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Molecular Epidemiology of Human Metapneumovirus in Riyadh Province, Saudi Arabia

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Key Words

Attachment glycoprotein · Fusion protein · Genotypic classification · Human metapneumovirus · Phylogeny · Riyadh

Abstract

Human metapneumovirus (HMPV) is an important cause of respiratory tract illness in children. Two HMPV subgroups, A and B, and four genotypes, A1, A2, B1 and B2, have been identified. Concurrent circulation of the different genotypes in yearly epidemics has been recorded globally, but not in Saudi Arabia. The current report was designed to study HMPV epidemiology in Saudi children and to analyze the genetic diversity and circulation patterns. Nasopharyngeal aspirates (n = 174) were collected from hospitalized children in Riyadh (2008–2009). The screening of samples using real-time RT-PCR identified 19 HMPV strains. The majority of the strains belonged to subgroup B, while all strains of subgroup A were members of genotype A2. In 2008, only subgroup B was recognized, whereas in 2009 both subgroups were identified to be cocirculating at similar rates. The full-length attachment (G) gene and a partial sequence of the fusion (F) gene of positive samples were sequenced. The G gene showed a high degree of genetic diversity and exhibited a variable number of positively selected sites in different lineages. In contrast, the F gene demonstrated an extensive genetic stability with a higher tendency toward purifying selection. This is the first report on HMPV genotype circulation in Saudi Arabia; however, the exact circulation kinetics requires further retrospective and prospective study.

Introduction

Human metapneumovirus (HMPV) is an important respiratory pathogen that was first identified in the Netherlands in 2001 [van den Hoogen et al., 2001]. HMPV mostly affects infants less than 5 years of age, immunocompromised patients and the elderly, causing different forms of respiratory tract illness that range from mild upper respiratory tract symptoms to bronchopneumonia [van den Hoogen et al., 2001, 2003]. The virus often produces seasonal outbreaks in winter and spring and accounts for 5–15% of respiratory tract infections (RTIs) worldwide [Legrand et al., 2011; Maggi et al., 2003]. Reinfection with HMPV is commonly observed throughout an individual's lifetime due to an incomplete immune response and the continuous emergence of new virus variants [Bruno et al., 2009].

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Table 1. Prevalent HMPV genotypes in the study population

Epidemic Year	HMPV genotype				
	A1	A2a	A2b	B1	B2
2008	0 (0.0%)	0 (0.0%)	0 (0.0%)	4 (67.7%)	2 (33.3%)
2009	0 (0.0%)	1 (10.0%)	4 (40.0%)	0 (0.0%)	5 (50.0%)
Total	0 (0.0%)	1 (6.3%)	4 (25.0%)	4 (25.0%)	7 (43.8%)

HMPV is the sole human virus in the genus *Metapneumovirus*, subfamily Pneumovirinae, family Paramyxoviridae. Similar to all family members, HMPV is an enveloped virus with a helical capsid and negative single-stranded nonsegmented RNA genome. The viral genome encodes nine proteins, among which the attachment (G) and fusion (F) glycoproteins are the principle surface peplomers [van den Hoogen et al., 2002]. In contrast to the G protein, which is weakly immunogenic, the F protein is the major antigenic determinant that elicits cross-reactive neutralizing and protective antibodies [Skiadopoulos et al., 2006]. Consequently, the F protein exhibits a high level of conservation due to the structural and functional constraints compared to the G protein, which is the most diverse viral protein [Agapov et al., 2006; Velez Rueda et al., 2013].

Genetic analyses of both the G and F proteins enabled the classification of HMPV strains into two subgroups, A and B, and four genotypes, A1, A2, B1 and B2 [Bastien et al., 2004; van den Hoogen et al., 2004]. Further subdivision of genotype A2 into two lineages, A2a and A2b, has been established [Huck et al., 2006]. All of the genotypes have been identified in most countries worldwide. The global circulation of different genotypes appears to be complex and unpredictable with no distinct geographical or temporal pattern. Concurrent annual circulation of all or different genotypes in the same geographic region with the predominance of a single genotype is a common observation. The predominant genotype always changes every 1–3 years due to the development of genotype-specific immunity [Papenburg and Boivin, 2010; Williams et al., 2010].

Since HMPV epidemiological characteristics vary both spatially and chronologically, community-based prospective studies are crucial to achieving an understanding of the genetic heterogeneity and to tracking the evolutionary kinetics of the virus. Unfortunately, the epidemiological and virological data on HMPV in Saudi Arabia are very limited [Al-Ayed et al., 2014; Al Hajjar et al., 2011]. In the present report, HMPV was identified in samples collected from a cohort of hospitalized children in Riyadh between 2008 and 2009. The G and F gene sequences of local virus strains were analyzed and compared to the international sequences in order to study the genetic variability and circulation pattern of HMPV in Saudi Arabia.

Results

Epidemiology of HMPV in the Study Population

During the study period, HMPV was identified in 19 (10.9%) samples as the third major cause of RTI. The prevalence of HMPV was significantly higher in males (14; 73.7%) than in females (5; 26.3%), $p = 0.039$. The majority of HMPV-positive specimens were obtained from young children aged 6–24 months (13; 68.4%) with a mean age of 22 months and a median age of 10 months. This allowed the ranking of HMPV as the second most common cause of RTIs in this age group, with a prevalence rate of 16.5%. HMPV was most frequently detected in winter and spring, particularly from January to March. The higher incidence rates of 2008 were shown during February and March (20 and 19.4%, respectively), while those of 2009 were evident in February (23.1%). Coinfection with other respiratory viruses was identified in 3 (15.8%) HMPV-positive samples: 2 with influenza A viruses and 1 with RSV type A.

Circulation Pattern of HMPV Genotypes and Subgenotypes

To identify the prevalent genotypes and circulation behavior of HMPV in Saudi Arabia during the study period, the nucleotide sequences of the entire G protein gene and a portion of the F protein gene were amplified and sequenced for all positive samples. Out of 19 HMPV-positive samples, 16 unique F gene sequences were retrieved. However, only 9 full-length G gene sequences could be analyzed due to the low yield of most RT-PCR products. Sequence and phylogenetic analyses showed that Saudi HMPV

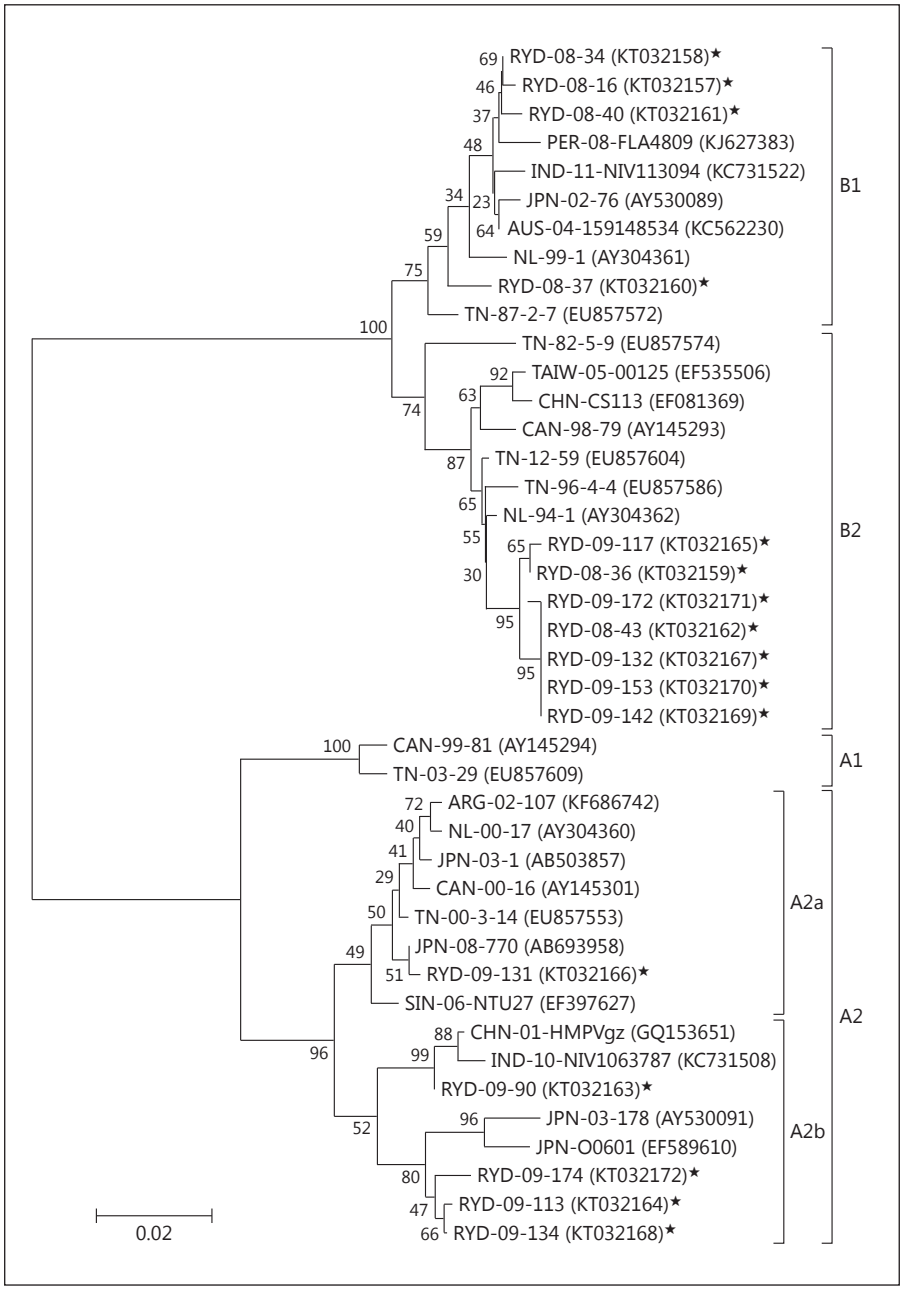

Fig. 1. Phylogenetic classification of HMPV strains using a 525-nucleotide-long fragment of the F gene. The tree was generated by the MEGA 6.0 program using the neighbor-joining method with bootstrap values of 1,000 replicates. The name and accession number of each strain are shown. Saudi strains are marked by stars, and genotypes are shown in parentheses. The scale bar at the bottom indicates substitutions per site.

strains were clustered into two subgroups, A and B, with the majority belonging to subgroup B (68.8%; fig. 1, 2). B2 was the most prevalent genotype (43.8%), followed by A2 (31.2%) and finally B1 (25%). Both lineages of genotype A2 were observed with a distinct predominance of the A2b lineage (80%). In 2008, subgroup B was only identified, with the majority belonging to genotype B1 (67.7%). In 2009, both subgroups were cocirculated concurrently at a similar rate. Although B1 was the predominant genotype in 2008, it was totally absent in 2009 (table 1).

Sequence Diversity among Different Genotypes

Pairwise alignment of Saudi HMPV sequences has indicated a higher genetic stability of the F gene compared to the G gene, with a mean nucleotide identity of 92.5 and 72.5%, respectively. At the amino acid level, the G gene exhibited a higher degree of sequence diversity (mean 56.4%), whereas the F gene did not (mean 97.7%). Similar findings have been reported for all HMPV subgroups, genotypes and lineages. In general, members of group B were more divergent than group A viruses. The extent of

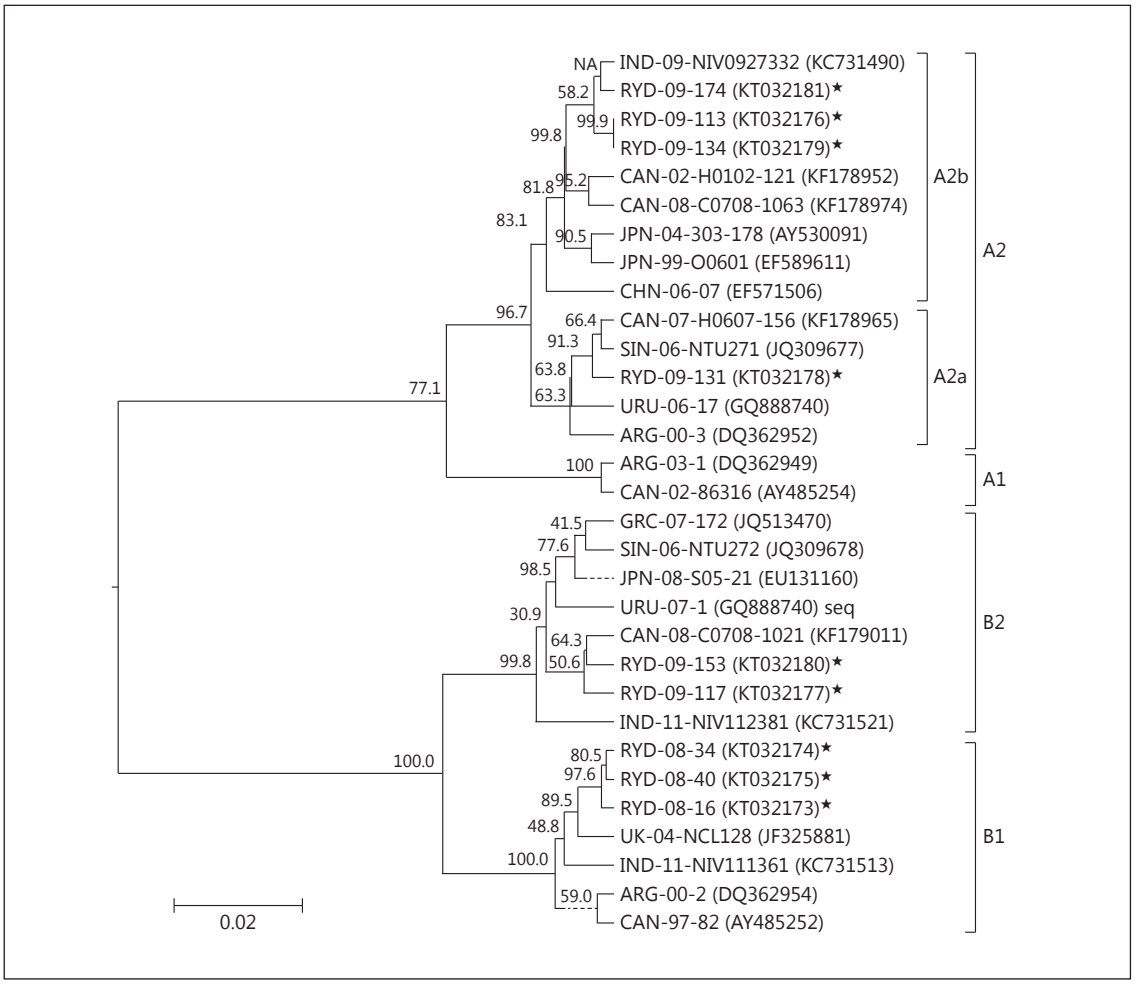

Fig. 2. Phylogenetic analysis of 31 full-length HMPV G gene sequences. Sequence alignment and tree construction were performed using the MegAlign program of Lasergene software (DNAStar). The name and accession number of each strain are shown. Saudi strains are marked by stars, and genotypes are shown in parentheses. The scale bar at the bottom indicates substitutions per site.

variation between the members of both groups was substantial, particularly for the G gene sequence. Genetic diversity was higher among members of genotype B2 than B1 for the G gene sequence, while the opposite was true for the F gene sequence (online suppl. table 1; see www.karger.com/doi/10.1159/000448374 for all online suppl. material).

Conserved and Divergent Features of G and F Proteins

The genetic variation within G and F genes of Saudi strains was further evaluated by comparing different sequences of Saudi and international strains on the amino acid level. As expected, the G gene possesses a wide range of variable amino acids between members of subgroups A and B, and therefore it is very difficult to allocate significant group-specific residues. Careful analysis of sequence alignments between the members of genotypes and lineages has enabled the identification of a conserved pattern of amino acid variation. Twenty-six amino acids at positions 26, 53, 57, 60, 62, 64, 66, 101, 114, 119, 123, 132, 135, 138, 142, 144, 159, 161, 169, 170, 186, 187, 189, 193, 203 and 215 were specific for either genotype of subgroup B strains. On the other hand, 5 amino acids at positions 78, 101, 150, 153 and 159 were only specific for either A2a or A2b lineages (online suppl. fig. 1). The number of predicted N- and O-linked glycosylation sites in Saudi strains generally ranged from 2 to 5 and 60 to 66, respectively. The pattern of N-linked glycosylation ap-

Table 2. Record of amino acid sequence variation in the F protein of Saudi HMPV strains

Amino acid position[1]	Genotype		
	A2	B1	B2
223	T	T[2]	N
233	N	Y	Y
280	D	D[2]	N
286	V	I	I
296	K	N	N
298	N	N	S[3]
312	Q	K	K
348	K	R	R

[1] Amino acid positions are shown relevant to the F protein sequence.

[2] Strain RYD-08-37 is the only member of genotype B1 that has amino acid (N).

[3] Strains RYD-08-36 and RYD-09-117 have amino acid (N) instead of (S).

pears to be genotype specific (online suppl. fig. 1; online suppl. table 2), whereas no distinct figure was found conserved for O-linked glycosylation in different classes.

In contrast to the G gene, the partial sequence of the F protein (175 amino acids) has indicated the well-conserved nature of its amino acid content. Only 5 amino acids, at positions 233, 286, 296, 312 and 348, were variable between subgroups A and B. There was no opportunity to study the variability between type A genotypes due to the unavailability of Saudi A1 strains. On the other hand, type B genotypes showed 3 variable amino acids at positions 223, 280 and 298. However, the distinction between the B1 and B2 genotypes was not absolute since few members of either genotype may exhibit the amino acid configuration of the other genotype, such as strain RYD-08-37 of B1 for amino acids 223 and 280, and strains RYD-08-36 and RYD-09-117 of B2 for amino acid 298 (table 2; online suppl. fig. 2). Only a single conserved N-linked glycosylation site at amino acid 353 and 23–24 O-linked glycosylation sites were recognized in the studied partial F protein sequence.

Positive and Negative Selection Attributes

The evolutionary pressure affecting the complete G and the partial F gene sequences was analyzed using the SNAP program. Both genes showed a higher tendency toward purifying selection with an average dN/dS ratio of 0.0826 (range 0.147–7.111) and 0.034 (range 0.0–0.137) for the G and F genes, respectively. No positively selected codons were identified in the F gene of Saudi strains, which confirms the conserved nature of this gene. In contrast, the G gene exhibited a variable number of positively selected sites in Saudi strains of different genotypes: A (46), B1 (5), and B2 (10). No common sites have been identified for the G gene.

Discussion

Little information is available in the Middle East region on the epidemiological characteristics of HMPV and the genetic diversity of the circulating genotypes. In the current report, real-time RT-PCR assay was utilized to identify HMPV in 19 (10.9%) clinical specimens collected from Riyadh (Saudi Arabia) in 2008 and 2009. The incidence rate of HMPV was highly variable worldwide and ranged from 1.7% in Cambodia between 2007 and 2009 [Arnott et al., 2013] to 23% in Taiwan between 2005 and 2010 [Wei et al., 2013]. The overall prevalence is basically affected by the geographical region, epidemic season, diagnostic assay, and the criteria of the patients from which samples are collected (age, sex, disease condition, immune status and risk factors). The HMPV incidence in this study was similar to that reported by studies in China (10.2%) [Xiao et al., 2013], Yemen (11%) [Al-Sonboli et al., 2006] and Brazil (11.4%) [Oliveira et al., 2009]. Several factors may justify the high HMPV prevalence in the current report compared to most of the relevant global studies, including: (1) all samples were gathered from children younger than 5 years of age (the most susceptible group of patients), (2) all patients were hospitalized with acute signs of respiratory illness, (3) most samples were obtained during March and April (the peak season of HMPV infection) and (4) the sample number was fairly restricted, which may occasionally give irrespective correlations.

The phylogenetic analysis that was performed on the full-length G gene and a partial sequence of the F gene revealed that all HMPV subgenotypes and lineages, except A1, circulated in Riyadh during the study period (fig. 1, 2). The cocirculation of different HMPV genotypes in the same geographic region in a given period of time is widely accepted. The global extinction of the prototype subgenotype A1 after the epidemic season of 2003–2004 and replacement of that old lineage with the emerging new lineages was proposed [Arnott et al., 2013; Papenburg et al., 2013]. However, two reports from Italy and Taiwan have shown that genotype A1 was identified

in a few samples collected between 2008 and 2010, and 2005 and 2010, respectively [Apostoli et al., 2012; Wei et al., 2013]. There have been no reports after 2010 to indicate that A1 may still exist.

Most of the strains identified in this study belonged to subgroup B (68.8%). Although different genotypes are circulating worldwide, it was notable that B is the prevalent subgroup in the Southern hemisphere, including Australia [Mackay et al., 2006], Brazil [Oliveira et al., 2009] and Uruguay [Pizzorno et al., 2010], while subgroup A is dominant in the northern hemisphere, including China [Zhang et al., 2012], Italy [Apostoli et al., 2012] and Japan [Nidaira et al., 2012]. The only exceptions that showed a dominance of subgroup B in the northern hemisphere came from Cambodia [Arnott et al., 2013], Egypt [Embarek Mohamed et al., 2014], Israel [Regev et al., 2006] and Saudi Arabia in this report. This proposed figure of subgroup dominance in both hemispheres does not essentially contradict the fact that dominance may change on a yearly basis.

A closer look at the temporal global prevalence of HMPV subtypes has shown that B was the prevalent subgroup during 2008. In contrast to this report that has revealed the dominance of the genotype B1 (67.7%; table 1) in 2008, all other studies agreed that B2 is the common genotype [Arnott et al., 2013; Embarek Mohamed et al., 2014; Papenburg et al., 2013; Pogka et al., 2013]. In 2009, cocirculation of both subgroups typified the observations recorded in many countries, such as Italy, Cambodia, Canada, China and, more specifically, India, which denoted an equal distribution of genotypes A2 and B2 [Apostoli et al., 2012; Arnott et al., 2013; Banerjee et al., 2011; Papenburg et al., 2013; Zhang et al., 2012].

To further investigate HMPV genetic variability, sequences of the F and G genes were aligned between Saudi strains and with selected international strains. Similar to previous reports [Boivin et al., 2004; Velez Rueda et al., 2013], a high degree of homology at both nucleotide and amino acid levels was observed for the F gene sequence among subgroups (nucleotide: 85.7–87.6%; amino acid: 95.4–97.1%), genotypes (nucleotide: 95.2–96.4%; amino acid: 97.7–99.4%) and lineages (nucleotide: 97.3–99.8%; amino acid: 100%; online suppl. table 1). These high levels of identity, along with the scarcity of the positively selected sites, confirmed that the F protein endures different structural and functional restraints that limits its evolution and, therefore, it represents a suitable target for the development of vaccines and diagnostic tests [de Graaf et al., 2008; Papenburg et al., 2013]. Nevertheless, it was shown that different regions of polymorphism that are distinct between subgroups and genotypes exist. In this study, four previously recorded substitutions at amino acids 286, 296, 312 and 348 [Arnott et al., 2013; van den Hoogen et al., 2004], and a newly defined substitution at residue 233, were able to distinguish between subgroups A and B. Other substitutions at residues 223, 280 and 298 may be important for the identification of members that belong to genotypes B1 and B2 (table 2; online suppl. fig. 2).

In contrast, the G gene sequence of Saudi strains demonstrated a substantial degree of genetic diversity among subgroups (nucleotide: 56.9–61.1%; amino acid: 33–37.8%), genotypes (nucleotide: 81.9–83%; amino acid: 66.6–67.1%) and lineages (nucleotide: 87.9–88.2%; amino acid: 93.6–99.5%; online suppl. table 1). The variation is basically an outcome of different genetic changes, including base substitution, deletion/insertion events and different stop codon usage (online suppl. fig. 1). No evidence of cumulative change over time (antigenic drift) was linked to the HMPV G protein. This study further confirms that G gene diversity may be related to immunogenic pressure, as has been proposed before [Galiano et al., 2006; van den Hoogen et al., 2004], through several observations: (1) the higher level of amino acid change compared to nucleotides (online suppl. table 1), (2) the instability of N- and O-linked glycosylation sites (online suppl. table 2) and (3) the existence of variable numbers of positively selected sites in different genetic groups.

In conclusion, this study is the first record in Saudi Arabia, and one of the few in the Middle East, to reveal the circulation of different HMPV genotypes simultaneously and the change in genotype predominance in two consecutive epidemic years. Further epidemiological and genetic studies are recommended to investigate HMPV prevalence and evolution over an extended period of time.

Experimental Procedures

Patients and Samples

Hospitalized children (males and females) aged 1–60 months at King Khalid University Hospital (KKUH), Riyadh, were recruited for the current study during the period extending from February 2008 until March 2009. A total of 174 nasopharyngeal aspirates were collected from the selected subjects after receiving informed medical consent from their parents. Samples were obtained by mechanical suction and were transported immediately in sustained cooling conditions to the Virology Research Laboratory at the College of Science, King Saud University, for analysis. The study protocols conformed to the 1975 Declaration of Helsinki and were approved by the Ethics Committee of King Saud University.

HMPV Detection by Real-Time RT-PCR

RNA was extracted from 140-μl aliquots of the nasopharyngeal aspirates using a QIAamp Viral RNA extraction kit (Qiagen, Valencia, Calif., USA) according to the manufacturer's guidelines. Identification of HMPV was accomplished using a Genesig standard one-step RT-PCR kit (PrimerDesign, Southampton, UK) in the ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, Calif., USA). Fluorogenic data were acquired after each cycle via FAM and VIC channels. Samples with CT values lower than or equal to 35 were considered positive, while those higher than 35 and less than 40 were retested.

Sequencing

The complete G gene and a partial sequence of the F gene (nucleotides 571–1097 of F ORF) were amplified using the following primers: SH7(+): 5′-TACAAAACAAGAAMATGGGACAAG-3′ and SH-8(–): 5′-GAGATAGACATTAACAGTGGATT-3′ for the G gene [van den Hoogen et al., 2004], and HMPV-3637-F(+): 5′-GTYAGCTTCAGTCAATTCAACAGAAG-3′ and HMPV-4164-R(–): 5′-CCTGTGCTRACT TTGCATGGG-3′ for the F gene [Embarek Mohamed et al., 2014]. Amplification was performed using the SuperScript® III One-Step RT-PCR system with Platinum® *Taq* High Fidelity (Invitrogen, Waltham, Mass., USA) in the GeneAmp 9700 PCR instrument (Applied Biosystems). The RT-PCR amplicons were gel purified using a QIAquick gel extraction kit (Qiagen) according to the manufacturer's instructions. DNA sequencing of both strands was carried out at GATC (Cologne, Germany). Sequences were aligned together and assembled using Bioedit software, version 7.2.5 (Ibis Biosciences, Carlsbad, Calif., USA) and the EditSeq program of the Lasergene software package, version 3.18 (DNAStar, Madison, Wisc., USA). All sequences generated and analyzed in the present study are available in the GenBank with the accession numbers KT032157 to KT032181.

Sequence and Phylogenetic Analysis

The G and F gene sequences of representative HMPV strains that are circulating globally and represent the diverse genotypic classification of the virus were acquired from GenBank. The aligning of nucleotide and deduced amino acid sequences of Saudi strains with their international counterparts was achieved using the Clustal W algorithm running within the MegaAlign program of Lasergene software. Potential N- and O-linked glycosylation sites were predicted using NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc) and NetOGlyc 4.0 (http://www.cbs.dtu.dk/services/NetOGlyc/), respectively. The ratio between nonsynonymous and synonymous mutations (dN/dS or ω) was obtained and evaluated according to the method of Nei and Gojobori [1986] using the SNAP program at the HIV database (http://www.hiv.lanl.gov/content/hiv-db/SNAP/WEBSNAP/SNAP.html). The phylogenetic tree of the F gene was constructed using the neighborhood joining method of MEGA 6.0 software, while the tree of the G gene was generated using the MegaAlign program due to the limited ability of MEGA 6.0 to develop phylograms based on unequal sequence data.

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Disclosure Statement

The author declares that he has no conflicts of interest.

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