JOURNAL OF PURE AND APPLIED MICROBIOLOGY, Sept. 2015. Vol. **9**(3), p. 2627-2634

Molecular Typing of *Salmonella* Typhimurium and *S.* Enteritidis Serovars from Diverse Origin by ERIC-PCR

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(Received: 15 April 2015; accepted: 03 June 2015)

Food borne infections due to *Salmonella* spp. are of high concern around the globe. The present study was undertaken with the aim of genotyping of *Salmonella* Typhimurium and *S.* Enteritidis from different sources by enterobacterial repetitive intergenic consensus- polymerase chain reaction (ERIC-PCR). A total of 69 strains comprising *S.* Typhimurium (49) and *S.* Enteritidis (20) obtained from the National Salmonella Centre, Indian Veterinary Research Institute, Izatnagar and Department of Veterinary Public Health, Bihar Veterinary College, Patna were subjected to molecular typing by ERIC-PCR. The isolates were revived and confirmed by PCR targeting *inv*A, *typh* and *ent* genes along with biochemical characterization. Based on the results of ERIC-PCR, *S.* Typhimurium isolates were categorized into 6 clusters (C1 to C6) with a discriminatory power (D) of 0.7764, while *S.* Enteritidis into 4 clusters (B1-B4) with a discriminatory power of 0.7368. Dendrogram analysis of *S.* Typhimurium and *S.* Enteritidis revealed 60% and 71% similarity, respectively between isolates. The isolates under study from different sources like animals, vegetables, humans, sewage, water and wild animal sources showed similar band patterns. The study revealed that *Salmonella* serovars share common source contamination and confirmed the zoonotic nature of this important pathogen with global significance.

Key words: *Salmonella* Typhimurium, *S.* Enteritidis, ERIC-PCR, dendrogram, discriminatory power.

Food borne diseases are the foremost public health problem worldwide with an estimated 6 million illnesses and approximately 9000 deaths each year[1]. Among several food borne bacterial pathogens *Salmonella* species has long been recognized as an important food borne pathogen and a major public health burden worldwide[2]. Although, as many as 2610 serovars of *S. enterica* have been identified[3], the most common serotypes attributed to food borne outbreaks are *S.* Typhimurium and *S.* Enteritidis, which account for more than 75% of reported cases[4]. Consumption of contaminated poultry, beef and egg products is believed to be responsible for 75% of the annual cases of human salmonellosis[5]. Due to its endemic nature, high morbidity and association with a wide range of foods, salmonellosis is considered as a zoonotic disease of high public health concern[6,7].

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Thus, a rapid typing method to differentiate *Salmonella* spp. is needed for epidemiological surveillance, monitoring and identification of food-borne outbreaks sources. Several genotyping methods have been employed to find out the epidemiological relationship between various isolates. Recent molecular techniques available for studying genetic diversities of bacterial pathogens include pulsed-field gel electrophoresis (PFGE), random amplification of polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), repetitive extragenic palindromic- PCR (REP-PCR), enterobacterial repetitive intergenic consensus polymerase chain reaction (ERIC-PCR) and multilocus sequence typing (MLST)[8,9,10,11]. ERIC sequences have been used for determining the genetic diversity among different pathogenic bacteria in the *Enterobacteriaceae* family including *Salmonella*[12,13]. This technique relies on the amplification of genomic DNA fragment using single primer pair, which is complimentary to the short repetitive sequences, and generation of reproducible and complex fingerprints[13]. In the present study, *S.* Typhimurium and *S.* Enteritidis serovars isolated at different time period from diverse sources and wide geographic area have been subjected to molecular typing by employing ERIC-PCR with the objective of determining their genetic diversity.

MATERIALS AND METHODS

Bacterial strains and revival

A total of 69 *Salmonella* isolates including *S.* Typhimurium (49) and *S.* Enteritidis (20) were obtained from the National Salmonella Centre, Indian Veterinary Research Institute, Izatnagar, Bareilly, U.P., India and Department of VPH, Bihar Veterinary College, Patna, Bihar, India (Table 1). All the isolates were revived in BHI broth (Himedia, India) by overnight incubation at 37°C followed by selective plating on Hektoen Enteric Agar (HEA, Difco, USA).

Molecular confirmation

A typical black colored colony surrounded by narrow green margin on HEA plate was selected for molecular confirmation for *Salmonella* spp. The DNA was extracted using blood and tissue kit (Qiagen, Germany) and screened by PCR standardized for determination of *inv*A (genus specific), *typh* (*S.* Typhimurium), *ent* (*S.* Enteritidis) genes. The three sets of primer used were as follows: *inv*A (forward (F): GTG AAA TTA TCG CCA CGT TCG GGCAA; reverse (R): TCA TCG CAC CGT CAA AGG AACC)[14]; *typh* (F: TTG TTC ACT TTT TAC CCC TGAA; R: CCC TGA CAG CCG TTA GAT ATT)[15] and *ent* gene (F: TGT GTT TTA TCT GAT GCA AGA GG; R: TGA ACT ACG TTC GTT CTT CTG G)[15]. The PCR reaction mixture for amplification of all targeted genes consisted of 12.5 µl of 2x PCR master mixtures (Thermo Scientific), 1 µl (10 pmol/µl) of each primer (Eurofins, India), 2 µl of DNA template and nucleus free water to make final volume up to 25 µl.

The amplification conditions for *inv*A gene consisted of 30 cycles with denaturation at 94°C for 60 s, annealing at 51°C for 60 s and extension at 72°C for 60 s; whereas, *typh* gene was amplified with 34 cycles with denaturation at 94°C for 60 s, annealing at 55°C for 90 s, and extension at 72°C for 90 s. The cyclic conditions for *ent* gene consisted of 34 cycles with denaturation at 94°C for 60 s, annealing at 58°C for 60 s and extension at 72°C for 60 s, with an initial denaturation for 5 min at 94°C and final extension for 7 min at 72°C for each cyclic conditions. The PCR products were resolved by agarose gel electrophoresis (1.2%) stained with 0.5% ethidium bromide and documented under gel documentation system (Alphaimager™ 2200).

ERIC-PCR

ERIC-PCR was standardized with published primers (ERIC1R: ATG AAG CTC CTG GGG ATT CAC) and ERIC2: AAG TAA GTG ACT GGG GTG AGC G[16]. The PCR reaction was optimized with 2.5µl of 10x *Taq* buffer, 2.5µl of $MgCl_2$ (25mM), 3 µl dNTP (2 mM), 30 pmol of each primer, 1U of *Taq* DNA polymerase, 3 µl of template DNA and nuclease-free water up to a final volume of 25 µl. The amplification cycles included initial denaturation at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 60 s, annealing at 40°C for 90 s and extension at 72°C for 60 s with a single cycle of final extension at 72°C for 7 min. The PCR products were electrophoresed in 1.5% agarose gel at 80 V for 95 min. The similarity banding pattern of different isolates was analyzed with Gel Doc IT imaging System using Image ILT Analysis Software

(UVP, U.K.) and clustering was studied employing the unweighted pair group method with arithmetic mean (UPGMA). D value was calculated with help of online available tool of discriminatory power calculator (http://insilico.ehu.es/mini_tools/discriminatory_ power/).

RESULTS AND DISCUSSION

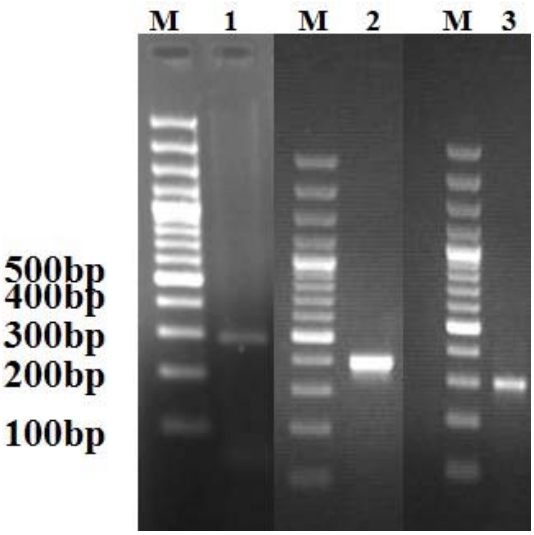
The PCR techniques have increasingly being used for rapid detection and confirmation of *Salmonella*[17]. All the 69 isolates of *Salmonella* revealed the presence of *inv*A gene and gave an

Table 1. List of isolates and their source of isolation

S. NO.	Isolates I.D.	Serotypes	Source
1.	E2385	*S.* Typhimurium	Broiler, Pune, 1992
2.	E2597	*S.* Typhimurium	Cheeta, Delhi, 1995
3.	E3715	*S.* Typhimurium	Calf, Mathura, 2004
4.	E3730	*S.* Typhimurium	Calf, Mathura, 2004
5.	E3739	*S.* Typhimurium	Calf, Mathura, 2004
6.	E3757	*S.* Typhimurium	Poultry, Pantnagar, 2004
7.	E3773	*S.* Typhimurium	Sewage, Bareilly, 2004
8.	E4043	*S.* Typhimurium	Human, Bareilly, 2005
9.	E4055	*S.* Typhimurium	Unknown, Ludhiana, 2005
10.	E4062	*S.* Typhimurium	Unknown, Bareilly 2005
11.	E4226	*S.* Typhimurium	Poultry, CARI, Izatnagar,2005
12.	E4230	*S.* Typhimurium	Poultry, CARI, Izatnagar, 2005
13.	E4231	*S.* Typhimurium	Poultry, CARI, Izatnagar, 2005
14.	E4233	*S.* Typhimurium	Fish, Mathura, 2005
15.	E4255	*S.* Typhimurium	Chicken Pizza, Izatnagar, 2005
16.	E4257	*S.* Typhimurium	Chicken Pizza, Izatnagar, 2005
17.	E4490	*S.* Typhimurium	Ganga water, Pantnagar, 2006
18.	E4659	*S.* Typhimurium	Water, CARI, 2007
19.	E4676	*S.* Typhimurium	Faeces, Srinagar, 2007
20.	E4811	*S.* Typhimurium	Egg, Pantnagar, 2008
21.	E4842	*S.* Typhimurium	Egg, Pantnagar, 2008
22.	E4936	*S.* Typhimurium	Cattle, Izatnagar, 2008
23.	E4938	*S.* Typhimurium	Human stool, Izatnagar, 2008
24.	E4946	*S.* Typhimurium	Calf diarrhoea, Izatnagar, 2008
25.	E4966	*S.* Typhimurium	Egg, Izatnagar, 2008
26.	E5137	*S.* Typhimurium	Unknown, Pantnagar, 2009
27.	E5587	*S.* Typhimurium	Chicken meat, Pantnagar,2014
28.	E5588	*S.* Typhimurium	Chicken meat, Pantnagar,2014
29.	E5591	*S.* Typhimurium	Chicken meat, Pantnagar,2014
30.	CS1	*S.* Typhimurium	Chicken meat, Patna, 2011-2013
31.	CS2	*S.* Typhimurium	Chicken meat, Patna, 2011-2013
32.	CS6	*S.* Typhimurium	Chicken meat, Patna, 2011-2013
33.	CS7	*S.* Typhimurium	Chicken meat, Patna, 2011-2013
34.	CS8	*S.* Typhimurium	Chicken meat, Patna, 2011-2013
35.	CS10	*S.* Typhimurium	Chicken meat, Patna, 2011-2013
36.	CS11	*S.* Typhimurium	Chicken meat, Patna, 2011-2013
37.	CS12	*S.* Typhimurium	Chicken meat, Patna, 2011-2013
38.	CS15	*S.* Typhimurium	Chicken meat, Patna, 2011-2013
39.	CS16	*S.* Typhimurium	Chicken meat, Patna, 2011-2013
40.	CS17	*S.* Typhimurium	Chicken meat, Patna, 2011-2013
41.	CS21	*S.* Typhimurium	Chicken meat, Patna, 2011-2013
42.	CS27	*S.* Typhimurium	Chicken meat, Patna, 2011-2013
43.	CS28	*S.* Typhimurium	Chicken meat, Patna, 2011-2013
44.	CS29	*S.* Typhimurium	Chicken meat, Patna, 2011-2013

45.	CS30	*S.* Typhimurium	Chicken meat, Patna, 2011-2013
46.	CS45	*S.* Typhimurium	Chicken meat, Patna, 2011-2013
47.	CS51	*S.* Typhimurium	Chicken meat, Patna, 2011-2013
48.	CS66	*S.* Typhimurium	Chicken meat, Patna, 2011-2013
49.	CS67	*S.* Typhimurium	Chicken meat, Patna, 2011-2013
50.	E 74	*S.* Enteritidis	Reference strain
51.	E2440	*S.* Enteritidis	Unknown, Banglore, 1993
52.	E2473	*S.* Enteritidis	Unknown, Banglore, 1993
53.	E2475	*S.* Enteritidis	Unknown, Banglore, 1993
54.	E 2478	*S.* Enteritidis	Calf, Pune, 1993
55.	E 2513	*S.* Enteritidis	Poultry heart blood, Bangalore, 1994
56.	E2575	*S.* Enteritidis	Piglet feces, Bangalore, 1994
57.	E2681	*S.* Enteritidis	Broiler liver, Pune, 1996
58.	E2684	*S.* Enteritidis	Broiler liver, Pune, 1996
59.	E2688	*S.* Enteritidis	Broiler liver, Pune, 1996
60.	E2691	*S.* Enteritidis	Guinea fowl liver, Izatnagar, 1996
61.	E2699	*S.* Enteritidis	Poultry liver, Pune, 1996
62.	E2755	*S.* Enteritidis	Poultry liver, Pune, 1996
63.	E5213	*S.* Enteritidis	Turnip, Izatnagar, 2010
64.	E5214	*S.* Enteritidis	Turnip, Izatnagar, 2010
65.	E5224	*S.* Enteritidis	Carrot, Izatnagar, 2010
66.	E 5229	*S.* Enteritidis	Carrot, Izatnagar, 2010
67.	E5232	*S.* Enteritidis	Carrot, Izatnagar, 2010
68.	E5233	*S.* Enteritidis	Carrot, Izatnagar, 2010
69.	E5236	*S.* Enteritidis	Carrot, Izatnagar, 2010

amplicon of 284 bp (Fig. 1). Amplification of *inv*A gene of *Salmonella* has been reported as an international standard for detection of genus *Salmonella* with potential diagnostic applications[18]. All 49 *S.* Typhimurium isolates used in this study amplified *typh* gene giving an amplicon of 401 bp (fig. 1). The 20 *S.* Enteritidis strains produced an amplified product of 304 bp for *ent* gene (Fig. 1). The successful employment of PCR based technique for detection of *S.* Typhimurium and *S.* Enteritidis serotypes, is an encouraging development, which may be applied to other serotypes[19].



M: 100 bp plus DNA ladder
Lane1: 284 bp of *inv*A gene of *Salmonella*
Lane2: 401bp of *typh* gene of *S.* Typhimurium
Lane3: 304 bp of *ent* gene of *S.* Enteritidis

Fig. 1. Agarose gel electrophoresis of PCR amplified products of *inv* A, *typh* and *ent* gene of *Salmonella*

ERIC-PCR is reported to be used for serovar-based typing of *Salmonella*[20]. In the present study, ERIC-PCR of *S.* Typhimurium strains yielded different patterns consisting of 4–6 band by which the strains were grouped into 6 clusters (C1 to C6) (Fig. 2) with a discriminatory power (D) of 0.7764. The *S.* Enteritidis serovars were grouped into 4 clusters (B1-B4) with a discriminatory power of 0.7368 (Fig. 3). Saxena *et al.*[21] studied strain differentiation of Indian isolates of *Salmonella* by ERIC-PCR and reported a discriminatory power of 0.856. Ammari *et al.*[22] reported that ERIC-PCR had a higher discriminatory power than PFGE profiling of *S.* Enteritidis isolated from human and foods. A study on *S.* Enteritidis isolates in Poland comparing ITS Profiling, REP- and ERIC-PCR concluded that ERIC-PCR has a high discriminatory power[23]. All

the isolates used in the present study were typeable and the technique showed a significant discriminatory power. The maximum similarities between isolates were 60%, which is in accordance with the findings of Chmielewski *et al.*[23].

The *S.* Typhimurium serovars E4811 and E4946 isolated from egg and calf diarrheal sample were clustered in the same group (C1) showing a dissimilarity of 6.7%. Similarly, isolates no. CS21, E3730, E2597, CS12, E4936, CS2 and CS51 isolated from chicken meat, calf diarrhea, cheetah and cattle were clustered together (C2); and isolates no. CS7, E5587, CS2, CS17, E4966, CS11, CS6, CS15, CS1, E4255, E4676, CS8, E3757 and CS29 from chicken meat, feces, egg and chicken pizza were clustered in the same group (C3) with a dissimilarity of 9.1%.

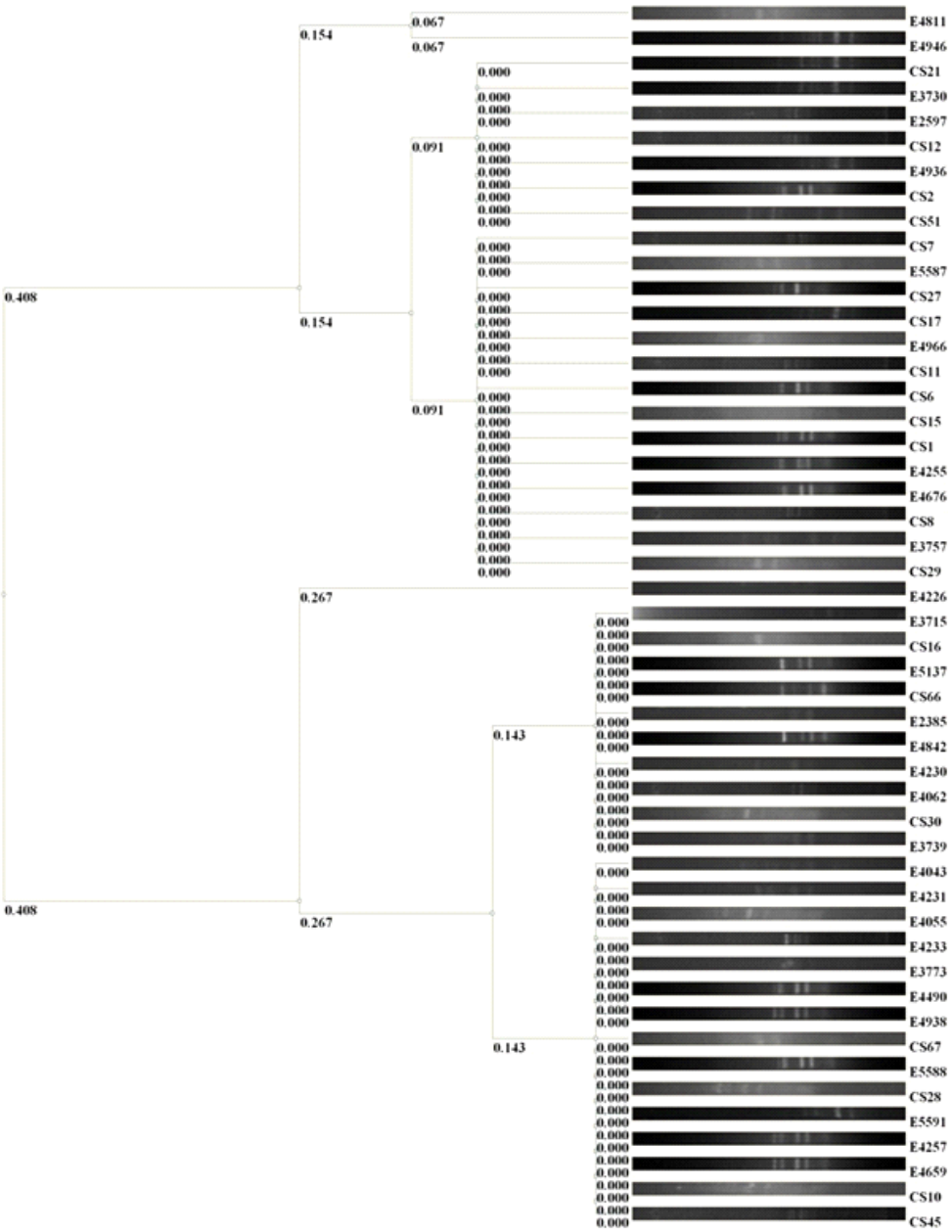


Fig. 2. Dendrogram showing genetic relationships among *Salmonella* Typhimurium serovars based on ERIC-PCR. The similarity was calculated from the Dice coefficient by the UPGMA method

The dendrogram analysis revealed 15.4% dissimilarity between C1, C2 and C3 clusters. The analysis of *Salmonella* isolates from meat fed to cheetah and those from feces of the same cheetah has earlier been reported to exhibit the similarity by RAPD fingerprinting [24].

Dendrogram profile revealed that E4226 isolate of poultry, Central Avian Research Institute, Bareilly was separately clustered (C4). E3715, CS16, E5137, CS66, E2385, E4842, E4230, E4062, CS30 and E3739 isolates from poultry, calf, egg and chicken meat were in a single cluster (C5); and E4043, E4231, E4055, E4233, E3773, E4490, E4938, CS67, E5588, CS28, E5591, E4257, E4659, CS10 and CS45 isolates from poultry, sewage, water, human stool, fish and meat products were clustered in the same group (C6). The dendrogram revealed 14.6% dissimilarity between clusters C5 and C6, and 26.7% dissimilarity between C4 and C5, C6. The two main clusters: C1, C2, C3 and C4, C5, C6 revealed a dissimilarity of 40.8%. Studies have shown that wild animals can share the same *Salmonella* serotypes and

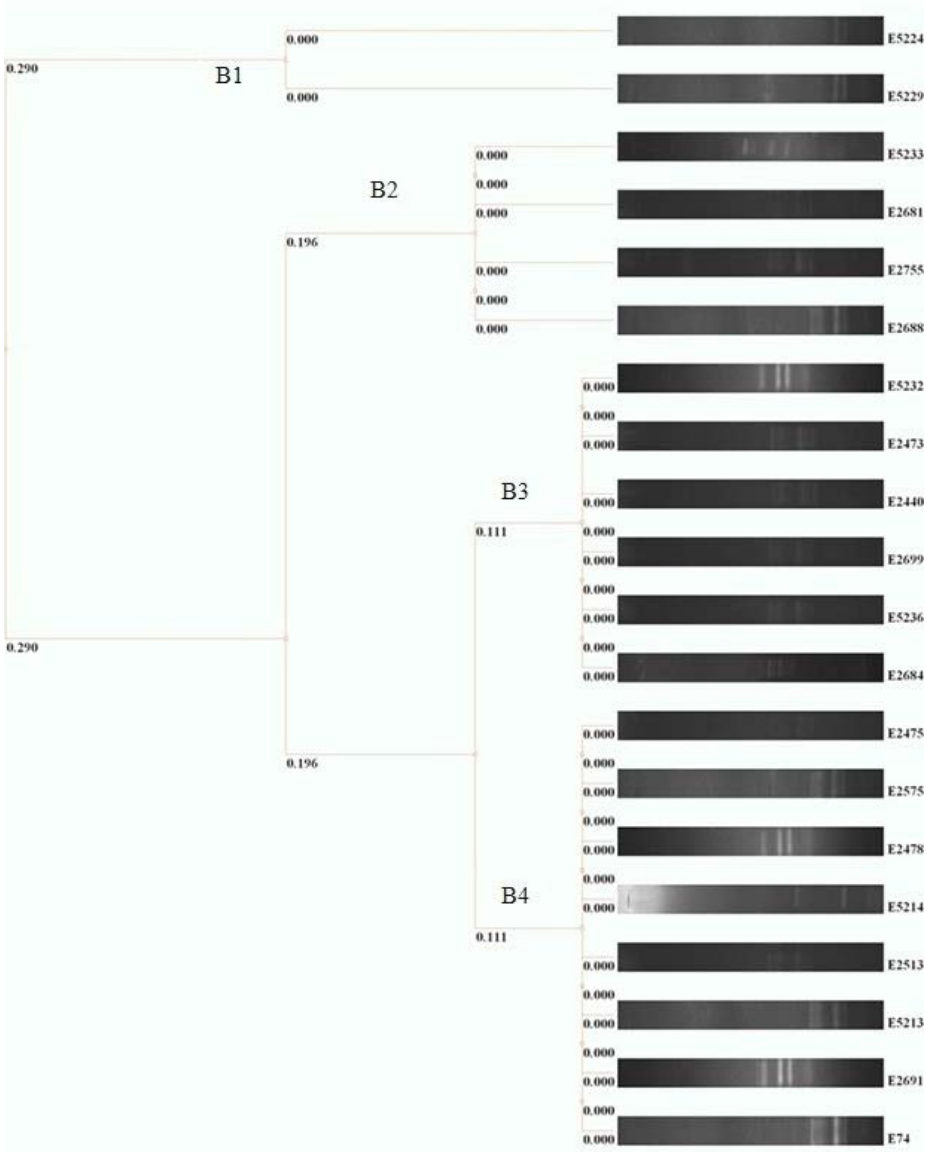


Fig. 3. Dendrogram showing genetic relationships among *Salmonella* Enteritidis serovars based on ERIC-PCR. The similarity was calculated from the Dice coefficient by the UPGMA method

potentially transmit them to humans. Similar types of *Salmonella* serovars have been isolated from wild animals and humans who live in close locality in African countries[25]. Nath *et al.*[26] reported 71 ERIC patterns from 89 *S.* Typhi strains isolated over a period of two decades (1987-2006) with D of 0.9821 and 100% reproducibility.

The *S.* Enteritidis strains E5224 and E5299 isolated from carrot were clustered (B1) together, while strains E5233, E2681, E2755 and E2688 isolated from carrot and poultry clustered (B2) in a different group. The dendrogram analysis revealed that strains E5232, E2473, E2440, E2699, E5236 and E2684 isolated from carrot, broiler liver and poultry were clustered (B3) in a single group. The strains E2475, E2575, E2478, E5214, E2513, E5213, E2691 and E74 isolated from poultry, turnip, calf, guinea fowl and piglet were clustered together (B4) with a dissimilarity of 11%. The dendrogram clearly shows a dissimilarity of 29% among the two main clusters:B1 and B2, B3, B4. Further, the dissimilarity between the cluster groups B2 and B3, B4 was 19.6%.

A previous study reported six ERIC types of *S.* Enteritidis isolated from foods and patients from northern Morocco[22]. The typing of 111 *S.* Enteritidis from southern Brazil revealed 3 ERIC types[27]. *Salmonella* strains from human stool, bone marrow, synovial fluid, blood, ascites and urine samples were differentiated by ERIC, RAPD, REP and BOX repeat-based (BOXAIR) PCR methods in a study in Iran during the years 2012-2013. The study reported 48 ERIC types with a D of 0.983 and recommended the use of a combination of ERIC and RAPD fingerprinting as the best method for differentiating *Salmonella* strains[28].

CONCLUSION

Various clusters generated in the ERIC-PCR dendrogram showed a similarity of isolates from diverse origin. The isolates from meat fed and from feces of wild animals were clustered in common groups inferring the probable source of infection to the wild animal by consumption of contaminated meat. The similar genotypes among human isolates and isolates from foods of animal origin, water, vegetables and sewage indicated the transmission of this important zoonotic pathogen through food and between human and animals.

ACKNOWLEDGMENTS

The authors are thankful to the Director, Indian Veterinary Research Institute, Izatnagar, U.P., for providing the facilities under the project fund entitled “Outreach Programme on Zoonotic Diseases” for this a part of PhD thesis work.

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