



Molecular Epidemiology and Antimicrobial Resistance of *Salmonella* spp. Isolated from Resident Patients in Mazandaran Province, Northern Iran

M.M. Soltan Dallal^{1,2*}✉, M. Khalilian¹, H. Masoumi Asl^{2,3}, R. Bakhtiari⁴, A. Davoodabadi⁵, M.K. Sharifi Yazdi^{6,7}, P. Torabi Bonabi²

1. Division of Food Microbiology, Department of Pathobiology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran

2. Food Microbiology Research Center, Tehran University of Medical Sciences, Tehran, Iran

3. Center for Communicable Disease Control, Ministry of Health and Medical Education, Tehran, Iran

4. Division of Microbiology, Department of Pathobiology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran

5. Department of Microbiology, Medical School, Babol University of Medical Sciences, Babol, Iran

6. Zoonosis Research Center, Tehran University of Medical Sciences, Tehran, Iran

7. Department of Medical Laboratory Sciences, School of Para Medicine, Tehran University of Medical Sciences, Tehran, Iran

HIGHLIGHTS

- *Salmonella enteritidis* was the most frequent serovar among salmonellosis outbreaks in Mazandaran province, Iran.
- All of the isolated *S. enteritidis* were resistant to tetracycline, nalidixic acid, and trimethoprim-sulfamethoxazole.
- No resistance was found to cefotaxime, chloramphenicol, ciprofloxacin, amoxicillin, and ceftazidime.

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Acronyms and abbreviations

PFGE=Pulsed-Field Gel Electrophoresis

REP-PCR= Repetitive Extragenic

Palindromic Sequence Polymerase

Chain Reaction

SI=Similarity Index

DI=Differentiation Index

ERIC=Enterobacterial Repetitive

Intergenic Consensus

ABSTRACT

Background: *Salmonella* is one of the causes of food-borne illnesses worldwide resulting in a wide range of human diseases. The aim of this research was to study molecular epidemiology and antimicrobial resistance of *Salmonella* spp. isolated from resident patients in Mazandaran province, northern Iran.

Methods: Totally, 51 stool samples were collected from patients in 20 outbreaks recorded in Health Care Center of Mazandaran province, North of Iran, during October 2013 to September 2014. Cultivation and biochemical methods were used for differentiation of *Salmonella*. Identified *Salmonella* isolates were confirmed with 16S rRNA PCR method and sequencing. Antibiotic susceptibility test was carried out for each isolate.

Results: Four of 51 samples (7.84%) were contaminated with *S. enteritidis*. Molecular analysis and sequencing verified the isolates. The delineation level for each isolate discrimination in subsequent analysis was set at 94% for the primer. All of the isolates were resistant to tetracycline, nalidixic acid, and trimethoprim-sulfamethoxazole. No resistance was found to cefotaxime, chloramphenicol, ciprofloxacin, amoxicillin, and ceftazidime.

Conclusion: *S. enteritidis* was the most frequent serovar among salmonellosis outbreaks in the Mazandaran province, Iran. Also, isolated *S. enteritidis* showed various rates of susceptibility for different antibiotics.

Introduction

Food has suitable conditions for bacteria to grow and therefore, it is very hard to control colonization of these

microorganisms from farm to fork chain (Newell et al., 2010). Food-borne diseases are occurred by consumption of food contaminated with microbial pathogens or their

* Corresponding author. ✉ soltanda@sina.tums.ac.ir

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toxins, causing gastrointestinal complications and sometimes hospitalization and even death. Every year, millions of people get a kind of food-borne illnesses caused by major pathogenic bacteria. On the other hand, antibiotic resistance makes this problem hard to resolve. The four most commonly reported bacterial enteropathogens in the United States including, *Campylobacter*, nontyphoid *Salmonella*, Shiga toxin-producing *Escherichia coli*, and *Shigella* are associated with an estimated cost of 7 billion dollars, annually (DuPont, 2007).

Salmonella is a genus of the Enterobacteriaceae family with a rod shape and negative in Gram staining. This bacterium is one of the causes of food-borne illnesses worldwide resulting in a wide range of human diseases like gastroenteritis, enteric fever, and bacteremia. It is estimated that 1.4 million infections occur annually in the United States (Kilic et al., 2010). There are many published researches in the literature showing various incidence rate and antibiotic resistance of *Salmonella* spp. isolated from foodstuffs distributed in Iran (Dallal et al., 2010; Jalali et al., 2008; Jamshidi et al., 2010; Salehi et al., 2005; Tajbakhsh et al., 2013) and the other countries such as Nigeria (Raufu et al., 2014), Saudi Arabia (Elhadi, 2014), France (Hue et al., 2011), Spain (Alvarez-Fernandez et al., 2012), Tunisia (Oueslati et al., 2016), China (Yan et al., 2010), Argentina (Favier et al., 2013), and Australia (Fearnley et al., 2011).

There are different genotyping methods for studying of the *Salmonella* serovars genetic diversity (Cho et al., 2008; Landeras et al., 1996; Pang et al., 2005). Pulsed-Field Gel Electrophoresis (PFGE) is a standard and suitable method for examining of related strains of *Salmonella* outbreak, but it is not suitable for sporadic cases (Foley et al., 2006). On the other hand, Repetitive Extragenic Palindromic Sequence Polymerase Chain Reaction (REP-PCR) targets the conserved and repetitive part of genome which found in eukaryotes and prokaryotes. The conserved region which places to the repetitive site of genome shows different sizes; therefore, it produces different fragments in length on agarose gel electrophoresis and provides a specific fingerprint of the organism (Anderson et al., 2010; Cleland et al., 2008).

The aim of this research was to study molecular epidemiology and antimicrobial resistance of *Salmonella* spp. isolated from resident patients in Mazandaran province, northern Iran.

Materials and methods

Sampling

Totally, 51 stool samples were collected from patients in 20 outbreaks recorded in Health Care Center of Mazandaran province, North of Iran, during October

2013 to September 2014. The main symptoms pertaining to each patient were recorded in data sheet.

Isolation and differentiation

Cultivation and biochemical methods were used for differentiation of *Salmonella*. The samples were inoculated in Cary-Blair transport media and immediately transferred to the Microbiology Laboratory of the Public Health Faculty, Tehran University of Medical Sciences. All the samples were inoculated in Selenit F medium (Merck KGaA, Darmstadt, and Germany) for 12 h in order to the enrichment, and then they were inoculated onto Hektoen enteric agar (Liofilchem, Italy) and incubated at 37 °C. Non-lactose-fermenter blue colonies were used to inoculate Kligler's iron agar (Liofilchem, Italy) tubes, and incubated at 37 °C for 24 h.

Antibiotic susceptibility tests

The antibiotic susceptibility test with the following antibiotics ampicillin, chloramphenicol, tetracycline, ceftazidime, cefotaxime, trimethoprim-sulfamethoxazole, ciprofloxacin, meropenem, nalidixic acid, and amoxicillin was carried out by disk diffusion method according to Wikler (2006). All of the antibiotic disks were obtained from MAST Company, England.

DNA extraction

DNA was extracted by boiling method. Briefly, one colony was cultured overnight and then boiled in 50 µl sterile STE buffer for 10 min, followed by centrifugation at 13300 rpm for 3 min.

16S rRNA PCR

ITS region of 16S rRNA considered as conserved region in all *Salmonella* serovars and therefore, PCR amplification was used for *Salmonella* detection and identification using specific primer pair that shown in Table 1 (Chiu et al., 2005). Each reaction mixture was consisted of 100 nM for each primer, 1.5 mM MgCl₂, 0.2 mM dNTPs, 100 ng genomic DNA, 1X PCR buffer, and 1.5 U Taq DNA polymerase (Sinaclon, Iran) in a final volume of 50 µl. The PCR program was set up with an initial denaturation at 94 °C for 2 min; subsequently, 30 cycles of denaturing at 94 °C for 30 s; annealing at 71 °C for 30 s; and extension at 72 °C for 50 s. Final extension in one cycle was done for 10 min at 72 °C. PCR products were analyzed by agarose gel electrophoresis (1.5% w/v) and stained with ethidium bromide and then visualized using Gel Documentation (ABI, USA).

Sequencing

For verification, the PCR products were sequenced

(Bioneer, Korea) and each 16S rDNA sequence was analyzed using nBLAST (Zhang et al., 2000).

REP-PCR

The reaction mixture consisted of 5 nM GTG5 primer, 1.5 mM MgCl₂, 0.2 mM dNTPs, around 100 ng genomic DNA, 1X PCR buffer, and 0.5 U Taq DNA polymerase (Sinaclon, Iran) in a final volume of 25 µl. The used primer was accorded to Rasschaert et al. (2005). The PCR program started with an initial denaturation at 94 °C for 5 min; subsequently, 30 cycles of denaturing at 94 °C for 30 s; annealing at 40 °C for 1 min; and extension at 72 °C for 8 min. The final extension in one cycle was done for 16 min at 72 °C cycle.

Analysis of amplification and dendrogram

Each amplicon fragment was separated on a 1.8% agarose gel. Briefly, 10 µl PCR product mixing with 2 µl loading dye buffer was loaded to each well alongside with DNA ladder. The PCR products were visualized by Gel Documentation. DNA fingerprint profiles were compared with each other using software gel compare II (Version 6.6). In general, “different”, “similar” and “in-distinguishable” were defined as <95%, <97%, and >95% similarity, respectively.

The variability in the clustering of the fingerprints introduced by the PCR run and preparation of lysates was assessed using the Similarity Index (SI) and Differentiation Index (DI). Lower SI and higher DI showed more variability. Also, net discriminating power (the difference between SI and DI) was considered as a measure for the discriminatory power of the strain (Johnson and Clabots, 2000). The last was calculated as 100–(SI–DI), which the higher value defined as the more net variability index.

Results

The results obtained from biochemical tests showed that four out of 51 samples (7.84%) were contaminated with *S. enteritidis*. Molecular analysis and sequencing verified the isolates. The main symptoms in patients were orderly diarrhea, abdominal pain, nausea, headache, and vomiting. Mortality did not occur among these patients.

The antibiotic resistant isolates and their antibiotic patterns are summarized in Table 2. All the isolates were resistant to tetracycline, nalidixic acid, and trimethoprim-sulfamethoxazole. No resistance was seen to ceftazidime, ciprofloxacin, chloramphenicol, amoxicillin, as well as cefotaxime. As shown in Fig. 1, the delineation level for each isolate discrimination in subsequent analysis was set at 94% for the primer.

Table 1: The primer pair used in this study with the target of 16S rRNA for *Salmonella* detection and identification

| Primer name | Oligonucleotide sequences | Reference |
|-------------|---------------------------------|--------------------|
| ITSR | 5'-TATAGCCCCATCGTGTAGTCAGAAC-3' | Chiu et al. (2005) |
| ITSF | 5'-TGCGGCTGGATCACCTCCTT-3' | |

Table 2: Antibiotic susceptibility of isolated *S. enteritidis* serovars

| Isolates | AMP | CA | TET | CAZ | CTX | SXT | CIP | MEM | NA | AMX |
|----------|-----|----|-----|-----|-----|-----|-----|-----|----|-----|
| 1 | MS | S | R | S | S | R | S | S | R | S |
| 2 | S | S | R | S | S | R | S | MS | R | S |
| 3 | S | S | R | S | S | R | S | R | R | S |
| 4 | S | S | R | S | S | R | S | MS | R | S |

-Antibiotics: ampicillin (AMP), chloramphenicol (CA), tetracycline (TET), ceftazidime (CAZ), cefotaxime (CTX), trimethoprim/sulfamethoxazole (SXT), ciprofloxacin (CIP), meropenem (MEM), nalidixic acid (NA), and amoxicillin (AMX)
 -S: sensitive; R: resistant; MS: middle sensitive

Discussion

Our results showed that *S. enteritidis* was the predominant *Salmonella* serovar isolated from outbreaks in Mazandaran province of Iran. Annually, in many countries in the world, *Salmonella* causes many illnesses and hundreds of death (CDC, 2007). The occurrence of typhoid salmonellosis is stable, however, the incidence of non-typhoid salmonellosis are increasing widespread. Also, serotype Enteritidis has been one of the most common isolated *Salmonella* causing food-borne outbreaks (Zheng et al., 2007). According to a report in United States, *S. enteritidis* and *S. typhimurium* have been the

common serotypes among hundreds of outbreaks occurred in the country (Newkirk and Hedberg, 2012). It has been reported that the main cause of salmonellosis outbreaks in Turkey was *S. enteritidis* (Kilic et al., 2010). Also, *S. enteritidis* was mainly associated with food-borne salmonellosis occurred in Europe in 2004 (Helwig, 2006).

In the current study, all the *S. enteritidis* isolates were resistant to tetracycline, nalidixic acid, and trimethoprim-sulfamethoxazole. Also, no resistance was found to ceftazidime, ciprofloxacin, chloramphenicol, amoxicillin, and cefotaxime. As a comparison, during 1999-2012 in

Brazil, the resistant to nalidixic acid were raised from 12.8% to 100%. It has been reported the highest sensitivity to tetracycline and chloramphenicol during 2001-2002 and no resistance to trimethoprim-sulfamethoxazole in 2014 (Tondo and Ritter, 2012). Researches in 10 European countries indicated that among 27000 cases of human salmonellosis infected by *S. enteritidis*, the most resistances were related to nalidixic acid, trimethoprim-sulfamethoxazole, and amoxicillin (Threlfall et al., 2003). It is known that misuse of antibiotics in both human and veterinary aspects leads to increase antimicrobial resistance in many *Salmonella* serovars. So, attention should be paid to the resistance of *Salmonella* serovars especially *S. enteritidis* as the predominant agent of human salmonellosis (Geimba et al., 2005).

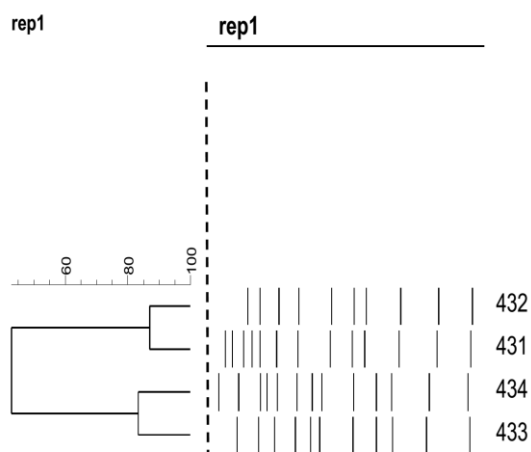


Fig. 1: Dendrogram of cluster analysis according to the REP-PCR fingerprinting

The REP-PCR method has good power for discriminate *S. enteritidis*. Rasschaert et al. (2005) has been previously reported that REP-PCR and Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR are equally able to discriminate *Salmonella* serotypes. They concluded that in epidemiological studies, discriminatory powers of the primer set ERIC and the primer (GTG)₅ are similar but the set primer of ERIC and the primer (GTG)₅ are complementary. Other studies have also reported about the ability of REP-PCR for *Salmonella* discriminating. Some of them (Burr et al., 1998; Milleman et al., 1996; Van Lith and Aarts, 1994) evaluated only the primer set of ERIC. These studies showed the different results. Van Lith and Aarts (1994) assessed 65 *Salmonella* isolates of 49 serotypes for discriminating the *Salmonella* serotypes that their results showed the possibility of using the primer set of ERIC1R-ERIC2. They reported that each serotype with unique fingerprint and the isolates within one serotype showed same patterns. Burr et al. (1998) analyzed 89 *Salmonella* isolates of 22 serotypes with the

set primer of ERIC and concluded that the fingerprints had not correlation with serotypes. According to Milleman et al. (1996) who had tested 56 serotype Typhimurium and 14 serotype Enteritidis strains using the ERIC primer set resulted that ERIC-PCR is not suitable for discriminating of *Salmonella* serotypes. They showed that all serotype Enteritidis isolates and some serotype Typhimurium isolates have the identical fingerprint. Popoff et al. (2004) confirmed that with increasing the annealing temperatures, the reproducibility and resolving power of REP-PCR with the primer set of ERIC2 and BOXA1R will be approved. In the last study 70 isolates of 15 serotypes were tested. We used the REP-PCR with high annealing temperature in a long time based on Popoff et al. (2004) because PCR program is the main factor, which explain the validity of REP-PCR for discrimination of *Salmonella* serotypes.

Having good knowledge about type of bacterial agent of food-borne diseases and also its antimicrobial resistance pattern plays very important role to reduce food-borne outbreaks. One problem in many developing countries such as Iran is that data on salmonellosis cannot be reported properly; because many patients with acute gastroenteritis do not visit a health care center or do not submit a specimen for laboratory testing. For reducing burden of food-borne diseases, the monitoring from farm to fork and encouraging all sectors of the food production chain is necessary.

Conclusion

The present study demonstrated that *S. enteritidis* was the most frequent isolate among salmonellosis outbreaks in the Mazandaran province, Iran. Also, isolated *S. enteritidis* showed various rate of susceptibility for different antibiotics.

Conflicts of interest

The authors declared that they had no conflicts of interest in this study.

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