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Development of multiplex real-time PCR for the rapid detection of five bacterial causes of community acquired pneumonia

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Abstract. Establishing a microbial diagnosis for patients with community-acquired pneumonia (CAP) is still challenging and is often achieved in only 30-50% of cases. Polymerase chain reaction (PCR) has been shown to be more sensitive than conventional microbiological methods and it could help to increase the microbial yield for CAP patients. This study was designed to develop, optimize and evaluate multiplex real-time PCR as a method for rapid differential detection of five bacterial causes of CAP namely *Streptococcus pneumoniae*, *Burkholderia pseudomallei* and atypical bacterial pathogens, *Mycoplasma pneumoniae*, *Chlamydophila pneumoniae* and *Legionella pneumophila*. Duplex and triplex real-time PCR assays were developed using five sets of primers and probes that were designed based on an appropriate specific gene for each of the above CAP pathogens. The performance of primers for each organism was tested using SYBR Green melt curve analysis following monoplex real-time PCR amplification. Monoplex real-time PCR assays were also used to optimize each primers-probe set before combining them in multiplex assays. Two multiplex real-time PCR assays were then optimized; duplex assay for the differential detection of *S. pneumoniae* and *B. pseudomallei*, and triplex assay for the atypical bacterial pathogens. Both duplex and triplex real-time PCR assays were tested for specificity by using DNA extracted from 26 related microorganisms and sensitivity by running serial dilutions of positive control DNAs. The developed multiplex real-time PCR assays shall be used later for directly identifying CAP causative agents in clinical samples.

INTRODUCTION

The etiologic diagnosis of CAP remains an uneasy task, with the causative organisms often identified in only up to 50% of cases. This is mainly due to difficulties in culturing and to the delayed results associated with conventional methods (serology and culture), which often allow a retrospective diagnosis only (Chan *et al.*, 2007; Nolte, 2008).

Molecular methods such as PCR offer a better approach for the rapid diagnosis of CAP (Benson *et al.*, 2008). Several conventional PCR assays have been developed for each individual respiratory pathogen and have demonstrated sensitivity and specificity equal to or even better than those of culture and/or serology (Boman *et al.*, 1999; Dorigo-Zetsma *et al.*, 1999). In addition, a number of investigators have successfully developed multiplex assays using conventional PCR (Miyashita *et al.*, 2004; McDonough *et al.*, 2005; Stralin *et al.*, 2005; Benson *et al.*, 2008).

With the added sophistication and modernization of amplification processes like real-time PCR, technology has enabled testing to be more rapid, efficient and precise. Real-time PCR allows us to monitor *in vitro* DNA amplification successively, eliminating

nonspecific amplification and the need for gel electrophoresis (Mackay, 2004). These advantages make real-time PCR the front-runner as a diagnostic test and superior to the conventional PCR (Espy *et al.*, 2006).

Individual monoplex real-time PCR assays have been established for *Mycoplasma pneumoniae* and *Legionella pneumophilia* (Wilson *et al.*, 2003; Daxboeck *et al.*, 2005). However, to be clinically useful, it would necessitate the running of multiple monoplex PCR tests for each patient. This would be both costly and inefficient. Therefore, there is a need for the development of a single diagnostic test capable of precisely detecting multiple pathogens simultaneously with exceptional sensitivity and specificity.

Multiplex real-time PCR methods could be useful for the rapid and simultaneous detection of multiple respiratory pathogens in a clinical specimen, offering an attractive alternative to conventional PCR in the clinical diagnostic laboratory (Benson *et al.*, 2008). Few investigators have developed multiplex real-time PCR assays targeting atypical bacteria and viruses (Welti *et al.*, 2003; Templeton *et al.*, 2005; Lassauniere *et al.*, 2010). These developed multiplex assays differ in the number and type of organisms targeted in each assay, the targeted genes for each organism, and also in the fluorescent chemistries used to monitor the amplification of the target sequence. To our knowledge, *Burkholderia pseudomallei* was not included in any multiplex assay with *Streptococcus pneumoniae* despite the fact that both of them are important causes of septicaemia and pneumonia in endemic areas (Cheng *et al.*, 2005; Raja, 2008).

Here, we describe the development, optimization and evaluation of two multiplex real-time PCR assays using the TaqMan^{R} methodology; a duplex assay for rapid differential detection of *Streptococcus pneumoniae* and *Burkholderia pseudomallei*, and a triplex assay for the atypical bacterial pathogens namely *M. pneumoniae*, *Chlamydophila pneumoniae* and *L. pneumophila*.

MATERIALS AND METHODS

This study consisted of three main phases: development, optimization and evaluation of the multiplex real-time PCR assays (duplex for the differential detection of *S. pneumoniae* and *B. pseudomallei*, and triplex for the atypical bacterial pathogens).

Development of the multiplex real-time PCR assays

Species-specific genes for each bacterium, reportedly used as PCR targets, were identified. For *S. pneumoniae*, the *lytA* gene sequence (GenBank accession no. M13812) was selected. The *lytA* gene encoding autolysin enzyme is specific and proven to be exclusively present in *S. pneumoniae* (Saukkoriipi *et al.*, 2002; Llull *et al.*, 2006; Morozumi *et al.*, 2006). For *Burkholderia pseudomallei*, the type III secretion system gene cluster (GenBank accession no. AF074878) was chosen as a PCR target based on its theoretical specificity and its apparent ubiquitous population distribution (Tomaso *et al.*, 2005; Meumann *et al.*, 2006; Supaprom *et al.*, 2007). A region of 548-bp of the *B. pseudomallei* type III secretion system gene cluster encompassing part of open reading frame 2 (*orf2*) was found to be present in *B. pseudomallei* and not in the related *Burkholderia mallei* or *Burkholderia thailandensis* (Winstanley *et al.*, 2000; Rainbow *et al.*, 2002). This open reading frame was later found to be present in all of a large number of Northern Australian *B. pseudomallei* strains (Smith-Vaughan *et al.*, 2003).

For *L. pneumophila*, the macrophage infectivity potentiator (*mip*) gene sequence (GenBank accession no. S72442) was chosen as a PCR target (Wellinghausen *et al.*, 2001; Wilson *et al.*, 2003; McDonough *et al.*, 2005). The *mip* gene is associated with intracellular invasion and survival and is identified in all *L. pneumophila* serogroups (serogroups 1–15) (Welti *et al.*, 2003; Benson *et al.*, 2008).

The 16S rRNA genes were chosen for *M. pneumoniae* (GenBank accession no. AY816340) and for *C. pneumoniae* (GenBank

accession no. Z49874), as those genes are conserved in a single gene copy in each of these two bacteria (Hardick *et al.*, 2004; Khanna *et al.*, 2005; Morozumi *et al.*, 2006; Otomo *et al.*, 2008).

Five pairs of primers and TaqMan^{R} probes were designed by using Beacon Designer software (Premier Biosoft, USA). The primers and probes were synthesized by Eurogentec AIT / Singapore. Primers and probes used in duplex and triplex real-time PCR are described in Table 1.

Optimization of the multiplex real-time PCR assays

Origin of the positive controls

Microbial DNA was obtained from *S. pneumoniae* (ATCC 49619), *L. pneumophila* (ATCC 33152) and *B. pseudomallei* (clinical isolate) after culturing them onto their suitable culture media with subsequent DNA extraction. DNA from *L. pneumophila* and *B. pseudomallei* was extracted using the QIAmpDNA mini kit (Qiagen, Germany), and DNA from *S. pneumoniae* was extracted using MasterPure™ Gram Positive DNA Purification Kit (Epicentre Biotechnologies, USA) according to the manufacturer's instructions. For *M. pneumoniae* and *C. pneumoniae*, two plasmids (pUC 57 vector with target genes incorporated inside) were synthesised (Eurogentec AIT / Singapore) and used as PCR positive control.

Testing primers performance using SYBR green melt curve analysis

The performance of the selected five sets of primers were tested by SYBR Green in five independent monoplex experiments with melt-curve analysis using CFX96 Real-time PCR detection system (Bio-Rad, USA) before combining them in multiplex assays.

Testing primers–probe Sets performance in monoplex real-time PCR

Primers-probe mix (0.5 µM forward and reverse primers and 0.2 µM probe) was prepared then 1.25 µL of this mix was used in a final PCR reaction volume of 25 µL. Reactions contained: 12.5 µL QuantiFast Multiplex PCR Master Mix (Qiagen, Germany), 1.25 µL primer–probe mix and 6.25 µL water. 5 µL of the targeted organism DNA (< 100 ng/reaction) was finally added. The performance of primers–probe sets was tested in five independent experiments using CFX96 Real-time PCR detection system (Bio-Rad, USA). The reaction included initial PCR activation step at 95°C for 5 min followed by 40 cycles of denaturation (at 95°C for 45

Table 1. Primers and probes used in duplex and triplex real-time PCR

Gene	GenBank accession no.	Sense primer (5'-3')	Antisense primer (5'-3')	Taqman Probe (5'-3')	Amplicon length
Duplex Assay					
S. pneumoniae lytA	M13812	CAACGAAGAAG GTGCCATGAAG	TGTTCCGTCTG GTTTGAGGTAG	FAM-AGCCTGTTCCGTCC GCTGACTGGA-BHQ1	148
B. pseudomallei type III secretion system	AF074878	TGGCCCTATGG ATTGCGAATC	TCGGTGTTCA GTGCGTTCTC	HEX-CGCCAATCCTCCGA CTCCGCCAC-BHQ1	117
Triplex Assay					
L. pneumophila MIP	S72442	TATAGCATTGG TGCCGATTTGG	GTCTTTCATTTG CTGTTCGGTTAG	FAM-AGCGCCACTCATAG CGTCTTGCAT-BHQ1	135
M. pneumoniae 16S rRNA	AY816340	CGTGGTGAAGT GAAACATCTCAG	AAGCCCTACAA CCCCTATCTAATG	HEX-CCTGTTCCGCTTTC GCTCGCCAC-BHQ1	119
C. pneumoniae 16S rRNA	Z49874	GCACTTTCGCC TGGGAATAAG	CCCTTTACGCCC AATAAATCCG	CY5-AGCACCCTCCGTAT TACCGCAGCT-BHQ2	145

sec) and combined annealing/extension (at 60°C for 45 sec). Subsequent data analysis was done using CFX manager software.

Testing primers–probe sets performance in multiplex real-time PCR

Duplex real-time PCR was used for the differential detection of *S. pneumoniae* and *B. pseudomallei*. The optimization process was done using positive control DNA. Primers-probe mixes (0.5 µM forward and reverse primers and 0.2 µM probe) were prepared for *S. pneumoniae* and *B. pseudomallei* then 1.25 µL of each mix was used in a final PCR reaction volume of 25 µL containing: 12.5 µL QuantiFast Multiplex PCR Master Mix (Qiagen, Germany), and 5 µL DNA/RNA free water. Finally, 5 µL of bacterial DNA was added (2.5 µL *S. pneumoniae* genomic DNA + 2.5 µL *B. pseudomallei* genomic DNA).

Triplex real-time PCR was used for the differential detection of *M. pneumoniae*, *C. pneumoniae* and *L. pneumophila*. The optimization process was done using positive control DNA. Primers-probe mixes (0.5 µM forward and reverse primers and 0.2 µM probe) were prepared for *M. pneumoniae*, *C. pneumoniae* and *L. pneumophila* then 1.25 µL of each mix was used in a final PCR reaction volume of 25 µL containing: 12.5 µL QuantiFast Multiplex PCR Master Mix (Qiagen, Germany), and 3.75 µL DNA/RNA free water. Template DNA mixture was prepared by the addition of 10 µL of *M. pneumoniae* plasmid DNA+10 µL of *C. pneumoniae* plasmid DNA+10 µl of *L. pneumophila* genomic DNA. Finally, 5 µL of this mixture was used in each reaction.

During the optimization process, different relative concentrations of each organisms' DNA were used (total DNA <100 ng/reaction) to ensure that the amplification of the less-efficient or less abundant target was not inhibited by the more-efficient or more-abundant target (Bio-Rad, 2006; Qiagen, 2009).

Recommended Qiagen protocol for duplex real-time PCR was followed, but with increasing the duration of both denaturation step and combined annealing/extension step from 30 seconds to 45 seconds as amplification was found to be better at the optimized increased duration. However, recommended Qiagen protocol for triplex real-time PCR was followed without any optimization.

Testing of primer–probe sets in duplex and triplex real-time PCR was done in duplicate using CFX96 Real-time PCR detection system (Bio-Rad, USA). The reaction included initial PCR activation step at 95°C for 5 min followed by 40 cycles of denaturation (at 95°C for 45 sec) and combined annealing/extension (at 60°C for 45 sec). Subsequent data analysis was done using CFX manager software.

Evaluation of the multiplex real-time PCR Assays

Multiplex real-time PCR specificity

Both duplex and triplex real-time PCR assays were tested for specificity by using DNA extracted from microorganisms listed in Table 2.

Multiplex real-time PCR sensitivity

The sensitivity of each multiplex real-time PCR assay was tested by running 10-fold serial dilutions of positive controls containing known amounts of microbial DNA. The resultant data were used to generate a standard curve by plotting Ct values against the log of the starting quantity of template for each dilution. Amplification efficiency and coefficient of determination (R^2) were automatically calculated by CFX manager software.

RESULTS

Real time PCR optimization

For each of the five tested sets of primers, a single peak was obtained in the SYBR green based melt curve analysis with the absence of any extra peak(s) as shown in Figure 1.

The first step in assembling each multiplex assay was to optimize the individual reactions. Thus, we tested each primers-probe set in monoplex then combined them in multiplex real-time PCR. Target amplification was successful in each of the monoplex assays.

Table 2. List of organisms used to determine the specificity of PCR

No.	Species	Source
1	*Streptococcus pypgenes*	ATCC 19615
2	*Enterococcus faecalis*	ATCC 29212
3	*Streptococcus viridians* (unidentified species)	*clinical isolate*
4	*Staphylococcus aureus*	ATCC 25923
5	*Staphylococcus epidermidis*	ATCC 12228
6	*Bacillus cereus**	ATCC 11778
7	*Bacillus subtilis**	ATCC6633
8	*Haemophilus influenzae*	ATCC 49247
9	*Haemophilus influenzae type b*	ATCC 10211
10	*Neisseria gonorrhoea*	ATCC 49226
11	*Proteus mirabilis*	ATCC 35659
12	*Salmonella typhimurium*	ATCC 14028
13	*Salmonella typhi*	*clinical isolate*
14	*Vibrio parahaemolyticus*	ATCC 17802
15	*Shigella sonnei*	ATCC 29930
16	*Klebsiella pneumoniae**	ATCC 700603
17	*Escherichia coli* *	ATCC 25922
18	*Psudomonas aerogenosa**	ATCC 27853
19	*Moraxella catarrhalis*	ATCC 25238
20	*Burkholderia cepacia*	*clinical isolate*
21	*Candida albicans*	ATCC 10321
22	*Burkholderia pseudomallei*	*clinical isolate*
23	*Streptococcus pneumoniae*	ATCC 49619
24	*Legionella pneumophila* *	ATCC 33152
25	*Chlamydia pneumoniae* **	synthesized gene segment
26	*Mycoplasma pneumoniae* **	synthesized gene segment

* Those bacteria were obtained from Microbiology Laboratory /Faculty of Medicine/International Islamic University Malaysia (IIUM) and all the others were obtained from Bacteriology Laboratory/ Hospital Tengku Ampuan Afzan (HTAA), Kuantan, Pahang, Malaysia.
** A gene containing PCR target of amplification was synthesized to be used as positive control.

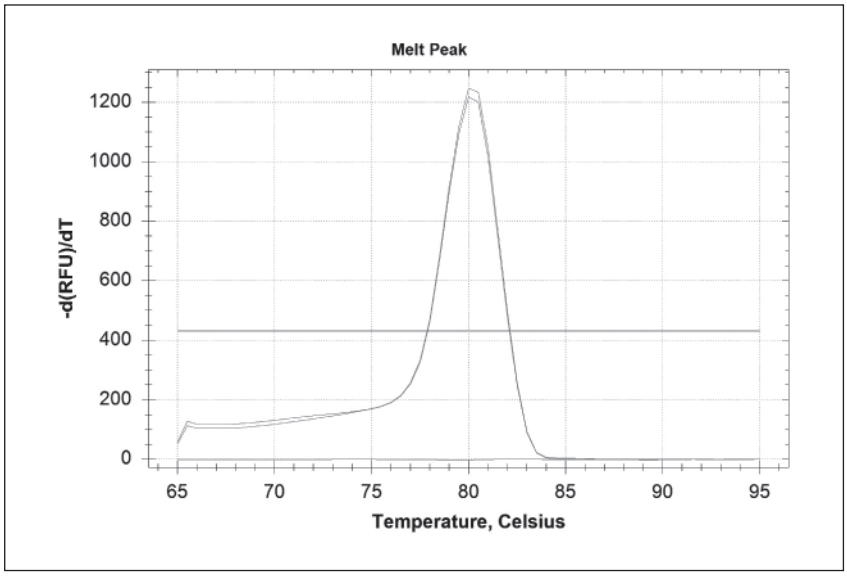

Figure 1. SYBR green based Melt-curve analysis. The negative first derivative of the change in fluorescence (-d(RFU)/dT) is plotted as a function of temperature. Similar curves were obtained from each of the five tested sets of primers specific for each targeted organism

The performance of primers–probe sets was tested in duplex real-time PCR (for *B. pseudomallei* and *S. pneumoniae*) and in triplex real-time PCR (for *L. pneumophila*, *M. pneumoniae* and *C. pneumoniae*). For duplex real-time PCR, amplification was optimized by increasing the duration of denaturation and annealing/extension steps from 30 (in manufacturer's recommended protocol) to 45 seconds. No optimization was required for triplex real time PCR manufacturer's recommended protocol. Amplification in both duplex and in triplex real-time PCR was successful.

Specificity of multiplex real-time PCR

For both duplex and triplex real-time PCR, no amplification was observed with the DNA extracted from any of the specificity panel tested organisms (Table 2). This indicates that the specificity of both duplex and triplex PCR is considered satisfactory.

Sensitivity of multiplex real-time PCR

To evaluate the sensitivity of the multiplex real-time PCR, 10-folds serial dilutions of the positive controls containing known amounts of microbial DNA were tested in duplicate by duplex and triplex real-time PCR. End point sensitivities of primers/probe sets in duplex and triplex real-time PCR are shown in Table 3 and 4 respectively.

According to the formula: 2^n = dilution factor, where n is the number of cycles between curves at the fluorescence threshold (the difference between the Ct values of the curves). Thus, with a 10-fold serial dilution of DNA, $2^n = 10$. Therefore, n = 3.32, and the Ct values should be separated by 3.32 cycles.

Average of spacing between mean Ct values was calculated and it was equal to 3.37 (±0.15) and 3.26 (±0.11) cycles for *S. pneumoniae* and *B. pseudomallei* respectively and equal to 3.38 (±0.19), 3.25 (±0.21) and 3.31 (±0.24) cycles for *L. pneumophila*, *M. pneumoniae* and *C. pneumoniae* respectively. All these values are not greatly different from the calculated standard value (3.32). This indicates the regular spacing between amplification curves of the dilution series and the exponential nature of the amplification.

Table 3. End point sensitivities of primers/probe sets in duplex real-time PCR

Target	Mean Ct value at the following dilutions of DNA :					
	10^{-1}*	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}#
Streptococcus pneumoniae	21.26	24.51	28.29	31.71	34.80	38.11
Burkholderia pseudomallei	21.87	25.07	28.44	31.73	35.07	38.18

* The starting concentration of *S. pneumoniae* was 10 ng and of *B. pseudomallei* was 14 ng of total genomic DNA.
# At the highest dilution, amplification was observed in only one of the duplicates tested.

Table 4. End point sensitivities of primers/probe sets in triplex real-time PCR

Target	Mean Ct value at the following dilutions of DNA :					
	10^{-1}*	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}#
Legionella pneumophila	21.35	24.62	28.18	31.70	35.19	38.28
Mycoplasma pneumonia	21.86	25.12	28.80	32.06	35.05	38.13
Chlamydophila pneumonia	22.34	25.61	29.32	32.94	35.76	38.88

* The starting concentration for *L. pneumophila* was 16 ng of total genomic DNA and was 3 ng of plasmid DNA for each of *M. pneumoniae* and *C. pneumonia*e.
# At the highest dilution, amplification was observed in only one of the duplicates tested.

Standard curves were constructed and amplification efficiencies (E), coefficients of determination (R^2) and curve slopes were autocalculated by the CFX manager software as shown in figures 2 and 3. These values were compared with standard values (amplification efficiency (E) (90-105%), optimal slope of the standard curve (-3.32), Coefficients of determination (R^2) $\geq$0.980). Amplification efficiencies (E) were equal to 95.7% and 100.7% for *S. pneumoniae* and *B. pseudomallei* respectively and were equal to 93.9%, 99.6% and 96.1% for *L. pneumophila*, *M. pneumoniae* and *C. pneumonia*e respectively. All these values are within the normal range of high amplification efficiency (90-105%).

Coefficients of determination (R2) values were equal to 0.998 and 1.000 for *S. pneumoniae* and *B. pseudomallei* respectively and were equal to 0.997, 0.996 and 0.991 for *L. pneumophila*, *M. pneumoniae* and *C. pneumonia*e respectively. All these values are greater than 0.980 which proves the linearity of the standard curves.

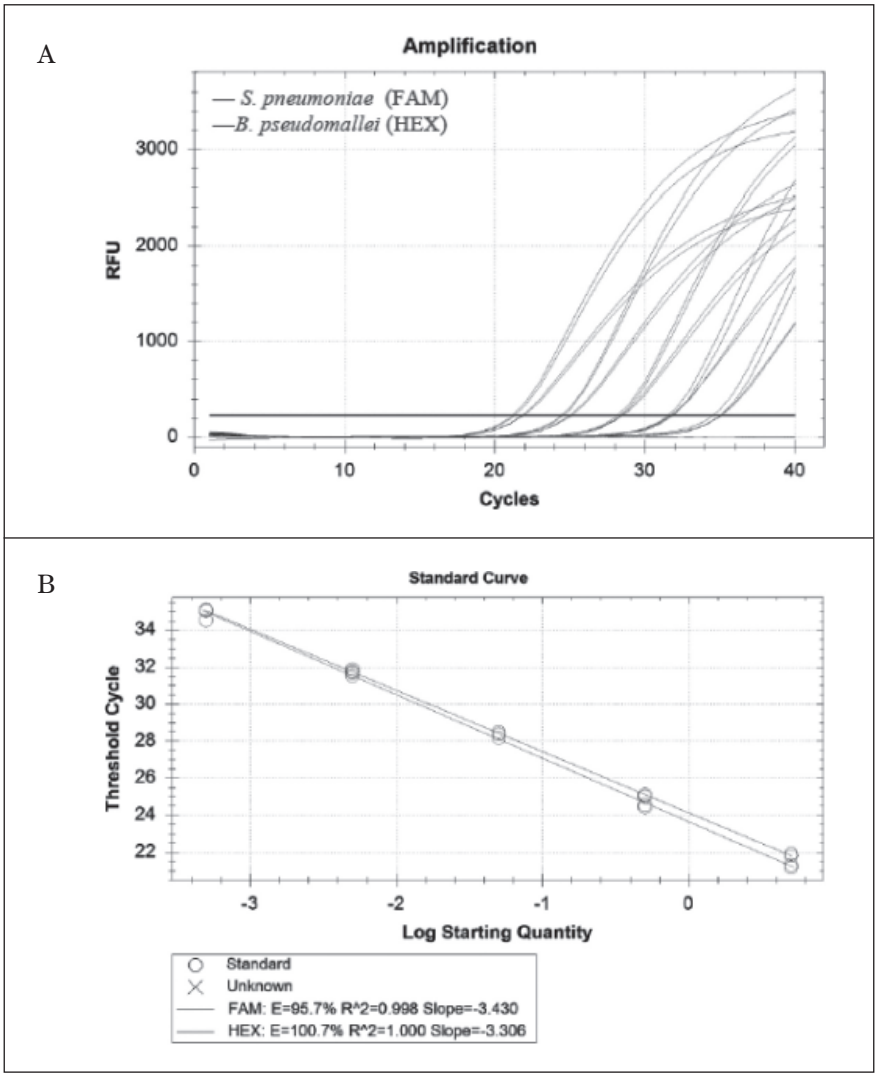

Figure 2. Duplex real-time PCR 10-fold dilution series. (A) Amplification curves of the dilution series. (B) Standard curves (Ct plotted against the log of the starting quantity of template for each dilution). Efficiency (E), coefficient of determination (R^2) and curve slope are shown in (B)

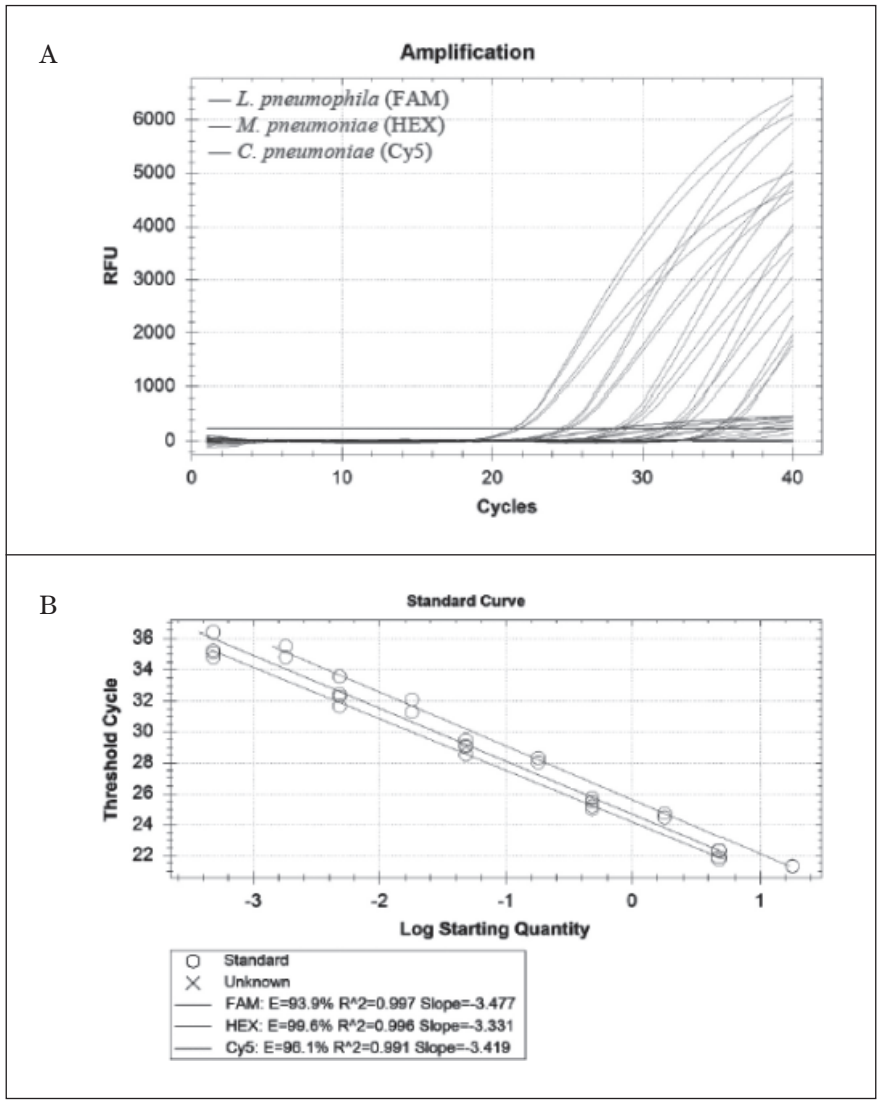

Figure 3. Triplex real-time 10-fold dilution series. (A) Amplification curves of the dilution series. (B) Standard curves (Ct plotted against the log of the starting quantity of template for each dilution). Efficiency (E), coefficient of determination (R^2) and curve slope are shown in (B)

Curve slopes of the duplex real-time PCR were equal to -3.430 and -3.306 for *S. pneumoniae* and *B. pseudomallei* respectively. For the triplex real-time PCR, they were equal to -3.477, -3.331 and -3.419 for *L. pneumophila*, *M. pneumoniae* and *C. pneumonia*e respectively. All these values are not greatly different from optimal slope of the standard curve (-3.32).

DISCUSSION

Despite the development of improved microbiological methods during the past few years, the etiology of CAP has still not been well characterized. Polymerase chain reaction (PCR) based methods have been developed for many pathogens causing respiratory tract infections. These methods

have been shown to be more sensitive than conventional microbiological methods and it could help to increase the microbial yield for patients with CAP (Jokinen *et al.*, 2001; Johansson *et al.*, 2010).

By multiplex real time PCR assay, it is possible to simultaneously detect and differentiate numerous causative agents of CAP rapidly, with high sensitivity and specificity and without being affected by antecedent antibiotics administration. Whereas traditional laboratory method may not always identify multiple pathogens in a single clinical sample, as identification from culture is based on the predominant organism and influenced by the use of selective culture media, the multiplex real-time PCR assay can simultaneously amplify multiple gene targets in the same sample saving both time and cost (Miyashita *et al.*, 2004; Khanna *et al.*, 2005; Brittain-Long *et al.*, 2008; Westh *et al.*, 2009).

In this study, we have successfully developed two multiplex real-time PCR assays. Duplex real-time PCR was developed for the simultaneous detection and differentiation of *S. pneumoniae* and *B. pseudomallei*, and triplex real-time PCR for the simultaneous detection and differentiation of *L. pneumophila*, *M. pneumoniae* and *C. pneumoniae*.

When the performances of primers' sets were tested, single peaks were obtained by SYBR green based melt curve anlaysis with absence of any extra peak (s). This indicates the absence of non-specific product(s) which proves that the five sets of primers are well designed and with good degree of specificity towards their targets (Robert *et al.*, 2006).

TaqMan assays require careful attention to temperature conditions. Instead of using the traditional three-step PCR cycle of denaturation, annealing, and extension, two-step PCR protocol was used. The protocol consists of a denaturation step followed by a combined annealing and extension step at 55–60ºC to ensure that each probe remains bound to its target during primer extension (Bio-Rad, 2006).

The first step in assembling each multiplex assay was to optimize the individual reactions. Thus, we tested each primers-probe set in monoplex then combined them in multiplex real-time PCR. Minimal optimization of QuantiFast Multiplex PCR Kit's recommended cycling conditions were required to achieve the best amplification.

On performing specificity testing, no amplification was observed with DNA extracted from any of our specificity test panel of 26 organisms. Thus, the specificity of both duplex and triplex PCR is considered satisfactory (Welti *et al.*, 2003).

In sensitivity testing of the two multiplex real-time assays, total genomic DNA extracted from fresh colonies of *B. pseudomallei*, *S. pneumoniae* and *L. pneumophila*, was used. However, for *M. pneumoniae* and *C. pneumoniae*, DNA from cloned plasmids harbouring synthesized gene segments targeted by the real-time PCR, was used. The suitability of plasmids for sensitivity testing and their use as PCR positive control was confirmed by Welti *et al.* in 2003 where they used plasmids for the first time for this purpose.

Both assays showed high amplification efficiency of (90-105%) with good linearity of the standard curves and regular spacing of the dilution series amplification curves. Thus, the hallmark of an optimized real-time PCR assay is applicable for both the duplex and triplex real time PCR assays (Bio-Rad, 2006).

As yet, no PCR assay has been approved by the US Food and Drug Administration for diagnosis of pneumonia, although commercial assays are becoming available. For this to happen, optimal standardized protocols need to be established and validated before PCR and other nucleic acid amplification methods become routine diagnostic tools. Several key parameters, notably sensitivity and specificity, reproducibility, and optimal sample types, need to be determined before PCR becomes part of the routine diagnostic workup for a particular pneumonia pathogen (Murdoch, 2003).

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