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Molecular identification of H9N2 subtype of avian influenza A virus in wild and domestic ducks in Basrah province, South of Iraq

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Abstract

Influenza A viruses spread naturally among aquatic birds, especially the wild ones. The aim of the current study is to investigate the avian influenza virus subtype H9N2 in the wild and domestic ducks in different geographical areas of Basrah Governorate, namely Shatt Al Arab, Abu Al-Khaseeb, Az Zubayr, and Al Qurnah. The presence of the virus was initially investigated generally using a pair of universal primers by performing the reverse transcriptase polymerase chain reaction. Subsequently, the virus subtype H9N2 was detected in both bird species. The results showed that the overall prevalence of the virus, regardless of subtype, was 66%. The total percentage in wild ducks was 78.6%, which showed significantly higher values than what was in domestic ducks, where it was 52.8%. Regarding the spread of the virus according to geographical location, the percentage of viruses in wild ducks was comparable in all areas involved in the study, while in domestic ducks it was higher in the Al Qurnah region, northern Basrah Governorate, compared to the rest of the regions.

Keywords: Ducks, Influenza A virus, Hemagglutinin 9, Neuraminidase 2, Polymerase Chain Reaction, Basrah

Introduction

Influenza viruses are members of the *Orthomyxoviridae* family that contain a fragmented an RNA genome into a number of pieces (1). Within this family, there are four main genera of influenza viruses: A, B, C, and D. The genus A is the only one known to infect a wide range of hosts, particularly birds (2). Within this genus, multiple subtypes are classified based on the composition of the external glycoprotein antigens of the virus, HA and NA. So far, there are 18 types of H and 11 types of N have been circulated. These viruses spread naturally among wild aquatic birds everywhere in the world and can infect most bird species, particularly the domestic poultry as well as other animals (3). In general, bird influenza viruses do not infect humans, however, sporadic human infections with bird influenza viruses have occurred from time to time causing great concern. Of the many subtypes of avian influenza, A viruses, only five subtypes have been documented as causing human infections: H5, H6, H7, H9, and H10. Among these, the most common subtypes causing human infections are the H5, H7, and H9 viruses (4,5).

Regarding the degree of pathogenicity of bird influenza, viruses can be classified into two main types: low pathogenicity avian influenza viruses (LPAI), which usually cause mild or even no clinical signs; and highly pathogenic avian influenza viruses (HPAI) which usually cause serious illness with severe clinical signs and high mortality rate (6,7). Not all subtypes of avian influenza viruses are classified as highly pathogenic, but there are limited types, especially certain strains of H5 and H7 are considered to be within this classification (8,9). On the other hand, avian influenza virus subtype H9N2 is of great concern as it has caused significant economic losses in the poultry industry in the Middle East and different parts of Eurasia, and it has also sporadically been spread to mammalian hosts including humans and pigs (10). Ducks, both wild and domestic, are the major reservoir for all subtype influenza viruses, including H9N2, as these birds play an important role in the development and spread of many species (11). It was found that the H9N2 virus was widespread in live bird markets and domestic duck farms in China (12).

Both types of ducks do not show signs of illness when infected with this subtype of the virus, but the infection may be transmitted to other poultry birds, including domestic and commercial chickens, causing a high percentage of deaths in chicken flocks (13). Due to the lack of information about the extent of the spread of the H9N2 avian influenza virus in our geographical area, we investigated the presence of this species in domestic and wild ducks in various areas of Basrah Governorate, southern Iraq. The results obtained from this study will help to take the necessary precautions to prevent the spread of this strain of viruses to other poultry birds, which can lead to significant economic losses.

Materials and Methods

Sample collection

During the period from April to October 2021, 430 cloacal swabs were obtained from 210 and 220 domestic and wild ducks, respectively, from four regions of Basrah governorate/ south of Iraq, which were Shatt Al Arab, Abu Al-Khaseeb, Az Zubayr, and Al Qurnah. Table 1 describes a full overview of the collected samples according to the geographic distribution, bird type, and sample number. Cloacal samples were collected using Dacron swabs and each sample was placed in a sterile tube containing phosphate buffer saline (PBS) with glycerol at a 1:1 ratio. The

samples were transported to the laboratory in appropriate cold conditions. All samples were spun at 1,000 × g for a minimum of 10 minutes after which the supernatant was collected and moved to new sterile tubes to prepare for RNA extraction.

Table 1: Types of birds and number of specimens according to geographical distribution.

Geographical area	Number of samples	
	Domestic ducks	Wild ducks
Shatt Al Arab	57	55
Abu Al-Khaseeb	48	53
Az Zubayr	47	52
Al Qurnah	58	60
Subtotal	210	220
Total	430	

Extraction and quantification of viral RNA

Samples were subjected to viral RNA extraction using the QIAamp Viral RNA Purification Kit, provided by Qiagen, Germany. The quality and quantity of extracted RNA were determined using a NanoDrop spectrophotometer. RNA was maintained at −20°C until further use.

Detection of viral nucleic acid by RT-PCR

The virus was detected using the conventional RT-PCR. A pair of previously designed universal primers was first used to identify the influenza A virus regardless of the virus subtype as in (14). H9 and H2 viral genes were identified utilizing a set of gene-specific primers designed through the National Center for Biotechnology Information (NCBI). (Table 2).

Table 2: Primers used during the study.

Gene	Primer sequence	Amplicon size	Reference
M	Forward: ATCGTCGCYTTAAATACGGT (20 bp) Reverse: CGTCAACATCCACAGCAYTC (20 bp)	108 bp	14
H9	Forward: TAATGGGATGCTGTGTGCGA (20 bp) Reverse: CCATTGGACATGGCCCAGAA (20 bp)	1491 bp	-
N2	Forward: CAATTTGCACTTGGGCAGGG (20 bp) Reverse: TGCGAAAGCTCATATCGGCA (20 bp)	1029 bp	-

M: Matrix protein, H9: Hemagglutinin 9, N2: Neuraminidase 2

The primers were specifically designed during the current study.

A One Step RT PCR kit supplied by Bioneer, Republic of Korea was utilized to synthesize and amplify target genes following the manufacturer protocol. The concentration of the extracted viral RNA used for the synthesis of cDNA was 150 ng/µl. The optimized PCR conditions were as follows: the synthesis of cDNA was conducted for 30 min. at 45°C, followed by one round of initial denaturation for 5 min. at 94°C. Subsequently, a total of 40 cycles of denaturation for 20 sec at 94°C, annealing for 35 sec at 58°C (for the use of universal primers), and 35 sec at 59°C (for H9 and N2 gene-specific primer), followed by extension for 1 min at 72°C. After these cycles, a single cycle of final extension was carried out for 5 min at 72°C. The reaction was then cooled down for 15 min at 4°C for stabilizing the reaction. The PCR was then detected by loading it on 1.5% agarose in TAE buffer stained with Nancy-520 fluorescent dye. The PCR bands were then visualized under a UV transilluminator.

Statistical Analysis

The data obtained in this study were statistically analyzed using the version 28 of Statistical Package for the Social Sciences (SPSS). To calculate the significance of different groups, the Chi-square test ($\chi 2$) was performed for this purpose. A P value less than or equal to 0.05 was considered statistically relevant among other variables.

Results

The results of viral gene amplification showed clear and sharp bands on the agarose gel with the expected corresponding sizes following the use of the universal primer to detect the viral M gene and the gene-specific primers to detect the viral H9 and N2 genes. The sizes of the bands were determined by comparing them with DNA ladders as references.

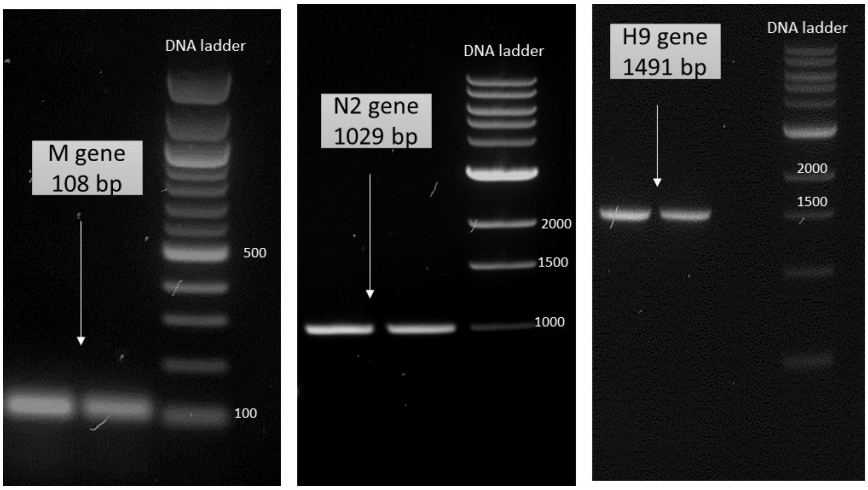

Figure 1: Detection of H9N2 avian influenza A virus subtype.

This figure shows the successful amplification with distinct bands on an agarose gel of a partial region of the viral M gene, H9 and N2 genes. The bands are consistent with the expected sizes and are easily distinguishable as observed by the DNA ladder used for comparison.

Overall, the results obtained from this study observed that the prevalence of infection with the avian influenza virus regardless of virus subtype (using a set of universal primers) was 66% (284/430). Among them, 52.8% (111/210) yielded positive results for the presence of the virus in domestic ducks, and 78.6% (173/220) were positive in the wild ducks. This result indicates that the prevalence of infection in wild ducks was significantly higher than in domestic ducks ($P < 0.05$), as shown in Table 3.

Table 3: Infection rate of influenza A virus in wild and domestic ducks.

Type of bird	Total sample number	Positive sample number	Rate of infection
Domestic duck	210	111	52.8%
Wild duck	220	173	78.6%
Total	430	284	66%
			P<0.05

According to the distribution of the positive results in the domestic ducks across the geographical areas, the significantly higher prevalence rates were noted in Al Qurnah district, accounting for 74.1%. The other districts within the studied region showed similar proportions of virus circulation ranging from 43.8% to 45.8%. In comparison, the prevalence of the virus in wild ducks was similar between different geographical regions ranging from 76.3 to 81.6 (Table 4).

Table 4: Influenza A virus infection rate in domestic and wild ducks depending on geographical distribution

Geographical area	Domestic ducks		Wild ducks	
	Number of positive samples	Percentage of infection	Number of positive samples	Percentage of infection
Shatt Al Arab	25/57	43.8%	/5542	76.3%
Abu Al-Khaseeb	22/48	45.8%	/5341	77.3%
Az Zubayr	21/47	44.6%	/5241	78.8%
Al Qurnah	43/58	74.1%	/6049	81.6%
Total	111/210	52.8%	173/220	78.6%
P value	P<0.05		P>0.05	

Among the total number of positive samples, which was 284, 94 samples (33.1%) were identified as positive for the presence of the H9N2 influenza virus subtype in both types of birds. In domestic ducks, the number and rate of infection with this virus subtype reached 20.7% (23/111). More importantly, the infection rate in wild ducks was much higher than that in domestic ducks, reaching 41% (71/173) as shown in Table 5. Regarding the prevalence of the H9N2 virus subtype across the different geographic regions, no significant association was found in the spread of the virus among wild ducks in any of these regions. In comparison, there was a significant difference in the spread of the virus among domestic ducks in Al Qurnah which was 32.5%, compared to prevalence rates ranging from 12% to 14.2% in the other regions (Table 6).

Table 5: Ratio of H9N2 influenza A virus subtype in wild and domestic ducks.

Type of bird	Number of positive influenzas	Number of positive H9N2	Ratio of H9N2 infection
Domestic duck	111	23	20.7%
Wild duck	173	71	41%
Total	284	94	33.1%
			P<0.05

Table 6: Rate of infection with H9N2 subtype of influenza A virus across the geographical areas.

Geographical area	Domestic ducks		Wild ducks	
	H9N2/ Total positive samples	Percentage of H9N2	H9N2/ Total positive samples	Percentage of H9N2
Shatt Al Arab	3/25	12%	17/42	40.4%
Abu Al-Khaseeb	3/22	13.6%	17/41	41.4%
Az Zubayr	3/21	14.2%	16/41	39%
Al Qurnah	14/43	32.5%	21/49	42.8%
Total	23/111	20.7%	71/173	41%
P value	P<0.05		P>0.05	

Discussion

Many studies demonstrate that almost all influenza A virus subtypes are naturally found in waterfowl, especially in wild ducks. Although these birds carry viruses, they typically do not show obvious symptoms of infection (11,15,16). The existence of viruses in ducks plays a big role in their spread to other birds, particularly domestic birds including other species of ducks, chickens, and turkeys, and typically has a big impact on the poultry industry (17, 18). In addition, there is a chance to pass these viruses from birds to humans causing serious illness with a high mortality rate. The occurrence of the influenza A virus subtypes in waterfowl across Iraq is not well documented, and this study is the first to identify subtype H9N2 in domestic and wild ducks. The study inspected this virus subtype in four different regions belonging to Basrah Governorate. The study revealed that above 50% of domestic ducks carry the influenza A virus. In contrast, among the wild ducks, more than three-quarters of birds carry the virus. Moreover, the presence of H9N2 was higher in the wild ducks than the domestic ones.

In general, the results obtained in this study showed a clear difference in the presence of the virus in domestic ducks according to geographical distribution, specifically in Al Qurnah region, where it was highest compared to other regions, while in wild ducks, this difference was not present according to the geographical distribution of the areas studied. This can be explained by the fact that this geographical region contains many water swamps compared to other areas. This aquatic nature creates an environment suitable for the presence of many birds, including wild ducks (the natural reservoir of viruses) and domestic birds. This coexistence between different birds plays a pivotal role in the transmission of viruses. This is consistent with many studies in different regions of the world, including Iraq, which confirm that the influenza virus in general is transmitted to a greater extent in the aquatic environment (14,19). Based on these repeated findings, it is possible to conduct further studies to identify other subtypes of the virus in these birds, especially H5N1 and H7N2 due to their potential to be highly pathogenic and cause serious problems in poultry (20). In addition to studying the presence of the virus or its antibodies in humans, which will give a picture of the extent of transmission of these viruses in communities, which, although rare, usually cause serious diseases and complications in human (21).

In this study, about one-third of the samples positive for avian influenza A virus belonged to the H9N2 subtype. It will be interesting to determine the pathogenicity of this subtype through further studies by sequencing the H9 gene. The resulting nucleotide sequence must be translated into amino acids to identify amino acid residues at the H9 proteolytic cleavage site. Typically, trypsin-like proteases cleave influenza viruses in their low-pathogenicity forms, while distinct proteases such as furin cleave the virus in its highly pathogenic form (22). In addition, another viral virulence-related gene, PB2, has specific amino acid positions 627, 155 and 292 that are responsible for converting the virus from low to highly pathogenic through increased replication (23). Pathogenicity and transmissibility can be significantly enhanced by combining HA and PB2 mutations (24). However, if the H9N2 virus is found to be a low pathogenicity subtype, this should not be neglected as influenza viruses are generally known to mutate over time and the accumulation of mutations can change viral

pathogenicity from low to highly pathogenic (25). Therefore, it is recommended to periodically investigate possible mutations in the virus genes. Hence, promising studies can be conducted to learn more about the molecular structure of this subtype of the virus and its potential negative impact on other birds and humans.

Conclusions

The current study concluded that the subtype H9N2 of the influenza A virus was significantly higher in the wild ducks than in domestic ducks. In addition, virus spread in the wild ducks was similar among the study areas in Basrah Governorate. In comparison, the distribution of the virus in domestic ducks was much higher in Al Qurnah region than in other regions.

Conflict of interest: All authors declare that there is no conflict of interest.

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التحديد الجزيئي للنوع الفرعي H9N2 من فيروس أنفلونزا الطيور A في البط البري والداجن في محافظة البصرة، جنوب العراق

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الخلاصة

نتشر فيروسات الأنفلونزا A بشكل طبيعي بين الطيور المائية، وخاصة البرية منها. الهدف من هذه الدراسة هو تقصي فيروس أنفلونزا الطيور من النوع الفرعي H9N2 في البط البري والداجن في مناطق جغرافية مختلفة من محافظة البصرة وهي شط العرب وأبو الخصيب والزبير والقرنة. تم التحقق من وجود الفيروس في البداية بشكل عام باستخدام زوج من البادئات العامة عن طريق إجراء تفاعل البلمرة المتسلسل العكسي. بعد ذلك، تم اكتشاف النوع الفرعي للفيروس H9N2 في كلا النوعين من الطيور باستخدام بريمرات متخصصة. وأظهرت النتائج أن معدل الانتشار الإجمالي للفيروس، بغض النظر عن نوعه الفرعي، بلغ 66%. وبلغت النسبة الإجمالية في البط البري 78.6% وهي أعلى بكثير مما كانت عليه في البط المنزلي والتي كانت 52.8%. وفيما يتعلق بانتشار الفيروس حسب الموقع الجغرافي، فقد كانت نسبة الفيروسات في البط البري متشابهة في جميع المناطق التي شملتها الدراسة، بينما في البط المنزلي كانت أعلى في منطقة القرنة التي تقع شمال محافظة البصرة، مقارنة ببقية المناطق.

الكلمات المفتاحية: البط، فيروس الانفلونزا أ، هيموغلوتنين 9، نيوراميديز 2، تفاعل البلمرة المتسلسل، البصرة.