Optimization of multiplex real-time RT-PCR for respiratory syncytial viruses detection

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Abstract

Background: Multiplex real-time RT-PCR (rRT-PCR) is a fast, sensitive and specific test to detect more than one target in single PCR reaction. In this study we developed multiplex rRT-PCR for RSV-A and RSV-B since those viruses are the most common pathogen found in respiratory tract. However, in order to gain optimal reaction for RSV-A and RSV-B detection, the optimization of primers and probes specific for RSV-A and RSV B are needed.

Method: The primers and probes of multiplex rRT-PCR for RSV-A and RSV-B were selected and optimized utilizing PerlPrimer software and BLAST to analyze the secondary structures and specificity, respectively. Further testing of selected primers and probes for rRT-PCR was done using annealing temperature based on in silico analysis as mentioned above. This includes sensitivity testing with the utilization of synthesized DNA of RSV-A and RSV-B and specificity testing targeting the common viruses found in respiratory tract.

Results: The primer set and probes selected for RSV-A and RSV-B detection were specific only for RSV-A and RSV-B and showed no secondary structure. Based on primer and probe criteria for rRT-PCR such as annealing temperature, no secondary structure formed, % GC content and limit of detection, the multiplex rRT-PCR test using selected primers and probes was able to detect synthesized DNA of RSV-A and RSV-B.

Conclusion: Multiplex rRT-PCR that employing primer sets and probes targeted N gene of RSV-A and RSV-B in this study were able to be detect RSV-A and RSV-B in single PCR reaction. ***(Health Science Journal of Indonesia 2021;12(2):66-73)***

Keyword: Multiplex, real-time RT-PCR, RSV-A, RSV-B

Abstrak

Latar belakang: *Multiplex real-time RT-PCR (rRT-PCR) merupakan metode yang cepat, sensitif dan spesifik untuk mendeteksi lebih dari satu target pathogen dalam satu reaksi PCR. Penelitian ini bertujuan untuk mengembangkan multiplex rRT-PCR virus RSV-A dan RSV-B yang merupakan patogen yang paling sering ditemukan di saluran pernafasan. Optimisasi dari primer dan probe dalam multiplex rRT-PCR diperlukan untuk mendapatkan reaksi yang optimal dalam deteksi virus RSV-A dan RSV-B.*

Metode: *Primer dan probe untuk multiplex rRT-PCR RSV-A dan RSV-B dipilih dan dioptimasi menggunakan software PerlPrimer dan BLAST untuk menganalisis adanya struktur sekunder serta spesifisitas dari primer dan probe. Uji multiplex rRT-PCR dilanjutkan berdasarkan suhu annealing berdasarkan hasil analisis menggunakan PerlPrimer. Uji sensitifitas dilakukan dengan menggunakan DNA sintetis dari RSV-A dan RSV-B dan uji spesifisitas dilakukan dengan mengetes primer dan probe terhadap virus-virus lain yang umumnya ditemukan di saluran pernafasan.*

Hasil: *Primer dan probe yang dikembangkan pada penelitian ini tidak membentuk struktur sekunder dan spesifik mengamplifikasi hanya RSV-A dan RSV-B. Berdasarkan kriteria primer dan probe untuk digunakan dalam rRT-PCR yaitu suhu annealing, tidak adanya pembentukan struktur sekunder, % GC content serta detection limit, uji multiplex rRT-PCR yang dikembangkan pada penelitian ini mampu mendeteksi DNA sintetis RSV-A dan RSV-B.*

Kesimpulan: *Multiplex rRT-PCR dengan menggunakan primer dan probe untuk RSV-A dan RSV-B dapat mendeteksi RSV-A dan RSV-B dalam satu reaksi PCR.* ***(Health Science Journal of Indonesia 2021;12(2):66-73)***

Kata kunci: *multiplex, real-time RT-PCR, RSV-A, RSV-B*

The contributions of viruses as the etiology of pneumonia emphasize the importance of a routine virus detection. Polymerase chain reaction (PCR) has been used globally to detect viral pathogen since PCR is more sensitive compared to culture, direct immunofluorescence test, rapid antigen test or even serology test.[1,2] The fast development of molecular technology enables the utilization of multiplex real-time RT-PCR (rRT-PCR) to detect viral pathogens within few hours. Since then Multiplex rRT-PCR becomes a reliable routine test and its application has significantly increased within this decade.[2,3]

Acute lower respiratory infection (ALRI) or pneumonia remains one of major heath problem in low and middle-income countries that cause high mortality and morbidity in children.[4,5] The incidences of ALRI among children age less than five years old were approximated to be 120 million cases in 2010[6]. Bacteria and viruses are the common etiologies of pneumonia, however 40-60% cases were caused by viruses.[7,8] Respiratory syncytial virus (RSV) has been reported as the major viral etiology associated with severe lower respiratory tract infections in children, meanwhile, the predominant agent causing pneumonia in adult was influenza viruses.[3,9,10] RSV is classified in two main groups, RSV-A and RSV-B, with multiple genotypes within each group.[11,12]

Detection of viruses provides essential data of viral etiology that could beneficial for public health authorities such as disease investigation and epidemiology. Moreover, the detection of viruses might contribute to study the relationship between clinical manifestations of ALRI and its etiologies, in which could improving clinical management of the patients.[7,8] The identification of RSV-A and B as an etiology of ALRI could reduce the use of inappropriate antibiotics in patients and therefor preventing antimicrobial resistance.[13]

This study aimed to optimize the primer and probe set for RSV-A and B that used previously as singleplex assay into multiplex assay to detect both RSV-A and RSV-B in one assay. This method will provide efficient and fast detection method since only single reaction is needed to detect two viruses. Further, the primer and probe sets for multiplex rRT-PCR assay presented in this study could be used as reliable and fast detection method to detect RSV-A and B that commonly reported as the viral etiology of ALRI in children.

METHODS

Primer and probe sequence of RSV-A and RSV-B

This study used nucleoprotein gene of RSV-A and RSV-B as the target of primers and probe for multiplex rRT-PCR. Nucleoprotein gene was selected since this gene was conserved among the other genes in RSV-A and RSV-B genome. Sequences of nucleoprotein gene of RSV-A and RSV obtained from Gene Bank (Accession number KJ817800, U39662, AY911262, U39661, DQ780565, DQ780568, NC001781, D00736, DQ780567, AF013254) were aligned together with primer and probe sequence to detect RSV-A and RSV-B in singleplex assay based on previous study [14] using BioEdit sequence alignment software .[15]

PerlPrimer v1.1.18 [16,17] was employed to calculate the melting temperature (Tm) and GC content of each primer and probe, which then was used to define the initial annealing temperature for optimizing the rRT-PCR condition. The optimum annealing temperature of the primers sets for rRT-PCR is approximately 5°C higher than the Tm given by PerlPrimer v1.1.18 calculation. The annealing temperatures with 5°C differences were tested to obtain optimum rRT-PCR reaction using synthesized DNA that covered nucleoprotein gene fragments of RSV-A and RSV-B as positive template. PerlPrimer v1.1.18 was also used to analyze secondary structures that could be generated between primers and probes. Basic Local Alignment Search Tool (BLAST) was used to analyze the primer and probe specificity.

Singleplex rRT-PCR optimization

The optimization of the primer and probe sets were performed by running the synthesized DNA that covered nucleoprotein gene fragments of RSV-A and RSV-B. Singleplex rRT-PCR assays of RSV-A and RSV-B were performed using primers and probes shown in Table 1. Primers were used at a final concentration of 40 µM while the probe at a concentration of 10 µM. Each reaction for RSV-A and RSV-B detection was performed using SuperScript ™ III One-Step qRT-PCR System with Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA) that consisted of 12.5 µl 2x reaction mix, 5.5 µl nuclease-free water, 0.5 µl Forward primer, 0.5 µl Reverse primer, 0.5µl probe and 0.5µl SuperScript ™ III RT and Platinum ®Taq enzyme mix. A total of 5 µL of synthesized DNA of nucleoprotein gene fragments

RSV-A and RSV-B were mixed into master mix of RSV-A and RSV-B, respectively. Synthesized DNA with concentration of 1 to 10^{-3} ng/µl was used for initial singleplex optimization, followed by 10^{-3} to 10^{-8} ng/µl in second optimization. Meanwhile, we used nuclease-free water for negative template control in all assay.

Singleplex rRT-PCR was performed on CFX96 Touch System Real-time PCR Detection System (Bio-Rad, Hercules, CA) in a total reaction volume of 25 µL. Singleplex rRT-PCR of RSV-A and singleplex for RSV-B were performed under the following conditions: reverse transcription at 55 °C for 30 min, hot start at 95 °C for 2 min and 45 cycles of PCR consisting of denaturation at 95 °C for 15 sec, primer annealing and data collection at 60 °C for 1 min. Singleplex rRT-PCR reaction was confirmed as positive if exponential amplification curve with Cycle Threshold (CT) value between 18-38 was observed.

Multiplex rRT-PCR optimization

Multiplex rRT-PCR assay was performed using primers and probes for RSV-A and RSV-B shown in Table 1 in one reaction assay. Primers were used at a final concentration of 40 µM while the probe at a concentration of 10 µM. Master-mix for multiplex rRT-PCR utilizing SuperScript ™ III One-Step qRT-PCR System with Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA) that consisted of 12.5 µl 2x reaction mix, 0.5 µl RSV-A Forward primer, 0.5 µl RSV-A Reverse primer, 0.5µl RSV-A probe, 0.5 µl RSV-B Forward primer, 0.5 µl RSV-B Reverse primer, 0.5µl RSV-B probe, 0.5µl SuperScript ™ III RT and Platinum ®Taq enzyme mix, and 4.0 µl nuclease-free water. A total of 5 µL of mixed synthesized DNA of nucleoprotein gene fragments RSV-A and RSV-B were added into master mix of multiplex RSV-A and RSV-B. Synthesized DNA with concentration of 10^{-5} ng/µl to 10^{-8} ng/µl were used in this assay and each reaction was performed in duplicates. Multiplex rRT-PCR was performed on CFX96 Touch System Real-time PCR Detection System (Bio-Rad, Hercules, CA) in a total reaction volume of 25 µL using the same rRT-PCR protocol for singleplex mentioned above. Reaction was confirmed as positive if exponential amplification curve with Cycle Threshold (CT) value between18-38 were observed. Nuclease-free water was used for negative template control in all assay.

Sensitivity and specificity assay

Different concentration of synthesized DNA fragment were used to optimize the sensitivity of Multiplex rRT-PCR assay. In this study, concentration of 10^{-5} ng/µl to 10^{-10} ng/µl concentration was employed and each reaction was performed in duplicates. In addition, specificity assay was performed using various known viruses that could be found in respiratory tract such as Influenza A/H1pdm, Influenza A/H3, Influenza A/H5, Influenza B (Victoria), Influenza B (Yamagata), MERS-CoV, Human Parainfluenza Virus 1-4 (HPIV 1-4), Human Coronaviruses (HCOV NL63, HCOV OC43, HCOV 229E, HCOV HKU1A) and SARS.

RESULTS

Primer and probe sequence of RSV-A and RSV-B

Figure 1 and 2 shows the alignment of N gene of RSV-A and RSV-B with the primer sets and probes. The target site of the primer sets and probes were located within conserved region of N gene both in RSV-A and RSV-B. BLAST analysis shows that primer sets and probes had 100% identity with RSV-A and RSV-B (data not shown). We found no other pathogen sequences that had similarity with the primers and probes sequence, indicating the specificity of the primers and probes. The secondary structure, the Tm and GC content calculation of primer sets and probes using PerlPrimer are shown in Table 1. The primer sets and probes for RSV-A and RSV-B had GC content within the range of 30 to 56% and the Tm were between 61°C to 70°C.

Singleplex rRT-PCR optimization

Positive reaction in singleplex real-time RT-PCR was presented as sigmoid amplification curve with CT value below 38 as shown in Figure 3 and Table 2. These results confirmed that annealing temperature in 60°C for both RSV-A and RSV-B primers and probe were able to amplify RSV-A and RSV-B in singleplex set up as expected. The decreasing of synthesized DNA concentration (10^{-4} to 10^{-8}ng/µl) was coherent with the increasing of CT values. As the CT values of synthesized DNA at concentration 1 to 10^{-3} ng/µl were below 20, we did not used these concentration for further testing in multiplex assay. No amplification or CT value was observed in negative template control.

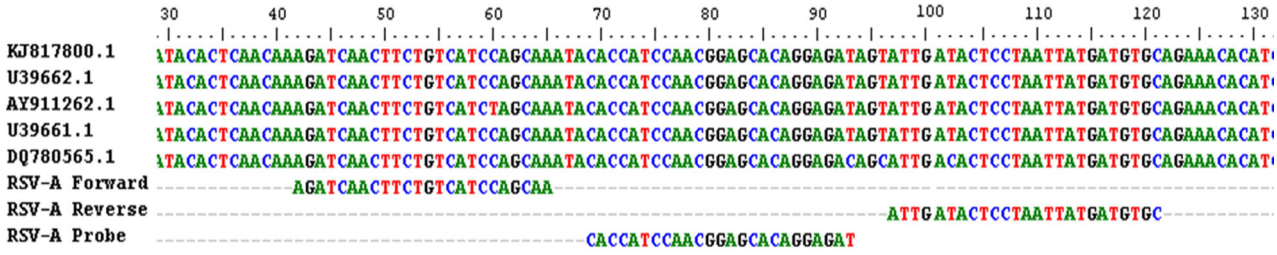

Figure 1. Alignment of N gene of RSV-A together with the primer sets and probe for singleplex rRT-PCR

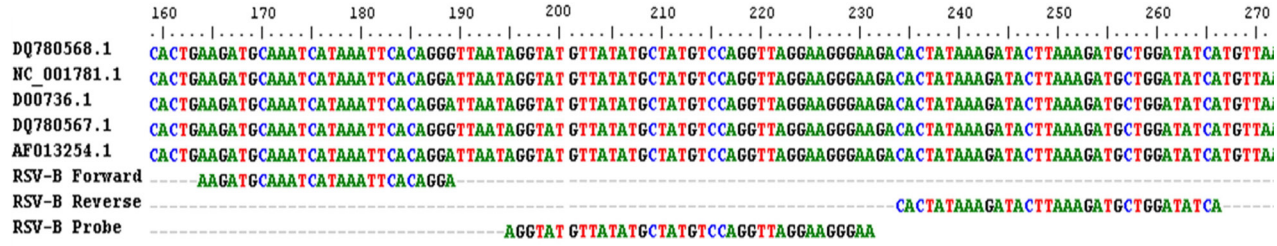

Figure 2. Alignment of N gene of RSV-B together with the primer sets and probe for singleplex rRT-PCR

Table 1. Primer and probe sequences for RSV-A and RSV-B

No	Primer/Probe	Sequence (5' to 3')	Tm (°C)	% GC Content	Secondary Structure (dimer, hairpin structure)
1	RSV-A Forward	AGATCAACTTCTGTCATCCAGCAA	64,34	41	None
2	RSV-A Reverse	GCACATCATAATTAGGAGTATCAAT	59, 96	32	None
3	RSV-A Probe	Cy5- CACCATCCAACGGAGCACAGGAGAT -Iowa Black RQ	70,54	56	None
4	RSV-B Forward	AAGATGCAAATCATAAATTCACAGGA	61,73	30	None
5	RSV-B Reverse	TGATATCCAGCATCTTTAAGTATCTTTATAGTG	62,97	30	None
6	RSV-B Probe	HEX-AGGTATGTTATATGCTATGTCCAGGTTAG GAAGGGAA -BHQ1	70,39	40	None

Multiplex rRT-PCR optimization

We performed multiplex rRT-PCR using synthesized DNA with concentration range of 10^{-4} to10^{-8} ng/µl. As shown in Table 2, the rising CT values were observed and consistent with the decreasing concentration of synthesized DNA. As expected in real-time PCR assay, the CT values had increased 3 points in each 10 fold diluted synthesized DNA. Interestingly, the same concentration of synthesized DNA in multiplex rRT-PCR gave higher CT values compared to singleplex. The negative template control of multiplex rRT-PCR, both of RSV-A and RSV-B, showed no amplification or CT values. The amplification curve of multiplex rRT-PCR was illustrated in Figure 4.

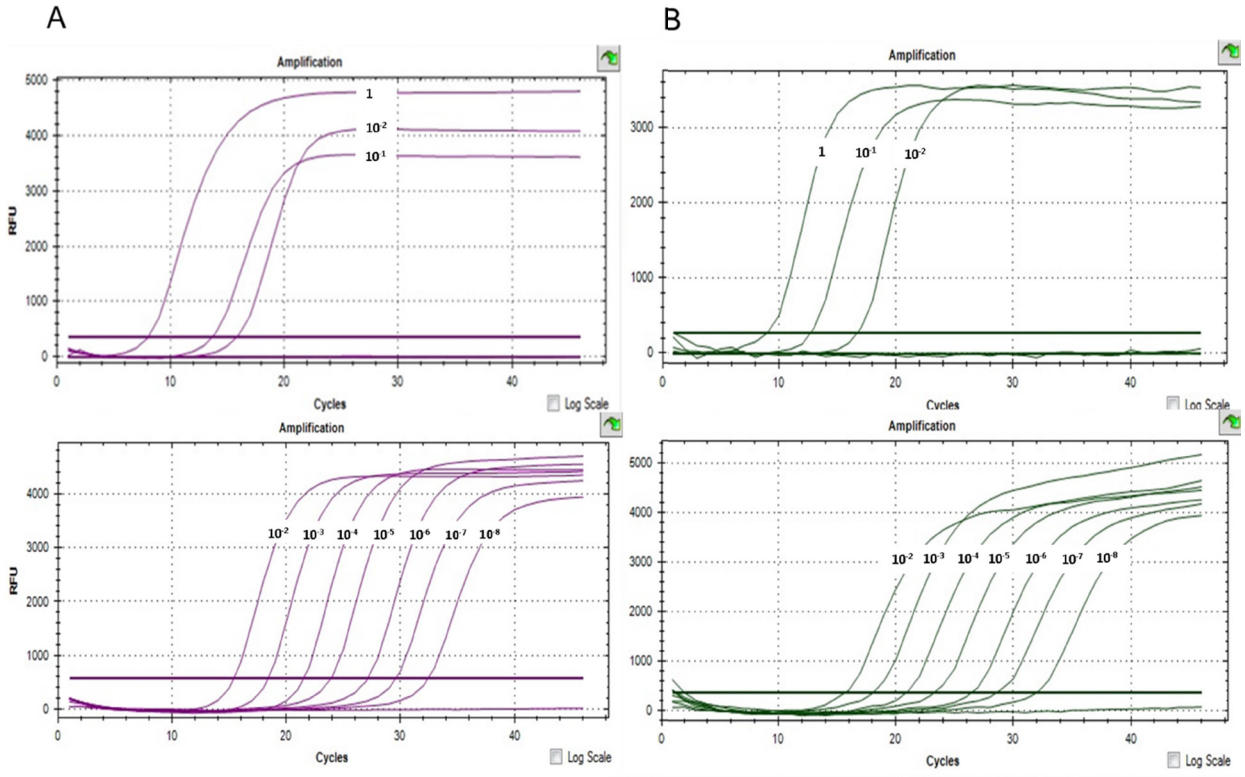

Figure 3. Amplification curve of singleplex rRT-PCR of RSV-A (Figure 3A) and RSV-B (Figure 3B) using concentration of synthesized DNA 1 to 10^{-8} ng/µl, respectively. The x axis illustrates the cycles of rRT-PCR while y axis shows the fluorescence intensity, displayed as Relative Fluorescence Unit (RFU).

Table 2. Average CT value in singleplex and multiplex rRT-PCR

Template concentration (ng/µl)	Singleplex		Multiplex	
	RSV-A	**RSV-B**	**RSV-A**	**RSV-B**
1	9,02	9,88	ND	ND
10^{-1}	13,73	13,36	ND	ND
10^{-2}	16,04	15,98	ND	ND
10^{-3}	18,37	17,86	ND	ND
10^{-4}	21,49	20,69	25, 76	27,87
10^{-5}	23,9	23,07	28,05	29,97
10^{-6}	27,11	26,24	31,34	33,97
10^{-7}	29,56	28,32	33,38	36,59
10^{-8}	32,39	31,92	38,7	39,5

ND: not done

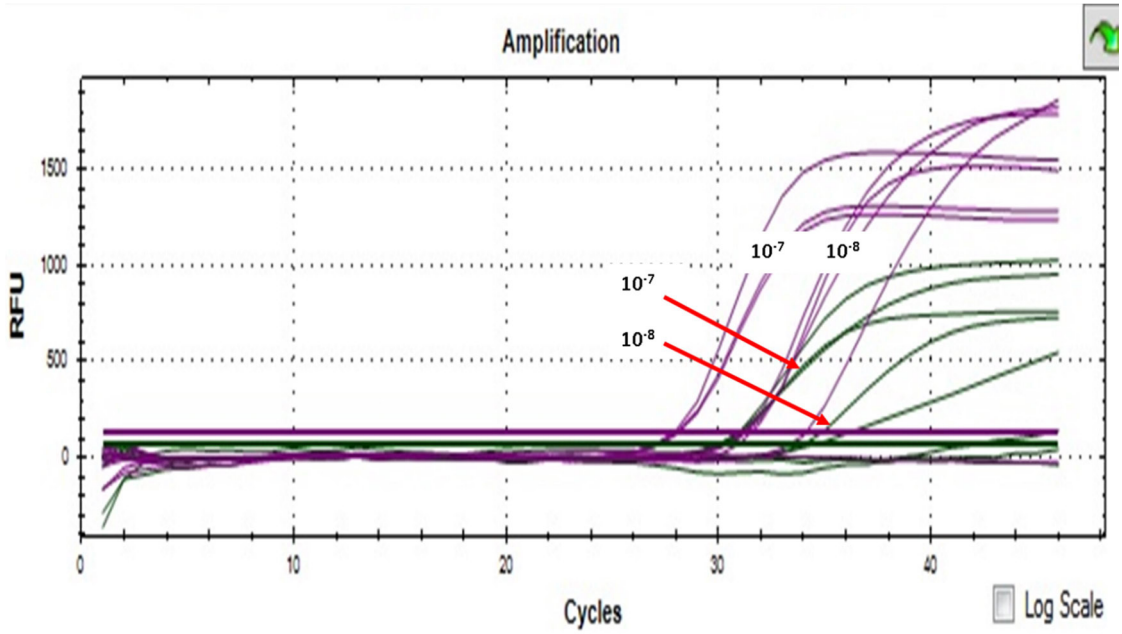

Figure 4. Amplification curve of multiplex rRT-PCR of RSV-A dan RSV-B .using synthesized DNA of 10^{-7} to 10^{-8} ng/µl. Amplification curve of RSV-A was in purple while RSV-B was in green. The x axis illustrates the cycles of rRT-PCR while y axis shows the fluorescence intensity, displayed as Relative Fluorescence Unit (RFU). Multiplex RT-PCR was done in triplicates

Sensitivity and specificity assay

Sensitivity test was carried out to understand the lowest concentration of template that could be detected using multiplex assay. In addition, the specificity testing was performed to examine other viruses in respiratory tract that could be detected using the primer set and probe for RSV-A and RSV-B. As seen in Table 3, concentration below 10^{-7} ng/µl was not able to detect both RSV-A and RSV-B. Meanwhile Table 4 shows that the multiplex assay for RSV-A and RSV-B detected no other viruses that could be found in respiratory tract such as Influenza A/H1pdm, Influenza A/H3, Influenza A/H5, Influenza B (Victoria), Influenza B (Yamagata), MERS-CoV, Human Parainfluenza Virus 1-4 (HPIV 1-4), Human Coronaviruses (HCOV NL63, HCOV OC43, HCOV 229E, HCOV HKU1A) and SARS.

Table 3. CT Value of Multiplex Sensitivity Test

Synthesized DNA concentration (ng/µl)	Average CT value	
	RSV-A	**RSV-B**
10^{-5}	29.13	29.03
10^{-6}	31.24	32.01
10^{-7}	34.13	34.63
10^{-8}	38, 08	37.97
10^{-9}	NA	41.07
10^{-10}	NA	NA

NA: Not available

Table 4. Specificity assay of Multiplex rRT-PCR for RSV-A and RSV-B

Virus	Type of template	Result
Influenza A/H1pdm	virus isolate	negative
Influenza A/H3	virus isolate	negative
Influenza A/H5	virus isolate	negative
Influenza B (Victoria)	clinical specimen	negative
Influenza B (Yamagata)	clinical specimen	negative
MERS-CoV	gene fragment	negative
HPIV 1	gene fragment	negative
HPIV 2	gene fragment	negative
HPIV 3	gene fragment	negative
HPIV 4	gene fragment	negative
HCOV NL63	gene fragment	negative
HCOV OC43	gene fragment	negative
HCOV 229E	gene fragment	negative
HCOV HKU1A	gene fragment	negative
SARS	gene fragment	negative

DISCUSSIONS

RSV groups are known to be the common cause of ALRI cases in children.[11] Conventional testing for respiratory viruses consist of various methods, including virus isolation in cell lines and serology tests. For diagnostic purposes, virus isolation requires days to perform whereas serology tests are not specific and sensitive enough.[18] The utilization of rapid detection is essential to enable implementation

of specific control measures and the limitation of virus spread.[18] The development of technique in advance molecular biology technologies have revolutionized the procedures for detection and characterization of pathogenic viruses.[19] One of the techniques that provide high throughput result and one of reliable technologies are multiplex real-time RT-PCR. In this study, we developed multiplex rRT-PCR for RSV-A and RSV-B detection in single reaction.

The initial setting for multiplex rRT-PCR is primer sets and probe. The primer and probe developed for multiplex rRT-PCR was based on basic rules of efficient primers that are 18-25 nucleotides in length, with 50-60% having G and C composition. In addition, primer set essentially are also designed to have no complementary sequence at the 3' ends between primer pairs that could form secondary structure such as primer dimer artifacts and hairpin loop.[20,21] The sequence of primer and probe in this study had been chosen to meet those criteria. Our analysis on primer probe sequences shows that no secondary structure was generated when combining two sets of primer and probes for RSV-A and RSV-B.

Detection of two or more gene target of viruses could be done with high sensitivity and specificity using multiplex rRT-PCR as this technology combines the polymerase chain reaction chemistry with the use of fluorescent reporter dye in order to observe the amplification during each PCR cycles. This study utilized the chemistry of TaqMan probe within the PCR reaction. The TaqMan probe is labeled with dual fluorescent dyes, the quencher and reporter dyes, which emit at different wavelengths.[22] As describes in Table 1, detection of two different target of viruses could be performed using this method as probes for RSV- A and RSV-B in this study were labeled with different reporter dyes, Cy5 and Hex, respectively.

The TaqMan probe sequence was specifically hybridized in the DNA target region of interest between the two PCR primers as seen in Figure 1 and 2. The probe was also designed to have higher annealing temperature compared to the PCR primers (Table 1), therefore the probes hybridized when extension (polymerization) of the primers begins. The Taq DNA polymerase with the 5'-exonuclease activity hydrolyzed the probe that have annealed previously in the target sequence. The hydrolysis of TaqMan probe caused reporter dyes no longer had close proximity to the quencher dye and consequently emitted fluorescent signal which captured by the computer system incorporated with thermal cycler.[18,22] These processes were repeated in each cycle and in the end of PCR reaction, the sigmoid amplification plot both of RSV-A and RSV-B were generated and could be analyzed.

We performed sensitivity testing in order to analyze the minimum number of copies in the sample that can be detected accurately with the optimized assay. The multiplex rRT-PCR was still able to detect synthesized DNA with concentration 10^{-7} ng/µl, suggesting that the multiplex rRT-PCR were sensitive to detect template with low concentration. The specificity testing both using in silico assay (BLAST) and rRT-PCR with real viruses or gene fragments of common viruses in respiratory tract suggested that the multiplex rRT-PCR was specific only to detect RSV-A and RSV-B. The specificity test was essential to employ in this study as this test refers to the assay that detects the appropriate target sequence instead of other or non-specific targets that present within the samples.[23]

This study did not perform the sensitivity and specificity examinations using real viruses of RSV-A and RSV-B or other respiratory viruses thus become the limitation of this study. This study utilized synthesized DNA of gene fragment that mimic the template for RSV-A and RSV B for sensitivity test or MERS-CoV, Human Parainfluenza Virus 1-4 (HPIV 1-4), Human Coronaviruses (HCOV NL63, HCOV OC43, HCOV 229E, HCOV HKU1A) and SARS for specificity test. Therefore the actual testing using clinical specimens are needed for ideal examination for further diagnostic purposes.

In conclusion, in this study we developed multiplex rRT-PCR for RSV-A and RSV-B since those viruses are the most common pathogen found in respiratory tract. Multiplex rRT-PCR is a fast, sensitive and specific test to detect more than one target in single PCR reaction. The optimization of primers and probes specific for multiplex rRT-PCR for RSV-A and RSV-B are essential therefore the optimal condition for PCR reaction to detect two targets in single PCR reaction is crucial to be accomplished. Based on in silico utilizing bioinformatics software and web analyses, the primer set and probes selected for RSV-A and RSV-B detection were specific and showed no secondary structure. Further, the optimization of the primer sets and probes was accomplished using gene fragments in a form

of synthesized DNA. Multiplex rRT-PCR that employing primer sets and probes targeted N gene of RSV-A and RSV-B in this study were able to be detect RSV-A and RSV-B in single reaction.

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