DEVELOPMENT OF MULTIPLEX REAL-TIME PCR ASSAY FOR SIX PATHOGENS: RAPID TOOL FOR DIAGNOSIS OF BACTERIAL MENINGITIS

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Abstract

Introduction: Bacterial meningitis is an acute central nervous infection with high mortality or permanent neurological sequelae if remained undiagnosed. However, traditional diagnostic methods for bacterial meningitis pose challenge in prompt and precise identification of causative agents. **Aims:** The present study will therefore aim to set up in-house PCR assays for diagnosis of six pathogens causing the disease including *H. influenzae type b, S. pneumoniae, N. meningitidis, S. suis* serotype 2*, E. coli* and *S. aureus.* **Methods:** in-house PCR assays for detecting six above-mentioned bacteria were optimized after specific pairs of primers and probes collected from the reliable literature resources and then were performed for cerebrospinal fluid (CSF) samples from patients with suspected meningitis in Hue Hospitals. **Results:** The set of four PCR assays was developed including a multiplex real-time PCR for *S. suis serotype 2, H. influenzae* type b and *N. meningitides;* three monoplex real-time PCRs for *E. coli, S. aureus* and *S. pneumoniae.* Application of the in-house PCRs for 116 CSF samples, the results indicated that 48 (39.7%) cases were positive with *S. suis serotype 2*; one case was positive with *H. influenzae* type b; 4 cases were positive with *E. coli*; pneumococcal meningitis were 19 (16.4%) cases, meningitis with *S. aureus* and *N. meningitidis* were not observed in any CSF samples in this study. **Conclusion:** our in-house real-time PCR assays are rapid, sensitive and specific tools for routine diagnosis to detect six mentioned above meningitis etiological agents.

Key words: *Bacterial meningitis, etiological agents, multiplex real-time PCR*

1. INTRODUCTION

Bacterial meningitis is a life threatening disease with high mortality and permanent neurological dysfunction. The number of estimated cases of bacterial meningitis is 1.2 million each year worldwide. The causative agents vary among the age groups and geographical regions. More than 95% cases of bacterial meningitis are caused by one of the following bacteria: *Neisseria meningitidis, Streptococcus pneumoniae, Streptococcus agalactiae, Staphylococcus spp., Escherichia coli, Haemophilus influenzae,* and *Listeria monocytogenes* [1]. In Southeast Asian, the incidence of bacterial meningitis varied from country to country, ranging from 18.3 to 24.6/100,000 populations [2]. In Viet Nam, *S. suis* serotype 2 and *S. pneumoniae* were main causative agents due to meningitis in adults meanwhile *S. pneumoniae*, *H. influenzae*, *N. meningitidis* and *E. coli* were the most common pathogens causing meningitis in children [3]–[6].

Immediate diagnostic and appropriate antimicrobial therapy is a prerequisite for disease management. Although CSF culture is still considered as "gold standard" for diagnosis of bacterial meningitis, CSF culture requires at least a day or more, and has limited sensitivity. With advances in molecular biology diagnostic, PCR have proved very helpful in the rapid and accurate diagnosis of causative agents thanks to amplification of species-specific genes. Therefore, this technique can resolve the limitation of culture assays.

Our study is aim to develop the set of in-house PCR assays to detect six bacteria causing meningitis (*H. influenzae* type b, *S. pneumoniae*, *N. meningitidis*, *S. suis* serotype 2, *E. coli*, and *S. aureus*) in the laboratories with available equipment for PCR.

2. MATERIALS AND METHODS

The experimental study was designed.

2.1. Preparation of bacterial suspension for DNA

Bacteria employed to positive control are the strains of American Type Culture Collection (ATCC): *S.*

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pneumoniae ATCC 27336, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *N. meningitidis* ATCC 10903, *H. influenzae* type b ATCC 10211 and *S. suis serotype 2* ATCC 31533. These ATCC strains were streaked on suitable culture media; these colonies and S. *suis serotype 2* strains isolated by culture (for MLST) were transferred into 100 µL sterile distilled water to prepare bacterial suspension for DNA extraction.

2.2. DNA extraction

The iVApDNA Extraction Kit (Viet A Technology Corporation, Ho Chi Minh City, Vietnam) was utilized for DNA extraction. Briefly, 100 µL of bacterial suspension (or 200 µL aliquot of the CSF sample pellet) were treated as recommended by manufacturer. After the extraction process, DNA pellet was resuspended in a final volume of 50 µL in MiliQ water. Concentration and purity of total DNA from bacterial suspension were evaluated by using NanoDrop 2000 spectrophotometer (Thermo Scientific, Massachusetts, USA). DNA samples were stored at – 20^{0}C until use. Concentration and purity of total DNA were evaluated by using NanoDrop 2000 spectrophotometer (Thermo Scientific, Massachusetts, USA). Pure DNA has an A260/A280 ratio of 1.8–2.0 [7].

2.3. Primers and TaqMan probes

Species-specific primers and probes for *S. suis serotype 2, H. influenzae* type b*, S. pneumoniae, N. meningitidis, E. coli,* and *S. aureus* were selected from the reliable literature resources (Table 2.2). Six pairs of primers and TaqMan probes were synthesized by IDT company (Singapore). The specificity of primers and probes were checked by performing a BLAST® search (www.ncbi.nlm.nih.gov/blast) [7].

Table 1: Sequence of primers and probes

Bacteria	Target Gene	Primer or Probe's Sequence	Product size (bp)	Ref.
S. suis serotype 2	*Cps2j*	Forward: 5'-GGTTACTTGCTACTTTTGATGGAAATT-3'	85	[8]
		Reverse: 5'-CGCACCTCTTTTATCTCTTCCAA-3'		
		Probe: 5'-FAM-CGCACCTCTTTTATCTCTTCCAA-3'		
H. influenzae type b	*Hpd*	Forward: 5'-TGTTCGCCATAACTTCATCTTAGC-3'	147	[9]
		Reverse: 5'-CTTACGCTTCTATCTCGGTGATTAATAA-3'		
		Probe: 5'-CY5-CACAAAACTTCTCATTCTTCGAGCCTA-3'		
N. meningitidis	*Sod C*	Forward: 5'-GCACACTTAGGTGATTTACCTGCAT-3'	127	[10]
		Reverse: 5'-CCACCCGTGTGGATCATAATAGA-3'		
		Probe: 5'-HEX CATGATGGCACAGCAACAAATCCTGTTT-3'		
S. pneumoniae	*Lyt A*	Forward: 5'-ACGCAATCTAGCAGATGAAGCA-3'	75	[11]
		Reverse: 5'- TCGTGCGTTTTAATTCCAGCT -3'		
		Probe: 5'-FAM-GCCGAAAACGCTTGATACAGGGAG-3'		
E. coli	*16S rRNA*	Forward: 5'-GGGAGTAAAGTTAATACCTTTGC-3'	204	[12]
		Reverse: 5'-CTCAAGCTTGCCAGTATCAG-3'		
		Probe: 5'-HEX- CGGTAATACGGAGGGTGCAA-3'		
S. aureus	*Spa*	Forward: 5'-TACATGTCGTTAAACCTGGTG-3'	224	[12]
		Reverse: 5'-TACAGTTGTACCGATGAATGG-3'		
		Probe: 5'-FAM-CATGGTTTGCTGGTTGCTTCT-3'		

2.4. Optimization of the multiplex real- time PCR assays

2.4.1. Performance of the primers and probes testing

Testing individual pairs of primers by using monoplex conventional PCR

The performance of the selected sets of primers were tested in six independent monoplex experiments based on the primer melting temperature (Tm) to perform temperature gradient experiments from 55^{0}C to 62^{0}C on Applied Biosystems® Veriti® 96-Well Thermal Cycler (Applied Biosystems Inc, Foster, USA) before combining them in multiplex assays. The optimal contents and conditions for PCR assays are listed in Table 2 and 3.

Table 2. The contents of conventional PCR mix

	Concentration	Volume
Master mix 2X (Thermo scientific)	2×	12.5 µl
Primer forward	10 nM	1.0 µl
Primer reverse	10 nM	1.0 µl
H_20		2.5 µl
DNA	(< 100 ng/reaction)	5.0 µl

Table 3. Temperature program for conventional PCR

Species	Thermal cycle
S. suis serotype 2	(95 ^{0}C: 5 minutes): 1 cycle (95 ^{0}C: 10 seconds, 60 ^{0}C: 1 minutes, 72^0: 30 seconds): 40 cycles
H. influenzae type b	(95 ^{0}C: 5 minutes): 1 cycle (95 ^{0}C: 10 seconds, 60 ^{0}C: 1 minutes, 72^0: 30 seconds): 40 cycles
N. meningitidis	(95 ^{0}C: 5 minutes): 1 cycle (95 ^{0}C: 10 seconds, 60 ^{0}C: 1 minutes, 72^0: 30 seconds): 40 cycles
S. pneumoniae	(95 ^{0}C: 10 minutes): 1 cycle (95 ^{0}C: 30 seconds, 60 ^{0}C: 1 minutes): 40 cycles
S. aureus	(95 ^{0}C: 5 minutes): 1 cycle (95 ^{0}C: 10 seconds, 60 ^{0}C: 1 minutes, 72^0: 30 seconds): 40 cycles
E. coli	(95 ^{0}C: 5 minutes): 1 cycle (95 ^{0}C: 10 seconds, 60 ^{0}C: 30 seconds, 72^0: 30 seconds):40 cycles

Testing the combination of specific probe and each pair of primes by using monoplex real-time PCR.

The performance of primers - probe sets was evaluated in six independent experiments on Stratagene Mx3000P qPCR system (Agilent Technologies Inc, Santa Clara, USA). Real-time PCR mix content of six monoplex was introduced in the Table 4.

Table 4. Real-time PCR mix content

PCR	Concentration	Volume
Master mix (Thermo scientific)	2×	12,5 µl
Primer forward	10 µM	1,0 µl
Primer reverse	10 µM	1,0 µl
Probe	5 µM	0,5 µl
H_20		5,0 µl
DNA	(< 100 ng/reaction)	5,0 µl

The optimal temperature of monoplex real-time PCR assays were similar the optimal temperature of monoplex conventional PCR (Table 3). Subsequent data analysis was performed by using MxPro qPCR 4.1 software.

2.4.2. Developing the set of in-house PCR assays

Following section (a) and (b), the set of in-house PCR was developed based on optimization of annealing temperature of each pair primers and probe and also frequency of bacteria causing meningitis.

2.5. Evaluation of the sensitivity and specificity of the in-house PCR assays

2.5.1. Specificity of in-house PCR assays

The multiplex real-time PCR and monoplex real-time PCRs were tested for specificity by using DNA extracted from ATCC bacteria strains listed in the able 5.

Table 5. The list of ATCC bacteria strain used to determine the specificity of PCR assay

No	Speices	Source
1	*N. meningitides*	ATCC 10903
2	*E. faecalis*	ATCC 29212

3	*P. vulgaris*	ATCC 49132
4	*E. coli*	ATCC 25922
5	*S. aureus*	ATCC 25923
6	*P. aeruginosae*	ATCC 27853
7	*S. pneumonia*	ATCC 27336
8	*K. pneumonia*	ATCC 700603
9	*H. influenzae* type b	ATCC 10211
10	*S. suis serotype 2*	ATCC 31533

2.5.2. Sensitivity of the in-house PCR assays

The sensitivity of each PCR assay was tested by running 10-fold serial dilutions (from 10^8 to 10^1) of extracted DNA of six ATCC strains and replicated 3 times.

For the real-time PCR assays, the raw 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 MxPro qPCR software (Aligent, CA, USA)

3. RESULTS

3.1. Developing the in- house PCR assays for six mentioned-above bacteria due to meningitis

The result of testing individual pairs of primers by using monoplex conventional PCR were checked by electrophoresis as shown in Figure 1.

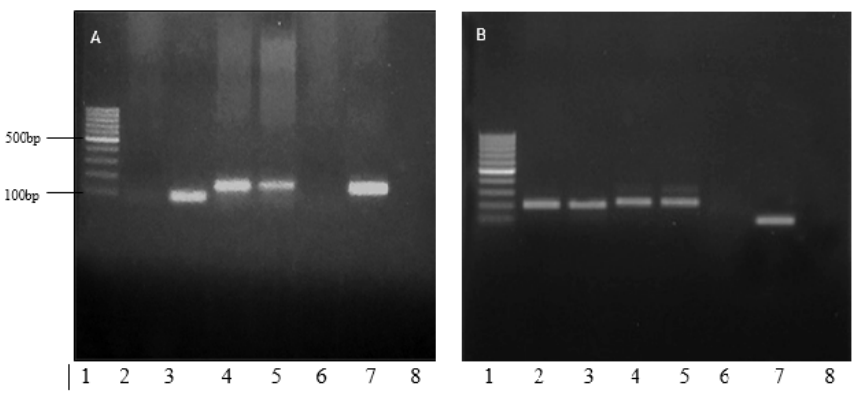

Figure 1: PCR products of six species-specific genes on 1% Agarose gel

***A**: lane 1: DNA Leader, lane 3: S. suis serotype 2 (75 bp), lane 4, 5: H. influenzae type b (147 bp), lane 7: N. meningitidis (127 bp). **B**: lane 1: DNA Leader, lane 2, 3: E. coli (204 bp), lane 4, 5: S. aureus (224 bp), lane 8: S. pneumoniae (75 bp).*

Following section (a) and (b), the set of *in-house* PCR was developed including 4 assays: one multiplex real-time PCR for three agents *S. suis* serotype 2, *H. influenzae* type b and *N. meningitidis* based on optimization of annealing temperature of each pair primers and probe and frequency of bacteria causing meningitis; three monoplex real-time PCR assays for *S. pneumonia, E. coli* and *S. aureus* (Table 6).

Table 6: The set of in-house real-time PCR

Multiplex real-time PCR	**Monoplex real-time PCRs**		
	1	**2**	**3**
S. suis serotype 2	*S. pneumoniae*	*S. aureus*	*E. coli.*
H. influenzae type b			
N. meningitidis			

The thermal profile of multiplex real-time PCR were the form of temperature cycle of monoplex of *S. suis* serotype 2, *H. influenzae* type b and *N. meningitidis.* Multiplex real-time PCR contents were introduced in Table 7.

Table 7. The component of multiplex real-time PCR

Component	Concentration	Volume
Master mix 2× (Thermo scientific)	2X	12,5 µl
Primer forward (*S. suis* serotype 2)	0.1 µM	1,0 µl
Primer reverse (*S. suis* serotype 2)	0.1 µM	1,0 µl
Probe(*S. suis* serotype 2)	0.05 µM	0,5 µl
Primer forward (*H. influenzae* type b)	0.1 µM	1,0 µl
Primer reverse (*H. influenzae* type b)	0.1 µM	1,0 µl
Probe (*H. influenzae* type b)	0.05 µM	0,5 µl
Primer forward (*N. meningitidis*)	0.1 µM	1,0 µl
Primer reverse (*N. meningitidis*)	0.1 µM	1,0 µl
Probe (*N. meningitidis*)	0.05 µM	0,5 µl
H_20		2,5 µl
DNA		5 µl

The set of real-time PCR assays was optimized successful with thermal profile as shown as Table 8.

Table 8. Thermal profile of the set of PCR assays

Species	Thermal cycle
S. suis serotype 2 *H. influenzae type b* *N. meningitidis*	(95 ^{0}C: 5 minutes): 1 cycle (95 ^{0}C: 10 seconds, 60 ^{0}C: 1 minutes, 72^0: 30 seconds):40 cycles
S. aureus	(95 ^{0}C: 5 minutes): 1 cycle (95 ^{0}C: 10 seconds, 60 ^{0}C: 1 minutes, 72^0: 30 seconds): 40 cycles
E. coli	(95 ^{0}C: 5 minutes): 1 cycle (95 ^{0}C: 10 seconds, 60 ^{0}C: 30 seconds, 72^0: 30 seconds):40 cycles
S. pneumoniae	(95 ^{0}C: 10 minutes): 1 cycle (95 ^{0}C: 30 seconds, 60 ^{0}C: 1 minutes): 40 cycles.

Specificity of in-house PCR assays

Testing the specificity of developed PCR assays by using DNA extracted from ATCC microorganisms showed that no amplification was observed with the DNA extracted from any tested organisms (Table 5). This indicates that the specificity of in-house PCR assays is considered satisfactory.

Sensitivity of in-house PCR assays

Using serially diluted quantities of genomic DNA of six ATCC, we assessed the detection limit, reproducibility and quantitative ability of the assay. Variance ranged of Ct values of different concentrations was calculated. 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.

Real-time PCR standard curves were constructed. Amplification efficiencies (E), coefficients of determination (R2) and curve slopes were generated by the MxPro software as shown in figure 3.3, 3.4, 3.5 and 3.6. These values were compared with standard values (amplification efficiency (E) (90-105%), standard curve slope real-time PCR range -3.3 to -3.8 (-3.32), coefficients of determination (R2) > 0.980) [13]. *S. aureus* were not detected with diluted concentration below 10^3 copies.

3.2. The rate of bacteria determined by in-house real-time PCR

Application of the in-house PCRs for 116 CSF samples, we determined 72 (62.1%) positive cases, of which 48 cases (39.7%) were positive with *S. suis* serotype 2, one cases (0.9%) of *H. influenzae* type meningitis; 04 positive cases (3.4%) of *E. coli* and 19 pneumococcal meningitis cases were determined by PCR assay. *N. meningitidis* and *S. aureus* meningitis were not detected in any of the samples (see Table 9).

Table 9. The rate of bacteria determined by PCR from CSF samples of patients with suspected meningitis

Microoganism	Positive PCR (N=116)	
	n	%
S. suis 2	48	39.7
H. influenzae type b	01	0.9
N. meningitidis	0	-
S. pneumoniae	19	16.4
E. coli	04	3.4
S. aureus	0	-
Total	72	62.1

4. DICCUSSION

4.1. Developing the in- house PCR assays for six mentioned-above bacteria due to meningitis

The six primers were tested by conventional PCR with agarose gel electrophoresis and the data shown that all primers amplified the expected amplicons. Primers-probe set in monoplex were checked by real-time PCR. The real-time PCR of detection of bacteria (*S. suis serotype 2, H. influenzae* type b, *N. meningitidis, E. coli*, and *S. aureus*) showed the good florescence signal. Florescence signal of *S. pneumonia test* were not suitable. This problem may derive from probe because of unsuitable signal florescence and good result of running electrophoresis for checking primers.

Monoplex real-time PCR were grouped into multiplex basing on the same annealing temperature introduced in Table 3.1 and the florescent dye linked to probe. Consequently, we created the set of *in-house* PCR assays for diagnosis six bacteria causing meningitis: a triplex real- time PCR assay for *S. suis* serotype 2, *H. influenzae* type b and *N. menigitidis,* three monoplex real-time PCR assays for *S. pneumonia, E. coli* and *S. aureus.*

On performing specificity testing, no amplification was observed with DNA of bacteria using for specific test introduced in Table 5. This indicate that the specificity of in-house PCR assays is considered satisfactory.

The sensitivity testing of the multiplex real-time PCR (*S. suis* serotype 2, *H. influenzae* type b and *N. menigitidis*) and two monoplex real-time PCRs for *S. pneumonia* and *E. coli* were low concentration, 10^2 copies by using Stratagene Mx 3000p. Additionally, multiplex and monoplex real-time PCR 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 multiplex and monoplex real-time PCR[14],[15]. In our study, the sensitivity of monoplex real-time PCR assay for *S. aureus* were limited at low concentration (below 10^3 copies/ml).

4.2. The rate of bacteria determined by in-house real-time PCR

S. suis serotype 2 is the most common agents leading meningitis in adults in this study, accounted for 39.7%. Earlier reports of Mai et al 2008, T.V. T Nga et al 2011and Nguyen et al 2015 indicated that *S. suis* serotype 2 were the most universal meningitis bacteria in adolescent and older people in Viet Nam with 33.6%, 42.4% and 45% respectively. The number of meningitis cases with *S. suis* serotype 2 (518 cases) in Viet Nam were higher than other countries in the Asian such as Thailand (292 cases) and China (245 cases) [16]. The reason is that Pork is the most important meat source with more 98% of house-hold consuming. Vietnamese consumers prefer to buy fresh pork from wet markets. In addition, many rural households have small number of pigs with the aim of poverty alleviation improve financial condition [17], [18].

S. pneumoniae is the second most common agent following *S. suis* serotype 2 in this study. Trends in pneumococcal meningitis is declined dramatically in children who were less than 5 years old because of implementation of childhood vaccination schemes.

Another causative agent caused meningitis in younger children in this study is *E. coli*. There are 4 positive cases and 3 of 4 cases found in children less than 1 month. On the other hand, the positive cases with *E. coli* were lower than other factors simply because number of CSF samples collected from children who were less than 1month were small in this study.

H. influenzae type b meningitis is a dramatic

reduction, just only 1 case (0.9%) were detected in this study. This is similarly to report of T.V.T Nga et al 2014. The main reason is that Ministry of Health in Viet Nam introduced *Hib* conjugate vaccine into the National expanded program on immunization from 2010 thanks to GAVI funding [19]. This is proved that developed countries reduced significantly and even eliminated this meningitis factors [20].

The remainder factors, *S. aureus* and *N. meningitis,* is not observed in 116 CSF suspected meningitis samples. Viet Nam might not be in the high epidemic risk with *N. meningitis* [21]. A majority of CSF samples are collected from acute meningitis patients without trauma so meningitis with *S. aureus* do not observed or inhibition of sensitivity of real-time PCR process for *S. aureus* confirmation.

5. CONCLUSION

We developed the set of *in-house* PCR tests for detected six common bacteria causing meningitis (*S. suis serotype 2, H. influenzae* type b*, N. meningitidis, E. coli, S. aureus,* and *S. pneumoniae*) in clinical samples. We advise that multiplex real-time PCR assay for determining *suis serotype 2, H. influenzae, N. meningitidis* should be priority choice for detecting bacterial meningitis, following step is conventional PCR for *S. pneumoniae.* With monoplex real-time for *S. aureus* and *E. coli,* we should depend on the ages of patient or diagnosis of physicians.

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