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Molecular typing of *Salmonella* Typhimurium isolates using repetitive sequence-based PCR: Insights into genetic relatedness beyond antibiotic resistance

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ABSTRACT

Multidrug resistant non-typhoidal *Salmonella* infections are a threat to food safety impacting human health in the form of hospitalizations and in severe cases, fatalities. Epidemiological investigations of an outbreak require accurate identification of the infection source and the transmission route for effective implementation of preventive measures against microbial food-borne pathogens. Therefore, the present study investigates the repetitive sequence-based PCR (rep-PCR) fingerprinting techniques for genotyping of multidrug-resistant isolates of *Salmonella* Typhimurium. ERIC, REP, BOX and GTG5-PCR fingerprinting generated reproducible band patterns ranging from 3-10, 1-5, 6-14 and 2-10 separate bands, respectively. Cluster analysis revealed 9 types from ERIC-PCR, 7 types from REP-PCR, 16 types from BOXPCR and 9 types from GTG5-PCR. Hundred percent typeability was obtained with all genotyping techniques except REP-PCR. The isolates' sharing similar band patterns is suggestive of genetic relatedness pointing towards the multifactorial involvement in the transmission of *Salmonella*. Faecal isolates and meat swab isolates sharing identical band patterns and grouped into a single cluster is indicative of a likely cross contamination. No unique pattern was observed in penta resistant (ACSSuT) profile isolates by our typing techniques. Discrimination index (DI) value of BOX-PCR was highest (0.940) followed by GTG5-PCR (0.862), REP-PCR (0.846) and ERIC-PCR (0.843). On comparing the techniques, the BOX-PCR exhibited good DI value, typeability and complex band pattern on gel in differentiating the isolates. Conclusively, the BOX-PCR fingerprinting technique was found useful in the genotyping of isolates thus suitable enough to find its application in an epidemiological investigation during an outbreak.

Keywords: Fingerprinting, Molecular genetics, Repetitive sequencing, Polymerase chain reaction, *Salmonella* Typhimurium

Non-typhoidal *Salmonella* is believed to be one of the world's leading causes of gastroenteritis and bacteremia, causing 93.8 million human cases and 1,55,000 deaths per year (Scallan *et al.* 2011). The non-typhoidal *Salmonella* serovars, responsible for salmonellosis (Bula-Rudas *et al.* 2015) affect both humans and animals and among the serovars, Typhimurium is prevalent in humans and domestic animals around the world (Tophdahl *et al*. 2005).

Epidemiological investigations of outbreaks of food borne illlnessses require accurate identification of the infection source and the transmission route for effective implementation of preventive measures against microbial food-borne pathogens. For this, techniques which can differentiate the isolates at genomic level are applied. Many traditional ways of typing (based on phenotypes), such as biochemical and serological characterizations, are tedious and expensive with limited epidemiological value (Fardsanei *et al.* 2016). However, genotyping methods such as repetitive element-based PCR (Rep) have been extensively used for characterization of bacterial foodborne pathogen. These repetitive elements include the repetitive extragenic palindromic sequence (Stern *et al.* 1984), the enterobacterial repetitive intergenic consensus sequence (Hulton *et al.* 1991), the BOX sequence (Martin *et al*. 1992) and the polytrinucleotides $(GTG)_5$ sequence (Doll *et al.* 1993). These Rep-PCR procedures provide a rapid diagnostic means of molecular fingerprinting bacterial strains, and allow global comparison of the fingerprints; being especially useful in the fields of disease epidemiology and disease control. As not all these procedures find equal applicability, the 45 MDR *S.* Typhimurium isolates belonging to diverse sources (poultry meat, poultry farm and their environment), were subjected to ERIC-, REP-, BOX- and GTG_5-PCR analysis and a comparison of these four techniques was made on the basis of discriminatory index and typability.

MATERIAL AND METHODS

Strains: Multidrug-resistant *Salmonella* Typhimurium isolates (n=45) (Table 1) belonging to various sources

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Table 1. Details of MDR *Salmonella* Typhimurium isolates from Uttarakhand

Isolate Id	Source	Place	Antimicrobial resistant pattern
S-1	Egg surface	Pantnagar	NA CIP CZ E
S-2	Feed	Pantnagar	CTX E TE SF
S-3	Feed	Pantnagar	E TE SF
S-7	Poultry caeca	Pantnagar	NA CIP LE GAT EX E AMP SF
S-8	Poultry caeca	Pantnagar	NA CIP LE GAT EX E AMP SF
S-10	Water	Pantnagar	NA E AMP
S-13	Water	Pantnagar	CIP GAT EX E AMP
S-14	Water	Pantnagar	NA CIP CTX E AMP TE SF
S-16	Litter	Pantnagar	CIP GAT EX E
S-18	Litter	Pantnagar	NA CIP CZ CX CTX E AMP
S-20	Poultry faeces	Pantnagar	NA EX CZ E AMP
S-21	Poultry faeces	Pantnagar	NA CIP CZ CX CTX E AMP
S-22	Poultry faeces	Pantnagar	NA CIP CZ CX E AMP
SH-35	Poultry faeces	Haldwani	E CTX SF TE NA
SH-55	Poultry faeces	Haldwani	E LE CIP CTX GAT SF TE NA
SH-56	Litter	Haldwani	E CTX SF TE CZ NA
D-1*	Poultry faeces	Shantipuri	AMP E LE CIP GAT SF TE CZ NA S C
D-2	Poultry faeces	Kiccha	AMP E LE CIP CTX GAT TE CZ NA S
D-3	Poultry faeces	Kiccha	AMP CX E CIP CTX SF TE CZ NA S
D-4	Poultry faeces	Haldwani	E CIP TE NA
D-5*	Poultry faeces	Haldwani	PAN RESISTANCE
D-6*	Poultry faeces	Kashipur	AMP CX E LE CIP CTX SF TE NA S C
L-1	Litter	Shantipuri	AMP E CIP CTX GAT TE NA
F-1	Feed	Ramnagar	AMP E CIP CTX GAT SF TE CZ NA S
M-1*	Meat swab	Pantnagar	AMP CX E CIP CTX GAT SF TE CZ NA S C
M-2	Meat swab	Pantnagar	AMP CX E CIP CTX GAT TE CZ NA
M-3	Meat swab	Pantnagar	AMP E LE CIP CTX GA SF NA S
M-4	Meat swab	Kiccha	AMP CX E CIP CTX SF TE CZ NA S
M-5	Meat swab	Lalkuan	E LE GAT SF TE NA S
M-6*	Meat swab	Lalkuan	PAN RESISTANCE
M-7	Meat swab	Rudrapur	AMP CX E LE CIP CTX GAT SF TE CZ NA
M-8	Meat swab	Rudrapur	AMP E LE CIP CTX GAT TE CZ NA
M-9	Meat swab	Rudrapur	AMP E LE CTX SF CZ NA C
M-10*	Meat swab	Haldwani	PAN RESISTANCE
M-11	Meat swab	Haldwani	E CIP GAT SF TE NA
M-12	Meat swab	Kashipur	AMP CX E LE CIP CTX GAT TE CZ NA
M-13	Meat swab	Kashipur	AMP E CIP GAT TE CZ NA S
W-1*	Drinking water	Shantipuri	AMP E LE CIP CTX GAT SF TE CZ NA S C
W-2	Drinking water	Haldwani	E SF TE NA
B-1	Chopping board swab	Lalkuan	AMP E CTX GAT SF CZ NA
B-3	Chopping board swab	Haldwani	AMP E CIP CTX GAT SF TE NA S
K-1	Knife swab	Pantnagar	AMP E CZ NA
K-2	Knife swab	Rudrapur	AMP CX E LE CIP CTX GAT SF CZ NA
H-1	Butchers hand swab	Kashipur	AMP E CIP CTX GAT SF CZ NA C
WS-1*	Rinsing water	Pantnagar	AMP E LE CIP CTX SF TE NA S C

*Penta (ACSSuT) resistant profile isolates, Antimicrobials- S: Streptomycin, NA: Nalidixic Acid, CIP: Ciprfloxacin, LE: Levofloxacin, GAT: Gatifloxacin, EX: Enrofloxacin, CZ: Cefazolin, CX: Cefoxitin, CTX: Cefotaxime, E: Erythromycin, AMP: Ampicillin, TE: Tetracycline, SF: Sulfisoxazole, C: Chloramphenicol

(poultry faeces (n= 11), meat swab (n=13), water (n=5), litter (n=4), feed (n=3), poultry caeca (n=2), chopping board swab (n=2), knife swab (n=2), rinsing water (n= 1), butcher hand swab (n=1) and egg surface (n=1)) were selected for the study. These isolates, stored in 20% glycerol stock and kept at -80°C deep freeze, were revived and checked for identity confirmation by cultural, phenotypic and biochemical test as previously described (Sharma *et al.* 2019). All the isolates were previously serotyped at National *Salmonella* Centre, Indian Veterinary Research

Table 2. Primers used for duplex PCR targeting *ompC* and *typh* genes

Gene	Primer specificity	Primer sequence (5'3)	Product size (bp)	Reference
ompC	*Salmonella* genus	F: ATCGCTGACTTATGCAATCG R: CGGGTTGCGTTATAGGTCTG	204	Alvarez *et al.* 2004
typh	*Salmonella* Typhimurium	F: TTGTTCACTTTTTACCCCTGAA R: CCCTGACAGCCGTTAGATATT	401	

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All the isolates were resistant to three or more than three class of antibiotics and therefore were categorized as Multi drug resistant (MDR) (CDC 2016).

Duplex PCR on MDR S. Typhimurium isolates: Duplex PCR was performed for genus and serovar confirmation as previously described (Alvarez *et al.* 2004) (Table 2). The DNA was extracted as per method suggested by Reischl *et al*. (2000). The extracted DNA was kept at −20°C until being further used.

BOX-, ERIC-, (GTG) 5-and REP-PCR Assays: To perform PCR reactions, the reactions were standardized with different concentrations of each primer set (Table 3). ERIC- and REP-PCR was carried out in 25 µL of volume using 2.5 µL of 10× Taq buffer (Tris HCL with 15mM $Mgcl_2$) (GeNei Bangalore, India), 2 µL of 2mM concentration of each dNTPs (GeNei Bangalore, India), 2.5 µL (25 pmol) of each forward and reverses primers, 1 U Taq Polymerase enzyme (GeNei Bangalore, India), 4 µL of DNA sample and nuclease-free water to make the final volume to 25 µL. The PCR condition for BOXA1R and $(GTG)_5$-PCR was carried out in 25 µL of volume using 2.5 µL of 10x Taq buffer (Tris HCL with 15 mM $MgCl_2$) (GeNei Bangalore, India), 2 µL of 2 mM concentration of each dNTPs (GeNei Bangalore, India), 2.5 µL (25 pmol) of primer, 1.25 U Taq polymerase enzyme (GeNei Bangalore, India), 4 µL of DNA sample and nuclease-free water to make the final volume to 25µL.

Thermocycling was performed in a thermal cycler (Gene GeneAMP® PCR System 9700; Applied Biosystems, Foster City, California, USA).

The cycling parameters for ERIC-PCR were initial denaturation at 95°C for 5 min followed by 34 cycles of denaturation at 95°C for 30 sec, primer annealing at 49°C for 1 min, extension at 72°C for 5 min and a final extension at 72°C for 10 min.The REP-PCR cycling conditions were initial denaturation at 95°C for 5 min followed by 30 cycles of denaturation at 90°C for 30 sec, primer annealing at 52°C for 1 min, extension at 72°C for 1 min and a final extension at 72°C for 8 min.

The BOX-PCR cycling parameters were initial denaturation at 95°C for 2 min followed by 30 cycles of denaturation at 94°C for 30 sec, primer annealing at 50°C for 1 min, extension at 72°C for 8 min and a final extension at 72°C for 10 min. The cycling parameters for $(GTG)_5$-PCR were involved initial denaturation at 95°C for 5 min followed by 30 cycles of denaturation at 94°C for 30 sec, primer annealing at 50°C for 1 min, extension at 72°C for 1 min and a final extension at 72°C for 7 min. The Rep-PCR's was repeated to determine the reproducibility of the banding patterns using same isolate strains in different PCR run.

To ensure reproducibility and consistency of the band patterns of the BOX-, ERIC-, (GTG) 5-and REP-PCR assays, each reaction was repeated at least three times.

Gel analysis: Amplified PCR products (10 µL) were mixed with 2 µL of 6X loading dye (GeNei Bangalore, India) and loaded on agarose gel (2%) pre-stained with ethidium bromide (EtBr) (0.5 µg/mL). The samples were run at 70 V for 3-4 h in the 1X TAE buffer (HiMedia, India). The band was analyzed visually under gel documentation system [Doc-It®LS Image Acquisition Software (UVP, UK)].The relative molecular weight of the amplicons was calculated against a low range 100 bp – 3 kb DNA ladder (GeNei Bangalore, India)**.**

Amplicon profile analysis and phylogenetic tree construction: Dendrogram was constructed using unweighted pair group method with arithmetic mean (UPGMA) clustering to determine the relatedness of the MDR Typhimurium isolates (Kumar 2016).

Determination of discrimination index: D value was calculated by online discriminatory power calculator (http://insilico.ehu.es/mini_tools/discriminatory power) (Kumar 2016). D value in a range of 0 (identical type) to 1.0 indicates that the typing method can distinguish among each member of a population (Pooncharoen *et al.* 2019).

Table 3. Primers used in the repetitive sequence-based PCR fingerprinting

Primer name	Primer sequence (5'-3')	Reference
ERIC1R ERIC2	ATGTAAGCTCCTGGGGATTCAC AAGTAAGTGACTGGGGTGAGCG	Nath *et al.* 2010
REP 1 REP 2	IIIGCGCCGICATCAGGC ACGTCTTATCAGGCCTAC	Hashemi and Baghbani-Arani 2015
BOXA1R	CTACGGCAAGGCGACGCTGACG	Hashemi and Baghbani-Arani 2015
$(GTG)_5$	GTGGTGGTGGTGGTG	Fardsanei *et al.* 2016

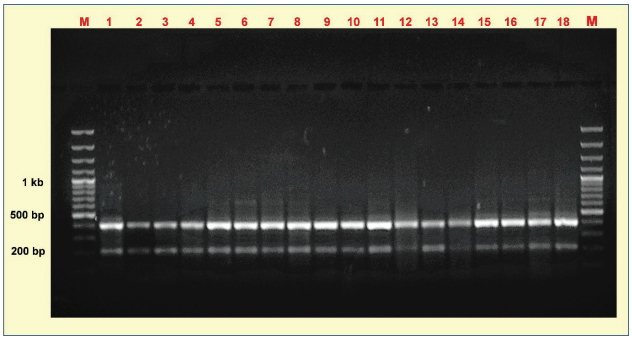

Fig. 1. PCR detection of *Salmonella* genus and *S.* Typhimurium serovar, Lane M: Molecular marker 100 bp Step DNA Ladder, Lane 1-18: Positive *Salmonella* isolates (S1, S2, S3, S7, S8, S20, S21, S22, D3, D4, D6, B1, WS1, M1, M2, M4, M5, M6)

RESULT AND DISCUSSION

Identity confirmation of Salmonella isolates: All the *Salmonella* strains on revival showed typical cultural and morphological characteristics of *Salmonella* genus on Xylose-Lysine-Tergitol 4 (XLT-4) media. On XLT-4 agar (BD Difco, USA) all the isolates produced smooth, black colonies with a red background. On biochemical characterization, all 45 isolates revealed positive TSI test [red (alkaline) slant, yellow (acid) butt along with H_2S production (blackening of agar)] and negative Urease test (no change in colour of slant). Our findings are in agreement with those of Sharma *et al*. (2019) who also performed urease and TSI test with characteristic results for biochemical confirmation.

Salmonella Genus and Serovar Confirmation of the Isolates: On subjection to duplex PCR, all the isolates (n=45) amplified 204 bp and 401 bp fragment for *ompC* and *typh* gene respectively, thus confirming *Salmonella* genus and Typhimurium serovar (Fig. 1).

The genes, *ompC* and *typh* have been previously targeted by many researchers (Ahmed *et al.* 2012, Anjay *et al.* 2015) who also relied on use of PCR as an effective tool for detecting the genus and serovar Typhimurium confirmation.

Molecular typing of the isolates: All 45 *S.*Typhimurium isolates were analyzed by the repetitive sequence element based-PCR techniques. The typability of ERIC-PCR, BOX-PCR and $(GTG)_5$-PCR were 100%, which means all the Typhimurium isolates were differentiated by these techniques. However, the REP-PCR could differentiate only 39 *S.*Typhimurium isolates out of 45 isolates (86.66%).

Comparative typing ability of BOX-, ERIC-, (GTG)5-, and REP-PCR: The different molecular typing methods, namely, BOX-, ERIC-, (GTG)5-, and REP-PCR generated amplicons of different sizes (170-1600bp) with BOX- PCR demonstrating maximum number of amplicons (6-14). Gel electrophoresis of 20 isolates have been shown (Fig 2(a,b,c,d)). The phylogenetic tree profiles showed BOX-PCR to have the highest discriminative index having D> 0.9 followed by the $(GTG)_5$-PCR (0.862) and ERIC _PCR

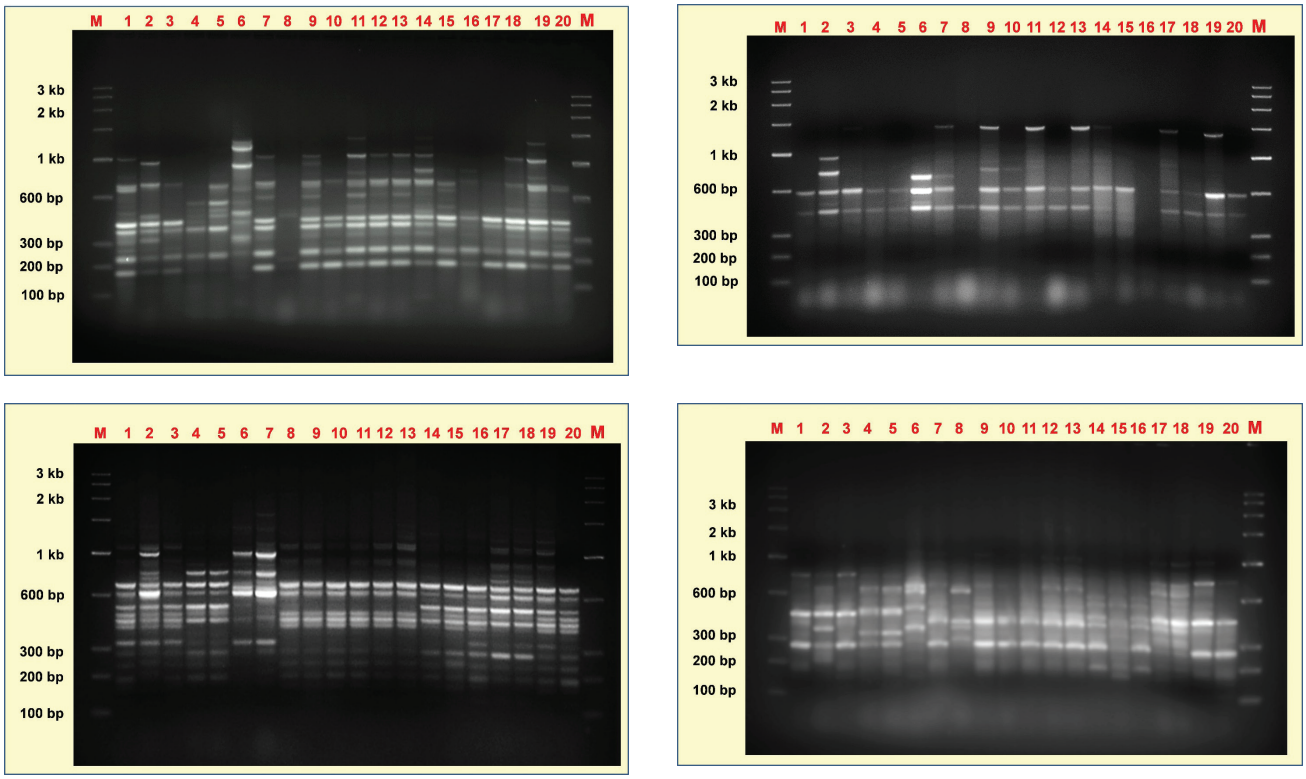

Fig. 2. Gel electrophoresis of profile of *Salmonella* Typhimurium isolates through ERIC-PCR (a), REP-PCR(b), BOX-PCR(c) and GTG_5-PCR(d), Lane M: Molecular Marker 100 bp – 3 kb DNA ladder, Lane 1-20: Isolates S1, S2, S3, S7, S8, S10, S13, S14, S16, S18, S20, S21, S22, SH35, SH55, SH56, W1, W2, K1 and K2 in respective lanes

(0.843)(Table 4).

The BOX-PCR technique was thus proved to be better in establishing an epidemiological investigation and relationship among isolates. Hunter *et al*. (1988) also recommended that a D value> 0.9 is desirable for good differentiation. Pooncharoen *et al*. (2019) constructed a phylogeny tree from BOX-PCR typing effectively differentiating *S*. 4,[5],12:i:- isolates as well as grouping them into different clusters according to their origin. The BOX elements are characterized as being conserved among diverse bacterial species and serve as potential targets for identifying genetic relatedness in Gram-negative bacteria. Another study (Shiroodi *et al.* 2019), proved that BOX AIR-PCR is a useful and effective tool for molecular typing and genetic diversity analysis of different *Salmonella* Enteritidis strains with high discriminative power.

Ability of the four molecular typing methods to differentiate clusters of Salmonella isolates belonging to different sources and location: In our study, all the four techniques differentiated the isolates with a heterogeneity of 40.2% (ERIC PCR), 40.8% (REP PCR), 49% (BOX PCR) and 26.5%$(GTG)_5$-PCR and put them into clusters, sub clusters and clades. Although, none of the repetitive PCR's could group isolates belonging to either same sample(source) or same location into one clade/cluster which suggests genetic relatedness among the samples grouped into a single cluster and warranting further investigations.

Table 4. Discriminative Index (D) and Typability of Techniques

Typing method	Number of bands min-max	Size (bp)	D value	Typability (%)
ERIC-PCR	3 to 10	170 bp - 1300 bp	0.843	100
REP-PCR	1 to 5	400 bp - 1400 bp	0.846	86.66
BOX-PCR	6 to 14	170 bp - 1600 bp	0.940	100
GTG_5-PCR	2 to 10	280 bp - 1000 bp	0.862	100

ERIC PCR grouped the isolates into two main clusters (A and B) and formed 9 clades (A1, A5, A6, A7, B1, B5, B6, B7 and B8) (Fig 3(a) and 3(b)). The major clade (A7) consisted of 12 isolates belonging to different sources [drinking water (n=1), feed (n=2), poultry faeces (n=3), egg surface (n=1), knife swab (n=2), butcher hand swab

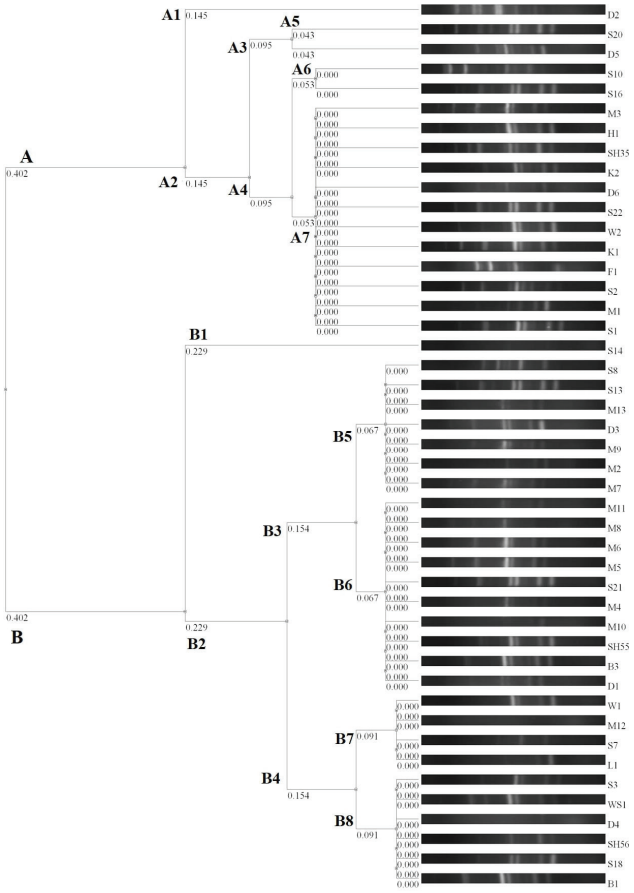

Fig. 3. (a) Dendrogram showing genetic diversity among 45 *S.* Typhimurium isolates from diverse sources as determined by ERIC –PCR) DNA fingerprint analysis

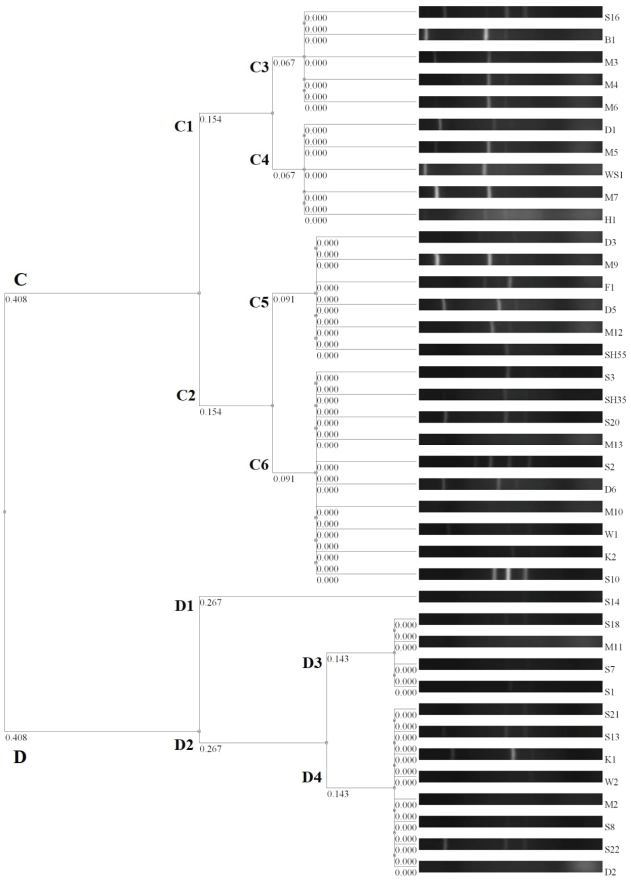

Fig. 3. (b) Dendrogram showing genetic diversity among 45 *S.*Typhimurium isolates from diverse sources as determined by REP-PCR DNA fingerprint analysis

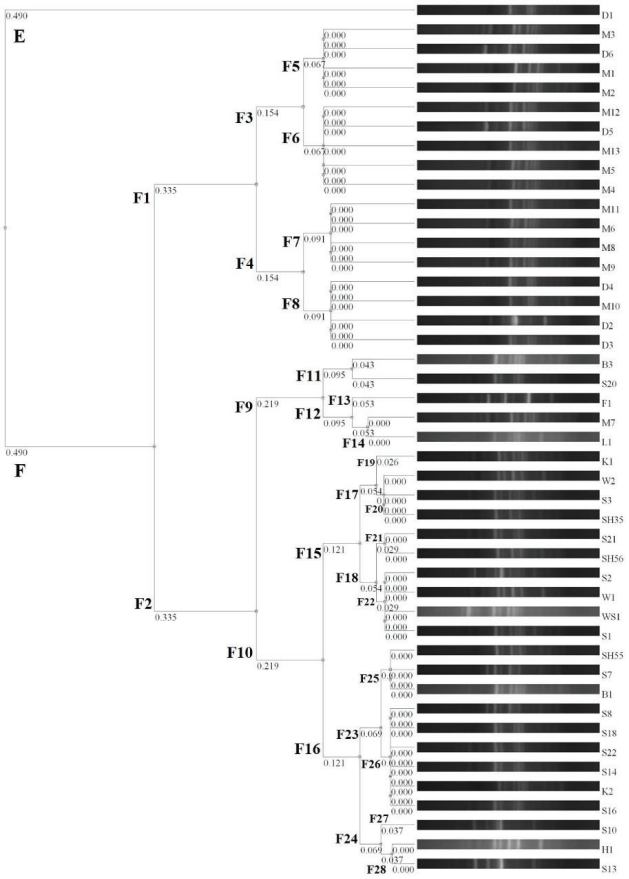

Fig. 3(c). Dendrogram showing genetic diversity among 45 *S.*Typhimurium isolates from diverse sources as determined by BOX –PCR DNA fingerprint analysis

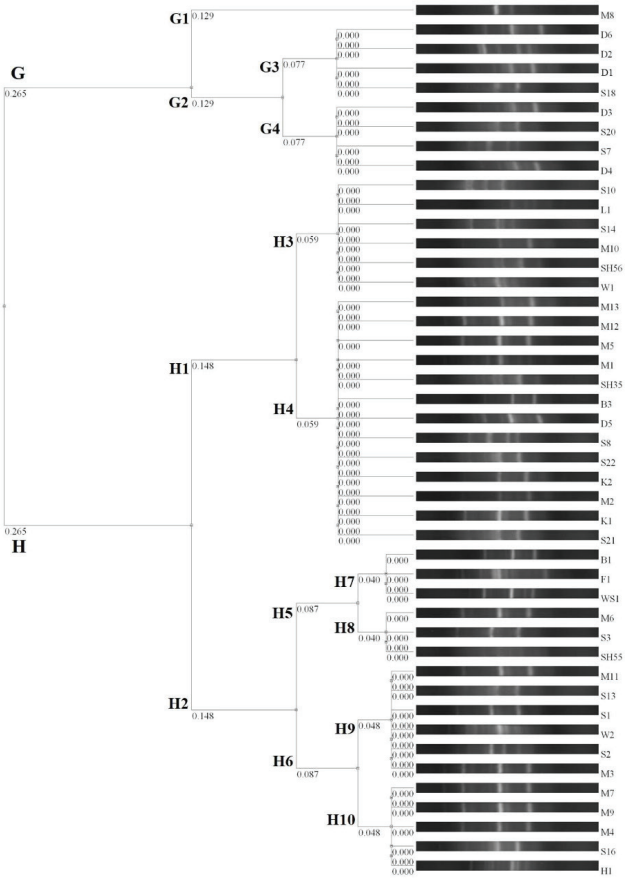

Fig. 3(d). Dendrogram showing genetic diversity among 45 *S.*Typhimurium isolates from diverse sources as determined by $(GTG)_5$-PCR DNA fingerprint analysis

(n=1) and meat swab (n=2)] irrespective of their locations.

The REP-PCR differentiated only 39 *S.*Typhimurium isolates which formed two main clusters (C and D) further divided into sub-clusters and forming 7 different clades (C3, C4, C5, C6, D1, D3 and D4) (Fig 3 b). All the 7 clades (C3, C4, C5, C6, D1, D3 and D4) consisted of isolates of varying sources. The major clade C6 consisted of 10 isolates of various sources [feed (n=2), poultry faeces (n=3), meat swab (n=3), drinking water (n=2) and knife swab (n=1)]. In clad C5 (n=6), poultry faeces (n=3), meat swab (n=2), feed (n=1) sample isolates with different location were grouped. The next major clade (D4) consisted of sample isolates (n=8) involving meat swab (n=1), poultry caeca (n=1), faeces (n=3), water (n=2) and knife swab (n=1) of different location to the retail meat shops. The D1 clade is a water sample isolate collected from Pantnagar.

Similarly, BOX-PCR grouped 45 Typhimurium isolates into two clusters (E & F) and further into 16 genotypes/ clades. The cluster (F) divided into sub-clusters formed 15 different clades (F5, F6, F7, F8, F11, F13, F14, F19, F20, F21, F22, F25, F26, F27 and F28). The cluster E contained a single isolate (D1) (Fig 3(c)). The major clade F26 (6 isolates) consisted of different sources of isolates with same location (Pantnagar). Visual comparison of band patterns and dendrogram also showed that the BOX-PCR differentiated the isolates irrespective of sources. Besides, the dendrogram showed that the different sources of isolates (farm isolates) with same location (Pantnagar) were grouped together in the major clade (F26), which indicates the significant genetic similarity was observed between isolates in that particular location.

$(GTG)_5$-PCR grouped the 45 S. Typhimurium isolates into two main cluster (G and H) forming 9 different clades (G1, G2, G4, H3, H4, H7, H8, H9 and H10) (Fig 3(d)). The clade H4 was the major one with 13 isolates. The dendrogram of $(GTG)_5$-PCR showed that the isolates were grouped irrespective of sources and location.

Pooncharoen *et al*. (2019) also found genetic relatedness among *Salmonella enteric* serotype 4,[5],12:i:- isolates from human and animal food samples. They also suggested that BOX-PCR provides a suitable molecular typing method for discriminating genetic relatedness among *Salmonella* spp. of the same and different serotypes and should be suitable for application in typing and tracking route of transmission in *Salmonella* outbreaks.

The methods under comparison rely on the distribution and copy number of specific repetitive sequences, which may not be uniformly present across all the *Salmonella* strains under study, which supports the difference in the ability of the four molecular typing methods to differentiate

clusters.

Ability of the four molecular typing methods to differentiate clusters of ACSSuT Salmonella isolates: ERIC PCR grouped the penta (ACSSuT) resistant types (n=8) in both the clusters A and B suggesting continuous surveillance of multidrug-resistant strains circulating in the area and taking adequate control measures to prevent the spread. REP –PCR, on the other hand, grouped 7 ACSSuT resistant types into the cluster C. However, it could not type one penta-resisistant type isolate (M1).

With BOX- PCR, the ACSSuT types were scattered among different clades (F4, F6, F7, F8 and F22) in cluster F. The cluster E having a single isolate was also ACSSuT type. While, $(GTG)_5$-PCR distributed all the ACSSuT types (n=8) in different clades indicating that the dendrogram cannot effectively group the isolates in single clade/ type based on ACSSuT resistant pattern.

Hence, none of the techniques could group ACSSuT isolates into a single clade/cluster. In an attempt to classify fecal streptococci isolates into groups (humans, dairy cattle, beef cattle, chickens, deer, and waterfowl) using antibiotic resistance patterns, Dombek *et al.* (2000) also found overlapping between the human and nonhuman (chicken) clusters.

Relatedness of Phylogenetic Tree with Antibiotic Resistant Pattern: In the present study, all the 45 *S.* Typhimurium isolates were multi-drug resistant with different antibiotic-resistant patterns. The molecular typing techniques grouped the isolates irrespective of AMR patterns. These typing methods did not identify particular/ similar patterns. This indicated antimicrobial resistant patterns did not relate to the typing techniques phylogenetic tree. The similar study was also conducted by Prasertsee *et al*. (2016) and they also observed resistant type of antimicrobial susceptibility testing and antimicrobial resistance genes could not identify specific patterns and cannot be used for classifying the source of *Salmonella.*

Our results indicate BOX PCR to be a promising tool for differentiation of the isolates. However, combining BOX PCR and $(GTG)_5$ PCR can also be studied for better results. The techniques could not identify ACSSuT types suggesting their inefficiency to discriminate the isolates based on resistance pattern. In the study, clonal relatedness was also observed among the isolates. The REP-PCR techniques require small amount of DNA and are thus useful in disease epidemiology and disease control.

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