

ABANT İZZET BAYSAL UNIVERSITY
THE GRADUATE SCHOOL OF NATURAL AND APPLIED
SCIENCES



ISOLATION, IDENTIFICATION AND DETERMINATION OF SOME
PATHOGENIC CHARACTERISTICS OF *SALMONELLA* SPP. FROM
RETAIL CHICKEN MEAT BY PHENOTYPIC AND GENOTYPIC
METHODS

MASTER OF SCIENCE

CEREN BETÜL EKEN

BOLU, MAY 2017

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APPROVAL OF THE THESIS

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To My Dear Family

DECLARATION

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

CEREN BETÜL EKEN



ABSTRACT

ISOLATION, IDENTIFICATION AND DETERMINATION OF SOME PATHOGENIC CHARACTERISTICS OF *SALMONELLA* SPP. FROM RETAIL CHICKEN MEAT BY PHENOTYPIC AND GENOTYPIC METHODS

MSC THESIS

CEREN BETÜL EKEN

ABANT IZZET BAYSAL UNIVERSITY GRADUATE SCHOOL OF
NATURAL AND APPLIED SCIENCES

DEPARTMENT OF BIOLOGY

(SUPERVISOR: PROF. DR. SEZA ARSLAN)

BOLU, MAY 2017

The genus *Salmonella* is a Gram-negative, rod belongs to the family of *Enterobacteriaceae*. The *Salmonella* genus consists of two species, namely *Salmonella enterica* and *S. bongori*. Particularly, *S. enterica* subspecies and serovars are known to cause infections in humans via contaminated foods or water. In this study, a total of 146 retail chicken meat samples were collected and investigated to determine the presence of *Salmonella* spp. Among the 146 chicken meat samples, 83 (56.6%) were found to be positive for *Salmonella* spp. A total of 116 *Salmonella* isolates were obtained from the 83 contaminated samples. Out of the 116 isolates, 76 (65.5%) were biochemically identified as *S. Typhimurium*, 27 (23.3%) as *S. enterica* subspecies *indica*, 9 (7.8%) as *S. Pullorum*, 2 (1.7%) as *S. enterica* subsp. *arizonae*, 1 (0.9%) as *S. enterica* subsp. *diarizonae* and 1 (0.9%) as *S. enterica* subsp. *salamae*. Some virulence characteristics (siderophore production and biofilm formation) were examined for all the isolates. Among the 116 *Salmonella* isolates, 95 (82.9%) were positive for the siderophore production. Biofilm formation of *Salmonella* isolates was investigated by using the microtiter plate assay. Biofilm formation was detected in 64 (55.2%) of all the examined isolates. The 52 (44.8%) isolates did not produce biofilm. All of the 116 *Salmonella* isolates were confirmed by using genus specific *invA* gene of *Salmonella*. In addition, the *Salmonella* isolates were also examined for the presence of *spvC*, plasmid virulence gene. The *Salmonella* isolates harbored the *spvC* gene was found to be 22 (19%). Besides, in this study, ampicillin (*bla*TEM), tetracycline (*tetA*),

sulfonamide (*sulI*) and class 1 integron resistance genes were investigated. The incidence of the genes, *bla*TEM, *tetA*, *sulI*, class 1 integron was 91.4%, 57.8%, 38.8% and 45.7%, respectively. Erythromycin resistance was observed in all *Salmonella* isolates. The antimicrobial resistance of the *Salmonella* isolates was as follows: tetracycline (77.6%), nalidixic acid (77.6%), streptomycin (58.6%) and chloramphenicol (44.8%). A multidrug-resistance was observed in 93 (80.2%) of the isolates to at least three or more antimicrobial agents. The present study is remarkable to include data on the isolation and identification, some virulence characteristics, antimicrobial resistance genes and antimicrobial resistance profiles of *Salmonella* spp., a significant foodborne pathogen, in chicken meat.



KEYWORDS: *Salmonella*, Chicken Meat, Isolation, Virulence Genes, Antimicrobial Resistance Genes, Antimicrobial Resistance

ÖZET

SALMONELLA TÜRLERİNİN PERAKENDE TAVUK ETİNDEN FENOTİPİK VE GENOTİPİK METOTLARLA İZOLASYONU, İDENTİFİKASYONU VE BAZI PATOJENİK ÖZELLİKLERİNİN BELİRLENMESİ

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Salmonella cinsi Gram-negatif, basil ve *Enterobacteriaceae* ailesine mensuptur. *Salmonella* cinsi *S. enterica* ve *S. bongori* olmak üzere 2 türden oluşur. Özellikle *Salmonella enterica* alt türleri ve serovarları insanlarda kontamine gıda veya su aracılığıyla enfeksiyonlara sebep olarak bilinirler. Bu çalışmada, *Salmonella* türlerinin varlığını belirlemek için toplam 146 adet perakende tavuk eti örneği toplanmış ve araştırılmıştır. 146 tavuk eti örneğinden 83'ü *Salmonella* türleri için pozitif bulunmuştur. Kontamine 83 örnekten toplam 116 *Salmonella* izolatu elde edilmiştir. 116 izolattan 76'sı konvansiyonel olarak *S. Typhimurium*, 27'si *S. enterica* subsp. *indica*, 9'u *S. Pullorum*, 2'si *S. enterica* subsp. *arizonae*, 1'i *S. enterica* subsp. *diarizonae* ve 1'i *S. enterica* subsp. *salamae* olarak tanımlanmıştır. Tüm izolatların bazı fenotipik virülans özellikleri (siderofor üretimi ve biyofilm oluşumu) incelendi. 116 *Salmonella* izolatu arasında 95'i (%82,9) siderofor üretimi için pozitif bulunmuştur. *Salmonella* izolatlarının biyofilm oluşum yetenekleri mikrotitre plak yöntemi kullanılarak araştırılmıştır. İncelenen tüm izolatların 64'ünde (%55,2) biyofilm oluşumu gözlenmiştir. Biyofilm üretmeyenlerin yüzdesi %44,8 olarak bulundu. 116 *Salmonella* izolatlarının tümü, *Salmonella* cinsine özgü *invA* geni kullanılarak teyit edildi. Ayrıca, *Salmonella* izolatları plazmid virülans geni olan *spvC*'nin varlığı açısından da incelenmiştir. *spvC* genini barındıran *Salmonella* izolatlarının sayısı 22 (%19) olarak bulunmuştur. Bu çalışmada ayrıca ampicilin (*blaTEM*), tetrasiklin (*tetA*), sülfonamid (*sulI*) ve sınıf 1 integron direnç genlerinin varlığı araştırılmıştır. Bu genlerin oranları sırasıyla %91,4, %57,8, %38,8 ve %45,7

olarak bulunmuştur. Tüm *Salmonella* izolatlarında eritromisine direnç gözlenmiştir. *Salmonella* izolatlarının antimikrobiyal direnci şöyledir: Tetrasiklin (%77,6), nalidiksik asit (%77,6), streptomisin (%58,6) ve kloramfenikol (%44,8). İzolatların 93'ünde (%80,2) en az 3 veya daha fazla antimikrobiyal ajana karşı çoklu ilaç dirençliliği gözlenmiştir. Bu çalışma, tavuk etinde önemli bir gıda kaynaklı patojen olan *Salmonella* türlerinin izolasyonu ve tanımlanmasına, bazı virülans özelliklerine, antimikrobiyal direnç genleri ve antimikrobiyal direnç profillerine dair verileri içerdiği için önemlidir.



ANAHTAR KELİMELEER: *Salmonella*, Tavuk Eti, İzolasyon, Virülans Genler, Antimikrobiyal Direnç Genleri, Antimikrobiyal Direnç

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LIST OF ABBREVIATIONS AND SYMBOLS

ATCC	: American Type Culture Collection
<i>bla</i>TEM	: Ampicillin Resistance Gene
CDC	: Centers for Disease Control and Prevention
cfu	: Colony Forming Units
CLSI	: Clinical and Laboratory Standards Institute
DNA	: Deoxyribonucleic Acid
EDTA	: Ethylenediaminetetraacetic Acid Disodium Salt Dihydrate
ISO	: International Organization for Standardization
<i>invA</i>	: Invasion Gene
nm	: Nanometer
PCR	: Polymerase Chain Reaction
SPI	: <i>Salmonella</i> Pathogenicity Island
<i>spvC</i>	: <i>Salmonella</i> Plasmid Virulence Gene
<i>sulI</i>	: Sulfonamide Resistance Gene
<i>tetA</i>	: Tetracycline Resistance Gene
TTSS	: Type Three Secretion System
WHO	: World Health Organization

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1. INTRODUCTION

Foodborne diseases are caused by consumption of water and foods including vegetables, dairy and meat products contaminated with pathogenic bacteria. Mainly, the products of animal origin such as poultry and meat products are the most significant reservoirs for many pathogens, particularly *Salmonella* (Bhunia, 2008; Akiyama et al., 2011; Nataro et al., 2011; Mezal et al., 2013). *Salmonella* is a non-spore forming, Gram-negative rod and an important zoonotic agent belongs to the family of *Enterobacteriaceae*. *Salmonella* includes more than 2,500 serotypes, belonging to two species: *Salmonella enterica* and *S. bongori*. *Salmonella enterica* is further divided into six subspecies as follow: subsp. *enterica* (I), subsp. *salamae* (II), subsp. *arizonae* (IIIa), subsp. *diarizonae* (IIIb), subsp. *houtenae* (IV), and subsp. *indica* (VI). *Salmonella enterica* has predominantly significant serovars including *Salmonella* Typhimurium, Enteritidis, and Typhi which cause diseases in humans (Popoff and Le Minor, 2005, Nataro et al., 2011).

Salmonella species can be present in the intestine of humans and warm-blooded animals such as ducks, chicken, turkey, bird, pig, cattle. *Salmonella* can also be isolated from intestinal tracts of cold-blooded animals such as reptiles, turtles, and insects. *S. enterica* subsp. *enterica* is mainly present in humans and warm-blooded animals and is responsible for about 99% of *Salmonella* infections in mammals. On the contrary, *S. bongori* and the *Salmonella enterica* subspecies except *S. enterica* subsp. *enterica*, generally found in the environment, and in the cold-blooded animals, and rarely in humans (Ellermeier and Schlauch, 2006; Bhunia, 2008; Eng et al., 2015).

Salmonella spp. are intracellular pathogens which can cause gastroenteritis (enterocolitis or salmonellosis), septicemia, and typhoid (enteric) fever (Marcus et al., 2000; Levinson and Jawetz, 2002; Bhunia, 2008). *Salmonella* is one of the major causes of foodborne infections in Europe. Gastroenteritis is the most common *Salmonella* infection worldwide, specifically *Salmonella* Typhimurium is the most common serovar that causes gastroenteritis in the United States. Non-typhoidal *Salmonella* strains usually cause intestinal infections with diarrhea, fever, and abdominal cramps, whereas less often non-typhoidal *Salmonella* strains can cause extraintestinal illnesses including septicemia, urinary tract infection, or osteomyelitis,

specifically in immunocompromised individuals (Rumeu et al., 1997; Levinson and Jawetz, 2002; Eng et al., 2015). In the developing countries from past to present, enteric fever is predominantly a serious problem for humans caused by *S. Typhi* and *Paratyphi*. *Salmonella* can cause infections in all people; however, the rate of infections is considerably high in infants, and children (Levinson and Jawetz, 2002; Bhunia, 2008; Nataro et al., 2011).

Pathogens have many virulence characteristics which are important for the pathogenesis, and they include iron uptake, invasion, adhesion, and survival in the host, damage in the host cell and tissue and causing infections. Iron uptake via siderophore is an important factor for the *Salmonella* pathogenesis, especially in low iron concentrations (Bhunja, 2008). Biofilm formation is other important virulence factor for the *Salmonella* pathogenicity. *Salmonella* have the ability to form biofilms on plastic surfaces, rubber, glass, stainless steel, medicinal equipments, epithelial cells of animals and humans (Lianou et al., 2012). The *invA* gene is an effective factor in the pathogenesis of *Salmonella*. The ability of invasion encoded by the *invA* gene allows *Salmonella* to enter the cells in the intestinal mucosa. On the other hand, *Salmonella* can survive in the macrophages due to the presence of plasmid virulence gene (*spvC*) (Wilson et al., 2002; Sallam et al., 2014).

The emergence of antimicrobial resistant bacteria since the widespread use of antimicrobials has led to represents serious global threat to modern medical practice. Overuse or misuse of antimicrobial agents in medicine, agricultural areas, and poultry is caused the increase of resistant bacteria (Bolton et al., 1999; Briggs and Fratamico, 1999; Cosby et al., 2015). The routine use of antimicrobials, specifically for preventing and treating diseases, and supporting growth as feed additives in animals of food-producing may cause increase in the frequency of antimicrobial resistant pathogens. However, the antimicrobial resistance genes have a significant role for the spread of antimicrobial resistance among the pathogens which are transferred from one organism to another between both the same species and different species. Furthermore, the spread of resistant bacteria can occur from animal to human by ingestion of contaminated food via food chain and cause serious health problems (Threlfall et al., 2000; White et al., 2001; Wang et al., 2015).

1.1 *Salmonella* spp.

1.1.1 Classification and Taxonomy

Salmonella was first isolated in 1885 from a pig by Daniel Elmer Salmon. *Salmonella* genus was formed by Lignieres (Ellermeier and Slauch, 2006). *Salmonella* nomenclature scheme has changed in many times based on conventionally methods which are biochemically and serologically and on principles of numerical taxonomy and DNA homology. In the early 1920's, White analyzed serologically *Salmonella* O and H antigens. Therefore, each *Salmonella* serovar was classified as a species by Kauffmann, because his concept was "one serovar-one species". Later DNA relatedness studies confirmed that *Salmonella* serovars formed single DNA hybridization group with seven subgroups described by studies of the thermal stability of hybrids. The seven DNA subgroups could be separated by biochemical characteristics. However, subspecies V might possibly signify a second species. Finally, as a result of multilocus enzyme analysis, *S. enterica* subsp. *bongori* was found to be significantly different from other subspecies. Based on this divergence and on DNA relatedness data, *S. enterica* subsp. *bongori* have been raised to species level as *S. bongori* in the new combination (Popoff and Le Minor, 2005).

1.1.2 The genus *Salmonella*

Salmonella is an important foodborne pathogen belongs to the *Enterobacteriaceae* family in the *Gamma-proteobacteria* subdivision. The *Enterobacteriaceae* family members are widely mesophilic, Gram-negative, non-sporeforming straight rods, facultative anaerobe, oxidase negative, and usually catalase positive (Popoff and Le Minor, 2005).

The *Salmonella* cell sizes are 0.7-1.5 μm x 2.0-5.0 μm . The genus *Salmonella* comprises two species: *Salmonella enterica* and *Salmonella bongori*. *Salmonella enterica* has six subspecies and these subspecies can also be shown in roman numerals as follows: *Salmonella enterica* subsp. *enterica* (I), *S. enterica* subsp. *salamae* (II), *S. enterica* subsp. *arizonae* (IIIa), *S. enterica* subsp. *diarizonae* (IIIb), *S. enterica* subsp. *houtenae* (IV), and finally, *S. enterica* subsp. *indica* (VI). The *Salmonella* genus has serovars and most of these serovars which are human

pathogens such as *S. Typhimurium*, *S. Typhi* and *S. Paratyphi* belong to subspecies *enterica* (Ellermeier and Schlauch, 2006; Nataro et al., 2011).

Salmonella species can grow in a range of temperature 5-45°C. On the other hand, their optimal temperature is 35-37°C, and they are mesophilic bacteria. The optimum water activity [a_w] for the growth of *Salmonella* is 0.995. They are very resistant to drying, but they die at the temperature of pasteurization. *Salmonella* is a chemoorganotrophic organism; having both a respiratory and fermentative type of metabolism. Mostly, the genus members are aerogenic; but *Salmonella Typhi* is an important exception and *S. Typhi* never produces gas (Popoff and Le Minor, 2005). The genus members are also mostly motile by peritrichous flagella. *S. Pullorum* and *S. Gallinarum* are different from other *Salmonella* species because they are non-motile organisms. The *Salmonella* genus utilizes citrate as a carbon source, mainly produces H_2S , reduces nitrate to nitrite, decarboxylase lysine and ornithine to cadaverine and putrescine, respectively. The production of urease, lipase, deoxyribonuclease, deamination of phenylalanine and tryptophan are negative for this genus (Popoff and Le Minor, 2005; Ellermeier and Schlauch, 2006; Josefsen et al., 2011).

1.1.3 Natural Habitats

The members of genus *Salmonella* are isolated from feces-contaminated foods or water, sewage and the intestinal tract of humans, cold-blooded animals (lizard and snake) and warm-blooded animals (chicken, duck, dog, bird and pig). Particularly, *Salmonella enterica* subspecies *enterica* can be found in warm-blooded animals and infect humans, whereas all other *Salmonella enterica* subspecies and *Salmonella bongori* are commonly found in cold-blooded animals and infrequently infect humans (Ellermeier and Schlauch, 2006; Bhunia, 2008; Nataro et al., 2011). *Salmonella enterica* subsp. *salamae*, subsp. *arizonae* and subsp. *diarizonae* are frequently isolated from the intestine of cold-blooded animals and infrequently obtained from humans and warm-blooded animals (Levinson and Jawetz, 2002; Popoff and Le Minor, 2005). *Salmonella* species and their habitats are given in Table 1.1.

Table 1.1. Natural habitat of *Salmonella* species and subspecies*

<i>Salmonella</i> species and subspecies	Natural Habitat
<i>S. enterica</i> subsp. <i>enterica</i> (I)	Warm-blooded animals
<i>S. enterica</i> subsp. <i>salamae</i> (II)	Cold-blooded animals and environment
<i>S. enterica</i> subsp. <i>arizonae</i> (IIIa)	Cold-blooded animals and environment
<i>S. enterica</i> subsp. <i>diarizonae</i> (IIIb)	Cold-blooded animals and environment
<i>S. enterica</i> subsp. <i>houtenae</i> (IV)	Cold-blooded animals and environment
<i>S. enterica</i> subsp. <i>indica</i> (VI)	Cold-blooded animals and environment
<i>S. bongori</i>	Cold-blooded animals and environment

*Modified from Popoff and Le Minor, 2005; Bhunia, 2008.

The *S. enterica* subsp. *enterica* serovars can be divided into three categories which are host-adapted, host-restricted and generalists. Host-adapted known as primarily infecting one host; however it can cause disease in others. Host-restricted serovars are known to infect only a single host. In contrast, the generalists are capable of infecting a variety of animals, although the course of the disease may differ in each host (Ellermeier and Schlauch, 2006). *Salmonella enterica* serovars and their specific hosts are listed in Table 1.2.

Table 1.2. Some of the *Salmonella* serovars and their targets*

Classification	<i>Salmonella enterica</i>	Target host
Host restricted	serovar Typhi	Humans
	serovar Paratyphi A	Human
Generalist	serovar Typhimurium	Humans, poultry
	serovar Enteritidis	Humans, poultry, swine
Host adapted	serovar Gallinarum	Poultry
	serovar Choleraesuis	Swine
	serovar Dublin	Cattle

*Modified from Ellermeier and Schlauch, 2006; Bhunia, 2008.

1.1.4 Serotyping

Salmonella are serotyped on the basis of the antigenic structure of the O antigen (heat stable-somatic antigen), the H antigen (heat labile; flagellin), and the V_i antigen (capsule), if present. Somatic antigen, O, is a cell wall antigen which is the outer polysaccharide part of the lipopolysaccharide. The heat labile (H) antigen is on the flagellar protein and only found in flagellated organisms have H antigens. Most of the *Salmonella* serotypes are diphasic, except *Salmonella* serovar Gallinarum. This serovar is non-motile organism due to have no expressed any flagella antigen. The V_i antigen is a capsular polysaccharide which is produced by serovar Typhi and Paratyphi C strains, and occasionally by strains of serovar Dublin (Levinson and Jawetz, 2002; Ellermeier and Schlauch, 2006).

Up to date, it has been reported that *Salmonella* has more than 2,500 serotypes. Some of the serotypes such as *S. Typhimurium* and *S. Enteritidis* can be widespread; however, other serotypes may regionally distribute. All serotypes are determined according to the Kauffmann-White scheme and antigenic formula of the serotypes are defined and conserved by the World Health Organization (WHO) Collaborating Centre for Reference and Research on *Salmonella* at the Pasteur Institute, Paris, France (WHO collaborating center). Moreover, in the annual updates, new serotypes are listed in the Kauffmann-White scheme. Serotyping is solely suitable on biochemically identified *Salmonella* strains because the antisera show cross-reaction with other bacteria (Brenner et al., 2000; Popoff and Le Minor, 2005; Ellermeier and Schlauch, 2006).

1.1.5 Virulence Factors of *Salmonella* spp.

1.1.5.1 Siderophore Production

Iron is one of the basic elements for the bacterial growth and survival. Bacteria must acquire iron from host. Furthermore, human body has low iron concentration since iron is bound in the form of lactoferrin, transferrin, or ferritin. Moreover, free iron (Fe³⁺) is also rarely found in environment, and bacteria can

secrete iron-complexing agents known as siderophores to satisfy their iron needs in poor iron concentration. Bacteria use siderophores to benefit the course of iron intake in the environment. Siderophore production can be main effect in the capacity of pathogens to cause illnesses, and they often stringent bound to many proteins, like hemoglobin, lactoferrin, and transferrin in tissue fluids. The free iron (Fe^{3+}) concentration is reduced to approximately one molecule per liter by these proteins (Hantke et al., 2003; Loudon et al., 2011).

1.1.5.2 Biofilm Formation

Bacteria can form layers on the surfaces of food contact, or in the mouth and in the intestine. Biofilm formation facilitates bacterial colonization in food processing or in the equipments, and in host tissues as well. Biofilm formation ability has been indicated to enhance pathogenic bacteria tolerance to under stress and their ability to escape from the immune system of host. Bacteria with biofilms are inherently protected from their surroundings and often exhibit increased resistance to host defence and antimicrobial agents by making these infections difficult or impossible to eradicate (Gong et al., 2013). Furthermore, biofilms are significant in human diseases such as intestinal colonization endocarditis and lung infections. The cells in biofilms are accepted a potential source for food sample contamination. Particularly, biofilms may have a critical role for the survival of *Salmonella* in adverse conditions of environment such as in animal slaughterhouses and processing plants (Bhunia, 2008; Wang et al., 2013).

1.1.5.3 *Salmonella* Pathogenicity Islands

Survival of bacteria in the host is related with a delicate balance of many genes acting in the correct location at the correct time. Pathogenicity islands are harbored large genes group provided to a specific virulence, which are generally expressed at a specific time during the infection process. SPIs are highly likely to have been acquired by horizontal transfer from phages or plasmids of unknown origin and are well conserved regions among *Salmonella* serotypes (Marcus et al., 2000).

Salmonella pathogenicity islands are necessary for the systemic infection, survival in macrophages, fimbrial expression, and iron and Mg^{2+} uptake. SPI-1 is primarily necessary for bacterial permeation of the epithelial cells. SPI-1 functions are invasion and iron uptake, however SPI-2 is responsible from invasion and systemic infection. SPI-3 and SPI-4 are necessary for growth and bacterial survival within the macrophages (Marcus et al., 2000). Moreover, SPI-5 can be responsible for the enteric infection. Besides, SPI-6 contains fimbria and invasion genes and it has genes which are unknown functions. The other locus is SPI-7 which is called the major pathogenicity island for the serovar Typhi and encodes gene for capsule antigen (Vi) and is specific for serovar Typhi and Paratyphi. SPI-8 is a specific locus for the serovar Typhi. Furthermore, SPI-9 is a locus which is found on the chromosome in *S. Typhimurium*, and also encodes the type 1 secretion system in the serovar Typhi. Lastly, SPI-10 is encoded genes for the fimbriae (Marcus et al., 2000; Bhunia, 2008; Gunaydin and Sen, 2011).

1.1.5.4 Type III Secretion System

Gram-negative bacteria have secretory channels known as secretion system. The type III secretion (T3SS or TTSS) is a complex system which has been isolated from *Salmonella*, *Yersinia*, *Shigella*, and *Escherichia*. TTSS is related with pathogenicity of bacteria and also known as the molecular injector (Ellermeier and Slauch, 2006). SPI-1 codes TTSS which plays a significant role in virulence of *Salmonella*; during a close contact with the host cells, bacteria use the TTSS to inoculate proteins of virulence into the cell. It is responsible for the transmission of virulence proteins directly into the host cells. SPI-1 effectors cause changes of the cytoskeleton in the eukaryotic cell by simplifying bacterial engulfment and attack of the intestine. TTSS is regulated highly, and proteins are only secreted when the bacteria sense specific environmental signals (Marcus et al., 2000; Gerlach and Hensel, 2007; Bhunia, 2008).

1.1.5.5 Chromosomal Encoded Virulence Factors

Some virulence genes of *Salmonella* can be found on chromosome. Most chromosomal encoded genes of virulence are found in the *Salmonella* pathogenicity islands (SPIs) (Swamy et al., 1996; Marcus et al., 2000). Lately, the investigation of virulence genes of *Salmonella* isolates has been widely used to characterize and determine the ability of pathogenicity. The invasion gene operon, *Inv*, found in SPI-1 is crucial for the virulence of *Salmonella*; it is thought to be necessary for the internalization of deeper tissues to attack. In most studies *invA* has been preferred to detect and characterize *Salmonella* using PCR assays in foods to target virulence genes which are related with genus specific genes (Bolton et al., 1999; Park et al., 2014).

1.1.5.6 Plasmid-Encoded Virulence Factors

The plasmid-associated virulence of *Salmonella* is a significant part for survival and growth in the host. Plasmid virulence is one of several *Salmonella* virulence determinants complicated in the host cells. The presence of virulence plasmids is limited in *Salmonella* genus and has been identified in *Salmonella* serovars Typhimurium, Enteritidis, Dublin, Pullorum, and Gallinarum. Several *Salmonella* loci are associated in macrophage survival but, specifically, virulence plasmids allow carrier strains to rapidly multiply within host cells and overthrow host defense mechanisms (Ellermeier and Schlauch, 2006; Swamy et al., 1996). The *spvC*, *Salmonella* plasmid virulence gene, can interfere with the host immune system and also it is essential for systemic infection (Bolton et al., 1999). This virulence gene promotes prolific growth of the *Salmonella* spp. in the reticuloendothelial host tissues. The *spvC* expression is induced when bacteria are in the stationary phase or under stress conditions such as low pH (Mohamed et al., 2014). Moreover, for the full expression of virulence of *Salmonella* Typhimurium the incidence of both *invA* and *spvC* are necessary for human typhoid and systemic disease (Swamy et al., 1996). Moreover, the virulence plasmid of *Salmonella* comprises a fimbrial operon (*pef*) encodes adhesion, which is implicated in the small

intestine colonization and enteropathogenicity of the organism (D'Aoust et al., 2001).

1.1.5.7 Other Virulence Factors

Salmonella virulence is also related with toxins and these toxins known as exotoxin and endotoxins. Acute inflammation is begin in the host epithelial cells when invasion by *Salmonella*. Lipopolysaccharide (LPS) is responsible for the acute inflammation and endotoxins are related with lipid A, which is a partial of the lipopolysaccharide (Peterson, 1996). Exotoxins comprise enterotoxins and cytotoxins. Enterotoxins are heat-labile enzymes, whereas cytotoxins are heat-stable toxins and can also inhibit the protein synthesis. Endotoxins have been found to stimulate a wide range of biological responses, while exotoxins have the capability to kill mammalian cells. *Salmonella* generally elaborate a thermolabile cytotoxic protein. Some *Salmonella* serovars may produce thermolabile cytotoxins, such as *S. Choleraesuis*, *S. Typhi*, *S. Enteritidis* (D'Aoust et al., 201; Foley et al., 2013).

1.1.6 Integrins and Antimicrobial Resistance Genes

Mobile genetic elements comprise of plasmids, transposons, and also integrins. The genes of antimicrobial resistance play a role in the spread of transferring horizontally or vertically as part of resistance plasmids or conjugative transposons and in the increase of multidrug-resistance (Khan et al., 2009).

Salmonella genomic island is a locus which is encoded genes for antibiotic resistance and consists of two integrins. Integrins have variable length that contain 5' conserved integrase gene (*intI*), mobile antimicrobial resistance genes are known as cassettes. An integration site for the gene cassette, attachment (*attI*) is also present in integrins. Integrins have been associated with the transmission of resistance and they can also play a role in the spread of antibiotic resistance determinants. These determinants cause the rapid evolution of multiple antimicrobial resistance pathogens, especially between different Gram-negative isolates from clinical sources. In some studies have reported integrins presence amongst Gram-positive species,

however, mobile integrons are commonly found in Gram-negative pathogens (Tosini et al., 1998; White et al., 2001; Rice and Bonomo, 2011; Domingues et al., 2015).

The most common integron types are class 1 and class 2 integrons which are identified in *Salmonella*. Furthermore, it has been reported that multi-drug resistance is related with the integrons. These integrons are significant in the dissemination of resistance genes among bacteria. Class 1 integron has been reported as the most common integron associated with antimicrobial resistance, especially in clinical isolates (Tosini et al., 1998; Briggs and Fratamico, 1999; Nataro et al., 2011; Silva et al., 2012; Mohamed et al., 2014; Domingues et al., 2015).

Integrons are an essential source for expand of the *bla* genes, and *tet* genes may be found included within integrons, and conjugative transposons. Moreover, plasmid-mediated and transferable resistance to sulfonamides has been informed in frequently Gram-negative bacteria. Besides, the sulfonamide genes (*sulI*, *sulII* and *sulIII*) are frequently combined into multidrug-resistance integrons (Rice and Bonomo, 2011).

1.2 Clinical Significance

Salmonella is one of the main problems of foodborne illnesses in both developed and developing countries and also causes typhoid fever, rarely bacteremia. This genus poses a serious risk to public health. The epidemiology of infections is related with the ingestion of food or water contaminated by feces and is generally resulted in gastroenteritis. Initial symptoms appear within 8 to 72 h as nausea, vomiting, abdominal cramp approximating appendicitis, chills and diarrhea followed by muscle pain and moderate fever. Persons of all ages can affected from *Salmonella* infections; however infants, young children, elderly and immunosuppressed individuals are more sensitive to infections than healthy adults. The infectious dose of *Salmonella* is considered to be $10^6 - 10^8$ cfu/g cells, particularly. In addition, *S. Typhi* can cause enteric fever and it is a significant problem in developing countries. Another disease is septicemia caused by *S. Choleraesuis* and it can rarely see in humans (D'Aoust et al., 2001; Levinson and Jawetz, 2002; Bhunia et al., 2008; Zorba, 2011).

1.2.1 Salmonellosis (Non-Typhoidal Form)

Non-typhoidal *Salmonella* strains that can cause intestinal infections are accompanied by diarrhea, abdominal cramps and fever frequently in the last week or longer (D'Aoust et al., 2001; Levinson, Jawetz, 2002; Nataro et al., 2011). The majority of the cases are sporadic; however, outbreaks are reported. Specifically, *Salmonella* subspecies *enterica* serovars can cause salmonellosis worldwide. *Salmonella* Typhimurium as the most common serovar cause gastroenteritis in the United States. Other enteric serovars related with gastrointestinal diseases are as follows: *S. Enteritidis*, and *S. Virchow*, *S. Heidelberg*, *S. Newport*, and *S. Dublin*. Mortality incidence for salmonellosis is 4.1%. Approximately 1.4 million people are infected by *Salmonella* every year in the U.S. and these cases result in hospital or death (Marcus et al., 2000; Levinson and Jawetz, 2002; Ellermeier and Schlauch, 2006; Su et al., 2006; Bhunia 2008; Erkmen and Bozoğlu, 2016).

1.2.2 Enteric Fever (Typhoidal Form)

In developing countries, enteric fever is accepted to be a significant cause of mortality arising from *S. Typhi* and *Paratyphi* (A, B, C). These are only for the human pathogen and *S. Typhi* transmission occurs only person-to-person. Other syndrome is similar to typhoid fever caused by paratyphoidal *Salmonella* strains, i.e., *Salmonella* serovar *Paratyphi* (A, B, C). Besides, *Paratyphi* B is a diverse serotype that is related with both paratyphoid fever and gastroenteritis (Levinson and Jawetz, 2002; Prager et al., 2003; Nataro et al., 2011; Erkmen and Bozoğlu, 2016). The typical symptoms are high fever and headache. In adults all the symptoms except diarrhea can be seen. Fatal complications of typhoid most commonly occur in the second or third week of the illnesses (Nataro et al., 2011). Treatment for enteric fever vaccines is available and effective, particularly in children. The enteric fever treatment is based on therapy with antibiotic usage which includes usage of chloramphenicol, ampicillin, or trimethoprim/sulfamethoxazole to reduce the systemic infection (D'Aoust et al., 2001).

1.2.3 Septicemia

S. serovar Choleraesuis is a host-adapted pathogen for the swine and can cause septicemia. Another host adapted serovar *S. Dublin* is a pathogen for the bovine and cause to bacteremia. These serovars rarely cause infections in humans. The symptoms of the septicemia caused by serovar *Choleraesuis* are fever, chilling, vomiting and anemia. Furthermore, infection when passing to other tissues can result in septic shock and death (Levinson and Jawetz, 2002; Bhunia, 2008; Zorba et al., 2011).

1.2.3.1 Mechanism of Pathogenesis

Human *Salmonella* infections depend on the ability of the invasion pathogen to attach and enter the intestinal epithelial cells. *Salmonella* can survive in several host environments and blockades to produce a productive infection. The first of these barriers is the acidic condition confronted in stomach. Motility could not be crucial for bacterial internalization, whereas the role of motility in the adherence and attack processes is indefinite (D'Aoust et al., 2001). After passing through the stomach, *Salmonella* can be colonized in the intestine in which it specially targets and attacks the most distal Peyer's patch of the small intestine. In gastroenteritis, pathogen spreads to organs (liver, spleen and bone marrow) where the bacteria continue to reproduce within the macrophages. At the end of the spread, pathogen causes septicemia and death of the host (Ellermeier and Schlauch, 2006).

1.2.3.2 Transmission

Direct or indirect contact with faeces of an infected human, animal, food or water can cause *Salmonella* transmission to humans. Specifically, *Salmonella* species can be transmitted by the fecal-oral route or rarely person-to-person and also by consumption of food or water contaminated with faeces (Fearnley et al., 2011). A variety of foods have been associated as vehicles transferring salmonellosis to humans including cheese, fruits, seafood and vegetables. Frequently, animal origin

foods such as poultry or meat products can contaminate with *Salmonella* and they can cause infections (Khan et al., 2009).

1.3 Antimicrobial Susceptibility

Antimicrobial therapy is not suggested for uncomplicated *Salmonella* enterocolitis. Antimicrobial resistance, particularly multiple-drug resistance has been noted in several non-typhoidal *Salmonella* serotypes. Mobile genetic elements such as gene cassettes have a critical role for the antibiotic resistant pathogens. Most antimicrobial resistance genes are transferred to each other via transposons or plasmids particularly in Gram-negative organisms (Tosini et al., 1998; Briggs and Fratamico, 1999).

Salmonella strains may be susceptible for fluoroquinolones, but some of the strains are resistant to nalidixic acid and result in treatment failure. Foods of animal origin such as domestic livestock are main sources to antimicrobial resistant pathogens. Some antimicrobial agents such as tetracyclines can be used for the treatment of some poultry diseases and also as feed additives, and they can lead to increase of the resistance. *Salmonella* Typhimurium is one of the multidrug-resistant *Salmonella* serovars. In 2001, *S. Typhimurium* strains received from the CDC were found to be resistant to at least one of the tested antimicrobial agents and 35% were found to be common resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracycline (ACSSuT) (Tosini et al., 1998; Briggs and Fratamico, 1999; Ellermeier and Schlauch, 2006; Jay et al., 2005; Nataro et al., 2011).

Trimethoprim/sulfamethoxazole, ampicillin or amoxicillin are the best choices for the treatment of the *Salmonella*. Since fluoroquinolones are not approved for the younger than 18 years of age, ceftriaxone and cefotaxime can be used (Singh, 2017).

1.4 Food Association of *Salmonella* Species

Many foodborne pathogenic bacteria are found in environmental generally found in soil, water, animals and plants. Besides, foods are mostly food animals and poultry, the most significant reservoirs for the pathogens. *Salmonella*, mesophilic

bacteria, is a significant foodborne pathogen. Fresh meats such as poultry, dairy products such as cheese, raw milk, eggs, and homemade ice cream containing fresh eggs and also seafood may carry *Salmonella*. Furthermore, the genus *Salmonella* can survive and grow inside the veins of plant tissues. Recently, human salmonellosis caused by consumption of fruits and vegetables contaminated with *Salmonella* has increased (D'Aoust et al., 2001; Tompkins et al., 2001; Bhunia, 2008; Nataro et al., 2011; Singh, 2017). The occurrence of *Salmonella* has been reported in a variety of meat products as chicken, turkey, beef, and duck. Compared to products such as ready-to-eat meat (3.1%), pasteurized eggs (14.6%), ground beef (7.5%), broiler chicken (20%), market hogs (8.7%) and steers and heifers (1%), *Salmonella* was detected in 49.9% of ground turkey and 44.6% of ground chicken meat (Rajan et al., 2016).

Mostly, poultry products can be heavily contaminated with *Salmonella*. Food animals may be infected with direct or indirect contact with especially *Salmonella* on the farm from birds, feed and infected food handlers. Raw foods of animal origin together with cross-contamination through inadequate processing and personal hygiene are significant related factors to cause foodborne outbreaks (Nayak et al., 2004).

Salmonella outbreaks were reported from chicken meat in many countries around the world. In fact, contaminated meat, poultry and dairy products are probably the most common cause of human salmonellosis. *Salmonella enterica* is an important pathogen which is a cause of foodborne illness in humans with 95,548 reported cases in the Europe in 2011. Milk-borne outbreaks by *Salmonella* are reported as 26% in the United States between 1973 and 1992. In Korea, all bacterial foodborne illnesses caused by *Salmonella* were at the rate of 23.8% between 1981 and 1995. Nevertheless, in Japan for during the same period, the cases were low in the rate (19.9%). Besides, *S. Enteritidis* has been a cause of a series of outbreaks in the U.S and in some parts of Europe. Likewise, ready-to-eat foods, predominantly those which are prepared by sellers in the streets and which are composed of meat and poultry have been recognized as potential sources of foodborne bacteria (Jay et al., 2005; Ahmed and Shimamoto, 2014).

2. THE AIM AND SCOPE OF THE STUDY

The aims of the present study were to investigate the prevalence of *Salmonella* spp. in chicken meat samples, to confirm *Salmonella* spp. using the phenotypic and genotypic methods, and to detect some virulence characteristics which play a significant role in the pathogenesis of *Salmonella* spp. based on biochemical and molecular methods. In addition, the study aimed to determine antimicrobial resistance genes (*bla*TEM, *tetA*, *sulI*, and class 1 integron) and antimicrobial resistance profiles of *Salmonella* spp. from chicken meat.



3. MATERIALS AND METHODS

3.1 Sample Collection

A total of 146 chicken meat products were collected from local butchers, supermarkets and groceries in Bolu, Turkey. All samples were transported to the laboratory under aseptically conditions in sterile bags and reserved in ice containers until they were analyzed in the laboratory.

3.2 Isolation and Identification of *Salmonella* spp.

Salmonella isolation and identification procedures from food samples were analyzed using the ISO 6579 'Microbiology of food and animal feeding stuffs- Horizontal method for the detection of *Salmonella* spp. (ISO, 2002). The detection method used for the isolation of *Salmonella* spp. from chicken meat products is shown in Figure 2.1.

ISO: 6579 STANDARD-HORIZONTAL METHOD

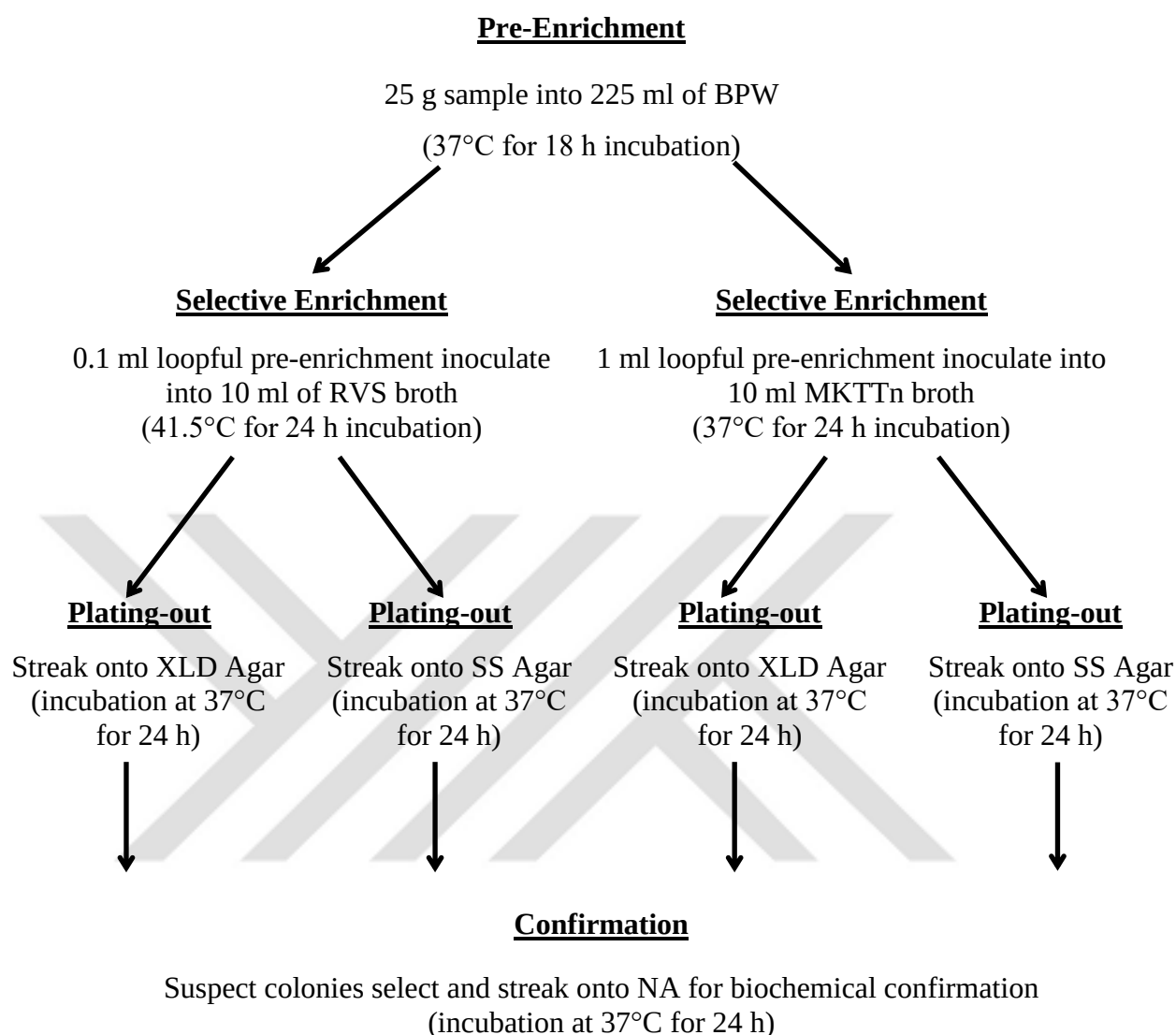


Figure 2.1. Scheme of the isolation and identification of *Salmonella* spp. according to ISO: 6579 method. BPW, Buffered Peptone Water; RVS, Rappaport Vassiliadis broth; MKTTn, Muller-Kauffmann Tetrathionate Novobiocin broth; XLD, Xylose Lysine Deoxycholate Agar; SS, Salmonella Shigella Agar; NA, Nutrient Agar.

In this study, chicken meat samples were analyzed to determine the incidence of *Salmonella* spp. according to ISO 6579:2002. A 25 g of chicken meat samples was aseptically added into 225 mL of BPW (Merck, Darmstadt, Germany) and homogenized by stomacher (Bagmixer 400, Interscience, Paris, France). The samples in BPW were incubated at 37°C for 18-20 h. RVS (Fluka, Steinheim, Germany) and MKTTn (Merck) Broths were inoculated respectively with the culture obtained from pre-enrichment. 0.1 mL of the culture was inoculated into 10 mL of the RVS Broth and incubated at 41.5°C for 24h. In parallel to RVS, 1 mL of the culture was transferred to 10 mL MKTTn Broth and incubated at 37°C for 24 h. After, a loopful of enriched RVS and MKTTn cultures were streaked onto both SS Agar (Merck) and XLD Agar plates (Merck). The SS Agar and XLD Agar plates were incubated at 37°C for 24 h. The suspicious colonies were detected usually with black center and colorless on the SS Agar plates. Pink, rarely with black center, or yellow, mucoid colonies on XLD Agar plates were observed as suspected *Salmonella* spp. At least five suspect colonies were selected from both SS and XLD Agar plates and streaked on Nutrient Agar for further identification. All the isolates for presumptive *Salmonella* spp. were confirmed with following biochemical tests: Gram-staining, oxidase and catalase tests, indole test, at Voges-Proskauer of acetoin production and acid reaction with methyl red, motility at 37°C, utilization of citrate, H₂S production on Triple-Sugar Iron (TSI) Agar. Besides, the ornithine and lysine decarboxylase, urease, growth in potassium cyanide (KCN) and carbohydrate utilization such as sucrose, sorbitol, lactose, dulcitol, melibiose, and xylose were used. *S. enterica* subsp. *enterica* (ex Kauffmann and Edwards) Le Minor and Popoff serovar Typhimurium (ATCC 13311) was used a reference strain in all microbiological experiments and for biochemical identification of *Salmonella* spp. Biochemical tests are used for identification of *Salmonella* isolates obtained from this study is shown in Table 2.1. All the isolates were identified according to The Prokaryotes (Ellermeier and Schlauch, 2006) and Manual of Clinical Microbiology (Nataro et al., 2011).

Table 2.1. Biochemical test key used for identification of *Salmonella* spp. in this study

Biochemical Tests	<i>S. enterica</i> subsp. <i>enterica</i> (I)		<i>S. enterica</i> subsp.			
	serovar Typhimurium	serovar Pullorum	<i>salamae</i> (II)	<i>arizonae</i> (IIIa)	<i>diarizonae</i> (IIIb)	<i>indica</i> (VI)
Gram stain	-	-	-	-	-	-
Oxidase	-	-	-	-	-	-
Catalase	+	+	+	+	+	+
H ₂ S from TSI	+	+	+	+	+	+
Indol production	-	-	-	-	-	-
Acetoin production (Voges-Proskauer)	-	-	-	-	-	-
Methyl Red	+	+	+	+	+	+
Urease	-	-	-	-	-	-
Motility	+	-	+	+	+	+
Lysine decarboxylase	+	+	+	+	+	+
Ornithine decarboxylase	+	+	+	+	+	+
Arginine dihydrolase	+/-	-	+	+/ -	+/-	+ /
ONPG	-	-	-/+	-	-	+ /

Table 2.1. Biochemical test key used for identification of *Salmonella* spp. in this study (cont'd)

Biochemical Tests	<i>S. enterica</i> subsp. <i>enterica</i> (I)		<i>S. enterica</i> subspecies			
	serovar Typhimurium	serovar Pullorum	<i>salamae</i> (II)	<i>arizonae</i> (IIIa)	<i>diarizonae</i> (IIIb)	<i>indica</i> (VI)
Fermentation of						
Glucose	+	+	+	+	+	+
Sucrose	-	-	-	-	-	-
Dulcitol	+	-	+	-	-	I
Lactose	-	-	-/+	-/+	+/-	I
Sorbitol	+	-	+	+	+	-
Xylose	+	+/-	+	+	+	+
Melibiose	+	-	-	+	+	+/-
Salicin	-	-	-	-	-	-

-, (0-10%); -/+, (11-30%); +/-, (70-89%); +, (90-100%); ONPG: o-nitrophenyl- β -D-galactopyranoside.

3.3 Examination of Phenotypic Virulence Characteristics of *Salmonella*

3.3.1 Siderophore Production

Chrome azurol S (CAS) blue plate was prepared based on the protocol of Schwyn and Neilands (1986) with some modifications made by Fiss and Brooks (1991). CAS solution prepared as follows: In 50 ml distilled water, 60.5 mg CAS was dissolved and mixed with 10 mL iron solution (1mM FeCl₃.6H₂O, 10 mM HCl). Under stirring this solution was slowly added to 72.9 mg

hexadecyltrimethylammonium bromide (HDTMA) dissolved in 40 mL distilled water. The dark blue liquid was autoclaved. The basal medium to which the CAS indicator solution was added consisted of per liter: KH_2PO_4 , 0.3 g; NaCl, 0.5 g; NH_4Cl , 1.0 g; PIPES (piperazine-*N*, *N'*-bis [2-ethanesulfonic acid]), 0.1 M, pH 6.8; L-asparagine, 5.0 g; and agar, 15 g. The sterile CAS indicator solution was mixed slowly followed with 20 mL of sterile glycerol, after the basal medium autoclaved. The CAS Agar plates were incubated at 37°C for 5 days. The presence of the orange halo surrounding the colonies was accepted as positive for siderophore production.

3.3.2 Biofilm Formation

Biofilm formation of the *Salmonella* spp. was performed according to microtiter plate assay described by Djordjevic et al. (2002) with some modifications. Overnight cultures of isolates were grown at 37°C in Tryptic Soy broth without NaCl. After incubation, isolates were diluted with TSB without NaCl to optical density (OD) equal to 0.5 McFarland turbidity i.e. 1.5×10^8 colony forming units (cfu) per mL. These suspensions were transferred to 96-well flat bottom tissue culture plates as 200 μL TSB. Thereafter, the microtiter plates were incubated statically for 48 h at 37°C. The well of negative control contained only sterile Tryptic Soy Broth. Following the incubation, wells were washed five times with sterile distilled water for removing weakly attached cells. After 45 min assigned for drying, 200 μL of 1% crystal violet solution was added and waited for another 45 min for staining. After, wells were washed five times more with sterile distilled water. 200 μL of ethanol-acetone solution (4:1) was added to the wells for the quantitative analysis of biofilm formation. The OD was measured at 595 nm for each well (Multiskan Spectrum, Thermo Electron Corporation). Six replicate wells were performed for each experimental parameter and each data point was averaged from these six. The result for each isolate was calculated by subtracting the OD_{595} of the control from the mean OD_{595} of the six replicates of sample. Biofilm formation abilities of isolates were categorized as strong, moderate and weak according to Stepanovic' et al. (2000). In this assay, *Staphylococcus aureus* subsp. *aureus* (ATCC 25923) and *S. Typhimurium* ATCC 14028 were used as positive control.

3.4 Molecular Analyses

In this study, the following molecular analyses were performed: DNA extraction of the 116 *Salmonella* isolates was obtained based on the organic extraction method of Ausubel et al. (1991). Phenotypically identified *Salmonella* isolates were confirmed with *Salmonella* specific primers targeting the *invA* gene. Moreover, all isolates were investigated to presence of the plasmid virulence gene of *spvC*, and *bla*TEM, *tetA*, *sulI* and class 1 integron antimicrobial resistance genes by PCR technique. In molecular analyses, *Salmonella* Typhimurium ATCC 13311, *S. Typhimurium* ATCC 14028, *S. Enteritidis* ATCC 13076 and also Paratyphi B were used as control strains for the detection of *Salmonella* spp. with the *invA* target gene and also for the detection of *spvC* virulence gene. Furthermore, *S. Typhimurium* ATCC 14028 and Paratyphi B were used as positive control for the investigated of *bla*TEM. All of these control strains were procured from Istanbul University Basic and Industrial Microbiology, Prof. Dr. Ayten Kimiran's culture collection. Besides, *Klebsiella pneumoniae* subsp. *pneumoniae* (ATCC 700603) were used as positive control for the detection of *tetA* gene. *Salmonella* Typhimurium T22/1 from our culture collection was used a positive control for amplification of the *sulI* gene. The selected and used primer pairs, primer sequences, and their product size of the genes of virulence and antimicrobial resistance are given in Table 2.2.

3.4.1 DNA Extraction

In this study, the genomic DNA of 116 *Salmonella* isolates was extracted based on the organic method according to Ausubel et al. (1991). All *Salmonella* isolates were grown on Nutrient Agar at 37°C for 24 h. Later, a single colony of the culture was inoculated into 5ml of Brain Heart Infusion Broth and it was incubated for 18 h, 37°C. The DNA extraction was stored at -20°C.

3.4.2 Examination of *Salmonella* Genotypic Virulence Characteristics

In this research, 116 *Salmonella* isolates were investigated for the presence of the *invA*, *spvC* virulence genes, and *bla*TEM, *tetA*, *sulI* and class 1 integron

antimicrobial resistance genes. The primer pair 139-142 of *invA* gene by Rahn et al. (1992), the genus *Salmonella* specific, was also used to detect of *Salmonella* spp. by using PCR method. All amplifications were performed on a XP Thermal Cycler (Bioer Technology Co., Ltd., Japan). The PCR protocol was performed for all amplifications as follows: 5 µL of 10X PCR buffer (100mM Tris-HCl pH 8.8, 500 mM KCl, 0.8% (v/v) nonidet P40; Vivantis Technologies Sdn. Bhd., Kuala Lumpur, Malaysia); 2 µL of 25 mM MgCl₂ (Vivantis); 1 µL of 10 mM dNTP mix (Vivantis); 0.75 µL 10 µM each of the primers (Biomers, Ulm, Germany); 1 µL of 50 ng/µL template DNA; 1 U of Taq DNA polymerase (Vivantis). Final volume of reaction was completed to 50 µL with molecular grade water. The thermal cycling conditions of the *invA* gene were as follows: initial denaturation (94°C for 5 min), 35 cycles of denaturation (94°C for 1 min), annealing (58°C for 2 min), extension (72°C for 2 min) and final extension (72°C for 7min). After PCR, 5 µL of PCR product was analyzed by using electrophoresis in a 1.5% Agarose gel. The expected size of the *invA* gene is 284 bp. They were visualized with UV transillumination. The amplification conditions for the *spvC* gene were: initial 94°C, 3 min; denaturation 94°C, 1 min; annealing 58°C, 1 min; extension 72°C, 2 min; final extension 72°C, 10 min and 30 cycles. The expected fragment has a size of 669 bp for the *spvC* (Swamy et al., 1996). DNA molecular marker-100 bp Plus DNA Ladder (Vivantis) was included as standard. In 1.5% Agarose gel, *invA* and *spvC* PCR products were visualized with UV transilluminator (DNR Minilumi Bio-imaging Systems Ltd., Mahale HaHamisha, Jerusalem, Israel). All primers, primer sequences, and their references are given in Table 2.2.

3.4.3 Antimicrobial Resistance Genes of *Salmonella* Spp.

In the present study, some of the antibiotic resistance genes were examined of the presence in 116 *Salmonella* isolates. Three antibiotic resistance genes *bla*TEM (Carlson et al., 1999), *sulI* (Chen et al., 2004), *tetA* (Ng et al., 1999) and class 1 integron (Lévesque et al., 1995) are investigated in the present study by the PCR method. All amplifications were performed on a XP Thermal Cycler (Bioer Technology Co., Ltd., Japan). The PCR protocol was performed for all amplifications as follows: 5 µL of 10X PCR buffer (100mM Tris-HCl pH 8.8, 500

mM KCl, 0.8% (v/v) nonidet P40; (Vivantis Technologies Sdn. Bhd., Kuala Lumpur, Malaysia); 2 μ L of 25 mM MgCl₂ (Vivantis); 1 μ L of 10 mM dNTP mix (Vivantis); 0.75 μ L 10 μ M each of the primers (Biomers, Ulm, Germany); 1 μ L of 50 ng/ μ L template DNA; 1 U of Taq DNA polymerase (Vivantis). The thermal cycling conditions of *bla*TEM gene were: initial denaturation (95°C, 5 min), 40 cycles of denaturation (95°C, 1 min), annealing (65°C, 30 sec), extension (72°C, 30 sec) followed by final extension (72°C, 10 min).

A hot start thermal cycler protocol for the *tetA* gene was carried out as follows: 95°C for 10 min, followed by 45 cycles of 95°C for 15 sec, 61°C for 30 sec, and 72°C for 30 sec, followed by a final extension step of 72°C for 10 min.

The thermocycling reaction conditions for the *suII* gene included an initial denaturation at 95°C for 10 min, followed by 30 cycles at 95°C for 30 sec, annealing at 65°C for 1 min, for 72°C for 1 min, followed by a final extension step of 72°C for 7 min.

The amplification conditions of class 1 integron were: an initial 94°C for 5 min, 35 cycles of denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec, elongation at 72°C for 2 min, and final extension at 72°C for 5 min. In 1.5% Agarose gel, all of the antimicrobial resistance PCR products were visualized with UV trans-illumination. All used primers, primer sequences, and their references are shown in Table 2.2.

Table 2.2. Primers used for detection of *Salmonella* spp. and detection of virulence and antimicrobial resistance genes

Target gene	Gene Function	Primer	Primer sequence (5'→3')	Amplicon size (bp)	Reference
<i>invA</i>	Genus specific and invasion	139	GTG AAA TTA TCG CCA CGT TCG GGC AA	284	Rahn et al., 1992
		141	TCA TCG CAC CGT CAA AGG AAC C		
<i>spvC</i>	Virulence plasmid	F	CCG AAA TAC CAT CTA CAA ATA	669	Swamy et al., 1996
		R	CCC AAA CCC ATA CTT ACT CTG		
<i>bla</i> TEM	Ampicillin resistance	TEM ^a	GCA CGA GTG GGT TAC ATC GA	310	Carlson et al., 1999
		TEM ^b	GGT CCT CCG ATC GTT GTC AG		
<i>tetA</i>	Tetracycline resistance	L ^c	GCT ACA TCC TGC TTG CCT TC	210	Ng et al., 1999
		R	CAT AGA TCG CCG TGA AGA GG		
<i>sul</i> I	Sulfonamide resistance	F	TCA CCG AGG ACT CCT TCT TC	331	Chen et al., 2004
		R	CAG TCC GCC TCA GCA ATA TC		
Class 1 integron	Multidrug- resistance	5' CS	GGC ATC CAA GCA GCA AG	Variable	Lévesque et al., 1995
		3' CS	AAG CAG ACT TGA CCT GA		

139, Forward for the *invA* gene; 141, Reverse primer for the *invA* gene; F, Forward; R, Reverse; ^a Forward primer for the *bla*TEM; ^b Reverse primer for the *bla*TEM; ^c Forward primer for the *tetA*; 5' CS, Class 1 integron forward primer; 3' CS, Reverse primer for the class 1 integron.

3.5 Antimicrobial Susceptibility Test

The antimicrobial susceptibilities of 116 *Salmonella* isolates were performed using the Kirby-Bauer disk diffusion assay (Bauer et al., 1966) in Mueller-Hinton Agar (Merck), according to the Clinical and Laboratory Standards Institute (CLSI, 2014). Eighteen antimicrobial agents were chosen for the study belonging to using ten different antimicrobial groups. The agents used were as follows, penicillins (ampicillin; 10µg), beta lactamase inhibitors (amoxicillin-clavulanic acid; 30µg), cephalosporins (cephalothin; 30µg, cefoxitin; 30µg, ceftriaxone; 30µg, cefepime; 30µg), quinolones (nalidixic acid; 30µg, ciprofloxacin; 5µg, levofloxacin; 5µg), carbapenems (imipenem; 10µg), aminoglycosides (amikacin; 30µg, gentamicin; 10µg, kanamycin; 30µg, streptomycin; 10µg), tetracyclines (tetracycline; 30µg), folate pathway inhibitors (trimethoprim/sulfamethoxazole; 25µg), phenicols (chloramphenicol; 30µg), and macrolides (erythromycin; 15µg).

The each bacterial suspension turbidity in Mueller-Hinton Broth (Merck) was adjusted to 0.5 McFarland, and then the suspensions were spread with a sterile swab confluent over the complete surface of Mueller-Hinton Agar. The antimicrobial agent disks were placed on the agar surface adequately separated so as to avoid overlapping of the inhibition zones. The plates were incubated at 37°C for 24 h. After, the inhibition zone of the growth around each antibiotic disk was measured in millimeters and results were classified as susceptible, resistant and intermediate according to CLSI (CLSI, 2014).

3.6 List of Culture Media

Buffered Peptone Water (BPW)..... (Merck 107228)

Peptone from casein, 10 g/L

Sodium chloride, 5 g/L

Potassium dihydrogen phosphate, 1.5 g/L

Di-sodium hydrogen phosphate dodecahydrate, 9 g/L

Mueller Kauffman Tetrathionate with Novobiocine Broth..... (Merck 105878)

Meat extract, 4.3 g/L

Peptone from casein, 8.6 g/L

Sodium chloride, 2.6 g/L

Calcium carbonate, 38.7 g/L

Sodium thiosulfate water-free, 30.5 g/L

Ox bile, 4.78 g/L

Brilliant green, 0.0096 g/L

Novobiocine sodium salt, 0.040 g/L

Rappaport-Vassiliadis Soy Broth..... (Fluka 17173)

Papaic digest of soyabean meal, % 4.5 g/L

Sodium chloride, 7.2 g/L

Monopotassium phosphate, 0.18 g/L

Magnesium chloride anhydrous, 28.6 g/L

Malachite green, 0.036 g/L

Salmonella Shigella Agar..... (Merck 107667)

Peptones, 10 g/L

Lactose, 10 g/L

Ox bile, dried, 8.5 g/L

Sodium citrate, 10 g/L

Sodium thiosulfate, 8.5 g/L

Ammonium iron (III) citrate, 1 g/L

Brilliant green, 0.0003 g/L

Neutral red, 0.025 g/L

Agar-agar, 12 g/L

Xylose Lysine Deoxycholate Agar..... (Merck 105287)

Yeast extract, 3 g/L

Sodium chloride, 5 g/L

D (+) Xylose, 3.75 g/L

Lactose, 7.5 g/L

Sucrose, 7.5 g/L

L (+) Lysine, 5 g/L

Sodium deoxycholate, 1 g/L

Sodium thiosulfate, 6.8 g/L

Ammonium iron (III) citrate, 0.8 g/L

Phenol red, 0.8 g/L

Agar-agar, 14.5 g/L

Nutrient Agar..... (Merck 105450)

Peptone from meat, 5 g/L

Meat extract, 3 g/L

Agar-agar, 12g/L

Brain Heart Infusion Broth..... (Merck 110493)

Nutrient substrate, 27.5 g/L

D (+) Glucose, 2 g/L

Sodium chloride, 5g/L

Disodium hydrogen phosphate, 2.5 g/L

Mueller-Hinton Agar..... (Merck 105437)

Beef, dehydrated infusion, 300 g/L

Casein hydrolysate, 17.5 g/L

Starch, 1.5 g/L

Agar-agar, 17 g/L

Mueller-Hinton Broth..... (Merck 110293)

Beef, dehydrated infusion, 300 g/L

Casein hydrolysate, 17.5 g/L

Starch, 1.5 g/L

SIM Medium..... (Merck 105470)

Peptone from casein, 20 g

Peptone from meat, 6.6 g

Ammonium iron (III) citrate, 0.2 g

Sodium thiosulfate, 0.2 g

Agar-Agar, 3 g

SIMMONS Citrate Agar (Merck 105470)

Ammonium dihydrogen phosphate, 1 g/L

Di-potassium hydrogen phosphate, 1 g/L

Sodium chloride, 5 g/L

Sodium citrate, 2 g/L

Magnesium sulfate, 0.2 g/L

Bromothymol blue, 0.08 g/L

Agar-agar, 13 g/L

Triple Sugar Iron Agar..... (Merck 103915)

Peptone from casein, 10 g/L

Peptone from meat, 10 g/L

Meat extract, 3 g/L

Yeast extract, 3 g/L

Sodium chloride, 5 g/L

Lactose, 10 g/L

Sucrose, 10 g/L

D (+) Glucose, 1 g/L

Ammonium iron (III) citrate, 0.5 g/L

Sodium thiosulfate, 0.5 g/L

Phenol red, 0.024 g/L

Agar-agar, 12 g/L

MR-VP Broth..... (Merck105712)

Peptone from meat, 7 g

D (+) Glucose, 5 g

Phosphate buffer, 5 g

Tryptone (Indol) Medium (Merck 110859)

Peptone from casein, 10 g/L

Sodium chloride, 5 g/L

Urea Broth..... (Merck 108483)

Yeast extract, 0.1 g/L

Potassium dihydrogen phosphate, 9.5 g/L

Disodium hydrogen phosphate, 9.5 g/L

Urea, 20g/L

Phenol red, 0.01 g/L

Phenol Red (Base) Medium..... (Merck 110987)

Peptone from casein, 5 g/L

Peptone from meat, 5 g/L

Sodium chloride, 5 g/L

Phenol red, 0.018 g/L

Decarboxylase Base Medium..... (Difco 287220)

Peptone, 5 g/L

Yeast extract, 3 g/L

Dextrose, 1 g/L

Bromocresol purple, 0.02 g/L

3.7 List of Chemicals, Reagents and Dyes

Agarose	(Merck 116801)
Boric acid.....	(Merck 100165)
Bromophenol blue.....	(BDH 200152E)
Chrome Azurol S.....	(Sigma 19532)
Cethyl Trimethyl Ammonium Biomide (CTAB).....	(Sigma H5882)
Chloroform.....	(Merck 102431)
Crystal Violet.....	(Merck 11594)
D (+) Glucose-Monohydrate.....	(Merck 108342)
EDTA.....	(BDH 280214S)
Ethanol.....	(Merck 100983)
Ethidium Bromide.....	(Applichem A1151)
Fuch sine.....	(Merck 115937)
Glycerol.....	(Merck 104092)
D (+) Glucose-monohydrate.....	(Merck 108342)
D-Sorbitol.....	(Fluka 85532)
D (+) Melibiose.....	(Fluka 63630)
Dulcitol.....	(Merck 105990)
HDTMA.....	(Merck 814119)
Immersion oil.....	(Pancreac 251002)

Iodine.....	(Merck 104741)
Isoamyl alcohol.....	(Fluka 59090)
Isopropanol.....	(Birpa 603001006)
Kovac's Reagent.....	(Merck 109293)
Lactose monohydrate	(Merck 107657)
L-Asparagine.....	(Applichem A3721)
L-Lysine Monohydrochloride.....	(Merck 105700)
L-Ornithine Monohydrochloride.....	(Merck 106906)
PIPES.....	(Merck 110220)
Proteinase K.....	(Thermo Scientific E00491)
Phenol Solution.....	(Sigma P4557)
Potassium Cyanide.....	(Riedel de-Haen 31252)
Potassium Iodide.....	(BDH 25850)
Sodium Chloride.....	(Merck 106404)
Sodium Hydroxide.....	(Merck 106495)
Sucrose.....	(Merck 107651)
Sodium Dodecyl Sulfate (SDS).....	(Fluka 71729)
Tris (hydroxyl-methyl) aminomethane.....	(Merck 108387)

3.8 List of Antimicrobial Agents

Ampicillin (10 µg).....	(Oxoid CT0003B)
Amoxicillin-clavulanic acid (30 µg).....	(Oxoid CT0223B)
Cephalothin (30 µg).....	(Oxoid CT0010B)
Cefoxitin (30 µg).....	(Oxoid CT0119B)
Ceftriaxone (30 µg).....	(Oxoid CT0417B)
Cefepime (30 µg).....	(Oxoid CT0771B)
Imipenem (10 µg).....	(Oxoid CT0455B)
Nalidixic acid (30 µg).....	(Oxoid CT0031B)
Ciprofloxacin (5 µg).....	(Oxoid CT0425B)
Levofloxacin (5 µg).....	(Oxoid CT1587B)
Amikacin (30 µg).....	(Oxoid CT0107B)
Gentamicin (10 µg).....	(Oxoid CT0024B)
Kanamycin (30 µg).....	(Oxoid CT0026B)
Streptomycin (10 µg).....	(Oxoid CT0047B)
Tetracycline (30 µg).....	(Oxoid CT0054B)
Trimethoprim-sulfamethoxazole (25 µg).....	(Oxoid CT0052B)
Chloramphenicol (30 µg).....	(Oxoid CT0013B)
Erythromycin (15 µg).....	(Oxoid CT0020B)

4. RESULTS

4.1 Prevalence and Identification of *Salmonella* spp. in Chicken Meat

In this study, 146 chicken meat samples were investigated for the presence of *Salmonella* spp. The contamination of *Salmonella* was detected in 83 (56.8%) of the analyzed samples. In our study, the 116 *Salmonella* isolates were obtained with at least one or more species in each sample. Out of the 116 *Salmonella* isolates, 76 (65.5%) were conventionally identified as *S. Typhimurium*, 27 (23.3%) as *S. enterica* subsp. *indica*, 9 (7.8%) as *S. Pullorum*, 2 (1.7%) as *S. enterica* subsp. *arizonae*, 1 (0.9%) as *S. enterica* subsp. *diarizonae*, and 1 (0.9%) as *S. enterica* subsp. *salamae*. The distribution of the 116 *Salmonella* isolates obtained was given in Figure 3.1.

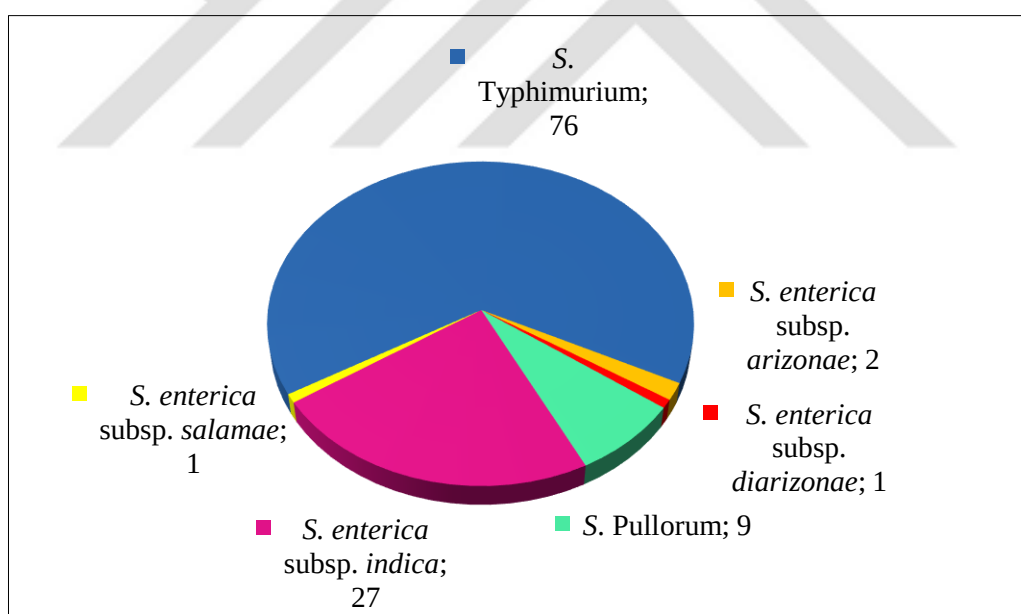


Figure 3.1. Distribution of the 116 *Salmonella* isolates in the 83 contaminated chicken meat samples.

Of the 116 isolates obtained, *S. Typhimurium* was the most common isolates, followed by *S. enterica* subsp. *indica*, *S. Pullorum*, *S. enterica* subsp. *arizonae*, *S. enterica* subsp. *diarizonae*, and *S. enterica* subsp. *salamae*.

4.2 Phenotypic Virulence Characteristics of *Salmonella*

In this study, some virulence characteristics of 116 *Salmonella* isolates were examined. Siderophore production was observed in the 95 (81.9%) isolates. Out of the 76 *S. Typhimurium* isolates, the 64 (84.2%) were found to be positive for the siderophore. The 23 (85.2%) *S. enterica* subsp. *indica* isolates were found positive for the siderophore production while only 3 (44.4%) *S. Pullorum* isolates were positive. In addition, siderophore was detected in all isolates of the *S. enterica* subsp. *arizonae*, *S. enterica* subsp. *diarizonae*, and *S. enterica* subsp. *salamae*. The positive and negative *Salmonella* isolates for siderophore production on the CAS agar plate were represented in the Figure 3.2. Nevertheless, 116 *Salmonella* isolates were examined for biofilm formation by using the microtiter plate assay. The 55.2% of isolates were biofilm producers, whereas 45.7% of the isolates were not detected as biofilm producers. The incidence of virulence characteristics including siderophore production and biofilm formation of the *Salmonella* spp. isolated from chicken meat samples was exhibited in Table 3.1. The results of biofilm formation among the *Salmonella* isolates were shown as weak producer, moderate producer, strong producer and no biofilm producer in Table 3.2. Figure 3.3 showed an illustrative photograph of 96-well microtiter plate indicated biofilm formation.

Table 3.1. The incidence of virulence characteristics of *Salmonella* spp.

Virulence characteristics	<i>S. Typhimurium</i>	<i>S. enterica</i> subsp. <i>indica</i>	<i>S. Pullorum</i>	<i>S. enterica</i> subsp. <i>arizonae</i>	<i>S. enterica</i> subsp. <i>diarizonae</i>	<i>S. enterica</i> subsp. <i>salamae</i>
Siderophore production	64 ^a (84.2) ^b	23 (85.2)	3 (44.4)	2 (100)	1 (100)	1 (100)
Biofilm formation	30 (39.5)	23 (85.2)	9 (100)	0	1 (100)	0

^a number of the positive *Salmonella* isolates; ^b Percentage of the positive isolates

Table 3.2. Distribution of biofilm producers as weak, moderate, strong and no biofilm producer among the *Salmonella* isolates

<i>Salmonella</i> isolates	No. of isolates tested	Weak producer	Moderate producer	Strong producer	No biofilm producer
<i>S. Typhimurium</i>	76	16 ^a (21.1) ^b	8 (10.5)	6 (7.9)	46 (60.5)
<i>S. Pullorum</i>	9	nd ^c	3 (33.3)	6 (66.7)	nd
<i>S. enterica</i> subsp. <i>indica</i>	27	7 (25.9)	3 (11.1)	13 (48.1)	4 (14.8)
<i>S. enterica</i> subsp. <i>salamae</i>	1	nd	nd	nd	1 (100)
<i>S. enterica</i> subsp. <i>arizonae</i>	2	nd	nd	nd	2 (100)
<i>S. enterica</i> subsp. <i>diarizonae</i>	1	nd	nd	1 (100)	nd
TOTAL (<i>Salmonella</i> spp.)	116	23 (19.8)	14 (12.1)	26 (22.4)	53 (45.7)

^a The number of the *Salmonella* isolates; ^b Percentage of the isolates; ^c none detected

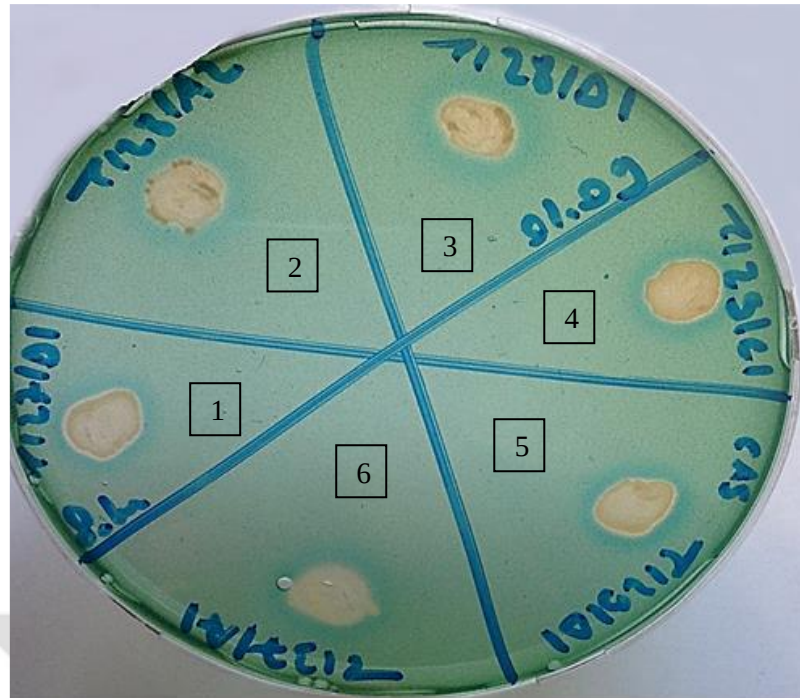


Figure 3.2. Screening of siderophore-producing *Salmonella* using the CAS Agar. Number 1 to 5: Positive isolates; number 6: Negative isolate

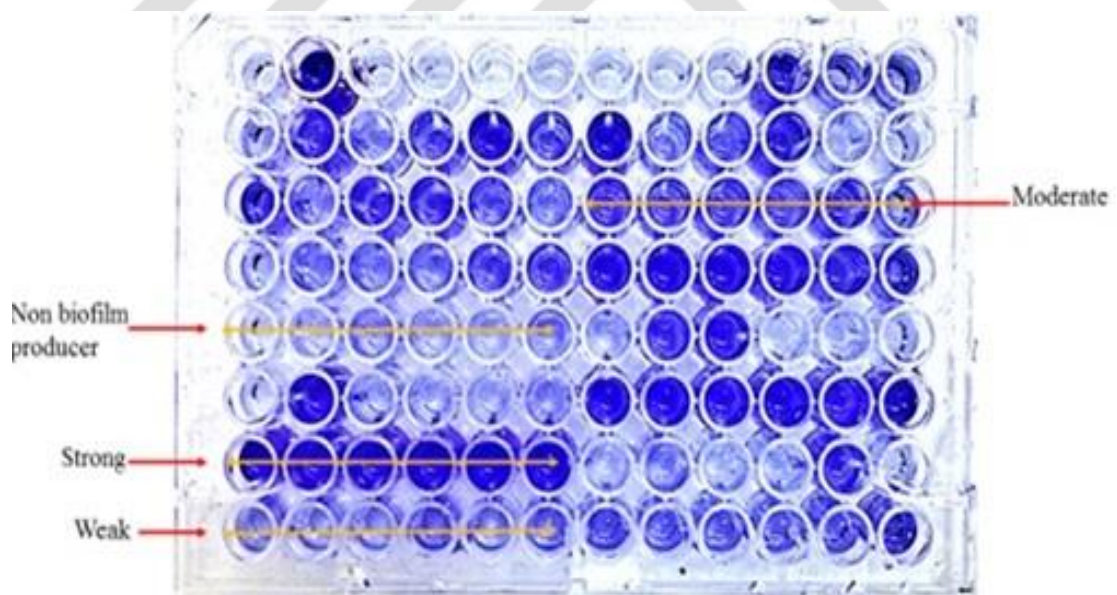


Figure 3.3. The photograph of biofilm formation of the *Salmonella* isolates. Biofilm formation was classified as weak, moderate, strong, and no biofilm producer on the microtiter plate. Six replicate wells were performed for each experimental parameter.

The 39.5% of *S. Typhimurium* isolates were found to be biofilm producers. These isolates were classified as follow: 21.1% as weak, 10.5% as moderate and 7.9% as strong. Of the 23 (85.2%) *S. enterica* subsp. *indica* isolates had ability of the biofilm formation and they were categorized as follow: 7 (25.9%) isolates as weak, 3 (11.1%) as moderate and 13 (48.1%) as strong. All the *S. Pullorum* isolates have the ability of biofilm production, and the 3 (33.3%) isolates were positive as moderate and 6 (66.7%) as strong. Besides, *S. enterica* subsp. *diarizonae* isolate was found to be positive as strong. In contrast, the *S. enterica* subsp. *salamae* isolate and 2 *S. enterica* subsp. *arizonae* isolates were no biofilm producers.

4.3 Genotypic Virulence Characteristics of *Salmonella*

The invasion gene, *invA* is an important virulence gene for the *Salmonella* pathogenesis which is a chromosomal encoded. This virulence gene was detected in all of the 116 *Salmonella* isolates. Besides, all *Salmonella* isolates were investigated for the presence or absence of the *Salmonella* plasmid virulence gene, *spvC*. The results showed that 19% of the isolates were positive for the *spvC*. The *spvC* was presented in 17 (77.3%) *S. Typhimurium*, 3 (13.7%) *S. enterica* subsp. *indica*, 1 (4.5%) *S. enterica* subsp. *diarizonae* and 1 (4.5%) *S. Pullorum*. The presence of *invA* (284 bp) and *spvC* (669 bp) virulence genes in the *Salmonella* isolates was shown on agarose gel in Figure 3.4.

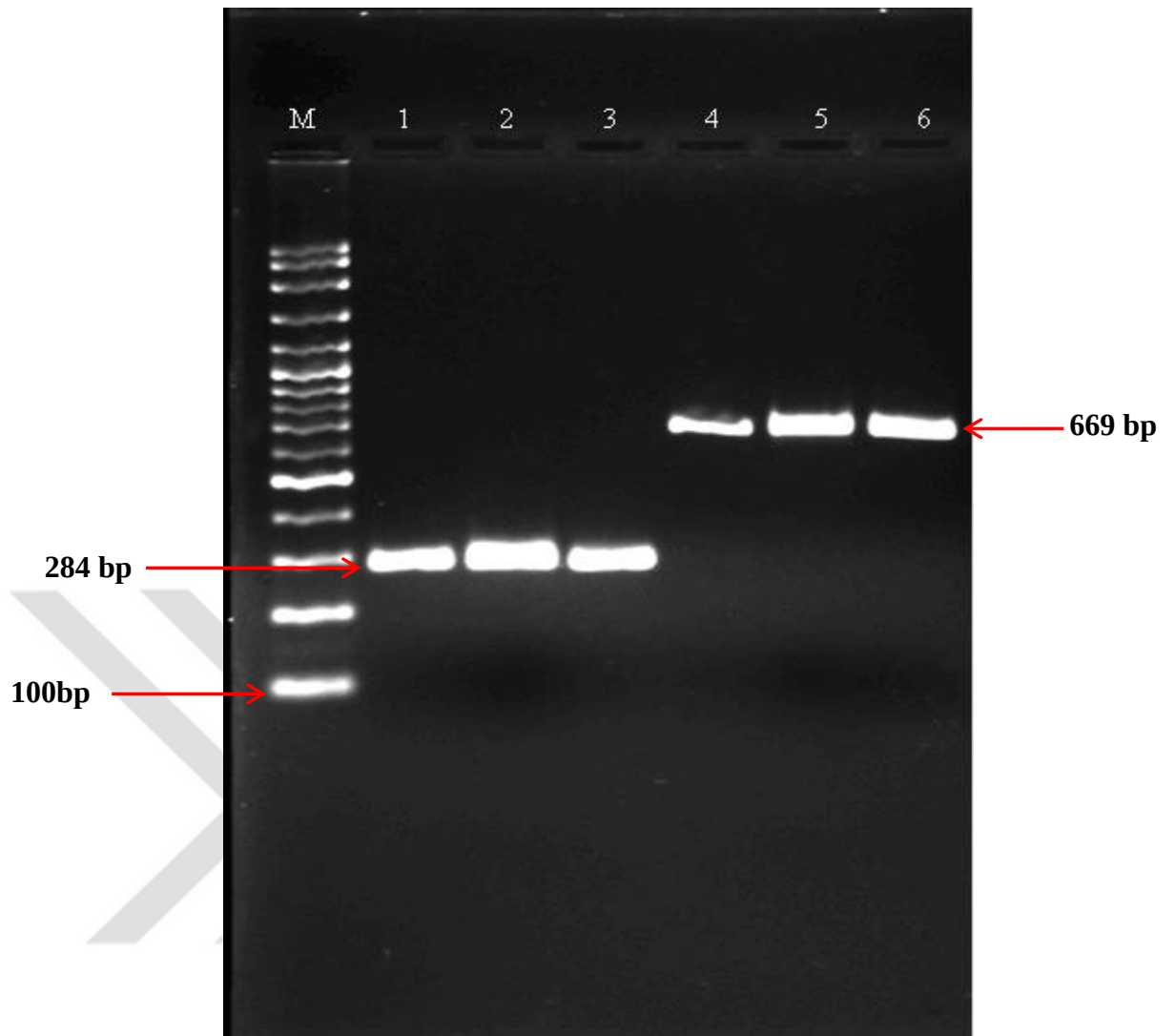


Figure 3.4. Amplification of the *invA* and *spvC* virulence genes from *Salmonella* isolates. Lane M: 100 bp molecular size marker. Lane 1: *Salmonella* Enteritidis ATCC 13076 positive control for the *invA*. Lanes 2 and 3: The *invA* positive *Salmonella* isolates in our study. Lane 4: *Salmonella* Typhimurium ATCC 14028 positive control for the *spvC* gene. Lanes 5 and 6: The *spvC* positive *Salmonella* isolates in our study.

4.4 Profiles of Antimicrobial Resistance Genes of *Salmonella*

In the present study, class 1 integron, ampicillin, tetracycline, and sulfonamide resistance genes were examined for the presence in *Salmonella* isolates. The incidence of resistance genes including *bla*TEM, *tetA*, class 1 integron and *sulI* were as follows: 91.4%, 57.8%, 45.7% and 38.8%, respectively. Out of the 90 tetracycline resistant isolates based on the disk diffusion, 62 (68.9%) had the *tetA* gene. Of the 37 ampicillin resistance isolates according to the disk diffusion method, 36 harbored the *bla*TEM. Among the 93 multidrug-resistant isolates (at least 3 antimicrobial agents) according to the disk diffusion method, 50 (53.8%) were positive for class 1 integron.

All of the antimicrobial resistance genes (*bla*TEM, *tetA*, *sulI* and class 1 integron) were detected in 16 (13.8%) isolates. In contrast, 3 (2.6%) isolates had no any resistance gene. Moreover, other resistance genes except class 1 integron were detected in 16 (13.8%) isolates. Class 1 integron, the *bla*TEM, and *tetA* genes were in 21 (18.1%) isolates, whereas the *bla*TEM and *tetA* genes were found in 9 (7.8%) *Salmonella* isolates. However, class 1 integron and the *bla*TEM genes were found to be positive in 12 (10.3%) isolates. All of the comprehensive results related to antimicrobial resistance genes of *Salmonella* isolates were seen in Table 3.3. Besides, representative agarose gel electrophoresis for PCR products was exhibited the expected molecular size of 331 bp for the *sulI* gene, 310 bp for the *bla*TEM gene, and 210 bp for the *tetA* gene in *Salmonella* isolates in the Figure 3.5.

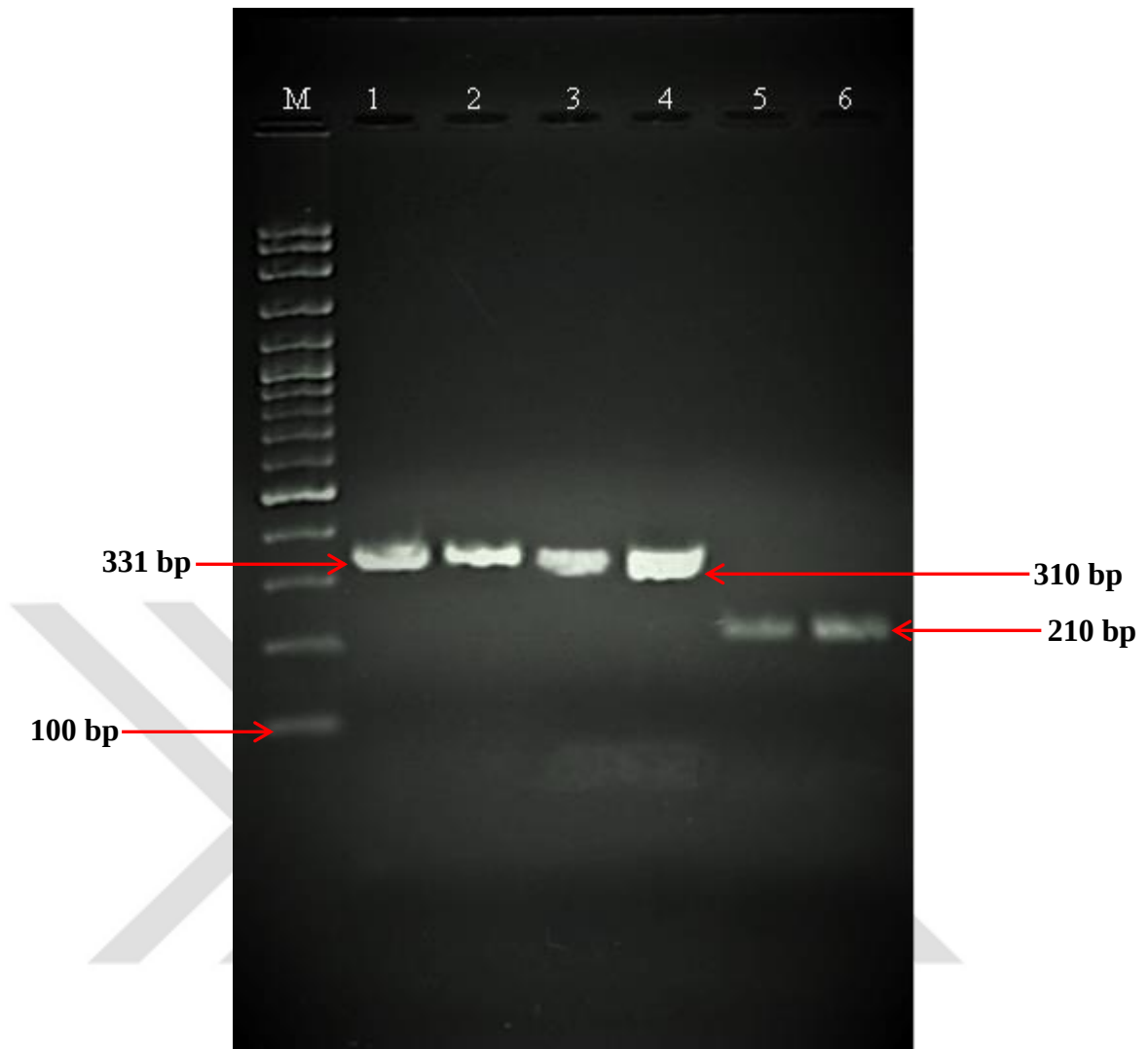


Figure 3.5. Amplification of the *bla*TEM, *tetA*, and *sulI* antibiotic resistance genes from the *Salmonella* isolates. M: 100 bp DNA Ladder (Vivantis). Lane 1: Positive control for the *sulI* gene from our laboratory collection. Lane 2: *sulI* positive *Salmonella* isolate from this study. Lane 3: Paratyphi B, positive control for the *bla*TEM. Lane 4: The *bla*TEM positive *Salmonella* isolate from this study. Lane 5: *Klebsiella pneumoniae* ATCC 700603, positive control for the *tetA* gene. Lane 6: The *tetA* positive *Salmonella* isolate from this study.

Table 3.3. Multidrug resistance profiles of *Salmonella* isolates designed by the classification of the antimicrobial resistance genes used in this study*

Antimicrobial resistance gene(s) ^a	No. of isolates (%)	Antimicrobial resistance patterns ^b
Class 1 integron, <i>bla</i> TEM, <i>sul</i> I, <i>tet</i> A	16	E, NA, TE AMP, E, NA, TE E, NA, S, TE C, E, NA, TE AMP, E, NA, TE, S AMP, CIP, E, K, NA, TE C, E, K, NA, S, TE AMP, E, NA, S, SXT, TE AMP, C, E, K, NA, S, TE C, E, K, NA, S, SXT, TE AMP, C, E, K, NA, S, SXT, TE AMP, C, E, FEP, KF, NA, S, TE AMP, CIP, E, KF, NA, S, SXT, TE
Class 1 integron, <i>bla</i> TEM, <i>tet</i> A	21	C, E, TE E, NA, S, TE E, NA, SXT, TE E, K, NA, S, TE AMP, E, K, NA, S, SXT C, E, K, NA, S, TE CIP, E, NA, S, SXT, TE E, K, NA, S, SXT, TE AMP, C, E, K, NA, S, TE C, E, K, NA, S, SXT, TE AMP, C, CIP, E, NA, S, SXT, TE AMP, E, K, KF, NA, S, SXT, TE AMP, C, E, IPM, K, NA, S, SXT, TE

Table 3.3. Multidrug resistance profiles of *Salmonella* isolates designed by the classification of the antimicrobial resistance genes used in this study (cont'd)*

Antimicrobial resistance gene(s) ^a	No. of isolates (%)	Antimicrobial resistance patterns ^b
Class 1 integron, <i>bla</i> TEM, <i>sul</i> I	2	AMP, C, CIP, E, NA, S, SXT
Class 1 integron, <i>sul</i> I, <i>tet</i> A	2	AMP, E, NA, S, TE C, E, IPM, S, SXT, TE
<i>bla</i> TEM, <i>tet</i> A, <i>sul</i> I	16	C, E, NA C, E, NA, TE E, NA, S, TE C, E, NA, S, TE C, CIP, E, NA, S, TE E, K, NA, S, SXT, TE AMP, CIP, E, K, NA, S, SXT AMP, C, E, K, NA, S, TE C, E, K, NA, S, SXT, TE AMP, C, E, K, NA, S, SXT, TE AMP, C, E, FEP, KF, NA, S, TE AMP, C, CIP, E, KF, LEV, NA, S, TE AMP, C, CIP, E, K, NA, S, SXT, TE AMP, CIP, E, IPM, K, LEV, NA, S, SXT, TE AMC, AMP, C, CN, CRO, E, K, KF, NA, S, SXT, TE
<i>bla</i> TEM, <i>tet</i> A	9	E, NA, S, TE E, K, NA, SXT, TE E, K, NA, S, TE C, E, NA, S, SXT, TE E, K, NA, S, SXT, TE C, E, K, NA, S, SXT, TE

Table 3.3. Multidrug resistance profiles of *Salmonella* isolates designed by the classification of the antimicrobial resistance genes used in this study (cont'd)*

Antimicrobial resistance gene(s) ^a	No. of isolates (%)	Antimicrobial resistance patterns ^b
<i>tetA, sulI</i>	2	E, NA, S, SXT, TE E, K, NA, S, SXT, TE
<i>blaTEM, sulI</i>	7	AMP, E, K, NA, SXT, TE AMP, C, CIP, E, K, NA, S, SXT AMP, C, CIP, E, NA, IPM, S, SXT, TE AMP, CIP, E, IPM, K, LEV, NA, S, SXT AMP, C, CN, E, IPM, K, NA, S, SXT, TE
Class 1 integron	1	E, IPM, TE

* Resistance profiles were formed based on the at least 3 antimicrobial agents, at least one or two resistance were ignored; ^a Isolates positive for genes of antimicrobial resistance; ^b Resistance to antimicrobial agents tested using disk diffusion method

In this study, the 54 (46.6%) *Salmonella* isolates harbored class 1 integron and six different class 1 integron profiles including about 3000 bp, about 2500 bp, about 2000 bp, about 1700 bp, about 1400 bp, and about 1100 bp were detected. Among the 93 multidrug-resistant isolates based on the disk diffusion method (at least 3 antimicrobial agents), 50 (53.8%) were positive for class 1 integron. The comparisons of class 1 integron profiles (IPs) with the results of disk diffusion method were given in Table 3.4. The photograph of 6 different class 1 integron profiles (IPs) (IP-I to -VI), with the fragments ranging from 1100 bp to 3000 bp in size were given in Figure 3.6.

Table 3.4. Comparison of class 1 integron profiles with the results of disk diffusion method

Number of Class 1 integron positive isolates (%)	Profiles of Class 1 integron (Approximate size)	Antimicrobial Resistance Profiles ^a
43 (79.6)	IP-I (1100 bp)	Resistance to at least 2 or more antimicrobials
1 (1.9)	IP-II (1400 bp)	Resistance to 8 antimicrobials
4 (7.4)	IP-III (1700 bp)	Resistance to at least 1 or more antimicrobials
1 (1.9)	IP-IV (2000 bp)	Resistance to 6 antimicrobials
1 (1.9)	IP-V (2500 bp)	Resistance to 2 antimicrobials
4 (7.4)	IP-VI (3000 bp)	Resistance to at least 6 antimicrobials

^a Antimicrobial resistance results based on the disk diffusion method

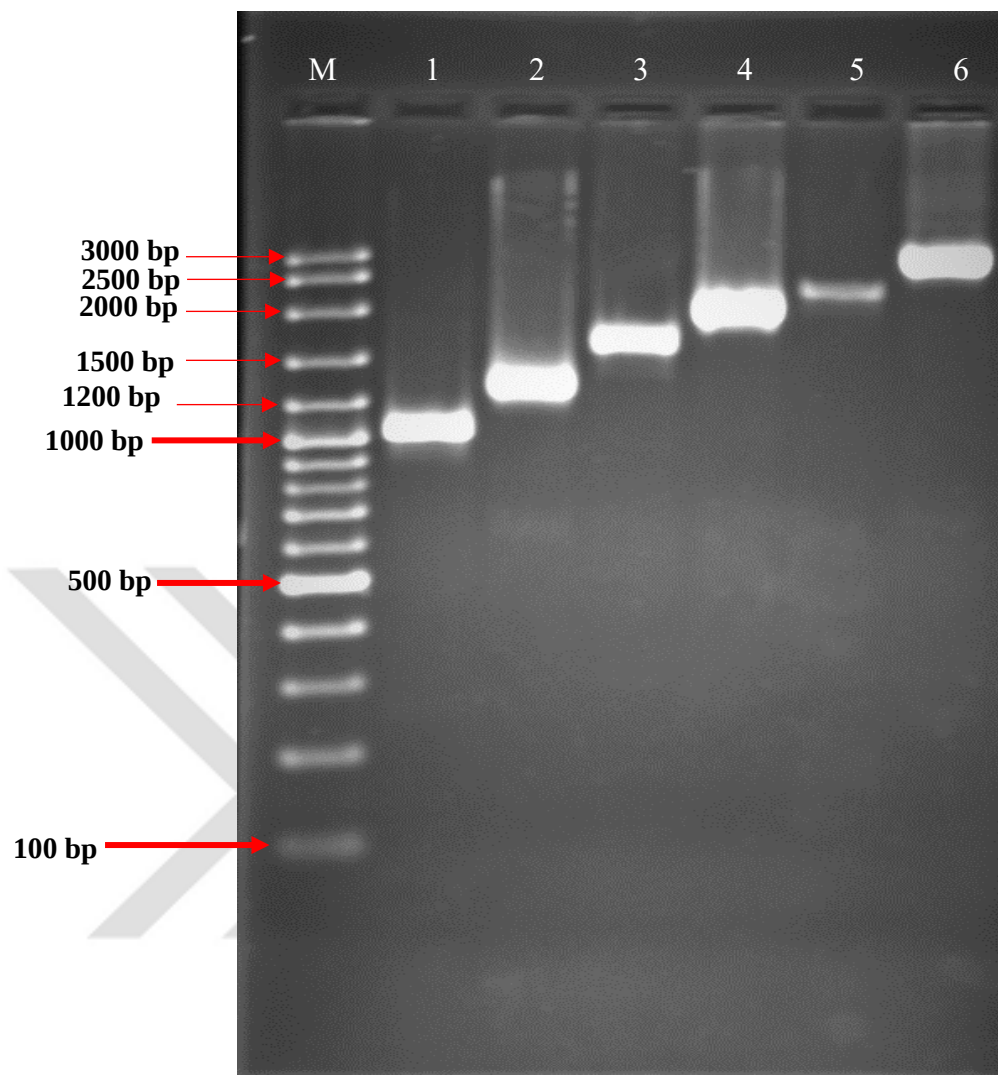


Figure 3.6. Analyses of class 1 integrons from *Salmonella* isolates from chicken meat. M: 100 bp DNA Ladder. Lane 1 to 6: Variable regions of about 1100 to 3000 bp.

4.5 Antimicrobial Resistance of *Salmonella* Species

In the present study, the antimicrobial susceptibility of the 116 *Salmonella* isolates from chicken meat products was examined with 18 different antimicrobial agents. A representative photograph of antimicrobial susceptibility test on Mueller-Hinton Agar was given in Figure 3.7. Among the *Salmonella* isolates the highest resistance after erythromycin was detected against tetracycline and nalidixic acid. Most *Salmonella* isolates with the rate of 77.6% were resistant to tetracycline and nalidixic acid, 58.6% to streptomycin, 44.8% to chloramphenicol and 44% to trimethoprim/sulfamethoxazole. The antimicrobials amikacin (99.1%), ceftiofur (95.7%), gentamicin (93.1%), levofloxacin (92.2%), amoxicillin/clavulanic acid (90.5%), and ceftriaxone (88.8%) were found to be effective against *Salmonella* isolates. Antimicrobial susceptibility rates of 18 antimicrobial agents tested against 116 *Salmonella* isolates from chicken meat samples are presented in Table 3.5.

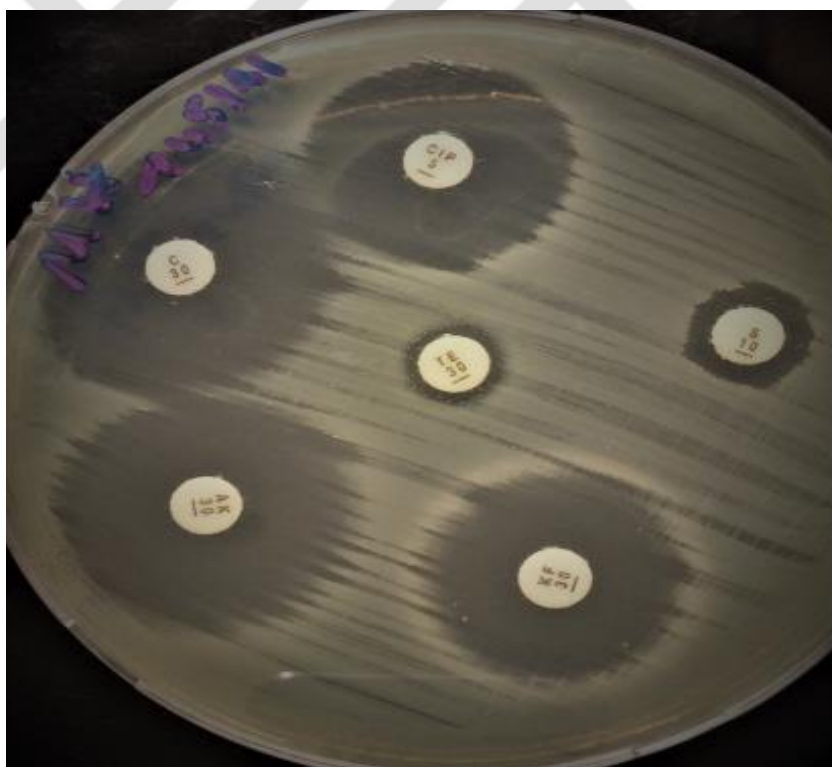


Figure 3.7. Representative *Salmonella* isolate on Mueller-Hinton Agar against tetracycline, streptomycin, amikacin, ceftriaxone, chloramphenicol, and ciprofloxacin.

Table 3.5. Antimicrobial susceptibility rates of the 116 *Salmonella* isolates

Antimicrobial agent groups	Conc. ^a (µg/disk)	Number of isolates (%)		
		Resistant	Intermediate	Susceptible
Penicillins				
Ampicillin	10	37 (31.9)	3 (2.6)	76 (65.5)
Beta lactamase inhibitors				
Amoxicillin-clavulanic acid	30	2 (1.7)	9 (7.75)	105 (90.5)
Cephalosporins				
Cephalothin	30	8 (6.9)	14 (12.1)	94 (81)
Cefoxitin	30	0	5 (4.3)	111 (95.7)
Ceftriaxone	30	2 (1.7)	11 (9.5)	103 (88.8)
Cefepime	30	2 (1.7)	13 (11.2)	101 (87.1)
Quinolones				
Nalidixic acid	30	90 (77.6)	1 (0.9)	25 (21.6)
Ciprofloxacin	5	20 (17.2)	79 (68.1)	17 (14.7)
Levofloxacin	5	5 (4.3)	4 (3.4)	107 (92.2)
Aminoglycosides				
Amikacin	30	0	1 (0.9)	115 (99.1)
Kanamycin	30	50 (43.1)	5 (4.3)	61 (52.6)
Gentamicin	10	4 (3.4)	4 (3.4)	108 (93.1)
Streptomycin	10	68 (58.6)	24 (20.7)	24 (20.7)
Tetracyclines				
Tetracycline	30	90 (77.6)	6 (5.2)	20 (17.2)
Folate Pathway Inhibitors				
Trimethoprim/sulfamethoxazole	25	51 (44)	0	65 (56)
Carbapenems				
Imipenem	10	13 (11.2)	20 (17.2)	83 (71.6)
Others				
Chloramphenicol	30	52 (44.8)	3 (2.6)	61 (52.6)
Erythromycin	15	116 (100)	0	0
Resistance to ≥ 1 antimicrobial		116 (100)		
Multidrug-resistance ^b		93 (80.2)		

^a Concentration of disks; ^b Resistance to at least three or more antimicrobial agents

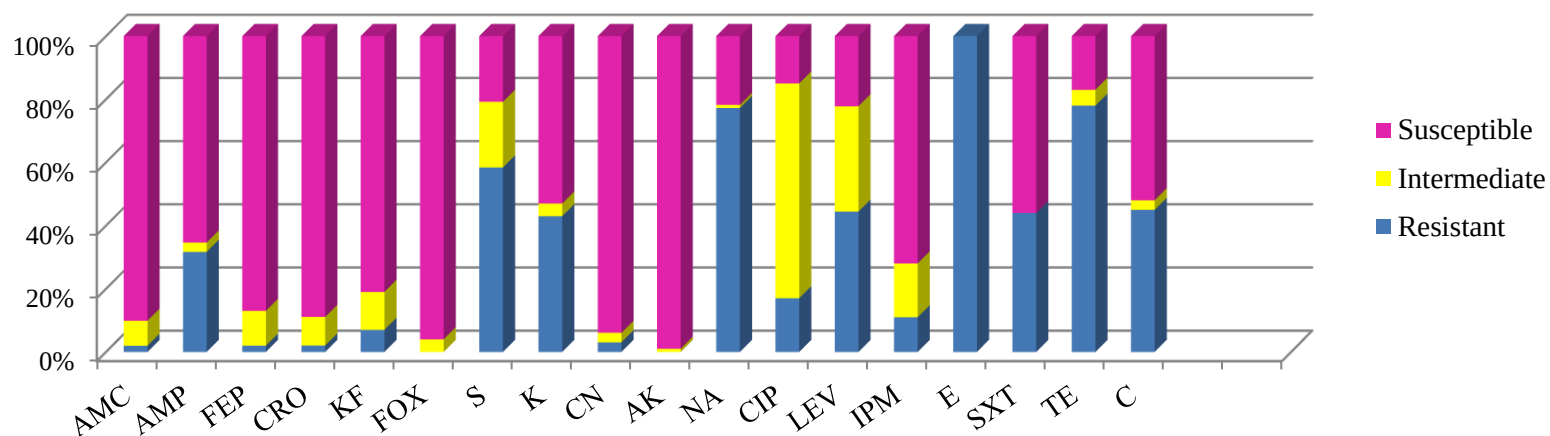


Figure 3.8. Antimicrobial susceptibility rates of 18 antimicrobial agents against the 116 *Salmonella* isolates in chicken meat. AMC, amoxicillin/clavulanic acid; AMP, ampicillin; FEP, cefepime; CRO, ceftriaxone; KF, cephalothin; FOX, cefoxitin; S, streptomycin; K, kanamycin; CN, gentamicin; AK, amikacin; NA, nalidixic acid; CIP, ciprofloxacin; LEV, levofloxacin; IPM, imipenem; E, erythromycin; SXT, trimethoprim/sulfamethoxazole; TE, tetracycline; C, chloramphenicol.

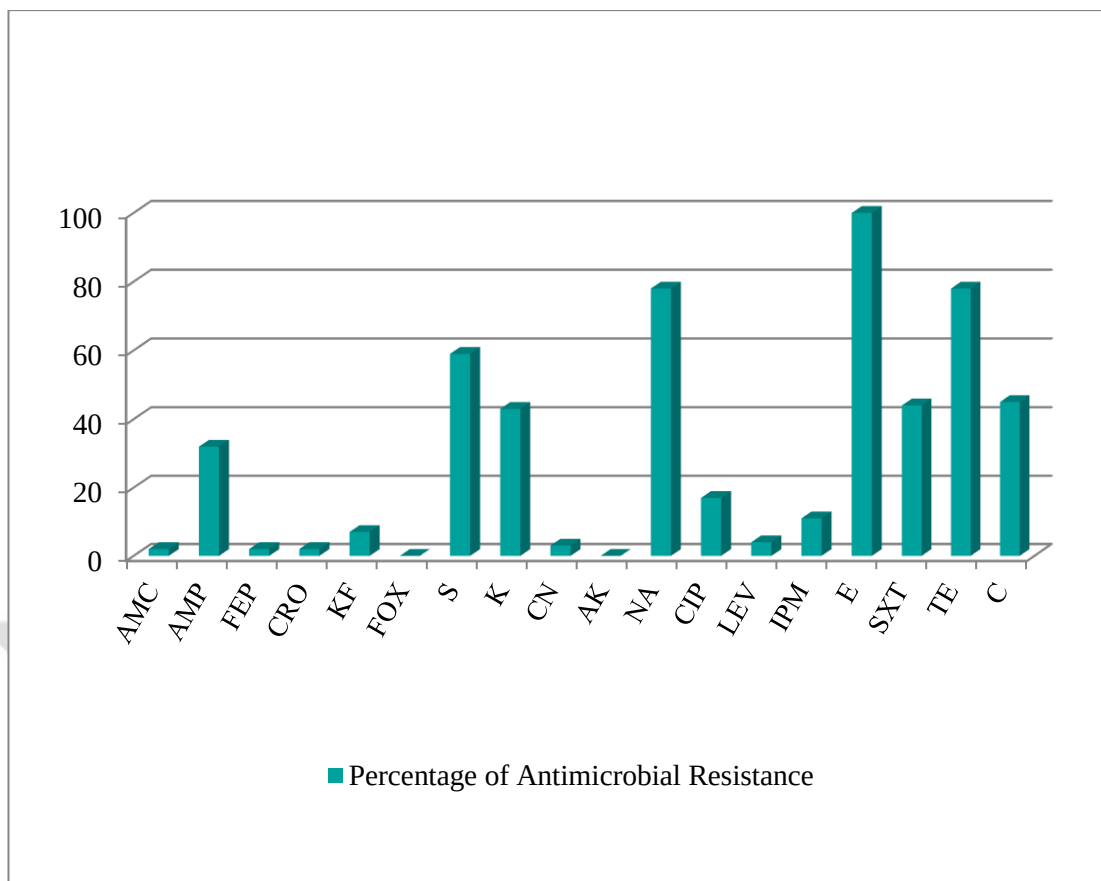


Figure 3.9. Resistance of the *Salmonella* isolates to antimicrobial agents. AMC, amoxicillin/clavulanic acid; AMP, ampicillin; FEP, cefepime; CRO, ceftriaxone; KF, cephalothin; FOX, ceftiofur; S, streptomycin; K, kanamycin; CN, gentamicin; AK, amikacin; NA, nalidixic acid; CIP, ciprofloxacin; LEV, levofloxacin; IPM, imipenem; E, erythromycin; SXT, trimethoprim/sulfamethoxazole; TE, tetracycline; C, chloramphenicol.

The resistance of *Salmonella* isolates to 18 antimicrobial agents was shown in Figure 3.9. Resistance to erythromycin was observed in all of the *Salmonella* spp. isolates. Furthermore, *S. Typhimurium* isolates generally had resistance to nalidixic acid (79%), whereas all *S. enterica* subsp. *arizonae*, *S. enterica* subsp. *diarizonae*, *S. enterica* subsp. *salamae* isolates were found to be resistant to nalidixic acid. The highest resistance rates to tetracycline were found in *S. enterica* subsp. *arizonae* (100%), *S. enterica* subsp. *diarizonae* (100%), and *S. enterica* subsp. *salamae* (100%) and *S. enterica* subsp. *indica* (81.4%) isolates. The resistance rate of *S. Typhimurium* isolates was found 61% for streptomycin. In all the *Salmonella* spp., resistance to tetracycline, nalidixic acid, kanamycin, chloramphenicol and trimethoprim/sulfamethoxazole and erythromycin was found. The distribution of antimicrobial resistance of *Salmonella* spp. isolates were given in Table 3.6.

In this study, a total of 80.2% of the *Salmonella* isolates were resistant to at least three or more antimicrobial agents. All *Salmonella* isolates were shown resistant to at least one antimicrobial agent, 10 to two, 7 to three, 12 to four, 13 to five, 22 to six, 17 to seven, 12 to eight, 6 to nine, 3 to ten and 1 to twelve. Multidrug-resistant *Salmonella* spp. in chicken meat samples are presented in Table 3.7.



Table 3.6. Distribution of antimicrobial resistance profiles of the *Salmonella* isolates

Antimicrobial agents	<i>Salmonella</i> species isolated from chicken meat (%)					
	<i>S. Typhimurium</i> (n=76)	<i>S. enterica</i> subsp. <i>indica</i> (n=27)	<i>S. Pullorum</i> (n=9)	<i>S. enterica</i> subsp. <i>arizonae</i> (n=2)	<i>S. enterica</i> subsp. <i>diarizonae</i> (n=1)	<i>S. enterica</i> subsp. <i>salamae</i> (n=1)
Ampicillin	21 ^a (27.6) ^b	10 (37)	4 (44.4)	0	1 (100)	1 (100)
Amoxicillin-clavulanic acid	1 (1)	1 (3.7)	0	0	0	0
Cephalothin	5 (7)	2 (7.4)	0	0	0	0
Cefoxitin	0	0	0	0	0	0
Ceftriaxone	1 (1)	1 (3.7)	0	0	0	0
Cefepime	2 (1.7)	0	0	0	0	0
Nalidixic acid	60 (79)	20 (7.4)	5 (55.5)	2 (100)	1 (100)	1 (100)
Ciprofloxacin	9 (12)	6 (22.2)	3 (33.3)	0	1 (100)	1 (100)
Levofloxacin	4 (5.3)	0	1 (11.1)	0	0	0
Streptomycin	46 (61)	14 (51.8)	5 (55.5)	2 (100)	1 (100)	0
Amikacin	0	0	0	0	0	0
Kanamycin	31 (41)	10 (37)	5 (55.5)	2 (100)	1 (100)	1 (100)
Gentamicin	1 (1.3)	2 (7.4)	1 (11.1)	0	0	0
Imipenem	4 (5.2)	6 (22.2)	3 (33.3)	0	0	0
Tetracycline	58 (76.3)	6 (22.2)	6 (66.6)	2 (100)	1 (100)	1 (100)
Chloramphenicol	30 (39.4)	16 (59.2)	6 (66.6)	1 (50)	1 (100)	1 (100)
Trimethoprim/ sulfamethoxazole	26 (34.2)	16 (59.2)	5 (55.5)	2 (100)	1 (100)	1 (100)
Erythromycin	76 (100)	27 (100)	9 (100)	2 (100)	1 (100)	1 (100)

^a Number of the resistant isolates; ^b Percentage of the resistant isolates; n: Number of the *Salmonella* isolates

Table 3.7. Multiple antimicrobial resistance profiles of the 116 *Salmonella* isolates

Antimicrobial agents	No. of antimicrobial agents	No. of <i>Salmonella</i> spp.
E, NA, TE	3	3
C, E, NA	3	1
E, IPM, TE	3	2
C, E, TE	3	1
E, NA, S, TE	4	7
E, NA, SXT, TE	4	1
E, IPM, NA, TE	4	1
C, E, NA, TE	4	2
AMP, E, NA, TE	4	1
AMP, E, NA, S, TE	5	2
C, E, IPM, NA, TE	5	2
C, CIP, E, LEV, NA	5	1
C, E, NA, S, TE	5	1
E, NA, S, SXT, TE	5	1
E, K, NA, SXT, TE	5	1
C, E, K, NA, TE	5	1
E, K, NA, S, TE	5	2
C, E, NA, SXT, TE	5	2
AMP, E, K, NA, S, SXT	6	1
AMP, E, NA, S, SXT, TE	6	1
AMP, CIP, E, NA, SXT, TE	6	1
AMP, CIP, E, K, NA, TE	6	1
AMP, E, K, NA, SXT, TE	6	1
AMC, AMP, E, IPM, KF, TE	6	1
C, E, IPM, S, SXT, TE	6	1
CIP, E, NA, S, SXT, TE	6	1
C, CIP, E, K, LEV, NA	6	1
E, K, NA, S, SXT, TE	6	7

Table 3.7. Multiple antimicrobial resistance profiles of the 116 *Salmonella* isolates (cont'd)

C, E, K, NA, S, TE	6	2
C, CIP, E, NA, S, TE	6	1
CIP, E, K, NA, S, TE	6	1
C, E, NA, S, SXT, TE	6	2
C, E, K, NA, S, SXT, TE	7	9
AMP, C, E, K, NA, S, TE	7	5
AMP, CIP, E, K, NA, S, SXT	7	1
AMP, C, CIP, E, NA, S, SXT	7	1
C, E, IPM, K, NA, SXT, TE	7	1
AMP, C, E, K, NA, S, SXT, TE	8	3
AMP, C, CIP, E, NA, S, SXT, TE	8	1
AMP, C, E, FEP, KF, NA, S, TE	8	2
AMP, E, K, KF, NA, S, SXT, TE	8	1
AMP, CIP, E, KF, NA, S, SXT, TE	8	1
AMP, C, CIP, E, K, NA, S, SXT	8	2
AMP, C, CIP, E, K, NA, SXT, TE	8	1
C, CRO, E, K, NA, S, SXT, TE	8	1
AMP, CIP, E, IPM, K, LEV, NA, S, SXT	9	1
AMP, C, CIP, E, KF, LEV, NA, S, TE	9	1
AMP, C, CN, E, K, NA, S, SXT, TE	9	1
AMP, C, CIP, E, K, NA, S, SXT, TE	9	1
AMP, C, E, IPM, K, NA, S, SXT, TE	9	1
AMP, C, CIP, E, IPM, NA, S, SXT, TE	9	1
AMP, C, CIP, CN, E, K, NA, S, SXT, TE	10	1
AMP, C, CN, E, IPM, K, NA, S, SXT, TE	10	1
AMP, CIP, E, IPM, K, LEV, NA, S, SXT, TE	10	1
AMC, AMP, C, CN, CRO, E, K, KF, NA, S, SXT, TE	12	1

5. DISCUSSION

Pathogenic bacteria have always been a serious risk for the food products from past to present. *Salmonella* is one of the significant foodborne pathogens isolated from various foods worldwide (Bhunia, 2008). In many countries, many researchers have investigated the occurrence of *Salmonella* in food products. The main foods contaminated with *Salmonella* are chicken, turkey, beef, eggs and dairy products. Chicken meat is one of the most consumed animal foods around the world. It constitutes an important *Salmonella* reservoir (Dahshan et al., 2010; Cui et al., 2016).

In our study, the prevalence rate of *Salmonella* was found 56.8% in chicken meat samples which were examined to presence of *Salmonella* spp. In different countries, the reported prevalence of *Salmonella* is varied in chicken meat products. Similarly, in Vietnam, the prevalence of *Salmonella* spp. reported 53.3% by Van et al. (2007) in the 180 retail raw food samples including chicken, pork, and beef. In some previous studies, the incidence of *Salmonella* spp. in chicken samples was found lower than our result. For instance, in Thailand, in a study by Akbar and Anal (2013), *Salmonella* prevalence was found as 5.3% in poultry meat. In Brazil, reported by Panzenhagen et al. (2015) the prevalence of *Salmonella* was 6.7% in chicken carcasses. Arslan and Eyi (2010) isolated *Salmonella* spp. (29.3%) from poultry products in Turkey. Dominguez et al. (2002) detected *Salmonella* (35.8%) from retail chicken meat in Spain. In another study from Turkey, Siriken et al. (2015) announced that 42.7% of all the chicken meat products contained *Salmonella* spp. In contrast to our result, the occurrence of *Salmonella* spp. in 85 poultry samples was reported as 71% by Avsaroglu (2007) in Turkey. In Egypt, the *Salmonella* spp. was found at the rate of 60% by Abdel-Maksoud et al. (2015) from chicken parts. This result was slightly higher than our result.

In the previous studies, different rates on the prevalence of *Salmonella* spp. in different food sources have been reported by several researchers. Compared with our result in these studies, the incidence of *Salmonella* in various food products including minced meat, pork sausages, liquid egg, ready-to-eat salads, mayonnaise, and pastry products, raw vegetables, milks, and eggs, cream cakes, fresh fruits, spices, cereal,

and fish, ranging from 0.6 to 20% was considerable lower (White et al., 2001; Zhao et al., 2001; Cetinkaya et al., 2008; Gallegos-Robles et al., 2009; Favier et al., 2013; Zhou et al., 2013; Can et al., 2014; Kramarenko et al., 2014; Sallam et al., 2014). In this research, the rate of *Salmonella* contamination is relatively high in comparison with several studies showing a low prevalence rate of different food products including animal origin such as fresh beef, ground beef, beef burger, eggs, and pork, ranging from 25 to 40% (Karmi, 2013; Miranda et al., 2009; Wang et al., 2013). In this study, the *Salmonella* prevalence is slightly higher than the other studies showing a low prevalence rate of various food products including beef products ranging from 40 to 50% (Yildirim et al., 2011; Thai et al., 2012). In contrast, *Salmonella* incidence (71%) observed in ground beef by Martinez-Chavez et al. (2015) was higher than our result. The prevalence of the *Salmonella* may vary depending on the number of samples examined, sampling methods, and the variability of sample types. The studies also show that *Salmonella* contamination in different food products and countries varies from one another (D'Aoust et al., 2001).

Salmonella is a foodborne pathogen bacterium which is more frequently isolated from chicken. Moreover, *S. Typhimurium* is one of the most common serovars in different types of samples worldwide and commonly reported in human diseases (Cosby et al., 2015). In the present study, the incidence of *S. Typhimurium* (65.5%) was manifestly much higher than the other species. *S. Typhimurium* has been reported as the dominant serovar in retail meat samples in the previous studies (Arslan and Eyi, 2010; Sallam et al., 2014). *S. enterica* subsp. *indica* is commonly found in cold-blooded animals, but it was the second most prevalent species in this study with 23.3%. *S. Pullorum* is an important pathogen for poultry and it could cause a great economic loss in the poultry industry. In fact, this serovar is a causative agent of pullorum disease in poultry, and an acute systemic disease in chickens (Gong et al., 2013). In the present study, *S. Pullorum* isolates were found at the rate of 7.8%. Besides, 1.7% *S. enterica* subsp. *arizonae*, 0.9% *S. enterica* subsp. *diarizonae* and 0.9% *S. enterica* subsp. *salamae* isolates which rarely found in the warm-blooded animals, and in humans were identified in chicken meat samples of this study (Ellermeier and Schlauch, 2006; Bhunia, 2008).

Salmonella species have certain virulence characteristics which play an important role in the pathogenesis of bacteria. *Salmonella* has mechanisms which

help its abilities to iron acquisition, adhesion, invasion, and survival in the host cells and tissues (Wilson et al., 2002). Siderophore which is needed for the iron uptake is one of the *Salmonella* virulence factors. Iron in the human body is at a very low rate, because iron is bound to proteins including lactoferrin, transferrin, ferritin, and hemin. During the iron uptake, pathogens can acquire iron by directly binding to host cells that contain iron on their surface (Hantke et al., 2003; Bhunia, 2008). The rate of siderophore producing isolates was detected as 81.9% in our study. In contrast, the production of siderophore was rather low (6.5%) in the study on *Salmonella* isolates by Akyıldız (2015).

Lately, biofilm formation of foodborne pathogens has become one of the central areas of food safety research because of the probability of potential cross-contamination and serious food safety problems (Wang et al., 2013). In addition, bacteria with biofilms making these infections difficult or impossible to eradicate are inherently protected from their surrounding environment and they often exhibit increased resistance to host defence and antimicrobial agents (Gong et al., 2013). *Salmonella* have the ability to form biofilms on plastic, rubber, glass, stainless steel, plants, animal cells, and also medicinal equipments (Bhunias, 2008; Lianou et al., 2012). According to Stepanovic' et al. (2000) the ability of the obtained *Salmonella* isolates to form biofilm was categorized as strong, moderate, weak and non-biofilm. As a result of this categorization, the biofilm formation data of the isolates were as follow: 26 (22.4%) as strong, 15 (12.9%) as moderate and 23 (19.9%) as weak. In a study by Nair et al. (2015), the 85% of the *Salmonella* isolates were found as biofilm producers. In another study, Lu et al. (2011) reported 63% biofilm formed from *S. Pullorum* strains. Biofilm formation in *S. Pullorum* isolates by Gong et al. (2013) was observed as 65.3%.

In the present study, all of the *Salmonella* isolates were identified by using the conventional method, and they were confirmed by using genus specific *invA* gene of *Salmonella*. The *invA* primer set 139-141 was used for the specific detection of *Salmonella* spp. (Rahn et al., 1992; Amini et al., 2010). In previous studies, the primer pair has been frequently used so as to genotypically confirm *Salmonella* spp. and to determine its virulence (Malorny et al., 2003; Nayak et al., 2004; Sunar et al., 2010; Langkabel et al., 2014; Wongsuntornpoj et al., 2014; Siriken et al., 2015). Furthermore, the *invA* gene found in the SPI-1 is a virulence gene responsible for the

invasion in the intestinal cells of the host (Swamy et al., 1996; Singh et al., 2015; Amini et al., 2010).

The plasmid-mediated *spvC* virulence gene is necessary for the *Salmonella* survival and growth in macrophages (Swamy et al., 1996). In this study, *Salmonella* isolates were investigated, and 19% were found to be positive for the presence of *spvC*. In a study by Borges et al. (2013), the presence of *spvC* was detected as 92%. The incidence of *spvC* detected by Swamy et al. (1996) in 208 (84.9%) of the 245 *Salmonella* isolates obtained from poultry, wastewater, and human sources. In another study, Rowlands et al. (2014) reported that the incidence of *spvC* was 48.1% in food sources. The results of the studies reported above were higher than our results.

Antimicrobial resistance has increased in many countries due to overuse or misuse of antimicrobials. Antimicrobial resistance genes have a significant role for the spread of antimicrobial resistance among the pathogens, especially in *Enterobacteriaceae* family, they can be transferred horizontally (Hall and Collis, 1998; Soltan-Dallal et al., 2010). In this study, the presence of antimicrobial resistance genes including *bla*TEM, *sulI*, *tetA* and class 1 integron was detected in *Salmonella* isolates. The results showing the relationship between antimicrobial resistance based on the disk diffusion method and presence of antibiotic resistance genes were presented in Table 3.3.

The *bla*TEM gene is a plasmid-encoded gene and responsible for the ampicillin resistance (Carlson et al., 1999). In our study, the *bla*TEM gene was the most frequently observed one among the 116 isolates, and the 95 (91.4%) isolates were detected positive for the *bla*TEM. Of the 37 ampicillin resistance isolates according to the disk diffusion method, 36 harbored the *bla*TEM. These results indicate that ampicillin resistance may be related to the *bla*TEM gene. In a study, the *bla*TEM detected in 85.7% of ampicillin resistant isolates (Mattiello et al., 2015). In Romania, Dan et al. (2015) reported that 3 out of the 5 *S. Enteritidis* isolates had *bla*TEM gene. In another study, the *bla*TEM was detected in all ampicillin resistant isolates by Zou et al. (2012). Hsu et al. (2015) announced that *bla*TEM incidence was 44.4%.

Tetracycline has been one of the most commonly used antimicrobial for animal production (Shahada et al., 2006). The *tetA* gene was commonly found in zoonotic isolates, and it can be easily transferred. It can spread among isolates due to mainly mobile genetic elements (Marshall et al., 1983; Abatcha et al., 2015; Agga et al.,

2015). In the present study, *tetA* was detected as the most common resistance gene after *bla*TEM gene. Dalibor et al. (2015) reported the *tetA* gene in poultry at a high rate of 64.3% in contrast with our result (57.8%). Similarly, Zou et al. (2012) detected *tetA* gene at a considerably high rate of 60%, unlike our result.

Sulfonamides have been one of the most commonly used antimicrobial groups for food animals around the world (Abdel-Maksoud et al., 2015). The *sulI* gene is a plasmid-mediated gene which is responsible for sulfonamide resistance and often found in Gram-negative clinical isolates (Hindi et al., 2013). In the present study, *sulI* gene was detected in the 45 (38.8%) *Salmonella* isolates. Likewise, Zhou et al. (2013) reported that the 50 sulfonamide resistant isolates, 19 (38%) were positive for this resistance gene. In another study, Hsu et al. (2015) obtained the incidence of *sulI* gene in percentage of 29.6% which was lower than our result. However, some other researchers have reported that this gene was found at slightly higher rates than our result. Soyer et al. (2013) announced the distribution of *sulI* gene was 47.9%. In a thesis by Acar (2015), the frequency of *sulI* gene was 58% in the isolates obtained from chicken meat and sheep ground beef.

Class 1 integrons have a significant role in the widely spread of antimicrobial resistance and they have been reported in the *Enterobacteriaceae* members isolated from humans and animals (Zou et al., 2012). In our study, 6 different types of class 1 integron were detected in the 54 (46.6%) positive isolates, including about 1100 bp, about 1400 bp, about 1700 bp, about 2000 bp, about 2500 bp, and about 3000 bp. Ribeiro et al. (2011) reported 3 different profiles of class 1 integron (700 bp, 1700 bp, 2700 bp). In Egypt, Abdel-Maksoud et al. (2015) obtained the 8 different profiles of class 1 integron from poultry. In that study, their obtained product was in the size of 700 bp, 1000 bp, 1100 bp, 1200bp, 1550 bp, and 1950 bp a combination of 950 bp and 1200bp, and a combination of 1100 bp and 1550 bp. Krauland et al. (2009) examined for the presence of class 1 integron in the non-typhoidal *Salmonella* isolates. It was reported that 86.5% isolates were positive and there were 16 different class 1 integron profiles. In another research, the 337 *S. Pullorum* isolates were tested, and 22.3% was found to have class 1 integron, but their profiles of class 1 integron were not reported (Gong et al., 2013).

Antimicrobial resistance has increased among the human pathogens, and this is a threatening issue for human health. Overuse and misuse of antimicrobials as feed additives and growth promotion in animal production were directly related to

increased resistance (Bilgili, 1994; Cianflone, 2008; Soltan-Dallal et al., 2010; Cosby et al. 2015). Bilgili (1994) indicated that some antimicrobial agents including erythromycin, tetracycline, nalidixic acid, ampicillin, streptomycin, chloramphenicol, sulfonamide, gentamicin, and penicillin are used for the treatment of poultry diseases. Also, it is known that tetracycline which is a broad-spectrum antimicrobial agent is widely used in treatment of humans and animals, as feed additives and growth promoters in poultry in the world (Soltan-Dallal et al., 2010; Marshall and Levy, 2011). In this study, antimicrobial susceptibility test for the *Salmonella* isolates was performed against 18 antimicrobial agents chosen. All the isolates were found to be erythromycin resistant. Moreover, tetracycline resistance was obtained relatively at a high rate in this study. The 77.6% of our isolates were found to be tetracycline resistant and similar to those reported by Douadi et al. (2010). However, a lower incidence of tetracycline resistant (12.9%) was announced by Mezali and Hamdi (2012).

Nalidixic acid is a quinolone group antimicrobial agent and commonly used for the treatment of human and animal infections and for poultry farms (Chen et al., 2004). Among the poultry originated *Salmonella* isolates, quinolones (nalidixic acid and ciprofloxacin) have a crucially high resistance level (Miranda et al., 2009). Our isolates showed a high resistance to this antimicrobial agent (77.6%), and these results affirm that nalidixic acid is excessively used in poultry. Based on the information about usage of antimicrobials in poultry reported by Bilgili (1994), our results also reflect more intensive use of these antimicrobial agents including tetracycline and nalidixic acid. We have obtained data showing that tetracycline, nalidixic acid, streptomycin, chloramphenicol, and trimethoprim/sulfamethoxazole were highly resistant. Our isolates were found to be resistant to streptomycin (58.6%), chloramphenicol (44.8%), trimethoprim/sulfamethoxazole (44%), and kanamycin (43.1%). However, none of our isolates showed resistance to amikacin and cefoxitin. Chen et al. (2004) obtained a high resistance to tetracycline, and nalidixic acid, high susceptibility to amikacin from chicken and pork samples, similar to our results. The results announced in previous studies have been supported our results that *Salmonella* in poultry were generally resistant to multiple antimicrobials, including tetracycline, nalidixic acid, and streptomycin (Chen et al., 2004; Zhao et al., 2007; Arslan and Eyi, 2010; Ribeiro et al., 2011; Siriken et al., 2015). In contrast to our study, high levels of resistance to ampicillin 93%, 60%,

83.3% were reported by Sallam et al. (2014) in from retail beef, by Ribeiro et al. (2011) in meat products and by Arslan and Eyi et al. (2010) in meat products, respectively.

The emergence of *Salmonella* isolates which are antimicrobial resistant can lead to serious human and animal infections (Wang et al., 2013). In the present study, the multidrug-resistance rates of *Salmonella* spp. to at least three or more antimicrobial agents were 80.2%. Our results were lower than those reported (88.9%) by Van et al. (2007). However, Pande et al. (2015) did not detect the multidrug-resistant *Salmonella*.



6. CONCLUSIONS

Foodborne illnesses are commonly caused by bacteria and are considered to be a reason for serious public health and economic problem worldwide. *Salmonella* is an important foodborne pathogen that can be isolated from variety of sources including water and food with contaminated faeces and intestines of various cold-blooded and warm-blooded animals, and humans. All *Salmonella* species may be pathogenic for human, and they can cause salmonellosis, typhoid fever and septicemia.

In many parts of the world, the incidence of *Salmonella* varies depending on geological features. Types of climate, temperature, moisture, and pH are important factors that influence the growth of *Salmonella*. Moreover, *Salmonella* can be transmitted to humans via contaminated food or water, especially with highly contaminated poultry products. In this study, the chicken meat samples were evaluated for the presence of *Salmonella* spp. The occurrence of *Salmonella* spp. was investigated according to ISO 6579 horizontal method for the detection of *Salmonella* spp. Some virulence characteristics (siderophore production, biofilm formation, invasion, and plasmid virulence) and the presence of antimicrobial resistance genes (*bla*TEM, *tetA*, *sulI* and class 1 integron) were noticed. In addition, the antimicrobial susceptibility results were examined by using the disk diffusion method. *Salmonella* was detected in the 83 (56.8%) chicken meat samples out of the 146 samples, and the 116 isolates were obtained. All the isolates were phenotypically and genotypically confirmed as *Salmonella* spp. Of the 116 isolates, 76 (65.5%) were biochemically identified as *S. Typhimurium*, 27 (23.3%) as *S. enterica* subsp. *indica*, 9 (7.8%) as *S. Pullorum*, 2 (1.7%) as *S. enterica* subsp. *arizonae*, 1 (0.9%) as *S. enterica* subsp. *diarizonae*, and 1 (0.9%) as *S. enterica* subsp. *salamae*. All of the isolates were tested for the production of siderophore and biofilm formation. The 116 *Salmonella* isolates harbored *invA* virulence gene while 22 (19%) *Salmonella* isolates were found to be positive for the *spvC* virulence gene. The *spvC* is interacted with the host immune system and responsible for the growth of *Salmonella* in cells, whereas *invA* gene is responsible for invasion in the epithelial tissues of animals and

humans intestine. Both of the *spvC* and *invA* genes are essential for human systemic disease.

In Turkey, there is limited number of studies related with the antimicrobial resistance gene profiles of *Salmonella* from different sources. In the present study, *bla*TEM (91.4%), *tetA* (57.8%), *sulI* (38.8%) and class 1 integron (45.7%) resistance genes were detected at a high rate. The 54 class 1 integron positive *Salmonella* isolates have six different sizes as follow: 1100 bp, 1700 bp, 2200 bp, 2500 bp, 2800 bp, and 3000 bp, respectively. Antimicrobial resistant bacteria are a potential public health threat, and multidrug-resistance pattern was observed in 80.2% of the *Salmonella* isolates to at least three or more antimicrobial agents.

Animal origin foods can highly contaminated with pathogenic bacteria. The results of the study indicate that the chicken meat creates a potential hazard to consumers. Moreover, the presence of virulence associated genes may contribute to invade and survive in the host cells, hence causing *Salmonella* infections.

Meat products, especially poultry should be separated from other raw foods. For instance, a separate cutting board for fresh raw meat must be used. Moreover, after cutting raw foods, the boards and knives should be thoroughly washed, as they may cause cross-contamination linked to raw and processed foods by food contact surfaces. The *Salmonella* contamination of the chicken products can be closely related to poor personal hygiene, inadequate cleaning, disinfection of both equipment and surfaces and untrained personnel. Protection against infections can be achieved by designing proper plants, personnel hygiene, training personnel, devising equipment that can be sanitized effectively and establishing an efficient cleaning and hygiene procedure.

Recently, drug resistant bacteria between foodborne pathogens have increased due to the excessive use and misuse of antimicrobial agents as a growth promotion and treatment of disease in animal feed. The resistant bacteria carried resistance genes can transfer to humans via food chain and may cause serious problems for the treatment of diseases. The awareness of appropriately antimicrobials use in animal farms should be increased. The strategies to prevent the environmental spread of resistant bacteria should be developed.

Foods should be produced by using proper sanitation and personal hygiene and should be reheated after refrigeration. Persons should not eat raw or undercooked eggs or meats. Consumers should be very careful for certain food, and had better take

necessary temperature and time combination into consideration to kill the pathogen before consumption. Specifically, meat products should be cooked at a core temperature of 74°C that can kill off the *Salmonella*.

Consequently, the wide range of efforts to achieve food safety include better monitoring and reporting of foodborne diseases by government agencies, routine food sampling and testing at food processing. In addition, the incidence of surveillance of food should be increased. Food workers trained in support of quality in the food production and consumer awareness for food safety are important. The use of antimicrobials as growth promotions and in the treatment of poultry diseases should be controlled in order to avoid overuse and misuse.



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