Bulgarian Journal of Veterinary Medicine, 2020, **23**, No 4, 456–466
ISSN 1311-1477; DOI: 10.15547/bjvm.2019-0016

Original article

MOLECULAR SURVEILLANCE OF AVIAN INFLUENZA A VIRUSES IN BASRAH AND WASIT, IRAQ

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Summary

Al-Badry, M. & F. Al-Mubarak, 2020. Molecular surveillance of avian influenza A viruses in Basrah and Wasit, Iraq. *Bulg. J. Vet. Med.,* **23**, No 4, 456–466.

The aim of this study was to detect influenza A virus in broiler chickens and wild ducks in different geographical regions of Basrah and Wasit provinces, Iraq. This study was authenticated by analysing the viral genome and designing a set of universal primers for the detection of all influenza A subtypes in a single enzymatic reaction through the amplification of a highly conserved region of viral M gene. A total of 157 and 155 oropharyngeal and cloacal swabs from broiler chickens and wild ducks, respectively, were analysed. The study shows that influenza A viruses were prevalent in these birds in all study regions with a significantly higher percentage in wild ducks compared to broiler chickens. The results showed that 92/157 samples (54/75 wild ducks and 38/82 broiler chickens) and 96/155 samples (38/75 wild ducks and 58/80 broiler chickens) in Basrah and Wasit, respectively, were positive for the viral M gene. In addition, there was no significant difference in virus prevalence between Basrah and Wasit provinces.

Key words: Basrah, chicken, duck, influenza A virus, RT-PCR, Wasit

INTRODUCTION

Influenza A viruses, which belong to the family *Orthomyxoviridae,* are enveloped with surface glycoprotein spikes and their genome consists of 8 segments of negative-sense single-stranded RNA (Samji, 2009). These viral segments are the polymerase basic 1 and 2 (PB1 and PB2), the polymerase acidic (PA), haemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix (M), and non–structural (NS) genes (Bouvier & Palese, 2008). Each gene encodes at least one protein to build up the complete virus particle. Influenza A viruses are subdivided into subtypes based on the two types of envelope proteins, haemagglutinin (HA or H) and neuraminidase (NA or N) (Van der Auwera *et al.*, 2014). Currently, there are 18 different HA subtypes (H1 through H18) and 11 different NA subtypes (N1 through N11) (Tong *et al.*, 2013). Avian influenza viruses are further classified into two groups based on the severity of the disease: high pathogenic avian influenza A viruses (HPAI) and low pathogenic

avian influenza A viruses (LPAI) (Swayne & Suarez, 2000).

Influenza A viruses infect a wide range of wild and domestic birds including wild and domestic waterfowl, chicken, and turkey causing serious outbreaks with high economic losses; and some mammals such as pigs, horses, dogs, and humans causing mild to severe infections (Peiris *et al.*, 2007; Pohlmann *et al.*, 2017). Typically, the viruses are transmitted from infected mammals through the air by sneezing or coughing (Herfst *et al.*, 2014), and from infected birds through their droppings (Achenbach & Bowen, 2011).

Migratory aquatic birds, in particular mallard ducks, are considered the main reservoir for the majority of influenza A virus subtypes (H1–H16 and N1–N9) (Germundsson *et al.*, 2010); and more recently, virus subtypes H17N10 and H18N11 have been identified in fruit bats (Tong *et al.*, 2012; 2013). The main route of transmission between waterfowl is orofaecal (van Dijk *et al.*, 2018); and the viruses replicate in the intestinal mucosa and are excreted at high concentrations from the cloaca into water (Slemons & Easterday, 1978). Ducks can harbour the infection with the virus without showing apparent clinical signs; and they rarely show subclinical signs following infection with certain subtypes of HPAI strains (Coker *et al.*, 2014). In addition, they might spread the infection to commercial poultry flocks, such as chicken causing serious infection with high mortality rates, which might reach 100% (Singh *et al.*, 2018). Moreover, mammals including humans are occasionally infected with the virus upon exposure to infected domestic poultry, which is usually associated with mild to severe disease (Peiris *et al.*, 2007).

Laboratory diagnosis of influenza virus has been achieved by the detection of nucleic acid by reverse transcriptase polymerase chain reaction (RT-PCR) along with different serological diagnostic tests (complement fixation (CF), haemagglutination inhibition (HI), and neutralisation tests). These tests have different sensitivity rates with some advantages and disadvantages (Dwyer *et al.*, 2006). Nucleic acid testing using RT-PCR is usually more specific and sensitive and is not time consuming. This test provides precise detection, and can be used for typing and subtyping of influenza A viruses (Ellis *et al.*, 1997). However, the frequent genetic changes of influenza viruses may result in a false-negative diagnosis of influenza A virus infection by performing the current protocols for PCR analysis (Trevino *et al.*, 2011). Therefore, using an updated PCR protocol for influenza virus detection would be valuable for virus surveillance and screening.

The aim of this study was to conduct a molecular survey on influenza A virus distribution in broiler chickens and wild ducks in Basrah and Wasit provinces, Iraq by determining the highly conserved region of influenza A virus genome using gene analysis software to design a pair of universal primers for detection of all influenza A subtypes in a single enzymatic reaction.

MATERIALS AND METHODS

Design of universal primers

In this study, a pair of universal primers was designed for the detection of all influenza A subtypes in a single enzymatic reaction. The complete nucleotide sequences of viral M gene were obtained from the influenza database, which is available online (www.fludb.org).

Selected nucleotide sequences of viral M gene among several strains of influenza

A viruses of different host species were used to design the universal primers by aligning them together to determine the highly conserved regions using Geneious Inspirational Software for Biologists (www.geneious.com). The site of forward and reverse primers was then determined by observing the similarities between nucleotides of the selected virus strains (Fig. 1).

The primers were designed using National Center for Biotechnology Information (NCBI) software (www.ncbi.nlm.nih. gov). The quality of the forward and reverse primers was then checked using another software program, which is Oligoanalyzer tool – Integrated DNA Technologies (www.idtdna.com). Forward primer ATCGTCGCYTTAAATACGGT and reverse primer CGTCAACATC CACAGCAYTC were designed to amplify a partial fragment (108 base pair) of viral M gene. They were supplied by Bioneer Corporation – Daejeon, Korea, Republic of South Korea.

Sample collection

The study was conducted from October 2017 to April 2018. A total of 157 and 155 oropharyngeal and cloacal swabs from broiler chicken and wild duck, respectively, were analysed. The tested samples were collected from healthy ducks and chickens suffering from respiratory disorders. Samples were collected from live bird markets of four different geographical regions in Basrah (82 broiler chickens and 75 wild ducks), and Wasit (75 broiler chickens and 80 wild ducks) provinces. The geographical regions included in both provinces and number of samples taken are shown in Table 1.

Each sample was collected in viral transport medium: sterile phosphate buffer

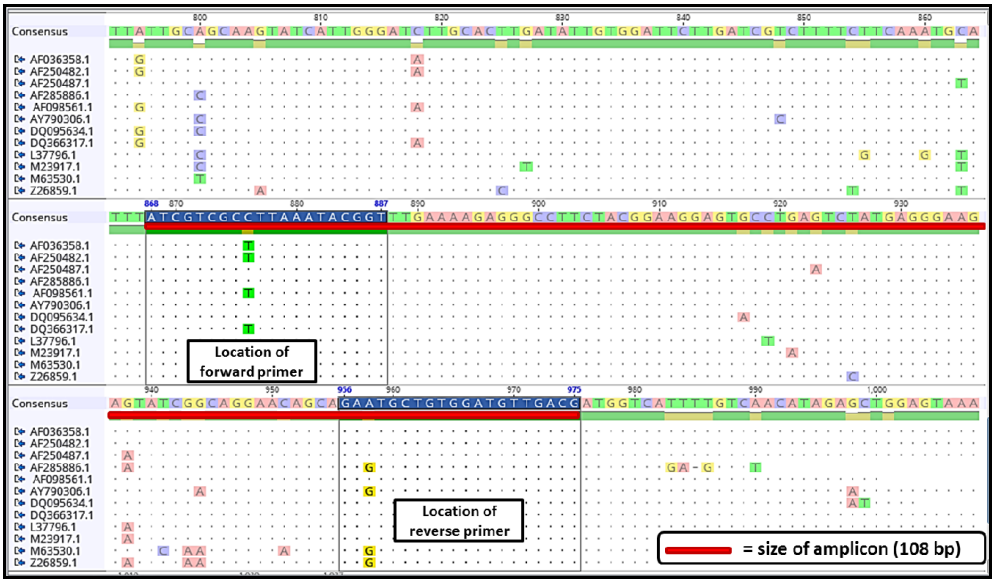

Fig. 1. Nucleotide sequences with their accession numbers of viral M gene of different host species were aligned to determine the highly conserved areas that were used for designing the PCR primers. The forward and reverse primers (highlighted in blue) are located between the nucleotides 868 to 887 and 956 to 975, respectively. The red line determines the expected amplicon size (108 bp).

Table 1. Geographical regions, types of birds, and number of samples used in the study

Geographical regions/Basrah	Wild ducks	Broiler chickens	Geographical regions/Wasit	Wild ducks	Broiler chickens
1. Abu Al-Khasseb	18	22	1. Al-Kut city	22	23
2. Shatt Al-Arab	20	20	2. Al-Hay	13	20
3. Zubair	17	20	3. Nomanea	24	18
4. Al-Qurnah	20	20	4. Sheikh Saad	21	14
Total	75	82	Total	80	75

saline (PBS) with glycerol (1:1). Samples were kept on ice during collection and then shipped to the laboratory immediately. They were centrifuged at 1000×*g* for 10 minutes, and the supernatants were gently collected and transferred to new-labelled tubes. They were then directly prepared for viral RNA extraction.

Viral RNA extraction and quantification

Viral RNA was extracted in the laboratory using QIAamp viral RNA extraction kit (Qiagen, Germany) following the manufacturer's instructions. The concentration of the purified RNA was determined using a NanoDrop spectrophotometer by UV absorption. The eluted viral RNA was then directly performed to RT-PCR.

Reverse transcriptase polymerase chain reaction (RT-PCR)

Viral nucleic acid was detected by performing RT-PCR assay that targeted influenza Matrix (M) gene using the designed universal primers. The viral M gene was amplified using one-step RT-PCR kit (Bioneer, South Korea) following the manufacturer's protocol. Both cDNA synthesis and PCR amplification were performed in a single tube using this system. Starting material of viral RNA used in cDNA synthesis was 100 ng/μL.

Two negative control samples (without RNA template, or without primers) were prepared in each PCR run along with one positive control which was represented by using viral RNA extracted form Gallimune inactivated vaccine against Newcastle disease and avian influenza (ND+Flu H9 M.E., Merial, France. The tube was capped and then placed in a thermal cycler. The following RT–PCR conditions were employed: cDNA synthesis at 45 ^{o}C for 30 minutes, initial denaturation at 95 ^{o}C for 5 minutes followed by 35 cycles of: denaturation at 95 ^{o}C for 30 seconds, annealing at 58 ^{o}C for 30 seconds, and extension at 72 ^{o}C for 1 minute. The reaction was then held at 72 ^{o}C for 5 minutes, and then cooled down at 4 ^{o}C for 5 minutes. The PCR product was detected using 2% agarose (Promega, USA) prepared with agarose in TBE buffer stained with ethidium bromide. The size of PCR products was determined by comparison with 100 bp DNA ladder.

Statistical analysis

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

RESULTS

Detection of influenza A virus by RT-PCR

The results of RT-PCR showed that a partial fragment of matrix (M) gene was suc-

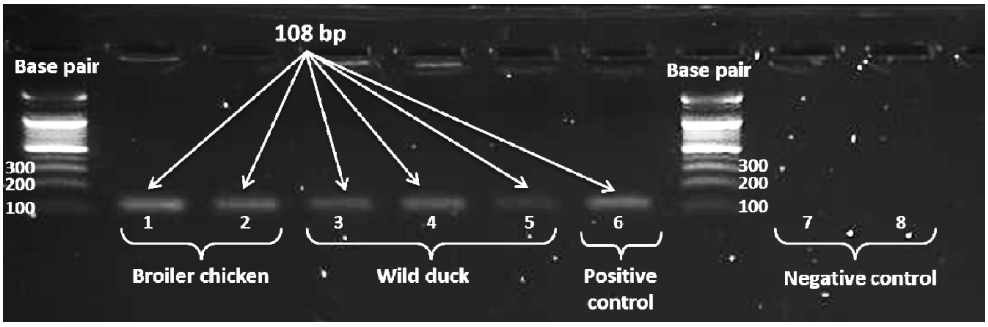

Fig. 2. PCR product of partial matrix (M) gene of influenza A virus on 2% agarose gel stained with ethidium bromide. Amplification of 108 bp from oropharyngeal and faecal specimens collected from broiler chickens (lane 1 and 2), wild ducks (lanes 3–5), and positive control (lane 6), respectively. Negative controls (lane 7 and 8) did not show any evidence of amplification.

Table 2. Percentages of infection with influenza A virus in wild ducks and broiler chickens in Basrah and Wasit province

Geographical regions	Type of bird	Number of samples taken	Number of positive samples	Percentage of infection
Basrah province	Wild duck	75	54	72.0%*
	Broiler chicken	82	38	46.3 %
	Total	157	92	58.5%
Wasit province	Wild duck	80	58	72.5%*
	Broiler chicken	75	38	50.6%
	Total	155	96	61.9%

* P<0.05 between wild ducks and broiler chickens in the respective province.

cessfully amplified from oropharyngeal swabs of broiler chickens and cloacal swabs of wild ducks, and from viral RNA extracted from Merial influenza vaccine. Single and clear bands of 108 bp were clearly visualised following the load of the PCR product on 2% agarose gel. The expected band size was determined by comparison with 100 bp DNA ladder (Fig. 2).

Avian influenza virus infection in Basrah province

The overall results obtained in Basrah province revealed that the total percentage of infection with influenza A virus in broiler chickens and wild ducks was 58.5% (92/157). The prevalence of infection in wild ducks was 72% (54/75), which was significantly higher (P<0.05) than that of broiler chicken which was 46.3% (38/82) (Table 2).

With regard to the geographical distribution, the highest virus prevalence was reported in Al-Qurnah region which was 75% (15/20) in wild ducks and 60% (12/20) in broiler chicken; and the average percentage of prevalence in both birds was 67.5% (27/40). The percentages of virus prevalence in wild ducks of the other three geographical regions were not significantly different from the Al-Qurnah region (70% to 72.2%) in wild ducks, but they were significantly lower (P<0.05) in broiler chicken ranging from 35% to 45.4% (Table 3).

Table 3. Percentages of infection with influenza A virus in wild ducks and broiler chicken at the study areas of Basrah and Wasit provinces

Geographical regions	Wild ducks			Broiler chickens		
	Number of samples	Positive samples	Infection percentage	Number of samples	Positive samples	Infection percentage
Basrah province						
Abu Al-Khasseb	18	13	72.2%	22	10	45.4%
Shatt Al-Arab	20	14	70.0%	20	7	35.0%
Al-Zubair	17	12	70.5%	20	9	45.0%
Al-Qurnah	20	15	75.0%	20	12	60.0%*
Total	75	54	72.0%	82	38	46.3%
Wasit province						
Al-Kut city	23	15	65.2%	23	13	56.5%
Al-Hay	13	9	69.2%	20	9	45.0%
Nomanea	24	18	75.0%	18	9	50.0%
Sheikh Saad	21	16	76.2%	14	7	50.0%
Total	80	58	72.5%	75	38	50.7%

* P<0.05 in wild ducks from Al-Qurnah region compared to the other 3 regions in Basrah province.

Avian influenza virus infection in Wasit province

The overall results obtained in Wasit province revealed that the total percentage of infection with influenza A virus in broiler chickens and wild ducks was 61.9% (96/155). The prevalence of infection in wild ducks was 72.5% (58/80), which was significantly higher (P<0.05) than that in broiler chickens (50.6%; 38/75) (Table 2).

With regard to the geographical distribution, the highest virus prevalence in broiler chickens was reported in Al-Kut city, which was 56.5% (13/23); while in wild ducks, the highest virus prevalence was reported in Sheikh Saad region, which was 76.1% (16/21). The percentages of virus prevalence in broiler chicken of the other three geographical regions were not significantly different f rom Al-Kut city (45% to 50%), and also in wild ducks, virus prevalence of the other three geographical regions were not significantly different from Sheikh Saad region (68.1% to 75%) (Table 3).

Table 4. Percentages of infection with influenza A virus in wild ducks and broiler chicken in Basrah and Wasit provinces.

Province	Percentage of infection	
	Wild ducks	Broiler chickens
Basrah	72.0% (54/75)	46.3% (38/82)
Wasit	72.5% (58/80)	50.6% (38/75)

The results of this study showed that there was no significant difference between the percentages of infection in Basrah and Wasit provinces in wild ducks and broiler chicken (Table 4).

DISCUSSION

Many studies have demonstrated that wild ducks are considered the main reservoir for all influenza A virus subtypes and that they can transmit the infection to domestic poultry, in particular chickens. In addition, ducks are usually resistant to infection in comparison to chickens, which are often susceptible (Kim *et al.*, 2009; Tang *et al.*, 2009; Smith *et al.*, 2015). In this study, avian influenza A virus was detected in wild ducks and broiler chickens in different geographical regions of Basrah and Wasit provinces, Iraq. The tested samples were collected from healthy ducks (because ducks do not show clinical signs following infection) and chicken suffering from respiratory disorders. According to the RT-PCR results, the general percentages of virus prevalence were significantly higher in wild ducks than broiler chicken in both provinces. In addition, according to the geographic distribution of birds in Basrah province, the highest virus prevalence was observed in Al-Qurnah region, which is located in the north part of that province. Moreover, the highest virus prevalence in Wasit province was observed in Sheikh Saad and Al-Kut city for wild duck and broiler chickens, respectively.

The role of universal primer in the detection of influenza A virus

The outer surface of influenza A virus particle must have haemagglutinin (HA) and neuraminidase (NA) proteins, which are encoded by HA and NA genes, respectively. To date, there are 18 HA and 11 NA subtypes described as H1–H18 and N1–N11 with amino acid sequences differing by 30% or more between subtypes (Tong *et al.*, 2012). Therefore, in theory, 198 different virus subtypes are possible by combinations of these proteins (Achenbach & Bowen, 2011). The sensitivity and specificity of PCR-based methods for the detection of virus subtype are most critically determined by the choice of primer sequences. Nucleic acid amplification techniques based on RT-PCR assays are regarded as a specific diagnosis to confirm the influenza virus infection (Fouchier *et al.*, 2000). The sequences of the primer sets that are routinely used for PCR-based detection of influenza A virus may be appropriate for the detection of some virus strains circulating in domestic poultry (Wang & Taubenberger, 2010). However, the use of the same primer sets over time would play a role in inhibiting virus detection and generation of false-negative results when the host is infected with an unknown or unexpected virus subtype (Kim & Poudel, 2013). In this study, a universal primer set for a conserved region of M gene was designed for the detection of all possible subtypes in a single enzymatic reaction. In addition, the molecular diagnostic method (RT-PCR), which was used in this study is more accurate than the serological diagnostic methods such as rapid detection test, haemagglutination inhibition, and complement fixation tests (Allwinn *et al.*, 2002). By performing the current PCR-based assay, diagnosis of influenza infection with any virus subtype would be achieved within a single PCR tube in a single working day, which would be a significantly faster tool than the previous PCR protocols for the diagnosis of bird influenza A virus infection.

Influenza A virus in birds and its potential threat to humans

Wild ducks are the reservoir of most influenza A subtypes and they support viral replication in the intestinal mucosa without showing any clinical signs following

infection (Smith *et al.*, 2015); it has been considered that these birds are the source of influenza A outbreaks in chickens and turkeys (Berhane *et al.*, 2009). Therefore, in this study, two important hosts (wild ducks and broiler chickens) were chosen to assess the distribution of viruses in these birds. In addition, these birds, particularly chickens, are closer to humans and might play a role in transmission of avian influenza to the community especially when they are infected with high pathogenic strains causing severe outbreak and even death (Peiris *et al.*, 2007).

The results of this study showed that the proportion of infection of wild ducks was not significantly different between the geographical regions of each province. This can be attributed to the fact that all wild birds may come from the same source and reach markets, possibly after bird hunting from marshes. The infection rate of chickens was significantly higher in Al-Qurnah in comparison to the other three regions of Basrah province. A recent study conducted on domestic ducks and geese in Basrah province also showed significantly higher virus prevalence in Al-Qurnah region in comparison to the other regions (Firas *et al.*, 2018). The Al-Qurnah region is located in the north part of Basrah, that is closest to marshes, which form an important region for breeding, and wintering of high number of different waterfowl that play a big role in producing viruses, which might reach to domestic poultry, including ducks, geese and chickens. In addition, the overall results of this study disagreed with another study conducted at different parts in the middle of Iraq, which showed similar virus distribution between wild ducks and broiler chickens (Karrar, 2015). The small size of marshes in some central areas of Iraq compared to Basrah may reduce the spread of the virus in the wild ducks because of the limited migration of wild birds to these areas.

Although wild ducks usually carry low pathogenic avian influenza (LPAI) viruses in their bodies, numerous studies have shown that high pathogenic avian influenza (HPAI) viruses could have evolved directly from LPAI strains following introduction into domestic birds, in particular chickens (Rohm *et al.*, 1995; Banks *et al.*, 2001; Monne *et al.*, 2014).

All samples used in this study were collected from bird markets from wild ducks and broiler chickens that are in contact with other birds and humans. Therefore, it is highly recommended to detect viruses from the other expected hosts using the current RT-PCR protocol, or by the detection of antibodies such as IgY and IgG in the domestic poultry and humans, respectively. In addition, further analysis such as virus subtyping may be required to obtain a clearer image about the distribution of high pathogenic subtypes such as H5, H7, and H9 using gene specific primers.

A study conducted at some geographic regions of Iraq on different wild birds including ducks, geese, flamingo and coot; and broiler chicken showed that H9 was the dominant subtype followed by H5, while H7 was not detected in these birds (Karrar, 2015). In addition, another recent study has shown that H5N1 was detected for the first time in peafowl in Kirkuk province, Iraq (Rashid *et al.*, 2017). Moreover, a more recent study showed that avian influenza A virus is highly prevalent in domestic ducks and geese in Basrah province, southern Iraq (Firas *et al.*, 2018). Therefore, this information gives a clear and real picture of the importance of studying the disease in our

geographic regions, and hence warrants further studies.

CONCLUSIONS

This study has concluded that the prevalence of avian influenza A viruses was significantly higher in wild ducks than broiler chickens in Basrah and Wasit provinces, Iraq. In Basrah province, the infection rate of wild ducks was similar in all geographic regions while the infection rate of broiler chickens was significantly higher in Al-Qurnah in comparison to the other geographic regions. In comparison, in Wasit province, the infection rate of both wild ducks and broiler chickens was similar in all regions that were included in the study.

ACKNOWLEDGEMENTS

The authors thank the Deanery of the College of Veterinary Medicine, University of Basrah for offering support to accomplish this research. They also thank all staff and technicians of the central laboratory in the college and the Department of Veterinary Microbiology and Parasitology for their kindness and useful advices.

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Paper received 02.02.2019; accepted for publication 04.04.2019

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