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MOLECULAR CHARACTERIZATION OF INFLUENZA B CIRCULATING IN CASABLANCA-MOROCCO DURING 2010-2013

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ABSTRACT

Influenza B is often regarded as the milder form of the disease. The aim of this study was to characterize the genetic diversity of the influenza B viruses identified in Casablanca during 2010-2013 among patients with influenza-like illness.

Materials and Method

Samples were collected from patients with influenza-like illnesses (ILI) and screened for influenza viruses by One-Step Real-Time RT-PCR, and Influenza B viruses identified were subsequently sequenced and analyzed phylogenetically.

Findings

Of the 760 nasopharyngeal swabs collected during 2010-2013, a total of 226 (30%) influenza viruses were identified by real time RT-PCR, 173 (23%) were influenza A and 53(7%) were influenza B. Molecular Characterization of the HA1 Gene of Influenza B identified during 2010-2011 showed that they belong to B/Victoria lineage and were closely related to the vaccine virus B/Brisbane/60/2008.

Influenza B activity remained very low during 2011-2012. In 2012-2013, a co-circulation of both lineages, Victoria and Yamagata, was detected. Most of the strains that belonged to the Victoria lineage were closely related to the B/Brisbane/60/2008 vaccine strain and influenza B identified belonging to Yamagata lineage were related to the vaccine virus B/Wisconsin/1/10.

Conclusion

The study confirmed the co-circulation of Victoria and Yamagata lineages of influenza B viruses. Therefore, surveillance and monitoring of genetic variations among circulating influenza B viruses is important for selection of annual vaccine strains as quadrivalent flu vaccine including influenza A (H1pdm09 and H3) and both lineages of influenza B.

INTRODUCTION

Influenza A and B viruses members of the family Orthomyxoviridae, cause annual epidemics in many regions of the world. In contrast to influenza A viruses, classified into different subtypes based on the Hemaglutinin (HA) and Neuraminidase (NA) genes and infecting a variety of host species (Tong *et al*., 2013). Influenza B viruses was isolated in 1940, designated B/Lee/40 (Nerome *et al*. 1998) and infect only humans and seals (Bodewes *et al*., 2013). Influenza B viruses have been divided into two antigenically and genetically distinct lineages, defined by the reference strains B/Victoria/2/87 (Vic87) and B/Yamagata/16/88 (Yam88) (Kanegae *et al*., 1990; Rota *et al*., 1990), which are named after the areas where they were first identified. Since the 1990s, the strains derived from the Yamagata lineage have circulated globally (Rota *et al*., 1990; McCullers, Saito, and Iverson 2004a; Shih *et al*., 2005). Viruses of the Victoria lineage were found primarily in the 1980s and sporadically found in the 1990s (McCullers, Saito, and Iverson 2004a). They re-emerged worldwide and co-circulated with viruses of the Yamagata lineage from 2001, leading the WHO to recommend the introduction of Victoria lineage (B/Hong Kong/330/01) in the vaccine strains. Viruses of these two

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lineages have been observed to circulate as seasonal influenza viruses simultaneously (P A Rota *et al*., 1990c) or individually in particular time periods and areas (McCullers, Saito, and Iverson 2004). Insertion and deletion of nucleotides (Lindstrom *et al*., 1999; McCullers *et al*., 1999) is a strategy of the influenza B viruses to generate genetic diversity (Nerome *et al*., 1998; McCullers *et al*., 1999). It is also well established that two lineages of influenza B viruses may co-circulate at the same time (Kanegae *et al*., 1990; Rota *et al*., 1990; Rota *et al*., 1992), thus favoring frequent reassortment events between the descendents of Vic87 and Yam88 viruses (Lindstrom *et al*. 1999; McCullers, Saito, and Iverson 2004; Matsuzaki *et al*. 2004). Therefore, analyses of the influenza B gene are an important part of the surveillance of these viruses from all parts of the world. Data of the influenza B viruses from Casablanca is not well documented. To follow the genomic characteristics and dynamics of the influenza B viruses in this city, we analyzed and compared the partial HA1 sequences of influenza B during 2010-2013.

MATERIALS AND METHODS

Samples collection

During flu seasons 2010–2013, a total of 760 nasopharyngeal swabs were collected in a transport medium HiViral ™ Transport Kit from patients with influenza-like illnesses (ILI) based on symptoms such as fever, sore throat, cough, nasal congestion, and myalgia. All the patients in this study have not been previously vaccinated. Virological test were performed at the laboratory of Medical Virology in Pasteur Institute of Morocco.

Virus Isolation

Virus was isolated by inoculation on Madin-Darby Canine Kidney (MDCK) cells. Cells were cultured in DMEM supplemented with 10 % fetal bovine serum, penicillin (100 U/mL), streptomycin (100 µg/mL), and amphotericin B (0.25 µg/mL) and incubated at 34 °C with 5 % CO_2. 100 µl of naso-pharyngeal swab supernatant was inoculated onto confluent Madin-Darby canine kidney cells grown in 24-well plates and incubated for 1 h to allow virus adsorption. The cytopathic effect (CPE) was monitored after 3–7 days of CO_2 incubation at 34°C. For the detection of influenza viruses, positive cultures were subsequently processed for real-time RT-PCR to detect the M gene of influenza A and B viruses. All the cultures showing CPE were stored at -80 °C for further studies.

RNA Extraction

Viral RNA was extracted from 200 µl of cell-culture supernatants using High Pure Viral RNA Isolation Kit (Roche) according to the manufacturer's instructions.

Virus collection and screening

A one-step real-time duplex RT-PCR was performed for detection of influenza A and B viruses, using SuperScript™III Platinium® One-step Quantitative RT-PCR System (Invitrogen). Real-time RT-PCR were carried out in an ABI 7500 Fast. To determine the quality of the sample, the presence of human ribonucleoprotein (RNaseP) was assessed for each specimen tested. Table I shows the oligo primers and probes used.

Table 1. Primers and probes used in one-step real-time RT-PCR for detection of A and B influenza viruses

Influenza A	InfA Forward	GAC GRA TCC TGT CAC CTC TGA C	CDC(WHO | CDC Protocol of Realtime RTPCR for Influenza A (H1N1) n.d.)
	InfA Reverse	AGG GCA TTY TGG ACA AAK CGT CTA	
	InfA Probe	TGC AGT CCT CGC TCA CTG GGC ACG	
Influenza B	InfB Forward	5'GAG ACA CAA TTG CCT ACC TGC TT3'	Ward *et al.* 2004
	InfB Reverse	5'TTC TTT CCC ACC GAA CCA AC3'	
	InfB Probe	VIC-5' AGA AGA TGG AGA AGG CAA AGC AGA ACT AGC3'-TAMRA	
RnP	RnaseP Forward	AGA TTT GGA CCT GCG AGC G	CDC(WHO | CDC Protocol of Realtime RTPCR for Influenza A (H1N1) n.d.) (H1N1)pdm09
	RnaseP Reverse	GAG CGG CTG TCT CCA CAA GT	
	RnaseP Probe	TTC TGA CCT GAA GGC TCT GCG CG	

Amplification and Sequencing of HA1 Gene

First-strand cDNA synthesis was carried out by mixing 10 µl of RNA with 1 µl of random primers (pd(N)6, Invitrogen), heated at 70°C for 5 min and quickly chilled on ice. The viral RNA was reverse transcribed into cDNA with SuperScript™ III Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. The resulting DNA was amplified for HA1 fragment with Primers for sequencing (Table II) using Taq DNA polymerase (Invitrogen) according to the manufacturer's instructions using 5µl of cDNA through the following thermo cycler program: after the initial ''hot start'' for 4 min at 95°C, 40 cycles of denaturation for 45 sec at 94°C, hybridization for 1 min and 30 sec at 55°C and elongation at 72°C for 2 min 30 sec and a final extension of 7 min at 94°C. In the nested PCR step, 3 µl of the initial reaction product was added to a second PCR reaction mixture, the initial denaturation at 95°C for 1 min followed by 40 cycles of denaturation for 45 sec at 94°C, hybridization for 1 min 30 sec at 55°C and elongation at 72°C for 2 min 30 sec and a final extension of 10 min at 72°C. The PCR products were detected by electrophoresis on 1% ethidium bromide-stained agarose gels, visualized and documented in a Biorad Gel Doc XR imager.

Table 2. Primers used in Nested- PCR for sequencing influenza B viruses

Region HA1	Primer	Sequence (5'> 3')	Ref
	B/s/86	5'-GAAGGCAATAATTGTACTACTC-3'	(Genset, Paris, France) (Barakat *et al*. 2010)
	B/-/1235	5'-CTCTTAAGGTCTGCTGCCACT-3'	
	B/S/403	5'-AATCTTCTCAGAGGATATGAA-3' Nested PCR	
	B/-/961	5'-GGCAATCTGCTTCACCAATTAA AGG-3 Nested PCR	

The positive PCR products were purified by exonuclease I and shrimp phosphatase alcaline enzymes (Promega) and

Bidirectional nucleotide (nt) sequences were obtained using gene specific forward and reverse primers for HA gene (Table II) using a BigDye Terminator Version 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and an ABI PRISM 3130 DNA automated sequencer (Applied Biosystems, Foster City, CA, USA).

Phylogenetic Analysis

Alignement results were edited using the BioEdit program (http://www.mbio.ncsu.edu/ BioEdit/) for amino acid variations, and sequences obtained were aligned with reference sequences for influenza B available on GenBank (http://www.ncbi.nih.gov/genomes /flu/swineflu.html) and the GISAID (http://gisaid.org/). Multiple sequence alignment by Clustal W and subsequent phylogenetic analyses were performed using Molecular Evolutionary Genetic Analysis MEGA version 5.0 software (Tamura *et al*. 2011). Phylogenetic tree was generated using the Neighbor-Joining method, and bootstrap analysis (1,000 replicates).

Gene Bank Accession Numbers

All the nucleotide sequences of influenza-B Casablanca strains have been deposited in GenBank under the accession numbers: CY099726-CY099739 and KC855464-KC855473.

RESULTS

Virological Surveillance

In Casablanca, 273, 280 and 207 nasopharyngeal swabs were collected respectively during the 2010-2011, 2011-2012 and 2012-2013 seasons (Fig. 1). In 2010–2011, a total of 84 (31%) influenza viruses were detected, 32 (12%) were subtyped as influenza B, 20 (7,3%) H3N2 and 29 (10,6%) H1N1pdm09. For 2011–2012 season, influenza A viruses predominated 48 (16,16%) H3N2 and 30 (10,10%) H1N1pdm09. During 2012–2013, a total of 64 (31%) influenza viruses were detected, 21 (10%) were subtyped as influenza B viruses, 30 (20,77%) H1N1pdm09. Two peaks of influenza B virus were detected in the 51th and 2th weeks of 2010-2011 and 2012-2013, however the number of influenza B isolates decreased after the 5th week.

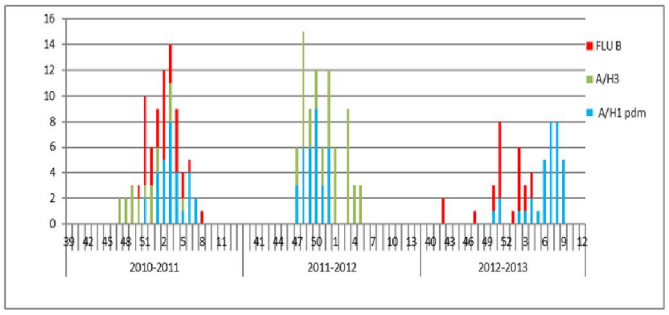

Figure 1. Weekly distribution of influenza isolates confirmed by the laboratory-based surveillance network in Casablanca during 2010-2013

Influenza B infection occurred in all age groups, In contrast, the 0-5 and 5-14 years age group were probably more affected. Meanwhile, lowest among adults aged 16-60 and none over 60 years of age (Fig.2).

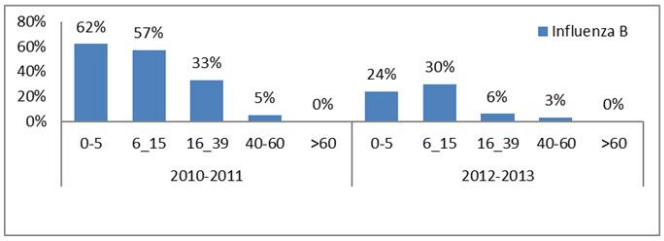

Figure 2. Age Group distribution of the influenza B positive cases

Molecular analysis

Twenty-tow influenza B viruses were sequenced in this study, 15 (28,3%) were related to the Victoria lineage and 7 (13,2%) to the Yamagata lineage. Victoria-lineage was identified throughout the season 2010–2011, whereas both Victoria and Yamagata-lineage viruses co-circulated throughout the season 2012-2013. The Comparison of the amino acid sequences of HA1 region of the Victoria lineage with the reference vaccine strain (B/Brisbane/60/2008) revealed some amino acid changes (Fig. 3). Genetically the majority of the B/Victoria lineage were from the B/Brisbane/60/2008 clade. The comparison of Victoria Casablanca strains with the reference strain revealed the following mutations: The Isoleucine residue at position 146 in B/Brisbane/60/2008 was substituted with Serine in all the Casablanca isolates and sporadic substitutions in various positions of hemagglutinin such as: N129K, G141R, N145K, Q187H, Y224H, Y263H, G266S and S277R (Table III). In addition, the HA genes of Victoria lineage isolates were closely related to B/Brisbane/60/2008, the vaccine strains of 2010/2011.

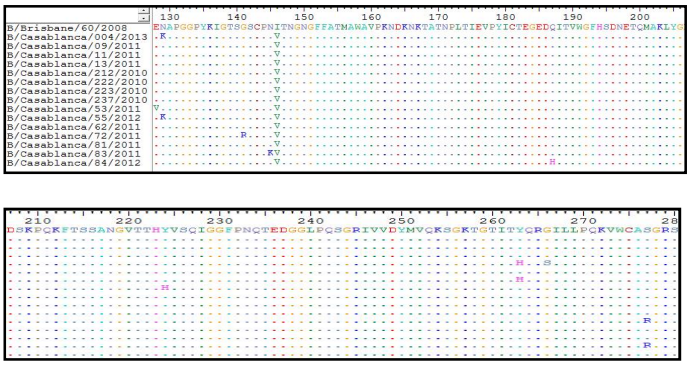

Figure 3. Comparison of amino acid sequences of HA protein domain 1 gene of Victoria lineage Casablanca influenza B strains with reference strain B/Brisbane/60/2008

Influenza B viruses of the Yamagata lineage fell in genetic clades 3 (represented by the vaccine virus B/Wisconsin/1/ 2010). The HA genes of viruses in clade 3 encode, I150, Y165, T181 and D229 (Table IV). Other substitutions defining the two Yamagata-like Casablanca groups were also identified: (T139S, A146T) and L172Q (Fig. 4). Sporadic mutations were also found in the Yamagata strains: L137I, C143W, K162Q, Y178L and I179P. The HA1 sequences of Casablanca influenza B isolates were compared with relevant virus sequences available in GenBank and GISAID databases. The phylogenetic analysis showed that all of these B-Victoria lineage viruses were related to the Brisbane/60 clade. Among the B viruses characterized during 2012-2013, those that were of the Yamagata lineage were antigenically related to the viruses recommended for the trivalent vaccine B/Wisconsin/1/ 2010. All these viruses displayed the S150I, N165Y and

Table 3. Amino Acid Changes in Influenza B HA1 Subunit in relation to B/Malaysia/2506/2004 (influenza B Victoria lineage)

Victoria lineage	Amino acid positions (B/Brisbane/60/2008)									
	128	**129**	**141**	**145**	**146**	**187**	**224**	**263**	**266**	**277**
B/Brisbane/60/2008	**E**	**N**	**G**	**N**	**I**	**Q**	**Y**	**Y**	**G**	**S**
B/Casablanca/004/2013	E	K	G	N	V	Q	Y	Y	G	S
B/Casablanca/09/2011	E	N	G	N	V	Q	Y	Y	G	S
B/Casablanca/11/2011	E	N	G	N	V	Q	Y	Y	G	S
B/Casablanca/13/2011	E	N	G	N	V	Q	Y	H	S	S
B/Casablanca/212/2010	E	N	G	N	V	Q	Y	Y	G	S
B/Casablanca/222/2010	E	N	G	N	V	Q	Y	H	G	S
B/Casablanca/223/2010	E	N	G	N	V	Q	H	Y	G	S
B/Casablanca/237/2010	E	N	G	N	V	Q	Y	Y	G	S
B/Casablanca/53/2011	V	N	G	N	V	Q	Y	Y	G	S
B/Casablanca/55/2011	E	K	G	N	V	Q	Y	Y	G	S
B/Casablanca/62/2011	E	N	G	N	V	Q	Y	Y	G	R
B/Casablanca/72/2011	E	N	R	N	V	Q	Y	Y	G	S
B/Casablanca/81/2011	E	N	G	N	V	Q	Y	Y	G	S
B/Casablanca/83/2011	E	N	G	K	V	Q	Y	Y	G	R
B/Casablanca/84/2011	E	N	G	N	V	H	Y	Y	G	S

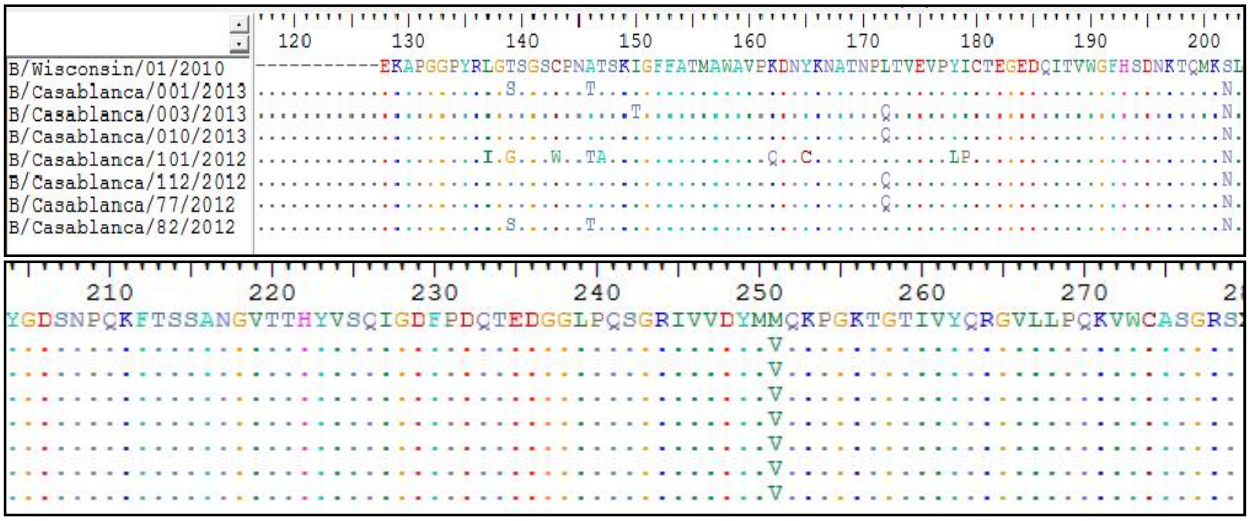

Figure 4. Comparison of amino acid sequences of HA protein domain 1 gene of Yamagata lineage Casablanca influenza B strains with reference strain B/Wisconsin/01/2010

Table 4. Amino Acid Changes in Influenza B HA1 Subunit in Relation to BB/Wisconsin/01/2010

Yamagata lineage	137	139	143	146	147	150	162	165	172	178	179	202	251
B/Wisconsin/01/2010	L	T	C	A	T	I	K	Y	L	Y	I	S	M
B/Casablanca/001/2013	L	S	C	T	T	I	K	Y	L	Y	I	N	V
B/Casablanca/003/2013	L	T	C	A	T	T	K	Y	Q	Y	I	N	V
B/Casablanca/010/2013	L	T	C	A	T	I	K	Y	Q	Y	I	N	V
B/Casablanca/101/2012	I	G	W	T	A	I	Q	C	L	L	P	N	V
B/Casablanca/112/2012	L	T	C	A	T	I	K	Y	Q	Y	I	N	V
B/Casablanca/077/2012	L	T	C	A	T	I	K	Y	Q	Y	I	N	V
B/Casablanca/082/2012	L	T	C	A	T	I	K	Y	L	Y	I	N	V

S229D substitutions. A M251V is present in influenza B viruses of the B-Yamagata lineage detected during the season 2012-2013. The Casablanca strains were genetically related to those of Morocco neighboring countries: Algeria, Tunisia and some African countries (Fig. 5).

DISCUSSION

Influenza viruses are the major respiratory viruses that cause yearly severe respiratory illness and associated with significant morbidity and mortality (Li, Choi, and Wong 2006). Surveillance and molecular characterization of circulating influenza viruses provide important information for annual selection of vaccine strains. In addition, identifying changes in the codons of the HA1 domain of the hemagglutinin gene, can facilitate the prediction of future lineages (Chi *et al*. 2005). In this present study we are reporting the activity and circulation of Influenza B viruses in Casablanca-Morocco during 2010-2013. During this surveillance study, a total of 760 clinical samples were collected, 53 (7%) were positive for influenza B viruses. The co-circulation of different influenza virus B strains during the same winter season, has been observed in this study. Influenza B causes disease in all age groups, but children and young adults tend to have higher rates of influenza B illness, which is consistent with the previous reports (Zaman *et al*. 2009). Molecular characterization of a

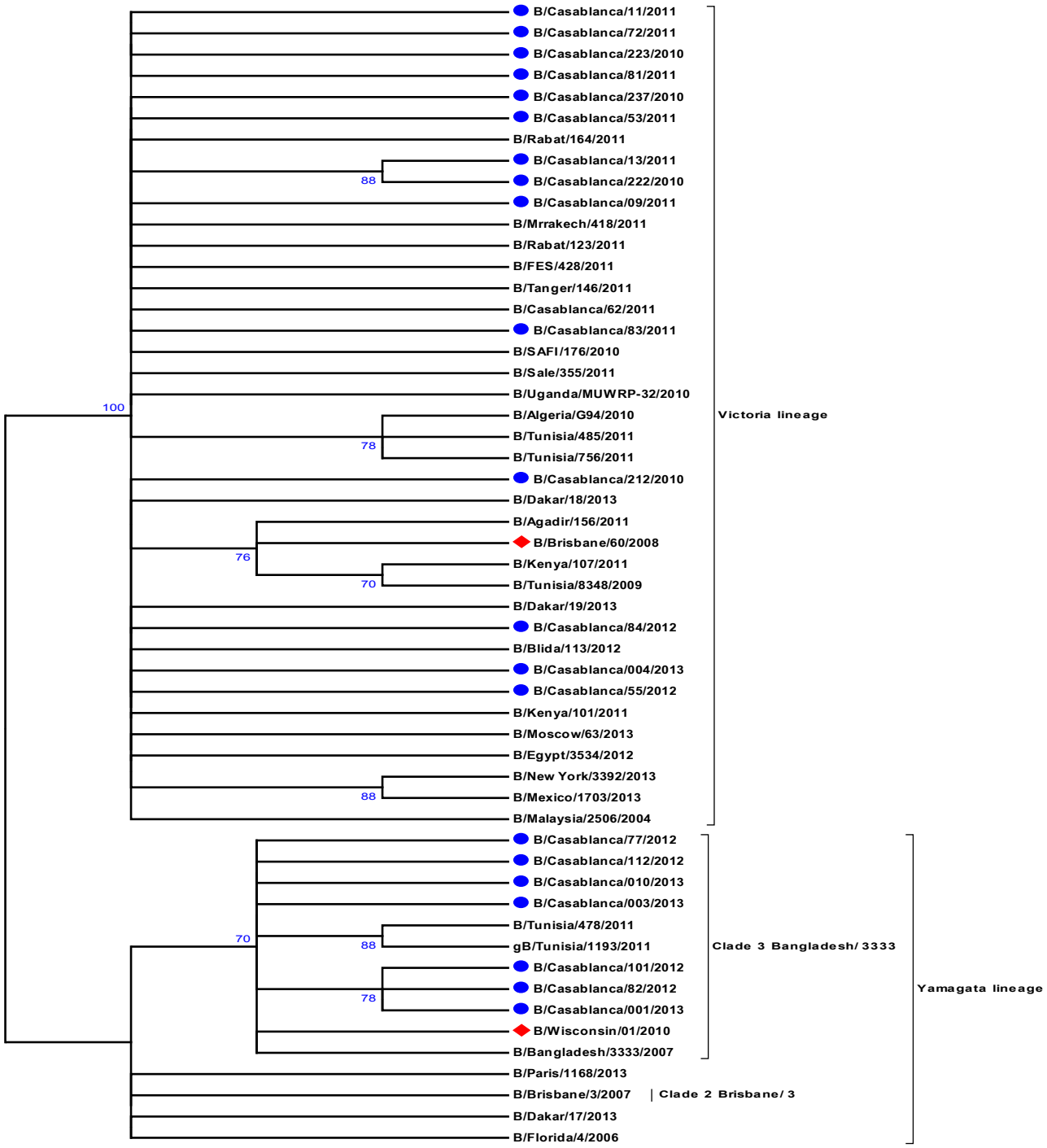

Figure 5. Phylogenetic relationship of the HA gene (HA1 domain). The tree was constructed using the neighbor-joining method with 1000 bootstrap replicates. The Casablanca strains are indicated with filled blue circles and the vaccine strains with filled red lozenge

partial domain HA1 of 22 influenza B viruses in Casablanca during the three seasons indicated the circulation of two lineages Victoria and Yamagata-lineag. During 2010–2011 season, Casablanca strains identified belonged to the Victoria lineage whereas most Tunisian strains belonged to the Yamagata lineage (El Moussi *et al*. 2013a). In 2012-2013, two lineages, B/Victoria and B/Yamagata have been co-circulating during the same winter season, a fact which is considered an important epidemiological characteristic as described (Paul A. Rota *et al*. 1990) and can cause wide spread epidemics in the population in general and mostly with high risk (Lin *et al*. 2007). Although influenza B viruses evolve at a slower rate when compared to influenza A, one mechanism of generating genetic diversity among influenza B viruses is by reassortment (Hay *et al*. 2001). The Co-circulation of multiple lineages B/Victoria lineage viruses and B/Yamagata lineage viruses have generated reassortants possessing Victoria HA and Yamagata NA, important evolutionary mechanism for influenza B viruses (McCullers *et al*. 1999) similar phylogenetic patterns among the basic polymerase 1 and basic polymerase 2, nucleoprotein and matrix protein genes, were observed, suggesting possible functional interactions among these proteins (Xu *et al*. 2004) (Zhu *et al*. 2013). All the results highlighted the importance of molecular monitoring of influenza B viruses for reassortment and antigenic drift. The particular epidemiology of these viruses allows strains to predominate during one season and virtually disappear from the population in the subsequent year with heterogeneity in

virus distribution (Ansaldi *et al*. 2003). The HA1 sequences of Casablanca B/Yamagata strains were classified in two groups. The first specific group of three viruses was characterized by two mutations (T139S and A146T). The second group of four viruses was defined by L172Q, as mentioned in the ECDC report (1204-TED-CNRL-Report.pdf n.d.). The Casablanca influenza B virus sequences were compared with World Health Organization (WHO) vaccine recommended for the period of study. Our Yamagata-like isolate of 2012-2013 was genetically closely related to the B/Wisconsin/01/2010 vaccine strains recommended (WHO). The Victoria-like strains showed similarities with the B/Brisbane/60/2008-like vaccine strain (Northern hemisphere season). Co-circulation of Victoria and Yamagata lineages of influenza B viruses observed world- wide (Barr *et al*. 2010)(Roy *et al*. 2011)(El Moussi *et al*. 2013b), this raise difficulties to select the next B strain for influenza vaccine composition. For years, flu vaccines have been trivalent, or designed to protect against three different flu viruses two A viruses and one B virus. WHO experts have had to choose between two very different B viruses for flu vaccine.

During the 10 seasons from 2001–02 through 2010–11, the predominant circulating influenza B virus lineage was represented in the trivalent vaccine in only five seasons (Ambrose and Levin 2012), this meant the vaccine did not help to protect against the second group of B viruses that was not included in the vaccine. By adding another B virus to the vaccine, quadrivalent vaccines may give broader protection (CDC - Quadrivalent Influenza Vaccine | Seasonal Influenza (Flu) n.d.). The quadrivalent vaccines recently approved by the FDA to be used in the United States in the 2013-2014 season may ensure a better correlation between the circulating influenza B viruses and the vaccine component in the world. (WHO | Recommended Composition of Influenza Virus Vaccines for Use in the 2014 Southern Hemisphere Influenza Season n.d.). In conclusion, the Surveillance and the monitoring of genetic variations among circulating influenza B viruses is important for public health surveillance and the selection of annual vaccine strains. The current report shows that the two genetically distinctive influenza B lineages; B/Vic and B/Yam, are co-circulating in Casablanca-Morocco. Therefore, there is an obvious need for the introduction of influenza vaccine including influenza A (H1N1 pdm09 and H3) and both lineage of influenza B strains to improve protection against both lineages of influenza B virus and decrease the morbidity infection.

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