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Molecular identification of avian influenza virus A subtypes H5 and H7 in domestic geese and ducks in Basrah, South of Iraq

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Abstract

Waterfowl are the main reservoir for most influenza A virus subtypes, and they can effectively transmit these viruses to other birds and humans. This study aims to identify two influenza virus subtypes, H5 and H7, in domestic geese and ducks in Basrah governorate, Southern Iraq. 310 cloacal swabs were obtained from 150 domestic geese and 160 domestic ducks from different geographical areas. The viruses were first detected by RT-PCR using a pair of universal primers. All positive samples underwent RT-PCR using gene-specific primers to identify H5 and H7 influenza virus subtypes. The results showed that the prevalence of influenza viruses detected through universal primers was 37.7%. Of these, 24.6% and 50% were positive for viruses in domestic geese and ducks, respectively. Regarding virus subtyping in geese, the infection rates with H5 and H7 were 43.2% and 29.7%, respectively, with 27% as a combination of the two, while in domestic ducks, the infection rate with H5 was 27.5%, and with H7 it was 15%. Interestingly, ducks had a high concurrent infection rate for both H5 and H7 subtypes, accounting for 57.5%. The study concluded that the two virus subtypes, individually or simultaneously, were present in domestic waterfowl in regions of Basrah, and they were higher in ducks than in geese.

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Introduction

Avian influenza is a common and widespread disease of birds in many areas worldwide, often resulting in significant economic losses (1). The responsible agent of this disease is influenza virus type A, which belongs to the family of *Orthomyxoviridae* (2). These viruses circulate naturally among wild waterfowl worldwide and can infect poultry and other bird species (3). In addition, the avian influenza virus can be transmitted to domestic animals and humans, causing severe diseases and high mortality rates (4,5). Influenza viruses contain an RNA genome fragmented into eight pieces of different lengths, and each viral segment encodes one or more viral proteins (2). The virus particles have variable shapes ranging from filamentous to spherical depending on the virus's genetic characteristics and the host type (6). The influenza A virus can be categorized into subtypes based on two types of virus surface glycoproteins: haemagglutinin (H) and neuraminidase (N). Currently, there are 18 and 11 distinct subtypes of hemagglutinin and neuraminidase, respectively, denoted as H1 through H18 and N1 through N11 (7). Regarding disease pathogenicity, avian influenza viruses are further divided into two main types: low-pathogenic and highly pathogenic AIV. Low-pathogenic viruses typically cause mild to moderate infections in domestic poultry, particularly chickens and turkeys, with moderate signs such as decreased egg production and ruffled feathers. In comparison, high-pathogenic viruses often lead to serious infections with a high mortality rate in chicken flocks. They often cause severe disease in poultry, affecting the internal organs with a fatality rate reaching 100% within 48 hours (8). Among the

subtypes of avian influenza viruses, there are only some subtypes, namely H5 and H7, which are classified as highly pathogenic viruses for birds (9-11). In the meantime, the majority of viruses belonging to those subtypes present in avian populations are classified as low-pathogenic viruses (12). Interestingly, waterfowls such as ducks and geese often do not show clinical signs after infection with any of these groups of viruses, and they can also be considered the main reservoirs for most of the influenza A subtypes (13). Avian influenza viruses are released from birds through feces and respiratory discharges, including saliva and nasal secretions. They can spread through direct contact with the secretions of infected birds, mainly through contaminated water and contact with virus-contaminated surfaces (14). They are known for their remarkable ability to survive for periods under low-temperature conditions. Moreover, they can be moved through farm equipment, facilitating their effortless transmission from one farm to another (15,16). Although most geese and duck infections with avian influenza virus are asymptomatic, it plays a significant role in transmission to other birds, such as backyard and commercial chickens, and humans, which can often be severe and cause high mortality rates (17). A previous study found high rates of avian influenza infection for both domestic ducks and domestic geese in different regions of Basrah province. The viruses were screened using a pair of universal primers, regardless of virus subtypes, and it gave a comprehensive view of the presence of the virus in birds (18).

In this study, we used gene-specific primers for the H5 and H7 subtypes to clarify the existence of these two important branches of the avian influenza virus.

Materials and methods

Ethical approval

All work and test analyses followed the official guidelines of the College of Veterinary Medicine, University of Basrah, Iraq with approval issue number 298 on April 5, 2022.

Sample collection

During the period from November 2022 to March 2023, 310 cloacal swabs were collected from 150 domestic geese and 160 domestic ducks in four different districts of Basrah/ southern Iraq, which were Abul Khasib, Al-Qurnah, Zubair, and Shatt Al Arab. Table 1 provides a comprehensive overview of the samples collected in terms of geographic location, number of samples, and type of birds.

The collected samples were put into sterile tubes containing phosphate buffer saline (PBS) and moved to the laboratory under cold conditions. Each sample was spun at 1,000 × g for 10 min and the supernatant was gently harvested and transferred into freshly labeled tubes to prepare for nucleic acid (RNA) extraction.

Table 1: Number of samples collected across geographical regions

Region	Number of collected samples	
	Domestic geese	Domestic ducks
Abul Khasib	42	48
Al-Qurnah	33	38
Zubair	30	30
Shatt Al Arab	45	44
Sub-total	150	160
Total	310	

Extraction and quantification of viral RNA

According to the manual guidelines, viral RNA was extracted from all collected samples by employing the QIAamp viral RNA purification kit from Qiagen. The extracted RNA was quantified utilizing a NanoDrop spectrophotometer. They were preserved at a temperature of -20 ˚C until it was used.

Detection of nucleic acid by RT-PCR

Virus detection was performed using the conventional RT-PCR method. Influenza A virus was first detected regardless of the subtype using a pair of universal primers (19). H5 and H7 virus subtypes were identified using gene-specific primers designed through the primer designing tool available at the National Center for Biotechnology Information (Table 2).

According to the manual's instructions, the synthesis of cDNA and gene amplification was performed in a single tube using Bioneer's One-Step RT-PCR kit (supplied in South Korea). The initial RNA concentration used for cDNA synthesis was 125 ng/µl. The conditions of the RT-PCR were as follows: cDNA synthesis was carried out at 45 °C for 30 min. Subsequently, a one-step initial denaturation at 95 °C for 5 min was performed, followed by a total of 35 cycles of denaturation at 95 °C for 15 sec, annealing at 58 °C for 30 sec (for the universal primer) and at 60 °C for 30 sec (for H5 and H7 gene-specific primers), and extension at 72 °C for 1 min. Following these cycling steps, a step of the final extension was performed at 72 °C for 5 min. After that, the reactions were cooled down at a temperature of 4 °C for 10 min to stabilize the reaction. The PCR products were detected after being loaded on a gel of 1.5% agarose in TBE buffer stained with the Nancy-520 fluorescent dye and then visualized under a UV transilluminator.

Statistical analysis

The obtained data were evaluated statistically using the Statistical Package for the Social Sciences (SPSS). The Chi-square ($\chi 2$) test was performed to calculate the significance between different groups. A statistically significant result was a P-value equal to or less than 0.05, indicating a significant difference or relationship between the variables examined.

Table 2: Primer sets used throughout the study

Gene	Primer set	Amplicon size
M	Forward: ATCGTCGCYTTAAATACGGT (20 bp) Reverse: CGTCAACATCCACAGCAYTC (20 bp)	108 bp
H5	Forward: GTGTAGCTGGATGGCTCCTC (20 bp) Reverse: CACGTTGCCCGTTTACTTGG (20 bp)	504 bp
H7	Forward: GGGAAACGGACAGTTGACCT (20 bp) Reverse: TGCTCTGCAGTTGAAACGGA (20 bp)	413 bp

Results

RT-PCR results showed successful amplification of target genes from cloacal swabs collected from ducks and geese. After loading the PCR product with a concentration of 1.5% agarose gel, sharp and visible bands with sizes corresponding to genes belonging to the viral M gene were visualized using the universal primer, also H5 and H7 subtypes (Figure 1). Band sizes were determined by comparing them with an appropriate reference DNA ladder.

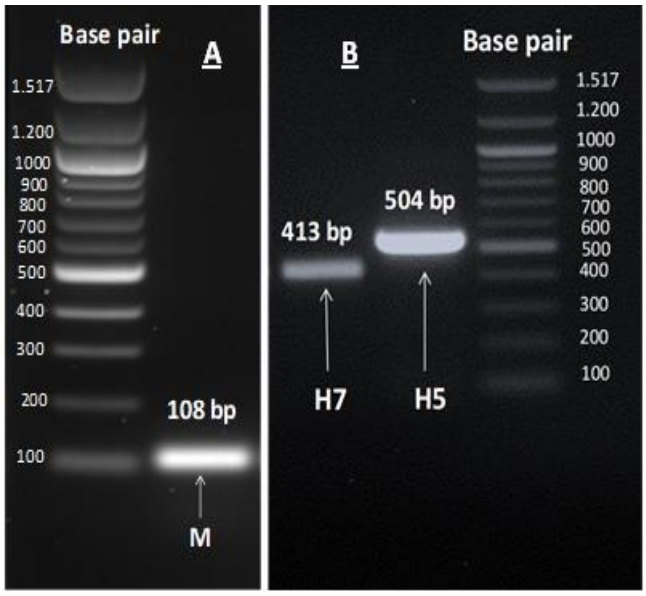

Figure 1: Detection of avian influenza A virus. The presented figure shows the existence of successful amplification of partial regions of the M gene (A) and H5 and H7 genes (B) with well-defined bands on an agarose gel. The bands are easily distinguishable and correspond to the expected sizes, as indicated by the DNA marker used for comparison.

Overall, the results showed that the prevalence of general avian influenza viruses detected through universal primers was 37.7% (117/310). Of these cases, 24.6% (37/150) were positive for the virus in domestic geese, while 50% (80/160) were positive in domestic ducks. The prevalence of infection in ducks was significantly higher than in geese (Table 3).

Table 3: The infection rate with influenza virus type A in domestic geese and ducks

Type of bird	Total number of samples	Number of positive samples	Percentage of infection
Domestic geese	150	37	24.6%
Domestic duck	160	80	50%
Total	310	117	37.7%
X^2= 13.333, P<0.05			

Among the total positive results (117 cases) for the general avian influenza virus, H5 and H7 virus subtypes were detected in both types of birds. In addition, concurrent infection with both H5 and H7 subtypes was detected in several birds. Specifically, in domestic geese, the infection rate with the only H5 subtype was 43.2% (16/37), and with the subtype H7 it was 29.7% (11/37). There was a concurrent infection rate for both H5 and H7 subtypes, accounting for 27% (10/37) of the total positive cases in domestic geese. On the other hand, in domestic ducks, the infection rate with either subtype was as follows: the infection with H5 was 27.5% (22/80), and with H7, it was 15% (12/80). Interestingly, there was a high simultaneous infection rate for both H5 and H7 subtypes, accounting for 57.5% (46/80) of the total positive cases in domestic ducks. Although the infection rate with the H5 or H7 virus was significantly higher in geese than in ducks, the matter is the opposite regarding double infections, as the presence of viruses together in ducks is significantly higher than in geese (Table 4).

Based on geographical distribution, Al-Qurnah district had the highest avian influenza A virus infection ratio, accounting for 42.4% (14/33) of cases in domestic geese and 71% (27/38) in domestic ducks. The other geographical areas revealed similar ratios of virus distribution ranging from 19% to 20% in domestic geese and 43.1% to 43.7% in domestic ducks. Virus distribution in Al-Qurnah region was significantly higher than in the other studied regions (Table 5).

Regarding the spread of viral subtypes based on geographical distribution, the results showed that the H5 had a higher rate among geese in three regions than the H7 (the fourth region showed an equal infection rate between the two

subtypes). In contrast, the average occurrence of the H5 subtype was higher than that of the H7 subtype in duck samples across all regions. The co-infection rates with both subtypes were significantly higher in ducks than in geese in either geographic area (Table 6).

Table 4: The infection rate with H5 and H7 influenza A virus subtypes A in domestic geese and ducks

Type of bird	H5 subtype	H7 subtype	Co-infection with H5 and H7	P value
Domestic geese	43.2% (16/37)	29.7% (11/37)	27% (10/37)	X^2=6.510, P<0.05
Domestic duck	27.5% (22/80)	15% (12/80)	57.5% (46/80)	X^2=42.864, P<0.05
Total	32.4% (38/117)	19.6% (23/117)	47.8% (56/117)	X^2=17.760, P<0.05
P value	X^2=5.133, P<0.05	X^2=6.452, P<0.05	X^2=19.060, P<0.05	

Table 5: The infection rate with influenza A virus in domestic geese and ducks according to the geographical distribution

Region	Domestic geese		Domestic ducks	
	Total / positive (n)	Infection (%)	Total / positive (n)	Infection (%)
Abul Khasib	8/42	19%	21/48	43.7%
Al-Qurnah	14/33	42.4%	27/38	71%
Zubair	6/30	20%	13/30	43.3%
Shatt Al Arab	9/45	20%	19/44	43.1%
Total	37/150	24.6%	80/160	50%
P value	X^2=19.855, P<0.05		X^2=22.991, P<0.05	

Table 6: Distribution of influenza A virus subtypes H5 and H7 across geographical regions

Region	Domestic geese (Subtype)			Domestic ducks (Subtype)		
	H5	H7	H5 + H7	H5	H7	H5 + H7
Abul Khasib	4/8 (50%)	2/8 (25%)	2/8 (25%)	7/21 (33.3%)	4/21 (19%)	10/21 (47.6%)
Al-Qurnah	6/14 (42.8%)	4/14 (28.5%)	4/14 (28.5%)	3/27 (11.1%)	2/27 (7.4%)	22/27 (81.4%)
Zubair	2/6 (33.3%)	2/6 (33.3%)	2/6 (33.3%)	4/13 (30.7%)	3/13 (23%)	6/13 (46.1%)
Shatt Al Arab	4/9 (44.4%)	3/9 (33.3%)	2/9 (22.2%)	8/19 (42.1%)	3/19(15.7%)	8/19 (42.1%)
Total	16/37 (43.2%)	11/37 (29.7%)	10/37 (27%)	22/80 (27.5%)	12/80 (15%)	46/80 (57.5%)
P value	X^2=6.07 P≤0.107	X^2=2.129 P≤0.546	X^2=3.427 P≤0.330	X^2=15.80 P<0.05	X^2=10.19 P<0.05	X^2=39.193 P<0.05

Discussion

Several studies prove that waterfowl, particularly ducks, are natural reservoirs for almost all influenza A virus subtypes. These birds usually do not present clear signs of infection, even though they carry viruses (20-22). The presence of viruses in waterfowl plays a crucial role in their transmission to other birds, especially domestic ones, and usually causes significant effects on the poultry industry (3). There is minimal information on the existence of influenza A virus subtypes in waterfowl throughout Iraq, and this study is the first ever to detect the presence of the subtypes H5 and H7 in domestic geese and ducks. This study studied these virus subtypes in four interesting areas in Basrah governorate, where many waterfowl reside. The study found that nearly a quarter of the samples taken from geese tested positive for influenza viruses. In contrast, in the case of ducks, the percentage of samples tested positive for the virus was significantly higher, with about half of the samples tested showing positive results. In addition, the presence of the H5 subtype was significantly higher than that of H7 in both geese and ducks.

In this study, a significant variation was observed between the prevalence rates of the virus in both geese and ducks, as its incidence in ducks was twice as high as that in geese. This was expected since many studies indicate that ducks are the primary reservoir hosts for most influenza A viruses. A study in Bangladesh focused on detecting the presence of the H5 subtype of avian influenza A in a particular type of duck. The study results indicated that nearly half of the samples examined were positive for the presence of the virus, which strongly agrees with what was reported in our study (23). This agreement may indicate that the prevalence of the H5 subtype may generally overwhelm other subtypes in a broader context and in different regions.

On the other hand, the findings of our study showed that the percentage of H5 is much higher than that of H7 in both goose and duck samples. Interestingly, co-infection

involving both H5 and H7 was readily observed in both types of birds, and this was more prevalent in ducks than in geese. Although we obtained these essential results in our study, the determination of the level of pathogenicity of the H5 and H7 subtypes should not be overlooked. Therefore, it is strongly recommended to conduct future studies to comprehensively understand the potential risks posed by these subtypes of influenza virus. If these viruses are highly pathogenic, they will pose a serious threat to other domestic birds, especially chickens, and the possibility of their transmission to humans, creating a pandemic threat (24). However, low pathogenic subtypes can mutate over time to become highly pathogenic and cause potential epidemics (25). Therefore, the results obtained in this study strongly recommend the need for ongoing monitoring to track any changes in the prevalence and pathogenicity of subtypes of the virus in both geese and ducks. This proactive approach will help identify potential outbreaks or emerging strains, enabling timely interventions and measures to protect human and animal health. On the other hand, if viruses reach poultry, the differential diagnosis from other respiratory infections using specific laboratory techniques should be considered, as many respiratory infections in birds share similar signs, such as Newcastle disease, infectious bronchitis, and some bacterial infections (26-29).

In this study, depending on the geographical location of the birds, the highest percentage of influenza virus presence, in general, was in Al-Qurnah district compared to other districts. In addition, the combined incidence of both H5 and H7 was also the highest in this region. This can be explained by the nature of the Al-Qurnah region, with the availability of water swamps and marshes, which provide opportunities for the entry of migratory waterfowl that may effectively transmit viruses to domestic waterfowl. The other geographical areas showed lower infection rates, which can be attributed to the nature of these areas, which lack water resources and natural swamps, where the presence of migratory birds is less. Although H5 and H7 subtypes were identified in several samples collected, screening for other virus subtypes, particularly H9, is highly recommended, as many studies have shown that this subtype is of concern (30-32).

Numerous studies confirm that domestic waterfowl can receive different subtypes of influenza viruses through migratory wild birds, which is always a cause for concern (33-35). The transmission of avian influenza viruses through water is well documented, notably the highly pathogenic subtypes, and this could play a significant role in the virus spreading over time (36). The acquisition of multiple viruses by the same host may play a pivotal role in the evolution of the virus through the processes of antigenic drift (minor change) (37,38) or antigenic shift, which is also called reassortment (significant change) (39), which probably plays an active role in transmitting the virus to the humans (40). Breaking the chain of virus evolution, especially in waterfowl, is challenging. Therefore, regular screening of virus subtypes and sequences of genes responsible for increased pathogenicity, especially the HA gene, is recommended for future work. Moreover, it would be interesting to investigate other infectious diseases among domestic ducks and geese in our geographical region. This could include examining infections with parasites like *Eimeria truncata* and other gastrointestinal parasites as were previously researched in the Nineveh governorate, Northern Iraq (41,42). To obtain a clearer picture of the spread of other avian viruses, the Newcastle disease virus is like the avian influenza virus in that both infect ducks without causing any symptoms in most cases (43). The use of universal primers for Newcastle disease viruses has a major role in detecting all strains of Newcastle virus in a single enzymatic test (44). Therefore, implementing the investigation plan for these viruses will ensure obtaining promising results about the extent of their spread in ducks and the possibility of their transmission to poultry birds

Conclusions

The current study concluded that the two avian influenza virus subtypes (H5 and H7), individually or simultaneously, were present in domestic waterfowl in the regions of Basrah governorate in varying proportions, where they were significantly higher in ducks than in geese. These results gave us a clear view of the spread of these types of viruses in our environment.

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Conflict of interest

The authors of this research declare that there is no conflict of interest.

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التحديد الجزيئي لفيروس أنفلونزا الطيور أ للأنواع الفرعية الهيموغلوتنين ٥ و الهيموغلوتنين ٧ في الوز والبط الداجن في البصرة، جنوب العراق

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الخلاصة

الطيور المائية هي المستودع الرئيسي لغالبية الأنواع الفرعية لفيروس الأنفلونزا أ، ويمكنها نقل هذه الفيروسات بشكل فعال إلى الطيور الأخرى والبشر. تهدف هذه الدراسة إلى التعرف على نوعين فرعيين من فيروس الأنفلونزا الهيموغلوتنين ٥ والهيموغلوتنين ٧ في الوز والبط الداجن في محافظة البصرة، جنوب العراق. تم جمع ٣١٠ مسحة مذرقية من ١٥٠ وزاً محليا و ١٦٠ بطة محلية من مناطق جغرافية مختلفة. تم الكشف عن الفيروسات في بداية الأمر بواسطة تفاعل البلمرة المتسلسل العكسي باستخدام زوج من البادئات الشاملة. خضعت جميع العينات الموجبة للتفاعل مرة أخرى باستخدام بادئات متخصصة بالجينات لتحديد الأنواع الفرعية لفيروس الإنفلونزا نوع الهيموغلوتنين ٥ والهيموغلوتنين ٧. وأظهرت النتائج أن نسبة انتشار فيروسات الأنفلونزا بشكل عام من خلال استخدام البادئات الشاملة ٣٧,٧٪. من بينها كانت ٢٤,٦ و ٥٠٪ إيجابية لوجود الفيروسات في الوز والبط على التوالي. فيما يتعلق بالتصنيف الفرعي للفيروس في الوز، كانت معدلات الإصابة بفيروس الهيموغلوتنين ٥ والهيموغلوتنين ٧ ٤٣,٢ و ٢٩,٧% على التوالي، مع ٢٧٪ كمزيج من الاثنين، بينما في البط الداجن، كان معدل الإصابة بفيروس الهيموغلوتنين ٥ والهيموغلوتنين ٧ ٢٧,٥ و ١٥% على التوالي. ومن المثير للاهتمام، أنه كان هناك معدل إصابة متزامن مرتفع في البط لكل من النوعين الفرعيين الهيموغلوتنين ٥ والهيموغلوتنين ٧، وهو ما يمثل ٥٧,٥٪. وخلصت الدراسة إلى أن النوعين الفرعيين للفيروسات بشكل فردي أو متزامن، كانا موجودين في الطيور المائية المحلية في مناطق البصرة، وكانا أعلى في البط منها في الوز.

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