3rd International Conference on Green Energy and Environmental Engineering (GEEE-2016)
Proceedings of Engineering & Technology (PET)
p. 140-145

Molecular epidemiology of influenza virus in Tunisia

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***Abstract*— Influenza is an infectious viral disease, which evolves in epidemic modal and causes thousands of deaths each year.**
The virus of influenza A is easily transmitted between species including the humans.
Its genome is still evolving. It is therefore essential to study the epidemiology and molecular evolution of this viral family to monitor genetic variations and detect the possible emergence of new mutant strains.
We collected all the protein sequences of virus strains isolated in Tunisia from 2009 to 2014 using data available in the public databases. Its sequences were compared with previously isolated sequences in nearby countries geographically of Tunisia. We did phylogenetic analyzes using Bayesian statistical approach, assuming a relaxed molecular clock model and a Bayesian skyline demographic model in coalescent analyses with the BEAST program.
Phylogenetic analyses identified three distinct populations and different subpopulations. This suggests a strong similarity between the virus strains in circulation in Tunisia, and only emerging in nearby countries. Phylogenies including sequences other than isolated in Tunisia, showed close genetic relationships among different viral subtypes studied. Phylogenies can provide reliable data of incidence in the types and subtypes virus in the country. We could identify patterns that seem to be related to the virulence of this virus family. The presence of conserved motifs in the sequences emerging and circulating strains suggests a relationship between these conserved motifs and viral pathogenicity.
These specific patterns can be targeted for drug design or in the context of the curative treatment such as the manufacture of vaccines to prevent and combat the flu.

***Keywords*— Influenzavirus- phylogeny- bayesien analysis, BEAST.**

I. INTRODUCTION

The causative agent of influenza is a respiratory virus, *Orthomyxoviridae* family, and influenza type. Influenza viruses are divided into three types: A, B and C. The influenza virus A is the best known, it has been isolated from different animal species, including mammals and birds, and has a potentially high pathogenicity.

It is responsible for outbreaks that occur seasonally, and infects hundreds of millions of people worldwide over the world every year [1]. Wild birds are the natural reservoir for influenza virus A. These viruses are usually not pathogenic for wild birds, but can result in significant morbidity and mortality in domestic poultry and occasionally humans [2, 3].
Influenza A viruses are classified into subtypes according to the antigenic properties of their two surface glycoproteins: the HA and NA. Sixteen antigenic types of HA and nine types of NA were isolated from wild water birds. [4]
Currently, two other antigenic types of HA and NA have been isolated from bats in the New World and classified H17, H18 and N10, N11 [5]. In Tunisia, every year the flu season occurs mainly during the winter months, in conjunction with the decrease in temperature.
In 2009, Tunisia has recognized the pandemic A / h1n1 pdm09 that was emerging in the world. Since, over the past five decades, the number of annual deaths attributable to influenza ranges from 1 to 29, with 29 for the 2009/2010 season, 13 for 2010/2011, one for 2011/2012, 22 for 2012/2013, 1 for 2013/2014 and 8 for 2014/2015 [6].
Recently, through the first week of April 2016, 30 cases of deaths from seasonal flu have been recorded in Tunisia, including 24 deaths due to the H1N1 virus [7]. Note that no pandemic was recorded in Tunisia since 2009 pandemic.
The influenza A virus is an enveloped virus segmented RNA [8,9] in eight gene segments that encode at least 11 viral proteins [10].
Previous studies have shown that several segments of the genome are believed had important roles during infection and replication [11-15], thus supporting the pathogenicity [15-17] virus. Two membrane glycoproteins: hemagglutinin HA and neuraminidase NA are used for classification of influenza virus and contribute to the genetic variability of influenza virus. According to HA and NA, several combinations are possible giving rise to different sub viral types.
The new subtypes of the virus cause annually considerable economic burden worldwide and the significant morbidity and cumulative mortality [18-20]. Given its characteristics described above, it is clear that knowledge of epidemiological and virological characteristics and mode of movement of virus is important to strengthen and develop control strategies and surveillance against influenza. Identification and characterization of virulence determinants of influenza A

ISSN: 2356-5608

virus, can provide insight genotypic signatures pathogenicity and a deeper understanding of the factors that give rise to pandemics.

In this study we performed a phylogenetic analysis of influenza strains isolated in Tunisia between 2009 and 2014, which included the 2009 pandemic.

We have included the complete sequences of the proteins belonging to the same viral family and isolated in nearby countries Tunisia and the reference sequence A / California / 07/2009, responsible for pandemic H1N1 2009.

Influenza viruses continually changing the origin of their antigenicity by point mutations [21].

Previous studies have suggested that recombination is largely involved in the virus strains of diversity, and virus strains of avian origin, circulating in Tunisia represent multiple reassortant strains with genes from Middle East strains.

The viral sub-type H9N2, isolated in Tunisia in migratory birds as well as in the domestic chicken, was characterized as a low pathogenic. But some internal genes appeared to have undergone extensive reassortment with other viral subtypes. Indeed viral strains circulating in Tunisia have recognized permanently substitutions and mutations, leading to their genetic variability. This diversity can be explained by an increased spread of these viruses following the migration of the avian species. Indeed, wild birds are the natural hosts of influenza virus A. Regularly, these viruses infect domestic birds, and the passage of the virus in wildlife industrial poultry is accompanied by the development of virulence in new hosts.

Monitoring is needed to prevent transmission to humans. In this respect, we compared them to those of Tunisia strains detected in circulation in the abovementioned countries. The study of molecular variation of circulating strains is of importance to the activation and strengthening of the monitoring system against the flu.

II. Materials and Methods

A. Collection of biological data

The protein sequences of influenza virus A isolated in Tunisia from 2009 to 2014 were collected from the data available in public databases. We searched through the interface of NCBI [22]. Only complete sequences were retained except a sequence which has been isolated recently (2014). The reference sequence A / California / 07/2009, responsible for the pandemic H1N1 2009, was added to achieve comparability. Besides the isolated protein sequences in Tunisia, we have included the full sequences protein of influenza A, isolated in four countries geographically closer to Tunisia, namely Algeria, Libya, Morocco and Egypt.

B. Multiple sequence alignment

The multiple alignment of all sequences was performed using Clustalw [23] version 2.1. Multiple alignments were performed to characterize the potential areas conserved and variable for each sequence group, as well as consensus sequences. Conserved regions provide information on the function and structure of a protein.

C. Phylogenetic analyses of protein sequences

The different data sets have been subjected to various exploratory analyzes until due phylogenetic analyzes were performed. Various surveys were conducted to describe the evolutionary relationships among the Tunisian viral strains.

A Sampling method (Monte Carlo Markov Chain (MCMC)) implemented in a Bayesian statistical framework in the BEAST v1.8.2 [24] program was used to estimate the phylogeny of the studied sequences.

Three molecular clock models were compared in a coalescing approach: a strict molecular model and two models of relaxed molecular clock assuming either uncorrelated lognormal or uncorrelated exponential priors distributions of substitution rates among lineage. For each model, seven models of demographic history were analysed: constant size, exponential growth (growth rate and doubling time), logistic growth, expansion growth, Bayesian skyline constant and , Bayesian skyline linear modal. Thus, 21 different analyses were performed to deduce the most adopted model.

Parameter estimates were obtained from runs consisting in 10 million MCMC iterations depending on the dataset

analyzed. The program default priors on the substitution model parameters were used in these analyses. For most analyses 10000 trees were sampled from the posterior distribution (one every 1000 generations). The trees were produced with FigTree v.1.4.2 [25].

D. Search conserved patterns

All the recovered and prepared protein sequences, and the same software [26] Version 4.11.1 [27] were used for pattern matching retained for all considered groups of sequences.

MEME (Multiple EM for Motif Elicitation)) is a tool that allows users to discover patterns, at several levels and without gaps, in a group of related nucleic or protein sequences [28] using the *expectation-maximization* (EM) algorithm. Only the most significant results have been preserved.

III. Results and Discussion

A. Data collection and preparation of the sequences

The protein sequences of virus strains isolated in Tunisia from 2009 to 2014 were collected by searching through the NCBI interface.

A total of 112 sequences isolated from four different species (human, porcine, chicken domestic and migratory bird), were found. A selection of the complete sequences allowed the retention of 19 (n = 19) sequences. the complete sequences of the proteins belonging to the reference strain California pdmH1N1 2009 were also harvested from protein database of the NCBI interface (n = 10). By adding the full protein sequences influenza A virus isolated in four other countries besides Tunisia: Algeria, Morocco, Libya and Egypt, a total of (n = 55) sequences was used in this study.

These sequences are distributed in 10 different countries. They belong to four different viral subtypes. The features of sequences and viral subtypes studied are shown in Table 1. Multiple alignments are a preliminary step which serves to highlight the similarity between the sequences of interest. It illustrates the conserved and variable sites in a family of sequences. Multiple alignments were performed by the software Clustalw.

ISSN: 2356-5608

B- Phylogeny results

Molecular clock and demographic model selection

The multiple sequence alignment was subjected to Bayesian coalescent analyses to investigate which molecular clock model (three models tested) and population coalescent priors (seven models tested) fit the sequence data.

The efficient convergence of the MCMC analyses to a stationary distribution for the parameters estimated was obtained in all analyses with the exception of those assuming either a logistic growth or an expansion demographic model.

After several tests, the strict model of the molecular clock was strongly rejected for all studied data.

Furthermore, the relaxed molecular clock model assuming an exponential distribution uncorrelated substitution rate better than the model relaxed molecular clock with uncorrelated lognormal distribution. In all the analyses assuming the best-fitting model for the molecular clock, the Bayesian skyline plot model performed better than the other models tested for estimating the demographic patterns.

The estimated tree topology depicted two main virus clusters (labelled C1 and C2 in Fig.1) and different subgroups. The sequences included within cluster C1 were distributed in clusters S1 and S2. Those included within cluster S2 were distributed in clusters S2-I and S2-II. The sequences included within cluster C2 were distributed in clusters SC2-I and SC2-II. The phylogenetic relationships between taxa within the various clusters were indicative that they were closely related to each other. Most recent virus isolated in Tunisia (AMK09409_2014) was included between the reference sequence AFM72836_A/California/07/200909 and an old isolated sequence in Algeria (ACF22135.1_Algiers_1972).

Tunisian virus sequences are well dispersed on different clusters primarily S2-II, S1 and SC2-II. This clustering reveals the circulation of the same virus subtypes in Tunisia since 2009.

The mosaic distribution of Tunisian sequences of avian origin between different clusters (AHA38674_A_migratory bird, AHA38672_A_chiken..) suggests the ease of transmission of avian influenza from wild birds to domestic species and then the possibility of transmission to humans, which can cause epidemics and serious pandemics, hence the need for active and continuous monitoring of avian flu. In recent years, continues to appear in H9 avian infections and causing a potential threat to poultry and human health.

California sequences are also well dispersed on the sets of groups and subgroups. This suggests the close phylogenetic relationships between the different circulating viral strains, and the ease of possible emergence of new strains.

Phylogenetic trees give much information on the evolutionary relationships among species. The clustarisation can help in the detection of conserved motifs in protein sequence other than HA and NA used in conventional classification viral strains. The conserved regions may have a relationship with the virulence of viral strains, and may have an important role in the synthesis of new antiviral treatments and vaccines for Seasonal Flu.

The significance of our study is to show the strong evolutionary relationships among viral strains of influenza circulating between Tunisia and neighboring countries. The search for conserved domains may be responsible for the pathogenicity of the virus strains, can help the classification of newly emerging strains that can cause widespread pandemics, early detection of these strains helps prevent transmission between species, and thus prevent its transmission to humans.

C- Search Results conserved motifs

The set of sequences subjected to the multiple alignments, were used as input to the same server, to detect regions conserved in these sequences. The conserved motif is associated with a clearly defined function.

MEME uses statistical modelling techniques to automatically choose the best width, number of occurrences, and description for each pattern. Given that MEME output gives us the number of units required by the imposed parameters, we then changed the settings several times to obtain reliable results.

We opted for a pattern of length between 10 and 50, the number of repeat unit has not been set (any number of recurrence) .The results are as sequences and Logo.

The analysis of the MEME result revealed that there were ten conserved motifs in most sequences.

Three motifs were reasonable and show the best and most significant distribution among all groups of sequences.

These patterns in logo format and the consensus sequences (best possible match) are presented in Table 2 and Figure 2.

Pattern 1 was stored in 20 sequences, including two viral proteins HA and M1, and distributed in different countries surveyed namely Tunisia (11 conservations), Algeria (2 conservations), California (2 conservations), Egypt (3 conservations), Libya and Morocco (1 conservation for each).

The pattern 2 and 3 are each observed in all 13 sequences corresponding to the HA with a distribution on the various aforementioned countries.

Previous studies have shown that HA and NA addition, other proteins namely PA, M1 and M2 are critical in the virus host cell interaction and virus survival in general [29]. However, these results do not exclude that these sequences can contain other variants of probabilistic found grounds.

A. Figures and Tables

Table 1. GenBank accession numbers and characteristics for sequences Influenza virus included in phylogenetic analysis (see next page).

Table 2. Patterns generated by MEME (see next page).

Fig. 1. Phylogeny Tunisia strains estimated with protein sequences.

Fig2. MEME patterns in logo format.

ISSN: 2356-5608

Table 2. Motifs generated by MEME.

Pattern Number	Start- end	Widht	BEST POSSIBLE MATCH
1	333-382	50	WTGMRNVPSKQTRGIFGAIAGFIEPGWEGMVDGWYGFHHQNSQGDGQAAD
2	474-523	50	NAEDMGNGCFKIYHKCDNACMGSIRNGTYDHDVYRDEALNNRFQIKGVEL
3	642-691	50	HQIEKEFSHVEGRIQDLEKYVEDTKIDLWSYNAELLVALENQHTIDYHDS

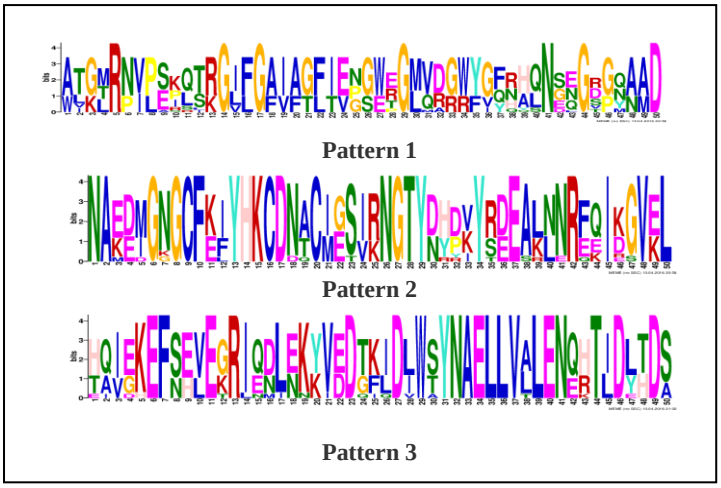

Fig2. MEME patterns in logo format

IV. Conclusions

In this study phylogenetic analysis of all protein sequences of all viral strains that circulated in Tunisia since the pandemic from 2009 to 2014 were carried out.

The search for conserved domains identified by the phylogeny, can help the issue of new more effective vaccines against emerging viral strains.

Phylogenetic analyzes must be wider by investigating evolutionary relationships among viral strains that have circulated in other countries mediterraniens. However, comparisons between different clusters obtained can help detect new conserved regions may be responsible for the pathogenicity and virulence of influenza type A. The phylogenies that include two other virus types B and C can also help determine the degree genetic divergence viral strains and thus evaluate their pathogenic potential.

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ISSN: 2356-5608

Table 1. GenBank accession numbers and characteristics for sequences Influenza virus included in phylogenetic analysis

References strains	*Accession number (Genbank)*	*Serotype*	*Protein*	*Length of sequences*
A/Tunisia/422/2011	ADV91010.1	H1N1	HA	566
A/Tunisia/197/2011	ADV91009.1	H1N1	HA	566
A/Tunisia/1987/2013	AGM16036.1	H3N2	HA	566
A/Tunisia/2635/2013	AGM16035.1	H3N2	HA	566
A/Tunisia/2494/2013	AGM16034.1	H3N2	HA	566
A/Tunisia/2334/2013	AGM16033.1	H3N2	HA	566
A/Tunisia/1199/2013	AGM16032.1	H3N2	HA	566
A/Tunisia/716/2012	AFV68720.1	H3N2	HA	566
A/migratory_bird/Tunisia/51/2010	AHA38675.1	H9N2	NEP	121
A/migratory_bird/Tunisia/51/2010	AHA38674.1	H9N2	NS1	230
A/migratory_bird/Tunisia/51/2010	AHA38673.1	H9N2	PA	716
A/chicken/Tunisia/12/2010	AHA38672.1	H9N2	PA	716
A/chicken/Tunisia/12/2010	ADX99483.1	H9N2	NEP	121
A/chicken/Tunisia/12/2010	ADX99482.1	H9N2	NS1	230
A/migratory_bird/Tunisia/51/2010	ADX99481.1	H9N2	M2	45
A/migratory_bird/Tunisia/51/2010	ADX99480.1	H9N2	M1	252
A/chicken/Tunisia/12/2010	ADX99479.1	H9N2	M2	45
A/chicken/Tunisia/12/2010	ADX99478.1	H9N2	M1	252
A/chicken/Tunisia/56/2014	AMK09409.1	H9N2	HA	364
A/California/07/2009	ACP44189.1	H1N1	HA	566
A/California/07/2009	ACQ63273.1	H1N1	PB2	759
A/California/07/2009	ACP41958.1	H1N1	PB1	757
A/California/07/2009	ACP41957.1	H1N1	PA	716
A/California/07/2009	ACP41955.1	H1N1	M2	97
A/California/07/2009	ACP41954.1	H1N1	M1	252
A/California/07/2009	ACT36688.1	H1N1	NA	469
A/California/07/2009	AFM72838.1	H1N1	NEP	121
A/California/07/2009	AFM72837.1	H1N1	NS1	219
A/California/07/2009	AFM72836.1	H1N1	NC	498
A/Egypt/42/2014	AJM70762.1	H1N1	NS1	219
A/Egypt/42/2014	AJM70760.1	H1N1	M1	252
A/Egypt/42/2014	AJM70759.1	H1N1	NA	469
A/Egypt/42/2014	AJM70757.1	H1N1	HA	566
A/Egypt/42/2014	AJM70755.1	H1N1	PA	716
A/Egypt/42/2014	AJM70754.1	H1N1	PB1	757
A/Egypt/42/2014	AJM70753.1	H1N1	PB2	759
A/chicken/Egypt/SCU8/2014	AIW63677.1	H9N2	PA	716
A/chicken/Egypt/SCU8/2014	AIW63683.1	H9N2	M1	252
A/chicken/Egypt/SCU8/2014	AIW63682.1	H9N2	NA	469
A/chicken/Egypt/SCU8/2014	AIW63679.1	H9N2	NS1	230
A/equine/Algiers/1/1972	ACF22135.1	H3N8	PB1_F2	90
A/equine/Algiers/1/1972	ACF22134.1	H3N8	PB1	757
A/equine/Algiers/1/1972	ACF22133.1	H3N8	PA	716
A/equine/Algiers/1/1972	ACF22132.1	H3N8	NEP	121
A/equine/Algiers/1/1972	ACF22131.1	H3N8	NS1	217
A/equine/Algiers/1/1972	ACF22130.1	H3N8	NC	498
A/equine/Algiers/1/1972	ACF22129.1	H3N8	NA	470
A/equine/Algiers/1/1972	ACF22128.1	H3N8	M2	97
A/equine/Algiers/1/1972	ACF22127.1	H3N8	M1	252
A/equine/Algiers/1/1972	ACF22126.1	H3N8	HA	565
A/equine/Algiers/1/1972	AAA43355.1	H3N8	NA	470
A/equine/Tiaret/1/2011	AGR54591.1	H3N8	HA	567
A/equine/Essaouira/3/2004	AFJ69911.3	H3N8	NS1	230
A/equine/Nador/1/1997	AFN69204.2	H3N8	NS1	230
A/avian/Libya/RV35D/2006	AFO83271.1	H9N2	HA	560

ISSN: 2356-5608

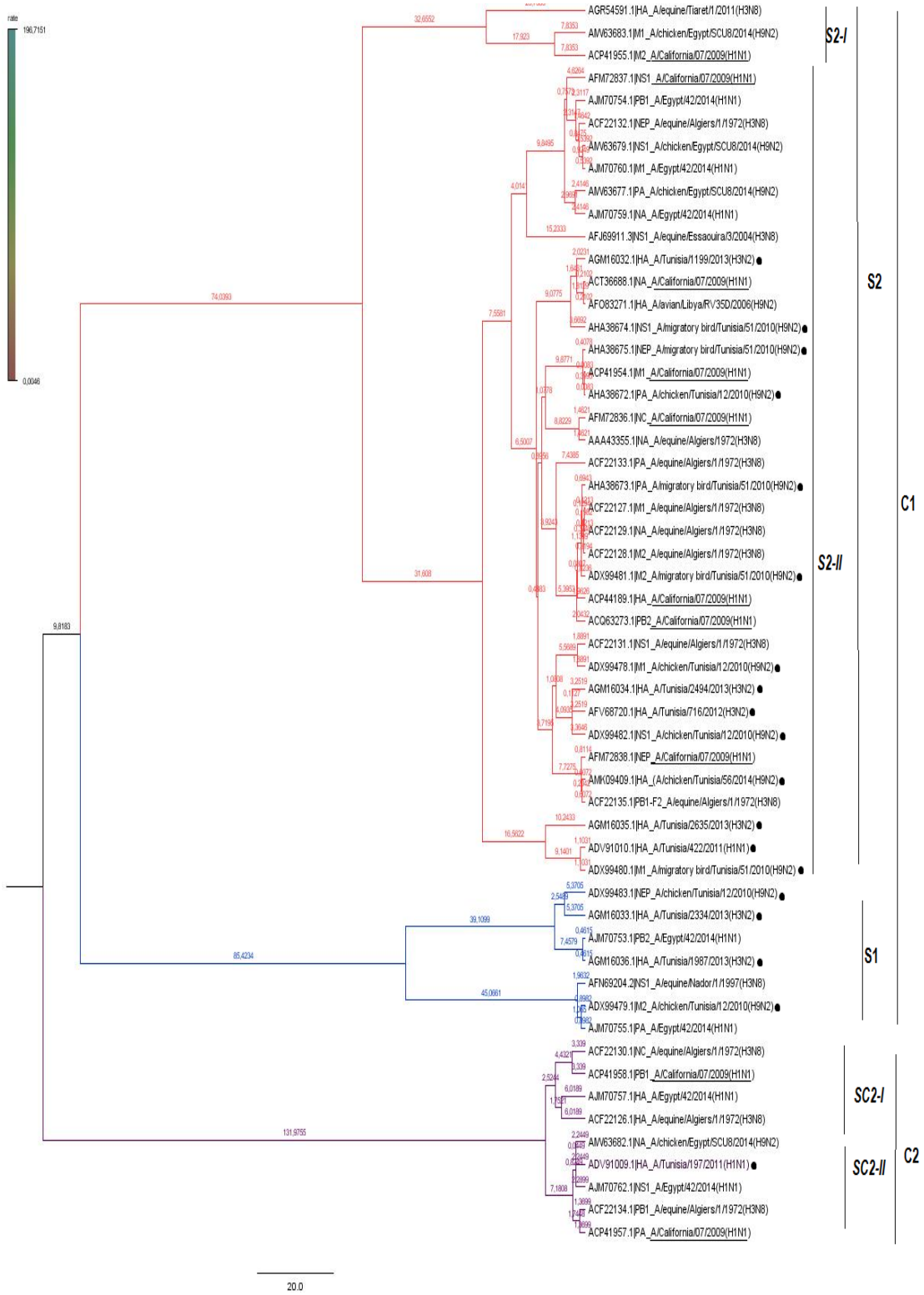

Fig. 1. Phylogeny Tunisia strains estimated with protein sequences. The phylogenetic relationships between 55 taxa were inferred with full-length using a Bayesian method (Program BEAST v1.8.2; Drummond and Rambaut, 2007). The phylogeny was inferred with a relaxed molecular clock model (Bayesian skyline method). The length of horizontal branches is drawn to the scale (proportion of number of substitutions per site per year).The Tunisian sequences used in the study are indicated with points. The references sequences for California strains are indicated by surligne.
Abbreviation used: C1: clade 1, C2: clade 2, S1, S2: sous clades.

ISSN: 2356-5608