Original Research

Journal of Advanced Veterinary Research
(2024) Volume 14, Issue 3, 466-469

Molecular detection of highly pathogenic avian influenza A (H5N8) virus isolated from domestic ducks and chickens in Egypt across 2018-2021

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ARTICLE INFO

Recieved: 21 October 2023

Accepted: 11 February 2024

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Keywords:

HPAI H5N8
RT-PCR
Sequencing
Phylogenetic analysis
Duck
Chicken

ABSTRACT

The present study was carried out to describe Egyptian H5N8 viruses isolated from vaccinated duck and chicken flocks in 2018-2021 from different provinces, Egypt. This study screened 10 vaccinated farms (five duck and five chicken) suffering from respiratory & nervous signs with high mortality rate (90%). Out of ten examined flocks, six flocks were positive for avian influenza virus (AIV) by virus isolation into embryonated chicken eggs (ECEs) for third blind passages. The initially positive samples were confirmed, identified molecularly using quantitative reverse transcriptase-polymerase chain reaction (RT-qPCR) using primers targeting M gene of influenza A. RT-qPCR positive isolates were subjected to partial amplification of hemagglutinin (HA) and neuraminidase (NA) genes using RT-PCR followed by sequencing and phylogenetic analysis for six isolates representative for one duck flock and five chicken flocks. Our findings proven that four flocks only were found positive for AIV/HPAI H5N8 virus by sequencing and phylogenetic analysis for HA and NA genes. The similarity between nucleotide sequencing for the four HA and NA was 99.8% and 98.1%, respectively, with H5N8 viruses previously detected in Russia, Iran, Israel and Iraq. As well as, the phylogenic analyses, focusing on HA and NA genes indicated that Egyptian H5N8 viruses clustered in group B Russian like reassortant H5N8 viruses of clade 2.3.4.4. In conclusion, the detection of the HPAI H5N8 virus in domestic birds even in vaccinated birds is a serious threat; therefore, this is needed for periodic molecular monitoring with vaccine efficacy evaluation and annual surveillance.

Introduction

Influenza A is the only species of genus *Alphainfluenavirus* of the family Orthomyxoviridae that can produce pandemic disease (Kaplan and Webby, 2013). Influenza A viruses have negative-sense ssRNA genomes composed of eight segments. Based on the combination of two surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), there are 16 HA and 9 NA subtypes have been identified in birds and two additional subtypes, H17N10 and H18N11, have been identified in bats (Ying *et al*., 2014). 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 (Neumann *et al*., 2007). HPAI H5N8 virus was 1st recoded and isolated in china in 2010 and outbreaks of HPAI H5N8 clade 2.3.4.4 were isolated from chickens and domestic ducks (Lee *et al*., 2014). Additionally, in early 2014 was recorded in South Korea (Li *et al*., 2014). HPAI H5N8 spread across Europe, North America, and East Asia late 2014 (Bouwstra *et al*., 2015). Later in 2016/2017, a new reassortant H5N8 virus of clade 2.3.4.4 of subtype was detected in wild birds in Russia and continued to spread by migratory birds in different localities in Europe, Asia, and Africa (OIE, 2017). Wild birds act as an asymptomatic natural reservoir for all AIV subtypes and disseminate the infection among domesticated bird globally (Alexander, 2007). The migratory birds along the Black Sea–Mediterranean and East African–West Asian migration flyways represented a main accused for introduction of HPAI H5N8 clade 2.3.4.4b in Egyptian poultry (Salaheldin *et al*., 2022). Multiple HPAI H5N8 isolates clade 2.3.4.4 were recorded in Egypt since 29 November 2016 with different reassortments in the internal genes originating from Europe and Asia (Kandeil *et al*., 2017) causing serious economic losses in poultry production. Therefore, we desperately need to implement vaccination programs against H5N8 to limit the huge economic losses in the Egyptian poultry industry. Clinically, HPAI H5N8 viruses are mainly characterized by neurological signs in ducks versus respiratory signs in chickens, in addition to lethargy, ruffled feathers, eyelids edema, comb cyanosis prior death (Pantin-Jackwood *et al*., 2017). Therefore, in the current study, tissue specimens were collected from different vaccinated duck and broiler chicken farms in different Egyptian provinces with a history of severe mortalities associated with respiratory and nervous manifestations for virus isolation, molecular detection and partially sequencing of the HA and NA of HPAI H5N8 viruses to recognize the circulating HPAI H5N8 strains during the period from 2018 to 2021.

Materials and methods

Ethical approval

The Animal Ethical Committee of the Faculty of Veterinary Medicine, Zagazig University, Egypt approved the design of the current study.

Clinical inspection and gross examination

The investigated ducks and chicken were inspected for presence of any abnormal signs. Internal organs were examined grossly, abnormal clinical and findings and gross lesions were recorded.

Collection of samples

Sampling was carried out into ten vaccinated chicken and duck

ISSN: 2090-6277/2090-6269/

flocks suspected to have avian influenza infection from different Egyptian provinces. The specimens were collected from freshly dead chickens and ducks including trachea, brain, liver, spleen and lung and submitted to the laboratory between November 2018 to March 2021 on ice and then preserved at -80°c till used.

Preparation of collected specimens and Isolation of the AIV using embryonated chicken eggs (ECEs)

Collected five organ specimens were pooled per each flock from five different birds and prepared. The supernatant of the prepared specimens (0.2 ml) was inoculated into the allantoic sac of 10-days-old ECEs (five ECEs for each specimen) from commercial non-vaccinated layers in a class II biosafety cabinet for closely blind 3rd passages and observed daily. According to the protocol (Ahmed, 2006 and OIE, 2018), the allantoic sacs were harvested after embryos deaths and checked using hemagglutination test and kept at -80°C until further studies. The harvested allantoic fluids were screened and checked using hemagglutination test against washed chicken erythrocytes for virus hemagglutination action by HA gene.

Molecular detection, confirmation and identification of AIV isolates from duck and chicken flocks using RT-qPCR

The positive isolated specimens were tested using RT-qPCR targeting M gene of influenza A virus using primers with the following sequences; Forward: 5′-ATGAGYCTTYTAACCGAGGTCGAAACG-3′ & Reverse: 5′-TGGACAAANCGTCTACGCTGCAG-3′ (Fujitsuka *et al*., 2011). The positive influenza A virus was further tested to determine HA and NA genes using multiplex PCR (Stewart *et al*., 2001) as following. Total RNA extraction was conducted using Gene JET viral RNA Purification Kit (Thermo Fisher Scientific Inc., USA). RNA from each specimen was reverse transcribed to produce cDNA using a QuantiTect Reverse Transcription Kit (Qiagen, Germany). Afterwards, PCR for amplification of 542 bp from haemagglutinin (HA) and 756 bp from neuraminidase (NA)gene, respectively were performed using designed primers with the following sequences: forward: 5′-ATGCAAACAACTCGACAGAGCA-3′ & reverse: 5′ GGATGTTGTGGAATGGCATACT-3′ for HA gene and forward: 5′-CCTTTCGTCTCWTGTTCACC-3′ & reverse: 5′- GTCTCTACACACGCATTCC - 3′ for NA gene. Amplification was done using Dream TaqTM Green PCR Master Mix (2X) (Fermentas, Glen Burnie, MD). The amplified fragments were separated on agarose gels (1.5%) and 1-kbp DNA marker (Fermentas, Glen Burnie, MD) was used as standard and the amplified products were visualized using ultraviolet light transilluminator (Spectroline). Tissues of the negative control group were included for detection of any contamination. The amplicons were purified using the QIA quick gel extraction kit (Qiagen, Germany) following the manufacturer's instructions, and kept at -20°C until sequencing.

Molecular confirmation and identification using Sequencing and phylogenetic analysis of the AIV HA and NA genes

The purified amplified HA and NA segments of four selected isolates was sequenced in both directions using Bigdye Terminator v3.1 cycle sequencing Kit (Perkin-Elmer, CA) utilizing the amplification primers in applied Biosystems 3130 genetic analyzer (ABI, USA). For every sequence, the blast search was conducted (http://www.ncbi.nlm.nih.gov/BLAST). Amino acid sequence identities and divergences were calculated utilizing Meg Align software (DNA STAR® Laser gene® version 7.2, USA). Phylogenetic tree was created via the maximum-likehood method employing the Kimura 2-parameter model in MEGA.6 software (Tamura *et al*., 2013) through a bootstrap of 1000 trials of the Clustal W alignment algorithm. The nucleotide sequences of HA and NA segments of Egyptian H5N8 viruses generated in this study and H5N8 viruses from Asia, Africa, middle east, Europe during the period 2011 to 2022 were compared to the sequences of the commercially available H5 vaccines in Egypt.

Results

Clinical findings and gross lesion of affected ducks and chickens

Clinical signs of examined birds including variable mortalities (90-100%), weakness, and lethargy. Clinical signs in chickens ranged from nasal discharges, dyspnea, coughing, sneezing, greenish diarrhea, ataxia, cyanosis and congestion of the comb and wattle with dark red to blue depressed areas of ischaemic necrosis (Figure 1A&C), as for shanks and feet showed hyperemia and hemorrhage (Figure 1B). As well as in ducks, ataxia and nervous signs (torticollis) including tremor of neck were observed (Figure 1D). Gross lesions in affected flocks included generalized congestion involving trachea, lungs, heart, liver, pancreas, intestine, spleen, kidneys and brain, in addition to, petechial hemorrhage on visceral organs and in muscles. Infected Muscovy duck showed enlarged and congested liver (Figure 2A). While infected chicken showed enlarged congested liver with multifocal white mottling pattern (Figure 2B). Petechial and ecchymotic hemorrhage was shown in pancreas and intestine of infected chicken (Figure 2C) and enlarged haemorrhagic ovarian follicles in infected layer chicken flock (Figure 2D).

Detection of AIV isolates from chicken and duck flocks using virus isolation and RT-qPCR

Out of 10 examined flocks, six flocks were positive for AIV by virus isolation into ECEs via allantoic sacs showed that the embryos died within 48 hours with hemorrhages and congestion (Figure 3B) in comparison with control embryo showed normal development (Figure 3A). The harvested allantoic fluids were tested for virus hemagglutination action by HA gene.

Molecular detection, confirmation and identification of AI viruses isolated from duck and chicken flocks using RT-qPCR

The initially positive isolates were confirmed, identified molecularly using quantitative reverse transcriptase-polymerase chain reaction (RT-qPCR) using primers targeting M gene of influenza A. The positive results of RT- qPCR with low Ct value (M gene was 18-21). The positive AIV flocks were geographically distributed as follow; two positive duck flocks from Alexandria governorate, four positive chicken flocks from Dakahl-

Table 1. Results of AIV detection using qRT-PCR and virus isolation into ECEs.

Flock #	Flock size	Breed	Locality (governorate)	Collection date	Age	Vaccination status
1	600	Duck (Muscovy)	Alexandria	22-Nov-20	90 days	+
2	4000	Broiler (Cobb500)	Sharqia	21-Dec-18	14 days	+
3	3600	Broiler (Avian 48)	Dakahlia	30-Jan-19	21 days	+
4	2560	Layer (Lohman brown)	Sharqia	12-Nov-21	33 weeks	+
5	5000	Broiler (Sasso)	Dakahlia	15-Mar-18	60 days	+
6	430	Duck (Muscovy)	Alexandria	12-Dec-21	75 days	+

ia and Sharqia governorate. The information regarding breed, age, flock size, vaccination status and collection date were summarized as shown in (Table 1).

Figure 1. Clinical signs of HPAIV H5N8 infected chickens and ducks; (A) dark red to blue depressed areas of ischaemic necrosis of comb and wattle of infected chicken. (B) hyperemic and hemorrhagic shank and feet of infected layer chicken. (C) cyanosis and congestion of the comb and wattle of chicken. (D) infected Muscovy ducks showed ataxia and nervous signs (torticollis) including tremor of neck.

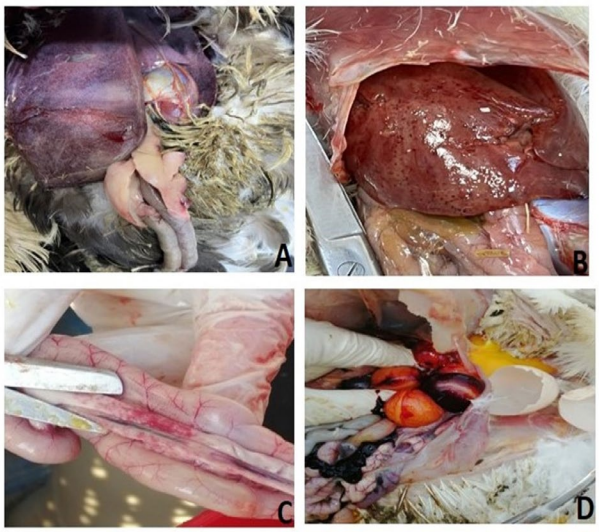

Figure 2. Post mortem changes of HPAIV H5N8 infected chickens and ducks; (A) Infected Muscovy duck showed enlarged and congested liver. (B) infected chicken showed enlarged congested liver with multifocal white mottling pattern (C) Petechial and ecchymotic hemorrhage was shown in pancreas and intestine of infected chicken (D) enlarged haemorrhagic ovarian follicles in infected layer chicken flock.

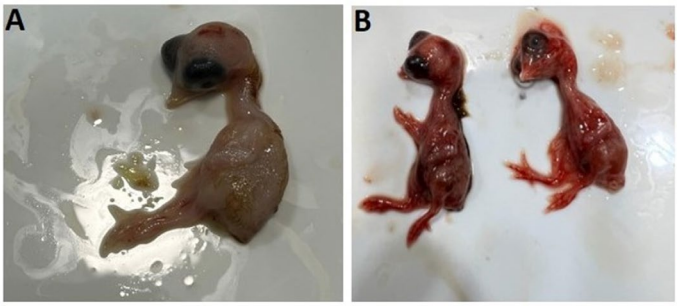

Figure 3. AIV isolated in ECEs via allantoic sac: (A) Normal embryo development (B) the embryos died within 48 hours with hemorrhage and congestion after third blind passage.

Sequencing and phylogenetic analysis of the AIV HA and NA genes

Four out of ten vaccinated flocks from different provinces in Egypt were tested positive for HPAI/A/H5N8 using RT-PCR for HA and NA genes (Figure 4). The amplified NA and HA were subjected to sequencing and phylogenetic analysis. These specimens represented 4 poultry flocks (broiler & layer chicken, Muscovy duck). All studied flocks were vaccinated once and/or 3 times. The sequences were submitted to genBank and assigned accession numbers OR569129-OR569132 for H5 sequences & OR569135-OR569138 for N8 sequences and then published by the National Center for Biotechnology Information (NCBI). The phylogenetic tree's topology based on the HA gene showed that the studied isolates (A/H5/duck/Alexandria/2020, A/H5/layer/Sharqia/2021, A/H5/broiler/ Sharqia/2018, A/H5/broiler/ Dakahlia/2019) belonged to clade 2.3.4.4b (Figure 5). The phylogenetic tree revealed that these isolates were resembling the Egyptian isolates from 2017 – 2021 with no major genetic differences, also they closely clustered in group B Russian like reassortant H5N8 viruses of clade 2.3.4.4 isolates with wild and domestic birds in Russia, Iran, Israel and Iraq. In addition to, the phylogenic analysis of the partial NA gene of the Egyptian H5N8 viruses also showed that it belonged to group B, and it was clustered with viruses from wild birds in Russia, India, Japan, Nigeria and Iraq (Figure 6). The amino acid sequences of the studied isolates showed that multiple basic amino acid motifs "PLREKRRKR/GLF" at HA cleavage sites of HPAIV H5N8 clade 2.3.4.4b which was detected by phylogenetic analysis. The similarity between the isolates in this study showed that H5N8 isolates have similarity between the other Egyptian isolates (2017 - 2021) ranging from (97.8% - 98.2%) and this cluster includes other H5N8 strains recently isolated from Asia, Europe and Africa. The nucleotide identity percent between the local vaccine strains and the field isolates showed relativity low distinct range ranging from (88.7% - 92.4%).

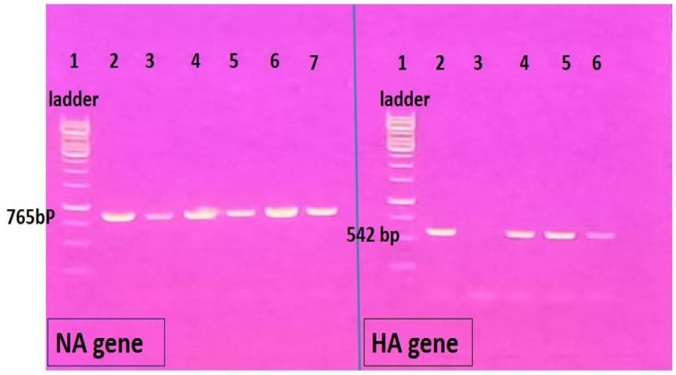

Figure 4. Agarose gel electrophoresis of the RT-PCR products of HA gene (right) and NA gene (left) showing bands of ~542 bp and 765 bp in size respectively. For NA gene: lane 1: Molecular Thermo Scientific™ GeneRuler 1 kb DNA Ladder (Fermantas), Lane 2: Ctrl +ve: Known Positive HPAIV N8, Lane 3-7: Positive specimens. For HA gene: lane 1: Molecular Thermo Scientific™ GeneRuler 1 kb DNA Ladder (Fermantas), Lane 2: Ctrl +ve: Known Positive HPAIV H5. Lane 3: Ctrl-ve: Control negative, Lane 4-6: Positive specimens.

Discussion

Highly Pathogenic Avian Influenza (HPAI) is a contagious avian disease, causing significant economic losses in the poultry production (Neumann, 2015). HPAI/ A/H5N8 viruses were introduced into Egypt by a wild bird in 2016. It has since spread rapidly in domestic birds, causing high economic losses in poultry production (Selim *et al*., 2017). The previously isolated A/H5N8 Egyptian viruses belong to clade 2.3.4.4b (Russian reassorted strain). There have been multiple introductions of reassorted AIVs into Egypt from Russian and Europe (Yehia *et al*., 2018). The present study aimed to characterize the genetic properties of HPAI subtype H5N8 in naturally infected chickens and ducks in Egyptian farms between 2018 -2021. Most of the examined specimens in this work were collected from vaccinated domestic chickens and ducks suffering ranged from nasal discharges, dyspnea, coughing, sneezing, greenish diarrhea, ataxia, cyanosis and congestion of the comb and wattle, hyperemic shanks, and feet as well as ataxia and nervous signs in ducks. Out of ten examined flocks, six flocks were positive for AIV by virus isolation into ECEs via allantoic sacs and were positive for virus hemagglutination assay that geographically distributed in Alexandria, Dakahlia and Sharqia province. Four out of ten vaccinated flocks from different provinces in Egypt were tested positive for HPAI/A/H5N8 by RT-PCR for HA and NA genes. As well as the amino acid sequences of the studied isolates showed that multiple basic amino acid motifs "PLREKRRKR/GLF" at HA cleavage sites of HPAIV H5N8. These

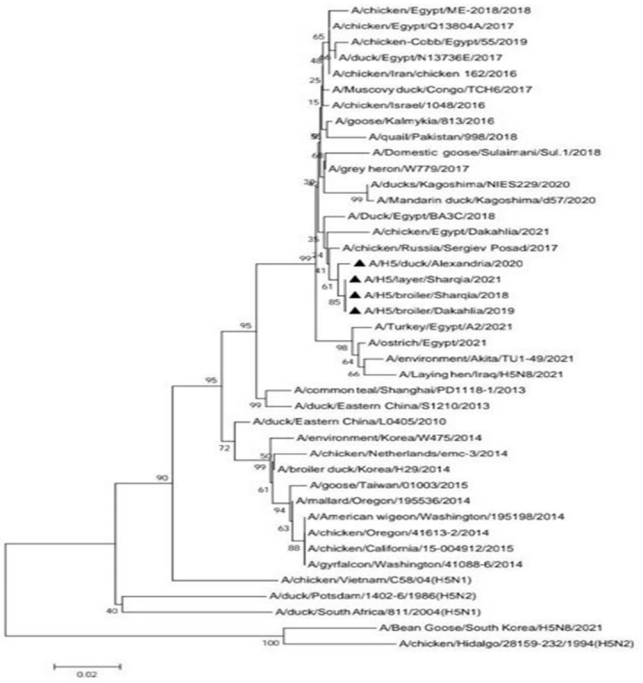

Figure 5. Phylogenetic tree of HA nucleotide sequences estimated with the Neighbor-Joining algorithm using MEGA.6.The topology was supported by bootstrap analysis with 1000 replicates. H5N8 isolates are depicted as black triangle. It fell in clade 2.3.4.4b.

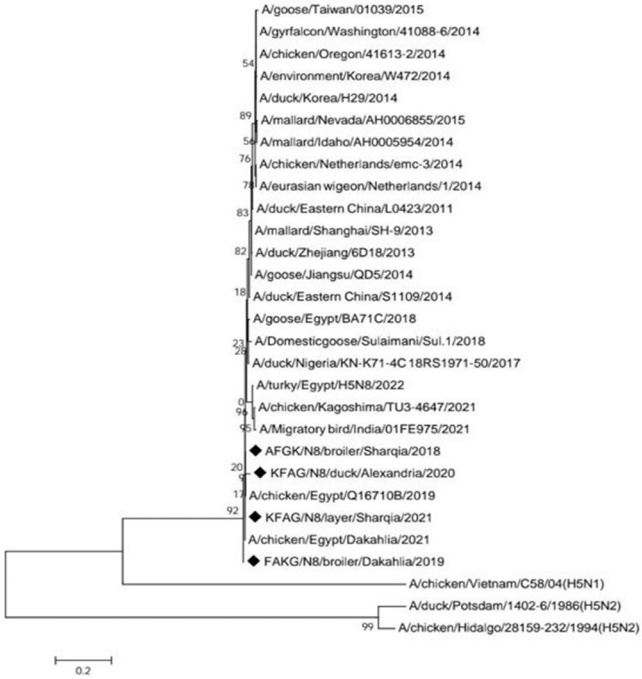

Figure 6. Phylogenetic tree of NA amino acid sequences estimated with the Neighbor-Joining algorithm using MEGA.6. The topology was supported by bootstrap analysis with 1000 replicates. H5N8 isolates are depicted as black rhombus. It fell in clade 2.3.4.4b

results are similar with the findings of Yehia *et al.* (2020) who revealed that the amino acid sequence of the protease cleavage site of HA protein from migratory bird have multiple basic amino acids, which is characteristic of HPAIV (Kandeil *et al*., 2017). In addition to, the phylogenetic analysis of the partial HA and NA genes of our sequenced viruses indicated that they clustered together in group B Russian like reassortant H5N8 viruses of clade 2.3.4.4 and grouped with a few other H5N8 viruses from the Middle East in 2017-2021. These results agreed with Kandeil *et al*. (2017) and Yehia *et al.* (2018) who demonstrated that the Egyptian HPAI H5N8 viruses were related to recently characterized reassortant H5N8 viruses of clade 2.3.4.4b isolated from different Eurasian countries. As well as previous studies revealed that multiple introductions of H5N8 from Russian and Europe with multiple reassorted viruses have occurred in Egypt (Salaheldin *et al*., 2018; Yehia *et al*., 2018). Furthermore, Yehia *et al*. (2020) confirmed that the importance of poultry in the global epidemiology of H5N8 clade 2.3.4.4b in Egypt along the migration flyways and as an endemic hotspot for H5N8 in the Middle East (Kilpatrick *et al*., 2006). Also, in the present study according to both HA and NA clusters, our sequenced viruses have a common ancestor with A/chicken/Sergiev/Posad/1/17/HA gene, isolated from chicken in Russia. Millions of wild birds use Egypt as a crucial stopover on their yearly migration between the Palearctic and Afrotropical ecozones (Denny, 1991).

Conclusion

According to the phylogenic study, the Egyptian H5N8 viruses isolated in 2018-2021from vaccinated flocks in the present study clustered in group B Russian like reassortant H5N8 viruses of clade 2.3.4.4 and grouped with a few other H5N8 viruses from the Middle East. In addition to, the bulk of approved vaccines used in the field are genetically distinct from the isolates in the present study. It is necessary to assess and improve the effectiveness of current vaccines in order to completely protect Egyptian flocks against H5N8 viruses and biosecurity measures should be improved.

Acknowledgments

This work was supported by Faculty of Veterinary Medicine, Zagazig University, Egypt.

Conflict of interest

The authors declare that there are no conflicts of interest.

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