Comparison of Epidemic and Endemic Group G Streptococci by Restriction Enzyme Analysis

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Restriction enzyme profiles of group G beta-hemolytic streptococci associated with a point source outbreak and an outbreak of sporadic pharyngitis in two different communities were compared. To assess the epidemiologic utility of this approach for studying group G streptococci, DNA fingerprints of strains responsible for a point source outbreak of pharyngitis associated with the consumption of contaminated food were compared with DNA fingerprints of pharyngeal isolates from children with pharyngitis seen at a pediatric practice during a 6-month period. In each epidemiologic situation, a single strain characterized by a unique restriction enzyme pattern predominated. The results are compatible with the conclusion that human infections could be limited to a few strains of group G streptococci which have the capacity to spread through a given population. The restriction enzyme profiles proved to be a highly specific and precise means of evaluating strain relatedness and of providing further understanding of the epidemiology of group G streptococcal infections.

Epidemic pharyngitis caused by group G streptococci (7, 14, 18, 19, 23, 26) is usually associated with food contamination but also, less frequently, with respiratory droplet exposure. In most cases, the symptoms and clinical features closely resemble those of group A streptococcal pharyngitis. Virulence factors have not been well defined for group G streptococci; however, asymptomatic pharyngeal carriage of group G streptococci occurs in up to 23% of humans (7, 20). It has been shown that some strains (2, 8, 16, 17) possess M protein-like antigens which are related to those of the group A streptococci. Furthermore, Simpson et al. (21) demonstrated that DNA from isolates of group G streptococci from humans hybridize to M protein-specific DNA probes, whereas DNA from isolates from animals lack homology to these molecular probes. In contrast to group A streptococci, there is no readily available method to identify strains of group G streptococci to the subspecies level, making epidemiologic study of these organisms impossible. Without appropriate markers, it is also not possible to distinguish strains of the normal flora from truly epidemiologically significant pathogens.

In this study, we examined the use of restriction endonuclease fingerprinting of chromosomal DNA to study the epidemiology of group G streptococcal infections and to assess the relatedness of group G streptococcal strains. This approach has proven to be useful as an epidemiologic tool for the identification of subspecies and tracking of group A and C streptococci (3, 22), as well as a variety of other pathogenic bacteria and fungi (4-6, 9-12, 15, 24, 25). Two separate epidemiologic situations were examined: strains derived from an epidemic outbreak of food-borne pharyngitis at a college cafeteria in Rochester, N.Y., and one from a community-wide respiratory outbreak of group G streptococcal pharyngitis in children in Danbury, Conn.

MATERIALS AND METHODS

Bacterial strains. One group of strains was isolated from individuals involved in an apparent food-borne outbreak of group G streptococcal pharyngitis in a college cafeteria (K. M. Bell, personal communication). Fifty isolates from students (strains 87-043 through 87-051 and R-1 through R-31), food handlers (strains 87-056 through 87-060), and a laboratory technician (strains 87-052 and 87-053) were studied. An additional 66 strains (strains 87-149 through 87-198 and 87-312 through 87-327) from children presenting with pharyngitis at a pediatrics practice in Danbury, Conn., over a 6-month period were also studied. All of the isolates from both sources were of the large colony type and were grown on blood agar plates prior to storage at -20°C in Todd-Hewitt broth with 5% sheep erythrocytes.

DNA extraction and digestion. The technique used for extraction and digestion was a modification of the method used in this laboratory for group A streptococci (3). Strains of group G streptococci were incubated in blood broth and then transferred to 10 ml of Todd-Hewitt broth with 1% yeast extract overnight at 35°C. Cells were washed twice with 0.2 M cold sodium acetate and suspended in TE-glucose buffer (25% glucose, 100 mM Tris hydrochloride, 10 mM EDTA) and digested with mutanolysin (Miles Laboratories, Inc., New Haven, Conn.) at 37°C for 1 h. Protoplasts were suspended in lysis buffer containing diethylpyrocarbonate and were incubated for 30 min at 70°C. Potassium acetate was added to a concentration of 5 M before protoplasts were frozen for 1 h. Bacterial debris was removed from the thawed lysate by microcentrifugation, and then 2 volumes of ethyl alcohol at -20°C was added to the supernatant to precipitate the DNA. The precipitated DNA was then dissolved in RNase-100 mM Tris-10 mM EDTA (pH 8.0) and stored at 4°C. DNA was diluted up to 30 µl with sterile deionized water, enzyme buffer, and *Hind*III restriction enzyme (Bethesda Research Laboratories, Inc., Gaithersburg, Md.), in that order. The DNA was digested for 2 h at

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FIG. 1. (A) Fingerprint patterns of isolates from representative food handlers in Rochester, N.Y., and a laboratory technician (Tech) who became infected during the Rochester outbreak. An example of each distinguishable pattern is shown. Lanes 1 through 8, Isolates 87-053, 87-055, 87-056, 87-057, 87-054, 87-058, 87-059, and 87-060, respectively. (B) Fingerprint patterns of representative isolates from students in Rochester, N.Y. An example of each distinguishable pattern is shown. Lanes 1 through 7, Isolates 87-043, 87-044, 87-045, 87-046, 87-049, 75, and 98, respectively. White lines between fingerprint patterns indicate that they were run on separate gels, and therefore, migration rates may not be identical.

37°C. Three microliters of tracking dye (5% glycerol, 0.025% bromophenol blue, and 0.01% sodium dodecyl sulfate) was added to stop the reaction.

Electrophoretic analysis. The DNA fragments were electrophoresed in a 0.7% agarose gel (Seakem-ME; FMC Corp., Marine Colloids Div., Rockland, Maine) at 55 V for 17 h in Tris borate buffer (pH 8.3) (3). The gels were then stained with 1 µg of ethidium bromide per ml for 30 min and cleared with 0.1 M magnesium sulfate for 30 min. DNA fragments were visualized with shortwave UV light and photographed (type 57 film; Polaroid, Cambridge, Mass.).

Antibiotic susceptibilities. Antibiotic susceptibilities were determined by a modification of the method of Bauer et al. (1) for streptococci. Organisms that were grown overnight in Todd-Hewitt broth with 5% sheep erythrocytes at 35°C were

TABLE 1. Occurrence and distribution of DNA fingerprint patterns obtained from isolates from individuals in Rochester, N.Y.

Fingerprint pattern designation	No. of isolates showing each fingerprint pattern		
	Students (n = 40) ^a	Food handlers (n = 8)	Laboratory technician (n = 2)
1a	37	5	2
1b	1	0	0
1c	1	0	0
2	0	2	0
3	0	1	0
4	1	0	0

^a Numbers in parentheses refer to the total number of isolates tested.

inoculated into fresh Todd-Hewitt broth, incubated at 35°C for 4 h, and then adjusted to an A_{620} of 0.02 to 0.06 with a spectrophotometer (Coleman Jr. II). They were then spread onto 150-mm-diameter Mueller-Hinton blood agar plates by using sterile cotton swabs. Disks containing 10 µg of penicillin, 30 µg of cephalothin, 30 µg of tetracycline, 15 µg of erythromycin, 10 µg of ampicillin, 2 µg of clindamycin, and 30 µg of chloramphenicol were applied; and the plates were incubated for 18 h at 35°C. Zone sizes for susceptibility, resistance, and intermediate susceptibility determinations were measured with a caliper.

Resistance to phagocytosis. Selected group G streptococcal strains were tested for their resistance to phagocytosis, which was presumably dependent on the presence of M protein, by previously described procedures (13).

RESULTS

The local public health department investigated an outbreak of pharyngitis among college students after a holiday banquet dinner in Rochester, N.Y. Sixty-nine students described acute onset of pharyngitis within 4 days after eating at the banquet (Bell, personal communication). Upon analysis of food histories, the ingestion of an imitation crab meat product correlated best with the incidence of disease. Although group G beta-hemolytic streptococci were isolated from the throats of ill students, it was not possible to confirm the presence of these bacteria in the food (Bell, personal communication).

A total of 50 isolates (40 isolates from students, 8 isolates from food handlers, and 2 isolates, separated by 3 weeks, from a laboratory technician who worked with the specimens) from the outbreak were analyzed for their restriction enzyme electrophoresis patterns in an attempt to identify and characterize the responsible strain.

Figure 1A shows one of two identical fingerprint patterns from the isolate from the laboratory technician and representative patterns of isolates from four of the five food handlers that had the predominant fingerprint pattern (designated pattern 1a). Distinctive patterns are numbered. Distinctive but closely related patterns were considered to be subgroups (assigned the letters a through d). The DNA patterns of isolates from the other three food handlers (designated types 2 and 3) are shown. The arrow points out the presence of a low-molecular-weight plasmid in this single isolate.

Figure 1B shows restriction profiles of representative isolates from students that gave the predominant pattern (designated pattern 1a, 1b, or 1c). One different isolate from a student that did not give the predominant pattern (designated pattern 4) is also shown.

TABLE 2. Comparison of DNA fingerprint patterns obtained from isolates from individuals in Rochester, N.Y.

Fingerprint pattern designation	% Similar fragments ^a					
	1a	1b	1c	2	3	4
1a	100					
1b	91	100				
1c	93	91	100			
2	72	65	63	100		
3	83	73	74	73	100	
4	74	69	80	57	74	100

^a The percentage of similar fragments was calculated by dividing the number of fragments shared by two fingerprint patterns by the total number of fragments counted.

Table 1 indicates the number of distinct patterns and their distributions among students, food handlers, and the laboratory technician. A total of 97.5% of the isolates tested had an identical or very similar pattern of *Hind*III fragments, suggesting that a single organism was the causative agent. By counting similar fragments between strains by visual inspection, we determined that there was a greater than 90% similarity between the patterns designated types 1a, 1b, and 1c (Table 2). Fingerprint patterns 2, 3, and 4 could be clearly distinguished from the epidemic strain. These strains may represent normal flora in those individuals.

The community-wide outbreak of group G streptococcal pharyngitis in Danbury, Conn., provided an opportunity to test whether disease was associated with dissemination of a single bacterial strain or a variety of strains with pathogenic potential. A total of 66 isolates from pediatric patients with acute pharyngitis cared for at a pediatrics practice in Danbury, Conn., were analyzed. Figure 2 shows the seven distinctive restriction enzyme patterns obtained from representative isolates. Note the low-molecular-weight plasmid pointed out by the arrow in the predominant pattern designated pattern 5.

FIG. 2. Fingerprint patterns of representative isolates from pediatric patients in Danbury, Conn., showing examples of each distinguishable pattern. Lanes 1 through 11, Isolates 87-157, 87-158, 87-153, 87-177, 87-178, 87-315, 87-163, 87-149, 87-324, 87-325, and 87-326, respectively. White lines between fingerprint patterns indicate that they were run on separate gels, and therefore, migration rates may not be identical.

TABLE 3. Occurrence and distribution of DNA patterns obtained from a community-wide outbreak

Fingerprint pattern designation	No. of isolates showing each fingerprint pattern
5a	47
5b	1
1d	12
6a	2
6b	1
7	2
8	1

Table 3 indicates the number of distinct patterns and their distributions among pediatric patients in Danbury, Conn. The pattern designated pattern 5 appeared in 72.7% of the 66 isolates. Those patterns designated 5a and 5b were 95% similar, and those patterns designated 6a and 6b were over 96% similar (Table 4).

Each patient had as many as four isolates during the acute or convalescent phase of infection. There were six sets of siblings among the pediatric patients. From the fingerprint patterns obtained, we were able to ascertain that three of six sets of siblings were infected with identical strains (two sets with pattern 1d and one set with pattern 5), while the remaining three sets of siblings were infected with similar but distinguishably different strains (in one set, one sibling was infected with pattern 5 and one was infected with pattern 1d). Of four patients with multiple group G streptococcal isolates, one yielded the original strain 1 month later, while three patients harbored similar but distinguishably different strains (DNA patterns 1d and 5).

There was 90% similarity between pattern 1d from the Danbury, Conn., isolates and pattern 1a from the Rochester, N.Y., isolates (Table 5). To obtain better resolution in the fingerprint patterns, these DNAs were also digested with restriction enzyme *Hae*III (Bethesda Research Laboratories). Because *Hae*III cuts occur more frequently along the DNA strand (it is a 4-base cutter, in contrast to the 6-base cutter *Hind*III), the differences between patterns 1d and 1a were more obvious (Fig. 3). Although not identical, pattern 1d and 1a strains were clearly clonally related.

In an attempt to identify additional markers and to characterize the strains further, antibiotic susceptibilities and the abilities of the strains to resist phagocytosis were examined. Strains representing eight fingerprint patterns were tested. All isolates tested were shown to be susceptible to ampicillin, chloramphenicol, erythromycin, clindamycin, cephalo-

TABLE 4. Comparison of similarity percentages between DNA fingerprint patterns obtained from epidemic isolates of group G streptococci

Fingerprint pattern designation	% Similar fragments ^a						
	5a	5b	1d	6a	6b	7	8
5a	100						
5b	95	100					
1d	80	NC ^b	100				
6a	80	NC	74	100			
6b	NC	NC	NC	96	100		
7	77	NC	76	66	NC	100	
8	75	NC	81	67	NC	69	100

^a Percent similarity was calculated as described in footnote a of Table 2.

^b NC, Not counted.

TABLE 5. Comparison of fingerprint patterns obtained from isolates in Rochester, N.Y., and Danbury, Conn.

Rochester, N.Y., fingerprint pattern	% fragments similar to the following Danbury, Conn., fingerprint patterns ^a				
	5a	1d	6a	7	8
1a	68	90	73	56	67
2	67	79	70	69	65
3	86	75	68	66	55
4	82	72	78	72	75

^a Percent similarity was calculated as described in footnote a of Table 2.

thin, and penicillin. Susceptibility was variable only with regard to tetracycline, but this did not prove to be a useful distinguishing feature.

All of the representative isolates proved to be resistant to phagocytosis by demonstrating a 21- to 705-fold increase in CFU following 3 h of incubation with fresh human blood. Again, there was no correlation between growth in blood and fingerprint patterns. Growth in blood suggested that these strains express M proteins and is in agreement with the findings of Simpson et al. (21), who have suggested that isolates of group G streptococci from humans have an M protein on their surfaces.

DISCUSSION

Group G streptococci are known to be associated with clinically symptomatic pharyngitis, sore throat, difficulty in swallowing, enlarged tonsils, pharyngeal exudate, and fever. Although frequently isolated from pharyngeal cultures, the relative importance of group G bacteria as an etiologic agent in patients with pharyngitis is unclear. The taxonomic confusion created by serogrouping, which places strains together that may have diverse biological properties, and the

lack of reliable methods to distinguish true pathogens from transient colonization by strains with low virulences is, in part, responsible. The intention of this study was to explore the possibility that restriction enzyme profiles of total DNA could serve to identify specific strains associated with disease in humans. This approach has been successfully used to subgroup and monitor the epidemiology of a variety of bacterial pathogens (3-6, 9-12, 15, 22, 24, 25). Among the group A streptococci, the DNA fingerprint is serotype specific and can even reflect the geographic origin of the strain (3). Comparisons of DNA fingerprints have advantages over methods that depend on phenotype (i.e., enzyme or antigen profiles), because the locations of restriction sites in DNA are not subject to culture conditions or dramatic genetic instability.

To evaluate the utility of DNA fingerprinting, strains of group G streptococci from two specific epidemiologic situations were analyzed. Examination of a group of strains isolated from students infected by consumption of contaminated food documented that a single strain produced the outbreak. Nearly all isolates (92%) from ill individuals showed the same or closely related restriction fragment profile, designated DNA pattern 1. Restriction fragment length polymorphism was observed in the higher-molecular-weight fragments; these were distinguished by the letters a through d. Greater than 90% of the fragments from these strains, however, were of the same size.

The indisputable association of pharyngitis with acquisition of this single group G streptococcal strain demonstrated that these bacteria have the capacity to cause significant disease in humans. This conclusion was further confirmed by the unknowing inoculation of a laboratory technician who subsequently developed pharyngitis. Furthermore, the fact that this technician became infected with the pattern 1 strain, presumably from a smaller dose, as this individual had not consumed the suspected contaminated food, suggests that this strain may be more virulent than other strains. This conclusion is also supported by the observation that this was the second most common DNA pattern associated with sporadic disease. The epidemiology of group G streptococcal disease may be comparable to that of type B meningococci. Kristiansen et al. (10) suggested that although large numbers of different B15 clones of meningococci are present in the population they studied, only one clone caused invasive disease. Perhaps the number of clones of group G streptococci that have the capacity to cause pharyngitis is limited.

In a Connecticut community, Gerber and Randolph (M. A. Gerber and M. Randolph, unpublished data) have witnessed an increase in the frequency of isolation of group G streptococci from children with documented clinically significant pharyngitis. We compared the DNA fingerprints of 66 strains isolated over a 6-month period from pediatric patients in that community. This collection of strains provided an opportunity to evaluate the relationship between strains of group G bacteria isolated from a community. Unexpectedly, these were not a heterogeneous group of unrelated organisms; instead, most exhibited one of two DNA restriction patterns. DNA pattern 5 and 1d strains were the most common, representing 72 and 18%, respectively, of the 66 cultures analyzed. The DNA pattern 1d strain appeared to be clonally related to the pattern 1a strain that was responsible for the food outbreak in Rochester, N.Y.; more than 90% of their fragments were identical in size. If DNA patterns 5a, 1d, and 6 with 80% similarity are considered to be clonally related, then this clone accounted for 95% of the infections over the

FIG. 3. Comparison of two similar but distinguishable fingerprint patterns with *Hind*III and *Hae*III. Lanes 1 and 5, Danbury isolate 87-177; lanes 2 and 6, Rochester isolate 87-044; lanes 3 and 7, Danbury isolate 87-165; lanes 4 and 8, Rochester isolate 3.

6-month period of collection. In general, our data suggest that in the population studied, a limited number of group G streptococcal strains have the capacity to cause disease in humans. To verify this observation, strains from sporadic cases of pharyngitis in other geographically separated communities should be compared.

The decision to separate strains into distinct DNA subpatterns is admittedly arbitrary; however, quantitative comparison of fragment similarity suggested a reasonable rationale (3). Cleary et al. (3) noted that strains of group A streptococci with the same serotype had more similar fingerprints than did those with different M proteins. Epidemiologically related strains of the same serotype exhibited *Hind*III fragments which were more than 85% similar, whereas heterologous group A strains with different M antigens had less than 75% similar *Hind*III restriction profiles (3). Unfortunately, this method is not amenable to instrumentation because of unequal compressions across the gel and day-to-day variations.

Categories for group G streptococcal strains were chosen with these percentages as a guide. The observation of polymorphism in high-molecular-weight fragments is curious and was also observed to occur in strains of group A streptococci (3). The variability in DNA patterns among the strains isolated from students infected by contaminated food is remarkable. It is highly unlikely that students transmitted this organism to one another. If we assume that the pattern 1a strains was the original strain contained in the contaminated food, then the various restriction fragment length polymorphisms arose during the infection of an individual student. This could be explained by the acquisition of a bacteriophage, transposon, or plasmid from the microbial flora of that individual. Indeed, one strain had, in fact, acquired what appeared to be plasmid DNA based on the high-intensity fluorescence of some DNA bands. The acquisition of extrachromosomal DNA sequences in this manner greatly expands the potential of these organisms to adapt to the host environment and could provide a means for relatively avirulent strains to acquire new genetic information. Perhaps genes that encode proteins such as M protein, the immunoglobulin G Fc receptor, and inhibitors of chemotaxis and that permit them to avoid the host's defenses are shuffled between species.

We were unable to ascertain whether there was a sharp boundary between strains that were relatively avirulent and those that were able to cause pharyngeal infection. Hybridization studies indicated that strains isolated from animals lacked M-protein genes and DNA sequences flanking this region of the streptococcal genome (21). We wonder whether this dichotomy could also exist among human isolates. A thorough comparison of restriction profiles of group G streptococcal strains isolated from healthy individuals with those described here would further test the possibility that truly pathogenic subspecies exist.

The placement of group G streptococci into one or more species is still under debate. Until this state of taxonomic flux is resolved, the analysis of restriction fragment length polymorphisms may be the only accurate means to distinguish one strain from another in an epidemiologically meaningful manner. The method we described here is reproducible and applicable to relatively large numbers of cultures. Our data illustrate the potential of restriction enzyme analysis as a high-resolution epidemiologic tool for the identification and surveillance of group G streptococcal infections.

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