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STUDY OF THE VIRAL PHYLOGENIC DIAGNOSIS OF AVIAN INFLUENZA DISEASE IN BROILER CHICKENS IN BABYLON PROVINCE, IRAQ

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Abstract

Avian influenza (AI) disease are infectious, highly contagious viral diseases infected the respiratory and digestive system that cause neurological signs and sudden death combined with high mortality (90%) in poultry industry. Assessment of viral phylogenic diagnosis of avian influenza disease in broiler chickens in Babylon province. Identification of AIV in Samples (lung, trachea, spleen and intestine) were done by indirect ELISA test and were conducted to identify (AIV) by using 18 samples of specific serum from infected broiler chick. The molecular method (qRT-PCR) test also confirmed for 7 samples infected with AIV (H9N2). The nucleotide sequencing and translation results for cleavage site of HA-gene for AIV showed that all these 2 samples are low pathogenic because they don't contain multiple basic amino acids at cleavage site and the sequencing of all these strains at cleavage site is (PAKSSR↓GLF). Isolated strains of AIV isolated strains were registered in NCBI is begun by accession number (A1908158.002) and ends (A1908158.003).

Key words : Avian influenza disease, poultry, broiler chickens H9N2 H-gene.

Introduction

Avian influenza (AI) is considered dangerous viral disease that infected numerous types of birds and it is highly contagious disease and that's caused by different serotypes of AIVs type A from Orthomyxoviridae family, and it is caused endemic, epidemic and pandemic disease among a wide variety of birds and mammals (Alexander and Capua, 2009). AIV is replicated in the nucleus of infected cells and it is has segmented negative sense single stranded RNA genomes. AIVs type A have a genome composed from eight different segments of RNA therefore it is exposed to high mutation rate, therefore when target cells are infected with two different AIV strains that lead to exchange and interference between these two viruses gens and that called genetic interaction (reassortment) in the same time the pandemic human influenza is arisen or created from re-assortment between two viruses (Adams *et al.,* 2013). Mutations were taken place in each replication cycle of AIV and the nucleotide exchange average for HA protein is about 10.15×10^{-3} exchange site in year, and this characteristic gives AIV ability for nucleotides insertions(Monne *et al.,* 2014). The AIV viruses were subtyped according to the heamagglutination (HA) glycoprotein characteristics and there are eighteen HA subtypes were identified, in the same time the AIVs are subtyped dependent on the neuraminidase (NA) glycoprotein characteristics and there are eleven NA subtypes (Tong *et al.,* 2013). All subtypes of AIV can infected birds and become carrier for this virus (Olson *et al.,* 2014). AIV, according to the molecular specifications of the haemagglutinin cleavage site (HACS), is classified into HPAI and LPAI, the HPAI viruses have a multi basic amino acids at cleavage site motif such as H5N1, while LPAI viruses lack of this privacy such as H9N2 (Schat *et al.,* 2012; Gohrbandt *et al.,* 2011). LPAI viruses are replicated in the respiratory and gastrointestinal epithelial target cells of susceptible birds but HPAI viruses replicated are in the respiratory and gastrointestinal system and also replicated systemically in multiple tissues and caused high mortality (Pantin-Jackwood and Swayne, 2009). In Iraq the first study for AIV identification was by (AL-Nasraoui, 2002). From layers flock, Poultry industry in Iraq still suffers from severe infections with

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respiratory and neurological signs accompanied by high mortality with sudden death so that poultry industry is exposed to highly economic losses, So, this study was design for detection of AIV from chicken in Babylon province.

Materials and Methods

Estimation of AIV by ELISA

ProFLOK® ELISA Kit (Synbiotics–USA) was used in to assessment the infection of AIV. The procedure used in this test was performed according to the manufacturer instructions listed in kit, which is a rapid serologic test for the detection of AIV antibody in chicken serum samples. It was developed primarily to aid in the detection of pre and post-vaccination AIV antibody levels in chickens.

Viral RNA Extraction

The total viral RNA was extracted from harvested AF by Automated total RNA extraction (Magnisia 16 close system) with using Magnesia viral nucleic acid extraction kit. The extracted RNA was normalized for (100 ng D ml) by nanodrop. The eluted RNA was converted directly in DNA amplification HA gene for AIV genes. Table 1 showing the primers and probe using in qRT-PCR:

Table 1: Primers and probe using in qRT-PCR.

Reagents name
Probe H645' FAM-TCAGGCCCCCCTCAAAGCCGA-TAMRA 3'
Primer H25F5'-AGATGAGTCTTCTAACCGAGGAGGTCG-3'
Primers H124R5'-TGCAAAAACATCTTCAAGTCTCTG-3'

In RNA samples, the concentration and purity were measured using Nanodrop devise and that detected the RNA concentration in ng D ml and the purity was detected by measuring the optical density ratio (OD) 260D 280 nm. The accepted purity of RNA sample was in range of (1.9 -2 ng/ml).

Tissue Sample

Tissue are appropriate spacemen for virus identification and nucleic acid based assay, tissue sample (Brian, lung, trachea, liver, spleen, kidney, intestine), collected in 7 months, were collected from broiler farm in the early stage of disease as soon as respiratory sings appeared with mortality and placed into sterol plastic bags labeled with a unique identifier with ice and stored in freezing refrigerator in (-20°C) and transferred to the central laboratory of the general state of veterinary Service for r RT-PCR diagnosis, samples of blood collecting the blood (3ml) was collected from jugular vein, (10-24) blood samples were collected from each farm to carry on Indirect ELISA test.

Phylogenetic Analysis

All nucleotides and amino acid sequences was alignment and comparison between strain isolated and selected strains representing AIV genotypes were performed using the software ApE- A plasmid Editor Program version 2.0.51. Phylogenetic analysis was accomplish using the MEGE5 software (Version, 6.0.6). The sequences was differentiated against for AIV, respectively, complete and near complete reference genomes sequences of virus strains obtainable and analyzed via BLAST search on the GenBank database.

Results

The Avian influenza virus (AIV) ELISA was able to detect specific AIV antibodies as early as one week post infection. After few days or hours from samples collecting, The ELISA results of serum analysis showed high titer for AIV in all these farms the mean of these result alternated between (330-5889) for AIV, and these results considered diagnostic results for AIV infection, 18 samples out of 24 were positive for AIV were as six samples showed mixed infection with IB, Therefore only the first 18 samples were used. Table 2 showing the mean titer of ELISA Test of infected farms with AIV without mixing with NDV and/or IBV.

Table 2: The mean titer of ELISA Test of infected farms with AIV without mixing with NDV and/or IBV.

No. of case	Owners	Result ELISA by Mean Titer	Age in collection serum
1	A	3198	25 day

After the recognition of seven samples positive with ELISA test for AIV (H9), The RT-PCR was carried on these positive sample for AIV by using specific primers for H-gene in sample showed positive result with AIV, The samples with positive RT-PCR showed highest levels of viral RNA amount in (63.84),(49.68) show in Fig. 1.

Discussion

In the last years, poultry industry in Iraq suffered from high mortality reached up to 90% in some farms or more with severe respiratory distress, neurological signs and sudden death, grossly characterized by tracheitis, lung congestion and Payer´s patches, cecal tonsils and proventriculus hemorrhage that lead to high economic losses in poultry industry. The ELISA results of serum analysis showed high titer for AIV in all these farms the mean of these result alternated between (330-5889) for AIV, and these result considered diagnostic result for AIV infection, 18 samples out of 24 were positive for AIV were as six samples showed mixed infection with

Table 3: The mean titer of ELISA Test of infected farms with AIV without mixing with NDV and/or IBV.

Sample	Location	Od	S/p Ratio	Titer	Group
1	G4	1.40	2.35	5486	2
2	G5	1.39	2.33	5437	2
3	G6	1.16	1.92	4101	2
4	G7	0.71	1.12	1857	2
5	G8	0.19	0.19	0	0
6	G9	1.46	2.46	5889	2
7	G10	1.42	2.39	5627	2
8	G11	0.75	1.19	2033	2
9	G12	0.83	1.34	2410	2
10	H1	1.06	1.74	3544	2
11	H2	0.28	0.34	330	1
12	H3	0.73	1.16	1953	2
13	H4	1.14	1.88	3957	2
14	H5	1.14	1.89	3985	2
15	H6	1.27	2.13	4748	2
16	H7	1.29	2.15	4819	2
17	H8	0.20	0.21	0	0
18	H9	0.60	0.92	1394	2

Samples: 18
Mean: 3198
GMT: 1231 Bleed Comment:
StDev: 1997
%CV: 49.53

Controls	1	2	3	Average
Positive	0.65 G1	0.64 G3	0.65 H11	0.65
Negative	0.08 G2	0.09 H10	0.09 H12	0.09
Corrected positive:				0.56

Table shows the result_of AIV antibody titers.

IB, Therefore only the first 18 samples were used, The result in this study was agreement with (Kariminejhad and Mehrabanpour, 2012) who collected (300) samples from 30 broiler farm that had respiratory sings with variable mortality rate and by using ELISA technique, 274 samples showed positive AIV antibodies, if the titer less than 396 considered negative and greater than 396 was considered positive. After the recognition of seven samples positive with ELISA test for AIV (H9), The RT-PCR was carried on these positive sample for AIV by using specific primers for H-gene in sample showed positive result with AIV, The samples with positive RT-PCR showed highest levels of viral RNA amount in (63.84),(49.68) and this method suitable for diagnosis and for the evaluation of viral load in filed specimens, this study agreed with (Alexander, 2006*)*. who reported that H9N2 infections of poultry have been reported since 2000 in the middle and East Asia and this study agreement with study by (Hassan, 2007; Jasim, 2009; Al-Nakshab andi, 2009) in Iraq those studied the detection of H9N2 AIV by RT-PCR in commercial poultry. The 2 positive samples for AIV are sent to Germany for making sequencing to showed the sequencing of amino acids at the cleavage site, Influenza A type H9 has been sequenced and was phylogenetically analyzed based on comparison to known reference strains, Sample No. (A1908158.002) and (A1908158.003), Also if cleavage site contain multiple basic amino acids in cleavage site of HA protein consider high pathogenic due to this protein was cleaved intra cellular but when the protein (HA-protein) did not contain multiple basic amino acids in cleavage site is (PAKSSR↓GLF), that lead to need trypsin like enzyme for cleavage and it is provide by tissues or by different microorganisms, The sequence post translation of cleavage site (PAKSSR↓GLF), for AIV HA- gene showed that all isolated strains have amino acids sequence did not contain multiple basic amino acid (Arginine and/ or lysine), so that isolated strains was consider low pathogenic strains, these results were came in line with (Tombari *et al.* 2011; Slomka *et al.,* 2013; and Kandeil *et al.,* 2014). The results showed that the amplified nucleotide sequence coding for the hemagglutinin (HA) glycoprotein of Influenza A Virus (type H9), the RNA extracted from sample numbers A1908158.002 and A1908258.003 is most related to A/chicken/Jiangxi/ X1289/2016(H9N2) (MK601116.1) (98,6% and 98,16%) and to A/Duck/Jiangsu/JS1144/2016(H9N2) (MK601211.1) (98,4% and 98,1%), and all the isolated strain have homology sequencing between them, All 2 registered strain formed a distinct adjacent to the all strain above coming from East Asian strain,, so that it may be take place re-assortment with another avian influenza

Table 4: Showed Highest Levels of Viral RNA Amount Spacemen Result of AIV.

Sample Read	Well ID	Name	Location	260 Raw	280 Raw	320 Raw	260Raw	280Raw	Purity of RNA260/280	Amount of RNA Present Ng/ml
1	SPL1	TracheaAIV	A2	0.115	0.08	0.045	0.062	0.029	2.105	49.68
1	SPL2	TracheaAIV	B2	0.064	0.052	0.093	0.018	0.008	1.146	14.08
1	SPL3	TracheaAIV	C2	0.089	0.07	0.049	0.034	0.018	1.88	26.84
1	SPL4	TracheaAIV	D2	0.09	0.068	0.048	0.036	0.016	2.205	28.84
1	SPL5	TracheaAIV	E2	0.09	0.078	0.065	0.019	0.01	1.922	14.84
1	SPL6	TracheaAIV	F2	0.0127	0.083	0.04	0.08	0.038	2.073	63.84

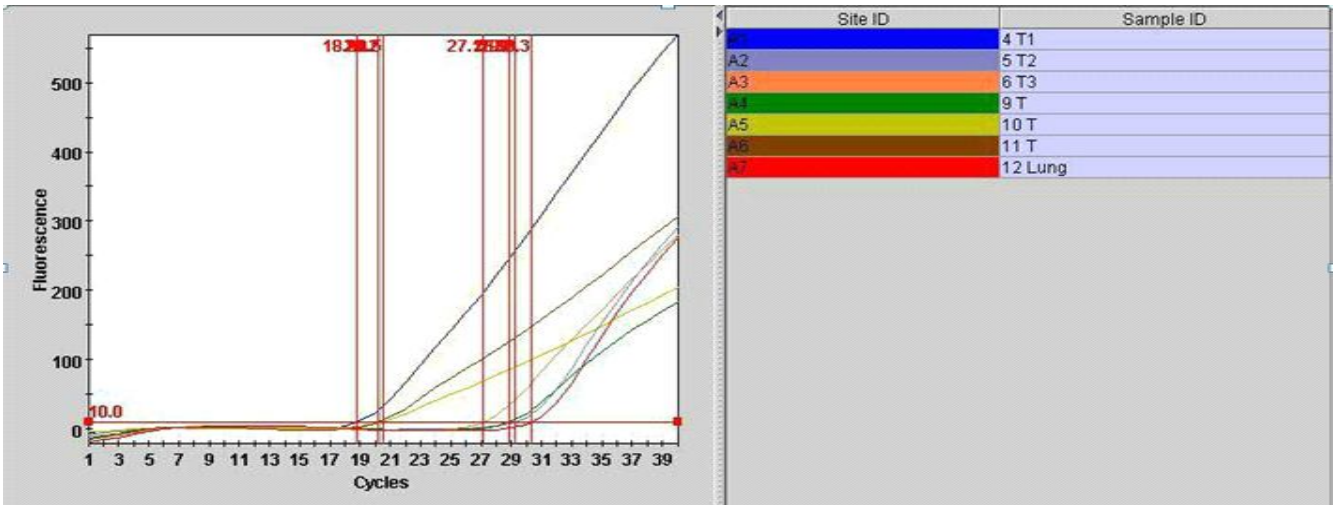

Fig. 1: H9 Gene Detection by qRT-PCR, Show curves of positive result of the sample of AIV.

virus like H5 serotypes or human H3N2 strains (Matrosovich *et al.,* 2001; Tombari *et al.,* 2011; Gohrbandt *et al.,* 2011). Some studies highlights the potential of avian to human transmission of H9N2 and this transmission may increase the chance of adaptation of avian influenza virus to human (Alizadeh *et al.,* 2009). Avian influenza virus subtype H9N2 is found in many bird species and poultry worldwide, mostly in chickens. However, it can also infect humans and mammals so that it is considered zoonotic disease, although H9N2 AIV is considered low pathogenicity, but previous studies have shown that serious disease with high morbidity and mortality with significant economic losses in broilers and layers is associated with secondary bacterial infection such as *E. coli* infection and other pathogens because *E. coli* supply AIV by protease like enzyme is necessary for HA protein cleavability and fission take place (Biswas *et al.,* 2008; Nagarajan *et al.,* 2009). The low pathogenic AIV (H9N2) can produce severe infection depending on the type of secondary pathogens present under field conditions. This may be explain the severity of infection and high mortality with the present H9N2 outbreak in the field (Bano *et al.,* 2003; Li *et al.,* 2018).

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