Phenotyping and Genotyping Characterization of 
*Salmonella* Strains Isolated from 
Retail Beef in Erzurum, Turkey

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In this study, phenotypic and genotypic characterization of *Salmonella* strains was carried out. Isolates were obtained from 140 different minced beef samples, taken from slaughterhouses and butcher shops in the vicinity of Erzurum, by using chromogenic media. For this purpose, fatty acid profiles, BIOLOG, ERIC-PCR, REP-PCR and (GTG)₃-PCR methods were used. As a result of isolation study, *Salmonella* spp. was detected in 4 samples out of 140 minced beef samples. Depending on the data of fatty acid analysis, it was detected that there were 14 different fatty acids in the examined 15 bacterial strains. In the results of analyses by using BIOLOG system, all of the test organisms gave identification result as *Salmonella* group 1 and it was determined that this method is inefficient for serotype level detection. Several primer sets, targeting the repetitive DNA elements of REP, ERIC and (GTG)₃, were evaluated and it was detected that for identification and taxonomic characterization of *S. typhimurium* strains, the ERIC primers were the most reliable technique. Hence, as a promising genotypic tool, rep-PCR fingerprinting using the ERIC and REP primers can be used for the identification and characterization of *Salmonella* isolates species to serotype level.

**Key words:** *Salmonella* strains, Ground meat, MIS, BIOLOG, rep-PCR.

Foodborne bacterial pathogens are so important in terms of food safety. In addition to this, correctly identification of these pathogens has also a significant importance for both the food quality and the traceability of contamination factors which may occur in the supply chain¹.

In recent years, there is a considerable increase in foodborne diseases as a result of bacterial contamination of foods. Although there are different views of science people regarding the incidence of these diseases in the world, it is predicted that foodborne diseases are observed in about the 30% of the population of industrialized countries and, this percentage is even more in developing countries²,³.

One of the most important worldwide public health issues is foodborne diseases, mainly caused by *Salmonella* spp., *Listeria monocytogenes*, *Escherichia coli* O157:H7 and
Yersinia spp. Above mentioned pathogenic bacteria have been present in different kinds of foods (fish, vegetables, dairy products, meat and meat products) related with outbreaks." Among these pathogenic microorganisms, Salmonella, which is responsible for the significant part of foodborne diseases observed in human beings and animals, is a member of Enterobacteriaceae family. There are two species belonging to this microorganism group (S. enterica ve S. bongori) and, type species is S. enterica. In addition to its being the main cause of several infections and outbreaks around the world, it is counted as one of the significant reason for human gastroenteritis. It has been detected that Salmonella-contaminated food products, responsible for human salmonellosis, are mainly originated from cattle and poultry.

For studying foodborne pathogens, the conventional methods such as phage typing, serotyping, biotyping, antibiotic resistance (R-type), antibiogram, and bacteriocin identification are well known ones. These mostly unreliable methods are laborious, nonsensitive and, slow serotyping and phages typing, as conventional methods, are widely used to type Salmonella. Serotyping of bacteria depends on antigen-antibody interaction. For Salmonella, there are 2 surface antigens, used to divide the bacteria into serogroups, flagella (H) and somatic (O). Up to now, over 2,500 Salmonella serovars have been identified in the world with respect to their O and H antigens. Lysing bacteria with bacteriophages provides a basis for phage typing method. Salmonella serotypes such as S. typhi and S. typhimurium have a surface envelope (Vi) antigen which makes it possible to identify by using specific phages.

Molecular methods have been developed by taking into account the differences arising from nature, variations and ratios of macromolecules, which are forming microorganisms. Molecular methods use carbohydrates, lipids, proteins and genetic materials (DNA and RNA) as study materials. By using one of these items or a combination, identification and characterization of microorganisms are carried out.

In recent years, for identification of microorganisms, molecular methods is being intensely used like serologic techniques.

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possible. It is possible to identify several bacteria at the subspecies and strain level by rep-PCR\textsuperscript{10,19}. The purpose of this study is to carry out the phenotypic and genotypic characterization of \textit{Salmonella} isolates, obtained from minced beef meats marketed in Erzurum city, by using FAMEs, BIOLOG, REP-, ERIC- and, (GTG)$_5$-PCR methods and, determination of which molecular method can successively be used in the identification of \textit{Salmonella} isolates.

**MATERIALS AND METHODS**

**Bacterial Strain**

\textit{Salmonella thyphimurium} ATCC 14028 was kindly provided by Sahan Guran at Firat University, Elazig, Turkey.

**Isolation of \textit{Salmonella} spp**

One hundred and forty meat samples were obtained from retail markets and butchers in Erzurum province, Turkey. The samples were collected into sterile plastic containers and kept in a refrigerator. For detection of \textit{Salmonella} spp., each 25 g meat sample was mixed with 225 mL of sterile buffered peptone water (BPW, Merck KGaA, 107228, Germany) and pummeled in a Stomacher (Laboratory Blender Stomacher 400, Seward Medical, London, UK) apparatus for 2 minutes; the mixture was then incubated overnight at 37°C. A 0.1 volume of this culture was then transferred to 10 mL of Rappaport Vassiladis broth (RVS Broth, Merck KGaA, 107700, Germany) and pummeled in a Stomacher (Laboratory Blender Stomacher 400, Seward Medical, London, UK) apparatus for 2 minutes; the mixture was then incubated overnight at 37°C. A 0.1 volume of this culture was then transferred to 10 mL of Rappaport Vassiladis broth (RVS Broth, Merck KGaA, 107700, Germany) and incubated over night at 42°C. 0.1 mL of samples are taken from pre-enriched culture and spread evenly on the CHROM-agar\textsuperscript{TM} \textit{Salmonella} (RTA, Kocaeli, Turkey) medium with a sterile spatula, left for incubation at 37°C for 18-24 hours. After incubation, different mauve colonies developed in the media were selected and purified by subculturing.

**Extraction and analysis of FAME**

FAME from whole cell fatty acids of bacterial strains were prepared and analyzed according to the described method of the manufacturer’s manual (Sherlock Microbial Identification System version 4.0, MIDI, Inc., Newark, DE, USA)\textsuperscript{20,21}. For the separation of FAMES, gas chromatography (HP6890, Hewlett Packard, Palo Alto, CA, USA), with a fused-silica capillary column (25 m × 0.2 mm) of cross-linked 5% phenyl methyl silicone, was used. The commercial databases were compared with the MIS software package to identify the FAME profiles of each bacterial strain. Bacterial strains were identified by means of computer comparison of FAME profiles of the unknown test strains with those in the library.

**Biolog Micro plate assay**

One or two days after \textit{Salmonella} spp. strains were streaked on TSA plates, the inoculation of Biolog GN2 plates (Biolog) was carried out. Each well of Biolog GN2 microtiter plates was inoculated with 125 µl of the Gram-negative bacterial suspension, respectively, and then, was adjusted to the proper density (108 cfu/ml) and incubated at 37°C for 24 and 48 h. A microplate reader with a 590 nm wavelength filter was used to record automatically the development of color. By using BIOLOG420/Databases/GN601 software with applying the automatic threshold option, identification (Biolog Microlog 34.20 database) and ASCII file output of test results were conducted\textsuperscript{5,22}.

**DNA extraction and polymerase chain reaction (PCR) amplification**

A modified method, previously described by Adiguzel\textsuperscript{23}, was used for the extraction of total genomic DNA from bacteria samples.

**rep-PCR**

Using primer sets corresponding to REP, ERIC and (GTG)$_5$ elements, rep-PCR genomic fingerprinting was performed for a total of 15 strains\textsuperscript{5,24-26}. For amplifying putative REP 1R (5’-IIIICGICGCATCIGGC-3’) and REP 2 (5’-ICGICTTATCIGGCTAC-3’) (where I is Inosine); ERIC 1R (5’-ATGTAAGCTCCTGGGGAT-3’) and ERIC 2 (5’-AGTAAGTGACTGCGGGGTGAGC-3’) and (GTG), were used, respectively. Concisely, as a template, approximately 50 ng of purified DNA was used in a 30 µl reaction mixture. 27 µl of reaction cocktail was prepared as follows: specify Gitschier Buffer 5 µl, Dimethyl sulfoxide 2.5 µl (100%, 20×), dNTPs (10 mM) 1.25 µl, bovine serum albumin 1.25 µl (20 mg/ml), primer/primers (5 µM) 3.0 µl, Taq polymerase (250 U) 0.3 µl, water 10.7 µl for (GTG), PCR, 13.7 µl. In each PCR assay, a negative control (no DNA) was included. A Corbett Research Palm Cycler (Corbett CG1-96 AG, Australia) was used to perform PCR amplification reaction under the following conditions: an initial denaturation at 95°C for 7 min (94°C, 7 min for
from central Erzurum. Then, for the identification have been isolated from 140 meat samples obtained Kocaeli, Turkey) culture media, 15 by using CHROM-agar foodborne microorganisms scientists for isolation and identification of chromogenic culture media are widely used by developments in biotechnology, in recent years, biochemical test. Especially by means of important selective solid culture media and, different enrichment, enrichment, purification by using works to verify the data obtained from pre-enrichment. These data show that fatty acid profiles of organisms are specific to the strain.5

For identifying the 15 Gram-negative isolates (using GN2 micro-plates) from 140 meat samples, isolated after MIS and CHROM-agar, the Biolog bacterial identification system was used. S. typhimurium ATCC 14028, the reference strain, included as control and was identified with GN2 microplate to figure out the specificity of the Microlog/Biolog microbial identification system. It was identified up to species level. The results of MIDI analysis were not similar to those obtained with the Biolog analyze for most cultures as it was denoted by Boulter et al.6. According to Jordan et al., this technique is incapable of discriminating between different Salmonella serovars and could only be used as general detection for Salmonella. In the light of the results, it was concluded that for identifying Salmonella serotype, MIS and Biolog identification systems alone are not yet accurate enough to serve as a primary method. According to our results, a single approach may not be sufficiently reliable to identify Salmonella strains.

Genotypic identification is counted as an alternative or complement for the established phenotypic methods. Genomic fingerprints are the procedures of analyzing the whole genome of the targeted organisms. For bacterial identification and characterization, one of the well-established genomic fingerprint methods is rep-PCR. As a simple technique, rep-PCR can provide differentiation between closely related strains of bacteria. Additionally, bacteria can be assigned up to the strain level by this technique based on the presence of repeated elements within the genome.
In this study, one single oligonucleotide primer, (GTG)$_3$, and two oligonucleotide primer pairs, REP1R-I/REP2-I and ERIC1R/ERIC2, were firstly tested to see whether they can type a subset of 15 strains or not. The ERIC1R/ERIC2 primer set notably gave banding patterns with the highest complexity with respect to the REP1R-I/REP2-I and (GTG)$_3$ primer sets. Including 10-16 fragments of 200–6000 bp (Fig.1), reproducible and differentiating fingerprints were obtained from the ERIC primer set generated. Using REP primers brought about a banding pattern containing approximately 7–9 (700-4000bp) visualized PCR products (Fig. 2). The (GTG)$_3$ primer generated fingerprints containing between 5 and 7 fragments ranged from 700 to 4000 bp (Fig.3). As a conclusion,
fingerprints, generated with the ERIC-PCR derived DNA fingerprints, exhibited the highest genetic polymorphism compared to (GTG)_5- and REP-PCR fingerprints.

Similar results have been reported in some other studies indicating that rep-PCR genomic fingerprint protocols demonstrating high sensitivity in the discrimination of Salmonella isolates. It was also indicated in their study that rep-PCR is an efficient method for differentiating Salmonella strains.

In their study, Anderson et al., examined the applicability of DGGE (Denaturing Gradient Gel Electrophoresis) and REP-PCR methods in Salmonella serotyping with respect to conventional antibody serotyping method, which is used as golden standard. They stated that REP-PCR method forms more discriminating fragments.

**Fig. 3.** GTG primer profile generated with the GTG primer. Lanes: 1) S1; 2) S2; 3) S3; 4) S4; 5) S5; 6) S6; 7) S7; 8) S8; 9) S9; 10) S10; 11) S11; 12) S12; 13) S13; 14) S14; 15) S15; 16) S. thyphimurium ATCC 14028; 17) N; Negative Control; M) Molecular Marker (10 kb)

**Fig. 4.** 1) S1; 2) S2; 3) S3; 4) S4; 5) S5; 6) S6; 7) S7; 8) S8; 9) S9; 10) S10; 11) S11; 12) S12; 13) S13; 14) S14; 15) S15; 16) S. thyphimurium ATCC 14028
with respect to DGGE and also, both methods can be used as an alternative for the conventional antibody method.

Performing cluster analysis was taken into account because of the higher number of polymorphic bands produced by ERIC-PCR. Salmonella isolates were classified in two main clusters (Fig. 4). Fourteen test strains and a reference strain (S. typhimurium ATCC 14028), which were discriminated by two sub-clusters (Fig. 4), represent the first main cluster (S1-S12, S14 and S15). It was detected that there was a 99% of genetic relatedness between isolates S14 and S. typhimurium ATCC 14028. Furthermore, there was a high degree of similarity (≥ 82%), among twelve isolates (S1-S12, S14 and S15) and a reference strain (Fig. 4). Second cluster, represented by one test strain (S13) and a reference strain of S. typhimurium ATCC 14028, had low similarity ratio (≥75%), respectively.

Burr et al.,42 assessed the applicability of rep-PCR fingerprinting for the genotypic differentiation of a broad range of Salmonella isolates. They found that the REP-PCR was able to discriminate among Salmonella isolates sharing similar serotypes. Gallardo et al.43 suggested that REP-PCR method provided an alternative, rapid and powerful genomic typing method for S. typhimurium. Woo and Lee44 reported that the REP-PCR method may represent an efficient and time-saving analysis tool for the genotyping of Salmonella serotypes, including S. typhimurium. Albufera et al.,4 compared RAPD with rep-PCR for determination of genetic diversity of Salmonella isolates and determined that rep-PCR had greater discriminatory power than RAPD method. Li et al.45 showed that ERIC-PCR method was an alternative, rapid and powerful genomic typing method for discrimination of Salmonella serotypes.

FAMEs, rep-PCR genomic fingerprint analysis methods and BIOLOG technics have been firstly used in this study to compare with each other for the molecular characterization of Salmonella strains isolated from meat samples that were obtained from retail markets and butchers. The results in this study indicated that the rep-PCR fingerprinting technique, using REP- and ERIC-PCR, was a rapid, easy-to-perform and, reproducible tool for differentiation of Salmonella strains at the serotype level, with a single-performance protocol. For the genotypic characterization and phylogenetic analysis of Salmonella strains, REP- and ERIC-PCR fingerprinting methods could be a good choice. Therefore, our results endorsed the previous studies42-44 suggesting that rep-PCR fingerprinting is powerful molecular technique for both estimating genetic relatedness and for identification and characterization of Salmonella isolates (Figs. 1, 4).

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