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Identification of *Haemophilus influenzae* Serotypes by Standard Slide Agglutination Serotyping and PCR-Based Capsule Typing

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To resolve discrepancies in slide agglutination serotyping (SAST) results from state health departments and the Centers for Disease Control and Prevention (CDC), we characterized 141 of 751 invasive *Haemophilus influenzae* isolates that were identified in the United States from January 1998 to December 1999 through an active, laboratory-based, surveillance program coordinated by the CDC. We found discrepancies between the results of SAST performed at state health departments and those of PCR capsule typing performed at the CDC for 56 (40%) of the isolates characterized: 54 isolates that were identified as a particular serotype by SAST were shown to be unencapsulated by PCR, and two isolates that were reported as serotypes b and f were found to be serotypes f and e, respectively, by PCR. The laboratory error most likely to affect the perceived efficacy of the conjugate *H. influenzae* type b (Hib) vaccine was the misidentification of isolates as serotype b: of 40 isolates identified as serotype b by SAST, 27 (68%) did not contain the correlating capsule type genes. The frequency of errors fell substantially when standardized reagents and routine quality control of SAST were used during a study involving three laboratories. An overall 94% agreement between SAST and PCR results showed that slide agglutination could be a valid and reliable method for serotyping *H. influenzae* if the test was performed correctly, in accordance with standardized and recommended procedures. An ongoing prospective analysis of all *H. influenzae* surveillance isolates associated with invasive disease in children less than 5 years old will provide more accurate national figures for the burden of invasive disease caused by Hib and other *H. influenzae* serotypes.

Since the widespread use of *Haemophilus influenzae* capsular type b (Hib) conjugate vaccines in the United States started in 1987 (1), the incidence of invasive Hib disease in children less than 5 years old has declined drastically (1, 3, 4, 7, 10), from about 100 per 100,000 children before 1987 to <1 per 100,000 in recent years (Centers for Disease Control and Prevention [CDC], unpublished data). From 1998 to 2000, only about 66 Hib cases per year were reported among children less than 5 years old (4). Because of the success of the vaccination program, Hib disease is now one of the few bacterial diseases that are slated for elimination (6), and *H. influenzae* invasive disease is now associated primarily with nontypeable (NT) *H. influenzae* or with *H. influenzae* of capsule types a, c, d, e, and especially f (2). Traditionally, *H. influenzae* serotypes have been identified by slide agglutination serotyping (SAST), which uses six antisera (a to f) that are specific for each of the six serotypes. Recently, however, inconsistencies were reported between Hib SAST results obtained for isolates in state health laboratories and results for the same isolates tested at the CDC. To help resolve these SAST inconsistencies, we used a PCR capsule typing method that was developed by Falla et al. (5); two studies have shown this method to have greater sensitivity and specificity than SAST (5; J. S. Turner, S. W. Satola, S. T. Terris, A. R. Franklin, N. Rosenstein, and M. M. Farley, Abstr. 99th Gen. Meet. Am. Soc. Microbiol. 1999, abstr. C-415, p. 189, 1999). This PCR approach first tests for the *bexA* gene that is responsible for transporting capsular material; its presence or absence determines whether an isolate is encapsulated or unencapsulated and therefore NT. Subsequent PCR assays are then used to determine the presence of each of the six individual capsule types, a to f. In this study, we evaluated discrepancies in SAST results from different laboratories by comparing SAST results from the state health laboratories with the results of both SAST and PCR capsule typing at the CDC for a large number of surveillance program *H. influenzae* isolates. An important goal of this study was to determine, as we progress toward Hib elimination, whether the burden of Hib disease may be significantly less than is currently estimated because of false-positive SAST results.

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MATERIALS AND METHODS

Active Bacterial Core Surveillance. As part of the Emerging Infections Program, the CDC coordinates active, laboratory- and population-based surveillance for *H. influenzae* disease in nine states through the Active Bacterial Core Surveillance (ABCS) program (9). A case of *H. influenzae* disease is defined as an illness in a resident of the surveillance area that is clinically compatible with invasive disease, with *H. influenzae* being isolated from a normally sterile site. Cases are reported to surveillance officers by contacts in each hospital or laboratory in the surveillance site, and report forms are completed for each case. From 1998 to 1999, all available isolates from seven states (with a population of 26,437,876 in 1999) were sent to the state health laboratories, which performed serotyping by standardized SAST (2, 8). The isolates were then forwarded to the CDC for further analysis.

Bacterial strains. All strains were maintained in sterile defibrinated sheep blood at −70°C until tested.

ABCS *H. influenzae* strains. From 1998 to 1999, 751 cases of *H. influenzae* disease were identified in ABCS sites; 487 isolates were serotyped at state health laboratories and then sent to the CDC. Upon receipt at the CDC, the isolates were subcultured onto Chocolate II agar plates (BD Bioscience, Cockeysville, Md.), incubated under 5% CO_2 at 37°C for 18 to 20 h, and serotyped by SAST with either CDC or Difco *H. influenzae* serotype-specific rabbit antisera (BD Bioscience, Franklin Lakes, N.J.). Of the isolates serotyped at state health laboratories and sent to the CDC, a convenience sample of 63 was evaluated, with special emphasis given to the isolates reported as serotype b. The CDC and state laboratory SAST results differed for 14 (22%) of these isolates: 13 were NT by SAST at the CDC but were reported by state laboratories as serotypes a (1 isolate), b (9 isolates), d (1 isolate), e (1 isolate), and f (2 isolates). The remaining discrepancy involved an isolate identified at the CDC by SAST as serotype e and at a state health laboratory as serotype f. An additional 78 *H. influenzae* strains were included to provide a representative sample of the diversity of all *H. influenzae* serotypes identified by SAST at the state health laboratories and collected through the ABCS program from 1998 to 1999; thus, a total of 141 *H. influenzae* isolates were included in this study.

Control *H. influenzae* strains. A set of 18 *H. influenzae* CDC reference strains, representing each of the serotypes, a, b, c, d, e, and f, and NT *H. influenzae*, were used as positive controls. All test and control strains were identified by standard microbiological procedures (2, 8).

Other control strains. A set of 34 bacterial strains, representing a range of respiratory pathogens and normal flora, were used as negative controls (Table 1).

DNA preparation. The isolates were recovered from frozen storage by being subcultured onto Chocolate II agar plates and incubated under 5% CO_2 at 37°C for 18 to 20 h. Rapid DNA extraction was performed on all test and control strains as follows: 15 to 20 colonies from each culture were suspended in 100 μl of Tris-HCl (10 mM), pH 8.0 (Gibco BRL Life Technologies, Rockville, Md.). The suspensions were boiled for 10 min and then centrifuged at 2,000 × *g* for 2 min. Finally, 80 μl of supernatant was collected and stored at −20°C until testing was performed.

PCR. Primers specific for the *bexA* gene (primers H1 and H2), required for capsular export, were used to differentiate NT isolates from capsulated isolates among the 141 test strains. Then, six primer pairs (each used in a separate PCR), specific for capsule types a through f, were used for all strains (5). Reactions were carried out in a 96-well MicroAmp plate (Perkin-Elmer, Norwalk, Conn.) with the use of a programmable thermal cycler (Gene Amp PCR system 9700; Applied Biosystems, Foster City, Calif.). Each 25-μl reaction mixture contained a 0.4 μM concentration of each oligonucleotide primer, 1 μl of template DNA, 200 μM deoxynucleotide mix (Roche Diagnostics, Indianapolis, Ind.), 14.75 μl of water, and the Expand High Fidelity PCR system (Roche Diagnostics), which includes 2.5 μl of Expand High Fidelity buffer (10× without magnesium chloride), 3.5 μM magnesium chloride, and 0.9 U of DNA polymerase. The cycling conditions used were described previously by Falla et al. (5), except that the annealing temperature was decreased to 55°C when primers specific for capsule type e were employed. Five microliters of each PCR product was resolved by performing electrophoresis for 45 min at 80 V on a 1.0% agarose gel (Bio-Rad, Hercules, Calif.). The products were visualized by using 50 mg of ethidium bromide (0.625 mg/ml; Amresco, Solon, Ohio). The R_f of the amplicons was compared with those of a positive control and a 1-kb DNA ladder (Gibco BRL Life Technologies). We identified products of approximately 250, 480, 250, 150, 1,350, and 450 bp for capsule types a, b, c, d, e, and f, respectively (5). Control strains, both encapsulated for serotypes a to f and unencapsulated, were included with each PCR. To evaluate specificity, the *bexA* PCR was also performed on a set of 34 bacterial strains that represented a range of respiratory pathogens and normal flora (Table 1). PCR using primers amplifying 16S rRNA genes (11) was performed as a control for DNA extraction and possible PCR inhibition on all *bexA*-negative strains and 34 control strains (Table 1). Each isolate for which there was a discrepancy between PCR capsule type and SAST results at a surveillance site or at the CDC was retested in a blinded fashion by PCR and SAST. Both CDC and Difco *H. influenzae* rabbit antisera types a through f were used for repeat SAST.

TABLE 1. Bacterial strains, representing a range of respiratory pathogens and normal flora, used as negative controls for *bexA* PCR

Species	Strain designation	*bexA* PCR	16S rRNA PCR
Corynebacterium accolens	CDC 1455	−	+
Corynebacterium aquaticum	CDC 1443	−	+
Corynebacterium bovis	CDC 529	−	+
Corynebacterium jeikeium	CDC 1457	−	+
Corynebacterium minutissimum	CDC 536	−	+
Corynebacterium mycetoides	CDC 1460	−	+
Corynebacterium pseudodiphtheriticum	CDC G2486	−	+
Corynebacterium striatum	CDC 530	−	+
Haemophilus aegyptius	CDC 5575	−	+
Haemophilus haemolyticus	CDC 3442	−	+
Haemophilus parainfluenzae	CDC 3438	−	+
Moraxella catarrhalis	CDC 4419	−	+
Moraxella catarrhalis	M6452	−	+
Neisseria cinerea	CDC 6451	−	+
Neisseria gonorrhoeae	M6450	−	+
Neisseria lactamica	CDC 6454	−	+
Neisseria meningitidis serogroup A	CDC 318	−	+
Neisseria meningitidis serogroup B	CDC 321	−	+
Neisseria meningitidis serogroup C	CDC 323	−	+
Neisseria meningitidis serogroup E29	CDC 330	−	+
Neisseria meningitidis serogroup W135	CDC 327	−	+
Neisseria meningitidis serogroup Y	CDC 326	−	+
Neisseria meningitidis serogroup Z	CDC 329	−	+
Neisseria meningitidis nongroupable	CDC 4631	−	+
Neisseria sicca	CDC 6453	−	+
Neisseria subflava	M6449	−	+
Staphylococcus aureus	ATCC 12598	−	+
Staphylococcus aureus	ATCC 25923	−	+
Streptococcus group A	CDC 2373–96	−	+
Streptococcus group B	CDC SS-615	−	+
Streptococcus group C	CDC SS-498	−	+
Streptococcus group D	CDC SS-1344	−	+
Streptococcus group G	CDC SS-175	−	+
Streptococcus pneumoniae	ATCC 49619	−	+

Interlaboratory comparison. We conducted an interlaboratory comparison study of *H. influenzae* serotyping results with three of the seven state health laboratories: laboratories A, B, and C. To ensure standardization of the key factors that could affect the results of the SAST, we provided each laboratory with vials of Difco *H. influenzae* rabbit antisera for each of the serotypes a through f, as well as a vial of polyvalent serum from the same lot. We also provided participating laboratories with a set of 32 *H. influenzae* strains, representing each of the six serotypes a through f and NT *H. influenzae*. Prior to being sent to the participating state health laboratories, each strain had been serotyped by SAST and capsule typed by PCR at the CDC. The strains were coded, and aliquots from one original cell suspension were shipped frozen to each laboratory. The participating laboratories were asked to perform the SAST according to their standard laboratory protocols.

RESULTS

Correlation between SAST and PCR capsule typing. One hundred forty-one isolates evaluated at state health laboratories by SAST were also evaluated at the CDC by SAST and PCR; this sample included the initial convenience sample of 63 isolates tested by SAST at the CDC. Of these 141 isolates, 62 (44%) contained the *bexA* gene and were subsequently identi-

TABLE 2. Correlation of SAST and PCR capsule typing results for 141 *H. influenzae* isolates

Serotype by SAST at surveillance sites	No. of isolates	Capsule type results by PCR					
		Agreement with SAST		Disagreement with SAST			
				NT		Typeable	
		No.	%	No.	%	No.	%
a	3	2	67	1	33	0	0
b	40	12	30	27	68	1	2[a]
c[b]	6	0	0	6	100	0	0
d	8	1	12	7	88	0	0
e	11	7	64	4	36	0	0
f	48	38	79	9	19	1	2[c]
NT	25	25	100	0	0	0	0
Total	141	85	60	54	38	2	1.4

[a] Identified as capsule type f.
[b] One of the six strains identified by SAST at the surveillance site as serotype c, at the CDC as serotype e, and as NT by PCR. This is the only instance where the results of SAST performed at the CDC during this study disagreed with the PCR capsule typing results.
[c] Identified as capsule type e.

fied by capsule-specific PCR as capsule type a, b, d, e, or f, while 79 (56%) isolates did not contain the *bexA* gene; all were positive for the presence of the 16S rRNA gene (Table 2). Incorrect SAST results varied substantially among the seven surveillance site laboratories (15 to 66%; median range, 43%). No strains were *bexA* negative and capsule type positive by PCR. For 85 (60%) of the 141 isolates, the results of SAST performed at state health laboratories agreed with the PCR results (Table 2). For the remaining 56 isolates whose SAST results and PCR results did not agree, two types of errors were found. The first type applied to 54 of these 56 isolates and involved the identification of a particular serotype by SAST at the state health laboratories when both *bexA*- and capsule-type-specific PCR results were negative. In particular, of the 40 *H. influenzae* isolates typed by SAST at state health laboratories as serotype b, only 12 (30%) were positive for capsule type b by PCR, whereas 27 (68%) were negative in both *bexA*- and capsule-type-specific PCR assays and 1 was serotype f. By comparison, of the 48 *H. influenzae* isolates that were identified by SAST at the state health laboratories as serotype f and evaluated by the CDC, the PCR results were concordant with the SAST results for 38 (79%) of them. The second type of laboratory error was simple mistyping: two *H. influenzae* isolates that were reported by surveillance sites as serotypes b and f were shown to be capsule types f and e, respectively, by PCR.

Interlaboratory comparison. State health laboratory A correctly serotyped 31 (97%) out of 32 blinded interlaboratory comparison control *H. influenzae* strains: it reported one isolate to be NT, whereas the PCR results showed it to be capsule type f. State health laboratory B correctly identified 30 (94%) out of 32 *H. influenzae* isolates: it reported two isolates as serotypes a and b, whereas the PCR results were negative for both isolates. State health laboratory C correctly identified 14 (88%) out of 16 tested *H. influenzae* isolates: two with negative PCR results were reported as serotype b. Overall, 94% of the isolates tested by SAST at these three laboratories were correctly serotyped.

DISCUSSION

The success of widespread use of conjugate vaccines has resulted in fewer *H. influenzae* isolates being submitted to state health laboratories for identification and typing (9). Consequently, laboratories are likely to perform once-routine methods less frequently. Recent discrepancies between SAST results from state health laboratories and those from the CDC (CDC, unpublished data) led us to study a large number of *H. influenzae* isolates from the United States, collected through active surveillance from 1998 to 1999.

We used as our reference method a two-step PCR approach that detects the *bexA* gene, whose product is responsible for capsular export, and six sequences that code for each specific capsule type (a to f). These assays, initially developed by Falla et al. (5), have been shown to be highly sensitive and specific (Turner et al., Abstr. 99th Gen. Meet. Am. Soc. Microbiol. 1999), and with their use we were able to correctly identify all of our *H. influenzae* controls. The most common and significant laboratory error with respect to measurable effects of implementing conjugate Hib vaccine was the misidentification of NT strains as those of serotype b (Table 2). Only 30% of the isolates identified at state health laboratories as serotype b isolates possessed the necessary capsular genes; consequently, these discrepancies could not be the result of down-regulation of gene expression. These discrepancies might result from the use by laboratories of antiserum specific only for serotype b, instead of the full set of *H. influenzae* serotype-specific antisera. This could lead the laboratories to interpret any form of agglutination observed with the serotype b-specific antiserum as a positive and specific reaction. However, if the laboratories tested the same isolate with the full set of serotype-specific *H. influenzae* antisera, they could determine whether agglutination occurred with one or more serotype-specific antisera (cross-reaction) or even with the control saline (rough isolate) and thus whether their initial interpretation of agglutination with the serotype b-specific antiserum was incorrect (false positive). The agreement between reported SAST results and PCR results varied from 0 to 79% for *H. influenzae* strains of serotypes other than b but was 100% for NT *H. influenzae* (Table 2). To determine the reasons for the observed discrepancies, we conducted an interlaboratory comparison of the results from three state health department laboratories and the CDC. The participating laboratories performed SAST on the same set of 32 strains, using a CDC-supplied complete set of *H. influenzae* type-specific antisera, according to their standard laboratory protocols. Of all the *H. influenzae* isolates tested by the three participating laboratories, 94% were correctly serotyped. This represents a significant increase in the proportion of *H. influenzae* isolates correctly serotyped by state laboratories, suggesting that standardized reagents and routine quality assurance practices are crucial for obtaining reliable and reproducible SAST results. Consequently, recommendations that laboratories adhere more strictly to quality assurance procedures, including the use of standardized reagents and protocols, should be emphasized. The PCR assays used in this study were more sensitive and specific than SAST and may continue to serve as a method to resolve SAST inconsistencies, but more extensive evaluation of this approach will be beneficial.

Discrepancies between the results of SAST and PCR capsule typing and our finding that two-thirds of the *H. influenzae* isolates reported to the CDC as those of serotype b were incorrectly

serotyped by SAST suggest that the number of actual Hib cases in the United States may be significantly lower than reported and that the national burden of Hib disease may be overestimated. However, since the percentages of SAST results that were incorrect differed substantially among the seven surveillance site laboratories (15 to 66%; median range, 43%), one must be cautious in extrapolating these findings beyond the ABCS sites. Therefore, to determine the extent to which such false-positive laboratory results have contributed to overestimates of the number of Hib cases in the United States, the CDC has initiated a prospective analysis of all *H. influenzae* surveillance site isolates associated with invasive disease in children less than 5 years old; in this analysis, *H. influenzae* serotypes will be identified by both SAST and PCR capsule typing. Such monitoring will serve to clarify the prevalence of *H. influenzae* serotype misidentification. As the burden of Hib disease declines in the United States, it becomes increasingly important to determine accurately the serotypes of all *H. influenzae* isolates associated with invasive disease, especially since Hib serves as a model for other vaccine-preventable diseases.

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