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RECENT UPDATES ON MOLECULAR DETECTION OF H9N2 AS LOW PATHOGENIC STRAIN OF AVIAN INFLUENZA VIRUS FROM POULTRY FARMS OF LAHORE, PAKISTAN

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ABSTRACT

H9N2 avian influenza outbreaks have caused great economic losses to the poultry industry in recent decades due to decreased egg production, high morbidity, and mortality. Due to different antigenic variants, the influenza virus has become problematic because it can cross the species barrier. As it is highly pathogenic, its diagnosis and vaccines are highly important. Hemagglutination inhibition (HI) test is mostly used for subtyping and detecting antibody titer against the virus. Furthermore, its continuous mutations in the HA gene transforms AIV subtype H9N2 (a low pathogenic subtype) into high pathogenic virus subtypes like H5N2 and H7N7 that may have pandemic potential. Thus, it is necessary to identify various antigenic variants of the influenza virus, so it is needed to study the HA gene, its attachment to host receptors, the release of genetic material, and pathogenicity. In the present study, virus samples from poultry were isolated. Both serological and molecular confirmation was carried out for 100 samples collected from a different area. They were properly labeled and prepared for the process of egg inoculation in embryonated eggs. The virus was grown in the amnioallantoic membrane of embryonated eggs, and harvested fluid then proceeded for confirmatory testing. Did hemagglutination and hemagglutination inhibition testing. RNA was extracted by the kit method, and cDNA was synthesized. Reverse transcriptase (RTPCR) was performed using specific primer sets, and then ran the PCR product on the agarose gel. Then sent bands obtained for sequencing.

Keywords: Avian Influenza virus, H9N2 strain, Poultry Industry, Economic Losses, Molecular Characterization, Polymerase chain reaction.

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1. INTRODUCTION

Avian influenza disease is caused by avian influenza viruses (AIVs) belonging to Orthomyxoviridae family. The Influenza viruses have caused serious diseases in commercial chickens and has many outbreaks worldwide and also became prevalent in Pakistan. Moreover, this virus has a zoonotic perspective that means it has the potential to infect humans as well (Spackman et al. 2002). Subtypes of AIV are H5, H7, and H9, which have been most commonly implicated diseases in poultry. In contrast to H5N1 strain which is highly pathogenic, the H9N2 strain is generally considered to have low pathogenicity in chickens but it can evolve into highly pathogenic strain due to its mutagenic ability, so eradicating H9N2 AIVs on farms is not a priority. During the last several decades, H9N2 AIVs have been circulating widely in poultry population and caused tremendous losses, especially in Asian countries like China and Pakistan. H9N2 AIVs can occasionally transmit to humans (Liu et al. 2016; Ali et al. 2018). The H9 subtypes cause mild conjunctivitis and respiratory disease in human. It poses a similar threat likes H5N1 as the mild infection may become virulent through antigenic drift or shift (Li et al. 2005). The H9N2 were frequently isolated from China. The H9N2 subtype was isolated in Hong Kong from 2 children, suffering from respiratory disease (Ahad et al. 2013).

The first occurrence of avian influenza in poultry dates back to 1878 when highly pathogenic avian influenza (HPAIV) was initially recognized as an infectious disease in chicken in Italy and was called fowl plague by Perroncito (Alexander 2000). Since then, an infection with subtype H9 is constantly reported in various parts of the world

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including China, North America, Middle East, Hong Kong, South Korea and South Africa. In 1998, the first case of H9N2 was testified in Pakistan (Fazel et al. 2014). Recently, an investigation of poultry disease has shown that another outbreak of AIV is responsible for huge economic losses in commercial poultry (Butt et al. 2010).

Influenza viruses live in a variety of host environments and use a limited catalogue of protein components. In contrast to viruses with stereotypical forms, influenza produces virions with significant morphological variability, even within clonal populations (Vahey and Fletcher 2019).

The Matrix 1 protein (with two specific amino acids) is considered to be important for controlling the morphology of the virion. The influenza A genome contains eight pieces of segmented negative-sense RNA (13.5 kilo bases) that encodes 11 proteins: HA (haemagglutinin); NA (neuraminidase); NP (nucleoprotein), M1; M2; NS1; NS2 (NEP); PA; PB1; PB1-F2 and PB2 (Webster and Rott 1987). All avian influenza viruses belong to type A. The glycoprotein in nature, HA the influenza virus, identify the sialic acid receptors. Beginning of virus infection involves multiple HAs binding to sialic acid receptors. These are present on carbohydrate side chains of cell-surface glycoproteins. HA activates the viral attachment to host cell receptors (sialic acid in nature) and aids the viral entry into the target cells. Broadly, HA has contained two subunits, HA 1 having a molecular mass of 55 kDa and HA2 having a molecular mass of 25KDa, host proteases cleave the HA0 which has molecular Mass of 75 kDa. This breakdown of HA0 into HA1 divided HA2 triggers virus infectivity. This cleavage is ultimately considered the backbone of virus pathogenicity in avian mammalian hosts (Garten et al. 2009). Avian influenza virus (AIV) subtype A (H9) flows in wild bird species worldwide and is endemic to domestic birds in many countries in Asia, the Middle East and Africa (Liu et al. 2016; Nagy et al. 2017). In spite of its low prominence as a pathogenic avian influenza (LPAI), subtype A (H9) is a problem for both the agricultural and health sectors. Although infected herds have only mild respiratory illnesses and the death rate is generally below 20% (Li et al. 2005; Ji et al. 2010; Gu et al. 2017).

Pathogenicity in influenza virus is a polygenic trait that depends largely on 'optimal' gene configuration affecting host and tissue tropism, replication efficacy and immune evasion mechanism. Host- and species-specific factors also contribute to the outcome of infection, which, after interspecies transmission, is therefore unpredictable. Most avian influenza viruses are LPAI viruses associated with mild disease in poultry. HPAI viruses, however, can cause severe illness and high mortality in poultry. HA0 that requires posttranslational cleavage by host proteases into the HA1 and HA2 subunits before becoming infectious (Webster and Rott 1987).

Exposure is the first step in the transmission and initiation of infection. Two types of vector play a role in the spread and transmission of avian influenza, which can be described as biological (infected birds) and mechanical (Fomites, air, and water). Avian Influenza virus can be spread to a poultry flock though: 1) Direct exposure from AI infected birds 2) Exposure from fomites contaminated with respiratory secretions or feces of infected birds 3) Personnel movement on the farm through contaminated shoes or clothing 4) Water contaminated with AI virus and 5) Airborne movement of the AI virus (Swayne 2006). Due to the current changes in HA gene, pandemic possible and species barrier crossing ability it is necessary to study HA gene. Characterization and identification of emerging H9N2 AIV endemic in Pakistan will help in producing a viral lineage. Sequence analysis of field isolates can open a new door with regards to AIV vaccine production and help ward off extremely loss in poultry zone (Cong et al. 2007).

2. MATERIALS AND METHODS

A total of 100 samples were collected from the different areas of Lahore. Birds showing respiratory signs were selected for sampling. Samples were collected from the dead as well as live birds. Trachea, lungs, and spleen were collected from dead birds while trachea swabs from live birds with antibiotics, 300µL, Ceftiofur sodium added. All samples were properly labeled and stored at 20 to 24℃ (Fereidouni et al. 2012), prior to processing.

2.1. Inoculation

To isolate the influenza virus from a clinical specimen, 9-11 days old embryonated eggs were inoculated by following mean i.e., fertile eggs were disinfected with pyodine, 0.2mL prepared sample was inoculated in 9-11 days old embryonated chicken eggs via allantoic sac route. The inoculation was done within Class 2 Biosafety cabinet. The eggs after inoculation were incubated at 37℃ for 48 hrs. Candling of eggs was done after every 12 hrs post inoculation to check for the embryonic death due to injury or contamination.

2.2. Harvesting of Amnioallantoic fluid

Amnioallantoic fluid (AAF) from eggs was collected after 48 hours of post inoculation. The eggs were chilled overnight at 4℃. After chilling, eggs were placed in Biosafety cabinet class 2 were disinfected using 70% alcohol. The eggshell portion over the air sac area was removed using the sterilized sharp blunt scissor. Air sac membrane was punctured and gently pressed with the help of sterilized forceps to pool out the fluid. The AAF was pooled out carefully using 5mL disposable syringe. It was poured in a separately labelled sterilized 15mL falcon tubes.

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2.3. Washing of RBC's

Approximately 10mL of healthy chicken blood was collected in a beaker containing 400µL anticoagulant (EDTA). Blood was gently mixed and shifted to 15mL falcon tube for centrifugation. The blood was centrifuged for 5 min at 3000rpm at room temperature. The supernatant was removed and settled RBS's were resuspend in normal saline to make a volume of 15mL. Again, centrifuged the tube for 5 min at 3000rpm at room temperature and repeated the same step thrice for proper washing of RBC's. After washing, 1mL of washed RBC's was re-suspended in 100 mL of normal saline to make 1% RBC's suspension for HA test.

2.4. Serological Confirmation of H9N2 AIV

Serological confirmation of H9N2 AIV was done by using Hemagglutination test (HA) and Hemagglutination Inhibition (HI) by following general protocols (Spackman and Sitaras 2020).

2.5. Molecular Confirmation

***2.5.1. RNA Extraction and Quantification*:** The first step in molecular confirmation is to extract RNA of H9N2 AIV from sample using a kit method (QIAGEN Cat No./ID: 74106). Both quality and quantity of extracted viral RNA were analyzed and Nano drops 2000 (Thermo Scientific company, USA). For this purpose, we used 1µL DEPC treated water as a blank. 1µL RNA sample loaded in the lower pedestal of Nano drops. With help of software provided by the company, quality and quantity of RNA were determined.

***2.5.2. cDNA Synthesis*:** Thermo Scientific Revert Aid First strand cDNA synthesis kit (Cat: K1822) was employed to synthesize cDNA from previously extracted Viral RNA.

2.5.3. Primer Designing: Gene sequence of HA gene was obtained from the NCBI database (http://www.ncbi.nlm.nih.gov). The sequence was employed to design the primer set for HA gene using Primer3 software (http://simgene.com/primer3) (Table 1).

Table 1: The sequence of primers is as given

Sr. No	Primer Name	Primer Sequence	Product Size (bp)
1	H9(773)F	5'-ATCTAATCGCTCCATGGTATGG-3'	287
	H9(1040)R	5'-TGACCAACCTCCCTCTATGA-3'	
2	N2(478)F	5'-CCAGCTCAAGTTGCCATGA-3'	483
	N2(940)R	5'-GATCTCTGCAGTTGCTGCT-3'	

***2.5.4. RT-PCR*:** Reverse transcriptase PCR (RT-PCR) was performed using design primers for quantification of HA gene H9N2. HA gene amplification was performed using thermo cycler in 25µL reaction mixture as final volume. Protocol in detail described by Tahir et al. (2016) was followed.

***2.5.5. Agarose Gel Electrophoresis and Amplicons Purification*:** Detailed method is described by Tahir et al. (2016). The PCR product was confirmed by running the PCR product on 1.2% Agarose gel. Electrophoresis was performed at 110 Volts for 30 minutes. The amplified PCR product was further purified using GeneJet Gel Extraction Kit (cat K0691) and instructions provided by the manufacturer.

3. RESULTS

H9N2 Avian influenza infection causes great economic losses to the poultry industry. Concerning the facts, the present study was designed to check the gene sequence, variation. All the objectives were properly obtained and analyzed. A total of 100 samples were processed. The major findings of the study are described below:

3.1. Hemagglutination test (HA)

The Allantoic Amniotic fluid was used to conduct Hemagglutination test. Out of 100 samples, 80 samples showed Hemagglutination. The HA activity of virus ranged from1:4 to 1:2048. The test was performed using 96-well U-shaped, microtiter plate. Positive samples agglutinated the RBC's and showed Hemagglutination. Sample 2022 showed agglutination up to 6th well. Sample 1991 showed agglutination up to 2nd well. Sample 2038 showed agglutination up to 6th well. Sample 2002 showed agglutination up to 8th well (Fig. 1).

3.2. Hemagglutination Inhibition Test (HI)

To detect the antibody titter against H9N2, HI test was performed. Specific monoclonal sera of H9N2 (GD Netherlands) were used to confirm H9N2 positive samples. Sample 2022 showed bead formation up to 2nd well.

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Sample 1991 showed bead formation up to 4th well. Sample 2038 showed bead formation up to 3rd well. Sample 2002 showed bead formation up to 5th well (Fig. 2).

3.2. RNA Extraction

RNA was extracted from Allanto Amniotic Fluid using FavroPrep Viral Nucleic Acid Extraction Kit Favrogen (Cat # FAVNK001-2). The concentration of extracted RNA was determined by using Nanodrop (Table 2).

Table 2: Concentration of extracted RNA

Sample ID	Conc (ng/µL)	Ratio 260/280
2038	1.78	1.23
2002	2.33	1.52
2022	1.83	1.15
1991	1.83	1.79

3.3. cDNA Synthesis and Quantification

cDNA was synthesized using Thermo Scientific RevertAid First Strand cDNA Synthesis kit (Cat: K1822. The concentration of cDNA was measured in ng/µL by using Nano drop 2000 spectrophotometer. The ratio of 260/280 nm wavelength was used as a measure of the purity of extracted cDNA. The ratio greater than 1.7 indicates its purity (Table 3).

Table 3: Concentration of cDNA after synthesis

Sample ID	Conc. ng/µL	Ratio 260/280
1991	2345.93	1.74
2022	2202.96	1.73
2038	3670.53	1.72

3.4. PCR Amplification and Purified Product

Initially, H9N2 was confirmed by using detection primers in PCR (polymerase chain reaction) thermo cycler. The PCR conditions were optimized at 54°C as annealing temperature. The PCR product was run on 1.2% agarose gel and visualized on Gel Doc (gel documentation system). The bands are shown in Fig. 3. The PCR product was purified by using Gene Jet Gel Extraction Kit (cat K0691). The bands were run on 1.2% agarose gel and visualized and photographed in Gel Doc system (Fig. 4).

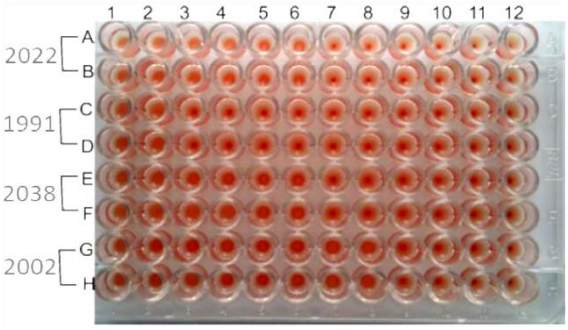

Fig. 1: HA titter of samples 2022, 1991, 2038 and 2002.

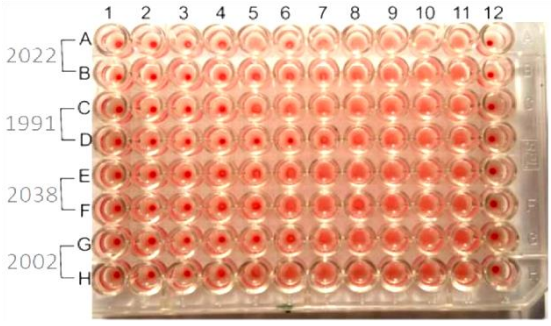

Fig. 2: HI titter of samples 2022, 1991, 2038 and 2002.

4. DISCUSSION

H9N2 AIV is considered a low pathogenic virus as it affects only those organs which contain trypsin-like proteases that are necessary to cleave H0 into H1 and H2. This study revealed that H9N2 AIVs evolved over a while thus, generating different antigenic groups (Munir et al. 2013; Chaudhry et al. 2017). Potential Glycosylation sites play a key role in increased virulence of the virus, either due to change in receptor configuration or modification in patterns of antigenicity (Islam et al. 2017). Many reports reveal the fact that H9N2 is continuously circulating in Asian Poultry. The virus has rapidly evolved antigenically and enhanced virulence than the past.

The absence of clinical signs in backyard poultry may strengthen the threat that the virus may expose to the human population and genetic re-assortment (Butt et al. 2010). H9N2 evolves rapidly in terms of antigenicity. Therefore, it was mandatory to evaluate the hemagglutinin (HA) protein. Antigenic variants of H9N2 were identified using monoclonal antibodies. 6 amino acid positions were identified by reacting monoclonal antibodies with both

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wild and local isolates. The positions were 92, 145, 166, 167, 168, and 197 in antigenic sites, among them the position 92 was not been reported previously. The study gave us the idea that this novel mutation at 92 position may become a molecular marker for viral evolution (Zhu et al. 2015).

Phylogenetic analysis of HA exposed that Asian H9N2 viruses can be divided into three subgroup lineages. Sequence analysis showed that at the haemagglutinin cleavage site of HA gene there was an additional basic amino acid. This sign basically supported that there is the difference in pathogenicity and various studies demonstrated diversity in prevalence of virus in this country (Fatima et al. 2017; Kausar et al. 2018). These isolates were found unique from the previous isolates as they differed antigenically (Munir et al. 2013; Islam et al. 2017). The idea gets more weightage by the fact that several H9N2 isolates are isolated from animals and humans in Southeast Asia. Pakistani isolates have much more similarity with those of Hong Kong due to the same antigenic sites. It depicts that same isolates circulated in Pakistan that caused human outbreaks in China during 1990. Currently, H9N2 in Pakistan is not established in the human population and is unable to cause human infection (Islam et al. 2017). From the above-mentioned discussion, it is obvious that there is great risk associated with H9N2 infections in Pakistan, especially regarding vaccine failure. Human life is on the verge of H9N2 Infections and prevalence estimates when this study compared with various studies conducted (Eladl et al. 2019).

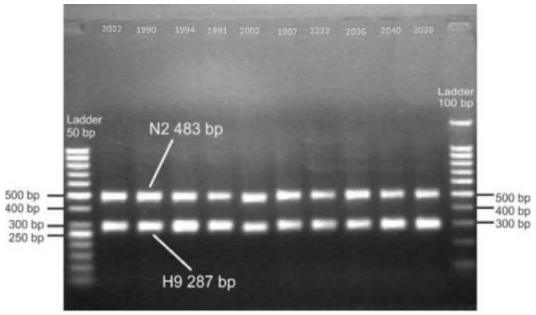

Fig. 3: Ethidium bromide-stained gel showing amplicons of H9N2

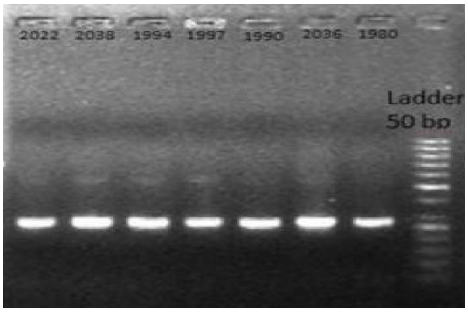

Fig. 4: Purified of PCR product of H9N2

Conclusion: A constant H9N2 prevalence revealed that the phenomenon adapted to the mouse model indicated about the virus adaption towards mammals. The situation is getting worse as undergoing backyard poultry practices are not up to the mark. Subtype AIV H9N2 (a low pathogenic subtype) into high pathogenic virus subtypes is a consequence of different kind of birds in vicinity and commercial poultry raising activities.

Author's Contribution: MMS has done work for his research purpose. MR supervised and prepared this manuscript. TY facilitated with lab and co-supervised this research. WF, FY and SN helped in execution of research and reviewing the manuscript. All authors approved final version of the manuscript.

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REFERENCES

Ahad A, Rabbani M, Yaqub T, Younus M, Mahmood A, Shabbir MZ, Fatima Z, Khalid RK and Rasheed M, 2013. Serosurveillance to H9 and H7 avian influenza virus among poultry workers in Punjab Province, Pakistan. Pakistan Veterinary Journal 33: 107-112.

Ali M, Yaqub T, Mukhtar N, Imran M, Ghafoor A, Shahid MF, Yaqub S, Smith GJ, Su YC and Naeem M, 2018. Prevalence and phylogenetics of H9N2 in backyard and commercial poultry in Pakistan. Avian Diseases 62: 416-424. https://doi.org/10.1637/11690-062117-ResNote.1

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Open Access Journal

Alexander DJ, 2000. A review of avian influenza in different bird species. Veterinary Microbiology 74: 3-13.

Butt AM, Siddique S, Idrees M and Tong Y, 2010. Avian influenza A (H9N2): computational molecular analysis and phylogenetic characterization of viral surface proteins isolated between 1997 and 2009 from the human population. Virology Journal 7: 319.

Chaudhry M, Ahmad M, Rashid HB, Sultan B, Chaudhry HR, Riaz A and Shaheen MS, 2017. Prospective study of avian influenza H9 infection in commercial poultry farms of Punjab Province and Islamabad Capital Territory, Pakistan. Tropical Animal Health and Production 49: 213-220. https://doi.org/10.1007/s11250-016-1159-6

Cong YL, Pu J, Liu QF, Wang S, Zhang GZ, Zhang XL and Liu JH, 2007. Antigenic and genetic characterization of H9N2 swine influenza viruses in China. Journal of General Virology 88: 2035-2041. https://doi.org/10.1099/vir.0.82783-0

Eladl AH, Alzayat AA and Ali HS, 2019. Comparative molecular characterization, pathogenicity and seroprevalence of avian influenza virus H9N2 in commercial and backyard poultry flocks. Comparative Immunology, Microbiology and Infectious Diseases 64: 81-89. https://doi.org/10.1016/j.cimid.2019.02.011

Fatima Z, Khan MA, Ahmad MUD, Muhammad K, Khwaja KN, Khan A, Anwar Z, Ahad A and Mahmood A, 2017. Cross sectional survey of live bird markets, and zoo birds for circulating influenza subtypes in Pakistan. Pakistan Veterinary Journal 37:185-189.

Fazel H, Shahsavandi S, Masoudi S, Ebrahimi M and Taghizadeh M, 2014. Evolutionary characterization of non-structural gene of H9N2 influenza viruses isolated from Asia during 2008–2012. Comparative Clinical Pathology 23: 523-528. https://doi.org/10.1007/s00580-012-1644-x

Fereidouni SR, Globig A, Starick E and Harder TC, 2012. Effect of swab matrix, storage time, and temperature on detection of avian influenza virus RNA in swab samples. Avian Diseases 56: 955-958. https://doi.org/10.1637/10146-033012-ResNote.1

Garten RJ, Davis CT, Russell CA, Shu B, Lindstrom S, Balish A and Deyde V, 2009. Antigenic and genetic characteristics of swine-origin 2009 A (H1N1) influenza viruses circulating in humans. Science 325: 197-201.

Gu M, Xu L, Wang X, and Liu X, 2017. Current situation of H9N2 subtype avian influenza in China. Veterinary Research 48: 49. https://doi.org/10.1186/s13567-017-0453-2

Islam A, Qayum MU, Islam K, Alam HMS, Chakraborty P, Shakil AA, Islam S, Hossain ME, Alamgir ASM, Rahman MZ and Flora MS, 2017. Circulation of H9N2 virus.in the backyard poultry at rural community in Northern part of Bangladesh. In: The sixth ESWI Influenza conference, 10-13 September, 2017 At Riga, Latvia.

Kausar A, Anwar S, Siddique N, Ahmed S and Dasti JI, 2018. Prevalence of avian influenza H9N2 virus among wild and domesticated bird species across Pakistan. Pakistan Journal of Zoology 50: 1347-1354. http://dx.doi.org/10.17582/journal.pjz/2018.50.4.1347.1354

Ji K, Jiang WM, Liu S, Chen JM, Chen J, Hou GY and Huang BX, 2010. Characterization of the hemagglutinin gene of subtype H9 avian influenza viruses isolated in 2007–2009 in China. Journal of Virological Methods 163: 186-189.

Li C, Yu K, Tian G, Yu D, Liu L, Jing B and Chen H, 2005. Evolution of H9N2 influenza viruses from domestic poultry in Mainland China. Virology 340: 70-83.

Liu YF, Lai HZ, Li L, Liu YP, Zhang WY, Gao R and Luo Q, 2016. Endemic variation of H9N2 avian influenza virus in China. Avian Diseases 60: 817-825. https://doi.org/10.1637/11452-061616-Reg

Munir M, Zohari S, Abbas M, Shabbir MZ, Zahid MN, Latif MS and Berg M, 2013. Isolation and characterization of low pathogenic H9N2 avian influenza A viruses from a healthy flock and its comparison to other H9N2 isolates. Indian Journal of Virology 24: 342-348.

Nagy A, Mettenleiter T and Abdelwhab E, 2017. A brief summary of the epidemiology and genetic relatedness of avian influenza H9N2 virus in birds and mammals in the Middle East and North Africa. Epidemiology & Infection 145: 3320-3333. https://doi.org/10.1017/S0950268817002576

Spackman E, Senne DA, Myers T, Bulaga LL, Garber LP, Perdue ML and Suarez DL, 2002. Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. Journal of Clinical Microbiology 40: 3256-3260.

Spackman E and Sitaras I, 2020. Hemagglutination Inhibition Assay. In: Spackman E. (eds) Animal Influenza Virus. Methods in Molecular Biology, vol 2123. Humana, New York, NY. https://doi.org/10.1007/978-1-0716-0346-8_2

Swayne DE, 2006. Microassay for measuring thermal inactivation of H5N1 high pathogenicity avian influenza virus in naturally infected chicken meat. International Journal of Food Microbiology 108: 268-271.

Tahir MS, Mehmood D, Sultan AU, Saeed MH, Khan AR, Ansari F, Salman MM and Majeed KA, 2016. A modified strategy of multiplex RT-PCR for simultaneous detection of H5, H7, and H9 subtypes of avian influenza virus based on common forward oligo. Journal of Applied Poultry Research 25: 322-327. https://doi.org/10.3382/japr/pfw014

Vahey MD and Fletcher DA, 2019. Low-fidelity assembly of influenza A virus promotes escape from host cells. Cell 176: 281-294. https://doi.org/10.1016/j.cell.2018.10.056

Webster RG and Rott R, 1987. Influenza virus A pathogenicity: the pivotal role of hemagglutinin. Cell 50: 665-666.

Zhu Y, Yang D, Ren Q, Yang Y, Liu X, Xu X and Liu X, 2015. Identification and characterization of a novel antigenic epitope in the hemagglutinin of the escape mutants of H9N2 avian influenza viruses. Veterinary Microbiology 178: 144-149. https://doi.org/10.1016/j.vetmic.2015.04.012