Iranian Journal of Virology 2021;15 (2): 82-87
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short communication

Detection of Influenza A viruses in migratory birds at live bird markets of Iran, 2019

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Abstract

Background and Aims: although wild aquatic birds are known to be a significant reservoir for avian influenza viruses (AIV), Live bird markets can become polluted with and become an origin of transmission for avian influenza viruses including the high and low pathogenic strains of avian Influenza (HPAI and LPAI). Many countries affected by the Avian Influenza virus have restricted resources for plans in environmental health, disinfection, and infection control in live bird markets. There are few recently published reports of surveillance directed at this group. Active surveillance for avian influenza (AI) viruses in wild migratory aquatic birds sold at live bird markets (LBMs) was conducted in Iran from October 2019 to February 2020.

Materials and Methods: molecular diagnostic tools were employed for high-throughput surveillance of migratory birds that were sold in the live bird markets of Iran. This study included 400 both cloacal (CL) and nasopharyngeal (OP) samples from two bird species belonging to the two ordersCoot (Fulica arta) (100 CL & 100 OP) and Eurasian teal (Anas crecca) (100 CL & 100 OP). The samples were mainly obtained from captured or hunted birds. Every 5 samples were pooled together.

Results: 1 CL and 3 OP samples of Coots and 2 CL samples of Eurasian teals were positive for the influenza A virus.

Conclusion: These data are useful for designing new surveillance programs and are particularly relevant due to increased interest in avian influenza in wild aquatic birds, and efforts should be made to promote practices that could limit the maintenance and transmission of avian Influenza viruses in Live Bird market.

Keywords: Avian Influenza; aquatic birds; Iran, Live Bird Market

Introduction

Wild migratory aquatic birds such as coot (*Fulica arta*), Euarsian teal (*Anas crecca*), Domestic goose (*Anser anser domesticus)*, Domestic duck (*Anas platyrhynochos domesticus*) and Eurasian woodcock (*Scolopax rusticola*) are the main natural reservoir for avian influenza A virus (AIV) [1]. The most combination of AIV subtypes, based on hemagglutinin (HA) and neuraminidase (NA), have been identified in these reservoirs. The *Anseriformes* and *Charadriiformes* are distributed through the world (except in arid region) and represent a wide range of AIV host species. Initially, influenza viruses infect the lining cells of intestinal tract and then high concentration of viruse excrete in feces.

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Table 1: rRT PCR test results for oropharyngeal (OP) and cloacal (CL) specimens collected during influenza surveillance in live bird markets by host species, Iran, October 2019 to February 2020.

Spices	Specimen OP	Specimen CL	Pooled Specimen OP	Pooled Specimen CL	No. of positive pooled(OP) specimens tested	No. of positive pooled(CL) specimens tested
Coots	100	100	20	20	1 (OP)	3(CL)
Eurasian teals	100	100	20	20	0 (OP)	2 (CL)

The fecal-oral route is the most important way for transmission; which seems to be an efficient way to transmit viruses among the waterfowls (the AIV shedding in feces lead to infect the surface and water). Additionally, some of the wild migratory aquatic birds could be hunted and transported to live bird markets (LBMs). LBMs may elevate the transition, circulating and maintenance of viruses specially zoonose viruse such as AIV [2]. The migratory aquatic birds are brought in LBMs daily, and freshly have been slaughtered.

Different species of these wild bird are stocked at a high density together; So they have sufficient time to transmit the viruses to the others. This issue convert the LBMs to an appropriate conditions for virus maintenance that lead to be a viral resorvire [2].

For detection of influenza virus in public health laboratories, the Reverse transcription–real-time PCR (rRT-PCR) is an important diagnostic tool. In September 2008, for diagnosis of the AIV, the Centers for Disease Control and Prevention (CDC) avian influenza virus rRT-PCR detection and characterization panel (rRT-PCR flu panel) was clarified by the U.S. Food and Drug Administration (FDA), although the gold standard method for AIV detection, isolation and propagation is Embryonated chicken egg inoculation [3].

Due to the role of LBMs in spreading of the AIV and public health importance, in this study, we investigated the presence of AIV by rRT-PCR assay with probes for the rapid screening of wild migratory aquatic birds' samples of LBMs for type A influenza.

Methods

Sample collection: The samples were mainly obtained from captured or hunted birds, Both nasopharyngeal and cloacal swabs were prospectively collected during October 2019 to February 2020 from wild migratory aquatic bird of LBMs at northren provinces of Iran [4]. This study included 400 both cloacal (CL) and nasopharyngeal (OP) samples from two bird species belonging to two ordersCoot (*Fulica arta*) (100 CL & 100 OP) and Eurasian teal (*Anas crecca*) (100 CL & 100 OP). Every 5 samples were pooled together. The sampling site comprised the most important wetlands of Iran, serving as wintering sites for migratory waterbirds. All of the samples had been collected from birds staging in the wetlands along the southern shores of the Caspian Sea at Mazandaran province, which form an important ecological site for wild migratory birds along the Central Asia flyway (Fig. 1).

RNA extraction and cDNA synthesis: Viral RNA was extracted from 200 µl of pooled samples by using the QIAamp Viral RNA Mini Kit. cDNA was then synthesized by using the Fermenas cDNA synthesis kit.

Real-time multiplex and conventional PCR assays: For detecting the presence of Influenza A Virus targeting Matrix gene, using rRT-PCR assay [5]. In this study, the M-gene probe and a pair of primers were used based on a conserved region in the matrix gene of influenza A virus [6].



Fig .1. Location of Mazandaran province, Iran. Where aquatic birds were tested for AIV.

Results

In the results of the fluorescent signals of the rRT-PCR, we considered the results from cycle threshold (Ct) 15 up to 40 as a positive sample; so each obtained results under Ct 15 or greater than Ct 40 estimated as a negative sample. The negative results obtained in this study was Ct 48 ± 1.3; While one results obtained of CL Coots samples was Ct 22 and the results of OP samples of Coots were Ct 25, that considered as positive samples. The positive results of the Eurasian teals were Ct 20 for CL samples. Briefly, 1 CL and 3 OP samples of Coots and 2 CL samples of Eurasian teals were positive for the influenza A virus. The sequencing of the M gene product confirmed the procedure (Table .1).

Discussion

Based on our results, we detected influenza A virus in wild aquatic birds traded in those LBMs in Iran. Influenza A viral RNA was detected in both Coots and Eurasil teal. In our study, the overall influenza A RNA detection rate among the birds sampled was 30% Surveillance studies on influenza viruses had recorded a variable prevalence of AIVs in birds which were traded in LBMs in different countries of world. Earlier, some researchers implemented several studies to isolated the AIV from different areas in Iran. For example, at 2010 a study was done by Fereidouni, S. R., et *al*. to determine the status of AIV infections in many different species of aquatic birds in Iran in 2003-2007. They implemented virological, molecular and serological examination in their study. They collected oropharyngeal and cloacal swab of all 1146 birds of 45 different species such as ducks, coots and shorebirds of 6 different provinces of most important wintering sites of migratory waterbirds. They demonstrated that 3.4% of the samples was positive for AIV. Among the samples, Mallard and Common Teal were indicated the highest number of positives results. They suggested

that these two species may have an important role in the preserving of AIV in that regions [7]. Migratory waterfowl is a major reservoir for AIV; To investigate the AIV antibody status in migratory waterfowl of Iran, some researchers collected 217 serum samples from 25 different species of waterfowl during 2003 and 2004. They were tested the serum samples by a competitive ELISA. They achieved to 77 positive samples (35.5%) from 14 different species. The seroprevalence of antibodies against type A influenza viruses was signify-cantly higher in Anseriformes (64%) than in Non-Anseriformes (12%) and in total birds (35.5%). They demonstrated that mallards, which presence in large numbers in winter in Iran, showed 87.5% positive reactions; The mallards might play an important role in the epidemiology of AIV in the sampling area, one of the most important wetlands of Iran, Ramsar Province [8]. Backyard poultry flocks chickens like the wild birds, could play an important role in the spread of the virus among industrial poultry; that can leading to considerable economic losses. To survey the newcastle disease virus (NDV) and AIV in the unvacci-nated backyard poultry in Bushehr province, Iran; Yousef, S., et al. (2014) implemented the HI test for antibodies against NDV and AIV (H9N2) in a total of 1530 blood samples, during 2012 to 2013. They observed that 614 (40.13%) and 595 (39.00%) were positive for NDV and AIV (H9N2) respectively [9]. A study in bird Parks of Tehran showed 14% of samples were positive for AIV. They were isolated from ducks and sparrows fecal samples [10].

In a study, researchers investigated in 50 domestic pigeons in Kavar area of Fars province, Iran. They collected and evauated the blood and faecal samples using HI and RT-PCR methods, respectively. Their results showed that any virus genome of AIV was not detected in faecal samples but 17 serum samples (34%) had antibody titres ≥ 2–5 against the H9N2 AI virus. They demonstrated that considerable percentage of domestic pigeons in the Kavar area were seropositive for AIV [11]. Another study in Fars Province, Iran, Hadipour, M. (2010) was propagated the determination of the seroprevalence of H9N2 AIV in in different human populations. They performed the HI test in 300 sera in five different population including workers of poultry farms, workers of slaughter house, veterinarians, patients who show clinical signs of respiratory disease, and clinically normal individuals, who were not or rarely in contact with poultry. They measured the antibodies against H9N2 AIV. They indicated the higher prevalences in poultry farm workers, slaughter house workers, and veterinarians. They suggested that this high prevalences in this populations was for their close and frequent contact with poultry [12].

LPAI epidemic (H9N2) occurred in the Iranian poultry industry, Since 1998; that caused mortality in broiler chicken farms. In a study in commercial chicken flocks in Dezful, southern Iran, researches were designed to investigate the prevalence of AIV H9N2 subtype. They were examined HI test for specific antibodies against AIV H9N2 subtype, in 160 broilers of 8 broiler flocks. They observed 7.3% of HI titre in the results [13]. In an investigation, in total of 310 blood samples were collected from 25 broiler flocks in slaughterhouses of West Azarbayjan, Iran. HI tests was implemented.

They observed high prevalence of AIV anti-bodies in serum of birds; The test showed 40.6% of srea were positive. They investigated that AIV has an important role in respiratory complexes in broiler chickens in this region, and probably throughout Iran [14]. In 2002, VASFI, M. M. and M. M. BOZORG (2002) indicated that non-highly pathogenic AIV strains (H9N2) were circulated in the Iranian layer, breeder and broiler flocks [15]. An epidemic of H9N2 AIV occurred in broiler chicken farms in Iran during 1998–2001. In that occurrence, the researchers observed that the mixed infections of AIV with with other respiratory pathogens, particularly infectious bronchitis virus and *Mycoplasma gallisep-ticum*, were thought to be responsible for such higher mortality, which resulted in great economic losses [16].

In a study conducted in Korea in 2003, 6% of chicken specimens were positive for AIVs [17], whereas 31% of ducks and 6.1% of and

geese samples were positive for AIV in Vietnamese markets in 2001 [18]. Also, the AIV was isolated from a LBM in an investigation in Guangdong Province in southern China. The researchers analyzed the complete genome of isolated strain [19]. In Kenya (2013) influenza A viruses circulated in birds population such as geese, turkeys, and chicken in LBMs [20]. A study in the USA elucidated that a HPAI was detected in 1.3% of hunted wild birds in the Pacific Flyway of the United States. The authors demonstrated that the AIV may detected in apparently healthy wild waterfowl without obvious clinical disease, which it showed that waterfowl species are susceptible to infection [21].

Because of HPAI outbreaks in poultry, we have to find their origin in LPAI present in waterfowl. Influenza A virus surveillance in wild birds could help to monitoring protocol of HPAI outbreaks for diagnostic purposes. It would also be helpful for representing the HPAI pandemic threats. Munster, V. J., et al. (2007) isolated HPAI of subtypes H5 and H7 from Mallards (*Anas platyrhynchos*) in northern Europe [22]. Another study showed that HPAI was isolated from common coots in Egypt, and it introducted into the Africa by migratory birds. In this study, they collected 19 oropharyngeal and cloacal swab samples from wild birds. Two samples of common coots were positive for AIV; The samples was indicated H5N8 subtype [23].

Researchers in Nigeria, collected samples from ducks (*Anas platyrhynochos domesticus*) to servey the presence of AIV. They observed that 13% of samples positive for avian influenza A virus [24]. In eastern Germany, some researchers isolated AIV A from aquatic birds. They directly obtained the isolation from wild ducks, feral ducks and white Pekin ducks. They observed considerable variability among species [25]. An investigation in southeastern Australia revealed LPAI was isolated from Chestnut teals (*Anas castanea*) which is a resident aquatic bird [26].

Conclusion

In the present study, we demonstrated that influenza A viruses regularly circulate in LBMs in Iran. Ongoing monitoring of AIV in birds of LBMs could helpful in detection of new isolation of AIVs in the birds population; It can have important role in public health and socioeconomic significance for the poultry industry and humans. Early detection of new potentially dangerous isolation of AIV could be useful for controlling protocols. We have to sequence the genome of circulating AIV in LBMs in the further study to help the further investigations of the epidemiology and molecular characteristics of AIV.

Acknowledgment

The authors would like to thank the PCR Veterinary Diagnostic Laboratory and Gahlyanchi Laboratory experts for their technical support.

Conflict of interest

The authors declare that they have no conflict of interest.

Funding

There are no undisclosed sources of funding for either our research or our manuscript itself.

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