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RESEARCH ARTICLE

Molecular Epidemiological Investigation of AIV H9N2 Subtype in Broilers in North and Northeast China

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ABSTRACT

Avian Influenza (AI) is the most significant disease problem of poultry being the cause of high mortality and is zoonotic as well. The virus mutates easily and thus generates new strains. There was a need to look at the current scenario of AI virus being circulating in certain parts of the China and its genetic distance from the vaccine strain. For this purpose, the study was carried out by collecting 864 tracheal and lung tissue samples showing lesions from commercial broiler farms, slaughterhouses, liver broiler markets, and cities of the China. Avian Influenza Virus was isolated from 67 lung + tracheal tissues, of these 35 strains of AIV were confirmed positive by PCR and sequencing. The results of genetic distance analysis revealed that 6 strains of the virus were grouped into H9.4.2.5.1 subfamily, while 29 strains were grouped in to H9.4.2.5.2 subfamily. The HA gene homology analysis revealed that 35 strains had 92.1 to 99.7% homology among themselves. The homology between the 35 strains and the vaccine strain (Y280) was 86.1 to 92% and the homology between the 35 strains and the SY/97 strain was 86.4 to 91.8%. The study showed that field virus has main two subfamilies, but both of these differ on basis of genetic analysis with the two vaccine strains.

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INTRODUCTION

Avian influenza virus (AIV) infection is the most significant respiratory viral disease recently emerged in the poultry (Arif *et al*., 2015; Zeynalova, *et al*., 2015). The disease can be symptomless to produce a range of symptoms including the drop-in egg production and very high mortality, as high as nearly 100% (Park *et al*., 2011). There are many serotypes of AIV (Sarwar *et al*., 2013). It has been speculated that the chicken was not the normal host of AIV, and had milder strains infecting the chicken over time. But, the most killing strains that now we have, has been developed from the milder strains of virus by repeated chicken to chicken passages (Abo-Elkhair *et al*., 2014). However, the AIV has a very high rate of mutation when it circulates in chicken. Among different subtypes of pathogenic avian influenza viruses, the H9 subtype spreads rapidly and is the low pathogenic avian influenza (LPAI) virus that infects the domestic poultry flocks (Park *et al*., 2011). The H9 viruses in poultry have been found associated with decreased egg production and causes moderate to high mortality in poultry birds in Eurasia and world (Lee *et al*., 2011). The AIV is a worldwide problem and cases have been reported from many countries (Baumer *et al*., 2010; Hansbro *et al*., 2010). Recently, a prevalence of 3.2% has been reported of H9N2 virus strain in free range poultry in eastern China (Caihui *et al*., 2018; Zhu *et al*., 2018). They further reported that the six representative H9N2 isolates sequenced showed that these viruses were genotypically similar at 99.0 to 100% and were closely clustered with Zhejiang H9N2 isolated from chicken (belonged to genotype G57) (Zhu *et al*., 2018). In China, 4212 samples collected from human working in live poultry markets in Changsha, showed that 25.81% (1087/4212) were H9N2-positive (Liu *et al*., 2018). It has been suggested that to gain a better understanding of the drivers of H9N2 spread, increased surveillance of the H9N2 virus is required in countries that are under-sampled in poultry birds and wild birds (Zhao *et al*., 2018). Another recent study from China, reported that H9N2 isolates were classified as genotype 57, and was a low-pathogenic virus (Han *et al.*, 2018). They also reported that there is a necessity for continued surveillance of H9N2 viruses in the environment. Keeping

in view the importance of H9N2 AIV and its continuous surveillance, the present study was carried out.

MATERIALS AND METHODS

Sample materials: During 2017-2019, epidemiological surveys of AIVs were conducted twice a year. A total of 864 samples were collected randomly from 38 live broilers markets (316 samples), 12 slaughter houses (213 samples) and 72 poultry farms (335 samples) from 12 cities of China (Hebei, Tianjin, Shandong provinces).

Virus isolation: The tissues of lungs and trachea with clear pathological changes were weighed and cut into small pieces. Four times by weight, physiological saline was added for grinding. After centrifugation at 5000 x g for 10 minutes, supernatant was collected. The SPF chicken embryo, 9-11 day old were inoculated with 0.02 mL of the supernatant through allantoic cavity and placed in an incubator at 37°C. The death of chicken embryo occurred within 72-96h of inoculation. The allantoic fluid was collected for harvesting of virus.

The haemagglutination inhibition titer was carried out for serological identification of AIV by using AIV antigen previously identified in the lab against serum collected from vaccinated flocks. The lab virus and isolated AIVs gave similar results of HI titers (range 8.6-9.2 $\log^2$).

Reverse Transcription-polymerase Chain Reaction (RTPCR): RNA was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany), and amplified with the Prime Script One-step RT-PCR Kit (TaKaRa, Dalian, China).

The primers (Hoffmann *et al*., 2001) used for virus identification were purchased locally (TaKaRa, Dalian, China). The serum for identification was purchased from Harbin Weike Biotechnology Development Company.

Reverse transcription primer sequences: 5 '-agcaaaagcagg-3'
Identification primer sequences of H9N2 HA:
H9-732 - u - TCAACAAACTCCACCGAAACTGT 5 '- 3'
H9-732 l 5 '- TCCCGTAAGAACATGTCCATACCA - 3'

RT-PCR was performed in a 50-mL reaction using the One-Step RT-PCR kit (TaKaRa, Dalian, China). Added 1 ng-1 µg total RNA into the precooled sterile non-RNase enzyme centrifuge tube. Added 1 µL Oligo (dT) 12-18 primer to this tube and added 1 µL of deionized water. After mixed, placed at 70°C for 10 min, place on ice for 2 min for rapid quenching. Then added with the following components: 0.5mm dNTP Mix (10 mM); 0.25 µL RNase Inhibitor (40 U/ µL) 0.5 µL m-mlv (200u/ µL); Hydrochloric acid. Kept warm at 42°C for 1 h, stored the cDNA solution at -20°C.

The primers were designed for amplification according to the standard NY/T 772-2004 protocol. The amplification was conducted according to the conditions already reported (Hoffmann *et al*., 2001). The PCR reaction conditions were as follows: 95°C for 5 minutes, then 30 cycles at 95°C for 1 min, 55°C for 1 min, 72°C for 90 sec; with final extension at 72°C for 10 min. After the reaction, 5 µL of product was taken and identified by running on 1.5% agarose gel by electrophoresis.

After the fragments were completely separated, the gel strips were quickly cut off and the DNA was eluted. Briefly, added 1 mL of buffer per gm gel and put at 65°C in a water bath. This was transferred to the Hibind DNA column and centrifuged for 1 min at 9000 RPM. Poured the filtrate and placed the DNA column back into the collecting tube. Then replaced the column with the collecting tube, added 700 µL wash buffer, centrifuged and discarded the filtrate. Placed the column on clean 1.5ml centrifuge tube, added 30-50 µL elution buffer to the column matrix, and let stand at room temperature for 2 min.

Sequencing and analysis: cDNA samples recovered after PCR were sent to BGI for sequencing. The sequencing results were collated and spliced to obtain the final HA gene sequence. The HA gene sequences of each virus were analyzed using MEGA (6.0) software, genetic distance, evolutionary tree and homology comparison were conducted, and the H9 influenza representative sequences were downloaded from NCBI for HA genetic variability analysis (Bao *et al*., 2008). The H9N2 subtype sequences of 35 avian influenza strains were analyzed using neighbour-joining tree method in MEGA (6.0) software to establish the phylogenetic Tree.

Ethical approval: The study was formally approved by the Ethical Committee of Department of Zoology, Lahore College for Women University Lahore, and all the protocols of Chinese Government were observed.

RESULTS

The Avian Influenza virus was isolated and cultured in SPF embryonated eggs, collected allantoic fluid and preliminarily identified by HA and HI tests. Sixteen strains were identified from the 38 (316 samples) live broiler markets, 8 (213 samples) strains from 12 slaughter houses and 11 (335 samples) strains from 72 poultry farms. A total of 35 different strains of AIV (H9N2 subtype) were identified from samples obtained from 12 cities of Hebei, Tianjin, Shandong provinces from broilers, as shown in Table 1. These strains were identified by HI titer, further by using RT-PCR with a band of 488 bp (Fig. 1) was obtained on agarose gel and then sequencing was done to confirm the AIV.

The isolated virus samples were also confirmed by HI titers against a known vaccinated flock and the titers ranged between 8.6-9.2 $\log^2$.

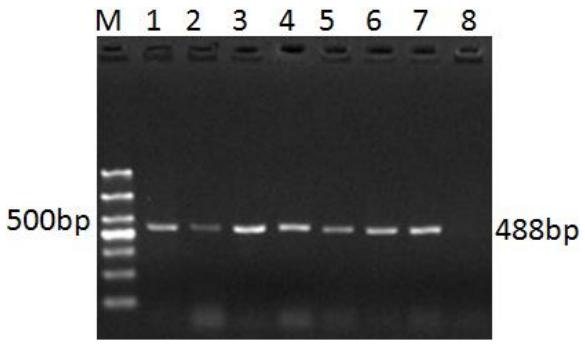

Fig. 1: The gel electrophoresis image of AIV H9 subtype recovered from 864 lungs and tracheal samples collected from broilers. Lane 8 is negative control, lane 7 is positive control and lane 1-6 are samples.

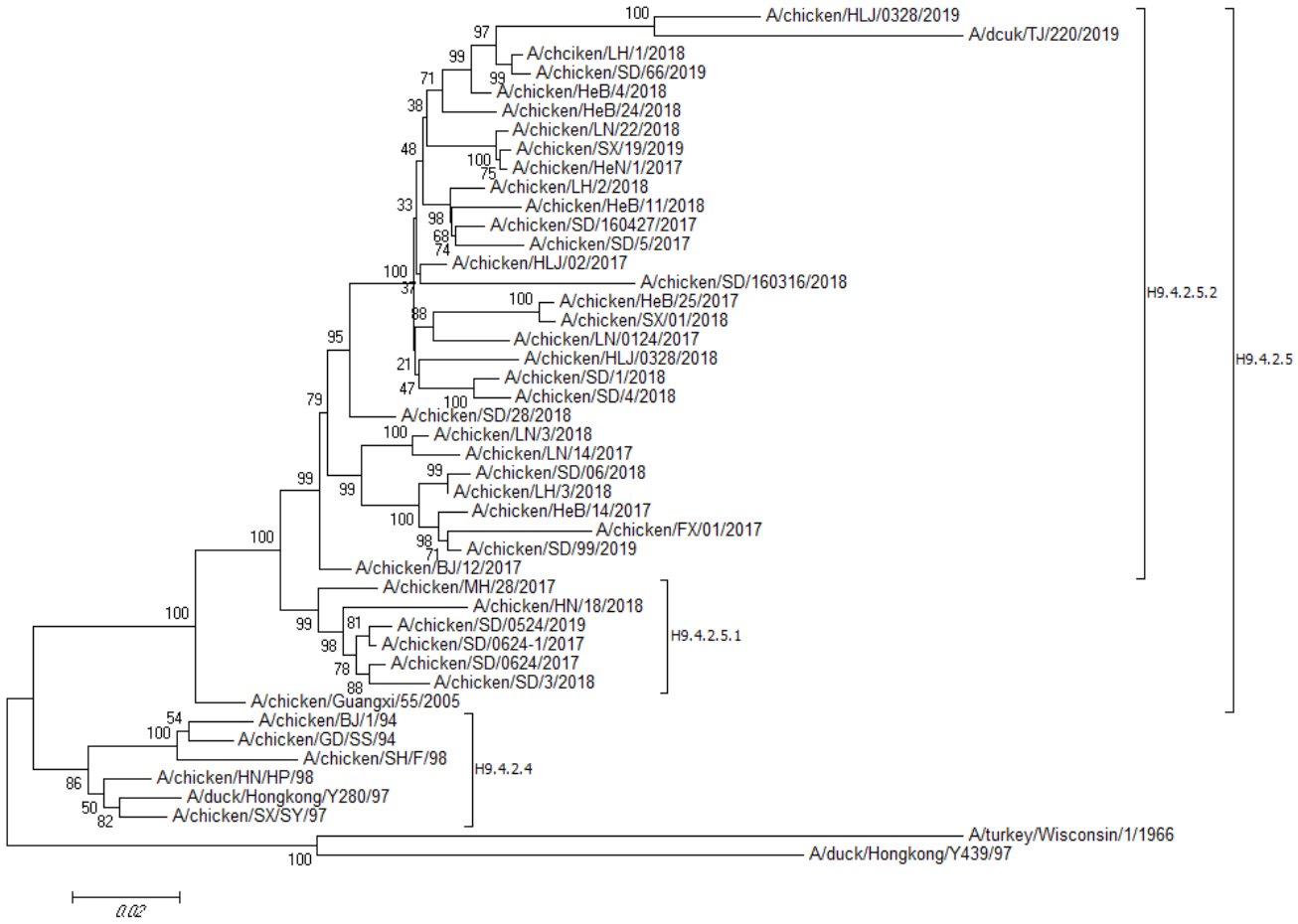

Fig. 2: Phylogenetic tree analysis of AIV H9 subtype 35 strains from 864 samples collected from broilers.

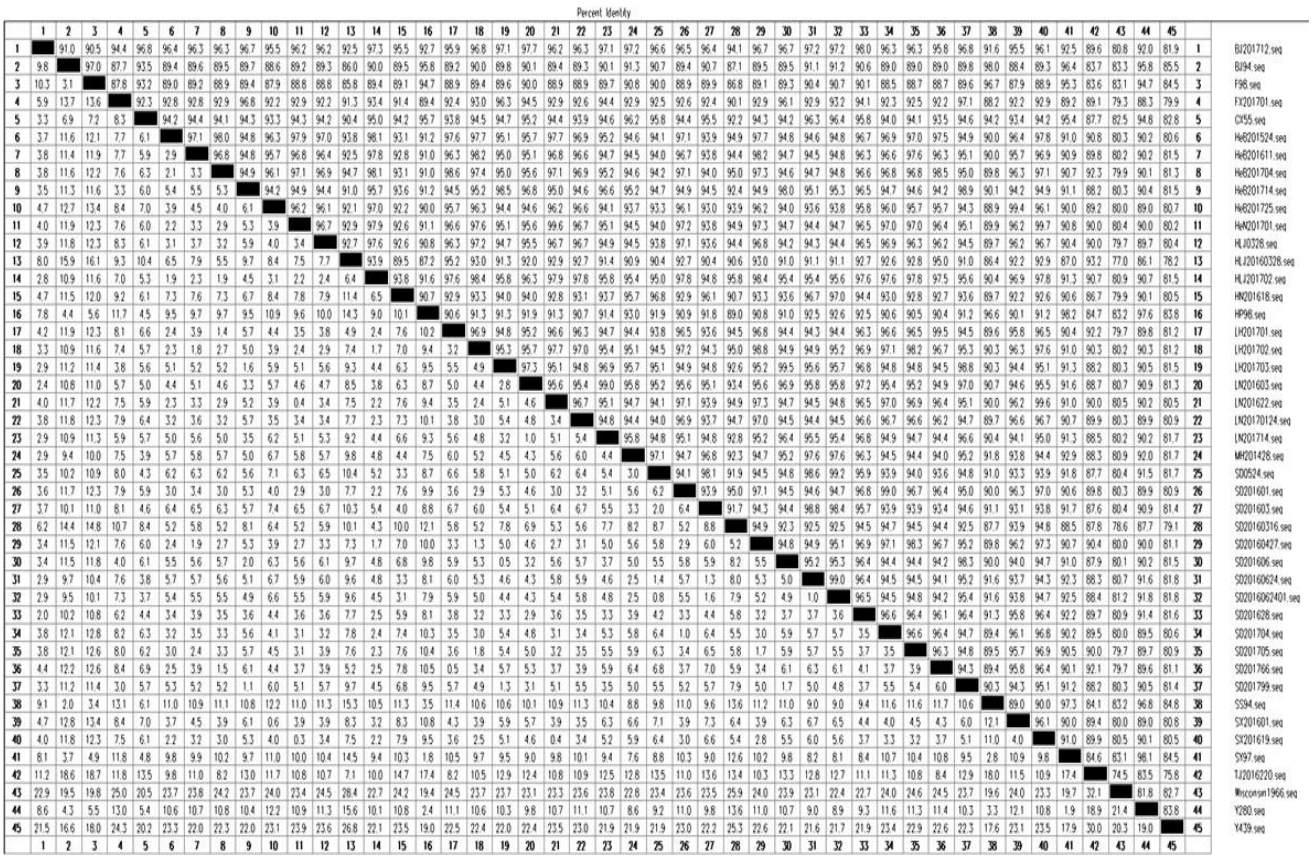

Fig. 3: Analysis of homology between virus isolates of present study and vaccine strains of AIV.

Table 1: Description of AIV of H9 subtype including 35 isolates from 864 samples collected from broilers is presented here under

Isolates	Place	Host	Time	H9 Subclass
A/chicken/SD/28/2018	Shandong	Broiler	2018	H9.4.2.5.2
A/chicken/HLJ/02/2017	Heilongjiang	Broiler	2017	H9.4.2.5.2
A/chicken/HN/18/2018	Hunan	Broiler	2018	H9.4.2.5.1
A/chicken/MH/28/2017	Shandong	Broiler	2017	H9.4.2.5.1
A/chicken/BJ/12/2017	Beijing	Broiler	2017	H9.4.2.5.2
A/chicken/SD/06/2018	Shandong	Broiler	2018	H9.4.2.5.2
A/chicken/SD/160316/2018	Shandong	Broiler	2018	H9.4.2.5.2
A/chicken/SD/160427/2017	Shandong	Broiler	2017	H9.4.2.5.2
A/chicken/LN/0124/2017	Liaoning	Broiler	2017	H9.4.2.5.2
A/chicken/HLJ/0328/2018	Heilongjiang	Broiler	2018	H9.4.2.5.2
A/chicken/SD/0524/2019	Shandong	Broiler	2019	H9.4.2.5.1
A/chicken/SD/0624-1/2017	Shandong	Broiler	2017	H9.4.2.5.1
A/chicken/SD/0624/2017	Shandong	Broiler	2017	H9.4.2.5.1
A/chicken/SD/3/2018	Shandong	Broiler	2018	H9.4.2.5.1
A/chicken/HeB/24/2018	Hebei	Broiler	2018	H9.4.2.5.2
A/chicken/HLJ/0328/2019	Heilongjiang	Broiler	2019	H9.4.2.5.2
A/chicken/SD/1/2018	Shandong	Broiler	2018	H9.4.2.5.2
A/chicken/LN/3/2018	Liaoning	Broiler	2018	H9.4.2.5.2
A/chicken/HeB/4/2018	Hebei	Broiler	2018	H9.4.2.5.2
A/chicken/SX/19/2019	Shanxi	Broiler	2019	H9.4.2.5.2
A/chicken/SD/4/2018	Shandong	Broiler	2018	H9.4.2.5.2
A/chicken/SD/5/2017	Shandong	Broiler	2017	H9.4.2.5.2
A/chicken/LN/22/2018	Liaoning	Broiler	2018	H9.4.2.5.2
A/chicken/HeN/1/2017	Hebei	Broiler	2017	H9.4.2.5.2
A/chicken/LN/14/2017	Liaoning	Broiler	2017	H9.4.2.5.2
A/chicken/HeB/11/2018	Hebei	Broiler	2018	H9.4.2.5.2
A/chicken/HeB/14/2017	Hebei	Broiler	2017	H9.4.2.5.2
A/chicken/HeB/25/2017	Hebei	Broiler	2017	H9.4.2.5.2
A/chicken/SX/01/2018	Shanxi	Broiler	2018	H9.4.2.5.2
A/chicken/FX/01/2017	Shandong	Broiler	2017	H9.4.2.5.2
A/chciken/LH/1/2018	Liaoning	Broiler	2018	H9.4.2.5.2
A/chicken/LH/2/2018	Liaoning	Broiler	2018	H9.4.2.5.2
A/chicken/LH/3/2018	Liaoning	Broiler	2018	H9.4.2.5.2
A/chicken/SD/99/2019	Shandong	Broiler	2019	H9.4.2.5.2
A/chicken/SD/66/2019	Shandong	Broiler	2019	H9.4.2.5.2

Genetic distance and homology analysis: The H9N2 subtype sequences of 35 strains isolated in 2017-2019 were compared with previous classical strains used in vaccine by genetic distance analysis using neighbor-joining tree in MEGA (6.0) software to develop the phylogenic tree. The parameters were as follows: set the repeat value of Bootstrap to 1.000; nucleotide variation was set to Kimura 2-parameter model; the site mutation rate was Gamma distribution and parameter was set to 4.0.

The genetic distance analysis was conducted among the 35 strains and the previous classical strains and the current vaccine strain on the market. It was showed that all HA genes of the isolates since 2017 belonged to the HA gene H9.4.2.5 subfamily (Fig. 2). Its representative strain was A/chicken/Guangxi/55/2005 virus, but there was a genetic distance. The 35 strains were mainly divided into 2 subtypes, among them 6 strains were classified as class H9.4.2.5.1 and 29 strains were classified as class H9.4.2.5.2. The main vaccine strain SY/97 belonged to H9.4.2.4 subfamily.

HA Gene Nucleotide Homology Analysis: The HA nucleotide homology between the 35 isolated strains of virus on the basis of gene sequences was 92.1 to 99.7%, while the nucleotide homology between the 35 isolated strains and the Y280 vaccine strains was 86.1 to 92%, and the nucleotide homology between the 35 isolated strains and SY/97 strains was 86.4 to 91.8% (Fig. 3).

DISCUSSION

The first AIV isolate was reported in the USA in 1966 (Homme & Easterday, 1970), since then it has been reported from all over the world including China (Xu *et al*., 2007). The surface of AIV is covered by haemagglutinin (HA) and neuraminidase (NA) glycoproteins (Ali *et al*., 2016). Type A Influenza virus infection in chicken is caused by either highly pathogenic avian influenza (HPAI) virus (mortality up to 100%) or by low pathogenic avian influenza (LPAI) virus (produce mild clinical signs) (AL-Barwary *et al*., 2012). The H9N2 avian influenza subtype lineages that are now endemic in poultry, likely to have several different origins (Lee *et al.*, 2000). Since the first H9N2 subtype was isolated in China in 1994, the epidemic situation of avian influenza H9N2 subtype in chickens is relatively serious, especially in broiler industry (Sun *et al*., 2013). The current preventive and treatment methods are mainly based on vaccine immunization which have achieved good results but because of the characteristics of H9N2 viruses that they mutate easily, the chance of genetic drift in vaccine strain has a high probability (Chen *et al.*, 2011; Shen *et al*., 2015). The difference of antigenicity result in that although it belongs to the same serotype H9N2 does not give ideal conditions of attack protection and the infection of H9N2 disease occurred frequently in immunized chicken. Keeping in view the mutation rate in AIV, it was felt important to study the current field strains of AIVs circulating in China and how do they differ from the vaccine strain as vaccine failure has frequently been reported in China.

The study found a total of 16 different strains from 316 samples collected from live broiler markets, eight strains from 12 slaughterhouse samples and 11 strains from samples collected from 72 poultry farms. This data reflects that maximum number of strains were from live broiler markets with second highest number being from poultry farms and least number of strains were from the slaughterhouses. This variation from different sources is always expected and may be associated with the supply chain to the live market and slaughterhouse, as broiler farms are the source of supply of the broilers to the market.

The 35 strains of AIV virus isolated from 864 samples collected in 2017-2019 when compared with previous classical strains and the vaccine strain which is present in the market showed that all the isolates belonged to the HA gene H9.4.2.5 subtype of its representative for A/chicken/Guangxi/55/2005 virus strains. The flu and the world health organization expert group pointed out A/duck/Hong Kong/Y280/97 is one of the major epidemic viruses since 2010 and explained that the flu HA gene mutations occur faster than expected, which may also be widely distributed in China (Park *et al*., 2011). The strains isolated from northeast, north China are more similar to virus strain A/chicken/Guangxi/55/2005 but has a certain genetic distance. At present, 35 strains were mainly divided into 2 subgroups, among which 6 strains were classified as class H9.4.2.5.1 and 30 strains were classified as class H9.4.2.5.2. The main vaccine strains, SY/97 belonging to H9.4.2.4, indicated that the virus strains had undergone great changes.

The HA nucleotide homology between the 35 strains of virus gene sequences was 92.1 to 99.7%, the nucleotide homology between 35 strains and Y280 vaccine strains was 86.1 to 92%, and the nucleotide homology between 35 strains and SY/97 strains was 86.4 to 91.8%. The most common reference strain is A/Duck/Hong Kong/Y280/

1997 being used as vaccine strain is extremely diverse and is referred to as the Y280 lineage (Sun *et al*., 2013; Li *et al*., 2017). Phylograms of strains isolated from Korea revealed that HA genes of the H9N2 viruses were grouped under the Ck/Korea/04116/ 04-like lineage with 95.7 to 99.2 % nucleotide homology (Moon *et al*., 2010), that drifted from vaccine strain Ck/Korea/01310/01 with 91.2 to 94.2% nucleotide homology. These results are in congruent with the present findings as for vaccine strain and the field virus homology is concerned. Both the studies indicate drift of field virus from vaccine strain. It has been reported that antigenic drift occur because of lack of a proof-reading mechanism for influenza A viral RNA polymerase thus leads to genetic variation due to mutations in the RNA (Kimble *et al*., 2010).

The results showed that the HA genes of 35 strains presently studied were far apart from the main vaccine strains. According to the results on HA gene encoding the base sequence it can be seen that the HA gene system of H9N2 subtype of avian influenza virus in parts of China is under the pressure of using homologous vaccine immunization. The results of the study clearly indicated that the vaccine strain belonged to H9.4.2.4 subfamily, while the field virus belonged to the H9.4.2.5.1 subfamily. Thus, there is need to think seriously about the use of vaccine strain and immunization protocol to protect the broiler industry in China. The conditions in the other parts of the world would not be different and there is need to carryout similar work in other countries so that the disease in poultry can be controlled.

Conclusions: The findings of the study indicated that the vaccine strain of Y280 and SY/97 belonged to different subfamily, while the field virus belongs to H9.4.2.5.1 subfamily and is different.

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Authors contribution: BY carried out the work, FM and CH are the supervisor and co-supervisor of Bai Yunhua and this research work was carried out under their guidance. MTJ also played significant role in research planning and writeup of the manuscript.

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