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Hemagglutinin and Neuraminidase Genes of Influenza B Viruses Circulating in Riyadh, Saudi Arabia During 2010–2011: Evolution and Sequence Analysis

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Influenza viruses are known as continuing threats to human public health every year worldwide. Evolutionary dynamics of influenza B viruses in humans are in a unique progression having two lineages; B/Yam and B/Vic-like viruses, which are circulating simultaneously worldwide. There is a considerable lack of data on influenza B viruses circulating in Saudi Arabia. During the winter-spring season of 2010–2011, 80 nasopharyngeal aspirates were collected from hospitalized patients with flu-like symptoms in Riyadh. Screening of samples by one-step RT-PCR identified three (3.8%) influenza B viruses. Sequencing of hemagglutinin (HA) and neuraminidase (NA) genes was performed to analyze influenza B viruses circulating in Riyadh as compared to the globally circulating strains. Several common and six unique amino acid substitutions were observed for both HA and NA genes of influenza B Saudi strains. Three unique substitutions (T182A, D196N, and K254R) were identified in HA gene of the B/Yam-like Riyadh strains. In NA gene, a unique common substitution (D53G) was found in all Riyadh strains, while two unique substitutions (L38P, G233R) were recognized only in B/Vic-like Riyadh strains. Riyadh strains were also found to contain N-glycosylation site in HA gene of both B/Vic and B/Yam lineages at positions 197–199 (NET) and 196–198 (NNK/DNK), respectively. The significance of these mutations on the antigenicity of both lineages is discussed herein. The unique changes observed in HA and NA genes of influenza B Riyadh strains support strongly the need for continuous surveillance and monitoring of new evolving strains that might pose threat to the Saudi community. ***J. Med. Virol. 86:1003–1016, 2014.*** © 2013 Wiley Periodicals, Inc.

KEY WORDS: influenza B virus; DNA sequencing; hemagglutinin; neuraminidase; phylogenetic Analysis; Saudi Arabia

INTRODUCTION

Influenza B viruses are members of family *Orthomyxoviridae*, which contains a segmented negative-sense RNA genome. The virus genome encodes for 11 proteins; namely polymerase PB1 (Seg-1), polymerase PB2 (Seg-2), polymerase PA (Seg-3), hemagglutinin (Seg-4), nucleoprotein (Seg-5), neuraminidase (NA), and NB protein (Seg-6), matrix protein 1 and BM2 protein (Seg-7), non-structural protein 1 and non-structural protein 2 (Seg-8) [Lamb and Choppin, 1983]. Influenza viruses including type A and B are the main respiratory viruses that cause worldwide annual epidemics with high economic loss and approximately 250,000–500,000 deaths every year [WHO, 2003; Thompson et al., 2004]. Three phylogenetically and antigenically distinct influenza virus types; A, B, and C are circulating globally in the human

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populations. Currently, influenza A and B viruses are the most common types, which are continuously causing recurrent epidemics associated with morbidity, mortality, and economic loss worldwide.

Influenza B virus was isolated for the first time at 1940 during an epidemic in USA, and the isolated virus was designated B/Lee/40 [Nerome et al., 1998]. Since that time, variant influenza B virus isolates were recovered and consequently they were divided, based on their antigenic and genetic properties, in two main lineages; Victoria lineage represented by the prototype strain B/Vic/2/87 and Yamagata lineage represented by the prototype strain B/Yam/16/88 [Kanegae et al., 1990]. Influenza B Vic-like viruses were first isolated and identified in USA during 1988 and 1989 epidemics, and later they were disseminated out to Asia and Europe. Whereas, Influenza B Yam-like viruses were isolated for the first time in Japan during the 1980s. These viruses were disseminated globally during the 1990s, and at present they are considered the most predominant strains in the world that are used in vaccine preparation [Nerome et al., 1998; Motta et al., 2006]. However, during 2001–2002, several studies had reported the re-emergence of Victoria lineage (represented by B/Hong Kong/330/01) in North America, Europe, Japan, China, and Taiwan, and have since been isolated infrequently with rare localized outbreaks globally [Shaw et al., 2002; Daum et al., 2006; Tsai et al., 2006; Chen et al., 2007; Lin et al., 2007].

Influenza viruses are characterized by two mechanisms that direct evolution of new variants; antigenic drift and genetic shift (also called reassortment) [Rota et al., 1992; Hay et al., 2001]. Although genetic shift is a common incident in influenza A viruses and contributes in sudden emergence of new subtypes [Treanor, 2004], previous reports have suggested that it plays no role in genetic variation of Influenza B viruses. For this reason, influenza B viruses are not involved commonly in worldwide pandemics [Treanor, 2004; Chen and Holmes, 2008]. Nevertheless, several mechanisms for evolution that include insertions, deletions, and substitutions are frequent among different lineages of influenza-B viruses [Nerome et al., 1998; McCullers et al., 1999; Shaw et al., 2002; Chi et al., 2005].

Hemagglutinin (HA) is the major viral surface glycoprotein, which performs two essential biological activities that include receptor binding and envelope fusion [Skehel and Wiley, 2000]. On the other hand, NA protein hydrolyzes terminal sialic acids of sialoglycans and promotes the release of progeny virions from infected host cells by destroying receptors [Shibata et al., 1993]. HA is a trimeric molecule that contains two basic subunits; HA1 and HA2. The monomeric HA molecule contains fusing domain and globular membrane-distal domain surrounded by receptor-binding sub-domain and vestigial esterase sub-domain. It was reported that four major epitopes (antigenic sites) of influenza B viruses exist on the membrane distal domain of HA1 subunit. On each subunit of HA gene, there are a total of ten potential glycosylation sites; seven on HA1 and three on HA2 [Wang et al., 2008].

In literature, influenza and other respiratory viruses from Saudi Arabia did not receive much concern, rather than monitoring of prevalence among the causes of acute respiratory illness in young children living in Riyadh and Abha cities, [Bakir et al., 1998; Al-Shehri et al., 2005] or in pilgrims during the Hajj seasons [Rashid et al., 2008]. Since few years back, a continuous surveillance system was initiated in Makkah region due to the higher chance of inter-lineage reassortment among influenza viruses, particularly during the Hajj season, and has been gradually expended up till now. Recently, a short report has discussed the isolation and partial identification of influenza B viruses from Riyadh [Almajhdi, 2010].

The current report shows that the two genetically distinctive influenza B lineages; B/Vic and B/Yam, are co-circulating in the community of Riyadh. It seems that the predominant circulating influenza B virus lineage is dissimilar from that contained in the vaccine; so there is limited or no protection. Therefore, there is an obvious need for the introduction of influenza vaccine consisting of influenza A and both lineage of influenza B strains to improve protection against both lineages of influenza B virus and reduce the morbidity of influenza B infection.

MATERIALS AND METHODS

Clinical Samples

During the winter-spring season of 2010–2011, a total of 80 nasopharyngeal aspirates were collected from patients hospitalized in different hospitals of Riyadh, Saudi Arabia. These subjects were included in this study with the symptoms of influenza-like acute respiratory infections along with history of first onset within 48 hr, fever ($T \geq 38^{\circ}C$), sore throat, cough, headache, runny nose, sneezing, and myalgia. Sample collection methodology was approved by the Ethical Committee of King Saud University. The informed consent forms were signed by the parents/guardians of patients. Aspirates were transported immediately in 2 ml viral transport medium containing minimal essential medium (MEM), 1,000 U penicillin, and 1 mg streptomycin, to the Virology Research Laboratory at College of Science, King Saud University. Upon receipt, samples were mixed by pulse-vortexing for 15 sec, centrifuged at 1,000*g* for 10 min at 4°C, divided into aliquots, and stored at −80°C until use for further screening/testing.

RNA Extraction

Viral RNA was extracted from the nasopharyngeal aspirates of patients using QIAamp Viral RNA extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's suggested protocol. The RNA was eluted from the spin columns in 60 μl of elution

buffer. Eluted RNA was divided into aliquots and stored immediately at −80°C till further use.

One-Step RT-PCR for Detection of Influenza B Virus

Identification of influenza B virus RNA in the extracts of clinical samples was achieved using QIAGEN one-step RT-PCR kit and a universal primer set described previously by Gröndahl et al. [1999]. This primer set was chosen from highly conserved sequences of NS2 gene of influenza B viruses (Table I). The RT-PCR mixture contained 5 μl of RNA, 5μl of 5 × Qiagen One-step RT-PCR buffer, 1 μl of one-step RT-PCR enzyme mixture, 1 μl of 5 mM dNTPs mix, 1 μl (0.6 μM) of each primer, 1 μl of RNase inhibitor, and 10 μl PCR grade water (Qiagen). The reaction tubes were incubated in the thermal cycler Gene-Amp 9700 (Applied Biosystems, Foster City, CA). Amplification of the target sequence was carried out using the following cycling protocol: reverse transcription at 50°C for 30 min; initial PCR activation at 95°C for 15 min; 35 cycles of denaturing at 95°C for 30 sec, primer annealing at 55°C for 30 sec, and extension at 72°C for 90 sec. The PCR ended with a final termination step at 72°C for 10 min. The RT-PCR amplified products were analyzed, along with 100 bp DNA ladder, by electrophoresis in 1.5% agarose gel stained with ethidium bromide.

Amplification and Sequencing of HA and NA Gene Fragments

Three influenza B Saudi strains; Riyadh/01/2010, Riyadh/02/2010, and Riyadh/03/2010 were selected for sequence analysis of HA and NA genes. Sequencing approach involved the design of three primer sets for HA gene and two sets for NA gene (Table I), which amplify overlapping fragments that span the entire genes' sequence. The sequencing PCR was performed using the FideliTaq RT-PCR master mix (GE Healthcare, Buckinghamshire, UK). Cycling conditions included one cycle of reverse transcription at 50°C for 30 min; one cycle of initial PCR activation at 95°C for 3 min; 35 cycles of denaturation at 95°C for 30 sec, primer annealing at 50°C for 30 sec, and extension at 68°C for 1 min; and a final extension step at 68°C for 5 min. Specific amplified products were excised from agarose gel using DNA gel recovery kit (GE Healthcare). The nucleotide sequence of the purified fragments were sequenced subsequently in both directions (GenArt, Rosenberg, Germany) using an automated DNA sequence analyzer.

Sequence and Phylogenetic Analysis of HA and NA Genes

Bidirectional nucleotide (nt) sequences were obtained using gene specific forward and reverse primers for HA and NA genes. All HA and NA gene sequence data were edited and assembled using BioEdit software (http://www.mbio.ncsu.edu/BioEdit). These sequences were aligned with other known international sequences (Tables II and III) selected from NCBI Influenza Resource (http://www.ncbi.nlm.nih.gov/genomes/flu/) using the ClustalW algorithm of MegAlign program, LASERGENE software package (DNAStar, Inc., Madison, WI). Multiple sequence alignment with the international strains available in Genbank was performed to determine the genetic diversity and relationship between influenza B Riyadh viruses and global/vaccine strains. A phylogenetic tree was generated using neighbor-joining method supported by 1,000 bootstrap replicates in the MegAlign program of the LASERGENE software [Saitou and Nei, 1987].

Gene Bank Accession Numbers

All the nucleotide sequences of influenza-B Saudi strains B/Riyadh/01/2010, B/Riyadh/02/2010, and B/Riyadh/03/2010 have been deposited in GenBank under the accession numbers: JN663826, JQ771075

TABLE I. Primer Sets used in the Study

Influenza B virus gene	Primer pair	Sequences	Product size
Non-structural 2 (NS-2)	INFB-Univ-F1	5′-ATG GCC ATC GGA TCC TCA AC-3′	238 bp
	INFB-Univ-R1	5′-TGT CAG CTA TTA TGG AGC TG-3′	
Hemagglutinin (HA)	INFB-HA-F1	5′-ATG AAG GCA ATA ATT GTA CTA CTC-3′	440 bp
	INFB-HA-R1	5′-GGC AA G AYC CTG AGG TTC C-3′	
	INFB-HA-F2	5′-CTT TCC TAT AAT GCA CGA CAG AAC-3′	677 bp
	INFB-HA-R2	5′-CAT ATT GGG CAA TTT CCT ATG GC-3′	
	INFB-HA-F3	5′-AGC AAG CCT TAC TAC ACA GG-3′	800 bp
	INFB-HA-R3	5′-CCT TAT AGA CAG ATG GAG CAA G-3′	
Neuraminidase (NA)	INFB-NA-F1	5′-TGA ACA ATG CTA CCT TCA AC-3′	851 bp
	INFB-NA-R1	5′-GCA AAT CCG CAT GAG CAT TC-3′	
	INFB-NA-F2	5′-CAA GAA AGT GCC TGC AAT TGC-3′	709 bp
	INFB-NA-R2	5′-GAA CAG AYT CAA CCA TTC CTC C-3′	

TABLE II. List of Influenza B Virus Strains included in Sequence Analysis of HA Gene

No	Strain	Origin	Genbank accession no.	Reference
1	B/Riyadh/01/2010	Saudi Arabia	JN663826	This study
2	B/Riyadh/02/2010	Saudi Arabia	JQ771075	This study
3	B/Riyadh/03/2010	Saudi Arabia	JQ771077	This study
4	B/Kol/2546/2009	India	JF965349	Roy et al. [2011]
5	B/Kol/2636/2010	India	JF693249	Roy et al. [2011]
6	B/Kol/194/2010	India	JF965353	Roy et al. [2011]
7	B/Kol/273/2010	India	JF965355	Roy et al. [2011]
8	B/Kol/2044/2008	India	JF693244	Roy et al. [2011]
9	B/Kol/1253/2007	India	JF693242	Roy et al. [2011]
10	B/Kol/673/2006	India	JF965342	Roy et al. [2011]
11	B/Lee/40	USA	J02093	Krystal et al. [1982]
12	B/Wisconsin/01/2010	USA	JN993010	Unpublished
13	B/Texas/04/2008	USA	EU779606	Unpublished
14	B/Florida/4/2006	USA	CY033876	Unpublished
15	B/Victoria/03/85	USA	X13553	Air et al. [1990]
16	B/Victoria/504/2000	USA	CY018653	Unpublished
17	B/Hawaii/13/2004	USA	CY018517	Unpublished
18	B/Brisbane/60/2008	Australia	CY115151	Unpublished
19	B/Brisbane/32/2002	Australia	EF566110	Unpublished
20	B/Hong Kong/1434/2002	Hong Kong	CY018685	Unpublished
21	B/Hong Kong/8/1973	Hong Kong	EF456778	Unpublished
22	B/Sichuan/379/99	China	EF566113	Unpublished
23	B/Malaysia/2506/2004	Malaysia	EU124274	Unpublished
24	B/Yamagata/16/88	Japan	M36105	Kanegae et al. [1990]
25	B/Nepal/1331/2005	Nepal	CY019523	Unpublished
26	B/Israel/95/2003	Israel	AJ784047	Unpublished
27	B/Tehran/80/2002	Iran	AJ784042	Unpublished
28	B/Oman/16291/2001	Oman	AF532566	Shaw et al. [2002]

and JQ771077 for HA gene, and JN663827, JQ771076 and JQ771078 for NA gene.

RESULTS

Detection of Influenza-B Viruses

Among 80 nasopharyngeal aspirates, 3 (3.8%) samples were positive for influenza B virus using one-step RT-PCR assay. These Saudi strains were designated as B/Riyadh/01/2010, B/Riyadh/02/2010, and B/Riyadh/03/2010.

Sequence and Phylogenetic Analysis of HA Gene

HA gene of influenza B Riyadh strains was sequenced and aligned with sequences of 25 global strains available in Genbank database using the Clustal W program (Table II). This alignment allowed grouping of strains into definite clusters. Two Riyadh strains (B/Riyadh/02/2010 and B/Riyadh/03/2010) were grouped with B/Yam-like viruses and showed maximum sequence homology with the vaccine strain of 2008–2009; B/Florida/4/2006 (94.6–97.1%) and the current vaccine strain; B/Wisconsin/01/2010 (95.6–99%). Whereas the other strain B/Riyadh/01/2010 was clustered with B/Vic-like viruses with a sequence homology of 98.5% with B/Malaysia/2506/2004 (vaccine strain of 2006–2008) and 98.9% with the current vaccine strain B/Brisbane/60/2008.

In the HA gene sequence of B/Vic-like Riyadh strain (B/Riyadh/01/2010), 44 (2.5%) nucleotide substitutions were found that lead to 9 amino acid changes. Similarly, in the HA gene sequence of B/Yam-like Riyadh strains (B/Riyadh/02/2010 and B/Riyadh/03/2010), 99–143 (5.6–8.1%) nucleotide substitutions that lead to 31–32 (5.3–5.4%) amino acid changes were observed. The divergence between Riyadh strains of both lineages was estimated as 7.4–11.7% and 1.0% at nucleotide and amino acid levels, respectively. Similarly, amino acid divergence was observed 1.0% between the B/Yam-like Riyadh strains and their respective vaccine strain B/Wisconsin/01/2010, while the deduced amino acid difference was estimated between the B/Yam-like Riyadh strains and B/Yam/16/88 ranging from 4.7 to 6.1%. On the other hand, the amino acid divergence between B/Vic-like Riyadh strain and its respective vaccine strain B/Vic/03/85 was estimated to be 3.8%.

TABLE III. List of Influenza B Virus Strains Included in Sequence Analysis of NA Gene

No	Strain name	Origin	Genbank accession no.	Reference
1	B/Riyadh/01/2010	Saudi Arabia	JN663827	This Study
2	B/Riyadh/02/2010	Saudi Arabia	JQ771076	This Study
3	B/Riyadh/03/2010	Saudi Arabia	JQ771078	This Study
4	B/Kol/2546/2009	India	JF965365	Roy et al. [2011]
5	B/Kol/2636/2010	India	JF693265	Roy et al. [2011]
6	B/Kol/194/2010	India	JF965369	Roy et al. [2011]
7	B/Kol/273/2010	India	JF965371	Roy et al. [2011]
8	B/Kol/2044/2008	India	JF693260	Roy et al. [2011]
9	B/Kol/1253/2007	India	JF693258	Roy et al. [2011]
10	B/Kol/673/2006	India	JF965358	Roy et al. [2011]
11	B/Lee/40	USA	J02095	Shaw et al. [1982]
12	B/Wisconsin/01/2010	USA	JN993012	Unpublished
13	B/Texas/04/2008	USA	EU779607	Unpublished
14	B/Florida/4/2006	USA	CY073896	Unpublished
15	B/Victoria/03/85	USA	M30639	Air et al. [1990]
16	B/Hawaii/13/2004	USA	CY018519	Unpublished
17	B/Victoria/02/1987	USA	AB036870	Unpublished
18	B/Brisbane/60/2008	Australia	CY073894	Unpublished
19	B/Brisbane/32/2002	Australia	EF566111	Unpublished
20	B/Hong Kong/1434/2002	Hong Kong	CY018687	Unpublished
21	B/Hong Kong/330/2001	Hong Kong	AY191502	Unpublished
22	B/Beijing/184/93	China	AJ784090	Unpublished
23	B/Malaysia/2506/2004	Malaysia	CY038289	Unpublished
24	B/Yamagata/16/88	Japan	AY139081	Shaw et al. [2002]
25	B/Nepal/1331/2005	Nepal	CY018527	Unpublished
26	B/Taiwan/110/2005	Taiwan	EF102950	Lin et al. [2007]
27	B/Aichi/5/88	Japan	AY581986	McCullers et al. [2004]
28	B/Taiwan/217/97	Taiwan	AY139053	Shaw et al., [2002]

Sequence analysis of nucleotide and deduced amino acid sequences of HA gene of Riyadh strains showed a considerable mutation rates that may predicts potential effect on the protein structure and function. In HA gene sequence of B/Malaysia/2506/2004, B/Brisbane/60/2008, and B/Vic-like Riyadh strain, there was E (glutamic acid) at position 48, however, B/Yam-like Riyadh strains had R (arginine). The HA gene sequence of B/Yam-like Riyadh strains and vaccine strain had L (leucine) at position 58, whereas B/Vic-like Kolkata strains and Riyadh strain had P (proline) at this position. In B/Malaysia/2506/2004 and B/Brisbane/60/2008 vaccine strains of B/Vic-like viruses, T (threonine) residue, at position 73 was substituted by M (methionine) in the B/Vic-like Riyadh strain. Similarly, vaccine strain and B/Yam--like Riyadh viruses had same V (Valine) residue at position 73. All B/Vic-like viruses differed from B/Hong Kong/1434/2002 by only two characteristic amino acid changes R116H and E164D. In B/Vic-like strains H (histidine) amino acid at 116 position was replaced by N (asparagine) in all the B/Yam-like representative strains of Riyadh except for B/Victoria/504/2000 and B/Sichuan/379/99, which had K (lysine), whereas B/Hong Kong/1434/2002 had R (arginine) amino acid at 116 (Table IV). In all B/Vic-like strains same substitution H122Q was observed by comparing with the sequence of B/Yam lineage-like viruses.

The antigenic alterations of an influenza virus occurs largely in the HA1 region of HA gene. Table IV lists the amino acid changes in the HA1 gene sequence of Riyadh strains in comparison with other reference strains from Genbank. In HA gene sequences of B/Vic-like Riyadh strain (B/Riyadh/01/2010), no unique amino acid changes were observed by comparing with B/Lee/40 and vaccine strain of 2010. However, 22 deduced amino acid changes (E48Q, K56N, P58F, K75N, I76T, R80K, H116N, H122S, N129T, I137V, N163D, D164N, K165N, I175V, T182S, E198K, A202E, K203R, S255P, T262V, D505N, and V555I) and two asparagine (N) insertions between amino acid positions 165 and 166 were found in the HA gene sequences of B/Riyadh/01/2010 by comparison with B/Lee/40 like strain (Table IV), which is the oldest group of influenza-B viruses circulating throughout the world for several years. The variation between the HA gene sequence of B/

TABLE IV. Amino Acid Comparison in HA Sequence of Influenza-B Riyadh Strains Along with Strains Representing Both Lineages and Vaccine Strains (in Bold)

Amino acid positions	Common sequence	B-Riyadh-1-2010	B-Kol-2636-2010	B-Kol-194-2010	B-Kol-273-2010	B-Kol-2044-2008	**B-Brisbane-60-2008**	B-Nepal-1331-2005	**B-Malaysia-2506-2004**	B-Hawaii-13-2004	B-Israel-95-2003	B-Brisbane-32-2002	B-Hong Kong-1434-2002	B-Tehran-80-2002	B-Oman-16291-2001	B-Victoria-03-85	B-Riyadh-2-2010	B-Riyadh-3-2010	**B-Wisconsin-01-2010**	B-Kol-2546-2009	**B-Texas-04-2008**	B-Kol-1253-2007	B-Kol-673-2006	**B-Florida-4-2006**	B-Victoria-504-2000	B-Sichuan-379-99	B-Yamagata-16-88	B-Hong Kong-8-1973	B-Lee-40
		B-Victoria Lineage															**B-Yamagata Lineage**												
40	H	H	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Y	Y	Y	Y	Y	Y	Y	Y	.	.	.	.	.
48	K	E	.	.	.	E	E	E	E	.	.	.	.	.	.	.	R	R	R	R	R	R	R	R	.	.	.	Q	Q
56	K	K	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D	D	D	D	D	D	D	D	T	.	N	N	N
58	L	P	P	P	P	P	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	F
71	K	K	.	.	.	.	.	.	.	.	.	.	.	.	.	.	M	M	M	M	M	M	M	M	M	M	M	.	.
73	T	M	.	.	.	.	.	.	.	.	.	.	.	.	.	M	V	V	V	V	V	V	V	V	V	V	M	M	M
75	T	K	K	K	K	K	K	N	N	N	N	N	N	N	N	.	.	.	.	.	.	.	.	.	I	I	.	.	N
76	I	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	T	T	T	T	T	T	T	T	T	.	.	T
80	K	R	R	R	R	R	R	R	R	E	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
81	V	V	.	.	.	.	.	.	.	.	.	.	.	.	.	A	A	A	A	A	A	A	A	A	A	A	A	A	.
116	N	H	H	H	H	H	H	H	H	H	H	H	R	H	H	.	.	.	.	.	.	.	.	.	K	K	.	.	.
122	H	H	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	.	R	S
126	N	N	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D	D	D	D	D	D	D	D	.	.	.	.	.
129	K	N	N	N	N	N	N	N	N	.	.	.	E	.	.	T	.	.	.	.	.	.	.	.	.	.	R	T	T
136	K	K	.	.	.	.	.	.	.	.	.	.	.	.	.	I	R	R	R	R	R	R	R	R	R	R	R	I	.
137	I	I	.	.	.	.	.	.	.	.	.	.	.	.	.	V	L	L	L	L	L	L	L	L	L	L	L	V	V
146	V	V	.	.	.	.	I	.	.	.	.	.	.	.	.	.	A	A	A	A	A	A	A	A	A	A	.	.	.
148	N	N	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	S	S	S	S	S	S	S	S	S	S	.	.
149	G	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	K	K	K	K	K	K	K	K	K	K	R	.	.
150	N	N	I	I	I	.	.	.	.	.	.	.	.	.	.	.	I	I	I	I	S	I	I	S	S	S	.	.	.
163	N	N	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D	D	D	D	D	D	D	D	D	D	D	—	D
164	D	D	.	.	.	.	.	.	.	.	.	.	E	.	.	N	N	N	N	N	N	N	N	N	N	N	—	—	N
165	N	K	K	K	K	K	K	.	.	.	.	.	.	.	.	.	Y	Y	Y	Y	.	Y	Y	.	.	.	—	.	.
166	—	N	N	N	N	N	N	N	N	N	N	N	N	—	N	N	—	—	—	—	—	—	—	—	—	—	—	—	—
167	—	K	K	K	K	K	K	K	K	K	K	K	K	—	K	K	—	—	—	—	—	—	—	—	—	—	—	—	—
168	T	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	N	N	N	N	N	N	R	N	.	.	.	.	.
175	V	I	I	I	I	I	I	I	I	I	I	I	I	I	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.
182	T	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	*A*	*A*	.	.	.	.	.	.	.	.	.	.	*S*
196	D	D	.	.	.	.	.	.	.	.	.	.	.	.	.	.	*N*	.	.	.	.	.	.	.	.	.	.	.	.
198	E	E	.	.	.	.	.	.	.	.	.	.	.	.	.	.	K	K	K	K	K	K	K	K	K	K	K	.	K
202	A	A	.	.	.	.	.	.	.	.	.	.	.	.	.	V	K	K	K	K	K	K	K	K	K	K	K	V	E
203	K	K	.	.	.	.	.	.	.	.	.	.	.	.	.	.	N	N	S	N	N	N	N	N	N	N	N	.	R
209	K	K	.	.	.	.	.	.	.	.	.	.	.	.	.	.	N	N	N	N	N	N	N	N	N	N	N	.	N
230	G	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D	D	D	D	S	D	D	S	.	.	D	.	.
233	N	N	.	.	.	.	.	.	.	.	.	.	.	.	.	K	D	D	D	D	D	D	D	D	E	D	.	.	.
254	K	K	.	.	.	.	.	.	.	.	.	.	.	.	.	.	*R*	*R*	.	.	.	.	.	.	.	.	.	.	.
255	S	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	P	P	P	P	P	P	P	P	P	P	P	P	P
262	T	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	V	V	V	V	V	V	V	V	V	V	V	A	V
267	I	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	V	V	V	V	V	G	V	V	.	.	V	V	.
479	E	E	.	D	.	.	.	.	.	.	.	.	.	.	.	D	D	.	D	.	.	D	D	.	D	.	D	D	.
505	D	D	.	.	.	.	.	.	.	.	.	.	N	.	.	N	N	.	N	.	N	N	N	N	N	.	N	N	N
551	I	I	.	M	.	.	.	.	.	.	.	.	.	.	.	.	L	.	L	.	L	L	L	L	.	.	.	.	.
555	V	V	.	.	.	.	.	.	.	.	.	.	.	.	.	I	I	.	I	.	I	I	I	I	I	.	I	I	I

The amino acid positions correspond to the B/Lee/40 strain. Common amino acids are indicated by dot (.), deletion represented by (–) and unique amino acid substitution represented in bold italic.

Riyadh/01/2010 and the two prototype B/Vic vaccine strains B/Brisbane/60/2008 and B/Malaysia/2506/2004 was three (L58P, T73M, and I146V) and four (L58P, T73M, T75K, and N165K) amino acid changes, respectively. On the other hand, five common amino acids D196, E479, D505, I551, and V555 were observed between B/Vic-like and B/Yam-like Riyadh strains except in B/Riyadh/02/2010 strain. Similarly, by comparing HA gene sequence of both B/Yam-like Riyadh strains B/Riyadh/02/2010 and B/Riyadh/03/2010 with the prototype vaccine strain B/Wisconsin/01/2010, three amino acid substitutions T182A, S203N and K254R were found along with other five variations D196N, E479D, D505N, I551L, and V555I, which were observed only in B/Riyadh/02/2010. So far, B/Yam-like Riyadh strains showed three unique amino acid changes T182A, D196N, and K254R by comparing with the highly homologous strains from the Genbank and also with concurrent vaccine strain of 2010.

In 120 loop of HA1 region, N (asparagine) at position 126 of all B/Vic lineage-like strains was replaced by D (aspartic acid) in most of the B/Yam-like strains. At position 129, N (asparagine) and K (lysine) were observed in B/Vic and B/Yam-like Riyadh strains, respectively. The amino acid position 137 is usually occupied by either I (isoleucine) or L (leucine) and in some instances by V (valine) in both lineages of influenza B viruses.

In HA gene, both antigenic lineages of Riyadh strains contains an N-linked glycosylation site (Fig. 3) at positions of 197–199 (NET) in B/Vic-like and 196–198 (NNK/DNK) in B/Yam-like viruses. The HA gene sequence of both circulating lineages of Riyadh viruses lacks the amino acid (R) at position 141, which plays an important role in stabilization of the glycosylation site without disturbing virus antigenicity. The loss of a potential glycosylation site at position 233–235 of HA1 region of the B/Yam lineage-like Riyadh strains was also observed.

Phylogenetic analysis of influenza B virus strains showed that strains isolated before 1983 are collected into distinct clusters represented by B/Lee/40 strain. However, all subsequent virus strains were grouped under B/Yam lineage (represented by B/Malaysia/2506/2004, B/Brisbane/32/2002, and B/Brisbane/60/2008 vaccine strains) or B/Vic lineage (represented by B/Texas/04/2008, B/Florida/4/2006, and B/Wisconsin/01/2010 vaccine strains). Similar to sequence analysis data, the constructed tree proved that B/Riyadh/02/2010 and B/Riyadh/03/2010 strains belong to B/Yam lineage and B/Riyadh/01/2010 is a member of B/Vic lineage (Fig. 1).

Sequence and Phylogenetic Analysis of NA Gene

The NA gene sequence of influenza B Riyadh strains was aligned with sequences of international strains available in Genbank database using ClustalW program. Sequence and phylogenetic analysis of NA genes indicated that influenza B virus strains are diverged into two genetically distinct lineages; B/Vic and B/Yam (Fig. 2). One (B/Riyadh/01/2010) of three influenza-B Riyadh strains belonged to the B/Vic-lineage represented by the vaccine strains B/Brisbane/32/2002 and B/Malaysia/2506/2004, to which the amino acid sequence divergence was estimated 0.9% and 2.5% and homology 97.8% and 98.9%, respectively. 0.8–0.9% amino acid divergence and 99.2% homology was found between B/Vic-like Riyadh and Kolkata strains. The remaining two Riyadh strains showed genetic proximity with the B/Yam-lineage represented by the two vaccine strains B/Wisconsin/01/2010 and B/Florida/04/2006, to which the sequence divergence 0.4–1.6% and identity 94.5–99% was observed. The difference between the amino acid sequences of both lineages of influenza-B Riyadh strains was ranging from 5.2 to 5.4%. A maximum sequence homology (99.2–99.7%) was demonstrated between the NA genes of both lineages of influenza-B Riyadh strains and Kolkata strains like B/Kol/2636/2010, B/Kol/194/2010, B/Kol/1253/2007, and B/Kol/673/2006.

A comprehensive analysis of the deduced amino acid sequences of NA gene of both lineages represented by Riyadh strains is given in Table V. Sequence analyses of NA gene, revealed several number of amino acid substitutions S27L, L38P, P51S, L73F, K125N, E148G, S198N, N199D, I204V, N220K, G233R, D235N, I248V, E320D, A358E, K373E, T389A, A395V, and E404K in B/Vic-like Riyadh strain, by comparing them with the international strains of influenza-B viruses (Table V). A gain of the glycosylation site at 233–235 in the NA gene of B/Vic lineage-like Riyadh strains was also observed.

The NA gene of B/Riyadh/01/2010 showed close proximity with the B/Brisbane/60/2008 vaccine strain of Victoria lineage through the identification of seven amino acid variations including S27L, L38P, P51S, L73F, N199D, G233R, and A395V. Similarly, several amino acid substitutions S27L, L38P, S42P, P51S, L73F, K125N, N199D, I204V, N220K, G233R, E320D, A358E, K373E, A395V, E404K, and N463D were found by comparing NA gene of B/Riyadh/1/2010 with the B/Malaysia/2506/2004 vaccine strain of B/Vic lineage.

Most of the Riyadh strains isolated during the 2010–2011 season, clustered with B/Yam lineage showing three amino acid substitutions P42R, D53G, and A218V in comparison with the vaccine strain B/Wisconsin/01/2010. Similarly, five amino acid substitutions P42R, D53G, T125K, K186R, and A218V were observed by comparing Riyadh strains with the B/Texas/04/2008. In addition to the above substitutions, Q42R, D53G, T125K, K186R, A218V, D463N, and A465T were also found in B/Yam-like Riyadh strains by comparing with B/Florida/04/2006 vaccine strain of 2008–2009. Based on the phylogenetic analysis of all the NA gene sequences, 32 common and one unique amino acid substitutions (D53G) were found

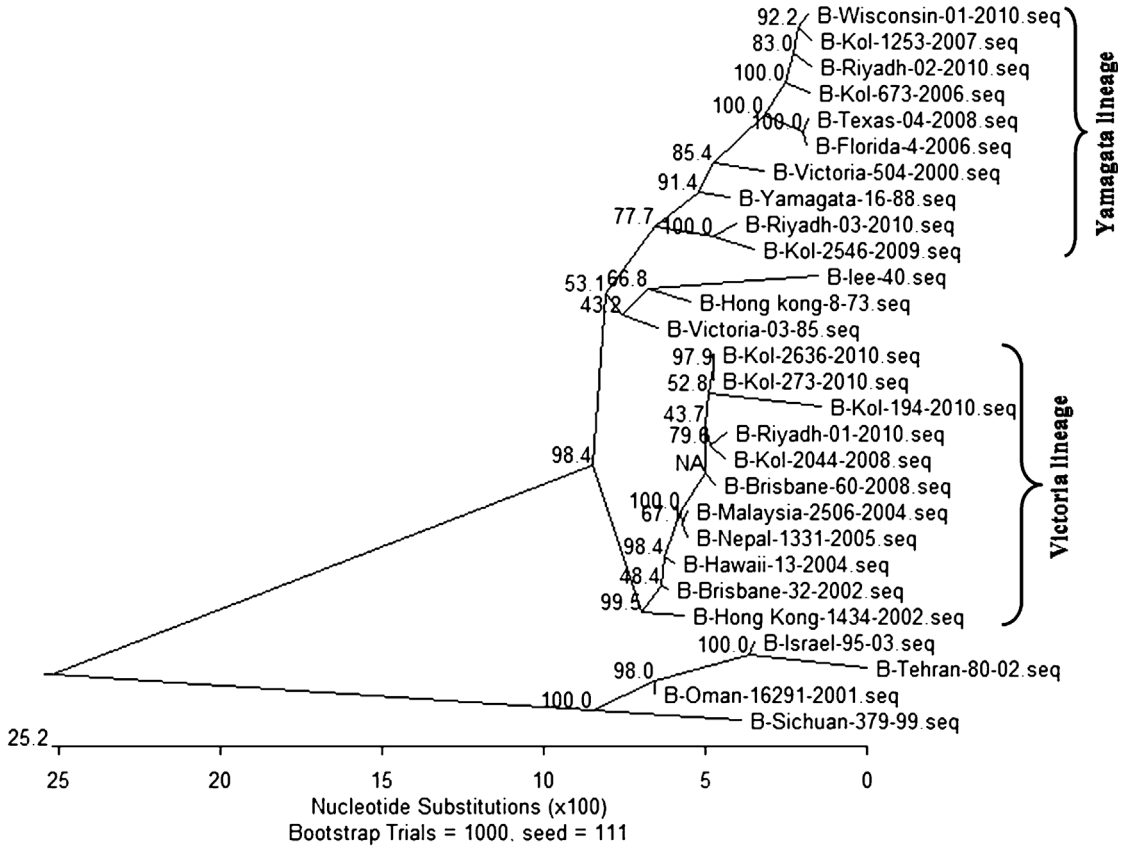

Fig. 1. Phylogenetic analysis of influenza B virus strains based on complete HA gene sequence. The tree was constructed by the neighbor-joining method using Clustal W of MegaAlign program, Lasergene software (DNAstar). Strains in the tree are shown by their names, geographic location, and year of isolation, according to the World Health Organization. Strains were grouped under B/Yam and B/Vic. Lineages. Others that were isolated before 1983 were not included in this classification. Numbers at each node of the tree show bootstrap percentages obtained after 1,000 replicates.

in B/Yam-like Riyadh strains (Table V). Similarly, the B/Vic lineage-like Riyadh strain (B/Riyadh/1/2010) showed two unique amino acid substitutions L38P and G233R through comparison with vaccine and global strains available in NCBI Genbank database.

DISCUSSION

Influenza viruses are the major respiratory viruses that cause severe respiratory illness. Saudi Arabia is at risk of imported and emerging viral disease infections due to the high rate of immigration of working force as well as major annual gathering in the Hajj event from all over the world. Such an environment might support viral spread and making Saudi Arabia a source for potential health problems and possible epidemics. Prior to this study, phylogenetic relationship of the influenza B viruses circulating in Saudi Arabia was largely unexplored. The aim of this study was to identify the genetic variations in the HA and NA genes of influenza B lineages circulating in Riyadh, Saudi Arabia during 2010–2011. During this surveillance episode, a total of 80 clinical samples were obtained from different hospitals of Riyadh, of which 3 samples (3.8%) were positive for influenza B viruses, that is statistically reliable with the already published data [Zaman et al., 2009].

Influenza B evolution is not understood clearly. The oldest classified group of influenza B viruses (prototype; B/Lee/40) was circulating all over the world for years. Although it was the main cause of an outbreak in Asia during the 1996–1997 seasons, B/Vic/2/87-like viruses did not initiate to recapture domination globally until the 2001–2002 season [Chen and Holmes, 2008]. Apart from a short break during the seasons of 2003 and 2004, Victoria lineage remained dominant until 2006–2007 season, when a raise in the number of B/Yam/16/88-like viruses was reported [Chen and Holmes, 2008]. Influenza viruses can spread between hemispheres and across the continents, and consequently new drift variants create a warning to global health. Especially, WHO has

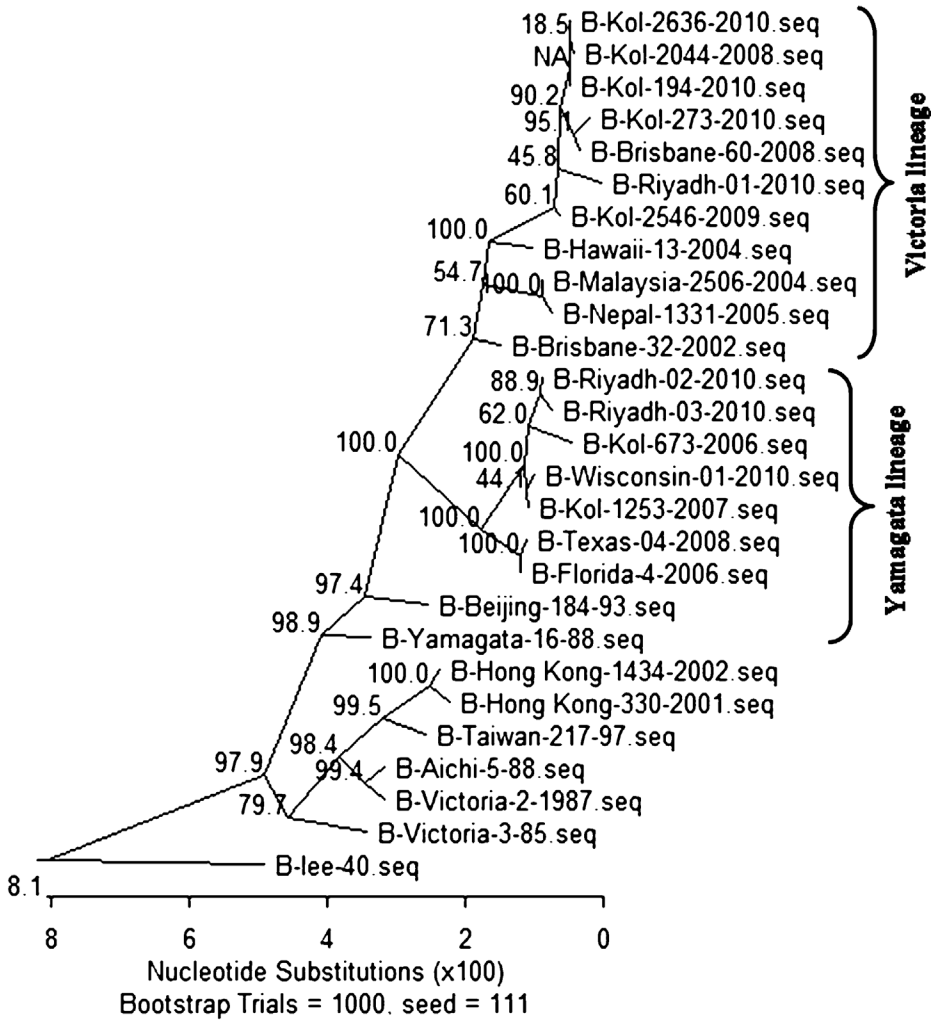

Fig. 2. Phylogenetic analysis of influenza B virus strains based on complete NA gene sequence. The tree was constructed by the neighbor-joining method using Clustal W of MegaAlign program, Lasergene software (DNAstar). Strains in the trees are shown by their names, geographic location and year of isolation, according to the World Health Organization. Strains were grouped under B/Yam and B/Vic. Lineages. Others that were isolated before 1983 were not included in this classification. Numbers at each node of the trees show bootstrap percentages obtained after 1,000 replicates.

been identified "East and Southeast Asian circulation network" as a leading migration route to Europe and North America [Russell et al., 2008].

Influenza B virus is known to be limited mainly to the human reservoir and co-circulation of different multiple lineages in single population increase the chances for reassortment and generating new viruses [Xu et al., 2004]. In addition, other epidemiological factors, such as multitype interference and accumulation of permissive reservoirs may control influenza B virus infection and epidemics [Osterhaus et al., 2000; Yang et al., 2012]. In this study, the co-circulation of B/Vic and B/Yam lineages during 2010–2011 season was recorded in Riyadh. Co-circulation of both B/Vic and B/Yam lineages in Middle East region might have resulted in reassortment of new viruses with selective advantages. These results are in support to the earlier assumptions based on comprehensive analyses of the entire genomes of several influenza B viruses [Chen and Holmes, 2008].

There are four major antigenic epitopes located on the *HA1* region of *HA* gene: the 120 loop (residues 116–137), the 150 loop (141–150), the 160 loop (162–167), and the 190 helix (194–202) [Wang et al., 2008; Shen et al., 2009]. The 120-loop epitope determines the HA antigenicity and appears to be one of the most commonly mutated regions. In 120 loop of *HA1* region, it was observed that the common amino acid pattern at positions 122, 126, 129, and 137 of B/Vic lineage Riyadh strains are H, N, N, and I, respectively, while in B/Yam lineage Riyadh strains, they are Q, D, K, and L. Amino acid change over at residues 129 and 137 of *HA* gene were found to change the antigenicity of field isolates [Lugovtsev et al., 2007].

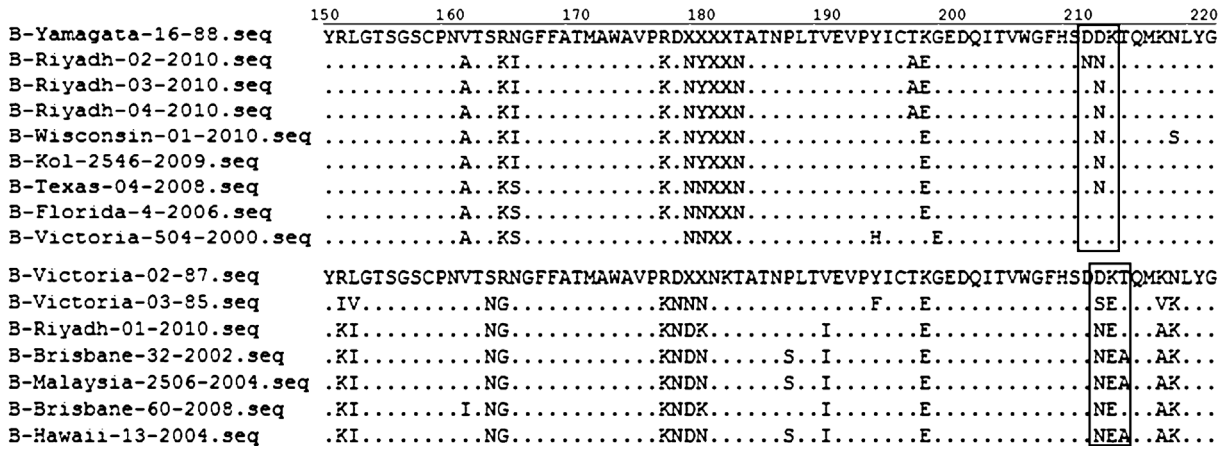

Fig. 3. Amino acid sequences of HA gene near the glycosylation site were aligned with that of both influenza-B lineages. The potential N-glycosylation site NNK in B/Riyadh/02/2010, DNK in B/Riyadh/03/2010 at position 196–198 were indicated (by box) in the Riyadh strains clustered with B/Yamagata lineage. Whereas, the glycosylation site NET at position 197–199 in B/Riyadh/01/2010 is indicated (by box) in Riyadh strain belonged to the B/Victoria lineage.

These amino acid variations may suggest potential alteration in the antigenicity of this loop among Riyadh strains. The 150 loop is an important epitope in influenza B viruses, specially for Yamagata lineage [Wang et al., 2008]. The antigenic properties of viruses in 150 loop were found to be changed at positions 146, 148, 149, and 150, though the polar neutral amino acid N was observed at residues 148 and 150 of B/Vic-like Riyadh strain (B/Riyadh/01/2010) and was substituted by the polar/hydrophobic amino acid S (serine)/I (isoleucine) in B/Yam-like Riyadh strains B/Riyadh/02/2010 and B/Riyadh/03/2010, respectively. These amino acid residues are positioned in close proximity in this loop of *HA* molecule and it may have an effect on the electrostatic interface of *HA* with its antibodies. A group of unique amino acid variations at positions 122, 136, 137, and 149 were demonstrated in both lineages of Influenza B viruses [Rota et al., 1990]. These amino acids lie within the proposed single immune-dominant epitope of influenza B *HA* gene [Berton et al., 1984; Berton and Webster, 1985] in regions of the molecule that may be analogous to antigenic sites of influenza A (H3N2) *HA* gene [Wiley et al., 1981]. These antigenic sites are found at the distal tip of the globular head portion of the influenza A (H3) *HA* molecule.

The 160 loop is the most important region in *HA* gene of influenza B Victoria lineage viruses, where insertions, deletions and amino acid changes were reported in field isolates [Nerome et al., 1998; McCullers et al., 1999]. In this study, amino acid substitutions and deletions were identified in this loop, suggesting that these alterations were considered as an effective way for influenza B viruses to survive for long periods of time without antigenic shifts [Nerome et al., 1998]. In *HA1* region of B/Yam-like Riyadh strains, D (aspartic acid), N (asparagine) and Y (tyrosine) amino acids were recognized at positions 163, 164, and 165, respectively (Table V). However, B/Vic-like Riyadh strain had N (asparagine) at 163, D (aspartic acid) at 164, and K (lysine) at 165. All the representative strains of Yamagata lineages including B/Yam-like Riyadh strains had deletions between amino acid positions 165 and 166 of *HA1* domain. These deletions present antigenic identity of B/Yam lineage, which is not reproduced by any strain of B/Vic lineage.

The 190 loop is an important part of the receptor binding site, and it is one of the most vital epitopes of HA molecule. Currently circulating antigenic lineages of influenza B Riyadh strains have an N-linked glycosylation site (Fig. 3) in the *HA*1 region at positions of 197–199 (NET) in B/Vic-like and 196–198 (NNK/DNK) B/Yam-like viruses. These notable variations in B/Yam and B/Vic lineages were reported to be involved in determination of antigenicity [Nakagawa et al., 2001, 2005]. This may justify the differential sensitivity of the *HA*1 region towards specific antibodies. In a previous study of Nakagawa et al. [2003], a substitution at the amino acid residue 197, from serine to lysine or asparagine, hindered the binding capacity of the monoclonal antibody 8E6. In this study, a substitution of the amino acids was determined at position 196 to D (aspartic acid) in B/Vic-like strains and to N/D (asparagine/aspartic acid) in B/Yam-like Riyadh strains. Antigenic stability of *HA* molecule was evaluated by single point mutation in several reports [Berton et al., 1984; Berton and Webster, 1985]. The current findings fully agree with this concept; therefore, one single substitution mutation may modulate the antigenicity of influenza virus *HA* for both circulated strains.

Glycosylation is a post-translation modification of the viral structural proteins, which modulate and play effective role during viral infection and

TABLE V. Amino Acid Comparison in NA Sequence of Influenza-B Riyadh Strains Along with Strains Representing Both Lineages and the Vaccine Strains (in Bold)

Amino acid number	Common sequence	B-Riyadh-01-2010	B-Kol-2636-2010	B-Kol-194-2010	B-Kol-273-2010	B-Kol-2546-2009	**B-Brisbane-60-2008**	B-Kol-2044-2008	B-Taiwan-110-2005	B-Nepal-1331-2005	**B-Malaysia-2506-2004**	B-Hawaii-13-2004	B-Brisbane-32-2002	B-Riyadh-02-2010	B-Riyadh-03-2010	**B-Wisconsin-01-2010**	**B-Texas-04-2008**	B-Kol-1253-2007	B-Kol-673-2006	**B-Florida-04-2006**	B-Hong Kong-1434-2002	B-Hong Kong-330-2001	B-Taiwan-217-97	B-Beijing-184-93	B-Yamagata-16-88	B-Aichi-5-88	B-Victoria-2-1987	B-Victoria-03-85	B-Lee-40
		B-Victoria Lineage																			**B-Yamagata Lineage**								
27	S	L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	L	L	L	.	.	L	L	.	.
38	L	***P***	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
42	P	P	.	.	.	.	.	.	.	S	S	.	.	R	R	.	.	R	R	Q	.	.	.	.	.	.	.	.	S
49	T	T	.	.	.	.	.	.	.	.	.	.	.	I	I	I	I	I	I	I	.	.	.	.	K	.	.	.	.
51	P	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	S	S	S	.	.	S	S	T	S
53	D	D	.	.	.	.	.	.	.	.	.	.	.	***G***	***G***	.	.	.	.	.	.	.	.	.	.	.	.	.	E
73	L	F	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	F	F	F	.	.	F	F	F	F
125	K	N	N	N	N	N	N	N	N	.	.	N	.	.	.	.	T	.	.	T	.	.	.	.	.	.	.	.	.
148	E	G	G	G	G	G	G	G	G	G	G	G	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	K
186	K	K	.	.	.	.	.	.	.	.	.	.	.	R	R	R	.	R	R	.	R	R	R	R	R	R	R	R	R
198	S	N	N	N	N	N	N	N	N	N	N	N	N	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	N
199	N	D	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D
204	I	V	V	V	V	V	V	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
218	A	A	.	.	.	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.
219	N	N	.	.	.	.	.	.	.	.	.	.	.	K	K	K	K	K	K	K	.	.	.	.	.	.	.	.	H
220	N	K	K	K	K	K	K	K	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
233	G	***R***	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
235	D	N	N	N	N	N	N	N	N	N	N	N	N	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
244	S	S	.	.	.	.	.	.	.	.	.	.	.	P	P	P	P	P	P	P	.	.	.	.	.	.	.	.	.
248	I	V	V	V	V	V	V	V	V	V	V	V	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.
320	E	D	D	D	D	D	D	D	.	.	.	.	.	.	.	.	.	.	.	.	.	.	K	.	.	.	.	.	K
329	D	D	.	.	N	.	N	.	.	.	.	.	.	N	N	N	N	N	N	N	.	.	.	.	.	.	.	.	.
358	A	E	E	E	K	E	E	E	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
373	K	E	E	E	E	E	E	E	E	.	.	E	.	.	.	.	.	.	.	.	.	.	.	E	E	E	E	E	N
389	T	A	A	A	A	A	A	A	A	A	A	A	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
392	D	D	.	.	.	.	.	.	.	.	.	.	.	E	E	E	E	E	E	E	.	.	.	.	.	.	.	.	.
395	A	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.	D	T
396	L	F	F	F	F	F	F	F	F	F	F	.	.	.	.	.	.	.	.	.	P	P	P	.	P	P	P	P	.
404	E	K	K	K	K	K	K	K	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	K	K	K	K	.
436	E	E	.	.	.	.	.	.	.	.	.	.	.	T	T	T	T	T	T	T	T	T	K	K	.	K	K	K	D
463	D	D	.	.	.	.	.	.	.	N	N	N	.	N	N	N	.	N	N	.	.	.	.	.	.	.	.	.	.
465	A	A	.	.	.	.	.	.	.	.	.	.	.	T	T	T	.	T	T	.	.	.	.	.	.	.	.	.	.

The amino acid positions correspond to the B/Lee/40 strain. Identical amino acids are indicated by dot (.) and unique amino acid substitution represented in bold italic.

replication [Sun et al., 2011]. The potential glycosylation site in the *HA*1 region lies at the amino acid position 196/197 [Chen et al., 2008], where the later appeared in isolates of mid 1990s, and became a dominant feature in influenza B isolates from thereon [Tsai et al., 2006]. The effect of glycosylation on growth and antigenicity of influenza B virus was systemically evaluated by Chen et al. [2008]. They demonstrated that glycosylation affects the virus binding to α-2, 3-linked sialic acid receptor, virus antigenicity and stability. On the other hand, the loss of the glycosylation site at this region of HA protein, in monoclonal antibody escape mutants, egg adaptated variants, and field isolates was found to cause significant antigenic alternations [Saito et al., 2004]. Moreover, it was noticed that R (arginine) residue at amino acid position 141, which is located far away from the glycosylation site, can stabilize the glycosylation site at 196/197 without affecting virus antigenicity. In this study, *HA* protein sequence of both influenza B lineages circulating in Riyadh lacks the residue R at position 141. Thus, R should be introduced at this position 141 into vaccine strains prepared from Riyadh strains to maintain the glycosylation site at 196–197.

It has also been observed that a potential glycosylation site at positions 233–235 of *HA1* region of the B/Yam lineage-like Riyadh strains is missing, which needs further investigation to elucidate its biological role. On the other hand, a concurrent gain of the glycosylation site at 233–235 in *NA* gene of B/Vic lineage-like Riyadh strain is an interesting occurrence. In such a way, only a single amino acid substitution like N/D (asparagine/aspartic acid) at position 233 may induce a big antigenic change. In influenza viruses, residues on this site 233–235 are known to participate in proper folding, assembly, and transport of the *HA* protein out of the endoplasmic reticulum [Roberts et al., 1993]. Appearances and disappearances of glycosylation sites during the evolution of influenza B viruses in human hosts have also been reported in numerous studies [Roberts et al., 1993].

Phylogenetic analysis of the *NA* genes showed that, one (B/Riyadh/01/2010) of three influenza-B Riyadh strains clustered with the B/Vic-lineage represented by the vaccine strains B/Brisbane/32/2002 and B/Malaysia/2506/2004, to which the amino acid sequence divergence was estimated 0.9–2.5% and homology ranged from 97.8% to 98.9%, respectively. The *NA* gene of B/Vic-like Riyadh strains showed 99.1% homology with B/Brisbane/60/2008 by presenting seven deduced amino acid substitutions S27L, L38P, P51S, L73F, N199D, G233R, and A395V (Table V). These two substitutions L38P and G233R appeared only in B/Vic-like Riyadh strains. Similarly, *NA* gene of B/Yam-like Riyadh strains showed 99.5–99.6% sequence homology with their vaccine strain B/Wisconsin/01/2010, with only one unique variation D53G. Hatakeyama et al. [2007] reported that few potential changes at the amino acid positions 198, 222, 250, and 402 of *NA* gene may result in reduced sensitivity towards NA inhibitors. In this analysis, no mutations were recorded at positions 222, 250, and 402 in *NA* gene of all Riyadh strains, while a substitution from S to N was observed only at position 198 of B/Vic-like Riyadh strain B/Riyadh/01/2010 (Table V).

Therefore, overall very few unique genetic changes were observed in the *HA* and *NA* genes of B/Riyadh viruses (B/Yam-like) by comparing protein sequences of the virus with vaccine and other global strains. In the present study, complete sequence data analysis of *HA* and *NA* genes of all B/Riyadh strains indicated the presence of collectively six unique along with several common amino acid substitutions. Depending on the site(s) of these substitution mutations, absolute affects cannot be anticipated and a few of these unique changes might somehow alter an antigenic site, which may reflect a viral escape from human immune pressure [Nakagawa et al., 2003]. Similarly, it is also unclear at which extent and for how long these antigenic changes may play a role in the epidemics of influenza-B viruses in Riyadh region of Saudi Arabia and neighboring countries.

Phylogenetic analyses of *HA* and *NA* genes indicated that both genes of influenza B viruses continued to evolve from many years in Riyadh. The appearance of maximum sequence identity (99.1–99.7%) between influenza B Riyadh strains of both lineages and Kolkata strains suggests that the currently circulating influenza B viruses in Saudi Arabia might have originated from the Indian continent. This suggestion is potentiated by the existence of a large workforce from India and neighboring countries in Saudi Arabia. Nevertheless, analysis of new strains representing different districts of Saudi Arabia is essential to justify the reliability of this conclusion. It is noteworthy that Saudi Arabia is the country with the earliest and maximum incidence of B/Yam-like viruses in the region of Middle East may be due to the huge immigration of workers from the Indian-subcontinent. Similarly, the WHO report of February 2012 supports, to a limited extent, the study findings that influenza-B viruses of both lineages circulated in some countries, but observing an increase in the prevalence of B/Yam/16/88 lineage-like viruses, although the number of viruses collected was relatively small.

In summary, the results of the current study revealed that both influenza B virus lineages are co-circulating in Riyadh, Saudi Arabia. Few significant variations exist in the deduced amino acid sequence of the *HA* and *NA* proteins of both B/Vic and B/Yam-like groups of influenza B Riyadh viruses. These genetic changes support strongly the need for continuous surveillance and monitoring to discover newly evolving strains that might pose a threat to the Saudi community. Furthermore, it has demonstrated the importance and need for rapid production

of quadrivalent influenza vaccine against circulating influenza A and both lineages of B viruses, as suggested by WHO, which will help measures to control influenza epidemics in the future.

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