

Development of PCR-ELISA for rapid detection of *Haemophilus influenzae* Type b in seeded cerebrospinal fluid samples

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- Objectives** : To develop a PCR-ELISA test for the simple, rapid, and reliable detection of *Haemophilus influenzae* type b (Hib) in seeded cerebrospinal fluids.
- Design** : A cross-sectional analytical study
- Setting** : Department of Microbiology, Faculty of Public Health, Mahidol University
- Materials/Methods** : A PCR-ELISA was set up with the use of a newly designed primer pair based on the gene coding for type-specific capsule of Hib. The PCR assay was optimized with the use of DNA from standard reference strains. A total of 311 blind bacterial isolates, which commonly cause meningitis, seeded into sterile and pooled CSF samples were tested for the presence of Hib by the PCR-ELISA method and by culture and slide agglutination with Hib specific antiserum as the "gold standard".
- Results** : Of the 311 bacteria seeded in CSF, 40 were identified as Hib positive, and 271 were identified as Hib negative by culture and slide agglutination with Hib antiserum. Compared with standard methods, the PCR-ELISA method using the OD 405 nm value at 0.15 as the cut-off point, exhibited high sensitivity, specificity and efficiency with 100, 99.63

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and 99.67%, respectively. One capsule-deficient type b mutant strain was detected by PCR-ELISA while the result was negative by the slide agglutination test. The sensitivity of detection of Hib spiked in sterile CSF by PCR-ELISA was 10 times less than that of which could be detected by PCR-agarose gel electrophoresis. However, the PCR-ELISA showed superior benefits for detection of Hib spiked in contaminated CSF whereas the result was negative by culture.

Conclusion : *The PCR-ELISA developed in this study showed rapidity, simplicity, high sensitivity and high specificity and may be used in a routine clinical microbiology laboratory as an alternative method for early diagnosis of H. influenzae type b meningitis.*

Key words : *PCR-ELISA, Haemophilus influenzae type b, Meningitis, Rapid detection, Cerebrospinal fluid.*

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- วัตถุประสงค์** : เพื่อพัฒนาเทคนิค PCR-ELISA เพื่อให้ได้เทคนิคที่ง่าย รวดเร็ว และนำเชื้อถือสำหรับตรวจหาเชื้อ *Haemophilus influenzae* type b (Hib) ในตัวอย่างน้ำไขสันหลังที่ใส่เชื้อ
- รูปแบบงานวิจัย** : การศึกษาเชิงวิเคราะห์ภาคตัดขวาง
- สถานที่** : ภาควิชาจุลชีววิทยา คณะสาธารณสุขศาสตร์ มหาวิทยาลัยมหิดล
- ตัวอย่างและวิธีวิจัย** : ทำการทดลองวิธี PCR-ELISA ด้วยไพรเมอร์ที่ออกแบบขึ้นใหม่ ซึ่งได้จากยีนที่ควบคุมการสร้างปลอกหุ้มเซลล์ที่จำเพาะต่อ Hib ปรับหาสภาวะเหมาะสมของวิธี PCR-ELISA โดยตรวจกับตัวอย่างดีเอ็นเอที่สกัดจากเชื้อสายพันธุ์มาตรฐาน แล้วจึงนำวิธีที่พัฒนามาตรวจวิเคราะห์หาเชื้อ Hib กับตัวอย่างน้ำไขสันหลังที่ได้ใส่เชื้อแบคทีเรียที่ไม่ทราบชื่อ ซึ่งมักก่อโรคเยื่อหุ้มสมองอักเสบจำนวน 311 สายพันธุ์ โดยอาศัยผล การตรวจโดยวิธีการเพาะเลี้ยงและพิสูจน์ด้วยการจับกลุ่มกับแอนติซีรัมเฉพาะต่อทัยปี บี เป็นวิธีมาตรฐาน
- ผลการศึกษา** : จากน้ำไขสันหลังที่ใส่เชื้อ จำนวน 311 ตัวอย่าง โดยวิธีมาตรฐานให้ผลบวกว่าเป็น Hib จำนวน 40 ตัวอย่าง และให้ผลลบ จำนวน 271 ตัวอย่าง ในขณะที่การตรวจโดยเทคนิค PCR-ELISA เมื่อใช้ค่าจุดตัดความเข้มแสงที่ 405 นาโนเมตร เท่ากับ 0.15 ได้ค่าความไว ความจำเพาะ และประสิทธิภาพร้อยละ 100, 99.63 และ 99.67 ตามลำดับ โดยมีเชื้อ 1 สายพันธุ์ที่ให้ผลบวกโดย PCR-ELISA แต่ให้ผลลบกับแอนติซีรัม ซึ่งคาดว่าอาจเป็น Hib สายพันธุ์ที่ไม่มีปลอกหุ้มเซลล์ ส่วนการทดสอบขีดความไวในการตรวจหา Hib ที่ใส่น้ำไขสันหลัง พบว่า การตรวจโดย PCR-ELISA มีความไวต่ำกว่า 10 เท่า เมื่อเปรียบเทียบกับ การตรวจโดยวิธี PCR-agarose gel electrophoresis อย่างไรก็ตาม เทคนิค PCR-ELISA สามารถตรวจหา Hib ในน้ำไขสันหลังที่มีการปนเปื้อนด้วยเชื้อแบคทีเรียอื่น ๆ ได้ โดยที่การตรวจด้วยวิธีการเพาะเลี้ยงเชื้อให้ผลลบ

สรุปผล

: เทคนิค PCR-ELISA ที่พัฒนาขึ้นในห้องปฏิบัติการนี้ ประสบผลสำเร็จในการตรวจหาเชื้อ Hib เทคนิคนี้มีความสะดวก รวดเร็ว มีความไว และความจำเพาะสูง จึงอาจเป็นทางเลือกอีกวิธีหนึ่งที่จะนำไปใช้ในงานตรวจประจำของห้องปฏิบัติการจุลชีววิทยาคลินิก เพื่อตรวจหาเชื้อ Hib ตั้งแต่ระยะเริ่มแรกของโรคเยื่อหุ้มสมองอักเสบได้

In Thailand, *Haemophilus influenzae* type b (Hib) remains a major contributor to serious and life threatening infections causing high mortality and morbidity in children between 6 months and 1 year old.⁽¹⁾ Although the mortality rate from Hib meningitis in developed countries was less than 5%, the incidence of serious permanent sequelae was about 19 - 45%, only 50 % of the patients were judged to function normally.⁽²⁾ Consequently, the need for a prompt and reliable detection method for Hib strain is critically needed to reduce the mortality rate and the neurological damage among survivors.

Direct microscopy of cerebrospinal fluid (CSF) by Gram staining is the fastest possible diagnosis, but it gives low sensitivity and specificity. It is thus necessary to culture and isolate the microbial pathogens. Limitations of the cultivation method are that it is time consuming and usually gives false negatives resulting from cases of fastidious organisms or antibiotic administration prior to treatment. Numerous reports have described the usefulness of various immunological techniques for detecting the capsular polysaccharide of Hib in serum, CSF, or urine.⁽³⁻⁶⁾ Of these methods, the latex agglutination (LA) test for detection of Hib antigen in CSF is most frequently performed in Thai laboratories. Despite its frequent use and documented sensitivity and specificity, questions remain about the clinical usefulness of the LA test which is shown to be unreliable due to nonspecific agglutination, false positives resulting from cross reaction with other organisms and less sensitivity for detection of small amounts of antigen.^(3,6) The most frequent cause of a misleading LA test result was recent immunization with Hib conjugate vaccine.⁽⁷⁾

Polymerase chain reaction (PCR) is recognized as a promising method for detection of Hib directly in CSF.⁽⁸⁻¹¹⁾ This assay provides high specificity and sensitivity for detecting small amounts of DNA in CSF.⁽⁸⁾ Although PCR diagnosis of Hib infection has been reported, the analysis of the PCR product was confined to gel electrophoresis which has certain disadvantages, including handling time, safety hazards, and risk of nonspecific banding patterns; thus the results must be confirmed by hybridization procedures on membranes. These tests are laborious and the readings are subjective, not suitable for the analysis of large numbers of samples, and therefore not practicable in all laboratories. Currently, the post amplification product procedure has been progressively developed as a simplified analytic method using colorimetric detection⁽¹²⁾ which is a specific, sensitive, quantitative, and practical procedure that might come into a routine use in the clinical microbiology laboratory.

In order to find an alternative assay which is reliable for rapid diagnosis of meningitis caused by Hib, we have developed a simplified PCR-ELISA without a hybridization step using our primers designed from capsule type-specific DNA sequences from the capsular gene cluster of Hib for the detection of Hib in seeded preparation of CSF samples, collected from patients with noninfective neurological disorders, and comparing results with those by culture and slide agglutination with Hib specific antiserum as the "gold standard."

Materials and Methods

Bacterial strains. A total of 311 bacterial isolates comprising 191 *H. influenzae* and 120 other

bacteria were tested. *H. influenzae* strains; containing type b ATCC 9795 and 10211, type c ATCC 9007, and type f ATCC 9833 provided by Dr. Takeshi Honda, Osaka University, Japan; and 21 of *H. influenzae* types a - f and nontypeable provided by Dr. Crook, John Radcliff Hospital, Oxford, UK, were used as controls. Another 166 *H. influenzae* strains were clinical isolates from the National Institute of Health, Ministry of Public Health, and from Children's Hospital. Another 120 bacterial species were isolated from clinical specimens comprising 18 *Streptococcus pneumoniae*, 23 *Streptococcus pyogenes*, 24 *Streptococcus agalactiae*, 26 *Staphylococcus aureus*, 6 *Mycobacterium tuberculosis*, 9 *Escherichia coli*, 3 *Pseudomonas aeruginosa* and 11 *Neisseria meningitidis*.

DNA extraction. Total purified DNA was isolated from reference ATCC strains; i.e., *H. influenzae* type b ATCC 9795 and type c ATCC 9007 for used as a positive and a negative control DNA in PCR amplification. DNA was extracted from pure culture isolates using DNAzol™ (Life Technologies, BRL) as described by the manufacturer and 10 ng of purified DNA was used in the PCR. For detection of Hib by PCR-ELISA in comparison with the culture, tube of seeded CSF samples was boiled for 10 min and then placed in wet ice for 5 min, after centrifugation at 10,000 X g for 5 min, 10 µl of supernatant was used in the PCR.

Oligonucleotide primers and probe. Primers corresponding to the central sequence of *cap* gene, region 2 of the Hib involved in the biosynthetic step required for the formation of the type b capsular polysaccharide⁽¹³⁾ were synthesized by the National Center of Genetic Engineering and Biotechnology, Ministry of Science, Technology and Environment. The

primer set (5' GATCGTGCGGATACTCGTTTA 3', complement of bases 4584 to 4604 and 5'GAACAA-GCTGTGTGCGGTAAC 3' complement of bases 4899 to 4879) of 21 bp in length was designed by using the PCR primer analysis software. The amplified region contains the nucleotide number +4584 to +4899 which results in a length of the amplified product of 316 bp. Probe of 18 bp in length (5' TGTCCTCGCCGTCTTGG 3' complement of bases 4745 to 4762) was designed from the amplified target on the criterion of 60% G+C and a melting point temperature of 58°C.

Conventional PCR. The reaction mixture for PCR (50 µl) consisted of 0.5 µM each of the primer, 1XPCR buffer (10mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin), 200 µM (each) the four deoxyribonucleoside triphosphates, 0.5 U of *Taq* polymerase (Promega) and 10 µl of sample. The DNA was denatured for 5 min at 94°C and 30 amplification cycles were performed with an automated thermal cycler (Perkin-Elmer Cetus). Each cycle consisted of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min, followed by a 10-min final extension at 72°C. The presence of the 316-bp amplification product was analyzed by agarose gel electrophoresis (AGE) and ethidium bromide staining and Hib PCR products were confirmed by Southern blot hybridization test using fluorescein labeled oligonucleotide probe as described earlier.⁽¹⁴⁾

PCR-ELISA. The principle of PCR-ELISA as described by Landgraf et al,⁽¹⁵⁾ was used with a little modification. The sense and anti-sense strand primers were labeled 5'end with biotin and fluorescein, respectively using the labeling kit (Promega) as described by the manufacturer. The specific PCR products were incorporated with biotin and fluorescein

on the either side of the 5'end. The biotinylated-fluorescein DNA was immobilized by avidin coated microtiter plates with biotin end and directly identified fluorescein end by using anti-fluorescein antibody alkaline phosphatase (AP) conjugate and substrate.

Optimization of PCR-ELISA. A total of 10 ng of Hib DNA was amplified by PCR using labeled primer pairs and optimized condition of conventional PCR as mentioned above. The amplified products were analyzed by 2% AGE before using to optimize an ELISA. The ELISA was optimized in microtiter plates (MaxiSorb™, Nunc) by checkerboard titration with varying concentrations of substances used in the reaction; i.e., avidin (0.5, 1.0, 1.5 and 2.0 µg/ml), anti-fluorescein-AP conjugate (1:5,000, 1:10,000 and 1:15,000). In addition, the amplification cycles were optimized at 25, 30, and 35 cycles, and also 1, 5, and 10 µl of the amplification products of those cycles were prepared for optimization in the same manner.

After many trials of various conditions, the following conditions were found to be optimal for PCR-ELISA. The microtiter plate was coated with 100 µl of 2.0 µg/ml avidin (13 U/mg, Sigma) diluted in coating buffer (50 mM Carbonate-bicarbonate buffer, pH 9.6). After incubation for 2 h at 37°C or overnight at 4°C in a humid atmosphere, the plate was washed 3 times with TBST buffer (50mM Tris; pH 8, 0.15N NaCl, 0.1% Tween 20). Ten µl of the PCR product with 35 cycles diluted in 40 µl of TBST was dropped into a well of a coated plate and incubated for 30 min at 37°C. After incubation, the plate was then washed 3 times with TBST to remove nonincorporated fluorescent primers and nonspecific PCR products. The specific PCR products bound via its biotin residue to the avidin coated plate were determined by incubating the plate

in the dark for 30 min at 37°C with 100 µl/well of anti-fluorescein-AP conjugate (Sigma) at a 1:5,000 dilution in TBST. The plate was subsequently washed 3 times with TBST. Then 100 µl of 1.0 mg/ml of para-nitrophenol phosphate in Tris buffer (Sigma) was added and the plate was incubated at 37°C for 30 min and then stopped by adding 25 µl of 3 N NaOH. The optical density at A_{405} was measured by an ELISA reader (Bio-Kinetics Reader EL321e, Biotex Instrument, USA).

Determination of limits of detection. For a rough estimation of detection limits of Hib in CSF by PCR-ELISA, aliquots of pooled and sterilized CSF samples were used in the following experiments:

a) The detection limit of Hib in sterile CSF. Some colonies of Hib reference strain were spiked into CSF to make a suspension of Mc Farland No. 0.5. Then the serial 10-fold dilution from 10^{-1} to 10^{-8} was performed and 10 µl of each dilution were spreaded immediately on chocolate agar plates by using standard calibrated inoculating loops. The DNA of each dilution was extracted by boiling, and after centrifugation 10 µl of supernatant was used as the template for 35 amplification cycles of PCR reaction with labeled primer pairs. After analysis by AGE and DNA hybridization, the PCR products of each dilution were identified by ELISA. The detection limit for each assay was determined by the number of colonies grown on the chocolate agar plates of the corresponding dilution.

b) The detection limit of Hib in contaminated CSF. In this experiment, *S. aureus* and *E. coli* were selected for creating artificial contamination of the CSF. Hib colonies were suspended in CSF at concentrations of 10^3 and 10^5 CFU/ml. Each dilution

was divided into four tubes and each group of dilutions was spiked with mixed *S. aureus* and *E. coli* at concentrations of approximately 10^3 , 10^4 , 10^5 , and 10^6 CFU/ml, respectively. Then 10 μ l of each suspension was subjected to culturing and analyzed by conventional PCR and PCR-ELISA as described above for the detection limit of Hib in sterile CSF.

PCR-ELISA for detection of Hib in seeded CSF samples. For preparation of seeded CSF samples, CSF from 25 randomly selected child patients aged under 5 years old with noninfective neurological disorders who had been admitted at Children's Hospital were used. All CSF specimens were pooled and sterilized by membrane filtering (0.45 μ m pore size, Millipore) and then divided into aliquots and each aliquot was seeded with colonies of *H. influenzae* or other bacteria.

To make a bacterial suspension that was suitable for testing, then 10 fold (1 dilution) higher than the detection limit was used. Therefore, after each bacterial suspension was adjusted to a turbidity of Mc Farland No. 0.5 ($\sim 10^7$ organisms per ml) with sterile CSF, then 10^{-3} dilution ($\sim 10^4$ organisms per ml) was prepared in the same solution, and 10 μ l ($\sim 10^2$ organisms) of prepared seeded CSF samples were first cultured onto chocolate agar plates. Then tube of 10^{-3} dilution was placed on boiling water bath for 10 min, and 10 μ l was used as the template for PCR-ELISA. The validity of the PCR-ELISA assay was determined by using the results of culture and agglutination with type b-specific antiserum as standard method.

All 311 seeded CSF samples were investigated in parallel by conventional culture and by PCR-ELISA. As a control, pool CSF after filtering was also

culture without seeding. After cultured onto chocolate agar plates and incubated overnight at 37°C with 5% CO₂, all bacterial isolates were identified by colony morphology and their growth requirement of X and V factors⁽¹⁶⁾ and serotyping of Hib was identified by slide agglutination test using type b-specific antiserum (ZM21; Murex, Merck).

Results

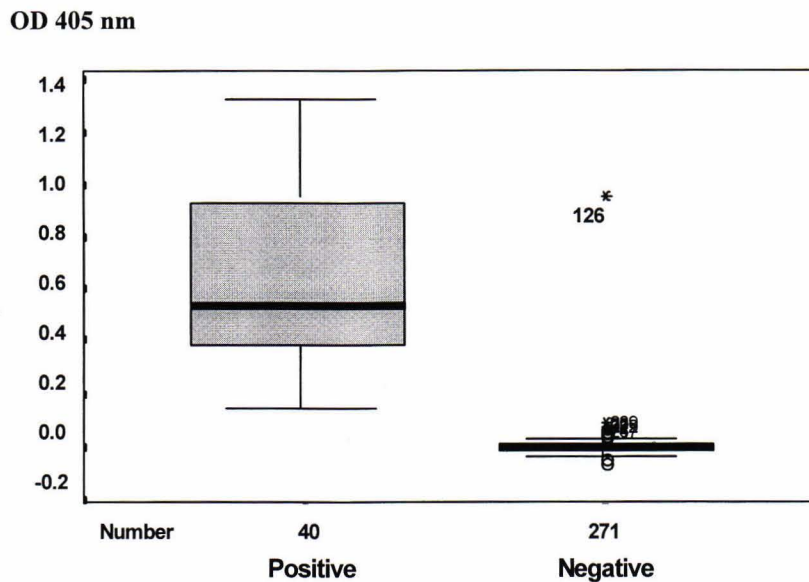
Validity of PCR-ELISA. Among all 311 bacteria seeded in CSF samples, 40 isolates were positive by colony morphology, their growth requirement of X and V factors and slide agglutination with Hib specific antiserum. Of the isolates, 151 were *H. influenzae* of other types and nontypeable (NT), and 120 isolates were other bacteria. When all blind seeded CSF samples were tested with optimized PCR-ELISA in triplicate, the results showed that the mean OD at 405 nm of the positive group was 0.6201 (95% CI = 0.5120, 0.7282) and of the negative group was 0.0071 (95% CI = -0.0003, 0.0145) (Table 1 and Fig. 1). The means OD of the positive group and negative groups were significantly different ($p = 0.00$). The lowest OD of the positive group was used as the cut off point (cut off OD 405 nm value = 0.15).

Of the 40 culture and slide agglutination-positive samples for Hib, all were positive by PCR-ELISA. Of the 271 culture and slide agglutination negative samples for Hib, 1 was positive by PCR-ELISA. The sensitivity, specificity and efficiency of PCR-ELISA when compared with the results of culture and slide agglutination with Hib antiserum were 100, 99.63 and 99.67%, respectively (Table 2). The agreement rate of PCR product detection by ELISA comparing with AGE was 1.0 by Kappa analysis (data not shown).

Table 1. The mean optical density, standard deviations and 95% confident interval (CI) of the positive group and negative group when blind DNA of 311 bacterial isolates seeded in CSF samples were analyzed by PCR-ELISA using culture and slide agglutination with Hib specific antiserum as the "gold standard".

	Positive group*	Negative group*
Numbers	40	271
Means of OD 405 nm	0.6201	0.0071
Standard deviations of OD 405 nm	0.3380	0.0616
Minimum OD 405 nm	0.153	-0.06
Maximum OD 405 nm	1.300	0.95
95% CI of OD 405 nm	0.5120, 0.7282	-0.0003, 0.0145

*Results by culture and slide agglutination with Hib specific antiserum, positive group (N = 40), and negative group (N = 271).



Results by Culture and Hib antiserum

Figure 1. Box plot of the optical density of PCR product detection by ELISA between the positive group and the negative group.

* : Sample no.126 which gave positive result for PCR-ELISA but negative result for culture and agglutination with Hib antiserum.

Table 2. Detection of Hib in seeded CSF samples by PCR-ELISA compared with detection by standard method (culture and slide agglutination with Hib specific antiserum).

PCR-ELISA	Standard method		
	Positive	Negative	Total
Positive	40	1	41
Negative	0	270	270
Total	40	271	311

Sensitivity	100.00 %
Specificity	99.63 %
Efficiency	99.67%

Limit of detection. The detection limit of the PCR assay for cultured Hib seeded in sterile CSF was 30 cells per reaction by PCR-ELISA, and 3 cells per reaction by ethidium bromide-stained AGE. Fig. 2 shows the OD of ELISA assay when Hib spiked in sterile CSF in 10-fold dilution from 10^{-1} - 10^{-8} . For contaminated CSF, the result showed that 10^5 CFU/ml of Hib spiked in CSF could be detected by PCR-ELISA, PCR-AGE and culture at every concentration of contamination with mixed bacteria. On the other hand, at 10^3 CFU/ml of Hib spiked in CSF could be detected by PCR-AGE only (Fig. 3). For culture, no growth of colonies of 10^3 CFU/ml Hib spiked in CSF was detected at any concentrations of mixed bacteria except for a concentration of 10^3 CFU/ml of mixed bacteria where one colony of Hib was found (Table 3).

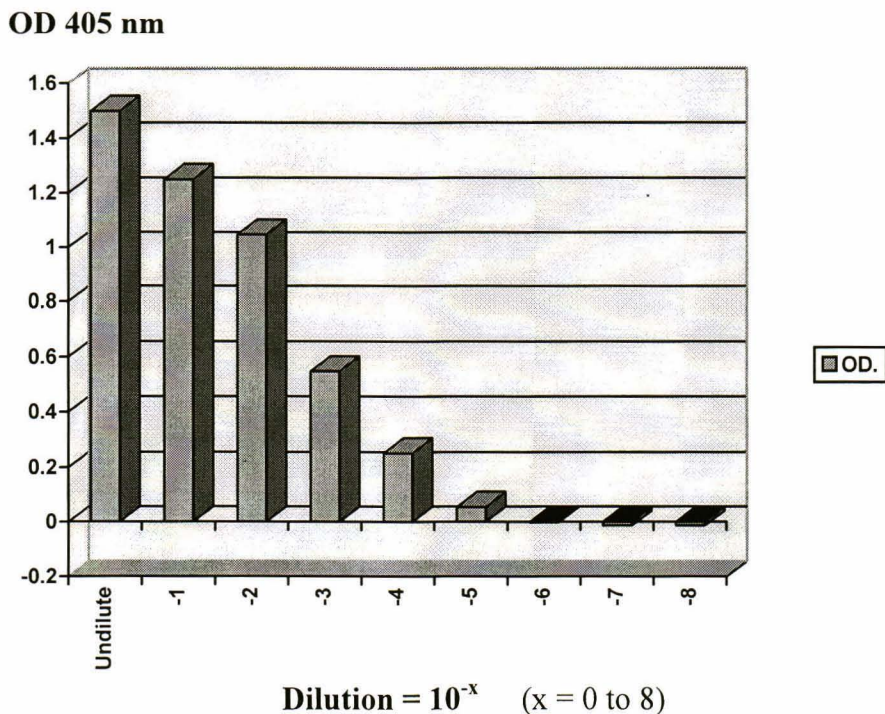


Figure 2. Graph showing the optical density of ELISA assay measured from PCR products of Hib colonies seeded in sterile and pooled CSF that serial ten fold dilutions from 10^{-1} through 10^{-8} were performed and used as the template.

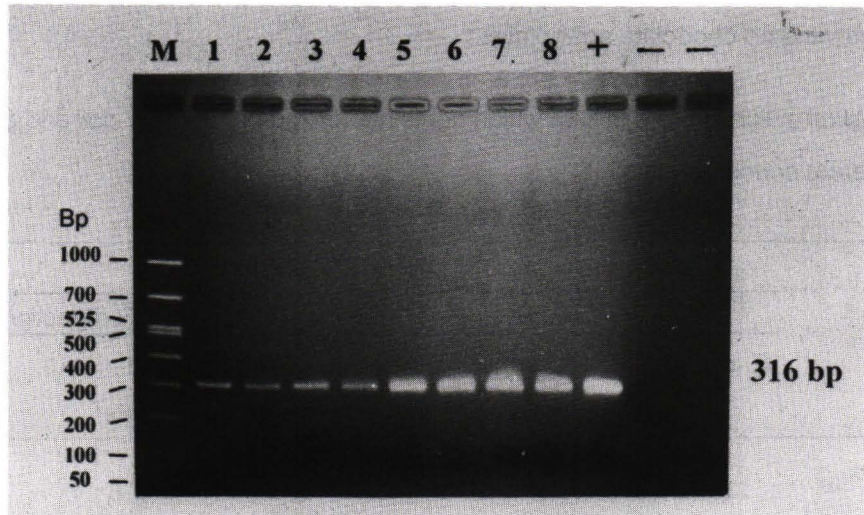


Figure 3. Sensitivity of detection by PCR-agarose gel electrophoresis for Hib seeded at 10^3 and 10^5 CFU/ml into CSF which contained mixed bacteria at different concentrations. Lane M contained DNA size marker, Gel Marker™ 50-1000 bp. Lanes 1 to 4 contained DNA of 10^3 CFU/ml of Hib with 10^3 , 10^4 , 10^5 , and 10^6 of mixed bacteria in CSF, respectively. Lanes 5 to 8 contained DNA of 10^5 CFU/ml of Hib with 10^3 , 10^4 , 10^5 , and 10^6 of mixed bacteria in CSF, respectively. Lanes 9 to 11 contained DNA of Hib ATCC 9795, *H. influenzae* type c ATCC 9007, and nuclease free water as positive control, negative control, and reagent control, respectively.

Table 3. The detection limit of Hib detection by PCR-ELISA and PCR-agarose gel electrophoresis compared to culture growing on chocolate agar plates.

(a) 10^5 CFU/ml of Hib seeded in CSF which contained mixed bacteria (*E. coli* and *S. aureus*) at different concentrations.

Organisms	Hib, 10^5 CFU/ml				
	Culture*			PCR detection by :	
	<i>E. coli</i>	<i>S. aureus</i>	Hib	Agarose gel electrophoresis	ELISA (OD 405 nm)
Mixed bacteria					
10^3 CFU/ml	+1	+2	+3	+	0.43
10^4 CFU/ml	+2	+3	+2	+	0.43
10^5 CFU/ml	+3	+4	+2	+	0.41
10^6 CFU/ml	+4	+4	+2	+	0.39

Table 3. The detection limit of Hib detection by PCR-ELISA and PCR-agarose gel electrophoresis compared to culture growing on chocolate agar plates.

(b) 10^3 CFU/ml of Hib seeded in CSF which contained mixed bacteria (*E. coli* and *S. aureus*) at different concentrations.

Organisms	Hib, 10^3 CFU/ml				
	Culture*		Hib	PCR detection by :	
	<i>E. coli</i>	<i>S. aureus</i>		Agarose gel electrophoresis	ELISA (OD 405 nm)
Mixed bacteria					
10^3 CFU/ml	+1	+1	±	+	.090
10^4 CFU/ml	+1	+1	NG	+	.067
10^5 CFU/ml	+2	+3	NG	+	.069
10^6 CFU/ml	+3	+3	NG	+	.062

NG: No growth.

± : only one colony was seen.

* Degree of growth on agar plates; +1 through +4 was graded from colony growth on streak lines, i.e. +4 means colonies can be seen from the first streak lines until the fourth streak lines, at least .

Discussion

At present, LA tests to detect the capsular polysaccharide antigen of Hib are frequently performed in most laboratories in Thailand and their use has become routine for evaluation of patients thought to have bacterial meningitis caused by Hib or other bacteria. The LA kits have the potential advantages of being rapid, technologically straightforward, specific, sensitive for detection of nanogram quantities of antigen, and positive despite prior antimicrobial therapy.^(3,4,17) However, there are several factors which limit the clinical utility of bacterial antigen tests and make their routine use controversial. Misleading positive LA test results are due to detection in urine or CSF of circulating Hib antigen

after vaccination with a conjugate Hib vaccine,^(7,18,19) contamination of urine with skin flora, and imperfect antigen specificity. Lack of specificity of the LA test has been noted in multiple previous publications in which the nonspecific agglutinations might result from infection or sample contamination with an organism bearing cross-reacting antigens.^(4,17,20,21)

There have been few studies of *H. influenzae* diseases in Thailand. From the existing reports, *H. influenzae* was the most common causative organism and the overall fatalities of childhood meningitis was 17.3% for patients who were admitted to Bangkok Children's Hospital during 1980-1990.⁽²²⁾ The latest document reported that 84% of bacterial meningitis during 1990 - 1995 was caused from Hib with the

incidence rate of Hib meningitis being 5.2-7.8 per 100,000 of the children under 5 years old.⁽¹¹⁾ However, the advent of routine immunization of infants with Hib conjugate vaccines may lead to a declining incidence of invasive Hib disease, and may even result in a lowering of the frequency of LA-detectable Hib vaccine antigen in CSF. If most laboratories in Thailand still use the LA test as a routine microbiological procedure for the rapid detection of Hib antigen in CSF, the false-positive LA test results may lead to additional costs, prolonged hospitalization, and some clinical complications.⁽²³⁾ However, most PCR has revolutionized molecular biology research and is currently considerably broadening the field of microbial diagnosis, including diagnosis of Hib infection. Most studies show that PCR can compete as an alternative diagnostic technique with culture and rapid antigen detection assays.

We had also developed a PCR assay using our designed primers⁽¹⁴⁾ based on the central sequence of *cap* gene; region 2 of the Hib which is 8.3 kb length. These sequences are involved in the biosynthetic step required for the formation of type b capsular polysaccharide.⁽¹³⁾ Our designed primer set showed high sensitivity and specificity in comparison to the results of culture and agglutination with Hib antiserum for detection of Hib culture isolates, and the sensitivities achieved with 2% AGE revealed the minimum limit of detection of 1 pg for this primer set based on 25 amplification cycles.

For post amplification detection, there are several different procedures for detection of the PCR products. The simplest version of product detection relies on agarose gel electrophoresis (AGE) which employs a hazardous chemical, ethidium bromide,

for staining of the DNA, and may be prone to nonspecific banding products needed to check the specificity of the PCR products by hybridization. However, this is not a desirable approach in a routine laboratory because the procedures are quite labor intensive and lengthy, which increases turnaround times as well as cost. Currently, the ELISA-based-method, a chromogenic detection system, is one of the most popular assays for post amplification detection. With this system, hazardous chemicals are avoided, the establishment of OD cutoffs removes subjectivity in reading results, it can be readily done on a large scale and the method is amenable to automation. Being a rapid, simple, and convenient procedure, ELISA makes this assay possible for general use in the microbiology laboratory or screening in epidemiologic studies.

In our study, a simple PCR-ELISA with-out hybridization steps was developed for Hib detection to generate a rapid, sensitive and economic assay for detection of Hib in Thailand. The assay designed in this study was modified from Landgraf's technique⁽¹⁵⁾ which also excluded a hybridization step, thus reducing time, labor, and cost. Our previous study⁽¹⁴⁾ found a perfect agreement (100%) between the PCR product detection by AGE and hybridization methods. This revealed that the analysis of Hib positive PCR products produced from highly specific primer pairs by AGE with DNA size of 316 bp was highly specific and sufficient to indicate the result without the need of DNA hybridization.

After optimizing the ELISA assay conditions, we found that it was necessary to increase the amplification cycles from 25 to 35 and increase the amount of PCR product to 10 μ l which produced more

favorable results and was also easier to differentiate between positive and negative PCR visually without the need of an ELISA reader. However, a non specific reaction was observed when 10 μ l of 35 amplification cycles PCR product was used. It was eliminated when blocking reagent (1% BSA in TBST buffer) was used as an additional step before adding PCR products but the reading time after adding the substrate should be extended to 1 h. The total ELISA assay time following PCR assay was between 2 to 3 h.

When the optimized ELISA condition was utilized to detect PCR products amplified from all bacteria seeded in CSF, the isolates with positive bands on agarose gel and positive slide agglutinations with Hib specific antiserum also exhibited an OD 405 value above the background level which resulted from negative isolates. This indicated that the PCR-ELISA can perform a distinctive analysis of Hib isolates from other types of *H. influenzae* and other bacterial strains. Using the cut off point at the lowest OD 405 value of positive group (cut off OD 405 nm = 0.15) as the criteria for positive Hib detection, there was only one questionable isolate which was identified as Hib negative by slide agglutination but Hib positive by PCR-AGE and PCR-ELISA. This condition was also found in the study of Falla et al. ⁽¹¹⁾ This isolate may be a capsule-deficient mutant of type b strain ⁽²⁴⁾ which can not produce positive reaction by Hib antiserum but can be detected by DNA-based assay. ^(11,24)

CSF is known to contain inhibitors of *Taq* DNA polymerase but the inhibitory constituents have not been characterized. That may be caused of differences between CSF samples and/or differences in numbers of organisms existing in CSF. The sterilized CSF sample pooled from 25 patients aged

under 5 years old was used as a representative of CSF sample in this study. In fact the CSF specimens may also be contaminated with other bacteria during collection of the specimen or from specimen containers and during storage of specimen before delivery to the laboratory. Most of the bacterial species contaminated in the specimen in hospital are *S. aureus* and *E. coli*. ⁽²⁵⁾ Thus in this study, these bacterial species were selected for making of artificial contamination to the CSF.

For the detection limit of Hib seeded in sterile CSF, we found that PCR-ELISA was 10 times less sensitive than PCR-AGE even though the same amount of PCR product, 10 μ l was used. This low sensitivity of PCR-ELISA may be explained by the design of the indicator system with only 1 molecule of fluorescein labeled at the 5'end of the primer. This is because the signal of this system depends on the amount of anti-fluorescein-AP conjugate that can be immobilized by incorporating with the fluorescein molecule labeled on PCR product. Thus anti-fluorescein incorporating with one labeled fluorescein can not amplify the signal when AP conjugated anti-fluorescein is detected by colorimetric substrate. Both PCR-ELISA and PCR-AGE also showed superior benefits for detection of Hib seeded in contaminated CSF where the culture result was negative.

In conclusion, the PCR-ELISA test developed in this study shows rapidity, simplicity, high sensitivity and specificity and it could be able to detect a capsule-deficient mutant of type b strain which is indistinguishable from nontypeable strains by traditional serotyping techniques. However, this assay has not been applied in clinical samples yet where the results may be different from the results in this study.

We are continuing our studies in order to evaluate the usefulness of this PCR-ELISA assay for the rapid detection of Hib in clinical CSF samples from young patients with meningitis that will give a clearer indication of feasibility in using this assay protocol on a routine basis for epidemiology or surveillance of Hib diseases.

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