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Novel Gene Mutations in Tunisian Isolate of Avian H9N2 Influenza Virus

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Abstract

A new strain of H9N2 avian influenza virus (AIV) was isolated from suspected broiler flocks and characterized using RT-PCR and sequencing techniques which have shown new interesting mutations as compared to previously characterized Tunisian strains of major clinical importance. Reverse transcription-PCR, nucleotide sequencing, and GenBank BLAST database analyses of external and internal genes of the virus demonstrated that the new isolate, designated A/CK/TUN/145/12, has the ^{333}P**S**RSSR*GLF^{341} motif at the cleavage site of its hemagglutinin (HA), different from that described in the older Tunisian strains, which possess the motif ^{333}P**A**RSSR*GLF^{34}, and others reported strains in the world. The presence of Leu at position 234 in the amino acid sequence of HA indicated the virus binding preference to the human cellular receptor α-2,6 sialic acid. Besides, such HA amino acid sequence showed two new mutations D280N and Y144S. The hemadsorption (HB) site of its neuraminidase (NA) did show three new mutations H441N, N342D and S331N in comparison to older Tunisian strains. Such mutations were reported for the highly pathogenic H5N2 subtype in Nigeria. Phylogenetic data allowed classification of the new Tunisian isolate in a new genetic group including the old Tunisian isolates.

Keywords: Characterization; Epidemiology; Phylogeny; Influenza virus

Abbreviations: AA: amino acid; AIV: Avian Influenza Virus; HA: Hemagglutinin; HB: Hemadsorption; HPAIV: High Pathogenic Avian Influenza Virus; LPAIV: Low Pathogenic Avian Influenza Virus; M: Matrix; NA: Neuraminidase; NP: Nucleoprotein; NS: Non Structural; PB2: Polymerase; PL: PDZ Ligand; RBS: Receptor Binding Site; RBD: RNA Binding Domain.

Introduction

The poultry sector is becoming an increasingly important agriculture sector, in Tunisia, This sector faces tough challenges due to viral infections in the region [1,2]. Influenza A viruses (IAV) belonging to the *Orthomyxoviridae* family, cause highly contagious respiratory diseases in chickens, leading to important economic losses related to mortality, severe egg drop and poor egg quality. The viral genome is a negative stranded RNA, made up of eight gene segments. These viruses have been divided in low pathogenic AI (LPAI) and high pathogenic AI (HPAI), on the basis of their capability to cause mild or severe disease in vulnerable birds, respectively. The subtypes H5, H7 and H9 cause respiratory and systemic diseases. The H9N2 subtype virus may infect chickens, turkeys, ducks and pigs and suspected to infect humans [3].

Phylogenetic analyses showed that avian H9N2 viruses are classified into three diverse groups: G1-like lineage, represented by G1 97, Y280-like lineage, represented by Y280 or A/Chicken/Beijing/1/94 (BJ 94) and Y439-like lineage, represented by A/Duck/Hong Kong/Y439/97 (Y439) [4]. Since 1998, a new common lineage of H9N2 viruses in eastern China is represented by the A/Chicken/Shanghai/F/98 lineage (F 98-like lineage) [5].

In Tunisia, where poultry industry is of great importance for its social and economic impacts, active surveillance of avian H9N2 virus and study of strain evolution is a great importance to effectively control the disease. The epizootiology of AIV in Tunisia is being documented. The disease has been reported since 2009 and the virus is still circulating causing severe economical losses. Thus, efforts are undertaken to better control the disease by isolating and typing AIV field strains that are very important not only for the study of emerging viruses and their evolution but also for adaptation of preventive measures.

Materials and Methods

Virus isolation

The H9N2 avian influenza virus was isolated and identified in 2012 during an avian influenza (AI) outbreak in the south of Tunisia. At least 10 cloacae and 10 trachea swab samples were collected from suspected chickens. Initial isolation was performed in 10-day-old specific-pathogen-free embryonated chicken eggs (ECE, inoculated via the allantoic route and incubated at 37°C for 72-96 h [6].

Hemagglutination inhibition test

Specific anti-H9N2 serum was prepared in chickens vaccinated with inactivated influenza vaccine (Nobilis H9N2-Intervet). Collected serum was titrated before its use in an inhibition hemagglutination test to confirm the identity of the isolated virus. The serum was heat treated at 56°C for 30 min and serially diluted in PBS (pH 7.4) in 96-well plate before adding an equal amount of 4HA units (25 µl) of A/Chicken/Tunisia/145/12 (H9N2) influenza virus in each well. After incubation for 30 min at room temperature, 25 µl of 1% chicken red blood cells were added to each well and the plate incubated for 30 min. A complete inhibition of hemagglutination indicates the identity of the virus tested (Table 1).

Viral nucleic acid extraction

Viral RNA was extracted using 200 µl of virus suspension following the Trizol method (Invitrogen, CA). The RNA was precipitated with

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absolute ethanol, centrifuged and the final pellet suspended in 20 µl RNase-free water then stored at -80°C.

Viral RNA amplification

Two step RT-PCR techniques were carried out to amplify well defined regions of the six gene segments using segment-specific primers. The cDNA was synthesized using appropriate upstream primers with SuperScript™ II Reverse transcriptase (Invitrogen, CA) then amplified by PCR using a mixture made of 5 µl of cDNA, 2.5 µl of 10 × PCR buffer, 2.5 µl of 2.5 mM dNTPs, 0.5 µl Taq DNA polymerase (5 units/µl, Invitrogen), 1 µl of each primer (10 µM each) (Table 2) [7], 2 µl of 5 Mm $MgCl_2$ (Invitrogen, CA) and 17.5 µl RNase free water (BioBasic, CA). The PCR program was run as follows: 45°C for 30 min, 95°C for 10 min, 40 cycles of 95°C for 60 s, 58°C for 20 s (62°C for HA1, NA, PB2, and NS), 72°C for 1 min followed by 72°C for 10 min. The obtained PCR products were purified with QIA quick PCR purification kit (Qiagen).

Sequencing

The purified PCR products were then partially sequenced using Amersham ET Dye terminator kit and analyzed with ABI 3730 DNA sequencer (Perkin-Elmer Applied Biosystems).

Sequencing analyses

The Bio-Edit program 5.0.6 software and the ClustalW alignment algorithm (Version 1.83) were used to compare and align nucleotide sequences. Phylogenetic trees were constructed by MEGA5.01 program, version 3.65, with the neighbor-joining method using a grouping strength of 1000 bootstrap resembling. The Blast software and the Bio-Edit program were utilized to determine the sequence similarity between the Tunisian identified strains. The nucleotide sequences obtained were deposited in the GenBank data library under the accession numbers: KP058446, KP058447, KP271003, KP271004, KP271005 and KP772312. The amino acid residues were numbered according to the HA sequences of Qu/HK/G1/97 (H9) with the GenBank accession number AF156378.

Results

Identification of AI virus isolate

The HA titer of the allantoic liquid of inoculated SPF embryonated chicken eggs was 512HA. The IH test allowed confirmation of the isolated virus as avian Influenza type A virus using a specific anti-H9N2 serum, showing a titer of 512 IH. The virus was further characterized using RT-PCR and sequencing.

Phylogenetic analysis of surface genes of H9N2 virus

To find out its genetic diversity, the isolated virus was amplified by RT-PCR and 6 out of its 8 gene segments were partially sequenced. These sequences were then aligned with different H9 viral sequences listed in the GenBank (Table 3).

The results showed that the viral HA gene was correlated to the Libyan and the Middle Eastern strains, especially A/Chicken/Libya/13VIR7225-5/2013, A/Chicken/Israel/1548/2006, A/Chicken/SaudiArabia582/2005, A/Chicken/Pakistan/47/2003. The HA and NA (neuraminidase) gene sequences showed high homology with Middle Eastern strains grouped in the G1 lineage, sharing the same ancestor with the isolate A/Quail/Hong Kong/G1/1997 (Table 4).

To study the evolutionary relationships between the new and the old Tunisian H9N2 isolates found in the Genbank (Table 3), phylogenetic analyses were carried out for the 6 considered viral gene segments (hemagglutinin (HA), neuraminidase (NA), polymerase (PB2), nucleoprotein (NP), matrix (M) and non structural (NS) genes) (Figures 1 and 2). The HA amino acid sequence of the newly identified strain A/Chicken/Tunisia/145/12 showed 95% to 99% concordance with that of other strains of the G1 lineage. Besides, the neuraminidase gene of this isolate (nucleotides 962 to 1372) clustered with the A/Qa/

Virus Name	Abbreviation	Host	HA titre	EID50/100 µl
A/Chicken/Tunisia/145/12	A/CK/TUN/145/12	Chicken	1/1024	$10^{6.75}$

Table 1: H9N2 virus isolated from infected chickens in Tunisia 2012.

Name	Sequence (5'- 3') a	Position	Expected products Size (pb)	Gene	Reference
AMF	CTTCTAACCGAGGTCGAAAC	7- 26	244	M	
AMR	AGGGCATTTTGGACAAAKCGTCTA	259-238		M	20
MF	CTCATGGAATGGCTAAAGACA	149-169	700	M	
MR	CGATCAADAATCCACAATATC	847-827		M	22
H9F	GAATCCAGATCTTTCCAGAC	426-445	384	HA	
H9R	CCATACCATGGGGCAATTAG	808-789		HA	23
NPF	CAGRTACTGGGCHATAAGRAC	1200-1220	326	NP	
NPR	GCA TTGTCTCCGAAGAAATAAG	1529-1510		NP	24
HAF1	GAATTGATTATTATTGGTCAGTA	710-732	550	HA	
HAR1	TCATCAATCT-TATTGTTGATCAT	1272-1249		HA	25
NAF	CTTGTTGGCGACACACCAAGRAA	961-983	410	NA	
NAR	GAGCCTGTTCCAT-AGGTACCTGA	1370-1348		NA	25
PB2F	TATTCAT-CRTCAATGATGTGGGA	1591-1613	540	PB2	
PB2R	GATGCTYAATGCTGGTCCATATC	2130-2108		PB2	25
NSF	AGCAAAAGCAGGGTGACAAA	1-20	890	NS	
NSR	AGTAGAAACAAGGGTGTTTT	890-871		NS	26

[a]Codes for mixed bases positions: D=G/A/T, H=A/C/T, K=G/T, R=A/G, Y=C/T.

Table 2: Primer sequences as used in the RT-PCR.

References strains	Abbreviations	HA	NA	PB2	NP	M	NS
A/Chicken/Tunisia/145/12[a]	Ck/TUN/145/12	KP058446	KP058447	KP271005	KP271003	KP271004	KP772312
A/chicken/Tunisia/848/2011	Ck/TUN/848/11	JQ952591.1	JQ952595.1	-	-	-	-
A/turkey/Tunisia/2068/2010	Ck/TUN/2068/2010	JQ952589.1	JQ952593.1	-	-	-	-
A/chicken/Tunisia/2019/2010	Ck/TUN/2019/2010	JQ952588.1	JQ952592.1	-	-	-	-
A/chicken/Tunisia/345/2011	Ck/TUN/345/2011	JQ952590.1	JQ952594.1	-	-	-	-
A/migratory bird/Tunisia/51/2010	MB/TUN/51/10	JF323007.2	JF323009.2	JF323011.2	JF323013.2	JF323015.1	KF751661.1
A/chicken/Tunisia/12/2010	Ck/TUN/12/2010	JF323006.2	JF323008.2	JF323010.2	JF323012.2	JF323014.1	JF323016.1
A/chicken/Libya/13VIR7225-5/2013	CkLi13VIR7225513	KM244121.1	-	-	-	-	-
A/chicken/Israel/1548/2006	CkIs14806	FJ464729.1	-	-	-	FJ464611.1	-
A/chicken/Saudi Arabia/582/2005	AvSA58205	JX273556.1	-	-	-	-	-
A/avian/SaudiArabia/910135/2006	AvSA91013506	GU050287.1	-	GU050294.1	-	GU050288.1	GU050291.1
A/chicken/Pakistan/47/2003	CKPa4703	JX273552.1	-	-	-	-	-
A/chicken/Israel/182/2008	CkIs18208	GQ120549.1	-	-	-	-	-
A/chicken/Egypt/D4907A/2012	CkEgD4907A12	JX912984.1	-	-	-	KF881678.1	-
A/quail/UnitedArabEmirates/1819/2006	CkEmR181906	-	F188376.1	-	-	-	-
A/chicken/Dubai/339/2001	CkDu33901	-	KF188354.1	EF063556.1	-	-	EF063542.1
A/chicken/Dubai/383/2002	CkDu38302	-	EF063522.1	EF063557.1	-	-	EF063543.1
A/chicken/Dubai/339/2001	CkDu33901	-	EF063521.1	KF188349.1	-	-	EF063542.1
A/Hong Kong/1074/1997	QuHKG197	AF156378	GU053180.1	AJ289872.1	AF255743.1	-	-

[a]Viruses whose HA, NA, PB2, NP, M and NS genes were sequenced in the present study; N.D, not done; b, - No sequence data available

Table 3: Abbreviations used and GenBank accession numbers for H9N2 Avian Influenza virus isolates included in the phylogenetic analyses.

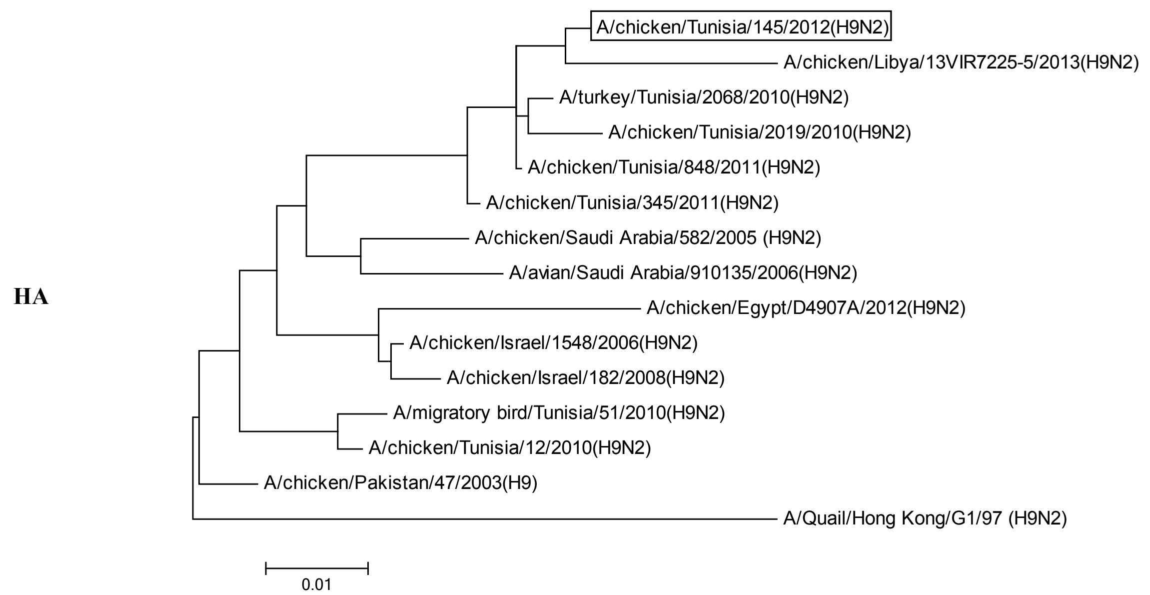

Figure 1: Phylogenetic Analysis of HA gene (878 bp from 392 to 1270) of Tunisian H9N2 virus and established Middle Eastern strains. The Tunisian isolate is framed.

HK/G1/97 lineage, as did isolates from Dubai, Hong Kong and United Arab Emirates.

Phylogenetic analyses of internal genes

Four internal genes (PB2, NP, M and NS) of the Tunisian H9N2 strain showed more than 91% nucleotide similarity with those of the Middle-Eastern strains isolated in 1997 and 2011 (Table 4). Phylogenetic studies of PB2 gene, using a 540- base-long nucleotide sequence (nucleotides 1591 to 2130) that codes the PB2 protein region between positions 530 and 710, showed a close similarity between the A/Ck/TUN/145/12 and the old Tunisian strains which have close relationship with the Middle Eastern isolates (more than 93% identity) (Figure 3).

Analysis of NP gene of A/Ck/TUN/145/12 allowed its classification in the same genetic group as the old Tunisian genotypes (Figure 4), showing a percentage of similarity of 98 to 97% with A/Hong Kong/1074/99 (H9N2) and A/chicken/China/27402/1997 (H5N1), respectively (Table 4).

The matrix gene (regions of the overlapping reading frames of M1-M2, nucleotides 7 to 845) of A/Ck/TUN/145/12 strain (Figure 5)

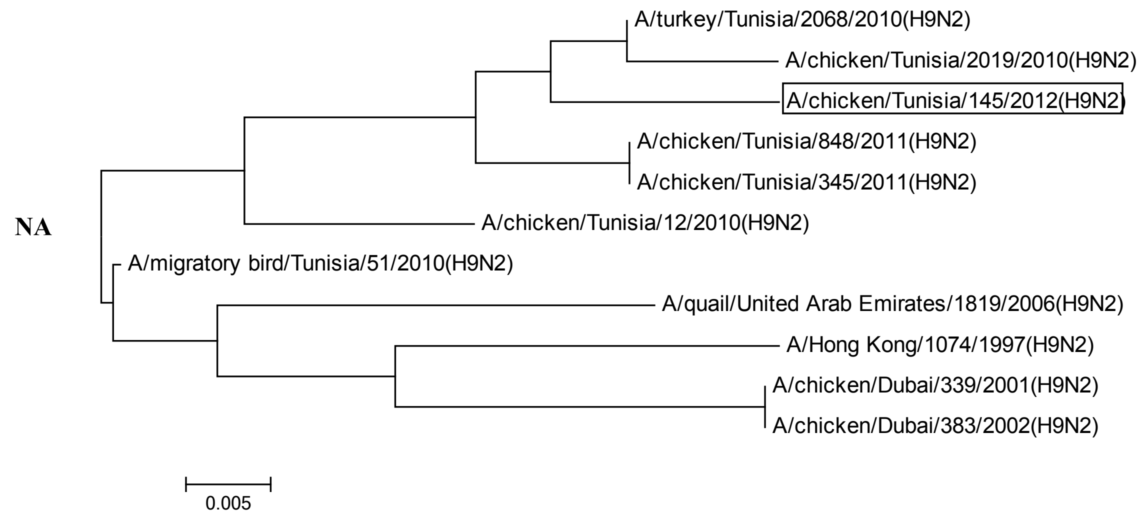

Figure 2: Phylogenetic Analysis of NA gene (410 bp from 962 to 1372) of Tunisian H9N2 virus and established Middle Eastern strains. The Tunisian isolate is framed.

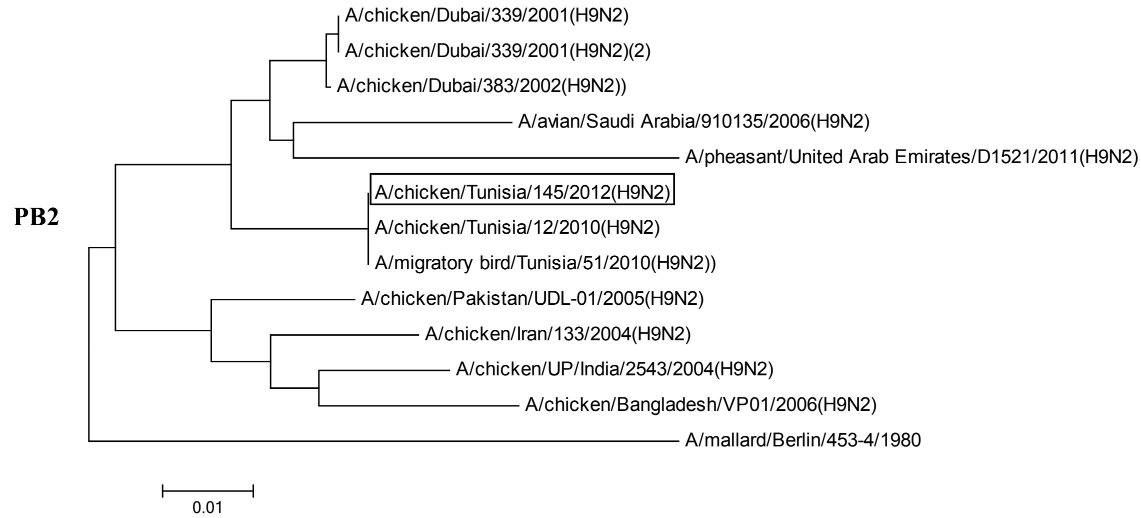

Figure 3: Phylogenetic Analysis of PB2 gene (540 bp from 1591 to 2130) of Tunisian H9N2 virus and established Middle Eastern strains. The Tunisian isolate is framed.

showed a close relationship with that of Middle Eastern isolates (96% to 98% similarity) and full identity with the Tunisian isolates previously identified. It has also demonstrated 96.93% and 96% similarities with other subtypes like A/Ck/KHNC/100/04 (H7N3) and A/Environment/Hong Kong/258/1997 (H5N1) strains, respectively.

The NS gene (nucleotides 1591 to 2130) of Ck/TUN/145/12 strain demonstrated an evidence of a reassortment with other viral subtypes, showing 96% similarity with that of A/mallard/Sweden/3240/2003(H8N4) strain (Figure 6).

Genetic characteristics of surface glycoproteins

Hemagglutinin: The molecular determinants of pathogenicity and virulence of influenza virus are the HA1/HA2 connecting (cleavage site) polypeptide sequence, the specific amino acid (aa) residues at the receptor binding site (RBS), and the presence or absence of glycosylation sites around the receptor binding site.

The HA cleavage site motif of the sequence of our isolate A/Ck/TUN/145/12 was ^{333}PSRSSR* GLF^{341}. Interestingly, this motif is different from that of other Tunisian H9N2 and Asian H9N2 viruses including those described in Libya, Israel, Pakistan, Saudi Arabia and Egypt which have the motif ^{333} PARSSR* GLF^{341}, meaning that our Tunisian isolate carries amino acid substitution in the cleavage site at position A334S.

Residues at positions 110, 161, 163, 191, 198, 234, 235 and 236 are known to be the major components of the receptor binding site of HA molecule [6]. Our isolate showed conservation of residues P110, W161, T163, H191, A198 and I235 in the receptor binding pocket in comparison with those of the old Tunisian isolates and other isolates from all over the world. However, the left edge (amino acid residues at

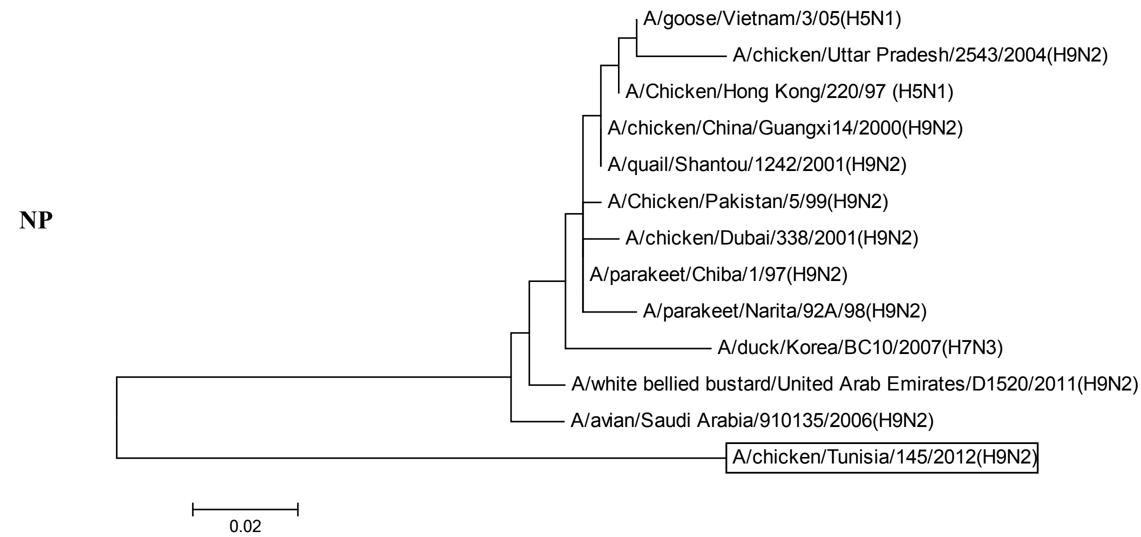

Figure 4: Phylogenetic Analysis of NP gene (326 bp from 1200 to 1529) of Tunisian H9N2 virus and established Middle Eastern strains. The Tunisian isolate is framed.

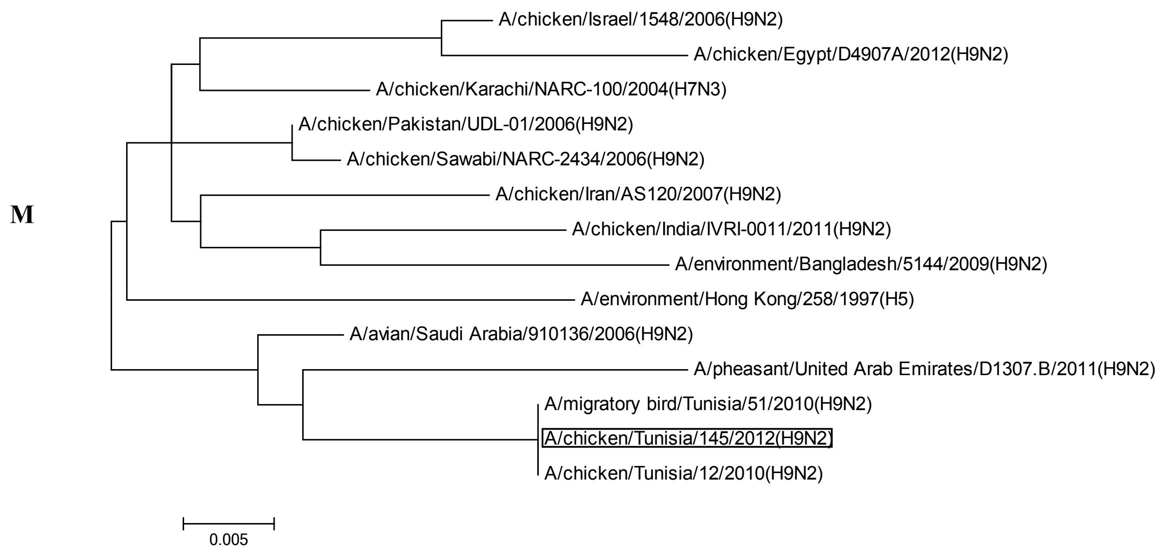

Figure 5: Phylogenetic Analysis of M gene (840 bp from 7 to 847) of Tunisian H9N2 virus and established Middle Eastern strains. The Tunisian isolate is framed.

positions 232-236) of the binding pocket motif was: NGLIG and the A/CK/TUN/145/12 H9N2 strain carried the amino acid substitutions (Y144S) and (D280N). The Y144 and D280 residues are shown to be engaged in both receptor binding and ligand interaction. Receptor mutations at this position (Y144S) showed dramatic impact on binding affinity and functionality [8].

Analysis of HA protein sequences indicated that the Tunisian H9N2 isolate has many potential glycosylation sites with an N-X-T/S motif (X can be any amino acid except Proline). In fact, X could be an S (Serine) at position 298-300 (NST) or any other substitutions as indicated in Table 5.

HB site of Neuraminidase: The HB site of neuraminidase is situated on the NA surface, far from the enzymatic site. Analysis of the neuraminidase and the framework sites of NA protein showed mutations in aa residues in the 3 loops that interact directly with the sialic acid. In the loop carrying amino acids at positions 367-370-372, three S (Serine) were substituted by KLA, respectively; the new substitutions H441N, N342D and S331N were also found. On the other hand, the framework site contained R371, A372, N402, and E425 (Table 6). The Tunisian isolate presented the same mutations found in previously characterized Tunisian strains except for the three new mutations (H441N, N342D and S331N).

Molecular characteristics of internal proteins: The novel Tunisian H9N2 strain has shown the same PB2, M, NP and NS protein sequences as the Tunisian isolates previously identified showing the same described mutations [9].

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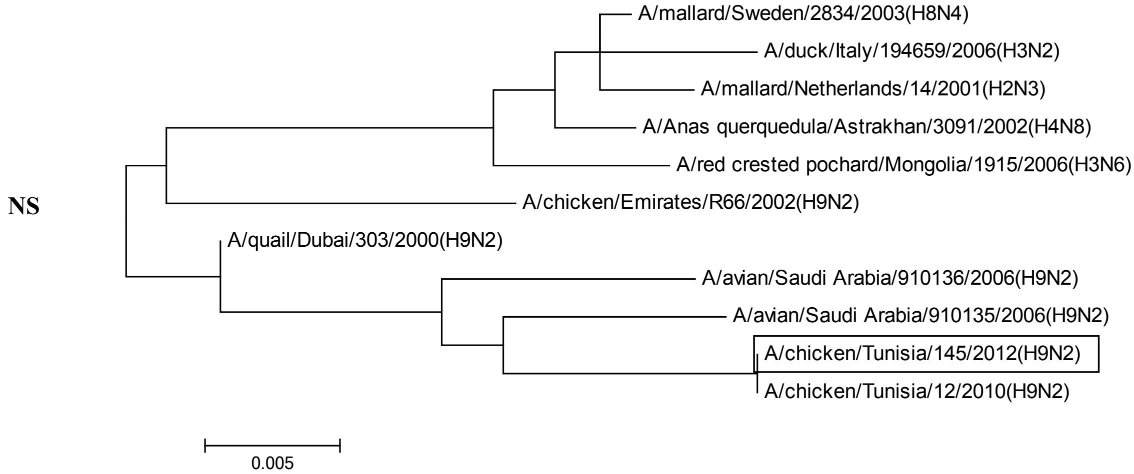

Figure 6: Phylogenetic Analysis of NS gene (890 bp from 1 to 890) of Tunisian H9N2 virus and established Middle Eastern strains. The Tunisian isolate is framed.

Gene	Nucleotide similarity	%	Lineage
PB2	A/chicken/Tunisia/12/2010(H9N2) A/chicken/Dubai/338/2001(H9N2) A/chicken/Dubai/383/2002 (H9N2) A/quail/Dubai/303/2000(H9N2) A/chicken/Pakistan/UDL-01/2005(H9N2) A/chicken/Iran/68/2006(H9N2) A/environment/Bangladesh/8463/2010(H9N2)	100 98 97 96 95 94 93	G1
HA	A/chicken/Tunisia/848/2011(H9N2) A/chicken/Libya/13VIR7225-5/2013(H9N2) A/avian/Libya/RV35D/2006 (H9N2) A/chicken/Israel/1548/2006 (H9N2) A/chicken/Pakistan/47/2003 (H9N2)	99 98 97 96 95	G1
NP	A/chicken/Tunisia/12/2010(H9N2) A/Hong Kong/1074/99(H9N2) A/chicken/China/27402/1997(H5N1)	100 98 97	G1
NA	A/chicken/Tunisia/2019/2010(H9N2) A/chicken/Tunisia/848/2011(H9N2) A/chicken/Tunisia/12/2010(H9N2) A/migratory bird/Tunisia/51/2010 (H9N2) A/quail/United Arab Emirates/1819/2006 (H9N2) A/white bellied bustard/United Arab Emirates/D1520/2011 A/quail/Shantou/1912/2001 A/Hong Kong/1074/1997(H9N2) A/quail/Shantou/308/2003 (H9N2) A/chicken/Iran/450/2001(H9N2)	99 98 96 95 94 93 92 92 91 91	G1
M	A/migratory bird/Tunisia/51/2010(H9N2) A/chicken/Tunisia/12/2010(H9N2) A/avian/Saudi Arabia/910135/2006(H9N2) A/chicken/Karachi/NARC-100/2004(H7N3) A/chicken/India/IVRI-0011/2011(H9N2)	100 100 98 97 96	G1
NS	A/chicken/Tunisia/12/2010(H9N2) A/avian/Saudi Arabia/910135/2006(H9N2) A/chicken/Emirates/R66/2002(H9N2) A/mallard/Sweden/3240/2003(H8N4)	100 98 97 96	G1

Table 4: Similarity indices of the Tunisian H9N2 (A/CK/TUN/145/12) genes at the nucleotide levels.

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Virus	RBS 110	RBS 161	RBS 163	RBS 191	RBS 198	RBS 202	RBS 203	Left-edge of Binding pocket 232-236	Connecting peptide aa sequence 152 333	Glycosylation site at position 338 168
A/Ck/TUN/145/12	P	W	T	H	**A**	L	Y	N**G** L **I**G	P **S** R S S R	+
A/Ck/TUN/12/2010	P	W	T	H	A	L	Y	NG L IG	P **A** R S S R	+
A/Ck/TUN/345/2011	P	W	T	H	A	L	Y	NG L IG	P A R S S R	+
A/Tu/TUN/2068/2010	P	W	T	H	A	L	Y	NG L IG	P A R S S R	+
A/Av/Libya/13VIR7225-5/2013	P	W	T	H	**A**	L	Y	NG L IG	P **S K** S S R	+
A/Ck/Israel/1548/2006	P	W	T	H	**T**	L	Y	NG L IG	P **A R** S S R	+
A/Av/SA/582/2005	P	W	T	H	T	L	Y	NG L IG	P A R S S R	+
A/Av/SA/910135/2006	P	W	T	H	**A**	L	Y	NG L IG	P A R S S R	+
A/Qu/HK/G1/97	P	W	T	H	**E**	L	Y	N**D** L**Q**G	P A R S S R	+
A/CK/TUN/848/2011	P	W	T	H	**A**	L	Y	N G L IG	P A R S S R	+
A/CK/TUN/51/2010	P	W	T	H	A	L	Y	N**GQ**IG	PA R S S R	+
A/Ck/Pak/47/2003	P	W	T	H	A	L	Y	NG L IG	P A R S S R	+
A/Ck/Is/182/2008	P	W	T	H	T	L	Y	NG L IG	P A R S S R	+
A/Ck/Eg/D4907A/2012	P	W	T	H	A	L	Y	NG L IG	P A R S S R	+

-/+ absence or presence of the glycosylation site.

Table 5: Analysis of the amino acid sequences of the HA protein of the Tunisian isolate A/Ck/TUN/145/12 in comparison with old Tunisian and reference strains.

Virus	Neuraminidase active site (HB) 366. 373	Framework Site NA 399. 406	Framework Site NA 431. 433	Framework Site NA 425
A/Ck/TUN/145/2012	IK**KDL**RAG	DSD**NW**SG**Y**	P**K**E	**E**
A/Ck/TUN/2019/2010	IKKDLRAG	DSDNWSGY	PKE	E
A/Tu/TUN/2068/2010	IKKDLRAG	DSDNWSGY	PKE	E
A/Ck/TUN/848/2011	IKKDLRAG	D**R**D**D**WSGY	PKE	E
A/Ck/TUN/345/2011	IKKDLRAG	DRDDWSGY	PKE	E
A/Ck/TUN/12/2010	IKKDLRAG	DSD**NW**SG**Y**	-	-
A/MB/TUN/51/2010	IKKDLRAG	DSDNWSGY	PQE	E
A/Ck/Du/339/2001	IKKDLRAG	DS - - - - - -	PQE	E
A/Ck /Du/338/2002	IKKDLRAG	DSDNWSGY	PQE	-
A/Ck/Em/1819/2006	IK**E**DLRAG	DSDNWSGY	PQE	E
A/Ck/HK/1074/1997	IK**KDS**RAG	DSDNWSGY	PQE	E

(-)=gap

Table 6: Analysis of the amino acid sequences of the NA protein of the Tunisian isolate in comparison with older Tunisian and reference strains.

Discussion

The study of the six genes of Tunisian H9N2 subtype of avian influenza reported, for the first time, new mutations that are not found in previously described Tunisian strains. Considering the sequences and the phylogeny analyses of Middle Eastern and older Tunisian viruses, it appeared that they are closely related and represent a single sub-lineage, the G1-like lineage, indicating similar origin. It might reflect geographical parameters responsible of virus restriction in these areas.

The genetic data of H9N2 subtype circulating in Tunisian poultry farms are presented. The results revealed that H9N2 virus infection is well established in many endemic areas of the country and allowed the characterization of our strain from a flock in southern of Tunisia. Thus, understanding the genetic and the biological characteristics of circulating H9N2 virus can give more comprehensive vision on the biology and the ecology of H9N2 virus, and the capacity of migratory birds to disperse AI viruses. Although infected birds might be able to spread the virus over short distances, during periods of cold weather, experiments in which birds are subjected to physiologic stresses associated with migration are needed to determine their capacity to spread virus over long distances. Indirect estimations of virus dispersal derived from knowledge of bird migrations could also provide complementary information related to the spread of avian influenza viruses [10].

The Blast analysis (NCBI) of the nucleotide sequences of HA and NA genes showed that A/Ck/TUN/145/12 is the closest strain (more than 92% identity) to the Middle Eastern isolates belonging to the G1-like lineage of H9N2 subtype.

The results of phylogenetic analyses were totally in accordance with blast data, and confirmed that our isolate fall, along the Middle Eastern isolates, into one cluster in relation to the G1 lineage; a result that may indicate that both strains have the same origin.

Based on the deduced amino acid sequences, the HA1-HA2 connecting peptides of the Tunisian H9N2 isolate did not harbor multiple basic amino acids with the motif PSRSSR/GL which is not exactly as found for the newly isolated H9 viruses in Middle East, having the motif PARSSR/GL [11,12]. A new substitution of Alanine, a non polar amino acid, by Serine, a polar amino acid, at position 334 is noticed. The biological significance and the role of such substitution are not yet known. This may reveal the LPAI nature of H9N2 strain, despite a motif identical to the RX RYK-R required for the highly pathogenic H5 and H7 subtypes [13]; noting that such new mutation was also found in highly pathogenic H5N2 subtype in Nigeria [14].

These genetic results indicated that our H9N2 virus may have the capacity to acquire basic amino acids in HA connecting peptide sequence, required to become highly pathogenic through the addition of a single basic amino acid. Moreover, A/Ck/TUN/145/12 possesses a Leu (L) at position 234 in its HA1 portion; the receptor binding site (RBS) residue being essential for the transmission of the H9N2 viruses in ferrets [13,15,16]. Besides, Q234L substitution, found in G1 lineage isolated in Hong Kong, was shown to allow H9N2 viruses to infect non ciliated cells and to grow more efficiently in human airway epithelial cell cultures, resulting in the increase of the severity of human infection [13].

Antigenic and phylogenetic analyses of the Tunisian isolate demonstrated that its surface glycoproteins are related to those of A/Qa/HK/G1/97 lineage, with the highest homology with A/chicken/Libya/13VIR7225-5/2013(H9N2) (98%), A/chicken/Israel/1548/2006 (96%) and A/chicken/Pakistan/47/2003 (H9N2) (95%) viruses. The two new mutations D280N and Y144S in HA gene and their significance are not yet known.

Three new mutations H441N, N342D and S331N are found in NA gene. The N342D mutation was found in the NA gene of H1N1 and H3N2 avian influenza viruses in Japan [14,15]. This may indicate the capacity of H9N2 virus to pass to humans. Besides, a potential additional glycosylation site was discovered at position 331 of NA gene in A/CK/TUN/145/2012 (S331N mutation); such mutation was also detected in H5N2 Nigerian isolate in 2007 [12]. The significance of H441N mutation is not known, yet. Therefore, all these new mutations may contribute to the change of a low pathogenic to a high pathogenic avian influenza H9N2 virus.

The Q432K substitution in NA gene is similar to that observed in other identified Tunisian strains. But, the biological meaning of this mutation is not known. All other mutations found in previous Tunisian strains are absent in A/CK/TUN/145/2012 isolate.

The E627K mutation in PB2 gene contributes to higher polymerase activity of influenza virus [4]. The PB2 E627K mutation has shown, *in vitro*, a promoting effect on virus growth in mammalian but not in avian cells [16].

The M1 protein showed a V15I substitution in all H9N2 lineages [17]. When the complete genome phenotypes of high-pathogenic strains were compared to those of low pathogenic ones, 5 amino acid differences were found and correlated with high-pathogenic strain phenotype. One of these changes was seen in M1 (V15I) of our strain, a change that has also been found in PR34 but not in Brevig18 strains [18].

Recently, NS1 protein of avian influenza A viral was shown to be a type I interferon (IFN) antagonist which plays a major role in viral pathogenesis [19]. Molecular analysis demonstrated that our isolates contain a NS1 protein with 230aa in length, typical of H9N2 viruses. In its RNA- binding domain of NS1, A/Ck/TUN/145/2012 isolate has incorporated R38 and K41 amino acids, which are shown to be critical for RNA binding. Similarly, amino acid residues P31, D34, R35, G45, R46, T49 and D55 also mediate NS1-dsRNA interaction and residue D55 is situated within the third alpha- helix (residues 54-70) of the dsRNA-binding domain RBD (residues 1-73) of NS1 [20]. However, NS1 D55G may stabilize the coiled-coiled helical structure. The old Ck/TUN/12/2010 Tunisian strain showed an Asp (N) at position 217 which is also found in our A/CK/TUN/145/12 strain but differs from other H9N2 strains, showing a K at this position. The biological significance of this substitution is not known yet. However, the Ck/TUN/145/2012 isolate didn't show the five amino acid deletion (80TIAS84) already described for avian strains isolated in 2001 in Hong Kong; their significance being still not understood [21]. Finally, our H9N2 Tunisian strain showed a PL motif "GSEV" as previously found in Tunisian strains isolated in 2010; the biological signification of this motif is not yet known [22]. Interestingly, the E227G mutation in NS1 introduces an S70I mutation into nuclear export protein.

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Disclosure

There is no conflict of interest.

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