1]	~ RT-qPCR Ct value	Laboratory A						Laboratory B					
		5 swab pool			10 swab pool			5 swab pool			10 swab pool		
		VI Result	Passage	Contamination	VI Result	Passage	Contamination	VI Result	Passage	Contamination	VI Result	Passage	Contamination
5.9 (High)	22	Positive	1	No	Positive	1	No	Positive	1	No	Positive	1	No
4.0 (Medium)	29	Positive	1	No	Positive	1	No	Negative	3	Yes[2]	Positive	3	Yes[2]
2.8 (Low)	32	Negative	2	Yes[2]	Negative	2	Yes[2]	Negative	3	Yes[2]	Negative	3	Yes[2]
2.7 (Low)	33	Negative	2	Yes[2]	Negative	2	Yes[2]	Negative	3	Yes[2]	Negative	3	Yes[2]

[1]Virus concentration was calculated using the standard linear regression formula y = -3.2329x+41.843 (Figure 3)

[2] Fungal and or bacterial contamination was present in samples

VI: Virus isolation

Table 5. Detection of influenza A virus by virus isolation in RT-qPCR positive samples from VTM and 50% v/v glycerol PBS transport medium

Transport medium (replicate)		RT-qPCR Ct value	Virus concentration (EID_{50}/ml)[1]	Passage 1				Passage 2 - reinoculation after sample filtration				Final VI Result
				Embryo morphology	HAT Result	Contamination	VI Result	Embryo morphology	HAT Result	Contamination	VI Result	
50% v/v glycerol PBS	(1)	27.09	4.56	Red	Positive	No	Positive	-	-	-	-	Positive
	(2)	26.13	4.86	Red	Negative	Yes[2]	Negative	Red	Positive	No	Positive	Positive
	(3)	27.57	4.41	Red	Negative	Yes[2]	Negative	Red	Positive	No	Positive	Positive
VTM	(1)	27.89	4.32	Red	Positive	No	Positive	-	-	-	-	Positive
	(2)	27.87	4.32	Red	Positive	No	Positive	-	-	-	-	Positive
	(3)	27.50	4.44	Red	Negative	Yes[2]	Negative	Red	Positive	No	Positive	Positive

[1] Virus concentration was calculated using the standard linear regression formula y = -3.2329x+41.843 (Figure 3)

[2] Fungal and or bacterial contamination was present in sample

VI: Virus isolation

HAT: Haemaglutinnation test

Currently, the standard transport media is prepared in bottles containing 1000ml to 2500ml of media for distribution to state veterinary officials. To facilitate the ease of use of virus transport media in the field, it is recommended that media should be prepared in smaller volumes of not more than 100 ml so that media can be frozen and thawed when required. This will ensure that only the required amount is defrosted to prepare aliquots three ml of VTM into sample tubes when sampling is scheduled on a farm, thereby reducing the possibility of contaminating the media and extending the shelf life.

5.4 Cost benefit analysis

AI surveillance testing may only be performed by accredited diagnostic laboratories in South Africa, and at the time of writing, this included two laboratories located in Gauteng province (ARC-OVR and N (Pty) Ltd), one laboratory located in Oudtshoorn (Deltamune (Pty) Ltd) and a provincial government laboratory located in Stellenbosch (Western Cape Provincial Veterinary Laboratory (WCPVL)); the latter two are located in close proximity to the ostrich farming areas in South Africa. In 2018, 4 857 influenza A virus RT-qPCR screening tests were performed on tracheal swabs pools for movement and outbreak response purposes in the Western Cape province (Dr L. Roberts [Animal Health, Department of Agriculture, Western Cape Province] pers. comm., 10 May 2019). Given that five tracheal swabs are pooled per sample, the number of ostriches sampled in this period is estimated to be approximately 24 285. Currently, the cost of performing these tests varies between R316 and R438 per test or sample pool, depending on the diagnostic laboratory performing the test (Table 6).

Based on the testing performed in 2018, test fees could be as high as R 2 127 366 per annum. These costs are carried by famers and industry such as the SAOBC as well as national and provincial government. Increasing the number of swabs pooled from five to ten per sample would decrease the cost of testing for AI surveillance in ostriches significantly (Table 7). In this study, it was suggested that the addition of antibiotics to the transport medium improves the efficiency of virus isolation from trachea swabs pools. The costs of preparing VTM and 50% v/v glycerol PBS, both with added antibiotics are comparable (0.13% percentage difference) (Table 7), thus it is recommended that VTM be used preferentially for sampling protocols given that it widely accepted and recommended and would improve the isolation of viruses form tracheal swab pools (Table 6). This increase in sampling costs, could be offset by increasing the number swabs pooled per sample from five to ten swabs.

Table 6. Test fees for routine and outbreak surveillance for AI RT-qPCR testing, where five and ten tracheal swab pools are used, based on the number of tests performed in 2018 (n = 24 285 ostriches).

	Five tracheal swab pool			Ten tracheal swab pool		
Laboratory	**WCPVL[1]**	**ARC-OVI[2]**	**Deltamune[3]**	**WCPVL[1]**	**ARC-OVI[2]**	**Deltamune[3]**
Total fee (n = 24 285)	R 1 534 812	R 1 699 950	R 2 127 366	R 767 406	R 849 975	R 1 063 683

[1] WCPVL fee per test: R316, WCPVL, provincial government laboratory fees are VAT exempt.

[2] ARC-OVR fee per test: R350, VAT included.

[3] Deltamune (Pty) Ltd fee per test: R438, VAT included.

Table 7. Comparison of sampling costs for routine and outbreak surveillance for influenza A virus RT-qPCR testing, where five and ten swab pools, and transport media with and without antibiotics for tracheal swabs are required, based on the number of tests performed in 2018 (n = 24 285 ostriches).

Transport media (pH 7.2)	**Transport media in 5ml tube[1]**	**13.64% estimated overhead costs**	**Transport media in 5ml tube[1], plus overhead costs**	**Five pooled swabs (4857 pools)[2]**	**Ten pooled swabs (2429 pools)[2]**
50% v/v glycerol PBS transport medium without antibiotics	R 2.75	R 0.37	R 3.12	R 29 745	R 22 160
50% v/v glycerol PBS transport medium with antibiotics	R 7.57	R 1.03	R 8.61	R 56 372	R 35 476
Virus transport medium (VTM) with antibiotics	R 7.58	R 1.03	R 8.62	R 56 421	R 35 500

[1] 5ml tube containing 3ml transport media

[2] The cost per rayon tipped swab was R3.00 (Copan Italia SPA, Brescia, Italy)

6. Conclusion

Sampling protocols used in surveillance programs for farmed ostriches should ensure the detection of influenza A virus at low virus titres by combining optimum swab pooling and transport media. The current study showed that increasing the number of swabs from five to ten per pool did not significantly reduce the detection of influenza A virus using RT-qPCR and could be applied to the sampling strategy for ostrich farms, ensuring the same coverage while significantly reducing the number of PCR tests by 50%. In addition, 5 ml sampling tubes with an internal diameter of at least 20 mm are well suited to pool ten swab tips in 3ml volume of transport media, ensuring sufficient immersion of the swab in the transport media while in transit.

Another important aspect of AI surveillance is to ensure that samples that test positive for influenza A virus can be characterised rapidly for immediate notification of pathogenicity by PCR and sequencing of PCR amplicons. Full characterisation of viral protein sequences is only possible if the virus is successfully isolated from samples. Several virus transport media have been evaluated and recommended for use, some favouring either a protein based virus transport media such as BHI, whilst others have shown that a glycerol PBS transport media can be used to isolate influenza A virus successfully (Spackman et al., 2013, USDA, 2016, Zhang et al., 2015). In the present study, while both transport mediums facilitated the isolation of influenza A virus, the protein based growth medium (VTM), containing antibiotics appeared to show more efficient and rapid isolation, as growth of contaminants could be reduced in the inoculum and the virus was isolated after one passage using embryonated chicken eggs. This suggests that VTM should contain antibiotics to limit contaminants in order to improve the efficiency of standard methods used to isolate virus.

Molecular based detection methods such as RT-qPCR have a high sensitivity, detecting up to $10^{2.5}$ EID_{50}/ ml (RT-qPCR Ct value ~ 35) virus titre compared to virus isolation, detecting 10^{4} to $10^{4.5}$ EID_{50}/ ml (RT-qPCR Ct-value 27- 29) virus titre, confirming that low virus titre swab samples detected by RT-qPCR and reported as positive (RT-qPCR Ct value > 29) may not be detected by standard virus isolation techniques regardless of the virus transport media used due to the intrinsic sensitivity limits of the assay and possible species barrier between ostrich-adapted viruses and the embryonated chicken egg propagation system.

It is therefore recommended that sampling protocols be adapted to ensure that the optimum virus transport media (VTM, with added antibiotics) is used to improve the stability of the virus in trachea swabs pools for the detection of influenza A virus by RT-qPCR and virus isolation. Increased sampling costs may be offset by increasing the number of swabs pooled in a single sample tube from five to ten,

as this did not significantly affect the detection or isolation of virus in trachea swabs pools where a single positive swab was present at a virus concentration of 10^4 to $10^{4.5}$ EID_{50}/ml. Resources and capacity should be focused at isolating virus from five or ten pooled swab samples with a virus titre of $\geq 10^4$ EID_{50}/ ml (RT-qPCR Ct value ≤ 29), followed by full molecular sequencing and characterisation of the viral isolate. It is difficult to obtain sufficient quantities of viable RNA that can be amplified from samples with a low virus titre $< 10^4$ EID_{50}/ ml and higher RT-qPCR Ct values (> 29). However, the most viable option is to characterise the virus in these samples by direct sequencing of PCR amplicons or type-specific RT-qPCR assays.

7. References

ABOLNIK, C. 2007. Molecular characterization of H5N2 avian influenza viruses isolated from South African ostriches in 2006. *Avian Diseases,* 51**,** 873-879.

ABOLNIK, C., BISSCHOP, S., GERDES, T., OLIVIER, A. & HORNER, R. 2007. Outbreaks of avian influenza H6N2 viruses in chickens arose by a reassortment of H6N8 and H9N2 ostrich viruses. *Virus Genes,* 34**,** 37-45.

ABOLNIK, C., LONDT, B. Z., MANVELL, R. J., SHELL, W., BANKS, J., GERDES, G. H., AKOL, G. & BROWN, I. H. 2009. Characterisation of a highly pathogenic influenza A virus of subtype H5N2 isolated from ostriches in South Africa in 2004. *Influenza and other Respiratory Viruses,* 3**,** 63-68.

ABOLNIK, C., OLIVIER, A., REYNOLDS, C., HENRY, D., CUMMING, G., RAUFF, D., ROMITO, M., PETTY, D. & FALCH, C. 2016. Susceptibility and status of avian influenza in ostriches. *Avian Diseases,* 60**,** 286-295.

ABOLNIK, C., OLIVIER, A. J., GREWAR, J., GERS, S. & ROMITO, M. 2012. Molecular analysis of the 2011 HPAI H5N2 outbreak in ostriches, South Africa. *Avian Diseases,* 56**,** 865-879.

ABOLNIK, C., PIETERSE, R., PEYROT, B. M., CHOMA, P., PHIRI, T. P., EBERSOHN, K., HEERDEN, C. J. V., VORSTER, A. A., ZEL, G. V. D., GEERTSMA, P. J., LALEYE, A. T., GOVINDASAMY, K. & RAUFF, D. L. 2018. The Incursion and Spread of Highly Pathogenic Avian Influenza H5N8 Clade 2.3.4.4 Within South Africa. *Avian Diseases***,** 63**,** 149-156.

ALLWRIGHT, D. M., BURGER, W. P., GEYER, A. & TERBLANCHE, A. W. 1993. Isolation of an influenza A virus from ostriches (Struthio camelus). *Avian Pathology : Journal of the World Veterinary Poultry Association,* 22**,** 59-65.

ARNOLD, M. E., SLOMKA, M. J., COWARD, V. J., MAHMOOD, S., RALEIGH, P. J. & BROWN, I. H. 2013. Evaluation of the pooling of swabs for real-time PCR detection of low titre shedding of low pathogenicity avian influenza in turkeys. *Epidemiology and Infection,* 141**,** 1286-1297.

BARROSO, J. M. 2011a. COMMISSION IMPLEMENTING REGULATION (EU) No 536/2011 of 1 June 2011. *In: Official Journal of the European Union.* Brussels, Belgium.

BARROSO, J. M. 2011b. COMMISSION IMPLEMENTING REGULATION (EU) No 991/2011 of 5 October 2011. *In: Official Journal of the European Union.* Brussels, Belgium.

BARROSO, J. M. 2014. COMMISSION IMPLEMENTING REGULATION (EU) No 166/2014 of 17 February 2014. *In: Official Journal of the European Union.* Brussels, Belgium.

DEPARTMENT OF AGRICULTURE, FORESTRY AND FISHERIES (DAFF). 2006. *Ostrich Protocol - Edition 1. 11 September 2006* [Online]. Available: https://www.nda.agric.za/vetweb/Disease%20Control/Protocols/Control%20Protocol%20-

%20Ostriches%20-%20Ed.1%20-%20Sep%2006%20-%20SIGNED.pdf. [Accessed: 4 April 2018].

DEPARTMENT OF AGRICULTURE, FORESTRY AND FISHERIES (DAFF). 2012a. *A profile of the South African ostrich market value chain* [Online]. Available: http://www.nda.agric.za/docs/AMCP/Ostri2012.pdf [Accessed 4 April 2018].

DEPARTMENT OF AGRICULTURE, FORESTRY AND FISHERIES (DAFF). 2012b. *VPN/04/2012-01 (Revision 6.0). Standard for the requirement, requirements, maintenance of registration and official control of ostrich compartments in South Africa* [Onine]. Available: https://www.nda.agric.za/vetweb/VPN%20&%20SOP/004-VPN%2004%20Standards%20for%20the%20registration%20of%20ostrich%20farm%20for%20export.pdf [Accessed 4 April 2018].

DONIS, R. O., SMITH, G. J. D., PERDUE, M. L., BROWN, I. H., CHEN, H., KAWAOKA, Y., MACKENZIE, J. & SHU, Y. 2008. Toward a unified nomenclature system for highly pathogenic avian influenza virus (H5N1). *Emerging Infectious Diseases,* 14**,** e1.

FEREIDOUNI, S. R., HARDER, T. C., GAIDET, N., ZILLER, M., HOFFMANN, B., HAMMOUMI, S., GLOBIG, A. & STARICK, E. 2012. Saving resources: avian influenza surveillance using pooled swab samples and reduced reaction volumes in real-time RT-PCR. *Journal of Virological Methods,* 186**,** 119-125.

FOUCHIER, R. A. M., BESTEBROER, T. M., HERFST, S., VAN DER KEMP, L., RIMMELZWAAN, G. F. & OSTERHAUS, A. 2000. Detection of influenza A viruses from different species by PCR amplification of conserved sequences in the matrix gene. *Journal of Clinical Microbiology,* 38**,** 4096-4101.

JUNCKER, J. 2015. COMMISSION IMPLEMENTING REGULATION (EU) 2015/1349 of 3 August 2015. *In: Official Journal of the European Union.* Brussels, Belgium.

KANDEIL, A., KAYED, A., MOATASIM, Y., WEBBY, R. J., MCKENZIE, P. P., KAYALI, G. & ALI, M. A. 2017. Genetic characterization of highly pathogenic avian influenza A H5N8 viruses isolated from wild birds in Egypt. *Journal of General Virology,* 98**,** 1573-1586.

LADMAN, B. S., SPACKMAN, E. & GELB, J., JR. 2012. Comparison of pooling 11 or 5 oropharyngeal swabbings for detecting avian influenza virus by real-time reverse transcription-PCR in broiler chickens. *Avian Diseases,* 56**,** 227-229.

LATORRE-MARGALEF, N., AVRIL, A., TOLF, C., OLSEN, B. & WALDENSTROM, J. 2016. How Does Sampling Methodology Influence Molecular Detection and Isolation Success in Influenza A Virus Field Studies? *Applied and Environmental Microbiology,* 82**,** 1147-1153.

LEE, D., BERTRAN, K., KWON, J. & SWAYNE, D. E. 2017. Evolution, global spread, and pathogenicity of highly pathogenic avian influenza H5Nx clade 2.3.4.4. *Journal of Veterinary Science,* 18**,** 269-280.

LOTH, L., PRIJONO, W. B., WIBAWA, H. & USMAN, T. B. 2008. Evaluation of two avian influenza type A rapid antigen tests under Indonesian field conditions. *Journal of Veterinary Diagnostic Investigation,* 20**,** 642-644.

LYCETT, S. J., BODEWES, R., POHLMANN, A., BANKS, J., BANYAI, K., BONI, M. F., BOUWSTRA, R., BREED, A. C., BROWN, I. H., CHEN, H. L., DAN, A., DELIBERTO, T. J., DIEP, N., GILBERT, M., HILL, S., IP, H. S., KE, C. W., KIDA, H., KILLIAN, M. L., KOOPMANS, M. P., KWON, J. H., LEE, D. H., LEE, Y. J., LU, L., MONNE, I., PASICK, J., PYBUS, O. G., RAMBAUT, A., ROBINSON, T. P., SAKODA, Y., ZOHARI, S., SONG, C. S., SWAYNE, D. E., TORCHETTI, M. K., TSAI, H. J., FOUCHIER, R. A. M., BEER, M., WOOLHOUSE, M., KUIKEN, T. & GLOBAL CONSORTIUM, H. N. R. 2016. Role for migratory wild birds in the global spread of avian influenza H5N8. *Science,* 354**,** 213-217.

MANVELL, R. J., HORNER, R., AKOL, G., ABOLNIK, C., ROMITO, M. & BROWN, I. H. 2005. Isolation of an influenza A virus subtype H5N2 from ostriches in South Africa in 2004, *In:* Carbajo, E. (ed), *Proceedings of the 3rd International Ratite Science Symposium of the World's Poultry Science Association (WPSA) & XII World Ostrich Congress*, Madrid, 14-16 October 2005, pp. 197-199.

MANVELL, R. J., JORGENSEN, P. H., NIELSEN, O. L. & ALEXANDER, D. J. 1998. Experimental assessment of the pathogenicity of two avian influenza A H5 viruses in ostrich chicks (Struthio camelus) and chickens. *Avian Pathology,* 27**,** 400-404.

MUNSTER, V. J., BAAS, C., LEXMOND, P., BESTEBROER, T. M., GULDEMEESTER, J., BEYER, W. E., DE WIT, E., SCHUTTEN, M., RIMMELZWAAN, G. F., OSTERHAUS, A. D. & FOUCHIER, R. A. 2009. Practical considerations for high-throughput influenza A virus surveillance studies of wild birds by use of molecular diagnostic tests. *J Clin Microbiol,* 47**,** 666-673.

NEUMANN, G., HATTA, M. & KAWAOKA, Y. 2003. Reverse genetics for the control of avian influenza. *Avian Diseases,* 47**,** 882-887.

OFFLU. 2013. OFFLU Strategy document for surveillance and monitoring of influenzas in animals, OIE-FAO [Online]. Available https://www.oie.int/doc/ged/D12209.PDF [Accessed 14 April 2018].

OFFLU. 2018. Influenza A Cleavage sites [Online]. Available: http://www.offlu.net/fileadmin/home/en/resource-centre/pdf/Influenza_A_Cleavage_Sites.pdf [Accessed 14 April 2018].

OIE. 2015. Avian influenza (infection avian influenza viruses). Chapter 3.3.4. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2019 [Online]. Available: http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/3.03.04_AI.pdf [Accessed 14 April 2018].

OIE. 2018a. Avian Influenza Portal. Follow-up report No.23. Report reference: HPAI_H5N8_2017_wild, Reference OIE : 26727, Report Date : 31/05/2018, Country : South Africa [Online]. Available: http://www.oie.int/wahis_2/temp/reports/en_fup_0000026727_20180531_180035.pdf [Accessed 24 June 2018].

OIE. 2018b. Immediate notification. Highly pathogenic influenza A viruses (terrestrial poultry), Nigeria [Online]. Available: http://www.oie.int/wahis_2/public/wahid.php/Reviewreport/Review?reportid=23728 [Accessed 1 July 2018].

OIE. 2018c. Immediate notification. Highly pathogenic influenza A viruses (terrestrial poultry), Zimbabwe [Online]. Available: http://www.oie.int/wahis_2/public/wahid.php/Reviewreport/Review?reportid=23935 [Accessed 1 July 2018].

OIE. 2018d. OIE Situation Report for Highly Pathogenic Avian Influenza updated 2018/05/31 [Online]. Available: http://www.oie.int/fileadmin/Home/eng/Animal_Health_in_the_World/docs/pdf/OIE_AI_situation_report/OIE_SituationReport_AI_31052018.pdf [Accessed 24 June 2018].

OIE. 2019a. *Avian Influenza Portal. Follow-up report No.41. Report reference: AI_H5N8_2017, Reference OIE : 29842, Report Date : 14/03/2019, Country : South Africa* [Online]. Available: http://www.oie.int/wahis_2/temp/reports/en_fup_0000029842_20190314_135548.pdf [Accessed 21 March 2019].

OIE. 2019b. *Avian Influenza Portal. Prevention and Control.* [Online]. Available: http://www.oie.int/en/animal-health-in-the-world/web-portal-on-avian-influenza/early-detection-warning-diagnostic-confirmation/ [Accessed 21 March 21 March 2019].

OIE. 2019c. *OIE Terrestrial Animal Health Code (2018). Volume 1. General provisions. Glossary* [Online]. Available: http://www.oie.int/index.php?id=169&L=0&htmfile=glossaire.htm [Accessed 21 March 2019].

OLSEN, B., MUNSTER, V. J., WALLENSTEN, A., WALDENSTRÖM, J., OSTERHAUS, A. D. M. E. & FOUCHIER, R. A. M. 2006. Global Patterns of Influenza A Virus in Wild Birds. *Science,* 312**,** 384-388.

PERDUE, M. L. 2008. Molecular Determinants of Pathogenicity for Avian Influenza Viruses. *In:* SWAYNE, D. E. (ed.). *Avian Influenza.* 1st ed. Ames, Iowa, USA: Blackwell Publishing. pp. 23-41.

PERDUE, M. L. & SUAREZ, D. L. 2000. Structural features of the avian influenza virus hemagglutinin that influence virulence. *Veterinary Microbiology,* 74**,** 77-86.

RAMEY, A. M., DELIBERTO, T. J., BERHANE, Y., SWAYNE, D. E. & STALLKNECHT, D. E. 2018. Lessons learned from research and surveillance directed at highly pathogenic influenza A viruses in wild birds inhabiting North America. *Virology,* 518**,** 55-63.

RAUFF, D., STRYDOM, C. & ABOLNIK, C. 2016. Evolutionary consequences of a decade of vaccination against subtype H6N2 influenza. *Virology,* 498**,** 226-239.

SAAD, M. D., AHMED, L. S., GAMAL-ELDEIN, M. A., FOUDA, M. K., KHALIL, F. M., YINGST, S. L., PARKER, M. A. & MONTEVILLEL, M. R. 2007. Possible avian influenza (H5N1) from migratory bird, Egypt. *Emerging Infectious Diseases,* 13**,** 1120-1121.

SIMS, L., HARDER, T., I., B., GAIDET, N., BELOT, G., VON DOBSHIETZ, S., KAMATA, A., KIVARA, F., PALAMARA, E., BRUNI, M., DAUPHIN, G., RAIZMAN, E. A. & LUBROTH, J. 2017. Highly pathogenic H5 avian influenza in 2016 and 2017- observations and future perspectives. *FAO-EMPRES FOCUS ON No. 11*.

SINCLAIR, M., BRUCKNER, G. K. & KOTZE, J. J. 2006. Avian influenza in ostriches: epidemiological investigation in the Western Cape Province of South Africa. *Veterinaria Italiana,* 42**,** 69-76.

SPACKMAN, E., CATTOLI, G. & SUAREZ, D. L. 2017. Avian Diagnostics and surveillance methods. *In:* SWAYNE, D. E. (ed.) *Animal Influenza.* 2nd ed. Ames, Iowa, USA: Blackwell Publishing. pp. 31-33.

SPACKMAN, E. & KILLIAN, M. L. 2014. Avian influenza virus isolation, propagation, and titration in embryonated chicken eggs. *Methods in Molecular Biology*, 1161, 125-40.

SPACKMAN, E., PEDERSEN, J. C., MCKINLEY, E. T. & GELB, J., JR. 2013. Optimal specimen collection and transport methods for the detection of avian influenza virus and Newcastle disease virus. *BMC Veterinary Research,* 9**,** 35.

SPACKMAN, E., SENNE, D. A., MYERS, T. J., BULAGA, L. L., GARBER, L. P., PERDUE, M. L., LOHMAN, K., DAUM, L. T. & SUAREZ, D. L. 2002. Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. *Journal of Clinical Microbiology,* 40**,** 3256-3260.

SSEMATIMBA, A., MALLADI, S., BONNEY, P. J., FLORES-FIGUEROA, C., MUNOZ-AGUAYO, J., HALVORSON, D. A. & CARDONA, C. J. 2018. Quantifying the effect of swab pool size on the detection of influenza A viruses in broiler chickens and its implications for surveillance. *BMC Vet Res,* 14**,** 265.

THRUSFIELD, M. V. 2007. *Veterinary epidemiology,* Oxford; Ames, Iowa, Blackwell Science. pp. 322-324.

USDA. 2016. *FY2016 HPAI Response Avian Sample Collection for Influenza A and Newcastle Disease* [Online]. Available: https://www.aphis.usda.gov/animal_health/lab_info_services/downloads/WIAV0020.pdf [Accessed 18 January 2019].

VAN HELDEN, L. S. 2016. Epidemiology Report 2016. Western Cape Government Veterinary Services Animal Health [Online]. Available: http://www.elsenburg.com/vetepi/epireport_pdf/2016Bundle.pdf [Accessed: 15 April 2018].

VAN HELDEN, L. S. & ROBERTS, L. 2017. Epidemiology Report 2017. Western Cape Government Veterinary Services Animal Health [Online]. Available: http://www.elsenburg.com/vetepi/epireport_pdf/2017Bundle.pdf [Accessed 15 April 2018].

VAN HELDEN, L. S., SINCLAIR, M., KOEN, P. & GREWAR, J. D. 2016. Description of an outbreak of highly pathogenic avian influenza in domestic ostriches (Struthio camelus) in South Africa in 2011. *Preventive Veterinary Medicine,* 128**,** 6-11.

WHO. 2006. Collecting, preserving and shipping specimens for the diagnosis of avian influenza A(H5N1) virus infection. Guide for field operations. WHO/CDS/EPR/ARO/2006.1 [Online]. Available: https://apps.who.int/iris/handle/10665/69392 [Accessed 18 January 2019].

WOOLCOCK, P. R. & CARDONA, C. J. 2005. Commercial immunoassay kits for the detection of influenza virus type A: evaluation of their use with poultry. *Avian Diseases,* 49**,** 477-481.

ZHANG, X. C., LIU, S., HOU, G. Y., ZHUANG, Q. Y., WANG, K. C., JIANG, W. M., WANG, S. C., LI, J. P., YU, J. M., DU, X., HUANG, B. X. & CHEN, J. M. 2015. Comparison of three media for transport and storage of the samples collected for detection of avian influenza virus. *Journal of Virological Methods,* 222**,** 202-205.

ZHOU, B., DONNELLY, M. E., SCHOLES, D. T., ST GEORGE, K., HATTA, M., KAWAOKA, Y. & WENTWORTH, D. E. 2009. Single-reaction genomic amplification accelerates sequencing and vaccine production for classical and Swine origin human influenza a viruses. *Journal of Virology*, 83, 10309-13.

8. Ethics approvals

The following ethics approvals were obtained for the project:

APPENDIX A

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**Evaluation of swab pooling and transport medium to improve the identification of avian influenza viruses in farmed ostriches in South Africa**
PROJECT NUMBER	**V015-18**
RESEARCHER/PRINCIPAL INVESTIGATOR	**R Pieterse**

STUDENT NUMBER (where applicable)	**U_18379941**
DISSERTATION/THESIS SUBMITTED FOR	**MSc**

ANIMAL SPECIES	**Ostrich *(Struthio camlus)***
NUMBER OF SAMPLES	**200-300 (6 swabs/bird)**
Approval period to use animals for research/testing purposes	**March 2018 – March 2019**
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 26 March 2018
CHAIRMAN: UP Animal Ethics Committee	Signature

S4285-15

APPENDIX B

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science
Animal Ethics Committee

5 June 2019

Approval Certificate
Annual Renewal - Extension 1

AEC Reference No.:	V015-18
Title:	Evaluation of swab pooling and transport medium to improve the identification of avian influenza viruses in farmed ostriches in South Africa
Researcher:	Miss R Pieterse
Student's Supervisor:	Prof C Abolnik

Dear Miss R Pieterse,

The **Annual Renewal** as supported by documents received between 2019-05-15 and 2019-05-27 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2019-05-27.

Please note the following about your ethics approval:

1. The use of species is approved:

Species and Samples
Additional 3 months approved to complete testing and analise data (September 2019)

2. Ethics Approval is valid for 3 months as requested
3. Please remember to use your protocol number (V015-18) on any documents or correspondence with the AEC regarding your research.
4. Please note that the AEC may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.
Yours sincerely

Prof V Naidoo
CHAIRMAN: UP-Animal Ethics Committee

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Fakulteit Veeartsenykunde
Lefapha la Diseanse tša Bongakadiruiwa

APPENDIX C

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Research Ethics Committee

PROJECT TITLE	**Evaluation of swab pooling and transport medium to improve the identification of avian influenza viruses in farmed ostriches in South Africa**
PROJECT NUMBER	**REC025-18**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Reneé Pieterse**

STUDENT NUMBER (where applicable)	
DISSERTATION/THESIS SUBMITTED FOR	**MSc**

SUPERVISOR	**Prof Celia Abolnik**

APPROVED	Date **5 June 2018**
CHAIRMAN: UP Research Ethics Committee	Signature A. M. Duncan

APPENDIX D

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

Enquiries: Mr Henry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HenryG@daff.gov.za
Reference: 12/11/1/7/9

Ms Reneé Pieterse
Western Cape Provincial Veterinary Laboratory
Helshoogte Road
Stellenbosch

Email: reneep@elsenburg.com

RE: PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO. 35 OF 1984)

Dear Ms Pieterse

Your application, dated 27 March 2018, requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers.

I am pleased to inform you that permission is hereby granted to perform the following study, with the following conditions :

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. All potentially infectious material utilised or collected during the study is to be destroyed at the completion of the study. Records must be kept for five years for audit purposes. A dispensation application may be made to the Director Animal Health in the event that any of the above is to be stored or distributed;
3. The study may not commence until a valid ethics approval has been obtained, in writing, from the relevant ethics approval body;
4. Exemption is hereby granted from compliance with the "PROCEDURE MANUAL: IMPORTATION OF A NEW TEST KIT OR REAGENT", approval date March 2018;

5. A South African veterinary import permit must be obtained prior to the importation of the ANIGEN Rapid AIV Ag Test Kits;
6. The test kits must be under the personal control and supervision of the principle researcher at all times;
7. The kits may only be used for the purposes of the study with any excess kits destoyed upon completeion of the study;
8. The researcher or research collaborators are not permitted to distribute any results obtained from any test which has not been appropriately validated for diagnostic purposes. All results must be submitted to the relevant state veterinarian.

Title of research/study: Evaluation of swab pooling and transport medium to improve the identification of avian influenza viruses in farmed ostriches in South Africa
Researcher: Ms Reneé Pieterse
Your Ref./ Project Number:
Institution: Western Cape Provincial Veterinary Laboratory, Stellenbosch
Our ref Number: 12/1/1/99
Expiry date: 31 December 2018

Kind regards,

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH

Date: 2018 -06- 25

SUBJECT: *PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO. 35 OF 1984)*

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

Enquiries: Mr Henry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HenryG@daff.gov.za
Reference: 12/11/1/7/9

Ms Reneé Pieterse
Western Cape Provincial Veterinary Laboratory
Helshoogte Road
Stellenbosch

Email: reneep@elsenburg.com

RE: DISPENSATION ON SECTION 20 APPROVAL IN TERMS OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984) FOR: "EVALUATION OF SWAB POOLING AND TRANSPORT MEDIUM TO IMPROVE THE IDENTIFICATION OF AVIAN INFLUENZA VIRUSES IN FARMED OSTRICHES IN SOUTH AFRICA"

A dispensation is hereby granted on Point 2 of the Section 20 approval that was issued for the above mentioned study (attached):

i) Isolated virus may be stored in the University of Pretoria's viral repository within the BSL3 facility;

ii) Isolated virus may not be outsourced or used for further research without prior written approval from DAFF.

Kind regards,

DR. MPHO MAJA
DIRECTOR: ANIMAL HEALTH
Date: 2018 -06- 25