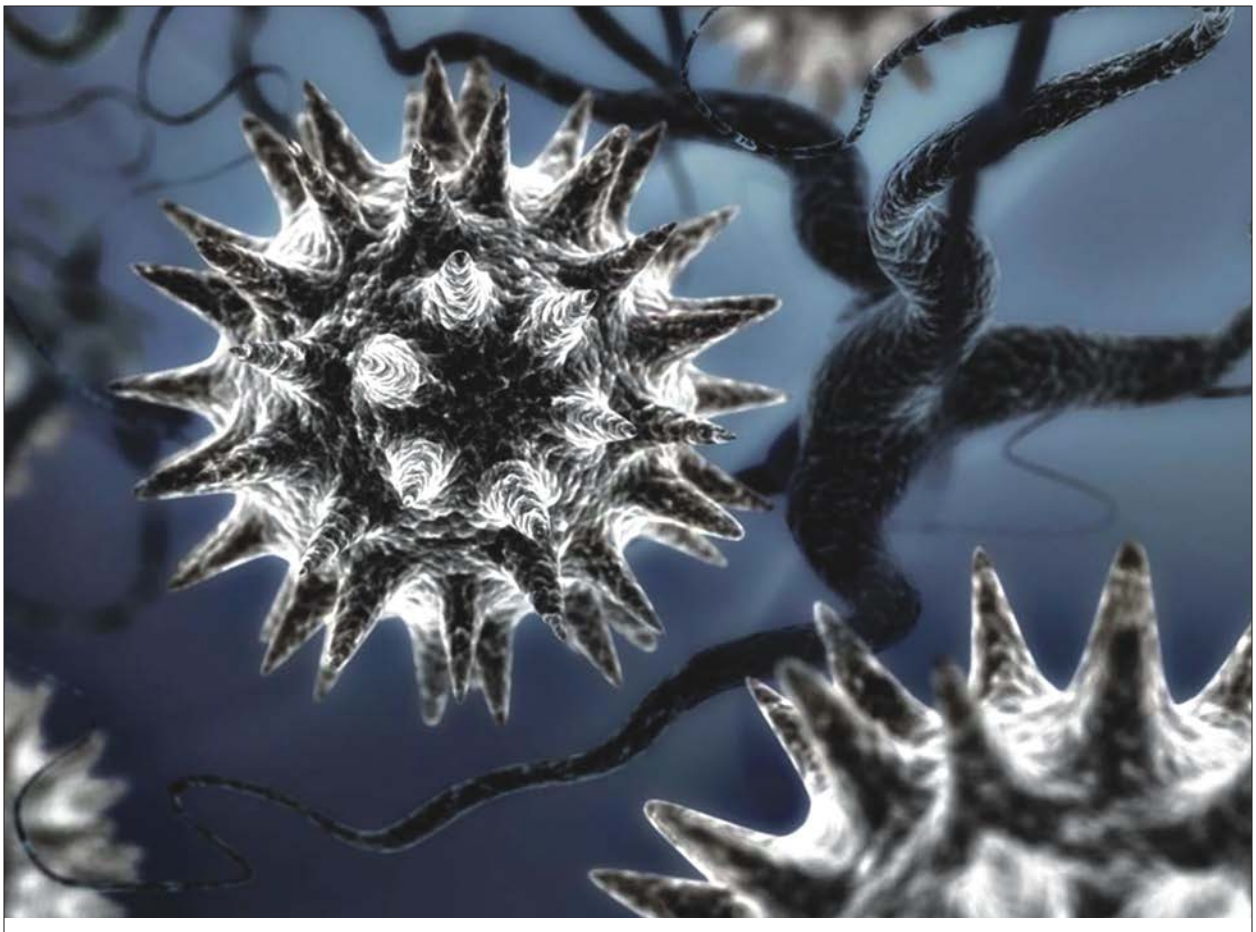

International Journal of Virology

ISSN 1816-4900

Academic Journals Inc.

www.academicjournals.com

International Journal of Virology 10 (2): 143-149, 2014
ISSN 1816-4900 / DOI: 10.3923/ijv.2014.143.149

Molecular Characterization of PB2 Gene of Highly Pathogenic *Avian influenza* H5N1 in Egypt

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ABSTRACT

Science 2006 Egypt has reported a severe crisis of highly pathogenic *Avian influenza* (H5N1) virus infections in poultry as well as a higher number of recorded cases in human than any other country. The H5N1 viruses continue to cause outbreaks in poultry with sporadic human infections. Viral determinants of virulence and transmissibility are still poorly understood, lysine at position 627 of the polymerase subunit PB2 (PB2-627K) is known to be important for *Avian influenza* virus adaptation to mammals. In the present study we molecularly characterize PB2 gene for 9 Egyptian H5N1 isolates form 2010 to 2011, phylogenic analysis of these viruses give genetic prospective of the virus circulating in Egypt, identifying genetic evolution and mutations associated with enhance replication and virulence in mammalian species.

Key words: Highly pathogenic *Avian influenza* H5N1, polymerase basic 2 (pb2) and virulence markers

INTRODUCTION

The genome of *Influenza a* viruses consists of eight single-stranded RNA segments of negative sense, *Influenza a* virus surface proteins (hemagglutinin (HA) and neuraminidase (NA) have been widely studied in contrast to the internal and nonstructural protein genes (PA, PB1, PB2, NP and NS), whose evolutionary pathways were established only recently. Influenza A viruses are classified into different subtypes on the basis of the antigenicity of their surface proteins (HA) and (NA). There were 17 different HA subtypes and 10 different NA subtypes have been identified in viruses isolated from wild birds, which are regarded as the natural hosts of *Influenza A* viruses. Of the numerous potential subtypes of viruses, only a few have adapted to and circulate among humans (H1N1, H2N2 and H3N2), pigs (H1N1 and H3N2) and domestic poultry (H9N2, H5 and H7).

The H5N1 viruses typically acquire critical changes in their genomes that allow them to adapt to or severely damage their host, Identification of these genetic changes will improve our understanding of the determinants of virulence and aid in the development of counter measures. Several determinants of *Avian influenza* Virus (AIV) virulence have been identified (Hatta *et al*., 2001; Jiao *et al*., 2008; Fan *et al*., 2009), The multiple-basic-amino-acid motif in the cleavage site of the HA gene is required for the systemic replication and lethal infection of the H5 and H7

subtypes of influenza viruses in chickens (Kawaoka *et al*., 1987) and mice (Hatta *et al*., 2001). In addition, mutations in the M1 protein contribute to the virulence of H5N1 influenza viruses in mice (Fan *et al*., 2009). Certain amino acids or regions of the NS1 protein play a key role in the ability of H5N1 influenza viruses to undermine the antiviral immune response of the host cell and are critical for the pathogenicity of H5N1 influenza viruses in mice (Jiao *et al*., 2008).

The polymerase proteins also play important roles in the pathogenicity of AIVs in different species. A single amino acid substitution, from glutamic acid (E) to Lysine (K) in position 627 in PB2 is a determinant of mammalian host range (Subbarao *et al*., 1993; Shinya *et al*., 2004). Most Avian isolates have E in this position. The substitution to K in this position converts a nonlethal H5N1 *Influenza a* virus isolated from a human to a lethal virus in mice (Shinya *et al*., 2004). Amino acid changes may be critical to confer transmissibility in humans. Lysine at position 627 of PB2 is a principal determinant of the high virulence of the 1997 Hong Kong H5N1 influenza viruses (Hatta *et al*., 2001; Chen *et al*., 2007).

Also amino acid at position 701 in PB2 plays a crucial role in the ability of H5N1 viruses of duck origin to replicate and be lethal in mice (Li *et al*., 2005). This amino acid also contributes to the increased lethality of an H7N7 AIV in a mouse model (Gabriel *et al*., 2005) while both of these PB2 amino acids contribute to the transmission of the H5N1 influenza viruses in guinea pigs (Gao *et al*., 2009; Steel *et al*., 2009).

PB2 residue 271 plays a role in the enhanced polymerase activity of *Influenza a* viruses in mammalian host cells (Bussey *et al*., 2010). Lastly, the importance of the amino acid at position 591 of PB2 for efficient replication of the pandemic H1N1 viruses in humans has recently been reported (Mehle and Doudna, 2009; Yamada *et al*., 2010).

The PB2 and PB1 polymerase subunits contribute to the high virulence of the human H5N1 influenza virus isolate A/Vietnam/1203/04 in mice and ferrets (Salomon *et al*., 2006). Recently, it was reported that differences in viral transcription and replication levels between mammalian and avian cells are determinants of both host specificity and pathogenicity of an H7N7 virus (Gabriel *et al*., 2007).

In this study, the molecular characterization of PB2 gene was conducted to give genetic prospective of the H5N1 viruses circulating in Egypt and report mutations that contribute to the virulence of H5N1 *Avian influenza* viruses in mice and human.

MATERIALS AND METHODS

Viral RNAs were extracted from the infective allantoic fluid of SPF fowls' eggs using QIAmp viral RNA mini kit and were reverse transcribed with the Access Quick RT-PCR using UN1-12 primer. Polymerase Chain reaction amplification was performed by using specific primers for PB2 gene (primer sequences available on request) using Reddy master mix (Thermo two-step) and with different annealing temperature. The sequences were generated using the Big Dye Terminator v 3.1 cycle sequencing kit (Applied Biosystem, Foster City, CA-USA). The products of the sequencing reactions were cleaned-up using Centrisep purification kit Analyzer (Applied Biosystem, CA-USA). Sequence data were aligned and compared with A/H5N1 sequences of viruses from Egypt and with representative sequences of viruses from Gaza, Israel, Sudan and the Middle East available on GenBank. Phylogenetic analyses were carried out using the neighbour-joining (N-J) method with laser gene software DNA Star (Table 1).

Table 1: Isolate identification of Egyptian H5N1 viruses under study

Isolate ID	Date	Governrate	Species	Breeding
A/chicken/Egypt/1063/2010(H5N1)	Feb-2010	Qalyobia	Chicken	Farm
A/duck/Egypt/1011d/2010(H5N1)	Feb-2010	Alexandria	Duck	Farm
A/turkey/Egypt/101474v/2010(H5N1)	Feb-2010	Giza	Turkey	Backyard
A/turkey/Egypt/10453F/2010(H5N1)	08-Aug-2010	Kafr ElShikh	Turkey	Backyard
A/chicken/Egypt/115AD/2011(H5N1)	26-Feb-2011	Behera	Chicken	Backyard
A/chicken/Egypt/1158SF/2011(H5N1)	27-Jan-2011	Fayoum	Chicken	Backyard
A/chicken/Egypt/113Q/2011(H5N1)	26-Jul-2011	Qalyobia	Chicken	Market
A/chicken/Egypt/1188/2011(H5N1)	25-Oct-2011	Giza	Chicken	Backyard
A/chicken/Egypt/1112/2011(H5N1)	10-Jan-2011	Monofia	Chicken	Backyard

Table 2: Difference of deduced amino acid sequence of PB2 gene between all isolates of Egyptian H5N1

	Amino acids differences						
	Egypt/ 2.2.1					Egypt/ 2.2.1.1	
Year	K80	T129	K197	I292	M315	D195	T559
2010	R	N	R	M	I	G[1]	A
2011	R	N	R	M	I	G	A
	R	N	R	M	I	G	A

[1]All viruses in the study contain "G" except A/chicken/Egypt/1063/2010 virus

Table 3: Conserved motifs of PB2 gene

Gene	Motif	Sequence	Length	H5N1 Start position	H5N1-End position	Repeated position on H5N1
PB2	1	TGATGTGGG	9	1604	1612	-
	2	GAAACG	6	2205	2210	2211-2216
	3	AGCATACTTAC	11	2224	2234	
	4	CAGACAGCG	9	2242	2250	-

Length of these 4 motifs ranged from 6-11 nucleotides and only motif No. 2 has a repeated position on PB2 gene from nucleotide No. 2211 to 2216

RESULTS

Molecular characterization of PB2 gene: Nine Egyptian *Avian influenza* H5N1 isolates collected during the period from 2010 to 2011, sequence of PB2 gene have done to determine the differences and similarity % of the whole length of the PB2 gene in comparison with some representative Egyptian strains from Gen bank between 2006 to 2009, A/Goose/Guangdong/1/96 and A/Duck/Fujian/1734/05. From sequence analysis and aligned amino acid of PB2 we find that the Egyptian viruses during this period have evolutional character like that of H5 gene which divide the isolates into 2 groups with presence of markers characterizing each group in relation to 2006 strains Table 2, 3.

It was found that all strains of subclade 2.2.1 (Egypt/2.2.1) contain K80R, T129N, K197R, I292M and M315I while all strains of subclade 2.2.1.1 (Egypt/ 2.2.1.1) contain T559A and D195G Except A/chicken/Egypt/1063/2010 (H5N1) contain D.

Analysis of sequence data indicates presence of 4 conserved motifs in all 9 strains as shown in Table 4.

Also there is very highly conserved region among all infiuenza viruses between PB1 and PB2 as there is binding between N terminus of PB2 (1-37 AA) with C terminus of PB1 (678-757) (Poole *et al*., 2007).

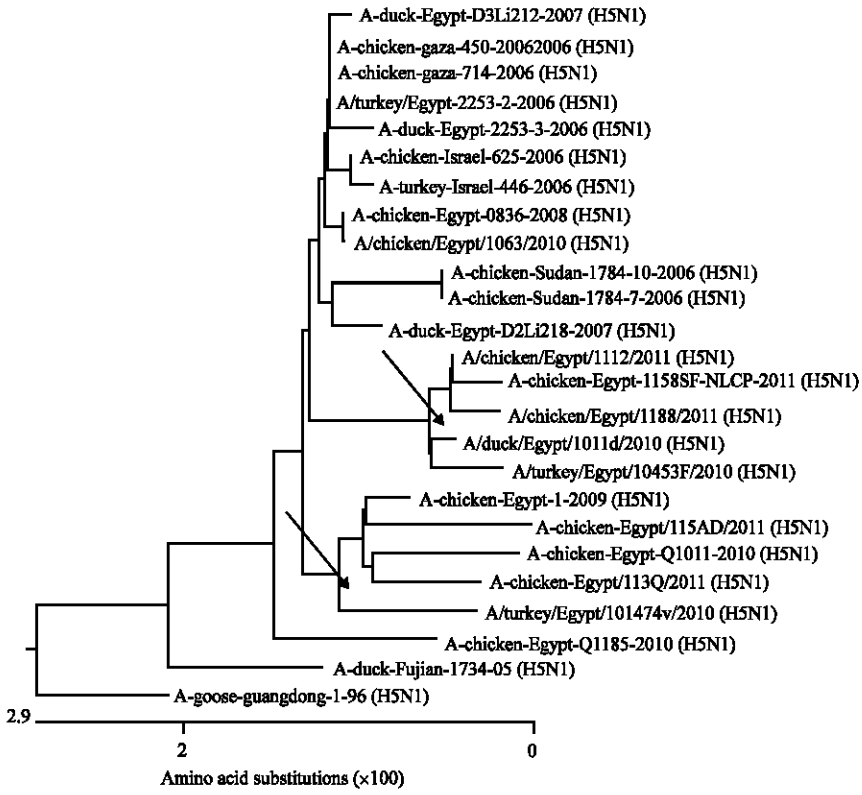

Fig. 1: Phylogenetic tree for the PB2 amino acid sequence of Egyptian strains under study in comparison to A/goose/guangdong/1/96 and other Egyptian H5N1 strains

Table 4: Deduced amino acids substitutions in PB2 protein among Egyptian isolates from 2010 to 2011

	Amino acids differences*												
Isolate ID	Q73H	K80R	V109A	T129N	R175K	K197R	I292M	M315I	D195G	M570I	R369K	T559A	E627K
A/duck/Egypt/2253-3-2006(H5N1)	--	--	--	--	--	--	--	--	--	--	--	--	+
A/chicken/Egypt/1063/2010(H5N1)	--	--	--	--	--	--	--	--	--	--	--	+	+
A/duck/Egypt/1011d/2010(H5N1)	--	+	--	+	--	+	+	+	--	+	--	--	+
A/turkey/Egypt/101474v/2010(H5N1)	--	--	+	--	--	--	--	--	+	+	--	+	+
A/turkey/Egypt/10453F/2010(H5N1)	+	+	--	+	--	+	--	+		+	--	--	+
A/chicken/Egypt/115AD/2011(H5N1)	R	--	--	--	+	--	--	--	+	+	--	+	+
A/chicken/Egypt/1158SF/2011(H5N1)	--	+	--	+	--	+	+	+	--	+	+	--	+
A/chicken/Egypt/113Q/2011(H5N1)	--		--	--	+	--	--	--	+	+	--	+	+
A/chicken/Egypt/1188/2011(H5N1)	--	+	+	+	--	+	+	+	--	+	+	--	+
A/chicken/Egypt/1112/2011(H5N1)	--	+	--	+	--	+	+	+	--	+	+	--	+

*Amino acids differences between Egyptian isolates were calculated in comparison with 2006 Egyptian strains from NCBI, --: Absence of substitution mutation, +: Presence of substitution mutation

It was found that all isolates under study possess the marker E627K in the PB2 gene which is well-known to be associated with increased virulence of A/H5N1 viruses for mice (Hatta *et al.*, 2001). Both A199S and D701N mutations are not recorded in all strains under study (Fig. 1).

Phlogenetic analysis of PB2 gene indicates that Egyptian viruses are closely related to Israeli and Gazian viruses and to Egyptian strains isolated from 2006.

Percent identity

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19		
1		99.9	99.2	98.7	97.6	98.3	99.1	99.9	98.9	98.8	99.1	98.8	98.5	98.8	98.8	98.8	98.9	98.7	98.8	1	A-turkey-Egypt-2253-2-2006(H5N1)
2	0.1		99.1	98.5	97.4	98.1	98.9	99.7	98.8	98.7	98.9	98.7	98.4	98.7	98.7	98.7	98.8	98.5	98.7	2	A-duck-Egypt-D3Li212-2007(H5N1)
3	0.8	0.9		98.5	96.8	97.4	98.5	99.3	98.9	98.3	98.5	98.3	98.8	98.3	99.1	98.3	98.4	98.1	98.3	3	A-chicken-Egypt-1-2009(H5N1)
4	1.4	1.5	1.5		97.0	97.4	98.1	98.8	98.3	97.8	98.1	97.8	97.8	97.8	98.1	97.8	98.0	97.7	97.8	4	A-chicken-Egypt-Q1185-2010(H5N1)
5	2.5	2.6	3.3	3.0		97.6	96.6	97.4	96.6	96.4	96.6	96.4	96.1	96.4	96.4	96.4	96.5	96.2	96.4	5	A-goose-Guangdong-1-96(H5N1)
6	1.8	1.9	2.6	2.6	2.5		97.3	98.1	97.2	97.0	97.3	97.0	96.8	97.0	97.0	97.0	97.2	96.9	97.0	6	A-duck-Fujian-1734-05(H5N1)
7	0.9	1.1	1.5	1.9	3.4	2.7		98.9	98.3	99.5	99.7	99.5	97.8	99.5	98.1	99.5	99.6	99.3	99.5	7	A-duck-Egypt-1011AD-NLQP-2010
8	0.1	0.3	0.7	1.2	2.6	1.9	1.1		99.1	98.7	98.9	98.7	98.7	98.7	98.9	98.7	98.8	98.5	98.7	8	A-chicken-Egypt-1663A-NLQP-2010
9	1.1	1.2	1.1	1.8	3.4	2.9	1.8	0.9		98.0	98.3	98.3	98.3	98.0	98.5	98.0	98.1	97.8	98.0	9	A-turkey-Egypt-101474V-NLQP-2010
10	1.2	1.4	1.8	2.2	3.7	3.0	0.5	1.4	2.0		99.5	99.2	97.7	99.2	97.8	99.2	99.3	99.1	99.2	10	A-chicken-Egypt-10543F-NQLP-2010
11	0.9	1.1	1.5	1.9	3.4	2.7	0.3	1.1	1.8	0.5		99.7	97.8	99.7	98.1	99.7	99.9	99.6	99.5	11	A-chicken-Egypt-1112A-NQLP-2011
12	1.2	1.4	1.8	2.2	3.7	3.0	0.5	1.4	1.8	0.8	0.3		97.6	99.5	97.8	99.5	99.6	99.3	99.2	12	A-chicken-Egypt-1188A-NQLP-2011
13	1.5	1.6	1.2	2.2	4.0	3.3	2.2	1.4	1.8	2.3	2.2	2.5		97.8	98.4	97.6	97.7	97.7	97.6	13	A-duck-Egypt-115AD-NQLP-2011
14	1.2	1.4	1.8	2.2	3.7	3.0	0.5	1.4	2.0	0.8	0.3	0.5	2.2		98.1	99.5	99.6	99.9	99.2	14	A-ostrich-Egypt-11139F-NQLP-2011
15	1.2	1.4	0.9	1.9	3.7	3.0	1.9	1.1	1.5	2.2	1.9	2.2	1.6	1.9		97.8	98.0	98.0	97.8	15	A-chicken-Egypt-113Q-NQLP-2011
16	1.2	1.4	1.8	2.2	3.7	3.0	0.5	1.4	2.0	0.8	0.3	0.5	2.5	0.5	2.2		99.6	99.3	99.2	16	A-chicken-Egypt-1158SF-NQLP-2011
17	1.1	1.2	1.6	2.0	3.6	2.9	0.4	1.2	1.9	0.7	0.1	0.4	2.3	0.4	2.0	0.4		99.5	99.3	17	A-chicken-Egypt-121A-NQLP-2012
18	1.4	1.5	1.9	2.3	3.9	3.2	0.7	1.5	2.2	0.9	0.4	0.7	2.3	0.1	2.0	0.7	0.5		99.1	18	A-chicken-Egypt-128S-NQLP-2012
19	1.2	1.4	1.8	2.2	3.7	3.0	0.5	1.4	2.0	0.8	0.5	0.8	2.5	0.8	2.2	0.8	0.7	0.9		19	A-chicken-Egypt-119S-NQLP-2011
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19		

Fig. 2: Identity and divergence percent of isolates under study with A/goose/guangdong/1/96 and A/duck/Fujian/1734/05

Identity Percent for Amino Acid sequence of whole PB2 gene of the 9 isolates under study in comparison with A/Goose/Guangdong/1/96(H5N1) ranging between 96.0 and 97.8% and with A-Duck-Fujian-1734-05(H5N1) ranging between 96.9-98.1 (Fig. 2).

DISCUSSION

Phylogenic analysis of 9 PB2 genes of Egyptian viruses showed that 2 main groups are present in 2010 and 2011 the classic group that related to 2006 and present widely in backyard as (A/duck/Egypt/1011d/2010, A/turkey/Egypt/10453F/2010, A/chicken/Egypt/1158SF/2011, A/chicken/Egypt/1188/2011 and A/Chicken/Egypt/1112/2011) and belonging to clade 2.2.1 and the variant group which present mainly in vaccinated farms and belonging to clade 2.2.1.1, as: (A/Chicken/Egypt/113Q/2011, A/turkey/Egypt/101474v/2010, A/chicken/Egypt/1063/2010 and A/Chicken/Egypt/115AD/2011.

It was found that there is interaction between PB1 and PB2 as there is binding between N terminus of PB2 (1-37 AA) with C terminus of PB1 (678-757) forming very highly conserved region among all infuenza viruses, PB1-PB2 interface that appears as target for novel anti-infuenza drugs of use against all strains of *Infuenza A* virus (Poole *et al*., 2007).

All isolates under study possess the marker E627K in the PB2 gene, which is well-known to be associated with increased virulence of A/H5N1 viruses for mice (Hatta *et al*., 2001).

E627K confers efficient replication in mammals (Neumann *et al.*, 2012). Beside, it is a recognized host determinant of influenza viruses. Single mutation at the amino acid position 627 of PB2 that leads to increased virulence of an H5N1 *Avian influenza* virus during adaptation in mice can be compensated by multiple mutations at other sites of PB2 (Li *et al.*, 2009). It was recorded that presence of basic amino acid at position 591 compensates for the lack of PB2-627K and substantially increased the lethality of an avian H5N1 virus in mice (Shinya *et al.*, 2004) which not detected in strains under study.

The substitution (D701N) considered one of the molecular determinants of H5N1 virus pathogenicity in mice and not recorded in strains under study. Steel *et al.* (2009) recorded that the transmission of influenza viruses in mammalian host is increased by PB2 amino acids 627K or 627E/701N, also A199S recorded as one of host specific marker which is conserved in seasonal human influenza viruses (Finkelstein *et al.*, 2007) and they did not found in all strains under study.

In conclusion, this study indicates the importance of the genetic characterization of PB2 gene and protein as it has a direct influence in virus pathogenicity and virus evolution in Egypt. The PB2 gene evolution in Egypt carry the same evolutional characters like H5 gene with clear markers characterizing each cluster. There was no geographical limitation based on PB2 gene sequences as the viruses isolated from different governorates in Egypt have the same grouping characters, also there is no characterization based on the type of breeding or poultry species. All isolates under study possess the marker E627K in the PB2 gene, which is well-known to be associated with increased virulence of A/H5N1 viruses for mice. There is no insertion or deletion mutations observed in PB2 gene but only substitution mutations. Presence of conserved regions with high percent will help for induction of new anti-influenza drugs to overcome the resistance of Tami flu and Amantadine.

REFERENCES

Bussey, K.A., T.L. Bousse, E.A. Desmet, B. Kim and T. Takimoto, 2010. PB2 residue 271 plays a key role in enhanced polymerase activity of *Influenza a* viruses in mammalian host cells. J. Virol., 84: 4395-4406.

Chen, H., R.A. Bright, K. Subbarao, C. Smith, N.J. Cox, J.M. Katz and Y. Matsuoka, 2007. Polygenic virulence factors involved in pathogenesis of 1997 Hong Kong H5N1 *Influenza* viruses in mice. Virus Res., 128: 159-163.

Fan, S., G. Deng, J. Song, G. Tian and Y. Suo *et al.*, 2009. Two amino acid residues in the matrix protein M1 contribute to the virulence difference of H5N1 *Avian influenza* viruses in mice. Virology, 384: 28-32.

Finkelstein, D.B., S. Mukatira, P.K. Mehta, J.C. Obenauer, X. Su, R.G. Webster and C.W. Naeve, 2007. Persistent host markers in pandemic and H5N1 influenza viruses. J. Virol., 81: 10292-10299.

Gabriel, G., B. Dauber, T. Wolff, O. Planz, H.D. Klenk and J. Stech, 2005. The viral polymerase mediates adaptation of an *Avian influenza* virus to a mammalian host. Proc. Natl. Acad. Sci., 102: 18590-18595.

Gabriel, G., M. Abram, B. Keiner, R. Wagner, H.D. Klenk and J. Stech, 2007. Differential polymerase activity in avian and mammalian cells determines host range of *Influenza* virus. J. Virol., 81: 9601-9604.

Gao, Y., Y. Zhang, K. Shinya, G. Deng and Y. Jiang *et al.*, 2009. Identification of amino acids in HA and PB2 critical for the transmission of H5N1 *Avian influenza* viruses in a mammalian host. PLoS Pathogens, Vol. 5. 10.1371/journal.ppat.1000709

Hatta, M., P. Gao, P. Halfmann and Y. Kawaoka, 2001. Molecular basis for high virulence of Hong Kong H5N1 *Influenza A* viruses. Science, 293: 1840-1842.

Jiao, P., G. Tian, Y. Li, G. Deng and Y. Jiang *et al.*, 2008. A single-amino-acid substitution in the NS1 protein changes the pathogenicity of H5N1 *Avian influenza* viruses in mice. J. Virol., 82: 1146-1154.

Kawaoka, Y., A. Nestorowicz, D.J. Alexander and R.G. Webster, 1987. Molecular analyses of the hemagglutinin genes of H_5 *Influenza* viruses: Origin of a virulent Turkey strain. Virology, 158: 218-227.

Li, J., M. Ishaq, M. Prudence, X. Xi, T. Hu, Q. Liu and D. Guo, 2009. Single mutation at the amino acid position 627 of PB2 that leads to increased virulence of an H5N1 *Avian influenza* virus during adaptation in mice can be compensated by multiple mutations at other sites of PB2. Virus Res., 144: 123-129.

Li, Z., H. Chen, P. Jiao, G. Deng and G. Tian *et al.*, 2005. Molecular basis of replication of duck H5N1 influenza viruses in a mammalian mouse model. J. Virol., 79: 12058-12064.

Mehle, A. and J.A. Doudna, 2009. Adaptive strategies of the *Influenza* virus polymerase for replication in humans. Proc. Natl. Acad. Sci., 106: 21312-21316.

Neumann, G., C.A. Macken, A.I. Karasin, R.A.M. Fouchier and Y. Kawaoka, 2012. Egyptian H5N1 influenza viruses-cause for concern? PLoS Pathogens, Vol. 8. 10.1371/journal.ppat.1002932

Poole, E.L., L. Medcalf, D. Elton and P. Digard, 2007. Evidence that the C-terminal PB2-binding region of the *Influenza A* virus PB1 protein is a discrete alpha-helical domain. FEBS Lett., 581: 5300-5306.

Salomon, R., J. Franks, E.A. Govorkova, N.A. Ilyushina and H.L. Yen *et al.*, 2006. The polymerase complex genes contribute to the high virulence of the human H5N1 *Influenza* virus isolate A/Vietnam/1203/04. J. Exp. Med., 203: 689-697.

Shinya, K., S. Hamm, M. Hatta, H. Ito, T. Ito and Y. Kawaoka, 2004. PB2 amino acid at position 627 affects replicative efficiency, but not cell tropism, of Hong Kong H5N1 *Influenza A* viruses in mice. Virology, 320: 258-266.

Steel, J., A.C. Lowen, S. Mubareka and P. Palese, 2009. Transmission of *Influenza* virus in a mammalian host is increased by PB2 amino acids 627K or 627E/701N. PloS Pathogens, Vol. 5. 10.1371/journal.ppat.1000252

Subbarao, E.K., W. London and B.R. Murphy, 1993. A single amino acid in the PB2 gene of *Influenza A* virus is a determinant of host range. J. Virol., 67: 1761-1764.

Yamada, S., M. Hatta, B.L. Staker, S. Watanabe and M. Imai *et al.*, 2010. Biological and structural characterization of a host-adapting amino acid in *Influenza* virus. PLoS Pathogens, Vol. 6. 10.1371/journal.ppat.1001034