SHORT COMMUNICATION

Molecular typing of colonizing *streptococcus agalactiae* strains by enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) in a Chennai based hospital

D. K. Bishi · S. Verghese · R. S. Verma

Abstract *Streptococcus agalactiae* is reported to be an asymptomatic vaginal colonizer in Indian women, although it is considered one of the major causes of neonatal infections in many European countries. DNA based molecular typing methods are more reliable than the conventional serotyping method for identification and typing of this pathogen. In the present study, we have evaluated genetic diversity among colonizing *S. agalactiae* strains (n=86) by using a PCR-based genotyping method i.e. Enterobacterial Repetitive Intergenic Consensus PCR (ERIC-PCR). With ERIC-PCR fingerprinting at 60% similarity level in a dendrogram generated by UPGMA cluster analysis, 10 different ERIC groups were identified, which were subdivided into 62 distinct genotypes at ≥ 95% similarity level. Based on these findings, we demonstrate that ERIC-PCR is a simple, rapid, and inexpensive tool with sufficient discriminatory power and is applicable for characterization and genotyping of a large number of clinical isolates of *S. agalactiae* at molecular level.

Keywords S*treptococcus agalactiae* · Colonization · ERIC-PCR · Genotyping · Dendrogram

D. K. Bishi [1, 2] · S. Verghese [2] · R. S. Verma [1] (✉)
[1] Department of Biotechnology,
Indian Institute of Technology Madras,
Chennai - 600 036, India
[2] Department of Microbiology,
Frontier Lifeline Pvt. Ltd.
Chennai - 600101, India
e-mail: vermars@iitm.ac.in

Introduction

Streptococcus agalactiae, also referred as Group B Streptococcus (GBS), is a Gram-positive, catalase negative, β-hemolytic, opportunistic pathogen. Common clinical manifestations of *S. agalactiae* include puerperal sepsis, meningitis, and pneumonia in neonates and urinogenital infections and colonization in pregnant and non-pregnant adults [6, 7, 8]. The incidence of GBS infections varies with the geographical regions with a lower colonization rate in Indian women and infants [15, 18]. During a 10 year study between 1988 and 1997 in Vellore, southern India, only 10 cases of neonatal GBS cases were identified, giving an incidence of 0.17 per 1000 live births [12]. In 2001, a colonization rate of 12.89% was reported among asymptomatic carrier women in Chennai, India [21].

Serotyping is the common, phenotypic method for typing *S. agalactiaea* and 9 antigenically distinct serotypes (types Ia, Ib, II-VIII) have been identified today. In India the most common isolates belong to types III, II, and Ib [15]. However, this phenotypic method is ambiguous and nowadays various DNA based typing methods e.g. Pulse field Gel Electrophoresis (PFGE), Multilocous Sequence Typing (MLST), Randomly Amplified Polymorphic DNA (RAPD) analysis etc. are emerging as reliable and quicker methods of genotyping *S. agalactiae* [10, 11, 13, 20].

Enterobacterial repetitive intergenic consensus (ERIC) sequences are 126bp intergenic inverted repeats; present mostly in Gram negative bacteria of the family Enterobacteriaceae e.g. *Salmonella typhimurium* and *E. coli* [8]. ERIC-PCR has been used for molecular typing of many Gram-negative [4, 19], and also some Gram-positive bacteria [22]. Presence of ERIC sequences have been demon-

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strated in *M. tuberculosis* [17] and ERIC-PCR was used as a genotyping tool [16]. ERIC-PCR has been tested for genotyping of *Staphylococcus epididermis* [23] *Streptococcus pyogenes* [14], Viridans group Streptococci [1] and group B Streptococcus [3] . In the present study, we applied ERIC-PCR to establish genetic relationships among unrelated strains of *S. agalactiae* (Indian isolates) and to validate its usefulness as a rapid and adequate genotyping tool.

Materials and methods

Bacterial Strains and Laboratory Methods: Eighty-six strains of Group B Streptococcus were supplied by the Department of Microbiology, Frontier Lifeline hospital, Chennai, India. Bacterial strains were earlier isolated mainly from vaginal swabs of asymptomatic female patients, and a few from urine cultures and pus samples, during 1999 to 2006. Strains were initially identified as GBS based on the following criteria: a narrow zone of beta-hemolysis on 5% sheep blood agar plate, Gram-positive cocci in pairs or short chains on Gram staining, a negative-catalase reaction, a positive reaction with Christie, Atkins, Munch-Peterson (CAMP) test, and positive hippurate hydrolysis reaction and Lancefield grouping with type B antiserum (Pastorex Strep Latex Kit).

Extraction of genomic DNA: Total genomic DNA was extracted from bacterial cells, according to the procedure followed by Bensing BA et al [2]. In brief, cells from a single colony were inoculated in Todd-Hewitt broth and were grown overnight. Bacterial culture was suspended in lysis buffer [containing Lysozyme (50 mg/ml), mutanolysin (200 U/ml) and TE buffer (50:5)] and incubated at 37 °C for 1hour. 20 % SDS and proteinase K was added to it. Suspension was mixed with 2 ml phenol: chloroform (1:1) and extracted with chloroform: isoamyl alcohol (24:1). Finally DNA was precipitated out by adding 40 μl of 3 M sodium acetate and 1 ml of ethanol. Then DNA pellets rinsed with 70% ethanol were resuspended in TE buffer (10:1) containing 0.5 μg DNase-free RNase. DNA concentration was quantified using UV-visible spectrophotometer at 280nm.

ERIC PCR: Primers used for ERIC PCR experiments were ERIC 1R (5'-ATG TAAGCTCCTGGGGATTCAC-3') and ERIC 2 (5'-AAG TAA GTG ACT GGG GTG AGCG-3'). The ERIC-PCR mixture consisted of 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 2 mM $MgCl_2$, each of the four dNTPs at a concentration of 125 mM, 100 pmol of primer, 1.5 U of Taq polymerase, and 100 ng of DNA solution made up to a final volume of 25 μl. PCR was carried out in an automated thermal cycler (Biorad, USA). PCR amplification condi-

tions were an initial denaturation cycle (5 min at 95°C) followed by 4 cycles of low stringency (1 min at 94°C, 1 min at 26°C, and 4 min at 72°C), 35 cycles of high stringency (1min at 94°C, 1.5 min at 52°C, and 8 min at 72°C) and final extension for 16 min at 72 °C.

Visualization of ERIC PCR fingerprints: The amplification products of ERIC PCR (10 μl) were analyzed with 1.5 % agarose containing 0.5 mg of ethidium bromide per ml and were separated electrophoretically on gels at 100V for 2 hour in TAE (Tris-Acetate -EDTA) buffer. A 100bp DNA ladder was used as a molecular size marker. The banding patterns (fingerprints) of all lanes were visualized and compared under UV-transilluminator.

Analysis of DNA fingerprinting Patterns: All of the ERIC PCR fingerprint patterns in the form of TIFF images were analyzed with the Windows version of GelCompar software (version 4.0; Applied Maths, Kortrijk, Belgium). The individual bands in each of the patterns were analyzed by applying the Dice coefficient to the peaks. For clustering, the Unweighted Pair Group Method with Arithmetic means (UPGMA) was used, and a band position tolerance of 1.06% was used for comparison of the DNA patterns. The analysis of banding patterns was undertaken in accordance with the instructions of the manufacturer.

Discriminatory Index analysis: The probability that two unrelated isolates sampled from the test population will be placed into different typing groups or clusters was assessed according to the Hunter-Gaston formula [9]. This probability, also called discriminatory index is calculated as:

$$D = 1 - \frac{1}{N(N-1)} \sum_{J=1}^{s} n_j(n_j - 1),$$

Where N is the total number of isolates in the sample population, s is the total number of ERIC PCR patterns described, and n_j is the number of isolates belonging to the jth type.

Results and discussion

Specificity of two primers, ERIC1R and ERIC2 were evaluated under different PCR conditions, such as annealing temperatures, $MgCl_2$ concentration gradients. It had showed that ERIC 2 primer produced better banding pattern than ERIC1R and hence we used ERIC 2 primer in the present study. ERIC-PCR, a PCR based genotyping method, was applied to the group B Streptococcus strains and the amplified products were analyzed. The banding pattern showed 4
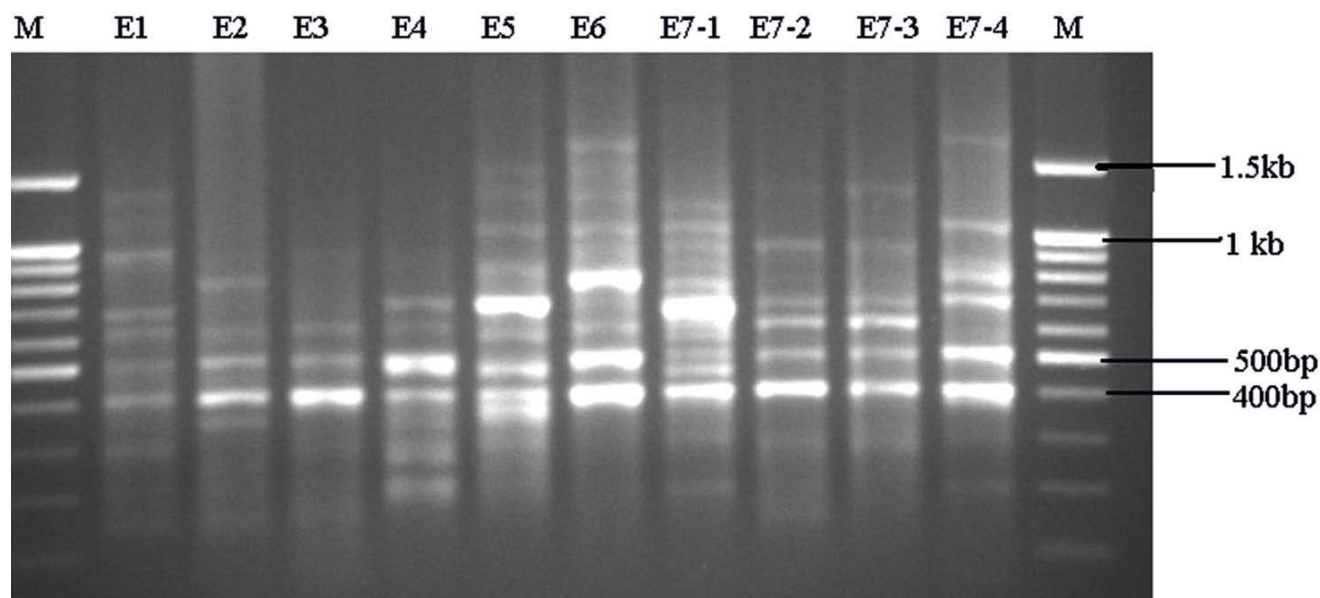
Fig. 1 1.5% agarose gel electrophoresis image of representative ERIC PCR patterns (E1 to E6 and E7-1 to E7-4); Lane M – 100bp DNA ladder

to 9 major bands with varying molecular sizes ranging from 200bp to 1.5 Kb. The position and intensity of the amplified PCR products varied, which showed the genetic diversity among different strains. The common amplified bands obtained at 400bp and 500bp were shared by almost all strains in our study. Besides, some strains also shared similar banding patterns having varied copy number, as could be seen by the intensity of the products (Fig. 1). These results exhibited the reproducibility of ERIC PCR profiles of few selected strains by repeating the experiments several times, which showed identical banding patterns.

The genetic relationship among all ERIC PCR patterns of *S. agalactiae* based on the data obtained with the ERIC2 primer is represented in the dendrogram (Fig. 2). Overall *S. agalactiae* isolates presented 45% similarity. ERIC PCR pattern of 86 strains upon cluster analysis at 60% similarity level, generated seven different groups called ERIC groups and were designated as E1 to E7. However, E7 included 41.2% of all strains and had a complex clustering pattern among its strains and hence it was further subdivided into 4 sub-clusters, E7-1 to E7-4. So, finally there are 10 ERIC groups. Each cluster or ERIC group was then further subdivided into different ERIC types at individual strain level based on more than 95% similarities between the strains. Strains showing differences in one or more bands were considered to be different ERIC types. Intensity of bands was also considered while differentiating between the strains. In this way 62 ERIC types were obtained form 10 ERIC groups among 86 strains (Table 1, Fig. 2). The ERIC groups

are designated by capital letter E, followed by a number and the individual ERIC types are designated by small letters. For example, "E5a" referred to ERIC group 5 and the ERIC type "a" under this group.

All strains in cluster E1 (n = 8) showed more than 75% similarity. It was subdivided into 4 ERIC types, E1a to E1d. Strain nos. 82 to 86 shows 100 % similarities among them and hence was considered as a single ERIC type, E1c (Fig. 2). Similarly, strain nos. 53 and 54, both obtained from vaginal swabs during the year 1999 were 100% similar. Strain nos. 46, 47 and 49, were from vaginal swabs of different patients, but did not show any strain differentiation. A high similarity was seen among strains obtained from vaginal swabs of the same year and hence they were included in same group. It was the same with the urine isolates. However, some of the urine isolates shows ≥ 60% similarity with vaginal isolates, although both were from different sources and of different patients.

Limited information is available on the epidemiology of Indian isolates of *S. agalactiae,* and to our knowledge only serotyping is carried out for typing purpose. Here, we applied ERIC-PCR to 86 nonserotyped strains, for the first time in India, since this method has not been used extensively to genotype *S. agalactiae*. Application of ERIC-PCR fingerprinting reaction for genotyping group B Streptococcus has been reported in Poland [3]. They identified 13 genotypes among 120 strains from various clinical samples tested. However they did not use any phylogenetic software and only on the basis of visualization of banding patterns
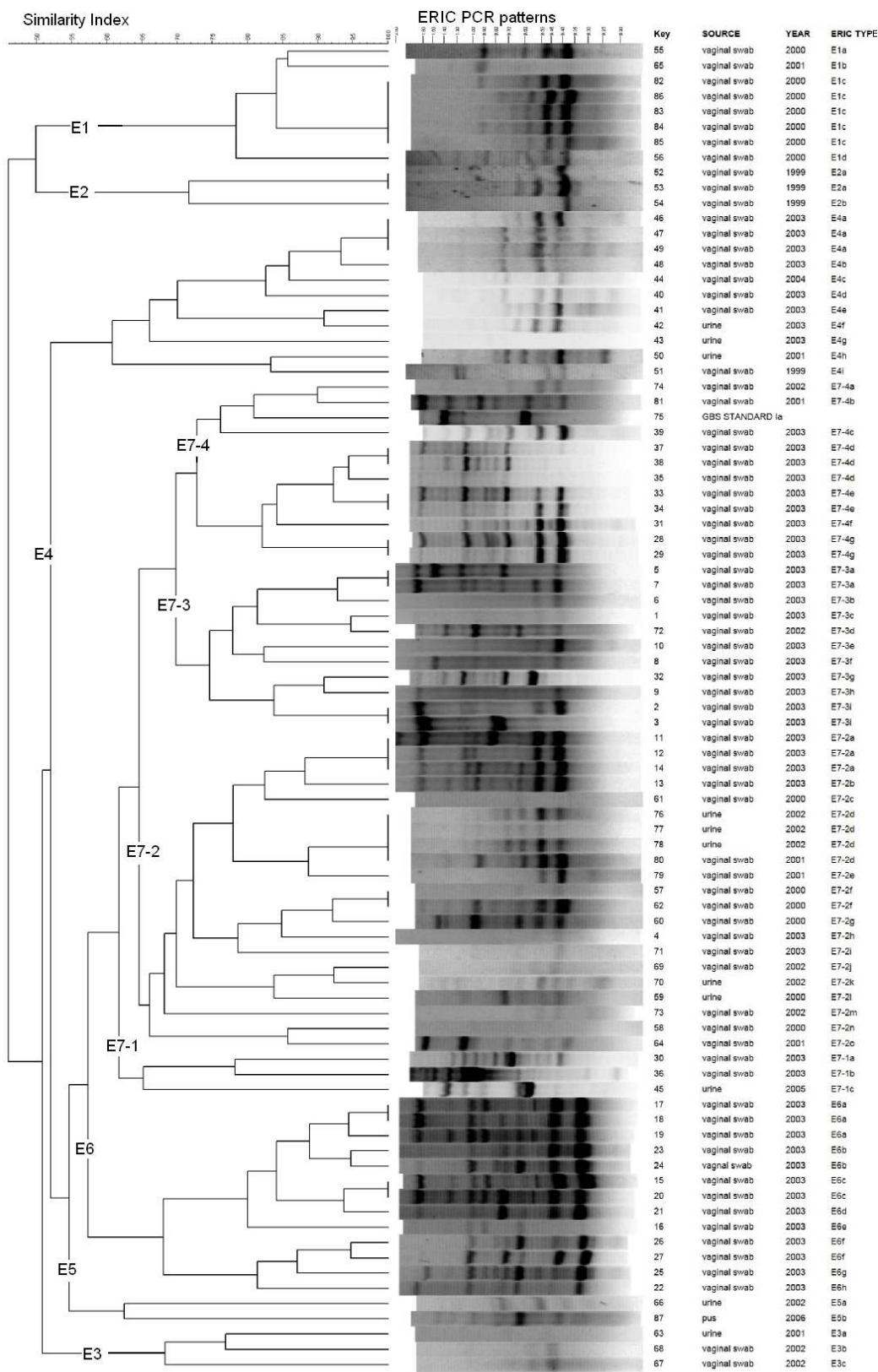
Fig. 2 Dendrogam showing relatedness between ERIC -PCR band patterns of 86 group B streptococci strains. Bands were analyzed by applying the Dice coefficient, and the matrix was clustered by the UPGMA method. Key refers to the strain No. along with their respective ERIC types. Source and year of isolation of strains are also shown

Table 1 *S. agalactiae* isolates differentiated into ERIC groups and ERIC types based on dendrogram pattern

ERIC groups	No. of isolates (%)	No. of ERIC types
E1	8 (9.3)	4
E2	3 (3.4)	2
E3	3 (3.4)	3
E4	11(12.7)	9
E5	2 (2.3)	2
E6	13 (15.1)	8
E7-1	3 (3.4)	3
E7-2	21 (24.4)	15
E7-3	11 (12.7)	9
E7-4	11 (2.7)	7
Total = 10	Total = 86	Total = 62

they have interpreted the genotyping results. In present study, we categorized 86 strains into 10 ERIC groups showing overall 47% similarity among all strains, which were further genotyped into 62 distinct ERIC types. Here, we report a high genetic diversity, which is evident from the fact that different strains were obtained from different patients and also from different clinical samples e.g. vaginal swabs, urine and pus. As, clearly observed from cluster analysis (Fig. 2), strains were clustered both year wise and sample wise into different groups.

However, the extraordinary discriminatory power of ERIC PCR on gram positive bacteria is lower when compared to that of gram negative bacteria, possibly because of presence or absence of ERIC sequences in bacterial genomes. ERIC-PCR probably works in *S. agalactiae* as RAPD-PCR, because the presence of ERIC or ERIC like elements has not been demonstrated yet in its genome. Gillings and Holley [5] reported that ERIC-PCR does not necessarily amplify bands directly from genuine ERIC sequences. ERIC primers may act as arbitrary or random primers as in RAPD or AP PCR [24]. The use of larger primer (22 nucleotides) and higher annealing temperature renders ERIC PCR less sensitive to changes in reaction conditions. Although the banding patterns were reproducible and were able to type the unrelated strains, the real basis of discrimination was not clear. Therefore, the sequences that act as targets for ERIC primer within *S. agalactiae* genome cannot be stated absolutely.

Irrespective of the presence or absence of ERIC elements in *S. agalactiae* genome and even if ERIC primers work on the principle of RAPD PCR, the ERIC-PCR based genotyping method is simpler, quicker, reproducible and advantageous over tedious and time consuming methods like PFGE and serotyping. The discriminatory capacity of the ERIC PCR typing was determined in order to evalu-

ate the suitability of the technique as a genotyping tool for *S. agalactiae*. Hunter and Gaston [9] proposed that Simpson's diversity index (D) greater than 0.900 would be desirable for a typing method. We defined 62 ERIC types among 10 ERIC groups for 86 isolates and a discrimination index (D) of 0.939 was obtained. which showed sufficient discriminatory power of ERIC PCR typing of *S. agalactiae*. Moreover, it can be applied for genotyping a large number of clinical isolates in laboratories. Therefore, we conclude that ERIC-PCR is simple, rapid, affordable, reproducible and is a highly discriminatory molecular typing method for genotyping *S. agalactiae* strains. Furthermore, this method may be used for identification of isolates belonging to a broad geographical distribution and can be used in epidemiological investigations of group B Streptococci.

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