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Genotyping of *Salmonella enterica* subsp. *enterica* serovar Entritidis, isolated from poultry, cattle and human in Iran by ERIC-PCR

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Abstract

Salmonellosis is a serious disease in human and animals. One of the most important serovars (among more than 2610 known serovars of *Salmonella*) is *Salmonella enterica* serovar Entritidis which is a common foodborne pathogen worldwide. Various molecular subtyping techniques have been applied for identification of *Salmonella*. Enterobacterial repetitive intergenic consensus (ERIC) PCR techniques [collections of ERIC1R–ERIC2 primers] were examined for the discrimination of *Salmonella enterica* seovar Entritidis isolates at the serotype level. Sixty four *S*.Entritidis isolates from both human and (non-human) sources (cattle and poultry) in Iran, which previously were described by common culture, biochemical and serological procedures, were chosen. Enterobacterial repetitive intergenic consensus (ERIC) were selected for rep-PCR to generate DNA fingerprints for the *S*.Entritidis isolates. These banding patterns provided DNA fingerprints of different isolates. A dendogram was made by using NTSYS software, Version 2.0 and represented that most human isolates were separately clustered from the non-human isolates. This experiment also revealed that PCR fingerprinting with the ERIC primers is suitable for typing of the *S*.Entritidis isolates from different origin and for epidemiological trace back and taxonomy.

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Introduction

Nontyphoidal salmonellosis is an important infection in human and animals (Malorny *et al.*, 2003;Brooks *et al.*, 2013;CDC, 2009). Nontyphoidal *Salmonella* (NTS) are inhabitant in the gastrointestinal lumen of a broad range of vertebrates (Fauci *et al.*, 2008; Gyles *et al.*, 2004). Over 2610 different serotypes (serovars) have been identified to date (Guibourdenche *et al.*, 2010) by Kaufmann–White-Le minor scheme. Throughout the world *Salmonella* Entritidis and *Salmonella* Typhimurium are the two most common serotypes that cause salmonellosis in human and animals and are often transmitted by infected animals and their products to humans (Andrews-Polymenis *et al.*, 2009; Smith *et al.*, 2011; Weigel *et al.*, 2004). According to reports about 1.3 billion cases of nontyphoidal salmonellosis occur annually worldwide (Porwollik *et al.*, 2002). The common route of transmition to humans is ingestion of contaminated poultry food products (e.g., eggs, meat) (Olson *et al.*, 2007; Calnek *et al.*, 1997).

Phenotypic methods such as biotyping, phage typing, serotyping, and antibiotic resistance patterns lack the ability to differentiate isolates and strains of the same serotype and these tests may be time consuming. In most countries serotyping is restricted to national reference laboratories and is expensive. In addition some of the human and animal isolates are not identified by serologic method (Rasschaert *et al.*, 2005). Therfore to investigate the source and relatedness among different strains more prcise tools than serotyping are needed. Enterobacterial repetitive intergenic consensus (ERIC) sequences are 124-127 bp imperfect palindromes that occur in multiple copies in the genomes of enteric bacteria and vibrios. ERIC sequences were first described in *Escherichia coli*, *Salmonella* Typhimurium and other members of the Enterobacteriaceae, as well as *Vibrio cholera* (Sharples and Lloyd, 1990). The widespread distribution and highly conserved nature of these repetitive elements in various microorganisms can be used for analysis of prokaryotic genomes (Ishii and Sadowsky, 2009).

The objective of this study was to use enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) for the analysis of genetic diversity among *S.*Entritidis strains isolated from human and cattle and poultry.

Materials and methods

Bacterial isolates, preparation and quantitation of genomic DNA

Briefly all the isolates of *S.*Entritidis in this research, originated from studies performed previously (Amini *et al.*, 2010). Isolates were selected from microbiology laboratory's freezer of the Islamic Azad University, Science and Research branch, Shahriyar, Tehran, Iran. The isolates had been identified previously as *Salmonella* according to standard methods of culture and serologic method with *Salmonella* Polyvalent Somatic (O) antiserum and *Salmonella* Polyvalent Flagellar (H) antiserum method (Kauffmann-White scheme) and multiplex- PCR. 64 isolates of *Salmonella enterica serovar* Entritidis selected and were diagnosed from different geographic areas of Iran. 64 isolates of *S.*Entritidis including 32 human isolates (m1 to m32) and 19 human isolates (H1 to H19) isolates and 13 cattle isolates (B1 to B13) that subjected to ERIC-PCR analysis after genomic DNA extraction.

First frozen cultures were removed from the freezer bank and placed at room temperature. Bacterial cultures were inoculated to 2mL of Brain Heart infusion (BHI) broth at 37°C for 24 h. After a review of turbidity, isolates were inoculated to LB agar (Luria-Bertani-agar) and were incubated at 37° C for 18-24 hours.

DNA extraction

The genomic DNA from each *Salmonella* isolate was extracted with the Fermentas genomic DNA purification Kit (Thermo Fisher Scientific - USA) according to the manufacturer's protocol. Component of kit comprised of lysis solution, precipitation solution (10X), NaCl solution (1.2M sodium chloride). The purity and amount of the genomic DNA in solution was checked and quantified by spectrophotometry at excitation and emission

wavelengths of 365 nm and 460 nm respectively using the DNA-specific dye. Strains were stored at -50 °C.

Table1. Primers used for ERIC-PCR amplification (Albufera *et al.*, 2009; Versalovic *et al.*, 1991).

ERIC-PCR assay

For ERIC-PCR, the (22-mer) primers ERIC-1R and ERIC 2 were used (Table 1). The PCR reaction was performed in Lyophilized PCR master mix (AccuPower PCR PreMix, 0.2 ml, 96tubes, with attached cap, 20 µl reaction, Bioneer Corporation Korea). A convenient lyophilized PCR master mix tube contains 1U Top DNA polymerase, 250 µM of dNTP, 10 mM Tris-HCl (pH 9.0), 30 mM KCl, 1.5 mM MgCl2, Stabilizer and tracking dye .The PCR reaction was performed in 20 µl solution . 30 ng of template DNA and primers (25 pmol of each of the two primers for ERIC) added to AccuPower® PCR tube. Then distilled water added to tubes to a total volume of 20 µl. The lyophilized blue pellet dissolved by vortexing, and briefly spined down. PCR amplification was performed in the BIORAD Thermo cycler with the following conditions: for ERIC-PCR, one cycle at 95 °C for 3 min; 30 cycles at 90 °C for 30 s, at 40 °C for 1 min, and at 72 °C for 1 min; and one cycle at 72 °C for 8 min (Albufera *et al.*, 2009). As shown above briefly an initial denaturation [95°C] followed by cycles of denaturation, annealing and extension with a single final extension [ERIC at 72 °C for 8 min]. Five microliters of the PCR or amplification product loaded into the wells. Seven microliters of 1kbp DNA ladder (CinnaGen) was loaded into one terminal wells or into the middle well of the gel. PCR products were electrophoresed in 1.5% agarose gels by horizontal electrophoresis in 1x TBE buffer at room temperature and 120 mV for 45 min to 1 hour. Then gels stained with 0.5 µg /ml ethidium bromide and visualized in a UV transilluminator (Gel Doc; Bio-Rad).

Construction of dendogram

The DNA bands or fingerprints obtained from ERIC-PCR amplification were first analyzed visually, and a score '1' was defined as the presence of a visible band of a given size, while a score '0' was defined as the absence of any band of the definite size. These scores were merged and by NTSYS (Numerical Taxonomy System), Version 2.0 computer software the genetic similarity index was calculated with simple matching coefficient and dendograms were drawn (fig 1).

Results

In the present study, the multiple DNA fragments obtained by rep-PCR was composed of visible, stable and reproducible bands. Profiles were determined on the basis of differences in numbers and size of electrophoresis bands. Briefly, in 64 different bacterial isolates of *S.*Entritidis by Eric-PCR 6 -17 bands observed which ranging in size from 100 to 2000 bp. The approximate sizes of bands observed in the present study are 100, 250, 300, 350, 400, 500, 550, 600, 750,900, 1000, 1200, 1300, 1400, 1500, 1800 and 2000 bp.

Table 1. Primers used for ERIC-PCR amplification (Albufera *et al.*, 2009; Versalovic *et al.*, 1991).

Primer	Sequence
ERIC-1R	5-ATGTAAGCTCCTGGGGATTCAC-3
ERIC 2	5-AAGTAAGTGACTGGGGTGAGCG-3

Table 2. Measurement of discriminatory power of ERIC-PCR fingerprinting method.

Method	No.of types (molecular profiles)	Size(%) of largest profile	Discriminaion index (1-D)
ERIC-PCR	31	5.76	0.9470

As shown in figure 1, by ERIC-PCR, 64 isolates of *S.*Entritidis were divided into three major clusters (E1, E2, E3) that represented 31 ERIC molecular profiles. The similarity among isolates of every cluster (E1, E2, E3) were about 84%, 80% and 79% respectively. E1 cluster included 13 profiles and

involved 32 poultry isolates (m1 to m32). E2 cluster included 9 profiles and involved 13 cattle isolates and one human isolate (H19) with 80% similarity among isolates. E3 cluster included 8 profiles and involved 17 human isolates (H1 to H11, and H13 to H18) with 86% similarity. H12 human isolate, made one profile. H19 a human isolate, was closely related to cattle isolates (about 80% similarity). Poultry isolates had more similarity with cattle isolates than human isolates by ERIC-PCR. In summary by using the descriptive statistics, mean (μ) and standard deviation or σ (sigma) of data was calculated for ERIC-PCR technique. The mean (μ) and standard deviation σ (sigma) of the number of molecular profiles were μ=11.09 and σ=2.02 for ERIC-PCR. In statistics, the coefficient of variation (CV) is a normalized measure of dispersion. CV is sometimes known as relative standard deviation (RSD). CV value was 18.21 for ERIC-PCR (Munro, 2004). Base on the results, index of discrimination (DI) or discrimination power for ERIC-PCR was 0.9470 (Table 2). The formula used to calculate the index of discrimination (DI) is shown here:

$$DI = 1 - \frac{\sum_{j}^{K} ni(ni-1)}{N(N-1)}$$

DI is index of discrimination, N=total number of strains or isolates in the sample population, K= the total number of types described, ni= the number of strains or isolates belonging to the ith type (Hunter and Gaston ., 1988).

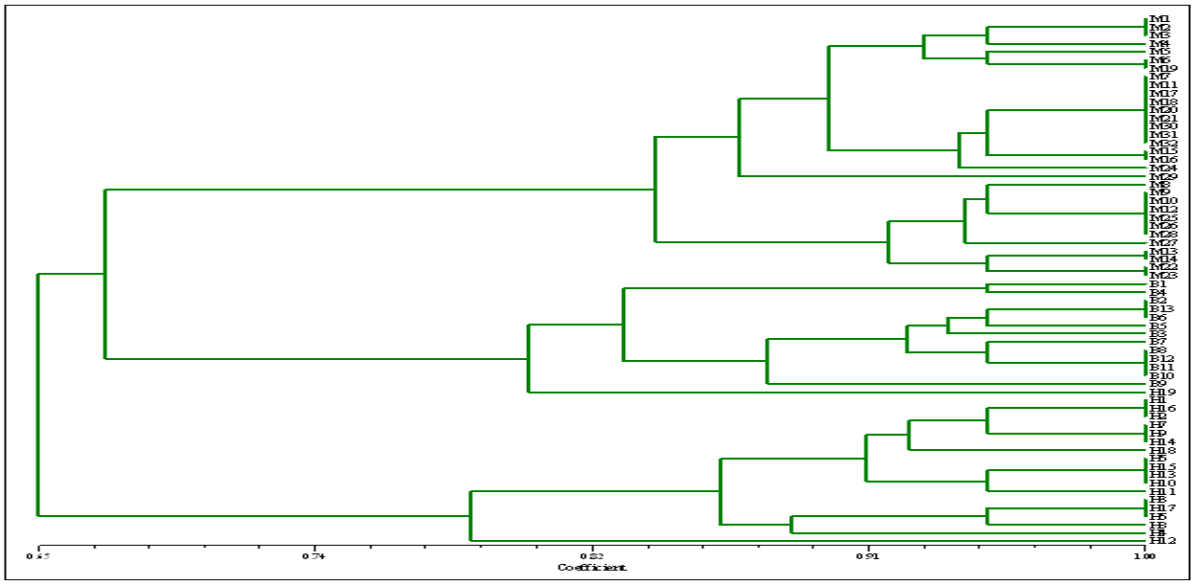

Fig. 1. Dendogram showing similarity and the relatedness of *S.*Entritidis strains isolated from human (H) and Cattle(B) and poultry (C) sources as determined by the DNA fingerprint analysis performed by ERIC-PCR using the NTSYS Version 2.0 computer software program. M1–M32: poultry isolates and H1–H19: human isolates and B1–B13: cattle isolates.

Table 2- Measurement of discriminatory power of ERIC-PCR fingerprinting method.

Discussion

In the present study, it has been proved that ERIC-PCR is useful to assess the genetic diversity and to discriminate strains of *Salmonella* Entritidis. A high discriminatory power also found by other investigations (Rasschaert *et al.*, 2005; Bhowmick *et al.*, 2012; De Oliveira *et al.*, 2007). Bhowmick *et al.* (2012) reported that the diversity index (DI) value obtained by ERIC-PCR was 0.96 . Their study was performed on 58 *Salmonella* isolates of 7 serotypes. The number of bands depends on the resolution of the electrophoretic system. We obtained 8-13 bands with the ERIC primer set with approximate sizes between 100 to 2000 bp. According to Versalovic *et al.* (1994) the optimal number of bands for rep-PCR is

8 to 15 bands. Rasschaert *et al.* (2005) found out 13 to 22 bands with ERIC1R–ERIC2 primers in 80 different serotypes belonging to 10 serotypes of *Salmonella*. Oliveria *et al.* (2007) observed 12 or 13 bands with ERIC-PCR in 111 isolates of *S.*Entritidis ranged from 190 to 1430 bp. According to Lith and Aarts (1994), it is possible to use the ERIC1R–ERIC2 primer set to discriminate *Salmonella* serotypes. Their study was performed on 65 *Salmonella* isolates of 49 serotypes. According to Burr *et al.* (1998), who tested the same primer set on 89 *Salmonella* isolates of 22 serotypes, the fingerprints obtained did not correlate with serotypes. The great heterogenicity found by Chmielewski *et al.* (2002) using REP- and ERIC-PCR in 31 *S.*Entritidis isolates conformed to our study.

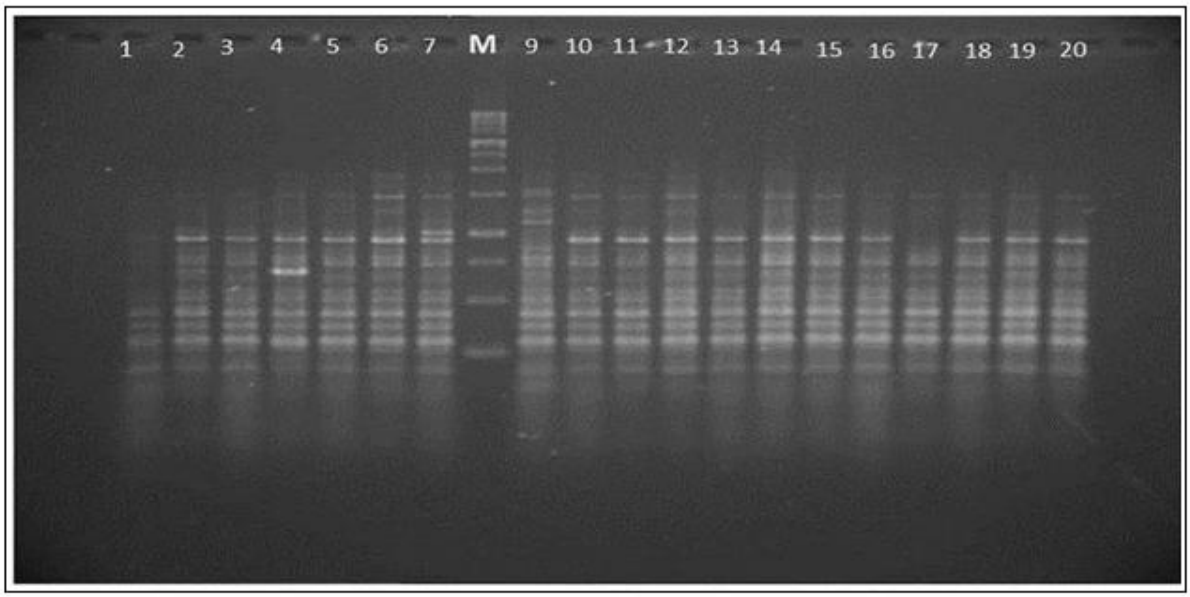

Fig. 2. ERIC-PCR DNA fingerprints of *S.*Entritidis isolates with ERIC-1R and ERIC 2 primer . From left to right, Lanes 1-7 and 9-20: human isolates; lane 8 or M is 1-Kb molecular weight DNA ladder.

In conclusion, the findings presented in this research, and the results of the molecular fingerprinting of the *S.*Entritidis strains revealed that ERIC-PCR is highly discriminatory techniques and most of the human isolates (with the exception of H19 isolate) were different from non-human isolates of *S.*Entritidis but had more relatedness and similarity with cattle isolates. Thus it was found that there is a high heterogeneity among analysed samples therefore it is necessary to do more surveys and researches.

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