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Short Communication

Biotyping, Capsular Typing, and Antibiotic Resistance Pattern of *Haemophilus influenzae* Strains in Iran

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SUMMARY: The aim of this study was to determine the capsular types of *Haemophilus influenzae* isolated from clinical specimens by slide agglutination serotyping (SAST) and PCR capsule typing methods. All the isolates were biotyped and their antibiotic resistance patterns also determined. Thirteen isolates of serotype b, 2 of serotype e, 4 of serotype f, and 19 nontypeable (NT) isolates were identified by SAST method in 38 *H. influenzae* culture-positive samples. Capsule typing by PCR increased the proportion of all invasive cases from 34.2% (by SAST) to 60.5%, and 6 culture-negative samples were identified as invasive *H. influenzae* (Hib) by this method. The discrepancy rate between SAST and PCR results were 41%. Biotypes I, II, and III were the prevalent biotypes whereas biotypes VI and VII were not found. The majority of capsule type b belonged to biotype II. The isolates were resistant to cotrimoxazole (47.1%) and ampicillin (43.6%). Multidrug resistance was observed in 7 of the isolates.

A number of available reports in Iran have indicated *Haemophilus influenzae* type b (Hib) to be responsible for meningitis, mainly in children below 4 years of age. These reports have been stated detection of Hib capsular isolated by traditional serotyping and PCR typing methods, mainly from the cerebrospinal fluids (CSF) of children suspected of having meningitis (1,2). However, increasing reports on cases of invasive disease due to nontypeable (NT) and non-Hib encapsulated strains have urged the need for a careful determination of capsule type (3,4).

For efficient diagnosis of encapsulated and nonencapsulated *H. influenzae*, standard slide agglutination serotyping (SAST) has normally been used (5). A number of inconsistencies arising for SAST results have urged the need for an effective and reliable capsular typing strategy. The detection of capsule type specific genes and a capsule-negative phenotype known as Hib-minus with a PCR approach has proved to be a highly specific and reliable method for capsular typing of the *H. infulenzae* isolates (4,6). In this study, we evaluated the accuracy of SAST by comparing the above-mentioned conventional serotyping method (SAST) with the PCR capsule typing method.

Initially, *H. influenzae* strains were isolated from CSF from children with meningitis, blood from patients with sepsis, eye mucus from patients with conjunctivitis, and nasopharyngeal specimens from individuals without meningitis. Thirty-eight (31.4%) of the 121 specimens were culture positive for *H. influenzae*, based on their morphology and growth requirements for hemin (factor X) and nicotinamide adenine dinucleotide (NAD or factor V). All the isolates were serotyped by SAST with *H. influenzae* polyvalent and serotype a to f specific antiserums (BD Biosciences, Franklin Lakes, N.J., USA) according to manufacturer's instructions. As seen in Table 1, 13 (34.2%) of the 38 *H. influenzae*-positive samples had positive agglutination reactions with type b specific antiserum and were recorded as Hib, while 4 isolates were typed as f and 2 as type e. Nineteen (50%) of the isolates were reported to be NT and the remaining 19 (50%) were encapsulated by this method. None of the isolated strains were serotyped as type a, c, or d by SAST method.

In the PCR assay, DNA primers targeting all 6 capsule-specific (*cap*) genes and the capsule export gene (*bexA*) as described by Falla et al. (3) were used. Isolates containing PCR products for both a *cap*-specific gene (a, b, c, d, e, or f) and the *bexA* gene were designated as the specific capsule type; those containing a *cap* gene but not *bexA* were designated as capsule-deficient variants or capsule-minus strains (e.g., Hib-minus). Isolates lacking both *bexA* and *cap* genes were considered to be NT *H. influenzae*. Confirmation of the primary product was performed by a second round of PCR with the third internal primer and one of the first round pair of primers.

The prevalence rate of different serotypes of *H. influenzae* by PCR was 52.2% for capsule type b, 38.6% for NT *H. influenzae*, 5.2% for capsule type f, and 5.2% for capsule deficient mutants (Hib-minus). Table 1 shows that capsule typing by PCR increased the proportion of all invasive cases from 34.2% (by SAST) to 60.5%; a difference of 26.3%. Six of the specimens that appeared as negatives in the culture method were positive by PCR analysis. Moreover, 2 Hib-minus variants were identified by the presence of Hib-specific capsule genes and the absence of the *bexA* gene by PCR amplification.

According to the results of the PCR capsule typing

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Table 1. Serotypes of *H. influenzae* isolated from clinical samples by SAST and PCR method

Sample	SAST			PCR		
	Hib	NTHi	Other type	Hib	NTHi	Other type
Serum	2	4	2 f	6	3	0
CSF	10	2	0	17	0	0
EM	—	0	1 e	0	0	1 f
NP	1	13	1 e, 2 f	0	14	1 f, 2 Hib$^-$ [1)]
Total	13	19	6	23	17	4
	38			44		

[1)]: *H. influenzae*-minus variant.
CSF, cerebrospinal fluid; EM, eye mucus; NP, nasopharynx secretions; Hib, *Haempohilus influenzae* type b; NTHi, nontypeable *H. influenzae*; e, *H. influenzae* type e; f, *H. influenzae type f.*

Table 2. Discrepancy rate between SAST and PCR results

No.	SAST	PCR	Total % discrepancy
1	1 b	1 NTHi	
2	2 e	1 f and 1 Hib$^-$	
3	3 f	3 NTHi	40.9
4	6 NTHi	5 b and 1 Hib$^-$	
5	6 –	6 b	

method, 19 NT isolates by SAST were NT *H. influenzae*, while 5 of the 6 serologically NT *H. influenzae* isolates gave PCR products consistent with an encapsulated genotype. Of the 19 isolates originally serotyped as encapsulated by SAST, 4 (1 of type b and 3 of type f) were shown to be NT *H. influenzae* by this method. Of the 19 unencapsulated (NT *H. influenzae*) by SAST, 5 were found to be of type b and 1 Hib-minus variants by PCR. One serologically type e from eye mucus contained the type f-specific capsule gene and was confirmed as capsule type f. Moreover, 6 culture-negative samples were identified as invasive *H. influenzae* (Hib) by PCR. None of the capsular types a, c, d, or e was found by PCR assay.

Table 2 shows the disagreement rate between the SAST and PCR methods in the serotyping of the *H. influenzae* isolates. According to the results, the discrepancy rate between the two methods was estimated as 41%.

The species *H. influenzae* has been subdivided into 8 biotypes or biovars on the basis of urease, ornithine decarboxylase activities, and indole production (7,8). In our results, biotype II (42.1%) was the dominant biotype followed by biotype I (18.4%), III (15.7%), VIII (10.0%), IV (7.8%), and V (5.2%). Overall, 7 of the isolates were biotype I, 16 biotype II, 6 biotype III, 3 biotype IV, 2 biotype V, and 4 isolates were biotype VIII. Biotype and strain isolation origins were characteristically associated. Table 3 shows the chronological distribution of biotypes based on different serotypes. Most of type b belonged to biotype II, whereas type f isolates belonged to type III. Biotypes VI and VII were not found in this study.

Antibiotic resistance in *H. influenzae* is more diverse and widespread than is commonly appreciated, and continued surveillance of antimicrobial susceptibility is

Table 3. Chronological distribution of biotypes based on serotypes of *H. influenzae* isolates

No.	Serotype	Biotype								Total
		I	II	III	IV	V	VI	VII	VIII	
1	Type b	4	10	3	0	0	0	0	0	17
2	Type f	0	0	2	0	0	0	0	0	2
3	Hib$^-$	0	2	0	0	0	0	0	0	2
4	NTHi	3	4	1	3	2	0	0	4	17
Total		7	16	6	3	2	0	0	4	38

recommended in order to monitor resistance trends and identify emerging resistance early (9,10). In our studies, we observed a high rate of antibiotic resistance in the isolated *H. influenzae* strains to cotrimoxazole (47.1%), ampicillin (43.6%), and tetracycline (38.28%), and the phenomenon of multi resistance was seen in 7 (18.4%) of the isolates, which were resistant (non-susceptible) to 3 or more antibiotics.

The burden of Hib disease in countries where children are not vaccinated against Hib is increasing drastically. Iran is at present among the countries which lacks a routine Hib vaccination program and thus increasing incidence of Hib meningitis, resulting in significant morbidity and mortality in children aged less then 5 years is reported. Moreover, with the increasing reports of antibiotic resistance among these isolates, the need for immediate immunization mainly children and the immune susceptible is highly required. Although the number of samples was low, the results of this study can be considered indicative of the prevalence of the disease in the country and the necessity for vaccination against the disease. The other serotypes of *H. influenzae*, namely serotype f, NT *H. influenzae*, and Hib-negative variant isolated in this research, also require further detailed investigation to determine the possibility of their involvement in disease.

Conflict of interest None to declare.

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