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Original Research Article

Molecular generation and characterization of an efficient recombinant vaccine for avian influenza A/H5N8 in Saudi Arabia

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Abstract

Purpose: *To characterize a highly pathogenic avian influenza (HPAI) H5N8 for engineering recombinant 6-+ 2 vaccine strain based on reverse genetic technology.*
Methods: *A total of 135 swab samples from various birds were collected from different parts of Saudi Arabia as part of an influenza surveillance activity. The samples were checked for influenza virus infection using reverse transcriptase-polymerase chain reaction (RT-PCR). Furthermore, Avian influenza H5N8 (A/chicken/KSA/1-NRC/2018), was used for the generation of H5N8 vaccine strain. The vaccine was tested on specific pathogen-free (SPF) chicken purchased from a local market.*
Results: *The results indicate that the candidate vaccine (rgH5N8/KSA) induced specific neutralizing antibodies in chicken, and thereby protected the chickens from subsequent infections of H5N8.*
Conclusion: *The study reinforces the development of a vaccine against avian influenza H5N8 virus isolated in Saudi Arabia, suggesting its possible application against the influenza virus associated with bird flu*

Keywords: *Avian influenza vaccine, H5N8, Reverse genetics, Infection*

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INTRODUCTION

Influenza viruses are segmented, and negative-strand RNA viruses from the Orthomyxoviridae family [1]. The virus family is categorized into A, B, C, and D genera [2]. Humans are considered as the main host for influenza B and C, whereas, influenza D affects mainly cattle [3]. Nevertheless, molecular characterization of these viruses have shown a common genetic

origin. Viruses from genera A and B contain eight vRNA segments, and genera C type of influenza viruses contain only seven segments [4]. Furthermore, about eight segments of influenza A genome encodes approximate 10 viral proteins [5].

Influenza A viruses are further characterized on the basis of a *subtype* of their surface glycoproteins such as the hemagglutinin (HA) and the neuraminidase (NA) [5]. Influenza A and influenza B viruses are the main causes of respiratory disease in humans. Moreover, as per the reports by World Health Organisation (WHO), it has been estimated that influenza causes about 1 billion infections, 3 – 5 million severe illnesses, and 3- 5 hundred thousand deaths per year [6].

Influenza A is clinically important and is the primary cause of severe epidemics in humans and domestic animals [7]. Thus, influenza A is responsible for seasonal infections and pandemic outbreaks that pose a major threat to human health [8]. Asian origin H5N8 is considered highly pathogenic avian influenza (HPAI) virus which has emerged in the Asian and European countries in 2014 and was isolated from wild birds and poultry in China, Taiwan, Hungary, and Sweden [9]. The H5N8 outbreak was also reported in the Middle East in Israel, Iran, Iraq, and Kuwait [10]. The HPAI H5N8 virus was also detected in domestic ducks and geese in Egypt in 2017 [11], and later the virus was also reported in Riyadh Saudi Arabia [12].

Traditionally, influenza vaccines are generated by the creation of a natural re-assortant of circulating viral strain and egg-adapted influenza A/PR/8/1934 (H1N1, PR8), followed by high yield re-assortants, expressing at least hemagglutinin (HA) domain of the circulating strain [13]. However, this method is time-consuming and expensive; therefore, this classical approach was completely replaced with reverse-genetics technology. Reverse genetics technique is a powerful method for the development of inactivated and live-attenuated influenza vaccines. In this technique, the influenza vaccine strain expresses the internal proteins of PR8 and the immunogenic (HA) and neuraminidase (NA) antigens from the circulating strains [14]. Influenza (HA) is comprised of two subunits; (i) the constantly evolving globular head or HA1, which is encoding the antigenic and receptor binding sites, and the highly conserved stalk domain or HA2 [15].

In December 2017, poultry infection with high pathogenic avian influenza H5N8 virus were observed and recorded in Saudi Arabia. There are no available stockpile of protective vaccines against HPAI H5N8 virus that prevent virus shelding. Considering these outbreaks, there is a great need for the development of an efficient vaccine based on locally isolated strains/clades. Therefore, a pre-pandemic vaccine strain of matching antigenicity and high productivity is required. In the current study, a recombinant 6 + 2 vaccine strain based on reverse genetic technology of influenza viruses was engineered, expressing HA and NA from actively/recently circulating H5N8 in Saudi Arabia to combat against the diseases associated with H5N8 avian influenza virus.

EXPERIMENTAL

Sample collection

A total of 135 oropharyngeal swabs were collected from poultry (86 local chicken, 4 black Hershey, 5 ducks, 1 white hepshy,1 Holland chicken,2 Roman chicken, 3 pigeons, and 4 quails) at Central Sheep and Birds Market, Jeddah, Saudi Arabia. Swabs were carefully collected and stored in a medium containing an equal amount of glycerol and phosphate buffer saline (PBS). In addition, the medium was supplied with the antibiotics like streptomycin (200 mg/L), penicillin (2 x 10^6 U/L), and amphotericin B (250 mg/L).

Detection, isolation, and subtyping of virus

For detection and isolation of viral infection, 50 mL of PBS containing antibiotics mixture was inoculated with the viral inoculant. Seven days grown specific-pathogen-free (SPF) chicken eggs were purchased from the local market, and were incubated in an egg incubator at 37 ^{o}C for a week until an embryo develop, which was confirmed under direct light. The viral inoculum prepared in specific media was carefully propagated in the allantoic cavity. The inoculated SPF eggs were incubated for two days in an egg incubator at 37 ^{o}C following the guidelines recommended by WHO [16]. The eggs were tested by hemagglutination assay for viral infection using chicken red blood cells (0.5 %). Samples that showed positive signs of infection were preserved in a nitrogen tank for further study. Using commercial viral RNA kit purchased from Qiagen, the RNA extraction was carried out from samples with positive signs infection. The real-time PCR was run using primer specific to the M gene by following the protocol described by WHO [16]. Samples that were positive for M gene were again subtyped using universal primers designed for hemagglutinin and

neuraminidase [17,18]. Avian influenza H5N8 (A/chicken/KSA/1-NRC/2018), was used for the generation of the H5N8 vaccine strain. In brief, MDCK cells were grown in DMEM medium in a CO_2 incubator at 37 °C. Once the cells reached confluence of 80% of medium was discarded followed by washing with PBS. Further, cells were infected with avian influenza virus (AI H5N8) at a multiplicity of infection (MOI) 0.01 for 1 h at 37 ^{o}C followed by addition of growth medium. Infected cells were observed daily for cytopathic effect (CPE). The medium containing the virus supernatant was harvested from the cells. Cell debris was cleared off by centrifuging the sample at 4000 g for 30 min and viral supernatant was stored at -80 ^{o}C for further use.

Characterization of Saudi Arabia avian influenza A/H5N8 isolates

The assembled HA sequence was subjected to NCBI blast analysis. BioEdit 7.0 was used for multiple sequence alignment was done using BioEdit 7.0. The amino acid and nucleotide homologies were evaluated using DNASTAR. A phylogenetic tree was constructed using Mega 7.0. The trees included currently assembled H5N8 HA virus sequences; all related viruses to current isolate are available on GISAID and GenBank till 2018.

Virus RNA extraction and amplification of full-length hemagglutinin (HA) and neuraminidase (NA)

RNA extraction from the virus was done using RNeasy kit (Qiagen, USA), by following instructions recommended by the manufacturer. The extracted RNA was quantified using Nanodrop. The cDNA synthesis was done as described by Mostafa *et al* [13]. A total of 10 µL of isolated RNA was mixed with 2 µL of primer for the full-length amplification of monobasic NA or HA segments. These segments were then amplified again in a discrete reaction using universal primers and SuperScript III One-Step RT-PCR Platinum Taq High Fidelity. Then amplicons of expected sizes were subsequently gel-purified using QIAGEN gel extraction kit (Qiagen, USA). Finally, the PCR products were sequenced at Macrogen Sequencing Facility, Macrogen, South Korea.

Plasmid construction and cloning for mutated HA (monobasic) and NA in pHW2000 mammalian expression vector

The amplified two HA fragments (HA1 and HA2) and full-length NA gene were digested with BsmBI (NEB, MA, USA). In brief, 12 µL PCR product (HA1 and HA2), 10 µL 10x buffer 3, 74 µL H_2O and 2 µL BsmBI restriction enzyme were incubated at 55°C overnight. The digested product was run and confirmed on 1 % agarose gel. The pHW2000 vector was linearized with 2 µl BsmBI restriction enzyme incubated overnight at 37 °C. Transformation of the ligated product was carried out in TOP10 chemically competent bacteria (Invitrogen; Germany) as per manufacturer's instructions. The plasmid was isolated in recombinant colonies and the insert was checked with Sanger sequencing.

Transfection and rescue of the recombinant vaccine strain

The transfection was carried out using the following methods described previously [13]. The recombinant H5N8 vaccine strain was generated by encoding HA and NA segments of H5N8/KSA with 1 µg of pHW2000-plasmid expressing proteins of PR8-H1N1 transfected into a mixture of MDCK-II/293T cells (1:3). The transfection medium used consist of Opti MEM, approximately 8 µg of plasmid DNA and Trans-IT2020 were incubated at room temperature for about 45 min before use.

Chicken immunization

Twenty SPF chickens (2-weeks-old) were purchased and segregated into two groups, test group and intact control group (n = 10 per group). The chickens were subjected to blood collection for H5N8 antibody detection in serum. In test group, each chicken was injected with 500 µL of recombinant vaccine via intramuscular route into the right thigh on the 14th day.

The second group was treated as an intact control without any vaccination or treatment. Post-vaccination, a blood sample as collected weekly for a period of three weeks to confirm antibody titers against H5N8 using hemagglulination inhibition assay. All the chickens were monitored on daily basis for morbidity and mortality.

Hemagglutination assay

The HA assay was carried out based on the WHO recommendation [16]. A 50µl of PBS was added to a 96 well plate. Further, an equal volume of virus suspension was added to first well and serially two-fold diluted. Then, 0.5 % chicken RBCs of approximately 50 µL were added to each well, followed by light shaking and incubation at room temperature for 30 min. Finally, the plates were examined for HA titer.

Hemagglutination inhibition assay

The HAI titers of collected serum samples were estimated against H5N8 isolates. A total of 25 µL serum was treated with 75 µL of the receptor-destroying enzyme (RDE II, Denka Seiken, Japan) and incubated at 37 ^{o}C overnight, followed by heat inactivation at 56 ^{o}C for 30 min. The serum was mixed with 5 % packed chicken RBCs for 1 h and incubated at 4 ^{o}C. The RBCs were then removed, and two-fold serial dilutions of the serum were prepared in 1X PBS. An equal volume of antigen containing 4 HAU/25 µL was added to the serum sample followed by incubation for 30 min at room temperature. Following that, 50 µL of 0.5 % chicken RBCs were added to all wells and incubated for 30 min. The HAI titer was the reciprocal of the uppermost serum dilution fully inhibiting hemagglutination and was reported using a $\log_2$ scale.

Biosafety and ethics

Experiments using influenza viruses of low or high pathogenicity were carried out at biosafety level 2 and 3. The local authorities at National Research Center, Cairo, approved the protocols and safety measures. The animals were handled as per international, national and institutional guidelines. No human subjects participated in the study.

RESULTS

Selected viral isolates

A total of 135 swab samples were collected from different places in Saudi Arabia. However, only three samples showed positive results for the subtype HPAI H5N8 virus infection, and all three strains were found in local chicken samples. All other samples from black hepshy, ducks, white hepshy, Holland chicken, Roman chicken, pigeons, and quails were negative for HAPAI H5N8. Figure 1 shows the PCR amplification of AI/H5 and AI/N8 subtypes.

Genetic characteristics of avian influenza A/H5N8 isolates

To investigate the genetic phenotype of the influenza A/H5N8 isolated from Saudi Arabia in 2018, phylogenetic analysis depicting the HA sequence was categorized below clade 2.3.4.4B and was genetically distinct from the circulating H5N8 clade 2.3.4.4A in the neighbouring countries (e.g. Egypt). The phylogenetic analysis results showed that the A/H5N8 isolates from Saudi Arabia formed one distinctly separate group within the clade 2.3.4.4B, which essentially

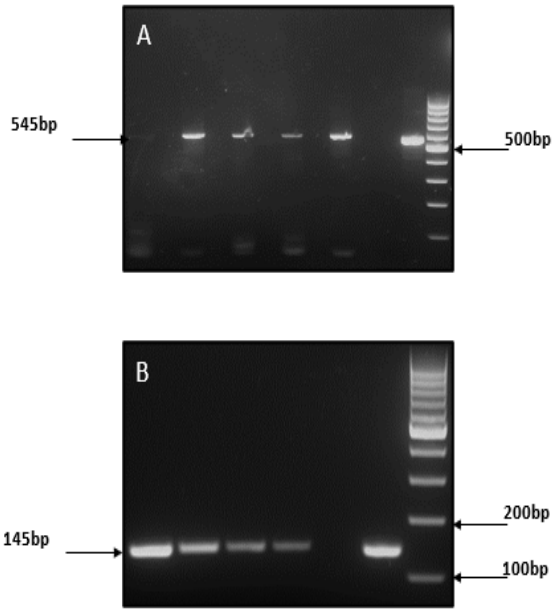

Figure 1: RT-PCR amplification for AI/H5 (545 bp) [A] and AI/N8 subtype (145 bp)

consist of isolates dominated from Africa or Europe. In addition to the isolates from Saudi Arabia, 2 isolates from Israel [A/grey goose/Israel/986/2016(H5N8) and A/turkey/ Israel/184/2017/(H5N8)], and 4 from China [(A/Cygnus_atratus/Hubei/2Z2-O/2016(H5N8); (A/Anser cygnoides/Hubei/FW44/2016 (H5N8); (A/Whooper Swan/Sanmenxia/01/2016 H5N8; and (A/Whooper Swan/Sanmenxia/01/2016 (H5N8)] were also grouped in this clade. These results reinforce the assortment between influenza viruses of Eurafrican and Asian lineages. In contrast, isolates clustered in clade 2.3.4.4A represent Eurasian linage as shown in Figure 2.

Immunization with rgH5N8/KSA elicits specific IgG titres against H5N8

The isolated H5N8 strain from Saudi Arabia (clade 2.3.4.4B) is genetically distinct from those circulating in neighbouring countries (e.g. Egypt; H5N8 clade 2.3.4.4A), a recombinant rgH5N8/KSA vaccine strain expressing the HA and NA proteins of A/chicken/KSA/1-NRC/2018 (H5N8) was generated. In order to assess the immunogenicity of the rgH5N8/KSA vaccine strain, SPF chicks were immunized. The HAI assay showed a significant increase in neutralizing antibody to H5N8 after the second week of immunization reflecting the high immunogenicity of the generated vaccine as shown in Figure 3.

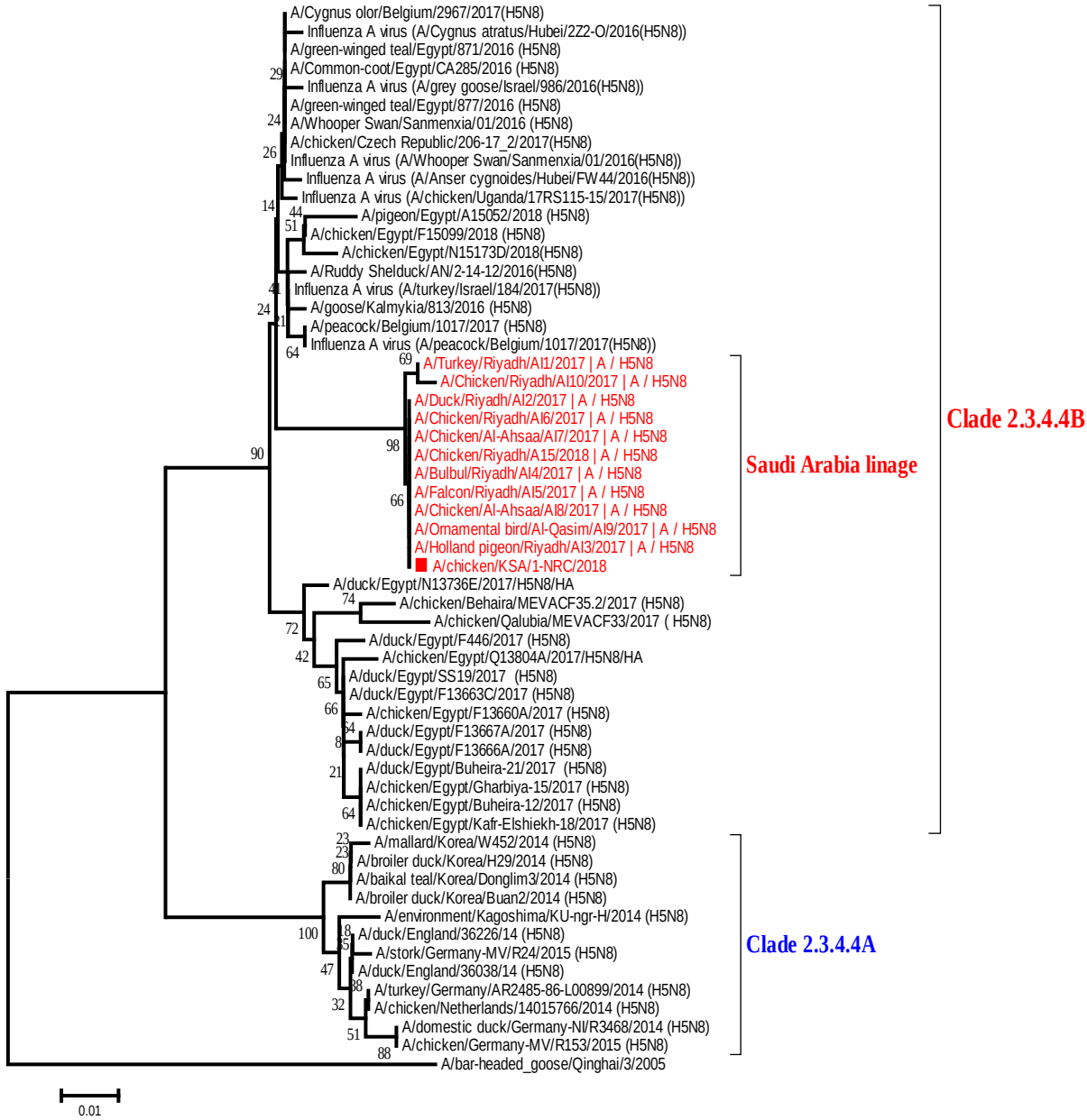

Figure 2: Phylogenetic analysis of the HA nucleotide sequence of Influenza A/H5N8 from 2014 to 2018. Saudi Arabia H5N8 HA sequence is represented with a quadrangle (■)

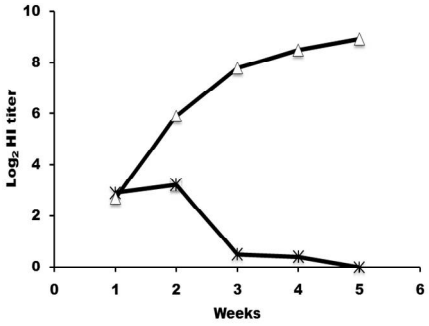

Figure 3: HAI titers of chicken immunized with rgH5N8/KSA influenza vaccine. Chickens were given intranasal administration of rgH5N8/KSA vaccine in combination with adjuvant on day 0 for 6 weeks and HAI titers were monitored weekly (Δ) rgH5N8/KSA, (×) intact control.

Immunized chickens with rgH5N8/KSA showed 100 % survival upon challenge with HPAI/H5N8

In order to investigate whether the induced antibody was protective against infection with highly pathogenic avian influenza (HPAI) H5N8 strains, immunized and control chickens (n = 3) were subjected in the 4th-week post-immunization to infection with HPAI H5N8 strain (A/chicken/KSA/1-NRC/2018 (H5N8)). Unlike the control group, all immunized chickens were protected and survived until the end of the experiment as shown in Figure 4.

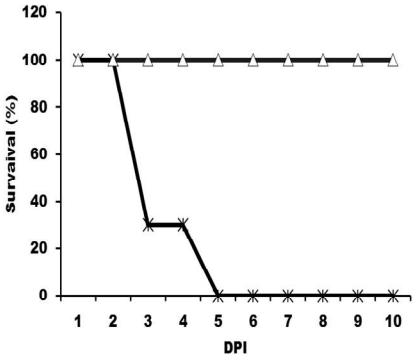

Figure 4: Efficacy of single dose vaccination against influenza A/chicken/KSA/1-NRC/2018 (H5N8) (∆) rgH5N8/KSA, (×) intact control

DISCUSSION

Highly pathogenic avian influenza (HPAI) H5N8 is a main cause for infectious in poultry, and is responsible for the loss of domestic poultry in Europe, Asia and Africa. In past, number of HPHAI H5N8 viral strains with different genetic characters have been reported from South Korea, China, Egypt, and France [19-22].

The avian influenza virus is capable of contaminating all types of avian species including poultry and domestic animals [23]. In Saudi Arabia, H5N8 (HPAI) virus was first reported on 19th December 2017 in a bird market in Riyadh, when dead birds were reported and diagnosed with positive signs for this subtype of H5N8 by the Department of Animal Resources Services, Ministry of Environment, Water, and Agriculture [12]. There was no vaccine available that would potentially prevent chickens from developing of virus shield. Therefore, to curb this epidemic, this study was carried out to investigate the genetic relationship of currently isolated H5N8 with other HPAI for the development of a candidate vaccine against avian influenza virus.

The H5 HA domain of the isolated (HPAI) H5N8 was sequenced and was phylogenetically analyzed with available reference sequences of H5N8. Phylogenetic analysis depicted that the H5 HA of this (HPAI) H5N8 isolate belong to clade 2.3.4.4B H5N8 viruses. The currently isolated strain H5N8 virus fell into a phylogenetic cluster together with Egyptian H5N8 isolates (group B, indicated in red in the phylogenetic. This confirms the previous reports that the H5N8 clade 2.3.4.4B is prevalent in Saudi Arabia [12].

The variability among influenza viruses is a major challenge for the development of effective vaccine against influenza. There are many techniques used for the development of a vaccine against this deadly disease and many new approaches are currently under investigation for the development of universal influenza vaccines. Reverse genetics systems is one of the techniques used in designing vaccines as it provides manipulation and study of RNA virus genomes [24].

In the current study, a reverse genetics technology-based approach was explored for the development of the vaccine against (HPAI) H5N8. Influenza A viruses were genetically engineered to a low pathogenic and highly growing locally isolated AIV H5N8 strain. A candidate vaccine strain was generated against H5N8 to be used for the development of an inactivated monovalent AI vaccine. The vaccine was evaluated for its immunogenicity and protective efficacy against HPAI H5N8 virus in chicken, which was recently isolated from Saudi Arabia in 2018. Interestingly, the results showed the high immunogenicity of the prepared H5N8 vaccine and its protective efficacy against the recently characterized H5N8 virus in Saudi Arabia. Thus, it could be postulated that the current vaccine developed may be the best candidate for anti-influenza A against HPAI H5N8 in Saudi Arabia and other affected countries.

CONCLUSION

The results showed the high immunogenicity of the prepared H5N8 vaccine and its protective efficacy against the recently characterized H5N8 avian influenza virus in Saudi Arabia. Thus, these findings postulate that the current vaccine developed in this study could be the best anti-influenza A candidate against HPAI H5N8 in Saudi Arabia and other countries affected with such avian influenza infections.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the

authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

REFERENCES

1. *Palese P, Shaw ML. Orthomyxoviridae: the viruses and their replication. In Knipe D, Howley P (Ed), Fields Virology, 5th edn. Lippincott Williams & Wilkins, Philadelphia. 2017; pp 1647–1690.*
2. *Long JS, Mistry B, Haslam SM, Barclay WS. Host and viral determinants of influenza A virus species specificity. Nat Rev Microbiol 2019; 17: 67–81.*
3. *Zhai SL, Zhang H, Chen SN, Zhou X, Lin T, Liu R, Lv DH, Wen XH, Wei WK, Wang D, Li F. Influenza D Virus in Animal Species in Guangdong Province, Southern China. Emerg Infect Dis 2017; 23: 1392-1396.*
4. *Mostafa A, Abdelwhab EM, Mettenleiter TC, Pleschka S. Zoonotic Potential of Influenza A Viruses. A Comprehensive Overview. Viruses 2018; 10: 497.*
5. *Bouvier NW, Palese P. The biology of influenza viruses. Vaccine 2008; 4: 49–53.*
6. *Krammer F, Gavin GJD, Fouchie RAM, Peiris M, Kedzierska K, Doherty PC, Palese P, Shaw ML, Treanor J, Webster RG, Sastre AG. Influenza. Nat Rev Dis Primers 2018; 4: 3.*
7. *Pleschka S. Overview of influenza viruses. Curr Top Microbiol Immunol 2013; 370: 1-20.*
8. *Ehrhardt C, Seyer R, Hrincius ER, Eierhoff T, Wolff T, Ludwig S. Interplay between influenza A virus and the innate immune signaling. Microbes Infect 2010; 12: 81-7.*
9. *World Health Organization. Assessment of risk associated with influenza A (H5N8) virus. (WHO, Geneva, 2016). Available at: http://www.who.int/influenza/human_animal_interface/avian_influenza/riskassessment_AH5N8_201611/en/ (accessed 17 April 2017).*
10. *World Organisation for Animal Health. Update on avian influenza in animals (types H5 and H7) [cited 2018 May 19]. http://www.oie.int/en/animal-health-in-the-world/update-on-avian-influenza/*
11. *Anis A, AboElkhair M, Ibrahim M. Characterization of highly pathogenic avian influenza H5N8 virus from Egyptian domestic waterfowl in 2017. Avian Pathol 2018; 47: 400-409.*
12. *Al-Ghadeer H, Chu DKW, Rihan EA, Abd-Allah EM, Gu H, Chin AWH, Qasim IA, Aldoweriej A, Alharbi SS, Al-Aqil MA. Circulation of Influenza A(H5N8) Virus, Saudi Arabia. Emerg Infect Dis 2018; 24: 10.*
13. *Mostafa A, Kanrai P, Petersen H, Ibrahim S, Rautenschlein S, Pleschka S. Efficient generation of recombinant influenza A viruses employing a new approach to overcome the genetic instability of HA segments. PLoS One 2015; 10: e0116917.*
14. *Mostafa A, Kanrai P, Ziebuhr J, Pleschka S. Improved dual promotor-driven reverse genetics system for influenza viruses. J Virol Methods 2013; 193: 603-610.*
15. *Mostafa A, Pleschka S. Influenza H3N2 Vaccines: Recent Challenges. Trends microbiol 2018; 26: 87-89.*
16. *WHO. Manual for the laboratory diagnosis and virological surveillance of influenza. 2011.*
17. *Lee MS, Chang PC, Shien JH, Cheng MC, Shieh HK. Identification and subtyping of avian influenza viruses by reverse transcription-PCR. J Virol Methods 2001; 97: 3-22.*
18. *Fereidouni SR, Starick E, Grund C, Globig A, Mettenleiter TC, Beer M, Harder T. Rapid molecular subtyping by reverse transcription polymerase chain reaction of the neuraminidase gene of avian influenza A viruses. Vet Microbiol 2009; 135: 253-260.*
19. *Kim SH, Hur M, Suh JH, Woo C, Wang SJ, Park ER, Hwang J, An IJ, Jo SD, Shin JW, et al. Molecular characterization of highly pathogenic avian influenza H5N8 viruses isolated from Baikal teals found dead during a 2014 outbreak in Korea. J Vet Sci 2016; 17: 299-306.*
20. *Ma L, Jin T, Wang H, Liu H, Wang R, Li Y, Yang G, Xiong Y, Chen J, Zhang J, et al. Two reassortant types of highly pathogenic H5N8 avian influenza virus from wild birds in Central China in 2016. Emerg Microbes Infect 2018; 7: 14.*
21. *Kandeil A, Kayed A, Moatasim Y, Webby RJ, McKenzie PP, Kayali G, Ali MA. Genetic characterization of highly pathogenic avian influenza A H5N8 viruses isolated from wild birds in Egypt. J GenVirol 2017; 98: 1573–1586.*
22. *Scoizec A, Niqueux E, Thomas R, Daniel P, Schmitz A, Bouquin SE. Airborne Detection of H5N8 Highly Pathogenic Avian Influenza Virus Genome in Poultry Farms, France. Front Vet Sci 2018; 5: 15.*
23. *Chatziprodromidou LP, Arvanitidou M, Guitian J, Apostolou T, Vantarakis V, Vantarakis A. Global avian influenza outbreaks 2010– 2016: a systematic review of their distribution, avian species and virus subtype. Systematic Rev 2018; 7: 17.*
24. *Nogales A, Martínez-Sobrido L. Reverse Genetics Approaches for the Development of Influenza Vaccines. Int J Mol 2017; 18: 20.*