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Experimental Study of Wild and Domestic Pigeons with Local Isolate of Influenza Virus H9N2

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وَقُل رَّبِّ زِدْنِي

عِلْمًا

صدق الله العظيم

{ سُورة طه: الآية ١١٤}

Dedication

To…the soul of my father and soft hearted mother

To the symbol of faithfulness "my wife"

To My dear sisters.

With Love

Ahmed

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Ahmed

Declaration Form

I hereby declare that this thesis entitiled **"Experimental Study of Wild and Domestic Pigeons with Local Isolate of Influenza virus H9N2 " presented to the College of Veterinary Medicine-University of Diyala in 2020,** is my original work**,** except for quotation and citations which have been duly acknowledged. I also declare that it has not been previously and is not concurrently, submitted for any other degree at University of Diyala or other Universities.

Ahmed Raad Rashid

Date: / / 2020

Abstract

Avian influenza (AI) is an infectious disease of poultry caused by Influenza A viruses (AIv) and it is classified into different antigenic subtypes according to their glycoproteins on the surface, the hemagglutinin (H) and neuraminidase (N) (Fouchier et. al., 2005).Avian influenza virus (AIV) are also classified, according to their pathogenicity to poultry, into highly pathogenic avian influenza viruses (HPAIv) and low pathogenic avian influenza viruses (LPAIv).(Spickler et al., 2008).

Low pathogenic avian influenza virus (LPAIV) H9N2 local isolates of pathogenic avian influenza were used in experiment infection of 50 domesticated (*Columba livia domestica*) and 50 feral pigeons of the same species.The aim of the expermintal was to explore the susceptibility of pigeons to H9N2 infections and to study its histopathological effects in vaccinated and unvaccinated pigeons with H9N2 commercial vaccine. Furthermore, conventional and real time PCR were used to detect the experimental virus and performed through specific primers to H9N2 genome in fecal samples and tissues collected from infected pigeons.

The local isolate of H9N2 was propagated in allantoic cavity of 9-11 days hen's chick embryo. The virus gave a titer of 1024 HAU/ 0.1 ml of processed stock virus when titrated by hemagglutination test (HA). Furthermore, it was identified as H9N2 by hemagglutination inhibition (HI) test using locally prepared hyper immune serum. The antibody titer of such serum was appeared as 2048 HIU/0.1 ml of stock serum. The use of infectious dose 50 in embryonated eggs (EID50), the virus gave a titer of $10^{10.5}$ EID_{50} / 0.1 ml of propagated stock H9N2 virus. Furthermore, hyper immune serum to H9N2 was prepared locally in rabbits using the H9N2 commercial virus vaccine.

Abstract

Antibodies to avian influenza virus type A were estimated pre and post vaccination by the use of ELISA. Pigeons included in study were divided into 5 groups, A, B, C, D, and E. Groups A and C were 20 each of feral pigeons, B and D were 20 each of domesticated pigeons. Group E was of 20 (10 feral and 10 domesticated pigeons) used as unvaccinated control. Groups A and B were vaccinated with H9N2 and NDV LaSota commercial vaccines. Group C and D were vaccinated with NDV LaSota vaccine only. All groups except group E were challenged with local isolate of H9N2. The results showed low antibody titer to AIV in all groups in pre-vaccination that ranged between 152.83 ± 42.01 to 337.00 ± 150.76 with no significant differences between them. In post vaccination antibody evaluation, anti AIV antibodies showed significant titer in groups A and B (740.13 ± 214.38 and 673.00 ±242.40) respectively in comparison to pre vaccination levels. Determination of HI antibodies 5^{th} day post infection (PI) revealed different levels of HI antibodies ranged from 256 HIU in group A followed by 128 HIU in group B and 64 HIU in group C and D.

Clinical signs were appeared in the 5^{th} day PI as mild to moderate respiratory signs, digestive disorders and conjunctivitis in some birds of all groups.

Using of conventional reverse transcriptase (RT-PCR) revealed the detection of H9N2 virus genome in coloacal and visceral tissue samples collected from infected birds at different times. The molecular weight of PCR resulted fragment appeared as 897 bp. The same results were confirmed by the use of most sensitive real-time one step (RT-qrPCR).

Histopathological changes in affected tissues appeared as moderate to severe multifocal necrosis diffused in the parenchymal cells of lung

tissues. There was also infiltration with mononuclear inflammatory cells were detected in some lung tissue area associated with mild to severe inflammation. Necrotic foci and mononuclear infiltration were also observed in trachea and liver of infected pigeons but mild changes were observed in intestine.

The virus was re-isolated in embryonated hen's eggs inoculated with samples collected from tissues and cloaca of infected birds. The re-isolated virus was detected by HA and identified by HI using locally prepared hyperimmune serum to H9N2 in rabbits.

In final conclusions, H9N2 local isolate of influenza A virus had the ability to infect domesticated and feral pigeons causing mild to moderate respiratory and digestive clinical signs. Detection and re-isolation of the virus in fecal samples of infected pigeons might be facilitate the transmission of the influenza virus to other susceptible birds and help in emerging of new A influenza strain of such birds were suffered from mixed infection with other A influenza virus. Furthermore, commercial H9N2 vaccine did not prevent the infection but might reduce the severity. It was recommended to apply further epidemiological serological and molecular studies to detect most circulating A viruses among human and animals in Iraq, and to apply more and strict control programs to avoid or prevent the spread of such viruses.

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List of Abbreviations

Abbreviations	Explanation
AIV	Avian influenza viruses
AGID	agar gel immunodiffusion
DCs	dendritic cells
ELISA	Enzyme linked immune sorbent assay
HPAI	High pathogenic avian influenza
HA	Hemagglutinin
HI	Hemagglutination inhibition
Ig	Immunoglobulin
IB	Infectious bronchitis
LPAI	Low pathogenic avian influenza
M	Matrix
NA	Neuraminidase
NS	non-structural proteins
NEP	nuclear export protein
NP	nuclear protein
NDV	Newcastle disease virus
ORF	Open reading frame
PBS	Phosphat buffer saline
PRRs	pattern recognition receptors
PPLO	Pleuro Pneumonia Like organisms

rNP	Ribonucleoprotein
RdRP	RNA-dependent RNA polymerase
RT-PCR	Reverse transcription chain reaction polymerase chain reaction
TLRs	toll like receptors

Introduction

Influenza viruses of avian origin spread widely in birds worldwide; They are contagious but variable in their severity. Accordingly, they were subdivided into low pathogenic avian influenza (LPAI) viruses and high pathogenic avian influenza (HPAI) viruses (Kalthoff *et al*., 2010; Imai *et al*., 2013). Their natural reservoirs are wild and domestic aquatic birds, but domesticated birds and mammalian are susceptible to the infection with both LPAI and HPAI. Such infections might cause severe disease in humans and high economic losses among poultry industry that lead to more than 90% mortality rate, and mostly associated with HPAV strains.

Some subtypes of LPAI viruses are reported to cause mild infections in human, poultry, and different birds (Samy and Naguib, 2018). These viruses carried specific protein spikes on their surfaces known as hemagglutinin (HA) and neuraminidase (NA). Differences in genetic buildup of avian influenza viruses (AIV) in their HA and NA lead to their classification into 16 HA (from H1 to H18) and nine NA (from N1 to N11) (MacLachlan and Dubovi, 2011; Shehata *et al*., 2015; Ahmed *et al*., 2018), H17 and H18 and N10 and N11 are isolated from bats as subtypes H17N10 and H18N11 (Tong, 2013). All these subtypes are grouped in genus *Influenzavirus A*. This genus and another seven genera (*Influenzavirus B, Influenzavirus C, Influenzavirus D, Isavirus and Thogotovirus , Quaranjavirus*) are classified within the family *Orthopoxviridae*. *Influenzavirus A* genus included viruses with linear single stranded, negative-sense and segmented (8 segments) RNA (MacLachlan and Dubovi, 2011).

Influenza viruses of A subtypes were continuously exposed, either to minor and gradual mutated changes which known as antigenic drift, or there were major changes in the genetic buildup of two influenza A viruses multiply in same host due to genetic reassortment. This will lead to major changes in HA and NA proteins, that might lead to emerging of new influenza A strain, resist or escape the immune responses of susceptible host. These changes are known as antigenic shift (MacLachlan and Dubovi, 2011; Arai *et al*., 2019).

Most HPAI are associated with subtypes H5 and H7 avian viruses can also cause infection in mammalian including man (Kim, 2018; Samy *et al*., 2018).

Low pathogenic avian influenza viruses like H9N2 were reported worldwide (OIE ,2015). In Iraq, H9N2 was endemic since 2004 (Kraidi *et al*., 2017; Kraidi *et al*.,2016; Mohamed *et al*., 2019). The virus was also reported to cause high mortality rates in broilers (70%) and in breeders and layers up to10% (Khamas, 2008). The surveillance of H9 and some other influenza A viruses were studied in Iraqi domestic and wild birds by Abdul-Sada (2015). Low pathogenic avian influenza H9N2 virus that was worldwide in its distribution was reported to be in two major linages of Eurasian linage and North American linage. Eurasian linage was subdivided into many clusters like A/duck/Honk Kong/Y280/1997 (Y280-like), A/chicken/Beijing/1/1994 (BJ/94-like), A/quail/Hong Kong/G1/1997 (G1-like), and so on (Gu *et al*., 2017)

Many studies concerned with H9N2 co-infection with other viruses like HPAI H5N1 (Arafa *et al.*, 2012) might lead to re-assortment and emerging of new influenza virus variant or strain (Gerloff *et al*., 2014; Lee *et al*., 2016; Kandeil *et al.*, 2017). Co-infection of H9N2 with other

viruses rather than influenza viruses was also reported like (IB) infectious bronchitis virus (Roussan *et al*.,2008; Hassan *et al.,* 2016; Arai, *et al*., 2019). It was suggested that open reading frame (ORF1a) of IB encoded for trypsin-like protease that might enhance the pathogenicity of H9N2 in chicken infected with both viruses (Liu *et al*., 1995). It was also reported that infection of recently IB vaccinated chicken with H9N2 may lead to prolonged severe disease and shedding of the virus (Haghighat-Jahromi *et al*., 2008).

Newcastle disease virus and H9N2 co-infection was reported by Umar *et al*., (2015), when they used LaSota NDV strain and field H9N2 (A/Chicken/Pakistan/UDL/08). They reported higher H9N2 and lower titer NDV of viruses re-isolated from cloacal or oropharyngeal swabs. Furthermore, they did not observe clinical signs of infected birds.

Most neighbor countries are endemic with H9N2. The virus was reported in chickens from Kuwait (Brown, 2010; Slomka, *et al*., 2013). It was also recorded for the first time in the region in chicken from Kingdom of Saudi Arabia (Alexander, 2003) and detected molecularly in pigeons, ducks, layers and broilers of Northern parts of KSA (Alkhalaf, 2010).

Great economic losses are recorded in Iran when H9N2 caused co-infection with infectious bronchitis, and many other pathogens; resulted in high morbidity and mortality (Nili and Asasi, 2003; Toroghi, 2006; Karimi-Madab *et al*., 2010). But experimentally, the virus caused mild infections with no mortality in many bird species including chickens, (Mosleh *et al*., 2009), Chukar partridges (Nili *et al*., 2013), and quails (Nili *et al*., 2007; Ebrahimi *et al*., 2011).

In Jordan, H9N2 was isolated from domestic ducks and chicken (Gharaibeh, 2008). Natural infection of chicken recorded 30% mortality, whereas no mortality was reported with experimentally infected chickens, but only loss of body weight and respiratory signs (Gharaibeh, 2008). A mortality rate of 35% was reported in H9N2 infected broilers in Lebanon, but infected layers and breeders showed 1-2% mortality (Barbour *et al*., 2006).

In Iraq H9N2 was first isolated for the first time by (النصراوي٢٠٠٢). HPAI virus H5 subtypes like H5N1 (WHO, 2006; Rashid *et al*., 2016; FAO report, 2016; Haji *et al*, 2017) and H5N8 (AIV-HP report, 2018) are recorded. Accordingly, and as the viruses H5N1, H5N8, and H9N2 are recorded in Iraq, the co-infection of H9N2 with H5 HPAI viruses in Iraq was possible that may lead to emerge of new influenza strain. Wild and domestic pigeons are available worldwide and can easily cross Iraqi borders like the aquatic wild birds, and sometimes pigeons closely lived with these aquatic wild birds. Many reports mentioned that aquatic wild and domesticated birds are reservoir for influenza A viruses (Abdelwhab *et al*., 2015; Nagy *et al.*, 2017; Kausar *et al*., 2018). The possibility of transmission of such viruses to pigeons is acceptable as many workers mentioned the natural infection of pigeons with H9N2 influenza virus (Gomaa, *et al*., 2015; Xu *et al*., 2015; Kandeil *et al*., 2017; Kausar *et al*., 2018;Tolba *et al*., 2018).

Experimental infection of pigeons was reported by Zhang et al., (2006), since their little data was available for such experimental infection. Accordingly, the aims of this study are:

1- Explorer the susceptibility of local wild and domesticated pigeons to the experimental infection with local isolate of LPAI H9N2 virus.
2- To assess the pathological lesion produced by experimental infection in different body organs
3- Detect H9N2 by molecular techniques in infected tissues and cloacal swabs to point out the possibility of spreading H9N2 by infected pigeons.
4- Evaluate the efficiency of H9N2 commercial vaccine on pigeons.