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POLYMERASE CHAIN REACTION AMPLIFICATION OF 16S-23S SPACER REGION FOR RAPID IDENTIFICATION OF *SALMONELLA* SEROVARS

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Polymerase chain reaction (PCR) was used to amplify the spacer regions between the 16S and 23S genes of rRNA genetic loci of *Salmonella* serovars for their rapid identification. These genetic loci revealed a significant level of polymorphism in length across the species/serovar lines. When the 16S-23S spacer region amplification products were subjected to agarose electrophoresis, the patterns observed could be used to distinguish all the serovars of *Salmonella* tested. Unique elements obtained in amplification products were mostly clustered at serovar level, although certain genus-specific patterns were also observed. On the basis of the results obtained, the amplification of 16S-23S ribosomal spacer region could suitably be used in a PCR-based identification method for *Salmonella* serovars.

Key words: Polymerase chain reaction, ribosomal DNA, spacer region, 16S-23S

The ribosomal RNA (rRNA) genetic loci in prokaryotes contain genes for all the three rRNA species, *viz.* 16S, 23S and 5S genes. A single genome contains multiple rRNA genetic loci. The spacer region, which separates these rRNA genes, exhibits a large degree of variation in length at the level of genus and species (Jensen et al., 1993). The diversity and the variation in length of spacer region is due to the variable number of tRNA sequences found within the spacers (Brosius et al., 1981; Lehner et al., 1984). It has been shown that the length and sequence polymorphism in the spacers within the rRNA loci could be used to discriminate between different species of prokaryotes (Barry et al., 1991). Spacer region polymorphism has also been applied for species identification of several fungi (Gardes et al., 1991). The most rapid means to study the polymorphism of rRNA spacers is to carry out PCR amplification of spacer regions using the primers designed from highly conserved flanking regions. In this report, we used a unified set of primers and cycling conditions to amplify the spacer region between 16S and 23S genes of rRNA gene loci of several serovars of *Salmonella* to

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facilitate genus- and species-level identification without the requirement for presumptive identification. In India *Salmonella* Enteritidis remains the most predominant serotype followed by *S.* Typhimurium, *S.* Gallinarum, *S.* Dublin, *S.* Choleraesuis, and *S.* Indiana. The rare serotypes such as *S.* Cerro, *S.* Senftenberg, *S.* Agona, and *S.* Newport are reported from a wide range of animals, man and environment (Verma et al., 2000). The serotypes of *Salmonella* selected for the present study were chosen so that both commonly occurring and rare serotypes of *Salmonella* are represented.

Materials and methods

Bacterial cultures

Cultures of *Salmonella* serovars *viz*. *S.* Enteritidis, *S.* Typhimurium, *S.* Indiana, *S.* Agona, *S.* Choleraesuis, *S.* Dublin, *S.* Senftenberg, *S.* Gallinarum, *S.* Newport, *S.* Cerro and *S.* Bareilly were obtained from the National Salmonella Centre, Indian Veterinary Research Institute (IVRI), Izatnagar. The cultures were revived in Luria Bertani broth, and streaked on brilliant green agar plates. Single colonies were picked up, propagated and maintained on 1% nutrient agar slants.

Preparation of genomic DNA

Genomic DNA of *Salmonella* serovars used in this study was prepared by the method of Wilson (1987). The DNA was dissolved in a buffer containing 10 mM Tris and 1 mM EDTA and its concentration was determined spectrophotometrically. DNA samples were dissolved to give a final concentration of 25 ng/µl. An aliquot of one microlitre of DNA was used for amplification by PCR.

DNA amplification

The amplification of the spacer region between 16S and 23S rRNA was carried out by the method of Jensen et al. (1993). Primer pair consisting of 15-mer forward primer G_1 (5'- GAA GTC GTA ACA AGG-3') and 15-mer reverse primer L_1 (5'- CAA GGC ATC CAC CGT-3') was used for amplification. A 50 µl reaction was set up containing 25 ng of genomic DNA, 5 µl of 10 × reaction buffer containing 500 mM KCl, 100 mM Tris-HCl (pH 8.8), 15 mM $MgCl_2$ and 0.17% gelatin, 2 µl of 2.5 mM dNTPs, 1.25 µl of each primer (50 ng/µl), and 1.5 units of Taq DNA polymerase (Promega Corporation, Madison, USA) and 38 µl of sterile distilled water. *Pasteurella multocida* genomic DNA was used as negative control. Thirty amplification cycles were performed in a thermocycler (DNA Engine, M.J. Research, USA). Each cycle consisted of an initial denaturation at 94 °C for 1 min, annealing at 55 °C for 2 min and an extension at 72 °C for 2 min. The final cycle was followed by an additional 7 min

at 72 °C to complete the partial polymerisation. At the end of the cycles a 10-µl aliquot of the amplified products was mixed with 2 µl of 6 × loading dye and electrophoresed on 1:3 Nusieve agarose (Sigma, USA). The gel was stained with ethidium bromide, observed under UV transilluminator and photographed. The sizes of the fragments were calculated by comparison with standard molecular weight markers (100 bp Ladder, Gibco BRL).

Results

The patterns of the 16S-23S spacer amplification products of the eleven serovars of the *Salmonella* tested were found to be unique for each serovar (Figs 1A, 1B and 1C; Table 1). Bands of 480 and 660 bp were observed in all the *Salmonella* serovars except *S.* Senftenberg and *S.* Bareilly. Additional bands of 590, 520 and 550 bp were observed in *S.* Enteritidis, *S.* Indiana, and *S.* Agona, respectively. *S.* Newport revealed a major band of 480 bp and three minor bands of 590, 660 and 680 bp. In *S.* Senftenberg and *S.* Bareilly only single amplification products of 370 and 490 bp were obtained, respectively. In *S.* Dublin and *S.* Typhimurium, in addition to primary fragments of 480 and 660 bp, weaker bands of 580, 680, and 510, 580, 680 bp, respectively, were also revealed. *S.* Choleraesuis amplified only two bands of 480 and 660 bp. *S.* Gallinarum strains amplified five bands of 480, 500, 560, 660 and 680 bp. *Pasteurella multocida,* which was used as an unrelated negative control, amplified products of 510 and 710 bp (Fig. 1B).

Table 1

Summary of sizes of 16S-23S spacer region amplification products obtained from *Salmonella* serovars

Serial no.	Serovars	No. of strains tested	Primary fragments (bp)	Secondary fragments (bp)
1	*S.* Enteritidis	25	480, 590, 660	
2	*S.* Newport	2	480	590. 660, 680
3	*S.* Indiana	3	480, 520, 660	
4	*S.* Agona	2	480, 550, 660	
5	*S.* Cerro	1	480	560, 660
6	*S.* Senftenberg	3	370	
7	*S.* Bareilly	2	490	
8	*S.* Dublin	4	480, 660	580, 680
9	*S.* Choleraesuis	4	480, 660	
10	*S.* Typhimirium	5	480, 660	510, 580, 680
11	*S.* Gallinarum	5	480, 500, 560, 660, 680	
12	*Pasteurella multocida*	1		510, 710

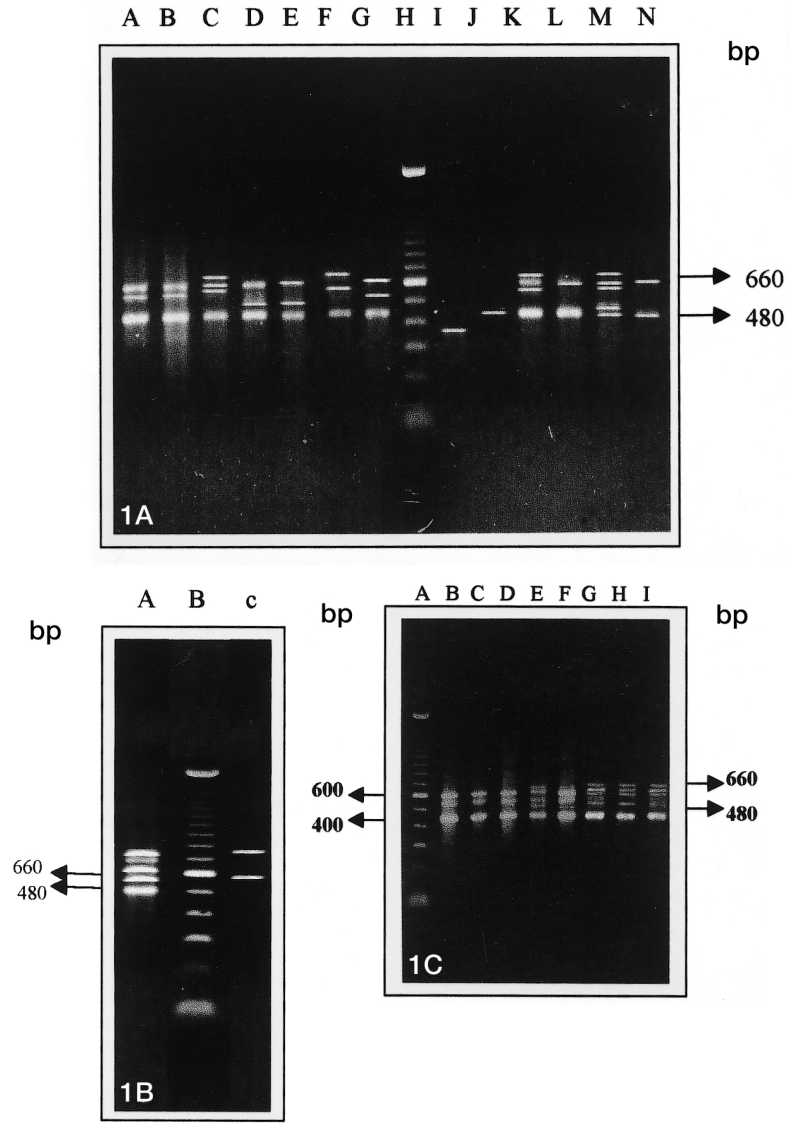

Fig. 1A, 1B and 1C. Agarose gel electrophoresis of PCR amplification products of 16S-23S spacer regions of various serovars of *Salmonella. Fig. 1A.* Lanes: A: E20, *S.* Enteritidis; B: E2681, *S.* Enteritidis; C: E2714, *S.* Newport; D: E2719, *S.* Indiana; E: E2741, *S.* Indiana; F: E2721, *S.* Cerro; G: E2725, *S.* Agona; H: Marker 100 bp Ladder; I: E2710, *S.* Senftenberg; J: E2717, *S.* Bareilly; K: E2492, *S.* Dublin; L: E97, *S.* Choleraesuis; M: E2723, *S.* Typhimurium; N: E2417, *S.* Choleraesuis. *Fig. 1B.* Lanes: A: E2440, *S.* Gallinarum; B: Marker 100 bp ladder; C: P52, *Pasteurella multocida. Fig. 1C.* Lanes: A: Marker 100 bp Ladder; B: E2688, *S.* Enteritidis; C: E2746, *S.* Enteritidis; D: E2441, *S.* Enteritidis; E: E77, *S.* Gallinarum; F: E2150, *S.* Gallinarum; G: E2175, *S.* Typhimurium; H: E2186, *S.* Typhimurium; I: E2188, *S.* Typhimurium

Discussion

Use of primer pair G1-L1 designed from highly conserved flanking sequences of 16S-23S boundaries for amplification of spacer region produced amplification products for almost all the serovars tested. The products were generated using unified amplification conditions irrespective of the origin of DNA. The multiple primary amplification products obtained suggests that the intergenomic spacer length polymorphism in *Salmonella* is characteristic of each serovar of *Salmonella* tested. Spacer DNA polymorphism in *Salmonella* published by Jensen et al. (1993) and many of our results agree. However, differences in the amplicons in some cases may be due to strain polymorphisms or due to undetected rearrangements of 'tRNA' genes spanning the spacer region. The variation in the size of amplicons observed in some cases could be due to the difference in the method being used for the extrapolation of sizes of the fragments with reference to standard markers (Southern et al., 1979; Schaffer and Sederoff, 1981).

In some cases, weak secondary amplification products in the size range of 490–700 bases were also generated. However, the yield of such fragments was significantly lower than the primary fragments. These secondary products provide additional information for the identification of *Salmonella* serovars. The majority of the *Salmonella* serovars with the exception of *S.* Senftenberg and *S.* Bareilly amplified two primary products of 480 and 660 bp. This pair of amplification products appears to be a common feature for all the *Salmonella* serovars tested and could be designated as unique to the genus *Salmonella*. Products of 335 to 380 and 570 to 655 bp have been described as genus specific for *Listeria.* No such amplification products have been reported either for *E. coli* or *Staphylococcus aureus* as these two genera do not yield uniform product profiles (Jensen et al., 1993).

Thus, the present study confirms that the PCR amplification of the 16S-23S spacer region could be a promising tool for identification of *Salmonella* serovars. The nucleotide sequence of the primers used for the amplification of spacer region appears to be sufficiently conserved throughout the *Salmonella* spp. to permit the PCR amplifications to be carried out with a single set of reaction conditions and amplification parameters without regard to serovars. Spacer DNA polymorphism could be a part of species or strain description. The amplification patterns of 16S-23S spacer region could be a definite way of checking the identity of the *Salmonella* serotypes. In a taxonomic study, the determination of spacer region patterns could help to identify the serotypes of *Salmonella* assuming that identical patterns are most certainly from the strains belonging to the same serotype.

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