



Investigation of the Antimicrobial Susceptibility Profile, Virulence Genes, and Epidemiologic Relationship of Clinical *Salmonella* Isolates

Klinik *Salmonella* İzolatlarında Antimikrobiyal Duyarlılık Profiline, Virülans Genlerinin ve Klonal İlişkinin Araştırılması

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ABSTRACT

Objectives: The objectives of this study were to investigate the epidemiologic relationship, prevalence of the beta-lactamase and virulence genes of clinical ampicillin-resistant *Salmonella enterica*.

Materials and Methods: *In vitro* ampicillin susceptibilities of 117 *Salmonella enterica* isolates obtained between 2011-2012 from Ege University Hospital, Bacteriology Laboratory of Medical Microbiology Department were examined using disc diffusion assays in accordance with the CLSI guidelines. The MIC levels in the ampicillin-resistant bacteria were determined using the broth microdilution method. The resistant strains were serotyped by the Public Health Institution. Epidemiologic relations of resistant strains were evaluated using ERIC-PCR. The presence of beta-lactamase genes and virulence factors were detected using PCR.

Results: The 117 *S. enterica* strains had ten isolates that were resistant to ampicillin, and the MIC range of ampicillin was found as 512-128 µg/mL. Ampicillin-resistant strains were susceptible to nalidixic acid, ciprofloxacin, cefotaxime, sulfamethoxazole/trimethoprim. Four different serotypes were identified and isolates were grouped into seven clusters. Five isolates carried *bla*_{TEM} and two carried the *bla*_{CTX-M} gene. However, it was determined that *bla*_{SHV} and *bla*_{PER} genes did not exist in these strains. Virulence genes *invA*, *pipD*, and *sopB* were found in all isolates. *sifA*, *pefA*, and *sopE* genes were found in seven, four, and three isolates, respectively.

Conclusion: Our data suggest that the rate of ampicillin resistance in *S. enterica* isolates was 8.5% in the two year period, but this ratio was generally lower than rates abroad. *bla*_{CTX-M} and *bla*_{TEM} genes could be responsible for ampicillin resistance. The *bla*_{SHV} gene, which is highly prevalent in our country, was not found in any strains. *sopB* and *pipD* genes, which might be associated with beta-lactam resistance, were found in all strains. It is also noteworthy that the three isolates containing the *sopE* gene, which is associated with epidemic cases, were of the same serotypes and epidemiologic clusters.

Key words: *Salmonella enterica*, beta-lactamase, virulence factors, ERIC-PCR

ÖZ

Amaç: Bu çalışmanın amacı ampisilin dirençli klinik *Salmonella enterica* izolatlarında epidemiyolojik ilişkinin, beta-laktamaz ve virülans genlerinin araştırılmasıdır.

Gereç ve Yöntemler: Ege Üniversitesi Hastanesi, Tıbbi Mikrobiyoloji Anabilim Dalı, Bakteriyoloji Laboratuvarı'nda 2011-2012 yıllarında izole edilen *S. enterica* kökenlerinin ampisilin duyarlılıkları CLSI önerileri doğrultusunda disk difüzyon yöntemiyle değerlendirildi. Ampisilin dirençli izolatlardaki ampisilin MİK değerleri yine CLSI kriterlerine göre sıvı mikrodilüsyon yöntemiyle belirlendi. Dirençli kökenler Türkiye Halk Sağlığı Kurumu tarafından serotiplendirildi. Kökenlerin epidemiyolojik ilişkisi ERIC-PZR ile incelendi. Beta-laktamaz ve virülans genlerinin prevalansı PZR ile tespit edildi.

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Bulgular: İzole edilen 117 *S. enterica* kökeninde 10 izolat ampisilin dirençli olarak saptandı ve bu izolatların ampisilin Mik aralığı 512-128 µg/mL olarak belirlendi. İzolatlar nalidiksik asit, siprofloksasin, sefotaksim ve sulfametoksazol/trimetoprim antibiyotiklerine duyarlı bulundu. Dört farklı serotip belirlenirken, izolatlar ERIC-PZR'ye göre 7 farklı epidemiyolojik grupta yer aldı. Kökenlerin 5 tanesinde *bla_{TEM}*, iki tanesinde *bla_{CTX-M}* geni saptandı. *bla_{SHV}* ve *bla_{PER}* genleri hiçbir izolatta saptanmadı. *invA*, *pipD*, *sopB* virülans genleri tüm kökenlerde belirlenirken, *sifA*, *pefA* and *sopE* genleri sırasıyla üç, dört ve yedi kökende belirlendi.

Sonuç: Verilerimiz, *S. enterica* izolatlarında iki yıllık dönemde ampisilin direnç oranının %8.5 olduğunu ortaya koymakla birlikte bu oranın genel olarak yurtdışındaki oranlardan düşük olduğu göze çarpmaktadır. *bla_{CTX-M}* ve *bla_{TEM}* genleri ampisilin direncinden sorumlu olabilir. Ancak ülkemizde oldukça yüksek oranda saptanan *bla_{SHV}* genine hiçbir izolatta rastlanmamıştır. Beta-laktam direnci ile ilişkili olabileceği düşünülen *sopB* ve *pipD* genleri tüm suşlarda bulunmuştur. Ayrıca epidemik olgularla ilişkilendirilen *sopE* genini içeren üç izolatin aynı serotipe ve epidemiyolojik sınıfa ait kökenler olması dikkat çekicidir.

Anahtar kelimeler: *Salmonella enterica*, beta-laktamaz, virülans faktörleri, ERIC-PZR

INTRODUCTION

Salmonella spp. are some of the most important agents that lead to enteritis in the world. Additionally, they can lead to more critical health problems such as bacteremia and enteric fever.¹ This group of bacteria include more than 2600 serotypes and consist of two species called *S. enterica* and *S. bongori*. *S. enterica* spp. are responsible for 99% of *Salmonella* infections and *Salmonella* Enteritidis and *Salmonella* Typhimurium are the most commonly isolated serotypes both in our country and in developed countries.² Virulence factors, which responsible for invasion, extraintestinal spread, and intracellular survival, are encoded by genes located in the *Salmonella* pathogenicity island (SPI). Although some pathogenicity islands seem to be preserved in *Salmonella* genus, others are unique for certain serotypes. Based on presence of SPI and SPI features, *Salmonella* serotypes differ from each other in terms of adaptation in host cells, virulence factors, and severity of infections.³

In most cases, resultant infections do not necessitate antibiotic treatment due to the self-limiting nature of disease. However, antibiotic treatment may be necessary for some situations such as invasive infections, advanced age, and immunosuppression.⁴ In such cases, ampicillin is widely used to treat *Salmonella* infections.⁵ Therefore, resistance to ampicillin has emerged and beta-lactam enzymes are primarily responsible of ampicillin resistance.⁶

The objectives of this study were to investigate beta-lactam resistance, the epidemiologic relationship, serotype distribution, and the prevalence of beta-lactamase genes, namely *bla_{TEM}*, *bla_{PER}*, *bla_{CTX-M}*, *bla_{SHV}* and the virulence genes of clinical ampicillin-resistant *S. enterica*.

EXPERIMENTAL

Antimicrobial susceptibility

Salmonella enterica strains isolated in the Bacteriology Laboratory between 2010 and 2012 were used in our study. *In vitro* antibiotic susceptibilities of 117 *S. enterica* isolates were examined using modified Kirby-Bauer disc diffusion assays in accordance with Clinical and Laboratory Standards Institute (CLSI).⁷ Standard ampicillin (10 µg), cefotaxime (30 µg), ciprofloxacin (5 µg) sulfamethoxazole/trimetoprim (23, 75/1.25 µg) (Oxoid, United Kingdom) discs were used to detect resistance. The other part of the study was performed in

ampicillin-resistant strains. Minimum inhibitory concentration (MIC) values of the isolates were also determined using broth microdilutions in accordance with the recommendations of the CLSI and *S. enterica* ATCC 04059 was used as a control strain.⁸

DNA isolation

DNA isolation was performed for use in polymerase chain reaction (PCR) studies. For this purpose, isolates were suspended and homogenized in 200 µL of sterile ultrapure water. The isolates were then incubated in a heat block at 95°C for 10 min. Microtubes were centrifuged at 13,000 rpm for 5 min. The supernatants were transferred to sterile microtubes and stored at -20°C for use in PCR studies.

Serotypes and epidemiologic relation

The strains were serotyped by the Turkish Public Health Agency, National Microbiology Reference Laboratory. The epidemiologic relations of the isolates were analyzed using PCR with enterobacterial repetitive intergenic consensus (ERIC)-2 and ERIC-1R primers.⁹ To evaluate similarity between these isolates, Jaccard coefficients were derived from the banding patterns. Dendrograms were constructed according to the unweighted pair group with arithmetic mean method, using Jaccard coefficients and MEGA software, version 4.0.

Beta-lactamase genes

bla_{CTX-M}, *bla_{TEM}*, *bla_{SHV}*, *bla_{PER}* genes were determined using primers targeting the relevant regions¹⁰⁻¹² using the conventional multiplex PCR method. PCR assays were run in 25 µL amplification mixtures composed of 5 µL bacterial DNA template, 2.5 µL Taq buffer, 1.5 mM MgCl₂, 200 µM dNTP, 30 pmol forward and reverse primers, and 1.25 U Taq polymerase.

Virulence genes

Six different virulence genes were analyzed in two different multiplex PCR reactions, using primers targeting the relevant genes.^{13,14} For this purpose, a 5-µL bacterial DNA template, 2.5 µL Taq buffer, 1.5 mM MgCl₂, 200 µM dNTP, 20 pmol forward and reverse primers, 1.25 U Taq polymerase were prepared in 25 µL volume.

RESULTS

As a result of the disc diffusion test, ten (8.5%) out of 117 *S. enterica* isolates were detected as resistant to ampicillin. These resistant strains were susceptible to ciprofloxacin, cefotaxime,

sulfamethoxazole/trimethoprim. The ampicillin MIC range of the isolates was found as 512-128 µg/mL. The strains were divided into seven different clusters based on ERIC-PCR results. The detected serotypes were as follows; *S. Enteritidis* (n=5), *S. Infantis* (n=2), *S. Typhimurium* (n=1), and *S. Corvallis* (n=1). One isolate could not be serotyped. Five strains involved *bla*_{TEM} genes, two strains contained *bla*_{CTX-M} genes. *bla*_{PER} and *bla*_{SHV} genes were not encountered. *InvA*, *pipD*, *sopB*, which are virulence genes, were detected in all strains, *sifA* in seven, *pefA* in four, and *sopE* in three strains.

DISCUSSION

The *Salmonella* genus comprises many members. Some *Salmonella* serotypes are known to be more commonly isolated. In light of this information and existing data; the most frequently isolated *Salmonella* serotype is known to be *S. Enteritidis*.¹⁵ In our study, five of the ampicillin resistant *Salmonella* strains were determined as *S. Enteritidis*. In 2014, Maraki and Papadakis⁴ determined *S. Enteritidis* as the most commonly isolated serotype of *Salmonella* (37.3%). In another study by Ozdemir and Acar,¹⁶ *S. Enteritidis* was determined as the most commonly isolated serotype from *Salmonella* isolates collected from 4 different provinces of Turkey. Our results show consistency with the literature.

Serotyping is the basic phenotypic method for epidemiologic investigation of isolates. Nonetheless, it cannot differentiate strains of the same serotype. Genotypic methods such as pulsed-field gel electrophoresis (PFGE), ERIC-PCR, and repetitive element palindromic PCR can distinguish the strains more effectively. Although PFGE is the gold standard method for fingerprinting, due to the lack of equipment and to avoid protocols lasting four-five days, a simpler method of ERIC-PCR was preferred.^{17,18} In our study, strains were divided into 7 unrelated clusters using ERIC-PCR with an acceptable (≥0.90) discriminatory index value of 0.92. When the literature and data obtained in this study are evaluated, ERIC-PCR is considered to be a useful and easily applicable method for genotyping of strains.

Recently, *Salmonella* strains have shown resistance against many antibiotic groups. Ampicillin, which is a member of the beta-lactam antibiotics, is the first-line agent used in the treatment of *Salmonella* infections. *Salmonella* strains that are resistant to ampicillin and other beta-lactams pose a risk for public health.⁵ According to our disc diffusion results, ampicillin-resistant strains are susceptible to other groups of antibiotics such as ciprofloxacin, cefotaxime, and sulfamethoxazole/trimethoprim.

According to data gained abroad, the rate of ampicillin resistance against *Salmonella* varies from one country to another. As for studies abroad; in India, ampicillin resistance in *S. enterica* isolates was detected as 25% in 2011.¹⁹ It was 33% in *Salmonella* isolated from children in Cambodia,²⁰ 46% in Korea,²¹ 55% in Spain,²² and 8% in the United States.²³ In Turkey, there have been a few studies of clinical *Salmonella* strains that were isolated from children's hospital. The rate of ampicillin

resistance in *Salmonella* strains were determined as 25.8% in 2012,²⁴ and 19% in 2014.²⁵ However, resistance rates were higher than in our study. According to our knowledge, the stress of starting antibiotic treatment empirically in pediatric patients, before culture results, might be responsible for higher ampicillin resistance rates than in our study.

In our study, five isolates with *bla*_{TEM} genes and two isolates with *bla*_{CTX-M} genes were found. *bla*_{CTX-M}-positive isolates were in the *S. Infantis* serotype. Four of five *bla*_{TEM} gene positive isolates were in *S. Enteritidis* serotype, and the remaining strain was in *S. Typhimurium*. Although the most common beta-lactamase genes of *Salmonella* isolates are variants of *bla*_{CTX-M} and *bla*_{SHV},²⁶ no *bla*_{SHV} gene was found in isolates used in our study. Different rates of beta-lactam resistance genes were reported in studies conducted abroad. Among 20 ampicillin-resistant *Salmonella* isolates, beta-lactamase genes were found to be *bla*_{SHV} 100%, *bla*_{TEM} 85%, *bla*_{CTX-M} 5% in one study;² and in 90 beta-lactam-resistant strains in Spain it was *bla*_{TEM} 22%, and *bla*_{CTX-M} 1%.²² In the Netherlands, a study with 34 *Salmonella* strains isolated from humans and the environment, Hasman et al.⁶ detected the *bla*_{TEM} gene in 19 (55%) strains, the *bla*_{CTX-M} gene in six (17%), and the *bla*_{SHV} gene in three (8%) strains; all 34 isolates were found to be resistant to penicillin. A study conducted on *S. Typhimurium* in 2011 in Turkey detected 23% *bla*_{CTX-M} gene, 76% *bla*_{TEM} gene, and 100% *bla*_{SHV}. The *bla*_{PER} gene could not be detected in any isolates.²⁷

Salmonella bacteria carry many different and complex virulence factors. Investigating the presence of virulence factors coded as different pathogenicity islands will guide us in the matter of discovering *Salmonella* pathogenesis. Our study is thought to be the first in Turkey to investigate the virulence factors of *Salmonella*, at least three virulence factors were detected in all strains. The *invA* gene was determined in all isolates involved in this study, and it was present independent from conditions such as serotype and resistance genes. Dione et al.²⁶ detected the *invA* gene in 99.5% of strains in a study. Another study conducted by Smith et al.⁵ in 2010 encountered the *invA* gene in all. The determination of high levels of the *invA* gene in different regions despite different serotypes and antimicrobial susceptibility profiles indicated the existence of a preserved region in this gene. Thus, the idea of using this gene for rapid diagnosis of *Salmonella* with PCR as a target region has arisen, and this idea has led to studies with positive outcomes.²⁸

Generally, the *sopE* gene, which has been shown to have the lowest prevalence, has been associated with epidemic cases.^{26,29} In our study, the presence of three strains that involve this gene in the same group according to ERIC-PCR and their isolation in a short period of time indicated that they may have been isolated after a community-onset epidemic.

Hughes et al.¹⁴ detected *pipD* and *sopB* genes in all strains and these virulence factors have been associated with enteritis. The detection of these genes in all resistant isolates indicated a possible relationship between these virulence factors and resistance. A study conducted by Dione et al.²⁶ and our data gave similar results. Also, Khoo et al.³⁰ detected alterations

in beta-lactam resistance as a result a mutations created in these two genes. Although our study did not directly show the relationship between resistance and virulence factors, it showed the necessity for further extensive studies about the relationship between these two factors.

The *pefA* gene, which was detected in four strains at the present study, may be located on the same or different plasmid with different virulence (*spv*) genes. Only a fraction of *Salmonella* serotypes carries different-sized plasmids, which are known as serovar specific. However, it is known that not every plasmid-carrying serotype includes the *pefA* gene. Therefore, it is thought that the *pefA* gene has a lower prevalence compared with other virulence factors.³¹ Hughes et al.¹⁴ showed that only three isolates out of 32 involved the *pefA* gene. Skyberg et al.¹³ performed a study on *Salmonella* of different serotypes, the *pefA* gene was found in 11 of 152 strains, and it was only present in serotypes of *S. Typhimurium* and *S. Enteritidis*. The low prevalence of the *pefA* gene in our study is compatible with the results of the other studies. Additionally, three out of four *pefA* gene-carrying strains belonged to the *S. Enteritidis* serotype, and this result is also consistent with the literature data.

The *sifA* gene, which enables the sustained vitality of *Salmonella* bacteria in macrophages, was detected in seven strains in our study. Hur et al.³² detected the *sifA* gene in all of 42 strains. Skyberg et al.¹³ determined the *sifA* gene in 137 out 158 isolates. The existence rate of the *sifA* gene in this study was similar to that of studies conducted abroad.

CONCLUSION

As a result, in light of studies both in our country and abroad, it is known that there are *Salmonella* strains that are resistant to antimicrobial agents, some of which are beta lactamase-producing. Further investigation on the resistance and virulence profiles of *Salmonella* strains will enable us to better understand the pathogenesis of infections and to be able to take better measurements and give proper treatments.

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