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Genotyping of group A streptococcus by various molecular methods

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***Background & objectives*: Group A streptococcus (GAS) causes a wide variety of life threatening diseases in developing countries like India. Characterization of GAS is therefore necessary for prevention and control of the disease. Genotypic analysis of GAS is largely lacking from India, therefore an attempt was made to study the genotype distribution of north Indian GAS isolates.**

***Methods*: Sixty clinical isolates of GAS, (52 collected from pharyngitis and 8 from RF/RHD patients) were genotyped by various molecular techniques like restriction enzyme analysis (REA), ribotyping, PCR-ribotyping and random amplification of polymorphic DNA (RAPD). A few isolates were also typed by *emm* gene sequencing for comparison.**

***Results*: REA using *Hind* III digestion differentiated the isolates into six different patterns. The same isolates were grouped into three ribotypes when analyzed for PCR – ribotyping of 16S- 23S rRNA region. However, RAPD fingerprints generated higher level of discrimination by AP4 and AP5 primers showing 12 rapdemes, followed by AP3, AP2 and API producing 11, 9 and 6 rapdemes respectively. A total of 78 RAPD fragments or rapdemes were generated, of which 48 (62%) were shared and 30 (38%) were unique. These unique RAPD fragments could be used as a genetic marker for identification of GAS. Representative isolates that produced 12 different rapdemes by AP5, on further confirmation by *emm* typing showed 11 different *emm* types.**

***Interpretation & conclusions*: The finding of our study demonstrated the RAPD profiling to be the most discriminatory for genotyping of group A streptococcus isolates as well as comparable to the most commonly used sophisticated technique of *emm* typing.**

Key words *emm* typing - GAS - pharyngitis - RAPD - REA - RF/RHD - ribotyping

The pathogenic role of group A β haemolytic streptococcus (GAS, *Streptococcus pyogenes)* has been well studied in a wide variety of suppurative infections like pharyngitis as well as in nonsuppurative sequelae like rheumatic fever/ rheumatic heart disease (RF/RHD)[1]. The clinical association between pharyngitis and the subsequent appearance of rheumatic fever has been long

recognized. However, all the cases of pharyngitis do not necessarily lead to RF/RHD, which is associated with significant morbidity and mortality, especially in developing countries[1-3]. In India itself the prevalence of RF/RHD varies from 0.3 to 5.4 per 1000 children[3]. Moreover no significant decline in the percentage of RF/RHD cases over past twenty years has been observed in the Indian perspective[4-6]. Hence, for prevention and diagnosis it becomes essential to characterize the GAS strains in a particular community.

The epidemiological investigations of streptococcus have been known to employ standard classical techniques based on serology[7]. Such methods however, have their own limitations, *i.e.,* these are associated with low specificity, high failure rate and involve high cost[7,8]. Therefore, a number of genotyping techniques like ribotyping[8-12], pulse field gel electrophoresis[13,14], random amplified polymorphic DNA (RAPD) analysis[8,14,15] Vir/*emm* typing[16-18] and multilocus sequence typing[19] are being applied worldwide for typing of GAS isolates.

In India, information regarding genotyping of GAS isolates is largely lacking. In an earlier study, we identified several antigenically distinct streptococcal erythrogenic toxin genes and their distribution among GAS isolates[20]. In a later study, type distribution among 40 GAS isolates was monitored by using expensive techniques of *emm* typing[18]. In the present study we attempted discrimination among GAS isolates obtained from north India by various easy to use and reproducible molecular methods.

Material & Methods

Bacterial isolates and genomic DNA preparation: Sixty clinical isolates of GAS were selected randomly from the samples collected from March 1995 to February 1996, from peri-urban slum area near Chandigarh. Of which, 52 isolates were collected from throat swabs of pharyngitis and eight were from RF/RHD patients[20]. Genomic DNA was prepared from overnight grown streptococcal cultures by modified SDS-phenol chloroform method[21].

Restriction endonuclease analysis (REA): 10 µg of genomic DNA was digested with 100 units each of *Hin*d III, *Hae* III and *Eco*RI restriction enzymes (Boehringer Mannheim, Germany) according to the manufacturer's instruction. Digested DNA was electrophoresed on 0.8% agarose gel and REA patterns were examined under UV transilluminator (San Gabriel, USA) for direct visual comparison.

Ribotyping: The electrophoresed DNA digests were blotted on Hybond N^+ nylon membrane and hybridized with 5'end ^{32}P labelled 16S rRNA oligo probe at 37 °C. The probe used was a synthetic oligonucleotide (5' AAGAGTTTGATCCTGGCTCAG3') from bacterial 16S rRNA (Biobasics, Canada). The hybridized membranes were washed and autoradiographed as described by Seppala *et al*[8].

PCR ribotyping: Genomic DNA was subjected to PCR amplification with primer (Southwest Scientific Resource Inc, USA) specific for 16S and 16S-23S rRNA (Table I). The PCR reaction was performed in a total volume of 25 µl in presence of 200 µM (each) dNTPs, 100 ng of primer, 1.25 unit of *Taq* DNA polymerase (Boehringer Mannheim, Germany) and 2 µl of template DNA (5ng/µl). Amplification was performed in an automated thermocycler (Perkin-Elmer Cetus, USA) programmed for denaturation at 94°C for 1 min, annealing at 49°C for 16S rRNA and 55°C for 16S-23S rRNA for 2 min each and extension at 72°C for 2 min. The amplification products were electrophoresed (12 µl) in 0.8 per cent agarose gel in TBE buffer containing ethidium bromide. PCR amplicons were digested with restriction enzyme (10 units each) *Hae*III and *Hae*III–BglI (Boehringer Mannheim, Germany) at 37°C for 2-3 h, according to the manufacturer's instructions. Digested products were run on 2 per cent agarose gel stained with ethidium bromide and visualized in an UV transilluminator.

Table I Primers used for PCR ribotyping and RAPD fingerprinting of GAS isolates

Primers used for PCR ribotyping

Gene	Primer sequence
16S rRNA	5'AAG AGT TTG ATC CTG GCT CAG '3'
	5'GGT TAC CTT GTT ACG ACT T'3'
16S-23S rRNA	5'TTG TAC ACA CCG CCC GTC A '3'
	5' GGT ACC TTA GAT GTT TCA GTT C '3'

Primers used for RAPD analysis

Primer	Primer sequences	G+C content (%)
AP1	5' TGC TTT GTC CGG GTT TTC TAC CGT CCC CCT 3'	57
AP2	5' AAG TAC AGG GCG GAC TCC 3'	61
AP3	5' ATG TCC CGC GTC AGG 3'	67
AP4	5' GCC CGA GCA ACA CCC 3'	73
AP5	5' CGG GTC ATT TAT TGT ACC CCT AGT CAC GGC 3'	53

RAPD fingerprinting : Five arbitrary oligonucleotide primers (AP1 to AP5) commercially synthesized from Southwest Scientific Resources Inc, USA, were used to initiate PCR amplification. The G+C content of the primers varied from 53 to 73 per cent (Table I). Amplifications were performed in a DNA thermal cycler (Perkin Elmer Cetus, USA) for 40 cycles with conditions, each consisting of denaturation at 94°C for 1 min, annealing at 30°C for 1 min and extension at 72°C for 1 min. Amplified products were resolved in 2 per cent agarose gel, stained with ethidium bromide and visualized in a UV transilluminator.

Discriminatory power of the typing methods : The discriminatory ability of each typing method was determined by calculating the numerical discrimination index (DI) by the method of Simpson[22]. A DI of 1.0 indicated the capability of typing method to distinguish each isolates from the test population. Conversely, a DI of 0 indicates that all the isolates of the test population are indistinguishable. An estimation of confidence intervals (CI) was also done following the methods of Grundmann *et al*[23] which has the ability to address the discriminatory potential of diverse typing systems (Table II).

emm typing: *emm* gene was amplified by using expand high fidelity PCR kit (Boehringer, Mannheim, Germany) by "all M" primers as described by Podbielski *et al*[24]. Approximately 30 ng of PCR product was sequenced by using primer (5'ATAAGGAGCA TAAAAATGGCT3') with the dye terminator mix and subjected to automated sequence analysis[25,26] on a 310 model autosequencer (Applied Biosystems, USA) as per manufacturer's instructions. The cycling parameters were 96°C for 30 sec, 50°C for 15sec and 60°C for 4 minute. *emm* gene sequence was subjected to homology search against CDC reference strains (*http://www.cdc.gov/ncidod/biotech/strep/strepblast.htm*) as well as by Blast search analysis (*http://www.ncbi.nlm.nih.gov/blast/Blast.cgi*). Isolates which showed >95 per cent homology with reference strain, were designated the particular parental *emm* type.

Table II. Discriminatory power of different typing techniques

Typing method	Number of types	Discrimination Index D1 (%)[21]	Confidence interval CI (%)[22]
REA	6	76.5	74.95-78.05
PCR–ribotyping	3	55.1	54.54-55.66
RAPD fingerprinting			
AP1	6	70.6	69.05-72.15
AP2	9	86.3	83.53-89.07
AP3	11	87.7	84.04-91.36
AP4	12	91.5	86.7-95.1
AP5	12	91.7	87.57-95.83

REA, restriction endonuclease analysis; RAPD, random amplified polymorphic DNA; Superscript numerals denote reference numbers

Results

Genotype distribution of 60 GAS isolates, was studied using different molecular techniques and were compared for epidemiological purpose.

REA, ribotyping and PCR ribotyping: Restriction endonuclease analysis of genomic DNA with *Hin*d III produced six REA patterns among 60 isolates (DI: 76.5%, CI: 74.95-78.05%, Table II). The pattern consisted of 10-17 DNA fragments, different from each other by one or more fragments. The fingerprinting patterns could not be used to discriminate pharyngitis and RF/RHD isolates based on profile and number of fragments generated. Similarly, the results obtained from *Hae* III and *EcoR* I digestion did not reveal any significant discrimination among the isolates. Ribotyping with *Hin*d III restriction endonuclease produced five identical fragments among all the isolates studied except one isolate from RF/RHD case, which generated a single extra fragment. Similarly *Hae* III ribotype profile also showed identical ribotypes among the isolates, thus revealing no polymorphism within the rRNA region.

On amplification of 16S region of the GAS rRNA operon, a PCR product of ~ 1.5 kb was obtained in all the isolates. Further *Hae*III restriction enzyme digestion of the PCR amplicon generated four equal sized DNA fragments in all the isolates including pharyngitis and RF/RHD isolates, hence failing to differentiate the isolates irrespective of their source. Further, the restriction enzyme digestion with the combination of *Hae* III- *Bgl* I provided similar ribotype profile except in one isolate which showed an extra band. The results largely showed lack of polymorphism present within 16S rRNA subunits of group A streptococcus isolates of north India.

When oligonucleotide primers from conserved region of 16S-23S rRNA spacer region of the rRNA gene were used to amplify the 16S- 23S rRNA, a single PCR amplicon of about 0.8 kb was generated from all the isolates (Fig. 1a). *Hae* III restriction endonuclease analysis of PCR product revealed three different banding patterns consisting of 2-3 DNA fragments

ranging from 0.1 to 0.6 kb in the isolates (Fig. 1b). Among the pharyngitis isolates, all the three banding patterns (1,2,3) were observed, with 50 per cent isolates showing pattern 2 (0.1 kb, 0.2 kb and 0.5 kb), followed by 37.5 per cent showing pattern 3 (0.2 kb and 0.6 kb and 12.5 per cent showing pattern 1 (0.2 kb and 0.55kb). However, all the RF/RHD associated isolates showed pattern 3. Analysis of GAS isolates by PCR-ribotyping showed a discriminatory power of 55.1 per cent with the CI (confidence interval) value of 54.54-55.66 per cent (Table II).

RAPD fingerprinting: In contrast to all other methods used for typing GAS in this study, RAPD fingerprinting showed highly polymorphic nature of the isolates. GAS isolates exhibited marked heterogeneity with each of the five arbitrary primers AP1-AP5 (Table I) used, generating number of RAPD patterns. The primer AP3, AP4 and AP5 revealed more discrimination as compared to AP1 and AP2. As shown in Fig. 2 (a-c), AP3 resulted in 11, AP4 and AP5 12 rapdems each, while AP1 and AP2 produced 6 and 9 patterns respectively (data not shown). Hence the primer AP3, AP4 and AP5 provided the higher level of discrimination with a DI value of 87.7, 91.5 and 91.7 per cent respectively (Table II).

Arrays of fragments ranging from approximately 0.15 kb to 2 kb in size were observed by the use of five arbitrary primers. A total of 78 amplified DNA fragments (rapdemes) were observed in all of which 48 (62%) were shared and 30 (38%) unshared or unique rapdemes representing a 38 per cent overall genetic heterogeneity among the isolates. However, RAPD patterns obtained from pharyngitis isolates were indistinguishable from RF/RHD pattern even by RAPD analysis. Two polymorphic markers of size about 0.55 kb and 0.8 kb were found to be conserved in both pharyngitis and RF/RHD associated isolates which can be used as RAPD marker among the isolates.

emm typing: Twelve representative isolates (seven from pharyngitis and five from RF/RHD cases) distinguished on the basis of 12 different rapdems by AP5 primer,

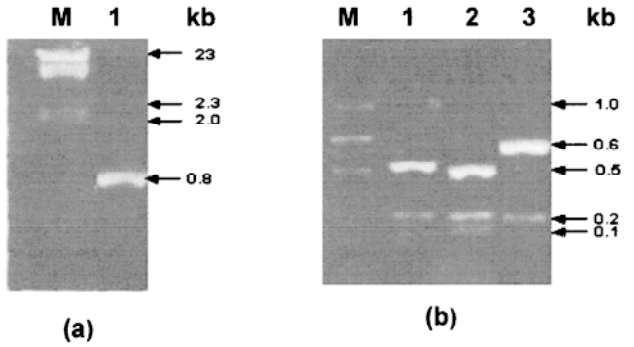

Fig. 1. 16S-23S rRNA PCR-ribotyping pattern. (a) lane M: λ *Hind* III DNA molecular weight maker; lane 1: 16S-23S rRNA PCR amplicon of representative isolates (b) lane M: 100 bp DNA molecular weight marker (Bangalore Genei, India); lane 1-3 : *Hae* III digested PCR amplicon of representative clinical isolates of GAS.

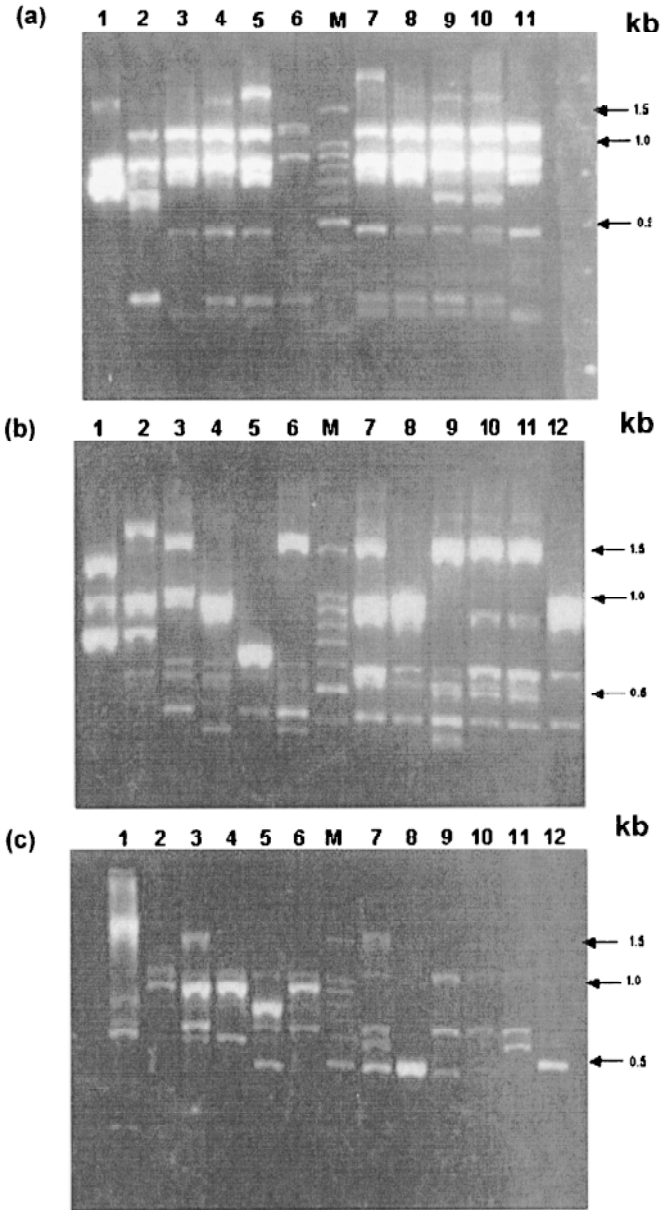

Fig. 2. RAPD–PCR amplification pattern of different subtypes of GAS isolates with (a) AP 3 primer, (b) AP 4 primer, (c) AP 5 primer. Lane M: 100 bp DNA molecular weight marker (Promega, USA), Lanes 1 to 12 : representative clinical isolates of GAS.

were selected for *emm* typing. All seven pharyngitis isolates showed seven different *emm* types (*emm* 1.2, 11, 49, 53, 68, 75, ST1731) whereas five RF/RHD isolates were typed in to four *emm* types (*emm* 43, 49, 80, 93). Two rapdems showed similar *emm* type *i.e*., *emm* 93.

Discussion

Serotyping based on M-protein has been the primary basis for the strain classification of GAS for decades[7,13]. Although serotyping provides valuable information, proportion of isolates remains untypeable even with the most comprehensive set of typing sera. Therefore the need for more reliable methods for strain differentiation led to the development of genotypic methods. The molecular typing methods such as REA, ribotyping, RAPD have been used for subtyping of GAS isolates mainly in western countries[8,9,12,14,15,27]. However, lack of data on molecular type distribution of GAS isolates from India led to the present study where various genotyping methods were compared in order to characterize GAS isolates.

In the present study, REA performed by three restriction endonucleases *Hin*d III, *Hae* III and *Eco* R I were used for the maximum discrimination of 60 GAS isolates. The results showed *Hin*d III to be the discriminatory for typing of GAS as compared to others, hence indicating the importance of selection of a suitable restriction enzyme[9]. However, the profiles of GAS isolates from pharyngitis and RF/RHD patients when compared could not give the distinct picture of any specific differentiation. This information was comparable with the findings of previous workers in which Mylvaganam *et al*[28] obtained characteristic and reproducible DNA fingerprints with *Eco*R1 restriction enzyme digestion, while Mencarelli and co-workers[29] from Italy observed the inability of *Hae* III and *Hin*d III restriction enzyme to distinguish REA patterns among the strains. The present investigation also reflected poor discriminatory power of ribotyping as identical patterns were obtained among all the isolates. The findings were similar to those reported by Bruneau *et al*[10] and Sriprakash & Gardiner[11] who achieved limited success while characterizing GAS isolates by ribotyping. However, Shundi *et al*[12] identified eight and eleven ribotypes successfully among 70 GAS isolates after digestion with *Hin*d III and *Pvu* II respectively.

Further, the possibility of PCR ribotyping of 16S rRNA gene with *Hae* III and *Hinf* 1 enzymes to study the genetic heterogeneity among the collected GAS isolates was explored. Use of the enzymes alone or in combination with *Hae*III-*Bgl*I could not differentiate the isolates. Further analysis of isolates by PCR-ribotyping with intergenic spacer region (16S-23S rRNA) revealed the existence of three PCR ribotypes type 1, 2 and 3 of which type 2 and 3 were the major types. It was interesting to find that all RF/RHD associated isolates produced ribotype pattern 3 though some pharyngitis isolates also belonged to this type. Sriprakash & Gardiner[11] observed only two major *Hae* III RFLP patterns of rRNA operons in GAS isolates. Thus results from the present investigations indicated the absence of intraspecies genetic variation at 16S rRNA subunit but documented variation in intergenic (16S-23S) spacer region.

Use of PCR based RAPD method for typing of GAS was found to be highly discriminatory. As reported earlier, selection of primers, optimization of PCR condition and combination of different primers play an important role in discriminating the isolates by RAPD[9,30]. Hence five arbitrarily selected primers with G+C contents varying from 53 to 73 per cent were tested. The majority of arbitrary primers used, produced distinctly reproducible patterns in all the isolates studied. However, the primers varied in the extent of information they generated with some primers (API and AP2) producing less polymorphic patterns, whereas other (AP3, AP4 and AP5) producing highly polymorphic and discriminatory patterns. However, no association could be observed between sensitivities and G+C content of the different primers used[9]. The RAPD assays were performed at constant DNA concentrations and the experiments were repeated several times by taking proper precautions. In the present study, RAPD results were reproducible when samples were run in large gels simultaneously. Thus, all these facts suggest the usefulness of this technique in disease outbreak detection.

Genotyping of Indian GAS isolates showed high DI values by RAPD fingerprinting confirming the analysis to be far discriminatory as compared to the other techniques used[9]. Since *emm* typing is widely used these days and is considered highly discriminatory hence to validate results of RAPD, *emm* typing was done for a few representative isolates. The 12 isolates which produced 12 different RAPD patterns showed eleven different *emm* types only. Comparative analysis of RAPD data with that of *emm* typing in this study gives an edge to RAPD technique, which can be used in laboratories in developing countries where *emm* typing

can rarely be performed for routine clinical use and epidemiological analysis of GAS[31]. Various workers[14,15] have observed RAPD to reproduce PFGE's discriminatory ability on the epidemiological analysis of GAS infections. Apart from discriminatory ability, RAPD profile could be effectively used as a supporting marker for taxanomic identification in routine laboratories[32]. In conclusion, the findings of this study demonstrated the benefit of RAPD fingerprinting in comparison to other molecular methods in identifying and characterizing GAS isolates obtained from pharyngitis and RF/RHD cases.

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