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# *Streptococcus pyogenes* Pharyngitis: Characterization of Strains by Multilocus Enzyme Genotype, M and T Protein Serotype, and Pyrogenic Exotoxin Gene Probing

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**Multilocus enzyme electrophoresis, serological characterization of M and T proteins, and probing for pyrogenic exotoxin A and C genes were used to investigate the bacteriologic epidemiology of strains of *Streptococcus pyogenes* recovered primarily from patients with recurrent pharyngitis. A total of 164 strains recovered from individuals living in nine states of the United States was analyzed. Two-thirds of the patients in our sample were infected with the homologous strain following antibiotic therapy and presumably represented treatment failures, whereas the other one-third of the patients were infected with a heterologous strain after therapy and probably represented reinfections. Multilocus enzyme electrophoresis was as efficacious in strain discrimination as serologic typing techniques were and, in addition, successfully characterized all organisms that were serologically nontypeable. Two clones of *S. pyogenes* responsible for most of the episodes of toxic shock-like syndrome in the United States are geographically widespread, but they vary by locality in the frequency of their occurrence. Compared with a sample of strains cultured from patients whose pharyngeal infections were eliminated by antimicrobial therapy, these two clones were statistically overrepresented among organisms that cause recurrent pharyngitis.**

Acute pharyngitis caused by *Streptococcus pyogenes* is a major health problem in children worldwide. One topic of special concern in recent years is the therapy of recurrent pharyngitis (2, 3, 5, 6, 9–12, 14–16, 19, 21). Although much work has been done to investigate the bacteriologic epidemiology of streptococcal pharyngitis, less information is available regarding recurrent disease episodes. For example, few studies have attempted to address whether first and subsequent attacks of streptococcal pharyngitis following antibiotic therapy are caused by the same or different organisms. The limited data (3, 5, 6, 11, 15, 21) that bear on this issue have been based virtually entirely on the serotyping of a small number of surface proteins, which may not adequately reflect strain relationships. Moreover, some of the studies (3, 11) have been conducted in the course of local pharyngitis outbreaks, and therefore, the results may not necessarily be generally applicable.

The primary purpose of this multicenter study was to examine the bacteriologic epidemiology of recurrent episodes of pharyngitis caused by *S. pyogenes* through the combined application of multilocus enzyme electrophoresis (18) and serotyping of M and T proteins. In addition, because of the recent increase in the frequency of severe infections caused by *S. pyogenes* strains that express one or more pyrogenic exotoxins (4, 8, 13), we examined a sample of isolates for sequences that hybridize with the structural genes for exotoxins A (*speA*) and C (*speC*) in order to generate baseline data on the occurrence of potentially toxinogenic strains that cause pharyngitis.

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## MATERIALS AND METHODS

**Patients.** Isolates were collected as part of a multicenter study that compared the efficacy and safety of an investigational broad-spectrum oral cephalosporin (ceftibuten) and penicillin V in the treatment of symptomatic pharyngitis, tonsillitis, or scarlet fever in children and adolescents 3 to 18 years of age. Patients with signs and symptoms compatible with acute *S. pyogenes* pharyngitis and/or tonsillitis were eligible for enrollment in the study. To qualify as an evaluable case, it was necessary for the initial pharyngotonsillar swab specimen to grow 10 or more colonies of *S. pyogenes* on a blood agar plate. Patients were excluded from the study if they were known to be chronic carriers of *S. pyogenes* or if they had deep tissue infection involving the upper respiratory tract (epiglottitis, glossitis, retropharyngeal abscess, etc.).

Patients were treated with either ceftibuten (9 mg/kg of body weight per day) or penicillin V (25 mg/kg/day) for 10 consecutive days. Pharyngotonsillar swab specimens were obtained between days 4 and 6 of treatment, between days 5 and 7 posttreatment, and if possible, 2 to 3 weeks posttreatment.

**Localities.** Between November 1988 and March 1990, we studied a total of 164 isolates recovered from patients living in Akron, Ohio ($n$ = 11 isolates); Detroit, Mich. ($n$ = 22 isolates); Scottsdale, Ariz. ($n$ = 24 isolates); Birmingham, Ala. ($n$ = 17 isolates); Salt Lake City, Utah ($n$ = 37 isolates); Cleveland, Ohio ($n$ = 2 isolates); Little Rock, Ark. ($n$ = 5 isolates); Rochester, N.Y. ($n$ = 32 isolates); and Chicago, Ill. ($n$ = 14 isolates). Of the 164 strains, 40 were obtained from patients whose bacteriologic response to therapy was classified as elimination.

**Definitions of bacteriologic responses to therapy.** Bacteriologic responses to therapy were classified as follows: (i) elimination, the absence of *S. pyogenes* from cultures obtained at days 5 to 7 and weeks 2 to 3 posttreatment; (ii) symptomatic persistence (treatment failure), continued signs and symptoms of pharyngotonsillitis and the continued presence of the same strain of *S. pyogenes* in pharyngotonsillar cultures obtained between days 4 and 6 of therapy and/or at 5 to 7 days after treatment; (iii) asymptomatic persistence (conversion to carrier state), recovery of the same causative *S. pyogenes* strain from the pharyngotonsillitis culture at 5 to 7 days posttreatment with or without the presence of the causative strain in the culture obtained after 4 to 6 days of treatment and in the absence of clinical signs and symptoms of pharyngotonsillitis; (iv) relapse, absence of the causative *S. pyogenes* strain and symptoms at 5 to 7 days posttreatment but recovery of the causative strain at greater than or equal to 10 colonies on a blood agar plate in any culture of a pharyngotonsillar specimen taken in the interval from 8 days after the end of treatment through 3 weeks posttreatment accompanied by signs and symptoms of *S. pyogenes* pharyngotonsillitis; (v) reinfection, absence of the causative strain of *S. pyogenes* in the culture at days 5 to 7 posttreatment but the presence of a different strain at greater than or equal to 10 colonies on a blood agar plate in any culture of a pharyngotonsillar specimen obtained during the interval from the end of treatment through 3 weeks posttreatment and the presence of signs and symptoms of *S. pyogenes* pharyngotonsillitis; and (vi) recolonization, the absence of the causative *S. pyogenes* strain from the culture at 5 to 7 days posttreatment but the subsequent isolation of the same or a different strain from any culture of a pharyngotonsillar specimen taken during the period from the end of treatment through 3 weeks posttreatment and without concurrent signs or symptoms of *S. pyogenes* infection. These definitions are similar to criteria used previously (15).

**Bacteriology.** Pharyngotonsillar specimens for culture were collected on a sterile throat swab, inoculated onto a 5% sheep blood agar plate, incubated at 37°C for 18 to 24 h in 5% $CO_2$, and examined for beta-hemolysis and colony morphology. Presumptive *S. pyogenes* colonies were isolated in pure culture on a fresh sheep blood agar plate, tested for their susceptibilities to bacitracin, and grouped by a commercial latex agglutination test. Because of the multicenter nature of the study, no attempt was made to ensure that materials used for *S. pyogenes* identification were obtained from the same commercial suppliers.

**Serotyping.** T protein types were identified by slide agglutination (1, 7) with rabbit antisera obtained from the Centers for Disease Control, Atlanta, Ga. Strains that did not react with T protein agglutination sera were considered to be nontypeable. Strains that reacted with T protein agglutination antisera were then examined for their M protein serotypes by microimmunodiffusion with acid extracts and antisera from the Centers for Disease Control (17).

**Multilocus enzyme electrophoresis.** Methods of protein extract preparation, electrophoresis of soluble enzymes, and selective enzyme staining have been described previously (13, 18). Allelic variation was assayed at 12 metabolic enzyme-encoding loci. Distinctive electromorphs (mobility variants) of each enzyme, numbered in order of decreasing rate of anodal migration, were equated with alleles at the corresponding structural gene locus. Isolates that lacked activity for a specific enzyme were assigned a null allelic state at the locus in question.

Each isolate was characterized by its combination of alleles at the 12 enzyme loci, and distinctive multilocus enzyme genotypes were designated as electrophoretic types (ETs) (18). The ET designations are identical to those used in a previous study of genetic variation in natural populations of *S. pyogenes* (13).

**Definition of a strain.** For the purposes of this study, we considered two isolates of *S. pyogenes* to be the same strain if they were recovered from a single individual and had identical multilocus enzyme genotypes and M and/or T protein serotypes.

**Exotoxin gene probing.** The presence of sequences that hybridized to genes encoding exotoxin A (*speA*) and exotoxin C (*speC*) was determined with a $^{32}P$-labeled DNA probe specific for each gene (8).

## RESULTS AND DISCUSSION

**M protein serotypes and T protein agglutination types.** T protein agglutination patterns were determined for 149 strains, and there were 10 distinct T types recorded (types 1, 2, 3, 4, 11, 11/12, 12, 13, 25, and 28). Fifteen isolates were nontypeable for the T protein agglutination pattern.

M protein serotypes were determined for 90 (55%) of the strains from 56 distinct patients. Of the remaining organisms, 72 (44%) strains from 51 patients were nonserotypeable for M protein, and 2 strains were not studied for their M protein serotypes. The strains in our sample produced M protein serotype 1, 2, 3, 4, or 12 antigens only.

**Multilocus enzyme genotypes.** In contrast to M protein serotyping, for which at least 44% of isolates were nontypeable, all isolates could be assigned a multilocus enzyme genotype. Twenty-two distinctive ETs were identified. There was a strong correlation between the multilocus enzyme genotype and the M protein serotype among strains in our sample, a result consistent with data reported elsewhere (13). For example, strains that expressed the M1 serotype were usually ET type 1 (ET 1), and similarly, strains typed as M3 were usually ET 2. However, three isolates expressing M1 and two isolates typed as M3 were not ET 1 and ET 2, respectively.

The primary advantage of the multilocus enzyme electrophoretic technique in studies such as this is that all isolates are fully typeable for enzyme genotype, and therefore, informative epidemiologic data can be generated for the entire sample of isolates.

**Serial isolates from patients.** Among the 54 patients from whom serial isolates were available, 36 individuals harbored the same strain (relapse patients) and 18 patients had at least two distinctive strains (reinfection patients). For all 18 of the reinfection patients, the multilocus enzyme genotypes of the initial and subsequent strains were not identical. In contrast, for two of these patients the M protein serotypes of the paired isolates were identical, for eight patients all isolates were serologically nontypeable for M protein, and for eight patients one of the strains was M typeable and one strain was M nontypeable. When T protein agglutination patterns were incorporated into the serotyping scheme, the initial and subsequent strains from 16 of 18 patients could be discriminated.

**Exotoxin gene analysis.** As a consequence of the recent significant increase in severe streptococcal disease reported in many areas of the United States (20) and elsewhere (4), we chose to examine a sample of isolates for the presence of genes encoding pyrogenic exotoxins A (*speA*) and C (*speC*). We studied predominantly strains typed as M1 and M3 because of the strong association of strains expressing these

serotypes with the ability to cause streptococcal toxic shock-like syndrome (13). Thirty-five strains recovered from 28 distinct patients were analyzed, including strains expressing serotypes M1 ($n$ = 12 patients), M3 ($n$ = 8 patients), M4 ($n$ = 2 patients), M12 ($n$ = 1 patient), and M nontypeable ($n$ = 8 patients). Sequences that hybridized to a probe specific for *speA* were identified in 15 isolates obtained in cultures of specimens from 11 patients. These organisms included strains expressing the M3 serotype ($n$ = 6 patients), the M1 serotype ($n$ = 4 patients), and M nontypeable ($n$ = 1 patient). Seven isolates contained sequences homologous with *speC*, including strains typed as M3 ($n$ = 2 patients); M nontypeable ($n$ = 2 patients); and M1, M4, and M12 ($n$ = 1 patient each). A single isolate of serotype M3 reacted with both probes. For all cases in which we studied duplicate strains from the same patient, both cultures were cognate for toxin gene hybridization reactivity. We did not examine isolates for sequences that hybridized with an exotoxin B gene probe because virtually all *S. pyogenes* isolates harbor *speB* (8, 13).

Inasmuch as strains that produce pyrogenic exotoxin A and that express the M1 and M3 serotypes account for an unusually large proportion of cases of streptococcal toxic shock-like syndrome in the United States (13), we consider it highly significant that, in the aggregate, for 50% of the patients harboring M1 and M3 strains in our sample, the strains hybridized with a probe specific for *speA*. Included in this probe-positive sample were strains recovered from patients in Detroit, Scottsdale, Salt Lake City, Cleveland, Little Rock, and Chicago. Hence, *S. pyogenes* strains of the same two clonal genotypes (ET 1 and ET 2 [13]) causing most of the toxic shock-like syndrome episodes and harboring a gene whose product has been implicated in the pathogenesis of this disease are geographically highly dispersed and are well represented among isolates from patients with pharyngitis. It is noteworthy that only four ET1 and M1-positive isolates and no ET2 and M3-positive isolates were recovered from patients in Rochester, N.Y., and none of these organisms was *speA* probe positive. These results mean that there is striking geographic variation in the frequency of occurrence of two highly pathogenic streptococcal clones, an observation that partially explains local increases in severe invasive disease episodes (20). Geographic variation in the frequency of clone recovery also implies that conclusions drawn from epidemiologic or other studies with strains cultured from patients living in one or a restricted number of communities may be erroneous as a consequence of significant sampling bias.

**Multilocus enzyme genotypes of elimination isolates and relapse isolates.** Of 40 strains recovered from patients that satisfied our definition of bacteriological elimination, only 4 (10%) were either ET 1 or ET 2. In striking contrast, 13 of 36 isolates cultured from patients classified as relapse cases were of either of these two genotypes ($P < 0.01$ by chi-square analysis). This result suggests that isolates of these two related (13) clonal genotypes are better able to survive antimicrobial therapy than those with other genotypes. Most isolates assigned to ET 1 and ET 2 produce proteins M1 and M3, respectively, and are the major clones that cause streptococcal toxic shock-like syndrome (13). Therefore, enrichment of these two clones among organisms recovered from recurrent cases of pharyngitis is of concern and warrants further study.

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