Genetic characterization of highly pathogenic avian influenza A (H5N8) virus isolated from domestic geese in Iraq, 2018

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Abstract

After the circulation of H5N1 in 2006, 2015 and 2016 in Iraq, a new H5N8 influenza virus emerged in Iraq in 2018. In this study, a HPAI virus subtype H5N8 was identified from backyard domestic geese in Kurdistan region, Iraq. Phylogenic analyses of the hemagglutinin (HA) and neuraminidase (NA) genes indicated that Iraq H5N8 viruses belonged to clade 2.3.4.4 group B and clustered with isolates from Iran, Israel and Belgium. Genetic analysis of HA of the Iraq H5N8 indicated molecular markers for avian-type receptors. Characterization of the NA gene showed that the virus had sensitive molecular markers for antiviral drugs. This is the first study ever on H5N8 in Iraq and it is crucial to understand the epidemiology of the viruses in Iraq and the Middle East. The results are suggestive of a possible role of migratory birds in the introduction of HPAI subtype H5N8 into Iraq.

Introduction

Influenza A is the only genus of the *Orthomyxoviridae* family that can produce pandemic disease [1]. The virus is categorized based on the surface glycoproteins into 16 hemagglutinin (HA) subtypes and 9 neuraminidase (NA) subtypes in birds [2]. Avian influenza viruses (AIV) are divided into highly pathogenic avian influenza (HPAI) and low pathogenic avian influenza (LPAI), based on the structure of the HA gene and the capability of the virus to produce clinical symptoms [3]. Since the first detection HPAI subtype H5N1 in China in 1996, the HA gene of the virus has evolved into ten main genetically distinct phylogenetic clades (clades 0–9) [4]. Due to increased diversity, clade 2 was subdivided into clades 2.1 to 2.5. These suborder clades are further divided into subclades. The HA gene of H5 subtypes underwent reassortment with various neuraminidase subtypes (1–9) to form AI H5NX viruses [5]

Hemagglutinin, the crucial antigen on the viral surface, is the principal target for neutralizing antibodies and is responsible for the viral binding to host receptors. Alteration in receptor specificity from avian α–2,3 sialic acid to human α–2,6 sialic acid is a major obstacle for influenza viruses to adapt to a new host [6].

The subtypes of H5N8 that belong to H5 clade 2.3.4.4 were first isolated from poultry farms in China in 2010 [7]. Outbreaks of H5N8 were then reported in South Korea in early 2014 in chickens and domestic ducks [8, 9]. By the end of 2014, H5N8 spread throughout Europe, North America and East Asia [10–12].

The first report of HPAI subtype H5N1 in Iraq was from humans in Sulaymaniyah Governorate, Kurdistan region in 2006 [13]. In May 2015, an isolated outbreak of H5N1 was detected in the backyard poultry in Sulaymaniyah Governorate, as it was stated by the Ministry of Agriculture in the Kurdistan Region [14]. In 2016, the World Organization for Animal Health (OIE) released 2 reports in Iraq indicating a total of 17 outbreaks of H5N1 in broiler farms. On January 7th, 2018, OIE declared the occurrence of first outbreaks of H5N8 in Iraq, followed by 10 reports indicating a total of 16 outbreaks in broiler chicken farms [15]. In 2019, OIE declared only one outbreak of H5N8 in April in the south of Iraq [16].

In 2018, H5N8 avian influenza was identified in backyard domestic geese in Sulaymaniyah Province. This is the first study analyzing the genetic characteristics of the Iraqi H5N8 viruses because it is the only H5N8 viral sequence available in GenBank databases. The phylogenetic analysis, focusing on HA and NA proteins,

provided information to identify the closely-related viruses to better understand the epidemiology of the virus in the area.

Methods

Outbreak history

Influenza infection was suspected in flocks of domestic greylag geese*Anser anser)*,, that were raised in a privately-owned farm in Sulaymaniyah city in November 2018. About 200 geese were raised in backyards in a populated area and were divided into several flocks. There were no other birds raised in the backyards. The symptoms were depression, anorexia, torticollis, convulsions, blindness, and death within 24–48 h after the onset of symptoms. The mortality rate in the flocks was about 30%. The suspected preliminary diagnosis was avian influenza based on clinical signs.

Sampling and RNA extraction

Tracheal mucus and lung tissue were collected from two 12-month-old geese suspected of being infected. The samples were pooled and subjected directly to total RNA extraction using a total RNA extraction kit (GeNet Bio, South Korea). The procedure was conducted following the instructions from the manufacturer.

Oligonucleotides

The *M* gene was used first to diagnose Influenza virus A [17]. For the identification and sequencing of *HA* and *NA* genes of the H5N8 virus, sets of primers were designed and used in this study (Table 1). All the primers were produced by Macrogen®, Korea.

RT-PCR amplification

The total RNA was subjected to a one-step RT-PCR. *M, HA* and *NA* genes were amplified separately by using SuPrimeScript RT-PCR Premix (GeNet Bio, Korea). The reaction was carried out in 0.2 mL PCR tubes. The constituents of the PCR tube were 10 µL master mix, 4 µL RNA, 1 µL (10 pmol) of each of the forward and reverse primers (Table 1). The volume was then completed to 20 µL by adding 4 µL diethylpyrocarbonate (DEPC)-treated water.

The thermal cycler was initially set at 50°C for 30 minutes. The PCR started with an initial denaturation at 95°C for 10 minutes. After that, 40 cycles of denaturation (95°C for 30 sec), annealing, and extension (72°C for 50 sec) were run. A final extension at 72°C for 4 min was also included. The annealing temperature for the *M* gene was set at 52°C for 30 sec, while the temperature for the sequencing primers of *HA* and *NA* gene was set at 57°C for 50 sec.

PCR products were analyzed by loading 7 µL on 1% agarose gel in 1× Tris/Borate/EDTA (TBE) buffer. The gel was stained with 10 µL safe dye. Electrophoresis was run at 130 volts for 1 hour on the Safe-Blue

Illuminator/Electrophoresis System. The amplicon of PCR products (Table 1) was analyzed according to the migration pattern of a 100 bp DNA ladder.

Direct sequencing

Forty microliters of PCR products were sequenced in the Macrogen Sequencing Facility in South Korea. The identity of each nucleotide was verified by the sequencing of nucleotide sequences from both ends via reverse and forward primer. The coding sequences were submitted to the GenBank Influenza virus database. The virus was named based on the WHO system of influenza viruses nomenclature by A/Domestic goose/Sulaimani/Sul.1/2018 [18] and received the accession numbers MK757595 and MK757597 for HA and NA, respectively.

Phylogenic Analysis

Phylogenic trees were generated based on partial HA gene 1065 bp and NA genes 885 bp of 175 strains of H5N8 virus (Figure 1 and Figure 2). The sequences obtained from Global Initiative on Sharing Avian Influenza Data (GISAID) and the National Institute of Allergy and Infectious Diseases (NIAID) Influenza Research Database (IRD) through the website http://www.fludb.org [19]. Multiple alignments of these sequences were performed with the Clustal W method [20]. MEGA 7 was used to perform phylogenetic analysis with Neighbor-Joining. A/turkey/Ireland/1378/1983 virus was used as an outgroup to root the trees. The bootstrap values were determined from 1000 replicates of the original data.

Ethics Approval and Consent to Participate

The study was conducted following the ethical guidelines of the Animal Care and Use Committee (ACUC) at the College of Veterinary Medicine, University of Sulaimani. The ACUC approved the study (approval number 19/5) and verbal consent to collect samples from the infected animals was provided by the owner of the farm. After confirmation that influenza infection has spread in the farm, the animals were humanely euthanized by an overdose injection of sodium pentobarbital and properly disposed of by burying in a permitted landfill.

Results

Genetic analysis of HA and NA

The topology of the phylogenetic tree based on the HA gene showed that the A/Domestic goose/Sulaimani/Sul.1/2018 viruses from Iraq belonged to clade 2.3.4.4 group B (Figure 1). The phylogenetic tree revealed that A/Domestic goose/Sulaimani/Sul.1/2018was closely clustered with isolates of wild and domestic birds in Iran and Israel, namely A/Crow/Aghakhan/2017, A/peregrine falcon/Israel/1086/2016, and A/turkey/Israel/1076/2016. The phylogenic analysis of the partial NA gene of the Iraq H5N8 virus also showed that it belonged to group B and it was clustered with viruses from wild birds in Belgium, namely A/Anas platyrhynchos/Belgium/1899/2017, A/Buteo buteo/Belgium/3022/2017, and A/Cygnus olor/Belgium/1567/2017.

The amino acid sequences of A/Domestic goose/Sulaimani/Sul.1/2018 virus showed that the virus had multibasic cleavage sites of HPAI in the motif PLREKRRKR.GLF, which is the molecular marker of HPAI. The receptor binding sites of the H5 contained H103, E186, N189, K192, K189, G221, Q222, R223 and G224 (H5 numbering). The HA gene was also characterized by having A133 and A156 amino acid residues. A/Domestic goose/Sulaimani/Sul.1/2018 had amino acids S94, V282 and I114, which is characteristic of 2.3.4.4 group B. Amino acid characterization of NA gene of A/Domestic goose/Sulaimani/Sul.1/2018 indicated that the gene possessed the amino acid residues V116, I117, R118, E119, Q136, V148, D151, R155, D198, I 222, S246, H277, E 276, R292, and N294 (N2 numbering).

Discussion

In this study, HPAI (H5N8) virus, A/Domestic goose/Sulaimani/Sul.1/2018, was detected in geese in Sulaymaniyah province in Kurdistan Region, Iraq. Phylogenetic analysis reveals that the virus fell in group B in clade 2.3.4.4 H5N8. The topology of the phylogenetic tree based on the HA gene indicated that the Iraq virus clustered with viruses isolated from Iran and Israel in 2016–2017. The topology of the phylogenetic tree based on the NA gene showed that the Iraq virus clustered with viruses in Iran and Belgium. According to both HA and NA clusters, A/Domestic goose/Sulaimani/Sul.1/2018 has a common ancestor with A/Crow/Aghakhan/2017, which was isolated from the migratory hooded crow in a national park in Esfahan province of Iran [21].

Because Iraq and Iran are located in the path of Black Sea-Mediterranean flyways and West Asian-East African flyway of migratory bird [22], it was suggested that both A/Domestic goose/Sulaimani/Sul.1/2018 and A/Crow/Aghakhan/2017 may have originated from the same source of migratory birds. Furthermore, according to previous database research on the transmission of H5N1 in Iraq, poultry trading is more likely associated with the transmission of avian influenza [22]. Iraq shares a long international border with Iran, and legal and illegal commercial and poultry trades take place between the two countries. Therefore, we cannot exclude that A/Domestic goose/Sulaimani/Sul.1/2018 may have been transmitted from Iran. As there were no reported HPAI cases in Sulaimani province and nearby provinces, it is difficult to estimate that the infection originated from indirect contacts with domestic birds and broiler farm chicken in Sulaiami Province.

According to the report of the World Organization for Animal Health (OIE), the first outbreak of H5N8 in Iraq was in January of 2018 [15]. However, the sequences of the viral HA and NA have not been identified previously. Hence, our study about the characterization of the Iraqi H5N8 in Greylag Geese is considered the first report of fully characterized HPAI H5N8 subtype.

Unfortunately, few sequences of H5N8 were available in the GenBank databases from the Middle East and only 34 H5N8 isolates were submitted to GISAID across the globe, which hindered the analysis of the avian influenza virus isolated in this study. On the other hand, the other six segments of H5N8 were deposited in GenBank much lower as compared to HA and NA, therefore, we depended only on the analysis of HA and NA in this study.

Multiple insertions of basic amino acids at the cleavage site of the HA gene were major determinants of pathogenicity of the H5 virus [23]. The sequence of the HA gene of A/Domestic goose/Sulaimani/Sul.1/2018 showed that the virus possessed the molecular markers for HPAI, with a polybasic amino acid cleavage site motif, PLREKRRKR/GLF. The receptor-binding site curtails the host range of influenza virus [24]. The single amino acid substitutions A132S, Q222 L, G224S, Q192H, Q192R, S223 N, and N220 K (H5 numbering) of HA

protein have been reported to increase the affinity of avian influenza virus to from α–2,3 sialic acidavian) to α–2, 6 sialic acid (human)[6, 25].

The receptor-binding domains of A/Domestic goose/Sulaimani/Sul.1/2018 were H103, E186, N189, K192, K189, G221, Q222, R223, and G224, which revealed the preference for classic avian α–2, 3 sialic acid specificity. In this study, the Iraq HPAI subtype H5N8 had two substitutions in HA at S133A and T156A, like most H5N8 subtypes. These substitutions increase the affinity for α2,6 sialic acid receptors in mammals [26, 27]. In spite of that, according to WHO and OIE, there were no reported cases of human infection with the H5N8 influenza virus so far [15, 28].

Susceptibility of avian influenza to antiviral drugs is associated with the sequence characteristic of NA protein [29]. Previous studies showed that molecular markers of resistance to zanamivir are V116A, R118K, E119G/A/D, Q136K, D151E, R152K, E277D, R292K and oseltamivir resistance markers are I117V, E119V, D198N, H274Y, R292K, and N294S (N2 numbering) [30]. Analysis of the NA-deduced amino acid of Iraq H5N8 showed that there were no markers for oseltamivir, zanamivir, and Peramivir. Therefore, A/Domestic goose/Sulaimani/Sul.1/2018 may be susceptible to antiviral drugs that act via the inhibition of NA.

Conclusions

The genetic characteristics of the HA gene of the Iraq H5N8 virus revealed molecular markers for the avian type receptor. The phylogenic analysis showed that Iraq H5N8 virus fell in clade 2.3.4.4 group B, and clusters with some of the Middle East H5N8 viruses. Genetic characterization of NA showed susceptibility of the virus to antiviral drugs. There was not enough information in the major sequence databases about the H5N8 viral sequence in the Middle East, especially in Iraq, which negatively affected our avian influenza research. Further surveillance on full-genome analyses is needed to determine the main risk factors for HPAI H5N8 viruses in Iraq.

Abbreviations

ACUC: Animal Care and Use Committee; AIV: Avian Influenza Virus; DEPC: diethyl pyrocarbonate; EDTA: ethylenediaminetetraacetic acid; GISAID: Global Initiative on Sharing Avian Influenza Data; HA: Hemagglutinin; HPAI: Highly Pathogenic Avian Influenza IRD: Influenza Research Database; LPAI: Low Pathogenic Avian Influenza NA: Neuraminidase; NIAID: National Institute of Allergy and Infectious Diseases; OIE: World Organization for Animal Health; TBE: Tris/Borate/EDTA; WHO: World Health Organization

Declarations

Ethics approval and consent to participate

The study was approved by the Animal Care and Use Committee at the College of Veterinary Medicine, University of Sulaimani. Verbal consent was taken from the owner of the animals to conduct the study. Because this was an observational study, the need for informed consent from the owner was not necessary.

Consent for publication

Not applicable

Availability of data and materials

The coding sequences of the HA and NA gene were submitted to the GenBank Influenza virus database. The virus was named by A/Goose/Iraq/Sul.1/2018. The accession numbers of the HA and NA genes are MK757595 and MK757597, respectively.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Nahla, MS and Peshnyar, MA designed and conceived this study and wrote the manuscript. Dyary, HO provided professional suggestions and wrote the manuscript. All authors read and approved the final manuscript.

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Not applicable

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Tables

Table 1. List of primers and amplified genes

Primer name	sequences	Gene	Purpose	Amplicon	Reference	Reference strain
M30F2/08F	ATGAGYCTTYTAACCGAGGTCGAAACG	M	Detection of AIV type A	244	[17]	26-52
M264R3/08R	TGGACAAANCGTCTACGCTGCAG	M	Detection of AIV type A		[17]	267-245
HA5-1F	AAAGTGATCAGATYTGCATTG	HA	Sequencing	420	[14]	44-64
HA5-1R	TGGTATGGRCATGCTGAGCTCA	HA	Sequencing		[14]	440-461
H5-2F	TCATTTTGAGAAGATTCTGATCATCC	HA	Sequencing	754	This study	375-400
H5-2R	CCCCTGCTCATTGCTATGGT	HA	Sequencing		This study	1128-1109
H5-3F	GGCAACGTGGAAGAATGGAC	HA	Sequencing	744	This study	617-636
H5-3R	ACTCGAAACAACCGTTACCC	HA	Sequencing		[17]	1360-1341
H5-4F	CATCCACCCTCTCACCATCG	HA	Sequencing		[14]	927-968
H5-4R	GCGATCCATTGGAGCACATC	HA	Sequencing		[14]	1681-1662
NA8-1F	AATAATGACCGTTGGCTCCA	NA	Detection & sequencing	616	This study	18-37
NA8-1R	AGTAGGCACCCCTCCGTAAT	NA			This study	633-614
NA8-2F	AAGTGGATGGCGATTGGTGT	NA	Sequencing	403	This study	567-586
NA8-2R	TGGGCAACCCTGCACATAAA	NA	Sequencing		This study	969-950

Figures

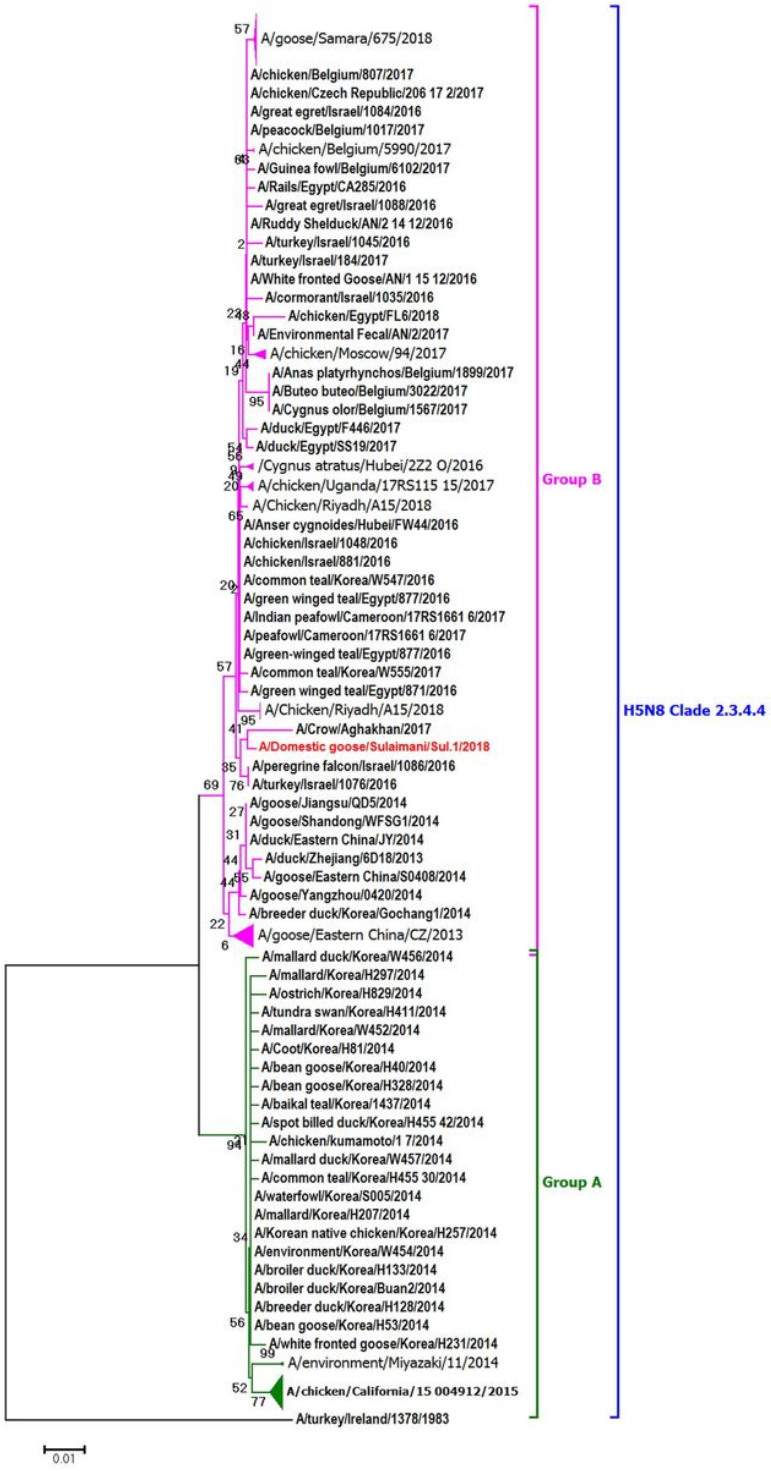

Figure 1

Phylogenetic tree of HA amino acid sequences estimated with the Neighbor-Joining algorithm using MEGA version 7. The topology was supported by bootstrap analysis with 1000 replicates. Iraq H5N8 viruses are depicted as red circles. It fell in clade 2.3.4.4 of group B.

Figure 2

Phylogenetic tree of NA amino acid sequences estimated with the Neighbor-Joining algorithm using MEGA version 7. The topology was supported by bootstrap analysis with 1000 replicates. Iraq H5N8 viruses are depicted as red circles. It fell in clade 2.3.4.4 of group B.