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LARGE- SCALE STUDY OF THE MOST COMMON AVIAN FLU VIRAL SUBTYPES IN WILD BIRDS THROUGHOUT IRAQ

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Abstract – Avian flu is a type a influenza virus which is important zoonotic pathogen causing marked public health and serious economic problems. Studying viral subtypes of avian flu in wild birds (natural reservoir) plays a key role in determination of viral spread and predilection of future epidemics and pandemics. Sample collection through taking either nasal swab or fresh tracheal swab in case of dead birds, 457 of wild birds belong to eleven different species from many provinces in Iraq included in this study, advance specified molecular techniques were used, Reverse Transcription Real Time PCR (rRT- qPCR) was done to investigate the presence of the most common viral subtypes. Avian flu was reported in 4.81% of the wild birds at Iraq; highest infection rate has been recorded in the Tufted duck, 11.11%, in the other hand, the infection was not showed in quail, gull, cormorant and heron; following subtypes were recorded: H5N1, H5N2, H7N9 and H9N2 where as other AIVs subtypes were not founded. Subtype H5N1 is the prevalent type which observed in 40.9% of positive samples, H97N2 ranked below in 27.2% of samples, whereas subtypes H5N2 and H7N9 came in 22.7% and 9% of positive samples, respectively. This study has demonstrated the incidence of avian flu in many different kinds of wild birds at Iraq; to the authors' knowledge, this is the first that recorded presence of H5N1, H5N2, H7N9 and H9N2 herein, that provide valuable information concern viral subtypes for researchers, veterinarians at our country in addition to public health importance of the virus.

INTRODUCTION

Avian flu is very important contagious disease caused by Avian Influenza A viruses (AIVs) which belong to *Orthomyxoviridae* family and continue to pose a real cosmopolitan threats for both human and animal health due to slight resemblance of their receptors at the respiratory tract in both birds and mammals. The single stranded, segmented RNA nucleic acid undergo two kinds of changes, small mutations at the same strand termed as genetic drift and strand exchanges called as genetic shift that approximately responsible for crossing of species barriers and pandemics (Fodor *et al*., 2019; Yousefi Naghani *et al*., 2019; Uyeki and Peiris, 2019).

According to the main virion antigens, Haemagglutinin (HA) and Neuraminidase (NA), there are 18 of HA and 18 of NA types; Theoretically, recombination between them conducive to forming thousands of subtypes (IIyushina *et al*., 2019).

In essence, the subtypes H1- H16 and N1-N9 are circulating among avian species causing avian flu worldwide, the wild birds particularly that belong to the orders *Anseriformes* and *Charadriiformes* are considered as main natural animal reservoir for the avian flu (Nagy *et al*., 2017).

The avian influenza viruses usually not pathogenic in wild birds, in spite of they may induce significant morbidity and mortality harms when transmitted to domestic poultry resulting in real economic loss and hygiene hazards (Kain and Fowler, 2019).

The worldwide spread of AIVs and epidemic infections ensue from the mi-gration of waterfowl; when, just an accidental cases of do-mestic birds and /or mammals infection have recorded, self-limiting or a sus-tained epidemics or even serious pandemics might be arise (Gomaa *et al*., 2018).

The infected wild birds perhaps transmit their contaminated infectious material either by active shedding, or transfer of water droplets mechanically by air or throughout drinking water that polluted with bird feces containing AIVs (Germeraad *et al*., 2019). Moreover, the wild birds sometimes enter poultry barns and could carry AIVs particles

directly inside the poultry flock or in many occasions they spending time nearby the poultry barns so contaminate the adjacent surfaces beside these barns from which AIVs can be carried into poultry barns by farm staff and workers, equipment, pets, many rodents or sometimes even insects (Suter *et al*., 2019). Many studies explain that at certain environmental conditions, AIVs in the water droplets, contaminated fecal material, or organic debris can survive and remain infectious for about several days to weeks (Kalil and Thomas, 2019).

According to our knowledge, there was only a single orphan study involve AIVs in wild birds at Iraq which recorded presence of H5 and H9 subtypes (Abdul-Sada, 2015).

Because of most global studies were emphasized that the wild birds, especially aquatic, considered as the ultimate source of influenza A viruses for both man and animals; thus, the information regarding epidemiology of AIVs among wild birds in our country is quit important to improve reliable surveillance concerning these viruses *in situ* besides to ensure a better clarify underlying factors related host-switching of AIVs, in addition to establishing of a real and beneficial informative data for predilection of future epidemics and preparedness of vaccine at our region.

Therefore, the aim of the present study is searching of the most common AIVs virus subtypes in wild birds at different geographical areas from our country.

MATERIALS AND METHODS

A team of well sophisticated veterinarians participated in sample collection from wild birds in different Iraqi provinces; Baghdad, Basra, Babylon, Karbala, Theqaar, Muthana, Najaf and Diwania.

About 457 Random samples were taken from wild birds at life birds markets (LBMs) in above provinces, the wild birds types that had been involved in the present study were: quail, teal duck, mallard, coot, gull, wild geese, swamp hen, cormorant, flamingo, tufted duck and heron, the study was conducted from the beginning of September 2018 to the end of October month 2019.

Specific Dacron tipped swabs were used for sample collection, Nasal swab had been taken from the each bird or fresh tracheal swab in case of newly dead birds, each sample was put in sealed tubes which contain certain sterile viral transport medium, which called M199 solution [0.5% (w/v) sterile bovine serum albumin (BSA), 26106 U/L penicillin, 200 mg/L streptomycin, 26106 U/L Polymyxin B, 250 mg/L Gentamycin and 60 mg/L Levofloxacin hydrochloride besides to 56105 U/ L Nystatin], these sealed tubes were kept on ice during collection and immediately preserved in a liquids nitrogen dry shipper for shipment to our laboratory.

All samples were preserved inside a specified deep freezer at -70 °C until use, at the laboratory, a part of each sample was injected inside the fluid of allantoic cavities that belong to 11-day old specific pathogen-free embryonated chicken eggs, then incubated for two days at 37 °C. After that, we take 100 µL of these allantoic fluids and tested them by using of hemagglutination test with addition about 0.5% of the chicken red blood cells (CDC, 2007; Kim *et al*., 2019; Saito *et al*., 2019). About 100 µL of each positive HA samples were prepared for RNA extraction and purification through using the AniGen viral RNA purification kit, Bionote, Korea.

Influenza A virus was screened in all of HA positive samples by using reverse transcription real time PCR assay (rRT-PCR) that targeted influenza Matrix gen, samples were amplified using One-step Reverse Transcription and Amplification Real Time Kit (Qiagen, Germany) for detection of type A avian influenza viruses using. Exicycler Thermal Block Real-Time PCR apparatus (Bioneer, Korea), that targeting matrix gene using following primers and probe; forward primer: 52 -AGA TGA GTC TTC TAA CCG AGG TCG-32, reverse primer: 52-TGC AAA AAC ATC TTC AAG TCT CTG-32and probe: 52 FAM-TCA GGC CCC CTC AAA GCC GA-TAMRA-3 (Spackman *et al*., 2002).

All rRT-PCR positive were subjected for further examination searching for most common avian influenza HA and NA types were performed using one-step RT-PCR kit (Enzynomics, Korea), through utilizing multiple sets of specified array from forward and reverse primers and probes (Lee *et al*., 2001; Fereidouni *et al*., 2009).

RESULTS

The surveillance of AIVs by use of use of specific rRT-qPCR after collection of random nasal and tracheal swab samples from wild birds at the study areas showed that the percentage of infection was 4.81% (22/457); the highest infection rate has been recorded in the Tufted duck,11.11% (3/27), whereas the lowest infection rate was reported in the

mallard, which is 3.33% (2/60), while the infection not found in quail, gull, cormorant and heron (Table 1).

Regarding the geographic distribution with AIVs, the highest infection rate was observed in Babylonprovince, 9.67%, while the lowest rate was recorded in Karbala province, 1.69%, in the other hand, the infection not recorded at Muthana province (Table 2).

Further specified examination for all positive samples through using of rRT-qPCR via multiple sets of primers searching of the major AIVs subtypes, had revealed that only following subtypes were recorded: H5N1, H5N2, H7N9 and H9N2 at the wild birds in the present study. While other AIVs subtypes were not founded.

Our study reported that the subtype H5N1is the prevalent type, which had been recorded in 9/22 (40.9%) of positive samples, followed by subtype H97N2 in 6/22 (27.2%) of AIVs samples, whereas subtypes H5N2 and H7N9 had been recorded in 5/

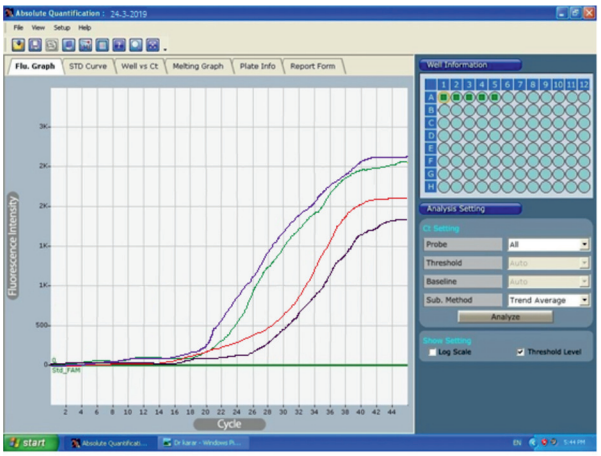

Fig. 1. Graphic Results for AIVs Positive Samples that Obtained by Reverse Transcription Real Time PCR (rRT-PCR), Thermo-cycler: Exicycler™ technique, Quantitative Thermal Block, Korea. New Version. Four Amplification curves of blue, black, green and red colors belong to subtypes: H5N1, H5N2, H7N9 and H9N2 respectively. Xaxis: cycle number, Y axis: Log. Fluorescence.

Table 1. Infection Rates with AIVs among different Kinds of the Wild Birds with distribution of the viral subtypes.

Kinds of Wild Birds	No. of Taken Samples	No. of Positive Samples	Rates of Infection	Samples with H5N1 Subtype	Samples with H5N2 Subtype	Samples with H7N9 Subtype	Samples with H97N2 Subtype
Swamp hen	81	5	6.17%	3	0	0	2
Wild geese	67	6	8.95%	3	1	0	2
Mallard	60	2	3.33%	1	1	0	0
Teal duck	57	2	3.50%	0	2	0	0
Quail	53	0	0.00%	0	0	0	0
Coot	47	3	6.38%	1	0	1	1
Gull	42	0	0.00%	0	0	0	0
Tufted duck	27	3	11.11%	1	1	0	1
Flamingo	14	1	7.14%	0	0	1	0
Cormorant	8	0	0.00%	0	0	0	0
Heron	1	0	0.00%	0	0	0	0
Total	457	22	4.81%	9	5	2	6

Table 2. Infection Rates with AIVs Subtypes in Wild Birds at Different Iraqi Governorates.

Governorates	No. of Taken Samples	No. of positive Samples	Rates of Infection	Samples with H5N1 Subtype	Samples with H5N2 Subtype	Samples with H7N9 Subtype	Samples with H97N2 Subtype
Baghdad	74	6	8.10%	3	1	1	1
Basra	63	3	4.76%	1	1	0	1
Babylon	62	6	9.67%	2	2	0	2
Karbala	59	1	1.69%	0	0	0	1
Theqaar	57	3	5.26%	2	0	0	1
Muthana	51	0	0.00%	0	0	0	0
Najaf	48	2	4.16%	1	0	1	0
Diwania	43	1	2.32%	0	1	0	0
Total	457	22	4.81%	9	5	2	6

22 (22.7%) and 2/22 (9%) of AIVs positive samples, respectively (Table 1, 2) (Figure 1).

DISCUSSION

In essence, AIVs are very important zoonotic agents which can easily pass the species barriers, imposing great challenges for human and animal hygiene also it induce great and marked economic losses toward the industry of poultry particularly during the past years and it is substantially continuing (Suttie *et al*., 2019).

Globally, the natural reservoir of all influenza A viruses are the wild birds particularly the waterfowl, In spite of the issues that it markedly found in wide number of avian and mammalian species as well(Li and Cao, 2017); therefore, the epidemiological studies concerning these viruses is quit important in the wild birds at our country, as it lie between two continents, Asia and Europe in addition to close vicinity from Africa, representing an important migratory line for these birds.

In the current study, we utilize specific rRT-qPCR assay in order to insure reliable and accurate results because this assay is highly sensitive and highly specific (Kim *et al*., 2019).

Searching of Avian flu in eleven different species of wild birds at our country emphasized that the infection was recorded in 22 out of 457 birds (4.81%); this percentage came slightly lower than that obtained by Abdul-Sada, 2015 in Iraq which was 6.42% (7/109).

In Egypt, El-Zoghby *et al*., (2013) reported higher incidence, 11.4% (108/944), in contrast to that, our finding is clearly higher than that registered by Kayed *et al*., 2019 in Egypt which was 1.37% (18/1316), Kirunda *et al*., 2014 in Uganda which was 1.3% (12/929) and result of Jiménez-Bluhm *et al*., 2018 in Chile, 2.84% (115/4036).

The variation in prevalence of avian flu in wild birds at different regions not uncommon and that could probably be associated with the kinds and diversity of wild birds, season of samples collection besides to the continuous migration of these birds, such finding came in alignment with the observation of Chatziprodromidou *et al.* (2018).

The current study emphasized the presence of only following subtypes: H5N1, H5N2, H7N9 and H9N2 in the wild birds at the study areas, whereas other subtypes not founded, future successive surveillances at consecutive times are required to determine updating profile of avian flu subtypes because new subtypes may be observe at any time and at any place to ensure complete readiness for probable harms.

According to our knowledge, this is the first study that recorded these subtypes in the wild birds at Iraq; there is just one previous study that founded Influenza A/ H5 and H9 subtypes herein only, Abdul-Sada, 2015.

It is crucially important to get information about most common avian flu subtypes in our country, as such findings is so beneficial for us to gain an important data about the disease, as wild birds are offering a good niche for viral spread and to predict the future outbreaks that originated from new viral subtypes in man or at poultry farms, in addition to preparation of a specific vaccine for these subtypes as soon as possible in order to prohibit or minimize of any future hygiene disasters.

The present study manifested that the subtype H5N1is the eminent type, which had been recorded in 9/22 (40.9%) of positive samples, followed by subtype H97N2 that ranked below in 6/22 (27.2) of AIVs samples, while the subtypes H5N2 and H7N9 had been recorded in 5/22 (22.7) and 2/22 (9%) of AIVs positive samples, respectively.

The domination of H5N1 subtype in this study came in alignment with many studies worldwide, for instance, the study of Chen *et al*., 2019 in China. Such scenario might be due to that this subtype is the original type in birds Venkatesh *et al*., 2018; However, contrast results were obtained by Bergervoet *et al*., 2019, who reported that subtype H13 is the dominant type (30% of all subtypes) isolated from wild birds in Netherlands.

The current study was referred to that the higher infection rat with avian flu had recorded in Tufted duck (11.11%) among all wild birds that involved in this study followed by that observed in Wild geese which was 8.95%, while the infection not recorded in quail, gull, cormorant and heron; This variation in aforementioned results may be due to ornithological issue, number of samples, immunity, health status and the original environment of these birds, this demonstration came in comparable to that explained by Naguib *et al*., 2019.

Interestingly, our country considered as stopover for a huge number of wild birds from different kinds as they pass the continents annually during their spring and autumn migration, that providing a probable assortment events among influenza A subtypes; frankly speaking, many of these birds are hunted or captured to be sold locally in a stochastic

way either died as a food or alive; most often, inside illegal live bird markets, moreover, the close vicinity of some commercial poultry farms to rivers, swamps and wetlands harboring huge numbers of wild migratory waterfowl can creates an ideal niche for wild-domestic transmission of avian flu, thereby, outbreaks can evoke and serious biological hazards may be establish at any time, make the offering of precise and sufficient information related viral subtypes of the avian flu in wild birds at our country are drasticallyneeded.

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