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MOLECULAR CHARACTERIZATION AND GENETIC DETECTION FOR CANINE INFLUENZA VIRUS GENOTYPE H3N8 IN IRAQ

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Abstract

Little is known about the genetic characteristics of canine influenza viruses (CIV) circulating in Iraq. Besides, influenza remains a major public health problem and an endemic disease in Iraq. Therefore, this study describes the genetic characteristics of the detected CIV H3N8 in Iraq during the 2018-2019. Full genome sequence of CIV H3N8 virus isolated from dogs in Iraq was assembled. Moreover, genetic analysis of Iraqi CIV H3N8 was primarily detected by reverse real-time quantitative polymerase chain reaction (RT-qPCR) test. While next-generation sequence (NGS) technology was used for CIV whole genome sequence. Phylogenetic analysis of surface glycoproteins (*Hemagglutinin* and *Neuraminidase* genes) and internal segments (*PB2, PB1, PA, NP, M* and *NS*) indicated that the Iraqi CIV H3N8 was closely related to equine-influenza viruses genotype H3N8 isolated from the USA strains. Active surveillance of CIV in canine training and qualification kennels should be adopted to monitor the genesis and emergence of new viruses in Iraq.

Key words: Canine Influenza, RT-qPCR, H3N8, Molecular detection, NGS, Iraq.

Introduction

Influenza A viruses can infect a varied range of hosts, from birds to mammals, and exhibits varying degrees of host alteration (Taubenberger and Kash, 2010, Crawford *et al.,* 2005). More attention to potential cross-species transmission capacity of influenza A viruses (Ma *et al.,* 2008) that given international communication among human, birds, pigs and further mammalian types (Brown, 2000). Epidemiological studies of CIVs have revealed several outbreaks of inter-species transmission, such as the equine-origin H3N8 (Crawford *et al.,* 2005) and the avian-origin H3N2 influenza viruses that crossed host barrier of the dog (Song *et al.,* 2008). Sialic acid (SA) receptors in dogs have a distribution similar to avian SA receptor (Ning *et al.,* 2012) and similar to equine SA receptor (Daly *et al.,* 2008), which enables influenza viruses to enter the respiratory epithelial cells. Infected dogs by influenza viruses showed respiratory clinical signs, which are susceptible to highly pathogenic influenza (Lyoo *et al.,* 2017). Avian and equine influenza viruses when they crossed the host barrier to give rise to the canine influenza virus (CIV). That indicates dogs co-infected with various influenza viruses may act as an intermediate host for avian and equine influenza viruses' re-assortment (Na *et al.,* 2016).

As well as avian-to-canine and equine-to-canine transmission, evidence has been reported for transmission of seasonal human H3N2 and pandemic H1N1 (pH1N1) virus to canines. Dogs had experienced seasonal H3N2 infection since 2008 and pH1N1 infection alone or in blending with H3N2 CIV after 2009 were revealed by Serum samples gathered from canines. Pathological variations in the lungs caused by infectivity of pH1N1 and migrant H3N2 viruses in canines proved by artificial injection of viruses with lively viral shedding (Song *et al.,* 2015). The possibility of re-assortment between the two viruses in canines was suggested by Studies on seroprevalence and artificial infection; subsequently, M segment-swapped CIV between pH1N1 and wild-type H3N2 CIV and H3N1 were isolated (Moon *et al.,* 2015; Song *et al.,* 2012).

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Materials and Methods

To display the prevalence of the equine-derivation CIV H3N8 in the dog population, four dog kennels were selected from four different cities from Iraq provinces. Regular inspections were conducted to assess the prevalence of CIV in dog kennels from October 2018 to September 2019. A total of 150 nasal swabs were collected from suspected dogs at a different sex and age from four dog kennels and collected in three different periods as groups (group 1 (G1) from October to November 2018, group 2 (G2) from February to May 2019 and group 3 (G3) from August to September 2019) showed in Fig. 1. Samples collected from four cities in Iraq included Baghdad, Babel, Karbala and Najaf. These samples were transferred in Viral Transport Media (VTM) after collection and were stored at -70°C before tested.

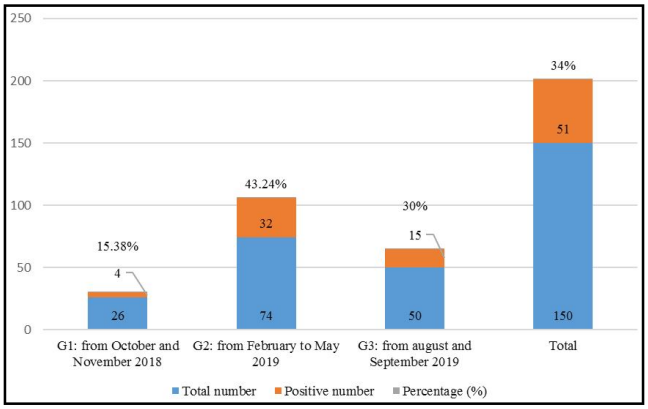

Fig. 1: Distribution of samples according to results of RT-qPCR.

The isolation of viruses from canine nasal swabs were carried out according to report protocols for the World Health Organization, (2006). Viral RNA was extracted by using the Ribo Kit Virus (Sacace, Italy). The subtyping experiments primers used for detection influenza A types by RT-qPCR in this study according to Terrier *et al.,* (2011). And reverse transcription RNA to synthesizes complementary DNA (cDNA) performed by using the Go Script™ Reverse Transcription (Promega, USA). cDNA delivered to generate through widely used next-generation sequence (NGS) technologies (Illumina) for whole-genome sequence (WGS).

Reading quality analysis and improvement: This unit is the first stage in almost WGS bioinformatics analyses and refers to the quality control and improvement of the raw sequencing data. Raw data accepted single-end and paired-end reads (fastq.gz format) to generate NGS technologies (Illumina). Reads' quality control in this study performed by using FastQC software (Andrews, 2010), while through Trimmomatic achieved quality improvement (Bolger *et al.,* 2014).

Variant uncovering and accord generation: This stage of the pipeline contains mapping the quality processed reads against user reference sequences, followed up by SNP/indel calling and annotation and generation of consensus nucleotide sequences.

The reference database of canine influenza includes reference sequences of equine-origin Influenza A virus (MK690099.2 Influenza A (H3N8) (HA) gene USA 2020). Reference sequences are publicly available at the National Centre for Biotechnology Information (NCBI). The reference file, both in ".fasta" and ".gbk" (GenBank) format annotation accomplished by using (Prokka) (Seemann, 2014), was prepared to fit amplicon-based schemas capturing the whole coding sequences (CDS) of the eight genes of influenza virus. ".Fasta", files uploaded and explained by using Prokka. In this unit, by Snippy advantage, an extremely elastic multi-program tool for fast readability mapping (by using Burrows-Wheeler Aligner – BWA) (Li and Durbin, 2009), SNP/indel calling (by using samtools and freebayes) (Li *et al.,* 2009; Garrison and Marth, 2012), variant annotation (by using SnpEff) (Cingolani *et al.,* 2012) and consensus generation (by using vcftools) (Danecek *et al.,* 2011).

The phylogenetic trees were generated with the MEGA program (version 6.0) by using neighbour-joining analysis (Tamura *et al.,* 2013). Besides, the Statistical Analysis System (SAS, 2012) program was used to detect the effect of different factors in study parameters.

Results

The recent genetic screening revealed that from 150 nasal swabs 51 (34%) was found positive by *M* gene-specific RT-qPCR in kennelled dogs Fig. 1. Kennelled dogs were examined by *M* gene-specific RT-qPCR for equine-origin H3N8 CIV from four cities in Iraq. Kennelled dogs from Baghdad were detected with the highest prevalence when tested by *M* gene-specific RT-qPCR (78.38%), followed by Babel, Najaf (8.1%) and Karbala (5.4%) Fig. 2. Phylogenetic tree was mainly divided into subgroups that correlative with equine lineages. As shown in the constructed phylogenetic trees, the CIV grouped with the newly isolated Equine influenza viruses H3N8 from France, Malaysia, Chile, and the USA. *Hemagglutinin* (*HA*), and *Neuraminidase* (*NA*) genes of CIV H3N8 closely relate and cluster in the same clade with the equine H3N8 viruses from the USA, while CIV from other countries isolated in different clades. Furthermore, the *HA* gene of the canine H3N8 strain from kennelled dogs seemed to be derived from the (A/equine/Oregon/78356/2012(H3N8)) strain Fig. 3 and table

1 was shown neighbour-joining relation compatibility tree data. The internal genes of_A/canine/Iraq/2020(H3N8) most closely connected to the (A/equine/Oregon/78356/2012 (H3N8)). Similarly, we compared the gene sequence of the *NA* gene from the CIV isolate compatible with the equine isolate from the USA and the most similar influenza virus strain was (A/Equus caballus/USA/154390/

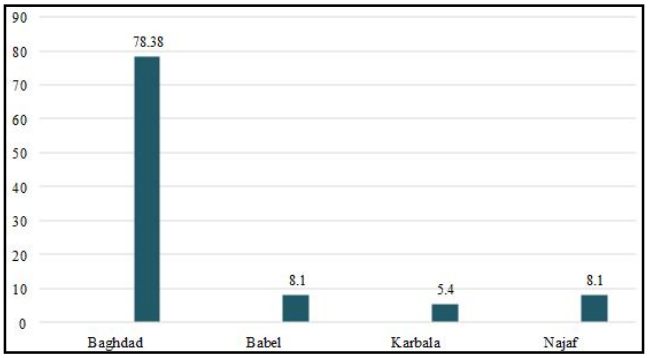

Fig. 2: Distribution of collected samples study according to Provinces.

Table 1: Neighbour-joining relation tree data of NCBI reference strains of influenza genotype H3N8 for H3 *HA gene*.

Accession	Data collection	Host	Country	Compatibility
MF173348.1	2012	*Equus caballus*	USA	91%
MH135223.1	28-Aug-2015	*Equus caballus*	Malaysia	90%
KR351249.1	21-Oct-2014	*Equus caballus*	USA	90%
MF173124.1	2016	*Equus caballus*	USA	92%
MG198999.1	08-May-2015	*Equus caballus*	Montana, USA	92%
MK501760.1	Dec-2018	*Equus caballus*	France	93%
MK690099.2	23-Aug-2018	*Equus caballus*	USA	93%
MK690123.2	28-Aug-2018	*Equus caballus*	USA	93%
MH347131.1	25-Feb-2018	*Equus caballus*	Chile	93%
MH346560.1	21-Feb-2018	*Equus caballus*	Chile	87%
MH346579.1	21-Feb-2018	*Equus caballus*	Chile	93%
MH347079.1	25-Feb-2018	*Equus caballus*	Chile	93%

Table 2: Neighbor-joining relation tree data of NCBI reference strains of influenza genotype H3N8 for N8 *NA gene*.

Accession	Data collection	Host	Country	Compatibility
KR351219.1	21-Oct-2014	*Equus caballus*	USA	90%
KR351243.1	21-Oct-2014	*Equus caballus*	USA	90%
MF173246.1	2016	*Equus caballus*	USA	91%
MK690121.2	28-Aug-2018	*Equus caballus*	USA	91%
MK501802.1	Jan-2019	*Equus caballus*	France	88%
MK690097.2	23-Aug-2018	*Equus caballus*	USA	92%
MH346616.1	21-Feb-2018	*Equus caballus*	Chile	91%
MH347096.1	15-Feb-2018	*Equus caballus*	Chile	91%
MH346708.1	15-Feb-2018	*Equus caballus*	Chile	91%
MH346583.1	15-Feb-2018	*Equus caballus*	Chile	91%

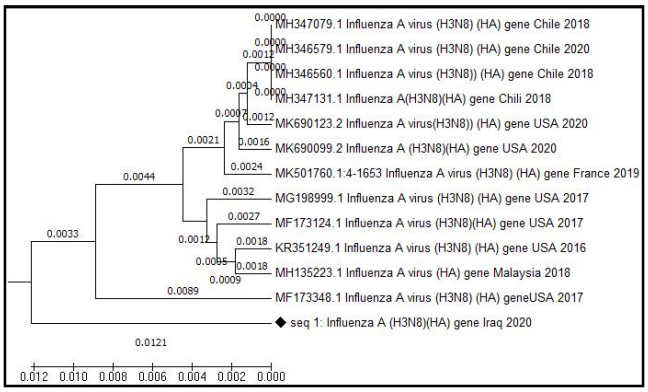

Fig. 3: Phylogenetic trees for the A/canine/Iraq/2020(H3N8) *HA* gene and those of other influenza A viruses. The analysis was based on the nucleotide sequences in the open reading frames of the *HA* gene. The trees were generated with the MEGAX program by using neighbour-joining analysis.

2018(H3N8)) strain Fig. 4 and table 2 showed neighbour-joining relation compatibility tree data. All these analyses revealed that few changes happened in CIVs after years of spreading.

The dogs showed similar symptoms of sneezing, copious nasal discharge, and subnormal to low fever 36°C-39.5°C when the dogs entered the clinics. Until now, no such genetically study about H3N8 CIV infections were carried out in kennelling dogs in Iraq. This study will provide an important insight into pathogenesis, transmission, and evolution of CIVs, which emerged recently in Iraq and help determine future counter measures.

Discussion

This is the first epidemiological survey to assess the risk of H3N8 CIV transmission among different dog populations in Iraq. One strain was isolated from a suspected pet dog in Baghdad veterinary hospital. This indicates that H3N8 CIV may currently be a common pathogen for dog populations in Iraq. Our study showed that the infection rate of this equine-origin canine influenza in kennelled dogs were (34%) from total suspected cases, as determined by *M* gene-specific RT-qPCR test in Fig. 1. The close contact is the most probable route for spreading that occurred between infected canines with CIV H3N8. Our findings strengthened data by showing that after long-term adaptation in dogs, the equine-origin CIV H3N8 has already circulated in various dog inhabitants in Iraq (Li *et al.,* 2010; Song *et al.,* 2008).

The benefits of qPCR in relation to conventional PCR include speed, reproducibility and quantitative ability. In addition to operational advantages, qPCR is

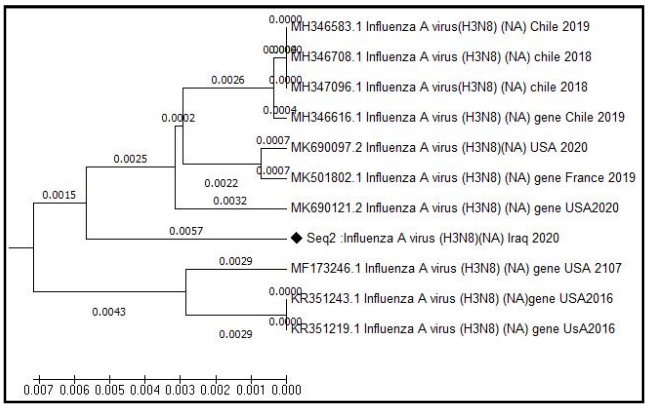

Fig. 4: Phylogenetic trees for the A/canine/Iraq/2020(H3N8) *NA* gene and those of other influenza A viruses. The analysis was based on the nucleotide sequences in the open reading frames of the *NA* gene. The trees were generated with the MEGA X by using neighbour-joining analysis.

more sensitive and reproducible (Paiva-Cavalcanti *et al.,* 2010). Besides, there is a problem in the association of primer with the target gene, and this may be due to the rapid emergence of changes in influenza viruses especially in *HA* and *NA* gene, which demands for the continuous development of primers to obtain the best results for RT-qPCR. This explanation is considerable with Inoue *et al.,* (2010) finding, this is a key reason for pushing researchers to develop new molecular marker as primer matching well with that new variation. Because of that variation, the WGS is preferred from the Sanger sequence for influenza virus. The evolutionary history for H3 was conducted using the UPGMA method by MEGAX software. The flanking region of Hemagglutinin Ha gene involving 1650 bps in the final dataset for the 13-nucleotide sequences, one of them belonged to local Iraqi isolate and others imported from NCBI gene bank. The optimal tree with the sum of branch length = 0.05063280 was shown in Fig. 3.

The phylogenetic tree was drawn for N8 segment six represent Neuraminidase, NA gene including 11 nucleotides sequences, one of them represents our genotype and 10 nucleotides sequences imported from NCBI was shown. The optimal tree with the sum of branch length = 0.02999746 was shown in Fig. 4. There were 1409 positions in the final dataset. Evolutionary analyses showed that all eight genes of the Iraqi virus were phylogenetically close to H3N8 EIVs from Fig. 3 and 4. With variable nucleotide sequence similarities between Iraqi isolate and the EIVs (HA, 94%; NA, 91%; PB2, 95%; PB1, 95%; PA, 95%; NP, 90%; MP, 84%; NS, 82%), this CIV H3N8 was most likely of equine-origin. Re-assortments were observed in this CIV H3N8. The Iraqi isolate for canine influenza virus was 94% identical, which blasted with NCBI standard species MK690099.2. Besides, the compatibility of the neighbour-joining relation tree was aligned between (87%-93%) for H3 as showed in table 1 and Fig. 3. On the other hand, the Iraqi isolate for canine influenza virus was (91%) identical, which blasted with NCBI standard species MK690121.2. Besides, the compatibility of the neighbour-joining relation tree was aligned between (88%-91%) for N8 as shown in table 2 and Fig. 4. As it is, known that dogs accompany humans, so direct contact with humans is possible and no direct transmission of CIV H3N8 from dogs to humans has been documented. Because of the similarity of the cellular receptors of the virus between dogs and humans, according to Song *et al.,* (2009); and Daly *et al.,* (2008); this is likely to cause CIV a new pandemic outbreak and this may pose a great threat to our human life. These findings highlight the importance of monitoring dogs in pet hospitals and on dog kennels.

Conclusion

As CIV H3N8 outbreaks among dogs continue in Iraq provinces, areas where are densely populated and with frequent animal trade, there are a non-stop dangers for pets CIV H3N8 infections and for mutations, or genetic re-assortment foremost to first-hand CIV strains with greater than before transmissibility between dogs. Furthermore, vaccine development for CIV H3N8 is very urgent. Further study is required as the CIV H3N8 confirmed in various dog inhabitants and poses a potential danger to community health. The kennelled dogs' population may serve as a more sensitive sentinel for monitoring emerging CIV H3N8 in the future.

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Author contributions

Yahya A. AbdulKareem, Oday K. Luaibi, and Nadira S. Mohamed conceived and designed the study; Yahya A. AbdulKareem and AbdulRaheem Wali collected samples; Yahya A. AbdulKareem and Nadira S. Mohamed performed the experiments and sequencing analysis. Yahya A. AbdulKareem, Oday K. Luaibi and Nadira S. Mohamed contributed to the writing and revision of the manuscript and approved the final one.

Ethical approval

This study does not cover any training with human/ animal members implemented by authors.

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