



Genetic analysis of human parainfluenza type 2 virus in Riyadh, Saudi Arabia

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Abstract

The extensive mass gathering of pilgrims from all over the world, as well as the constant flow of foreign workers via country entry crossings, raises the likelihood of respiratory virus outbreaks spreading and evolving in Saudi Arabia. Here, we report the sequence and phylogenetic analysis of the human parainfluenza type-2 (HPIV-2) in nasopharyngeal aspirates (NPAs) collected from Riyadh, Saudi Arabia, from 2020/21 to 2021/22 seasons. RNA was extracted from the clinical samples and subjected to RT-PCR analysis for the detection of IAV and IBV. The full-length HN gene of HPIV-2 was amplified and sequenced. Multiple sequence alignments (both nucleotides and deduced amino acids) were aligned using Clustal W, MegAlign program of Lasergene software, and MEGA 7.0. HPIV-2 was found in (4; 2% of 200) NPAs. Sequence and phylogenetic analysis results showed that indicated a genotype shifting from G3 to G4a with 83% sequence homology 62-M786 from Japan, which was prominent throughout the winter seasons of 2008/09. Multiple amino acid sequence alignment revealed 25 sites of possible difference between G3 genotypes and G4a. A total of twenty- two of these locations were shared by the other G4a genotypes, whereas three positions, 67 V, 175 S, and 377Q, were exclusively shared by G3. Only eight conserved N-glycosylation sites were found at amino acids 6(NLS), 286(NTT), 335(NIT), 388(NNS), 498(NES), 504(NPT), 517(NTT), and 539(NGT) in four Riyadh isolates. Our findings also revealed that the G4a genotype of HPIV-2 predominated in our samples population during the winter seasons of 2020/21 and 2021/22. Further research with a larger sample size covering numerous regions of Saudi Arabia throughout different epidemic seasons is needed to achieve an improved knowledge of HPIV-2 circulation.

Keywords Respiratory tract Infections · Human parainfluenza · HPIV-2 · Phylogenetic analysis · Glycosylation

Introduction

Human parainfluenza viruses (HPIV) are one of the primary causes of lower respiratory tract infections (LRTI), accounting for 13% of LRTI cases, 4–14% of LRTI hospitalizations, and 4% of LRTI mortality in children under the age of five worldwide among children younger than 5 years [1]. These viruses were first isolated from children with croup in the late 1950s and were known as croup-associated viruses [2, 3].

The HPIV virion contains a single-stranded negative-sense RNA genome of 15,462 nucleotides that encode six genes and eight proteins. The two surface glycoproteins that are responsible for infecting host cells are the hemagglutinin-neuraminidase (HN) and fusion (F) proteins [4]. HN is responsible for binding to sialic acid residues on the surface of host epithelial cells and F protein induces fusion of the viral envelope with the host cell membrane [5]. The HN gene has been chosen as the optimal target gene region for HPIV phylogenetic analysis due to its antigenicity and variability [6, 7]. HPIVs belong to the family *Paramyxoviridae*, of the order *Mononegavirales* [3]. HPIVs are classified into four serotypes (HPIV-1, 2, 3, and 4) in two genera: *Respirovirus* (HPIV-1 and HPIV-3) and *Rubulavirus* (HPIV-2 and HPIV-4) [8].

Of the HPIVs detected, the prevalence of HPIV-2 was ranked third behind HPIV3 and HPIV1, accounting for 2.6 to 14% of HPIV infections [1, 9, 10]. Although the fact that

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HPIV-3 has been identified as a major viral cause of acute respiratory infections in newborns and young children [11]. Infections with HPIV-1 and HPIV-2 were more prevalent in older children [12]. Furthermore, Villaran et al. discovered that the detection rate of HPIV-3 was four times higher in children under the age of five than in those over the age of five [13].

The epidemiology and economic effects of HPIV infections in Saudi Arabia are mostly unclear. During the winter seasons of 2003 and 2004, among 282 screened samples of NPAs obtained from infants and young children admitted to the Buraidah Maternity and Pediatric Hospital in Al-Qassim, Saudi Arabia, and clinically diagnosed with acute lower respiratory tract infections, the frequency of HPIV-1, -2, and -3 was 9 (3.2%), 4 (1.4%), and 1 (0.4%), respectively [14]. In Riyadh, we previously show that HPV-3 was detected in 42 cases (8%) of 1429 Saudi infants hospitalized at King Khaled University Hospital with LRTIs between April 1993 and March 1996. We also discovered that HPIV-3 could be detected in all months, with outbreaks occurring from June to August when the air temperature was 40 °C [15]. Only a few surveillance studies have been conducted to detect HPIV-2 in clinical samples collected from hospitalized patients in various districts. We previously demonstrated that HPIV-2 had been observed in Riyadh over the winter-spring season. We previously showed that only one sample (0.56%) was HPIV-2 positive using nested RT-PCR among the 180 nasopharyngeal aspirates (NPAs) collected from possible infections in Riyadh [16]. This study aimed to examine the molecular characteristics of HPIV-2 isolates throughout admitted children in Riyadh during two seasons, 2020/21 and 2021/22.

Materials and methods

Sample collection

A total of 200 NPAs were collected from all children admitted to King Khalid University Hospital (KKUH) in Riyadh, Saudi Arabia (120 between October and January 2020/21 and 80 in 2021/22). After receiving informed consent from their parents, samples were collected from children's hospitals with acute respiratory illness and respiratory infection (fever, cough, sore throat, wheezing, or apnea), and the study protocol was approved by the institutional review board (IRB) of KKUH, Riyadh (Ethics Reference No. 14/4463/IRB 03).

Virus detection

Viral RNA was extracted from samples using kits from (Qiagen, Hilden, Germany) as directed by the manufacturer. In brief, the 140 µl samples were thawed and lysed in 560 ml of lysis solution containing carrier RNA. To disperse any visible precipitate, one volume of ethanol (96–100%) was added, and QIAamp Mini column binding was performed by centrifuging the lysate at 8000 rounds per minute (rpm) for 30 s. Columns were washed with 500 µl wash buffers with ethanol at 8000 rpm for 15 s. Columns were dried, and Viral RNA was eluted in 60 µl elution buffer by centrifugation at room temperature for 2 min at 8000 rpm.

The presence of HPIV-2 RNA in extracted RNA was evaluated using the One-step Ahead RT-PCR Kit with Taq High Fidelity DNA Polymerase (Qiagen, Hilden, Germany) and the primer pair HPIV2-Cdna-F/HPIV2-R (Table 1). The PCR amplification procedure was followed exactly as specified in the kit's instruction manual. MM Mix 2.5×(6 µl), Reaction Mix (25X) (1 µl), RNase-free water (10 µl), forward primer (F) (0.6 M, 1.5 µl), and reverse primer (R) (0.6 M, 1.5 µl) were combined to make the master mix. Individual tubes were filled with template RNA extracts (5 µl). The template RNA extracts (5 µl) were added to the individual tubes. The runs were performed in a 25-µl reaction mixture in a GeneAmp 9700 thermal cycler (Applied Biosystems, Foster City, CA) using the manufacturer's specified reaction conditions: 30 min of reverse transcription at 50 °C, followed by 35 cycles of 15 min at 95 °C denaturation, 30 s of annealing at 52 °C, and 2 min of extension at 72 °C. The final extension was performed for two minutes at 72 °C.

DNA sequencing

The sequences of the whole positive sample (4 isolates) from each of the two epidemic years (2020/21 and 2021/22) were designated (Riyadh 40/2020, Riyadh 74/2020, Riyadh 25/2021, and Riyadh 37/2021) used for sequencing and phylogenetic analysis of the complete HN gene. Using a GeneAmp 9700 thermal cycler, the same kit (Step Ahead RT-PCR Kit with Taq High Fidelity DNA Polymerase) was utilized to amplify HN antigenic glycoprotein. Three overlapping primer sets were utilized to get the full HN gene, as indicated in Table 1. RT-PCR products were purified from agarose gel with the Illustra GFX PCR DNA Kit (GE Healthcare) and sequenced on both strands with a commercial service (Macrogen Inc, Seoul, South Korea).

Table 1 Primer sets were used in the study

Type/Subtype	Oligo Name	5' - Oligo Seq - 3'	Amplicon (bp)
Universal Primer for Parainfluenza	HPIV-2-cDNA F	AACAATCTGCTGCAG-CATT	508
	HPIV-2-R	ATGTCAGA-CAATGGGCAAAT	
Parainfluenza nested primers for typing	HPIV-2-nF	CCATTTACCTAAGTGATG-GAAT	204
	HPIV-2-nR	GCCCTGTTGTATTTG-GAAGAGA	
Sequencing Parainfluenza Fragment 1	HPIV-2-HN-F1	CGAACCCTTAAGGTGTC-GTAACGTC	886
	HPIV-2-HN-R1	GGTATAGCAGTGACT-GAACAGC	
Sequencing Parainfluenza Fragment 2	HPIV-2-HN-F2	GACCCATTGGTGTTA-CACTACAATG	840
	HPIV-2-HN-R2	GTTGCATTGCATGGCAT-GACTC	
Sequencing Parainfluenza Fragment 3	HPIV-2-HN-F3	GGTACCGTCCTAT-CAAGTTCC	426
	HPIV-2-HN-R3	GAATCTG-GAGTCTTCATTCAATAG	

Sequence data analysis and phylogeny

The HN gene sequence data was edited and assembled for the selected strains using the Bioedit tool (Ibis Biosciences, Carlsbad, CA). Multiple sequence alignment was used to compare the acquired sequences to the sequences of 39 reference and vaccination strains from GenBank (Table 2).

Divergence analysis, mutation site identification, and amino acid change prediction were performed with the EditSeq and MegAlign programs, Lasergene software 3.18 (DNASTar Inc., Madison, WI). NetNGlyc 1.0 was used to predict probable N-linked glycosylation sites (Asn/X/Ser/Thr, where X is any amino acid other than proline) and O-linked glycosylation sites (Ser or Thr) (<http://www.cbs.dtu.dk/services/NetNGlyc>) [17] and NetOGlyc 3.1 (<http://www.cbs.dtu.dk/services/NetOGlyc>), [18], respectively. MEGA 7.0’s neighbor-joining approach [19] was used to create a phylogenetic tree based on a complete nucleotide sequence of the HN gene with bootstrap values of 1,000 replicates.

Table 2 Accession numbers of HPIV-2 strains in the current study

No.	Strain	Origin	GenBank accession No.		
			Accession no.	Year	Clade
1.	Riyadh 105/2009	Saudi Arabia	HM460888	2009	G3
2.	Oklahoma/94/2009	USA	JF912194	2019	G3
3.	Zagreb.HR/49.11(17,034)	Croatia	MG836422	2011	G3
4.	Zagreb.HR/42.12(2995)	Croatia	KT595233	2012	G3
5.	Zagreb.HR/50.12(3263)	Croatia	MG836424	2012	G3
6.	Zagreb.HR/38.12(2858)	Croatia	MG836423	2012	G3
7.	Zagreb.HR/42.14(1472)	Croatia	MG836425	2014	G3
8.	Zagreb.HR/03.17(228)	Croatia	MG460784	2017	G3
9.	86–391	Japan	AB189949	2004	G1B
10.	76–86	Japan	AB189950	2004	G1B
11.	4–80	Japan	AB189951	2004	G2
12.	TC-6482	Japan	AB189952	2004	G2
13.	TC-6558	Japan	AB189953	2004	G2
14.	Oklahoma/3955/2005	USA	JF912196	2011	G2
15.	62-M786	Japan	AB189948	2004	G4a
16.	Greer-J1	Japan	AB367954	2007	G4b
17.	London	UK	D00865	1990	G4b
18.	HPIV2-1990	Japan	NC_003443	1990	G4b
19.	Lyon/26,056/1997	France	DQ072589	2005	G1a
20.	LYON/18,620/2001	France	DQ072586	2008	G1a
21.	Lyon/20,283/2001	France	DQ072587	2005	G1a
22.	20,435/Lyon/2001	France	DQ072588	2008	G1a
23.	V9412-6	USA	AF213352	2000	G1a
24.	Oklahoma/283/2009	USA	JF912195	2011	G1a
25.	Zagreb.HR/49.15(1849)	Croatia	MG836418	2015	G1a
26.	Zagreb.HR/47.15(1779)	Croatia	MG836417	2015	G1a
27.	Zagreb.HR/49.16(2400)	Croatia	MG836419	2016	G1a
28.	Zagreb.HR/50.16(2513)	Croatia	MG836420	2016	G1a
29.	V94	USA	AF533010	1994	G1a
30.	Zagreb.HR/50.16(2514)	Croatia	MG460781	2016	G1a
31.	Zagreb.HR/44.16(2115)	Croatia	MG460773	2016	G1a
32.	Zagreb.HR/50.16(2469)	Croatia	MG460779	2016	G1a
33.	Zagreb.HR/41.16(1939)	Croatia	MG460770	2016	G1a
34.	Zagreb.HR/40.16(1916)	Croatia	MG460769	2016	G1a
35.	Zagreb.HR/44.16(2085)	Croatia	MG460772	2016	G1a
36.	Zagreb.HR/47.14(1688)	Croatia	KT315667	2016	G1a
37.	Zagreb.HR/28.16(1623)	Croatia	MG460767	2016	G1a
38.	Zagreb.HR/07.17(705)	Croatia	MG460785	2017	G1a

Statistical analysis

The Fisher’s Exact test was performed to identify significant differences in the prevalence of HPIV2 isolates across gender and age groups. The Z-test with Bonferroni adjustment was used for the posthoc comparison. P<0.05 was considered significant.

Results

Prevalence of HPIV-2

HPIV-2 was found in (4; 2% of 200) NPAs collected throughout two epidemic seasons (2020/21 and 2021/22). Based on gender, HPIV-2 isolates were more prevalent ($P < 0.05$) in males (3; 2.83% of 106) than females (1, 1.06% of 94 cases) ($P < 0.05$). Infants aged 1–2 years were the most ($P < 0.05$) afflicted age group, accounting for (2; 2.56% of 78 cases as demonstrated in (Table 3).

Amino acid sequence analysis of the HN gene

Analysis of HN Gene amino acid residues, 571 amino acids with a TAA as a stop codon similar to the prototype HPIV-2 strain (NC_003443) with the other genotypes. Specific amino acids were assigned to G3 genotypes, including 67 V, 175 S, and 377Q (Table 4). The four Riyadh isolates shared an amino acid sequence with the 62-M786 strain except for eleven positions: 48 V, 101P, 111I, 118 S, 117T, 164 N, 316 K, 345Q, 351 N, and 360 N.

N- and O-Glycosylation site analysis in amino acid sequences

Analyzing the N-glycosylation sites at the HN gene of four Riyadh isolates revealed only 8 conserved N-glycosylation sites at residues at amino acids 6(NLS), 286(NTT), 335(NIT), 388(NNS), 498(NES), 504(NPT), 517(NTT), and 539(NGT). Conversely, the HN Gene is extensively glycosylated with O-linked carbohydrates at serine and threonine residues. All isolates from this study had a total

of 40 to 45 potential O-linked glycosylation sites, as shown in (Fig. 1).

Genetic analysis of HOPV strains

We examined the phylogenetic tree generated by the neighbor-joining method for HPIV-2 positive samples to other reference sequences from GenBank, including local and international strains. Sequence and phylogenetic analyses revealed a genotype shift from G3 to G4a genotype with sequence homology of 83% 62-M786 from Japan, which was prevalent during the winter seasons of 2008/09, as shown in (Fig. 2).

Discussion

HPIVs primarily impact children under the age of five, with the pathogenic range including upper and lower respiratory tract infections. They cause 30–40% of all acute respiratory tract infections in infants and children under the age of five, causing a variety of clinical symptoms such as colds, croup, bronchiolitis, and pneumonia [3]. HPIV-2 is responsible for 60% of all infections in children under the age of two and immunocompromised patients, and so cannot be ignored. However, features of this virus's epidemiology and evolutionary dynamics are currently being challenged. As a result, the purpose of this study was to examine the seasonality, circulation pattern, genetic diversity, and evolutionary dynamics of HPIV-2 in Riyadh, Saudi Arabia over an extended period (2020/21 and 2021/22).

Only four (4; 2% of 200) clinical samples collected throughout the two epidemic seasons 2020/21 and 2021/22 were positive for HPIV-2. HPIV infections are identified at a low incidence throughout the year [20]. HPIV-1, -2, and -3 were found in 9 (3.2%), 4 (1.4%), and 1 (0.4%) of the 282 NPAs screened from newborns and young children admitted to the Buraidah Maternity and Pediatric Hospital in Al-Qassim, Saudi Arabia, during the winter seasons of 2003 and 2004 [14]. The circulation pattern of HPIVs in Saudi patients treated at King Khaled University Hospital in Riyadh was studied in our laboratory. RT-PCR was used to screen for HPIV-2 in NPAs collected from hospitalized children during two consecutive seasons (2007/08 and 2008/09). Only one (0.56%) of the 180 samples tested positive for HPIV-2 [16]. During an early investigation in Beijing, China, from 2004 to 2012, HPIVs were found in 1675 (6.50%, 1675/25,773) individuals, including 261 (1.01%, 261/25,773) for HPIV-1, 28 (0.11%, 28/25,773) for HPIV-2, and 1388 (5.39%, 1388/25,773) for HPIV-3. [11]. In a recent study, 21 HPIV-2 HN sequences were acquired from LRTI pediatric cases in seven Chinese provinces (Henan,

Table 3 The demographic and clinical information and the prevalence of HPIV-2

Seasonal and demographic details		Number of samples	Number of positive samples (%)
Winter season	2020/21	120	2(1.66)
	2021/22	80	2(2.5)
	Total	200	4(2)
Age in years	< 1	56	1(1.78)
	1–2	78	2(2.56) ^a
	3–4	45	1(2.2)
	≥ 5	21	0(0)
Gender	Male	106	3 (2.83) ^b
	Female	94	1(1.06)

Note: Data presented as number (%). Data are displayed as percentages (%).

^a Significantly different ($p < 0.05$) from age groups < 1, 3–4, and ≥ 5 years.

^b Significant difference ($p < 0.05$) to females

Table 4 Amino acid changes among isolates of the HPIV2 genotype, dots refer to identical amino acids to the consensus

Isolates / Site	18	48	50	54	67	100	164	175	186	275	279	316	232	332	345	351	360	367	375	377	443	479	497	513	514
HPIV2	R	V	S	D	I	F	N	I	M	F	V	S	K	K	Q	S	N	V	M	H	Y	P	R	S	A
Riyadh 40/2020	.	.	P	.	.	L	H	.	I	.	I	N	E	T	.	G	Y	.	T	.	.	L	K	N	S
Riyadh 74/2020	.	A	.	.	.	L	H	.	I	.	I	N	E	T	R	G	Y	.	T	.	H	L	K	N	S
Riyadh 25/2021	.	.	.	N	.	L	H	.	I	.	I	N	E	T	R	G	Y	.	.	.	H	L	K	N	S
Riyadh 37/2021	.	A	.	N	.	L	H	.	I	.	I	N	E	T	R	G	Y	L	K	N	S
62-M786	L	.	.	I	.	I	K	E	T	.	N	K	N	S
Riyadh 105/2009	K	A	P	N	V	L	H	S	I	.	.	N	E	T	R	G	Y	I	.	Q	H	L	K	N	S
Zagreb.HR/42.14(1472)	.	A	.	N	V	L	H	S	I	.	.	N	E	T	R	G	Y	I	.	Q	.	L	K	N	S
Zagreb.HR/50.12(3263)	.	.	.	N	V	L	H	S	I	.	.	N	E	T	.	G	Y	I	.	Q	.	L	K	N	S
Zagreb.HR/38.12(2858)	.	.	.	N	V	L	H	S	I	.	.	N	E	T	R	G	Y	I	.	Q	.	L	K	N	S
Zagreb.HR/03.17(228)	.	.	.	N	V	L	H	S	I	.	.	N	E	.	R	G	Y	I	.	Q	.	.	K	N	S
Oklahoma/94/2009	.	.	.	N	V	.	H	S	I	.	.	N	E	T	R	G	Y	I	.	Q	.	L	K	N	S
Zagreb.HR/49.11(17,034)	.	.	.	N	V	L	H	S	I	.	.	N	E	T	R	G	Y	I	.	Q	.	L	K	N	S
Zagreb.HR/42.12(2995)	.	.	.	N	V	L	H	S	I	.	.	N	E	T	R	G	Y	I	.	Q	.	L	K	N	S

Jilin, Gansu, Chongqing, Shandong, Anhui, and Hebei) in children aged 1 to 12 years between 2011 and 2017 to 2021. [21].

In addition, we sequenced the complete *HN* gene to investigate the genetic characteristics and phylogeny of Riyadh HPIV-2 strains. Riyadh HPIV-2 isolates were belonging to the G4a genotype with sequence homology of 83% 62-M786 from Japan. Our earlier work of Phylogenetic analysis of HPIV-2 was classified in cluster G3 related to a strain reported in the US state of Oklahoma during the winter seasons of 2008/09 [16], as shown in (Fig. 2). Despite this, there are little studies on the virus’s epidemiology and evolutionary dynamics. Phylogenetic analysis indicated two groups within HPIV2 isolates, but due to the small number of isolates, the phylogenetic tree was insufficiently informative [6]. Later, a phylogenetic tree was created using more diverse international HPIV-2 isolates, which revealed the existence of four clusters (G1-4) [16]. A previous investigation of HPIV-2 genetic diversity in Croatia between 2011 and 2014 predicted that the G1a genotype will supplant the G3 genotype in this period [22]. Although the G1a genotype is currently dominant, the G3 genotype can still be identified irregularly among the isolates. [23].

For instance, an examination of the deduced amino acid sequence alignment revealed 25 possible locations of difference between G3 genotypes and G4a (Table 3). Twenty-two of these positions were shared by the other G4a genotypes, whereas three positions, 67 V, 175 S, and 377Q, were solely shared by G3. In comparison to the 62-M786 strain, the four Riyadh isolates shared an amino acid sequence with eleven points of potential variation: 48 V, 101P, 111I, 118 S, 117T, 164 N,316 K,345Q,351 N, and 360 N. The effect of these changes on the antigenic characteristics of the mature HN protein, however, could not be established because the positions of the antigenic sites for HPIV-2 have not been determined yet. The two glycoproteins HN and F proteins are the most common targets of neutralizing antibodies [23]. These two proteins were thoroughly examined. HN glycoprotein is organized as a tetramer and trimer on the surface of the viral envelope with the host cell membrane [5].

Glycosylation is an essential protein modification process that involves the enzymatic process of adding a carbohydrate chain to a protein, resulting in a posttranslational modification that modifies protein folding, antigenicity, and biological activity [24, 25]. A wide range of viruses rely on O-linked and N-linked glycosylation to carry out essential biological activities. Viruses frequently contain integral proteins that contribute to host-cell interactions such as adhesion to receptors and membrane fusion. Fusion proteins from a variety of viruses, including HPIVs, and influenza viruses, share several common characteristics that are increased by glycosylation. Each of these viruses has

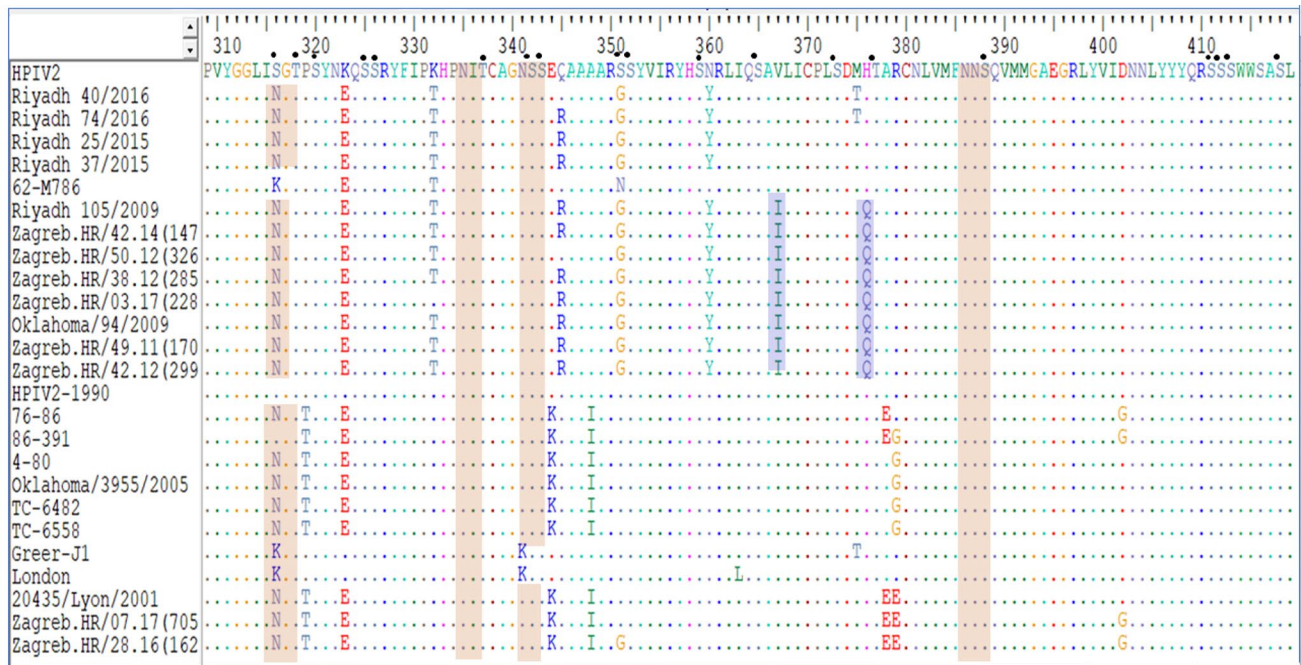


Fig. 1 Alignment of deduced amino acid sequences of the *HN* gene of HPIV-2 isolates. The amino acid positions matched to amino acid positions in the prototype HPIV-2 strain (NC_003443) sequences. Various amino acids are assigned a color, and similar residues are shown by

dots. Maroon rectangles surround predicted N-glycosylation sites. The blue rectangle represents amino acids assigned to G3 genotypes. Small filled circles relate to O-glycosylation sites predicted

several glycosylation sites that must be processed and modified by the host post-translational machinery to be fusogenically active. Glycosylation is involved in the biogenesis, stability, antigenicity, and infectivity of most viruses [26]. The N-glycosylation sites at the HN gene of four Riyadh isolates revealed only eight conserved N-glycosylation sites at amino acids 6 (NLS), 286 (NTT), 335 (NIT), 388 (NNS), 498 (NES), 504 (NPT), 517 (NTT), and 539 (NGT). These findings are consistent with a recent work that found four N-glycosylation sites (N6, N115, N142, and N272) to be substantially conserved in all HN sequences of Chinese HPIV2 strains [21].

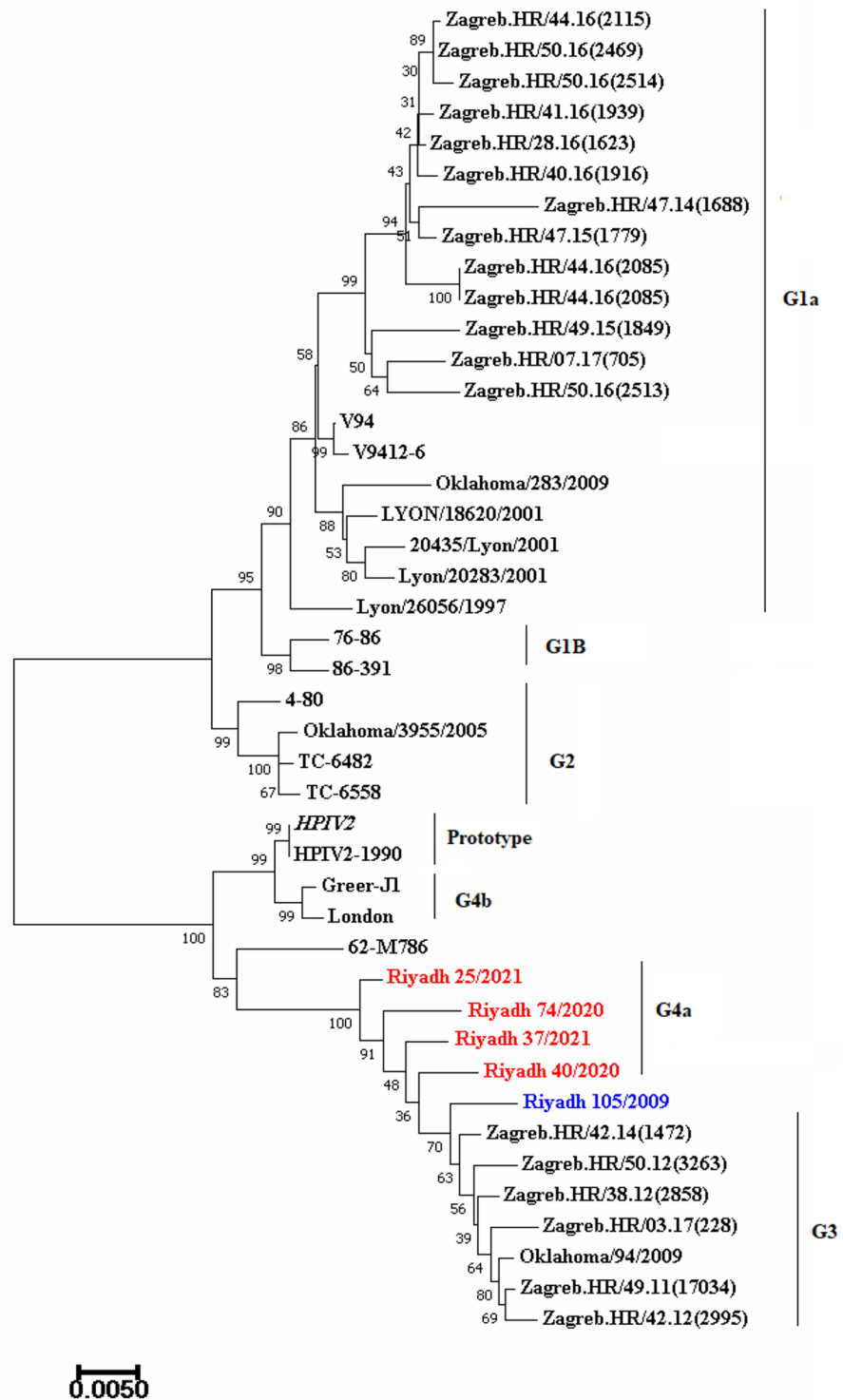
This study was limited by a small sample size, geographical and chronological distribution. Additional thorough studies with larger sample sizes encompassing various regions of Saudi Arabia throughout different epidemic seasons are needed to gain an improved knowledge of HPIV-2 circulation.

Conclusion

In conclusion, we present data on the genetic diversity of HPIV-2 in the HN protein gene in Riyadh throughout the winter seasons of 2020/21 and 2021/22. In addition, *HPIV-2* was identified only in (4; 2% of 200) clinical samples. A sequencing and phylogenetic investigation of the HPIV-2

full-length HN gene indicated a genotype shifting from G3 to G4a with 83% sequence homology 62-M786 from Japan, which was prominent throughout the winter seasons of 2008/09. Multiple amino acid sequence alignment revealed 25 sites of possible difference between G3 genotypes and G4a. A total of twenty-two of these locations were shared by the other G4a genotypes, whereas three positions, 67 V, 175 S, and 377Q, were exclusively shared by G3. Only eight conserved N-glycosylation sites were found at amino acids 6(NLS), 286(NTT), 335(NIT), 388(NNS), 498(NES), 504(NPT), 517(NTT), and 539(NGT) in four Riyadh isolates. The variation in the glycosylation profile, as well as the into genotypic and intergenotypic changes across HPIV-2 isolates, may increase the virus's ability to avoid the host immune response. Our findings also revealed that the G4a genotype of HPIV-2 predominated in Riyadh during the winter seasons of 2020/21 and 2021/22. Further research with a bigger sample size covering numerous regions of Saudi Arabia throughout different epidemic seasons is needed to achieve an improved knowledge of HPIV-2 circulation.

Fig. 2 HPIV-2 phylogenetic trees. For alignment, the *HN* gene was employed. The phylogenetic tree was generated using the MEGA7 program and the neighbor-joining method. The numbers at the tree's internal nodes are the bootstrap values from 1,000 replicates. Only values greater than 60% are displayed. Isolates from seasons 2020/21 and 2021/22 are in red font, whereas isolates from seasons 2008/09 are in blue font



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Author contributions Conceptualization A.N.A. and I.M.A.; methodology, A.N.A.; software, N.A.A. and I.M.A.; validation, A.N.A., N.A.A., I.M.A., and F.N.A.; formal analysis, A.F.A.; investigation, F.N.A.; resources, F.N.A.; data curation, A.N.A and I.M.A.; writing original draft preparation, A.N.A and I.M.A.; writing review and ed-

iting, F.N.A.; review and editing, visualization, I.M.A.; supervision, F.N.A.; project administration, F.N.A.; funding acquisition, N.A.A. All authors have read and agreed to the published version of the manuscript.

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Data Availability All the data is provided.

Declarations

Institutional Review Board Statement The study was conducted in accordance with the Declaration of Helsinki, and approved by Research Ethics Committee (RES) at the King Saud University, Riyadh, Saudi Arabia (Ethics Reference No. 21/4463/IRB 03).

Informed consent Informed consent was obtained from all subjects involved in the study.

Competing interests The authors declare no conflict of interest.

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