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ORIGINAL PAPERS

Rapid Detection of *Haemophilus influenzae* Type b in Bangladeshi Children with Pneumonia and Meningitis by PCR and Analysis of Antimicrobial Resistance

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ABSTRACT

A polymerase chain reaction (PCR) assay with primers from *'bexA'* gene was compared with culture for the detection of *Haemophilus influenzae* type b (Hib) in clinical samples from children with pneumonia and meningitis. Of 200 sera (180 from pneumonia, 20 from non-pneumonia patients) tested by PCR (serum-PCR), Hib was detected in 15 of 16 blood culture-positive and in 6 blood culture-negative pneumonia cases. When compared with the results of blood culture, serum-PCR had sensitivity, specificity, and accuracy index of 93.7%, 96.7%, and 96.5% respectively. Of 120 cerebrospinal fluid (CSF) samples from meningitis patients tested by culture and PCR (CSF-PCR), the latter method could detect Hib in all 15 culture-positive and in 8 of 105 culture-negative cases, showing sensitivity, specificity, and accuracy index of 100%, 92.4%, and 94.4% respectively. The PCR result was available within a day. Antimicrobial susceptibility of Hib was determined by the disc-diffusion method. High rate of resistance to ampicillin (54.8%), chloramphenicol (48.4%), and co-trimoxazole (80.6%) was observed among 31 invasive Hib isolates with resistance to all 3 drugs (multiresistance) in 48.4% of the isolates. All the Hib isolates were susceptible to ceftriaxone. The study has shown that PCR is a rapid, sensitive and specific diagnostic test for Hib from clinical samples, and a combination of culture and PCR is necessary for the detection of Hib infections to the maximum extent for case management to reduce morbidity, mortality, and complications of the invasive Hib infections. A high prevalence of multiresistant Hib strains is a matter of concern.

Key words: *Haemophilus influenzae*; Pneumonia; Meningitis; Polymerase chain reaction; Culture media; Antibiotic resistance; Drug resistance, Microbial; Serum; Cerebrospinal fluid; Bangladesh

INTRODUCTION

Haemophilus influenzae type b (Hib) is an important cause of meningitis, community-acquired pneumonia, and septicaemia, resulting in significant morbidity and mortality in children aged less than five years in Bangladesh and other countries where children are not vaccinated against Hib (1-2). A recent retrospective hospital-based study in a children hospital in Dhaka, Bangladesh, during 1987-1997 showed that there was an increase of 700% in the annual number of *H. influenzae* isolates from cerebrospinal fluid (CSF) of meningitis patients (1). The exact burden of invasive Hib diseases in Bangladesh is unknown, since optimum and rapid laboratory facilities are not routinely available for detecting aetiologic agents of meningitis and pneumonia–two most important diseases caused by Hib. In many industrialized countries, the incidence of

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invasive Hib diseases has fallen sharply with the introduction of Hib conjugate vaccines into routine immunization programmes (3). Despite the success of the Hib vaccination programme, the re-emergence of invasive Hib diseases has been noted in a well-vaccinated population, implying the necessity for continuous surveillance in the post-vaccination period (4).

Blood cultures remain the gold standard with which the effectiveness of other techniques is compared, and positive cultures may be as low as 20% in case of patients with pneumonia due to Hib. The figure may even be as low as about 10% in paediatric populations having the highest morbidity and mortality rates (5-7). Secretions from the lower respiratory tract are difficult to obtain and are often contaminated with upper respiratory tract flora, making interpretation of culture results difficult for the diagnosis of Hib infections. Attempts to diagnose Hib infections by detecting antibody responses to the type b capsular antigen have been reported from several countries (2,5-7), but the sensitivity and specificity of this method are unknown. The clinical and epidemiological value of this approach is also limited by the need to obtain convalescent sera from young children.

Diagnosis of acute bacterial meningitis by direct microscopy is rapid but non-specific and has a low sensitivity. On many occasions, it fails to provide definitive evidence of infection due to a particular species. Traditional laboratory diagnostic methods of culture of CSF take up to 36 hours or more and may show negative results in approximately 30% of cases due to antibiotic treatment prior to sampling (8). The need for rapid, sensitive and specific methods for the diagnosis of meningitis and pneumonia due to Hib is becoming more urgent, since strains of Hib, resistant to ampicillin, chloramphenicol, and co-trimoxazole, emerged in different parts of the world, questioning the appropriateness of empirical antimicrobial therapy. Moreover, the estimation of burden of the Hib diseases and the efficacy of Hib vaccination are imprecise without an optimum diagnostic test.

The development of a PCR assay for target gene amplification has enabled the detection of low numbers of pathogens in clinical samples (9-10). We carried out the study to detect Hib directly from clinical samples of patients with pneumonia and meningitis by culture and PCR assay, and to assess antibiotic resistance patterns among Hib isolates in Bangladesh.

MATERIALS AND METHODS

Study population

The study population included children, aged less than five years, admitted to the Clinical Research and Service Centre of ICDDR,B: Centre for Health and Population Research, Dhaka, and to a general hospital in Dhaka. The patients were categorized into three groups: 180 pneumonia cases, 20 non-pneumonia cases (9 febrile, 11 diarrhoeal patients), and 120 meningitis cases according to their clinical illnesses. Children were assessed for pneumonia according to the WHO criteria: cough or difficult breathing and a respiration rate of 50 breaths per minute or more in children aged 2-11 months or 40 breaths per minute or more in children aged 12-59 months. A child with signs and symptoms of meningitis, such as fever, lethargy, convulsion, bulged fontanelle, neck rigidity, etc., was considered a case of meningitis, and a spinal tap on that child was obtained.

Clinical samples

Three types of clinical samples were collected for the detection of Hib. These were blood and serum from pneumonia and non-pneumonia patients for culture and PCR respectively and CSF from suspected meningitis patients for both PCR and culture.

Microbiological identification of *H. influenzae* type b

Blood samples (1-2 mL, one sample per patient) were obtained from patients by venipuncture and inoculated directly into 20 mL of trypticase soy broth containing sodium polyanthol sulfonate (Becton Deckinson, Cockeysville, MD, USA) for aerobic cultures at 37 °C. Patients with suspected bacterial meningitis were subjected to lumber puncture for collecting CSF. Blood culture broth showing haemolysis, turbidity, or gas and all CSF were cultured on a supplemented chocolate agar (blood agar base with 2% haemoglobin and 1% isovitalex containing X and V factors) plate. The suspected bacterial colonies were identified by typical Gram stain morphology, catalase, oxidase, and requirement tests of X and V factors (Difco, Detroit, MI, USA). The *H. influenzae* isolates were serotyped by slide agglutination using Hib-specific antiserum (Denka-Seiken Co. Ltd, Tokyo, Japan). Antimicrobial susceptibility of *H. influenzae was* determined by the disc-diffusion method using Mueller-Hinton agar II supplemented with 1%

haemoglobin, X and V factors, and commercial antibiotic discs (Becton Deckinson, Cockeysville, MD, USA): ampicillin (10 μg), chloramphenicol (30 μg), co-trimoxazole (25 μg), and ceftriaxone (30 μg). Blood culture broth showing no haemolysis, turbidity, or gas was subcultured on supplemented chocolate agar on day 7 of incubation.

Detection of *H. influenzae* type b by PCR

DNA extraction from clinical samples

Serum samples: Serum was collected from 1 mL of blood. DNA was extracted from the serum samples following the procedure of Salo *et al.* (11). Serum (100 μL) was mixed with 100 μL of 0.5% sodium dodecyl sulphate (SDS) solution containing 0.1 M NaOH, and 2 M NaCl. The mixture was incubated for 15 minutes at 95 ˚C in a hot dry bath; 100 μL of 0.1 M Tris-HCl (pH 8.0) was added to the mixture. The mixture was vortexed for 30 seconds. DNA was extracted with phenol:chloroform:isoamyl alcohol in the proportion of 25:24:1 (Sigma, St. Louis, MO, USA). Finally, DNA was precipitated with cold isopropanol (stored at -20 ˚C). It was dissolved in 30 μL of sterile, deionized water, and 15 μL was used as template DNA for PCR (12).

CSF specimens: One hundred μL of CSF was taken in a microcentrifuge tube and was heated in a boiling water bath for 10 minutes and centrifuged at 14,000 rpm for 30 seconds. Fifteen μL of supernatant was used as template DNA (13).

PCR primers

The selection of primers was based on published work of Hassan-King *et al.* (14). The primers were constructed from the *bexA* gene-encoding capsulation-associated Bex A protein, most probably involved in the intracellular transportation of capsular polysaccharide (15). The primers for detecting Hib were Hib 1 [GCG-AAA-GTG-ACC-TCT-TAT-CTC-TC] and Hib 2 [GCT-TAC-GCT-TCT-ATC-TCG-GTG-AA], and were similar and complementary to nucleic acid sequence of conserved regions of the *bexA* gene specific for b serotype of *H. influenzae*. Both the primers were of 23 bases.

PCR assay

PCR was performed in a reaction mixture of 100 μL containing 50 mM KCl, 100 mM Tris-HCl (pH 8.3), 5 mM $MgCl_2$, 0.01% (W/V) gelatin, 200 μM of dNTPS each, 2.5 U of *Taq* polymerase (Perkin-Elmer Cetus) per reaction tube, and primers. The reaction mixture was overlaid with 1 or 2 drop(s) of mineral oil and processed in a programmable DNA thermal cycler (Perkin Elmer Cetus Corp., Norwalk, Conn, USA) consisting of 40 cycles with the following parameters: (a) denaturation for two minutes at 95 ˚C, (b) annealing for two minutes at 45 ˚C, and (c) extension of primers for two minutes at 72 ˚C. At the end of 40 cycles, the samples were incubated for further 10 minutes at 72 ˚C and stored at 4 ˚C until these were analyzed. PCR products (480 bp) for Hib were detected by 1.5% agarose gel electrophoresis.

RESULTS

Over a period of one year (April 1998-March 1999), 320 children, aged less than five years, were included in the study. Two hundred blood samples (180 from pneumonia, 20 from non-pneumonia patients) and 120 CSF specimens from suspected meningitis cases were cultured. Hib was isolated from 16 of the 180 (8.9%) blood cultures from pneumonia patients, none from non-pneumonia, and from 15 of the 120 CSF (12.5%) cultures. Of the 31 Hib isolates tested for antimicrobial susceptibility, 6 (19.3%) were susceptible to ampicillin, chloramphenicol (Cm), co-trimoxazole (Sxt), and ceftriaxone. Co-trimoxazole resistance was detected in 25 (80.6%), ampicillin (Ap) in 17 (54.8%), and chloramphenicol in 15 (48.4%) isolates. All the isolates were susceptible to ceftriaxone. Three types of resistance patterns (R types) were observed. Eight (25.8%) Hib isolates were resistant only to co-trimoxazole and two isolates (6.4%) to both ampicillin and co-trimoxazole (R type ApSxt). Fifteen isolates (48.4%) were simultaneously resistant to choramphenicol, ampicillin and co-trimoxazole (R type CmApSxt).

DNA was extracted from 200 serum (180 from pneumonia, 20 from non-pneumonia) and 120 CSF samples. Of the 180 sera from pneumonia patients, 21 (11.7%) were serum-PCR-positive for Hib. PCR products (480 bp) from serum specimens are shown in the figure. None was positive for Hib among 20 sera from the non-pneumonia patients by PCR. Of the 120 CSF samples, 23 (19.2%) were CSF-PCR-positive for Hib. When compared with blood culture from the pneumonia cases, serum-PCR could detect Hib in 15 of the 16 blood culture-positive samples. Of the 184 culture-negative samples, six were serum-PCR-positive, and three of them received antibiotics. Thus, serum-PCR detected six more Hib compared to the culture method (Table 1). Fifteen of the 120 CSF samples were positive

for Hib by both culture and CSF-PCR. CSF-PCR also detected Hib in 8 (7.6%) of 105 culture-negative CSF samples, and two of them received antibiotics (Table 1).

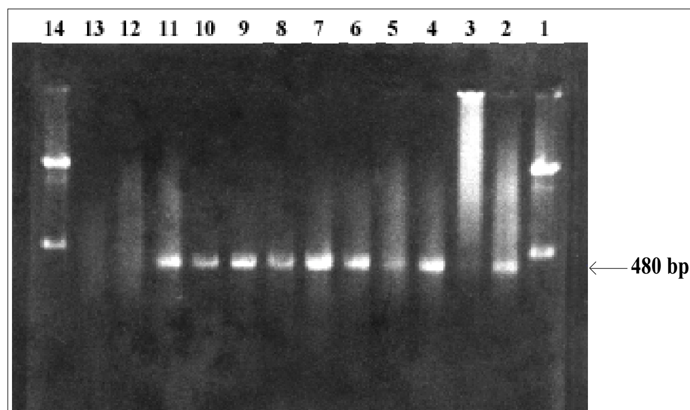

Fig. Agarose gel electrophoresis of PCR products from serum samples. Lane 1,14=molecular weight marker (100 bp ladder), lane 2-11=culture-positive samples, lane 12-13=culture-negetive samples

Table 1. Results of culture and PCR for detection of *H. influenzae* type b in clinical samples of children with pneumonia, non-pneumonia, and meningitis

Disease (no. of patients)	Clinical samples (no.)	Culture results		PCR results	
		Positive	Negative	Positive	Negative
Pneumonia (180)	Blood* (180)	16	164	ND	ND
	Serum* (180)	ND	ND	21	159
Non-pneumonia (20)	Blood* (20)	0	20	ND	ND
	Serum* (20)	ND	ND	0	20
Meningitis (120)	Cerebrospinal fluid (120)	15	105	23	97

* Blood was cultured, and serum, instead of blood, was tested by PCR for *H. influenzae* type b in children with pneumonia and non-pneumonia

ND=Not done

Compared to the blood culture results, serum-PCR yielded six false-positive and one false-negative results (Table 2). The sensitivity of serum-PCR was 15 (93.7%) of 16 for a positive Hib culture, and the specificity was 178 (96.7%) of 184 for a culture-negative Hib. The test had a positive predictive value for 15 (71.4%) of 21 and a negative predictive value for 178 (99.4%) of 179. The accuracy index was 193 (96.5%) of 200.

Compared to the CSF culture results, CSF-PCR yielded 8 false-positive and no false-negative results (Table 3). The sensitivity and specificity of CSF-PCR were 100% and 92.4% respectively.

DISCUSSION

PCR is increasingly being used in clinical microbiology laboratories for the detection of agents of infectious diseases, since the conventional diagnostic methods, such as microscopy, culture, and serology, fail to identify responsible pathogens in many occasions (16). In the present study, we found PCR to be a useful technique for the detection of Hib capsular DNA in the serum and CSF samples from pneumonia and meningitis patients respectively. The *bexA* gene which encodes Hib capsulation-associated protein has been used as target DNA for amplification by PCR. The primers were sensitive and specific for the detection of Hib in the clinical samples. The selected primers showed excellent specificity with the non-Hib strains. PCR was also negative with other common agents of pneumonia and meningitis. It provided a result within a day from the clinical samples, whereas the culture methods took up to 36 hours or more. In the present study, the DNA extraction method of Dagan *et al.* (17) was used for the serum samples. However, the yield of bacterial DNA was low. The technique of DNA extraction of Salo *et al.* (11) was used subsequently for extracting DNA from the serum samples, which produced satisfactory results.

Pneumonia is the most common fatal infection in the world. No aetiologic agent can be identified in more than 35% of cases of community-acquired pneumonia, using conventional diagnostic methods, such as culture and serology (16). We used PCR in search of a better diagnostic method for pneumonia. Serum-PCR increased the detection of Hib in 3.6% of the pneumonia cases. It was negative in one culture-positive case. It is possible that the serum-PCR-negative samples might have contained PCR inhibitors (haeme, etc.) that interfered with DNA amplification. PCR was negative in 20 non-pneumonia patients showing its good specificity. Hassan-

King *et al.* also found similar results and detected 11 more cases of pneumonia due to Hib that were negative by culture (10). The sensitivity of PCR was 93.7%, and

Table 2. Comparison of results of PCR from serum with blood culture for detection of *H. influenzae* type b from patients with pneumonia and non-pneumonia

PCR	Culture		Total
	Positive (n=16)	Negative (n=187)	(n=200)
Positive	15	6	21
Negative	1	178	179

The sensitivity of PCR from serum was 15 (93.7%) of 16 for a culture-positive *H. influenzae* type b (Hib), and the specificity was 178 (96.7%) of 184 for a culture-negative Hib. The test had a positive predictive value for 15 (71.4%) of 21 and a negative predictive value for 178 (99.4%) of 179. The accuracy index was 193 (96.5%) of 200.

the specificity was 96.7% compared to the culture method. This finding is similar to the results of other studies (10-11). In fact, six samples that were positive for Hib by PCR and negative by culture are likely to be true positive, since PCR is found to be 100% specific and sensitive for detecting known culture of Hib and non-Hib pathogens of pneumonia and meningitis. PCR was also negative in all negative control sera.

The limitation of the study is that we used agarose gel electrophoresis for screening PCR products, which appears to be a less-sensitive method for detecting PCR products than hybridization (12). DNA extraction by a commercial kit may also increase the yield of DNA sufficiently for PCR amplification. The use of serum samples as a source of Hib template DNA might have contributed to the lower sensitivity of the PCR test in our study. However, Salo *et al*. found serum sample to be a good source of pneumococcal template DNA for amplification of pneumolysin gene by PCR (11).

It has been observed in our clinical microbiology laboratory, as in many other laboratories (8,9), that there is a growing discrepancy between the numbers of clinically-suspected and culture-confirmed cases of bacterial meningitis. To address this problem, non-culture method, such as CSF-PCR, has been employed and shown to detect 7.6% additional cases of Hib meningitis in our study. CSF-PCR showed a sensitivity of 100% compared to the conventional culture method. Thus, the sensitivity of CSF-PCR was superior to that of bacterial culture. It is a good diagnostic test for the detection of Hib in the CSF samples. It is relatively simple and requires a short period of time compared to culture. However, the determination of antibiotic susceptibility of Hib isolates is a problem in the PCR technique. Further development of a multiplex PCR for detecting both Hib and antibiotic resistance genes would be extremely useful (18). The limitation of CSF-PCR results is that, in our study, we did not evaluate other diagnostic tests for Hib, such as Gram stain, cytology, and biochemical analysis of CSF, and latex agglutination for Hib in CSF.

Both culture and PCR test detected Hib in the blood and CSF samples. We did not detect other serotypes of *H. influenzae* by culture from blood and CSF in our samples, suggesting that Hib is the primary cause of invasive infections, such as meningitis and pneumonia, among children in Bangladesh.

Following diagnosis of meningitis and pneumonia, administration of early therapy with a proper antimicrobial agent is the key factor to reduce mortality and morbidity and sequelae of invasive Hib diseases, particularly meningitis. We found a high rate of resistance to co-trimoxazole (80.6%), ampicillin (54.8%), and chloramphenicol (48.4%) among the invasive Hib isolates in our study. A high multiresistance to conventional first-line antibiotics among Hib isolates is a matter of great concern. However, no resistance to third-generation cephalosporin, such as ceftriaxone, was observed among the Hib isolates in our study. Thus,

Table 3. Comparison of results of PCR with that of culture for detection of *H. influenzae* type b from cerebrospinal fluid of suspected meningitis cases

PCR	Culture		Total
	Positive (n=15)	Negative (n=105)	(n=120)
Positive	15	8	23
Negative	0	97	97

The sensitivity of PCR from CSF was 15 (100%) of 15 for a culture-positive *H. influenzae* type b (Hib). The specificity was 97 (92.4%) of 105 for a culture-negative Hib. The test had a positive predictive value for 15 (65.2%) of 23 and a negative predictive value for 97 (100%) of 97. The accuracy index was 112 (93.3%) of 120.

ceftriaxone appears to be the drug of choice for the treatment of invasive Hib infections in Bangladesh, particularly in the case of meningitis. Multiresistant strains of Hib were recently reported in India (20).

A rapid, sensitive, and specific PCR test will be useful for early case detection and proper management of Hib

infections to reduce high morbidity and mortality. However, the emergence of multiresistant Hib strains cause increasing difficulties in selecting proper antibiotics for the treatment of pneumonia and meningitis. A real-time multiplex PCR that provides a result in 30 minutes is likely to offer physicians a powerful new weapon for simultaneous detection of Hib and antibiotic resistance of genes promptly and directly in clinical samples in the near future (18-19). Thus, a rapid, low-cost, sensitive, and specific multiplex PCR gives the hope of improved management of meningitis and pneumonia in children.

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