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Phylogenetic Analysis of Respiratory Syncytial Virus Identified at Universiti Kebangsaan Malaysia Medical Centre

(Analisis Filogenetik Virus Pernafasan Sinsitium di Pusat Perubatan Universiti Kebangsaan Malaysia)

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ABSTRACT

Human respiratory syncytial virus (RSV) is an important cause of acute respiratory tract infection in infants and young children. Phylogenetic analysis for RSV in Malaysia has not been reported before. We investigated the genetic features of RSV in respiratory specimens from March to August 2011 with molecular methods. From a total of 130 throat swab and nasopharyngeal aspirate specimens, 54 (41.5%) were positive with RSV, identified by in-house real-time reverse transcriptase polymerase chain reaction (rRT-PCR) assay. Thirty-four out of 54 (63.0%) RSV positive patients were children below two years old and two (1.4%) were adults. Phylogenetic analysis showed 39 isolates were genotype GA5, 13 genotypes GA2, one genotype GA1 and one genotype GA7. The findings indicated four genotypes of RSV circulating in the country and the predominant genotype is GA5.

Keywords: Genotype GA1, GA2, GA5 and GA7; phylogenetic analysis; respiratory syncytial virus

ABSTRAK

Virus pernafasan sinsitium (RSV) adalah punca utama yang menyebabkan jangkitan saluran pernafasan akut pada bayi dan kanak-kanak. Analisis filogenetik untuk RSV di Malaysia belum pernah dilaporkan. Kami mengkaji ciri-ciri genetik RSV daripada spesimen yang dikumpulkan bermula dari bulan Mac hingga bulan Ogos 2011 dengan cara pengesanan molekular. Daripada jumlah sebanyak 130 spesimen swab tekak dan cecair nasofarink, 54 (41.5%) adalah RSV positif selepas diuji dengan asai dalaman masa nyata transkriptase balik tindak balas berantai polimerase (rRT-PCR). Tiga puluh empat daripada 54 (63.0%) pesakit dengan RSV positif adalah kanak-kanak di bawah dua tahun dan dua (1.4%) adalah dewasa. Analisis filogenetik menunjukkan bahawa 39 virus RSV adalah genotip GA5, 13 genotip GA2, satu genotip GA1 dan satu genotip GA7. Hasil penyelidikan ini menunjukkan empat genotip RSV yang sedang merebak di negara kita dan genotip utama adalah GA5.

Kata kunci: Analisis filogenetik; genotip GA1, GA2, GA5 dan GA7; virus pernafasan sinsitium

INTRODUCTION

Respiratory syncytial virus (RSV) is an enveloped, negative-sense RNA virus belonging to Paramyxoviridae family. It is the major cause of lower respiratory tract illness in young children (Hall 2001). Annually, it causes an estimated 1 million death worldwide, primary in children younger than 5 years of age (Garenne et al. 1992). In the United States, an estimated 75000-125000 hospitalizations related to RSV occur yearly among children aged <1 year and RSV infection results in approximately 1.5 million outpatient visits among children aged <5 years (Hall et al. 2009; Shay et al 1999).

A survey was conducted previously at Universiti Kebangsaan Malaysia Medical Centre (UKMMC) and it was reported that viral aetiologies were confirmed in 23.4% paediatric patients with lower respiratory tract infections. The authors identified RSV as the most common respiratory pathogen followed by parainfluenza virus, influenza virus and adenovirus (Zamberi et al. 2003).

Antigenic characterization of RSV strains with monoclonal antibodies has identified two distinct groups of the virus, RSV group A and B, which circulate worldwide (Anderson et al. 1985; Mufson et al. 1985). Many investigators have studied the molecular evolution of virus isolates of group A (Cane et al. 1994; Sanz et al. 1994; Zlateva et al. 2004). Most investigators have studied a gene segment of about 300 nucleotides, coding for the C-terminal end of the protein. The studies have shown the existence of eight genotypes, named GA1 to GA7 and South Africa A1 (SAA1) (Madhi et al. 2003; Peret et al. 1998; Venter et al. 2001).

One or few genotypes can dominate one epidemic season, but later, there is a replacement by other genotype/or genotypes (Cane et al. 1994; Choi & Lee 2000; Frabasile et al. 2003; Peret et al. 1998). In contrast to these findings, GA2 genotype was reported dominance for two seasons in South Africa (Venter et al. 2001) and most of the predominant genotypes were isolated in more than two consecutive

epidemics (Choi & Lee 2000). In a recent study from Belgium on 112 virus strains, it was reported that the GA2 and GA5 genotypes dominated and co-circulated in 10 of 19 epidemics between 1983/1984 and 2000/2001. In six and three epidemics, respectively, GA5 or GA2 strains were absent. As no such data is available in Malaysia, therefore, the objective of this study was to detect RSV by rRT-PCR and their phylogenetic analysis. This study was approved by the Research and Ethics Committee, UKMMC (FF-320-2011).

Materials and Methods

Study population and specimens

RSV positive samples identified by the previous study of by in-house RT-PCR were used in the present study. The specimens were those of patients with respiratory illness seen at the various units or departments at UKMMC during the period of March 2011 to August 2011. All specimens were collected as part of the standard of care for laboratory diagnosis purposes. The patients' information including age, gender and clinical diagnoses were stated on the forms that accompanied the specimens. Throat swabs were collected on swabs with a synthetic tip (such as nylon, polyester or Dacron®) and an aluminium or plastic shaft. The appropriate swabs were provided to the wards, since swabs with cotton tips, wooden shafts and swabs made of calcium alginate were not acceptable, as they may interfere with PCR. Throat swabs were submitted in Viral Transport Medium (VTM) and nasopharyngeal aspirates in sterile containers, placed in ice or cold packs for transport to the laboratory. The specimens were sent immediately to the virology laboratory for investigations and identification of viruses. All specimens were immediately tested or kept at 2-4°C (≤72 hours) or frozen at –70°C until tested. Specimens which did not fulfil the requirements for specimen collection, transport and storage requirements were excluded.

RNA extraction

A volume of 200 µL of the sample either nasopharyngeal aspirates or viral transport medium of throat swabs added to 400 µL lysis/binding buffer, containing a chaotropic salt, poly(A) and proteinase K and were incubated. Then, these were applied to the glass fibre fleece in a high pure spin filter tube and centrifuged for 15 s at 8000× g. After that 500 µL inhibitor removal buffer was added to remove the inhibitor. Subsequently, brief wash-and spin steps were performed to remove those contaminants with 450 µL wash buffer twice. The remaining, purified nucleic acid was then eluted in 50 µL of elution buffer (High Pure Viral RNA Kit, Roche, Germany).

Real-time reverse transcription polymerase chain reaction

For rRT-PCR, the RealTime ready RNA Virus Master kit, Roche, Germany was utilized for specific real-time one-step RT-PCR analysis of viral RNA. An amount of 5 µL of purified viral RNA was added to a reaction mixture of 15 µL containing 0.4 µL enzyme blend 50-fold concentration, 4 µL reaction buffer 5-fold concentration, 3 µL primer and probe mix (Table 1) and 7.6 µL water. A single negative control consisting of water as template and a single positive control consisting of plasmid control were included for each set of the rRT-PCR reactions. The rRT-PCR was performed using LightCycler® 2.0 system (Roche Diagnostics, Germany) using LC-rRT-PCR protocol with PCR parameters as listed in Table 2.

Nucleotide sequencing

The purified rRT-PCR products were sequenced by the automated sequence analyzers CEQ™ 8000 Genetic Analysis System, Beckman Coulter with GenomeLab™ Dye Terminator Cycle Sequencing (Quick Start Kit). The nucleotide sequence data (base-call) produced by automated sequence analyzers CEQ™ 8000 Genetic Analysis System were compared with RSV sequences in Genbank to define the genotype of strain obtain from the study.

Phylogenetic analysis

Phylogenetic analysis was performed to each virus sequence using software Bioedit with Neighbour joining method to create a dendogram for cluster analysis.

Statistical analysis

Data analysis was done using SPSS 15.0. Analysis using Chi-square test for qualitative data and P value less than 0.05 is considered significant.

TABLE 1. Primers and probes designed and used for detection of RSV

Primer	Sequence	Gene target	Location (bp)	Genbank
Forward primer	5'-ATCTTCTACCAGAGGTGGCAGT-3'	NS1	1829-1850	U39661
Reverse primer	5'-GCTTGCACACTAGCGTGTCC-3'	NS1	1975-1956	U39661
Probe 1	5'-CTGGATTGTTTATGAATGCCTATGGTGC AGGGC-Fluorescein-3'	NS1	1870-1902	U39661
Probe 2	5'-LC Red 705-GTGATGTTACGGTGGGGGG TCTTAGCAAAATCAGT-Phosphate-3'	NS1	1905-1939	U39661

TABLE 2. LC-rRT-PCR protocol for the LightCycler® 2.0 System

Analysismode	Cycles	Segment	Target temp. (°C)	Hold time (hh:mm:ss)	Ramp rate (°C/s)	Acquisition mode
			Reverse transcription			
None	1	1	58	00:08:00	20	none
Initial denaturation						
None	1	1	95	00:00:30	20	none
			Amplification			
Quantification	45	Denaturation	95	00:00:01	20	none
		Annealing	60	00:00:20	20	single
		Extension	72	00:00:01	2	none
			Cooling step			
None	1	1	40	00:00:30	20	none

NUCLEOTIDE SEQUENCE ACCESSION NUMBER

Nucleotide sequence accession numbers obtained for this study are: JF920061.1, JF920055.1, JF920070.1 and JF920068.1.

RESULTS

From a total of 130 respiratory specimens during the period from March 2011 through August 2011 tested, 54 specimens were positive for RSV. All 54 positive samples identified were sequenced and phylogenetic analysis was performed. Age distribution is shown in Table 3 and gender distribution is shown in Table 4. Statistical analysis did not show any significant differences with respect to age and gender.

After sequencing, all 54 positive RSV samples were placed under 4 clusters in phylogenetic tree with the neighbour joining method. Genotype GA2 were found in 13 isolates and genotype GA5 in 39 isolates. Genotypes GA2 and GA5 formed their own branch. Genotypes GA1 and GA7 were in separate branches where only 1 genotype GA1 and 1 genotype GA7 were determined. The phylogenetic tree is shown in Figure 1.

DISCUSSION

RSV is well known as the major respiratory agent in infants and young children worldwide, and may also be a significant pathogen in adults. Thirty-four out of 54 (63.0%) RSV positive patients were children below 2 years and two (1.4%) were adults. In the United States, an estimated 11000-17000 adults die of RSV infection annually (Walsh & Falsey 2012).

Data from studies of pathogens in respiratory infections showed that RSV was detected in 93% of hospitalized children younger than 24 months in the United States (Suryadevara et al. 2011). In China, in children under 14 years of age with acute respiratory tract infections, 38.14% (341/894) of samples were positive for RSV, where 60.4% of the selected 227 RSV strains were GA2, 34.4% were BA, 4.8% were GB2 and 0.4% were GB3 (Zhang et al. 2010).

This study of RSV virus showed 4 clusters in phylogenetic characteristics of Malaysian isolates with the neighbour joining method. Genotype GA2 were found in 13 isolates and genotype GA5 in 39 isolates. Genotypes GA2 and GA5 formed their own branch. Genotypes GA1 and

TABLE 3. Age distribution among study population (p value = 0.117)

Age (years)	rRT-PCR positive (n=54)	rRT-PCR negative (n=76)
0-1 (<2)	34	43
2-3	11	23
3-4	0	1
5-6	3	3
7-12	4	0
>12	2	6

TABLE 4. Gender distribution among study population (p value = 0.917)

Gender	rRT-PCR positive (n=54)	rRT-PCR negative (n=76)
Male	36	50
Female	18	26

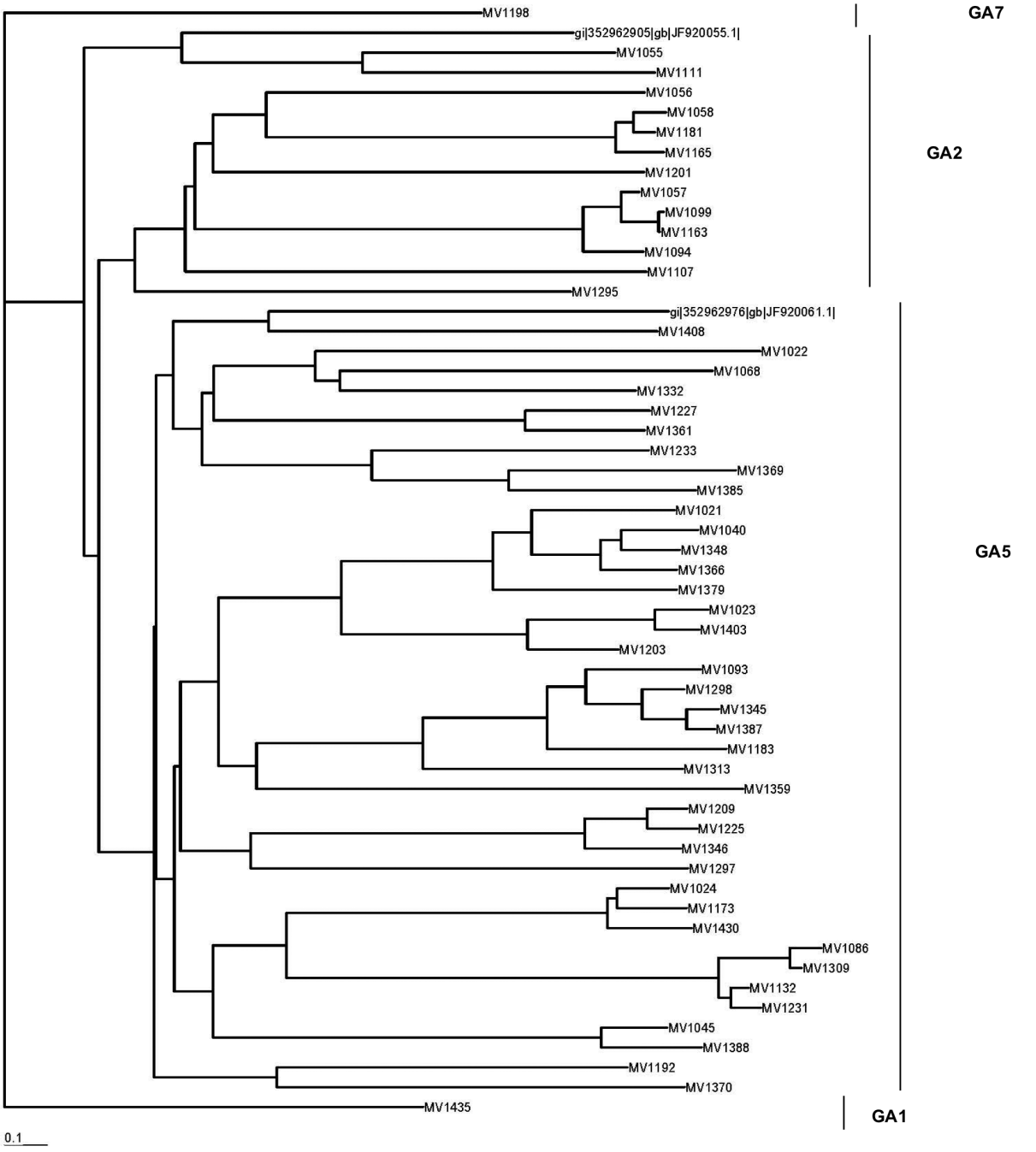

FIGURE 1. Phylogenetic tree for RSV nucleotide sequences contructed by using BioEdit version 7.0.9.0, Protdist – neighbor phylogenetic tree. Reference Genbank sequences of strains were included to compare the strains. The scale bars show the proportions of nucleotide substitution. Distribution of specimens selected for analysis by the LC-rRT-PCR. The majority of the isolated virus cluster with the GA5 genotype

GA7 were in separate branches where only one genotype GA1 and one genotype GA7 were determined. There was no isolate belonging to subgroup B in this population. Due to non-availability of literature on similar studies in the country, we could not compare the results in the light of Malaysian data. Similar studies were conducted in other countries on RSV group A and reported eight different genotypes, GA1 to GA7 and SAA1, on the basis of classification of the C-terminal variable region of the G protein (Frabasile et al. 2003; Peret et al. 1998; Venter et al. 2001).

In Japan during the 2009/2010 season, phylogenetic tree of RSV were detected in 50 Japanese children with acute respiratory infection showed that 34 strains were classified into subgroup A and 16 into subgroup B, where they were further subdivided into genotypes GA2 and BA, respectively (Zhang et al. 2010). While in Sweden, 152 out of 234 RSV isolates from respiratory samples

belonged to subgroup B and 82 to subgroup A. Genotypes within subgroup A were GA2 (25) and GA5 (57) and within subgroup B viruses were GB3 (137) and SAB1 (15) strains (Yoshida et al. 2012).

Dominance of the GA2 and GA5 genotypes during the last decade has been described from Belgium, Mozambique, South Africa and South America (Frabasile et al. 2003; Ostlund et al. 2008; Venter et al. 2001; Zlateva et al. 2004). In the studies from Belgium and South America, co-circulation of the GA2 and GA5 were found, but in some years, only one of the genotypes was isolated (Frabasile et al. 2003; Zlateva et al. 2004).

A study found several clinical observations in infants with RSV infection suggesting that A-subtype infections were more severe than B-subtype infections (Roca et al. 2001). In Canada, comparison of clinical data between 106 children infected with group A RSV (96 GA2 genotypes) and 94 children infected with group B RSV (62 GB3 genotypes) found Group A and genotype GA2 strains were associated with greater severity of RSV disease than group B strains (McConnochie et al. 1990). In another study which characterized RSV isolates by phylogenetic analysis of the RSV G gene, the authors reported that subgroup A and B did not differ in terms of severity of illness caused. However, the GA3 clade was associated with more severe illness than GA2 and GA4 (Gilca et al. 2006).

Presently, there is no effective vaccine available against RSV. In order to produce a broadly reacting RSV vaccine, it is necessary to know more about the circulation of different RSV genotypes in different parts of the world and to increase the knowledge about the antigenic relationship that exists between them (Rafiefard et al. 2004).

It is well known that an effective vaccine must have similar genomic nature against which the causal agent it is being vaccinated. This can be achieved by molecular analysis of the vaccine strain as well as infecting stains. Sequence analysis and phylogenetic assay must be performed before preparation of effective vaccine against which the vaccine is developed (Rahman 2011). Therefore findings of the present phylogenetic analysis of Malaysian strains of RSV would help in developing knowledge of circulating strains in the country as well as to help in developing suitable vaccines in future.

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