Original Article

Influenza monitoring in Sardinia, Italy identifies H3 subtype in Mediterranean wild migratory birds

Alyson A. Kelvin[1,6,], Dionigia Meloni[1], Paola Sansonetti[1], Ilaria Borghetto[1, 5], Thomas Rowe[2], Rosaria Santangelo[3], Danilo Pisu[4], Cheryl M. Cameron[2], Bianca Paglietti[5], David Banner[2], Amber Farooqui[1], Patrizia Marongiu[5], Antonella Santona[1], Giovanni Fadda[3], David J. Kelvin[1,2, 5], Salvatore Rubino[1,5]

[1]SaRD - Sardinia Research and Development, University di Sassari, Sassari, Italy
[2]University Health Network, Toronto, Ontario, Canada
[3]Istituto di Microbiologia, Facoltà di Medicina e Chirurgia, Università Cattolica del Sacro Cuore, Rome, Italy
[4]Centro Studi Fauna, Porto Torres SS, Italy
[5]Dip. di Scienze Biomediche University di Sassari, V. le San Pietro 43/b, 07100 Sassari, Italy
[6]IDR, Immune Diagnostics & Research, Toronto Medical Discovery Tower (TMDT), Toronto, Ontario, Canada

Abstract

Introduction: Wild migratory birds are global distributors of pathogens. Sardinia, Italy, is the second largest Island in the Mediterranean and is a land bridge between Europe and Africa.

Methodology: We designed a surveillance protocol to investigate wild migratory birds for presence, frequency, and type of avian influenza viruses. We collected over 4,000 avian samples and compared three sampling methods, fecal, cloacal, and tracheal, to determine the most productive for virus identification. To determine frequency of infection, RNA was extracted and RT-PCRs for avian influenza virus genes were run. Positive samples were cultivated for live virus, sub typed and sequenced.

Results: Forty-four samples were positive for influenza nucleoprotein gene. We identified two previously unidentified H3 subtype strains and found cloacae to have the highest rate of virus identification and fecal sampling to provide quality RNA and repeatable results for determination of virus presence.

Conclusion: Our investigation provides information on the frequency of Mediterranean avian influenza viruses, and validates the initiation of an avian influenza surveillance protocol. Taken together with global avian influenza findings, these results give insight into infectious disease distributions which is important for viral pandemic monitoring and design of preventative measures.

Introduction

Influenza type A, a negative strand virus member of the orthomyxoviridae family, can infect various animal species [1,2]. Influenza A is characterized by immunologically important proteins HA and NA [3] of which there are 17 HA antigens (H1 – H17) and 9 NA (N1 – N9) antigens [2,4,5]. Although both the HA and the NA are immunologically relevant, it is the HA isotype that determines host infectivity. Subtypes H1 – H16 have been detected in waterfowl, the natural reservoir for Influenza A viruses [6]. Unless the influenza strain is a highly pathogenic variant of H5 or H7 (High Pathogenic Avian Influenza (HPAI)), the disease caused by LPAI A (Low Pathogenic Avian Influenza) infection in avian species presents only mild symptoms although a more severe disease and active immune response occur when the virus is passed to swine or humans [7–9]. LPAI viruses infect the intestinal tract of avian species typically through the fecal-oral route, whereas the HPAI viruses with mutated polybasic amino acids in the HA cleavage site can infect a range of tissues and organs outside of the gut [8,9]. Studies have implicated migratory birds in the spread of pathogenic organisms including the influenza virus, although controversy exists surrounding the ability of migratory birds to disseminate HPAI strains and the clinical disease caused by infection [7,8,10–17]. Furthermore,

understanding the relationships among the environment and weather, migratory patterns, wild avian species and domesticated birds with the influenza virus remains a significant hurdle in the field of influenza research.

For interspecies transmission to occur, the virus must undergo a genetic alteration either by antigenic shift or antigenic drift [18,19]. Previously, it was thought that humans were unable to be directly infected with avian influenza and that reassortment events were required to occur in transition vessels such as pigs prior to human infection [19]. Previously only H1, H2, and H3 influenza antigens have been thought to infect humans but more recently there has been evidence showing that other HA antigens, such as H5, H7 and H9 viruses, can cross the species barrier and infect humans and cross-species transmission of H5N1 from poultry to humans causes high mortality [20–22]. These examples suggest that influenza interspecies transmission between birds and humans does not always require a transitional species.

Prior human pandemics have been caused by viruses with the HA antigens H1, H2, and H3. Currently H1 and H3 are circulating in the human population, including the pandemic 2009 strain of H1N1 [19,23–27]. With recent evidence indicating avian influenza can directly infect humans, a new worry of another global influenza pandemic has emerged [19,20,28,29]. To cause a pandemic, an influenza strain must be highly pathogenic but also easily transmissible from person to person. The 1997 and current H5N1 viruses are unable to be passed from person to person, decreasing its pandemic potential. Although the current H5N1 virus can only be acquired from contact with infected birds, the rapid mutation rate of the influenza virus leads many researchers to believe that H5N1 will become a human global threat in the near future [19,29,30]. The new threat of a highly pathogenic transmissible strain of avian influenza has provoked the need for monitoring influenza strains circulating in wild migratory birds [31]. Furthermore, it is commonly thought that early detection of avian influenza is essential for economic security in the poultry industry and maintenance of public health [32]. Previously, avian influenza has been monitored in wild birds, including those in Italy and those crossing the Europe/North America land bridge [11,33–37]. We investigated for the first time the influenza strains currently affecting the migratory birds landing in the island of Sardinia, a land bridge between Europe and Africa. Furthermore, Italy has the highest number of avian influenza outbreaks in the EU and has been affected by H5 and H7 influenza outbreaks in avian populations [38–41]. We compared three avian sampling methods, fecal, cloacal, and tracheal, and report an effective and economical surveillance system for influenza virus monitoring in avian species. We found avian influenza infectivity to be 3.4% and we detected two previously unidentified H3N8 strains. Our results provide valuable information on the current influenza strains of Mediterranean birds which may be used for the development of influenza therapeutics.

Methodology
Bird capture net construction
The design used to build the experimental traps for aquatic birds capture was based on the protocols followed by the National Institute for Wildlife Management (now known as ISPRA, http://epanet.ew.eea.europa.eu/european_epas/countries/it) for the purpose of scientific ringing and ensuring the safety at all stages of the specimens captured, with special interest for the systematic families anatidae and rallidae. Cages consisted of 18 wooden supporting poles (9 or 12 cm in diameter and either 3 or 4 meters in length) placed at equal distances on the circumference of a circle measuring 60 square meters in area. The walls of the cage were 2 meters in height and covered with galvanized 2.88 mm plastic mesh (openings 2.5 cm x 5 cm). The roof was covered by a polyethylene net ("Tenax" model, Cintoflex M, Baltimore, USA) 17 x 24 mm rectangular plastic netting. The materials were chosen to avoid animal injury. Birds entered the side of the cage facing the pond (Figure 1).

Sample collection
Live birds from capture nets were tracheal and cloacal swabbed. All live bird samples collected were obtained from a ringed animal and the details of the bird's species and health were recorded. Swabs were placed in viral transport medium 199, consisting of tissue culture medium 199 containing 0.5% bovine serum albumin, benzylpenicillin (2 x 106 IU/litre), streptomycin (200 mg/litre), polymyxin B (2 x 106 IU/litre), gentamicin (250 mg/litre), nystatin (0.5 x 106 IU/litre), ofloxacin hydrochloride (60 mg/litre), and sulfamethoxazole (0.2 g/litre). Specimens were immediately chilled to 4°C and then frozen at −70°C at the laboratory. Samples numbers are recorded in Table 1.

A large tarp was used to collect fecal matter. Subsequently, fecal specimens were swabbed and the

Table 1. Total number of avian samples collected during 2007 and 2008 collection

	Number Collected
Tracheal	921
Cloacal	1085
TOTAL Live Bird	**2006**
Fecal	2297
TOTAL Samples Collected	**4253**

swab was placed in a cryogenic vial containing 1 ml of virus stabilization media and vortexed. Samples were placed on ice then frozen. Each fecal specimen was swabbed twice to obtain a duplicate of each sample. Sample collection occurred between December 2007 and December 2008. Samples numbers are recorded in Table 1 and bird species seen at fecal collections are recorded in Table 2.

RNA extraction and RT-PCR

Reverse Transcriptase (RT) –PCR and real-time PCR have previously been used to screen wild avian populations for the presence of avian influenza [34,35]. As RT-PCR was sufficient and a cost effective method of testing for the presence or absence of Influenza A, it was chosen to test the collected avian samples for the presence of influenza A. RNA was extracted from avian samples using the TRIzol (Invitrogen, Milan, Italy) method from Invitrogen adapted from the manufacturer's instructions. Briefly, samples were placed in 1.6 ml of TRIzol and rested for 5 minutes, then 400 µl of chloroform was added and the samples were vigorously shaken for 30 seconds and left for 2 minutes. After centrifugation, the upper aqueous layer was removed and RNA was precipitated using 600 µl ice cold isopropanol. After centrifugation, the pellet was washed with 70% ethanol. The RNA pellet was dried for 5 minutes and the pellet was resuspended in 100 µl of DEPC H20 and measured by spectrophotometry.

For cDNA synthesis, 600 ng of RNA was used for the M-MLV reverse transcription kit from Invitrogen using random hexamer primers. Following cDNA synthesis, PCR was performed using Invitrogen's Platinum® Taq DNA Polymerase PCR kit according to the manufacturer's instructions with in-house designed or previously described primers (Table 3). The following basic program was used: 95° C for 3 minutes, denaturing 95°C for 30 seconds, annealing X° C for 40 seconds, extension 72°C for X seconds,

repeat to 95oC for 35 cycles, 72°C for 10 minutes, 4° C. To control for RNA and PCR quality, we designed duck beta-actin primers to be used as a housekeeping control. Primer specific annealing temperature, extension time and predicted amplicon are described in Table 3. PCR products were run on 1.5% agarose gels with ethidium bromide in 1 x TRIS, Acetate, EDTA (TAE) buffer to visualize product bands. Gels were electrophoresed at 80 V using a Powerpac 200 (BioRad, Milan, Italy) and were visualized using a Gene Genius Bio Imaging System (Syngene, Cambridge, UK).

Sequencing

PCR reactions were run using specialized sequencing primers (described in Table 3). The product was cleaned using a Qiagen PCR Clean-up kit (Qiagen, Milan, Italy). The purified PCR product was sent for sequencing at BMR Genomics (BMR Genomics, Padua, Italy). An NCBI *BLAST* search was performed with the returned sequence. Alignments and phylogenetic trees were created using the *ClustalW* program from the European Bioinformatics Institute [42] (http://www.ebi.ac.uk/Tools/clustalw2/).

Virus culture and virus typing

The duplicates of a portion of live and fecal bird samples collected were sent to the BSL-3 facility at Università Cattolica del Sacro Cuore, Rome, Italy, for culture. Specimens were inoculated into and cultured in 10-day-old embryonated chicken eggs for 48 hours. Allantoic fluids were harvested and frozen in aliquots at −70°C, tested for HA activity and positive virus isolates were subtyped by the HAI test, using a panel of reference antisera against 15 HA subtypes obtained from the National Institutes of Allergy and Infectious Diseases (Bethesda, Md.) reagent repository using 4 HAU of virus and 0.5% turkey red blood cells. Briefly, the HAI test is described as follows: 25 µl of PBS was added to appropriate wells of a microtitre plate, then 50 ul of each serum from panel of reference

Table 2. Common avian species identified at fecal sample collections

Order	Family	Scientific name	English name
Podicipediformes	*Podicipedidae*	*Tachibaptus ruficollis*	Little Grebe
Anseriformes	*Anatidae*	*Anas plathyrhynchos*	Mallard
Anseriformes	*Anatidae*	*Anas acuta*	Pintail
Anseriformes	*Anatidae*	*Anas crecca*	Teal
Anseriformes	*Anatidae*	*Anas clypeata*	Shoveler
Gruiformes	*Rallidae*	*Rallus aquaticus,*	Water Rail
Gruiformes	*Rallidae*	*Gallinula chlorpous*	Moorhen
Gruiformes	*Rallidae*	*Fulica atra*	Coot

Table 3. Primers used during the Avian Influenza Study

Primer name	Sequence	T_m	Size	Source
Duck β-actin_F	CAGACATCAGGGTGTGATGG	54°C	275 bp	This study
Duck β-actin_R	GGGGTGTTGAAGGTCTCAAA	52°C		This study
NP_F	CAG(A/G)TACTGGGC(A/T/C)ATAAG(A/G)AC	52°C	330 bp	Lee M.S. *et al.,* 2001
NP_R	GCATTGTCTCCGAAGAAATAAG	51°C		Lee M.S. *et al.,* 2001
NPsequencing_F	GTAAAACGACGGCCAGTCAG(A/G)TACTGGGC(A/T/C)ATAAG(A/G)AC	NA	330 bp	This study
NPsequencing_R	GGAAACAGCTATGACCATGGCATTGTCTCCGAAGAAATAAG	NA		This study
HA_F	GGAATGATHGAYGGNTGGTATGG	50°C	Approx. 640 bp	Phipps L. P. Et al., 2004
HA_R	AGTAGAAACAAGGGTGTTTT	50°C		Hoffman E. *et al.,* 2001
HAseq_F	GGAATGATHGAYGGNTGGTATGGAGCAAAAGCAGGAGTGA	NA	Approx. 640 bp	This study
HAseq_R	AGTAGAAACAAGGGTGTTTTAGTGAAACAAGGAGTT	NA		This study

Figure 1. Live bird capture for sampling.

A

B
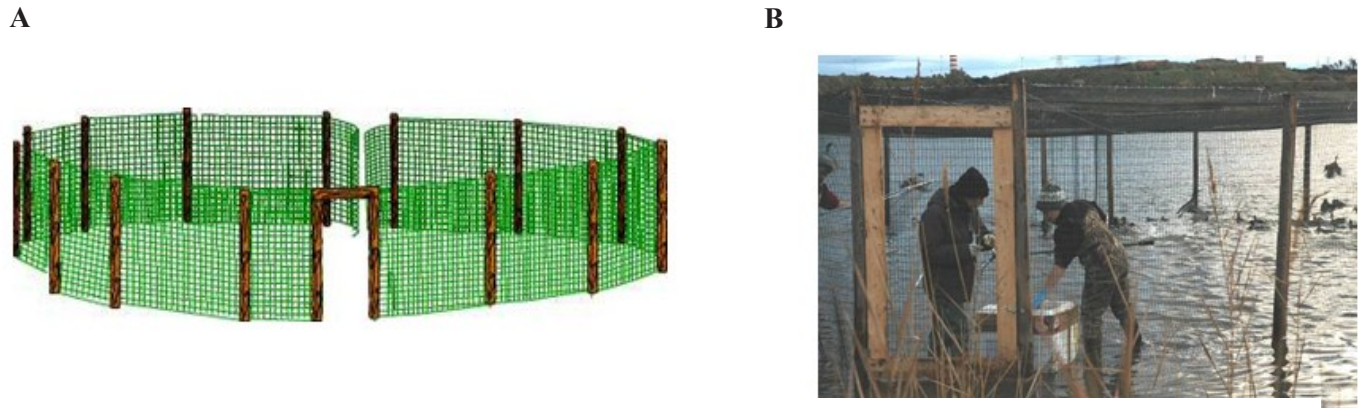
A. Cartoon schematic showing the plan of construction for bird capture houses
B. A photo of a constructed bird capture house with sampling taking place
Captured birds were sampled and their information was recorded before they were released back into the wild.

Figure 2. Clustal Alignment of SaRD43 and SaRD44 H3 nucleotide sequence with a published H3 nucleotide sequence

Sequencing PCRs were performed using general primers for the HA gene of the influenza virus on samples SaRD43 and SaRD44. The returned sequence was blasted on the NCBI database and both closely matched the H3N8 2007 H3 sequence as the top match GenBank ID:CY041346.1, (A/Turnstone/Netherlands/1/2007/H3N8). Green shows where SaRD43 and SaRD44 differ from the reference sequence. Pink shows when SaRD43 and the reference sequence differ from SaRD44. Blue shows when SaRD44 and the reference sequence differ from SaRD43.

Figure 3. Clustal alignment of SaRD43 and SaRD44 HA nucleotide translated sequences

```
SaRD43|   GMIDGWYGFRHQNSEGTGQAADLKSTQAAIDQINGKLNRVIEKTNEEFHQIEKEFSEVEG 60
SaRD44|   GMIDGWYGFRHQNSEGTGQAAELKSTQAAIDQINGKLNRVIEKTNEKFHQIEKEFSEVEG 60
          ********************.********************.************

SaRD43|   RIQDLEKYVEDTKIDLWSYNAELLVALENQHTIDLTDSEMNKLFEKTRRQLRENAEDMGN 120
SaRD44|   RIQDLEKYVEDTKIDLWSYNAELLVALENQHTIDLTDSEMNKLFEKTRRQLRENAEDMGN 120
          ************************************************************

SaRD43|   GCFKIYHKCDNACIESIRNGTYDHDIYRDEALNNRFQIKGVELKSGYKDWILWISFAISC 180
SaRD44|   GCFKIYHKCDNACIEPIRNGTYDHDIYRDEALNNRFQIKGVELKSGYKDWILWISFAISC 180
          ***************.********************************************

SaRD43|   FLLCVVLLGFIMWACQRGNIRCNICI 206
SaRD44|   FLLCVVLLGFIMWACQRGNIRCNICI 206
          **************************
```

After obtaining the nucleotide sequence of our SaRD43 and SaRD44 H3 samples they were translated into amino acids. Following translation the sequences were aligned with each other showing that the nucleotide differences were translated to amino acid substitutions. Three amino acid substitutions were seen at positions 22, 47 and 136 of the SaRD sequences (highlighted in yellow).

antisera against 15 HA subtypes diluted 1:10 was added to the first well of the appropriate column. Serial two-fold dilutions of the treated sera were prepared, and 25 µl of standardized control antigen or virus test antigen was added to wells containing diluted treated sera. Plates were agitated manually, covered and incubated at room temperature for 15 minutes, then 50 µl of standardized RBCs was added to all wells and agitated as before, and plates were covered to allow the RBCs to settle at room temperature for 30 minutes. HAI titres were recorded. As well as for NA typing, live viruses were typed using a CDC-Developed Diagnostic Lab Test for Flu.

Results

Avian sample collection

To determine the presence and frequency of avian influenza in the wild bird population of Northern Sardinia, live bird capture nets and traps were built at five different locations including the ponds Stagno di Pilo, Stagno di Casaraccio Sponda Nord, Cuile Issi, Abbagur, and Platamona to collect both live bird samples and fecal samples. A total of 4,303 live bird and fecal samples were collected and are described in Table 1. Specifically, 2,006 live bird samples were taken as follows: 921 tracheal samples, 1,085 cloacal samples, and 2,297 fecal samples were collected. Bird species seen at each fecal sample collection were recorded and are presented in Table 2. The species recorded only refer to the species observed at time of sample collection and do not necessarily correspond to the species fecal sampled, although it is probable that these species are represented.

RT-PCR screening and virus typing

RT-PCR was performed for the influenza A NP gene [35] on 2,023 samples screened to determine the presence or absence of influenza in the avian sample. Of the 2,023 samples screened, 44 samples were amplified under NP PCR conditions, which is a positivity percentage of 2.2% positive out of the beta-actin positive PCR samples. PCR reactions for duck beta-actin were 64% positive out of the screened samples. Of the three sample types, we found that tracheal samples had the lowest NP positivity. The cloacal samples had the highest NP detection. Fecal samples showed 3% positivity for NP, suggesting that non-live bird fecal sampling produced quality RNA and could be used to identify influenza infection.

Subsequent to the identification of the NP positive samples, amplicons were sequenced to verify NP gene amplification. NCBI *BLAST* search was performed with returned sequences and confirmed the presence of the NP and Avian Influenza (data not shown).

Virus culture

To determine if live influenza virus was present in collected live bird and fecal samples, duplicates of NP positive fecal samples and live bird samples were sent to the BSL-3 facility at Università Cattolica del Sacro Cuore, Italy, for virus culture. In total 32 bird samples in 111 chicken embryonated eggs were screened for live virus. These samples, two samples (designated SaRD43 and SaRD44) were found positive for live influenza virus. These results suggested that our PCR method of screening for Avian Influenza could identify both live and dead influenza virus.

The allantoic fluid from the cultured viruses were subsequently used for HA and NA subtyping of the two live viruses. Using this method it was found the viruses were H3N8 influenza sub-types.

Hemagglutinin sequencing

Since the HA antigen is often the main determinant of influenza severity, we sub-typed the HA gene using sequence analysis to confirm our HA protein typing. Degenerate HA typing primers that have been previously used for influenza HA typing

Figure 4. Clustal alignment of SaRD43 H3 amino acid sequence with and H3 amino acid sequence from a Northern Pintail in Alaska


```
SaRD43|      --------------------------------------------GMIDGWYGFRHQ  12
ACE73389.1|  VNKITYGACPKYVKQSTLKLATGMRNVPEKQTRGLFGAIAGFIENGWEGMIDGWYGFRHQ 360
                                                         ***********

SaRD43|      NSEGTGQAADLKSTQAAIDQINGKLNRVIEKTNEEFHQIEKEFSEVEGRIQDLEKYVEDT  72
ACE73389.1|  NSEGTGQAADLKSTQAAIDQINGKLNRVIEKTNEKFHQIEKEFSEVEGRIQDLEKYVEDT 420
             **********************************:*************************

SaRD43|      KIDLWSYNAELLVALENQHTIDLTDSEMNKLFEKTRRQLRENAEDMGNGCFKIYHKCDNA 132
ACE73389.1|  KIDLWSYNAELLVALENQHTIDLTDSEMNKLFEKTRRQLRENAEDMGNGCFKIYHKCDNA 480
             ************************************************************

SaRD43|      CIESIRNGTYDHDIYRDEALNNRFQIKGVELKSGYKDWILWISFAISCFLLCVVLLGFIM 192
ACE73389.1|  CIESIRNGTYDHDIYRDEALNNRFQIKGVELKSGYKDWILWISFAISCFLLCVVLLGFIM 540
             ************************************************************

SaRD43|      WACQRGNIRCNICI 206
ACE73389.1|  WACQRGNIRCNICI 554
             **************
```
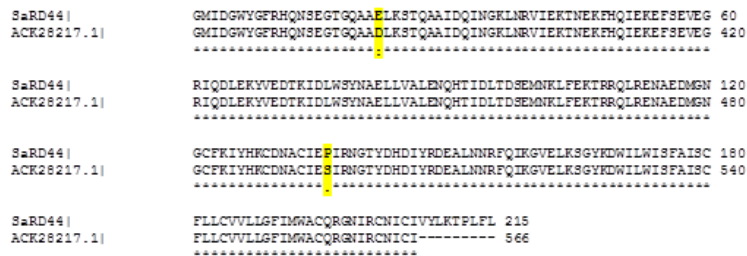
A BLAST search was performed using the translated SaRD43 H3 sequence. The top match an H3N8 sequence isolated from a Northern pintail in Alaska was used to create a clustal alignment with the ClustalW program. The highlighted text shows the SaRD43 H3 E substitution found to be K at aa 394 in other H3 sequences examined, including the Northern pintail H3 sequence GenBank ID: ACE73389.1, (A/northern pintail/Alaska/44202-126/2006(H3N8)).

Figure 5. Clustal alignment of SaRD44 H3 amino acid sequence with H3 amino acid sequence from a Green-wing teal in Alaska


```
SaRD44|      GMIDGWYGFRHQNSEGTGQAAELKSTQAAIDQINGKLNRVIEKTNEKFHQIEKEFSEVEG  60
ACK28217.1|  GMIDGWYGFRHQNSEGTGQAADLKSTQAAIDQINGKLNRVIEKTNEKFHQIEKEFSEVEG 420
             *********************:**************************************

SaRD44|      RIQDLEKYVEDTKIDLWSYNAELLVALENQHTIDLTDSEMNKLFEKTRRQLRENAEDMGN 120
ACK28217.1|  RIQDLEKYVEDTKIDLWSYNAELLVALENQHTIDLTDSEMNKLFEKTRRQLRENAEDMGN 480
             ************************************************************

SaRD44|      GCFKIYHKCDNACIEPIRNGTYDHDIYRDEALNNRFQIKGVELKSGYKDWILWISFAISC 180
ACK28217.1|  GCFKIYHKCDNACIESIRNGTYDHDIYRDEALNNRFQIKGVELKSGYKDWILWISFAISC 540
             ***************:********************************************

SaRD44|      FLLCVVLLGFIMWACQRGNIRCNICIVYLKTPLFL 215
ACK28217.1|  FLLCVVLLGFIMWACQRGNIRCNICI--------- 566
             **************************
```

A *BLAST* search was performed using the translated SaRD44 H3 sequence. The top match an H3N8 sequence isolated from a Green-wing teal in Alaska was used to create a clustal alignment with the *ClustalW* program. The highlighted text shows the SaRD44 H3 E substitution found to be D in other H3 sequences examined at aa 381 and the SaRD44 H3 P found to be S in other H3 sequences at aa 496, including the Green-wing teal H3 sequence GenBank ID:ACK28217.1, (A/green-winged teal/Interior Alaska/1/2007(H3N8)).

[43,44] were used with the SaRD43 and SaRD44 RNA template. The amplicons were sent to BMR genomics for sequencing and a *BLAST* was performed with the returned sequences. Both the sequences (GenBank IDs: HM802759 and HM802760 for SaRD43 and SaRD44, respectively) matched closely to H3 sequence (GenBank ID: CY041346.1 (A/Turnstone/Netherlands/1/2007/H3N8)) segment 4, complete sequence) with significant identity. A clustal alignment was created with the H3 sequences and the published HA sequence using *ClustalW* (Figure 2). The results showed significant similarity between SaRD43 and SaRD44 H3 sequences with the published H3N8 sequence indicating that our sequences were H3 influenza viruses. Furthermore, although the clustal alignment showed the same top match, our SaRD43 and SaRD44 samples differed from each other in their nucleotide sequence. Pink highlighting shows when SaRD43 and the reference sequence differ from SaRD44 and blue shows when SaRD44 matches the reference sequence but differs from SaRD43. Green highlight shows where SaRD43 and SaRD44 both differ from the reference sequence. These results suggested SaRD43 and SaRD44 sequences to be H3 influenza subtypes but they were not the same influenza virus.

Following H3 sequence identification, the nucleotide sequence was translated into amino acids. A clustal alignment was created between SaRD43 and SaRD44 amino acid sequences (Figure 3) and showed the translated sequences were not identical. The sequences differed at three positions, namely 22, 47 and 136 (highlighted in yellow), corresponding to aspartic acid to a glutamic acid (D to E) (both negative amino acids); glutamic acid to lysine (E to K) (negative amino acid to a positive amino acid); and serine to proline (S to P) (uncharged to special), respectively. These results further suggested that sample SaRD43 and SaRD44 were of different H3 influenza viruses.

Since the viruses were not identical and neither 100% matched nucleotide sequences in the NCBI database, we queried the aa sequences to determine if these H3 virus strains had been previously reported. SaRD43 did not have 100% identity with any aa sequences found in the NCBI database (Figure 4). The top match for the SaRD43 aa sequence was an H3N8 virus from an Alaskan Northern pintail. The clustal alignment showed 1 amino acid substitution. The highlighted text shows the SaRD43 H3 glutamic acid (E) substitution at position 47 to be lysine (K) in the Northern pintail H3 sequence. This K to E

substitution is a positive to negative amino acid change. Since the substitution of K to E has not been previously reported these results indicated that the SaRD43 H3 sequence was a unique H3 virus strain.

The same methodology was followed for the amino acid sequence of sample SaRD44 (Figure 5). When the amino acid sequence was queried the top 100 matches all were H3 avian influenza sequences, the top match being an H3 sequence from a Green-wing Teal. The clustal alignment of SaRD44 H3 amino acid sequence with the Green-wing Teal H3 sequence showed two amino acid substitutions (Figure 5). The highlighted text shows the first shift to be a negative to negative amino acid change at position 22. The 44 H3 E (glutamic acid) was found to be D (aspartic acid) in other H3 sequences examined. The second shift at position 136 was a P (proline) in SaRD44 H3 sequence and an S (serine) in other H3 sequences, a change from an uncharged to an amino acid of special properties. These results also suggest that SaRD44 H3 sequence had not been reported previously.

After analysing the clustal alignments, we created phylogenetic trees to determine the evolutionary relationship of the sequences with previously described influenza H3 sequences. Ten H3 influenza sequences from swine, avian and human species were used for the phylogenetic trees (European Bioinformatics Institute, (European Bioinformatics Institute 2010))[42]. Results showed the swine, avian, and human sequences created species clusters/branches (Figure 6). Furthermore, SaRD43 and SaRD44 both clustered with the avian H3 sequences depicting an avian origin. As well, both sequences showed a marked distance from the other avian sequences, which further suggested the novelty of both sequences.

Discussion

The spread of influenza is currently a concern for countries worldwide. Recently the first pandemic of the second millennium was declared and H1N1 virus monitoring and eradication is of immediate importance [23–27]. Avian species are prime global carriers of influenza strains since birds easily and frequently relocate carrying diseases to distant locations. Here we have established a surveillance program for monitoring influenza subtypes in migrating birds that compared different sampling methods. As islands of the Mediterranean Sea are land bridges between Europe and Africa, it is an important surveillance point to establish disease migration. Our study illuminated the importance of using various sampling methods and identified H3 influenza strains circulating in avian species in the Mediterranean. These findings and our establishment of an economical surveillance protocol in the Mediterranean will lead to improved influenza break-out detection as well as influenza therapeutics.

Previously, avian influenza has been investigated in the mainland Mediterranean area. Lebarbencho and colleagues reported the presence of avian H9N2 in France [45]. In the Lebarbenchon study, 72 birds were tested and 2 were found to be influenza positive for a positivity of almost 3% [45]. These results support our findings of a 2.2% influenza positivity rate in the Sardinian bird samples although wild avian influenza positivity of 15% by RT-PCR has been previously reported in Italy [41]. Conversely, we isolated H3N8 virus subtypes compared to the H9N2 virus subtype identified in 2006 in France. Presence of different influenza strains may reflect the yearly changes of circulating influenza viruses, which supports the need for yearly avian influenza monitoring to identify new and threatening influenza subtypes.

Fecal sampling has previously been used for influenza virus identification by PCR based methods [46]. In total we found 44 fecal, tracheal, and cloacal samples to be RT-PCR positive for the NP gene, a positivity percentage of 2.2%. Interestingly, the fecal samples identified 20 positive influenza samples but none was able to be cultured for virus, which is supported by previous findings that RT-PCR is more sensitive than live virus culture screening [47]. Since live virus was not detected in any of the fecal samples this suggests that fecal sampling may not be an effective method of sampling for live influenza. Conversely, fecal sampling may in fact be an efficient method of screening for influenza presence. Furthermore, fecal sampling is an easy and cost-effective method which does not require specialized animal handlers to capture and sample animals. As well, the use of standard PCR opposed to Real-Time PCR methods is most cost effective to identify influenza positive samples. Therefore, this method can be used in times of funding shortages. As well, fecal sampling remains an effective way to sample large populations in a short time when the goal of the study is to identify current strains of circulating influenza and not culturing live virus.

Before pandemic H1N1 2009, there had been three pandemics caused by influenza A viruses over the last century. The first and second pandemics which occurred in 1918 and 1957 were caused by H1N1 and

Figure 6. Phylogentic trees of H3 influenza sequences including SaRD43 (A) and SaRD44 (B) H3 sequences
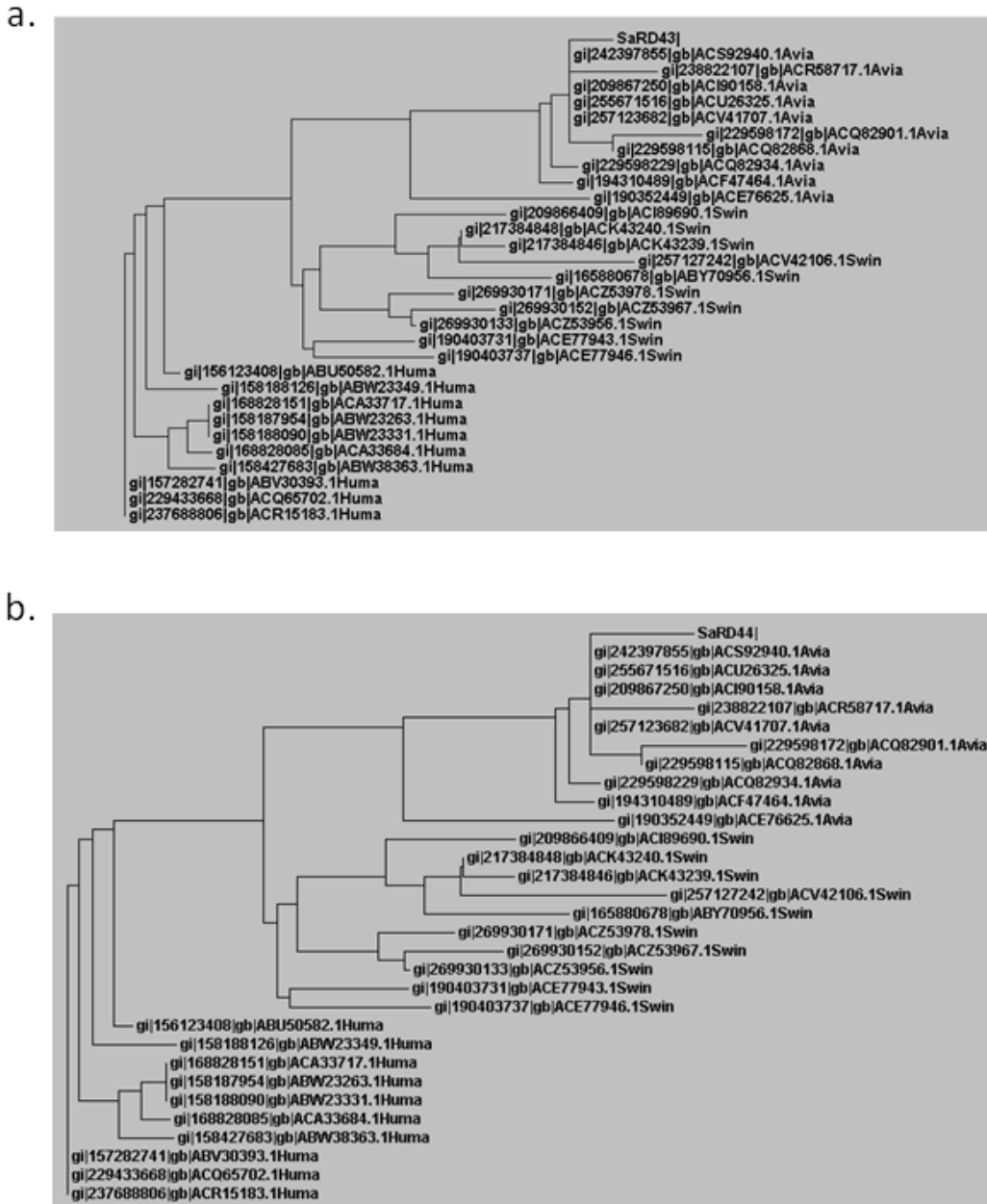
Phylogenetic trees were created using the ClustalW program from EBI. H3 sequences from avian, swine and human species were used to place the SaRD43 and SaRD44 sequences.

H2N2 viruses, respectively. The Hong Kong pandemic of 1968 caused by a H3N2 virus was the most recent influenza pandemic prior to 2009 H1N1 [19]. Although the H3 subtype is typically associated with human infections, as all influenza viruses, H3 viruses are also found waterfowl [48,49]. Since H3 influenza subtypes have caused global infection, it is a potential threat to human and domesticated animal health. Although not of avian origin, we have previously characterized H3 influenza illness in ferrets and shown that H3 influenza strains can cause differing clinical symptoms with severe to moderate illness indicating the significance of the H3 subtype [50]. Moreover, European pigs have been found to be infected with avian strains of the H3 subtype of influenza viruses [51]. As well, in another study sampling waterfowl, H3 viruses were the most common influenza HA subtype in environmental reservoirs [49]. In an investigation following an outbreak of H3 influenza in Italian chickens during the winter of 1994-1995, it was reported that the chickens contracted the virus from waterfowl in the area [52]. Importantly, this study showed that migrating waterfowl could infect domestic birds with a potentially deadly subtype of influenza. Since chickens are domesticated birds this presents an opportunity for H3 influenza to be passed to humans. In this previous Italian study, three separate strains of H3 influenza were isolated from the chickens during this period suggesting that there were numerous strains of H3 viruses circulating in the wild birds of Italy. These findings are in accordance with our results which identified two differing strains of H3 influenza circulating in wild birds. Furthermore, H3N8 influenza strains have been isolated previously in Italy [37,41]. Taken together these observations indicate the importance of monitoring influenza subtypes in wild birds and also highlight the importance of characterizing the H3 subtype.

Both of the live influenza samples identified in our study were found to be H3 viruses by PCR typing and live virus screening. Once we had confirmed the nucleotide sequence to be that of H3, we translated it to amino acids to determine if the viruses had been previously reported and if there were any significant amino acid changes that may influence virus pathogenicity. Since lysine, a positively charged amino acid, was changed to the negatively charged glutamic acid in the SaRD43 sequence there is a possibility that the substitution would lead to a change in antigenicity. Both polar amino acids are commonly found on the surface of proteins and the change in

charge from positive to negative therefore may inhibit or increase typical protein-protein interactions or lead to conformational changes of protein folding. Similar substitutions have been shown to change the biological activity of proteins [53]. Importantly a substitution of glutamic acid to lysine in the influenza virus PB2 protein has been shown to transform a nonlethal H5N1 virus to lethal form indicating the potential threat a K to E shift could bring [54]. Furthermore, a D to E amino acid shift (negative to negative) was reported in the non-structural protein NS1 of H5N1 influenza strains and caused increased virulence and cytokine resistance [54]. Taken together, these findings suggested that the H3N8 virus we identified could have significant impact on the health of infected species.

Aneriformes and Charadiiformes are typically considered the natural reservoir for LPAI viruses [8,55] and LPAIs have been isolated from over 105 different wild bird species. We were able to observe several aneriformes species during our study with eight avian species in total. Furthermore, these have been previously reported to carry influenza viruses in Italy [37,41]. Specifically, De Marco and colleagues found influenza antibodies were detected in 52% of all ducks screened and 7% in coots. It would be important in future influenza surveillance studies to determine the influenza A antibody seroprevalence and subtype in the wild birds of Sardinia.

In summary, we found a 2.2% influenza infectivity rate among wild migrating birds and identified H3N8 viruses circulating in the Mediterranean. We compared three types of bird sampling, fecal, tracheal and cloacal, and found that fecal sampling produces quality RNA for large batch sample screening. Furthermore, we determined that cloacal sampling provided the highest virus identification and tracheal the lowest, suggesting that tracheal may not be the most productive sampling method. These results are important for the understanding of avian influenza movement in wild birds of the Mediterranean and suggest Sardinia to be an important site for avian influenza surveillance.

References

1. Kim JK, Negovetich NJ, Forrest HL, Webster RG (2009) Ducks: the "Trojan horses" of H5N1 influenza. Influenza Other Respi Viruses 3: 121-128. IRV084 [pii];10.1111/j.1750-2659.2009.00084.x [doi].

2. Munster VJ and Fouchier RA (2009) Avian influenza virus: of virus and bird ecology. Vaccine 27: 6340-6344.

3. Zambon MC (1999) Epidemiology and pathogenesis of influenza. J Antimicrob Chemother 44 Suppl B: 3-9.

4. Tong S, Li Y, Rivailler P, Conrardy C, Castillo DA, Chen LM, Recuenco S, Ellison JA, Davis CT, York IA, Turmelle AS, Moran D, Rogers S, Shi M, Tao Y, Weil MR, Tang K, Rowe LA, Sammons S, Xu X, Frace M, Lindblade KA, Cox NJ, Anderson LJ, Rupprecht CE, Donis RO (2012) A distinct lineage of influenza A virus from bats. Proc Natl Acad Sci U S A 109: 4269-4274. 1116200109 [pii];10.1073/pnas.1116200109 [doi].

5. Rohm C, Zhou N, Suss J, Mackenzie J, Webster RG (1996) Characterization of a novel influenza hemagglutinin, H15: criteria for determination of influenza A subtypes. Virology 217: 508-516. S0042-6822(96)90145-2 [pii];10.1006/viro.1996.0145 [doi].

6. Webster RG, Shortridge KF, Kawaoka Y (1997) Influenza: interspecies transmission and emergence of new pandemics. FEMS Immunol Med Microbiol 18: 275-279. S0928-8244(97)00058-8 [pii].

7. Feare CJ and Yasue M (2006) Asymptomatic infection with highly pathogenic avian influenza H5N1 in wild birds: how sound is the evidence? Virol J 3: 96. 1743-422X-3-96 [pii];10.1186/1743-422X-3-96 [doi].

8. Olsen B, Munster VJ, Wallensten A, Waldenstrom J, Osterhaus AD, Fouchier RA (2006) Global patterns of influenza a virus in wild birds. Science 312: 384-388. 312/5772/384 [pii];10.1126/science.1122438 [doi].

9. Alexander DJ (2007) An overview of the epidemiology of avian influenza. Vaccine 25: 5637-5644. S0264-410X(06)01187-X [pii];10.1016/j.vaccine.2006.10.051 [doi].

10. Reed KD, Meece JK, Henkel JS, Shukla SK (2003) Birds, migration and emerging zoonoses: west nile virus, lyme disease, influenza A and enteropathogens. Clin Med Res 1: 5-12.

11. Foti M, Rinaldo D, Guercio A, Giacopello C, Aleo A, De LF, Fisichella V, Mammina C (2011) Pathogenic microorganisms carried by migratory birds passing through the territory of the island of Ustica, Sicily (Italy). Avian Pathol 40: 405-409. 10.1080/03079457.2011.588940 [doi].

12. Horimoto T, Kawaoka Y (2001) Pandemic threat posed by avian influenza A viruses. Clin Microbiol Rev 14: 129-149. 10.1128/CMR.14.1.129-149.2001 [doi].

13. Hubalek Z (2004) An annotated checklist of pathogenic microorganisms associated with migratory birds. J Wildl Dis 40: 639-659. 40/4/639 [pii].

14. Humair PF (2002) Birds and Borrelia. Int J Med Microbiol 291 Suppl 33: 70-74.

15. Kurtenbach K, De MS, Sewell HS, Etti S, Schafer SM, Holmes E, Hails R, Collares-Pereira M, Santos-Reis M, Hanincova K, Labuda M, Bormane A, Donaghy M (2002) The key roles of selection and migration in the ecology of Lyme borreliosis. Int J Med Microbiol 291 Suppl 33: 152-154.

16. Wallace JS, Cheasty T, Jones K (1997) Isolation of vero cytotoxin-producing Escherichia coli O157 from wild birds. J Appl Microbiol 82: 399-404.

17. Hernandez J, Bonnedahl J, Waldenstrom J, Palmgren H, Olsen B (2003) Salmonella in birds migrating through Sweden. Emerg Infect Dis 9: 753-755. 10.3201/eid0906.030072 [doi].

18. Chen J, Deng YM (2009) Influenza virus antigenic variation, host antibody production and new approach to control epidemics. Virol J 6: 30. 1743-422X-6-30 [pii];10.1186/1743-422X-6-30 [doi].

19. Hampson AW and Mackenzie JS (2006) The influenza viruses. Med J Aust 185: S39-S43. ham10884_fm [pii].

20. Suarez DL, Perdue ML, Cox N, Rowe T, Bender C, Huang J, Swayne DE (1998) Comparisons of highly virulent H5N1 influenza A viruses isolated from humans and chickens from Hong Kong. J Virol 72: 6678-6688.

21. Subbarao K, Klimov A, Katz J, Regnery H, Lim W, Hall H, Perdue M, Swayne D, Bender C, Huang J, Hemphill M, Rowe T, Shaw M, Xu X, Fukuda K, Cox N (1998) Characterization of an avian influenza A (H5N1) virus isolated from a child with a fatal respiratory illness. Science 279: 393-396.

22. Zhou NN, Shortridge KF, Claas EC, Krauss SL, Webster RG (1999) Rapid evolution of H5N1 influenza viruses in chickens in Hong Kong. J Virol 73: 3366-3374.

23. 2010 July) WHO Global Alert and Response, GAR: Pandemic H1N1 2009. July 2010. PrFont34Bin0BinSub0Frac0Def1Margin0Margin0Jc1Indent1 440Lim0Lim1http://www.who.int/csr/disease/swineflu/en/ .

24. Girard MP, Tam JS, Assossou OM, Kieny MP (2010) The 2009 A (H1N1) influenza virus pandemic: A review. Vaccine 28: 4895-4902.

25. CDC (2009) Outbreak of swine-origin influenza A (H1N1) virus infection: Mexico, March-April 2009. MMWR Dispatch 58: 1-3.

26. CDC (2009) Update: swine influenza A (H1N1) infections: California and Texas, April 2009. MMWR Dispatch 25: 1-3.

27. World Health Organization (WHO) (2009) New Influenza A(H1N1) virus: global epidemiological situation, June 2009. Weekly Epidemiological Record 25: 249-260.

28. Shortridge KF, Zhou NN, Guan Y, Gao P, Ito T, Kawaoka Y, Kodihalli S, Krauss S, Markwell D, Murti KG, Norwood M, Senne D, Sims L, Takada A, Webster RG (1998) Characterization of avian H5N1 influenza viruses from poultry in Hong Kong. Virology 252: 331-342. S0042-6822(98)99488-0 [pii];10.1006/viro.1998.9488 [doi].

29. Koh G, Wong T, Cheong S, Koh D (2008) Avian Influenza: a global threat needing a global solution. Asia Pac Fam Med 7: 5. 1447-056X-7-5 [pii];10.1186/1447-056X-7-5 [doi].

30. Juckett G (2006) Avian influenza: preparing for a pandemic. Am Fam Physician 74: 783-790.

31. Lam PY (2008) Avian influenza and pandemic influenza preparedness in Hong Kong. Ann Acad Med Singapore 37: 489-496.

32. Comin A, Stegeman A, Marangon S, Klinkenberg D (2012) Evaluating surveillance strategies for the early detection of low pathogenicity avian influenza infections. PLoS One 7: e35956. 10.1371/journal.pone.0035956 [doi];PONE-D-11-22632 [pii].

33. Ferro PJ, Budke CM, Peterson MJ, Cox D, Roltsch E, Merendino T, Nelson M, Lupiani B (2010) Multiyear surveillance for avian influenza virus in waterfowl from wintering grounds, Texas coast, USA. Emerg Infect Dis 16: 1224-1230.

34. Van BS, Steensels M, Ferreira HL, Boschmans M, De VJ, Lambrecht B, van den Berg T (2007) A universal avian

endogenous real-time reverse transcriptase-polymerase chain reaction control and its application to avian influenza diagnosis and quantification. Avian Dis 51: 213-220.

35. Lee MS, Chang PC, Shien JH, Cheng MC, Shieh HK (2001) Identification and subtyping of avian influenza viruses by reverse transcription-PCR. J Virol Methods 97: 13-22. S0166093401003019 [pii].

36. Ip HS, Flint PL, Franson JC, Dusek RJ, Derksen DV, Gill RE, Jr., Ely CR, Pearce JM, Lanctot RB, Matsuoka SM, Irons DB, Fischer JB, Oates RM, Petersen MR, Fondell TF, Rocque DA, Pedersen JC, Rothe TC (2008) Prevalence of Influenza A viruses in wild migratory birds in Alaska: patterns of variation in detection at a crossroads of intercontinental flyways. Virol J 5: 71. 1743-422X-5-71 [pii];10.1186/1743-422X-5-71 [doi].

37. De Marco MA, Campitelli L, Foni E, Raffini E, Barigazzi G, Delogu M, Guberti V, Di TL, Tollis M, Donatelli I (2004) Influenza surveillance in birds in Italian wetlands (1992-1998): is there a host restricted circulation of influenza viruses in sympatric ducks and coots? Vet Microbiol 98: 197-208. 10.1016/j.vetmic.2003.10.018 [doi];S037811350300364X [pii].

38. Comin A, Stegeman JA, Klinkenberg D, Busani L, Marangon S (2011) Design and results of an intensive monitoring programme for avian influenza in meat-type turkey flocks during four epidemics in northern Italy. Zoonoses Public Health 58: 244-251. JVB1343 [pii];10.1111/j.1863-2378.2010.01343.x [doi].

39. Capua I, Marangon S, Cancellotti FM (2003) The 1999-2000 avian influenza (H7N1) epidemic in Italy. Vet Res Commun 27 Suppl 1: 123-127.

40. Capua I, Marangon S (2000) The avian influenza epidemic in Italy, 1999-2000: a review. Avian Pathol 29: 289-294. 713651177 [pii];10.1080/03079450050118403 [doi].

41. De Marco MA, Foni E, Campitelli L, Delogu M, Raffini E, Chiapponi C, Barigazzi G, Cordioli P, Di TL, Donatelli I (2005) Influenza virus circulation in wild aquatic birds in Italy during H5N2 and H7N1 poultry epidemic periods (1998 to 2000). Avian Pathol 34: 480-485. V5325N072X543274 [pii];10.1080/03079450500368185 [doi].

42. European Bioinformatics Institute (2010) http://www.ebi.ac.uk/Tools/clustalw2/.

43. Phipps LP, Essen SC, Brown IH (2004) Genetic subtyping of influenza A viruses using RT-PCR with a single set of primers based on conserved sequences within the HA2 coding region. J Virol Methods 122: 119-122. S0166-0934(04)00239-3 [pii];10.1016/j.jviromet.2004.08.008 [doi].

44. Hoffmann E, Stech J, Guan Y, Webster RG, Perez DR (2001) Universal primer set for the full-length amplification of all influenza A viruses. Arch Virol 146: 2275-2289.

45. Lebarbenchon C, Chang CM, van der Werf S, Aubin JT, Kayser Y, Ballesteros M, Renaud F, Thomas F, Gauthier-Clerc M (2007) Influenza A virus in birds during spring migration in the Camargue, France. J Wildl Dis 43: 789-793. 43/4/789 [pii].

46. Pannwitz G, Wolf C, Harder T (2009) Active surveillance for avian influenza virus infection in wild birds by analysis of avian fecal samples from the environment. J Wildl Dis 45: 512-518. 45/2/512 [pii].

47. Gilbert M, Jambal L, Karesh WB, Fine A, Shiilegdamba E, Dulam P, Sodnomdarjaa R, Ganzorig K, Batchuluun D, Tseveenmyadag N, Bolortuya P, Cardona CJ, Leung CY, Peiris JS, Spackman E, Swayne DE, Joly DO (2012) Highly Pathogenic Avian Influenza Virus among Wild Birds in Mongolia. PLoS One 7: e44097. 10.1371/journal.pone.0044097 [doi];PONE-D-12-05982 [pii].

48. Zhang W, Jiang Q, Chen Y (2007) Evolution and variation of the H3 gene of influenza A virus and interaction among hosts. Intervirology 50: 287-295. 000104788 [pii];10.1159/000104788 [doi].

49. Lang AS, Kelly A, Runstadler JA (2008) Prevalence and diversity of avian influenza viruses in environmental reservoirs. J Gen Virol 89: 509-519. 89/2/509 [pii];10.1099/vir.0.83369-0 [doi].

50. Huang SS, Banner D, Fang Y, Ng DC, Kanagasabai T, Kelvin DJ, Kelvin AA (2011) Comparative analyses of pandemic H1N1 and seasonal H1N1, H3N2, and influenza B infections depict distinct clinical pictures in ferrets. PLoS One 6: e27512. 10.1371/journal.pone.0027512 [doi];PONE-D-11-09202 [pii].

51. Campitelli L, Donatelli I, Foni E, Castrucci MR, Fabiani C, Kawaoka Y, Krauss S, Webster RG (1997) Continued evolution of H1N1 and H3N2 influenza viruses in pigs in Italy. Virology 232: 310-318. S0042-6822(97)98514-7 [pii];10.1006/viro.1997.8514 [doi].

52. Campitelli L, Fabiani C, Puzelli S, Fioretti A, Foni E, De MA, Krauss S, Webster RG, Donatelli I (2002) H3N2 influenza viruses from domestic chickens in Italy: an increasing role for chickens in the ecology of influenza? J Gen Virol 83: 413-420.

53. Tsuji T, Inoue T, Miyama A, Okamoto K, Honda T, Miwatani T (1990) A single amino acid substitution in the A subunit of Escherichia coli enterotoxin results in a loss of its toxic activity. J Biol Chem 265: 22520-22525.

54. Li M, Wang B (2007) Homology modeling and examination of the effect of the D92E mutation on the H5N1 nonstructural protein NS1 effector domain. J Mol Model 13: 1237-1244. 10.1007/s00894-007-0245-0 [doi].

55. Lang V, Marjuki H, Krauss SL, Webby RJ, Webster RG (2011) Different incubation temperatures affect viral polymerase activity and yields of low-pathogenic avian influenza viruses in embryonated chicken eggs. Arch Virol 156: 987-994. 10.1007/s00705-011-0933-z [doi].

Corresponding author
Dr. Alyson Ann Kelvin, PhD.
Scientific Projects Director,
Immune Diagnostics and Research,
Toronto Medical Discovery Tower,
101 College Street, 3rd Floor Room 913
Toronto, Ontario M5G 1L7
Tel: 416-581-7605
Email: akelvin@immunediagnosticsresearch.com

Conflict of interests: No conflict of interests is declared.