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A multiplex touchdown PCR for detection of *Streptococcus pneumoniae*, *Haemophilus influenzae* type b and *Mycobacterium tuberculosis* complex in sputum samples

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Abstract. Rapid and accurate detection of *Streptococcus pneumoniae* (*Sp*), *Haemophilus influenzae* type b (Hib) and *Mycobacterium tuberculosis* complex (MTBC) in sputum by conventional methods remains problematic. Primers based on capsular polysaccharide biosynthesis gene (*cpsA*), the region II of the capsulation locus (*cap*), the insertion sequence IS*6110* were designed for *Sp*, Hib, MTBC respectively. These primers were incorporated in a multiplex touchdown PCR assay for simultaneous detection of *Sp*, Hib and MTBC. The multiplex touchdown PCR assay was evaluated using standard strains and clinical sputum samples. The multiplex touchdown PCR assay showed 100% specificity in identifying *Sp*, Hib, MTBC from pure culture of standard strains. The sensitivities of the multiplex touchdown PCR assay were 94%, 98%, 98% for detection of *Sp*, Hib and MTBC respectively based on culture results while evaluated using 492 consecutive qualified clinical sputum samples; the specificities were all 100%. This highly sensitive and specific multiplex touchdown PCR assay offers a rapid and simple method for detection of *Sp*, Hib and MTBC in clinical sputum samples.

INTRODUCTION

Streptococcus pneumoniae (*Sp*) and *Haemophilus influenzae* type b (Hib) are major causes of community-acquired pneumonia (CAP) (Apisarnthanarak & Mundy, 2005; Anh *et al.*, 2009). *Sp* and Hib vaccines are not included in the routine immunization schedule of China. *Mycobacterium tuberculosis* complex (MTBC) refers to a genetically closely related group of *Mycobacterium* species that can cause tuberculosis (TB), including *Mycobacterium tuberculosis*, *Mycobacterium bovis*, *Mycobacterium bovis BCG* (*Bacillus Calmette-Guerin*), *Mycobacterium africanum*, *Mycobacterium microti*, *Mycobacterium canettii*, *Mycobacterium pinnipedii* and *Mycobacterium mungi*. Seventeen hundred thousand people died from TB worldwide in 2009, equal to 4 700 deaths per day (WHO, 2011) . Conventional detection of *Sp*, Hib and MTBC is based on culture. However, culture methods are time consuming (it requires weeks for MTBC identification) and relatively low sensitive, especially during ongoing antibiotic treatment. Recently, polymerase chain reaction (PCR) based techniques have improved sensitivity of bacteria detection. A number of PCR based assays for *Sp*, Hib and MTBC detection have been reported (Hellyer *et al.*, 1996; Morrison *et al.*, 2000; Greiner *et al.*, 2001; Shoma *et al.*, 2001; Marty *et al.*, 2004; Morozumi *et al.*, 2006; Billal *et al.*, 2007; Abdeldaim *et al.*, 2008; Gopinath & Singh, 2009; El Aila *et al.*, 2010a; Kim *et al.*, 2010; Park *et al.*, 2010; Leung *et al.*, 2011; Reddington *et al.*, 2011;

Zbinden *et al.*, 2011). Previously reported amplification targets include *spn9802* (Abdeldaim *et al.*, 2008), *spn9828* (Suzuki *et al.*, 2005), *pneumolysin* (*ply*)(Greiner *et al.*, 2001), *psaA* (Morrison *et al.*, 2000), *lytA* (Morozumi *et al.*, 2006), *capsular polysaccharide biosynthesis* (*cpsA*)(Kim *et al.*, 2010; Park *et al.*, 2010), *recA* (Zbinden *et al.*, 2011), and *16S rRNA* (El Aila *et al.*, 2010a) gene for *Sp* detection; region II of the capsulation locus (*cap*)(Marty *et al.*, 2004), *bexA* (Shoma *et al.*, 2001), *cpsb* (Billal *et al.*, 2007) for Hib detection; insertion sequence IS*6110* (Hellyer *et al.*, 1996, Leung *et al.*, 2011), *cfp10* (Gopinath & Singh, 2009), *lepA* gene (Reddington *et al.*, 2011) for MTBC detection. However, El Aila *et al.* (2010b) and Kim *et al.* (2010) found *spn9802*, *lytA* and *ply* based PCRs failed to distinguish *Sp* from *Streptococcus pseudopneumoniae*.

However, assays for directly simultaneous detection of *Sp*, Hib, and MTBC in respiratory tract samples are very rare. Wang *et al.* (2008) developed a multiplex PCR-based reverse line blot hybridization (mPCR/RLB) assay for simultaneous detection of bacterial respiratory pathogens (including *Sp*, Hib and *M. tuberculosis* etc.). No other PCR based assays have been reported. Touchdown (TD) PCR offers a simple way to increase specificity, sensitivity of PCRs (Korbie & Mattick, 2008).

In the present study, we developed a highly sensitive and specific multiplex touchdown PCR (TD-PCR) assay for simultaneous detection of *Sp*, Hib and MTBC. The performance of the assay was evaluated using clinical sputum specimens.

MATERIAL AND METHODS

Bacterial strains

The bacterial strains used in this study included: *Acinetobacter baumannii* ATCC® 19606™, *Moraxella (Branhamella) catarrhalis* ATCC® 25238™, *H. influenzae* ATCC® 10211™ (from American Type Culture Collection, ATCC); *H. influenzae* ATCC® 49247™, *S. pneumoniae* ATCC® 49619™ (kindly provided by Dr. Shi Lining); *Staphylococcus aureus* ATCC® 25923™, *Escherichia coli* ATCC® 25922™, *Pseudomonas aeruginosa* ATCC® 27853™, *Klebsiella pneumoniae* ATCC® 700603™, *Enterococcus faecalis* ATCC® 29212™ (kindly provided by Dr. Sun Guangming); *M. bovis*, *M. tuberculosis* (clinical isolates, kindly provided by Dr. Sun Chunhong, The Third Hospital of Zhenjiang, Jiangsu, China); *M. smegmatis*, *M. bovis BCG* (our laboratory).

Sample collection

A total of 492 consecutive qualified sputum samples having more than five leukocytes per epithelial cell were tested. The samples were collected from the clinical laboratory at Affiliated Hospital of Jiangsu University between June 2010 and May 2011. These samples were washed with 0.9% NaCl twice and digested with 1% trypsin at 37°C for 90 minutes for subsequent bacterial culture, then were stored at -70°C for DNA extraction.

Bacterial culture

Ten microliter digested samples were cultured on Chocolate agar supplemented with 15 mg L^{-1} NAD, Blood agar, MacConkey agar, Lowenstein-Jensen medium and incubated at 5% CO_2 in 35°C. The bacterial identifications were performed according to standard microbiological methods (D., 2007).

DNA extraction

DNA extraction was performed with Takara minibest bacterial genomic DNA extraction (Takara, China) according to manufacturers' protocol. Extracts were stored at -20°C until required for analysis.

Primer design

After comparing several amplification targets, primers were designed based on the region II of the capsulation locus (*cap*) of *H. influenzae* type b (Hib), the capsular polysaccharide biosynthesis gene (*cpsA*) of *S. pneumoniae* (*Sp*), the insertion sequence *IS6110* of *M. tuberculosis* complex (MTBC) using PrimerPlex 2 (PremierBiosoft, US). The specificity of the designed primers was tested using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The primer-pairs used in this study are listed in Table 1.

Table 1. Primer pairs used in this study

Species	Target sequence	Amplicon size (bp)		Primer sequence	Reference
Sp	*cpsA*	653	F	AGTGGTAACTGCGTTAGTCCTA	This study
			R	GTGGCGTTGTGGTCAAGAG	
Hib	*cap*	177	F	ATGTTAGATCGTGCGGATACTC	This study
			R	GCGAGGAACAGAACCATCAG	
MTBC	*IS6110*	494	F	GGTAGCAGACCTCACCTATGT	This study
			R	CTCTGGCGTTGAGCGTAGTA	

Simplex Touchdown PCR

Simplex PCRs were performed in a volume of 25 µl, with each reaction mixture containing 1 X PCR buffer (20mM Tris-HCl, 50 mM KCl), 2.0 mM $MgCl_2$, 0.2 mM each deoxyribonucleotide triphosphates (dNTP) (Fermentas, Lithuania), 0.4 mM each primer, 1 U *Taq* DNA polymerase (Fermentas, Lithuania), 0.1 µg μl^{-1} BSA (NEB, US) and 2 µl DNA template. Sterile molecular-biology-grade water in place of DNA template was used as a negative control, and genomic DNA extracted from *S. pneumoniae* ATCC® 49619™, *H. influenzae* ATCC® 10211™ and *M. tuberculosis* clinical isolate *w*as used as positive control in each reaction batch. The PCR was carried out on 2720 Thermal Cycler (ABI, US) using the following conditions: an initial denaturation at 94ºC for 5 min, followed by 20 cycles of 94ºC for 30 s, 65ºC (0.5ºC decrease per cycle) for 30 s, 72ºC for 1 min, then another 20 cycles of 94ºC for 30 s, 55ºC for 30 s, 72ºC for 1 min, and a final extension step at 72ºC for 7 min. The PCR products were separated on 2% agarose gels in Tris-acetate-EDTA (TAE) buffer stained with ethidium bromide and visualized by GeneGenius Bio Imaging System (Syngene, UK).

Multiplex Touchdown PCR

The multiplex touchdown PCR was performed using the same protocol as the individual PCR reactions described above, with modified primer concentrations (0.5 µM of *cpsA* primer set, 0.1 µM of *cap* primer set, 0.4 µM of IS*6110* primer set, listed in Table 1).

Limit of detection

DNA extracted from cultured *S. pneumoniae* ATCC® 49619™, *H. influenzae* ATCC® 10211™ and *M. tuberculosis* strain were 10-fold serially diluted in DNA extracted from a pool of 20 individual *Sp*, Hib and MTBC negative sputum samples. The PCR assay was performed as described above using the diluted DNA template. The lowest DNA concentration that produced visible band was considered as the limit of detection (LOD).

DNA sequence analysis of amplicons

PCR amplicons generated by the different primer sets were sequenced to validate their identities if PCR results were inconsistent with culture status. In general, PCR products were purified using DNA purification kit (Generay Biotechnology, China) according to the manufacturer's protocol and sequenced by Invitrogen Company (Life Technologies, China) using the same primers of the multiplex touchdown PCR. The DNA sequences obtained were then compared with the GenBank database using BLAST (http://www.ncbi.nih.gov).

RESULTS

Simplex Touchdown PCR

The individual Simplex PCRs produced specific band sizes. No cross amplification with other species was observed (data not shown). The LODs were 6.3 pg, 0.2 pg, and 0.02 pg DNA per reaction for *Sp*, Hib and MTBC respectively.

Multiplex Touchdown PCR

The multiplex touchdown PCR produced specific band sizes of *Sp*, Hib and MTBC. No cross amplification with other species was observed (Figure 1). The LODs of the multiplex touchdown PCR were 6.3pg, 0.2 pg and 0.02 pg DNA per reaction for *Sp*, Hib and MTBC respectively, just same as the simplex ones.

The multiplex touchdown PCR assay was evaluated using DNA extracted from 492 sputum samples from 254 patients. All tests were repeated twice. Performance of the multiplex touchdown PCR was calculated against culture results (Table 2). Specific bacterial DNAs were successfully amplified in all 44 culture positive samples (*Sp* 25, Hib 7, MTBC 12). Additional 46 samples (*Sp* 27, Hib 9, MTBC 10) were tested positive in 448 culture negative samples. The amplicons of these 46 samples were sequenced and shown 99% (98-100%, data not shown) identical to the targeting sequences (*cpsA*, *cap*, IS*6110* respectively).

The specificities for *Sp*, Hib, MTBC detection were 98%, 94%, 98% respectively, the sensitivities were all 100% compared to culture results (Table 2).

DISCUSSION

Wang *et al.* (2008) previously developed a multiplex PCR-based reverse line blot hybridization (mPCR/RLB) assay for simultaneous detection of bacterial respiratory pathogens (including *Sp*, Hib and *M. tuberculosis* etc.). However, clinical samples used in that study were naso-pharyngeal aspirate and blood, sputum samples were not included.

This study describes the development and evaluation of a single-tube, three-target, multiplex touchdown PCR assay which can simultaneously detect *Sp*, Hib and MTBC directly on clinical sputum samples.

CpsA gene was recently found target for specific detection of *Sp* which could distinguish *Sp* from *S. pseudopneumoniae* (Kim *et al.*, 2010; Park *et al.*, 2010) and was chose in our study. Targets for PCR-based detection of Hib in published literatures

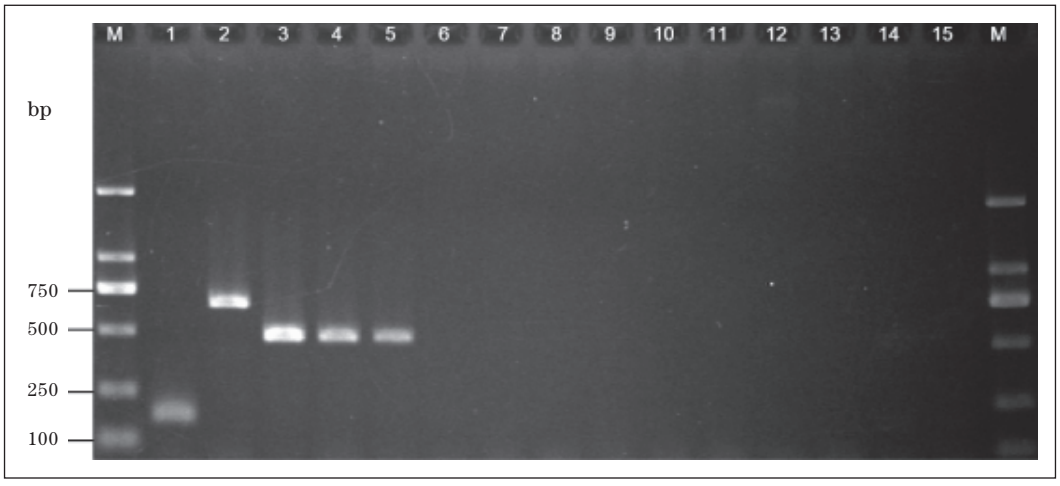

Figure 1. Specificity evaluation of the multiplex touchdown PCR performed on DNA form pure cultures.

Lane M: DL2,000 DNA Marker (Takara, China); Lane 1: *Haemophilus influenzae* ATCC® 10211™ (Hib); Lane 2: *Streptococcus pneumoniae* ATCC® 49619™; Lane 3: *Mycobacterium tuberculosis* clinical isolate; Lane 4: *Mycobacterium bovis* clinical isolate; Lane 5: *Mycobacterium bovis BCG*; Lane 6: Negative control; Lane 7: *Escherichia coli* ATCC® 25922™; Lane 8: *Staphylococcus aureus* ATCC® 25923™; Lane 9: *Pseudomonas aeruginosa* ATCC® 27853™; Lane 10: *Klebsiella pneumoniae* ATCC® 700603™; Lane 11: *Acinetobacter baumannii* ATCC® 19606™; Lane 12: *Moraxella (Branhamella) catarrhalis* ATCC® 25238™; Lane 13: *Enterococcus faecalis* ATCC® 29212™; Lane 14: *Haemophilus influenzae* ATCC® 49247™; Lane 15: *Mycobacterium smegmatis*

Table 2. Performance of the multiplex TD-PCR compared to culture results

Species	Multiplex TD- PCR	Culture		Sensitivity (%)	Specificity (%)
		Positive +	Negative –		
Sp	Positive +	25 (a)	27 (b)	100	94
	Negative –	0 (c)	440 (d)		
Hib	Positive +	7 (a)	9 (b)	100	98
	Negative –	0 (c)	476 (d)		
MTBC	Positive +	12 (a)	10 (b)	100	98
	Negative –	0 (c)	470 (d)		
Total	Positive +	44 (a)	46 (b)	100	82
	Negative –	0 (c)	402 (d)		

Sensitivity = a / (a + c)
Specificity = d / (b + d)

are relatively rare, we chose region II of the capsulation locus of Hib as target for the detection of Hib. IS*6110* is an ideal and widely used target for the detection of MTBC, and was chosen for our study. The LOD of the multiplex touchdown PCR for *Sp* detection (6.3 pg reaction^{-1}) was consistent with Tzanakaki *et al.* (2005) study on a multiplex PCR assay (10 pg reaction^{-1}) but higher than Wang *et al.* (2008) study on a mPCR/RLB assay (0.5 pg reaction^{-1}); for Hib detection, it (0.2 pg reaction^{-1}) was lower than Tzanakaki *et al.* (2005) (5 pg reaction^{-1}) and Wang *et al.* (2008) (50 pg reaction^{-1}); for MTBC detection, it (0.02 pg reaction^{-1}) was lower than Wang *et al.* (2008) (50 pg reaction^{-1}).

The performance of this multiplex touchdown PCR assay was evaluated using 492 qualified consecutive sputum samples. The specificities for *Sp*, Hib, MTBC detection were 94%, 98%, 98% respectively. The sensitivities were all 100% compared to culture results. This result was consistent with previously reported PCR-based assays (Stralin *et al.*, 2006; Armand *et al.*, 2011) for direct detection of *Sp*, MTBC in sputum samples. To our knowledge, this is the first reported PCR-based assay for direct detection of Hib in sputum samples compared on culture. The multiplex touchdown PCR detected out 46 positive results from the 448 culture negative samples. There were some possible reasons for this result: 1) false negative results of culture resulted from antibiotic pretreatment before sampling; 2) lower LODs of the multiplex touchdown PCR assay than culture; 3) false positive results of the multiplex touchdown PCR assay which probability was very small considering sequencing results of the amplicons.

It should be noted that this assay using sputum samples could not distinguish infection from colonization. Further evaluation of this assay using other clinical samples, including pleural fluid and cerebrospinal fluid, is indicated to determine its role in clinical diagnosis. However the multiplex touchdown PCR assay we developed expands the application of multiplex touchdown PCR in detection of *Sp*, Hib, MTBC especially in sputum samples. And with high sensitivity, high specificity, short turnaround time, it is very useful for clinical and epidemiological study of *Sp*, Hib, MTBC.

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