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Typing of Group A Streptococci by Random Amplified Polymorphic DNA Analysis

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Random amplified polymorphic DNA (RAPD) analysis was evaluated in comparison with restriction endonuclease analysis (REA) of genomic DNA and serotyping in the typing of 160 epidemiologically unrelated group A streptococci (GAS). Amplification of genomic DNA of GAS was performed with a single primer with an arbitrarily selected nucleotide sequence of 12 nucleotides. In total, 31 RAPD patterns and 15 REA patterns were observed among the isolates studied. The results of RAPD analysis were in accordance with the results of REA for 86% of the isolates, as both methods identified 15 different strains among 138 isolates. However, RAPD analysis differentiated 16 additional strains among 22 isolates. RAPD analysis was somewhat better than REA for differentiation of isolates of the same and different serotypes. However, not all of the serotypes were differentiated by RAPD analysis either. In conclusion, RAPD analysis provides a practical alternative for genomic typing of GAS. It can be recommended for the typing of GAS, especially if used in parallel with serotyping.

Group A streptococcus (GAS; *Streptococcus pyogenes*) is an important cause of bacterial infections in both adults and children throughout the world (3). Accurate identification of GAS strains is important for the study of the epidemiology, pathogenesis, and therapy of infections with GAS.

For more than half a century, serotyping of T and M antigens has been the standard method used for typing of GAS (13). A major limitation of serotyping is that a fairly large proportion of isolates, 15 to 40% in our experience (18, 19), are nontypeable. During the last 6 years, DNA polymorphism assays, such as restriction endonuclease analysis (REA) of genomic DNA and analysis of DNA restriction fragment length polymorphism of rRNA genes (ribotyping), have been applied to the typing of GAS (1, 2, 5–7, 9, 15, 19). These methods have been shown to differentiate clones among GAS isolates of the same serotype (6, 19).

The DNA polymorphism assay most recently introduced for the typing of GAS is random amplified polymorphic DNA (RAPD) analysis (22), which has also been called arbitrarily primed PCR. This method is faster, technically easier, and more economical than the older genomic typing methods, i.e., REA and ribotyping. Unlike conventional PCR, data on the DNA sequence of the organism are not a prerequisite for RAPD analysis. In RAPD analysis, the genomic DNA is amplified with a single primer with an arbitrarily selected nucleotide sequence. Binding of the primer to the template DNA is favored by a low annealing temperature. The multiple PCR products are separated according to size by conventional agarose gel electrophoresis. The resulting RAPD patterns of different isolates can then be compared.

In our studies, REA has proven to be a reproducible and discriminatory method for typing of GAS. However, we have used it together with serotyping since GAS isolates with different serotypes are sometimes not differentiated by REA alone (18, 19). Studies of other organisms have previously shown RAPD analysis to discern different strains as well as REA (4, 12, 20, 21). With GAS, however, these methods have not been compared. In this study, we compared the typing of GAS by RAPD analysis with that by REA and serotyping.

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MATERIALS AND METHODS

Bacterial isolates. A total of 160 epidemiologically unrelated GAS isolates were studied. They were collected from blood cultures of hospitalized patients throughout Finland from January 1988 through December 1990 (18). Identification of the organisms was done as previously described (19).

Serotyping. Serotyping was performed by using standard techniques as previously described (19).

DNA preparation and restriction endonuclease analysis. Genomic DNA was extracted from the GAS isolates as previously described (19). The DNA was cleaved with endonuclease *Hin*dIII under conditions recommended by the manufacturer (Boehringer GmbH, Mannheim, Germany). The digested DNA was separated by agarose gel electrophoresis, made visible, and photographed as previously described (19). The REA patterns were examined by eye as previously described (19).

Selection of a primer. The DNAs of selected isolates were initially amplified with seven different primers. Universal sequencing primer M13 (TTATGTAAAACGACGGCCAGT), with a G+C content of 43%, has previously been used for RAPD analysis of GAS (22). The other six 12-mer oligonucleotide primers were designed in our laboratory and were originally selected and tested for use in RAPD analysis of *Bordetella pertussis* (11). Two of these were randomly selected from the pertussis toxin gene, and four were from the repeated gene element of *B. pertussis*. Four primers had a G+C content of 83%, one had 74% G+C, and one had 92% G+C. The sequences of the six primers were considered to be random in relation to the GAS genome. After the seven primers were tested with different reagent concentrations under different reaction conditions, four primers were found to produce reproducible RAPD patterns. The ability of these primers to

differentiate epidemiologically unrelated strains of GAS was further evaluated. After testing of 20 unrelated isolates, RAPD patterns produced by primers M13 and H2 (CCTCCCGC CACC) seemed to discern the strains similarly. However, testing of 30 additional isolates with the two primers showed that primer H2, when used alone, produced the most discriminative RAPD patterns, i.e., patterns with the best ability to differentiate unrelated strains. Consequently, this primer was used to amplify the DNAs of all isolates.

PCR amplification and detection of PCR products. After PCR conditions had been optimized, amplification was performed by using a DNA thermal cycler (HB-TR1; Hybaid Ltd., Middlesex, United Kingdom). The 50-μl reaction mixture contained 50 mM KCl, 10 mM Tris-HCl (pH 8.8), 1.5 mM $MgCl_2$, 0.1% (vol/vol) Triton X-100, 200 μM deoxyribonucleotides, 50 pmol of primer, 2 U of DynaZyme polymerase (Finnzymes Oy, Espoo, Finland), and 20 to 50 ng of DNA extracted from GAS. A total of 40 amplifying cycles, each consisting of denaturation at 94°C for 30 s, annealing at 38°C for 1 min, and synthesis at 72°C for 1 min, were carried out with the thermal cycler. After the amplification, 20 μl of the reaction mixture was run in a 1.5% agarose gel (FMC Bio Products, Rockland, Maine). A 100-bp ladder (Gibco-Bethesda Research Laboratories, Gaithersburg, Md.) was used in each gel as a DNA fragment size marker. After staining with ethidium bromide, PCR products were photographed with type 667 film (Polaroid, Cambridge, Mass.) in UV light. The arbitrarily primed PCR patterns were examined by direct visual comparison of the patterns and the 100-bp ladder.

In repeated analyses of selected GAS isolates, the results obtained with RAPD analysis were found to be reproducible. When the results of RAPD analysis and REA disagreed, RAPD analysis was repeated at least once.

RESULTS

RAPD analysis was compared with REA and serotyping in the typing of 160 epidemiologically unrelated GAS isolates. Thirteen different serotypes (Table 1) were represented among the 160 GAS isolates studied. Sixteen (10%) isolates were nontypeable by serotyping. The most common serotypes were T1M1 (25% of all isolates), T12M12 (9%), T11 (12%), T28 (11%), and T6 (8%).

Fifteen of the previously found (19) REA patterns (A to O in Table 1) were represented among the GAS isolates studied. The most common REA patterns were A (24% of all isolates), E (22%), F (8%), G (8%), K (8%), and H (7%).

Amplification of genomic DNAs from the GAS isolates with primer H2 resulted in RAPD patterns consisting of two to nine distinct DNA fragments, generally ranging from approximately 190 to 1,500 bp. However, in RAPD pattern Oa, the size of one fragment exceeded 2,000 bp (Fig. 1 and 2).

A total of 31 different RAPD patterns (Aa to Ob in Table 1 and Fig. 1) were found among the 160 GAS isolates studied. The results of RAPD analysis and REA were in accordance for 86% of the GAS isolates, as both methods identified the same 15 different strains among 138 isolates. Among 22 isolates, RAPD analysis differentiated 16 additional strains.

All of the 15 REA patterns were distinguished by RAPD analysis (Table 1). The results of RAPD analysis were identical to those of REA among the isolates with five REA patterns (G, H, I, J, and M), as all of the isolates with a REA pattern had the same RAPD pattern (Table 1). Among the isolates with 10 REA patterns (A, B, C, D, E, F, K, L, N, and O), up to four different RAPD patterns were observed within a REA pattern (Table 1). The isolates with REA patterns B, C, D, N, and O

TABLE 1. RAPD patterns of GAS isolates in comparison with REA patterns and serotypes

Serotype	No. of isolates studied (%)	No. of isolates with RAPD pattern indicated and following REA pattern:														
		A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
T1M1	40 (25)	36 Aa, 2 Ab												2 Ma		
T12M12	14 (9)				4 Da, 1 Db							9 Ka				
T12M22	9 (6)				1 Db		2 Fa					1 Ka	2 La	1 Ma	2 Na	
T11M12	6 (4)					6 Ea										
T14M49	6 (4)						6 Fa								1 Nb	
T2M65	1 (<2)															
T11	20 (12)					20 Ea										
T28	18 (11)			1 Ca, 1 Cb		2 Ea, 1 Eb			11 Ha	1 Ia					1 Nc	
T6	13 (8)							12 Ga				1 Kb				
T22	8 (5)						2 Fa				4 Ja		1 Lb			1 Oa
T4	4 (2)	1 Ab													1 Na, 1 Nb, 1 Nd	
T13	3 (<2)				1 Dc, 1 Dd							1 Kb				
T9	2 (<2)					1 Ea	1 Fb									
None[a]	16 (10)		2 Ba, 1 Bb, 1 Bc			6 Ea	1 Fc				2 Ja					1 Oa, 2 Ob
Total	160	39	4	2	8	36	12	12	11	1	6	12	3	3	7	4

[a] Nontypeable by serotyping.

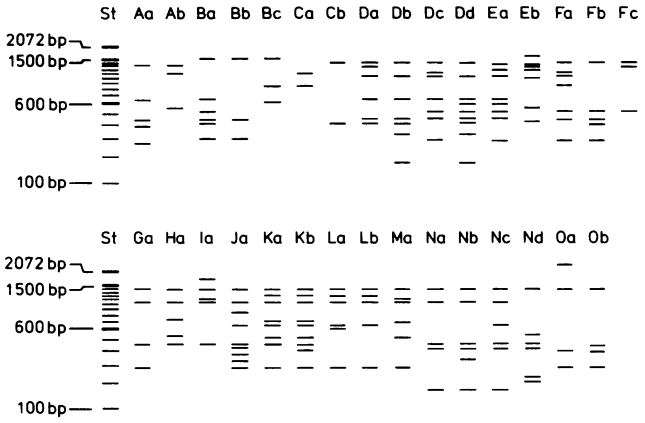

FIG. 1. Schematic drawing of the 31 different RAPD patterns found among 160 GAS isolates. St denotes the 100-bp DNA ladder.

were the most heterogeneous, since no more than 43 to 50% of the isolates with a REA pattern shared a RAPD pattern. REA patterns B and O belonged mainly to isolates nontypeable by serotyping (Table 1). In contrast, the isolates with REA patterns A, E, F, and K were much less heterogeneous, since 83 to 97% of the isolates with a REA pattern shared a RAPD pattern.

The correlation between the results of RAPD analysis and REA was high among isolates of the same serotype. Among isolates with eight serotypes (T12M22, T11M12, T14M49, T2M65, T11, T6, T22, and T9), the results of RAPD analysis and REA were identical (Table 1). The results of these two methods were in accordance for 88 to 94% of isolates with serotypes T1M1, T12M12, and T28 and for 33 to 50% of isolates with serotypes T4 and T13. Within these serotypes, RAPD analysis differentiated one or two more pattern types than did REA (Table 1). In total, RAPD analysis produced up to seven patterns (Table 1, serotype T28) and REA produced up to six different patterns among isolates with the same serotype (Table 1, serotype T12M22). The results of RAPD analysis were in accordance with those of REA for 13 (81%) of the 16 nonserotypeable isolates. Among the nonserotypeable isolates, RAPD analysis differentiated eight strains and REA differentiated five strains (Table 1).

RAPD analysis was better than REA at differentiating between isolates with different serotypes. However, not all of the serotypes were distinguished by RAPD analysis either (Table 1 and Fig. 2b). For example, RAPD pattern Ea was found within serotypes T11M12, T11, T28, and T9 and among nontypeable isolates and RAPD pattern Fa was found within serotypes T12M22, T14M49, and T22.

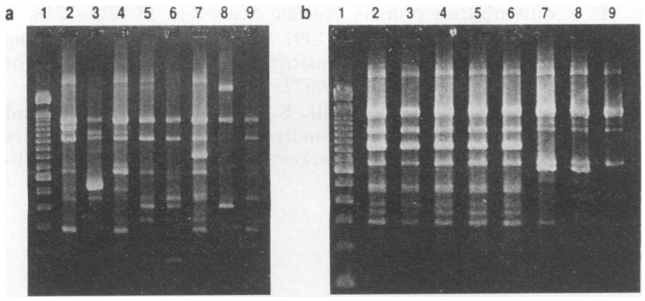

FIG. 2. RAPD patterns of selected isolates with different serotypes. (a) Lanes (RAPD pattern/serotype): 1, 100-bp DNA ladder; 2, Ka/T12M22; 3, Fa/T12M22; 4, Ma/T12M22; 5, Db/T12M22; 6, Na/T12M22; 7, Ja/T22; 8, Oa/T22; 9, Ga/T6. (b) Lanes: 1, 100-bp DNA ladder; 2, Ja/T22; 3, Ja/T22; 4, Ja/T22; 5, Ja/nonserotypeable; 6, Ja/nonserotypeable; 7, La/T12M22; 8 La/T12M22; 9, Lb/T22.

DISCUSSION

REA is a useful method for genomic typing of GAS, since it can add to the discriminatory power of serotyping, the standard method for typing of GAS (19). RAPD analysis is a new molecular typing method with several advantages over REA. It is faster, technically less demanding, and more economical. The amount of DNA needed for RAPD analysis is much smaller than that needed for REA, and digestion by restriction endonuclease enzymes is not necessary. Because of the smaller number and better resolution of DNA fragments in agarose gel, analysis of RAPD patterns is easier than analysis of REA patterns. In addition, our study indicates that with an appropriate primer the discriminatory power of RAPD analysis can be equal or even superior to that of REA. This finding is in accordance with studies on other organisms comparing RAPD analysis with REA (4, 12, 20, 21).

In our study, the results of RAPD analysis with primer H2 were in accordance with the results of REA with endonuclease *Hin*dIII for 86% of the isolates studied. Within 8 of the 13 serotypes, the results of these two methods were identical. However, within five serotypes, as well as the nontypeable isolates, RAPD analysis identified more different strains than did REA (Table 1). Small DNA sequence changes, e.g., mutations, insertions, or deletions not involving restriction sites, may not be detected by REA. These changes may, however, produce a new RAPD pattern by creating or destroying a target site of the PCR primer (24). This could explain why this RAPD analysis was slightly more efficient at detecting genomic differences than was REA with restriction enzyme *Hin*dIII. A technical reason could be that better resolution of the amplified fragments in agarose gel may reveal differences located in the shortest restriction fragments and therefore not noticed in REA.

Although RAPD analysis was better than REA at differentiating between isolates with different serotypes, it did not differentiate all serotypes either. This gives further support to the concept that some serotypes are phylogenetically more closely related than others (10). The genetic heterogeneity found by RAPD analysis within serotypes is probably due to factors similar to those responsible for the genetic heterogeneity of REA patterns, which are discussed in detail elsewhere (19).

Selection of an appropriate primer and optimization of PCR conditions are of great importance for maximization of the discriminatory power of RAPD analysis (8, 23). Primers that work for some bacteria may not work for others. We tested seven primers with G+C contents varying from 43 to 92%. Four of these, including universal sequencing primer M13, produced reproducible RAPD patterns. The differences in the discriminatory powers of these primers became apparent only after analysis of a relatively large number of isolates, which showed primer H2 to be superior to the others (data not shown). It is thus important to compare each primer with other molecular typing methods with a sufficiently high number of unrelated isolates.

It has been shown to be possible to increase the discriminatory power of RAPD analysis by using two primers together (16, 23). However, in the present study we achieved good discrimination by using only one primer. This was also true in

the study of *Streptococcus uberis* isolates by Jayarao et al. (12). Van Belkum et al. (20) have reported obtaining even better discrimination among *Legionella pneumophila* isolates with one primer than with the same primer combined with another.

A drawback of RAPD analysis in general is that the RAPD patterns produced by a given primer may be highly dependent on the specific conditions of the reaction and the concentrations of the reagents. Especially, variations in the annealing temperature, the primer-template concentration ratio, and the magnesium concentration have been reported to affect the result (8, 14). The sensitivities of different primers, even primers with the same G+C content, to variations in these parameters may be different (11). When using a sensitive primer, even the use of a different thermal cycler may cause variation (11, 17). Of the seven primers tested in this study, H2 proved to be the least sensitive to variation in the reaction parameters (data not shown). Thus, this primer is likely to be suitable for use in other laboratories also.

In conclusion, RAPD analysis is a new molecular method which provides a practical alternative for genomic typing of GAS. In this study, RAPD analysis had somewhat better discriminatory power than REA. Since RAPD analysis did not differentiate between all GAS isolates with different serotypes, we recommend that it be used in parallel with serotyping, unless a primer or combination of primers with even better discriminatory power is discovered.

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