Molecular characterization of recently isolated highly pathogenic avian influenza virus (H5N1) in Egypt

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Abstract: Highly Pathogenic Avian Influenza (HPAI) H5N1 virus is an ongoing public health and socio-economic challenge, particularly in Egypt. H5N1 is now endemic in poultry in many countries, and represents a major pandemic threat and have caused multiple human infections. Merging the epidemiological and genetic data is important to understand the transmission, persistence and evolution of the virus. This work describes the molecular characterization of the hemagglutinin (HA) gene of 5 isolates collected from 2015, 2016. In this study, sequence analysis of five HPAI H5N1 viruses of chicken during 2015 and 2016 revealed that were highly pathogenic due to the presence of multiple basic amino acids in the cleavage site through sequence of full HA gene. Also, we found that it belonging to clade 2.2.1 (3 samples) and 2.2.1.1. (2 samples). Our samples showed double mutation at HA residues 134 deletion and I167T which increased viral binding affinity to alpha 2, 6 SA receptor and infectivity in the human lower respiratory tract.
[Saleh M.M, Selim A, Shahera A.A., El -Shahidy M.S. and El-Trabilli M.M. **Molecular characterization of recently isolated highly pathogenic avian influenza virus (H5N1) in Egypt.** *Nat Sci* 2017;15(6):103-109]. ISSN 1545-0740 (print); ISSN 2375-7167 (online). http://www.sciencepub.net/nature. 11. doi:10.7537/marsnsj150617.11.

Keywords: Avian Influenza, H5N1, Sequencing, Phylogenetic tree, Egypt

1. Introduction

Influenza-A viruses contain eight segments of single strand RNA (ssRNA) and they are continuously evolving overtime. Point mutations can introduce small changes known as genetic drift which mainly occurs because the virus polymerase lacks the proofreading property. These changes are thought to be selected by pressures that force the virus to mutate. Highly pathogenic avian influenza viruses of the H5N1 subtype caused severe outbreaks in 1996/97 in southern China and Hong Kong **(Chen and Holmes, 2006).** In recent years, the H5N1 viruses spread from Asia to Europe and then to Africa, becoming endemic in poultry in parts of Asia and Egypt with frequent transmission to humans.

In February 2006, avian influenza virus (AIV) of subtype H5N1, clade 2.2.1, was introduced into Egypt presumably by wild birds **(Saad et al., 2007; Aly et al., 2008).** Since then, these viruses have caused devastating losses in the poultry industry and threaten public health **(Abdelwhab and Hafez, 2011).** Although data from surveillance and research on the Egyptian H5N1 viruses have been accumulating in the last few years, there are still large gaps in knowledge regarding the evolution and epidemiology of these viruses.

Despite of poultry vaccination in Egypt, a new H5N1 viruses was emerged due to escape mutants. Resulting from antigenic drift of the viruses **(Swyene and Kapczynski, 2008).** These escape mutants are less susceptible to vaccine induced neutralizing antibodies. Theoccurrence of escape variants in vaccinated poultry has been described in Egypt **(Hafez et al., 2010).** Vaccine escape is a serious problem in influenza virus eradication programs for poultry, especially for HPAIV H5N1 **(Grund et al., 2011).** Antigenic epitopes in HA protein of AI H5N1 virus of escape mutants were identified by sequencing and structural mapping using monoclonal antibodies or polyclonal (rabbit- or mouse-derived) antiserum **(Lambkin et al., 1994).**

This work was initiatedto analyzethe molecular properties of five H5N1 virusesthat have caused outbreaks in poultry in Egypt during 2015 and 2016 using sequencing of the HA gene and to compare them with publishedsequences from H5N1 viruses obtained from GenBank database.

2. Material and methods

Sample preparation and virus isolation

At the laboratory, each flock pooled samples were processed routinely for virus isolation and RNA extraction. Individual pooled samples were homogenized in tissue homogenizer and centrifuged for 5 minute at 5000 rpm/minutes. The supernatant fluids were separated and used for virus isolation. At the day 10 of incubation, the eggs were inoculated with 100 ul of inoculum by allatoic sac route and incubated again at the proper conditions and examined daily for embryonic death. The eggs were opened for

embryos examination and harvesting the allantoic fluids for examination of haemagglutinating activity (**OIE, 2008).**

Sequencing of HA gene

The H5N1 HPAI of five virus isolates were collected in Egypt during the period from 2015and 2016. They were collected from different localities in Egypt, different commercial poultry farms **(Table 1).** The ribonucleic acids (RNAs) of virus isolates were extracted using QiaAmp viral RNA extraction kit (Qiagen, Germany) according to the manufacturer's instructions. A one-step RT-PCR was conducted on the extracted RNAs using specific primers for H5 gene **(Table 2).** The PCR products were purified using a QiaAmp purification kit (Qiagen, Germany). The full length HA gene sequencing was done using a Bigdye Terminator Kit (version 3.1; Applied Biosystems, Foster City, CA) on a 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). The sequencing of the HA gene was conducted and the data were regularly submitted to the GenBank and are available at the National Center for Biotechnology Information (NCBI)**(Table 1).**

Table 1: Influenza A (H5N1) viruses included in this study and collected from poultry, Egypt.

Isolate name	Age/day	Governorates	Genotype	H5N1 strain	Accession no.
A/chicken/Behera/1/2015	28	Dakahlia	**2.2.1**	**Classic**	**KY951986**
A/chicken/Dakahlia/2/2015	22	Behera	**2.2.1.1**	**Variant**	**KY951987**
A/chicken/Giza/3/2015	35	Giza	**2.2.1**	**Classic**	**KY951988**
A/chicken/Sharkia/4/2015	30	Sharqia	**2.2.1.1**	**Variant**	**KY951989**
A/chicken/Gharbia/5/2016	42	Gharbia	**2.2.1**	**Classic**	**KY951990**

Table 2: Primers of H5 AI used in conventional one step PCR

Primer	Direction	Target gene	TM	Sequence (5′ - 3′)
HGGT	Forward	**H5**	60˚C	CTC TTC GAG CAA AAG CAG GGG T
H5-KH3	Reverse	**H5**	62.4˚C	TAC CAA CCG TCT ACC ATK CCY TG
H5F4	Forward	**H5**	55.3˚C	AGT AAT GGA AAT TTC ATT GCT CCA GAA
Bm-NS 890R(HR)	Reverse	**H5**	66˚C	ATA TCG TCT CGT ATT AGT AGG AAA CAA GGG TGT TTT

Primers obtained from the Veterinary laboratory Agency (VLA) available at.www.defra.gov.uk/vla/.

3. Results

Results RT-PCR for H5N1 isolates

Fig 1: electrophoretic pattern of five H5N1 strains isolated from five province in Egypt, Amplified product of 1100 base pair for 5 isolates (lane 1 Dakahlia, lane 2 Giza, lane 3 Gharbia, lane 4 Sharkia lane 5 Bhira, Lane –ve is the negative control and lane +ve is positive control).

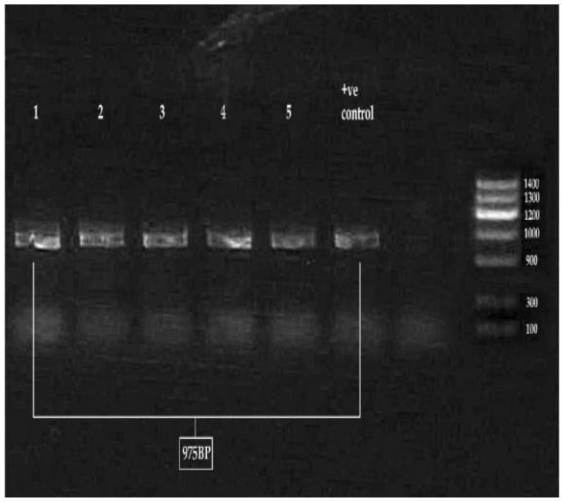

Fig 2: electrophoretic pattern of five H5N1 strains isolated from five province in Egypt, Amplified product of 975 base pair for 5 isolates (lane 1. Dakahlia, lane 2. Giza, lane 3 Gharbia, lane 4. Sharkia lane 5. Behira, Lane –ve is the negative control and lane +ve is positive control).

A comparative analysis of deduced amino acids and nucleotide sequences of the HA gene was created using the CLUSTAL W Multiple Sequence Alignment Program, version 1.83 of Meg Align module of Lasergene DNA Star software **(Madison, WI).** Amino acids phylogenetic tree was drawn for the sequenced isolates along with other vaccine and reference strains available in the GenBank database using MEGA version 6 **(Tamura et al., 2013),** to determine nucleotide and amino acid sequence similarities and relationships. AIV sequences used for the alignments were obtained from the GenBank and EMBL database using multi sequence alignment and sequences were then presented using BOXSHADE 3.21 (http://www.ch.embnet.org/software/BOX_form.html).

Using set of primers specific for amplification of HA gene in 2 fragments for the selected isolates of study, the PCR products run in agar gel 1.5% which give 2 specific band at 975pb and at 1100pb in weight measured against 100 plus ladder (Qiagene – Germany), all of the 5 selected isolates are positive for HA gene (figure 1 and 2).

Sequence and Phylogenetic analysis of H5 gene

Sequencing of haemagglutinin (HA) indicates that all isolates are AIV subtype H5, isolates belong to Clade 2.2.1.1 (variant) and 2.2.1(classic). Amino acid identity between the variant isolates obtained in this study 98.5% (**Fig. 4**). Amino acid identities between classic isolates ranged from 93.3% to 99.3% among the three isolates **(Fig. 4).** And shared 94%-95%, in the amino acids sequences between variant and classic isolates **(Fig. 4).**

Regarding the H5 amino acid similarity between Egyptian isolates in this study and Hongkokong1994 H5N1 was ranged between 82%-84% and Mexico 1994 H5N2 was ranged between 72-80% which are used as the seed virus strains in the preparation of H5N1 and H5N2 vaccines used for poultry vaccination in Egypt.

Table 3: Amino acid pattern of cleavage site of the five isolates

Isolate no.	Cleavage site pattern
AI.sqn A/chicken/Behera/1/2015	PQGERRRKKRGLF
AI.sqn A/chicken/Dakahlia/2/2015	PQGEKRRKKRGLF
AI.sqn A/chicken/Giza/3/2015	PQGEKRRKKRGLF
AI.sqn A/chicken/Sharkia/4/2015	PQGERRRKKRGLF
AI.sqn A/chicken/Gharbia/5/2016	PQGEKRRKKRGLF

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	
1		96.7	96.2	93.5	98.1	97.7	95.8	98.2	97.2	95.2	81.0	87.8	96.7	97.5	97.5	97.2	91.4	1
2	3.3		98.1	95.2	95.4	95.0	96.9	95.6	94.4	96.9	83.7	92.8	94.2	94.8	95.0	94.4	93.8	2
3	4.0	1.9		94.4	96.3	96.0	97.9	96.5	95.4	97.9	83.7	90.6	93.5	95.8	96.0	93.7	94.8	3
4	6.9	5.0	5.8		92.3	92.1	94.0	92.7	91.5	94.0	80.0	88.9	94.6	91.9	92.1	94.8	91.0	4
5	2.0	4.8	3.7	8.1		99.6	97.8	99.5	99.1	97.3	79.3	84.4	95.4	99.5	99.5	96.0	93.1	5
6	2.3	5.2	4.2	8.3	0.4		97.8	99.5	99.1	97.3	79.7	83.9	95.0	99.5	99.5	95.6	93.1	6
7	4.3	3.1	2.1	6.2	2.2	2.2		97.6	97.3	99.2	82.7	90.0	93.1	97.6	97.8	93.6	94.7	7
8	1.8	4.6	3.5	7.7	0.5	0.5	2.4		98.9	97.1	80.0	84.4	95.6	99.3	99.3	96.1	93.0	8
9	2.9	5.8	4.8	9.0	0.9	0.9	2.8	1.1		96.7	79.0	83.9	94.4	98.9	98.9	95.1	93.0	9
10	5.0	3.1	2.1	6.2	2.7	2.7	0.8	2.9	3.3		83.0	90.6	92.7	97.1	97.7	92.9	96.3	10
11	22.0	18.5	18.5	23.3	24.2	23.8	19.8	23.3	24.7	19.3		86.7	77.7	79.3	79.3	77.0	80.0	11
12	13.4	7.6	10.1	12.1	17.5	18.2	10.8	17.5	18.2	10.1	14.7		83.3	84.4	84.4	82.2	84.4	12
13	3.3	6.0	6.9	5.6	4.8	5.2	7.3	4.6	5.8	7.7	26.6	18.9		95.2	95.0	98.5	94.2	13
14	2.5	5.4	4.4	8.6	0.5	0.5	2.4	0.7	1.1	2.9	24.2	17.5	5.0		99.3	96.1	93.3	14
15	2.5	5.2	4.2	8.3	0.5	0.5	2.2	0.7	1.1	2.3	24.2	17.5	5.2	0.7		95.4	93.5	15
16	2.9	5.8	6.6	5.4	4.2	4.5	6.7	4.0	5.1	7.5	27.5	20.3	1.6	4.0	4.7		89.6	16
17	9.2	6.4	5.4	9.7	7.2	7.2	5.5	7.4	7.4	3.7	23.3	17.5	6.0	7.0	6.8	11.2		17
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	

1 (A/chicken/Egypt/06959-NLQP/2006(H5N1
2 A/chicken/Egypt/07118/2007
3 A/Egypte/0896/2008
4 A/chicken/Egypt/0963S/2009
5 (A/chicken/Egypt/10513S/2010(H5N1)
6 (A/quail/Egypt/1171SG/2011(H5N1))
7 (A/chicken/Egypt/1219s/2012(H5N1)
8 (A/duck/Egypt/14VIR784-4-133AD/2013(H5
9 A/goose/egypt/1439FAOS/2014(H5N1)
10 A/DUCK/EGYPT/1532SG/2015
11 MEXICO 1994 (H5N2)
12 hongkong 1994 (H5N1)
13 AI-CHICKEN/Behera-Egypt-1-2015
14 AI/CHICKEN/Dakahlia-Egypt-2-2015
15 AI/CHICKEN/Giza-Egypt-3-2015
16 AI/CHICKEN/Sharkia-Egypt-4-2015
17 AI/CHICKEN/Gharbia-Egypt-5-2016

Fig 4: H5 amino acid identities and divergence of the isolated five Egyptian duck H5N1 strains in comparison with chicken strains isolated in Egypt from 2006 till 2015.

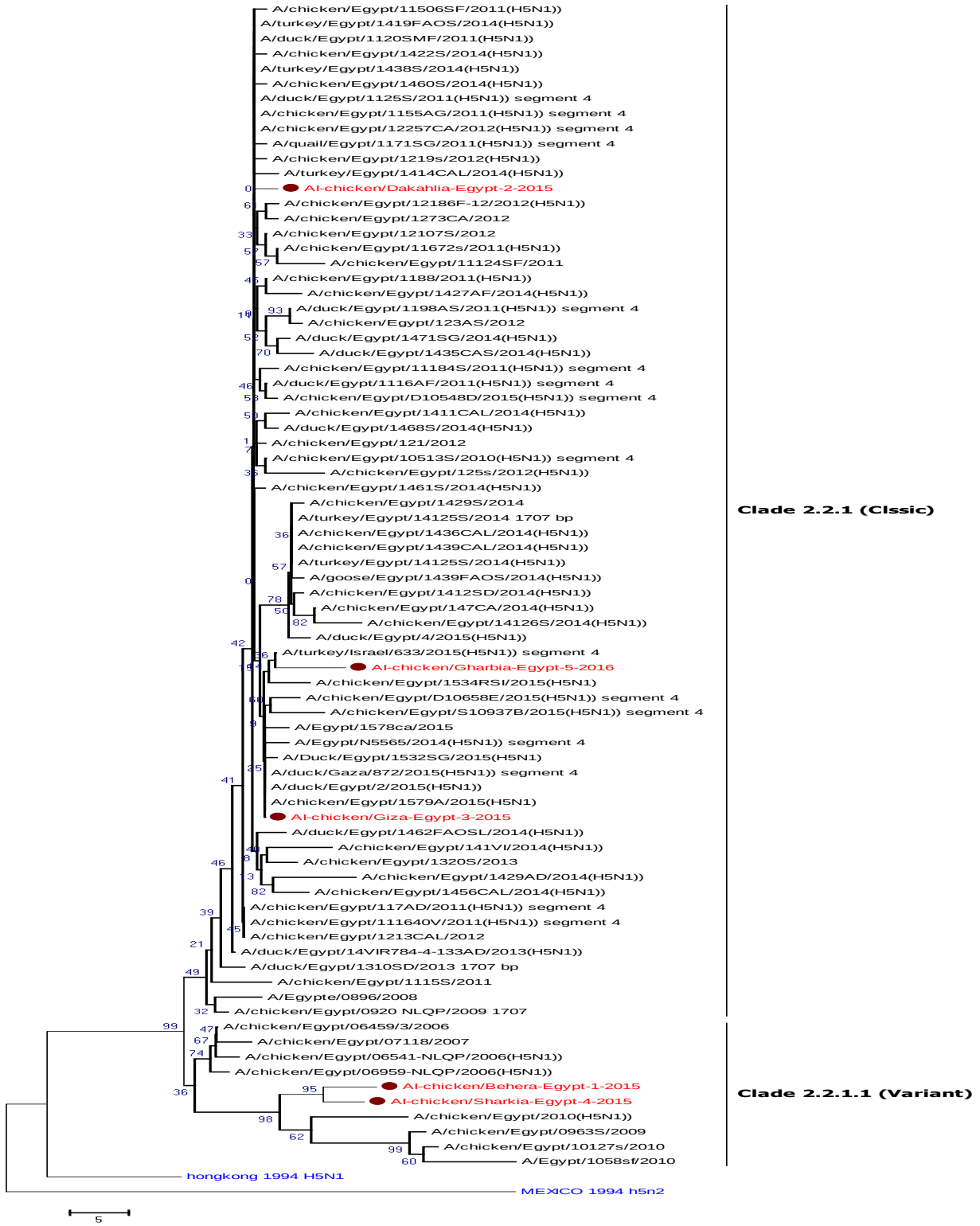

Fig 3: Phylogenetic tree of the full length of hemagglutinin H5 gene of Egyptian strains using MEGA 6 software was used for the phylogenetic tree construction by applying the neighbor-joining method with Kimura's two-parameter distance model and 1000 bootstrap replicates indicate presence of 2 groups, the classic group of sub clade 2.2.1 and the variant group of 2.2.1.1.

Sequence of receptor binding and antigenic sites:

The most characteristic change in the receptor binding site of the Egyptian viruses included in this study was the observation of one amino acid deletion at site 143 (143Δ) that was not recorded in the ancestral strain (A/chicken/Egypt/06959-NLQP/2006). Another important change in the receptor binding site S129L substitution was not recorded in our isolates. The substitution was more pronounced in 2.2.1.1variant cluster and was linked with another substitution (P90S) which represented in our study in Behera and Sharkia isolates **(Table 4).** The deletion in 143Δ and substitution in I167T in the classic isolates (Dakahlia, Giza, Gharbia) was not found in the variant strain 2.2.1.1(Behira, Sharkia). S136D were recorded in classic isolates (Dakahlia, Giza), meanwhile, S136E were recorded in classic isolate (Gharbia) which isolated in 2016**(Table 4).**

Table 4: Mutations of amino acids in antigenic sites and receptor binding site in the five isolates in relation to (A/chicken/Egypt/06959-NLQP/2006) strain.

Isolate	Genotype	Receptor binding sites changes	Antigenic sites changes
A/chicken/Behera/1/2015	Variant (2.2.1.1)		P90S,R178K,R156G, S157L
A/chicken/Sharkia/4/2015			P90s,R178K,R156G,S157P
A/chicken/Dakahlia/2/2015	Classic (2.2.1)	Δ143	S136D,I167T, D170N, R178K
A/chicken/Giza/3/2015			S136D,I167T, D170N,R178K
A/chicken/Gharbia/5/2016			S136E,I167T, D170N,R178K

Sequence of glycosylation sites

Seven N-linked potential glycosylation sites (26NNS, 27NST, 39NPT, 181NNT, 209NPT, 302NSS and 500NGT), where 181NNT were not recorded in both Behira and Sharkia isolate and 209NPT was not recorded in Gharbia isolate. All isolates contain mutations at D170N and N171D.

4. Discussion

AI H5N1 viruses were isolated from ducks, chickens, and humans in Egyptian households and clustered into a distinct genetic group designated as 2.2.1. The majority of viruses derived from vaccinated poultry in commercial farms belonged to the 2.2.1.1 clade of variant viruses (**Abdel-Moneim et al., 2009**). In a situation like Egypt, the genetic diversity of viruses leads to the production of heterogeneous genotypes (**Arafa et al., 2010**). However, the mechanisms associated with the genotype diversity of H5N1 viruses have still not been investigated (**Vijaykrishna, 2008**).

Avian influenza virus disease was identified in Egypt since 2006 causing a huge economic losses in chicken industry owing to high mortality transmit to human by contact with infected birds (**Aly et al., 2008**). Although intensive vaccination programs are well adopted, the disease still catastrophic not only in commercial chicken farms but also extended to household and backyard chickens (**Arafa et al., 2010**). So far, searching for sustainable poultry breed with sustainable genetic resources which has relatively better resistance to infectious disease is important issue.

Herein, 5 provinces of Egypt (Giza, Dakahlya, Sharkia, Gharbia and Behera), were involved in this study because of suffering from recurrent outbreaks of avian influenza H5N1 in chicken farms. Five isolates of AI H5N1 virus were successively isolated in embryonated chicken eggs and identified by HA and RT-PCR **(Fig.1, 2).**

In this study, Sequence analysis of five HPAI H5N1 viruses of chicken during 2015 and 2016 revealed that were highly pathogenic due to the presence of multiple basic amino acids in the cleavage site through sequence of full HA gene. Also, we found that it belonging to clade 2.2.1 and 2.2.1.1. AI H5N1 isolated from Dakhalia, Giza and Gharbia were classic strains of clade 2.2.1 while Behera and Sharkia strains belong to variant strains of clade 2.2.1.1 **(Table 1, 3).** These viruses are related to viruses isolated during 2014, and constitute an endemic cluster in Egypt **(Arafa et al., 2015).** HPAI influenza A (H5N1) viruses of clade 2.2.1 and their descendants have been circulating in Egyptian poultry populations since 2006, causing sporadic human infections **(Abdelwhab et al., 2011).** In 2015, a new cluster originated from 2.2.1.2 were emerged and causes more deaths **(Arafa et al., 2015).**

Increased evolutionary rate of AIV might be accelerated either by the immune pressure (due to prior immunization or natural infection) exerted on the replicating viruses in different hosts and/or jumping of the virus from one species to another (**Yassine et al., 2010).** Like our result, double mutation at HA residues 134 deletion and I167T were reported in other studies who found such mutations which increased viral binding affinity to alpha 2,6 SA receptor and

infectivity in the human lower respiratory tract but not in the larynx in combination with residual affinity for alpha 2,3 SA receptor **(Watanabe et al., 2011).** Alternatively, there were mutations in antigenic sites (S136D, I167and R178K) which may cause evolving of escape mutant viruses affecting immune response to vaccine **(Arafa et al., 2016).** The deletion Δ143 in receptor binding domain of haemagglutinin detected in the isolated viruses could facilitate the interspecies and intra-species transmission **(Auewarakul et al., 2007).** Amino acids similarities between variant and classic isolates shared 94% -95% **(Fig. 4).**

The antigenic analysis of the earlier H5N1 variant strains in Egypt demonstrated antigenic variation (**Beato et al., 2013**), which was driven by multiple mutations primarily occurring in the major antigenic sites at the globular head of HA (**Cattoliet al., 2011)**. Other studies showed that the classic clade 2.2.1 strains are antigenically related and crossreactive to the ancestral Asian H5N1 strains, but demonstrated weak cross-reactivity with the Egyptian variant 2.2.1.1 strains (**Watanabe Y et al 2012**). The majority of these mutations, alongside the other 19 amino acid mutations, were located within or adjacent to the receptor binding domain (RBD) in the HA1 that may affect the virus replication and transmission.

The viruses with 143Δ were found in the majority of human infections in Egypt in 2009 and have been found in all H5N1 human infections afterwards (**Watanabe et al 2011**). The presence of the 143Δ mutation may affect the binding of the virus to human receptors. The loss of HA154–156 glycosylation site was shown to enhance H5N1 virus binding to terminally α-2,6 sialic acid receptors and so increased the transmissibility to mammals (**Neumann et al., 2012**).

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