CHARACTERIZATION OF PLASMIDS FROM MULTI DRUG RESISTANT *SALMONELLA* INFANTIS ISOLATES

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ABSTRACT

CHARACTERIZATION OF PLASMIDS FORM MULTI DRUG RESISTANCE *SALMONELLA* INFANTIS ISOLATES

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Foodborne *Salmonella* infection is a worldwide challenge to human health and food economy. *Salmonella* pathogenicity depends on different factors involved in virulence that help the pathogenic organism in adhesion and invasion mechanisms. Resistance to antimicrobials is a challenge for treatment strategies of salmonellosis. Plasmids, small, circular, self-replicating DNA elements, often capable of transfer via conjugation, are frequently associated with drug resistance by *Salmonella* strains. Plasmids are classified by incompatibility (Inc) groups, which are named as such, because two members of the same Inc group cannot be stably maintained in a bacterium during cell division. Current study aims to identify and characterize genes responsible for drug

resistance associated with *Salmonella* plasmids. For this purpose, plasmid purification was performed to 70 multidrug resistant (MDR) *Salmonella* Infantis isolates collection. Eight plasmid presences were observed with the size between 40-47 kb in 8 isolate out of 70 MDR *Salmonella* Infantis. To characterize plasmids, antimicrobial resistance gene screening, Multi Locus Sequence Typing (pMLST), and whole genome sequencing were performed. High number of antimicrobial resistance genes in the plasmids showed that the conjugation might be the major way of transmission of antimicrobial resistance among the isolates. pMLST scheme included *repI, ardA, trbA, sogS, pilL, smr0018, smr0199, FII, FIA, FIB, FIC, repN, traJ, korA* genes. Among 14 pMLST genes, only 3 different genes (*ardA, pill, sogS*) belonging to the InclI group, were found in 8 plasmids. To confirm wet lab results, 5 representative plasmids were whole genome sequenced. Interestingly, the result of whole genome sequencing of 5 plasmid isolates showed the evidence of bigger plasmids, such as 131 kb. This conflict might be due to the difficulties of isolating larger plasmids with low copy numbers. The results of this study provides a better understanding of molecular distribution of plasmids in the recently emerged serotype, Infantis, found in poultry samples, as well as how molecular paths of gaining multidrug resistance by *Salmonella* isolates. In a big picture this study provides a detailed information that can be used in human salmonellosis infection control and therapeutic strategies.

Keywords: food-borne pathogen, plasmid, *Salmonella*, chicken, isolation, antimicrobial resistance

ÖZ

ÇOKLU İLAÇ DİRENÇLİ *SALMONELLA* İNFANTİSLERİN PLAZMİDLERİNİN KARAKTERİZASYONU

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Gıda kaynaklı *Salmonella* infeksiyonu insan sağlığı ve gıda ekonomisi için dünya çapındaki bir sorundur. *Salmonella* patojenitesi, patojenik organizmanın yapışma ve istilasına yardımcı olan virülansı içeren farklı faktörlere dayamaktadır. Antimikrobiyallere direnclilik, salmonellosis tedavi yöntemlerine karşı zorluk çıkartmaktadır. Plazmidler küçük, dairesel, konjugasyon yoluyla aktarılabilen, kendi kendine çoğalabilen DNA parçacıklarıdır ve *Salmonella* türlerindeki ilaç dirençliliğiyle ilişkilendirilmektedirler. Plazmidler uyuşmazlık (Inc) gruplarına göre sınıflandırılmaktadırlar çünkü aynı Inc grubuna ait iki plasmid hücre bölünmesi sırasında bir bakteri hücresinde bulunamazlar. Bu çalışma *Salmonella* Infantis

plazmidlerindeki ilaç dirençliliğinden sorumlu genlerin belirlenmesi ve karakterize edilmesini amaçlamaktadır. Bu amaçla 70 coklu ilaç direncliliğe sahip *Salmonella* Infantis izolatı plasmid varlığı tepiti için incelenmiştr. Çoklu ilaç dirençliliğine sahip 70 *Salmonella* Infantis içinden 8 tanesinde boyutları 40 ile 47 arasında değişen 8 adet plasmid gözlenmiş, plasmid karakterizasyonu için, antimiktobiyel dirençlilik geni taraması, plasmid Multi Lokus Sekans Tiplendirme ve tüm genom sekanslamaları yapılmıştır. Plazmidlerdeki yüksek miktarda antimikrobiyal direnç genleri, konjügasyonun, izolatlar arasında antimikrobiyal direncin ana yolunun olabileceğini belirginleştirmiştir. Multi lokus sekans tiplendirme (pMLST) repI, *ardA, trbA, sogS, pilL, smr0018, smr0199,* FII, FIA, FIB, FIC, repN, traJ, korA genlerini içermektedir. 14 pMLST geni arasından çalışmamızda varlığı tespit edilen 8 plazmid izolatında IncII grubuna ait sadece 3 farklı gen (*ardA, pilL, sogS)* bulundu. Laboratuvar sonuçlarımızı doğrulamak için tespit edilen 5 plazmid izolatı tüm genom analiziyle diziletilmiştir. İlginç bir şekilde, 5 plazmid izolatında tüm genom dizilemesi sonucu, 131 kb gibi büyük plazmidlerin varlığı gözlemlenmiştir. Bu karşıtlık düşük kopya sayısından ötürü daha büyük plazmidlerin izole edilmesinin zorluklarından kaynaklandığı tahmin edilmektedir. Bu çalışmanın sonuçları, *Salmonella* izolatları ile çoklu ilaç direnci kazanmanın moleküler yollarının yanı sıra, kanatlı örneklerinde bulunan en yaygın serotipin (*Salmonella* Infantis) plasmid moleküler dağılımının daha iyi anlaşılmasını sağlayacaktır. Büyük pencereden bakıldığında bu çalışma insanlardaki salmonellosis infeksiyonlarını kontrol etme ve tedavi yöntemlerinde kullanılabilecek detaylı bir bilgi sağlayacaktır.

Anahtar kelimeler: gıda kaynaklı patojen, plazmid, *Salmonella,* tavuk, izolasyon, antimikrobiyel dirençlilik

To my family…

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CHAPTER I

INTRODUCTION

1.1 Foodborne Diseases

As human population increases, the concerns of providing healthy and hygienic food are increased. According to World Population Prospects the 2017 Revision, the word population had reached to 7.6 billion as of mid-2017 (Department of Economic and Social Affairs, DESA, 2017) and it continues to increases over year, hence it is a major concern to take the essential precaution for a healthy global food distribution to the increasing word population (DESA, 2017) .

Foodborne disease or illness is any illness caused by contaminated food. Majority of the foodborne diseases are infectious ones due to pathogenic bacteria, viruses or parasites, while other foodborne diseases are caused by intoxication although different foodborne pathogens cause various symptoms, diarrhea, nausea, vomiting and abdominal cramps are common symptoms for many foodborne diseases (Addis and Sisay, 2015).

There are many issues that pose a risk to food safety around the world, as well as in Turkey due to industrialization and mass production, the arise of longer and more complex food chains, fast food consumption, street vendors and increasing international trade and tourism. In addition, long-term inflation and other economic reasons; advertisements, increasing fast foods and restaurant meals eating habits are possible

causes of increased food safety problems in Turkey (Baş et al., 2006, Baş et al. 2007). In 2016, 4,786 foodborne outbreaks have been reported, including waterborne outbreaks. *Salmonella* was the most frequently confirmed causative agent. One out of six outbreaks was through *Salmonella* followed by other bacteria, viruses and bacterial toxins (EFSA, 2017).

Despite the increased capabilities of detection methods, less than 50 % of all epidemic causes are detected due to limited diagnostic capability. Viruses are probably the most common cause of foodborne illnesses, but there are very limited studies on viruses, due to the short duration of the viral infection and the nature of viruses. In addition, the cost of subsequent viral investigations is higher, thus resulting in a lack of clinical investigation and therefore inadequate reporting. On the other hand, foodborne pathogenic bacteria are the most documented foodborne agents (Altekruse et al, 1996). *Campylobacter* spp., *Salmonella* spp., *Shigella* spp., and *Escherichia coli* O157: H7 are the most common identified bacterial pathogens, which have more cold, heat, and acid tolerance than most common ancestors, as well as resistance to multiple antimicrobial In addition, prolonged infection due to these pathogens has resulted in increased drug resistance in these bacteria (Varma et al, 2002).

In Turkey, the number of foodborne cases has not be reachable due to different reasons; the number of cases has not been released by the Ministry of Health and the causative agents have not been identified from cases. Therefore, there are limited data, reporting foodborne cases in Turkey. WHO (World Health Organization) reported that 26,156 foodborne diseases, including 175 people deaths, were detected between 1993 and 1998 with a peak in 1995 (WHO, 2004). Another study from Turkey reported that 84,340 and 77,515 food-borne illness cases occurred in 1999 and 2000, respectively (Soner and Özgen, 2002). The most common known foodborne illness in Turkey is salmonellosis caused by *Salmonella* spp., similar to the other countries in 1999, 28884

foodborne cases and in 2000, 24498 cases of salmonellosis were detected. (WHO, 2004).

1.1.1 *Salmonella* and Salmonellosis

The genus *Salmonella* are members of the *Enterobacteriaceae* group and contains two species; *Salmonella enterica*, *Salmonella bongori*. *Salmonella enterica* consists of 6 subspecies *enterica* subsp. *enterica*, *enterica* subsp. *salame*, *enterica* subsp. *arizonae*, *enterica* subsp. *diarizonae*, *enterica* subsp. *houtanae* and *enterica* subsp. *indica* (Guibourdenche et al., 2010). Currently more than 2,600 *Salmonella* serovars have been described by the Kauffmann-White scheme and listed in the White-Kauffmann-Le Minor (WKL) scheme (Issenhuth-Jeanjean et al, 2014). The majority of serovars are represented by *enterica* subsp. *enterica* (Table 1). Among all recognized *Salmonella* serovars, only 22 belongs to *bongori* and others belonging to *enterica* (Porwollik, S., 2004).

Table 1: Number of serovars represented by *Salmonella* species, subspecies according to White -Kauffmann-Le Minör Scheme (Issenhuth-Jeanjean et al, 2014)

Salmonella species and subspecies	No. of serovars
enterica	
subsp. *enterica* (I)	1586
subsp. *salamae* (II)	522
subsp. *arizonae* (IIIa)	102
subsp. *diarizonae* (IIIb)	338
subsp. *houtenae* (IV)	76
subsp. *indica* (VI)	13
bongori (V)	22
Total	2659

Salmonella enterica subspecies *enterica* (*Salmonella*) is a zoonotic agent that can live in the gastrointestinal tract of warm and cold blooded animals and cause diseases in human being *Salmonella* spp.s are Gram-negative rod shaped, zero-tolerant, mesophilic and facultative anaerobic bacteria that are able to survive without oxygenic atmospheres and can tolerate the low temperature, such as temperatures below 15°C (Table 2).

Table 2: Factors effecting the growth of *Salmonella* spp.s (ICMFS, 1996)

Conditions	Minimum	Optimum	Maximum
Temperature (ºC)	5.2	35 – 43	46.2
pH	3.8	7 – 7.5	9.5
Water activity (a_w)	0.94	0.99	>0.99

Salmonella (*Salmonella*) serovars can cause various diseases depending on the serovar *Salmonella* Typhi causes severe disease, called as typhoid fever, with an incubation period in 3-60 day. Symptoms of typhoid fever are anorexia, malaise, high fever, myalgia and headache; sometimes diarrhea or constipation. Similarly, genetically closely related serovars to Typhi, such as Paratyphi A, causes typhoid like symptoms, but milder than typhoid fever. Other serovars, non-typhoid serovars, can cause a milder disease, called as salmonellosis. Symptoms of salmonellosis are vomiting, nausea, diarrhea, abdominal pain, vomiting and mild fever. Incubation period of salmonellosis might be observed in the period of 6 hours to 10 day Depending on the economic status of the countries, the prevalence of *Salmonella* causing diseases are different. In the developed countries, the number of typhoid fever cases is very low so they can be even negligible. However, salmonellosis cases have been commonly observed, even the hygienic conditions have been improved. On the other hand, typhoid fever is still a big problem in undeveloped countries, such as African countries. According to EFSA 2016 report, a total of 94,625 confirmed salmonellosis cases were reported by 28 European Union (EU) Member State (MS) in 2015 (European Food Safety Authority European Centre for Disease Prevention and Control, 2016). In Turkey, the number of outbreaks have also increased dramatically in the past 30 years and Enteritidis and Typhimurium are the most commonly reported serotypes in Turkey, but we have limited data the confirmed salmonellosis cases of these isolates in Turkey. (Dolapçı İ., et all, 2015).

Distribution of serovars is varied to geographical region. The four most frequently reported *Salmonella* serovars among humans in 2015 were Enteritidis and Typhimurium, followed by monophasic Typhimurium, 1,4,[5],12:i:- and Infantis. These four serotypes caused 72,1%, of confirmed 69,663 human cases in the European Union country (EU) (Table 3).

Table 3: Distribution of reported confirmed cases of human salmonellosis in the EU/EEA, 2013–2015, by the 20 most frequent serovars in 2015 (EFSA, 2016)

Serovar	2015			2014			2013		
	Cases	MSs*	%	Cases	MSs	%	Cases	MSs	%
Enteritidis	31.829	26	45,7	32.874	27	44,4	29.090	27	39,5
Typhimurium	10.997	26	15,8	12.866	27	17,4	14.852	27	20,2
Monophasic Typhimurium 1.4.[5].12:i:-	5.770	15	8,3	5.773	13	7,8	6.313	14	8,6
Infantis	1.585	24	2,3	1.841	26	2,5	2.225	26	3,0
Stanley	763	22	1,1	757	23	1,0	813	21	1,1
Newport	725	19	1,0	752	20	1,0	714	21	1,0
Derby	648	21	0,9	753	23	1,0	818	21	1,1
Kentucky	506	18	0,7	605	21	0,8	651	23	0,9
Virchow	504	21	0,7	509	22	0,7	571	22	0,8
Paratyphi B var. Java	434	17	0,6	388	15	0,5	348	16	0,5
Agona	374	15	0,5	378	23	0,5	581	24	0,8
Bovismorbificans	372	20	0,5	440	21	0,6	412	20	0,6
Napoli	366	13	0,5	333	14	0,4	434	14	0,6
Oranienburg	305	15	0,4	261	17	0,4	274	17	0,4
Saintpaul	274	17	0,4	374	19	0,5	401	19	0,5
Thompson	262	17	0,4	167	18	0,2	255	19	0,3
Chester	260	13	0,4	294	18	0,4	111	13	0,2
Panama	258	13	0,4	244	15	0,3	352	16	0,5
Braenderup	238	15	0,3	276	17	0,4	245	19	0,3
Hadar	235	19	0,3	286	16	0,4	267	20	0,4
Other	12.958	-	18,6	13.845	-	18,7	13.900	-	18,9
Total	69.663	26	100,0	74.016	27	100,0	73.627	27	100,0

*: MSs: The number of Member States

Besides these serovars, there are numerous serovars of *Salmonella* are pathogenic to both human and animal. The most common serovars were different 10 years ago. There are some serovars, emerging and becoming to top of list. For example, in the EU, the top five serovars, which commonly cause salmonellosis in humans were Enteritidis, Hadar, Infantis, Typhimurium, and Virchow (Galanis et al., 2006). In last 5 years, monophasic Typhimurium, 1,4,[5],12:i:- and Infantis serovars have been seen more commonly all over the world, including Turkey. In our previous studies, we observed that the most commonly seen serovar among the food samples, especially in poultry samples, was Infantis Interestingly, the prevalence of Enteritidis and Typhimurium was lower in the poultry samples, representing a shift in *Salmonella* serovar distribution to Infantis (Durul et al., 2015).

1.1.2 *Salmonella* enterica subspecies enterica serovar Infantis (*Salmonella* Infantis)

Salmonella enterica subspecies *enterica* serovar Infantis (*Salmonella* Infantis), a nontyphoidal serovar, commonly transmitted from animal products to humans via contaminated food. *Salmonella* Infantis has become one of the main causes of human salmonellosis in 5 year. In the United States, *Salmonella* Infantis is one of the dominant serovars in swine among livestock (Janecko et al., 2015; Walton et al, 2005). Similarly, Infantis is one of the common causes of salmonellosis in humans in the European Union (Janecko et al., 2015; Walton et al, 2005; Galanis et al., 2006). As previously mentioned EFSA report (2016) by European Centre for Disease Prevention (ECDC), Infantis is the fourth most common *Salmonella* serovars in human in 2015 in the EU. According to same EFSA report, the most frequent serovar among reports from *Gallus gallus* (poultry) was Infantis, accounting for 33.6 % of all reported isolate. In details, EFSA (2016) reported that as for poultry (*Gallus gallus*), Infantis was isolated in 38.7% from

all visited broilers in EU (N=28) and serovars Infantis and Enteritidis were the two most common serovars isolated from broiler meat, accounting for 54.1% and 12.4%, respectively (EFSA, 2016).

The most frequent serovar among reports from poultry was Infantis, accounting for 1,859 or 33.6% of all reported isolates, followed by Enteritidis (875 isolates; 15.8%) and *Salmonella* Mbandaka (373 isolates; 6.7%). These serovars were confirmed for the fourth year as those most frequently isolated serovars from poultry (*Gallus gallus*) (EFSA, 2016).

The increase of Infantis in poultry and poultry products have also affected the human cases. Researchers reported that there have been increased salmonellosis cases due to Infantis in human in several countries, e.g. Japan, Israel and Hungary, Germany (Bassal et al, 2012, Miller et al, 2010).

1.2 Antimicrobial Resistance of Foodborne Pathogens

Agents that inhibit bacterial growth or kill them are called as antimicrobial. The majority of antimicrobials is originated from natural sources, but today semi-synthetic or full-synthetic forms are used as drug. There are mainly two categorizations in antimicrobials such as bacteriostatic and bactericidal. Bacteriostatic antimicrobials prevent or reduce in replicating of bacteria, while bactericidal ones kill bacteria. On the other hand, when used high doses of antimicrobials defined as bacteriostatic, they might have the ability to kill bacteria (Nemeth et al., 2014).

The number of antimicrobial resistant bacteria which are isolated from human and animals have increased over the last two decades. The global pandemic of antimicrobial

resistant bacteria has been contributed by the misuse and overuse of antimicrobials in both human and veterinary medicine (Walsh et al, 2008).

Some strains of *Salmonella* show different antimicrobial resistance profiles and is attracting researchers around the world. The resistance profile may vary depending on time, serovar, subtype, microorganism source and also the geographical region of the origin of the isolate. It was seen that multidrug resistance (MDR) phenotype is emerging in *Salmonella* serovars. In the recent studies, serovars such as Typhi, Paratyphi, Infantis, Uganda, Agona, and Newport, Hadar, Heidelberg showed multidrug resistance. Antimicrobial resistance in the serovar Infantis isolates from human and animal sources in various European countries has emerged over the last few years. As a result, this serovar, along with Kentucky, is a major contributor to the number of multidrug-resistant (MDR) *Salmonella* in Europe (Nógrády et al, 2008; Dionisi et al., 2011). In addition, antimicrobial agents that Infantis isolates from human and animal sources are resistant to are varied (Hindermann et al., 2017; Tate et al., 2017).

Multidrug resistance is defined as a condition, in which a disease-causing organism is enable to live and give harm to the host organism by resisting to drugs or chemicals used for killing or stabilizing of their growth. Bacteria have developed different ways to survive from the antimicrobial effects, for example (Dzidic et al., 2008):

- Antibiotic inactivation
- Target modification
- Efflux pumps and permeability changes of outer membrane
- Target bypass

Multidrug resistance in bacteria developed by the action of multidrug efflux pumps, each of which can pump out more than one drug type or by the accumulation, on

resistance (R) plasmids or transposons, of genes, with each coding for resistance to a specific agent (Nikaido et all, 2009). In bacteria, multi-drug resistance (MDR) occur usually the result of acquisition of mobile genetic element that containing multiple resistance genes occurs. Nucleotide sequence analysis of multiresidue integrons shows that the added resistance gene cassettes differ markedly in codon usage, indicating that antibiotic resistance determinants have different origins. (Nirdnoy et all., 2005) Bacteria that have evolved separately up to 150 million years have the strongest influence on the development of antibiotic resistance in bacterial pathogens. (Vulic et all., 1997). The DNA movement can be within the genomes (intra-genomic mobility) or between the genomes (inter-genomic mobility) (Frost et al, 2005). There are three forms of DNA transfer in prokaryotes; transformation, conjugation and transduction, which are considered as horizontal gene transfer (HGT) (Burmeister, 2015).

1.2.1 Mobile Genetic Elements and Plasmids

Mobile genetic elements (MGEs) are DNA fragments that encode enzymes and protein to control the movement of DNA within the genome. Plasmids, the mobile genetic elements, are small DNA molecules that are separated from a chromosomal DNAs. Plasmids found in all three domains; Archaea, bacteria and eukaryote can be transferred through transformation or conjugation, between cells. The other types of MGEs can be ordered as transposons, bacteriophage and integrons.

Transposable elements (TEs), also called jumping genes or transposons, are double-stranded DNA sequences that are able to change their position in the genome. They are found in variable sizes and have been identified in all three domains and can occupy a high ratio of a species' genome. For instance, TEs consist of nearly 50% of the human genome and 90% of the maize genome (SanMiguel, 1996). Because of have not own replication system transposons need to integrate into replication-proficient DNA

molecule in the cells. The movement of TEs is termed transposition or retrotransposition, according to their nature mobility (Muñoz-López et al 2010).

Importance of transposons on bacterial cells might be:

- helping organisms to rearrange genetically,
- causing heritable changes,
- giving new genetic functions and
- contributing organisms to their spreading in bacterial cells.

Enteric Gram-negative bacteria and the constitution of their plasmids are taken into consideration to state the role of transposons in evolvement of bacteria. Bacteria that recovered in pre-antimicrobial period, had plasmids, but they had no resistance agents were located in them. R plasmids currently isolated belong to same incompatibility groups similar to plasmids determined before, but they have gained multi-drug resistance genes after some evolutionary events. There are close correlations among replicons of R plasmids. This relationship might become a strong proof that current R plasmids improved from older plasmids by gaining of resistance agents. Some resistant plasmids have one transposon with more than one resistance factors, while others have more than one resistance transposons placed at different sites (Holmes et al., 1996).

Plasmids, other type of mobile elements, are non-chromosomal double stranded DNA molecules. Most plasmids are circle physically, because covalent bond is forming in their two ends; others are linear. They can multiply themselves independently. The size of plasmids naturally found in host can vary from 1 kb to 1000 kb. The copy number of plasmids naturally found in one host can show varieties from one to several hundred (Novick, 1987). There are genes between 5 and 100 in one plasmid. These genes not vital for regular growth of bacteria but they come up with abilities to live in harsh environmental conditions and resistance to antimicrobials for bacteria.

Plasmids can exist in different families as archaea and eukaryote, mostly in bacteria (Shintani, 2015). Conjugation is the one of major ways to transferring genetic material between cells (Frost and Koraimann, 2010). Genetic elements in bacterial cells can be spread effectively by conjugation (Guglielmini et al., 2011). About % 14 of the sequenced plasmids were showed to have the conjugative ability (Smillie et al., 2010). Plasmids are factors that increase their genetic diversity by lateral transfer by conjugation process (Carattoli, 2009). Plasmids have importance due to they are leading in bacterial communication with rapid evolution and adaptation abilities (Aminov, 2011). Most of plasmids carry antimicrobial resistance genes phenotypically observable (Carattoli, 2009).

National Center for Biotechnology Information (NCBI) Plasmid Genome database had 4602 completely sequenced plasmids in 2014: 4418 are from bacteria, 137 are from archaea and 47 are from eukaryote. Nucleotide sequence data relieved a better understanding how plasmids spread among bacteria related with the knowledge of the correlation among host taxonomy and plasmid properties (Shintani, 2015).

There are some features of known plasmids which of helping successful outspread of plasmids originated from different sources and origins. Plasmids can be identified by phenotypes of the bacterial host that depend on existence of their virulence or antimicrobial resistance genes (Carattoli, 2014). The prevalence of multidrug resistant bacterial clones might be changing due to the gaining of plasmids containing antimicrobial resistance genes. To examine the molecular epidemiology of transferable plasmids is as least important as the molecular epidemiology of dissimilar bacterial clones.

There are different ways to characterize the bacterial plasmids:

- molecular determination of the incompatibility groups
- plasmid MLST
- plasmid genome sequencing.

1.2.1.1 Molecular Determination of Incompatibility in Plasmids

Plasmids have codes for replicating themselves, controlling their copy number and promoting inheritance of them during each cell division (Frost, 2005). The phenomenon stated as ‘incompatibility’ (Inc) is the impossibility of plasmids with the same multiplication mechanism remains in the same cell during cell division. Initial classification of plasmids is conducted by Inc groups (Couturier, 1988).

Inc group classification of plasmids has been both a significant method to detect the spread of plasmids featuring antimicrobial resistance and to monitor the distribution and development of coming out plasmids (Anderson et. al, 1977). There are about 28 different incompatibility group determined among *Enterobacteriaceae* plasmids. Every incompatibility group has their own marker genes for determination (Carattoli, 2009).

1.2.1.2 Plasmid Multi Locus Sequence Typing (pMLST)

Multilocus Sequence Typing (MLST) is genetically typing method that aims to be robust and rapid method for the characterization of bacterial isolates at the molecular level (Maiden et al, 1998). The MLST technique is a molecular characterization technique that is based on population genetic approaches that underlined with a similar point of view to that of multilocus enzyme electrophoresis (MLEE) (Selander et al, 1986). MLST basically depends on the sequencing of the previously determined 450-500 base pair fragments 7 housekeeping genes and the detection of the nucleotide

differences of these fragments. For each house-keeping gene, the different sequences in the bacterial species are assigned as different alleles and for each of the 7 loci for each isolate define the allelic type (AT) or sequence type (ST). For instance, in the most commonly used MLST scheme of *Salmonella enterica*, including seven genes, ST 32 which is the sequence type of the majority of serovar Infantis isolates, corresponds to seven-gene (*aroC-dnaN-hemD-hisD-purE-sucA-thrA*) AT profile: '17-18-22-17-5-21-19' which further corresponds to housekeeping gene profile (Patcharapan et al, 2017).

MLST is also adapted to plasmids for identifying related plasmid scaffolds. IncF, IncI1, IncHI2 and IncHI1 plasmids are currently subtyped by plasmid Multi Locus Sequence Typing (pMLST) (http://pubmlst.org/plasmid/; García-Fernández et al.,2008; García-Fernández and Carattoli, 2010; Villa et al., 2010). The plasmid multilocus sequence typing (pMLST) scheme identified similar plasmids in bacteria from different sources and from different countries, facilitating comparative interlaboratory studies and supported molecular studies on antimicrobial resistance epidemiology (Carattoli et al, 2011). The web-based sequence definition database, pubmlst.org, is curated by Alessandra Carattoli, Minh-Duy Phan and Steven Hanckok. Primers and protocols of InclI, IncHI2, IncF and IncN groups is given obviously in this website. For the first time, multilocus sequence typing (MLST) have been used to analyze and characterize IncI1 plasmids identified in β-lactamase *E. coli* and *Salmonella* producer from animal and human sources in Europe and the USA. The characterization of plasmids belonging to IncI1 family with different sequence types (STs) was performed. (García-Fernández et al, 2008). *Repl1, ardA, trbA-pndC, sogS, pilL,* which are listed in Table 4, were used as corresponding genes for IncI1 plasmids in García-Fernández study (2008).

Table 4: pMLST scheme regarding to Inc groups (Garcia et al., 2008).

Inc group	Locus	Functions	Amplicon size (bp)	Locus size (bp)
IncI1	*repI*	Replicase gene	142	105
	ardA	Type I restriction-modification enzyme	501	343
	trbA	Involved in maintenance and plasmid transfer	507	485
	sogS	DNA primase	291	254
	pilL	Type IV pilus biogenesis	316	254
IncHI2	*smr0018*	encodes an open reading frame similar to the *Salmonella typhi* putative surface exclusion protein	364	330
	smr0199	Type I restriction-modification enzyme	536	460
IncF	*FII*	The copA region of the FII replicon	258	154
	FIA	The region comprising the iterons and the replication protein repE gene of the FIA replicon	462	408
	FIB	The replication protein repB gene of the FIB replicon	683	373
	FIC	The copA region of the FIC replicon	262	200
IncN	*repN*	replicase gene	514	
	traJ	mobilization of the plasmid during the conjugative transfer	636	
	korA	negatively regulating the synthesis of the conjugal pilus and mating pore during the conjugation process	278	

Until now, 1497 plasmids, 16,9% (254 plasmids) from *Salmonella* species, have been collected at the pMLST database. Among these, 178 were IncI1 plasmids encoding beta-lactamase genes assigned to 48 different sequence types. The most frequent IncI1 sequence type (ST) identified was ST12 as 26,9%. In details, database (pubmlst.org/plasmid) contains 76 *Salmonella* plasmids isolated from poultry samples. According to the database, 83 % of *Salmonella* plasmids were classified in IncI1 group.

1.2.1.3 PCR-Based Replicon Typing

PCR- Based replicon typing is a method depending on hybridization of distinct replicons by using 19 DNA probes to diagnose the main replicons of plasmids in the *Enterobacteriaceae* was developed by Coutrier et al in 1988 (Coutrier et al., 1988).

It is obviously hard to perform that huge number of strains by the help of this PCR-based method depending on hybridization and conjugation-based methods are restricted with the time consuming and troublesome work requirement.

1.2.1.4 Whole Genome Sequencing

Whole genome sequencing (WGS) or complete genome sequencing is the application of defining the entire DNA sequence of an organism at a time. WGS have been rise as an important molecular tool that based high-throughput sequencing technologies. WGS serves the highest genomic resolution possible. WGS offers an advantageous alternative to traditional subtyping methods to report the relationship of bacterial isolates during outbreak or cluster surveys (Vincent et al., 2018; Allard et al., 2012). Whole plasmid sequencing is also a part of WGS application. Recent studies have shown that massive amount DNA sequencing data gathered from WGS can be used to detect among closely related isolates. In addition molecular differences between the isolate groups can

identify by WGS By increased accessibility and lower costs, WGS-based vehicles are quickly taking the place of PFGE typing (Wilson et al., 2016).

WGS varieties are listed in the most basic way in terms of their purpose and techniques. Large Whole-Genome Sequencing is informative about large genomes (such as plant, human or animal genomes) for studies of population genetics or diseases. This method is used for larger than 5 Mb sequences. Small Whole-Genome Sequencing includes sequencing of small organisms smaller than 5 Mb genome in one-pieces. De Novo Sequencing enables sequencing of new genomes without any reference sequence by distinguishing of alleles on homologous chromosomes. This method is used for genetic diseases.

1.3 Aim of the Study

MDR *Salmonella* isolates lead to severe diseases in Turkey and all over the world. *Salmonella* can cause illness from mild gastroenteritis to serious illnesses in advances, especially at risk groups (infected patients, infants, elderly and pregnant women). Serovars of *Salmonella* have been evolving regarding to the environmental conditions, host species, therefore different serovars dominate the *Salmonella* population in different geographical regions. However, there are some serovars frequently dominate population, for example Typhimurium. It has been observed that less commonly isolated serovar, Infantis, emerged recently and become one of the major serovars (4th most common serovar) among poultry, as well as human cases, all over the world, including Turkey. Here, we hypothesis that this emerge of Infantis isolates is due to their plasmids and their ability of conjugation that leads multi-drug resistant strains. To test this hypothesis, our aim is to assess the distribution of plasmids in *Salmonella enterica* subs. *enterica* serovar Infantis by screening antimicrobial genes on plasmids, plasmid multilocus sequence typing (pMLST) and genome sequencing.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Bacterial Isolates

In this study, a total of 70 *Salmonella* Infantis was used. 15 *Salmonella* Infantis isolates were collected in this study, rest of them (N=55) was already collected from different projects and were stored at -80°C in Food Safety Laboratory (FSL, Soyer Laboratory) at Food Engineering Department at Middle East Technical University (METU). 15 of 55 *Salmonella* Infantis isolates were collected from chicken samples following TS EN ISO 6579 taken from Şanlıurfa between the years 2012 and 2013 in our laboratory for a previous project, funded by TUBITAK (Project number 111O192). Phenotypic and genotypic subtyping (PFGE, MLST, antimicrobial resistance typing and serotyping) of these isolates (N=15) were already completed with the scope of the TUBITAK 1110192. In addition, 1 Infantis isolate isolated from chicken in Ankara region in 2005 was added to the study.

Rest of 55 *Salmonella* Infantis (39) were collected from chicken meat sold commercial markets following TS EN ISO 6579 in Ankara region between 2012 and 2014 for Scientific Research Project (BAP), funded by Middle East Technical University (METU) (BAP Project number: BAP-03-14-2013-001). Phenotyping (ie., serotyping

and antimicrobial susceptibility) and genotyping (ie., PFGE subtyping) were completed for these isolates in the scope of BAP-03-14-2013-001.

To determine band sizes on the gel electrophoresis, *E.coli* 39R861 isolate that was kindly supplied by Dr. Belkis Levent from Turkiye Halk Sagligi Kurumu was used.

All mentioned above isolates were frozen in glycerol under specific IDs and stored at -80°C in our isolate bank in Food Safety Laboratory (FSL, Soyer Laboratory) at Food Engineering Department at Middle East Technical University (METU) (Appendix A).

2.1.2 Chemicals

Analytical grade chemicals were used in this study. Chemicals with their suppliers are listed in the Appendix B. Sequences of primers and amplified region sizes are given in Appendix D.

2.1.3 Solutions and Buffers

The Appendix B is included the information about solutions and buffers used in.

2.1.4 DNA Analysis Software

To analyze raw sequence data (.abi files) for pMLST, DNAStar (DNASTAR Inc, Madison, USA) was used. For determining of pMLSTs, pMLST web-site (https://pubmlst.org) was used. BioNumerics Software was performed to find the size of DNA bands from PFGE gel pictures (Applied Maths, Kortrijk, Belgium).

2.2 Methods

2.2.1 Isolation of *Salmonella*

Chicken samples (N=40) were collected from markets in Ankara. Isolation was conducted according to the TS EN ISO 6579. Each sample was weighted as 25 g in the stomacher bag and mixed with Buffered Peptone Water (BPW) in the stomacher for 30 seconds. Stomacher bags were incubated at 37°C for 18 ± 2 hours for non-selective enrichment. For selective enrichment step, 100 µl sample was transferred to to the tube containing Rappaport Vassiliadis Soy (RVS) Broth. For this step, duplicated samples were prepared per a chicken sample and they were incubated at 42°C for 24 hours. 10 µl incubated sample taken from the RVS broth was plated on Brillant Green Agar (BGA) and XLD Agar to incubate in the incubator at 37°C for 24 hours. Presumptive colonies, black colonies with reddish zone from XLD agar and pink colonies from BGA, were streaked in triplicate on to Brain-Hearth Infusion Agar (BHI) and incubated at 37°C for 24 hours.

2.2.2 Confirmation of *Salmonella*

Salmonella suspected colonies were confirmed by Polymerase Chain Reaction (PCR) of the *invA* gene. One colony was transferred from BHI agar to the tube containing 95 µl double deionized distilled water (ddH_2O). *Salmonella* isolate (MET S1-713) was used as the positive control. Caps of tubes were closed and they were microwaved at 90V for 30 seconds to obtain cell DNA inside the cell membrane. Master mixture solution was prepared in a sterile micro centrifuge tube with reagents listed in the Table 5.

Table 5: Master mix for PCR of the *invA* gene

Master mix	Primer Sequences 5’ - 3’	Concentrations	Volume (µl)
ddH_2O	-		
Mg-free Taq Buffer	-	10X	
$MgCl_2$	-	20 mM	1.85
dNTPs	-	10 mM	0.5
invA – F	GAATCCTCAGTTTTTCAACGTTTC	12.5 mM	1
invA – R	TAGCCGTAACAACCAATACAAATG	12.5 mM	1
Taq DNA Polymerase	-	5U	0.125
TOTAL			24.5 µl

Prepared master mix solution was distributed in the amount of 24 µl to labelled 0.2 ml PCR tubes. 1 µl of dirty lysate was mixed with 24 µl master mix solution and tubes were put into the thermocycler for conditions listed in the Table 6:

Table 6: PCR condition for *invA*

Temperature (°C)	Time	Cycles
94	8min.	x 1
94	30 sec.	
72	30 sec.	x 34
72	1 min.	
72	5 min.	x 1
4	∞	x 1

Amplification of PCR was evaluated by gel electrophoresis. 1.5% agarose gel was dissolved in 0.5 x TBE buffer by microwaving. Samples were loaded onto solidified agarose gel in 6 µl (5 µl PCR product with 1 µl 6 x loading dye volume). First well was filled with 1 µl DNA Marker-H1 (100 bp-1000 bp) Ready-to-Use (Bio Basic Inc., Canada). Last two wells were filled with positive and negative controls. Agarose gel was placed into electrophoresis tank to run at 110 V for 30 min. Et-Br solution was used for staining step for 5 min. De-staining step was done by 30 minutes using ddH_2O. PCR results were observed under the UV light (Gel Doc XR Gel Documentation System, California).

2.2.3 Isolation of *Escherichia coli*

Escherichia coli isolates were also collected from same chicken samples to check if *Salmonella* Infantis isolates share transfer antimicrobial genes with *E. coli* isolates, found in the same environment. Isolation of *E.coli* was performed according to TS ISO 7251: Microbiology of food and animal feeding stuffs from chicken samples (N=13). 25 gr meat sample from chicken was taken by sterilized spoon and knife and put into the stomacher bag containing of 225 ml Lauryl Sulfate Broth for non-selective pre-enrichment step. Samples in the stomacher bag were incubated at 37ºC for 24 hours. Second day of the isolation procedure, 0.1 ml pre-enriched sample was transferred into the 10 ml EC Broth (reference) to incubate at 44ºC for 48 hours for selective enrichment. After two days, 10µl of inoculum was streaked onto the Brilliant Green Agar (BGA) and incubated at 37ºC for 24 hours. *Escherichia coli* colony has yellow to greenish-yellow color on BGA agar. *Escherichia coli* colonies were picked from BGA plate and put into Brain Hearth Infusion (BHI) agar and incubated at 37ºC for 24 hours. After *Escherichia coli* conformation with PCR method, the *Escherichia coli* colonies from BHI Agar were incubated in 5 ml BHI broth at 37 ºC overnight. Labelled vials were filled with 850 µl incubated BHI broth and 150 µl pre-sterilized glycerol solution. The vials were mixed gently up and down to ensure mixing of the bacterial culture and the glycerol. Lastly, the vials were taken to at –80°C.

2.2.4 Serotyping of *Salmonella* Isolates

Serovars were determined by Kaufmann-White scheme, conducted in Turkish Ministry of Health Laboratory (Turkiye Halk Sagligi Kurumu). Serotyping was only conducted for the *Salmonella* isolates that showed different PFGE patterns than Infantis isolate.

2.2.5 Pulsed Field Gel Electrophoresis (PFGE)

PulseNet Protocol was used for PFGE analysis. Colonies, grown on BHI agar at 37°C for overnight, were mixed in tubes containing 4 ml cell suspension buffer with cotton swabs. Optical density of 1300µl mixture was measured by using spectrophotometer. Samples having optical densities between 1.3 and 1.4 at 610 nm were kept in ice. 400 µl sample was transferred from tube to microcentrifuge tube to prepare plug. 400 µl of 1% Seakem Agarose prepared with 1% SDS was mixed with samples to cast plugs. Plugs were transferred quickly and cooled to room temperature for 30 minutes.

Solution of Cell Lysis Buffer (CLB) containing Proteinase K (20mg/ml) was prepared for lysing of the cell. Plugs, removed carefully from the plug mold using sterile spatula" were put in 50 ml Falcon tube containing 5 ml CLB – Proteinase K solution. Tubes were shaken for 2 hours at 54°C in the shaking incubator at 200 rpm. Washing steps of plugs were performed in the shaking incubator at 50°C with 70 rpm. Screw caps having small holes were used to keep plugs in the tube. Each solution in the tube were replaced with solution at 50°C every step. Washing steps and chemicals were given below:

- Step 1: CLB was poured and 10 ml ddH_2O was added to be waited at 50°C with 70 rpm for 10 min.
- Step 2: Old water was poured and new 10 ml ddH_2O was added to be waited at 50°C with 70 rpm for 10 min.

- Step 3: Water was removed and 10 ml TE Buffer was added to be waited at 50°C with 70 rpm.
- Step 3 was repeated for Step 4, 5 and 6. Lastly, 5ml TE Buffer was added to the tube to keep plugs at 4°C for storage.

DNA in the plugs were restricted by *XbaI*. Plugs were sliced in 2 mm thick and put into the labelled minicentrifuge tube containing 200 ml H-buffer solution. H-buffer solution was prepared mixing 20µl H-buffer with 180µl ddH_2O for each sample. After tubes were waited in 37°C for 10 minutes, liquid in tubes was replaced with *XbaI* enzyme solution to incubate at 37°C for 4 hours. Enzyme solution was prepared at given portions for each samples: 5 µl *XbaI* enzyme, 20 µl H-Buffer and 175 µl ddH_2O.

Agarose gel was prepared in 1% SKG agarose (BIORAD,) concentration for 150 ml of 0.5 x TBE by microwaving. 1.5 g of SKG agarose was mixed with 7.5 ml 10 x TBE and 142.5 ml ddH_2O. After the mixture was microwaved, it was waited in 55°C water bath for 15 minutes. Mold, black frame and 15-well-comb were adjusted with bubble level before pouring the agarose gel. Solidifying of gel was taken about 20 minutes. Plugs were placed into wells on the agarose gel carefully. Sealing agarose was poured onto filled wells. The loaded gel was performed with CHEF-DR III system with given conditions at Table 7:

Table 7: Electrophoresis condition for PFGE

DNA size interval	30 kb – 700 kb
% agarose	% 1
Voltage	6.0 v/ cm
Run time	19 h
Temperature set	14°C
Included angle	120°
Initial switch time	2.2 s
Final switch time	63.8 s
Pump speed	70 (1L/ min)

Electrophoresed agarose gel was stained with Et-Br solution for 30 min and washed in ddH_2O for 45 min. Results were observed under the trans UV light.

2.2.6 Antimicrobial Susceptibility Test

All *Salmonella* Infantis (N=70) and *E.coli* (N=19) isolates were characterized phenotypically by using method of disc diffusion. 18 different antimicrobial agent were worked at this method by using standards of Clinical Laboratory Standards Institute to see antimicrobial resistance (AR) profiles of all isolates (Table 8). *E.coli* ATCC 25922 was used the control organism for tests of disk diffusion. Isolates were grown in 4 ml Mueller-Hinton Broth at 37°C for 18 h. Incubated cultures were transferred to Mueller-Hinton (MH) Agar in the amount of 100 µl to spread with cotton swab after checking microbial density by 0.5 McFarland solution (~1-2x10^{8} cfu/ml). Disks containing antimicrobial agent were placed onto MH Agar to incubate at 37°C for 18 hours.

Table 8: Diameters of antimicrobial disks used for antimicrobial susceptibility test (AST) by disk diffusion method

Antimicrobial group	Antimicrobial agent	Disk Content	Zone Diameter (mm)		
		(µg)	S	I	R
Aminoglycosides	Amikacin [1]	30	≥17	15-16	≤14
	Gentamicin [1]	10	≥15	13-14	≤12
	Kanamycin [1]	30	≥18	14-17	≤13
	Streptomycin [1]	10	≥15	12-14	≤11
Beta lactams	Ampicillin [1]	10	≥17	14-16	≤13
	Ceftiofur[2]	30	≥21	18-20	≤17
	Cefoxitin [1]	30	≥18	15-17	≤14
	Ceftriaxone [1]	30	≥23	20-22	≤19
	Cephalothin [1]	30	≥18	15-17	≤14
	Amoxicillin-clavulanic acid [1]	20/10	≥18	14-17	≤13
	Ertapenem [1]	10	≥23	20-22	≤19
	Imipenem [1]	10	≥23	20-22	≤19
Phenicols	Chloramphenicol[1]	30	≥18	13-17	≤12
Quinolones and	Nalidixic acid [1]	30	≥19	14-18	≤13
Fluoroquinolones	Ciprofloxacin [1]	5	≥21	16-20	≤15
Tetracyclines	Tetracycline [1]	30	≥15	12-14	≤11
Sulfanomides and	Trimethoprim-sulfamethoxazole[1]	1.25/23.75	≥16	11-15	≤10
trimethoprims	Sulfisoxazole [1]	300	≥17	13-16	≤12

2.2.7 Detection of Antimicrobial Resistance Genes of Isolates

Antimicrobial resistant genes were screened in the cell and the plasmids in each resistant isolate. A total of 21 genes, encoding 5 antimicrobial groups, was screened in each isolate by PCR were performed in addition to thermocycler conditions of each primer set (Table 9).

Table 9: Antimicrobial groups of primers encoding antimicrobial resistance in bacteria and thermocycler conditions were tempered to annealing temperatures of each primer set

Antimicrobial group	Gene	Most founded place	Sequences of Primers	Annealing temperature (°C)	Reference
Aminoglycosides	*strB*	Plasmid	ATCGTCAAGGGATTGAAACC GGATCGTAGAACATATTGGC	57	Gebreyes and Altaier 2002
	aacC2	Plasmid	GGCAATAACGGAGGCAATTCGA CTCGATGGCGACCGAGCTTCA	57.9	Chen et al. 2004
	aphA1-Iab	Plasmid	AAACGTCTTGCTCGAGGC CAAACCGTTATTCATTCGTGA	54	Frana et al. 2001
Trimethoprim	*dhfrI*	Plasmid	CGGTCGTAACACGTTCAAGT CTGGGGATTTCAGGAAAGTA	51.7	Chen et al. 2004
	dhfrXII	Plasmid	AAATTCCGGGTGAGCAGAAG CCCGTTGACGGAATGGTTAG	57.9	Chen et al. 2004
Sulfonamides	*sulI*	Chromosome	TCACCGAGGACTCCTTCTTC CAGTCCGCCTCAGCAATATC	55.6	Chen et al. 2004
	sulII	Plasmid	CCTGTTTCGTCCGACACAGA GAAGCGCAGCCGCAATTCAT	56	Chen et al. 2004
Tetracyclines	*tetA*	Plasmid	GCGCCTTTCCTTTGGGTTCT CCACCCGTTCCACGTTGTTA	57.7	Chen et al. 2004
	tetB	Plasmid	CCCAGTGCTGTTGTTGTCAT CCACCACCAGCCAATAAAAT	58.4	Chen et al. 2004
	tetG	Plasmid	AGCAGGTCGCTGGACACTAT CGCGGTGTTCCACTGAAAAC	60	Chen et al. 2004

Table 9: (continued)

Antimicrobial group	Gene	Most founded place	Sequences of Primers	Annealing temperature (°C)	Reference
Beta-lactamases	*blaTEM-1*	Plasmid	CAGCGGTAAGATCCTTGAGA ACTCCCCGTCGTGTAGATAA	53.9	Chen et al. 2004
	blaPS1E-1	Chromosome	TGCTTCGCAACTATGACTAC AGCCTGTGTTTGAGCTAGAT	52.4	Chen et al. 2004
	blaCMY-2	Plasmid	TGGCCGTTGCCGTTATCTAC CCCGTTTTATGCACCCATGA	60.8	Chen et al. 2004
	ampC	Plasmid	AACACACTGATTGCGTCTGAC CTGGGCCTCATCGTCAGTTA	60	Perez-Perez and Hanson 2002
Chloramphenicols	*cat1*	Plasmid	CTTGTCGCCTTGCGTATAAT ATCCCAATGGCATCGTAAAG	TD 55-45	Chen et al. 2004
	cat2	Plasmid	AACGGCATGATGAACCTGAA ATCCCAATGGCATCGTAAAG	60	Chen et al. 2004
	flo	Chromosome	CTGAGGGTGTCGTCATCTAC GCTCCGACAATGCTGACTAT	54.4	Chen et al. 2004
	cmlA	Plasmid	CGCCACGGTGTTGTTGTTAT GCGACCTGCGTAAATGTCAC	58.5	Chen et al. 2004
Aminoglycosides	*aadA1*	Plasmid	TATCAGAGGTAGTTGGCGTCAT GTTCCATAGCGTTAAGGTTTCATT	53.6	Randall et al. 2004
	aadA2	Chromosome	TGTTGGTTACTGTGGCCGTA GATCTCGCCTTTCACAAAGC	57.3	Randall et al. 2004
	strA	Plasmid	CTTGGTGATAACGGCAATTC CCAATCGCAGATAGAAGGC	51.8	Gebreyes and Altaier 2002

2.2.8 Plasmid Isolation

Plasmids in all Infantis and *Escherichia coli* isolates were isolated by using QIAGEN Plasmid Mini Kit. One fresh colony was picked from Brain Hearth Infusion Agar (BHI Agar) after incubation at 37°C for 18 hours and inoculated to Luria Bertani Broth (LB broth) containing appropriate antimicrobial (Tetracycline). Inoculated 3 ml LB medium was incubated for 8 hours at 37°C in 300 rpm shaking incubator. Dilution to 1/500 was done by transferring 3 µl incubated medium to 3 ml fresh LB broth. Inoculated LB-broth culture was incubated at 37°C for 18 hours. Plasmid isolation buffers were placed as stated in the protocol of kit to increase yield. P1 stored at 4ºC was put at room temperature. SDS in buffer P2 was dissolved by warming to 37ºC. Buffer P3 was pre-chilled at 4ºC. QF buffer was pre-warmed at 65ºC. 3 ml incubated LB-broth was separated into two labelled mini centrifuge tubes to harvest bacterial cells by centrifugation for 15 min at 6000 x *g* at 4ºC. Total 0.3 ml RNase added P1 solution was used to mix well with pellets of one sample. 0.3 ml P2 buffer was mixed gently and observe blue color. If there were any non-homogeneous region, tube should have mixed more. Samples were incubated at room temperature for 5 min. Before adding Buffer P3, ice tubes were put on the bench. Buffer P3 was added to sample tubes and tubes were waited on ice for 5 min. After samples were centrifuged at maximum speed for 10 min, supernatant having plasmid DNA promptly transferred to the QIAGEN-tip 20 equilibrated by applying 1 ml Buffer QBT. It was allowed supernatant to enter the resin by gravity flow. QIAGEN-tip 20 was washed by 2 ml Buffer QC twice. Plasmid DNA was eluted to 1.5 ml microcentrifuge tube by adding 0.8 ml of Buffer QF. 0.56 ml isopropanol was added to tube before tubes were centrifuged immediately at 15,000 x g rpm for 30 min. Supernatant was removed carefully. DNA pellet was washed with 1 ml 70% ethanol and centrifuged at 15,000 g for 10 min. Supernatant was removed carefully and pellet was air-dried for 10 min. 100 µl TE Buffer was added to the tube and mixed with pellet to stock at -20ºC. The plasmid isolation was verified with the agarose gel

electrophoresis at 90V for 180 minutes and visualized with Bio-Rad Universal Hood II gel imager (Bio-Rad Laboratories, Hercules, CA).

2.2.9 Plasmid Size Determination

Two different methods were used to determine plasmid size. Agarose gel electrophoresis and PFGE with *S1 nuclease* were methods.

2.2.9.1 Agarose Gel Electrophoresis

Agarose gel electrophoresis was performed to observe plasmid sizes after the plasmid isolation on the 0.7% agarose gel λ-pUC ladder (Thermo Fisher Scientific Inc., Waltham, USA) was used. *E.coli* 39R861 was used as a positive control and their plasmid sizes (147, 63, 36, 7 in kb) were used as a ladder (Macrina et al., 1978).

2.2.9.2 PFGE with *S1 Nuclease*

Plasmid isolates were examined with PFGE by using of *S1 nuclease.* Enzyme solutions were prepared in amount of 30 µl in given amounts in Table 10. 15 µl of plasmid sample from one isolate was mixed in 30 µl enzyme solution in 200 µl – tubes. Mixtures of enzyme – plasmid sample were incubated at 37°C for 60 minutes in water-bath (Barton et al., 1995).

Table 10: Enzyme mixture prepared with *S1 nuclease*

Master mix	Concentrations	Volume (µl)
ddH_2O	-	20.85
S1-buffer	5x	9
S1 nuclease	100u/µl	0.15
DNA Template		15
TOTAL		45

Incubated enzyme-plasmid sample solutions were loaded on the 1% SKG Agarose gel prepared with using 0.5xTBE solution. Operation conditions were indicated the Table 11.

Table 11: Electrophoresis conditions in the BIORAD CHEF-DR III using for plasmid size determination by *S1 nuclease*

Condition	Value
Low KB	30KB
High KB	700KB
% Agarose	1%
Gradient	6.0 V/cm
Run Time	13h
Included Angle	120°
Initial Switch Time	1s
Final Switch Time	12s
Pump Speed	70

2.2.10 Plasmid Characterization

2.2.10.1 Plasmid MLST

Plasmid isolates were characterized by Plasmid Multilocus Sequence Typing (pMLST) method. Sequences which primers were given in the Table 12 were searched in plasmid isolates that gave any band at the plasmid size determination part by using PCR.

Table 12: Plasmid MLST primers of IncI1, IncHI2, IncF and IncN groups

Location	Genes	Sequences	Annealing Temperatures	Reference
IncI1	*repI*	F:CGAAAGCCGGACGGCAGAA R: TCGTCGTTCCGCCAAGTTCGT	60	García-Fernández A et al. 2008
	ardA	F:ATGTCTGTTGTTGCACCTGC R: TCACCGACGGAACACATGACC	60	
	trbA	F:CGACAAATGCTTCCGGGGT R:TCTTACAATCGACAGCCTGT	60	
	sogS	F:TTCCGGGGCGTAGACAATACT R:AACAGTGATATGCCGTCGC	60	
	pilL	F:CCATATGACCATCCAGTGCG R:AACCACTATCTCGCCAGCAG	60	
IncH2	*smr0018*	F:ATAATGATTCACCGGGGTAG R:CTTCAGGCTATCGTTTCG	56	García-Fernández A et al. 2008
	smr0199	F:TGTTTACACCACCAGCAG R: TTTAACAACAGGAGTCGGG	56	
IncF	*FII*	F:CTGATCGTTTAAGGAATTTT R:CACACCATCCTGCACTTA	54	Villa L. et al. 2010
	FIA	F:CCATGCTGGTTCTAGAGAAGGTG R:GTATATCCTTACTGGCTTCCGCA G	60	
	FIB	F:TCTGTTTATTCTTTTACTGTCCAC R:CTCCCGTCGCTTCAGGGCATT	60	
	FIC	F:GTGAACTGGCAGATGAGGAAGG R:TTCTCCTCGTCGCCAAACTAGAT	60	
IncN	*repN*	F:GTCTAACGAGCTTACCGAAG R:ACGGTCATTTAACCAAGCATG	60	García-Fernández A et al. 2011
	traJ	F:CTTCTTCCATAGTTACTGTGCT R:CATCCACGGCTAAATACCTG	60	
	korA	F:GGAACGTTTGTAYCTTGTATTG R:ACTCACTATCTTCTGTTGATTG	60	

Master mixture concentrations were given in the Table 13. 2 µl of plasmid isolate sample was mixed with 98 µl the prepared mixture. Proper conditions of PCR were listed in the Table 10.

Table 13: Master mix solution for PCR used for pMLST

PCR Content	Volume[µl]
ddH_2O	71.5
10X PCR Buffer Solution	10.0
$MgCl_2$ [25mM]	6.0
dNTPs [10mM]	2.0
Primer - F [12.5µM]	4.0
Primer - R [12.5µM]	4.0
Taq DNA Polimerase Enzyme	0.5
TOPLAM	98.0

2.2.10.2 Plasmid Sequencing and Comparison Analysis

Genome sequencing of plasmids isolation of different genomic characterized *Salmonella* Infantis plasmids were performed at BMLabosis (Ankara, Turkey) with Illumina MiSeq platform by reading on prepared DNA library containing 150 bp DNA. Once the short and poor quality readings have been extracted from the analysis, De novo sequence of plasmid samples have been performed using the Velvet (EMBL-EBI, Cambridgeshire, UK) alignment module in the Geneious (Biomatters Ltd, New Zealand) program and results were derived from the most contiguous contigs. The contigs were analysed by SeqMan Pro (Lasergene 8.0, DNAStar, Madison, WI). After

that, aligment of these contigs were by Local Alignment Search Tool (BLAST) method at National Center for Biotechnology information (Han et al., 2010).

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Prevalence of *Salmonella* and *Escherichia coli* in poultry samples

In this study, 19 *Escherichia coli* and 19 *Salmonella* were isolated from 40 chicken sample from grocery stores (Tables 14 and 15). The prevalence of *Salmonella* and *Escherichia coli* were defined as 47.5 % for both organisms.

Table 14: The list of *Salmonella* isolates collected from 40 poultry samples

Isolate ID	Genus	Source	Brand*	Date of isolation	City
MET S1-750	*Salmonella*	Chicken wing	Z	26.1.2015	Ankara
MET S1-753	*Salmonella*	Chicken heart	V	26.1.2015	Ankara
MET S1-759	*Salmonella*	Chicken Rib	Y	28.1.2015	Ankara
MET S1-765	*Salmonella*	Chicken breast	Z	28.1.2015	Ankara
MET S1-774	*Salmonella*	Chicken rib	W	1.2.2015	Ankara
MET S1-777	*Salmonella*	Chicken drumstick	P	1.2.2015	Ankara
MET S1-780	*Salmonella*	Chicken wing	P	1.2.2015	Ankara
MET S1-782	*Salmonella*	Chicken wing	P	1.2.2015	Ankara

Table 14: continued

MET S1-785	*Salmonella*	Chicken drumstick	P	1.2.2015	Ankara
MET S1-788	*Salmonella*	Chicken breast	M	1.2.2015	Ankara
MET S1-792	*Salmonella*	Chicken heart	Q	2.2.2015	Ankara
MET S1-795	*Salmonella*	Chicken breast	Q	2.2.2015	Ankara
MET S1-798	*Salmonella*	Chicken heart	Q	26.2.2015	Ankara
MET S1-801	*Salmonella*	Chicken breast	Q	26.2.2015	Ankara
MET S1-804	*Salmonella*	Chicken wing	Q	26.2.2015	Ankara

Table 15: The list of *Escherichia coli* isolates collected from 40 poultry samples

Isolate ID	Genus species	Source	Brand*	Date of isolation	City
MET A1-001	*Escherichia coli*	Chicken breast	W	1.2.2015	Ankara
MET A1-002	*Escherichia coli*	Chicken drumstick	P	1.2.2015	Ankara
MET A1-003	*Escherichia coli*	Chicken wing	Q	2.2.2015	Ankara
MET A1-004	*Escherichia coli*	Chicken drumstick	P	1.2.2015	Ankara
MET A1-005	*Escherichia coli*	Chicken drumstick	J	27.1.2015	Ankara
MET A1-007	*Escherichia coli*	Chicken wing	W	1.2.2015	Ankara
MET A1-008	*Escherichia coli*	Chicken breast	Q	2.2.2015	Ankara
MET A1-009	*Escherichia coli*	Chicken rib	Q	2.2.2015	Ankara
MET A1-010	*Escherichia coli*	Chicken wing	P	1.2.2015	Ankara
MET A1-011	*Escherichia coli*	Chicken wing	Q	2.2.2015	Ankara
MET A1-012	*Escherichia coli*	Chicken wing	Q	2.2.2015	Ankara
MET A1-014	*Escherichia coli*	Chicken wing	Z	27.1.2015	Ankara
MET A1-015	*Escherichia coli*	Chicken drumstick	Q	2.2.2015	Ankara
MET A1-016	*Escherichia coli*	Chicken wing	P	1.2.2015	Ankara
MET A1-017	*Escherichia coli*	Chicken rib	W	1.2.2015	Ankara
MET A1-018	*Escherichia coli*	Chicken drumstick	P	1.2.2015	Ankara
MET A1-019	*Escherichia coli*	Chicken wing	Q	2.2.2015	Ankara
MET A1-020	*Escherichia coli*	Chicken drumstick	W	1.2.2015	Ankara
MET A1-021	*Escherichia coli*	Chicken drumstick	P	1.2.2015	Ankara

*Every letter shows different brand.

A study conducted by Mahmoud from Egypt showed that the total incidence of *Salmonella* contamination of the examined 75 samples was 6.6%, with a higher percentage of *Salmonella* isolated from the liver samples (13.3%) followed by gizzard, wings and thighs (6.6 %) and breast samples gave negative result. In Ecuador, 62 (16%) *Salmonella* were isolated from 388 broiler samples (Vinueza-Burgos et al., 2016). *Salmonella* was isolated from 88.46% of 182 isolates with 100% of 15 chicken carcasses and 15 chicken cuts in Malaysia (Nidaullah et al., 2017). Kalaba et al. found that the presence of *Salmonella* in poultry samples and products was more than 8% in 1321 samples in Republic of Srpska.

3.2 Diversity of *Salmonella* isolates

Subtyping of 19 *Salmonella* isolated was performed by gold standard method, PFGE in this study (Figure 3-1). Serotypes of 19 *Salmonella* isolates were appointed by PFGE subtyping. Out of 19 *Salmonella* isolates, 15 (78,9%) represented the same PFGE patterns *with Salmonella* isolates in our database (Figure 3-2). Therefore, these isolates were appointed as Infantis and used in our further studies to test our hypothesis. Other 4 *Salmonella* isolates shared the same PFGE with Enteritidis isolates (Figure 3-3), therefore they were assigned as serovar Enteritidis and excluded from our further studies.

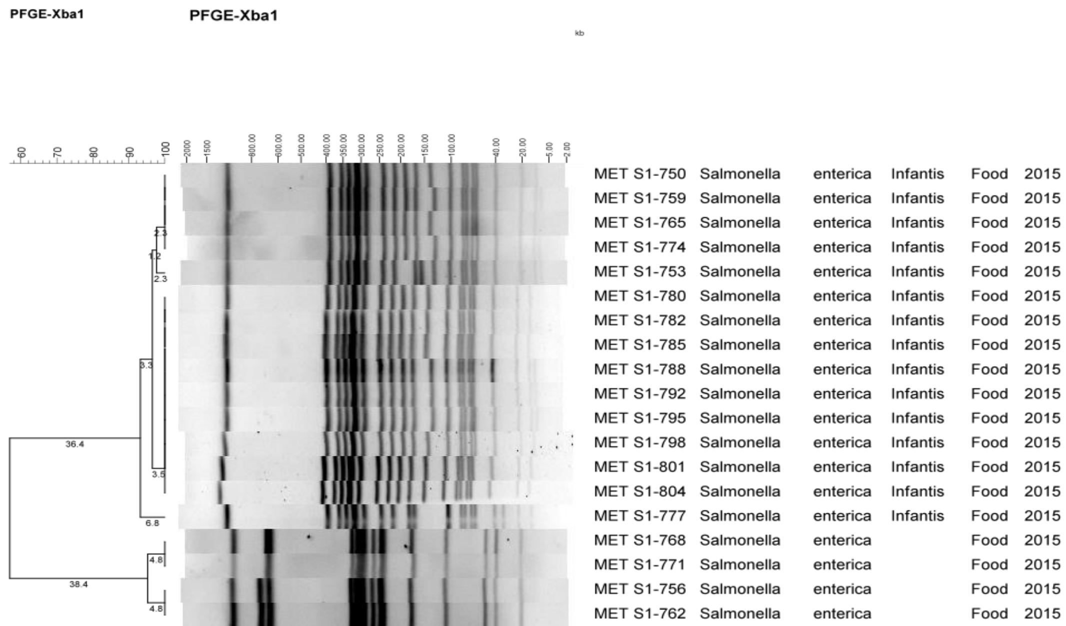


Figure 1: PFGE dendrogram built from 19 *Salmonella* isolates

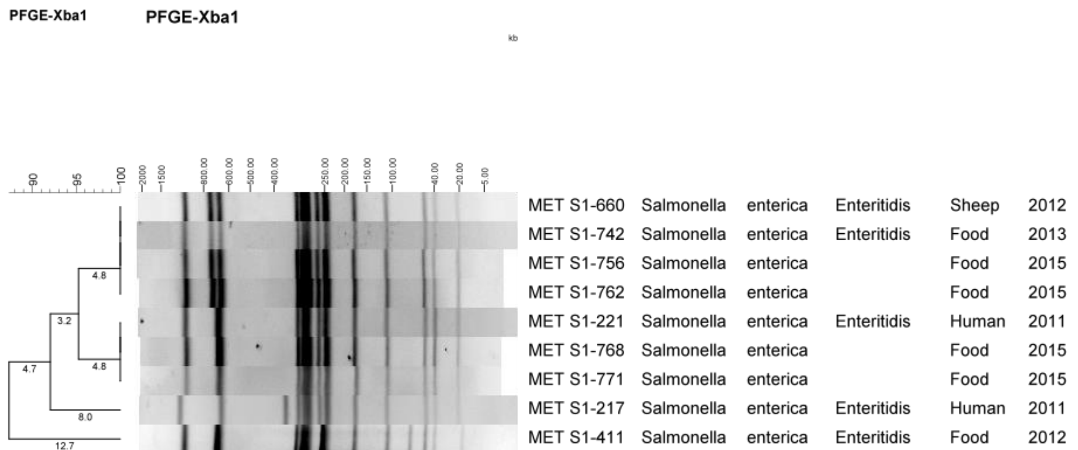


Figure 2: PFGE dendogram of 4 *Salmonella* isolates matched with *Salmonella* Enteritidis isolates in Food Safety Laboratory isolate bank

To test our hypothesis, we also used 55 *Salmonella* Infantis isolates collected from our other studies (TUBITAK111O192 and BAP-03-14-2013-001). All these *Salmonella* Infantis isolates were stored at -80 ^{0}C in our pathogen database. Phenotypic (serotyping, antimicrobial resistance) and genotypic characterization (MLST, PFGE, antimicrobial gene screening) of 55 *Salmonella* Infantis isolates were already performed and published (Acar et al., 2017; Cengiz et al. in progress) (Table 16). Acar et al. (2017) reported that the prevalence of *Salmonella* serovar Infantis was were 30.0 % in food-related samples (i.e., cattle meat, poultry, cheese, etc.) and isolates, representing serotype Infantis, were only collected from chicken samples (breast, wing, offal). *Salmonella* serovar Infantis were not observed in different animal samples (cattle, sheep, chicken) and clinical human samples in this study. (Acar et al., 2017). In another study conducted in our research laboratory, *Salmonella* Infantis isolates were collected from every poultry sample that we purchased from grocery stores in 2012 (Cengiz et al. in progress). Also a study conducted by Kalaba and his colleagues, 29 *Salmonella* isolates (27%) were appointed as *Salmonella* Infantis out of 108 *Salmonella* isolates (Kalaba et al, 2017).

Table 16: Phenotyping and genotyping characterization of 55 *Salmonella* Infantis isolates

METU ID	Source	Brand	Date of Isolation	City	PFGE Type	MLST	Reference	Study or Project *
MET S1-006	Chicken meat	-	7.11.2005	Ankara	PT08	32	Avsaroğlu, D., 2008	1
MET S1-050	Chicken meat	A	11.4.2012	Şanlıurfa	PT08	32	Acar, S., 2015	2
MET S1-056	Chicken meat	B	11.4.2012	Şanlıurfa	PT08	32	Acar, S., 2015	2
MET S1-088	Chicken meat	C	11.4.2012	Şanlıurfa	PT08	32	Acar, S., 2015	2
MET S1-092	Chicken meat	D	11.4.2012	Şanlıurfa	PT08	32	Acar, S., 2015	2
MET S1-103	Chicken meat	E	11.4.2012	Şanlıurfa	PT08	32	Acar, S., 2015	2
MET S1-142	Chicken meat	F	11.4.2012	Şanlıurfa	PT08	32	Acar, S., 2015	2
MET S1-150	Offal	G	11.4.2012	Şanlıurfa	PT08	32	Acar, S., 2015	2
MET S1-329	Chicken meat	H	18.7.2012	Şanlıurfa	PT09	32	Acar, S., 2015	2
MET S1-345	Chicken meat	D	18.7.2012	Şanlıurfa	PT07	32	Acar, S., 2015	2
MET S1-351	Chicken meat	C	18.7.2012	Şanlıurfa	PT08	32	Acar, S., 2015	2

MET S1-492	Chicken meat	H	7.11.2012	Şanlıurfa	PT08	32	Acar, S., 2015	2
MET S1-498	Chicken meat	J	7.11.2012	Şanlıurfa	PT07	32	Acar, S., 2015	2
MET S1-510	Chicken meat	K	7.11.2012	Şanlıurfa	PT08	32	Acar, S., 2015	2
MET S1-597	Chicken meat	A	7.12.2012	Şanlıurfa	PT08	32	Acar, S., 2015	2
MET S1-606	Chicken meat	C	7.12.2012	Şanlıurfa	PT08	32	Acar, S., 2015	2
MET S1-668	Chicken breast	L	28.11.2012	Ankara	PT08	32	Cengiz et al., *in progress*	3
MET S1-669	Chicken wing	L	12.12.2012	Ankara	PT07	32	Cengiz et al., *in progress*	3
MET S1-671	Chicken breast	M	12.12.2012	Ankara	PT08	32	Cengiz et al., *in progress*	3
MET S1-672	Chicken skin	L	19.12.2012	Ankara	PT08	32	Cengiz et al., *in progress*	3
MET S1-673	Chicken wing	N	19.12.2012	Ankara	PT08	32	Cengiz et al., *in progress*	3
MET S1-674	Chicken wing	O	19.12.2012	Ankara	PT08	32	Cengiz et al., *in progress*	3

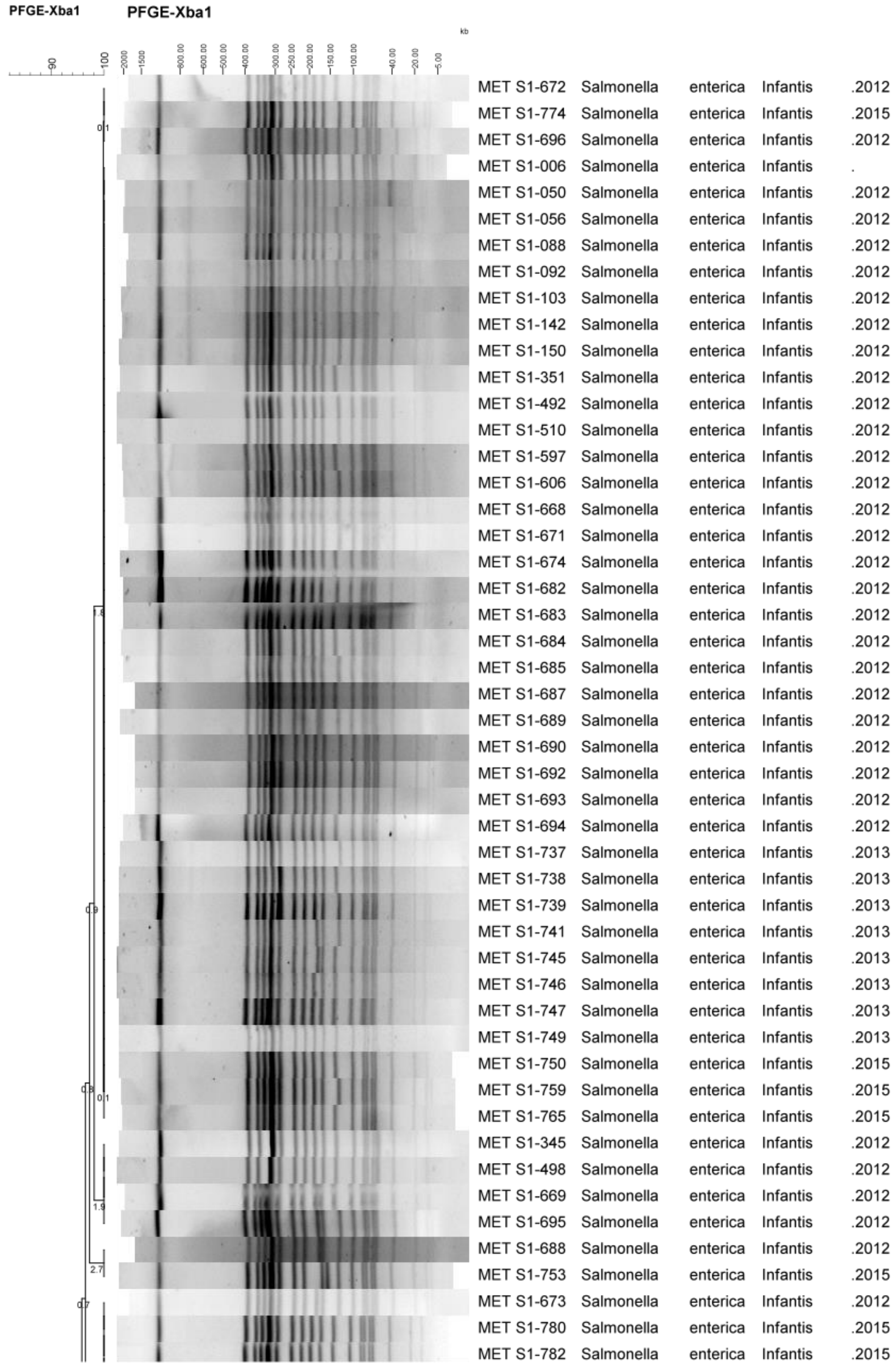
MET S1-676	Chicken leg	P	14.11.2012	Ankara	PT49	32	Cengiz et al., *in progress*	3
MET S1-677	Chicken skin	P	14.11.2012	Ankara	PT47	32	Cengiz et al., *in progress*	3
MET S1-678	Chicken wing	P	14.11.2012	Ankara	PT47	32	Cengiz et al., *in progress*	3
MET S1-679	Chicken liver	M	21.11.2012	Ankara	PT47	32	Cengiz et al., *in progress*	3
MET S1-680	Chicken leg	P	21.11.2012	Ankara	PT47	32	Cengiz et al., *in progress*	3
MET S1-682	Chicken wing	P	21.11.2012	Ankara	PT08	32	Cengiz et al., *in progress*	3
MET S1-683	Chicken liver	L	21.11.2012	Ankara	PT08	32	Cengiz et al., *in progress*	3
MET S1-684	Chicken skin	P	21.11.2012	Ankara	PT08	32	Cengiz et al., *in progress*	3
MET S1-685	Chicken wing	L	21.11.2012	Ankara	PT08	32	Cengiz et al., *in progress*	3
MET S1-686	Chicken liver	R	28.11.2012	Ankara	PT46	32	Cengiz et	3

							al., *in progress*	
MET S1-687	Chicken skin	M	28.11.2012	Ankara	PT08	32	Cengiz et al., *in progress*	3
MET S1-688	Chicken leg	M	28.11.2012	Ankara	PT45	32	Cengiz et al., *in progress*	3
MET S1-689	Chicken skin	M	28.11.2012	Ankara	PT08	32	Cengiz et al., *in progress*	3
MET S1-690	Chicken liver	S	28.11.2012	Ankara	PT08	32	Cengiz et al., *in progress*	3
MET S1-691	Chicken liver	L	12.12.2012	Ankara	PT08	32	Cengiz et al., *in progress*	3
MET S1-692	Chicken leg	L	12.12.2012	Ankara	PT46	32	Cengiz et al., *in progress*	3
MET S1-693	Chicken skin	L	12.12.2012	Ankara	PT08	32	Cengiz et al., *in progress*	3
MET S1-694	Chicken skin	T	12.12.2012	Ankara	PT08	32	Cengiz et al., *in progress*	3
MET S1-695	Chicken skin	U	12.12.2012	Ankara	PT08	32	Cengiz et al., *in*	3

							progress	
MET S1-696	Chicken leg	L	19.12.2012	Ankara	PT08	32	Cengiz et al., *in progress*	3
MET S1-697	Chicken leg	N	19.12.2012	Ankara	PT08	32	Cengiz et al., *in progress*	3
MET S1-698	Chicken skin	N	19.12.2012	Ankara	PT48	32	Cengiz et al., *in progress*	3
MET S1-699	Chicken breast	N	19.12.2012	Ankara	PT48	32	Cengiz et al., *in progress*	3
MET S1-700	Chicken liver	O	19.12.2012	Ankara	PT47	32	Cengiz et al., *in progress*	3
MET S1-701	Chicken skin	O	19.12.2012	Ankara	PT48	32	Cengiz et al., *in progress*	3
MET S1-737	Chicken liver	M	6.5.2013	Ankara	PT48	32	Cengiz et al., *in progress*	3
MET S1-738	Chicken skin	M	6.5.2013	Ankara	PT08	32	Cengiz et al., *in progress*	3
MET S1-739	Chicken wing	M	6.5.2013	Ankara	PT08	32	Cengiz et al., *in progress*	3

MET S1-741	Chicken liver	L	2.7.2013	Ankara	PT08	32	Cengiz et al., *in progress*	3
MET S1-745	Chicken liver	R	19.8.2013	Ankara	PT08	32	Cengiz et al., *in progress*	3
MET S1-746	Chicken liver	P	19.8.2013	Ankara	PT08	32	Cengiz et al., *in progress*	3
MET S1-747	Chicken skin	P	19.8.2013	Ankara	PT08	32	Cengiz et al., *in progress*	3
MET S1-749	Chicken wing	P	19.8.2013	Ankara	PT08	32	Cengiz et al., *in progress*	3

*: 1: Avsaroğlu, D., 2008 – Thesis, 2: TUBITAK 111O192, 3: BAP-03-14-2013-001


PFGE-Xba1
PFGE-Xba1
MET S1-672 Salmonella enterica Infantis .2012
MET S1-774 Salmonella enterica Infantis .2015
MET S1-696 Salmonella enterica Infantis .2012
MET S1-006 Salmonella enterica Infantis .
MET S1-050 Salmonella enterica Infantis .2012
MET S1-056 Salmonella enterica Infantis .2012
MET S1-088 Salmonella enterica Infantis .2012
MET S1-092 Salmonella enterica Infantis .2012
MET S1-103 Salmonella enterica Infantis .2012
MET S1-142 Salmonella enterica Infantis .2012
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MET S1-351 Salmonella enterica Infantis .2012
MET S1-492 Salmonella enterica Infantis .2012
MET S1-510 Salmonella enterica Infantis .2012
MET S1-597 Salmonella enterica Infantis .2012
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MET S1-782 Salmonella enterica Infantis .2015

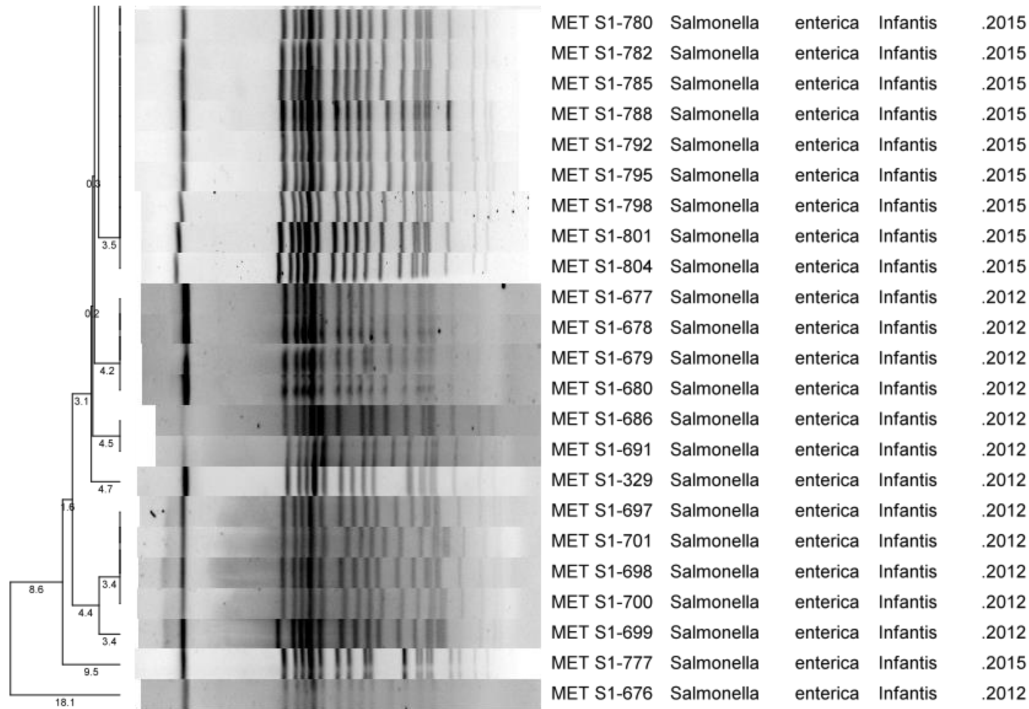

Figure 3: PFGE dendrogram for 70 *Salmonella* Infantis isolates, used in this study

3.3 Diversity of *Escherichia coli* isolates

The diversity of 19 *Escherichia coli* isolates, collected in this study, was determined by PFGE (Figure 4 and 5). 100 % diversity was found for these isolates; every *E. coli* isolate represented a unique PFGE pattern (Figure 6).

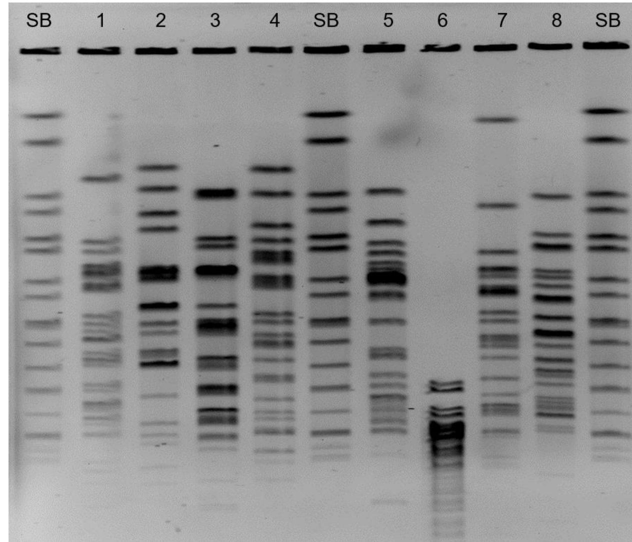

Figure 4: PFGE result of 7 *Escherichia coli* isolates (1: MET A1-001, 2: MET A1-002, 3: MET A1-003, 4: MET A1-004, 5: MET A1-005, 6: MET A1-006 is not *E.coli,* 7: MET A1-007, 8: MET A1-008, SB: *Salmonella* Braenderup H9812)

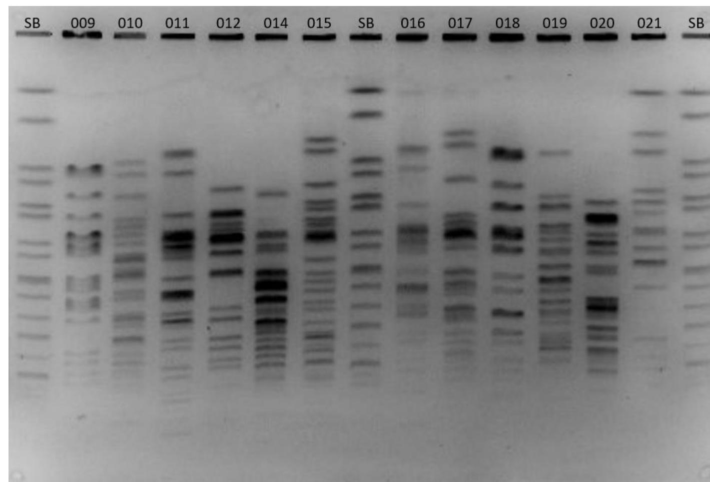

Figure 5: PFGE result of 12 *Escherichia coli* isolates (009: MET A1-009, 010: MET A1-010, 011: MET A1-011, 012: MET A1-012, 014: MET A1-014, 015: MET A1-015, 016: MET A1-016, 017: MET A1-017, SB: *Salmonella* Braenderup H9812)

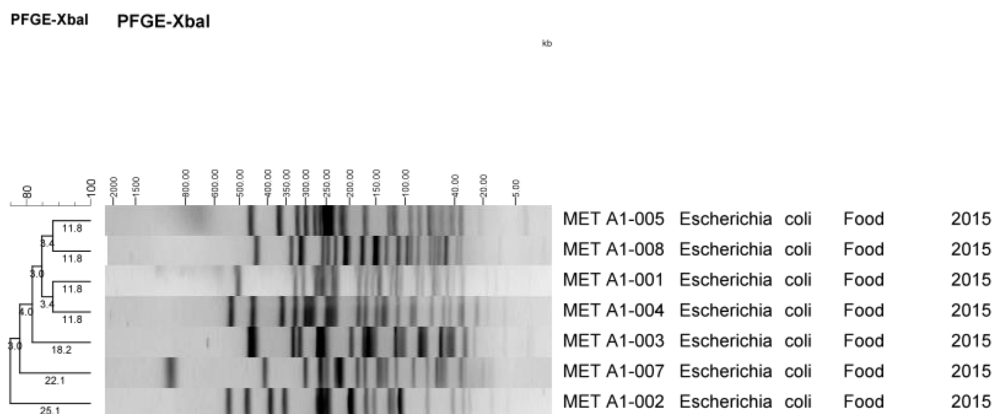

Figure 6: PFGE dendrogram created with 7 *Escherichia coli* isolates

3.4 Antimicrobial resistance profiles of *Escherichia coli* and *Salmonella* Infantis isolates

Antimicrobial resistance profiles of isolates were investigated with disk diffusion method and screening of genes encoding antimicrobial resistance by Polymerase Chain Reaction (PCR).

3.4.1 Disk diffusion

Among 19 *Escherichia coli* isolates, only 3 isolates (MET A1-014, MET A1-017 and MET A1-018) were susceptible against all the antimicrobials, used in this study (Table 11). Only MET A1-008 and MET A1-019 *Escherichia coli* isolates shared the same antimicrobial resistant profile (i.e., SfSxtCKSCipNAmpTKf), other than these isolates, all isolates posed different antimicrobial phenotypes including two or more antimicrobials, except one isolate represent antimicrobial resistance against cephalothin (Kf). However, some isolates (MET A1-003, MET A1-005, MET A1-008, MET A1-010, MET A1-015, MET A1-016, MET A1-019) showed the similar resistance profiles, including SfSxtCCipNAmpT. Resistance against ciproflaxin (Cip) and/or nalidixic acid (N) was frequently observed; 11 *E. coli* isolates were resistant to ciproflaxin (Cip)

and/or nalidixic acid (N). Interestingly, one isolate (MET A1-021) showed resistance against ertapenem, showing the emergent resistance against the extended spectrum beta-lactamases (Table 17).

Table 17: Antimicrobial resistance profiles of *Escherichia coli* isolates by disk diffusion method.

Isolate Code	Organism	AR Profiles*
MET A1-001	*Escherichia coli*	CroEftAmpAmcFoxKf
MET A1-002	*Escherichia coli*	AmpAmcFoxKf
MET A1-003	*Escherichia coli*	SfSxtCCnKSCipNAmpT
MET A1-004	*Escherichia coli*	CipN
MET A1-005	*Escherichia coli*	SfSxtCnKCipNAmpT
MET A1-007	*Escherichia coli*	SAmpKf
MET A1-008	*Escherichia coli*	SfSxtCKSCipNAmpTKf
MET A1-009	*Escherichia coli*	Kf
MET A1-010	*Escherichia coli*	SfSxtCCnSCipNAmpT
MET A1-011	*Escherichia coli*	SfSCipNT
MET A1-012	*Escherichia coli*	SfKCipNT
MET A1-014	*Escherichia coli*	*Susceptible*
MET A1-015	*Escherichia coli*	SfSxtCSCipNAmpT
MET A1-016	*Escherichia coli*	SfSxtCCnSCipNAmpTKf
MET A1-017	*Escherichia coli*	*Susceptible*
MET A1-018	*Escherichia coli*	*Susceptible*
MET A1-019	*Escherichia coli*	SfSxtCKSCipNAmpTKf
MET A1-020	*Escherichia coli*	SfT
MET A1-021	*Escherichia coli*	CipNEtp

*: Cro: Ceftriaxone , Eft: Ceftiofur, Sf: Sulphisoxazole, Sxt: Sulphamethaxazole/trimethoprim, C:Chloramphenicol, Imp: Imipenem, Ak: Amikacin, Cn: Gentamicin, K: Kanamycin, S:Streptomycin, Cip: Ciprofloxacin, N: Nalidixic acid, Amp: Ampicillin, Amc: Amoxicillin-clavulanic acid, T: Tetracycline, Fox: Cefoxitin, Kf: Cephalothin, Etp: Ertapenem

Unlike *E. coli* isolates, 19 *Salmonella* Infantis showed similar antimicrobial resistant profiles (Table 18). Only one isolate (MET S1-753) showed resistance against one antimicrobial, nalidixic acid (N), but other *Salmonella* Infantis isolates:

- shared the backbone multi-resistant profile (SfSxtNT),
- showed resistant to at least 5 different antimicrobials,
- represented 10 different antimicrobial resistant profiles.

Table 18: Antimicrobial resistance profiles of *Salmonella* Infantis isolates by disk diffusion method.

Isolate Code	Organism	AR Profiles*
MET S1-750	*Salmonella* Infantis	SfSxtKNT
MET S1-753	*Salmonella* Infantis	N
MET S1-759	*Salmonella* Infantis	SfSxtNT
MET S1-765	*Salmonella* Infantis	SfSxtKNT
MET S1-774	*Salmonella* Infantis	SfSxtKSNT
MET S1-777	*Salmonella* Infantis	SfSxtSCipNT
MET S1-780	*Salmonella* Infantis	SfSxtKNT
MET S1-782	*Salmonella* Infantis	SfSxtKSNT
MET S1-785	*Salmonella* Infantis	SfSxtCSNT
MET S1-788	*Salmonella* Infantis	SfSxtCSCipNT
MET S1-792	*Salmonella* Infantis	SfSxtSNT
MET S1-795	*Salmonella* Infantis	SfSxtNT
MET S1-798	*Salmonella* Infantis	SfSxtCSNT
MET S1-801	*Salmonella* Infantis	SfSxtCKSNT
MET S1-804	*Salmonella* Infantis	SfSxtCKNT

AR Profiles*: Cro: Ceftriaxone , Eft: Ceftiofur, Sf: Sulphisoxazole, Sxt: Sulphamethaxazole/trimethoprim, C: Chloramphenicol, Imp: Imipenem, Ak: Amikacin, Cn: Gentamicin, K: Kanamycin, S: Streptomycin, Cip: Ciprofloxacin, N: Nalidixic acid, Amp: Ampicillin, Amc: Amoxicillin-clavulanic acid, T: Tetracycline, Fox: Cefoxitin, Kf: Cephalothin, Etp: Ertapenem

The diversity percentage of antimicrobial resistance profile of poultry isolated *Salmonella* Infantis and *Escherichia coli* isolates are showed in Figure 7. The antimicrobial agents; Ampicillin (Amp), Cephalothin (Kf), Amoxicillin-clavulanic acid (Amc), Cefoxitin (Fox), Gentamicin (Cn), Ceftriaxone (Cro), Ceftiofur (Eft), Ertapenem (Etp), Imipenem (Imp), Amikacin (Ak) were observed to be effective on the *Salmonella* Infantis isolates from Turkey. The results show that any *Salmonella* Infantis and *Escherichia coli* isolates are resistant to Imipenem (Imp), Amikacin (Ak).

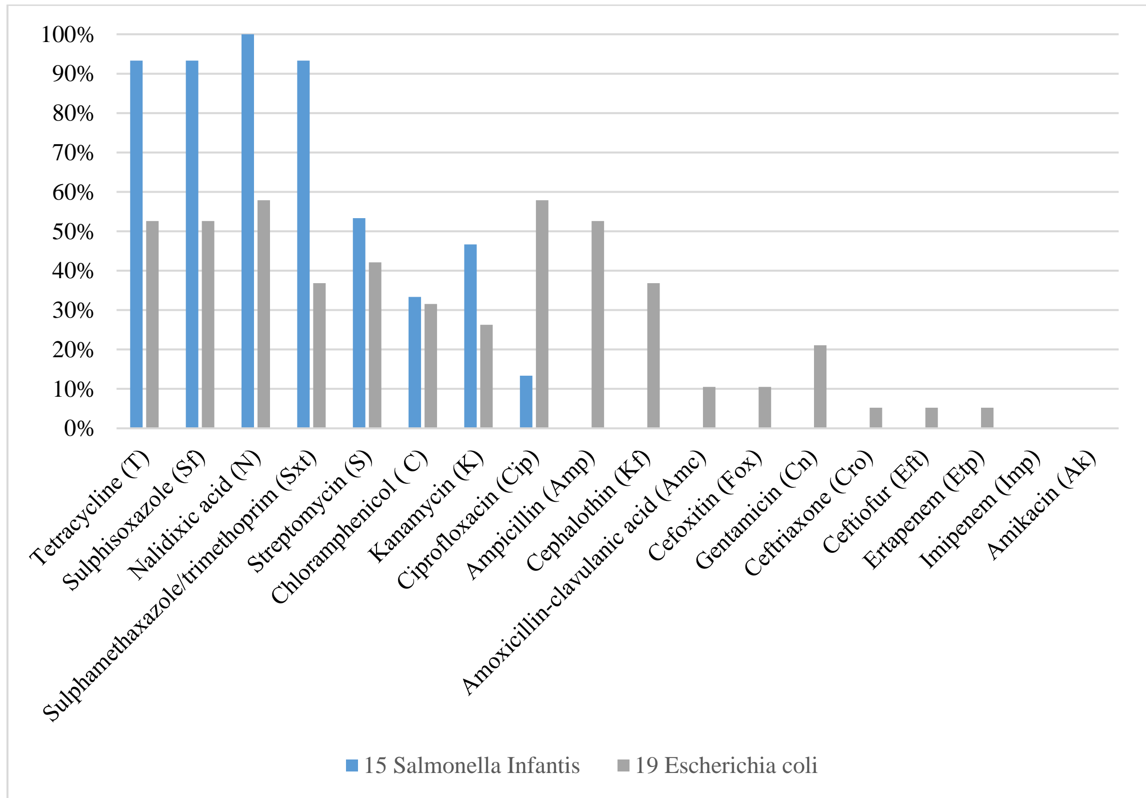

Figure 7: The diversity percentage of antimicrobial resistance profile of poultry isolated *Salmonella* Infantis and *Escherichia coli* isolates

Salmonella Infantis isolates were observed as 100% resistant to nalidixic acid and amoxicillin. There were 68.97% of the *Salmonella* Infantis poultry isolates having resistance to four or more antimicrobials (Kalaba et al., 2017).

3.4.2 Screening of genes encoding antimicrobial resistance

In this study, antimicrobial resistance profiles were also investigated as genotypic method with polymerase chain reaction (PCR). The phenotypic antimicrobial resistance has been also investigated by genotyping the gene screening (Table 19 and Table 20).

Table 19: Genotypic antimicrobial resistance profile of *Escherichia coli* isolates.

METU ID	Phenotypic AR Profile	Genetic AR profile
MET A1-001	CroEftAmpAmcFoxKf	*blaCMY-2*
MET A1-002	AmpAmcFoxKf	ND
MET A1-003	SfSxtCCnKSCipNAmpT	*bla_{TEM-1}, flo, aadA1, aadA2, aphA1-Iab, dhfrI, tetA, sul1, strB, sulII*
MET A1-004	CipN	ND
MET A1-005	SfSxtCnKCipNAmpT	*bla_{TEM-1}, aadA1, aadA2, aphA1-Iab, tetA*
MET A1-007	SAmpKf	*bla_{TEM-1}, aadA1*
MET A1-008	SfSxtCKSCipNAmpTKf	*bla_{TEM-1}, cat1, aphA1-Iab, strB, sulII*
MET A1-009	Kf	ND
MET A1-010	SfSxtCCnSCipNAmpT	*bla_{TEM-1}, flo, aadA1, dhfrI, tetA, sul1, strB, sulII*
MET A1-011	SfSCipNT	*tetA, strB*
MET A1-012	SfKCipNT	*aadA1, aadA2, tetA*
MET A1-014	*Susceptible*	*aadA2*
MET A1-015	SfSxtCSCipNAmpT	*bla_{TEM-1}, flo, aadA1, aphA1-Iab, tetA, aadA2, sul1, sulII*
MET A1-016	SfSxtCCnSCipNAmpTKf	*flo, aadA1, aphA1-Iab, dhfrI, tetA, sul1, strB, sulII*
MET A1-017	*Susceptible*	*bla_{TEM-1}*
MET A1-018	*Susceptible*	*bla_{TEM-1}, aphA1-lab*
MET A1-019	SfSxtCKSCipNAmpTKf	*bla_{CMY-2}, cat1, strB, sulII*
MET A1-020	SfT	*tetA*
MET A1-021	CipNEtp	*bla_{TEM-1}, aadA2*

*: ND= not detected

AR Profiles*: Cro: Ceftriaxone, Eft: Ceftiofur, Sf: Sulphisoxazole, Sxt: Sulphamethaxazole/trimethoprim, C: Chloramphenicol, Imp: Imipenem, Ak: Amikacin, Cn: Gentamicin, K: Kanamycin, S: Streptomycin, Cip: Ciprofloxacin, N: Nalidixic acid, Amp: Ampicillin, Amc: Amoxicillin-clavulanic acid, T: Tetracycline, Fox: Cefoxitin, Kf: Cephalothin, Etp: Ertapenem

Table 20: Genotypic antimicrobial resistance profile of *Salmonella* Infantis isolates.

Isolate code	Phenotypic AR Profiles	Genetic AR Profiles
MET S1-750	SfSxtKNT	*aadA1, aphA1-IAB, sul1, tetA*
MET S1-753	N	*aadA1, aphA1-IAB, tetA*
MET S1-759	SfSxtNT	*bla_{TEM-1}, aadA1, aphA1-IAB, sul1, tetA*
MET S1-765	SfSxtKNT	*aadA1, aphA1-IAB, bla_{TEM-1}, cmlA, sul1, tetA*
MET S1-774	SfSxtKSNT	*aphA1-IAB, sul1, tetA*
MET S1-777	SfSxtSCipNT	*aadA1, sul1, tetA*
MET S1-780	SfSxtKNT	*aadA1, aphA1-IAB, sul1, tetA*
MET S1-782	SfSxtKSNT	*aadA1, aphA1- IAB, strA, sul1, tetA*
MET S1-785	SfSxtCSNT	*aadA1, sul1, tetA*
MET S1-788	SfSxtCSCipNT	*aadA1, strA, sul1, tetA*
MET S1-792	SfSxtSNT	*aadA1, aphA1-IAB, sul1, tetA*
MET S1-795	SfSxtNT	*aadA1, strA, sul1, tetA*
MET S1-798	SfSxtCSNT	*aadA1, tetA*
MET S1-801	SfSxtCKSNT	*aadA1, aphA1-IAB, sul1, tetA*
MET S1-804	SfSxtCKNT	aadA1, aphA1-IAB, sul1, tetA

AR Profiles*: Cro: Ceftriaxone, Eft: Ceftiofur, Sf: Sulphisoxazole, Sxt: Sulphamethaxazole/trimethoprim, C: Chloramphenicol, Imp: Imipenem, Ak: Amikacin, Cn: Gentamicin, K: Kanamycin, S: Streptomycin, Cip: Ciprofloxacin, N: Nalidixic acid, Amp: Ampicillin, Amc: Amoxicillin-clavulanic acid, T: Tetracycline, Fox: Cefoxitin, Kf: Cephalothin, Etp: Ertapenem

In the Acar et al. study, it was stated that resistivity profiles may be specific to their geography. Molecular characterization of antimicrobial resistance in *Salmonella* Infantis

isolates collected from Turkey region might be difficult because features may encoded in different genes (Acar et. al, 2017).

The antimicrobial resistance profile detected in *Escherichia* coli isolates is much more diverse than *Salmonella* isolates. This is because the *Escherichia* coli isolates are much more diverse. While studying with active gene segments, some antimicrobial resistance profile has been explained, some antimicrobial resistance profile has not been explained. For example, the AmpAmcFoxKf profile of the MET A1-002 isolate did not overlap with any of the genes studied. This may be because the primers that used in this study are specific to *Salmonella* isolates. Even if the gene region is the same, changes in the primer binding site make it difficult to determine gene presence.

3.5 Plasmid detection among *Salmonella* Infantis and *Escherichia* coli isolates

Plasmid isolation from MDR *Salmonella* Infantis and *Escherichia coli* isolates is completed by using QIAprep spin minikit (Qiagen Finland, Helsinki, Finland).

Agarose gel and size determination results show that 8 *Salmonella* Infantis isolates from 70 have plasmid; MET S1-050, MET S1-056, MET S1-669, MET S1-785, MET S1-788, MET S1-798, MET S1-801, MET S1-804. Except MET S1-050, MET S1-788 and MET S1-669, other 5 *Salmonella* Infantis isolates have similar size and it is 47 kilobases. MET S1-788 and MET S1-669 isolates have 45, 45 and 40 kilobases plasmid size respectively (Table 21).

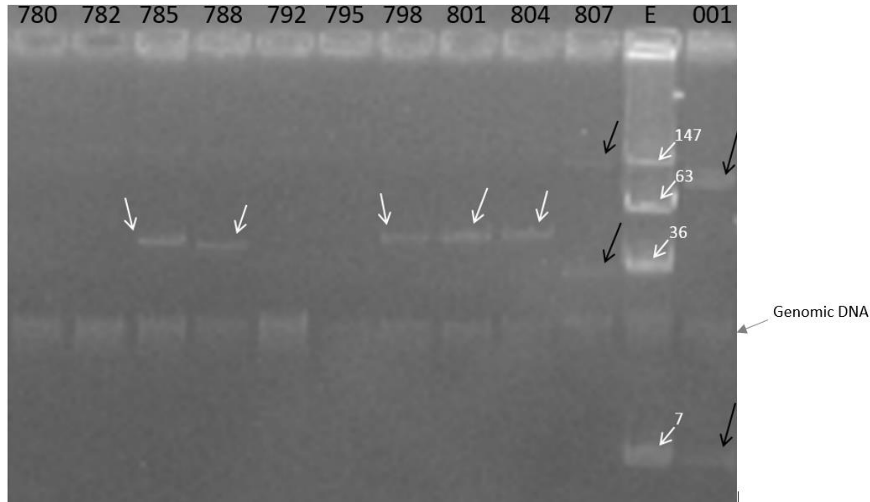

Figure 8: Plasmid Agarose Gel Image

The molecular sizes of the 7, 36, 63, 147 kb bands of *Escherichia coli* 39R861 are indicated in the Figure 8. The numbers above the wells represent the numbers in the isolate code. For example, 780 means MET S1-780. *Salmonella* Infantis isolates identified as carrying plasmid and *Escherichia coli* 39R861 isolate were indicated by white arrow marking. Plasmids were placed to compare the isolates shown with black arrows. MET S1-807 and MET A1-001 isolates are isolates of *S*. Infantis (MET S1-807) and *Escherichia coli* (MET S1-001) resistant to various phenotypic antibiotics found in our bank. The band seen in all isolates refers to genomic DNA.

Table 21: Plasmid presence results of *Salmonella* Infantis isolates

Isolate code	Organism	Plasmid	Plasmid sizes

Table 21. (continued)

		presence	(Kilobases)
MET S1-006	*Salmonella* Infantis	Negative	-
MET S1-050	*Salmonella* Infantis	Positive	47
MET S1-056	*Salmonella* Infantis	Positive	45
MET S1-088	*Salmonella* Infantis	Negative	-
MET S1-092	*Salmonella* Infantis	Negative	-
MET S1-103	*Salmonella* Infantis	Negative	-
MET S1-142	*Salmonella* Infantis	Negative	-
MET S1-150	*Salmonella* Infantis	Negative	-
MET S1-329	*Salmonella* Infantis	Negative	-
MET S1-345	*Salmonella* Infantis	Negative	-
MET S1-351	*Salmonella* Infantis	Negative	-
MET S1-492	*Salmonella* Infantis	Negative	-
MET S1-498	*Salmonella* Infantis	Negative	-
MET S1-510	*Salmonella* Infantis	Negative	-
MET S1-597	*Salmonella* Infantis	Negative	-
MET S1-606	*Salmonella* Infantis	Negative	-
MET S1-668	*Salmonella* Infantis	Negative	-
MET S1-669	*Salmonella* Infantis	Positive	40
MET S1-671	*Salmonella* Infantis	Negative	-
MET S1-672	*Salmonella* Infantis	Negative	-
MET S1-673	*Salmonella* Infantis	Negative	-
MET S1-674	*Salmonella* Infantis	Negative	-
MET S1-676	*Salmonella* Infantis	Negative	-
MET S1-677	*Salmonella* Infantis	Negative	-
MET S1-678	*Salmonella* Infantis	Negative	-
MET S1-679	*Salmonella* Infantis	Negative	-
MET S1-680	*Salmonella* Infantis	Negative	-

Table 21: (continued)

MET S1-682	*Salmonella* Infantis	Negative	-
MET S1-683	*Salmonella* Infantis	Negative	-
MET S1-684	*Salmonella* Infantis	Negative	-
MET S1-685	*Salmonella* Infantis	Negative	-
MET S1-686	*Salmonella* Infantis	Negative	-
MET S1-687	*Salmonella* Infantis	Negative	-
MET S1-688	*Salmonella* Infantis	Negative	-
MET S1-689	*Salmonella* Infantis	Negative	-
MET S1-690	*Salmonella* Infantis	Negative	-
MET S1-691	*Salmonella* Infantis	Negative	-
MET S1-692	*Salmonella* Infantis	Negative	-
MET S1-693	*Salmonella* Infantis	Negative	-
MET S1-694	*Salmonella* Infantis	Negative	-
MET S1-695	*Salmonella* Infantis	Negative	-
MET S1-696	*Salmonella* Infantis	Negative	-
MET S1-697	*Salmonella* Infantis	Negative	-
MET S1-698	*Salmonella* Infantis	Negative	-
MET S1-699	*Salmonella* Infantis	Negative	-
MET S1-700	*Salmonella* Infantis	Negative	-
MET S1-701	*Salmonella* Infantis	Negative	-
MET S1-737	*Salmonella* Infantis	Negative	-
MET S1-738	*Salmonella* Infantis	Negative	-
MET S1-739	*Salmonella* Infantis	Negative	-
MET S1-741	*Salmonella* Infantis	Negative	-
MET S1-745	*Salmonella* Infantis	Negative	-
MET S1-746	*Salmonella* Infantis	Negative	-
MET S1-747	*Salmonella* Infantis	Negative	-
MET S1-749	*Salmonella* Infantis	Negative	-

MET S1-750	*Salmonella* Infantis	Negative	-
MET S1-753	*Salmonella* Infantis	Negative	-
MET S1-759	*Salmonella* Infantis	Negative	-
MET S1-765	*Salmonella* Infantis	Negative	-
MET S1-774	*Salmonella* Infantis	Negative	-
MET S1-777	*Salmonella* Infantis	Negative	-
MET S1-780	*Salmonella* Infantis	Negative	-
MET S1-782	*Salmonella* Infantis	Negative	-
MET S1-785	*Salmonella* Infantis	Positive	47
MET S1-788	*Salmonella* Infantis	Positive	45
MET S1-792	*Salmonella* Infantis	Negative	-
MET S1-795	*Salmonella* Infantis	Negative	-
MET S1-798	*Salmonella* Infantis	Positive	47
MET S1-801	*Salmonella* Infantis	Positive	47
MET S1-804	*Salmonella* Infantis	Positive	47

Lastly in 19 *Escherichia coli* isolates; plasmids were observed in every isolate, except 5. Size distribution of plasmids was varied in *E. coli* isolates. In addition, most of the *Escherichia coli* isolates (10) included multiple plasmids. Other *Escherichia coli* isolates have got different number and variable plasmid sizes (Table 22).

Table 22: Plasmid presence results of *Escherichia coli* isolates obtained from Ankara

Isolate code	Organism	Plasmid Presences	Plasmid sizes (kb)
MET A1-001	*Escherichia coli*	Positive	58.22, 44.35, 37.99, 4.49
MET A1-002	*Escherichia coli*	Positive	4.46
MET A1-003	*Escherichia coli*	Positive	147
MET A1-004	*Escherichia coli*	Positive	29.49, 1.95, 1.78
MET A1-005	*Escherichia coli*	Positive	100, 172
MET A1-007	*Escherichia coli*	Positive	46.54
MET A1-008	*Escherichia coli*	Positive	2.49
MET A1-009	*Escherichia coli*	*Negative*	-
MET A1-010	*Escherichia coli*	*Negative*	-
MET A1-011	*Escherichia coli*	*Positive*	75.77, 6.98, 4.23, 2.19, 2.00
MET A1-012	*Escherichia coli*	*Positive*	316, 129, 7.95, 2.04
MET A1-014	*Escherichia coli*	*Negative*	-
MET A1-015	*Escherichia coli*	*Positive*	160, 2.30
MET A1-016	*Escherichia coli*	*Positive*	4, 2.50
MET A1-017	*Escherichia coli*	*Positive*	169, 74.2, 2.5
MET A1-018	*Escherichia coli*	*Negative*	-
MET A1-019	*Escherichia coli*	*Positive*	156, 97.2
MET A1-020	*Escherichia coli*	*Negative*	-
MET A1-021	*Escherichia coli*	*Positive*	83.8, 3.97

Plasmid size were detected both in agarose gel electrophoresis and pulsed field gel electrophoresis (PFGE). More accurate results were obtained in PFGE analysis because both bands stand apart from each other in the gel and reference bands used were more effective with the bioinformatics tools. In our agarose gel electrophoresis and PFGE results, all plasmid samples, except MET A1-001 sample, showed the same fingerprint

results. So plasmid showed same size and numbers both in agarose gel electrophoresis and pulsed field gel electrophoresis. Probably the reason of that might be absence of sequence region for S1 Nuclease.

3.6 Detection of plasmid originated antimicrobial resistance genes in *Salmonella* Infantis plasmids

The plasmids were screened in 70 *Salmonella* Infantis from Turkey and antimicrobial resistance genes screening were performed in the plasmids. The result of antimicrobial resistance gene profiles of 8 isolates observed plasmid presence in agarose gel images are showed in Table 23.

Table 23: Antimicrobial resistance gene profiles of isolates observed plasmid existence by gel image

Isolate code	Plasmid size in kb	Genetic profile of antimicrobial resistance of bacteria	Antimicrobial resistance profile of plasmid isolate
MET S1-050	47	*aadA1, aphA1-iab, tetA, bla_{TEM-1}, sul1*	*aadA1, aphA1-iab, bla_{TEM-1}*
MET S1-056	45	*aadA1, aphA1-iab, tetA, bla_{TEM-1}, sul1, cmlA*	*aadA1,aphA1-iab, bla_{TEM-1}*
MET S1-669	40	*aadA1,$bla_{TEM-1,,}$sul1*	*aadA1, bla_{TEM-1}*
MET S1-785	47	*aadA1, flo, strB, sul1, tetA*	*aadA1, flo, strB, sul1, tetA*
MET S1-788	45	*aadA1, flo, strA, strB, sul1, tetA*	*flo, strA, strB, sul1, tetA*
MET S1-798	47	*aadA1, flo, strA, strB, sul1, tetA*	*aadA1, dhfrI, flo, strB, sul1, tetA*
MET S1-801	47	*aadA1, aphA1-iab, flo, strB, sul1, tetA*	*aadA1, flo, strB, sul1, tetA*
MET S1-804	47	*aadA1, aphA1-iab, flo, strA, strB, sul1, tetA*	*aadA1, aphA1-iab, flo, strA, strB, sul1, tetA*

Although place of antimicrobial resistance gene reported as *flo* in chromosome (Chen et al, 2004), *flo* antimicrobial resistance gene also was detected in plasmid. *aadA1 gene was* detected in all isolate resistance gene screening. According to the Table 3.8 in MET S1-788 isolate *aadA1, in* MET S1-798 *strA and in* MET S1-801 *aphA1-IAB* were found in bacterial chromosome.

Plasmid presence was observed in 8 isolates. There are 3 ampicillin resistance and 5 chloramphenicol resistance isolates in 70 *Salmonella* Infantis isolates.

3.7 Molecular characterization of *Salmonella* Infantis plasmids

3.7.1 Plasmid Multi Locus Sequence Typing (pMLST)

pMLST analysis was performed for the molecular characterization of plasmids. For this analysis, the existence of the genes mentioned in Table 24 was investigated among Infantis isolates by PCR. Among 4 different Inc groups, total 11 genes were studied. Only 3 genes (*ardA, sogS,* and *pilL)* gave positive results in the plasmids of the isolates tested (Table 24). The PCR results for all the genes are listed in the Appendix C. Three genes are found in the IncI1 region.

Table 24: Results of pMLST genes in plasmid isolates from *Salmonella* Infantis

Plasmid Isolates	IncI1				
	repI	*ardA*	*trbA*	*sogS*	*pilL*
MET S1-050	-	+	-	-	-
MET S1-056	-	+	-	-	-
MET S1-669	-	+	-	+	+
MET S1-785	-	+	-	+	-
MET S1-788	-	+	-	+	+
MET S1-798	-	+	-	+	+
MET S1-801	-	+	-	+	-
MET S1-804	-	-	-	+	-

PCR products shown positive result of listed genes were sent to sequencing to complete pMLST analysis. The results of the sequencing of the 3 genes shown in Table 25 for the *Salmonella* isolate are given in Appendix D. Identified genes are located in IncI1 group.

For the positive results, these regions were replicated and sequence information were obtained. Sequences of the 3 genes for 8 plasmid isolates are given in Appendix D. According to the sequence results, allelic types of these genes were identified using bioinformatic tools in the plasmid MLST database (pubmlst.org). These genes were found to be same alleles in the detected plasmids.

Table 25: Allelic types of Plasmid MLST genes detected in *Salmonella* Infantis izolates

METU ID	*ardA* (Allelic Type)	*sogS* (Allelic Type)	*pilL* (Allelic Type)
MET S1-050	2	-	-
MET S1-056	2	-	3
MET S1-669	2	9	3
MET S1-785	2	9	-
MET S1-788	ND*	9	3
MET S1-798	-	9	3
MET S1-801	2	9	-

* ND: Not Detected

The study conducted by Cesur, primers of plasmid replicon typing were used to check plasmid groups. In contrast, the study resulted as plasmid isolates used this study (MET S1-050, MET S1-056, MET S1-669, MET S1-785, MET S1-788, MET S1-798, MET S1-801, MET S1-804) belonged to IncP group (Cesur, A., 2018).

Each of the gene sequences of plasmid isolates had been found to have the same domain all within itself. The six *Salmonella* Infantis plasmids, containing the *ardA* gene, were matched with the allele number 2, in the plasmid database on the pubmlst.org web site, except for the plasmid of the MET S1-788 isolate. The MET S1-788 isolate was inconsistent with the sequences in the indicated site of the isolate, indicating that there was a new allele type for the *ardA* gene in this study (Table 25).

It was determined that 5 plasmids contained the *pilL* gene (Table 25). When the *pilL* sequences of the plasmid isolates in the plasmid MLST database (pubmlst.org) were examined, it was determined that the plasmids in the progeny overlapped with the number 3 allele. The difference between the *pilL* allele type 3 and the *pilL* allele type 27 is extra bases in allele type 3. For this reason, *pilL* allele type 3 also includes allele type 27.

The 7 plasmid isolates in which the *sogS* gene is detected overlap with the 9 alleles from the sequences given in the website (Table 25). In studies conducted by Franco et al. in Italy, plasmids isolated from *Salmonella* Infantis isolates were found to have same allels, *ardA, sogS* and *pilL* genes when examined with primers from the pubmlst.org website (Franco et al., 2015). Three of the two plasmid isolates registered on the database website constructed by Alessandra Carattoli had the same allele as the genes obtained in our project. The isolates reported in the website had all the 5 alleles in the Incl1 group, whereas only 3 of these genes were found in the isolates isolated within the scope of the project. The two isolates on the database website are also isolated from *Escherichia coli* and are identical to the allele numbers in the isolates obtained in the study of the Hungarian origin isolate *trbA* allele Italy. Another similar isolate at the mentioned website is from *Escherichia coli* plasmid isolate in a study in Germany (Table 26).

Table 26: General information of Plasmid Sequence Types (Plasmid ST) given alleles of *ardA, sogS* ve *pilL* respectively 2, 9 and 3 from pubmlst.org

ID	Plasmid	Country	Year	Source	Plasmid ST	*IncI1*				
						repI1	*ardA*	*trbA*	*sogS*	*pilL*
1003	686-3	Hungary		poultry	209	1	2	21	9	3
1005	3255-12	Germany	2012	poultry	213	1	2	2	9	3

The phylogenetic trees explaining the genetic linkage of the existing isolates to the 3 plasmid MLST genes are shown below Figure 9, 10, 11.

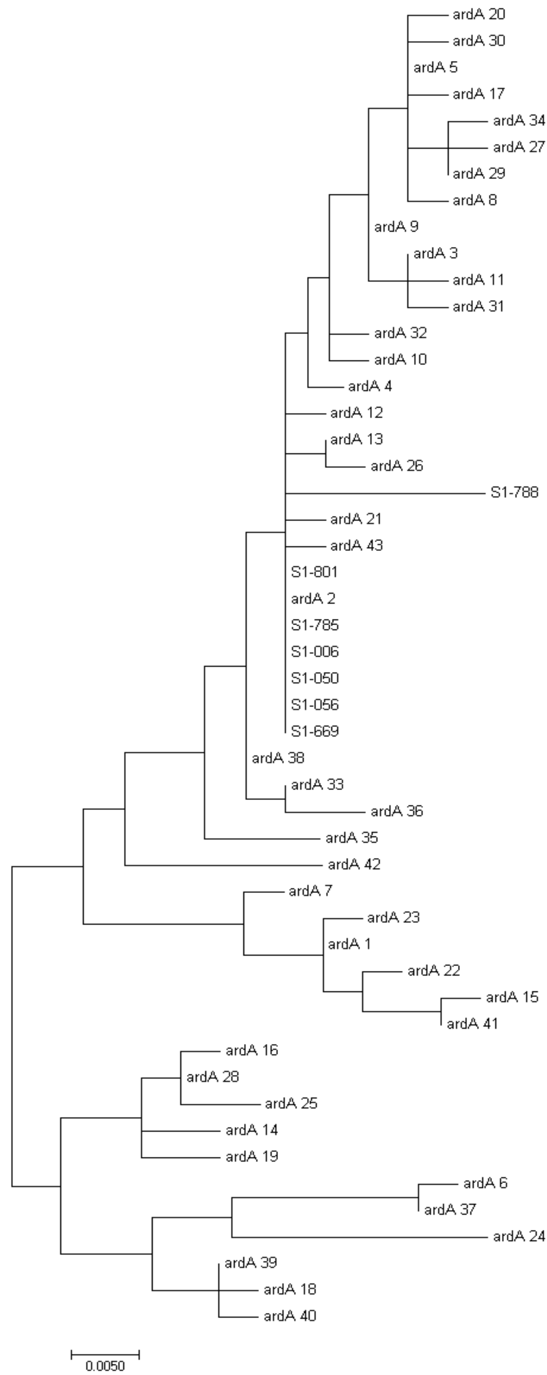

Figure 9: The phylogenetic tree constructed with sequences of ardA gene given in pubmlst.org

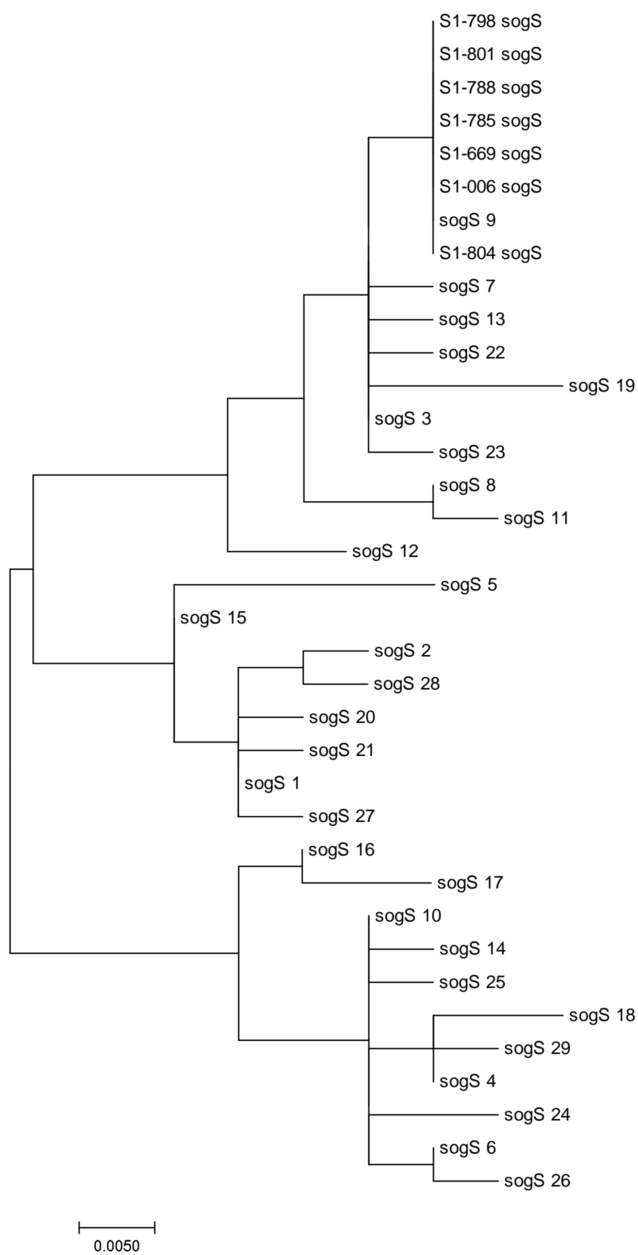

Figure 10: The phylogenetic tree of sequences of *sogS* gene

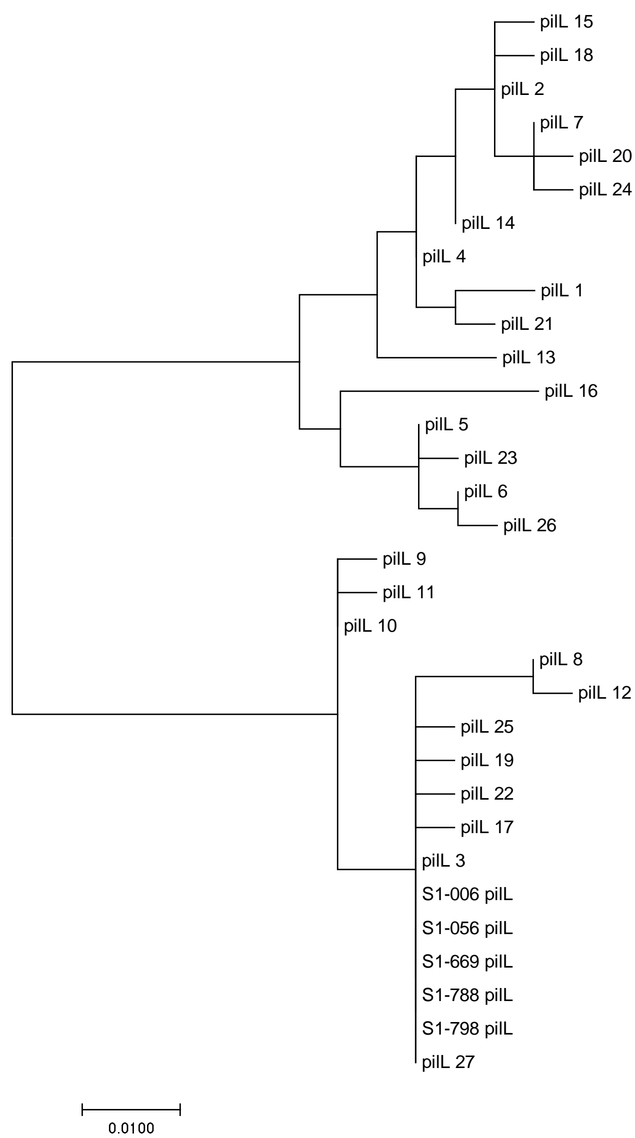

Figure 11: The phylogenetic tree constructed with sequences of *pilL* gene

3.7.2 Plasmid Genome Sequencing

By genome sequencing (GS), all the contigs with plasmid evidence were collected and summed up. Therefore, small sized ones were also added to the larger one, if it were present. In this study, 8 plasmids were characterized into larger plasmids than the ones determined by gel electrophoresis and PFGE (Table 27). This might be due to different reasons:

- Larger plasmids with low copy numbers were not able to be visualized by gel electrophoresis and PFGE,
- Contigs from GS included all the evidence of plasmids summed up in one plasmid sequence.

Table 27: Size classification of plasmids by genome sequence (WGS) result of plasmids

Isolate ID	Size, bp				Total size of plasmid DNA, kbp
	15000-20000	20000-50000	50000-100000	>100000	
MET S1-050		40675		131274	171
MET S1-056	13945 and 15354				28
MET S1-669			92039	131274	223
MET S1-785				156628	156
MET S1-788			79487	144930	224
MET S1-798		38800		156269	195
MET S1-801		38051 and 38805		144365	221
MET S1-804		38711	92845		131

Sequences of contigs can be seen in the given link:
https://drive.google.com/open?id=1Z2sV9xo0x6KPS5lbgQqpguyoOzldOyFr

Contigs and blast analyses results showed that most of the plasmid showed the exact similarity, meaning sharing the backbone of the plasmids. The Table 28 showed the plasmids, which were at least have one similar alignment result with another plasmid or plasmid fragment.

Table 28: Alignment results of plasmid sequences by different colors*

Plasmid Isolates	Sizes, kb						
MET S1-050	13.50	26.50					131.00
MET S1-056	13.50	0.50		15.00			
MET S1-669	92.00						131.00
MET S1-785					25.00		131.00
MET S1-788	38.70	38.00					
MET S1-798	38.70				25.00		131.00
MET S1-801	38.80		38.00		14.00		131.00
MET S1-804	38.70				25.00	67.00	

*: Same color showed the alignment sequences of plasmids or plasmid fragments.

In the literature, large plasmids, carrying antimicrobial resistant genes, were reported in Israel, Hungary and Italy (Szmolka et al., 2017; Franco et al., 2017; Aviv et al., 2016). Approximately 277 kb sized plasmids were found from pSI54/04 strains by Szmolka et al. Indistinguishably, Franco et al, designated their *S.* Infantis strains size approximately between 280–320 kb. Interestingly all the plasmids from different geographical regions showed similarities, showing parallel evolution regardless of geographical regions. Also, being different geographical regions may be the reason of plasmid size variants. In our study, we found that Turkish *S.* Infantis plasmids also overlapped with plasmids mentioned above.

CHAPTER 5

CONCLUSION

Characterization of *Salmonella* isolates collected from chicken meat from different groceries provided better understanding to distribution and ecology of *Salmonella* Infantis in Turkey.

From our knowledge, our study is pioneering work that had been carried out using phenotypic and genotypic characterization of plasmids as well as *Salmonella* Infantis isolates from chicken meat that collected various groceries by using different methods such as disc diffusion, plasmid isolation and conventional agarose, Pulsed Field Gel Electrophoresis (PFGE), antimicrobial gene determination by PCR, Plasmid Multi Locus Sequence typing (pMLST) and plasmid genome sequencing.

Salmonella Infantis isolates except one, were all multi-resistant isolates and were found to have low molecular diversity. Whereas *E. coli* isolates isolated from chicken isolates identified as high molecular varieties.

In our project, we studied *Salmonella* Infantis, which has increased in severity in the last five years and investigated why serotypes spread more rapidly than other serotypes, virulence and plasmid characteristics. We showed that the molecular diversity of Infantis isolates and plasmids was low, but the antimicrobial resistance of Infantis isolates was high. Also, we observed higher frequency of Infantis *Salmonella* among serovars in poultry samples. In addition, this frequency was found higher than other studies in the literature.

Even though, the antimicrobial usage in the feeds is forbidden in animal farms, one might propose that the antimicrobials have been used in feeds due to the high antimicrobial resistance among *Salmonella*, especially predominant serotype Infantis.

Our study is also showed that the Infantis isolates suppress the other *Salmonella* serovars and dominated the population in chicken products. In the future it is important to conduct more detailed investigations in this regard. The outputs of our project provide data to prevent *Salmonella* cases in the chain to the fork from the farm.

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APPENDIX A

Phenotypic and Genotypic Profiles of 70 *Salmonella* Infantis

Table 29: Phenotypic and Genotypic Profiles of 70 *Salmonella* Infantis

METU ID	Source	Brand	Date of Isolation	City	PFGE Type	MLST	AR Profiles	AR Genes	Plasmid size	Reference	Study or Project *
MET S1-006	Chicken meat	-	7.11.2005	Ankara	PT08	32	*aadA1, aphA1-IAB, tetA*	STSfN	-	Avsaroğlu, D., 2008	1
MET S1-050	Chicken meat	A	11.4.2012	Şanlıurfa	PT08	32	*blaTEM-1, aadA1, aphA1-IAB, sul I, tetA*	KSTAmpSfN	45	Acar, S., 2015	2
MET S1-056	Chicken meat	B	11.4.2012	Şanlıurfa	PT08	32	*blaTEM-1, aadA1, aphA1-IAB, cmlA, sul I, tetA*	KSTAmpSfN KfSxtC	47	Acar, S., 2015	2
MET S1-088	Chicken meat	C	11.4.2012	Şanlıurfa	PT08	32	*aphA1-IAB, sul I, tetA*	KSTSfN	-	Acar, S., 2015	2
MET S1-092	Chicken meat	D	11.4.2012	Şanlıurfa	PT08	32	*aadA1, sul I, tetA*	STSfN	-	Acar, S., 2015	2
MET S1-103	Chicken meat	E	11.4.2012	Şanlıurfa	PT08	32	*aadA1, aphA1-IAB, sul I, tetA*	KSTSfN	-	Acar, S., 2015	2
MET S1-142	Chicken meat	F	11.4.2012	Şanlıurfa	PT08	32	*aadA1, strA, sul I, tetA*	STSfN	-	Acar, S., 2015	2
MET S1-150	Offal	G	11.4.2012	Şanlıurfa	PT08	32	*aadA1, sul I, tetA*	STSfN	-	Acar, S., 2015	2
MET S1-329	Chicken meat	H	18.7.2012	Şanlıurfa	PT09	32	*aadA1, strA, sul I, tetA*	STSfN	-	Acar, S., 2015	2
MET S1-345	Chicken meat	D	18.7.2012	Şanlıurfa	PT07	32	*aadA1, aphA1-IAB, sul I, tetA*	KSTSfN	-	Acar, S., 2015	2
MET S1-351	Chicken meat	C	18.7.2012	Şanlıurfa	PT08	32	*aadA1, strA, sul I, tetA*	STSfN	-	Acar, S., 2015	2

Table 29: (continued)

METU ID	Source	Brand	Date of Isolation	City	PFGE Type	MLST	AR Profiles	AR Genes	Plasmid size	Reference	Study or Project *
MET S1-492	Chicken meat	H	7.11.2012	Şanlıurfa	PT08	32	*aadA1, tetA*	STN	-	Acar, S., 2015	2
MET S1-498	Chicken meat	J	7.11.2012	Şanlıurfa	PT07	32	*aadA1, aphA1-IAB, sul I, tetA*	KSTSfN	-	Acar, S., 2015	2
MET S1-510	Chicken meat	K	7.11.2012	Şanlıurfa	PT08	32	*aadA1, aphA1-IAB, sul I, tetA*	KSTSfN	-	Acar, S., 2015	2
MET S1-597	Chicken meat	A	7.12.2012	Şanlıurfa	PT08	32	*aadA1, aphA1-IAB, sul I, tetA*	KSTSfN	-	Acar, S., 2015	2
MET S1-606	Chicken meat	C	7.12.2012	Şanlıurfa	PT08	32	*aadA1, sul I, tetA*	STSfN	-	Acar, S., 2015	2
MET S1-668	Chicken breast	L	28.11.2012	Ankara	PT08	32	*aadA1, sul I, tetA*	SSfN	-	Cengiz et al., *in progress*	3
MET S1-669	Chicken wing	L	12.12.2012	Ankara	PT07	32	*blaTEM-1, aadA1, sul I, tetA*	SAmpKfN	40	Cengiz et al., *in progress*	3
MET S1-671	Chicken breast	M	12.12.2012	Ankara	PT08	32	*aadA1, aphA1-IAB, sul I, tetA*	KSTSfN	-	Cengiz et al., *in progress*	3
MET S1-672	Chicken skin	L	19.12.2012	Ankara	PT08	32	*aadA1, aphA1-IAB, sul I, tetA*	KSTSfN	-	Cengiz et al., *in progress*	3
MET S1-673	Chicken wing	N	19.12.2012	Ankara	PT08	32	*aadA1, tetA*	TN	-	Cengiz et al., *in progress*	3
MET S1-674	Chicken wing	O	19.12.2012	Ankara	PT08	32	*aadA1, aphA1-IAB, sul I, tetA*	KSTSfN	-	Cengiz et al., *in progress*	3
MET S1-676	Chicken leg	P	14.11.2012	Ankara	PT49	32	*aadA1, aphA1-IAB, sul I, tetA*	KSTSfN	-	Cengiz et al., *in progress*	3

Table 29: (continued)

METU ID	Source	Brand	Date of Isolation	City	PFGE Type	MLST	AR Profiles	AR Genes	Plasmid size	Reference	Study or Project *
MET S1-677	Chicken skin	P	14.11.2012	Ankara	PT47	32	*aadA1, aphA1-IAB, sul I, tetA*	KSTSfSxtCip	-	Cengiz et al., *in progress*	3
MET S1-678	Chicken wing	P	14.11.2012	Ankara	PT47	32	*aadA1, aphA1-IAB, sul I, tetA*	KSTSfN	-	Cengiz et al., *in progress*	3
MET S1-679	Chicken liver	M	21.11.2012	Ankara	PT47	32	*aadA1, sul I, tetA*	TSfN	-	Cengiz et al., *in progress*	3
MET S1-680	Chicken leg	P	21.11.2012	Ankara	PT47	32	*aadA1, aphA1-IAB, sul I, tetA*	KSTAmcKfSf N	-	Cengiz et al., *in progress*	3
MET S1-682	Chicken wing	P	21.11.2012	Ankara	PT08	32	*aadA1, aphA1-IAB, sul I, tetA*	KSTSfSxtN	-	Cengiz et al., *in progress*	3
MET S1-683	Chicken liver	L	21.11.2012	Ankara	PT08	32	*aadA1, sul I, tetA*	TSfN	-	Cengiz et al., *in progress*	3
MET S1-684	Chicken skin	P	21.11.2012	Ankara	PT08	32	*aadA1, aphA1-IAB, sul I, tetA*	KSTSfEftN	-	Cengiz et al., *in progress*	3
MET S1-685	Chicken wing	L	21.11.2012	Ankara	PT08	32	*aadA1, sul I, tetA*	STSfN	-	Cengiz et al., *in progress*	3
MET S1-686	Chicken liver	R	28.11.2012	Ankara	PT46	32	*aadA1, aphA1-IAB, sul I, tetA*	KSTSfN	-	Cengiz et al., *in progress*	3
MET S1-687	Chicken skin	M	28.11.2012	Ankara	PT08	32	*aadA1, aphA1-IAB, sul I, tetA*	KTSfN	-	Cengiz et al., *in progress*	3

Table 29: (continued)

METU ID	Source	Brand	Date of Isolation	City	PFGE Type	MLST	AR Profiles	AR Genes	Plasmid size	Reference	Study or Project *
MET S1-688	Chicken leg	M	28.11.2012	Ankara	PT45	32	*aadA1, sul I, tetA*	TSfN	-	Cengiz et al., *in progress*	3
MET S1-689	Chicken skin	M	28.11.2012	Ankara	PT08	32	*aadA1, sul I, tetA*	TSfN	-	Cengiz et al., *in progress*	3
MET S1-690	Chicken liver	S	28.11.2012	Ankara	PT08	32	*aadA1, sul I*	SSfN	-	Cengiz et al., *in progress*	3
MET S1-691	Chicken liver	L	12.12.2012	Ankara	PT08	32	*aadA1, aphA1-IAB, sul I, tetA*	KSTEftSfSxtN	-	Cengiz et al., *in progress*	3
MET S1-692	Chicken leg	L	12.12.2012	Ankara	PT46	32	*aadA1, aphA1-IAB, sul I*	KSTSfN	-	Cengiz et al., *in progress*	3
MET S1-693	Chicken skin	L	12.12.2012	Ankara	PT08	32	*aadA1, aphA1-IAB, sul I, tetA*	KSTSfEftN	-	Cengiz et al., *in progress*	3
MET S1-694	Chicken skin	T	12.12.2012	Ankara	PT08	32	*aadA1, aphA1-IAB, tetA*	KSTSfN	-	Cengiz et al., *in progress*	3
MET S1-695	Chicken skin	U	12.12.2012	Ankara	PT08	32	*aadA1*	STSfN	-	Cengiz et al., *in progress*	3
MET S1-696	Chicken leg	L	19.12.2012	Ankara	PT08	32	*aadA1, aphA1-IAB, sul I, tetA*	TSfN	-	Cengiz et al., *in progress*	3
MET S1-697	Chicken leg	N	19.12.2012	Ankara	PT08	32	*aadA1, sul I, tetA*	SKf	-	Cengiz et al., *in progress*	3

Table 29: (continued)

METU ID	Source	Brand	Date of Isolation	City	PFGE Type	MLST	AR Profiles	AR Genes	Plasmid size	Reference	Study or Project *
MET S1-698	Chicken skin	N	19.12.2012	Ankara	PT48	32	*aadA1, sul I, tetA*	STCipSfN	-	Cengiz et al., *in progress*	3
MET S1-699	Chicken breast	N	19.12.2012	Ankara	PT48	32	*aadA1, sul I*	STSfN	-	Cengiz et al., *in progress*	3
MET S1-700	Chicken liver	O	19.12.2012	Ankara	PT47	32	*aadA1, aphA1-IAB, sul I, tetA*	KSTSfN	-	Cengiz et al., *in progress*	3
MET S1-701	Chicken skin	O	19.12.2012	Ankara	PT48	32	*aadA1, aphA1-IAB, sul I, tetA*	KTSfN	-	Cengiz et al., *in progress*	3
MET S1-737	Chicken liver	M	6.5.2013	Ankara	PT48	32	*aadA1, aphA1-IAB, sul I*	KTSfN	-	Cengiz et al., *in progress*	3
MET S1-738	Chicken skin	M	6.5.2013	Ankara	PT08	32	*aadA1, sul I*	STSfN	-	Cengiz et al., *in progress*	3
MET S1-739	Chicken wing	M	6.5.2013	Ankara	PT08	32	*aadA1, sul I*	STSfN	-	Cengiz et al., *in progress*	3
MET S1-741	Chicken liver	L	2.7.2013	Ankara	PT08	32	*aadA1, tetA*	STSfN	-	Cengiz et al., *in progress*	3
MET S1-745	Chicken liver	R	19.8.2013	Ankara	PT08	32	*aadA1, sul I, tetA*	STSfN	-	Cengiz et al., *in progress*	3
MET S1-746	Chicken liver	P	19.8.2013	Ankara	PT08	32	*aadA1, aphA1-IAB, sul I, tetA*	KTSfN	-	Cengiz et al., *in progress*	3

Table 29: (continued)

METU ID	Source	Brand	Date of Isolation	City	PFGE Type	MLST	AR Profiles	AR Genes	Plasmid size	Reference	Study or Project *
MET S1-747	Chicken skin	P	19.8.2013	Ankara	PT08	32	*aphA1-IAB, sul I*	KTSfN	-	Cengiz et al., *in progress*	3
MET S1-749	Chicken wing	P	19.8.2013	Ankara	PT08	32	*aadA1, aphA1-IAB, sul I*	KTSfN	-	Cengiz et al., *in progress*	3
MET S1-750	Chicken wing	Z	26.1.2015	Ankara	PT08	32	*aadA1, aphA1-IAB, sul I, tetA*	SfSxtKNT	-	TUBITAK 114O180	
MET S1-753	Chicken heart	V	26.1.2015	Ankara	PT08	32		N	-	TUBITAK 114O180	
MET S1-759	Chicken Rib	Y	28.1.2015	Ankara	PT08	32	*aadA1, sul I, tetA*	SfSxtNT	-	TUBITAK 114O180	
MET S1-765	Chicken breast	Z	28.1.2015	Ankara	PT08	32	*aadA1, aphA1-IAB, sul I, tetA*	SfSxtKNT	-	TUBITAK 114O180	
MET S1-774	Chicken rib	W	1.2.2015	Ankara	PT08	32	*aadA1, sul I, tetA*	SfSxtKSNT	-	TUBITAK 114O180	
MET S1-777	Chicken drumstick	P	1.2.2015	Ankara	PT08	32	*aadA1, sul I*	SfSxtSCipNT	-	TUBITAK 114O180	
MET S1-780	Chicken wing	P	1.2.2015	Ankara	PT08	32	*aadA1, aphA1-IAB, sul I, tetA*	SfSxtKNT	-	TUBITAK 114O180	
MET S1-782	Chicken wing	P	1.2.2015	Ankara	PT08	32	*aadA1, aphA1-IAB, sul I, tetA*	SfSxtKSNT	-	TUBITAK 114O180	
MET S1-785	Chicken drumstick	P	1.2.2015	Ankara	PT08	32	*aadA1, strB, flo, sul I, tetA*	SfSxtCSNT	47	TUBITAK 114O180	
MET S1-788	Chicken breast	M	1.2.2015	Ankara	PT08	32	*aadA1, strA, strB, flo, sul I tetA*	SfSxtCSCipNT	45	TUBITAK 114O180	
MET S1-792	Chicken heart	Q	2.2.2015	Ankara	PT08	32	*aadA1, sul I, tetA*	SfSxtSNT	-	TUBITAK 114O180	
MET S1-795	Chicken breast	Q	2.2.2015	Ankara	PT08	32	*aadA1, sul I, tetA*	SfSxtNT	-	TUBITAK 114O180	

Table 29: (continued)

METU ID	Source	Brand	Date of Isolation	City	PFGE Type	MLST	AR Profiles	AR Genes	Plasmid size	Reference	Study or Project *
MET S1-798	Chicken heart	Q	26.2.2015	Ankara	PT08	32	*aadA1, strA, strB, flo, sul I, tetA*	SfSxtCSNT	47	TUBITAK 114O180	
MET S1-801	Chicken breast	Q	26.2.2015	Ankara	PT08	32	*aadA1, strB, aphA1-IAB, flo, sul I, tetA*	SfSxtCKSNT	47	TUBITAK 114O180	
MET S1-804	Chicken wing	Q	26.2.2015	Ankara	PT08	32	*aadA1, strA, strB, aphA1-IAB, flo, sul I, tetA*	SfSxtCKNT	47	TUBITAK 114O180	

*: 1: Avsaroğlu, D., 2008 – Thesis, 2: TUBITAK 111O192, 3: BAP-03-14-2013-001

APPENDIX B

CHEMICALS AND SOLUTIONS

Medium	Preparation
Buffered Peptone Water	20g Buffered Peptone 1000 mL deionized water Autoclave at 121°C for 15min
Rappaport Vassiliadis Soya Peptone (RVS) Broth	13.37g RVS medium 500 mL deionized water Distribute 10 mL/test tubes (50 tubes) Autoclave at 115°C for 15 min
Xylose Lysine Deoxycholate (XLD) Agar	26.5g XLD medium 500 mL deionized water Mix until boiling Cool at 50°C water bath for 30min Distribute 10 mL into sterile petri dishes
Brilliant Green (BGA) Agar	25g Brilliant Green medium 500 mL deionized water. Mix until boiling Cool at 50°C water bath for 30min Distribute 10 mL into sterile petri dishes
Brain Heart Infusion (BHI) Broth	18.5g BHI Medium 500 mL deionized water Distribute 5 mL/ test tubes (50 tubes) Autoclave at 121°C for 15min

Brain Heart Infusion (BHI) Agar	18.5g BHI Medium 7.5g Agar Bacteriological (No.1) 500 mL deionized water Autoclave at 121°C for 15min Cool at 50°C water bath for 30min Distribute 10 mL into sterile petri dishes
	Table E.1 (continued)
0.5M EDTA Stock Solution	pH=8.0 93.05g EDTA 450 mL deionized water Adjust pH 8.0 by 10M NaOH Dilute 500 mL Autoclave at 121°C for 15min 10M NaOH 40g NaOH 100 mL deionized water
10X Tris-Borate-EDTA Stock (TBE) Solution	54g Tris 27.5g Boric Acid 4.65g EDTA 500 mL deionized water Autoclave at 121°C for 15min
0.5X Tris-Borate-EDTA (TBE) Solution	25 mL 10X TBE 475 mL sterile deionized water 1M Tris Stock Solution, pH=8.0 78.8 Tris-HCl 450 mL deionized water Adjust pH 8.0 by 10M NaOH Dilute 500 mL Autoclave at 121°C for 15min
Cell Suspension Buffer (CSB)	20 mL 1M Tris-HCl, pH=8.0 40 mL 0.5M EDTA, pH=8.0 140 mL deionize d water Autoclave at 121°C for 15min

Cell Lysis Buffer (CLB)	25 mL 1M Tris-HCl, pH=8.0 50 mL 0.5M EDTA, pH=8.0 5g N-Lauryl sarcosine sodium salt 400 mL sterile deionized water Heat 60°C and mix for 30min Add 25 mL sterile deionized water
Tris-EDTA (TE) Buffer, pH=8.0	5 mL 1M Tris-HCl, pH=8.0 1 mL 0.5M EDTA, pH=8.0 450 mL deionized water Adjust pH 8.0 by 10M NaOH Dilute 500 mL Autoclaved at 121°C for 15min
10mg/ mL Ethidium Bromide Solution	40µL Et-Br 400 mL distilled water Agarose Gel (for PCR) 1.5g Agarose 100 mL 0.5M TBE Buffer 171 Table E.1 (continued)
20mg/ mL Proteinase K (ProK) Solution	0.001g Pro K 500µL sterile deionized water
20 % Sodium Dodecyl Sulfate (SDS) Solution	10g SDS 500 mL sterile deionized water Heat 45°C and mix thoroughly
1 % SeaKem Gold (SKG) Agarose: 1 % SDS	0.15g SKG 14.1 mL TE Buffer, pH=8.0 Microwave and dissolve completely Cool in 55°C water bath for 10min Pre-warm 20 % SDS Solution in 55°C water bath

	for 10min Add 750µL 20 % SDS to agarose, gently swirl
CLB/ProK solution	5 mL CLB 25µL 20mg/ mL Pro K H Buffer Solution 180µL sterile deionized water 20µL H buffer
XbaI Enzyme Solution	175µL sterile deionized water 20µL H buffer 5µL XbaI Enzyme SKG Agarose 1.5g SKG 7.5 mL 10X TBE 142.5 mL deionized water and mark the water level Add 8 mL deionized water Microwave until 8 mL evaporates Cool agarose in 55°C water bath for 10min
Running Buffer	110 mL 10X TBE buffer 2090 mL deionized water
10mg/ mL Thiourea solution	0.5g Thiourea 50 mL sterile deionized water

APPENDIX C

pMLST RESULTS OF PCR CONFIRMATION OF EIGHT PLASMID ISOLATES

Plasmid Isolates	Organism	Plasmid size	IncI1					IncH12		IncF				IncN		
			repI	*ardA*	*trbA*	*sogS*	*pilL*	*smr0018*	*smr0199*	*FII*	*FIA*	*FIB*	*FIC*	*repN*	*korA*	*traJ*
MET S1-050	*Salmonella* Infantis	45kb	-	+	+	-	-	-	-	-	-	-	-	-	-	-
MET S1-056	*Salmonella* Infantis	47kb	-	+	+	-	-	-	-	-	-	-	-	-	-	-
MET S1-669	*Salmonella* Infantis	40kb	-	+	+	+	+	-	-	-	-	-	-	-	-	-
MET S1-785	*Salmonella* Infantis	47kb	-	+	-	+	-	-	-	-	-	-	-	-	-	-
MET S1-788	*Salmonella* Infantis	45kb	-	+	-	+	+	-	-	-	-	-	-	-	-	-
MET S1-798	*Salmonella* Infantis	47kb	-	+	-	+	+	-	-	-	-	-	-	-	-	-
MET S1-801	*Salmonella* Infantis	47kb	-	+	-	+	-	-	-	-	-	-	-	-	-	-
MET S1-804	*Salmonella* Infantis	47kb	-	-	-	+	-	-	-	-	-	-	-	-	-	-

Table 30: pMLST results of PCR confirmation of eight plasmid isolates

APPENDIX D

pMLST SEQUENCES OF PLASMID ISOLATES

ardA sequences of plasmid isolates:

MET S1-050 *ardA*

GCTGTATACGTTGGAACCTGGCACAAATACAACTGTGGAAGCATCGCCGGA
CGCTGGTTTGACCTGACCACGTTTGATGATGAGCGCGACTTTTTCGCCGCCT
GCCGTGCTCTTCACCAGGATGAAGCCGATCCTGAACTGATGTTTCAGGATTA
TGAGGGATTCCCGGGGAATATGGCCTCTGAATGCCATATCAACTGGGCCTG
GGTTGAAGGCTTCCGCCAGGCACGGGATGAAGGCTGCGAAGAGGCTTATCG
TCTCTGGGTGGAGGATACCGGTGAGACGGATTTTGACACCTTCCGCGATGCC
TGGTGGGGCGAGGCTGACAGTGAGGAGGCTTTTGCGGTTGAGTTCGCCAGT
GATACCGGCCTGCTGGCTGACGTGCCGGAGACGGTGGCGCTCTATTTTGACT
ATGAGGCGTATGCGCGGGATTTATTCCTGGACTCCTTCACCTTTATTGACGG
TCAT

MET S1-056 *ardA*

GTCTGTTTTTGCACCTGCTGTATACGTTGGAACCTGGCACAAATACAACTGT
GGAAGCATCGCCGGACGCTGGTTTGACCTGACCACGTTTGATGATGAGCGC
GACTTTTTCGCCGCCTGCCGTGCTCTTCACCAGGATGAAGCCGATCCTGAAC
TGATGTTTCAGGATTATGAGGGATTCCCGGGGAATATGGCCTCTGAATGCCA
TATCAACTGGGCCTGGGTTGAAGGCTTCCGCCAGGCACGGGATGAAGGCTG
CGAAGAGGCTTATCGTCTCTGGGTGGAGGATACCGGTGAGACGGATTTTGA
CACCTTCCGCGATGCCTGGTGGGGCGAGGCTGACAGTGAGGAGGCTTTTGC
GGTTGAGTTCGCCAGTGATACCGGCCTGCTGGCTGACGTGCCGGAGACGGT

GGCGCTCTATTTTGACTATGAGGCGTATGCGCGGGATTTATTCCTGGACTCC
TTCACCTTTATTGACGGTCATG

MET S1-669 *ardA*

ACCTGCTGTATACGTTGGAACCTGGCACAAATACAACTGTGGAAGCATCGC
CGGACGCTGGTTTGACCTGACCACGTTTGATGATGAGCGCGACTTTTTCGCC
GCCTGCCGTGCTCTTCACCAGGATGAAGCCGATCCTGAACTGATGTTTCAGG
ATTATGAGGGATTCCCGGGGAATATGGCCTCTGAATGCCATATCAACTGGG
CCTGGGTTGAAGGCTTCCGCCAGGCACGGGATGAAGGCTGCGAAGAGGCTT
ATCGTCTCTGGGTGGAGGATACCGGTGAGACGGATTTTGACACCTTCCGCGA
TGCCTGGTGGGGCGAGGCTGACAGTGAGGAGGCTTTTGCGGTTGAGTTCGC
CAGTGATACCGGCCTGCTGGCTGACGTGCCGGAGACGGTGGCGCTCTATTTT
GACTATGAGGCGTATGCGCGGGATTTATTCCTGGACTCCTTCACCTTTATTG
ACGGTCATGTGT

MET S1-785 *ardA*

ACCTGCTGTATACGTTGGAACCTGGCACAAATACAACTGTGGAAGCATCGC
CGGACGCTGGTTTGACCTGACCACGTTTGATGATGAGCGCGACTTTTTCGCC
GCCTGCCGTGCTCTTCACCAGGATGAAGCCGATCCTGAACTGATGTTTCAGG
ATTATGAGGGATTCCCGGGGAATATGGCCTCTGAATGCCATATCAACTGGG
CCTGGGTTGAAGGCTTCCGCCAGGCACGGGATGAAGGCTGCGAAGAGGCTT
ATCGTCTCTGGGTGGAGGATACCGGTGAGACGGATTTTGACACCTTCCGCGA
TGCCTGGTGGGGCGAGGCTGACAGTGAGGAGGCTTTTGCGGTTGAGTTCGC
CAGTGATACCGGCCTGCTGGCTGACGTGCCGGAGACGGTGGCGCTCTATTTT
GACTATGAGGCGTATGCGCGGGATTTATTCCTGGACTCCTTCACCTTTATTG
ACGGTCATGTGTTCCCGTCGGTG

MET S1-788 *ardA*

TGTCTGTTGTTGCACCTGCTGTATACGTTGGAACCTGGCACAAATACAACTG
TGGAAGCATCGCCGGACGCTGGTTTGACCTGACCACGTTTGATGATGAGCG
CGACTTTTTCGCCGCCTGCCGTGCTCTTCACCAGGATGAAGCCGATCCTGAA
CTGATGTTTCAGGATTATGAGGGATTCCCGGGGAATATGGCCTCTGAATGCC
ATATCAACTGGGCCTGGGTTGAAGGCTTCCGCCAGGCACGGGATGAAGGCT
GCGAAGAGGCTTATCGTCTCTGGGTGGAGGATACCGGTGAGACGGATTTTG
ACACCTTCCGCGATGCCTGGTGGGGCGAGGCTGACAGTGAGGAGGCTTTTG

CGGTTGAGTTCGCCAGTGATACCGGCCTGCTGGCTGACGTGCCGGAGACGG
TGGCGCTCTATTTTGACTATGAGGCGTATGCGCGGGATTTATTCCTGGACTC
CTTCACCTTTATTGACGGTCATGTGTTCCGTCGGTG

MET S1-798 *ardA*

GCCACCGTCTCCGGCACGTCAGCCAGCATGCCGGGATCACTGGCAAACTCA
ACCGAAAAAGCCTCCTCACTGTCAGCCTCGCCCCACCAGGCATCGCGAAAG
GTGTCAAAATCCGTCTCACCGGTATCCTCCACCCAGAGACGATAAGCCTCTT
CGCAGCCTTCATCCCGTGCCTGACGGAAGCCTTCAACCCAGGCCCAGTTGAT
ATGGCATTCAGAGGCCATATTCCCCGGGAATCCCTCATAATCCTGAAACATC
AGTTCAGGATCGGCTTCATCCTGGTGAAGAGCACGGCAGGCGGCGAAAAAG
TCGCGCTCATCATCAAACGTGGTCAGGTCAAACCAGCGTCCGGCGATGCTTC
CACAGTTGTATTTGTGCCAGGTTCCAACGTATACAGCAGGTGCAAC

MET S1-801 *ardA*

CCTGCTGTATACGTTGGAACCTGGCACAAATACAACTGTGGAAGCATCGCC
GGACGCTGGTTTGACCTGACCACGTTTGATGATGAGCGCGACTTTTTCGCCG
CCTGCCGTGCTCTTCACCAGGATGAAGCCGATCCTGAACTGATGTTTCAGGA
TTATGAGGGATTCCCGGGGAATATGGCCTCTGAATGCCATATCAACTGGGCC
TGGGTTGAAGGCTTCCGCCAGGCACGGGATGAAGGCTGCGAAGAGGCTTAT
CGTCTCTGGGTGGAGGATACCGGTGAGACGGATTTTGACACCTTCCGCGATG
CCTGGTGGGGCGAGGCTGACAGTGAGGAGGCTTTTGCGGTTGAGTTCGCCA
GTGATACCGGCCTGCTGGCTGACGTGCCGGAGACGGTGGCGCTCTATTTTGA
CTATGGAGGCGTATGCGCGGGATTTATTCCTGGACTCCTTCACCTTTATTGA
CGGTCATG

pill sequences of plasmid isolates:

MET S1-056 *pilL*

TGCGTTGATGCCATGCTTTCGCATTTTGTTTCTTCTGCCCACTTAATAATGTT
TTCCCTTAATGTAGTGCCTGCCGGCGCACGCCACTCTTTACCCTGAGATACC
GGTTTGACAGGTGTCCCGGTCATGAGTGGGATAGACTTGACTGTAGAGCCG
GTCGGAGTCGGGATTGCTGCGGGCGTAGACGGAGATACGCTGTTTCCCCTG

AATGGGTTTCGTGGTTTGTTCTGGCTATTTGCTGTCGTTGAAGACTCCGGGG
AAAGTGGATGTGGTTACCATGGCTGCTGG

MET S1-669 *pilL*

AGTGCGTTGATGCCATGCTTTCGCATTTTGTTTCTTCTGCCCACTTAATAATG
TTTTCCCTTAATGTAGTGCCTGCCGGCGCACGCCACTCTTTACCCTGAGATA
CCGGTTTGACAGGTGTCCCGGTCATGAGTGGGATAGACTTGACTGTAGAGC
CGGTCGGAGTCGGGATTGCTGCGGGCGTAGACGGAGATACGCTGTTTCCCC
TGAATGGGTTTCGTGGTTTGTTCTGGCTATTTGCTGTCGTTGAAGACTCCGG
GGAAGTGGATGTGGTTACCATGGCTGCTGGCGAGATAGTGG

MET S1-788 *pilL*

TGCGTTGATGCCATGCTTTCGCATTTTGTTTCTTCTGCCCACTTAATAATGTT
TTCCCTTAATGTAGTGCCTGCCGGCGCACGCCACTCTTTACCCTGAGATACC
GGTTTGACAGGTGTCCCGGTCATGAGTGGGATAGACTTGACTGTAGAGCCG
GTCGGAGTCGGGATTGCTGCGGGCGTAGACGGAGATACGCTGTTTCCCCTG
AATGGGTTTCGTGGTTTGTTCTGGCTATTTGCTGTCGTTGAAGACTCCGGGG
AAGTGGATGTGGTTACCATGGCTGCTGGCGAGATAGTGGTT

MET S1-798 *pilL*

ATGCCATGCTTTCGCATTTTGTTTCTTCTGCCCACTTAATAATGTTTTCCCTTA
ATGTAGTGCCTGCCGGCGCACGCCACTCTTTACCCTGAGATACCGGTTTGAC
AGGTGTCCCGGTCATGAGTGGGATAGACTTGACTGTAGAGCCGGTCGGAGT
CGGGATTGCTGCGGGCGTAGACGGAGATACGCTGTTTCCCCTGAATGGGTTT
CGTGGTTTGTTCTGGCTATTTGCTGTCGTTGAAGACTCCGGGGAAGTGGATG
TGGTTACCATGGCTGCT

sogS sequences of plasmid isolates:

MET S1-669 SOGS

TTCCGGGGGGGTGACAATACTGTCAGGTATCGGTGGAGTCGTCGTGGTTTCC
GCTGAGGGCGTGGGATCACTGTTCTCATGCGCCTGTGAATCCGTTTTTTTAC

GCGTAAAAAGGCCACGCGCTTTGTCGAGAAACGATGAAGTATTATCAGAAG
GTGATGTGCTCTGAACAGGTTGCTGCGGAGTGGGTTCATCCCGGACAGCCG
GTTCTATAGTGGCTGTTGTGGCCTGAAGTTCTGACTCATCTGCCTGAACGGG
GCCTGTGTCCGGTTGCGA

MET S1-785 *sogS*

ATACTGTCAGGTATCGGTGGAGTCGTCGTGGTTTCCGCTGAGGGCGTGGGAT
CACTGTTCTCATGCGCCTGTGAATCCGTTTTTTACGCGTAAAAAGGCCACG
CGCTTTGTCGAGAAACGATGAAGTATTATCAGAAGGTGATGTGCTCTGAAC
AGGTTGCTGCGGAGTGGGTTCATCCCGGACAGCCGGTTCTATAGTGGCTGTT
GTGGCCTGAAGTTCTGACTCATCTGCCTGAACGGGGCCTGTGTCCGGTTGCG
A

MET S1-788 *sogS*

AATACTGTCAGGTATCGGTGGAGTCGTCGTGGTTTCCGCTGAGGGCGTGGG
ATCACTGTTCTCATGCGCCTGTGAATCCGTTTTTTACGCGTAAAAAGGCCA
CGCGCTTTGTCGAGAAACGATGAAGTATTATCAGAAGGTGATGTGCTCTGA
ACAGGTTGCTGCGGAGTGGGTTCATCCCGGACAGCCGGTTCTATAGTGGCTG
TTGTGGCCTGAAGTTCTGACTCATCTGCCTGAACGGGGCCTGTGTCCGGTTG
C

MET S1-798 *sogS*

TGACAATACTGTCAGGTATCGGTGGAGTCGTCGTGGTTTCCGCTGAGGGCGT
GGGATCACTGTTCTCATGCGCCTGTGAATCCGTTTTTTACGCGTAAAAAGG
CCACGCGCTTTGTCGAGAAACGATGAAGTATTATCAGAAGGTGATGTGCTCT
GAACAGGTTGCTGCGGAGTGGGTTCATCCCGGACAGCCGGTTCTATAGTGG
CTGTTGTGGCCTGAAGTTCTGACTCATCTGCCTGAACGGGGCCTGTGTCCGG
TTGCGAC

MET S1-801 *sogS*

TACTGTCAGGTATCGGTGGAGTCGTCGTGGTTTCCGCTGAGGGCGTGGGATC
ACTGTTCTCATGCGCCTGTGAATCCGTTTTTTTACGCGTAAAAAGGCCACGC
GCTTTGTCGAGAAACGATGAAGTATTATCAGAAGGTGATGTGCTCTGAACA
AGGTTGCTGCGGGAGTGGGTTCATCCCGGACAGCCGGTTCTATAAGTGGCT
GTTGTGGCCTGAAGTTCTGACTCATCTGCCTGAACGGGGCCTGTGTCCGGTT
GCGAC

MET S1-804 *sogS*

TACTGTCAGGTATCGGTGGAGTCGTCGTGGTTTCCGCTGAGGGCGTGGGATC
ACTGTTCTCATGCGCCTGTGAATCCGTTTTTTTACGCGTAAAAAGGCCACGC
GCTTTGTCGAGAAACGATGAAGTATTATCAGAAGGTGATGTGCTCTGAACA
GGTTGCTGCGGAGTGGGTTCATCCCGGACAGCCGGTTCTATAAGTGGCTGTT
GTGGCCTGAAGTTCTGACTCATCTGCCTGAACGGGGCCTGTGTCCGGTTGCG
ACGGCATATCA

APPENDIX E

SAMPLES OF FIGURES OF AGAROSE GEL RESULTS

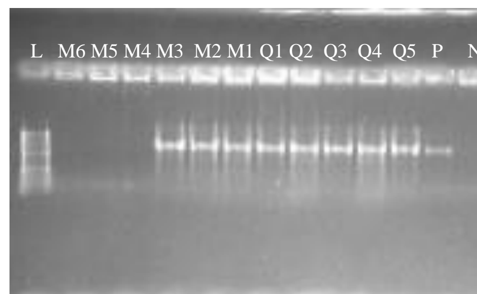

Figure 12: Agarose gel results of PCR of *invA* gene(678 bp) of presumptive colonies. (L: 100 bp ladder, M and Q: each letter shows different brand, each number shows different presumptive colony from different plates. P: positive control, N: negative control. M4, M5, M6 are negative, M1, M2, M3, Q1, Q2, Q3, Q4, Q5 are positive)

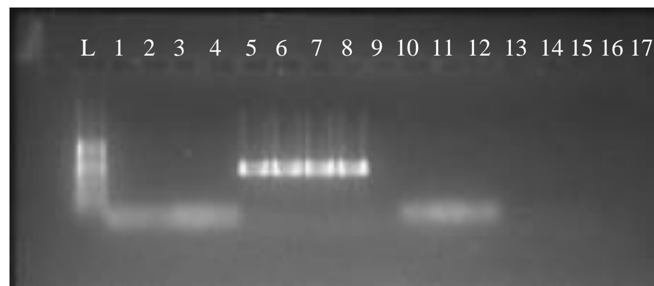

Figure 13: PCR results of pMLST genes of plasmid isolates.
(L: Ladder, 1: MET S1-050 *repI* gene (-), 2: MET S1-056 *repI* gene (-), 3: MET S1-669 *repI* gene (-), 4: MET S1-785 *repI* gene (-), 5: MET S1-050 *ardA* gene (+), 6:MET S1-056 *ardA* gene (+), 7:MET S1-669 *ardA* gene (+), 8: MET S1-785 *ardA* gene (+), 9:negative control of *ardA* gene, 10: MET S1-050 FIA gene (-), 11: MET S1-056 FIA

gene (-), 12: MET S1-669 FIA gene (-), 13: negative control of FIA gene, 14: MET S1-050 *repI* gene (-), 15: MET S1-056 *repI* gene (-), 16: MET S1-669 *repI* gene (-), 17: negative control of *repI* gene

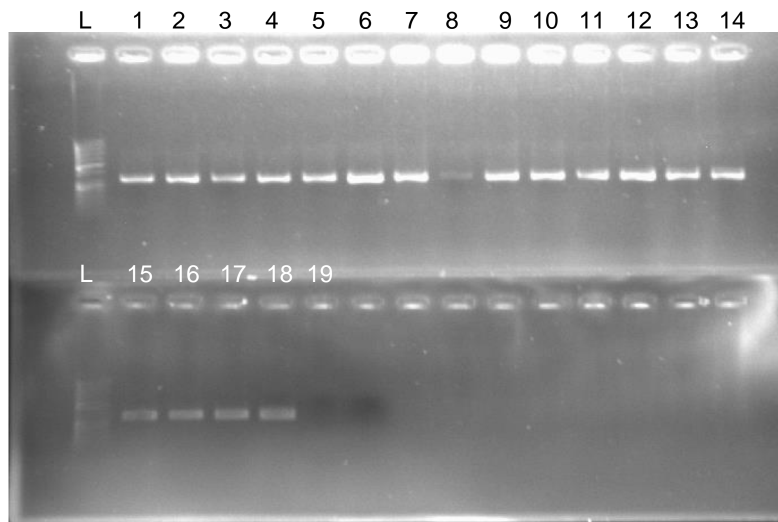

Figure 14: PCR results of *sulI* gene in plasmid isolates
(L: ladder, 1: MET S1-050 (+), 2: MET S1-056 (+), 3: MET S1-668 (+), 4: MET S1-684 (+), 5: MET S1-697 (+), *6: E. coli 39R861* (+), 7: MET S1-750 (+), 8:MET S1-753 (-), 9:MET S1-759 (+), 10:MET S1-765 (+), 11:MET S1-774 (+), 12*:E. coli 39R861* (+), 13:MET S1-056 (+), 14:MET S1-669 (+), 15:MET S1-677 (+), 16:MET S1-685 (+), 17:MET S1-689 (+), 18:*E. coli 39R861* (+), 19: negative control)