SUBGROUP DETERMINATION OF RESPIRATORY SYNCYTIAL VIRUS BY RT-PCR

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Abstract. PCR optimization for differentiation of RSV subgroup A (RT-PCR-1) and RSV subgroup B (RT-PCR-2) were developed. Various conditions of RT-PCR-1 and RT-PCR-2 were summarized. These methods are highly specific and sensitive to differentiate RSV subgroup A and RSV subgroup B from the other respiratory viruses.

INTRODUCTION

Respiratory syncytial virus (RSV) is one of the most common causes of acute respiratory tract infection in infants and young children around the world (Kim *et al,* 1971). The epidemiology of infection can be caused by both RSV subgroups A and B but the RSV subgroup A are more predominant and severe than the RSV subgroup B. The occurence of infection can vary according to time and geographical areas (Anderson, 1991: Thawatsupha, 1993). Characterization of RSV isolates is important for epidemiological study which can be done by using monoclonal antibodies or RT-PCR. In this study, various amplification conditions of RT-PCR for differentiation RSV subgroup A from subgroup B were developed for determination of RSV subgroups from nasopharyngeal aspirates of suspected RSV infections.

MATERIALS AND METHODS

Virus strain

RSV subgroup A Long strain and RSV subgroup B HC 306/91, Thai strain were used as prototype for RSV subgroups A and B respectively. Twenty strains of RSV subgroup A and 13 strains of RSV subgroup B were isolated since 1998 to 1994 at Virus Research Institue, Department of Medical Science, Ministry of Public Health, Thailand were also used to determine the specificity and sensitivity. Nasopharyngeal aspirates (NPAs) were collected from 50 children and infants with suspected of respiratory infections at the Children's Hospital, Bangkok, during June to July, 1992.

Tissue culture

RSV subgroup A virus, RSV subgroup B virus and NPAs were propagated in HEp-2 cells culture in Eagle's minimal essential medium, 5% fetal bovine serum and antibiotics. The infected cells were incubated at 37°C in a CO_2 incubator, The presence of CPE was observed daily, adenovirus, influenza virus, parainfluenza virus and uninfected HEp-2 cells were used as the controls. Adenovirus, parainfluenza virus, and influenza virus were propagated in HEp-2 cells and MDCK cells, respectively.

Preparation of samples for RT-PCR

Total RNA was extracted from RSV infected HEp-2 cells according to the procedure described by Chomczynski and Sacchi (1987) with a slight modification. An aliquot of RSV subgroup A Long strain or RSV subgroup B (HC 306/91, Thai strain) was extracted by adding 0.5 ml of lysis buffer (4 m guanidium thiocyanate, 25 nm sodium citrate, pH 7.0, 0.5% of sarcosyl, 0.1 M 2- mercaptoethanol), 50 µl of 2 M sodium acetate, pH 4.0, 0.5 ml of phenol and 0.1 ml of chloroform to the virus preparation. The preparation was mixed and centrifuged at 12,000*g* for 15 minutes at 4°C. The aqueous phase was further extracted with an equal volume of

Table 1

Comparison of RT-PCR conditions for differentiation of RSV subgroup A (RT-PCR-1) and RSV subgroup B, (RT-PCR-2).

	RT-PCR-1	RT-PCR-2
Tris HCl pH8.3	10 mM	10 mM
Mg Cl_2	1.5 mM	2 mM
Annealing temperature	58°C	58°C
Formamide concentration	5%	1.25%
Taq DNA polymerase	2.5 units	2.5 units
Primer concentration	1 µM	2 µM

isopropanol, kept in -70°C for 10 minutes, then centrifuged at 12,000g at 4°C for 15 minutes. The pellet was suspended in 200 µl of diethylpyrocarbonate (DEPC) treated water and 15 µl of 2 M sodium acetate pH 5.2, precipitated with 2 volumes of ethanol and washed with 500 µl 70% ethanol. The RNA pellet was dissolved in 4.5 µl DEPC treated water and kept for RT-PCR.

Reverse transcription and PCR

The cDNA was synthesized in 10 µl of the reaction mixture containing 50 mM Tris HCl pH8.3, 75 mM KCl, 3 nM $MgCl_2$, 10 mM dithiothreitol (DTT) 0.5 mM each of four dNTPs (Boehringer Mannheim, Germany), 5 units of human placental ribonuclease inhibitor (BRL, USA), 1 µM each of either two primers, P_6 (5′-AGATCAAGAACACAACCCC-3′) and P_7 (5′-CAGGTTGGATTGTTGCTGC-3′) for subgroup A or P_3 (5′CATCTCTGCCAATCA- CAAAG-3′) and P_5 (5′CCACATATACTACA-GGGAAC(GA)A-3′) for subgroup B, followed by addition of 100 units of SUPERSCRIPT reverse transcriptase (BRL, USA). The mixture was incubated at 42°C for 15 minutes. The reaction mixture was made up to 50 µl by adding 10 mM Tris HCl pH8.3, 50 mM KCl, 1.5 mM Mg Cl_2, 100 µM each of four dNTP's, 0.5 µM each of P_6 and P_7 for RSV subgroup A or P_3 and P_5 for RSV subgroup B, 2 µl of DNA template and 2.5 units of Taq DNA polymerase (Promega, USA). The final volume was adjusted to 50 µl with distilled water and overlaid with 1 drop of mineral oil. The reaction mixture was subjected to 1 amplification cycle, consisting of sample denaturation at 94°C for 45 seconds, primer annealing at 56°C for 45 seconds and primer extension at 72°C for 45 seconds. The amplification was repeated 44 more cycles.

The optimization scheme to determine the best amplification conditions for a specific template and set of primers were set up. The PCR reaction was amplified by varying pH of buffer, magnesium chloride concentration, annealing temperature, formamide concentration, primer concentration, and the concentration of Taq DNA polymerase.

Subgroup determination of RSV isolated from NPAs were subjected to RT-PCR by using PCR optimized condition. The specificity of RSV subgroups A and B by RT-PCR was assessed by comparing RSV with adenovirus, influenza virus, and parainfluenza virus. The sensitivity were performed by RT-PCR using ten fold dilutions of RSV subgroups A and B and primers P_6 and P_7 for subgroup A and P_3 and P_5 for subgroup B to evaluate the detection limit.

Analysis of amplified products

The amplified products were analyzed by 3% agarose gel electrophoresis containing ethidium bromide for 30 minutes. The gels were photographed under UV illumination.

Southern blot hybridization

The DNA amplified products were transferred from agarose gel to a Hybond-N nylon membrane (Amersham, UK). DNA sample was denatured on the membrane for 10 minutes in 0.4 M NaOH and soaked in 5 x SSC for 1 minute, then fixed under UV light. The membrane bound DNA was hybridized with oligonucleotide probes P_8 (5′-GAATCCCCAGCTTGGAATC-3′) for subgroup A or P_9 (5′-AGTCCCACCAGAAAGGGTT-3′) for subgroup B and labeled for chemiluminescence according to the method of enchanced chemiluminescence (ECL) 3-oligolabeling and detection systems (Amersham, UK). The hybridized DNA membrane was then exposed to X - ray film.

RESULTS

PCR optimization for differentiation of RSV subgroup A (RT-PCR-1)

The optimization scheme to determine the best PCR amplification condition for differentiation of RSV subgroup A was found. The highest yield of PCR product was obtained at pH 8.3 of PCR buffer and 1.5 mM of $MgCl_2$ concentration. The annealing temperature, formamide concentration, primer concentration, and the amount of Taq DNA polymerase were 58°C, 5% 1μM, and 2.5 units respectively.

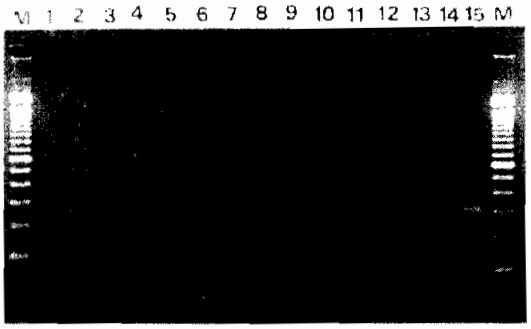

Fig 1–Specificity of RT-PCR-1 in differentiation of RSV subgroup A. Nucleic acids of virus infected HEp-2 cells or MDCK cells were subjected to RT-PCR-1 on 3% agarose gel. The size of the PCR product was 301 bp. Lane M is 100bp DNA ladder as a size marker 100, 200, 300,.....2,072 bp from bottom to top respectively.

a. Lanes1 to 13 are RSV subgroup B
Lane 14 is uninfected HEp-2 cells
Lane 15 is RSV subgroup A

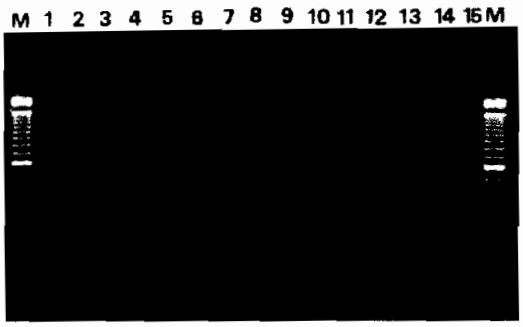

Fig 1– b. Lane 1 is Influenza A virus
Lanes 2 to 5 are Influenza B virus
Lanes 6 to 9 are Parainfluenza virus
Lanes 10 to 13 are Adenovirus
Lane 14 is uninfected HEp-2 cells
Lane 15 is RSV subgroup A

PCR optimization for differentiation of RSV subgroup B (RT-PCR-2)

The best PCR amplification condition for differentiation of RSV subgroup B was found. The highest yield of PCR product was obtained at pH 8.3 of PCR buffer and 2 mM of $MgCl_2$ concentration while the annealing temperature, formamide concentration, primer concentration, and the amount of Taq DNA polymerase were 58°C, 1.25%, 2 μM and 2.5 units respectively.

Sensitivity and specificity of RSV subgroups A and B were illustrated in Figs 1,2,3,4,5 respectively. Subgroup determination of RSV subgroups A and B from various RSV isolates are demonstrated in Figs 6,7, and 8.

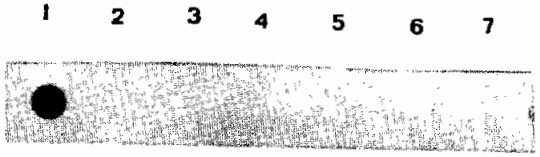

Fig 2–Confirmation of RT-PCR by dot-blot hybridization with P_8 probe.

1 = RSV subgroup A
2 = RSV subgroup B
3 = Influenza A virus
4 = Influenza B virus
5 = Parainfluenza virus
6 = Adenovirus
7 = Uninfected HEp-2 cells

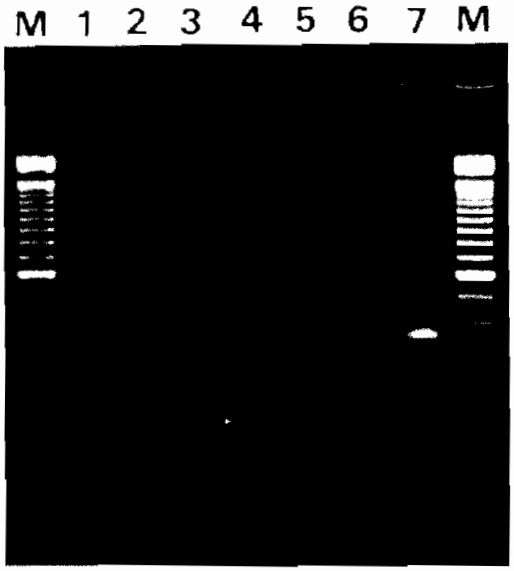

Fig 3–Specificity of RT-PCR-2 in differentiation of RSV subgroup B nucleic acids of virus infected HEp-2 cells or MDCK cells were subjected to RT-PCR-2 on 3% agarose gel . The size of the PCR product was 345 bp. Lane M is 100 bp DNA ladder as a size marker (100, 200, 300,.....2,072 bp from bottom to top, respectively).

(a) Lanes 1 to 5 are RSV subgronp A
Lane 6 is uninfected HEp-2 cells.
Lane 7 is RSV subgroup B

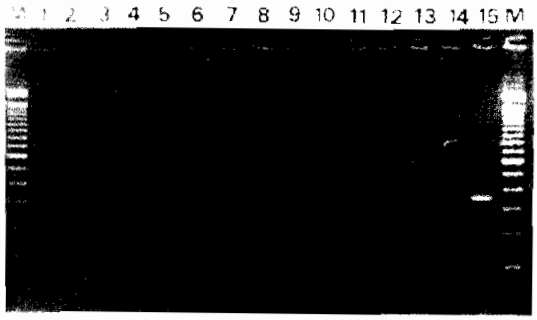

Fig 3–(b) Lane 1 is Influenza A virus
Lanes 2 to 5 are Influenza B virus
Lanes 6 to 9 are Parainfluenza virus
Lanes 10 to 13 are Adenovirus
Lane 14 is uninfected HEp-2 cells.
Lane 15 is RSV subgroup B

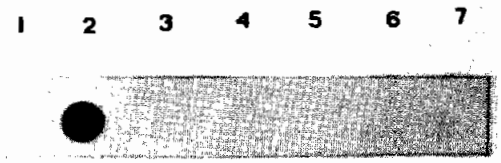

Fig 4–Confirmation of specificity of RT-PCR-2 by dot blot hybridization with P_9 probe.

1 = RSV subgroup A
2 = RSV subgroup B
3 = Influenza A virus
4 = Influenza B virus
5 = Parainfluenza virus
6 = Adenovirus
7 = Uninfected HEp-2 cells.

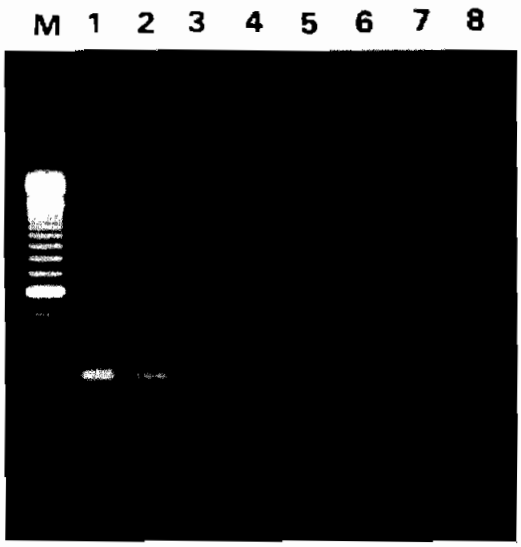

Fig 5–Sensitivity of RT-PCR-1 and RT-PCR-2. Serial tenfold dilutions of RSV subgroups A and B were subjected to RT-PCR-1 and RT-PCR-2, and electrophoresis on 3% agarose gel. The size of the PCR product were 301 bp and 345 bp respectively. Lane M is 100 bp DNA ladder as a size marker (100, 200, 300,.....2,072 bp from bottom to top respectively).

(a) Lanes 1 to 7 are Longstrain infected HEp-2 cells diluted 10^{-1} to 10^{-7}. Lane 8 is uninfected HEp-2 cells.

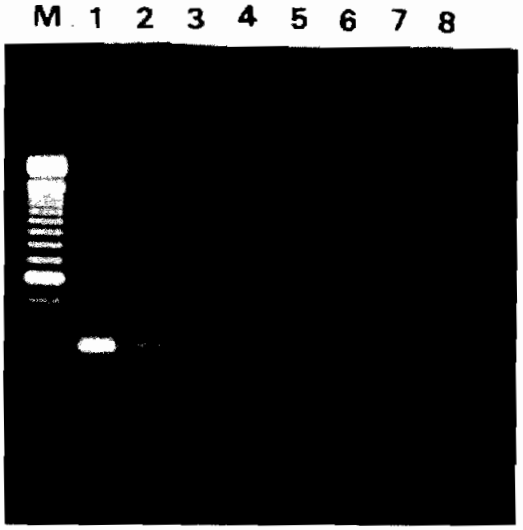

Fig 5–(b) Lanes 1 to 7 are HC 306/91 strain infected HEp-2 cells diluted 10^{0} to 10^{-6}. Lane 8 is uninfected HEp-2 cells.

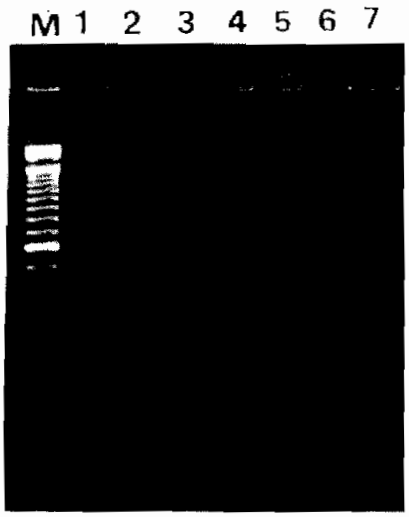

Fig 6–Subgroup determination of RSV subgroup A isolate by RT-PCR. Nucleic acids of RSV subgroup A and B infected HEp-2 cells were subjected to RT-PCR-1 on 3% agarose gel. The size of the PCR product was 391 bp. Lane M is 100 bp ladder as a size marker (100, 200, 300,.....2,072 bp from bottom to top respectively). Lanes 1 to 5 are RSV subgroup A infected HEp-2 cells. Lane 6 is uninfected HEp-2 cells. Lane 7 is RSV subgroup B infected HEp-2 cells.

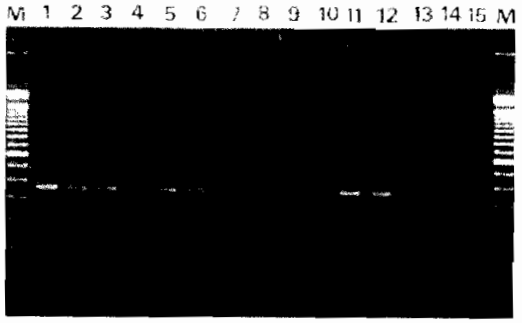

Fig 7–Subgroup determination of RSV subgroup B isolates by RT-PCR-2. Nucleic acids of RSV subgroups A and B infected HEp-2 cells ware subjected to RT-PCR-2 on 3% agarose gel. The size of the PCR product was 345 bp. Lane M is 100 bp DNA ladder as a size marker 100, 200, 300, 400,-500, 600, ...2,072 bp from bottom to top respectively. Lanes 1 to 13 are RSV subgroup B infected HEp-2 cells. Lane 14 is uninfeeted HEp-2 cells. Lane 15 is RSV subgroup A infected HEp-2 cells.

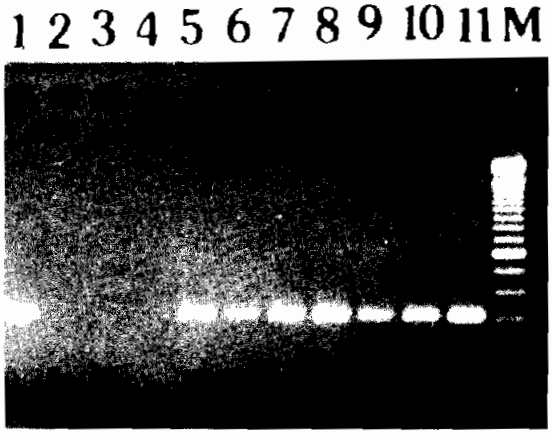

Fig 8–Subgroup determination of RSV from NPAs by RT-PCR-1 and RT-PCR-2. Amplified products were analyzed by 3% agarose gel electrophoresis. Lane M is 100 bp by DNA ladder as a size marker (100, 200, 300,.....2,072 bp from bottom to top, respectively). Lane 1 is positive control. (RSV subgroup A, Long strain). Lane 2 is uninfected HEp-2 cells. Lane 3 and 4 are amplified products of RSV with negative result by both tissue culture and RT-PCR-1. Lanes 5 to 7 are amplified products of RSV with negative tissue culture but positive by RT-PCR-1. Lanes 8 to 11 are amplified products of RSV with positive tissue culture and RT-PCR-1.

DISCUSSION

The RT-PCR assay is a highly specific and sensitive method for detection of RSV from clinical specimens. The reason for this is due to the specific primers from F_1 gene conserved region of RSV that use for amplification of RSV- cDNA (Johnson and Collins, 1988; Paten *et al,* 1992). At the present time, epidemiology of RSV is a steadily growing field, therefore, differentiation of RSV subgroup is strongly needed for epidemiological study. Subgroup determination can be done by monoclonal antibodies or RT-PCR. Development of monoclonal antibodies is not always possible in some laboratories due to the limited budget, and commercial monoclonal antibodies specific for RSV G protein is not always certain. RT-PCR can be a method of choice, but one must be concentrated on the various amplification conditions in order to obtain the best yield, high sensitivity and specificity.

The optimal reaction conditions of a PCR method depend on the template DNA and the respective primer. The various parameters that may vary between different application are the magnesium concentration, pH of the reaction buffer, annealing temperature, formamide concentration, primer concentration of Taq DNA polymerase enzyme.

The insufficient or excess magnesium concentration will reduce the yield or enzyme fidelity (Eekert and Kunkel, 1996) and increase the accumulation of non-specific amplification products (Williams 1989; Ellsworth *et al,* 1993; Saiki, 1989). The optimal magnesium concentration and pH of PCR buffer for the amplification of RSV subgroup A and B in this study are consistent with most of the amplification reaction (Innis and Gelfand, 1990; Saiki, 1989). Annealing temperature is depend on the length, GC content, and the sequences of the primers. In this study, the optimal annealing temperature for PCR amplification of RT-PCR-1 and RT-PCR-2 was 58°C which is higher than the annealing temperature of the PCR amplification that use to detect RSV from clinical specimens. The reason for this due to the higher temperature will help to minimize the nonspecific primer annealing, increasing the amount of specific product, and reducing the amount of primer dimer (Saiki, 1989; Innis and Gelfand, 1990).

Addition of casolvents such as dimethyl sulfoxide (DMSO), formamide, and tetamethylammonium chloride (TMACl) in low concentration will improve reaction yields and specificity (Hung *et al,* 1990; Sarkar *et al,* 1990; Bookstein *et al,* 1990). In this study, 5% and 1.25% formamide were added for amplification of products, RSV subgroup A, and RSV subgroup B respectively.

The optimal primer concentration is also important for amplification. Since higher primer concentrations may promote mispriming and accumulation of nonspecific product and may increase the probability of generating a template-independent artifact or primer dimer. The nonspecific products and primer dimer artifacts act as the substrates for PCR and compete with the desired product for enzyme, dNTPs, and primers resulting in a lower yield of the desired product (Innis and Gelfand, 1990).

The amount of Taq DNA polymerase is another important factors for the PCR reaction. The high amount of enzyme will result in nonspecific PCR products while the low amount of enzyme will result in insufficient amount of desired product (Rolfs *et al,* 1992).

The RT-PCR-1 is highly specific for differentiation RSV subgroup A while RT-PCR-2 is also specific for differentiation of RSV subgroup B from the other respiratory viruses. The results obtained from both RT-PCR-1 and RT-PCR-2 were in accordance to the subgroup determination by monoclonal antibodies which suggested that RT-PCR-1 and RT-PCR-2 can be used as an alternate method for subgroup determination.

The sensitivity of both RT-PCR-1 and RT-PCR-2 were lower than those reported by Van Milaan *et al* (1994). This low sensitivity may due to the use of primer from G gene. However, the lower limit of sensitivity of this study is still in the range of the RSV titer present in the nasal secretions of infected children (10^4 to 10^6 TCID 50/ ml) (Hall *et al,* 1975; Hall *et al,* 1976).

ACKNOWLEDGEMENTS

The authors would like to express our sincere thanks to Faculty of Tropical Medicine, Mahidol University, SEAMEO TROPMED, and The National Research Council of Thailand for their financial support. Thanks also go to Mrs Pranee Thawatsupha for providing virus isolates.

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