

## The Study of Genetic Relationship Among Third Generation Cephalosporin-resistant *Salmonella enterica* Strains by ERIC-PCR

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**Abstract:** *Background and Objectives:* *Salmonella* is an important food-borne pathogen responsible for disease in humans and animals. The aim of this study was to investigate the genetic relationship among third generation cephalosporin-resistant *Salmonella enterica* strains by Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR.

*Methods:* The study included all *Salmonella* isolates obtained from clinical cases in a pediatric hospital in Tehran, Iran during 2006 to 2009. Antimicrobial susceptibility testing was performed according to the Clinical and Laboratory Standards Institute. The genetic relationship between third generation cephalosporins-resistant *Salmonella enterica* strains was determined using ERIC-PCR.

*Results:* Of 136 *Salmonella enterica* isolates recovered from pediatric patients, six isolates including four *Salmonella enterica* serotype Infantis and two *Salmonella enterica* serotype Enteritidis showed an extended-spectrum cephalosporins resistant phenotype. ERIC-PCR differentiated *Salmonella enterica* serotypes Infantis and Enteritidis into 2 distinct clusters arbitrarily named as E1 and E2. Profile E1 was found in two *Salmonella enterica* serotype Enteritidis isolates, and profile E2 was found in four *Salmonella enterica* serotype Infantis isolates.

*Conclusion:* Extended-spectrum cephalosporins resistant *Salmonella* could be attributed to a few predominant serotypes including Enteritidis and Infantis in this study. Genetic analysis using ERIC-PCR showed that closely related clones are responsible for the occurrence of extended-spectrum cephalosporins resistant *Salmonella* infection in Tehran.

**Keywords:** *Salmonella enterica*, Enterobacterial Repetitive Intergenic Consensus, Antimicrobial susceptibility testing.

### INTRODUCTION

*Salmonella* is a genus of Gram-negative rod-shaped bacteria of the family *Enterobacteriaceae* [1]. It has been the leading cause of many outbreaks and infections around the world [2]. *Salmonella* species are important food-borne pathogens responsible for disease in humans and animals [3]. They cause a wide range of human diseases such as gastroenteritis, enteric fever, and septicemia [4].

Infections caused by *Salmonella* spp. are increasing in Iran [5-7]. Antibiotic treatment is not required for straightforward *Salmonella* gastroenteritis but becomes essential to treat invasive salmonellosis [8, 9]. Fluoroquinolones are the drugs of choice for an invasive extra intestinal infection in adults, whereas extended-spectrum cephalosporins (ESCs)

such as ceftazidime, cefotaxime, and ceftriaxone are drugs of choice for treatment of salmonellosis in children [10]. However, the use of these drugs is becoming compromised by the increasing development of ESCs and quinolone resistance all over the world. *Salmonella* strains resistant to ESC have been reported since the late 1980s, and their numbers have increased ever since.

Treatment of salmonellosis is of particular concern among children, because fluoroquinolones should not be used in this age group [11]. Treatment failures due to *in vivo* acquisition of an extended-spectrum  $\beta$ -lactamase (ESBL) gene or a reduced susceptibility to ciprofloxacin in *Salmonella* are now well established [12, 13].

Epidemiological studies are done for the prevention and treatment of infections. The ability to distinguish isolates of *Salmonella* may be very important to trace the source of outbreaks. The methods that have been used for deciphering the relatedness amongst the isolates are biotyping, antibiogram, plasmid typing, phage typing, serotyping, ribotyping and

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PCR fingerprinting. Some of the above techniques have low discriminatory potential, whereas others demand considerable amount of expertise, time and equipment.

In recent years, many DNA-based genotyping techniques have been utilized to delineate epidemiological relationships between various isolates [14-19]. Many reports are now available demonstrating the utility of PCR for typing of many organisms, including *Salmonella* [20-22]. The application of PCR based techniques has had a revolutionary impact in the diagnosis and epidemiology of infectious diseases. Utility of commonly used primers, and ERIC sequence elements in typing and differentiation of *Salmonella* isolates has been reported earlier [23, 24].

The ERIC sequences, also known as intergenic repeat units (IRUs), are present in many copies in genomes of members of the family [23]. The length of these elements is 126 bp and they are highly conserved at nucleotide level and also include a central core inverted repeat. The position of ERIC elements in enterobacterial genomes varies between different species and has been used as a genetic marker to characterize isolates within a bacterial species [25, 26].

ERIC sequence was first described in *Escherichia coli*, *Salmonella Typhimurium* and other enterobacteria [23]. This method uses consensus primers to amplify DNA sequences located between successive repetitive elements for subtyping Gram-negative enteric bacteria such as *Salmonella* spp. [23].

The objective of this study was to determine genetic relationship between extended-spectrum cephalosporins resistant *S. enterica* strains using ERIC-PCR.

## MATERIALS AND METHODS

The study included all *Salmonella* isolates recovered from clinical cases in a pediatric hospital in Tehran, during 2006-2009. The isolates had been identified by the conventional biochemical methods and serotyped by slide agglutination with commercial antisera (Staten Serum Institut, Copenhagen, Denmark).

Antimicrobial drug resistance was determined by using the disc diffusion method on Muller-Hinton agar (Merck Ltd, Darmstadt, Germany), according to the Clinical and Laboratory Standards Institute's recommendations [27] using disks (Oxoid, Basingstoke, Hampshire, United Kingdom) includ-

ing aztreonam (30µg), cefepime (30µg), cefotaxime (30µg), ceftazidime (30µg), ceftizoxime (30µg), ceftriaxone (30µg), and cephalothin (30µg).

Phenotypic confirmation of extended-spectrum cephalosporins resistant *Salmonella* was determined by the double disk synergy test using both cefotaxime and ceftazidime alone and in combination with clavulanic acid (Mast Group Ltd., Merseyside, UK). The 0.5 McFarland turbidity of bacteria were spread on the surface of Muller-Hinton agar. Double disk synergy test was performed by comparing the inhibition zone of disks containing cefotaxime or ceftazidime with and without clavulanic acid. The organisms used for quality control were *Escherichia coli* (ATCC 25922; American Type Culture Collection).

The relationship between strains was determined using ERIC-PCR. Genomic DNA was extracted as described previously [28]. The PCR amplifications were conducted by adding a mixture (20 µl per reaction) of 12 µl of sterile distilled water, 2 µl of 10x PCR buffer, 1 µl of 50 mM MgCl<sub>2</sub>, 1 µl of 10 mM dNTPs, 1 µl of each of primers, ERIC 1R: 5'-ATG TAA GCT CCT GGG GAT TCA-3' and ERIC 2: 5'-AAG TAA GTG ACT GGG GTG AGC G-3' (25), 1 µl of Taq polymerase and 1 µl of the template DNA to the reaction. ERIC-PCR was done as described previously [28, 29] with some modifications in temperature profile: An initial denaturation at 94°C for 4 minutes was followed by 35 cycles of 94°C for 1 minute, annealing at 52°C for 1 minute, extension period at 65°C for 8 minutes, and a final extension at 65°C for 15 minutes.

Amplified products were separated by electrophoresis on 1% agarose gel and photographed under uv illumination. A 100 kbp DNA ladder was used as the molecular size marker.

## RESULTS

One hundred-thirty-six *S. enterica* isolates had been recovered from children in a major pediatric hospital in Tehran, during 2006-2009. All extended-spectrum cephalosporins resistant strains were selected for further analysis by ERIC-PCR in order to assess an epidemiological link among the isolates.

As shown in Table 1, six isolates including four *S. enterica* serotype Infantis and two *S. enterica* serotype

**Table 1. Characteristics of the Extended-spectrum Cephalosporins Resistant *Salmonella enterica* Serotypes Enteritidis and Infantis**

Strains	<i>Salmonella</i> Serotype	Sex of Patient	ERIC Pattern	Resistance Pattern <sup>1)</sup>
1	Enteritidis	Male	E1	AM, PIP, TIC, CF, CR, CX, CZ, FE, CT
2	Enteritidis	Female	E1	AM, PIP, TIC, CF, CR, CX, CZ, CT, SXT, S, TE, NA
3	Infantis	Male	E2	AM, PIP, TIC, CF, AT, CR, CX, CZ, CT, SXT, S, TE, NA
4	Infantis	Female	E2	AM, PIP, TIC, CF, AT, CR, CX, CZ, CT, SXT, S, K, TE, NA
5	Infantis	ND	E2	AM, PIP, TIC, CF, AT, CR, CX, CZ, CT, SXT, S, TE, NA
6	Infantis	Female	E2	AM, PIP, TIC, CF, AT, CR, CX, CZ, CT, SXT, S, TE, NA

<sup>1)</sup>AM: Ampicillin, CF: Cephalothin, CR: Ceftriaxone, CX: Cefotaxime, CZ: Ceftazidime, CT: Ceftizoxime, K: Kanamycin, NA: Nalidixic Acid, PIP: Piperacillin, SXT: Sulfamethoxazole-trimethoprim, TIC: Ticarcillin, S: Streptomycin, TET: Tetracycline.  
ND, not determined

Enteritidis showed an extended-spectrum cephalosporins resistant phenotype (i.e. simultaneous resistant to ceftriaxone, cefotaxime, and ceftazidime). Each *S. enterica* serotype Enteritidis isolates were recovered from one female and one male pediatric patient and three strains of *S. enterica* serotype Infantis were belonged to 2 female and one male patient. The sex of the patient from which one of the isolates came from was not determined.

ERIC-PCR differentiated *S. enterica* serotypes Infantis and Enteritidis into 2 distinct clusters. The number of ERIC-PCR bands produced by PCR ranged from 6 to 17, with sizes ranging from 200 to 3200 bp. ERIC-PCR patterns were arbitrarily named as two different profiles (E1 and E2). Profile E1 was found in two *S. enterica* serotype Enteritidis isolates, and profile E2 was found in four *S. enterica* serotype Infantis isolates (Fig. 1).

## DISCUSSION

Infections caused by *Salmonella* spp. are endemic in Iran [30, 31]. *Salmonella enterica* serotypes Enteritidis and Infantis are among the most prevalent serotypes of salmonellae in humans in Iran [32-34]. *Salmonella* strains resistant to extended-spectrum cephalosporins are noteworthy because extended-spectrum cephalosporins are the most commonly used antimicrobial agent for the treatment of *Salmonella* infections in children in our country.

The strains of *Salmonella* spp. are usually defined phenotypically by serotyping and antimicrobial resistance tests in microbiology laboratories of Iran. Since phenotyping data suggest the clonal diffusion of a limited number of local strains, there is a need for practical techniques to discriminate *Salmonella* isolates, and to evaluate the association of

*Salmonella* strains in epidemiological studies [35]. Here, we presented the results of a molecular study of extended-spectrum cephalosporins resistant *Salmonella* strains isolated from pediatric patients in Iran. We performed ERIC-PCR using ERIC primers and obtained distinguishable DNA band patterns for each *S. enterica* serotypes including Enteritidis and Infantis.

The results indicated that ERIC-PCR could differentiate the *S. enterica* isolates at serotype level. However all strains within each serotype of Enteritidis and Infantis have shown similar ERIC-PCR pattern.

Application of ERIC-PCR fingerprinting for genotyping *S. enterica* strains has been reported in South Korea by Lim *et al.* [36]. They identified 50 genotypes among 57 *Salmonella* strains. Oliveira *et al.* have also reported some similar observations [24].

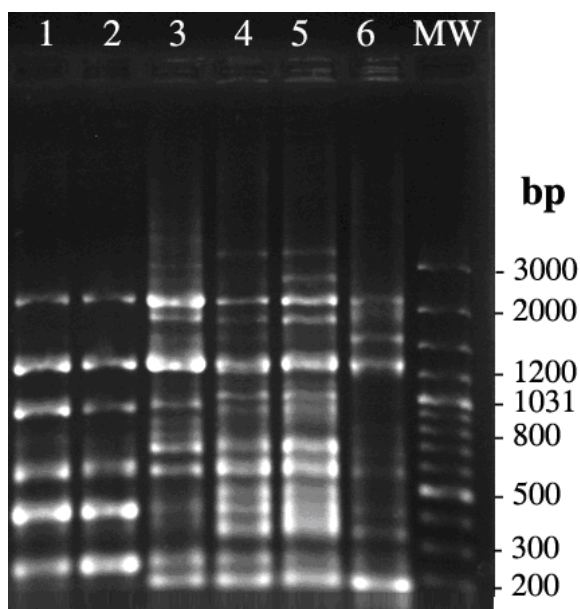
Our result is consistent with findings of Van Lith [37], who found each serotype was characterized by a unique DNA profile however Burr *et al.* characterized *Salmonella* serotypes and found that every isolate had a unique fingerprinting but the serotypes were not grouped together in major branches, thus, serotypes were not identified by ERIC-PCR [38].

The greatest advantages of genotyping by ERIC-PCR lie in its accessibility, speed, relative ease of use, and general stability. Unfortunately, ERIC-PCR does not always offer a complete picture of genetic relatedness. Identical bands are based on their size, and not necessarily their genetic make-up. It is possible that two genetically diverse segments between two ERIC segments can be counted as equal, provided that the ERIC targets are the same distance apart on DNA.

In conclusion, our results suggest that extended-spectrum cephalosporin resistant *Salmonella* could be attributed to a few predominant serotypes including Enteritidis and Infantis in this study. Genetic analysis using ERIC-PCR showed that closely related clones are responsible for the occurrence of extended spectrum cephalosporin-resistant *Salmonella* infection in Tehran.

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**Fig. (1).** Electrophoresis of ERIC-PCR products on 1% agarose gel stained with ethidium bromide. Lanes 1 and 2 are amplification products from DNA of *Salmonella enterica* serotype Enteritidis, lane 3, 4, 5, and 6 are amplification products from DNA of *Salmonella enterica* serotype Infantis. Lane MW is 100 bp molecular size marker.

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