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Evolution of the Hemagglutinin Gene of the Influenza A(H1N1)pdm09 Virus in Morocco During Two Influenza Seasons 2009–2011

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Abstract To study genetic evolution of Moroccan influenza A(H1N1)pdm09 virus strains, we conducted a molecular characterization of the hemagglutinin gene subunit 1 (HA1) of 36 influenza A(H1N1)pdm09 virus strains. The stains were collected from patients in Rabat and Casablanca during two influenza seasons 2009–2010 and 2010–2011. Nucleotide and amino acid sequences of 14 influenza A(H1N1)pdm09 virus strains from 2009 to 2010 were ~97 and 99 %, respectively, similar to the reference strain A/California/07/2009 (H1N1). Phylogenetic analysis of 22 influenza A(H1N1)pdm09 virus strains from 2010 to 2011 revealed a co-circulation of three well-described different genetic groups. Most important, none of the identified groups showed significant changes at the antigenic site of the virus HA1 subunit which may alter the efficacy of California/07/2009 (H1N1) vaccine.

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Introduction

The first emergence of the pandemic influenza A(H1N1) was reported in Mexico and United State in April 2009 [7]. On 11 June 2009, the World Health Organization (WHO) declared phase six of influenza A(H1N1) pandemic [influenza A(H1N1)pdm09] which quickly spread globally after human-to-human transmission [32]. Influenza A(H1N1)pdm09 virus shows distinct biological advantage in replication, transmission, tropism, and pathogenesis when compared to both seasonal influenza A(H1N1) and A(H3N2) viruses [3, 25, 33]. Whereas, the majority of patients experienced mild illness, severe disease, and high mortality rates were reported among certain risk groups including pregnant women, diabetes, and obesity patients [33].

Morocco is a country located in the extreme North Western Africa with a population of about 32.5 million inhabitants and urbanization rate of around 60 % in 2012 [13]. The first laboratory-confirmed influenza A(H1N1)pdm09 case in Morocco was identified on 10 June 2009 in a 19-year-old woman traveling from Canada [1]. From June to October 2009, cases were either imported or were part of local clusters linked to imported cases. Low level human-to-human transmission was observed until late October. The period from November 2009 to January 2010 was characterized by an elevated number of locally acquired infections, indicating sustained community transmission. The epidemic influenza A(H1N1)pdm09 virus number of cases peaked in Morocco in late November 2009 and ended by the last week of January 2010 [1, 17]. During the first pandemic wave, influenza A(H1N1)pdm09 virus was reported on about 40 % of patients with influenza-like illness (ILI) mainly among young people [1, 17]. On March 10, 2010, 2890 laboratory-confirmed cases with 64 deaths reported in the country [21].

A serological survey after the influenza season 2010–2011 showed that 67 and 53 % of the participants in Rabat and Meknes had antibodies against influenza A(H1N1)pdm09 [10]. Since its emergence in 2009, influenza A(H1N1)pdm09 virus continues to co-circulate with influenza A(H3N2) and influenza B. This co-circulation varied between regions and seasons resulting in death in some cases [34].

The aim of this work is to study the HA1 genetic diversity of influenza A(H1N1)2009 virus circulating in Morocco during two influenza seasons: 2009–2010 and 2010–2011.

Materials and Methods

Patients and Samples

Since May 2009, we initiated a surveillance of influenza A(H1N1)pdm09 infections in Mohammed V Military Teaching Hospital (MVMTH), Rabat, Morocco for patients who showed ILI. ILI is defined as the presence of at least two of the following symptoms: fever (≥38 °C), cough, muscular pain, headache, rhinorrhoea, and dyspnoea. Nasopharyngeal swabs were immediately shipped in transporting medium on ice to L3 laboratory in MVMTH. Standard questionnaire upon sampling included information about age, sex, date of onset of the symptoms, sampling date, place of residence, clinical features, travel history, vaccination/antiviral treatment history. During the period 2009–2011, we selected 22 influenza A(H1N1)pdm09 representatif positive samples (excluding imported and grouped cases, select samples from patients from different regions) for the sequencing of the HA1 subunit of the Hemagglutinin gene.

Laboratory Tests

Detection of Influenza A(H1N1)pdm09 Virus

Viral RNA was extracted from nasopharyngeal swabs using the High Pure Viral RNA Isolation Kit (Roche, Germany) according to the manufacturer's instructions. One-step real-time RT-PCR was performed at a LightCycler 2.0 Instrument (Roche, Germany) using RealTime ready Influenza A/H1N1 Detection Set and RealTime ready RNA Virus Master (Roche, Germany) as described before [17].

One Step RT-PCR for the Amplification of HA Gene

Hemagglutinin segments obtained from 22 influenza A(H1NI)pdm09 positive patients were partially amplified. Samples belonged to different phases (pre-epidemic, epidemic, and post-epidemic) in two seasonal influenza: 2009–2010 and 2010–2011. RNA extraction from clinical samples was performed using the viral RNA mini kit (Qiagen, Germany) according to the manufacturer's instructions. Briefly, extracted RNA from positive samples was then reverse transcribed and HA was amplified using SuperscriptTM one step RT-PCR with Platinum® Taq kit (Invitrogen, UK). Total volume of reaction 50 µl consists of: 25 µl reaction Mix (2×), 5 µl of extracted RNA, 1 µl of (10 µM) forward primer (SwHA-F1 5′-AGCAAAAG CAGGGGAAAATAAAA-3′), 1 µl of (10 µM) reverse primer (SwHA-R1165 5′-TATCCTGACCCCTGCTCATT-3′), 1 µl (1 U) of RT/Platinum® Taq Mix, and 1 µl of Magnesium Sulfate (50 mM). The primers used in this study are new designed using Primer 3 software [16, 31]. Reverse transcription was at 50 °C for 30 min and 95 °C for 5 min. PCR amplification included 40 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 90 s and final extension consists of 1 cycle of 72 °C for 10 min

Hemagglutinin Partial Sequencing

PCR products were purified using EXOSAP-IT (USB, USA) and bidirectional sequenced using BigDye Terminator version 3.1. The same primers (above) were used for the amplification on an ABI 3130xl automated sequencer (Applied Biosystems, USA). Analysis of the produced electropherogram was done with the sequencing Analysis Software version 5.3.1 (Applied Biosystems, USA). HA sequences have been deposited in the GenBank database. The obtained aminoacid sequences were compared with 14 Casablanca influenza A(H1N1)pdm09 strains, available in the NCBI GenBank database. Aminoacid numbering was carried out excluding the signal peptide.

Phylogenetic Analysis

Molecular phylogenetic analysis was performed by neighbor-joining method using the MEGA version 5.0 Software [29]. Thirty-six Moroccan Sequences (Table 1) were aligned with selected influenza A(H1N1)2009 sequences downloaded from GenBank and Global Initiative on Sharing All Influenza Data (GISAID) using Clustal W program implemented in MEGA version 5. Confidence values for the tree clades were provided by bootstrap analysis of 1,000 datasets.

Results

Between June 2009 and May 2011, the influenza A(H1N1)pdm09 was detected in 373 (31 %) out of 1,183 suspected cases tested using Real Time RT-PCR Assay (Table 2).

Table 1 Clinical data and GenBank accession numbers for the HA1 subunit (segment 4) of Moroccan influenza A(H1N1)pdm09 circulating strains from August 2009 to February 2011

InfluenzaA (H1N1)pdm09 stains name	Origin	Date of collection	Genbank accession	Age (years)	Sex	Clinical severity
A/Rabat/RH825/2009*	Rabat	05-Aug-2009	JX204753	33	M	Mild
A/Rabat/LAI812/2009*	Rabat	12-Aug-2009	JX204767	19	M	Mild
A/Rabat/FH819/2009*	Rabat	19-Aug-2009	JX204766	31	M	Moderate
A/Rabat/RF1026/2009*	Rabat	26-Oct-2009	JX204754	15	F	Moderate
A/Rabat/RA1028/2009*	Rabat	28-Oct-2009	JX204755	15	M	Mild
A/Rabat/LA1105/2009*	Rabat	05-Nov-2009	JX204765	32	F	Moderate
A/Rabat/RS113/2009*	Rabat	13-Nov-2009	JX204761	37	M	Mild
A/Rabat/RAb1113/2009*	Rabat	13-Nov-2009	JX204756	18	M	Mild
A/Rabat/KF1117/2009*	Rabat	17-Nov-2009	JX204757	18	M	Moderate
A/Rabat/TB1125/2009*	Rabat	25-Nov-2009	JX204764	12	F	Mild
A/Rabat/KM1231/2009*	Rabat	31-Dec-2009	JX204758	33	F	Mild
A/Rabat/AA119/2010*	Rabat	09-Jan-2010	JX204759	37	M	Mild
A/Rabat/FB110/2010*	Rabat	10-Jan-2010	JX204760	32	M	Mild
A/Rabat/HR1230/2010*	Rabat	Feb-2010	JX204762	30	F	Mild
A/Rabat/BF1229/2010*	Rabat	29-Dec-2010	JX204752	40	F	Moderate
A/Rabat/RR1231/2010*	Rabat	31-Dec-2010	JX204751	22	M	Mild
A/Rabat/AA104/2011*	Rabat	13-Jan-2011	JX204750	27	F	Mild
A/Rabat/RY113/2011*	Rabat	04-Jan-2011	JX204768	27	F	Mild
A/Rabat/RO120/2011*	Rabat	20-Jan-2011	JX204763	25	F	Mild
A/Rabat/SK131/2011*	Rabat	31-Jan-2011	JX204748	26	M	Mild
A/Rabat/044/2011*	Rabat	04-Feb-2011	JQ319710	36	F	Mild
A/Rabat/SN208/2011*	Rabat	08-Feb-2011	JX204749	36	F	Mild
A/Casablanca/211/2010	Casablanca	20-Dec-2010	CY099741	15	M	–
A/Casablanca/23/2011	Casablanca	13-Jan-2011	CY099750	3	F	–
A/Casablanca/26/2011	Casablanca	13-Jan-2011	CY099752	24	M	–
A/Casablanca/28/2011	Casablanca	13-Jan-2011	CY099753	10	M	–
A/Casablanca/60/2011	Casablanca	20-Jan-2011	CY099757	43	F	–
A/Casablanca/63/2011	Casablanca	20-Jan-2011	CY099758	47	F	–
A/Casablanca/69/2011	Casablanca	26-Jan-2011	CY099760	11	F	–
A/Casablanca/73/2011	Casablanca	26-Jan-2011	CY099763	43	F	–
A/Casablanca/76/2011	Casablanca	26-Jan-2011	CY099765	13	M	–
A/Casablanca/91/2011	Casablanca	02-Feb-2011	CY099766	15 months	M	–
A/Casablanca/92/2011	Casablanca	02-Feb-2011	CY099768	52	F	–
A/Casablanca/103/2011	Casablanca	07-Feb-2011	CY099742	5	M	–
A/Casablanca/107/2011	Casablanca	09-Feb-2011	CY099743	–	F	–
A/Casablanca/117/2011	Casablanca	15-Feb-2011	CY099745	10 months	_	_

The strains sequenced in this study are indicated by asterisks (*)

Table 2 Real-time PCR Assay data on respiratory samples and sequenced Influenza A(H1N1)pdm09 collected from August 2009 to February 2011

Season	Sample tested	Influenza A(H1N1) pdm09 positive sample	Sequenced strains
2009–2010	856	293 (34 %)	14
2010–2011	327	75 (23 %)	08
Total	1,183	368 (31 %)	22

All sampled patients from Rabat fully recovered without any complications or sequelae and none of them required further hospitalization. Following molecular analysis, the HA1-encoding regions of Moroccan strains were comparable to that of the vaccine strain A/California/7/09 (H1N1) with a number of detected variations (Table 3).

The molecular characterization of the HA1 subunit of the HA gene revealed that all of the Moroccan strains from Rabat (22) and Casablanca (14) collected between 2009

Table 3 Amino-acid substitutions in the HA1 subunit (segment 4) of Moroccan influenza A(H1N1)pdm09 circulating strains from August 2009 to February 2011

InfluenzaA H1N1pdm)09 strains	8	19	185	31	44	47	57	83	95	97	116	128	173	176	197	198	216
A/California/07/2009	H	V	S	N	L	V	I	P	F	D	I	S	V	L	A	Y	I
A/Rabat/RH825/2009*								S				P					
A/Rabat/LAI812/2009*								S									
A/Rabat/FH819/2009*								S									
A/Rabat/RF1026/2009*								S									
A/Rabat/RA1028/2009*								S									
A/Rabat/LA1105/2009*								S			M						
A/Rabat/RS113/2009*								S								D	
A/Rabat/RAb1113/2009*								S									
A/Rabat/KF1117/2009*								S	L								
A/Rabat/TB1125/2009*								S			M						
A/Rabat/KM1231/2009*								S									
A/Rabat/AA119/2010*						I		S		N							
A/Rabat/FB110/2010*								S						I	T		
A/Rabat/HR1230/2010*								S									
A/Rabat/BF1229/2010*								S		N							V
A/Rabat/RR1231/2010*								S		N							V
A/Rabat/AA104/2011*								S									V
A/Rabat/044/2011*								S		N							V
A/Casablanca/23/2011								S		N							V
A/Casablanca/91/2011	L							S		F							V
A/Casablanca/117/2011								S		N							V
A/Rabat/RY113/2011*			T					S		N							
A/Rabat/RO120/2011*			T					S		N	M						
A/Rabat/SK131/2011*			T					S		N							
A/Rabat/SN208/2011*			T					S		N							
A/Casablanca/211/2010			T					S		N							
A/Casablanca/26/2011			T					S		N			I				
A/Casablanca/28/2011			T					S		N							
A/Casablanca/60/2011			T					S		N							
A/Casablanca/63/2011			T					S		N							
A/Casablanca/69/2011			T					S		N							
A/Casablanca/73/2011	L		T					S		N							
A/Casablanca/92/2011		E	T	T	Q		V	S		N							
A/Casablanca/103/2011			T					S		N							
A/Casablanca/107/2011			T					S		N							
A/Casablanca/76/2011								S									

InfluenzaA H1N1pdm)09 strains										Antigenic sites						
	221	234	249	256	267	274	295	297	321	*Sa 125*	*Sb 185*	*Ca 138*	*Ca 170*	*Ca 203*	*Ca 205*	*Ca 222*
A/California/07/2009	R	V	V	A	I	D	I	P	I	*N*	*S*	*H*	*G*	*S*	*R*	*D*
A/Rabat/RH825/2009*		I							V					*T*		
A/Rabat/LAI812/2009*							V		V					*T*		
A/Rabat/FH819/2009*									V					*T*		*E*
A/Rabat/RF1026/2009*									V					*T*		
A/Rabat/RA1028/2009*									V					*T*		

Table 3 continued

InfluenzaA H1N1pdm)09 strains										Antigenic sites						
	221	234	249	256	267	274	295	297	321	*Sa 125*	*Sb 185*	*Ca 138*	*Ca 170*	*Ca 203*	*Ca 205*	*Ca 222*
A/Rabat/LA1105/2009*									V					*T*		
A/Rabat/RS113/2009*									V					*T*		
A/Rabat/RAb1113/2009*									V					*T*		
A/Rabat/KF1117/2009*								S	V					*T*		
A/Rabat/TB1125/2009*					L				V					*T*		
A/Rabat/KM1231/2009*									V					*T*		
A/Rabat/AA119/2010*									V					*T*		
A/Rabat/FB110/2010*									V					*T*		
A/Rabat/HR1230/2010*														*T*		
A/Rabat/BF1229/2010*			L						V					*T*	*K*	
A/Rabat/RR1231/2010*			L						V					*T*	*N*	
A/Rabat/AA104/2011*			L						V					*T*	*K*	
A/Rabat/044/2011*			L						V					*T*	*K*	
A/Casablanca/23/2011			L						V					*T*	*K*	
A/Casablanca/91/2011			L						V					*T*	*K*	
A/Casablanca/117/2011									V			*Q*		*T*	*K*	
A/Rabat/RY113/2011*									V		*T*			*T*		
A/Rabat/RO120/2011*						N			V		*T*			*T*		
A/Rabat/SK131/2011*									V		*T*			*T*		
A/Rabat/SN208/2011*									V		*T*			*T*		
A/Casablanca/211/2010									V		*T*			*T*		
A/Casablanca/26/2011									V		*T*			*T*		
A/Casablanca/28/2011									V		*T*			*T*		
A/Casablanca/60/2011									V		*T*			*T*		
A/Casablanca/63/2011									V		*T*			*T*		
A/Casablanca/69/2011	K								V		*T*			*T*		
A/Casablanca/73/2011				T					V		*T*			*T*		
A/Casablanca/92/2011									V		*T*			*T*		
A/Casablanca/103/2011									D		*T*			*T*		
A/Casablanca/107/2011									V		*T*		*R*	*T*		
A/Casablanca/76/2011							V		V	*D*				*T*		

Italicized refer to antigenic sites

and 2011 exerted the S203T mutation located at the antigenic site Ca1 (characteristic of clade 7 viruses). In addition, all Moroccan viruses carried lysine at position (−15) which is another specificity of strains belonging to clade 7.

The most common variations compared to vaccine strain A/California/07/09 (H1N1) were P83S (36/36), I321V (34/36), D97N (20/36), and S185T (14/36) substitutions observed in sequenced Moroccan strains.

Strains from influenza season 2009–2010 ($n = 14$) showed high conservation and were very close (99 % homology) to the reference strain A/California/07/09 (H1N1). The number of amino acid differences in HA between the Moroccan strains and the vaccine strain ranged from 3 to 5.

There was a trend of increasing frequency of cumulative numbers of amino acid substitutions progressively to the evolution of the influenza season (Table 3). Among these mutations, we found one substitution D222E located at the HA receptor-binding site.

Influenza strains of the season 2010–2011 accumulated many additional mutations compared to the reference strains (Table 3).

The phylogenetic analysis suggested that Moroccan circulating influenza A(H1N1)2009 virus during the season 2010–2011 could be classified into three different genetic groups (Fig. 1).

The first genetic group contains 14 strains (64 %) characterized by S185T substitution and all of them carried

D97N mutation (Table 3). In this group, 93 % strains shared V321I substitution. Several strains exerted some additional mutations: three strains had one substitution (V173I, R221K, or G170R), two strains had two variations (I116M/D274N or H8L/A256T) and one strain (A/Casablanca/92/2011(H1N1)) carried four mutations (V19E, N31T, L44Q, and I57V).

The second group, included five strains, is characterized by D97N, R205K, I216V, and V249L changes (Table 2). Two additional strains are group-related but presented a sequence that differs by one substitution of the three characteristic mutations of this group. A/Rabat/RR1231/2010 (H1N1) which presented R205N substitution and A/Casablanca/117/2011(H1N1) which kept valine at position 249. Then this brings the number of strains related to this group to 7 which represents 32 % of Moroccan circulating strains studied.

Finally, the third group is represented by one strain A/Casablanca/76/2011 (H1N1) which showed the characteristic amino acid substitution N125D. This strain had three additional associated variations (T25P, I295V, and I325V).

Discussion

In few weeks, influenza A(H1N1)pdm09 virus showed a sufficient diversity to form 7 distinct clades [22]. Clade 7 rapidly became the most prevalent worldwide, but other clades continued to circulate in many countries due to the multiple introductions of different clade-variants [2, 4, 11, 14, 23, 26, 28]. This first molecular study demonstrates that all strains circulated in Morocco between August 2009 and February 2011 belonged to clade 7 with recognized changes at S203T and K at position (−15). This finding might be attributed to the relatively late introduction of influenza A(H1N1)pdm09 in Morocco. The first introduction of the virus was on 11 June 2009, however, the indigenous transmission started from the second half of November 2009 [1, 17] at the time when clade 7 became dominant worldwide.

We found that the circulating virus during the first influenza season 2009–2010 was very close to the vaccine strain A/California/07/2009 (H1N1) and showed only few mutations. This result suggests that after its introduction in Morocco, the virus circulated widely without a pressure of host immune response. This is because of the limited number of vaccinated people in the country. Moroccan people have shown low interest in vaccination against influenza A(H1N1)pdm09, and the vaccination rate was only up to 7 % in 2011 [10]. Of mutations found in the Moroccan influenza A(H1N1)pdm09 strains in 2009, only one amino acid substitution D222E was located within the antigenic site. Such mutation can disrupt receptor(s)-HA interaction. Several studies reported on HA D222E mutation [12, 27], including predictive structural studies [30]. It has been suggested that this residue likely plays an important structural role for the recognition and attachment of influenza virus to its host receptor [15, 18, 20]. D222E substitution was detected in samples from mild and severe cases of Spanish virus, in a frequency of 17.21 and 9.75 % and was associated with the severity of respiratory disease [18]. For us, this mutation was detected in one patient who has experienced a moderate ILI and recovered after oseltamivir treatment. No further strain carrying this mutation was isolated during the years 2010 and 2011. This finding is in accordance with a French study in Reunion Island that showed infections with D222E variants result in less severe infection than other variants D222G/N [24].

Strains ($n = 23$) from the second influenza season 2010–2011 carried more mutations compared to the previous season. Fourteen strains (64 %), belonging to the first genetic group characterized by S185T substitution at the antigenic site Sb [28] and all of them carried D97N. This group, represented by A/England/676/2010 (H1N1) and A/St Petersburg/27/2011 (H1N1) [5, 6, 9] was previously identified in Northern Hemisphere and detected in many countries around the world [5, 6, 9]. This genetic group was also widely spread in Spain in 2011 including adjacent areas of Morocco from where a large population movement occurs daily [19].

The second genetic group included seven strains (32 %) and characterized by D97N, R205K (located at the antigenic site Ca1), I216V, and V249L. These changes were previously described and observed in at least ten countries including Europe and Middle East (the cluster represented by A/Astrakhan/1/2011 (H1N1) and A/Trieste/11/2011 (H1N1)) [5, 6]. In addition, this group circulates widely in Spain and was responsible for ∼28 % of influenza A(H1N1)pdm09 infections in 2011 [19]. Finally, only one strain (A/Casablanca/76/2011) belonged to the third genetic group was characterized by the N125D mutation located at the antigenic site Sa. This group was observed originally as an emerging genetic group in Southern Hemisphere which next spread in Northern Hemisphere (f.e.g., A/Christchurch/16/2010 (H1N1)) [5, 6]. This result is different from data reported in Spain where influenza A(H1N1)pdm09 virus from this group was responsible for ∼27 % of influenza A(H1N1)pdm09 infections [19]. This finding suggests limited circulation of strains belonging to this subgroup in morocco, whereas this difference could more likely be due to sampling bias.

Moreover, the three genetic groups identified in Morocco shows no antigenic variation capable of reducing vaccine response as has been demonstrated by several studies of inhibition of hemagglutination [5, 6, 19]

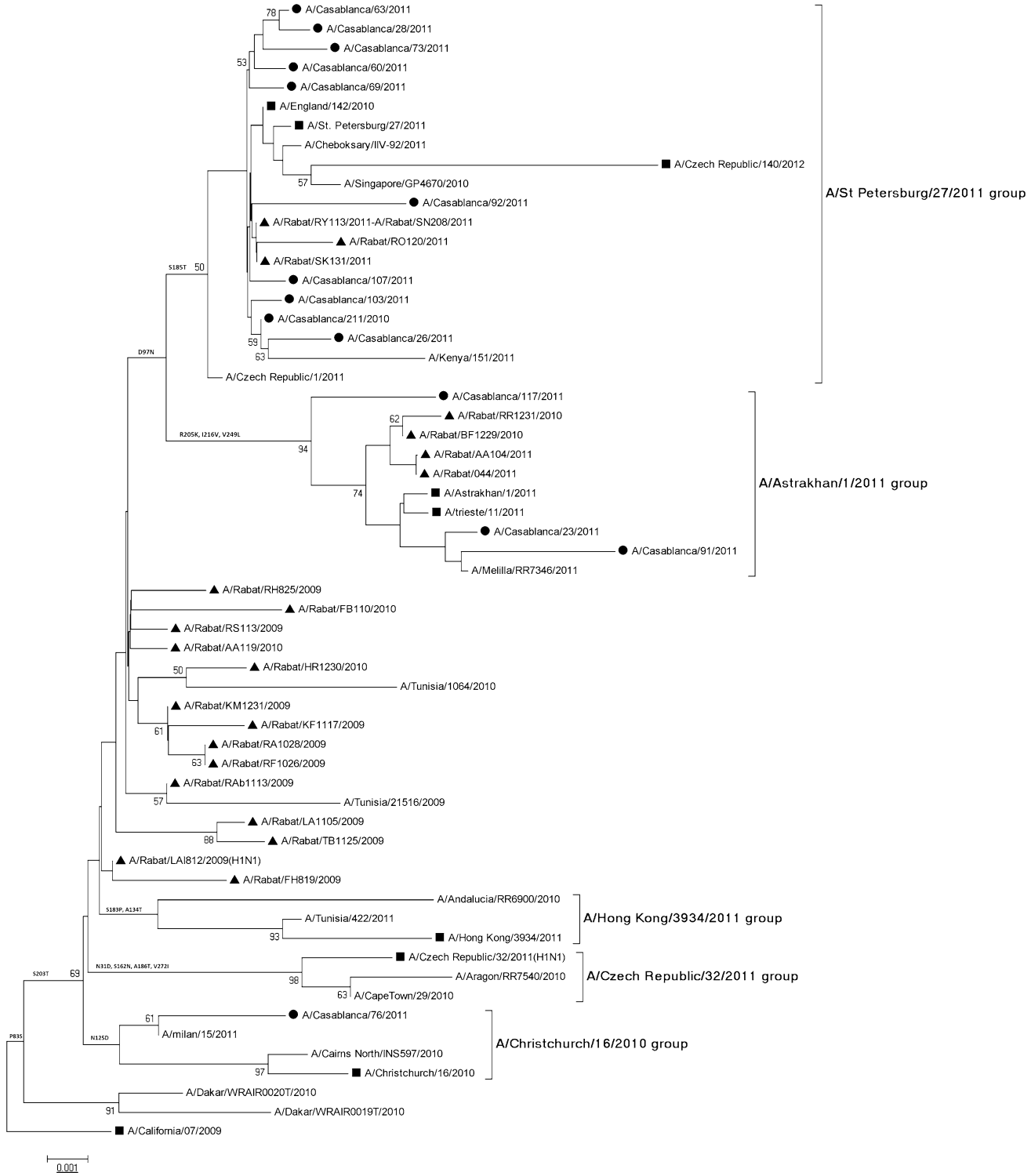

Fig. 1 Phylogenic relationship of HA1 subunit of the hemagglutinin of Moroccan influenza A(H1N1)2009 strains circulating in Morocco during the two seasons (2009–2010/2010–2011) performed using neighbor-joining method with 1,000 replications. The tree was rotted with A/California/07/2009 (vaccine strain) as outgroup. Branch values of more than 50 are indicated. HA1 subunits of strains sequenced in this study are shown in *closed triangles*. Other Moroccan strains are shown in *closed circles*. References strains are shown in *closed squares*. Five genetic groups were presented in the phylogenic tree according to data previously reported around the world [30, 31]

Conclusion

Since its first introduction in Morocco, all circulating influenza A(H1N1)pdm09 virus strains belonged to clade 7. Strains from the first influenza season 2009–2010 were very close to the reference strain A/California/07/2009 (H1N1) and carried very few amino acid substitutions. During the second influenza season 2010–2011, three different circulated groups were defined by specific amino acids changes with a predominance of the genetic group represented by A/St Petersburg/27/2011 (H1N1). None of these genetic groups seem to show significant antigenic differences with the vaccine strain which can alter the efficacy of the vaccine.

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