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ORIGINAL ARTICLE

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Molecular detection of influenza A viruses and H5 subtype among migratory Amur falcons (*Falco amurensis*) and captive birds of prey

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Abstract

Influenza A viruses (IAVs) and Newcastle disease viruses (NDVs) are major human and animal health threats with geographic differences in prevalence, characteristics and host populations. Currently, there is sparse information on IAVs and NDVs in avian species in South Africa. Because raptors feed on live wild birds which are the reservoir hosts of IAVs and NDVs, we considered them a good sentinel for surveillance. Therefore, in addition to other resident birds of prey, migratory Amur falcons (*Falco amurensis*) were screened for IAVs and NDVs. Oropharyngeal and cloacal samples were collected from raptor species at three sampling sites in KwaZulu-Natal Province and samples were screened for IAVs and NDVs using molecular and virus isolation methods. IAV-positive samples were further screened for the presence of H5, H7 and H9 viruses. A total of 14 samples from 11 birds (45.8% of all sampled birds) were IAV positive with C_t lower than 36 in duplicate tests. Five out of 24 birds (20.8%) were positive for IAV RNA in duplicate testing, albeit at low concentrations. Among raptor samples, three out of 24 birds (12.5%) were positive for IAVs with viral RNA detected in both cloacal and oropharyngeal swabs. One IAV-positive sample was also positive for H5 subtype (4.1%); all other samples were H5, H7 and H9 negative. Besides, all samples were NDV negative. Overall, our results support the development of more intensive and expanded influenza and other emerging virus studies in raptor species.

KEYWORDS

Asia, Avian influenza, birds of prey, China, falcons, influenza A virus, migratory, Mongolia, raptors, real-time reverse transcription-polymerase chain reaction, RT-PCR epidemiology, South Africa, surveillance

1 | INTRODUCTION

Influenza A viruses (IAVs) are members of the genus Alphainfluenza virus, species IAVs in the family *Orthomyxoviridae*. Wild aquatic birds serve as the natural reservoirs of IAVs (Clark & Hall, 2006; Krauss & Webster, 2010; Munster et al., 2007). Newcastle disease viruses (NDVs) also known as avian paramyxoviruses type 1, are members of the genus Orthoavulavirus, species Avian orthoavulavirus 1 in the family *Paramyxoviridae*. Avian influenza (AI) and Newcastle disease (ND) are important viral diseases of wild birds and poultry (Jørgensen et al., 2004; Kaleta & Baldauf, 1988; Webster et al., 1992). AI and ND are ongoing risks to domestic, pet, exotic and wild bird populations. In addition, AI is an emerging viral disease of zoonotic potential and presents a continuous threat to human public health. Because of their feeding habits, birds of prey, also known as raptors, are particularly susceptible to IAV and NDV subsequent infections (Temple, 1987). Raptors have been known to prey upon shorebirds (Ward & Laybourne, 1985), which are known to harbour IAVs (Krauss & Webster, 2010). In addition, the unusually long flight paths of some raptors may provide a conduit for the intercontinental movement of viruses. Viral diseases have been reported in raptors (Forbes et al., 1997); however, data are scarce due to laborious surveillance efforts, cost and limited interest. Nevertheless, IAV and NDV infections have been reported in raptors from different countries including the USA (Goyal et al., 2010; Jindal et al., 2010; Redig & Goyal, 2012; Shearn-Bochsler et al., 2019), Germany (Kohls et al., 2011; Schettler et al., 2003), Japan (Shivakoti et al., 2010), Saudi Arabia (Khan et al., 2009), the Republic of Korea (Choi et al., 2013), Belgium (Steensels et al., 2007; Van Borm et al., 2005), the Netherlands (Kleyheeg et al., 2017), the United Arab Emirates (Khan et al., 2009; Manvell et al., 2000; Marjuki et al., 2009; Naguib et al., 2015), Egypt (Aly et al., 2010) and Bulgaria (Marinova-Petkova et al., 2012). In addition to low-pathogenic IAV, raptors including Saker falcon (*Falco cherrug*), common kestrel (*Falco tinunculus*), peregrine falcon (*Falco peregrinus*), buzzards (family *Accipitridae*), sparrow hawk (*Accipiter nisus)*, northern goshawk (*Accipiter gentilis*) and Hodgson's hawk eagle (*Nisaetus nipalensis)* have been reported to be infected with highly pathogenic IAV (Alexander, 2007; Capua & Alexander, 2007; J. G. Choi et al., 2013; Lierz et al., 2007; Shivakoti et al., 2010).

Amur falcons (*Falco amurensis*) are a small migratory raptor species which breed mainly in northern China but also in the east of Mongolia and Siberia (Meyburg & Meyburg, 2010; Meyburg et al., 2017; White et al., 1994) and overwinter in southern Africa during the months of November–December. Amur falcons migrate from northern China, Mongolia and eastern Russia (fewer birds come from Mongolia and eastern Russia) to the South African coasts. Amur Falcons cover more than 14,500 km in about two months as they travel between breeding and wintering areas including a non-stop portion over the Indian Ocean (Meyburg & Meyburg, 2010; Meyburg et al., 2017).

IAV infection in wild birds and poultry was reported from different countries in Africa including Burkina Faso, Egypt, Niger, Cameroon, Nigeria, Cote d'Ivoire, Djibouti, Ghana, Kenya, Congo, Sudan and South Africa (Ducatez et al., 2007; Freidl et al., 2015; Ofula et al., 2013; Twabela et al., 2018; Wade et al., 2018). Although IAV infection was reported from an ostrich farm in Western Cape in South Africa (Venter et al., 2017), limited information is available on the ecology and prevalence of IAV in wild birds in South Africa. No data are available on the prevalence of IAVs in raptors from South Africa. Recently, we have reported the detection of low-pathogenic IAV in waterfowl in South Africa (Poen et al., 2019). We hypothesized that Amur falcons, due to their expansive geographic range and predation, would be an ideal sentinel species for a pilot virus surveillance study. The scope of the present investigation was, thus, to screen Amur falcons for possible infection with IAVs and NDVs with the long-term goal of establishing surveillance to provide useful insights for disease epidemiology, which is essential for making recommendations for wildlife disease management in South Africa.

2 | MATERIALS AND METHODS

2.1 | Ethics and permits

Bird catch, ring and release permits were obtained from Ezemvelo KwaZulu-Natal Wildlife (Permit numbers OP40/2018). Ethical approvals were obtained from the Animal Research Ethics Committee of the University of KwaZulu-Natal (Reference AREC 071/017 and AERC 014/018). The field sampling protocols, sample collection from animals, and the research were conducted in full compliance with Section 20 of the Animal Diseases Act of 1984 (Act No 35 of 1984) and were approved by the South African Department of Agriculture, Forestry and Fisheries (Section 20 approval Reference. 12/11/1/5). In addition, the research was conducted in compliance with the South African Council for Non-proliferation of Weapons of Mass Destruction (Ref number NPC018/416).

2.2 | Birds capture

Amur falcons roost in large aggregations on the wintering grounds, often many thousands of birds together. We trapped Amur falcons at the largest known roost in Newcastle (Figures 1 and 2) in KwaZulu-Natal Province, South Africa, using high altitude mist nets.

2.3 | Virologic sampling

The samples were collected from birds captured at three sites in KwaZulu-Natal Province in South Africa in February, 2018, as shown in Figures 3 and 4. Additional samples were collected from wild raptors which were temporarily kept in captivity in Durban and Pietermaritzburg in KwaZulu-Natal Province between March and August 2018. Cloacal and oropharyngeal swab samples were collected using sterile polypropylene swabs (Puritan Medical Products) and immediately placed into 2 ml viral transport medium (VTM) containing brain-heart infusion broth, streptomycin (10,000 μg/ml), penicillin (10,000 units/

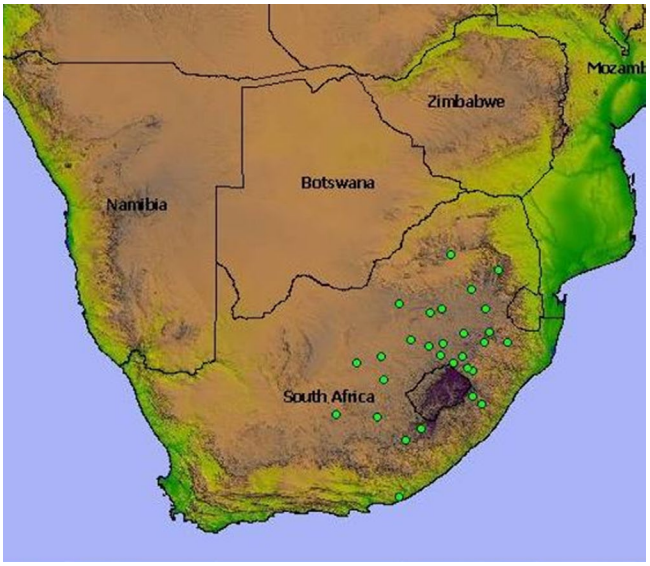

FIGURE 1 The known roosts of Amur falcons in Newcastle, KwaZulu-Natal Province, South Africa (photo was reproduced with permission from Bernd-Ulrich Meyburg)

ml), amphotericin B (50,000 μg/ml) and gentamicin (5,000 μg/ml) (El Zowalaty et al., 2011). Samples were stored at −80°C until shipping. The cryovials were placed into a cryogenic container and transported to the Division of Virology, St. Jude Children's Research Hospital under cold chain for virologic analysis.

2.4 | RNA extraction

Total RNA was extracted using Qiagen RNeasy Mini Kit (Qiagen) according to manufacturer's instructions. The frozen cloacal and oropharyngeal samples were thawed on ice. The samples were briefly vortexed, then 200 μl of the sample was added to the 350 μl of lysis buffer containing 2-mercaptoethanol. Equal volume of 70% ethanol was added to this solution, and the mixture was loaded to the RNeasy column. After washing steps with buffers RW1 and RPE, the RNA was eluted into 1.5 ml microcentrifuge tube using 50 μl of RNase free water. RNA was stored at −80°C for downstream processing.

2.5 | AIV real-time quantitative reverse transcription-PCR (RT-qPCR)

The RNA extracts obtained from the cloacal and oropharyngeal swab samples were screened for IAV using matrix-gene-based specific primers and TaqMan probe using real-time quantitative reverse transcription-polymerase chain reaction (RT-qPCR) as was previously described (Spackman et al., 2002) in a Quant Studio 5 real-time PCR machine. TaqMan® Fast Virus 1-Step Master Mix (Applied Biosystems, Thermo Fisher Scientific) was used for the RT-qPCR assay. Briefly, 1 μl primer-probe mix was prepared for each reaction using 40 μM of primer, 10 μM of the TaqMan probe and nuclease-free water. Then, TaqMan Master Mix, primer-probe mixture, nuclease-free water and RNA template were included in a 20 μl reaction for samples under investigation. A known RNA template was used as the positive control with cycle threshold (C_t) value of approximately 20. Nuclease-free water was used as the template for negative control. A C_t value of less than 36 was used to determine positivity (Agüero et al., 2007). Due to the high C_t values for some of the samples, a second RNA extraction was conducted on positive samples and subjected to RT-qPCR using a second set of primer probes (US Centers for Disease Control and Prevention). Samples positive in both RT-qPCR assays are reported as positive for IAV in this study.

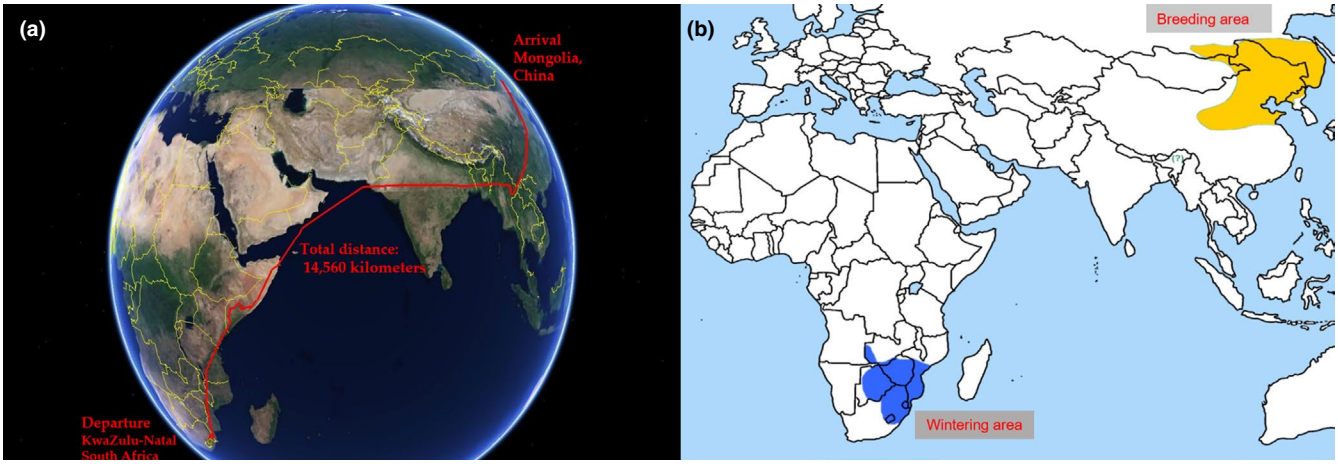

FIGURE 2 Migratory route of Amur falcons. (a) Map showing the first-ever recorded transoceanic migration route of an adult female Amur falcon in spring 2010 fitted with a 5 g solar-powered platform transmitting terminal (PTT 95773) manufactured by Microwave Telemetry Inc. from its wintering area in Newcastle, KwaZulu-Natal, South Africa, to its breeding area in northeast China. (b) The principal breeding (mainly northeast China) and wintering area (mainly South Africa) ranges are separated by both 70° of latitude and longitude (photos were modified and reproduced with permission from Bernd-Ulrich Meyburg)

FIGURE 3 Virologic sampling of Amur falcons in the present study a) Adult female Amur falcon fitted with a 5 g Platform Transmitter Terminal (PTT) (b) The ringing of birds and deployment of the PTTs. (c) Trapping of Amur falcons using mist nets. (d) Data capture of trapped birds (e) Sampling of Amur falcons for influenza A and Newcastle disease viruses (photos were reproduced with permission from Bernd-Ulrich Meyburg and Mohamed Ezzat El Zowalaty)

2.6 | DNA Sequencing

Influenza A virus positive samples were submitted to Hartwell Sequencing Centre facility, St. Jude Children's Research Hospital, for sequencing using Illumina Techniques (Illumina). A multisegment reverse transcription-PCR (M-RTPCR) was performed using gene-specific primers according to the previously published protocol to amplify the whole influenza viral genome (Zhou et al., 2009). PCR products were then gel-extracted and purified using GE Healthcare illustra™ GFX PCR DNA and Gel Band Purification Kit (Sigma Aldrich). Library preparation of samples was performed using Illumina's Nextera XT DNA Sample Preparation kit (Illumina) according to the manufacturer's protocol. Amplicons were sequenced on the Illumina's MiSeq platform using the paired-end approach. Sequencing reads were demultiplexed, quality-trimmed and filtered prior to consensus sequence generation using the Pallas pipeline, developed by the Hartwell Genomics Centre at St. Jude Children's Research Hospital. Final analysis and the generation of consensus sequences were completed using CLC Genomics Workbench (v.11.0.1).

2.7 | Molecular subtyping using RT-qPCR for H5, H7 and H9 influenza A viruses

Samples that were IAV positive by matrix-gene specific primers were further screened for H5, H7 and H9 viruses using subtype-specific primers and TaqMan probes for one-step real-time RT-PCR (Spackman et al., 2002) in a Quant Studio 5 real-time PCR machine. TaqMan® Fast Virus One-Step Master Mix purchased from Applied Biosystems (Thermo Fisher Scientific) was used for the real-time RT-PCR assay. Briefly, 1 μl primer-probe mix was prepared for each reaction using 20 μM of primer, 40 μM of the TaqMan probe and nuclease-free water, followed by a 20 μl reaction including primer-probe mix, TaqMan Master Mix and nuclease-free water for the matrix-gene positive IAV confirmed samples of Amur falcons. Known RNA templates for IAVs, A/duck/Bangladesh/19097/2013(H5N1), A/Netherlands/219/2003(H7N7) and A/Hong Kong/1073/99(H9N2) were used as positive controls. Nuclease-free water was used as the template for negative control.

2.8 | Molecular detection of Newcastle disease virus using conventional RT-PCR

All RNA samples were screened for NDV using two-step RT-nested PCR using as previously described primers (Kho et al., 2000) with modifications. First-strand cDNA was synthesized using Superscript III (Invitrogen, Life Technologies). The reaction mix was prepared using 2 μl RNA extracted using RNeasy Mini Kit (Qiagen), 5μl RNase free water, 1 μl (10 mM) dNTP, 1 μl (20 μM) FOP1 primer and 1 μl (20 μM) FOP2 primer. The mixture was incubated at 65°C for 5 min, then placed on ice for at least

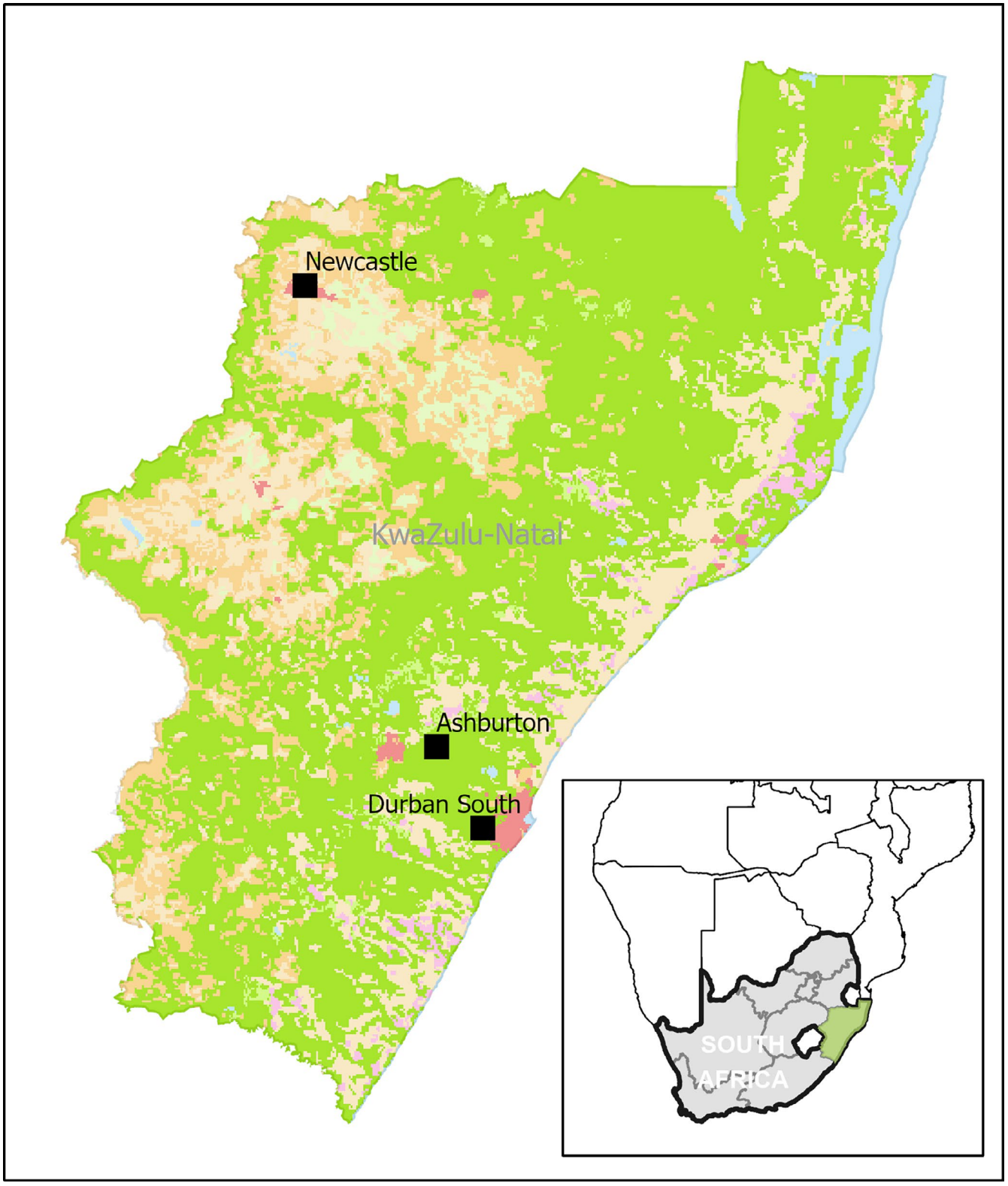

FIGURE 4 Map of KwaZulu-Natal Province in South Africa showing the sampling sites in the current study

1 min. The cDNA synthesis mixture was prepared by adding the following reagents in the following order: 2 µl 10× RT Buffer, 4 µl (25 mM) MgCl2, 2 µl (0.1 M) DTT, 1 µl RNase OUT (40 U/µl), 1 µl Superscript III RT (200 U/µl). Ten µls of cDNA synthesis mixture was added to RNA/primer mixture and incubated as follows: 50°C for 50 min, reaction was terminated at 85°C for 5 min and chilled on ice for at least 1 min. Samples were briefly centrifuged to collect the reactions at the bottom of the tube, and 1 µl RNase H was

added to each reaction and incubated at 37°C for 20 min. The cDNA was stored at −20°C for subsequent PCR reactions. For the first PCR amplification, 2 µl of cDNA was added to the following PCR mixture using the Phusion® High Fidelity PCR Master Mix with HF Buffer (New England Biolabs): 25 µl 2× Phusion MM w/ HF Buffer, 1 µl (20 µM) FOP1, 1 µl (20 µM) FOP2 and 21 µl water. The first-round amplification conditions were as follows: 94°C for 3 min, 94°C for 30 s (denaturation), 67°C for 30 s (annealing), 72°C for 30 s (extension), cycle step 2 20×, 72°C for 7 min (prolonged extension), 4°C on hold and then samples were placed on ice for at least 1 min. The second PCR amplification was carried out in the same tube of the first PCR. An additional 30 µl reaction mixture consisting of 13 µl H_20, 15 µl 2× Phusion Master mix with HF Buffer, 1 µl (20 µM FIP1), 1 µl (20 µM FIP2) was added to the previous PCR product. The second PCR reaction conditions were followed for an additional 30 cycles: 94°C for 30 s (denaturation), 55°C for 30 s (annealing), 72°C for 30 s (extension), 72°C for 7 min (prolonged extension) and kept on hold at 4°C. The reaction was visualized using 1.5% agarose for the detection of band size of 532 bp (FOP1/FOP2) and 280 bp (FIP1/FIP2). A positive control NDV RNA (P/Chicken/Bangladesh/23057/2014) was used as a positive control.

2.9 | Virus isolation in embryonated chicken eggs

Samples were cultured for virus isolation by inoculation in embryonated chicken eggs (ECEs). A volume of 100 µl from each swab sample was injected into the allantoic fluid (AF) of three 10-day-old ECEs per sample and incubated at 35°C. After 72 hr, the AF was collected and tested for hemagglutination using 0.5% turkey erythrocytes. All the first passage in ECE (E1) AF samples were passaged in ECEs for a second (E2) passage. All E1 and E2 passages AF samples were tested using the Flu Detect® (Zoetis Inc.).

3 | RESULTS

The Amur falcons and other wild-caught birds of prey in this study were sampled at time of banding in 2017–2018. For IAV screening, 48 cloacal and oropharyngeal samples from 24 birds (total number of sampled birds) were collected from birds of prey including 24 samples from 12 migratory Amur falcons and two samples from one red-footed falcon. Other samples were collected from additional wild captive raptor species including brown snake eagle, African goshawk, African wood owl, black sparrow hawk, yellow-billed kite, spotted eagle owl and peregrine falcon (Table 1). All birds appeared healthy at the time of sampling except one brown snake eagle which was sick at the time of sampling. A total of 14 samples from 11 birds (45.8% of all sampled birds) were IAV positive with C_t lower than 36 in duplicate tests. Among the Amur falcons, five (41.7%) out of the 12 birds were IAV positive with C_t values lower than 36. One Amur falcon was positive in both cloacal and oropharyngeal swabs. Two other birds (a yellow-billed kite and an African wood owl) were also positive for IAV in both cloacal and oropharyngeal swabs. The other positive samples came from a black sparrow hawk, a spotted eagle owl and an African goshawk. Due to the high C_t values of the positive samples, we re-extracted viral RNA from 10 of the positive samples (insufficient material was available for the remaining four samples). Seven of these samples (from five birds) were again positive after the additional freeze and thaw adding confidence to our determinations.

All IAV-positive samples were further screened for H5, H7 and H9 subtypes. One sample collected from a healthy female spotted eagle owl was found to be H5 positive using RT-qPCR. No samples were found to be H7 or H9 positive. Unfortunately, no virus isolates or sequence information could be retrieved from the samples. In addition, all samples were negative for NDV by RT-PCR. The two samples which were collected from a brown snake eagle, which was reported to be sick at the time of sampling, were negative for both IAV and NDV excluding these two aetiological agents.

TABLE 1 Detection of influenza A viruses in nine species of captured birds of prey in South Africa in 2018

Bird species	No. of birds captured	No. of tested cloacal samples	No. of tested OP samples	No. IAV-positive samples by RT-qPCR	Location of collected Samples
African goshawk (*Accipiter tachiro*)	2	2	2	1 (OP)	Ashburton
African wood owl (*Strix woodfordii*)	2	2	2	1 (OP) 2 (C)	Ashburton
Amur falcon (*Falco amurensis*)	12	12	12	2 (OP) 4 (C)	Newcastle
Brown snake eagle (*Circaetus cinereus*)	1	1	1	0	Durban
Black sparrow hawk (*Accipiter melanoleucus*)	2	2	2	1 (OP)	Durban
Peregrine falcon (*Falco peregrinus*)	1	1	1	0	Ashburton
Red-footed falcon (*Falco vespertinus*)	1	1	1	0 (C)	Newcastle
Spotted eagle owl (*Bubo africanus*)	2	2	2	1 (C)	Ashburton
Yellow-billed kite (*Milvus aegyptius*)	1	1	1	1(OP) 1 (C)	Durban
Total	24	24	24	14	48

4 | DISCUSSION

The title for undertaking the most arduous of all raptor migrations belongs certainly to the Amur falcon, which is a complete transcontinental, transequatorial, long-distance flocking migrant (Meyburg & Meyburg, 2010; Meyburg et al., 2017). The principal breeding (mainly northeast China) and wintering (mainly South Africa) ranges are separated by 70° of both latitude and longitude. Several migratory bird species, including Amur falcons, overwinter in the Southern hemisphere each year (Cade, 1982; Tarboton & Allan, 1984). With its migration route of over 14,500 kilometres, the Amur falcon has one of the longest migration routes of any raptor in the world (Meyburg & Meyburg, 2010; Meyburg et al., 2017), perhaps matched only by that of the Swainson's Hawk (*Buteo swainsoni*) in the New World and probably some peregrine falcon (*F. peregrinus*) populations.

We found one Amur falcon to be positive for IAV RNA in both cloacal and oropharyngeal swabs. With their arrival to Newcastle in South Africa (Figures 1 and 4), there is, thus, an elevated risk of introduction of IAVs into resident bird populations. IAV surveillance has long been a neglected area of research in South Africa despite repeated detections of the virus in ostrich and poultry populations (Venter et al., 2017). It was previously reported that IAVs spread rapidly from Qinghai Lake in China after the 2005 AI outbreak and were introduced into falcons in Saudi Arabia and Kuwait (Marjuki et al., 2009).

There are considerable knowledge gaps including the prevalence of IAVs in South African wild birds, the source of outbreaks in ostriches, and whether IAVs are transmitted from South Africa to other regions of the world. In this study, we sampled primarily Amur falcons, as examples of migratory birds of prey covering large geographic areas, piggybacking on banding activities. The population of birds we sampled breed in northeast Asian countries, including central Mongolia (Figure 2), where IAV detections have been reported over the past decade (Li et al., 2004; Sakoda et al., 2010). In the present study, we investigated for the first time whether Amur falcons visiting the South African coasts were infected with IAVs. In addition, we screened wild birds of prey which were temporarily kept in captivity for the presence of IAVs before their release to the wild. It was found that 45.8% of the sampled birds (41.7% of Amur falcons) were positive for IAVs in two independent tests. Although the origin and likely source of IAVs infecting these birds was not determined in this study, the large percentage of positive birds strongly suggests that steps should be taken to minimize the interaction of migratory and captive wild-caught raptors with domestic or commercial birds including poultry to avoid the possible introduction and spread of IAVs in other susceptible hosts. In the present study, no bird species was infected with NDVs when screened using RT-qPCR; however, raptor species have been infected with NDVs as was previously reported (K.-S. Choi et al., 2008; Haddas et al., 2014; Jindal et al., 2010; Schettler et al., 2003; Umali et al., 2016). Further investigation of raptors and other avian species in South Africa is warranted to further dissect the epidemiology of the disease in this country.

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DISCLAIMER

No other person has any role in the study concept, study design, data collection, experimental work, data analysis, data interpretation or the decision to publish. Any use of commercial names, commercial diagnostic products or firm names is for descriptive purposes only and does not imply endorsement by the National Institutes of Health or the United States Government. Any opinions, findings and conclusion or recommendations expressed in this material are those of the authors and do not necessarily reflect the view of the National Institutes of Health or the United States Government.

CONFLICT OF INTEREST

None to declare.

AUTHOR CONTRIBUTIONS

Conceptualization, M.E.E.Z. and R.J.W.; sample collection, M.E.E.Z.; methodology, M.E.E.Z., J.D.B., T.J., J.F. and R.P.; software, M.E.E.Z., K.F.; validation, M.E.E.Z., T.J., R.G.W. and R.J.W.; formal analysis, M.E.E.Z. and R.J.W.; investigation, M.E.E.Z. and R.J.W.; resources, M.E.E.Z. and R.J.W.; data curation, M.E.E.Z., R.J.W. and J.D.B.; writing—original draft preparation, M.E.E.Z. and R.J.W.; writing—review and editing, M.E.E.Z., T.J., S.G.Y., K.F., R.J.W. and R.G.W.; critical revisions and editing, R.J.W. and M.E.E.Z.; visualization, M.E.E.Z. and R.J.W.; supervision, M.E.E.Z. and R.J.W.; project administration, J.D.B and M.E.E.Z.; funding acquisition, R.J.W. and M.E.E.Z.

DATA AVAILABILITY STATEMENT

Not applicable.

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