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EXPERIMENTAL INFECTION OF CHICKENS BY AVIAN INFLUENZA H9N2 VIRUS: MONITORING OF TISSUE TROPISM AND PATHOGENICITY

By

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ABSTRACT

The low pathogenic avian influenza (LPAI) H9N2 virus became endemic in Egypt since 2011 and causing several losses in commercial poultry flocks either broilers, layers or broilers breeders flocks. The disease always present in complication with other pathogens either viral pathogen as Infectious bronchitis virus, Newcastle disease virus, infectious laryngeotrachitis virus or bacterial pathogen as Escherichia coli, Ornithobacterium and mycoplasma species. The aim of this work was to monitor the tissue tropism of the LPAI H9N2 virus in different bird tissues and check the proper organs to detect and isolate the virus on time manner interval in addition to check the pathogenicity of recent isolated LPAI H9N2 virus. The study has been done on one hundred SPF one-day old chicks and a recent isolated H9N2 virus used for challenge. The results of this study showed that, the H9N2 still low pathogenic and could not cause mortalities as a sole pathogen. The virus, could be detect starting from the 2nd to 11th day post infection on different birds' tissues. In conclusion, the H9N2 virus has pneumotropic, nephrotropic and viscerotropic properties and could be detected on those tissues in addition to some lymphoid tissue. The virus could be isolated starting from the 2nd day PI from respiratory organs and until 11th day post-infection (PI) from the kidney tissues

and the highest virus load in the birds' tissues was in respiratory organs and cecal tonsils at the 3-5 days PI.

Keywords:

Tissue tropism - Nephrogenic Virus - pneumotropic Virus - H9N2- Chickens.

INTRODUCTION

Avian influenza (AI) is a contagious viral disease, classified as Orthomyxoviridae family member; "where it is segmented, single strand negative sense RNA virus. Avian influenza divided into three distinct types; A, B, and C based on serologic reactions to the internal proteins, principally NP and M1 proteins [1, 2]". Avian influenza viruses belong to type-A group and are differentiated into several subtypes based on the antigenic relationship and structure in the surface glycoprotein. Avian influenza viruses (AIV) are classified into 18 hemagglutinin (HA) and 11 neuraminidase (NA) different type with variable combinations. They may be classified based on pathogenicity into two different types commonly known as a highly pathogenic avian influenza virus (HPAIV) and a low pathogenic avian influenza virus (LPAIV) [2-5]. The low pathogenic H9N2 avian influenza virus is considered one of the major viral problem affecting poultry industry in Egypt since its first official reporting from clinically healthy commercial bobwhite quail flock reported at May 2011 until now [6]. The virus infection in poultry causes high economic losses in different poultry flocks [7]. The virus caused mild disease in chicks usually manifested as nasal discharge, gasping, conjunctivitis, facial edema, reduced feed intake and mortality [8, 9]. It was reported that, the H9N2 virus cause immunosuppression in poultry farms in Egypt, in addition it make alteration to the blood biochemical and hematological parameters [10]. The LPAI H9N2 virus considers one of the potential avian influenza virus candidates for the next human widespread epidemic disease. Twenty-eight laboratory-confirmed cases of human infection with avian influenza A (H9N2) viruses, none fatal have been detected globally. In most human cases, the associated disease symptoms have been mild and there has been no evidence of human-to-human transmission; several human cases of H9N2 infection have been recorded since 1997 from Hong Kong and China in children and adult sex habiting influenza like symptoms and mild upper respiratory tract infections [11-14]. Human sera positive for H9 subtype were identified in China, India, Iran, Thailand, Cambodia, Romania, Egypt and Pakistan. In Egypt, the low pathogenic AI H9N2 virus infected human where the first case

reported on January 2015 and the sero-surveillance data indicated occupational exposure of humans to AI H9N2 virus in Egypt [15, 16]. According to phylogenic analysis, all AI H9N2 viruses isolated from poultry farms in Egypt science it is emerging until today, grouped in the G1/97-like lineage in one group with other Egyptian strains and other related strains that circulating in the Middle East countries [17, 18]. The low pathogenic AI H9N2 virus causes severe losses in layers and breeders broilers flocks reflected in drop in egg production and sever losses in broiler sectors associated with complication with other pathogen. LPAI H9N2 virus infection can cause severe losses in poultry farms if combined with other pathogen either if it is other viral pathogen as infectious bronchitis virus (IBV) or Newcastle disease virus (NDV), Adenovirus, infectious laryngeotrachitis virus (ILT), pneumovirus or bacterial pathogen as Mycoplasma,Escherichia coli (*E-Coli*),Ornithobacteriumor fungus as Aspergillus [19-22]. The mixed infection of H9N2 virus with the infectious bronchitis virus (IBV) results in higher economic losses in poultry farms and increase the shedding rate of IBV in mixed infected flocks [20, 23]. The coinfection of chickens with AI H9N2 virus and NDV can exaggerate the effect, as the previous infection with H9N2 virus can decrease the infected dose required by Newcastle disease virus to cause disease. Coinfection of Ornithobacterium rhinotracheale with AI H9N2 virus can elevate the economic losses and mortality in poultry farms [24]. AIV nucleoprotein detected in epithelium cells of respiratory organs (Lung, Air sacs and trachea) in addition to intestinal cells [25-27]. The low pathogenic avian influenza virus (LPAIV) needs trypsin-like proteases enzyme as essential elements for their replication; where such enzymes play a vital role in the cleavage of crude hemagglutinin (HA0) segment to Hemagglutinin segment-1 (HA1) and hemagglutinin segment-2 (HA2) protein so develop infectious virus particles; since the respiratory and digestive organs and other epithelial organs like kidney are rich in this enzyme, so it considers the proper site for replication of this virus [1, 4, 28, 29]. There are some reports on 2016 indicated that there are change some mutation occurred in the H9N2 virus circulating in Egypt and it reflected on its antigenicity and provided some evidence on new variant H9N2 virus in Egypt[30, 31]. The aim of this study was trying to assess the recent H9N2 virus isolates pathogenicity and if there are change in the virus, pathogenicity in experimental chickens (SPF chicken) associated with the recent detected mutation and antigenic drift in the virus at 2016 - 2017 in Egypt. in addition to monitoring the virus spreading and tropism in various organs of the infected SPF chickens at different days post challenge (DPC) to provide expected proper organs and time interval

guide for virus isolation and detection from suspected infected flocks in the field, employing the RT-PCR test for detection the presence of the virus in different body tissues, oral and cloacal discharge at different DPC. This might further help us to investigate the virus pathogenesis, propose right time, and place for virus isolation and detection associated with the 2016 isolates.

MATERIAL AND METHODS

Experiment design:

Challenge Virus strain:

LPAI H9N2 virus (A/chicken/Egypt/Elfeil-26/2017(H9N2) that was purified isolated from infected chicken flock in Egypt (unpublished data), was used in this investigation. The virus was propagated twice on 9-11 day old SPF ECE through intra-allantoic inoculation, then incubated at 37 °C for 3-5 days. The allantoic fluids harvested from the inoculated embryonated chicken eggs and tested for virus hemagglutination properties using 4 HAU based on the HA assay [32]. The birds from infected group challenged with 100 µl of allantoic fluid containing with 10^6 EID_{50}/bird.

Birds:

One hundred 3-week-old SPF chickens were randomly divided in two groups; infected group contain 80 birds and control non-challenged group contain 20 birds. Both groups were housed in the same condition in two separate isolated rooms. Feed and water were supplied ad libitum to both groups.

Pre-challenge monitoring:

Five chickens from infected group were sacrificed as well as organs collected, and investigated using RT-PCR to ensure birds are free from any H9N2, NDV, and IBV viruses' infection just before H9N2 virus challenge. Serum samples were collected before killing the birds, to ensure that, the birds in the challenged / infected group free from any specific humoral immune response against AI H9N2, NDV and IBV viruses examined by Hemagglutination inhibition assay (HI) according to OIE manual using 4-HAU [32].

Post challenge monitoring:

The birds in the infected group (75 birds) were inoculated with 100µl of infectious allantoic fluid containing 10^6 EID_{50} of H9N2 virus strain diluted in sterile PBS solution via intranasal routes. The birds in the control group were received sterile PBS with the same route. All the birds in both infected and control groups were monitored daily for 15 days post challenge

(DPC) to investigate against AI H9N2 Clinicopathological pictures. On daily basis from 1-DPC until 15-DPC; five chickens from the infected group and one chicken from the control group were randomly selected, then scarified and any pathological gross lesions were recorded; then samples from trachea, thymus, lung, proventriculus, kidney, spleen, brain, cloaca and Bursa of Fabrics were collected under aseptic condition for virus detection by RT-PCR. All tissue samples were stored at -70°C until used.

Serology:

Serum samples were collected on the pre-Challenge (at day of challenge)and on first to fifteenth days post challenge from all chickens and were evaluated the specific antibodies to H9 antigen by using Hemagglutination Inhibition (HI) assay [32].

Detection of H9N2 virus RNA by PCR tool:

The specimen subject to quantitative Real Time PCR (RRT-PCR) for virus titration; where the standard curves were generated with control viral RNAs and the Ct values of samples were converted into EID_{50}/ml by interpolation as previously described [33, 34]. The virus titer was calculated as virus titer/ gram specimen as a mean of five bird per group. The RNA extracted from different birds' tissue and blood samples using ABT Total RNA Mini Extraction Kit following the manufacturer's instructions (Cat. No. ABT002; Applied Biotechnology Co. Ltd, Egypt). The ABT cDNA synthesis kit (Cat. No. ABT00A2; Applied Biotechnology Co. Ltd, Egypt) used to synthesis the cDNA following the manufacturer's instructions. A set of oligonucleotides primers were used as showed in (Table 1) [35]. A Light Cycler® 96 Real-Time PCR system used to conduct the RRT-PCR assay (Roche Molecular Bio-chemicals, Mannheim, Germany) using previously described primers and probe. The total mixture exposed to 95 °C for 3 min as initial step, followed by 45 thermal cycles of its condition were 95 °C for 15s, 60 °C for 30 s and 72 °C for 30s,. The probe labeled with the 6 - carboxyfluorescein (FAM) reporter dye at the 5′ end and with the 6-carboxytetramethylrhodamine (TAMRA) quencher dye at the 3′ end as shown in (Table1).

RESULTS

Clinical signs:

No mortalities recorded in either challenged group with AI H9N2 virus or non-challenged control group. All birds in the control non-infected /non-challenged group with AI H9N2 virus did not develop any signs of clinical illness all over the 15 days of monitoring on daily basis. The birds from challenged infected chicken group developed clinical illness

manifestation starting from the 3rd day post challenge, which started to decline from the 7th day post challenge and complete cession at 12th day post challenge. The Major observed clinical manifestations were; facial swelling and edema, broken feathers, reduction in the feed consumption rate, general depression and watery diarrhea.

Gross Lesions:

In the challenged / infected group with AI H9N2 virus, postmortem examination showed pathological lesions of congestion in small intestine, mild degree of congestion in trachea and lungs, pancreas, and swollen kidneys. No pathological changes could be detected in control group.

HI test:

The results of HI test in serum samples from infected (challenged) group showed elevated titers for specific humoral immune response (HI geometric mean titer for the antibody-GMT). It started to increase at the 5th day post challenge and reached the highest titer (2 log11) at the 15th day post challenge, while no change in control non-infected (non-challenged) group as shown in (Table 2).

PCR results:

All samples were negative for IBV and NDV from both infected and non- infected groups that exclude risk of mixed infections.

The RRT-PCR test detect LPAI H9N2 virus RNA in various chicken organs; the sample analysis data showed the presence of LPAI H9N2 virus RNA in the following organs; Trachea, Lung, Kidney, Spleen, pancreas, Cecal Tonsil and Cloaca while could not detect in blood, brain or heart tissues. The LPAI H9N2Virus RNA detected firstly at trachea starting from 2nd day post challenge (DPC) at 20% of examined birds (1/5) until 7th day post challenge at 20% of examined birds (1/5) with highest titer at the 5th day post-challenge at 100% of examined birds (5/5) as shown in (Table.2). In lung tissues the AI H9N2 virus RNA was detected from the 2nd day post-challenge in 20% from examined birds until 6th day post-challenge in 20% (1/5) from examined birds and highest level was in the 5th day post challenge in 60% of examined birds; starting from the 7th day post challenge the LPAI H9N2 virus RNA could not be detected in lung tissue as shown in (Table 2). The LPAI H9N2 virus RNA was detected in kidney starting from the 3rd day post challenge in 20% of examined birds until 11th day post challenge; starting from the 12th day post challenge the virus RNA couldn't be detected in kidney tissue; at the 7th day post challenge the AI H9N2 virus RNA

detected in 100% (5/5) of examined birds as shown in (Table 2). In spleen tissue the LPAI H9N2 virus RNA could detected starting from the 3rd post challenge in 20% (1/5) of examined birds until the 5th day post challenge in 20% (1/5) of examined bird and reach the highest level at the 4th day post challenge in 60 % of examined birds and starting from the 6th day post challenge the RNA could not detected in spleen tissue as shown in (Table 3). In pancreas tissues; the LPAI H9N2 virus RNA could detected starting from the 3rd day post challenge in 20% of the examined birds until the 5th day post challenge in 20% of the examined birds and reach its peak at the 4th day post challenge in 40% (2/5) from the examined birds and no detection starting from the 6th day post challenge as shown in (Table3). The LPAI H9N2 virus RNA could detected in thymus tissue starting from the 4th day post challenge in 20% (1/5) of examined birds until the 7th day post challenge in 20% (1/5) of examined birds and reach maximum at the 6th day post challenge in 60% (3/5) of examined birds and starting from the 2nd 8th day post challenge couldn't detect LPAI H9N2 virus RNA as shown in (Table 3). In cecal tonsil, the LPAI H9N2 virus RNA start detected at the 2nd day post challenge in 20% of examined birds until the 7th day post challenge at 20% of examined birds and starting from the 8th day post challenge could not detect the virus RNA as shown in (Table 3). The LPAI H9N2 virus RNA could detected in cloaca starting from the 4th day post-challenge in 40% of examined birds until the 8th day post challenge in 40% of examined birds and reach maximum at the 6th day post challenge in almost 100% of examined birds and staring from the 9th day post challenge; could not detect the virus RNA as shown in (Table 3).

DISCUSSION

AI H9N2 virus recorded in Egypt for the first time at 2011, since then the virus become endemic. Then in 2016 there, some reports indicate evidence for presence of variant isolate from H9N2 virus and several genetic markers that enhance virulence in poultry and transmission to humans were detected; in addition to some isolates showed antigenic drift [30, 31, 36]. This study focused on evaluation of H9N2 pathogenicity, tissue tropism and dissemination throughout the chickens various organs after intranasal inoculation of the field virus, in addition to check if the current circulating H9N2 virus (2016 isolate) has elevated pathogenicity degree and causes higher mortalities levels. Results clinical finding and postmortem examination indicated no change in the virus pathogenicity (2016 H9N2 isolate) which accords with the previously published reports [26, 37-39] and did not cause mortalities

as a single challenge pathogen which agreed with the data obtained with **Bijanzad *et al* (2013)** but disagree with data obtained by **Abdel Hamid *et al* (2016);** who detect 20% mortalities assocaited with laboratory challenge of chickens with low pathogenic H9N2 isolates from Egypt at 2015 as a single pathogen or sole caustive agent [40]. Although some recent report indicates, high mortalities associated with H9N2 virus outbreak in Middle East in commercial chicken flocks. The current study finding disagree with **Elhouadfi *et al* (2016);** finding as he reports recent outbreaks of H9N2 in Middle east region with mortalities range from 40-60% in commercial flocks outbreaks, may be this mortalities due to complicated of H9N2 virus with other disease condition in the commercial flocks [41]. The results of serological monitoring matched with previous reports by **Mosleh *et al* (2009)** [35]. In the current study, the virus RNA was detected in the respiratory, urinary and digestives organs, which indicate that, the virus has pneumotropic, viscerotropic and nephrotropic tropism. Regarding the respiratory system, the trachea was the first organs to detect the virus RNA starting from 2nd day post infection till the 7th day post infection on daily basis; while in lung detected at the 3rd day post-infection and on daily basis till the 7th day post-infections which agreed with data reports by **Choi *et al*,** who detected the virus RNA from trachea and lung at the same days [42]; while other reports detected the virus RNA in respiratory organs either at 3rd or 5th day post-challenge [27, 35, 43]. Regarding the digestive system, the virus RNA detected staring from the 2nd day at cecal tonsil until 8th day in cloacal swabs. The virus was detected in the cecal tonsil as first gastrointestinal element (GIT) from 2nd to 7th day post infection with highest level at 4th day. Such result agreed with data obtained by **kwon *et al* (2005) and Hablolvarid *et al* (2004)** and disagreed with **Manjili *et al* (2011)** who can't detect the virus RNA in the cecal tonsils following intranasal inculcation of the AI H9N2 virus [27, 43, 44]. The virus RNA was detected in pancreas at 3rd -5th day PI. A result that agreed with previous reports [27, 43], which indicate the ability of virus to replicate in it and may associated with alternation of the pancreatic secretion which confirmed results of **Abdel Hamid *et al* (2016) and Sultan *et al* (2015);** who reported an alternation in food conversion rate and weight gain associated with H9N2 infection [10, 40]. Regarding the virus detection in the cloaca, the current study reported the virus RNA in cloacal swabs starting from 4th to 8th days PI. Such result agreed with the data obtained by **Kwon *et al* (2005)** and differ in days with data obtained from **Mosleh e*t al* (2009) and Manjili *et al* (2011)** as they detect it from 5th-7th days PI [27, 35, 43]. Regarding urinary system the virus

detected in Kidney from the 3rd to 11th day PI in kidney tissues with highest level at 3-5 days PI; which indicate the nephrotropic tropism of the LPAI H9N2 virus following intranasal inoculation in SPF Chicks. Previous report by **Swayne and Slemons (1994)** illustrated that the LPAIVs were nephrotropic pneumotropic following IV inoculation of the virus. while pneumotropic only following intranasal inoculation, as they did not detect the virus antigens in kidney using immunohistochemistry assay; also **Swayne and beck (2005)** didn't detect the H9N2 in kidney parenchyma following intranasal inoculation of the virus [26, 45] while recent report by **known *et al* (2005),Manjili *et al* (2011),Mosleh *et al* (2009),Gharagouzlou *et al.,* (2002); Hablolvarid *et al* (2004) and Bijanzad *et al* (2013)** detected the virus in the kidney following intranasal inoculation of the virus and agreed with the current study results [27, 35, 39, 43, 44, 46] which confirm the nephrotropic properties of the LPAI H9N2 virus. Regarding the lymphoid organs the virus detected in thymus and spleen at 3rd -5th days PI, where **Manjili *et al.* (2011)** didn't not detect the virus in the lymphoid organs while it detected by **kwon *et al* (2005) and Gharagouzlou *et al. (*2002)** in lymphoid tissues and agreed with the current study results [27, 43, 46]. AI H9N2 detection in lymphoid tissue might confirmed the previous reports regarding immunosuppression properties of H9N2 virus in chickens. The LPAI H9N2 virus RNA could not detect in the blood samples in the current study and also some other reports could not detect the virus in the bloods and this may another sampling and tropism protocol to investigate the hematogenous spread of the LPAI H9N2 virus in birds [35, 43, 45].

CONCLUSION

LPAI H9N2 virus still low pathogenic and could not cause mortalities as a single pathogen in chickens and the mutations and antigenic drifts associated in 2016 isolates did not reflect on the virus pathogenicity toward chickens. AI H9N2 virus has pneumotropic, nephrotropic and viscerotropic properties and could replicate in some lymphoid tissue and the virus can isolate starting from the 2nd day PI from respiratory organs and till 11th day PI from the kidney tissues and the highest virus load in the birds tissues was in respiratory organs and cecal tonsils at the 3-5 days PI.

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Table (1): RT-PCR and real-time PCR primer and probe sequences.

Specificity	Primer/Probe	Sequence
CDNA Synthesis Influenza-A	Forward	5′ TCTAACCGAGGTCGAAACGTA 3′
Real Time PCR for H9N2	Forward	5′ AAGACCAATCCTGTCACCTCTGA 3′
	Reverse	5′ CAAAGCGTCTACGCTGCAGTCC 3′
	Probe	5′ FAM-TTTGTGTTCACGCTCACCGT-TAMRA 3′
VNDV	ND+ RRT	5'-TCCGGAGGATACAAGGGTCT-3'
	ND- RRT	5'-AGC TGT TGC AAC CCC AAG-3'
	ND-Prob-RRT	5'-/56-FAM-AAgCgT TTCTgTCTCCTTCCTCCA-TAMRA-3'
IBVS1	IBS1+ RRT	5'-GCTTTTGAGCCTAGCGTT-3'
	IBS1- RRT	5'-GCC ATG TTG TCA CTG TCT ATT G-3'
	IBS1-Prob-RRT	5'-/56-FAM- CACCACCAgAACCTgTCACCTC-TAMRA-3'

Table (2): H9N2 serum antibody titration (Geometric Mean titer) of the test and control groups of chickens experimentally infected with H9N2 AI virus.

DPI	HI TITER														
	D0	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D13	D14	D15
Test Group	0	0	0	0	0.4	2	4	5	7	8	9	10	10	11	11
Control Group	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table (3): AI H9N2 RNA detection in different examined tissues by RRT-PCR techniques.

Tissue DPC	Trachea	Lung	Kidney	Spleen	Pancreas	Thymus	Cecal Tonsil	Cloaca
D0	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
D1	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
D2	1/5 (1.5)[a]	1/5 (1.1)[a]	0/5	0/5	0/5	0/5	1/5 (0.5)[a]	0/5
D3	3/5 (2.1±0.9)[a]	2/5 (1.2±0.4)[a]	1/5 (0.5)[a]	1/5 (0.2)[a]	1/5 (0.2)[a]	0/5	3/5 (1.1±1.1)[a]	0/5
D4	4/5 (2.3±1.9)[a]	3/5 (1.3±0.9)[a]	2/5 (0.7±0.4)[a]	3/5 (0.7±0.6)[a]	2/5 (0.3±0.2)[a]	1/5 (0.6)[a]	5/5 (2.7±1.9)[a]	2/5 (1.9±1.5)[a]
D5	5/5 (2.9±1.8)[a]	3/5 (1.2±1.1)[a]	3/5 (1.1±1.4)[a]	1/5 (0.3)[a]	1/5 (0.3)[a]	2/5 (1.3±1.2)[a]	3/5 (2.9±2.4)[a]	5/5 (2.6±2.8)[a]
D6	3/5 (1.5±0.6)[a]	1/5 (0.9)[a]	3/5 (1.7±1.1)[a]	0/5	0/5	3/5 (1.2±1.1)[a]	1/5 (2.1±1.1)[a]	5/5 (3.1±2.8)[a]
D7	1/5(0.6)[a]	0/5	4/5 (1.1±0.7)[a]	0/5	0/5	1/5 (0.3)[a]	1/5 (0.6)[a]	3/5 (2.6±2.3)[a]
D8	0/5	0/5	5/5 (0.8±.0.9)[a]	0/5	0/5	0/5	0/5	2/5 (1.4±0.5)[a]
D9	0/5	0/5	3/5 (0.4±0.5)[a]	0/5	0/5	0/5	0/5	0/5
D10	0/5	0/5	2/5 (0.4±0.2)[a]	0/5	0/5	0/5	0/5	0/5
D11	0/5	0/5	1/5 (0.2)[a]	0/5	0/5	0/5	0/5	0/5
D12	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
D13	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
D14	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
D15	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5

a Log EID50 ±S.D.. * $P < 0.05$.