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Molecular Characterization of Avian Influenza H9N2 Viruses Detected in Broiler Chickens in the Middle Region of Saudi Arabia

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ABSTRACT

Background: Since its first introduction in 1998 to Saudi Arabia (SA), Low pathogenic avian influenza virus (LPAIV) H9N2 has a widespread in poultry flocks in all regions inducing serious economic losses.

Methods: Thirteen broilers' farms from the middle region in SA of ages ranged between 11:23 days-old, suffering from respiratory distress, with variable mortality percentage were investigated. All tested flocks were vaccinated for (AIV) H9N2, Newcastle disease virus (NDV), and Infectious bronchitis virus (IBV). All flocks were screened for the presence of AIV (H9, H5 and H7) subtypes, NDV and IBV using real time RT-PCR (rRT-PCR) followed by complete hemagglutinin (HA) and neuraminidase (NA) sequencing by next generation sequencing (NGS) for one selected sample.

Results: Eleven out of the thirteen tested flocks were positive for AIV H9N2 and NDV detection, while all the flocks were negative for H5 and H7 AIVs. Only four flocks were positive for IBV. Sequence analysis of the HA gene revealed cleavage site motif sequences similar to low pathogenic AIV. The aa identity percentage of HA gene was high between the current study strain in 2022 and the SA H9N2 2020 strain (99.4%), however it was about 92%, and 91% with the SA strains in 1998 and 2002 respectively. The aa identity percentage of NA gene was about 90% and 87% when compared with the early SA H9N2 strains in 1998 and 2006 respectively with altered glycosylation patterns. The NA protein harbors five amino acids substitutions in the hemadsorping (HB) and frame active sites (K368T, S370L, D401N, R403W and Q432R). These 5 substitutions were not detected in older published data from SA till 2006.

Conclusions: The current study highlights the severe endemic situation of H9N2 virus in poultry flocks in SA with continuous evolution. Multiple aa mutations reported in

recently circulating SA H9N2 viruses might alter virus antigenicity, increase the virus virulence and increase receptor affinity toward human receptor with increased human infection possibility.

Keywords: H9N2, LPAIV, Saudi Arabia, Broilers.

INTRODUCTION

In 1966, the low pathogenic avian influenza virus (LPAIV) H9N2 was initially reported at a turkey farm in the United States. (Homme and Easterday, 1970), since then, the disease was reported in many countries around the world. Between 1992 and 1994, there were reports of H9N2 AIV in China, primarily affecting poultry farms (Chen et al., 1994), and it was reported in South Korea in 1996 (Lee et al., 2016). From 1998 the virus spread to several countries in the Middle East and North Africa like Iran (Nili and Asasi, 2003), Saudi Arabia (Monne et al., 2008), Jordan (Roussan et al., 2008), the United Arab Emirates (Wernery et al., 2013), Egypt (Kandeil et al., 2014), Morrocco (El Houadfi et al., 2016), and Algeria (Jeevan et al., 2019).

Influenza viruses are enveloped RNA viruses which are members of the Orthomyxoviridae family which is considered relatively small viruses. The genomic RNA is negative sense single stranded. Till now, Orthomyxoviridae family contains five genera: Influenza virus A, B, C, Thogovirus and Isavirus (Hause et al., 2014).

At least ten viral proteins, including hemagglutinin (HA), neuraminidase (NA), polymerase acidic protein (PA), polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2), matrix proteins (M1 and M2), nucleoprotein (NP), and non-structural proteins (NS1 and NS2 or the nuclear export protein [NEP]), are encoded by its segmented genome, which is made up of eight ssRNA segments (Mostafa et al., 2018). Influenza A viruses (IAVs) can evolve quickly due to the RNA polymerase high error rate, strong immune-driven natural selection, and the ability of genome segments to be exchanged by reassortment **(**Webster et al., 1992**).**

Infection with LPAI H9N2 virus caused significant economic losses in poultry industries due to increased mortalities and reduced egg production (Lee and Song, 2013). The H9N2 LPAI viruses occasionally produced clinical respiratory disorders in humans and frequently caused severe clinical symptoms with moderate mortality in domestic poultry in several Eurasian nations, although the basis for their pathogenesis remains uncertain particularly in chickens (Kwon et al., 2008). Despite the virus's moderate pathogenicity for ducks, quail, and turkeys, it did exhibit variations in seroconversion, shedding patterns, and clinical outcomes (Świętoń et al., 2020). Significant economic losses could arise from complications with additional bacterial or viral illnesses (Huang et al., 2017).

Six genetically different clades of H9N2 LPAIV have been identified in Eurasia by phylogenetic and antigenic analyses: the G1-like, Y280-like, G9-like, BJ94-like, Y439-like, and Korean-like clades (Xu et al., 2007). The subclades A, B, C, and D make up the G1-like clade; interestingly, groups A and B are highly prevalent in the Middle East. (Fusaro et al., 2011).

In 2024, a recent classification suggestion for AIV H9 divided it into three primary lineages (B, G, and Y). The G lineage, formerly known as G1 or h9.4.1, the B lineage, formerly known as BJ/94, Y280, G9, or h9.4.2, and the Y lineage, which includes the formerly known Y439 (or

Korean, h9.3) and American (or h9.1–h9.2) lineages (Fusaro et al., 2024).

In 1998, AIV H9N2 was firstly isolated from Saudi Arabian chickens (Alexander, 2003), also it was isolated from chickens in 1999 (Banks, 2000), and in 2003–2005 (Alexander, 2007). Molecular and serological methods used to detect AIV H9N2 in several poultry species in 2006–2007 showed that the H9N2 AI virus was widely distributed, primarily in the northern regions (Alkhalaf, 2010).

In the current study, epidemiological surveillance of common viral respiratory pathogens in Saudi Arabia during 2022 was performed, in addition to genetic analysis of the full genome sequence of H9N2 AIV.

MATERIALS AND METHODS

Ethical approval:

Ethical approval for this study was given by the Institutional Animal Care and Use Committee (IACUC), Faculty of Veterinary Medicine, University of Sadat City, Egypt under the No. VUSC – 057-1-22.

Sampling

Thirteen broiler chicken farms suspected to be infected with H9N2 AIV were sampled from Saudi Arabia (Middle region) during 2022. All sampled farms showed clinical signs of respiratory distress with medium to high mortalities. Tracheal swabs from 5 birds/flock were scraped on FTA card to be sent to laboratory for molecular detection. Samples processing and testing were performed in the central laboratory of diagnosis of viruses, Faculty of Veterinary Medicine, University of Sadat City.

History of examined farms

Thirteen broiler farms in the middle region of Saudi Arabia during the period from October to December 2022 were examined. Different broiler ages from 11-day-old to 23-day-old were sampled showing respiratory signs with medium to high mortality rates. All tested flocks were vaccinated with killed vaccines for H9N2 and NDV plus 2 vaccinations with live attenuated vaccines of NDV and IBV at 1st and 12-14-day-old., the history of these sampled broiler farms is shown in Table 1.

Table 1. History of the sampled broiler farms.

Farm* No	Sample Lab ID	Age at sampling (days)	Sampling date	Respiratory distress	Mortality
1	A1	18	10-2022	Yes	High
2	A2	20	10-2022	Yes	High
3	A3	21	10-2022	Yes	High
4	A4	23	11-2022	Yes	High
5	A5	22	11-2022	Yes	High
6	A6	22	10-2022	Yes	High
7	A7	22	10-2022	Yes	High
8	A8	22	10-2022	Yes	High
9	A9	22	12-2022	Yes	High
10	S1	11	10-2022	Yes	Medium
11	S2	12	11-2022	Yes	Medium
12	S3	11	12-2022	Yes	Medium
13	S4	15	12-2022	Yes	Medium

*All farms vaccinated at 0 day-old with inactivated H9N2 vaccine. IBV and NDV was vaccinated at 0-day-old and a second vaccination given at 12-14 day-old with live attenuated and lentogenic strains, respectively.

Molecular detection of the causative agent(s) by real time RT-PCR

Total viral RNA was extracted from FTA card suspensions using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Real time reverse transcriptase polymerase chain reaction (rRT-PCR) was performed using Thermo Scientific superscript III platinum One-Step qRT-PCR kit (Invitrogen, USA) with gene specific primers for detection of influenza A virus, AI H9 virus, AI H5 Virus, AI H7 virus, Newcastle disease virus (NDV), and infectious bronchitis virus (IBV) as shown in the following table.

Table (2): Primers used in the current study:

Virus	Target gene	Forward primer	Reverse primer	Probe	References
AIV (A)	Matrix (M)	AGATGAGTCT TCTAA CCG AGG TCG	TGCAAAAAC ATC TTCAAG TYT CTG	FAM-TCA GGC CCC CTC AAA GCC GA-BHQ1	Spackman et al., 2002
AIV (H9)	HA	GGAAGAATT AATTATTATT GGTCGGTAC	GCCACCTTT TTCAGTCTG ACATT	[FAM]AACCAGGCCAGACATTGCG AGTAAGATCC[TAMRA]	Ben Shabat et al., 2010
AIV (H5)	HA	ACATATGACT AC CCACAR TATTCA G	AGA CCA GCT AYC ATG ATT GC	FAM-TCWACA GTGGCGAGTTCC CTAGCA - BHQ1	Lòndt et al., 2008
AIV (H7)	HA	TTTGGTTTAG CTTCGGG	ACATGATGC CCCGAAGCT AAAC	FAM- CATCATGTTTCATACTT CTGGCCAT-BHQ	Monne et al., 2008
NDV	Matrix (M)	AGTGATGTGC TCGGACCTTC	CCTGAGGAG AGGCATTTG CTA	[FAM]TTCTCTAGCAGTGGGACAG CCTGC[TAMRA]	Wise et al. 2004
IBV	Untranslated region UTR	ATGCTCAACC TTGTCCCTAG CA	TCAAACTGC GGATCATCA CGT	FAM-TTGGAAGTAGAGTGACGCCCAAA CTTCA-BHQ	Callison et al., 2001

Table (3): The thermal profile used in rRT-PCR using one step qRT-PCR kit shown in the following table.

Virus	RT (reverse transcription	Initial denaturation	PCR *		
			Denaturation	Primer annealing	Extension
AIV (A)	50 ^{0}C / 15 mi	95 ^{0}C / 2 mi	95 ^{0}C / 30 sec	52.5 ^{0}C / 30 sec.	72 ^{0}C / 30 se
AIV (H9)	50 ^{0}C / 15 mi	95 ^{0}C / 2 mi	95 ^{0}C / 30 se	48 ^{0}C / 30 se	72 ^{0}C / 30 se
AIV (H5)	50 ^{0}C / 15 mi	95 ^{0}C / 2 mi	95 ^{0}C / 30 se	52 ^{0}C / 30 se	72 ^{0}C / 30 se
AIV (H7)	50 ^{0}C / 15 mi	95 ^{0}C / 2 mi	95 ^{0}C / 30 se	52 ^{0}C / 30 se	72 ^{0}C / 30 se
NDV (M)	50 ^{0}C / 15 mi	95 ^{0}C / 2 mi	95 ^{0}C / 30 se	54 ^{0}C / 30 se	72 ^{0}C / 30 se
IBV	50 ^{0}C / 15 mi	95 ^{0}C / 2 mi	95 ^{0}C / 30 se	46 ^{0}C / 30 se	72 ^{0}C / 30 se

*The number of PCR cycles was 45 cycles for all viruses.

Genomic sequencing

The extracted RNA for one selected sample was sent to Ceva laboratory (Phylaxia, Hungary) for next generation sequencing (NGS) for full HA and NA genes sequencing.

Sequence analysis

Sequence assembly and editing were performed using Bioedit® software package version 7.7.1.0 (Hall, 1999). Confirmation of identity and homology were performed using BLAST http://www.ncbi.nlm.nih.gov. To determine the molecular epidemiological relationships of our AIV H9N2 field strain, some AIV H9N2 reference strains isolated from Middle East region at different time periods were obtained from GenBank. These reference strains were representative of all the different AIV H9N2 strains. Phylogenetic and molecular evolutionary analysis were conducted using MEGA version 10 (Kumar et al. 2018). Phylogenetic trees of all genes based on amino acid sequences were constructed by the Neighbor-Joining method with the Jones-Taylor-Thornton model at 1000 bootstrap replicates.

RESULTS

Molecular identification of different respiratory agents by rRT-PCR

Eleven out of thirteen examined flocks were positive for AIV H9N2 and NDV while all examined flocks were negative for AIV H5 and H7 by using gene specific primers and probes. Four flocks (A3, A5, A8 and S1) were positive for IBV while other 7 flocks were IBV negative, as shown in Table 4.

Table 4. Results of rRT-PCR (Ct values) for different respiratory viruses.

Sample ID	Ct values for different tested viruses*					
	AIV (A)	H9	H5	H7	NDV	IBV
A1	19	17	- ve	- ve	35	- ve
A2	20	17	- ve	- ve	36	- ve
A3	19	18	- ve	- ve	34	36
A4	18	17	- ve	- ve	36	- ve
A5	28	29	- ve	- ve	36	33
A6	19	17	- ve	- ve	35	- ve
A7	18	16	- ve	- ve	35	- ve
A8	32	33	- ve	- ve	21	36
A9	22	20	- ve	- ve	36	- ve
S1	33	35	- ve	- ve	36	24
S2	- ve	- ve	- ve	- ve	- ve	- ve
S3	34	36	- ve	- ve	21	- ve
S4	- ve	- ve	- ve	- ve	- ve	- ve

*Negative result by real time RT-PCR (ct value more than 40) was indicated as (-ve).

Genetic analysis of HA gene of H9N2 AIV strains:

Full HA and NA Genes sequencing of the selected sample was successfully done by NGS. The cleavage site motif sequences of the HA of the current Saudi Arabian strain was HARSSR/GLF which have been associated with low pathogenicity as shown in Table 5. Analysis of the aa sequences at the HA cleavage sites as compared with the G1 ancestor (A/Quail/Hong Kong/G1/97) reveals the substitution (P333H) which was reported in Saudi Arabia H9N2 strains since 2020 and remained in 2022 strain sequenced in this study.

Table 5. Amino acid residues detected within cleavage sites compared to the ancestral G1/97 H9N2 strain.

H9N2 strains	**Cleavage sites**								
	333	**334**	**335**	**336**	**337**	**338**	**339**	**340**	**341**
A/Quail/Hong Kong/G1/97	P	A	R	S	S	R	G	L	F
A/chicken/Saudi Arabia/CP7/1998	-	-	-	-	-	-	-	-	-
A/chicken/Saudi Arabia/EPD-22-01/2002	-	-	-	-	-	-	-	-	-
A/chicken/Saudi Arabia/ASH-98.3/2020	H	-	-	-	-	-	-	-	-
A/Chicken/SA/A4/2022(H9N2)	H	-	-	-	-	-	-	-	-
A/chicken/Egypt/114940v/2011(Vaccine)	-	-	-	-	-	-	-	-	-
A/chicken/Egypt/A-chicken-1/2017	-	-	-	-	-	-	-	-	-
A/chicken/Dubai/D2506.A/2015	H	-	-	-	-	-	-	-	-
A/chicken/Iran/av1221/1998 (Vaccine)	-	-	-	-	-	-	-	-	-
A/Chicken/Shanghai/F/98(Vaccine)	-	-	-	-	-	-	-	-	-
A/chicken/Jordan/MQA-N-6/2021	H	-	-	-	-	-	-	-	-
A/chicken/Tunisia/H9_20-057/2020	H	-	-	-	-	-	-	-	-
A/chicken/Korea/01310_CE20/2001 (Vaccin	-	-	T	-	G	-	-	-	-

Analysis of the aa residues at the antigenic sites in our strain reveals aa substitutions from the G1/97 ancestral strain (A/Quail/Hong Kong/G1/97), as shown in Table 6. Four aa substitutions (G153D, S158N, E198A and N206T) which were reported previously in older S.A strains. Analysis of the aa residues at the receptor binding sites reveals aa substitution at the position 198 (E198A). Three aa substitutions were reported in the right edge: I147T, R149k and A150S. Other substitutions in the left edge presented as D233G, Q235I and Q234L, all these substitutions were previously reported in older S.A H9N2 strains as shown in Table 7.

Table 6. Amino acid residues detected within known antigenic sites compared to the ancestral G1/97 H9N2 strain.

H9N2 strains	Antigenic sites													
	143	165	170	153	201	234	14	197	206	58	93	158	182	198
A/Quail/Hong Kong/G1/97	T	K	P	G	N	L	T	T	N	M	E	S	K	E
A/chicken/Saudi Arabia/CP7/1998	-	-	-	-	S	Q	-	-	T	-	-	-	-	-
A/chicken/Saudi Arabia/EPD-22-01/2002	-	-	-	D	-	-	-	-	T	-	-	N	-	A
A/chicken/Saudi Arabia/ASH-98.3/2020	-	-	-	D	-	-	-	-	T	-	-	N	-	V
A/Chicken/SA/A4/2022(H9N2)	-	-	-	D	-	-	-	-	T	-	-	N	-	A
A/chicken/Egypt/114940v/2011(Vaccine)	-	-	-	D	-	Q	-	-	T	-	-	N	-	A
A/chicken/Egypt/A-chicken-1/2017	-	-	-	D	-	-	-	-	T	-	-	N	-	A
A/chicken/Dubai/D2506.A/2015	-	-	-	D	-	-	-	-	T	-	-	N	-	A
A/chicken/Iran/av1221/1998 (Vaccine)	-	-	-	-	-	Q	-	-	-	-	-	-	-	A
A/Chicken/Shanghai/F/98(Vaccine)	S	-	-	D	-	Q	-	-	T	-	K	-	-	A
A/chicken/Jordan/MQA-N-6/2021	-	-	-	D	-	-	-	-	T	-	-	N	-	A
A/chicken/Tunisia/H9_20-057/2020	-	-	-	D	-	-	-	-	A	-	-	N	-	A
A/chicken/Korea/01310-CE20/2001 (Vaccine)	-	-	-	-	-	Q	G	-	A	-	-	-	-	-

Table 7. Amino acid residues detected within known receptor binding sites compared to the ancestral G1/97 H9N2 strain.

H9N2 strains	**receptor binding sites**																		
	Binding sites							Right edge					Left edge						
	110	161	163	191	198	202	203	146-150					232-237						
A/Quail/Hong Kong/G1/97	P	W	T	H	E	L	Y	G	I	S	R	A	N	D	L	Q	G	R	
A/chicken/Saudi Arabia/CP7/1998	-	-	-	-	-	-	-	-	T	-	K	-	-	G	Q	-	-	-	
A/chicken/Saudi Arabia/EPD-22-01/2002	-	-	-	-	A	-	-	-	T	-	K	-	-	G	-	-	-	-	
A/chicken/Saudi Arabia/ASH-98.3/2020	-	-	-	-	V	-	-	-	T	-	K	S	-	G	-	I	-	-	
A/Chicken/SA/A4/2022(H9N2)	-	-	-	-	A	-	-	-	T	-	K	S	-	G	-	I	-	-	
A/chicken/Egypt/114940v/2011(Vaccine)	-	-	-	-	A	-	-	-	T	-	K	S	-	G	Q	I	-	-	
A/chicken/Egypt/A-chicken-1/2017	-	-	-	-	A	-	-	-	T	-	K	S	-	G	-	I	-	-	
A/chicken/Dubai/D2506.A/2015	-	-	-	-	A	-	-	-	T	-	K	S	-	G	-	I	-	-	
A/chicken/Iran/av1221/1998 (Vaccine)	-	-	-	-	A	-	-	-	T	-	K	-	-	G	Q	-	-	-	
A/Chicken/Shanghai/F/98(	-	-	-	N	A	-	-	-	T	-	K	-	-	G	Q	-	-	-	

Vaccine)																					
A/chicken/Jordan/MQA-N-6/2021	-	-	-	-	A	-	-	-	T	-	K	S	-	G	-	I	-	-
A/chicken/Tunisia/H9_20-057/2020	-	-	-	-	A	-	-	-	T	-	K	S	-	G	-	I	-	-
A/chicken/Korea/01310-CE20/2001 (Vaccine)	-	-	-	-	-	-	-	-	T	-	K	-	-	G	Q	-	-	-

The identity percentage of HA gene based on aa sequences between the current study strain and the first S.A strain in 1998 was 92.1%, and it was 91.6% with the S.A strain in 2002, while it was above 99% with the S.A strain 2020 (Table 8).

Table 8. Amino acid identity percent of the obtained sequences of hemagglutinin gene from Broilers and representative global H9N2 strains and the commercial vaccinal strains.

	Strain	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1	**A/Qu/H K/G1/97**	ID	**96.4**	**95.3**	**90.5**	**90.5**	**91.4**	**96.6**	**90.5**	**90.3**	**85.3**
2	**A/Ck/S.A/CP7/98**	**96.4**	ID	**96.6**	**92.1**	**92.1**	**93.6**	**98.6**	**92.3**	**92.1**	**86.6**
3	**A/Ck/S.A/EPD-22-01/200**	**95.3**	**96.6**	ID	**91.4**	**91.6**	**92.9**	**96.8**	**92.0**	**91.6**	**85.5**
4	**A/Ck/S.A/ASH-98.3/2020**	**90.5**	**92.1**	**91.4**	ID	**99.4**	**94.2**	**91.6**	**88.8**	**97.9**	**83.4**
5	A/Ck/SA/A4/2022(H9N2)	**90.5**	**92.1**	**91.6**	**99.4**	ID	**94.4**	**91.8**	**88.8**	**97.7**	**83.2**
6	**A/ck/Egy/114940v/2011 (V)**	**91.4**	**93.6**	**92.9**	**94.2**	**94.4**	ID	**93.3**	**89.9**	**94.6**	**84.2**
7	**A/Ck/Iran/av1221/98(V)**	**96.6**	**98.6**	**96.8**	**91.6**	**91.8**	**93.3**	ID	**92.1**	**91.8**	**86.4**
8	**A/Ck/Shanghai/F/98(V)**	**90.5**	**92.3**	**92.0**	**88.8**	**88.8**	**89.9**	**92.1**	ID	**89.0**	**88.1**
9	**A/Ck/Jordan/MQA/2021**	**90.3**	**92.1**	**91.6**	**97.9**	**97.7**	**94.6**	**91.8**	**89.0**	ID	**83.8**
10	**A/Ck/Korea/CE20/2001(**	**85.3**	**86.6**	**85.5**	**83.4**	**83.2**	**84.2**	**86.4**	**88.1**	**83.8**	ID

Analysis of the potential glycosylation sites (PGS) of the HA gene of the current study strain (Table 9) reveals that this strain harbors seven PGS, which are identical to the PGS of the HA gene of SA strains (2002 and 2020). However, the SA strain 1998 contains the same PGS of the HA gene with additional site 166-168 (NGS).

Table 9. Amino acid residues detected within known neuraminidase active sites and frame work sites compared to the ancestral G1/97 H9N2 strain.

H9N2 strains	**Neuraminidase AA residues**																			
	Neuraminidase active – hemadsorping (HI sites																**Framework sites**			
	366-373									**399-406**								**431-43**		**425**	
A/quail/Hong Kong/G1/1997	**I**	**K**	**K**	**D**	**S**	**R**	**S**	**G**		**I**	**S**	**D**	**I**	**R**	**S**	**G**	**S**	**P**	**Q**	**E**	**E**
A/Duck/Hong Kong/Y280/97	-	-	**E**	-	-	-	-	-		-	-	-	N	**W**	-	-	Y	-	-	-	-
A/Duck/Hong Kong/Y439/97	-	S	-	-	-	-	-	-		-	N	N	N	**W**	-	-	Y	-	-	-	-
A/turkey/Wisconsin/1/1966	-	S	-	-	-	-	-	-		-	-	N	N	**W**	-	-	Y	-	-	-	-
A/chicken/Hong Kong/G9/19	-	-	-	-	-	-	-	-		-	-	-	N	**S**	-	-	Y	-	K	-	-
A/chicken/Saudi Arabia/CP7/1998	-	-	-	-	-	-	**A**	-		-	-	-	N	**L**	-	-	Y	-	-	-	-

Genetic analysis of NA gene of H9N2 AIV strains

Analysis of aa residues at neuraminidase active sites and framework sites reveals the presence of five aa substitutions (K368T, S370L, D401N, R403W and Q432R) which were reported in the current study strain and not detected in older S.A strains while they were reported in some neighboring countries strains as shown in table 10.

Table (10): Analysis of aa residues at neuraminidase active sites and framework sites

A/Chicken/Saudi Arabia/532/99	-	-	-	-	-	-	**A**	-		-	-	-	N	**L**	-	-	Y	-	-	-	-
A/avian/Saudi Arabia/910134/2006	-	-	**E**	**E**	-	-	**A**	-		-	-	-	N	**L**	-	-	Y	-	-	-	-
A/Chicken/SA/A4/2022(H9N2)	-	-	**T**	-	**L**	-	**A**	-		-	-	**N**	N	**W**	-	-	Y	-	**R**	-	-
A/chicken/Iran/av1221/1998(H9N2))(V)	-	-	-	-	-	-	**A**	-		-	-	-	N	**L**	-	-	Y	-	-	-	-
A/chicken/Egy/114940v/2011(H9N2)(V)	-	-	-	-	-	-	**A**	-		-	-	-	S	**W**	-	-	Y	-	-	-	-
A/chicken/Egypt/N14782D/2017(H9N2))	-	-	-	-	-	-	**A**	-		-	-	-	S	**W**	-	-	Y	-	-	-	-
A/chicken/Emirates/R66/2002	-	-	**E**	-	**L**	-	**A**	-		-	-	-	N	**W**	-	-	Y	-	-	-	-
A/pheasant/UAE/D1521/2011	-	-	R	-	**L**	-	**A**	-		-	-	-	N	**W**	-	-	Y	-	**R**	-	-
A/chicken/Dubai/D2506.A/2015	-	-	-	-	**L**	-	**A**	-		-	-	-	N	**W**	-	-	Y	-	**R**	-	-
A/chicken/Algeria/225/2017(	-	-	-	-	**L**	-	**A**	-		-	N	-	N	**W**	-	-	Y	-	**R**	-	-

H9N2))																				
A/chicken/Morocco/AS77/2019	-	-	-	-	**L**	-	**A**	-	-	-	-	N	**W**	-	-	Y	-	**R**	-	-
A/chicken/Uganda/MUWRP-200169/2017	-	-	**R**	-	**L**	-	**A**	-	-	-	-	N	**W**	-	-	Y	-	**R**	-	-
A/chicken/Jordan/88/2005	-	-	-	-	-	-	**A**	-	-	-	-	N	**L**	-	-	Y	-	-	-	-
A/quail/Lebanon/272/2010	-	-	-	-	-	-	**A**	-	-	-	-	N	**W**	-	-	Y	-	-	-	-

The aa identity percent of NA gene of our strain was 90.5 % and 87.3 % when compared with S.A strains in 1998 and 2006, respectively (Table 11).

Table 11. Amino acid identity percent of the obtained sequences of Neuraminidase gene from Broilers and representative global H9N2 strains and the commercial vaccinal strains.

	Strain	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
1	**A/Qu/H K/G1/97**	ID	**94.5**	**97.3**	**92.3**	**89.2**	**96.7**	**92.1**	**89.9**	**90.8**	**89.7**	**92.3**
2	**A/Duck/H K/Y280/97**	**94.5**	ID	**95.1**	**91.6**	**88.8**	**94.5**	**90.8**	**89.0**	**89.4**	**88.4**	**90.8**
3	**A/ck/S.A/CP7/1998**	**97.3**	**95.1**	ID	**93.6**	**90.5**	**99.3**	**92.9**	**90.8**	**91.9**	**90.8**	**92.9**
4	**A/avi/S.A/910134/2006**	**92.3**	**91.6**	**93.6**	I	**87.3**	**92.9**	**90.5**	**88.6**	**88.1**	**87.5**	**90.3**
5	**A/Chicken/S.A/A4/2022**	**89.2**	**88.8**	**90.5**	**87.3**	I	**90.1**	**86.4**	**85.7**	**96.4**	**95.6**	**85.9**
6	**A/ck/Iran/av1221/98(V)**	**96.7**	**94.5**	**99.3**	**92.9**	**90.1**	ID	**92.3**	**90.1**	**91.4**	**90.3**	**92.3**
7	**A/ck/Egy/114940v/2011(**	**92.1**	**90.8**	**92.9**	**90.5**	**86.4**	**92.3**	ID	**96.4**	**88.4**	**87.3**	**97.3**
8	**A/ck/Egy/N14782D/2017**	**89.9**	**89.0**	**90.8**	**88.6**	**85.7**	**90.1**	**96.4**	ID	**87.7**	**86.6**	**94.7**
9	**A/ck/Dubai/D2506.A/201**	**90.8**	**89.4**	**91.9**	**88.1**	**96.4**	**91.4**	**88.4**	**87.7**	ID	**98.9**	**88.1**
10	**A/ck/Moroc/AS77/2019**	**89.7**	**88.4**	**90.8**	**87.5**	**95.6**	**90.3**	**87.3**	**86.6**	**98.9**	ID	**87.0**
11	**A/qu/Lebanon/272/2010**	**92.3**	**90.8**	**92.9**	**90.3**	**85.9**	**92.3**	**97.3**	**94.7**	**88.1**	**87.0**	ID

Analysis of the PGS of the NA gene of the current study strain showed altered glycosylation patterns with additional sites and loss of some sites when compared to older SA strains, which indicate continuous evolution.

Table 12. Potential glycosylation sites detected within the HA gene of the current study strains in comparison with G1/1997 strain and old strains of saudi Arabia.

strain	29	105	141	166	206	218	298	305	492	551	Total N
A/Qu/HK/G1/199	NST	NGT	NVT		NDT	NRT	NST	NIS	NGT		8
A/Ck/SA/CP7/199	NST	NGT	NVT	NGS			NST	NIS	NGT	NGS	8
A/Ck/SA/EPD-22/2002	NST	NGT	NVT				NST	NIS	NGT	NGS	7
A/Ck/SA/ASH/20	NST	NGT	NVT				NST	NIS	NGT	NGS	7
A/Ck/SA/A4/2022	NST	NGT	NVT				NST	NIS	NGT	NGS	7

Table 13. Potential glycosylation sites detected within the NA gene of the current study strains in comparison with G1/1997 strain and old strains of saudi Arabia.

strain	44	48	61	69	70	86	146	200	234	329	402	Total No
A/Qu/HK/G1/1997			NIT	NNT	NTT	NWS	NGT	NAT	NGT		NRS	8
A/Ck/SA/CP7/1998			NIT	NNT	NTT	NWS	NGT	NAT	NGT		NLS	8
A/Ck/SA/532/1999			NIT	NNT	NTT	NWS	NGT	NAT	NGT		NLS	8
A/Ck/SA/910134/20		NHT	NVT	NNT		NWS	NGT	NAT	NGT		NLS	8
A/Ck/SA/A4/2022	NLS		NKT		NIT		NGT	NAT	NGT	NDS	NWS	8

Phylogenetic analysis

The phylogenetic trees based on HA and NA gene sequences showed that H9N2 strain sequenced in this study was clustered with recently isolated H9N2 strains from middle east countries but the earlier detected H9N2 strains (1998-2005) from SA and middle east countries were closely clustered with each other and G1 ancestral strain which indicates significant evolution of H9N2 viruses in SA and middle east (Fig. 1 & 2).

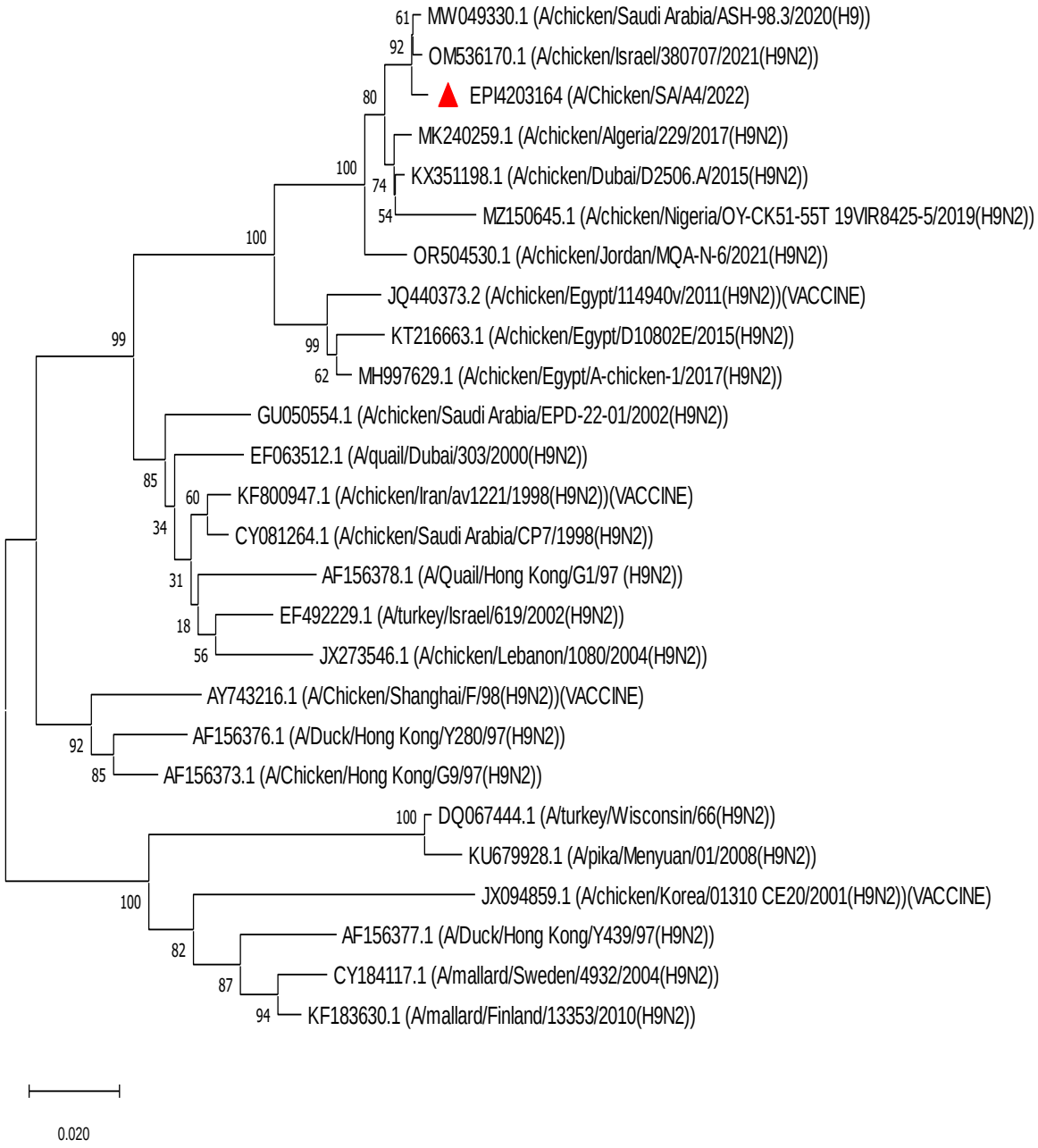

Figure 1. Phylogenetic tree based on aa sequence of full HA gene from G1 lineage. The tree was generated by the Neighbor-Joining method with the Jones-Taylor-Thornton model at 1000 bootstrap replicates with the MEGA 10 program.

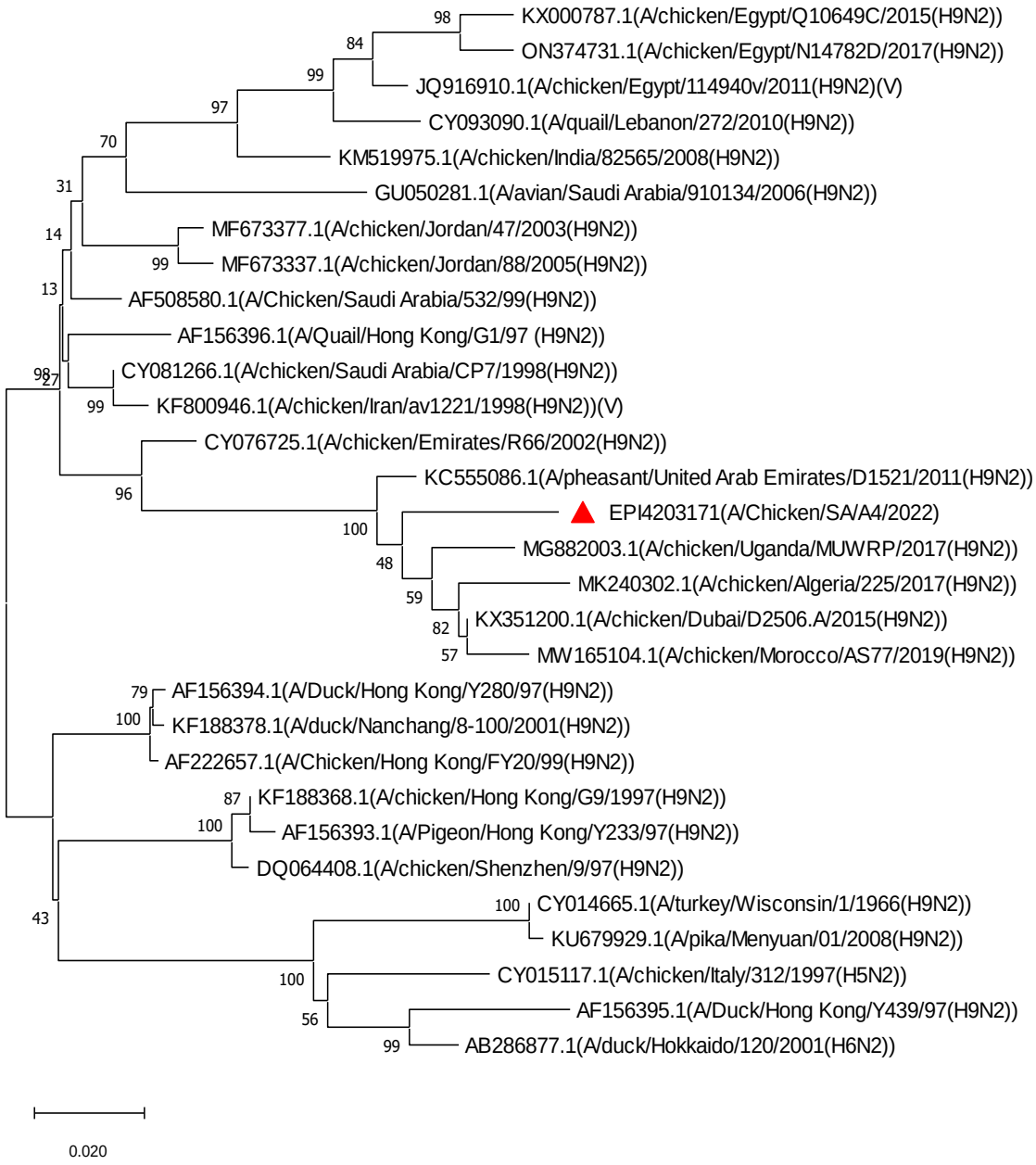

Figure 2. Phylogenetic tree based on aa sequence of full NA gene from G1 lineage The tree was generated by the Neighbor-Joining method with the Jones-Taylor-Thornton model at 1000 bootstrap replicates with the MEGA 10 program.

DISCUSSION

With endemic conditions in most Middle Eastern countries, the low pathogenic H9N2 AIV virus has spread globally and affects various forms of poultry industry, resulting in significant economic losses. Remarkably, there have been previous reports of H9N2 AIV virus infections in humans (Wan et al., 2008). Data about the circulating H9N2 AIVs in SA including virus epidemiology and the available sequences for strains from every year is so limited. Therefore, in the current study we molecularly screened 13 broilers farms located in the middle region of SA during late autumn and early winter of 2022 for the presence of different respiratory viruses then molecular characterization of the detected H9N2 AIV performed through full HA and NA genes sequencing.

The results of rRT-PCR for tested broiler farms showed 84% prevalence for H9N2 AIV, while previous study showed only 3.5% prevalence for 115 tested broiler chicken farms in the Eastern area of SA during 2012-2014 (Almubarak, 2018). Interestingly, an earlier study conducted in the Northern Region of Saudi Arabia showed that the prevalence of H9N2 in tested broiler and layer chicken farms was (21%) (Alkhalaf, 2010). This highlighted the endemic situation of recently circulating H9N2 AIVs during 2022 and the virus epidemiology may have variations between different geographical regions, involved chicken type and time (year) of study.

H9N2 viruses typically have minimal pathogenicity and can spread through poultry without causing any symptoms, or infected birds may exhibit minor respiratory and/or gastrointestinal symptoms (Peacock et al., 2019). However, several data indicated that some strains of H9N2 were linked to high-mortality epidemics in certain poultry species (El Houadfi et al., 2016; Jeevan et al., 2019). This was clear in the examined 13 broiler farms as all flocks showed respiratory signs, also the mortality rates were different between flocks despite detection of H9N2 AIV in most of the examined flocks. According to other investigations, AIV H9N2 viruses have the potential to result in significant economic losses when they co-infect poultry with other respiratory diseases, such as bacteria like Mycoplasma or viruses. (Sid et al., 2015; Hassan et al., 2016). NDV was detected in 11 out of 13 tested flocks in this study in addition to IBV in four farms, however mortalities were different between flocks which might be due to the effect of other bacterial infections and/or managemental factors like stocking density, ventilation, temperature, humidity and ammonia level inside the poultry house. The detected NDV may be velogenic strains or vaccinal strains which might cause this variable mortality. Although in the case of NDV, it could be vaccinal strain but the interaction between AIV H9N2 and vaccinal strains of NDV (Lasota strain) could lead to increased clinical signs, mortality rates, lesions and viral shedding (Ellakany et al., 2018). Additionally, the same case could happen with IBV even with vaccinal strains as it can lead to increased clinical outcome and increased mortality caused by AIV H9N2 viral infections (Hassan et al., 2017).

The genomes of LPAI H9N2 strains have been phylogenetically divided it into two lineages: North American and Eurasian. The majority of H9N2 strains in Eurasian lineage were divided into two clades (G1 and Y280), despite that the Eurasian lineage has multiple clades. Based on the obtained HA and NA genes sequences, the detected H9N2 strain in this study phylogenetically clustered with the recently detected H9N2 strains from SA and the Middle East in a

separate monophyletic group within the G1 clade. While the earlier detected H9N2 strains from SA were closely related to the ancestral G1 strain, this indicates the extensive evolution of H9N2 viruses over time. Sequence of the HA cleavage motif of the current SA H9N2 strain was RSSR/GLF, which indicated that this strain possesses low pathogenicity, this previously reported in Egyptian H9N2 strains (Bedair et al., 2024).

Substitutions of the amino acids located in the antigenic sites of the H9N2 AIV HA protein are important because they may alter the virus's antigenicity, which could influence the effectiveness of vaccinations or the virus's ability to attach to cell receptors (Matrosovich et al., 2001; Meng et al., 2016). Previous research demonstrated that any alterations to the aa in the three main antigenic sites (A, B, and overlapping site) of the H9N2 AIV HA protein resulted in the emergence of a novel variants (Kaverin et al., 2004). In this study, four substitutions (G153D, S158N, N206T and E198A) were retained. In addition to the substitution (Q234L) which could alter antigenicity or increase the binding affinity with erythrocytes which are expressing α 2,6-linked sialic acid that means that this strain might has higher zoonotic significance (Naguib et al., 2017).

For receptor specificity, the aa residues 191, 198, 234, 235, and 236 in the HA protein's RBS are essential (Iqbal et al., 2009), with the most important residue at 234 for host specificity. The residue 198 influences α [2–6] receptor binding and accordingly mammalian infections (Matrosovich et al., 2001). Previously, other substitutions at residue 198 were described, including 198 T/V/A (Zhu et al., 2018). However, the SA H9N2 strain has 198A that allows higher affinity to the human-like receptors as compared to residue T at the same site (Matrosovich et al., 2001).

Additionally, three aa substitutions were reported in the right edge, I147T, R149k and A150S in addition to Other aa substitutions in the left edge presented as D233G, Q234L and Q235I which were reported in earlier SA H9N2 strains. Recently detected H9N2 strains from Algeria have Q234L and Q235I aa mutations in the RBS similar to SA H9N2 recently circulating strains (Barberis et al.,2020), in addition to the presence of aa L instead of Q at the position 234 which could enhance the affinity alteration from avian to human-like receptors (Weis et al., 1988). Taking in mind all these aa substitutions in the RBS, the SA H9N2 strains might have the binding affinity shift toward human-like receptors.

Analysis of aa residues at neuraminidase active sites and framework sites of SA H9N2 strain in comparison to the older S.A H9N2 strain reveals the occurrence of five aa substitutions: K368T, S370L, D401N, R403W, and Q432R. Most of these aa substitutions were also reported in H9N2 strains detected in Emirates, Lebanon, Egypt, Algeria, Morocco and Uganda. The S372A and R403W aa substitutions were reported in the NA protein of Algerian H9N2 and also detected in earlier H9N2 strains from Middle East and Asia, and especially in H2N2 and H3N2 AIV strains which caused human pandemics (Barberis et al., 2020). The HB sites of the NA protein harbor six aa which interact directly with sialic acids; this involves three residues S at the positions 367–372, 400 N, and 432 K, in addition to 403 W which was present in current study strain (Varghese et al., 1997). So, any change in these residues could change sialic acid binding and specificity of host receptor.

The two aa substitutions (S372A and R403W) which were reported in the current study were known to enhance the jump of the species barrier and consequently the adaptation to mammalian hosts (Barberis et al., 2020).

Our strain harbours aa E (Glutamic acid) at position 277 and R (Arginine) at position 292 in addition to H (Histidine) at position 274 these residues are recognized to be correlated with sensitivity to neuraminidase inhibitors (El Zoghby et al., 2012; Hurt, et al., 2009)

Analysis of the potential glycosylation sites (PGS) of the HA gene of the current study strain reveals that this strain harbors seven PGS, which are identical to the PGS of the HA gene of SA strains (2002 and 2020). However, the SA strain 1998 contains the same PGS of the HA gene with additional site 166-168 (NGS).

Variations in the PGS patterns of the influenza virus's HA gene have been shown to affect the pathogenicity, receptor affinity and the binding specificity (Kaverin et al., 2004; Iqbal et al., 2009) and probably infection of a new host. Additionally, it was proposed that the adaptation of AIV to poultry is significantly influenced by glycosylation patterns (Matrosovich et al., 1999).

Analysis of the PGS of the NA gene of the current study strain showed altered glycosylation patterns with additional sites and loss of some sites when compared to older SA strains, as shown in table 10. These altered glycosylation patterns could have many consequences like affecting pathogenicity (Cameron et al., 2000) or increased virulence (Hulse-Post et al., 2007) through alteration of the sialidase activity or the antigenicity (Iqbal et al., 2009) and also the escape from the immune defenses (Igarashi et al., 2008). However, the limited data availability makes it difficult to compare our strains with recent strains from S.A.

In conclusion, this study highlighted the severe endemic situation of LPAI H9N2 virus in poultry, especially broiler chicken flocks in SA. However, more studies including different poultry species and different production types, also different geographical regions and different seasons of the year are needed particularly due to the limited publications. Multiple aa mutations in the H9N2 AIV genes were reported in recently circulating SA H9N2 viruses specially from 2020, Further studies are needed to study the possible effects of these substitutions on the virulence of H9N2 virus. However, some detected aa mutations might increase receptor affinity toward human receptor with increased human infection possibility. These results urge prompt actions to control H9N2 viruses like vaccines update, continuous surveillance studies, strict hygienic and biosecurity measures.

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