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Original Article

Molecular and demographic analysis of respiratory syncytial virus infection in patients admitted to King Chulalongkorn Memorial Hospital, Thailand, 2007

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Objectives: To preliminary preview the molecular character and its possible clinical correlation of RSV subgroups in Thailand.

Design: Cross-sectional analytic design.

Setting: Admitted acute lower respiratory tract infection patients of King Chulalongkorn Memorial Hospital, Bangkok, during Jun–Dec, 2007 were recruited.

Sample: Nasopharyngeal aspirations were collected.

Main outcome measures: All samples were analyzed for the presence of RSV glycoprotein G gene by reverse transcription PCR. Molecular character of each subgroup was determined by sequencing. Admission records were also analyzed for clinical correlations.

Results: Equal infectivity and severity of both RSV subgroups to the patients was shown. Mixed infection was shown to be as common as each single infection, higher than previously reported. GA2 of subgroup A and BA-IV of subgroup B were the most widespread genotypes and showed their monophyletic origins. From admission records, either type of infection did not show significantly preference in demographic record or clinical severity. Comorbidity, however, was statistically significant that more congenital heart disease was found in negative RSV cases, while more chronic pulmonary disease was in positive cases. Nevertheless, the clinical severity was insignificantly different suggesting that only patients with chronic pulmonary underlying were prone to be infected with RSV.

Conclusions: This preliminary RSV study showed prevalence of subgroups, types of infection, and common genotypes in an epidemic, uncorrelated to demography or clinical severity.

Keywords Genotype, molecular, respiratory syncytial virus, Thailand.

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Introduction

Respiratory syncytial virus (RSV) is the single most important cause of severe lower respiratory tract infections (LRTIs) in infants and young children worldwide.[1] The RSV disease spectrum includes a wide array of respiratory symptoms, ranging from rhinitis to bronchiolitis and pneumonia. Both bronchiolitis and pneumonia are associated with substantial global morbidity and mortality. In the USA, the virus is responsible for 70 000–126 000 hospitalized infant regarding pneumonitis or bronchiolitis annually.[1] RSV is a member of order *Mononegavirales*, family *Paramyxoviridae*, genus *Pneumovirus*. The envelope contains three virally encoded transmembrane surface glycoproteins, the major attachment protein G, the fusion protein F, and the small hydrophobic SH protein.[2] Glycoprotein (G) gene is commonly used to diversify subtype specificity.

Human RSV G is composed of 282–319 amino acids depending on the strain. It is a type II transmembrane glycoprotein and two-thirds of its C terminal is extracellular.[2] Two subgroups, A and B, were first characterized by monoclonal antibody technology. Later, nucleotide sequencing technology showed divergent evolution between these two subgroups.[3] Subgroup A can be classified into genotype GA1 to GA7, whereas subgroup B is divided into genotype BA, GB1-4, and SAB 1-4.[4,5] Discovered by Trento *et al.*, genotype BA has an additional 60-nucleotide insertion from the original subgroup B.[6] Starting from 1999, BA genotype currently consists of six subgenotypes (BA-I to VI).

Respiratory syncytial virus epidemic period in Thailand was previously reported as an annual outbreak from July to November.[7] The annual pattern was similar to reports from North[8] and South America,[9] Belgium,[10] and Japan;[5]

however, the biennial pattern was found in Croatia and Germany.[11] Molecular epidemiology of RSV is regularly studied in many countries. Overall, switching between 1–2 years of RSV subgroup A and 1 year of RSV subgroup B predominance was noted.[3] Moreover, different genotypes have shown their predominance in which no relation to geographical location or epidemic period was observed.[3–6,12–14]

In Thailand, Siritantikorn, *et al.* showed that RSV subgroup B predominance detected by monoclonal antibodies from November 1998 to February 2001 followed by subgroup A in a subsequent year.[15] Yet, RSV analysis for genotypic predominance in Thailand has not been studied. This study aims primarily to investigate the diversity of RSV subgroups as well as genotypes by means of RT-PCR and partial nucleotide sequencing of the G gene from samples sent to Virology laboratory, King Chulalongkorn Memorial Hospital, as a preliminary of subsequent epidemiology study. The demographic profile was also analyzed to search for a possible association with the subgroup. Furthermore, the result was compared and contrasted with the existing global strains.

Materials and methods

Clinical specimens

Laboratory record in 1988–2004 from Division of Virology, Department of Microbiology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand[7], showed that RSV infection is prevalent in June to December. As a result, 103 nasopharyngeal aspiration (NPA) specimens of admitted patients with clinical symptom were retrieved within June to December 2007. Demographic records, 77 records from 73 patients, were also retrieved and analyzed with genotypic records (Table 1).

RT-PCR for RSV glycoprotein G gene

Viral RNA was extracted directly from undiluted 200 μl of NPA sample by using RNA extraction kit (Nucleospin, Düren, Germany). The RNA specimens were stored at −70°C.

The cDNA was synthesized by using random hexamer primers and M-MuLV Reverse transcriptase of RevertAid First Strand cDNA Synthesis Kit (Fermentas, Glen Burnie, Maryland, USA). The seminested PCR amplification protocol was adjusted from Parveen S, *et al.*, 2006.[4] In brief, the external PCR was produced by the external forward primer, ABG-490, and the reverse primer, F-164 (Proligo, Boulder, Colorado, USA). Amplification of 3 μl of cDNA was carried out in 25 -μl total reaction mixture containing 20 mm $MgCl_2$, 2·5 u Pfu DNA polymerase (Fermentas), 200 μm of dNTP, and 25 pmole of each primer. The DNA amplification was performed in a Thermal cycler (Takara, Shiga, Japan). The PCR condition was initially with 94°C for 1 minute, then entered 35 cycles of denaturing at 94°C for 40 seconds, annealing at 50°C for 45 seconds, and extension of 72°C for 45 seconds. The final extension requires 72°C for 10 minutes. In second round PCR, the first PCR product (3 μl) was amplified by AG-655 and BG-517 as seminested forward primers for subgroup A and B, respectively, and F-164 as a reverse primer. The protocol was similar to the first round despite 25 cycles at 58°C. The amplified products were determined by 1·5% agarose gel electrophoresis (BRL, New York, USA) in 0·5× TBE (44.5 mM Tris, 44.5 mM Boric acid, 1mM EDTA) buffer. The final product sizes should be 450/585/645 bp for subgroup A/subgroup B/genotype BA.

Cloning and DNA sequencing

The PCR product from 15 mixed infected samples were cloned into *E.coli* DH52 using GeneJET PCR cloning kit (Fermentas). Twenty-five clones from each sample were randomly selected and checked for existence of the insert with RT-PCR. Only positive clones were then extracted by Plasmid DNA purification kit (Nucleospin), and PCR products were further purified with PCR clean-up (Nucleospin) before DNA sequencing. The DNA sequencing using both forward and reverse primers was carried out by Macrogen Inc., Seoul, Korea.

Phylogenetic analysis

All DNA sequences obtained in this study had already been submitted to GenBank for accession number, i.e., FJ489656–73for RSV subgroup A and FJ490351–62 for RSV subgroup B. The nucleotide sequence data were edited with sequence navigator and aligned with a few representatives from each RSV genotype retrieving from GenBank database using Bioedit program. MEGA4 was used to construct the phylogenetic trees of subgroup A and B by neighbor-joining method. The statistical significance of the tree topology was tested by bootstrapping (10 000 replicas).

Record review

Seventy-seven admission records of 73 hospitalized patients were reviewed. From 43 positive samples, 30 records were retrieved; similarly, 47 records were retrieved from 60 negative samples. Demographic data focusing on age and underlying comorbidities were noted. Other possible microorganisms isolated during admission were also retrieved. The bacteria and fungi were identified by using standard routine cultivation and identification carried out by Bacteriology Division, Department of Microbiology, Faculty of Medicine, Chulalongkorn University. Clinical severity score (range 0–6)[16] was assessed and calculated by addition of factors including positive pressure ventilation (two credits), hospitalization (one credit), length of stay

Table 1. Demographic data of the samples comparing between positive and negative groups

RSV	Positive cases *N* = 30	Negative cases *N* = 43	*P*-value
Gender male/female	17:13	22:21	
Age (months)			
Range	1–81	1 day – 114	0·48
Mean ± SD	17·71 ± 19·95	21·85 ± 27·43	
Median	10	10	
Comorbidity			
Congenital heart disease			
Incidence (%)	10 (33·33)	27 (57·45)	<0·05
Mean ± SD Clinical severity score	3·4 (0·97)	4·19 (1·30)	0·06
Chronic pulmonary diseases			
Incidence (%)	7 (23·33)	4 (8·51)	>0·05
Mean ± SD Clinical severity score	2·71 (0·76)	4 (1·41)	0·16
Clinical symptoms			
Mean ± SD body temperature (°C)	38·41 ± 0·99	38·30 ± 0·96	0·65
Mean ± SD length of hospitalization (days)	10·86 ± 10·17	22·23 ± 38·68	0·06
Mean ± SD clinical severity score (total = 6)	2·97 ± 1·03	3·51 ± 1·30	0·05
Diagnosis	28*	13**	
Viral pneumonia	2	1	
Viral bronchitis/bronchiolitis	0	26***	
Bacterial pneumonia	0	1	
Fungal pneumonia	0	1	
Unknown cause	0	1	

RSV, respiratory syncytial virus.
*Seven cases were bacterial infection.
**The other viruses apart from RSV.
***One case was with septicemia.

>5 days (one credit), O_2 saturation <87% (one credit), O_2 supplement use (one credit). Statistical analysis comparing among RSV-positive and between RSV-positive and RSV-negative groups was performed by non-parametric test, *Chi-square*, and Mann–Whitney *U* test. The statistic significance was noted when *P*-value ≤0·05.

Results

Genetic variation among RSV G genotypes

Phylogenetic analysis

From 103 NPA specimens, 43 (41·7%) positive RSV-RNAs were detected by RT-PCR. Respiratory syncytial virus subgroup A (30/43, 70%) predominated over RSV subgroup B (28/43, 65%). Fifteen of 43 (35%) mixed infection of subgroup A and B were demonstrated. Twenty-three RT-PCR products from single infection, 12 of subgroup A, and 11 of subgroup B were directly sequenced after purification. Fifteen mixed infected RT-PCR products were cloned, six of subgroup A and one of subgroup B were successfully retrieved and further analyzed for their sequences. Totally, 30 nucleotide sequences were recovered from the experiment, which was 18 of subgroup A and 12 of subgroup B (Figure 1). Analysis of nucleotide sequencing revealed that the viruses were grouped into genotypes characterized by previous RSV epidemiology studies.[4,5] In subgroup A, 221 bases (position 680–900) in second variable region of glycoprotein gene were aligned with representing sequences from each genotype. Genotype GA2 was responsible for 16/18 (88·89%) cases, while genotype GA5 was for the rest, 2/18 (11·11%) (Figure 2). All GA2s were monophyletic with its branch length 0·02 and possessed a rim of the phylogenetic tree. The two GA5s, S16a and S28a, could not be characterized for its specialty because few isolates were recovered; however, they were distributed among the existing strains worldwide (Figure 2).

Phylogenetic analyses of 12 subgroup B samples, 324 bases (position 637-900) of G gene were recovered. A 60-nucleotide insertion was found between position 260 and 261. All Thai strains were classified in genotype BA (Figure 3) and specified to BA-IV subgenotype (Figure 4). Most samples (11/12, 91·67%) were monophyletic in a branch length of 0·01.

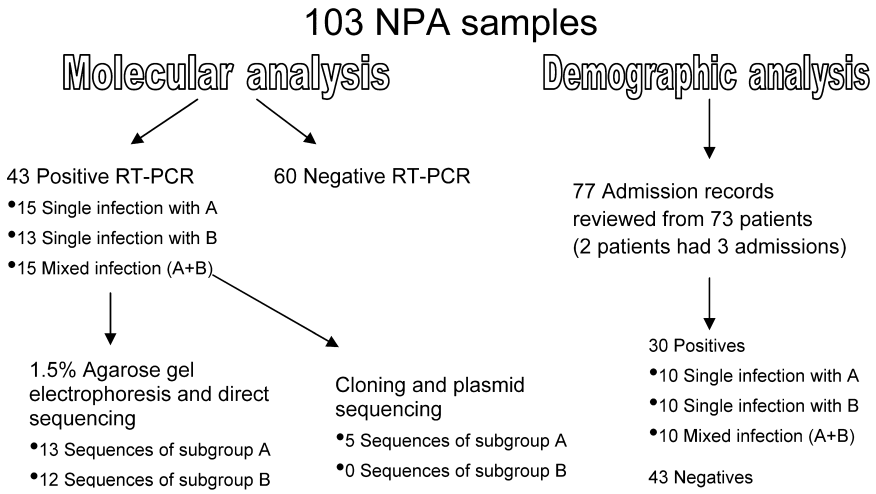

Figure 1. Molecular and demographic analysis of data collection from nasopharyngeal samples.

Intra- and intergenotypic variations

GA2 and GA5 nucleotide sequences were analyzed within their own grouping for intragenotypic variations of subgroup A. Concerning GA2, six nucleotide sequence patterns were observed. Nine sequences (n1a, s2a, s13a, s17a, s25a, s27a, s30a, s36a, and s37a) were exactly identical and were considered the majority of GA2s. The differences among those patterns varied from one to five nucleotides such as G805A, C694T, A745G, T788C, A673C, G683A, A712C, and C728A, resulting mostly in missense mutations; E272K, P235S, N252D, L266P, T228P, E233K, K241Q, T246N, respectively. s26a was excluded from this observation because only missense mutation (S290Y) was shown among five nucleotide shifts (G681A, A828G, C860A, C873T, A897T). In the case of GA5s, s28a had 13 nucleotides (T690A, T705C, T729C, T747C, C761T, T774C, T800C, C803T, T810C, C813T, C878T, T887A, and C893T) contrasting to s16a that resulted in four missense mutations (D233E, P257L, T296I, L299Q).

Seven nucleotide patterns were observed in BA-IV genotype. s12b was apparently outgroup from the majority (Figure 4) and excluded from this intragenotypic variation analysis. Among the six patterns, two patterns had maximal identical nucleotide sequences: s4b, s21b, s29b and n16b, s9b, s13b. When a group of s4b, s21b, and s29b was selected as a sequence to compare, the contrast of the other patterns varied from one to nine nucleotides and most were considered missense mutations such as T678A, G757A, C799T, T806C, and C811T resulting in D226E, D253N, H267Y, I269T, and P272S, respectively. Moreover, 1–2 nucleotide substitutions (T27C, C57T) observed in insertive area led to all silent mutation.

Intergenotypic variations between GA5 and GA2 indicated 19 distinct bases (T671C, G703A, C707T, A715C, T742A, C743A, A746G, A791T, A799T, C814A, T815C, A822T, G829A, T859C, T868C, A883G, A885C, T886C, and A888G) with 11 transitions. The differences had been retrieved from comparing sequences to the most commonly found GA2 (nine identical sequences) and the common feature of the two GA5s. No intergenotypic analysis of subgroup B samples was performed according to the fact that all were also categorized as genotype BA.

Amino acid analysis of Thai sequences

Amino acid sequences of second variable region in G protein were aligned with reference sequence[4] and analyzed, the percentage of amino acid homology was 90·5–100% among RSV genotype GA2, 90·6% in GA5, and 87% among BA. Figure 5 indicated the position of change in amino acids in 16 strains of RSV subgroup A comparing to other RSV sequences previously reported. All of them had a predicted G protein of 298 amino acids. T238D, Q263E, P275L, and T293S were observed as intragenotypic amino acid variations of Thai RSV genotype GA2 (Figure 5) to GA2s from other geographical locations showing monophyletic origin of Thai sequences. Similarly, the majority (11/12, 91·67%) of RSV genotype BA (Figure 6) showed monophyletic pattern branching from BA-IV genotype.

Demographic profile of the patients

RSV subgroup distribution

In 7 months of sample collection, both subgroups were isolated throughout a period. Totally, 15 cases were found to be single infected with subgroup A, 13 cases with subgroup B, and 15 cases were mixed infected with both subgroups. Most cases (15/43, 34·88%) were observed in August, consisting of nine mixed infection, three subgroup A single infection, and three subgroup B single infection. Single

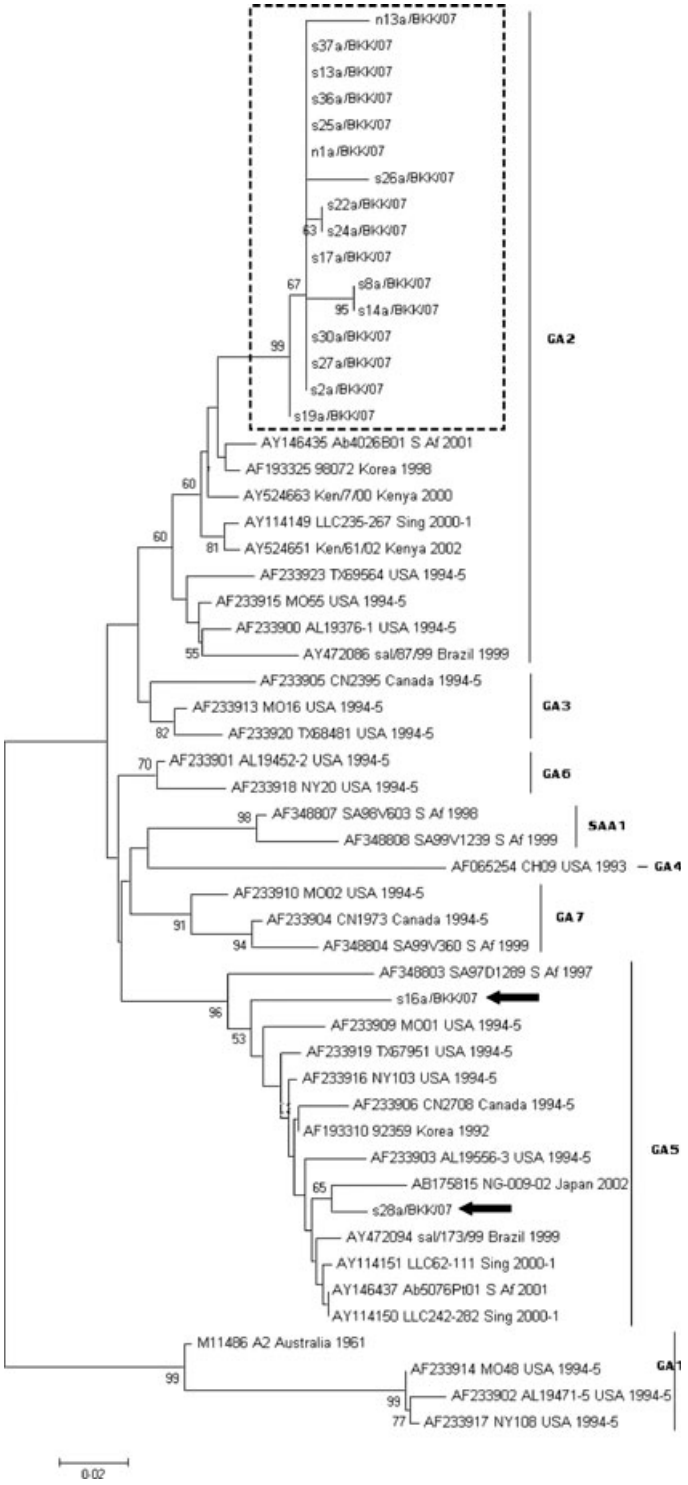

Figure 2. Phylogenetic tree of respiratory syncytial virus subgroup A sequences. Partial nucleotide sequences of the G protein gene, obtained from 18 clinical samples, were used to construct the phylogenetic tree by neighbor-joining method (see Materials and methods). The length of horizontal lines is proportional to the genetic distance between viruses. The bar represents 0·02 nucleotide substitutions per site, and the tree is unrooted. Numbers at the internal nodes represent the bootstrap probabilities (10 000 replicates). Only bootstrap values >50 are shown. Genotypes are indicated at the right by brackets. The sequences retrieved from GenBank are included here for comparative purpose. Our samples were shown in dot square box and arrow.

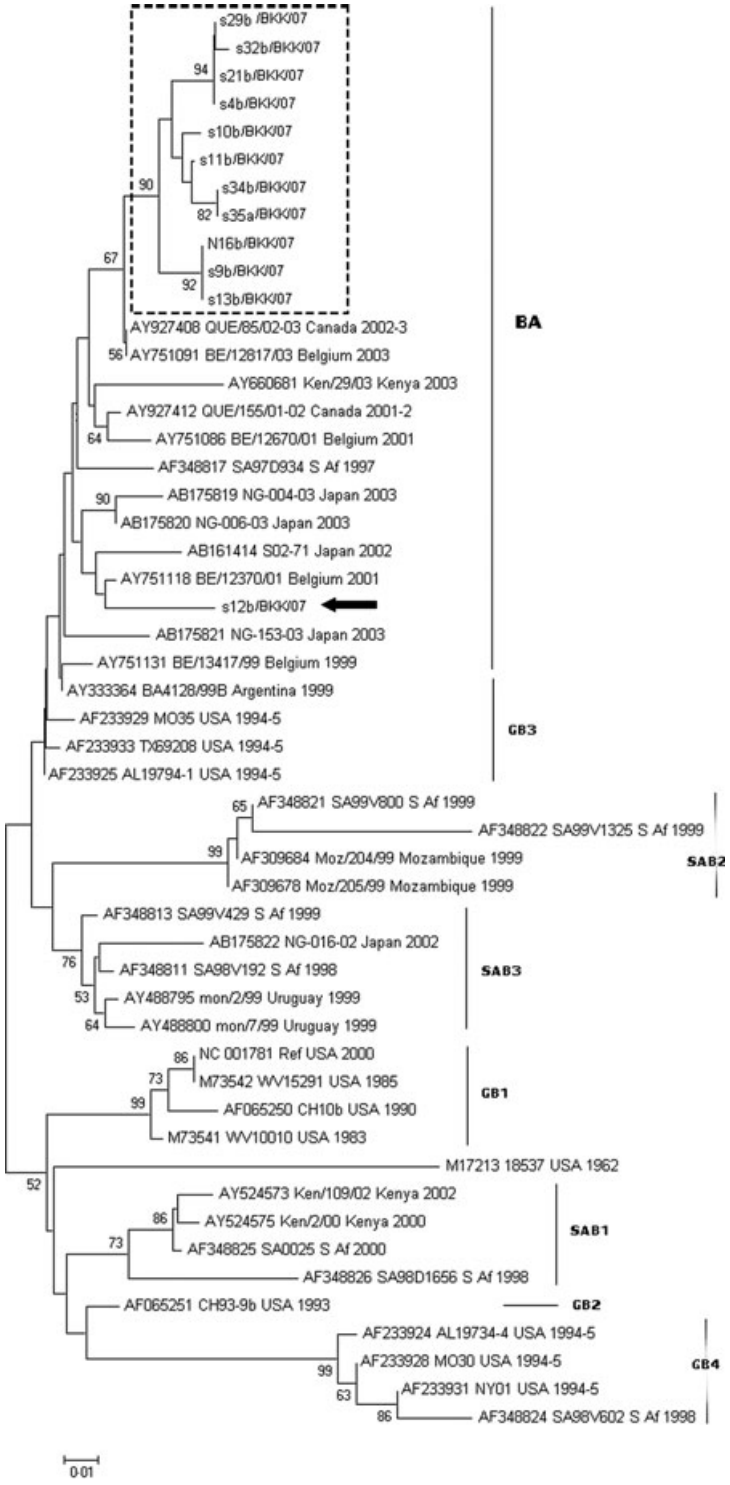

Figure 3. Phylogenetic tree of respiratory syncytial virus subgroup B sequences. Partial nucleotide sequences of the G protein gene, obtained from 12 clinical samples, were used to construct the phylogenetic tree by neighbor-joining method (see Materials and methods). The length of horizontal lines is proportional to the genetic distance between viruses. The bar represents 0·01 nucleotide substitutions per site, and the tree is unrooted. Numbers at the internal nodes represent the bootstrap probabilities (10 000 replicates). Only bootstrap values >50 are shown. Genotypes are indicated at the right by brackets. The sequences retrieved from GenBank are included here for comparative purpose. Our samples were shown in dot square box and arrow.

infection was also seen in July, five subgroup As and four subgroup Bs, and October, four subgroup As and four subgroup Bs, while mixed infection was rarely observed in these months.

Demographic data analysis

Seventy-seven admission records of 73 patients were grouped into positive and negative RSV RNA by reverse transcription PCR (Table 1). All presented with fever and

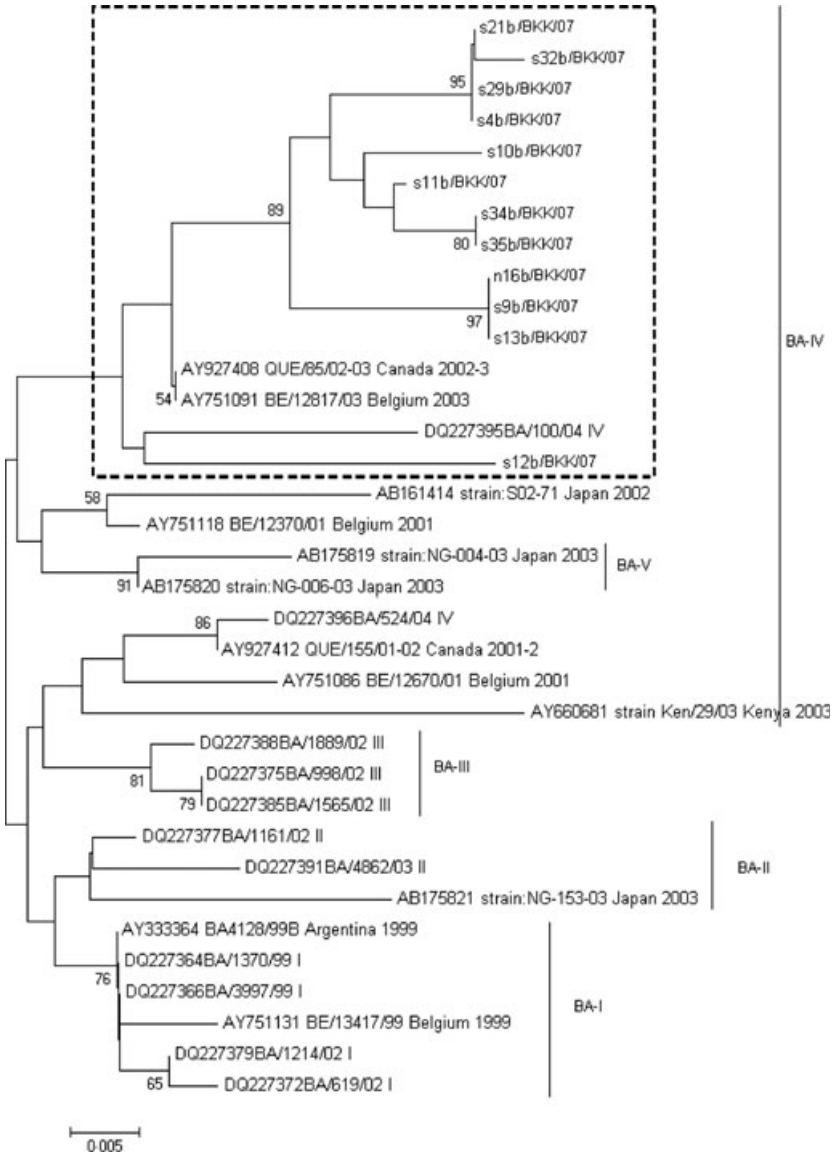

Figure 4. Phylogenetic tree of respiratory syncytial virus genotype BA sequences. Partial nucleotide sequences of the G protein gene, obtained from 12 clinical samples, were used to construct the phylogenetic tree by neighbor-joining method (see Materials and methods). The length of horizontal lines is proportional to the genetic distance between viruses. The bar represents 0·005 nucleotide substitutions per site, and the tree is unrooted. Numbers at the internal nodes represent the bootstrap probabilities (10 000 replicates). Only bootstrap values >50 are shown. Genotypes are indicated at the right by brackets. The sequences retrieved from GenBank are included here for comparative purpose. All our samples were in genotype BA-IV, which were showed in dot square box.

lower respiratory symptoms. It was noted that three admission records were observed in two patients. The infection was seen equally distributed in both genders. Patients with less than 2 years of age were found to be predominating (57/73, 78·57%). Comorbidity and clinical severity were paralleled and two groups, positive and negative RSV cases, were compared. Congenital heart disease was significantly more prevailed in negative RSV cases ($P < 0·05$), contrasting to chronic pulmonary disease that was significantly noted in positive cases. However, clinical severity score (maximum 6) was not significantly different, 3·4 for positives and 4·19 for negatives in congenital heart comorbidity and 2·71 for positives and 4 for negatives in chronic lung underlying.

Interestingly, among the 28 RSV-positive patients who were diagnosed as viral pneumonia or viral bronchiolitis, bacteria were also found in 7 (25·0%), which were *Pseudomonas* spp, *Haemophilus influenzae*, gram-positive cocci in

	230	238 240	250	260	263 270	275 280	290	293 299 300
AF013254_wt strain B1_USA_1997	TTN	PTKKPTLTTT	ERDTSTSQST	VLDTTTLEHT	IQQQSLHSTT	PENTPNSTQT	PTASE-PSTS	NSTQNTQSHA
AY146435_Ab4026B01_S AF_2001	..K	..E...IN..	KTNIR.TPL.	SNT.GNP...	S.KET.....	S.GNLS.S.V	Y.T..YL.Q.	L.PS..TKW*
AY472086_sal/87/99_Brazil_1999	..K	..E...IN..	KT.IR.TLE.	SNT.GNP...	S.KET.....	S.GN.SPS.V	Y.T..YL.Q.	LFPS..TN**
AF193325 98072_Korea_1998	..K	..E...IN..	KTNIR.TLL.	SNT.GNP...	S.KET.....	S.GNLSPS.V	Y.T..YL.Q.	L.PS..TKW*
AY524663_Ken/7/00_Kenya_2000	..K	..E...IN..	KTNIR.TLL.	PNT.GNP...	S.KET.....	S.GN.SPS.V	Y.T..YL.Q.	P.PS..TKW*
AY114149_LLC235-267_Singapore	..K	..E...IN..	KTNIK.TLL.	SNT.ENP...	S.KET....	S.GN.SPS.V	Y.T..YL.Q.	L.PS..TKW*
AF233915_MO55_USA_1994-5	..K	..E...IN..	KTNIK.TLL.	SNT.GNP...	S.KET.....	S.GN.SPS.V	Y.T..YL.Q.	L.PS..TN**
AF233923_TX69564_USA_1994-5	..K	L.E...IN..	KTNIK.TLL.	SNT.GNP...	S.KEI.....	S.GNLSPS.I	Y.T..NL.Q.	L.PS..TN**
AY524651_Ken/61/02_Kenya_2002	..K	..E...IN..	KTNIK.TLL.	SNT.GNP...	S.KET.....	S.GN.SPS.V	Y.T..YL.Q.	L.PS..TKW*
AY472094_sal /173 /99_Brazil_199	..K	..E...INI.	KPNIR.TLL.	NST.GN....	S.EET....S	S.GNTSPS.I	Y.T..YL.QP	P.PS.ITDQ*
AF233900_AL19376-1_USA_1994-5	..K	S.E...IN..	KTNIK.TLL.	SNT.GNP...	S.KET.....	S.GN.SPS.V	Y.T..YL.Q.	L.PS..TN**
n1a/BKK/07	..K	..E...ID..	KTNIR.TLL.	SNT.GNP...	S.EET.....	S.GNLSPS.V	Y.T..YL.Q.	P.SS..TK**
n13a/BKK/07	P.K	......ID..	QTNIRNTLL.	SNT.GNP...	S.EET.....	S.GNLSPS.V	Y.T..YL.Q.	P.SS..TK**
s2a/BKK/07	..K	..E...ID..	KTNIR.TLL.	SNT.GNP...	S.EET.....	S.GNLSPS.V	Y.T..YL.Q.	P.SS..TK**
s8a/BKK/07	..K	..E.S.ID..	KTNIR.TLL.	SDT.GNP...	S.EETP....	S.GNLSPS.V	Y.T..YL.Q.	P.SS..TK**
s13a/BKK/07	..K	..E...ID..	KTNIR.TLL.	SNT.GNP...	S.EET.....	S.GNLSPS.V	Y.T..YL.Q.	P.SS..TK**
s14a/BKK/07	..K	..E.S.ID..	KTNIR.TLL.	SDT.GNP...	S.EETP....	S.GNLSPS.V	Y.T..YL.Q.	P.SS..TK**
s17a/BKK/07	..K	..E...ID..	KTNIR.TLL.	SNT.GNP...	S.EET.....	S.GNLSPS.V	Y.T..YL.Q.	P.SS..TK**
s19a/BKK/07	..K	..E...ID..	KTNIR.TLL.	SNT.GNP...	S.EET.....	S.GNLSPS.V	Y.T..YL.Q.	P.SS..TK**
s22a/BKK/07	..K	..E...ID..	KTNIR.TLL.	SNT.GNP...	S.EET.....	SKGNLSPS.V	Y.T..YL.Q	P.SS..TK**
s24a/BKK/07	..K	..E...ID..	KTNIR.TLL.	SNT.GNP...	S.EET.....	SKGNLSPS.V	Y.T..YL.Q.	P.SS..TK**
s25a/BKK/07	..K	..E...ID..	KTNIR.TLL.	SNT.GNP...	S.EET.....	S.GNLSPS.V	Y.T..YL.Q.	P.SS..TK**
s26a/BKK/07	..K	..E...ID..	KTNIR.TLL.	SNT.GNP...	S.EET.....	S.GNLSPS.V	Y.T..YL.QY	P.SS..TK**
s27a/BKK/07	..K	..E...ID..	KTNIR.TLL.	SNT.GNP...	S.EET.....	S.GNLSPS.V	Y.T..YL.Q.	P.SS..TK**
s30a/BKK/07	..K	..E...ID..	KTNIR.TLL.	SNT.GNP...	S.EET....	S.GNLSPS.V	Y.T..YL.Q.	P.SS..TK**
s36a/BKK/07	..K	..E...ID..	KTNIR.TLL.	SNT.GNP...	S.EET.....	S.GNLSPS.V	Y.T..YL.Q.	P.SS..TK**
s37a/BKK/07	..K	..E...ID..	KTNIR.TLL.	SNT.GNP...	S.EET.....	S.GNLSPS.V	Y.T..YL.Q.	P.SS..TK**

Figure 5. Partial amino acid sequences of the glycoprotein genotype GA2. The 16 sequences were aligned with Bioedit program. The translated amino acids are compared with a reference strain (AF013254 wt strain B1) and other GA2s retrieved from GenBank. Identical residues are indicated by dots. Stop codons are shown by asterisks. Bold numbers represent locations to identify Thai uniqueness from other GA2 protein products.

	213	60 nt(20aa) insertion	272	293 300
NC_001781_Ref_USA_2000	KRDPKTPA KTTKKETTTN	PTKKPTLTTT ERDTSTSQST VLDTTTLEHT	---------- ----------	IQQQSLHSTT PENTPNSTQT PTASEPSTSN STQNTQSHA*
AY927408_QUE/58/02-03_Canada	......L. ..P.....I.	......PK.. ......P... ......SK..	ERDTSTSQSI VLDTTTSKHT	......Y... ....KL..Y.*
DQ227364BA/1370/99 I	......L. ..L.....I.	......PK.. ....SK..	ERDTSTSQST VLDTTTSKHT	....KL*.Y.*
DQ227366BA/3997/99 I	......L. ..L.....I.	......PK.. ....SK..	ERDTSTSQST VLDTTTSKHT	....KL*.Y.*
DQ227379BA/1214/02 I	......L. ..L.....I.	......PK.. ...A...... ......SK..	ERDTSTSQST VLDTTTSKHT	....KL*.Y.*
DQ227372BA/619/02 I	......L. ..L.....I.	......PK.. ...V...... ......SK..	ERDTSTSQST VLDTTTSKHT	....KL*.Y.*
DQ227388BA/1889/02 III	...S..L. ..L.....I.	......PK.. ......P... ......SK..	ERDTSTSQSI VLDTTTSKHT	.... .N.KL*.Y.*
DQ227375BA/998/02 III	...S..L. ..L.....I.	......PK.. ......P.F. ......SK..	ERDTSTSQSI VLDTTTSKHT	.... .N.KL*.Y.*
DQ227385BA/1565/02 III	...S..L. ..L.....I.	......PK.. ......P.F. ......SK..	ERDTSTSQSI VLDTTTSKHT	.... .N.KL*.Y.*
DQ227396BA/524/04 IV	......L. ..L.....I.	....L.PK.. ......P.T. ......SK..	ERDTSTSQSI VLDTTTSKHT	......Y... ....*KL*.Y.*
AY927412_QUE/155/01-02-Canada	......L. ..L.....I.	....L.PK.. ......P... ......SK..	ERDTSTSQSI VLDTTTSKHT	......Y... ....*KL*.Y.*
AB161414_S02-71_Japan	------L. ..L..D..I.	......PK.. ......P... ......SK..	ERDTSTPQST VLDTTTPKHT	....S.. ....KLR.Y.*
AY751086_BE/12670/01_Belgium	......L. ..L.....I.	......PK.. ......P... ......SK..	ERDTSTSQSI VLDTTTSKHT	......Y... ....*KF*.YT*
AY751091_BE/12817/03_Belgium	......L. ..P.....I.	......PK.. ......P... ......SK..	ERDTSTSQSI VLDTTTSKHT	......Y... ....KL..Y.*
AY751118_BE/12370/01_Belgium	......L. ..L.....I.	......PK.. ......P... ......SK..	ERDTSTPQST VLDTTTSKHT	....KL..Y.*
AY751131_BE/13417/99_Belgium	......L. ..L.....I.	......PK.. ....SK..	ERDTSTSQST VLDTTTSKHT	....T..... ....KL*.Y.*
AB175819_NG-004-03_Japan	......L. ..L.....I.	Q.....PK.. ......P... ......SK..	ERDTSTSQFI VLDTTTSKHT	....T..... ....KL..YG*
AB175820_NG-006-03_Japan	......L. ..L.....I.	Q.....PK.. ......P... ......SK..	ERDTSTSQSI VLDTTTSKHT	....KL..Y.*
AB175821_NG-153-03_Japan	....L.....I.	......PK.. ......P... ......SK..	ERDTSTSQPT VLDTTTSKHT	......Y... .D........ ....KL**Y.*
AY333364_BA4128/99B_Argentina	.....KL. ..L.....I.	......PK.. ....SK..	ERDTSTSQST VLDTTTSKHT	....KL*.Y.*
AY660681_Ken/29/03_Kenya	......LT ..L...N.I.	......PK.. ......P... ......SK..	ERDTSTSQSI VLDTTTSKHT	......Y... LK........ ....P. ...RL*----
DQ227395BA/100/04 IV	......L. ..P.....I.	......PK.. ......P... ......SK..	ERDPSTLQSI ALDTTTSKHT	......Y... ....F... ....*KL..Y.*
n16b/BKK/07	......L. ..P.....I.	......PK.. ......P... ..N...SK..	ERDTSTSQSI VLDTTTSKHT	......Y... SG........ ....*KL..Y.*
s12b/BKK/07	......L. ..P.....I.	......HK.. ......P... ......SK..	ERDTSTPQST ALDTTTSKNT	....D....... .....L.... ...KL.*Y.*
s4b/BKK/07	......L. ..P..D..I.	......PK.. ......P... ......SK..	ERDTSTSQSI VLDTTTSKHT	........I. .G........ ....*KL..Y.*
s9b/BKK/07	......L. ..P.....I.	......PK.. ......P... ..N...SK..	ERDTSTSQSI VLDTTTSKHT	......Y... SG........ ....*KL..Y.*
s10b/BKK/07	......L. ..P.....I.	......PK.. ......P..I ......SK..	ERDTSTSQSI VLDTTTSKHT	......Y... .G.....S.. ....*KL..Y.*
s11b/BKK/07	......L. ..P.....I.	......PK.. ......P... ......SK..	ERDTSTSQSI VLDTTTSKHT	.... .G........ ....*KL..Y.*
s13b/BKK/07	......L. ..P.....I.	......PK.. ......P... ..N...SK..	ERDTSTSQSI VLDTTTSKHT	......Y... SG........ ....*KL..Y.*
s21b/BKK/07	......L. ..P..D..I.	......PK.. ......P... ......SK..	ERDTSTSQSI VLDTTTSKHT	........I. .G........ ....*KL..Y.*
s29b/BKK/07	......L. ..P..D..I.	......PK.. ......P... ......SK..	ERDTSTSQSI VLDTTTSKHT	........I. .G........ ....*KL..Y.*
s32b/BKK/07	......L. ..P..D..I.	......PKI. ......P... ......SK..	ERDTSTSQSI VLDTTTSKHT	........I. .G........ ....*KL..Y.*
s34b/BKK/07	......L. ..P.....I.	.I....PK.. ......P... ......SK..	ERDTSTSQSI VLDTTTSKHT	......Y... .G........ ....*KL..Y.*
s35b/BKK/07	......L. ..P.....I.	.I....PK.. ......P... ......SK..	ERDTSTSQSI VLDTTTSKHT	......Y... .G........ ....*KL..Y.*

Figure 6. Partial amino acid sequences of the glycoprotein genotype BA-IV with 20 amino acids' duplication. The 12 sequences were aligned with Bioedit program. The translated amino acids are compared with standard reference strain (NC 001781), and other BA-IVs are retrieved from GenBank. Identical residues are indicated by dots. Stop codons are shown by asterisks. Bold numbers represent locations to identify Thai uniqueness from other GA2 protein products. Duplication area is labeled.

cluster, and gram-negative rod. Nevertheless, *Streptococcus pneumoniae*, *Klebsella pneumoniae*, *Mycoplasma pneumoniae*, and *Mycobacterium tuberculosis* were common in bacterial pneumonia, which were found in 26/43 (60·47%) RSV-negative patients. Viral infection other than RSV was found in about one-third of the negatives, 14/43 (32·56%).

Table 2. Characterization among subgroups of respiratory syncytial virus (RSV)-infected patients indicating insignificant difference in demography and clinical severity

RSV/group	Positive cases *N* = 30 A (10)	B (10)	Mixed (A+B) (10)
Gender male/female	6:4	5:5	6:4
Age (months)			
Range	3–81	2–64	1–28
Mean ± SD	18·10 ± 24·80	24·80 ± 22·07	10·40 ± 8·07
Median	8·5	18	9
Comorbidity			
Congenital heart disease (%)	4 (40)	2 (20)	4 (40)
Chronic pulmonary diseases (%)	3 (42·86)	2 (28·57)	2 (28·57)
Clinical symptom			
Body temperature (°C)			
Range	37·5–40	30·1–40·8	36·5–40·3
Mean ± SD	38·21 ± 0·79	38·62 ± 1·06	38·40 ± 1·15
Median	38·0	38·4	38·55
Length of Hospitalization (days)			
Range	3–26	5–38	3–35
Mean ± SD	9·1 ± 7·84	14·3 ± 12·47	9·2 ± 9·81
Median	6·5	8·5	5
Clinical severity score (total 6)			
Range	2–5	2–4	1–6
Mean ± SD	3·0 ± 0·94	3·2 ± 0·63	2·7 ± 1·42
Median	3	3	2
Complication with bacterial infection	1	3	3

Aspergillus and an unknown infectious agent were responsible for two remaining negative cases.

Subgroups were identified and characterized (Table 2). From available parameters, no significant difference was detected in mixed infection to single infection, either subgroup A or B. Similarly, differences in clinical severity of single infection with subgroup A or B were undetectable by statistics.

Discussion

Epidemiology reports of RSV genotyping show multiple lineages in most populations.[17–19] However, a single subgroup should predominate in each epidemic season. In Thailand, the RSV infection is detected every year, particularly in every rainy season and early winter (June–December).[7] Approximately 90% of Thai children experience an RSV infection by the age of 5.[20] Samransamruajkit, R and his colleague had grouped RSV among Thai Children admitted in King Chulalongkorn Memorial Hospital from 2001 to 2002 by monoclonal antibodies. The result showed predominance of RSV subgroup B in 2001, while subgroup A took its turn in 2002; nevertheless, the mixed infection was not identified.[21] In this study, RSV subgroups were identified by reverse transcription PCR and sequencing, instead of monoclonal antibody. The result revealed mixed infection, both subgroups identified in particular samples, at 15/43 (35%), while none were reported in the previous findings.[21] The discrepancy in the reports should be owed to the sensitivity of the methods rather than the periods of collecting samples. This study also indicated that RSV subgroup A and B coexisted in the epidemic period. From our data, subgroup predominance could not be detected, unlike the reports of South Africa,[22] Tunisia,[23] Germany,[24] Italy,[25] or India.[4] However, larger-scale samplings should be applied to the following study. This is the first molecular study of RSV in Thailand, previewing the preliminary picture of circulating genotypes.

Evidence showed that certain subgroups had evolved divergently for a period of time.[3] Therefore, two phylogenies were constructed separately within each subgroup. During the 7 months of the study (June–December 2007), genotype GA2 was predominant (89%), while GA5 was also detected in subgroup A lineage. The two genotypes were also reported in other countries such as India,[4] Japan,[5] Sweden,[26] and China.[13] Genotype GA5 was predominant in 2000–2001 and 2003–2004 in Sweden,[12] 2001–2002 and 2003–2004 in Japan,[5] 2002–2003 and 2003–2004 in India.[4] Unfortunately, no Thai genotypic information is provided to compare during those period; however, Thai 2007 data

still displayed a GA5 setting as seen in the two detected isolates. From the inadequate data, the introduction of GA5 to Thailand was hardly predicted. GA2, on the contrary, was found in 2007 sampled from the hospital (Figure 2). The GA2s genotype showed monophyletic origin. However, to confidentially argue the observation in[4,5] that distinct pattern was regionally developed, larger sample size from selected community and hospitals should be recognized of sufficient data sampling. Intragenotypic analysis of GA2 that mostly showed missense mutation along with changing the side-chain property indicated non-random mutation despite changing the total protein length of 298 amino acids. As only two GA5s were detected in this study, increase of GA5 sample size would be required for further analysis.

Interestingly, Thai subgroup B viruses were all classified into genotype BA, which was recently identified by Trento, *et al.*[6] An apparent character of genotype BA is that G protein sequence contains 60-nucleotide duplication. Some were evident as local isolates like BA-III in Buenos Aires, Argentina and BA-V in Niigata, Japan while the others were found in five continents such as BA-IV and BA-VI. The presence of genotype BA in Thailand was similar to the reports from countries such as China,[13] India,[4] Japan[5], and South Africa.[22] BA-IV, the only BA genotype discovered in Thailand, was hypothesized its introduction to Belgium in 1999, and then crossed the Atlantic to Canada in 2001–2003, to Argentina in 2002–2004, to Kenya in 2003, and to India in 2003–2004, but not to Japan before 2004.[6] The strain had shown high adaptability to the worldwide population by its unconfirmed selective advantage. Our data were insufficient for describing when BA-IV was first imported to Thailand or whether it actually developed its distinctive character. Compared to the strains worldwide, Thai BA-IV viruses showed monophyletic pattern described in Result (Figure 4); however, additional data are necessary for supporting the observation. Moreover, the deduced amino acid sequence of G protein was also shown (Figure 6) that most of them had a shortened G protein of only 312 amino acids. Regarding intragenotypic variation, nucleotide shifts also caused mostly missense mutations by affecting amino acid side-chain properties.

Admission records of the patients given out the NPA samples were reviewed. No significant difference was found in clinical severity between single or mixed infection, as well as between subgroup A or B. This observation corresponds with previous findings[27,28] that no difference in clinical severity was observed between subgroups or mixed infected cases, but contrast to Walsh, *et al.*[16] Moreover, the outgroup records such as s16a, s28a, and s12b were attentively reviewed; however, distinctive character in these patients was not observed.

Prior reviews[29] indicate neuromuscular impairment as one of the risk factors for increasing severity. In this study (Table 1), congenital heart disease and chronic pulmonary disease were recorded in comorbidity session. Congenital heart disease was a statistically significant comorbidity observed in the negative RSV group, yet the clinical severity score was unchanged, indicating that congenital heart disease is not a predisposing factor for RSV infection. In contrast, chronic pulmonary diseases were significantly found in RSV-positive cases, implying that chronic lung patients should be more susceptible to RSV infection.

This study provides the first molecular approach of RSV by means of its grouping, genotypic identification, sequence variation, and its relation to clinical settings. The study also reveals a RSV preliminary picture in the Thai population and is fundamental information to design the following large-scale epidemiological study of RSV in Bangkok, Thailand.

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