JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 2003, p. 4415–4417 Vol. 41, No. 9
0095-1137/03/$08.00+0 DOI: 10.1128/JCM.41.9.4415–4417.2003

Confirmation of Nontypeable *Streptococcus pneumoniae*-Like Organisms Isolated from Outbreaks of Epidemic Conjunctivitis as *Streptococcus pneumoniae*

Maria Gloria S. Carvalho,[1,2] Arnold G. Steigerwalt,[1] Terry Thompson,[1] Delois Jackson,[1] and Richard R. Facklam[1]*

Centers for Disease Control and Prevention, Atlanta, Georgia,[1] and CNPq, Rio de Janeiro, Brazil[2]

Received 28 April 2003/Returned for modification 18 May 2003/Accepted 9 June 2003

Eleven isolates representing five distinct outbreaks of pneumococcal conjunctivitis were examined for phenotypic and genetic characteristics. None of the strains possessed capsules, and all strains were susceptible to optochin, bile soluble, and Gen-Probe AccuProbe test positive. All 11 isolates were confirmed as *Streptococcus pneumoniae* by DNA-DNA reassociation experiments.

There is no "gold standard" for the identification of alpha-hemolytic streptococci as *Streptococcus pneumoniae* in the clinical laboratory. Most clinical laboratories use the optochin susceptibility test or the bile solubility test for presumptive identification (5, 15). Confirmation of alpha-hemolytic streptococci as *S. pneumoniae* requires demonstrating that the culture has a polysaccharide capsule, preferably by the Quellung reaction with a type-specific antiserum (5). While optochin susceptibility and bile solubility tests are in most instances very useful for presumptive identification of *S. pneumoniae*, there are exceptions, such as instances when optochin-susceptible viridans group streptococci and bile-insoluble *S. pneumoniae* are being tested (12, 13). The AccuProbe pneumococcus test (Gen-Probe, San Diego, Calif.), which is based on the rRNA gene sequence, is also used to identify refractory strains suspected of being *S. pneumoniae.* Several studies, including some of our own unpublished data, indicate that the AccuProbe test is reasonably accurate in identifying *S. pneumoniae* (5, 8, 12). However, only small differences of less than 1% between the 16S rRNA genes of *Streptococcus mitis* (11 bp) and *Streptococcus oralis* (14 bp) and that of *S. pneumoniae* may raise the question of its true specificity. Our findings, which are based on the examination of thousands of sterile-site pneumococcal isolates, indicate that atypical results with optochin susceptibility, bile solubility, and AccuProbe tests and the absence of capsules are very rare (5, 10). Approximately 0.5% of more than 25,000 sterile-site isolates failed to react with Centers for Disease Control and Prevention (CDC) pneumococcus typing antisera. On the other hand, when working with nonsterile-site isolates, sputum, oral pharyngeal isolates, or nasopharyngeal isolates, it is not uncommon to find that 10% of the isolates fail to react with pneumococcus typing antisera. Isolates that are optochin susceptible, bile soluble, and Gen-Probe positive appear to be nontypeable (NT) *S. pneumoniae*. It is intriguing that these NT *S. pneumoniae* isolates are frequently isolated from nonsterile sites but are isolated very rarely, if at all, from sterile sites (5, 10). The identification of NT *S. pneumoniae* isolates from very large, explosive outbreaks of conjunctivitis and the nature of the spread of these unusual strains led us to question whether or not these organisms were truly *S. pneumoniae* (3, 10, 16). Encapsulated *S. pneumoniae* isolates are found in epidemic situations but do not spread like the NT *S. pneumoniae* isolates identified during the large, rapidly spreading epidemics of conjunctivitis. Investigators have used a variety of molecular techniques to include these NT *S. pneumoniae* isolates in the taxon *S. pneumoniae*, including 16S ribosomal DNA gene sequencing (10) and PCR for the pneumolysin gene (9). The objective of this study was to confirm the true identity of these bacteria by DNA-DNA reassociation experiments, which is the only molecular technique with set standards for establishing bacterial species (17, 19).

All isolates used in this study were taken from the culture collection of the CDC *Streptococcus* Laboratory. The representative isolates used were from five different conjunctivitis outbreaks caused by NT *S. pneumoniae*-like organisms in the following states: New York (two strains, 1980), California (three strains, 1981), Illinois (two strains, 1981), New Hampshire (two strains, 2002), and New Jersey (two strains, 2002). The isolates from 1980 and 1981 were stored at −70°C in defibrinated blood. Isolates from recent outbreaks were stored in serum-tryptone-glucose-glycerol medium for 1 year. Serotyping was performed with the Quellung test as previously described (6). Tests were performed according to the instructions described in the 8th edition of the *Manual of Clinical Microbiology* (15). AccuProbe *S. pneumoniae* tests were purchased from Gen-Probe, Inc., and were performed according to the manufacturer's instructions. Isolates were examined for capsules by the colloidal carbon wet-mount capsule-staining procedure (4). Harvesting and lysis of the bacterial cells were performed as previously described (18). Extraction and purification of DNA and the determination of DNA relatedness by the hydroxyapatite hybridization method were done as described by Brenner et al. (2). DNA hybridization experiments were performed at 55°C for optimal DNA reassociation and at the stringent DNA reassociation temperature of 70°C. The levels of divergence within related sequences were determined by assuming that each degree of heteroduplex instability was

* Corresponding author. Mailing address: Centers for Diseases Control and Prevention, 1600 Clifton Rd., Mail Stop C02, Atlanta, GA 30333. Phone: (404) 639-1379. Fax: (404) 639-3123. E-mail: rrf2@cdc.gov.

TABLE 1. Phenotypic and genetic characteristics of conjunctivitis isolates of *S. pneumoniae*

Strain	Sample no.	Source or place of isolation[a]	Type	Optochin susceptibility[b]	Bile solubility[b]	AccuProbe reaction (RLU)[c]	Capsule production[b]	DNA-DNA reassociation		
								55°C (%)	ΔT_m[d] (°C)	70°C (%)
S. pneumoniae ATCC 33400^T		ATCC	35A	+	+	616,924	+	82		74
S. mitis NCTC12261^T	1303	NCTC		−	−	1,152	−	64		48
1138–80	165	NY	NT	+	+	427,409	−	85		84
1139–80	166	NY	NT	+	+	257,364	−	83		81
61–81	168	CA	NT	+	+	439,684	−	81		79
62–81	169	CA	NT	+	+	505,025	−	82		78
63–81	170	CA	NT	+	+	504,810	−	70	0.5	70
1718–81	171	IL	NT	+	+	312,508	−	71	4.0	65
1721–81	172	IL	NT	+	+	371,492	−	72	4.0	62
1852–02	245	NH	NT	+	+	112,030	−	73	1.0	75
1853–02	246	NH	NT	+	+	176,795	−	84		80
2136–02	247	NJ	NT	+	+	160,890	−	82		79
2137–02	248	NJ	NT	+	+	140,421	−	84		81

[a] ATCC, American Type Culture Collection; NCTC, National Collection of Type Cultures; NY, New York; CA, California; IL, Illinois; NH, New Hampshire; NJ, New Jersey.
[b] +, positive; −, negative.
[c] Relative light unit (RLU) values higher than 50,000 are considered a positive result.
[d] T_m, melting temperature.

caused by approximately 1% of unpaired bases. Divergence, expressed by the change in melting temperature, is the decrease in thermal stability (°C) of the heterologous DNA duplex relative to that of the homologous duplexes. Divergence was calculated to the nearest 0.5%.

All conjunctivitis isolates from epidemics investigated in the early 1980s as well as those investigated within the last 12 months were susceptible to optochin, were bile soluble, and reacted positively in the AccuProbe pneumococcus test. None of the isolates had capsules based on examination with CDC pneumococcus typing antisera or the colloidal carbon wet-mount procedure (Table 1). The results of the DNA-DNA reassociation studies, shown in Table 1, indicate that all NT *S. pneumoniae* conjunctivitis isolates belong to the taxon *S. pneumoniae*. All strains were more than 70% homologous under optimal reassociation conditions (55°C), and only two strains were less than 70% homologous to the type strain of *S. pneumoniae* under stringent reassociation conditions (70°C). The divergence in related sequences of all strains was less than 4%. The two isolates from the Illinois conjunctivitis outbreak were somewhat more divergent than the others, but according to the criteria established by the ad hoc committee on bacterial systematics, they belong to the *S. pneumoniae* taxon (17, 19).

NT *S. pneumoniae* isolates have been reported for many years. It is interesting that Finland and Barnes reported that isolates from eye swabs are less likely to be typeable than isolates from any other source (99% for cerebral spinal fluid, 96% for pleural fluid, 93% for otitic fluid, and 78% for eye cultures) during the years 1935 to 1974 (7). NT pneumococcal isolates are not limited to the United States; Medeiros et al. reported that more than 51% of epidemic conjunctivitis isolates from patients living in Brazil were NT (11). Investigators have reported sporadic cases of conjunctivitis as well (1, 14, 20). Identification of these unusual isolates is controversial. Some investigators have assumed the Gen-Probe AccuProbe pneumococcus test to be the gold standard for their studies (8, 12). This assumption has led to the inclusion of optochin susceptibility and bile solubility variants into the taxon *S. pneumoniae*. At least one other investigator has reported that the AccuProbe test should not be used as a gold standard (9). These investigators concluded that neither the AccuProbe nor a probe developed to identify the pneumolysin gene was useful in the final identification of atypical pneumococci. The heterogeneity of *S. pneumoniae*, *S. mitis*, and *S. oralis* was elegantly shown in the multilocus sequence typing data published by Whatmore et al. (20). These investigators showed that multiple isolates of each of the three species clustered after neighbor-joining analysis. In fact, the *S. pneumoniae* cluster was more homologous than that of either *S. mitis* or *S. oralis*. Also, there were several isolates of alpha-hemolytic streptococci included in the study that did not join any of the three clusters but that were closely allied to the *S. pneumoniae* cluster. None of these strains possessed capsules, and results of the tests for optochin susceptibility and bile solubility and of the AccuProbe reactions varied. This leads to the conclusion that there are isolates that are similar to *S. pneumoniae* with similar phenotypic characteristics that cannot be included in the taxon *S. pneumoniae*. Nevertheless, the data presented in this study clearly show by DNA-DNA reassociation that the isolates from several large outbreaks of conjunctivitis are *S. pneumoniae*, a finding which confirms the results obtained by optochin susceptibility, bile solubility, and AccuProbe tests. We cannot comment on the true identity of isolates from sporadic outbreaks of conjunctivitis or from sterile sites or nonsterile sites (nasopharyngeal or oral pharyngeal). DNA-DNA reassociation is the only method that can be confirmatory for inclusion of an isolate into any taxon (17, 19), but these results suggest that for identification of NT *S. pneumoniae* isolates involved in conjunctivitis outbreaks, clinical laboratories can rely upon the conventional physiological tests. The absence of atypical results for optochin susceptibility, bile solubility, or AccuProbe tests for these isolates can be explained in part by the fact that the majority of the isolates belong to a clonal group (10). As staff members of a reference laboratory, we will be using this method in the future to expand this study to include isolates other than those from outbreaks of conjunctivitis that are NT.

M. G. S. Carvalho was supported by a "Conselho Nacional de Desenvolvimento Científico e Tecnológico-CNPq" postdoctoral fellowship.

REFERENCES

1. **Barker, J. H., D. M. Musher, R. Silberman, H. M. Phan, and D. A. Watson.** 1999. Genetic relatedness among nontypeable pneumococci implicated in sporadic cases of conjunctivitis. J. Clin. Microbiol. **37:**4039–4041.
2. **Brenner, D. J., A. C. McWhorter, J. K. L. Knutson, and A. G. Steigerwalt.** 1982. *Escherichia vulneris*: a new species of *Enterobacteriaceae* associated with human wounds. J. Clin. Microbiol. **15:**1133–1140.
3. **Centers for Disease Control and Prevention.** 2003. Pneumococcal conjunctivitis at an elementary school—Maine, September 20–December 6, 2002. Morb. Mortal. Wkly. Rep. **52:**64–66.
4. **Chapin, K.** 1995. Clinical microscopy, p. 33–51, *In* P. R. Murray, E. J. Barron, M. A. Pfaller, F. C. Tenover, and R. H. Yolken (ed.). Manual of clinical microbiology, 6th ed. American Society for Microbiology, Washington, D.C.
5. **Facklam, R., and N. Pigott.** 1994. Description of phenotypic characteristics to aid in the identification of *Streptococcus pneumoniae*, p. 415–417. *In* A. Totollian (ed.), Pathogenic streptococci: present and future. Lancer Publications, St. Petersburg, Russia.
6. **Facklam, R. R., and J. A. Washington II.** 1991. *Streptococcus* and related catalase-negative gram-positive cocci, p. 238–257. *In* A. Balows, W. J. Hausler, Jr., K. L. Herrmann, H. D. Isenberg, and H. J. Shadomy (ed.), Manual of clinical microbiology, 5th ed. American Society for Microbiology, Washington, D.C.
7. **Finland, M., and M. W. Barnes.** 1977. Changes in occurrence of capsular serotypes of *Streptococcus pneumoniae* at Boston City Hospital during selected years between 1935 and 1974. J. Clin. Microbiol. **5:**154–166.
8. **Geslin, P., A. Fremaux, C. Spicq, G. Sissa, and S. Georges.** 1997. Use of a DNA probe test for identification of *Streptococcus pneumoniae* nontypeable strains. Adv. Exp. Med. Biol. **418:**383–385.
9. **Kaijalainen, T., S. Rintamaki, E. Herva, and M. Leionen.** 2002. Evaluation of gene-technological and conventional methods in the identification of *Streptococcus pneumoniae.* J. Microbiol. Methods **51:**111–118.
10. **Martin, M., J. H. Turco, M. E. Zegans, R. R. Facklam, S. Sodha, J. A. Elliott, J. H. Pryor, B. Beall, D. D. Erdman, Y. Y. Baumgartner, P. A. Sanchez, J. D. Schwartzman, J. Montero, A. Schuchat, and C. G. Whitney.** 2003. An outbreak of conjunctivitis due to atypical *Streptococcus pneumoniae.* N. Engl. J. Med. **348:**1112–1121.
11. **Medeiros, M. I., P. da Silva, J. O. Silva, A. M. M. Carneiro, M. C. Carloni, and M. C. C. Brandileone.** 1998. *Streptococcus pneumoniae* and *Haemophilus influenzae* as etiological agents of conjunctivitis outbreaks in the region of Ribeirao Preto, SP, Brazil. Rev. Inst. Med. Trop. Sao Paulo **40:**7–9.
12. **Mundy, L. S., E. N. Janoff, K. E. Schwebvke, C. J. Shanholtzer, and K. E. Willard.** 1998. Ambiguity in the identification of *Streptococcus pneumoniae* optochin, bile solubility, Quellung, and the AccuProbe DNA probe tests. Am. J. Clin. Pathol. **109:**55–61.
13. **Munoz, R., A. Fenoll, D. Vicioso, and J. Casal.** 1990. Optochin-resistant variants of *Streptococcus pneumoniae.* Diagn. Microbiol. Infect. Dis. **13:**63–66.
14. **Pease, A. A., C. W. I. Douglas, and R. C. Spencer.** 1986. Identification of non-capsulate strains of *Streptococcus pneumoniae* isolated from eyes. J. Clin. Pathol. **39:**871–875.
15. **Ruoff, K. L., R. A. Whiley, and D. Beighton.** 2003. *Streptococcus*, p. 405–421. *In* P. R. Murray, E. J. Barron, M. A. Pfaller, J. H. Jorgensen, and R. H. Yolken (ed.), Manual of clinical microbiology, 8th ed. American Society for Microbiology, Washington, D.C.
16. **Shayegani, M., L. M. Parsons, W. E. Gibbons, Jr., and D. Campbell.** 1982. Characterization of nontypable *Streptococcus pneumoniae*-like organisms isolated from outbreaks of conjunctivitis. J. Clin. Microbiol. **16:**8–14.
17. **Stackebrandt, E., W. Frederiksen, G. M. Garrity, P. A. D. Grimont, P. Kampfer, M. C. J. Maiden. X. Nesme, R. Rossella-Mora, J. Swings, H. G. Truper, L. Vauterin, A. C. Ward, and W. B. Whitman.** 2002. Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. Int. J. Syst. Evol. Microbiol. **52:**1043–1047.
18. **Teixeira, L. M., R. R. Facklam, A. G. Steigerwalt, N. E. Pigott, V. L. C. Merquior, and D. J. Brenner.** 1995. Correlation between phenotypic characteristics and DNA relatedness within *Enterococcus faecium* strains. J. Clin. Microbiol. **33:**1520–1523.
19. **Wayne, L. G., D. J. Brenner, R. R. Colwell, P. A. D. Grimont, O. Kandler, M. I. Krichevsky, L. H. Moore, W. E. C. Moore, R. G. E. Murray, E. Stackebrandt, M. P. Starr, and H. G. Trüper.** 1987. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. Int. J. Syst. Bacteriol. **37:**463–464.
20. **Whatmore, A. M., A. Efstratiou, A. P. Pickerill, K. Broughton, G. Woodard, D. Sturgeon, R. George, and C. G. Dowson.** 2000. Genetic relationships between clinical isolates of *Streptococcus pneumoniae*, *Streptococcus oralis*, and *Streptococcus mitis*: characterization of "atypical" pneumococci and organisms allied to *S. mitis* harboring *S. pneumoniae* virulence factor-encoding genes. Infect. Immun. **68:**1374–1382.