Streptococcus pneumoniae Isolates Expressing a Capsule with Epitopes of Both Serotypes 6A and 6B

Carmen L. Sheppard,1 Bruno Pichon,1 Robert C. George,1 and Lucinda M. C. Hall2*

Respiratory and Systemic Infection Laboratory, Health Protection Agency Centre for Infections, 61 Colindale Avenue, London NW9 5EQ, United Kingdom,1 and Queen Mary University of London, Centre for Immunology and Infectious Disease, Blizard Institute of Cell and Molecular Science, Barts and The London School of Medicine and Dentistry, 4 Newark Street, London E1 2AT, United Kingdom2

Received 29 July 2010/Returned for modification 7 September 2010/Accepted 20 September 2010

Four Streptococcus pneumoniae isolates expressing both 6A and 6B capsular serotypes were detected by a multiplex immunoassay. The sequence of WciP, a GT2-family glycosyltransferase, indicates that point mutation has compromised linkage specificity, allowing two alternative oligosaccharides to be synthesized. This finding highlights that mutation as well as recombination can mediate serotype change.

Widespread use of conjugate vaccines covering a limited number of serotypes represents strong selection pressure on the Streptococcus pneumoniae population. The switching of capsular serotype provides a mechanism by which pneumococci can escape from vaccine control. Most attention has focused on the replacement of capsule genes by recombination, but point mutation also has the potential to modify capsule synthesis through alteration of glycosyltransferase specificity.

S. pneumoniae strains of serogroup 6 are commonly found in invasive disease. The group includes serotypes 6A and 6B, which both have a capsule composed of oligosaccharide subunits of rhamnose-ribitol-galactose-glucose. These serotypes differ only at the rhamnose-ribitol linkage, which is 1→3 in 6A and 1→4 in 6B. (Serotypes 6C and 6D, with replacement of galactose by glucose, have also been described recently [7, 14].) The 7-valent vaccine in widespread use includes the 6B polysaccharide, while both 6A and 6B are included in the newer 13-valent vaccine.

We have developed a multiplex immunoassay for serotype determination and direct detection of capsular antigen in clinical samples (1, 18). Antigen was captured by serotype-specific monoclonal antibodies (for 6A, Wyeth Pn10-2.1, and for 6B, Wyeth Pn36-1, both supplied by Pfizer [formerly Wyeth Vaccines Research]) immobilized on differentially labeled Luminex xMAP beads and was detected by using polyclonal serogroup-specific sera (from Statens Serum Institut) and then incubated with a goat anti-rabbit IgG–R-phycoerythrin conjugate to generate a fluorescence signal. The serotype was determined by identifying the properties of the beads to which antigen was bound, and the identification was performed with a Bio-Plex suspension array instrument.

During development of the multiplex immunoassay, one isolate, designated PN6AB1 (Health Protection Agency [HPA] reference no. H055380438), was reported to be positive for both 6A and 6B. At that time, 22 6A and 23 6B isolates had been typed by the system with no ambiguity in the results. Identifications by both our standard slide agglutination serotype test and the Quellung reaction with PN6AB1 were recorded as serotype 6A. The isolate was replated, and the same results were obtained with each of 10 individual colonies.

The differential sugar linkages distinguishing 6A from 6B capsular polysaccharides are formed by a rhamnosyltransferase encoded by wciP (also referred to as cps6aS and cps6bS) in the serogroup 6 capsule locus (5, 6, 12). A single amino acid polymorphism in WciP has to date correlated fully with serotype: Ser195 is present in all 6A isolates, and Asn195 is present in all 6B isolates (Table 1) (5, 12, 13). Two other polymorphisms also correlate very strongly with serotype: Ala192 and Arg254 are present in 6A isolates, and Ser192 and Gly254 are present in 6B isolates (5, 12). We determined the sequence of wciP across this region in PN6AB1 by using previously described primers (12). A unique pattern of residues—Ser192, Ser195, and Gly254—was encoded (Table 1), including the critical 6A amino acid at 195, with the typical 6B amino acids at 192 and 254. Sequence reads were unambiguous (as determined by PHRED [4]), excluding the possibility that the isolate could contain two different copies of wciP.

Subsequently, the multiplex immunoassay was used for routine serotyping in our laboratory. From December 2005 to December 2007, 1,093 serogroup 6 isolates were analyzed and 3 further isolates (0.27%) presented a dual serotype, with multiple single colonies testing positive for both 6A and 6B capsules. PN6AB2 (HPA reference no. H062560519) and PN6AB3 (HPA reference no. H07260027) carried the same WciP amino acid combination at key residues as PN6AB1 and were identified as 6A by conventional serotyping. In contrast, PN6AB4 (HPA reference no. H073640551) was identified as 6B by conventional serotyping and had a different pattern of residues, Ala192, Cys195, and Arg254. Remarkably, the critical residue at 195 differs from both the 6A and 6B paradigms (Table 1), while the secondary amino acids are both typical for 6A. It should be noted that the sequence at codon 195 in PN6AB4 prevents recognition by either 6A- or 6B-specific
TABLE 1. Multilocus sequence types, amino acids at key positions in WciP (and corresponding codons), and serotypes for serogroup 6 pneumococci

| Isolate      | Sequence type | Amino acid (codon) at position: | Multiplex serotype | Standard serotype | Reference(s) |
|--------------|---------------|---------------------------------|--------------------|-------------------|--------------|
|              |               | 192                             | 195                | 254               |              |
| Consensus 6A | Ala (GCT)     | Ser (AGT)                        | Arg (AGG)          | 6A                | 5, 12, 13    |
| Consensus 6B | Ser (TCT)     | Asn (AAT)                        | Gly (GGG)          | 6A                | 5, 12, 13    |
| PN6AB1       | Ser (TCT)     | Ser (AGT)                        | Gly (GGG)          | 6A + 6B           | This study   |
| PN6AB2       | Ser (TCT)     | Ser (AGT)                        | Gly (GGG)          | 6A + 6B           | This study   |
| PN6AB3       | Ser (TCT)     | Ser (AGT)                        | Gly (GGG)          | 6A + 6B           | This study   |
| PN6AB4       | Ala (GCT)     | Cys (TGT)                        | Arg (AGG)          | 6A + 6B           | This study   |
| H051240098   | Ser (TCT)     | Asn (AAT)                        | Gly (GGG)          | 6B                | This study   |
| APH-10       | Ala (GCT)     | Asn (AAT)                        | Arg (AGG)          | 6B                | 12           |
| AAU-19       | Ala (GCT)     | Asn (AAT)                        | Arg (AGG)          | 6B                | 12           |

primers designed to distinguish the Ser or Asn codon, respectively (7). (All four dual-serotype isolates tested negative by PCR for the novel gene wciN, present in 6C and 6D [7], and negative with 6d factor serum for detection of 6C [Statens Serum Institut]).

WciP belongs to the GT-2 family of glycosyltransferases, a family that includes SpsA from Bacillus subtilis, for which the crystal structure has been determined previously (2). Based on structure prediction with Phyre software (8), Asp194 of WciP is expected to correspond to the active site residue Asp191 of SpsA and, hence, to form a hydrogen bond with the glycosyl acceptor (i.e., ribitol for WciP) and catalyze the glycosylation reaction (9). A critical role for amino acids at 192 and 195 in positioning the acceptor relative to the donor nucleotide sugar is thus highly plausible. The position of residue 254 cannot be predicted due to a lack of homology with SpsA in this region.

Our conclusion is that in isolates with a dual serotype the substitutions at position 195 of WciP have resulted in disruption of enzyme specificity and an incomplete serotype switch; a complete change in specificity would be achieved only if amino acids at 192 and/or 254 were also replaced. We cannot exclude the possibility that the enzyme alteration could also permit other linkages to form. The polymerases that link oligosaccharide subunits in 6A and 6B are indistinguishable (5, 6), so it is to be expected that polysaccharide chains in the capsules would comprise a mixture of both subunit types. This arrangement is in concord with the enhanced detection of the minor serotype in our multiplex immunoassay, since chains captured by monoclonal antibodies recognizing the less frequent oligosaccharide subunit would nevertheless be strongly detected by the secondary polyclonal serogroup serum.

Multilocus sequence typing (3, 16) revealed that PN6AB1 had a novel sequence type, ST4784, and was part of a small cluster of serotype 6B isolates from several areas of the world. PN6AB2 and PN6AB3 both belonged to ST176, part of a large clonal complex comprising mainly serotype 6B isolates. In each case, it is deduced that a single point mutation in codon 195 has given rise to a switch from 6B to a mixed serotype in which 6A predominates. PN6AB4 had ST2465, a sequence type shared by a serotype 6B isolate that we had characterized previously (HPA reference no. H051240098) and a single-locus variant of ST176 (Table 1). We determined a partial wciP sequence for the H051240098 isolate and found it to encode the expected amino acid sequence for serotype 6B, with Ser192, Asn195, and Gly254 at the critical positions (all of which differ from PN6AB4) (Table 1). The most parsimonious explanation of the evolution of PN6AB4 is a switch from 6B to 6A by recombination followed by point mutation in codon 195 of the 6A locus (or vice versa). It is probable that the four isolates with a dual serotype represent at least three independent mutational events. Mavroidi et al. (12) investigated the evolution of serogroup 6 pneumococci and sequenced part of the capsule loci from 102 isolates. Several examples of serotype switching between 6A and 6B (in both directions) were observed, the majority of which were best explained by horizontal transfer involving the capsule loci. Two 6B isolates (APH-10 and AAU-19) had the sequence Ala192, Asn195, and Arg254, the exact complement to PN6AB1 in terms of serotype association (Table 1), and both were deduced to have switched from 6A to 6B by point mutation; it is possible that these isolates may also express a mixture of capsular epitopes.

Application of a multiplex immunoassay using monoclonal antibodies has enabled us to detect four isolates of S. pneumoniae that simultaneously produce both serotype 6A and 6B capsular epitopes. Serotype 6A predominates in three isolates, and 6B predominates in the fourth. We believe this to be the first description of clinical isolates expressing two capsule serotypes. Genetically binary isolates described previously contained capsule genes at two different loci, but only one locus was functional and only one capsule type was produced (11). Single amino acid changes have been demonstrated previously to broaden the specificity of glycosyltransferases to include alternative sugar donors (17) but to our knowledge have not been shown to affect the position of the linkage to the acceptor.

Despite the relatedness of serotypes 6A and 6B, the inclusion of serotype 6B polysaccharide in the 7-valent vaccine has not proven fully effective in protection against serotype 6A (10, 15, 19, 20); hence, the new 13-valent vaccine includes both serotypes. A switch in serotype between 6B and 6A could be advantageous to a pneumococcus in evading the immune system in a host previously exposed to one of the serotypes through carriage, infection, or immunization. It is likely, based on standard serotype results, that isolates with a dual serotype would be relatively protected from recognition by antibodies specific to the minor serotype. Our results highlight the ability of a single nucleotide change to alter the specificity of a glycosyltransferase and hence the antigenicity of a pneumococcal capsule.
Nucleotide sequence accession numbers. Nucleotide sequences described herein have been deposited in GenBank with accession numbers HQ260721 to HQ260725.

We thank David Griffiths, Lisa Miyashita, Katherine Brown, Siobhan Martin, and Seyi Eletu for their contributions to this work.

REFERENCES
1. Ceyhan, M. I. Yildirim, C. L. Sheppard, and R. C. George. 2010. Pneumococcal serotypes causing pediatric meningitis in Turkey: application of a new technology in the investigation of cases negative by conventional culture. Eur. J. Clin. Microbiol. Infect. Dis. 29:289–293.
2. Charnock, S. J., and G. J. Davies. 1999. Structure of the nucleotide-diphospho-sugar transferase, SpcA from Bacillus subtilis, in native and nucleotide-complexed forms. Biochemistry 38:6380–6385.
3. Enright, M. C., and B. G. Spratt. 1998. A multilocus sequence typing scheme for Streptococcus pneumoniae: identification of clones associated with serious invasive disease. Microbiology 144:3049–3060.
4. Ewing, B., L. Hillier, M. C. Wendl, and P. Green. 1998. Base-calling of automated sequencer traces using phred. I. Accuracy assessment. Genome Res. 8:175–185.
5. Griffiths, D. B. 2003. The capsular polysaccharide loci of serogroup 6 Streptococcus pneumoniae. Ph.D. thesis. University of London, London, United Kingdom.
6. Jiang, S. M., L. Wang, and P. R. Reeves. 2001. Molecular characterization of Streptococcus pneumoniae type 4, 6B, 8, and 18C capsular polysaccharide gene clusters. Infect. Immun. 69:1244–1255.
7. Jin, P., F. Kong, M. Xiao, S. Oftadeh, F. Zhou, C. Liu, F. Russell, and G. L. Gilbert. 2009. First report of putative Streptococcus pneumoniae serotype 6D among nasopharyngeal isolates from Fijian children. J. Infect. Dis. 200:1375–1380.
8. Kelley, L. A., and M. J. Sternberg. 2009. Protein structure prediction on the Web: a case study using the Phyre server. Nat. Protoc. 4:363–371.
9. Lairson, L. L., B. Henriissat, G. J. Davies, and S. G. Withers. 2008. Glycosyltransferases: structures, functions, and mechanisms. Annu. Rev. Biochem. 77:521–555.
10. Lee, H., M. H. Nahm, R. Burton, and K. H. Kim. 2009. Immune response in infants to the heptavalent pneumococcal conjugate vaccine against vaccine-related serotypes 6A and 19A. Clin. Vaccine Immunol. 16:376–381.
11. Llull, D., R. Munoz, R. Lopez, and E. Garcia. 1999. A single gene (fts) located outside the cap locus directs the formation of Streptococcus pneumoniae type 37 capsular polysaccharide. Type 37 pneumococci are natural, genetically binary strains. J. Exp. Med. 190:241–251.
12. Mavroidi, A., D. Godoy, D. M. Aanensen, D. A. Robinson, S. K. Hollingshead, and B. G. Spratt. 2004. Evolutionary genetics of the capsular locus of serogroup 6 pneumococci. J. Bacteriol. 186:8181–8192.
13. Pai, R., J. Limor, and B. Beall. 2005. Use of pyrosequencing to differentiate Streptococcus pneumoniae serotypes 6A and 6B. J. Clin. Microbiol. 43:4820–4822.
14. Park, I. H., D. G. Pritchard, R. Cartee, A. Brandao, M. C. Brandileone, and M. H. Nahm. 2007. Discovery of a new capsular serotype (6C) within serogroup 6 of Streptococcus pneumoniae. J. Clin. Microbiol. 45:1225–1233.
15. Pileshvili, T., C. Laxa, M. M. Farley, J. Hadler, L. H. Harrison, N. M. Bennett, A. Reingold, A. Thomas, W. Schaffner, A. S. Craig, P. J. Smith, B. W. Beall, C. G. Whitney, and M. R. Moore. 2010. Sustained reductions in invasive pneumococcal disease in the era of conjugate vaccine. J. Infect. Dis. 201:32–41.
16. Platt, S., B. Pichon, R. George, and J. Green. 2006. A bioinformatics pipeline for high-throughput microbial multilocus sequence typing (MLST) analyses. Clin. Microbiol. Infect. 12:1144–1146.
17. Qasba, P. K., B. Ramakrishnan, and E. Boeggeman. 2005. Substrate-induced conformational changes in glycosyltransferases. Trends Biochem. Sci. 30:53–62.
18. Sheppard, C. L., T. G. Harrison, M. D. Smith, and R. C. George. Development of a sensitive, multiplexed immunoassay using xMAP beads for detection of serotype-specific Streptococcus pneumoniae antigen in urine samples. J. Med. Microbiol., in press.
19. Whitney, C. G., T. Pileshvili, M. M. Farley, W. Schaffner, A. S. Craig, R. Lynfield, A. C. Nyquist, K. A. Gershman, M. Vazquez, N. M. Bennett, A. Reingold, A. Thomas, M. P. Glode, E. R. Zell, J. H. Jorgensen, B. Beall, and A. Schuchat. 2006. Effectiveness of seven-valent pneumococcal conjugate vaccine against invasive pneumococcal disease: a matched case-control study. Lancet 368:1495–1502.
20. Yeh, S. H., A. Gurtman, D. C. Hurley, S. L. Block, R. H. Schwartz, S. Patterson, K. U. Jansen, J. Love, W. C. Gruber, E. A. Emini, and D. A. Scott. 2010. Immunogenicity and safety of 13-valent pneumococcal conjugate vaccine in infants and toddlers. Pediatrics 126:e493–e505.