Ann Microbiol (2014) 64:177–183
DOI 10.1007/s13213-013-0650-9

ORIGINAL ARTICLE

PCR-RFLP analysis of *fliC*, *fimH* and 16S rRNA genes in *Salmonella* Typhimurium isolates of varied origin

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Received: 30 October 2012 / Accepted: 8 April 2013 / Published online: 15 May 2013

Abstract Restricted fragment length polymorphism (RFLP) was used in analyses on the typing and heterogeneity, typeability and polymorphism of the 16S rRNA, *fliC* and *fimH* genes in *Salmonella* Typhimurium isolates of varied origin. The digestion of PCR products with restriction enzymes *Eco*RV, *Cla*I, *Hae*III and *Sca*I (*fliC* genes), *Hinc*II, *Cla*I, *Eco*RV and *Mlu*I (*fimH* genes) and *Eco*RI, *Sma*I and *Hae*III (16S rRNA genes) generated two to four bands of ranging in size from 100 to 1,104 bp. Of all the restriction profiles obtained, only the *Cla*I profile for *fimH* could be used to classify *Salmonella* Typhimurium isolates into different groups. According to this profile, pattern A with uncut *fimH* was observed in eight isolates (36.36 %) and pattern B with 755- and 253-bp bands was observed in 14 isolates (63.63 %). No pattern was allotted for a special region or source. These results demonstrate that PCR-RFLP based on these genes showed good typeability but low discriminatory power. Moreover, the highly conserved nature of *fliC*, *fimH* and 16S rRNA illustrated in our study suggests the importance of these genes as immunization and diagnostic factors in *Salmonella* Typhimurium. Simultaneously, our results also illustrate the potential of *Cla*I-based *fimH* analysis as a marker for the subserotype level differentiation of *Salmonella* Typhimurium isolates.

Keywords RFLP-PCR · *Salmonella* Typhimurium · Heterogeneity · 16S rRNA · *fliC* · *fimH*

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Introduction

Non-typhoidal salmonellae remain the major food-borne pathogens in both industrialized and developing countries even a century after their discovery (Goldman and Green 2009). The most common of the total of 2,668 *Salmonella enterica* serovars (Popoff et al. 2004) is *Salmonella* Typhimurium (Rahman 2002), and the number of Typhimurium infections in human and animals are dramatically increasing (Mikasova et al. 2005). Thus, discrimination of *Salmonella* Typhimurium between and within the same serotype is necessary (Goldman and Green 2009) for effective epidemiological surveillance and control (El-Sebay et al. 2012). Conventional approaches used for these purposes are lengthy, laborious and costly (Gallegos-Robles et al. 2008), necessitating the development and standardization of economical, easy-to-use and more rapid molecular methods to type and subtype *Salmonella* Typhimurium isolates.

In a study on the molecular diversity of *Salmonella* Typhimurium, Gebreyes et al. (2006) showed that among the various approaches used restriction fragment length polymorphism (RFLP) provided the best discriminatory index. Moreover, it has the added advantages of ease of performance, relatively low cost and high reproducibility (Olsen 2000; Mergulhao et al. 2008). rRNA has often been the target of many bacterial heterogeneity studies (Olsen et al. 1992). It has also been shown that genotypic diversity existing in flagellin genes (*fliC* and *fljB* encoding phase-1 and phase-2 flagellin, respectively) and FimH protein (fimbrial adhesin) of *S. enterica* makes them excellent candidates for heterogeneity studies (Guo et al. 2007; Jong et al. 2010; Kisiela et al. 2012). Paradoxically, Dilmaghani et al. (2010) found that *fljB* gene in *Salmonella* Typhimurium is highly conserved. Based on these findings, we have analyzed the conserved nature or polymorphism of the *fliC*, 16S rRNA and *fimH* genes in *Salmonella* Typhimurium isolates of varied origin using PCR-RFLP.

Materials and methods

Bacterial cultures A total of 22 *Salmonella* Typhimurium isolates of varied origin (Table 1) were obtained from the National Salmonella Centre, Indian Veterinary Research Institute, Izatnagar. After sub-culturing each isolate on MacConkey agar, we characterized the colonies morphologically by Gram staining and serologically with poly "O" sera (Kauffmann 1964). Molecular confirmation was done using *fimA* (*Salmonella* specific) (Cohen et al. 1996) and *STM4497 fr3* (Typhimurium specific) primers (Kim et al. 2006). After characterization, a glycerol stock was made of each culture which was kept at −20 °C.

Isolation of genomic DNA Genomic DNA of all isolates was isolated using the CTAB method (Wilson 1987). The concentration and purity of the isolated DNA was estimated spectrophotometrically, and the integrity of the DNA was assessed by electrophoresis in a 0.7 % agarose gel.

Table 1 Host and epidemiological sources of *Salmonella* Typhimurium isolates

Host	Culture code (Source)
Poultry	E2394[a] and E2393[a] (chick), E2375 [a](broiler), E2622[a] (liver), E4638[b] (egg), E4227[b] (poultry), E4841[c] and E4809[c] (eggs), E4885[d] and E4896[d] (chicken), E4256[b] (chicken pizza)
Bovine	E2416[b] (liver), E5158[b], E4935[b] and E4946[b] (faeces), E4242[e]
Human	E4938[b] (stool)
Feed	E4231[b] and E4658[b]
Water	E4659[b], E4490[c]
Coriander leaves	E2677[c]

Place of isolation: [a] Pune, [b] Bareilly, [c] Pantnagar, [d] Mumbai, [e] Babugarh

PCR-RFLP analysis

PCR amplification of fliC, fimH and 16S rRNA genes Amplification of the 1,488-bp *fliC* (forward: 5′-AAG GAA TTC ATC ATG GCA CAA G-3′, reverse: 5′-GAA GAA TTC AAC GCA GTA AAG AGA G-3′), 1,008-bp *fimH* (forward: 5′-GGA TCC ATG AAA ATA TAC TC-3′, reverse: 5′-AAG CTT TTA ATC ATA ATC GAC TC -3′) and 572-bp 16S rRNA fragment (forward: 5′-TGT TGT GGT TAA TAA CCG CA-3′, reverse: 5′-CAC AAA TCC ATC TCT GGA-3′) was performed using specific primers. PCR reactions were carried out in a 25-μl volume of reaction mix containing 600 ng of template DNA and 1× PCR assay buffer [$(NH_4)_2SO_4$, 1.0 mM $MgCl_2$, 125 μM dNTPs, 25 picomol of each primer, 1 U of *Taq* DNA polymerase]. The cycling conditions for *fliC* amplification were 94 °C for 4 min, 25 cycles of 94 °C for 1 min, 52 °C for 1 min and 72 °C for 1 min 30 s followed by 72 °C for 10 min. The cycling conditions for the *fimH* and the 16S rRNA genes were the same except for the annealing temperature which was 51 °C and 55 °C, respectively. The amplification products were checked for the presence of the desired bands in the agarose gel.

Restriction endonuclease digestion of PCR products All three genes were checked using the Lasergene software package (DNASTAR, Madison, WI), and based on the results suitable restriction enzymes were selected (Table 2). In each restriction digestion reaction, 10 μl of PCR-amplified product was digested with 10 U of each enzyme according to the manufacturer's instructions (Fermentas, Thermo Fischer Scientific, Vilnius, Lithuania). Digested PCR products were separated by agarose gel electrophoresis and photographed under a UV illuminator. Gel images were analyzed for molecular heterogeneity by searching for changes in the banding pattern between various isolates. The numerical index of discrimination (D) of restriction enzymes which can result in discrimination among *Salmonella* Typhimurium isolates was calculated using Simpson's index of diversity (Hunter and Gaston 1988).

Results

Characterization of Salmonella Typhimurium isolates All 22 isolates showed typical cultural, morphological and serological characteristics. During molecular confirmation using PCR all gave positive results with both genus and serotype primers.

Table 2 Restriction enzymes and their respective sites for the *fliC*, 16S rRNA and *fimH* genes

Restriction enzyme	Recognition site	Position of restriction in *fliC*	[a]Position of restriction in 16S rRNA gene	Position of restriction in *fimH*
*Eco*RI	G…AATTC	–	674	–
*Sma*I	CCC…GGG	–	614	–
*Eco*RV	GAT…ATC	472	–	103, 640
*Mlu*I	A…CGCGT	–	–	534
*Hinc*II	GTPy…PuAC	–	–	388
*Cla*I	AT…CGAT	468, 474	–	755
*Hae*III	GG…CC	384,1424	46,213,417	–
*Sca*I	AGT…ACT	604	736,898,932,1141	–

[a] Checked with full 16S rRNA gene sequence (1,234 bp) available at GenBank (NCBI)

Evaluation of quality and purity of genomic DNA The genomic DNA samples showing intact bands were selected for PCR amplification and analysis, while those showing smearing were discarded. DNA concentrations ranged from 300 to 1,500 ng/μl. The samples having an optical density (OD) ratio between 1.7 and 1.9 were used in subsequent experiments.

Amplification of fimH, fliC and 16S rRNA genes PCR of the *fimH fliC* and 16S rRNA fragment genes successfully amplified fragments of the expected sizes, namely, 1,008, 1,488 and 572 bp, respectively. The band size detected in all isolates was consistent for all three genes as analyzed by agarose gel electrophoresis (Fig. 1). It should be noted that all isolates produced a single PCR amplicon in each reaction.

PCR-RFLP analysis Of the four enzymes used for digesting *fimH*, *Eco*RV, *Hinc*II and *Mlu*I produced same banding pattern with all of the isolates (Figs. 2, 3), but *Cla*I failed to cut the *fimH* gene in eight isolates (Fig. 3). Thus, according to the *Cla*I profile, isolates E4638, E4809, E4885, E4231, E4935, E4896, E4256 and E4659 were placed in one group (pattern A), all having an uncut *fimH*. The other 14 isolates belonged to separate group (pattern B) comprising bands of 755 bp and 253 bp (Table 3). The discriminating power of this enzyme was calculated to be 0.485, but, no pattern could be allotted for a special region or source (Table 2). *Eco*RV produced only two major bands with an approximate molecular size of 640 and 368 bp in all isolates even though it had two restriction sites on *fimH*.

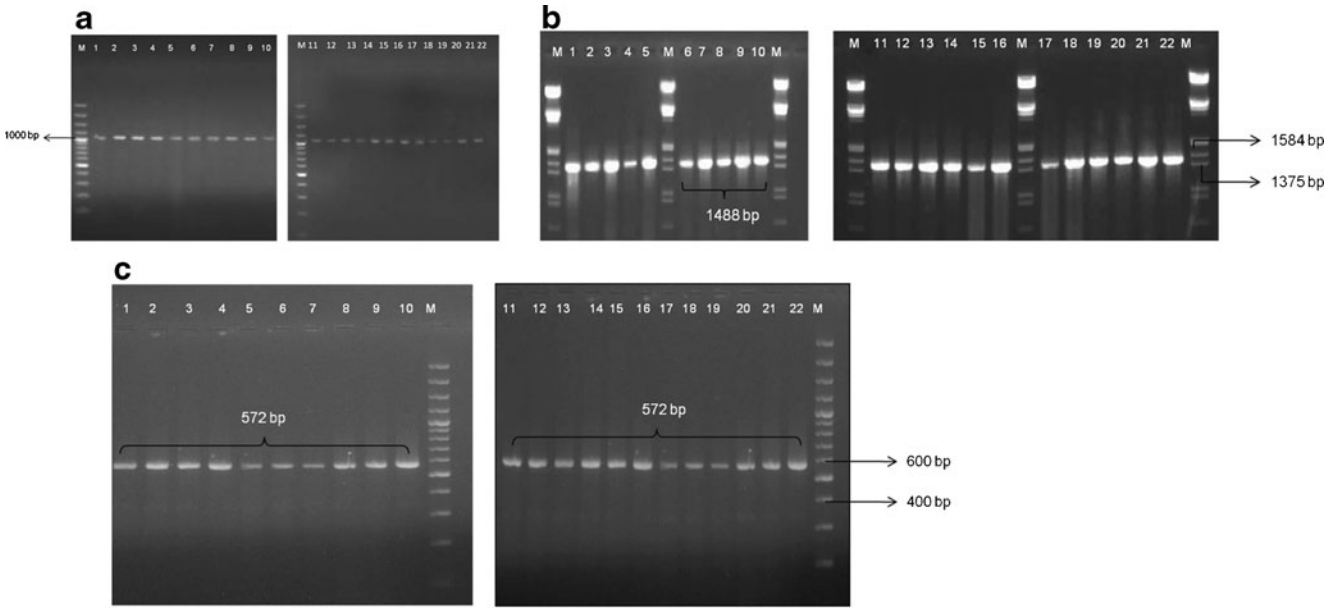

Fig. 1 PCR amplification of the *fimH*, *fliC* and 16S rRNA genes of *Salmonella* Typhimurium isolates. **a** PCR amplification of *fimH* gene (1,008 bp), **b** PCR amplification of *fliC* gene (1,488 bp), **c** PCR amplification of 16S rRNA fragment (572 bp size). *Lanes*: *M* Molecular weight markers, *1* E2394, *2* E4638, *3* E4809, *4* E2375, *5* E2393, *6* E4885, *7* E2416, *8* E4242, *9* E4938, *10* E4231, *11* E4227, *12* E4841, *13* E4659, *14* E4490, *15* E4658, *16* E2677, *17* E5158, *18* E4935, *19* E4896, *20* E4256, *21* E2622, *22* E4946

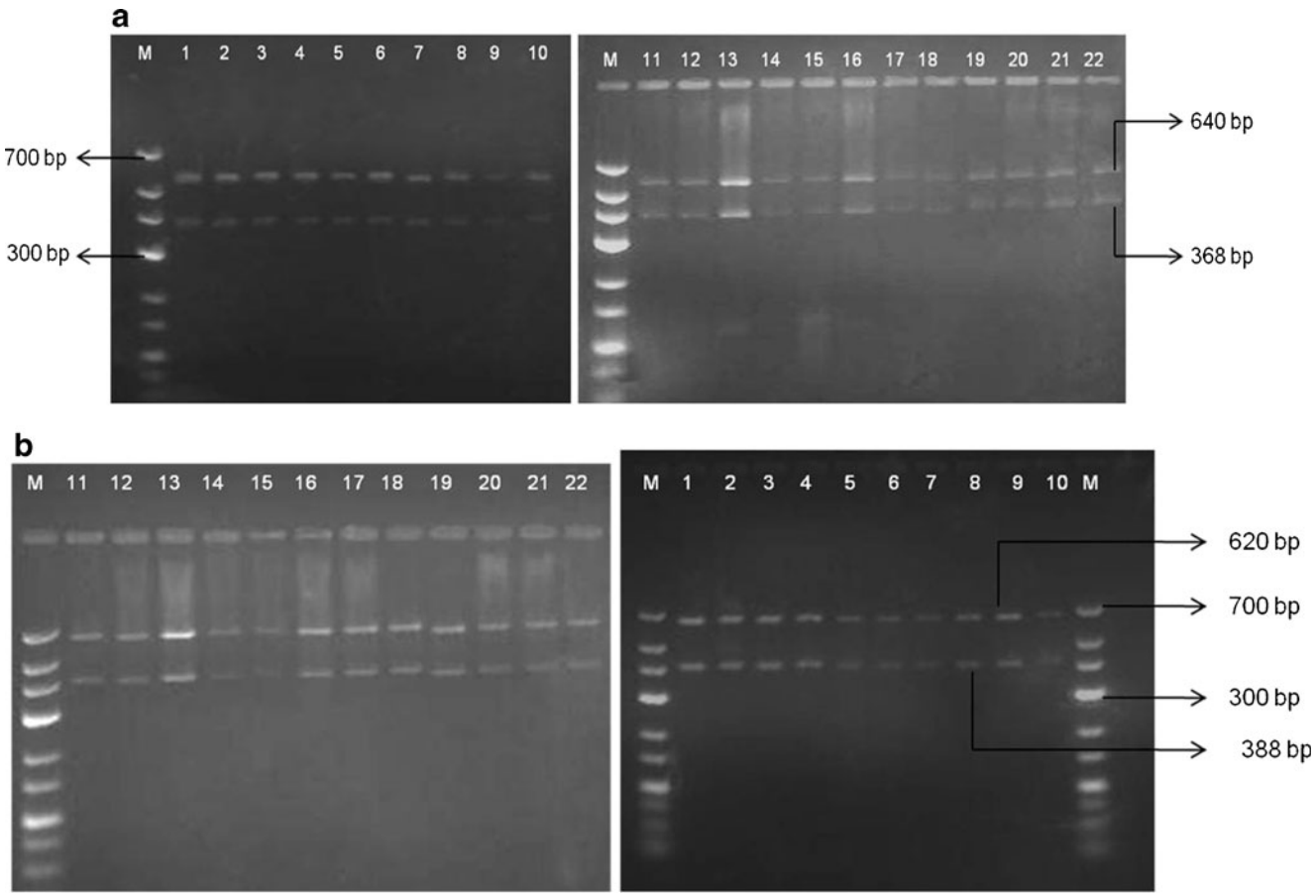

Fig. 2 Restriction fragment length polymorphism (RFLP) patterns of the *fimH* gene. **a** Restriction enzyme digestion of *fimH* with *Eco*RV, **b** restriction enzyme digestion of *fimH* with *Hinc*II. *Lanes*: *M* Molecular weight markers, *1* E2394, *2* E4638, *3* E4809, *4* E2375, *5* E2393, *6* E4885, *7* E2416, *8* E4242, *9* E4938, *10* E4231, *11* E4227, *12* E4841, *13* E4659, *14* E4490, *15* E4658, *16* E2677, *17* E5158, *18* E4935, *19* E4896, *20* E4256, *21* E2622, *22* E4946

*Hinc*II and *Mlu*I produced two major bands with an approximate molecular size of 388 and 620 bp, and 534 and 474 bp, respectively. Typing and sub-typing of *Salmonella* Typhimurium between and within the same serotype is very important (Goldman and Green 2009) for the determination of potential infective sources and effective epidemiological surveillance and control (El-Sebay et al. 2012). Among the various techniques that are used in heterogeneity analyses of Typhimurium isolates, RFLP has been found to have the best discriminatory index (Gebreyes et al. 2006) with the added advantage of high reproducibility (Olsen 2000; Mergulhao et al. 2008). Many authors (Olsen et al. 1992; Guo et al. 2007; Jong et al. 2010; Kisiela et al. 2012) have suggested that rRNA, FimH fimbrial adhesion and flagellin genes are excellent candidates for heterogeneity studies of *Salmonella*. We therefore have used PCR-RFLP based on the amplification and restriction digestion of the *fliC*, 16S rRNA and *fimH* genes to check the typeability and discriminating power of this method within *Salmonella* Typhimurium isolates.

All four enzymes (*Eco*RV, *Hae*III, *Sca*I and *Cla*I) used for digesting *fliC* produced same banding pattern with all 22 isolates (Fig. 4). *Eco*RV and *Sca*I produced two major bands of molecular size 472 and 1,016 bp, and 604 and 884 bp, respectively. Although *Hae*III was predicted to have two restriction sites on *fliC*, it produced two fragments of 384 and 1,040 bp with all of the isolates studied. *Cla*I, which has three restriction sites in *fliC*, digested the gene into four fragments with approximate sizes of 468, 1,014 and 6 bp. The 6-bp fragment was not visualized in the gel due to its very small size.

For the 16S rRNA, *Eco*RI and *Sma*I had a single site and thus produced two fragments of approximately 360 and 210 bp, and 410 and 160 bp, respectively, in all of the isolates (Fig. 5). *Hae*III digested the particular segment of 16S rRNA (572 bp) into four fragments with an approximate size of 280, 160, 100 and 30 bp, respectively, although the 30-bp fragment was not clearly visualized in the gel due to its very small size(Fig. 5). Overall, the restriction enzymes used against the 16S rRNA fragment revealed the

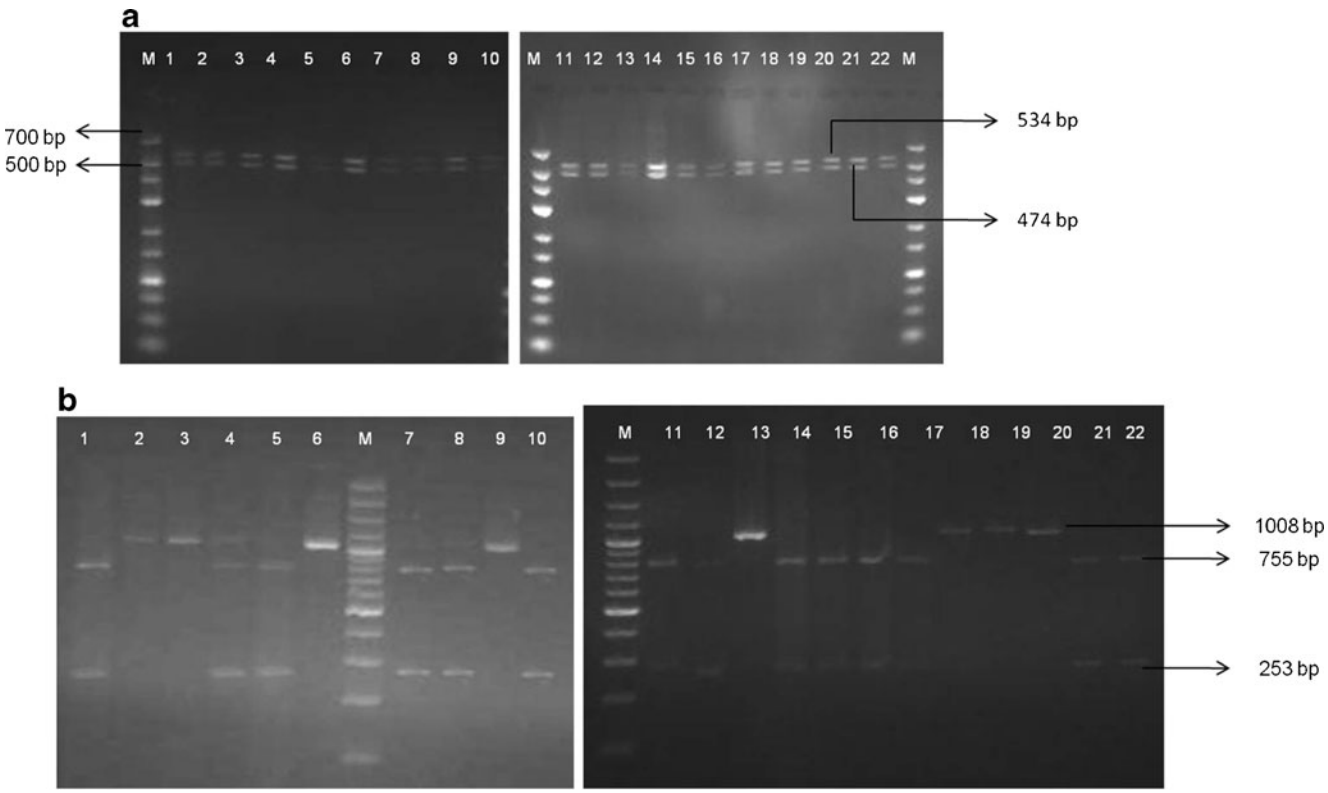

Fig. 3 RFLP patterns of the *fimH* gene. **a** Restriction enzyme digestion of *fimH* with *Mlu*I. **b** Restriction enzyme digestion of *fimH* with *Cla*I. *Lanes*: *M* Molecular weight markers, *1* E2394, *2* E4638, *3* E4809, *4* E2375, *5* E2393, *6* E4885, *7* E2416, *8* E4242, *9* E4938, *10* E4231, *11* E4227, *12* E4841, *13* E4659, *14* E4490, *15* E4658, *16* E2677, *17* E5158, *18* E4935, *19* E4896, *20* E4256, *21* E2622, *22* E4946

same banding pattern for all *Salmonella* Typhimurium isolates.

Discussion

Since a well-characterized panel of bacterial isolates is necessary for heterogeneity studies (Lin et al. 1996), we characterized all 22 members by cultural, morphological, serological and molecular techniques. During amplification, the components of the PCR reaction mixture were optimized with a high amount of template DNA, lower amount of dNTP and a reduced number of polymerization cycles to minimize the error produced by *Taq* DNA polymerase. Amplification of *fimH*, *fliC* and 16S rRNA gene fragment generated products with the desired size of 1,008, 1,488 and 572 bp, respectively, with no variation in gene size between isolates. In an RFLP analysis the number of fragments should be sufficient to enable good discrimination but not so many as to result in a crowded fingerprint which is difficult to interpret (Towner and Cockayne 1993). Therefore, restriction enzymes having one to three restriction sites in the target genes were utilized for our study. Of all the restriction profiles produced, with the exception of the *Cla*I profile against *fimH*, all provided uniform banding patterns for all of the *Salmonella* Typhimurium isolates, clearly indicating the highly conserved nature of these genes within this serotype. Similar interpretations regarding the 16S rRNA gene fragment and *fimH* on *Salmonella* Gallinarum isolates have been given by Habtamu et al. (2011). Jong et al. (2010) stated that genotypic diversity existing in flagellin genes (*fliC* and *fljB* encoding phase-1

Table 3 Distributions of RFLP profiles among *Salmonella* Typhimurium isolates of varied origin

Origin	No. of isolates	RFLP profile	
		A	B
Poultry	11	5	6
Bovine	5	1	4
Human	1	–	1
Feed	2	1	1
Water	2	1	1
Coriander leaves	1	–	1
Total	22	8	14

RFLP, Restriction fragment length polymorphism

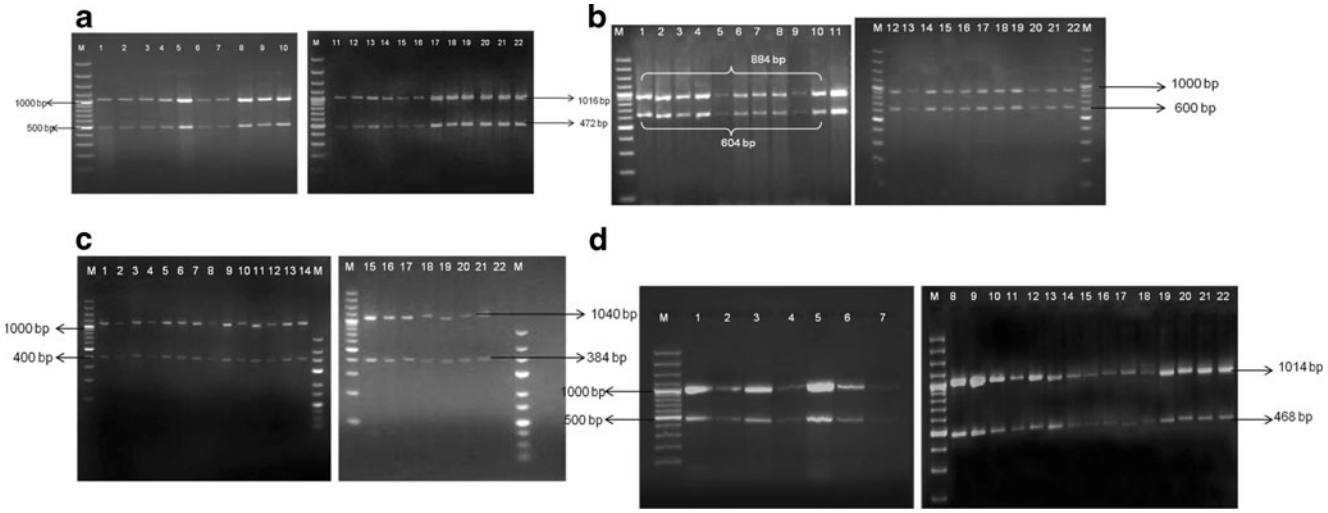

Fig. 4 RFLP patterns of the *fliC* gene. **a** Restriction enzyme digestion of *fliC* with *Eco*RV. **b** Restriction enzyme digestion of *fliC* with *Sca*I. **c** Restriction enzyme digestion of *fliC* with *Hae*III. **d** Restriction enzyme digestion of *fliC* with *Cla*I. *Lanes*: *M* Molecular weight markers, *1* E2394, *2* E4638, *3* E4809, *4* E2375, *5* E2393, *6* E4885, *7* E2416, *8* E4242, *9* E4938, *10* E4231, *11* E4227, *12* E4841, *13* E4659, *14* E4490, *15* E4658, *16* E2677, *17* E5158, *18* E4935, *19* E4896, *20* E4256, *21* E2622, *22* E4946

and phase-2 flagellin) of *S. enterica* can be used for subtyping. Paradoxically, in our study the restriction profiles in *fliC* were similar in all isolates, again showing the conserved nature of this gene in *Salmonella* Typhimurium. Our observations corroborate earlier observations of Dilmaghani et al. (2010) who showed that the *fljB* gene is highly conserved among *Salmonella* Typhimurium isolates from different geographical regions. The observed variation in the *fimH*, 16S rRNA gene and *fliC* RFLP profiles, as mentioned earlier by various authors (Olsen et al. 1992; Guo et al. 2007; Jong et al. 2010; Kisiela et al. 2012), might be due to the different serotype studied, different geographical origin, different restriction enzymes used and some point mutations, among other factors, in these studies; this variation has to be studied in detail by sequencing or the PCR-single strand confirmation polymorphism method. However, the *Cla*I profile against *fimH* classified *Salmonella* Typhimurium isolates into two groups. According to this profile, pattern A with uncut *fimH* was observed in eight isolates (36.36 %) and pattern B with 755- and 253-bp bands was observed in 14

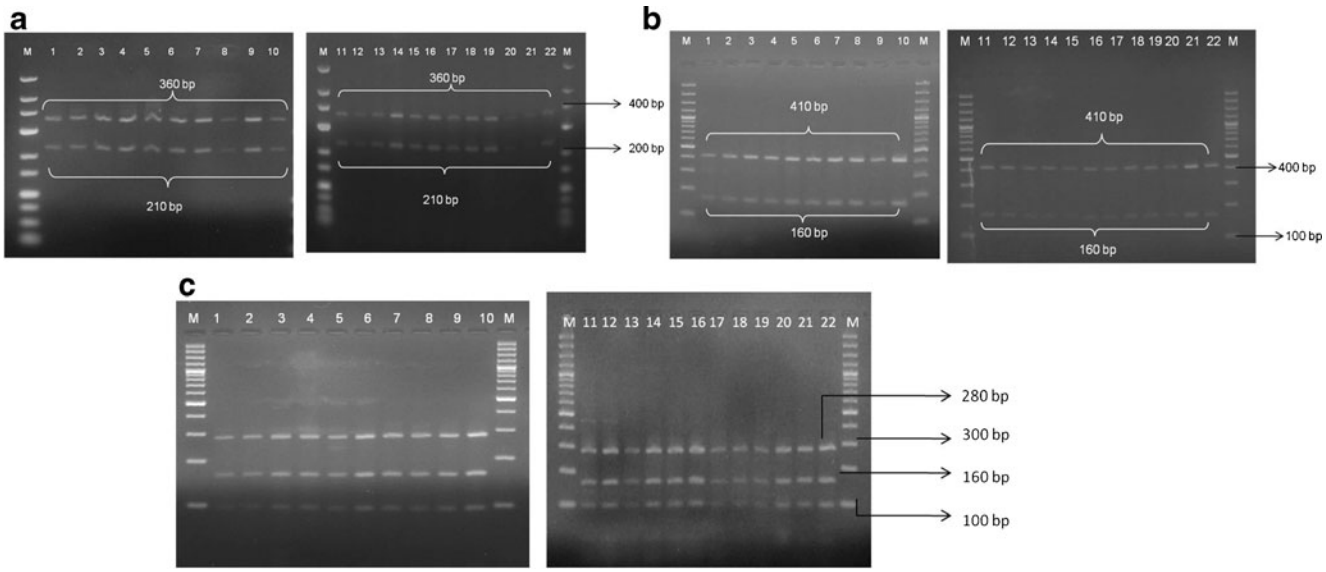

Fig. 5 RFLP patterns of the 16S rRNA fragment. **a**–**c** Restriction enzyme digestion of the 16S rRNA fragment with *Eco*RI (**a**), *Sma*I (**b**) and *Hae*III (**c**). *Lanes*: *M* Molecular weight markers, *1* E2394, *2* E4638, *3* E4809, *4* E2375, *5* E2393, *6* E4885, *7* E2416, *8* E4242, *9* E4938, *10* E4231, *11* E4227, *12* E4841, *13* E4659, *14* E4490, *15* E4658, *16* E2677, *17* E5158, *18* E4935, *19* E4896, *20* E4256, *21* E2622, *22* E4946

isolates (63.63 %). This result shows the potential of *Cla*I to be used in the differentiation of *Salmonella* Typhimurium isolates. No pattern was allotted for a special region or source which is similar to the observation of Dilmaghani et al. (2010) in the *fljB* gene.

Thus, in our study, the results obtained demonstrate that PCR-RFLP has good typeability but low discriminatory power due to its inability to produce a different banding pattern within *Salmonella* Typhimurium isolates. This result is in accordance with the interpretation of Cheah et al. (2008) that the discriminatory power of PCR-RFLP is generally not as good as that of other amplification-based techniques, such as randomly amplified polymorphic DNA, primarily due to the limited region of the genome that can be examined.

In conclusion, PCR-RFLP analysis based on analyses of the *fimH*, *fliC* and 16S rRNA genes in *Salmonella* Typhimurium showed good typeability but low discriminatory power. Simultaneously, our results also illustrate the potential of *Cla*I-based *fimH* analysis for the differentiation of *Salmonella* Typhimurium isolates. Our findings also reveal the highly conserved nature of the *fliC*, *fimH* and 16S rRNA genes among *Salmonella* Typhimurium isolates of different host species and geographical regions, suggesting the importance of these genes in the survival of these organisms under different conditions. These genes may therefore be good candidates to develop vaccines and diagnostic techniques against *Salmonella* Typhimurium and other *Salmonella* spp. which harbor the same conserved regions. These possibilities have to be achieved through further studies on the sequences of the flagellin and fimbrial genes.

Acknowledgements The authors are grateful to the Director and Joint Director (Academic and Research) of the Institute for providing the necessary research facilities to carry out the present investigation. TGS thanks the Indian Council of Agricultural Research (ICAR) for providing financial support in the form of a Junior Research Fellowship.

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