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Molecular Detection of Influenza A virus in Domestic Ducks and Geese in Basra Province, Southern Iraq

Firas T. Mansour* Hazim T. Thwiny* Harith A. Najim**

*Department of Microbiology – College of Veterinary Medicine – University of Basra
**Department of Pathology and Poultry Diseases – College of Veterinary Medicine – University of Basra

***Email:** Dr.frass77@gmail.com

Abstract:

Avian influenza is one of the important viral diseases in poultry that can cause serious economic losses in many countries around the world, and occasionally infect human causing mild to severe illness. It is caused by avian influenza A viruseswhich have been isolated from avian species, particularly waterfowls that are considered the main reservoir for all influenza A virus subtypes. The aim of this study was to detect influenza virus from domestic ducks and geese in different geographical regions(Abu Al-Khasseb, Shatt Al-Arab, Zubair, and Al-Qurnah) of Basrah province, Southern Iraq. The study was conducted on 115 cloacal swabs collected from 60 ducks and 55 geese distributed in different regions of Basrah province. Samples were processed for RT-PCR to amplify matrix (M) gene that is conserved between all influenza A subtypes by using a set of universal primer. We demonstrated that influenza A viruses is prevalent in domestic waterfowls with a significantly higher percentage in ducks than geese; 42 samples (29 ducks and 13geese) were positive to viral M gene. In addition, the virus prevalence was significantly higher in the North of Basrah (Al-Qurnah region) than the other geographical regions. The overall findings of this study obtained a primary picture about the distribution of avian influenza viruses in our region, which is importantto prevent the spread of infection to poultry and eventually minimize the risk of acquiring infection to humans. According toour knowledge, this is the first study aimed to identify avian influenza viruses in waterfowls in Basrah province, Southern Iraq.

Key words: Influenza A virus, duck, geese, polymerase chain reaction.

1. Introduction:

Influenza A viruses infect a wide range of domestic and wild birds including aquatic birds, chicken, and turkey causing dangerous outbreaks with high economic losses[Alexander,2000]; and some mammals such as pigs, horses, and humans causing mild to severe infections[Herfest et al., 2014]. They belong to the *"Orthomyxoviridae"* family and they have segmented RNA genome with surface glycoprotein spikes [Bouvier and Palese, 2008]. The genome of the virus is organized into 8 segments of different lengths, which are the polymerase basic (PB1 and PB2), the polymerase acidic (PA), hemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix (M), and non–structural (NS) genes[[Bouvier and Palese, 2008]. They are subdivided into subtypes based on the two types of envelope proteins, hemagglutinin (HA) and neuraminidase (NA)[Shtyrya, Mochalova, and Bovin, 2009]. Currently, there are 18 different HA subtypes (H1 through H18) and 11 different NA subtypes (N1 through N11),so, in theory, 198 different combinations of these proteins are possible [Tong, et al. 2013]. Influenza A viruses in birds are further classified into two pathotype groups: high pathogenic avian influenza A viruses (HPAI) and low pathogenic avian influenza A viruses (LPAI) based on the severity of the disease in the infected birds[Rebel et al., 2011].

Aquatic birds, in particular wild mallard ducks, are considered the main reservoir for most influenza A virus subtypes (H1-H16 and N1-N9) [Olsen et al., 2006]; and more recently, virus subtypes H17N10 and H18N11 have been identified in fruit bats [Rebel et al., 2011; Tong et al., 2012].In waterfowls, the main transmission route in waterfowl is oro-faecal [Vandigi et al., 2017]; and avian influenza viruses replicate in the

intestinal mucosa and are excreted at high concentrations from the cloaca into water [Slemons and Easterday, 1978].Avian influenza virus strains are stable in water [Webster et al., 1992; Stallknecht et al. 1990] and have been isolated from the surface of ponds containing a large number of waterfowl [Hinshow, Webster and Tumer 1979; Hinshow, Webster and Tumer 1980]. Ducks show no clinical signs following infection with low pathogenic virus strains;and rarelythey show subclinical sings following infection with certain subtypes of high pathogenic virus strains[Kim et al. 2009]. They might spread the infection to domestic poultry flocks such as chicken and turkey causing serious infection with high mortality rates reach to 100%[Akpinar and Saatci, 2006]. In addition, mammals including humans are occasionally infected with the virus upon exposure to infected domestic poultry[Peiris, et al., 2007].Human infection with avian influenza subtypes is usually associated with mild to severe disease. Therefore, the reservoirs of these viruses should be identified to prevent infection of poultry and eventually minimize the risk of acquiring infection to humans[Verhagen, et al., 2017].

The aim of this study was to detect influenza A viruses in domestic ducksand geese populations that are in close contact with the chicken and human. This is important to obtain a picture of avian influenza virus prevalence in our region.

2. Materials and methods:

2.1 Sample collection:

The study was conducted from November 2016 to March 2017.A total of 115 cloacal swabswere collected from 60 domestic ducks and 55 domestic geese live in backyardsof four different geographical regions in Basra governorate: Abu Al-Khasseb (17 duck and 16 geese), Shatt Al-Arab (12 duck and 12 geese),Zubair (13 duck and 12 geese), and Al-Qurnah (18 duck and 15). Cloacal samples were taken from each bird, and each sample was collected in a sterile phosphate buffer saline (PBS). Samples were kept on ice during collection and then shipped to the laboratory immediately. They were centrifuged at 1000 xg for 10 minutes, and the supernatants were gently collected and moved to new-labelledtubes. They were then directly prepared for viral RNA extraction.

2.2 Viral RNA extraction and quantification:

Viral RNA extraction was performed in the laboratory by using QIAamp viral RNA extraction kit (Qiagen, Germany) following the manufacturer's instructions.The concentration of purified RNA was determined using NanoDrop spectrophotometer by UV absorption. Eluted viral RNA samples were either processed directly for RT-PCR or preserved at –20˚C until further use.

2.3 Real time-Polymerase chain reaction:

Viruses were detected byperforming RT-PCR assay that targeted influenza Matrix (M) gene using a set of universal primers. A forward primer: 5′-AGA TGA GTC TTC TAA CCG AGG TCG-3′, and a reverse primer: 5′-TGC AAA AAC ATC TTC AAG TCT CTG-3′ mentioned by[19]were used to amplify a 101 bp fragment of M gene. Samples were amplified using one-step conventional RT-PCR kit (Bioneer, South Korea) following the manufacturer's instructions. The RT–PCR conditions were as follows: cDNA synthesis at 55˚C for 30 minutes, initial denaturation at 95˚C for 5 minutes followed by 35 cycles of: denaturation at 95˚C for 30 seconds, annealing at 60˚C for 30 seconds, extension at 72˚C for 1 minute. The reaction was then held at 72˚C for 5 minutes. The amplified PCR product was then detected using 1.5% agarose gel prepared with agarose (Promega) in TBE buffer stained with ethidium bromide. The size of the band was determined by comparison with a standard 100 bp DNA ladder (New England Biolabs).

2.4 Statistical analysis:

Statistical package for social science (SPSS) was used to analyze the data, and Chi-square (X^2) test was used to assess the significance between groups. P value ≤ 0.05 was considered to be statistically significant.

3. Results:

The current study revealed that the total percentage of infection with Influenza A virus in domestic ducks and geese was 36.5% (42/115). The prevalence of infectionin ducks, which was 48.3% (29/60) was significantly higher ($P<0.05$) than domestic geese which was 23.6% (13/55), Table 1.

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Table (1): Percentages of infection with influenza A virus in domestic ducks and geese

Type of bird	NO. of samples taken	NO. of positive samples	Percentage of infection
Domestic duck	60	29	48.3%
Domestic geese	55	13	23.6%
Total	115	42	36.5%

(P<0.05)

With regard to the geographical distribution, the highest virus prevalence was reported in Al-Qurnah region,which was 66.66% (12/18) in domestic ducks and 40% (6/15) in domestic geese; and the average percentage of prevalence of both birds was 54.54% (18/33). The other three geographical regions showed similar percentages of virus prevalence ranged from 38.4% to 41.1% in domestic ducks, and 16.6% to 18.7% in domestic geese, which were significantly lower than Al-Qurnah region (P<0.05)(Table 2).

Table (1): Percentages of Infection with influenza A virus in domestic ducks and geese at the study areas.

Geographical regions	Domestic ducks			Domestic geese		
	NO. of samples taken	NO. of positive samples	Percentage of infection	NO. of samples taken	NO. of positive samples	Percentage of infection
Abu Al-Khasseb	17	7	41.1%	16	3	18.7%
Shatt Al-Arab	12	5	41.6%	12	2	16.6%
Zubair	13	5	38.4%	12	2	16.6%
Al-Qurnah	18	12	66.66%	15	6	40%
Total	60	29	48.3%	55	13	23.6%

(P<0.05)

The RT-PCR results showed that matrix (M) gene was successfully amplified from faecal samples of domestic ducks and geese. Single and clear bands of 101 bp were clearly visualized following the load of the PCR product on 1.5% agarose gel. The expected size of the band was determined by comparison with 100 bp DNA ladder (Figure 1).

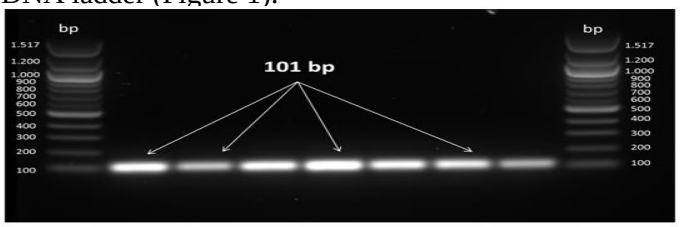

Figure 1: PCR product of partial matrix (M) gene of influenza A virus on 1.5% agarose gel stained with ethidium bromide. The results showed the amplification of 101 bp from faecal specimens collected from domestic ducks and geese.

4.Discussion:

In this study, avian influenza A virus was detected in domestic duck and geese in different geographical regions of Basrah province, southern Iraq. Although all tested samples were collected from healthy birds, showed positive results on agarose gel. According to the RT-PCR results, the general percentages of virus prevalence were significantly higher in ducks than geese. In addition, according to the geographic distribution of birds, the highest virus prevalence was observed in Al-Qurnah region, which is located in the north part of Basrah province.

Although the serological diagnostic methods such as rapid detection test, haemagglutination inhibition, and complement fixation tests are commonly used to confirm the infection with the influenza virus[Allwinn et al., 2002], the molecular diagnostic methods such as polymerase chain reaction-based strategy are more accurate and has been successfully used for detection of many pathogens including influenza A viruses [Yacoup et al. 2009; Tagayama et al., 2015]. In the current study, PCR technique was performed to identify viruses using a set of universal primers of M gene, which can be used to amplify all influenza virus subtypes in a single enzymatic reaction. Waterfowls are the reservoir of the majority of avian influenza A subtypes; and it has been suggested that these birds are the source of avian influenza outbreaks in domesticated poultry including chicken, turkey, ducks, and geese [Behane et al., 2009; De wet et al., Smith et al., 2015]. In addition, ducks and other waterfowls support viral replication in the intestinal tract without any outward clinical signs of infection [Smith et al. 2015]. Therefore, in this study, we chose two types of domesticated waterfowls (ducks and geese), which are more contact with human and other domesticated poultry in order to assess the distribution of avian influenza viruses in these birds.It is very important to obtain a picture of virus prevalence in the resistant birds (duck and geese) because, after disease transmission to other poultry, the viruses might become highly pathogenic and replicate systemically in the chicken causing severe disease with high mortality rate [Alexander, 2000]. Moreover, poultry might spread the infection to human causing severe outbreak and sometimes death [Jourdain et al., 2010].

The current results showed that the prevalence of avian influenza viruses was higher in ducks than geese. This is agreed with many studies

confirm that ducks are the natural hosts for all influenza A viruses [Kim et al., 2009;Jourdain et al., 2010;Groit and Hoop, 2007] in comparison with geese, which play a minor role as a reservoir for the virus[Harris et al., 2010]. In addition, the proportion of infection was similar in three geographical regions (Abu Al-Khasseb, Shatt Al-Arab, and Zubair) while it was higher in Al-Qurnah region, which is located in the north part of Basrah province. This can be attributed that Al-Qurnah is the nearest region to marshes, whichform an important region for breeding, and wintering of wild birds. During the winter (from November through February), marshes in the South of Iraq are shared by large number of migratory waterfowl and domestic waterfowl. As a result, the domestic waterfowl might acquire avian influenza viruses from the migratory waterfowls and might act as a natural reservoir of avian influenza viruses without showing clinical disease. Therefore, domestic waterfowl may play a major role in the ecology of avian influenza viruses in the South of Iraq and may act as potential vessels for their genetic reassortment and thus demand active surveillance.

In this study, all samples were collected from domestic ducks and geese that are in contact with other domestic poultry and human. Therefore, it is highly recommended to detect viruses from the other expected hosts using RT-PCR technique, or by detection of antibodies such as IgY and IgG in the domestic poultry and human, respectively. This will obtain a clearer image regarding to the possibility of transmitting the disease from waterfowls to other hosts. Furthermore, virus subtyping should not be overlooked. This can be achieved by designing specific primers for the most common virus subtypes such as H5, H7, and H9, which play a critical role in induction of severe outbreaks in the domestic poultry as well as the human.

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