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Molecular typing of *Brucella* species by PCR-RFLP and SSCP

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ABSTRACT

PCR-RFLP and PCR-SSCP help to understand variabilities in *Brucella* spp. genome which in turn assist in planning epidemiological strategies for control of brucellosis in animal population and thereby human transmission. Restriction fragment length polymorphism (RFLP) analysis was carried out for BCSP31 (223 bp) gene of 15 isolates using restriction enzyme HaeII and BsaBI. All isolates yielded a similar restriction pattern as that of reference strains i.e. 189 + 34 bp and 164 + 60 bp fragments with HaeII and BsaBI restriction enzyme, respectively. PCR-SSCP analysis of BCSP 31 gene of 33 *Brucella* spp. isolates was carried out. Seven different SSCP band patterns designated from "A" to "F" were observed. Out of 33 isolates of *Brucella* spp., 19 isolates showed SSCP profile similar to ATCC reference strains of *Brucella* spp. designated as band pattern "A" indicating no polymorphism. Whereas, 14 isolates showed polymorphism in SSCP band pattern designated from "B" to "G". Band pattern "A" was the most common in 19 (57.58 %) isolates followed by "B" in 6 (18.18 %) isolates and "G" in 4 (12.12 %) isolates, whereas "C","D","E", and "F" band patterns were found in each of 1 (3.03 %) isolate. SSCP band pattern "A" was observed most commonly in buffalo (10) followed by human (6) and cattle (3). The SSCP band pattern "B" was found in equal proportion in cattle and buffalo (3 each). The "C" and "E" SSCP band patterns were observed in cattle, whereas "D" and "F" SSCP band pattern was noticed in buffalo and human isolates, respectively. The "G" SSCP band pattern was observed in 3 cattle and 1 goat isolate. In cattle-ABCEG, buffalo-ABD, goat-G and in human-AF band patterns were seen. Comparative results of RFLP and SSCP in 15 isolates showed that, PCR-SSCP is more sensitive than RFLP for detection of polymorphism in BCSP31 gene.

Key words: *Brucella* spp., PCR, RFLP, SSCP

Strain identification in bacteria though difficult and complex, but is of paramount importance in studying its origin, evolution, distribution and pathogenicity. Molecular approaches, capable of addressing variability, are more precise and can be used reliably to detect base level mutations, but they should be performed with at most precision than biochemical methods. PCR-RFLP analysis shows excellent typing ability, reproducibility, stability and epidemiological concordance. PCR-RFLP assays of specific gene loci can serve as tools for diagnostic, epidemiological, taxonomic, and evolutionary studies. If insertion, deletion, or rearrangement events occur, the electrophoretic pattern of the cleavage fragments may also vary (Al Dahouk *et al*. 2005).

In recent times, polymerase chain reaction-single strand conformation polymorphism is most commonly used physical method for detection of minor mutation. Use of SSCP was first reported by Orita *et al.* (1989). Single strand conformation polymorphism offers a simple, inexpensive and sensitive method for detecting whether or not DNA fragments are identical in sequence, and hence can greatly reduce the amount of sequencing necessary (Orita *et al.* 1989, Ranjan *et al*. 2011). PCR-SSCP analysis is an appropriate, reliable and reproducible tool for detection of structural gene polymorphism that is primarily due to point mutations (Hayashi 1991, Neibergs *et al.* 1993, Sheffield *et al.* 1993, Nair *et al.* 2002). Neibergs *et al*. (1993) stated that PCR-SSCP analysis is the technique of choice for screening point mutations and minor deletions within a given fragment but it is necessary to optimize conditions for each case.

MATERIALS AND METHODS

Bacterial strains: Reference strains were *B. abortus* 544*, B. abortus* S19 and *B. melitensis* Rev1. A total of 15 field isolates recovered from animals during present research work and previous 18 field isolates of *Brucella* spp. maintained in Department of Microbiology were also included.

PCR-RFLP analysis of BCSP31 gene: The amplification product generated from BCSP31 (223 bp) gene PCR assays of 15 field isolates recovered from animals were subjected to restriction enzyme digestion using restriction enzymes

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Table 1. Reaction mixture for RE digestion of PCR product

Particulars	Concentration (µl)
Sterile distilled water	17
10× buffer	2.0
Restriction enzyme	1.0
PCR product	10
Total	30

namely BsaBI and HaeII. The reaction mixtures for digestion of products using different restriction enzymes are given in Table 1. The mixtures were spinned for few seconds and incubated at 37° C in water bath for 2.5 h. The reaction was stopped by adding 4 µl of 6× loading dye.

Analysis of RE digests: The restriction enzyme digests were evaluated by agarose gel electrophoresis using 3% agarose gel containing ethidium bromide at final concentration of 0.05 µg/ml. The samples were loaded in the wells and electrophoresis was carried out at 90 V, until the dye migrated three fourth of distance in the gel. A 50 bp DNA marker was electrophoresed simultaneously to know the size of restriction fragments generated. The results of RFLP were visualized and documented using computerized automatic gel documentation system using UV light source. The size of restriction fragments generated was estimated with the help of Image Lab (Version 4.1) software available with the gel documentation system.

Single strand conformational polymorphism (SSCP) analysis: Apart from reference strains, 15 field isolates recovered from animals during present research work and previous 18 field isolates of *Brucella* spp. maintained in Department of Microbiology were also included for PCR-SSCP analysis so as to know geographical relations in variable strains.

Sample preparation: Five µl of the amplified DNA and 5 µl of SSCP loading dye was added to a micro centrifuge tube and the contents of the tube were gently mixed. The micro centrifuge tubes were placed in a thermal cycler and heated at 95°C for 7 min and snap chilled on ice, kept the micro centrifuge tubes at –20°C for about 5 min. The sample was loaded in 10µl quantity into the wells of 8% acrylamide/ bis gel (37.5:1), containing 7% glycerol.

SSCP analysis of the PCR products was performed as per Orita *et al.* (1989) with minor modifications. The notched glass plate and outer glass plate of the vertical gel electrophoresis assembly were cleaned thoroughly with mild detergent using soft sponge and by using 70% ethanol i.e. rectified spirit.The spacer with 0.1 mm thickness was positioned and the gel sandwich was assembled. PAGE solution was prepared by mixing arylamide/bis solution, glycerol, 10× TBE stock buffer solution and distilled water. The solution was gently swirled while adding APS and TEMED and then poured into gel plate assembly with syringe/ micro-pipette. Quickly, comb was placed straight and gel was kept undisturbed at least 45 min for polymerisation. After polymerisation, the comb was removed and wells were flushed with 1× TBE buffer. The reservoir chamber and lower chamber were filled with running buffer and the sample was loaded in the respective wells. Electrophoresis was performed in 1× TBE buffer at constant 5 Watt for 4–5 h. During running, the temperature of running buffer was maintained low either by placing ice behind the reservoir chamber or by using pre-chilled buffer. On termination of electrophoresis, the plates were removed carefully from the apparatus and kept on blotting paper. Spacers were removed and plates were separated carefully using spatula so that gel was retained on smaller plate. Thereafter, gel was subjected to silver staining to visualize SSCP band patterns.

Silver staining of SSCP gel: The gel was immersed in a tray of appropriate size filled with 10% acetic acid (250 ml) overnight for fixing DNA bands. The acetic acid was decanted and 250 ml of distilled water was poured in the tray and rinsed thoroughly by placing the tray on oscillatory automatic shaker for 15 min. Meanwhile, 250 ml of 0.1% silver nitrate solution was prepared in amber colour bottle and 375 µl of 37–41% formaldehyde was added and mixed. Distilled water was gently decanted from tray. The gel was stained by merging it for 45 min in formal silver nitrate solution with constant shaking in a dark room or covering the tray with black cloth or by wrapping tray with aluminium foil. The gel was briefly rinsed twice with distilled water for 10–20 sec. Working quickly, distilled water was decanted from the tray. The freshly prepared and chilled 3% formal sodium carbonate solution containing 1.5 ml of 37–40% formaldehyde (w/v) and 60 ml of freshly prepared 1% sodium thiosulphate/ litre of sodium carbonate solution was transferred to the tray. The gel was kept immersed till the bands developed sharply (nearly 10 min). Further staining was stopped by giving the gel 10% acetic acid treatment for five min. Then, 250 ml distilled water was added to the tray. Finally, the gel was washed with distilled water for five min and drained.

Evaluation/interpretation of SSCP analysis: The gel after completion of silver staining procedure, was placed on a white tray of gel documentation system so that it produces its digital image and it can be evaluated by the software of the gel documentation system. The SSCP bands patterns were recorded and observations were noted.

RESULTS AND DISCUSSION

Molecular typing methods are the tool for species differentiation, tracing of strains and the detection of mutations influencing phenotypic characteristics. Considering the advantages of molecular techniques, characterization of *Brucella* spp. isolates for detection of polymorphism in BSCP31 gene was carried out by employing PCR-RFLP and PCR-SSCP techniques.

Restriction fragment length polymorphism (PCR-RFLP) analysis: NEB cutter software (http://tools.neb.com/NEBcutter2/enzcut.php) detected the restriction site for the selection of appropriate restriction enzymes. The single restriction site for restriction enzyme HaeII resulted in 2 fragments of 189 and 34 bp each. In Agarose gel

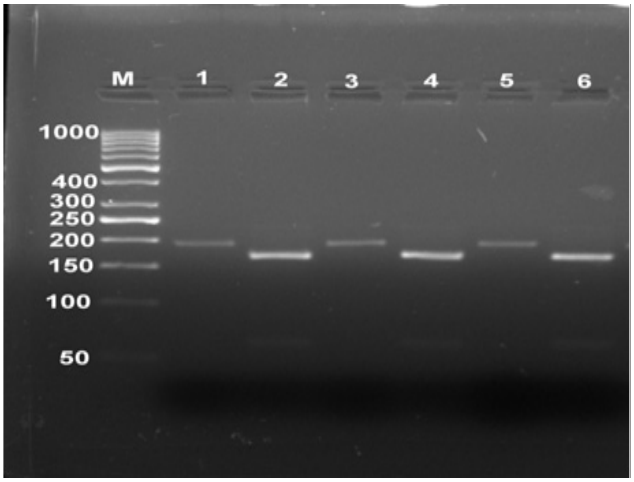

Fig. 1. PCR-RFLP of BCSP31 PCR assay using HaeII and BsaBI restriction enzyme. Lane M, 50 bp DNA ladder; 1, HaeII digest *B. abortus* 544; 2, BsaBI digest *B. abortus* 544; 3, HaeII digest *B. melitensis* Rev1; 4, BsaBI digest *B. melitensis* Rev1; 5, HaeII digest of field isolate; 6, BsaBI digest of field isolate.

electrophoresis, only one band of 189 bp fragment was clearly visible, however band of 34 bp fragment could not be seen clearly due to its small size. Whereas, 2 fragments of 163 and 60 bp each yielded by restriction enzyme BsaBI were visible in agarose gel electrophoresis (Fig. 1).

The analysis of RE digests of BCSP31 gene PCR product in all 15 field isolates of *Brucella* spp. by agarose gel electrophoresis revealed similar RFLP patterns as in reference strains of *B*. *Abortus* 544*, B. Abortus* S19 and *B. melitensis* Rev1, showing their monomorphic nature.

RFLP technique is useful to differentiate the closely related organisms by analyzing their DNA cleavage patterns. RFLP of whole genomes is supposed to be a reproducible and easily performable method for detection of polymorphism (Al Dahouk *et al*. 2005). PCR-RFLP analysis was applied to various *Brucella* genes, i.e. *omp*22, *omp*25, *omp*31, *omp*2, *rps*L, *dna*K, and *ery.*BCSP31, 16S rRNA, *gro*EL, *dna*J, *htr*A were investigated using PCR-RFLP with and without limited differential identification of *Brucella* species (Da Costa *et al*. 1996, Al Dahouk *et al*. 2005).

The results of PCR-RFLP revealed that the RFLP patterns of all 15 *Brucella* isolates were as per the prediction of software. The HaeII restriction enzyme produced fragments with molecular weight 189 bp and 34 bp. The small fragment of 34 bp could not be resolved due to its lower molecular weight (Al Dahouk *et al*. 2005). BsaBI restriction enzyme produced fragments of molecular size 164 and 60 bp. The result of RFLP with both the above enzymes displayed similar RFLP patterns amongst all 15 *Brucella* isolates and reference strains, indicating non-existence of polymorphism in BCSP31 gene of all 15 isolates.

In present study, the findings of lack of polymorphism in *Brucella* spp., may be due to poor sensitivity of RFLP in detecting polymorphism (Verger *et al.* 1985) although this technique is used for detection of polymorphism in different genes of *Brucella* spp. effectively (Vizcaino *et al.* 1997, McDonald *et al.* 2006).

Table 2. Details of geographical location of isolates analysed in PCR-SSCP analysis

Geographical location	No of isolates analyzed				Total
	Cattle	Buffalo	Goat	Human	
Thane					
Palghar	06	06	-	-	12
Titwala	-	01	-	-	01
Murbad	-	02	-	-	02
Kalyan	-	01	-	-	01
Mumbai	-	01	-	-	01
Pune	03	-	-	-	03
Kolhapur	-	03	-	-	03
Satara	01	-	-	02	03
Ahamdnagar	01	-	01	-	02
Northern Karnataka	-	-	-	05	05
Total	11	14	01	07	33

The ability of detection of polymorphism by PCR-RFLP depends upon occurrence of mutation at the restriction target. Therefore, in present study polymorphism was not detected. Barroso *et al.* (1999) reported that allelic mutations do not alter restriction targets for any commercially available endonuclease and result in non-detection of polymorphism by RFLP. They reported the non-feasibility of PCR-RFLP as a reference technique.

Single strand conformational polymorphism analysis (PCR-SSCP): Single strand conformational polymorphism analysis (PCR-SSCP), originally developed by Orita *et al.* (1989) is the most widely used method for detection of mutation in DNA. The SSCP technique has made possible the detection of both known and unknown single point mutations resulting in polymorphisms.

A total of 33 *Brucella* spp. field isolates (7 human, 14 buffalo, 11 cattle and 1 goat) from different geographical origins of Maharashtra and Karnataka state were characterized by PCR-SSCP (Table 2).

As primers B4 and B5 for BCSP31gene ensured highly efficient and consistent PCR amplification of all *Brucella* isolates, these PCR products were further analysed using SSCP. A 223 bp fragment of genus specific BCSP31gene was successfully amplified in all *Brucella* spp. isolates. PCR-SSCP of the amplified fragment was performed for

Table 3. Species wise SSCP band pattern of *Brucella* isolates

Species	SSCP band pattern/variants							Total
	A	B	C	D	E	F	G	
Cattle	03	03	01	-	01	-	03	
Buffalo	10	03	-	01	-	-	-	
Goat	-	-	-	-	-	-	01	
Human	06	-	-	-	-	01	-	
Total	19	06	01	01	01	01	04	33

characterization and detection of mutation/polymorphism in BCSP31 gene.

The SSCP amplimers of *Brucella* spp. were resolved on non-denaturing polyacrylamide gel. Seven variants (band patterns) designated as "A", "B", "C", "D", "E", "F" and "G" were observed in 33 *Brucella* spp. isolates analyzed. Variants were grouped on the basis of changes in position and number of SSCP bands in comparison with reference strains produced in polyacrylamide gels.

In PCR-SSCP analysis (Table 3), out of 33 isolates of *Brucella* spp., 19 (57.58%) isolates showed SSCP profile similar to the ATCC reference strains of *Brucella* spp. designated as band pattern "A" (Figs 2,4). Whereas 14 isolates differed in their SSCP band pattern from reference strains of *Brucella* spp. and were designated from "B" to "G". Out of 14 variants of *Brucella* isolates, 6 (18.18%) were having SSCP band Pattern B (Fig. 3), 4 (12.12%) isolates had band pattern G (Fig. 5), whereas in remaining 4 isolates, each (3.03%) had unique SSCP profile designated as variant "C" (Fig. 2), "D" (Fig. 3), "E" (Fig. 4) and variant "F" (Fig. 5).

Maximum variants were observed amongst Thane district isolates (cattle and buffalo) showing B to E PCR-SSCP band patterns. Band pattern "B" was observed in 06 isolates whereas "C", "D", and "E" were observed in each of single isolate. One isolate (human) with SSCP band pattern "F" belonged to district Satara. Pune district isolates (Cattle-3) showed only single "G" SSCP band pattern, whereas similar pattern was also observed in one isolate (goat) from Ahamadnagar district. Moreover, in district Kolhapur, only A band pattern was observed. From above results, it is observed that, the polymorphic nature of

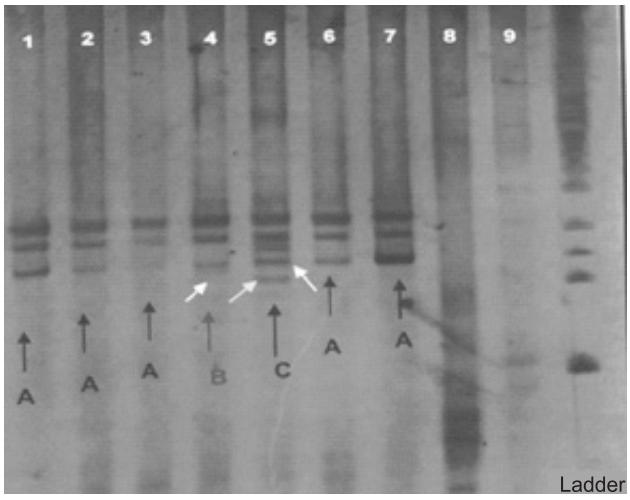

Fig. 2. Lane 1, *B. abortus* 544; lane 2, 3, 6, 7, SSCP pattern similar to *B. abortus* 544 without any mutation; lane 4, 5, Mutations; lane 8, 9, IS711 AB & OMP as positive control; lane 10, 100 bp ladder. White arrow, an extra SSCP band. Yellow arrow, an extra and shifted band.

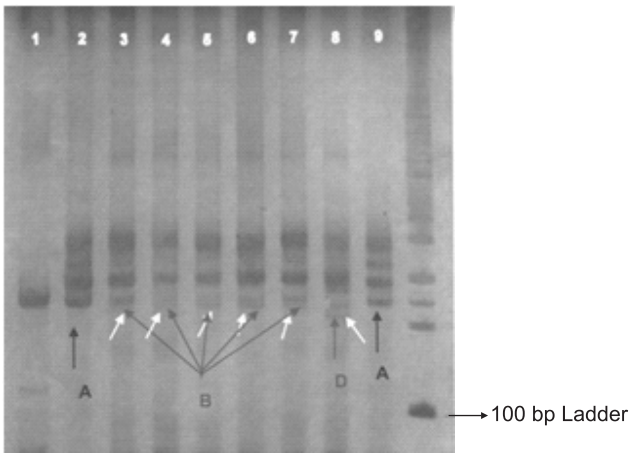

Fig. 3. Lane 1, PCR product; lane 2 to 8, *Brucella* isolates showing A, B and D SSCP band pattern; lane 9, *B. abortus* S19; Lane 10, 100 bp ladder. White arrow indicates an extra SSCP band. Orange arrow indicates mobility in SSCP band.

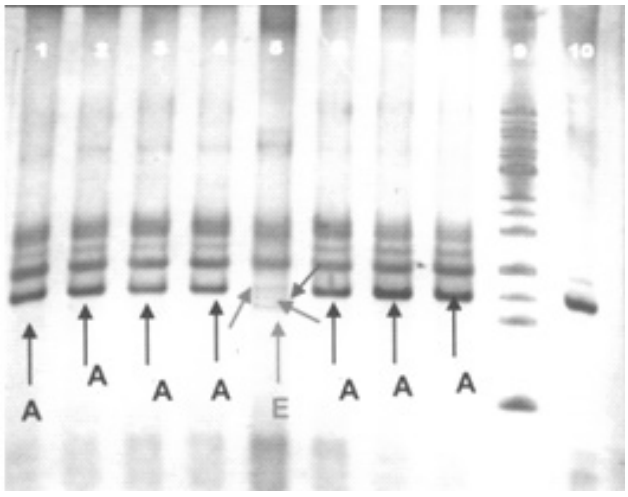

Fig. 4. Lane 1, *B. abortus* 544; lane 2, *B. abortus* S19; lane 3, *B. melitensis* Rev1; lane 4 to 8, *Brucella* isolates; lane 9, 100 bp ladder; Lane 10, PCR product. Red arrow indicates an extra SSCP band. Blue arrow indicates position of absent band.

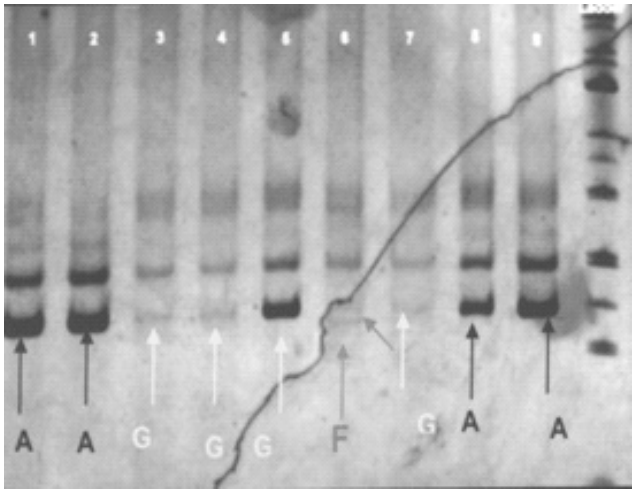

Fig. 5. Lane 1 to 8, *Brucella* isolates showing A, F and G SSCP pattern; lane 9, *B. abortus* 544; lane 10, 100 bp ladder. Green arrow indicates mobility shift of SSCP band.

BCSP31 gene of *Brucella* correlated to geographical location and source of origin.

Since, the use of greater than 250 bp PCR product is not considered suitable for SSCP (Orita *et al*. 1989, Kalvatchev and Draganov 2005), the PCR product of species specific *Brucella* primers could not be used for SSCP in present analysis. As shown in lanes 8 and 9 (Fig. 2), PCR products of IS711/AB (498 bp) and omp (853 bp) genes, respectively could not yield proper separate band due to their high molecular weights, therefore, SSCP analysis could not be done using above species specific PCR product. Hence, genus specific BCSP31 PCR products were used for same since the suitabilitity of this technique lies for the PCR product between 100–300 bp. Nair *et al.* (2002), Kalvatchev and Draganov (2005) also suggested that the PCR products used for SSCP should have a length <400 bp, ideally this should be around100–300 bp.

Varsada (2003) carried out PCR-SSCP targeting BSCP31 gene in DNA samples extracted from 77 whole human blood samples. The difference in mobilities in electrophoresis was recorded as a band "pattern". He observed only single SSCP band pattern by PCR-SSCP analysis indicating lack of variation in amplified PCR products. Pipalia *et al.* (2004) used PCR-SSCP for investigation of BoLA-ORB3 gene polymorphism and found 16 different SSCP band patterns designated as "1" to "16". Whereas, Anand *et al.* (2006) employed this technique for rapid identification of *E. coli*, which resulted in strain specific 22 SSCP patterns. Moreover, Ranjan *et al.* (2011) carried out PCR-SSCP on *Slc11a1* gene of Nimari and Kenkatha (Indian cattle breeds) and revealed 3 SSCP patterns.

Our findings are in agreement with the findings of the above authors, wherein SSCP was found sensitive in detection of polymorphism used for analysis of mutation, strain detection, etc.

Comparative efficacy of PCR-RFLP and PCR-SSCP: PCR-RFLP results showed similar RFLP patterns amongst all 15 *Brucella* isolates and reference strains, indicating non-existence of polymorphism. Whereas, results of PCR-SSCP analysis of same isolates revealed 5 types i. e. ABCDE band patterns amongst same 15 isolates, indicating presence of polymorphism in BCSP31 gene of these isolates. Evaluation of PCR-RFLP and PCR-SSCP analysis of BCSP31 gene of *Brucella* thus revealed that, the polymorphism detected in isolates by PCR-SSCP could not be detected by RFLP, proving PCR-SSCP as more sensitive and superior technique to RFLP for exploring polymorphism successfully due to point mutation and thereby in detecting strain variation.

Orita *et al.* (1989) developed mobility shift analysis of single-stranded DNA on neutral polyacrylamide gel electrophoresis i.e. SSCP. They stated that nucleotide substitution at any point in a fragment may be detected by SSCP analysis, but DNA polymorphism could be detected more frequently by SSCP analysis than by RFLP analysis. Like RFLP analysis, SSCP analysis is simple and does not require complicated instruments or specialized techniques. Nair *et al.* (2002) carried out PCR-RFLP and PCR-SSCP analysis on the *groEL* gene from 41 strains of 10 different *Salmonella* serovars. Whereas RFLP did not discriminate between the serovars, however, SSCP produced 14 SSCP profiles. Similar non-correlating results of RFLP and SSCP was reported by Pipalla (2004) wherein he stated inability to correlate a particular SSCP pattern with any specific restriction enzyme RFLP pattern, since RFLP pattern responds to a nucleotide change only at a specific point on DNA chain; on the other hand, SSCP patterns are generated as a result of nucleotide sequence change at 1 or several positions together.

Our result also indicated no correlation between RFLP and SSCP analysis and moreover better sensitivity of SSCP is in agreement with the findings of above authors.

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