

COMPARISON OF TWO SLIDE AGGLUTINATION SEROTYPING METHODS AND PCR-BASED CAPSULE TYPING FOR THE CHARACTERIZATION OF *HAEMOPHILUS INFLUENZAE* SEROTYPES

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SHORT COMMUNICATION

ABSTRACT

Ninety-three nasopharyngeal *Haemophilus influenzae* isolates were serotyped by two slide agglutination methods (SAST 1 and SAST 2) and the results compared with those obtained by capsule type-specific PCR. SAST 1 presented a low correlation with results obtained by PCR (75.2%) while SAST 2 showed a better agreement with the molecular technique results (93.5%). These findings suggest that SAST 2 could be an alternative method for adequate detection of *H. influenzae* type b.

Key words: *Haemophilus influenzae*, serotyping methods, PCR

Widespread use of *Haemophilus influenzae* type b (*Hib*) conjugate vaccines in industrialized countries has led to nearly complete elimination of *Hib* invasive diseases (6,9,12). However, *H. influenzae* (*Hi*) remains an important etiological agent of meningitis, septicemia and pneumonia in infants from countries where the *Hib* vaccine is not accessible (9). In regions where the availability of *Hib* conjugate vaccines is low, attendance at a day-care center (DCC) is recognized as a risk factor for invasive *Hib* disease (11). DCC was and still is considered a risk factor, as reflected by the increased opportunities for the sharing of respiratory pathogens between children in such settings (3,5,8).

Hi can express six capsule polysaccharides (serotypes a to f), traditionally identified by slide agglutination serotyping (SAST). Recently however, cases of misidentification of *Hi* serotypes by this method have been reported (1,7). To help to overcome this mishap, a SAST method (SAST 2) has been developed in the Adolfo Lutz Institute (1).

A PCR capsule typing method, developed by Falla *et al.* (4), has been shown to present higher sensitivity and specificity than SAST. The first PCR reaction detected the *bexA* gene responsible for the transport of capsular material; its presence or absence determines whether an isolate is encapsulated or nonencapsulated and therefore, nontypeable (*NTHi*). The second PCR assay using capsule type specific primers (a to f) is then used to determine the capsule types of the isolates (1,4,7). The aim of the present study was to verify the discrepancy between slide agglutination methods and the PCR method in the identification of the serotypes of *Hi* isolates from healthy carriers of the bacteria.

Between November 2002 and November 2003, a total of 114 children, of either sex aged from 18-36 months, attending three municipal DCCs in Ribeirão Preto, State of São Paulo, were recruited and specimens collected. The research protocol was approved by the Ethics Committee of the School of Dentistry of São Paulo University at Ribeirão Preto, and the study was

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initiated with the agreement of the Municipal Service of Health and Education and the approval of the DCC managers. Signed consent was obtained from parents or guardians of the subjects.

Nasopharyngeal samples obtained by using a twisted rayon tipped swab applicator (Copan, Brescia, Italy) in Amies media without charcoal, and plated on the same day, on a selective medium of 10% sheep blood-chocolate brain heart infusion agar plates, supplemented with 300 mg/L bacitracin (Difco Laboratories, Detroit, Michigan, USA). After incubation under a CO₂-enriched atmosphere at 36°C, plates were inspected for growth of *Hi*. Isolates were identified by colony and cell morphology and by the demonstration of growth requirements for Y and X factors (2). All strains were kept frozen at -80°C in brain heart infusion broth (Difco) with 40% glycerol, until further analysis. Isolates were recovered from frozen samples by subculture on chocolate agar plates. Chromosomal DNA was obtained by the Pitcher *et al.* method (10). Briefly, broth cultures were harvested at the end of the exponential growth phase by centrifugation, pellets were resuspended in Tris-HCl / EDTA buffer and cells were lysed with guanidium thiocyanate (Sigma) plus sarkosyl. DNA was extracted with ammonium acetate and cold isopropanol. A rapid DNA extraction method (7) was also performed; both DNA obtained were used for the PCR reactions to check results.

The isolates were blindly tested by three methods (SAST 1, SAST 2 and PCR). Each isolate was pre-tested with a 0.85% NaCl solution containing 0.5% formol to prevent autoagglutination. Monovalent serotype-specific rabbit antisera (specific for serotypes a through f), (Difco) were utilized. Method 1 (SAST 1) that uses a serotype b-specific antiserum for screening, was performed by suspending a full loop of bacterial growth into 50 µl of a serotype b-specific antiserum, and thoroughly mixed by

rocking the slide for 1 min. In this method, the serotype b-specific antiserum was used for the initial screening; a positive agglutination result was considered to indicate serotype b. If the isolate was not reactive, it was tested with the remaining antisera (a through f). When a negative reaction towards all antisera in the set was observed, the strain was considered *NTHi*. Method 2 was performed with all antisera in parallel, as indicated in the Difco serological antiserum sheets: initially with *Hi* polyvalent antiserum and afterwards with *Hi* monovalent antisera type a to f, in parallel. For both SAST methods, the intensity of the reaction was recorded by symbols representing the absence of, or different grades of agglutination: (-, negative; +, slow and weak; +++++, rapid, with formation of large clumps). Positive agglutination was defined as the production of large clumps (+++ or +++) with one specific antiserum only. The isolate was considered *NTHi* when negative agglutination results with the complete set of antisera or weak reactions with at least two antisera (nonspecific reaction) were observed. When positive agglutination occurred with at least two specific antisera, the isolate was considered polyagglutinated (1).

Primers specific for the *bexA* gene (primers H1 and H2) (4), required for capsular export, were used to differentiate *NTHi* from encapsulated isolates. Primers and PCR conditions have been previously described (4,7).

Table 1 shows the prevalence of capsule types found among *Hi* by the three methods studied. Significant differences in the prevalence rates were found for capsule type b SAST 1 (25.8%) versus SAST 2 (1.1%) and PCR (1.1%) respectively. Also, divergent rates of *NTHi* isolates were identified by SAST 1 (74.1%) compared with SAST 2 (72.1%) and PCR (93.5%). Serotypes d, e and f were identified by SAST 2 but not by

Table 1. Comparison between the frequencies of capsule types for 93 *H. influenzae* nasopharyngeal isolates obtained from children attending day-care centers in Ribeirão Preto from November 2002 to November 2003, according to the SAST 1, SAST 2 and PCR methods.

<i>H. influenzae</i> capsule type	No. (%) of isolates with the indicated capsule type by:			No. (%) of isolates which PCR results agree with:	
	SAST method 1	SAST method 2	PCR	SAST method 1	SAST method 2
a	0	0	0	0 (100)	0 (100)
b	24 (25.8)	1 (1.1)	1 (1.1)	1 (4.1)	1 (100)
c	0	2 (2.1)	0	0 (100)	0 (0)
d	0	3 (3.2)	1 (1.1)	0 (0)	1 (33.3)
e	0	2 (2.1)	1 (1.1)	0 (0)	1 (50.0)
f	0	2 (2.1)	3 (3.2)	0 (0)	2 (66.6)
<i>NTHi</i>	69 (74.1)	67 (72.1)	87 (93.5)	69 (100)	67 (77.0)
Polyagglutination	0	16 (17.3) ^a	0	NA ^b	NA ^b
Total isolates	93	93	93	70 (75.2)	72 (93.5) ^c

^a sixteen isolates identified as polyagglutinating by SAST 2 were identified as *NTHi* by PCR; ^b Not applicable; ^c sixteen isolates polyagglutinated were excluded from the agreement rate.

SAST 1 method. Sixteen isolates identified as polyagglutinated were typed as NTHi by PCR. The overall rate of agreement in capsule typing of *Hi* isolates between SAST 1 or 2 and PCR was of 75.2% or 93.5% respectively (Table 1). Thus an increase of 18.3% in the agreement rate was achieved by using SAST 2. The most important discrepant result was of the 23 isolates falsely identified as serotype b by SAST 1, and afterwards identified as NTHi or another serotype by PCR. Five isolates classified as NTHi or serotype b by SAST 1 were identified by PCR as type f (3 isolates), type d (1 isolate) and type e (1 isolate). When the results of SAST 2 and PCR were compared, 6 discrepant types were found: 1 isolate identified as NTHi by SAST 2 was typed as type f by PCR; 2 isolates identified as type d were typed as NTHi by PCR, 2 isolates identified as type c were typed as NTHi by PCR and 1 isolate identified as type e was classified as NTHi by PCR (Table 1).

These results demonstrate the limitations of SAST1 for *Hi* serotyping in comparison with the results provided by PCR. This difference was attributed to the use of a single type b-specific antiserum for screening, leading us to consider every form of agglutination as a *Hib* positive reaction, a finding complicated by the weak intensity of agglutination in the nasopharyngeal isolates from healthy subjects. Thus, SAST 1 overestimated the serotype b isolates, a finding which explains the poor agreement between SAST 1 and PCR for serotype b. This result is in accordance with those reported by La Claire *et al.* (7) and Bokerman *et al.* (1). However, as previously reported (1), the use of all antisera in parallel (SAST 2), significantly improved capsule type agreement with PCR.

The impact of the Brazilian program of *Hib* vaccination, initiated in 1999, should be evaluated among healthy children. We therefore stress the importance of using the SAST 2 method for the accurate estimation of *H. influenzae* type b in colonized children, and the use of PCR to confirm the results.

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RESUMO

Comparação de dois métodos de sorotipagem de cápsula por aglutinação em lamina e o método de PCR para a caracterização de sorotipos de *Haemophilus influenzae*

Noventa e três isolados nasofaringeanos de *H. influenzae* foram sorotipados através de 2 métodos de aglutinação em

lamina (SAST 1 e SAST 2) e os resultados foram comparados com a sorotipagem por PCR. SAST 1 apresentou uma baixa correlação com os resultados obtidos por PCR (75,2%) enquanto que SAST 2 mostrou uma melhor concordância com os resultados da técnica molecular (93,5%). Estes resultados indicam que SAST 2 pode ser um método alternativo para a correta detecção de *H. influenzae* tipo b.

Palavras-chave: *Haemophilus influenzae*, métodos de sorotipagem, PCR

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