Genotyping of *Salmonella enterica* subsp. *enterica* serovar Enteritidis, 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 Enteritidis 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* serovar Enteritidis isolates at the serotype level. Sixty-four *S*.Enteritidis 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*.Enteritidis 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*.Enteritidis isolates from different origin and for epidemiological trace back and taxonomy.
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 Enteritidis 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 transmission 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). Therefore to investigate the source and relatedness among different strains more precise 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. Enteritidis 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. Enteritidis 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 Enteritidis selected and were diagnosed from different geographic areas of Iran. 64 isolates of S. Enteritidis 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.

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

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>ERIC-1R</td>
<td>5-ATGTAAGCTCCTGGGGATTCAC-3</td>
</tr>
<tr>
<td>ERIC 2</td>
<td>5-AAGTAAGTGACTGGGTTGAGCG-3</td>
</tr>
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Table 2. Measurement of discriminatory power of ERIC-PCR fingerprinting method.

<table>
<thead>
<tr>
<th>Method</th>
<th>No.of types (molecular profiles)</th>
<th>Size(%) of largest profile</th>
<th>Discrimination index (1-D)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERIC-PCR</td>
<td>31</td>
<td>5.76</td>
<td>0.9470</td>
</tr>
</tbody>
</table>

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:

$$DI = 1 - \frac{\sum_{i=1}^{K} n_i(n_i-1)}{N(N-1)}$$

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. Enteritidis 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 heterogeneity found by Chmielewski et al. (2002) using REP- and ERIC-PCR in 31 S. Enteritidis isolates conformed to our study.

Fig. 2. ERIC-PCR DNA fingerprints of S. Enteritidis 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. Enteritidis 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. Enteritidis 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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