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SCREENING THE VIRULENCE GENES AND PROTEIN EXPRESSION PROFILES OF CLINICAL ISOLATES OF RESISTANT *SALMONELLA TYPHI*

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DİRENÇLİ *SALMONELLA TYPHİ* KLİNİK İZOLATLARININ VİRÜLANS GENLERİNİN VE PROTEİN EKSPRESYON PROFİLLERİNİN TARANMASI

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The M.Sc. Thesis entitled **"Screening the virulence genes and protein expression profiles of clinical isolates of resistant *Salmonella typhi*"** has been prepared in accordance with Erciyes University Graduate School of Natural and Applied sciences Thesis Preparation and Writing Guide

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ÖZET

Salmonella enterica serovar typhi'nin neden olduğu enterik ateş, özellikle gelişmekte olan ülkelerde en yaygın hastalıklar arasındadır. Bu çalışma, *Salmonella typhi'*nin genomik ve proteomik profilini izole etmeyi ve tanımlamayı amaçlamıştır. Irak'ın Bağdat kentinde tifo geçirmiş kişilerden 100 kan örneği alındı. *S. typhi*'yi test etmek için örnekler *Salmonella Shigella* agar üzerinde büyütülmüş ve şüpheli koloniler toplanmıştır. İzolatların bakteri tanımlaması ve antibiyotik duyarlılıkları VITEK® 2 Compact sistem kullanılarak yapılmıştır. İlgili genleri taramak için polimeraz zincir reaksiyonu metodu kullanılmıştır. SDS-PAGE metoduyla protein çalışmaları yapılmıştır. Kültür plakalarında siyah koloniler görülmüştür. VITEK® 2 Compact kullanılarak 100 örnekten 40'ının *S. typhi* olduğu tespit edilmiştir. Mevcut araştırmadan elde edilen veriler, *S. typhi* suşlarının çoklu ilaca dirençli olduğunu göstermiştir. Tüm *S. typhi* suşları, penisilin sınıfı antibiyotiklere (tikarsilin ve piperasilin), 4. kuşak sefalosporinlere (seftazidim, sefepim), monobaktam (aztreonam) ve aminoglikozitlere (amikasin, tobramisin, gentamisin) direnç göstermiştir. Tüm suşlar dirençli sülfonamidlere (trimetoprim/sülfametoksazol), karbapenemlere (imipenem ve meropenem) ve tetrasiklinlere (piperasilin/tazobaktam) karşı duyarlılık göstermiştir. Moleküler analizler tüm izolatların *aadA* geni taşıdığını, ancak hiçbirinin bla_{SHV} geni taşımadığını ortaya koymuştur. SDS-PAGE analizinde tüm izolatlar benzer bant profili göstermiş olup aynı tür içerisinde olduğunun önemli bir göstergesidir.

Anahtar Kelimeler: Enterik ateş, çoklu ilaca dirençlilik, *Salmonella typhi*, VITEK® 2 compact, SDS- PAGE

SCREENING THE VIRULENCE GENES AND PROTEIN EXPRESSION PROFILES OF CLINICAL ISOLATES OF RESISTANT *SALMONELLA TYPHI*

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ABSTRACT

Enteric fever, caused by *Salmonella enterica* serovar *typhi* is among the most prevalent diseases, particularly in developing countries. This study aimed to isolate and identify the genomic and proteomic profile of *Salmonella typhi* isolates. In Baghdad, Iraq, 100 blood samples were taken from people who had typhoid. To test for *S. typhi*, the specimens were grown on *Salmonella Shigella* agar and suspicious colonies were collected. Bacterial identification and antibiotic susceptibility of isolates were performed using the VITEK® 2 Compact system. Polymerase chain reaction was used to search genes of interest. Through SDS-PAGE, a proteomics studies was carried out. On cultured plates, black colonies were seen. Using VITEK® 2 Compact, 40 isolates out of 100 samples were found to be *S. typhi*. Data from the current investigation showed that *S. typhi* strains were multidrug resistant. All *S. typhi* strains showed resistance to penicillin class of antibiotics (ticarcillin and piperacillin), 4th generation cephalosporins (ceftazidime, cefepime), monobactam (aztreonam), as well as aminoglycosides (amikacin, tobramycin, gentamicin). All resistant strains displayed sensitivity to sulfonamides (trimethoprim/sulfamethoxazole), carbapenems (imipenem and meropenem), and tetracyclines (piperacillin/tazobactam). Molecular analysis revealed that all isolates carried *aadA* gene, but none of them carried bla_{SHV}gene. Results of band patterns on SDS-PAGE showed a similar pattern of protein bands of all isolates. This suggests that all isolates belonged to the same species of bacteria due to their similarity in protein profiles.

Keywords: Enteric fever, multidrug-resistance, *Salmonella typhi*, VITEK® 2 compact, SDS- PAGE

İÇİNDEKİLER

SCREENING THE VIRULENCE GENES AND PROTEIN EXPRESSION PROFILES OF CLINICAL ISOLATES OF RESISTANT *SALMONELLA TYPHI*

ABBREVIATIONS LIST

ABC METHOD : Activity-Based Costing

AFLP : Amplified Fragment Length Polymorphism

Ak : Amikacin

ARMS : Amplification Refractory Mutation System

AST : Antimicrobial Susceptibility Testing

Azt : Aztreonam

Caz : Ceftazidime

CIP : Ciprofloxacin

Cpe : Cefepime

DNTPs : Deoxy Nucleotide Triphosphate

GM : Gentamicin

ID-GN : Identification Gram Negative

Imp : Imipenem

kDa : kilodalton

MDR : Multi-Drug Resistance

Mer : Meropenem

MIN : Minocycline

PCR : Polymerase Chain Reaction

Pi : Piperacillin

RAPD : Random Amplified Polymorphic DNA

RPM : Revolutions Per Minute

S. typhi: Salmonella typhi

SCV : Small Colony Variants

SDS PAGE: Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis

SPIs : *Salmonella* Pathogenicity Islands

SS AGAR : *Salmonella Shigella* Agar

SXT : Trimethoprim/sulfamethoxazole

T3SS **:** Type III secretio n systems

TCV: Typhoid Conjugate Vaccine

TIC : Ticarcillin

TIM : Ticarcillin/Clavulanic Acid

TO : Tobramycin

Tzp : Piperacillin/Tazobactam

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

There are over 2,500 serotypes described within the genus of *Salmonella enterica* to date [4]. In developing countries, enteric fever caused by *Salmonella enterica* serovar *typhi* (*S. typhi*) and *Salmonella enterica* serovar *paratyphi* A (*S. paratyphi* A) also continues to be a major public health concern. It was estimated that typhoid fever caused 21.6 million diseases and 216,500 deaths worldwide, and an estimated 5.4 million diseases were caused by less serious paratyphoid fever in 2000 [5]. Most are harmless to humans, but one serotype, the enterica serovar typhi subspecies of *Salmonella enterica*, causes typhoid fever, a serious and life-threatening systemic infection in humans. Worldwide, out of 26.9 million new cases each year, typhoid fever causes 269,000 deaths [6]. Children, Travelers, immune-compromised individuals and the elderly are especially at risk [7]. Some febrile diseases are close to the clinical forms of typhoid fever. Diagnosis based on clinical signs and symptoms alone is also hard [8]. The development of multi-drug resistant *S. typhi* strains and typhoid carrier state growth have further complicated Typhoid Fever Treatment [9]. One of the most important problems the world has been faced in recent years is resistance of bacteria to antibiotics. *Salmonella* is one of them because of the extensive and wrong use of antibiotics both in human treatment and also in animal feeding to improve their growth [2]. *Salmonella* is a gram-negative Bacillus, belong to the Enterobacteriaceae. There are multiple types of this bacteria, but the two most important that causes human infection are *Salmonella typhi* and *Salmonella enterica* [1]. *Salmonella* colonizes in proximal colon and distal ileum after eating contaminated food or water and amount of *Salmonella* becomes enough to cause disease. Quantity after *Salmonella* infection ranges between 10^5 to 10^6. *Salmonella* use flagella for movement and use chemotaxis in order to target the host cells. *Salmonella* infection led to inflammation due to pro-inflammatory cytokines emission which cause acute inflammatory responses leading to ulceration, diarrhea, and

the demolition of the mucosa cells [10]. The name of *Salmonella* comes from the name of the scientists, Daniel Elmer Salmon, who discovered it. *Salmonella* widespread and difficult to control except for specific preventive measures. The symptoms of *Salmonella* for humans vary from person to person.

Some people who are not accompanied by symptoms but the others show infection such as vomiting, diarrhea, fever, abdominal pain, bloody stools, stomach cramps, cold, muscle pains, headache, dizziness, nausea and some people suffer from arthritis. Despite the infection of this bacteria in people who do not have health problems is not life menacing and the hydration is sufficient in this state but in immunodeficiency situation it must be resorted to antibiotics to get rid of them. Perhaps the most important sources of infection are animal-based foods. The transmission of *Salmonella* to humans may be via water or through the breeding of pets such as birds, livestock or wild-animals as well as eating meat containing this bacterium. More susceptible to these infections are people working in farms, slaughterhouses and veterinarians for direct contact with the infected animal, they must be careful and take safety precautions. The modern methods of slaughter that make animals close to each other during slaughter increased the chances of transmission of these bacteria from one sacrifice to another [10]. The issue is complicated by the resistance of *Salmonella* to antibiotics [2]. *Salmonella* lives in the gastrointestinal tract of cold- and hot-blooded animals with and can invade the bloodstream when it infects humans and causes intestinal typhoid fever and excreted through feces [1,2]. *Salmonella* has different virulence factors which play different roles in the pathogenesis of this bacteria. Factors of virulence included capsule, flagella, adhesion systems, plasmids, and *Salmonella* pathogenicity island that encode type 3 secretion systems [12]. *Salmonella enterica* produce many different virulence determinants, such as invasins, fimbriae, adhesins, endotoxins, exotoxins and hemagglutinins which are related to adhesion systems [13]. Virulence factors in combination with others or alone permit the colonization of *Salmonella* to the host via attaching, invading, passing and surviving the defense system of the host such as acidity of the gastric, gastrointestinal proteases, as well as intestinal microbiome aggressions [14,15]. *Salmonella* pathogenicity islands (SPIs) are clusters of genes located in specific regions of the chromosomes. SPIs can be found on the chromosomes or the plasmids. SPI have a variable G/C content and are surrounded by repeat sequences [16].

The polymerase chain reaction is a major tool for the detection and quantification of DNA or RNA. By using this method, we can realize and get accurate results; PCRis one the most potent type of genetic method. The aim of this study is the isolation and characterization of clinical *Salmonella typhi* in terms of antibiotic resistance, virulence genes and protein expression profiles through comparing with non-resistant isolates.

CHAPTER 1

GENERAL INFORMATION AND LITERATURE REVIEW

1.1. General information

Salmonella enterica is one of the gram-negative species. For humans, only the *Salmonella enterica* subspecies enterica is clinically relevant. Enteric fever is caused by the human-restricted serovar Typhi [1]. *Salmonella* is flagellated rod-shaped bacterium, serologically positive for the lipopolysaccharide antigens (O9, O12), and the polysaccharide capsular antigen Vi. In comparison to Vi positive strains, antigen Vi-negative strains appear to be less contagious and virulent [4]. *S. typhi* is a well preserved serovar of the *S. enterica* species. *S. typhi* is classified as Group D by the Kaufmann-White classification system, with Vi capsule positive and O-antigen type O9-12, phase1 flagellin type H: d. As a result, *S. typhi* is typically monophasic. The majority of *S. typhi* isolates are classified as Kaufmann-White, however a few are Vi negative [2]. *Salmonella* is a non-fastidious pathogen because it may live and replicate in a variety of environments outside of living hosts. The majority of serotypes develop and grow in temperatures ranging from 5 to 47 °C, with an optimum of 32 to 35 °C. Few serotypes, however, may thrive at temperatures as low as 2-4°C and as high as 54°C. Typhoid fever is a prevalent disease in areas where there is a lack of economic development and public health facilities. Human infection with *S. typhi* is usually transmitted through contaminated water or food. The pH required for *Salmonella* growth varies from 4 to 9, with a 6.5 to 7.5 as a preferred range. *Salmonella* can survive in water with a 0.2 water activity, such as in dried foods, but they need a high-water activity of 0.99 to 0.94 in order to survive [21]. As one of the public health challenges in many impoverished and emerging countries, enteric fever is a major concern.

Globally, approximately 21 million cases and 700,000 deaths are recorded each year, primarily in Africa, Southeast Asia and Latin America, due to inadequate and poor waste disposal, unplanned urbanization, and a lack of potable water supply [3]. In healthy persons, an infectious dosage of *Salmonella typhi* ranges between 1000 and 1 million organisms, however this is dependent on the host's defense mechanisms. Despite proper antibiotic therapy, from 1 to 5% of individuals will become chronic carriers of *Salmonella typhi*. A chronic carrier is characterized as a person who has bacterial excretion in the stool or urine for more than one year after an acute infection [5].

1.2. *Salmonella typhi* genome

Salmonella is a genus of enteric bacteria that contains *Escherichia coli* and *Shigella* species, and belongs to the Enterobacteriaceae family. With a few exceptions, the basic life cycle of these microorganisms includes colonization of the lumen of the intestine of animals and transmission between hosts via the external environment. The majority of Enterobacteriaceae are commensal microbes that are found in the normal intestinal microflora, while some have increased pathogenic potential. With a few exceptions, the life cycle of these bacteria includes colonization of the animal’s lumen of the intestine and transmission between hosts via the external environment. The majority of Enterobacteriaceae are commensal microbes that are found in the normal intestinal microflora, while some have increased pathogenic potential [9]. Genome comparison of different enteric bacteria reveals several key similarities. All have a single chromosome with a size of 4.3–5.0 Mb [10]. Extrachromosomal DNA in the form of plasmids may also be found in different strains. Plasmids are rapidly developing gene pools that often carry genes related with virulence or drug resistance. Different enteric bacteria's chromosomes compared finds a collection of "core genes" that are shared by all enteric organisms [11]. Core genes encode common structural proteins, having a function in central metabolism as well as polysaccharide biosynthesis [9]. *Salmonella enterica* comparative genomics has revealed distinct genetic fingerprints linked to invasive pathology and host adaptation. A comparison of typhoidal *Salmonella* genomes revealed the existence of virulence factors such as Vi polysaccharide pilus associated proteins, as well as typhoid-specific protein families.

Moreover, *in silico* comparison revealed 469 gene of *Salmonella* genomes involved in central anaerobic metabolism that were functional in non-typhoidal salmonellosis but decayed in extra-intestinal infections like in *Salmonella typhi* [1]. Antibiotic resistance in *S. typhi* is frequently caused by plasmids. Resistance to chloramphenicol, trimethoprim, ampicillin, tetracycline and sulfonamides is frequently encoded by plasmids from the *IncHI* group [3]. Plasmids are circular double-stranded deoxyribonucleic acid molecules that can be found as incorporated into the bacterial chromosome but may be exist and proliferate independently within a bacterial cell. Plasmids act as vectors for the lateral movement of genetic material between bacterial cells, boosting their spread and long-term viability inside a bacterial niche under changing environmental conditions [12].

Genes encoding multiple aspects in bacteria are carried in plasmids and expressed when the bacteria come into contact with a hostile environment, particularly those caused by human activities such as antibiotic use, such phenomenon has a significant role in the evolution of antibiotic resistant bacteria, especially among gram-negative Enterobacteriaceae, resulting in treatment failure and the persistence of infection in population. Plasmids are classified based on their persistence through transmission, and their incompatibility categories are investigated. The failure of two plasmids to coexist stably over a number of generations in the same cell line of bacteria is referred to as incompatibility grouping, and plasmids that are incompatible with each other are grouped together [13]. The incompatibility groups have been sub-grouped into four major clusters: IncF, IncP, TiI and ncI groups which include “IncF, IncS, IncC, IncD, IncJ“, “IncP, IncU, IncM, IncW“, “IncX, IncH, IncN, IncT“ and “IncI, IncB and IncK“, respectively [14]. The majority of these plasmids have been linked to MDR isolates from clinical and animal studies [15]. Competition within the normal flora, responding with variable nutrition sources in the host and environment, and pressure from the host immune system, to mention a few, all such condition provides selective pressure on the genomes of enteric bacteria. As a result, it appears that the genomes of enteric bacteria reveal evidence of these evolutionary forces. Blocks of genes or, in some cases, single genes scattered along the core genome have low or no similarity with the core genome. Furthermore, these genes may indicate significant divergence between enteric species or even within the same species [9].

These unique genes may, nevertheless, share certain similar characteristics and functions. They might, for example, collaborate to boost a species' pathogenicity potential. Furthermore, these genes may be unrelated or show significant divergence between enteric species or even within the same species. These unique genes may, nevertheless, share certain similar characteristics and functions. They might, for example, collaborate to boost a species' pathogenicity potential. The association of such gene combinations are often referred to as pathogenicity islands.

These gene clusters frequently have different GC content than the core genome, implying that they were gained more recently and separately through a horizontal gene-transfer event. Moreover, these genes are predominantly associated with plasmid and phage genes, and they are occasionally integrated into excessive transfer RNA genes that can operate as foreign DNA receptor sites [16]. The first complete genome sequence of an MDR *S. typhi* strain called was published in 2000 [17]. *S. typhi* has a transferable R plasmid called pHCM1 that encodes resistance genes for all first-line antibiotics used to treat typhoid fever, as well as an IncHI1 plasmid that was obtained from *S. enterica* in the 1960s. When compared to R27, pHCM1 has gained more genes, primarily at two locations, with 18 genes expressing antibiotic and heavy metal resistance. Around these two areas, several transposon-like and integrases/transposases are encoded, indicating a recombination hotspot. Genes encoded on pHCM1 include dhfr1b, sul2 cat, Ibla, tetA/tetC and strAB for antibiotic resistance against trimethoprim, sulphonamide, chloramphenicol, TEM-1; ampicillin, tetracyclines, and streptomycin, respectively [18]. In some *S. typhi* isolates with the MDR phenotype, IncHI1 plasmids are not always present. Instead, sections of the composite transposon seen on IncHI1 plasmids have inserted themselves into the *S. typhi* chromosome, in fully two independent chromosomal loci. This was worrying since, compared to plasmids, chromosomally integrated DNA is more durable and less likely to be lost [21]. Point mutations in the genome of bacteria or horizontal transfer of genetic elements encoding resistance genes can both lead to antimicrobial drug resistance. Resistance can be spread by cloning drug-resistant organisms or horizontally transferring genetic elements that code for resistance determinants [22].

Integrons are DNA elements which can integrate and express the genes that induce resistance to antimicrobials are mostly contain one gene or more encoding antimicrobial

resistance, integrons are identified in transposons and plasmids. The integrons are classified to four classes which are the classes 1, 2, 3, and 4. Class 1 is the most abundant among the members of *Enterobacteriaceae* in the non-pathogenic and pathogenic microbiota of animals. Most of *Salmonella* clinical isolates carry class 1 integron and resistant to different antimicrobials [11]. Integrons are thus ideally suited for the dissemination and recombination of antibiotic resistance genes. Integrons are common in *S. enterica* and make an important contribution to the extent of antimicrobial resistance in this species [23].

S. typhi resists antimicrobial action in three ways: deactivation of the antimicrobial agent, efflux of the antimicrobial, alteration of the antimicrobial target site, and decreased antimicrobial permeability. A mutation in the gene that codes for either the drug's target or perhaps the transport system as in membrane that controls the drug's uptake has been linked to chromosomal resistance [24]. The rate of spontaneous mutations is usually between 10^{-7} and 10^{-9}, which is lesser than the rate of plasmid acquisition. As a result, chromosomal resistance is a less serious clinical issue than plasmid-mediated resistance. As a result of selection pressure on the bacterial population due to their inappropriate use, chromosomal-mediated drug resistance to fluoroquinolones has recently been discovered. A point mutation in the quinolone resistance determining region of the topoisomerase gene *gyrA*, which encodes DNA gyrase, has been blamed for this [25]. Table (1.2) show some different genes of *salmonella typhi*

1.3. Pathogenesis of *Salmonella typhi*

To enter the small intestine, *Salmonella typhi* must pass across the stomach acid barrier, as well as a low gastric pH is a crucial defense mechanism. The infective dose is reduced by using proton-pump inhibitors, histamine H2-receptor antagonists or significant quantities of antacids. The bacteria attach to mucosal cells in the small intestine and eventually enter the mucosa. M cells, specialized epithelial cells that cover Peyer's patches, are most likely the location of *S. typhi* ingestion and transfer to the underlying lymphoid tissue.

After penetrating the intestinal lymphoid follicles and leaving mesenteric lymph nodes, some of invading microorganisms pass to reticuloendothelial cells of spleen and liver.

Salmonella pathogens can survive and multiply in mononuclear phagocytic cells of the spleen, lymphoid follicles as well as liver [6]. *Salmonella* discharged into the bloodstream from intracellular sit. Incubation lasts 7 - 14 days on average. The spleen, liver, gallbladder, bone marrow and Peyer's patches of the terminal ileum are the most prominent sites of secondary infection.

Count of *Salmonella typhi* in patients with acute typhoid fever is about 1 and 10 cell per milliliter in blood and bone marrow, respectively [7]. Despite the fact that *S. typhi* produces a strong endotoxin, the treated individuals have a death rate of less than 1%. In patients with severe typhoid, recent studies have found higher amounts of circulating anti-inflammatory, proinflammatory and cytokines, as well as a lower capacity of whole blood to generate inflammatory cytokines [8]. Typhoid causes systemic and local humoral as well as cellular immune responses, but these only provide a partial barrier to reinfection. In severe disease, the interplay of host immunologic mediators and bacterial components in infected tissue may contribute to Peyer's patch necrosis [5].

Fimbriae play a critical role in *Salmonella* pathogenicity, and it has recently been discovered to be a generator of variety among *Salmonella* serovars [19]. Fimbriae are the most frequent adhesion mechanisms, that are expressed differently and in varied patterns according on the serovar [20], as well as they play a set of functions, including biofilm formation, seroconversion, hemagglutination, cellular invasion, and macrophage interactions, in addition to mediating *Salmonella* adherence to host cells. Normally, the fimbrial systems are arranged into gene clusters of 4 to 15 genes that code for regulatory, assembly, as well as structural proteins. Several fimbriae are conserved among *Salmonella* serovars, except a few fimbriae that are only found in certain serovars. *Salmonella* pathogenicity islands (SPIs) are gene clusters that encode numerous virulence factors, adhesion and invasion genes as well as are found in specific locations of the chromosomes in bacterial cells [21]. SPIs are known to be frequently associated with transfer RNA (tRNA) and mobile genetic elements such as transposons or phage genes, and they have a base makeup that differs significantly from core genomes [26].

Different SPIs have been reported for several *Salmonella* serovars including SPI (1 to 5), the most typically detected in numerous *Salmonella* serovars and others being less

widely distributed across the serovars [21,27]. Generally , the SPIs play several roles during *Salmonella* pathogenesis, briefly, the SPI-1 is necessary for the host cells invasion and macrophage apoptosis activation, SPI-2 for systemic infections and replication inside macrophages, SPI-3 for macrophages survival as well as for *Salmonella* growth in low-magnesium environments, SPI-4 harboured secretion toxin and apoptosis genes as well as survival from macrophage, SPI-5 have a role in genes that encode effector proteins (T3SS) and SPI-6 is found as result of external stimulations in order to transport proteins into host cells [27]. Due to a lack of surveillance efforts in many places, the variability of disease presentation, and the difficulty in establishing the diagnosis, the true global disease burden of typhoid fever remains unknown [28]. According to several modeling studies, the illness burden worldwide ranges from 12 million to 21 million cases per year, with 129, 000 to 145, 000 fatalities per year. Antimicrobial resistance has been a persistent problem in the management of enteric fever since the first reports of chloramphenicol resistance in *S. typhi* in 1970s [29]. The recent advent of severely drug-resistant *S. typhi* strains, which are resistant to fluoroquinolones and third-generation cephalosporins as well as first-generation antibiotics, has made typhoid fever treatment difficult and costly [30].

Adults and children above the age of two were formerly advised to get typhoid immunizations. The live attenuated Ty21a vaccine was approved in Europe and the United States in 1983 and 1989, respectively; the Vi-polysaccharide vaccine was approved in the United States for the first time in 1994 [29]. Different virulance factors of *Salmonella typhi* were given in Table1.1 and Figure1.1 [31].

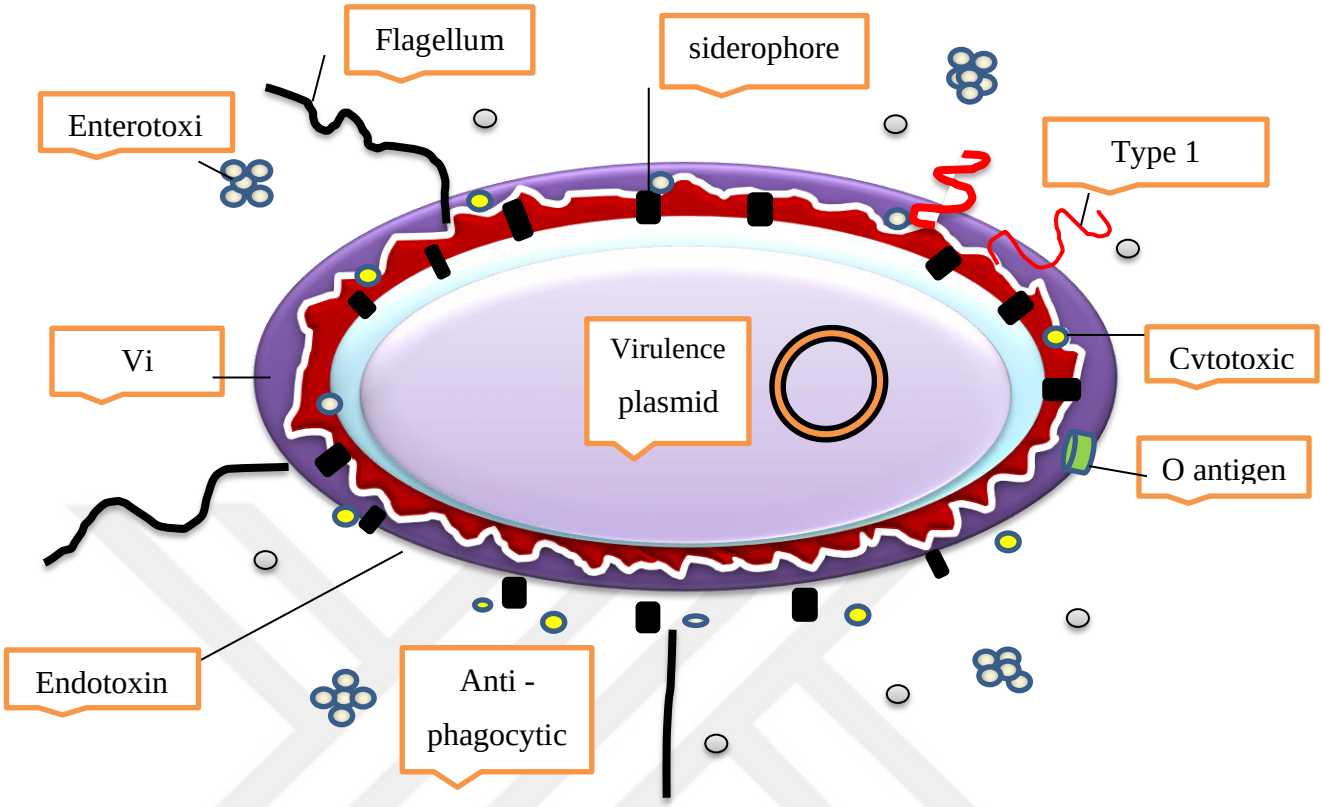


Figure 1.1. Different virulence factors of *Salmonella typhi* [59].

Table 1.1. Function of some virulence factors of *Salmonella typhi*

Virulence factor	**Function**
Siderophores	Salmochelin and Enterobactin and synthesis as well as iron chelators
Flagellum	Movement and chemotaxis
Enterotoxin	Caused diarrhea
Vi capsule	Complement binding inhibition
Endotoxin	Caused fever
Anti -phagocytic protein	Thriving within cells
O antigen	Inhibition of phagocytosis
Cytotoxin	Inhibition of protein synthesis of the cell and help in adherence
Type 1 fimbria	Assist in adherence

The World Health Organization (WHO) prequalified Typbar TCV, a typhoid conjugate vaccine (TCV), in January 2018. Bharat Biotech International Limited manufactures Typbar TCV, which is approved for use in people aged 6 months to 45 years. Following that, the WHO Strategic Advisory Group of Experts (SAGE) suggested that TCV be implemented first in countries with the highest burden of typhoid disease or antibiotic resistance to *S. typhi* [32]. TCV, along with other vaccines, should be given routinely at 9 months of age or in the second year of life, depending on the local situation in endemic areas. TCV vaccination as part of a standard immunization program is one of the most effective therapies for children under the age of five. Under the rapid introduction scenario, the yearly demand for TCV might rise to 160 million doses, depending on immunization techniques and country adoption rates [33].

1.4. Resistance mechanisms of *Salmonella typhi*

Chloramphenicol was the most efficient and widely used antibiotic for typhoid fever when it was developed in 1948. After two years Chloramphenicol-resistant *S. typhi* isolates were discovered from England within, owing to its widespread and unregulated usage [27]. Most of the drug resistance occurs as a result of a genetic alteration in the organism, such as a chromosomal mutation or the transfer of a plasmid / transposon.

Antibiotics resistant in *S. typhi* occur in different ways including inactivation and efflux of antibiotics as well as alteration of the antibiotic target site and minimize the permeability of the antibiotics [26,28].

S. typhi resistance against antibiotics is plasmid or chromosomal mediated. Incompatibility group plasmids which IncHI1 are important antibiotic resistance vectors in *S. typhi*. Plasmid frequently code for enzymes that destroy antibiotics such as penicillin hydrolysis as well as chloramphenicol and aminoglycoside acetylation. Nevertheless, some genes of the plasmid have been involved in aminoglycosides resistance and other antibiotics [26]. A mutation in the gene that codes for either the antibiotics target or the transport system in the membrane that regulate the antibiotics uptake has been linked to chromosomal resistance [28]. As a result of selective pressure on the bacterial population due to misuse of fluoroquinolones, the chromosomal-mediated resistance has lately been reported.

This has been related to a point mutation in the topoisomerase gene at quinolone resistance determining region, that encodes *gyrA* DNA [29]. To screen the in vitro antimicrobial activity of microorganisms, a range of laboratory approaches can be applied. The disk-diffusion and agar or broth dilution procedures are the most well-known and basic approaches [30]. Antibiotic susceptibility can be determined quickly through using a variety of methods, including molecular techniques, microarrays as well as commercial methods used in the workplace, colorimetric methods, immunochromatographic methods, MALDI-TOF mass spectrometry, nephelometry and flow cytometry [31]. Through its quick and automated fluorescence-based technology, the VITEK 2 system has revolutionized antimicrobial susceptibility testing. The VITEK 2 system's accuracy in identification and antimicrobial susceptibility testing as well as the greatly reduced handling time, which improves the clinical microbiology laboratory's workflow [32], nevertheless, genetic material, including deoxyribonucleic acid and ribonucleic acid, can be detected using molecular methods. The polymerase chain reaction is the molecular approach with the highest diagnostic value, as it not only allows the infectious agent to be precisely identified, but it is also the most common way for determining its resistance and virulence genotypes [31].

Table 1.2. Different genes of *Salmonella typhi*, origin and their roles

Gene name	Origin	Role
$\boldsymbol{bla_{TEM}}$	Plasmid	β-lactamase [33]
aadA	Plasmid	Encodes a spectinomycin/ streptomycin adenylyltransferase [34]
$\boldsymbol{bla_{CTX\text{-}M}}$	Plasmid	β-lactamase [33]
intI1	Plasmid or chromosome	Multiple antibiotic resistances [35]
qnrA	Plasmid or chromosome	Quinolone resistance gene [36]
qnrB	Plasmid or chromosome	Quinolone and Betalactamase resistance [36]
$\boldsymbol{bla_{SHV}}$	Chromosome or plasmid	β-lactamase [33]
dfr	Plasmid or transposons	Trimethoprim resistance [37]

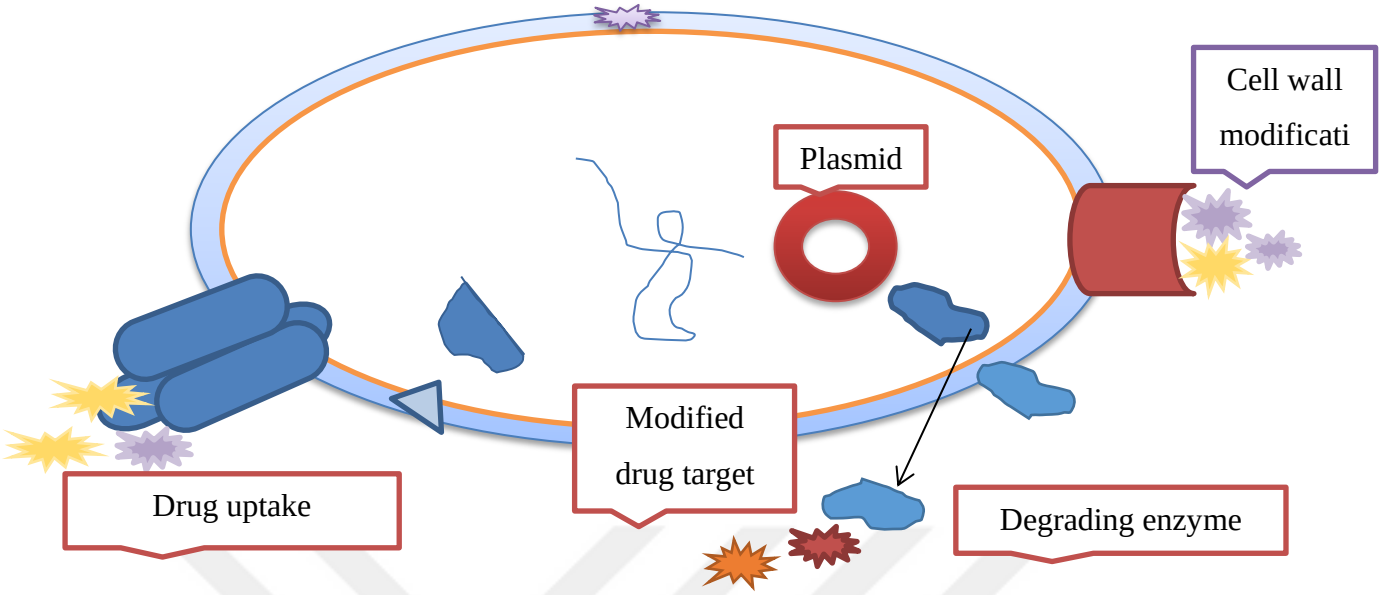


Figure 1.2. General mechanism of antimicrobial resistance in *Salmonella typhi* [95]

1.5. *Salmonella typhi* identification strategies

Bacterial identification is critical for scientists working in a wide range of applied research and industry fields, from clinical microbiology to food production. There are several criteria for classifying the numerous methods employed in the field of bacteria identification, however they may generally be divided into direct in which bacteria are isolated and cultured, then their phenotypic traits are determined, it can be used to identify non-culturable bacteria as well as individual microbes in a mixed population, and indirect procedures that include microscopic techniques, for example, are effective instruments for identifying microorganisms by visualizing their distinctive structures, however, microscopy identification is not enough for bacteria for various reasons such as small size of cells result in difficulties for identification as well as prokaryotes differ in size, in addition, the resolution of optical microscope is powerless to magnify some cells [33]. Furthermore, the microscopes have a severe drawback in that none of them disclose the phylogenetic diversity of the microorganisms found in the study area. Serological methods, which contain monoclonal antibodies against the Vi antigen, could be utilized to replace traditional polyclonal antisera for serotyping and fast identification of *S. typhi* [36].

From isolated patient samples the Vitek technique is effective for bacterial identification and antimicrobial susceptibility testing profiles [34]. Previously, morphological, and biochemical approaches that dependent on preliminary isolation as well as culture were used to identify and characterize species of bacteria. While traditional methods are still used in some situations, molecular-based techniques have given researchers novel insights into bacterial identification and typing [35]. Both of PCR and automated DNA sequencing in the last two decades ago, in addition to subsequent work on 16S rDNA sequencing of bacteria, all such techniques result in accumulation of a major amount of rDNA genes sequence data of the ribosomes smaller subunit of the different organism's type. The rDNA gene sequences are substantially preserved within live same genus and species as well they vary between other genera and species, based on comparison of these sequences. involving the rDNA gene sequences assist the phylogenetic studies in classification life domains in to the three classes which Archaea, Eukarya and Bacteria, in contrast to the conventional organism's classification that include prokaryotes and eukaryotes only [36,37].

1.5.1. Gram stain

Gram stain, originally used because Gram-positive and Gram-negative organisms have different responses to some antimicrobial agents, this stain was originally developed to help clinicians distinguish bacteria from host cells in tissue. It has evolved into a key assay to help clinicians decide which antibiotics should be used to treat infections. While the purple versus pink staining does not provide any phylogenetic information, it does highlight the various biologies of these two groups of organisms and relationships between numerous therapeutically significant bacteria as determined by 16S rRNA gene-based investigations [38].

1.5.2. Polymerase chain reaction

PCR (polymerase chain reaction) is a scientific process used in molecular biology to make hundreds to millions of copies of a single DNA sequence to amplify a single or few copies of DNA across several orders of magnitude.

The polymerase chain reaction is performed in a reaction mixture that contains an accumulation of four deoxyribonucleoside triphosphates in a buffer solution, as well as DNA extract (template DNA), Taq polymerase, primers, and the four deoxyribonucleoside triphosphates (dNTPs). In the heating block of a thermal cycler, the tubes carrying the mixed reaction are subjected to repeated temperature cycles numerous times. The equipment allows the duration and sequence of temperature step cycles to be programmed. A variety of PCRs were developed to identify a range of phenomena that emerge in various diseases and cases, allowing scientists to investigate these situations and diagnose the causes. AFLP, RAPD, ARMS, Colony, Multiplex PCR and Nested, as well as other forms of PCR, disclose the genetics and heredity of numerous traits and diseases [40]. Routine molecular biology research require a mechanism for quickly identifying microbial transformants using PCR analysis. Colony PCR is a crucial technology for such a reason because of its speed, flexibility, and low cell count requirements. Colony PCRs are frequently used in gene amplification, detection of recombinant integrants and plasmid constructions, taxonomy screening, and diagnostics in addition to identifying microbial transformants [41]. Colony PCR is most typically used to screen transformants bacteria for a certain DNA sequence.

Because not all colonies that emerge after transformation has a plasmid with the appropriate DNA insert. Furthermore, the DNA insert may also be oriented incorrectly for transcription and translation. Colony PCR is an effective approach for quickly identifying bacteria that contain the appropriate DNA including the desired insert orientation. The fact that colony PCR does not require DNA purification is a significant benefit. Simply, a tiny aliquot of intact bacteria for example, a colony is mixed with a PCR mix which involves particular primers, heat-stable DNA, dNTPs polymerase and buffer as well as PCRd. The initial PCR step of heating the bacteria to 94°C is enough to lyse them and liberate their DNA [42].

1.5.3. SDS PAGE

Separation strategies focus on differences in protein molecule solubility, size, charge, and adsorption properties. Ion-exchange chromatography, affinity chromatography, dialysis, ultrafiltration, size-exclusion chromatography, and SDS-PAGE electrophoresis , capillary electrophoresis and isoelectric focusing are some of the most commonly

utilised protein separation methods. Chromatography is a vital biophysical technique for qualitative and quantitative analysis that allows for the identification separation as well as purification of the components of a mixture. Size and shape, total charge, hydrophobic groups on the surface, and binding capacity with the stationary phase are all factors that can be utilised to purify proteins. Ion exchange, surface adsorption, partition, and size exclusion are four separation approaches based on molecule properties and interaction type [43]. SDS-PAGE is the most widely used technology for obtaining high precision analytical separation of protein mixtures [44]. Initially, component proteins are denaturated with an anionic detergent which also binds to them, giving every proteins a negative charge equal to their molecular mass. Following this, proteins are separated with fine resolution based on of molecular mass using electrophoresis via a porous acrylamide gel matrix. This approach, which has remained mostly unchanged since its invention in the early 1970s as well as such method works well in applications that do not need the preservation of specific protein structure or function.

The purposeful denaturation of proteins prior to electrophoresis is an obvious restriction of SDS-PAGE. On SDS-PAGE-isolated proteins, enzymatic activity, protein binding relationships, detection of protein cofactors, and so on are often impossible to identify [43].

1.6. Proteomic characteristic of *Salmonella typhi*

Protein secretion in bacteria is a promising technique for heterologous protein expression because it preserves bacterial hosts' high titres and tractability while simplifying downstream processing.

Traditional intracellular manufacturing methods necessitate cell lysis and isolation of the protein product from chemically identical cellular components, which is usually a multi-stage process that includes an expensive refolding step [45]. The growing variety of commercial protein products, combined with the high cost of developing these products, is motivating engineering attempts to create low-cost, easy-to-manipulate manufacturing methods. Traditional intracellular expression systems provide various hurdles, despite the fact that bacterial hosts are resilient, genetically tractable, and cheap to grow. Several downstream purification processes are required to recover products

from a complex lysate mixture [46]. Furthermore, insoluble inclusion bodies are frequently formed when heterologous proteins are overexpressed intracellularly. Although inclusion bodies have a better initial purity, re-solubilisation and refolding techniques must be created for each product [47]. These limitations are removed by engineering bacteria to secrete heterologous proteins into the extracellular space. T3SS stands for type III secretion system, which is a multimeric protein needle complex that bridges the bacterial cell's inner and outer membranes and secretes proteins from the cytoplasm to the extracellular space in a single step [48]. Protein expression has yet to be linked to the variations observed at the DNA level. As a matter of fact many of the horizontally acquired serovars-specific genes are not always translated into proteins, likely because these exogenous elements are incompatible with the cell's translational machinery [49]. The genome of *S. typhi* encodes over 4500 proteins, according to protein analysis. Toxic proteins are harmful chemicals that can cause sickness when they come into touch with or are absorbed by the host's body tissues. These proteins bind to biological macromolecules like enzymes or cellular receptors, causing the host immune system to malfunction [50]. The typhoidal *Salmonella enterica* serovars *typhi* and Paratyphi produce typhoid toxin, which is a unique virulence factor [51, 52]. Typhoid toxin is a virulence factor that is encoded by *S. typhi* , but is totally absent from *Salmonella enterica* serovars that induce self-limiting gastroenteritis (i.e. non-typhoidal serovars) [53].

Homologs of the toxin are also found in a small number of clade B serovars that are rarely linked to human disease, however these homologs have different biological activities.

Typhoid toxin is unique in that it is only produced when *S. typhi* is present in mammalian cells. A specific type X secretion mechanism transports the toxin from the bacterium to SCV (lumen of the *Salmonella*-containing vacuole. The toxin is subsequently packed and transported to the extracellular space by vesicle carriers [54]. The exocytic mechanism that carries the toxin from the SCV to the extracellular space is little understood, despite its importance in *S. typhi* pathology. PltB's glycan-binding site is changed by one amino acid, resulting in a toxin that is trapped within the SCV after the bacteria secrete it [55]. This finding shows that toxin export is regulated via a glycosylated receptor on the luminal side of the *S. typhi*-containing vacuolar membrane,

which is most likely regulated by a glycosylated receptor. The nature of such a potential receptor, however, is uncertain [54]. The etiology of human typhoid fever, a systemic disease that is still a serious public health concern around the world [52]. Typhoid toxin, when given to experimental animals, can mimic many of the pathognomonic acute symptoms of typhoid fever [51]. Typhoid toxin's biology is specifically tailored to *Salmonella's* intracellular existence. In fact, the toxin is only produced by bacteria that are found within cells [53]. The activity of TtsA, a specific muramidase that enables the translocation of typhoid toxin from the *cis* to the *trans* side of the peptidoglycan layer of the bacterial envelope, is required for typhoid toxin secretion from the bacterial cell [56, 57]. The typhoid toxin is generated by the SCV and discharged into the extracellular space. Typhoid toxin is implicated in the poisoning of infected and uninfected cells via autocrine and paracrine pathways once it is exported [58, 59]. Many virulence components, including Vi capsular polysaccharides, typhoid toxin and the Type III secretion system *Salmonella* Pathogenicity Island (SPI)-1 and SPI-2 effector toxins, help to coordinate the infection cycle of *S. typhi* [60]. The majority of these virulence factors stay on the bacterial surface or in the cytoplasm of *S. typhi*-infected host cells, changing host responses predominantly at the infection site and in infected cells. Typhoid toxin, on the other hand, is an exotoxin that targets host cells both locally and distantly in order to benefit the pathogen by modifying host immune cell numbers and activities [61, 62, 60]. Food safety can be threatened by the presence of bacteria or their toxins in food. Although the majority of microorganisms are harmless in nature, some are dangerous and can cause serious illness.

Several bacterial strains have been identified as the primary cause of food-borne illnesses, making them high priority targets for detection for example *E. coli* (infectious dosage of 10^1-10^2 CFU/ml), *Listeria monocytogenes* (infectious dosage of Staphylococcus (infectious dosage of > 10^6 CFU/ml) and *Salmonella* (infectious dose of 10^4-10^7 CFU/ml) [63]. Bacteremia, diarrhea, cholecystitis, pneumonia, cholangitis, campylobacteriosis, listeriosis, staph infections, and newborn meningitis are among disorders caused by pathogenic bacteria [63].

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

Tables below include the materials, kits and instruments used in bacterial identification processes till protein analysis.

2.1.1. Kits and materials for molecular studies

Materials	Manufacturer
Presto™ Mini gDNA Kit	Geneaid, Taiwan
Absolute Ethanol	ROMIL pure chemistry, UK
Agarose, Ethidium Bromide Solution (10mg/ml), GoTag Green Master Mix, Nuclease Free Water, TAE 40X and Quantifluor dsDNA System	Promega, USA
Primers	Macrogen, Korea

2.1.2. Primer pairs and properties

Primer Name	Sequence	Amplification size	Reference
***bla*$_{TEM}$**	F 5`-TTTTCGTGTCGCCCTTATTCC-3` R 5`-CGTTCATCCATAGTTGCCTGACTC-3`	798	[106]
aadA	F 5`-GTGGATGGCGGCCTGAAGCC-3` R 5`-ATTGCCCAGTCGGCAGCG-3`	528	[136]
***bla*$_{CTX-M}$**	F 5`-CGCTGTTGTTAGGAAGTGTG-3` R 5`-GGCTGGGTGAAGTAAGTGAC-3`	403	[137]
intI1	F 5`-GGGTCAAGGATCTGGATTTCG-3` R 5`-ACATGGGTGTAAATCATCGTC-3`	483	[106]
qnrA	F 5`-ATTTCTCACGCCAGGATTTG-3` 5`-GATCGGCAAAGGTTAGGTCA-3`	516	[108]
qnrB	F 5`-GATCGTGAAAGCCAGAAAGG-3` R 5`-ATGCCTGGTAGTTGTCC-3`	469	[108]
***bla*$_{SHV}$**	F 5`-GGTTATGCGTTATATTCGCC-3` R 5`-TTAGCGTTGCCAGTGCTC-3`	885	[106]
dfr	F 5`-GTGAAACTATCACTAATGG-3` R 5`-TTAACCCTTTTGCCAGATTT-3`	474	[106]
16s rRNA	F 5'- AGAGTTTGATCCTGGCTCAG- 3' R 5'- GGTTACCTTGTTACGACTT- 3'	1250	[138]

2.2. Methods

2.2.1. Bacterial identification and susceptibility test

Identified 100 clinical isolates with *Salmonella* spp collected from different geographical areas in Iraq. For identification, SS agar (*Salmonella Shigella* agar) is used which is a selective medium used to cultivate, isolate, as well as differentiate *Salmonella* spp. Gram stain used for microscopic study for *Salmonella typhi* isolations. Also, for specific identification VITEK 2 compact system was used.

2.2.1.1. Bacterial activation and SS Agar identification step

Salmonella typhi isolates were selectively grown on *Salmonella Shigella* (SS) media. The medium was made by dissolving 63.53 grams of powder in 1000 ml of distilled water and then heating to boiling without autoclaving, as directed by the manufacturer (Solarbio-china). When the media had cooled to 42-45°C, it was poured into plate. Bacterial isolates were taken from a liquid bacterial suspension and cultured with a sterile disposable loop spread on the plate by ABC method. Then incubated for 24 hours at 37˚C. Culturing method repeated twice for positive isolates to get pure colonies. (colorless black center colony had been selected from SS Agar)

- Brain Heart Infusion Broth (BHI)

BHI is a liquid medium used to cultivate fastidious and non-fastidious microorganisms from clinical specimens, food, as well as environmental samples, including aerobic and anaerobic bacteria. BHI Medium is a highly nutritive medium that was used to cultivate S. typhi isolates for analysing the growth curves, DNA extraction, soluble intracellular and secreted protein used in SDS-PAGE analysis.

The medium was made according to the manufacturer's instructions (HIMEDIA-India) by dissolving 37 g in 1000 ml, then autoclaving at 15 lbs pressure (121°C) for 15 minutes and using at the same day.

- Nutrient broth

It is a liquid medium used for the cultivation of a wide variety of organisms from clinical specimens. Nutrient Broth was employed for general cultivation, and it was also used to compare the growth curve characterization of S. typhi in comparison to BHI broth growing conditions.

The media was made according to the manufacturer's instructions (HIMEDIA-India) by suspending 13 g in 1000 ml distilled water, which was then heated to completely dissolve the medium, and then autoclaved at 15 lbs pressure (121°C) for 15 minutes.

2.2.1.2. Gram stain differentiation step

Specific colony has been picked from SSagar and fixed with heating on glass slid the staining procedure includes following steps:

1. Crystal violet staining dye is applied to cells. Afterwards, to create a complex between the iodine and the crystal violet, iodine and potassium iodide which is known as a Gram's iodine solution is added. This compound is insoluble in water and has a higher molecular size than the classic crystal violet stain with iodine.

2. The sample is treated with ethyl alcohol as decolorizes, which causes the peptidoglycan layer to dehydrate, shrink, and tighten. the thin layer of peptidoglycan layer in Gram negative cells and the damaged outer membrane of the bacteria prevent the retention of the crystal violet-iodine combination, resulting in the loss of color.

3. The sample is stained red by the addition of a counterstain, for instance the weakly water soluble safranin. The safranin does not alter the purple hue of Gram positive cells since it is less intense than crystal violet. The decolored Gram-negative cells, however, are stained red.

After that the prepared slide has been identified via light microscope.

2.2.1.3. VITEK 2-compact system

The Vitek 2 Compact system uses a 64-well card barcoded with information on card type, expiration date, lot number, and unique card identification number for organism

identification and a turbid metric approach for susceptibility testing. ID-GN and AST-GN, gram negative Bacillus identification and gram negative susceptibility test kits have been used, respectively. According to the manufacturer's instructions (bioMérieux, France), both of biochemically and antibiotic susceptibility tests for the 50 suspected colonies were examined using a Vitek 2 compact.

A sterile swab was used to transfer sufficient morphologically comparable colonies to the 3 ml saline tube and by using the VITEK 2 DensiCHEK Plus, a homogeneous suspension with a density equivalent to the required McFarland standard was prepared for ID identification, for AST detection 145µL was transferred to the second tube filled with 3 ml of normal saline. Both tubes injected to each specific card, then both cards uploaded to the system. The results were interpreted according to the manufacturer's instructions using the Vitek 2 compact system's specific software. The susceptibility data were assessed using the Medical Laboratory Standards Institute standards. The antibiotics used in this research were Ticarcillin, Ticarcillin/Clavulanic Acid, Piperacillin, Piperacillin/Tazobactam, Ceftazidime, Cefepime, Aztreonam, Imipenem, Meropenem, Amikacin, Gentamicin, Tobramycin, Ciprofloxacin, Minocycline and Trimethoprim/Sulfamethoxazole.

2.3. Molecular analysis

Molecular studies were carried out in two ways because not all of genes detected in first molecular analysis, used for undetected gene in order to ensure that if the isolations are carried or not for such genes colony PCR had been as confirmatory molecular test negative results, in order to confirm whether the negative results are true or not. All three isolates were mixed and treated as one isolate. Two methods were used; the first depends on cell blasting to obtain the plasmid, and the second method depends on DNA extraction of the isolates. One isolate was choosen randomly for 16s rRNA identification, as well as others later.

2.3.1. DNA Extraction for antibiotics resistance gene detection

Genomic DNA was extracted from bacterial cells using the Geneaid Extraction technique, which included the following steps:

- Suspension and protein digestion

An overnight culture was centrifuged for 3 minutes at 12000rpm to obtain cell pellet. After that, the supernatant was discarded. 180 µl of GT buffer was poured to the pellet and stirred by vortexing for suspending. Proteinase K (20 µl) was added to the suspension for protein digestion. All of the mixtures were incubated for at least 10 minutes at 60°C.

- Cell lysis

200 µl of GB buffer was added to the sample for cell lysis, and then it was homogenized via vortexing for 10 seconds. To verify that the sample lysate is clear, the combination was incubated at 70°C for at least 10 minutes. After that, samples were spun for 10 seconds in a centrifuge to eliminate air bubbles.

- Binding

200 µl of 100% ethanol was added to the sample lysate for DNA binding and mixed directly by continuous shaking. The mixture was then transferred to the GD column and centrifuged at 12,000rpm for 1 minute, including any insoluble precipitate. The flow-through GD collection tube was discarded, and the GD column was inserted in a new GD collection tube.

- Washing

For washing, 400 µl of W1 buffer was added to the GD column, centrifuged for 30 seconds at 12,000rpm, and the flow-through was discarded. the GD column was placed back in the 2ml collection tube, and 600 µl of Wash buffer was added to the GD column and centrifuged at 12,000rpm for 3 minutes.

The flow-through was then discarded, and the GD column was re-inserted into the 2ml collection tube. To dry the column matrix, the empty column matrix was centrifuged for 3 minutes at 12,000rpm.

- Elution

A clean 1.5ml microcentrifuge tube was used to transfer the dried GD column. In the center of the column matrix, an aliquot of 100 µl of pre-heated elution buffer1 was

added. Then allowing at least 3 minutes for elution buffer to absorb entirely. The pure DNA was eluted by centrifugation for 3 minutes at 9,000rpm.

2.3.2. DNA Quantitation

In order to detect the quality of samples for downstream applications, a Quantus Fluorometer was utilized to detect the concentration of extracted DNA. 199 µl of diluted Quantifluor Dye was combined with 1 µl of DNA. DNA concentration values were determined after a 5-minute incubation period at room temperature.

2.3.3. Primer preparation

These primers were lyophilized by the Macrogen Company. As a stock solution, lyophilized primers were dissolved in nuclease-free water to a final concentration of 100pmol/l. To make a working primer solution, 10 µl of primer stock solution (stored at -20 C) was combined with 90 µl of nuclease-free water to make a 10 pmol/l working primer solution.

2.3.4. Reaction Setup and Thermal Cycling Protocol

In order to prepare a 20 µl of a total volume for each sample, 10 µl of Master Mix, 1 µl of forward primer (100 nM), 1 µl of reverse primer (100 nM), 6 µl nuclease free water and 2 µl of DNA was added, respectively. Table 2 illustrate PCR reaction (temperature, time duration and cycle number.

2.3.6. Preparation of agarose gel

A flask was filled with 100 ml of 1X TAE and 1.5 g of agarose was added to prepare 1.5% solution. The solution was brought to a boil in the microwave until all of the gel particles were dissolved. Then 10 µl of ethidium bromide (10 mg/ml) was added. Then the solution was allowed to cool to 50-60 C°.

2.3.7. The casting of the horizontal agarose gel

After sealing both edges with cellophane tapes, the agarose solution was poured into the gel tray and allowed to set at room temperature for 30 minutes. After carefully removing the comb, the gel was deposited in the gel tray. 1X TAE-electrophoresis buffer was poured into the tray until it reached 3-5 mm above the gel's surface.

2.3.8. DNA loading

The PCR products (5 µl) were directly loaded into each well. For 60 minutes, electrical power was turned on at 100 V and then a gel imaging equipment was used to visualize the ethidium bromide-stained bands in the gel.

2.4. Colony PCR method

Some genes in the first molecular analysis couldn't be detected. Colony PCR method was used in order to ensure that the isolates contain such specific genes. Colony PCR is a method for rapidly screening colonies of yeast or bacteria that have grown up on selective media following a transformation step, to verify that the desired genetic construct is present, or to amplify a portion of the construct. Individual bacterial isolates were cultured on *SS*-agar.

A single colony was selected and suspended in 10 µl ddH2O, which was then heated 95ºC for 3 minutes using a heat block to get cell lysate. Each isolate's solution was then centrifuged, and working concentration which includes 8 µl of the lysate was combined with 1 µl of forward and 1 µl of reverse primers, as well as 10 µl of 2X Taq Plus PCR MasterMix (Solarbio). The PCR program was set according to the supplier's instructions

2.4.1. DNA extraction

The Bacterial Genomic DNA Extraction Kit (Solarbio D1600) was used to extract genomic DNA, which was done according to the instructions. The PCR was set according to the supplier's instructions for gene detection by PCR utilizing bacterial chromosome (Solarbio).

2.5. Protein Screening by SDS PAGE

SDS-PAGE is characterized as a major method for size-based protein separation and analysis. We used this method to analyze intracellular and secreted proteins of *Salmonella typhi* isolates in this study.

- **Cell lysate preparation**

The bacteria from glycerol stock culture were initially cultured on *SS*-agar media. A single colony was inoculated in 3.0 ml of BHI broth and incubated at 37 °C with 200 rpm overnight.

In a 250 ml conical flask, 50 ml of freshly prepared BHI or NB was inoculated with 1 percent of overnight culture. Bacterial broth was allowed to grow for 5 hours at 37°C with 200 rpm.

One milliliter of cell culture was centrifuged for 5 minutes at 4°C and 5000 rpm. The cell pellet was washed twice with distilled water containing 0.5 M NaCl and 50 mM Tris (pH 7.6). The pellet was then frozen at -20°C until it was used. To suspend the frozen cells, 100 ul of the same buffer supplemented with 10 mM EDTA was employed; from this point until the end of the procedure, all conditions were performed on ice. The suspension was supplemented with an appropriate amount of 0.1g glass beads 425-600 m (provided by Solarbio-china)in order to rupture the cell as well as to permits the removal of intracellular content.

After waiting 15 minutes on ice, the mixture was vortexes for 20s, followed by one-minute intervals of ice incubation (these steps were repeated 7-10 times). The solution was centrifuged for 10 minutes at 10,000 rpm. Each isolate's cell lysate was transferred to a fresh Eppendorf tube, which was subsequently kept at -20 °C after total protein content was quantified.

- **Protein quantification**

To determine protein concentration, absorbance values was obtained at 280 nm. The absorbance values were divided by path length Equals concentration. BSA was used to produce the standard curve and was used to measure protein concentrations using Bradford reagent. Typically, 10 ug of protein was combined with 5X SDS loading buffer and -mercaptoethanol, then boiled for 5 minutes before loading on SDS-PAGE gel.

2.5.1. Denaturing SDS-PAGE gel electrophoresis

SDS-PAGE is a process in which proteins are denatured and linearized before being passed through a thin gel and separated by size. The gel was made according to the method of Laemmli (1970). The resolving and stacking gels were made as given in Table 2,1. The resolving gel was initially poured between the glass plates, leaving ¼ of the area for the stacking gel. Isopropanol (50%) was used to prevent air contact for rapid condensation. After discarding the isopropanol and rinsing twice with water, the stacking gel was poured and wait for a while till polymerization. The comb was

removed once the gel had polymerized, and the gel was transferred to the electrophoresis tank, which was filled with 1X SDS running buffer. Loading dye was mixed in a 1:5 ratio with samples, then boiled for 5 minutes at 100 °C before being loaded separately in wells with a protein ladder. Table 2,2 shows preparation of loading dye and running buffer.

Electrophoresis was run at 50mA till the proteins were separated. The glass plates were then removed, and the gel was dyed for 1 hour using Commassie Brilliant blue stain solution, Coomassie R250 staining solution (0,1 % Coomassie Blue R250 (w/w), 30 % methanol, 5 % acetic acid) To prepare a staining solution, 1 g of Coomassie was dissolve to methanol (300) ml.

Then 650 ml and 50 ml of MQ water and acetic acid had been added, respectively. Stir the solution on a magnetic stirrer for 2 h. The solution then filtered via a Whatman No. 1 paper in order to remove insoluble dye. The solution. Destain solution 1 (30 % methanol, 5 % acetic acid) Add 300 ml of methanol and 50 ml of acetic acid to 650 ml of MQ water. Both of The Coomassie stain and destain solution can be stored at 20 ºC for months. The stain solution was then washed away, and the gel was left to dry overnight. 18 isolates was picked to analyse them with SDS-PAGE in different pH conditions, In the presence of 100 mM KH2PO4, nutrient broth was prepared at three different pH levels (5.5, 6.5, and 7.5). Each sample was loaded on SDS-PAGE with 100 ul of cell free broth that had been boiled in the presence of loading dye and reducing agents.

Table 2.1. Preparation of resolving and stacking gels

Material	15% Resolving gel (ml)	5% Stacking gel (ml)
dH_2O	2.7	6.95
30% ($^w/_v$) Acrylamide	3.8	1.7
3 M Tris-HCl (pH 8.3)	0.95	-
0.5 M Tris-HCl (pH 6.8)	-	1.25
10% ($^w/_v$) TEMED	0.02	0.02
10% ($^w/_v$) SDS	0.1	0.1

Table 2.2. Preparation of loading dye and running buffer

5X SDS loading dye		1X SDS running buffer	
Tris-HCl pH 6.8	0.225 M	Tris	3 g
Glycerol ($^v/_v$)	50%	SDS	1 g
SDS ($^w/_v$)	5%	Glycine	14.4 g
Bromophenol blue ($^w/_v$)	0.05%	dH_2O to 1 litre	
2-Mercaptoethanol	0.25 M		

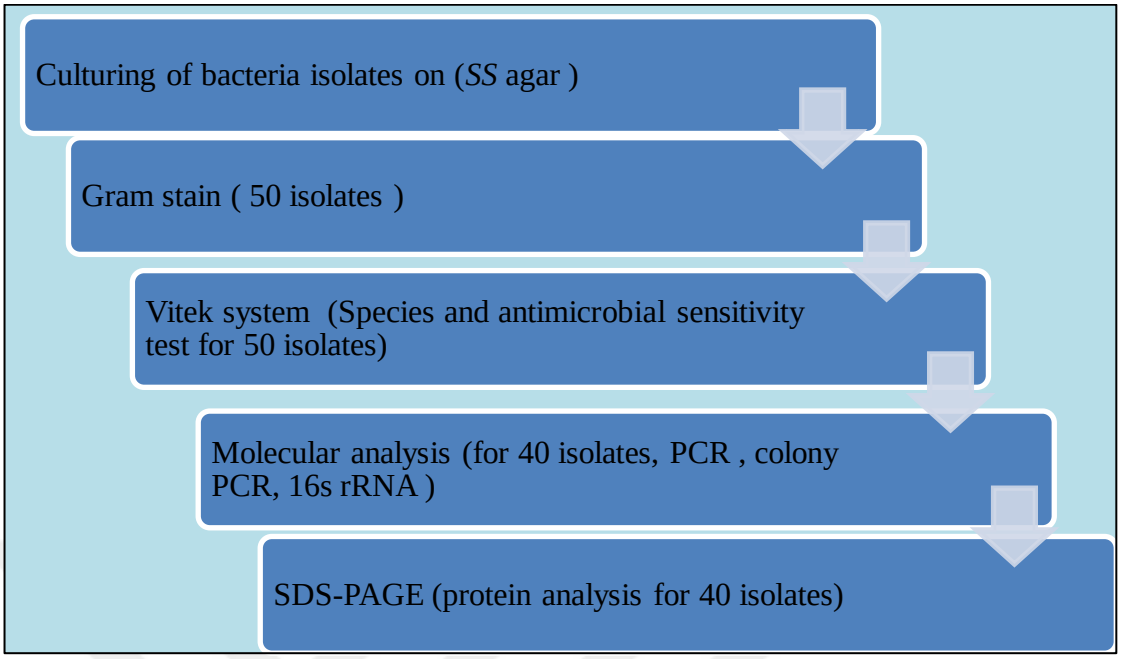


Figure 2.1. Summary of work follow

CHAPTER 3

RESULTS

3.1. Identification and Isolation of *Salmonella typhi*

Salmonella typhi isolates were evaluated in this investigation from February to December 2021. A total of 100 isolates were chosen at random from various geographic areas throughout Iraq. The morphological diagnosis of bacterial isolates using agar and Gram stain revealed that *Salmonella* spp. were found in 50 out of 100 isolates. After an incubation time, 50 of the 100 isolates on *SS* agar were positive (provided black centre with colourless colony) and 50 of them had distinct morphological forms. The gram stain revealed that 50 *Salmonella* isolates are rod-shaped and have a reddish-pink appearance, indicating that they are gram-negative. The Vitek system's bacterial identification at species level identified 40 (80 %) of the 50 isolates as *Salmonella typhi*, while the remaining 10 (20 %) were identified as *Salmonella* spp. (Figure3.1).

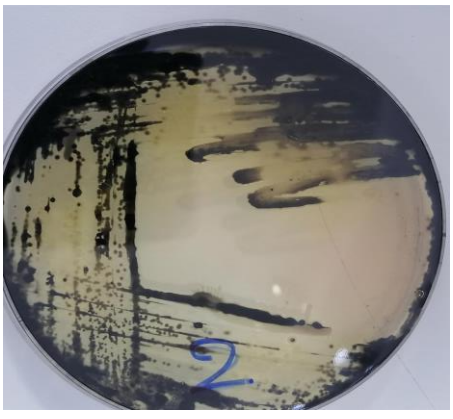


Figure 3.1. *S. thypi* on *SS* Agar

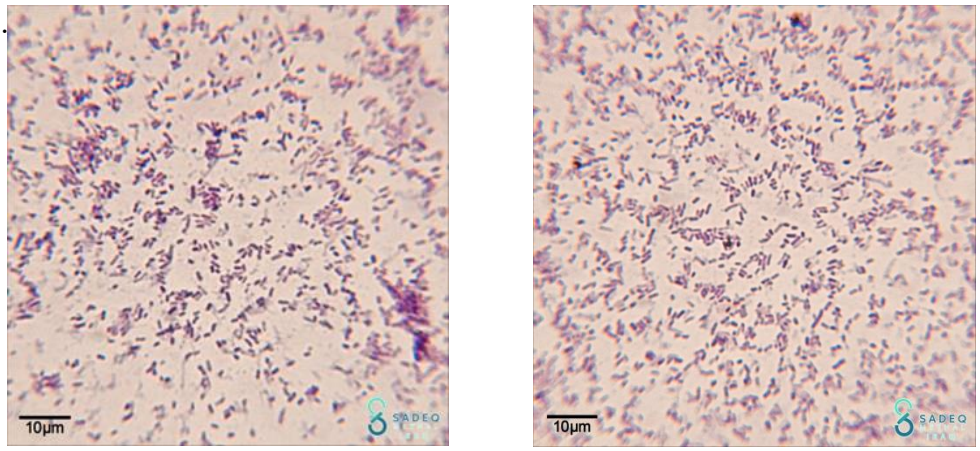


Figure 3.2. The findings of the gram stain identification as well as the morphological identification of the bacterial isolates.

3.1.1. Determination of antibiotics susceptibility of isolates

Vitek 2 compact system (bioMérieux, France) was used to test the antibiotic susceptibility of isolates using the GN cassette depending on the manufacturer's instructions. Table-3.3 illustrates the antibiotic susceptibility pattern of *S. typhi* isolates. Among the antibiotics studied, *S. typhi* strains showed resistance to 11 drugs from different antibiotics family. *S. typhi* strains were shown to be MDR, according to the data, Ticarcillin, Piperacillin, Ceftazidime, Cefepime, Aztreonam, Amikacin, Gentamicin and Tobramycin resistance were all found in all *S. typhi* strains. Other antibiotics, Ticarcillin/Clavulanic, Ciprofloxacin and Minocycline were found to have significant sensitivity rates at 30 %, 30 % and 70 %, respectivly. *S. typhi* isolates showed 100 % sensitivty against the remaining 4 antibiotics, Imipenem, Meropenem Piperacillin/Tazobactam and Trimethoprim/Sulfamethoxazole. Ticarcillin, Ticarcillin/Clavulanic Acid and Piperacillin had the highest levels of resistance with ≥128 MIC among the antibiotics tested.

Table 3.1. Antimicrobial Susceptibility Results of isolates

Antibiotics	Family	Resistance	Sensitivity
TIC	Penicillin	100 % (40)	
Pi		100 % (40)	
TIM		70 % (28)	30 %(12)
Tzp			100 % (40)
Caz	Cephalosporins	100 % (40)	
Cpe		100 % (40)	
Azt	Monobactam	100 % (40)	
Imp	Carbapenems		100 % (40)
Mer			100 % (40)
Ak	Aminoglycosides	100 % (40)	
TO		100 % (40)	
GM		100 % (40)	
SXT	Sulfonamides		100 % (40)
CIP	Fluoroquinolone	70 % (28)	30 % (12)
MIN	Tetracycline	30 % (12)	70 % (28)

3.2. Molecular studies

The first PCR method for *Salmonella typhi* genes revealed that 70 % of isolates carried the bla_{ctxm} gene, with 30 % of *S. typhi* carrying the *qnrB* gene and all isolates carrying the *aadA* gene. On a 1.5% agarose gel electrophoresis stained with ethedium bromide and a 100bp ladder marker, the amplification of the *S. typhi* gene was fractionated. This detection with PCR revealed that high level of Aminoglycosides (aadA) and β-lactams (bla_{ctx}) resistance genes with respective 100 % and 70% were observed with moderate level of fluroquinolone resistance gene (30 %).

When we compered our AST test with PCR analysis it was conflicting, therfore for the other 5 (bla_{TEM}, *intI1*, *qnrA*, bla_{SHV}, *dfr*) gene that was not detected with first PCR method, colony PCR was used as confirmative anaylsis for such genes.

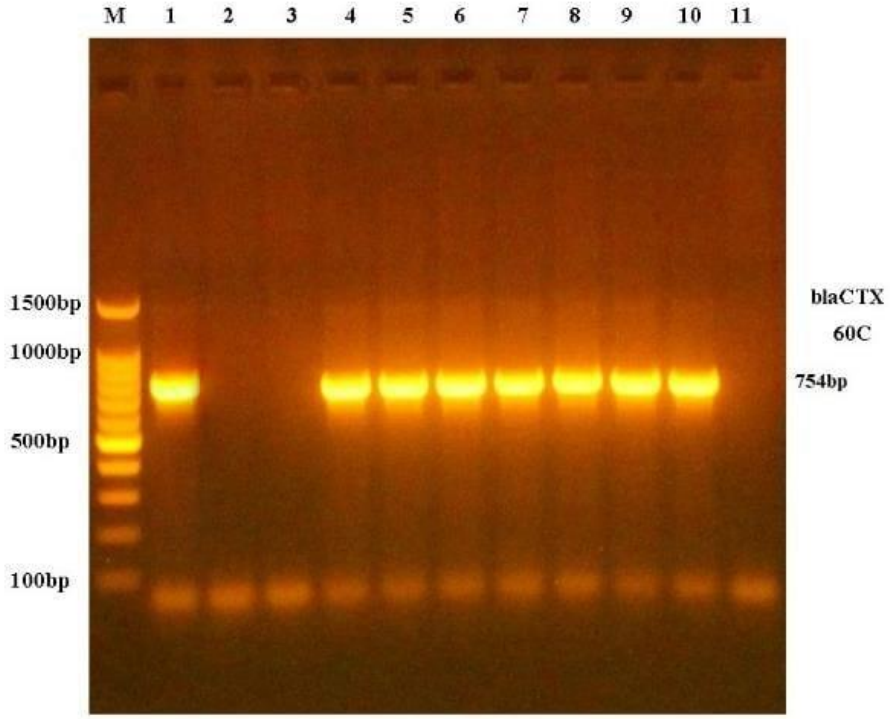
The positive results of cell blasting confirmed the presence of resistance genes in the isolates. But in order to confirm whether the genes carried on the plasmid or on chromosome, we used genes of such isolates.

The positive results of the blasting and the genetic extract give several possibilities, namely that the gene is carried on the plasmid or the chromosome or is carried on both. But the positive results for blasting extracted and negative extracted confirmed that the gene carried on the plasmid only. The results of colony PCR showed that 9 out of 40 (22.5%) isolates were positive for bla_{TEM} gene and *qnrA*. 12 (30%) isolates carried dfr genes as well as 15 (37.5%) isolates detected with *int1* gene. The results revealed different locations for the genes, 3 bla_{TEM} was located on plasmid and 2 of them carried on chromosomes. qnr gene showed different possibilities. 4 isolates show positive results for blasting and DNA extraction method that mean the gene is carried on chromosome and may be the gene is carried on plasmid also because in blasting method both of the plasmid and chromosome will be produced from the bacterial cell, all of the positive results of *dfr* and *int1* genes appeared that they are carried on the chromosome only. Table 3.2 shows the findings of the molecular analysis.

Table 3.2. The results of the molecular and antibiotics resistance findings

Genes	**Results of PCR analysis(%)**
(*aadA*) Aminoglycosides resistance	40 (100%)
(bla_{ctx}) β-lactams resistance	28 (70%)
(bla_{shv}) β-lactams resistance	None
(bla_{tem}) β-lactams resistance	9 (22.5)
(*qnrA*) fluroquinolone resistance gene	9 (22.5%)
(*qnrB*) fluroquinolone resistance gene	12 (30 %)
(*İntI1*) multiple antibiotic resistances	15 (37.5%)
(*dfr*) trimethoprim resistance	12 (30%)

bla$_{CTX}$



aadA

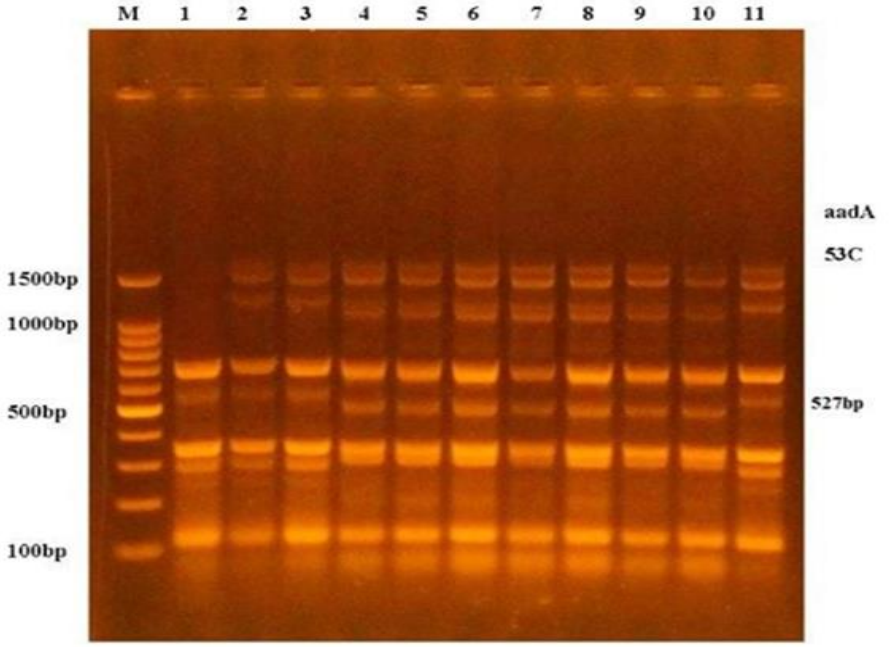
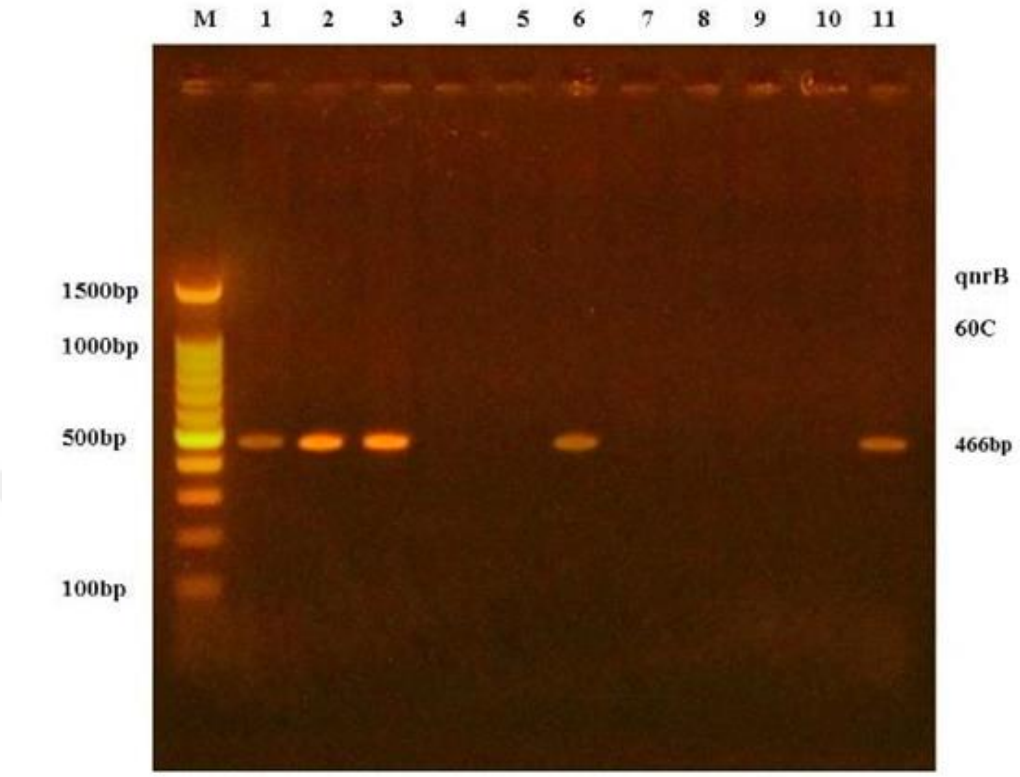


Figure: 3.3. Gene (PCR) Results .Continue

qnrB



bla$_{TEM}$

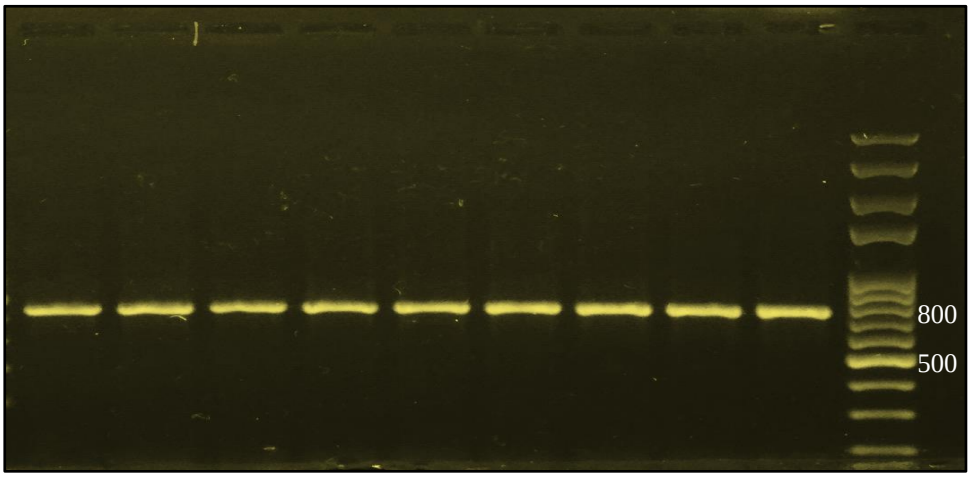
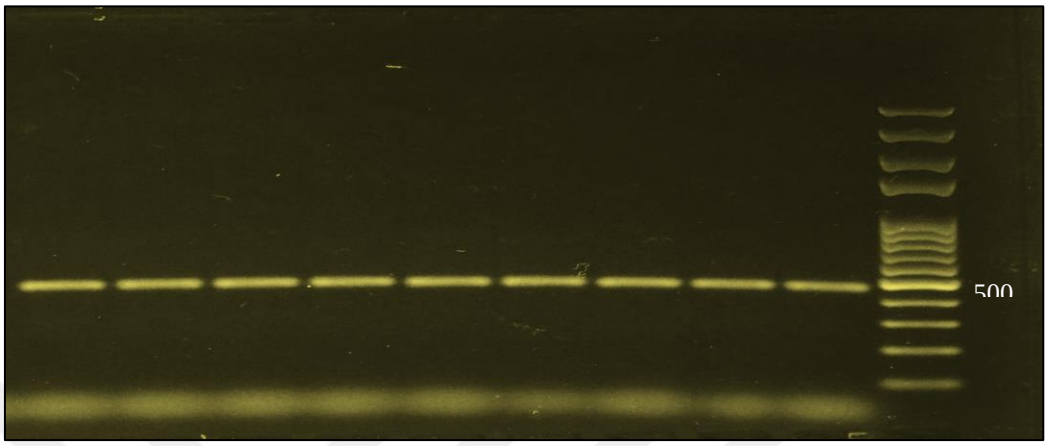
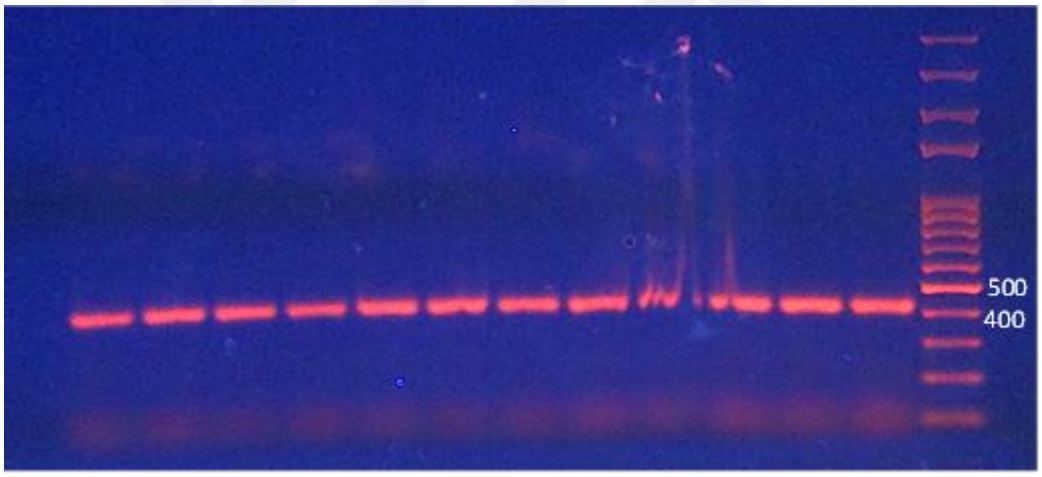


Figure: 3.3. Gene (PCR) Results. Continue

qnrA



dfr



intI1

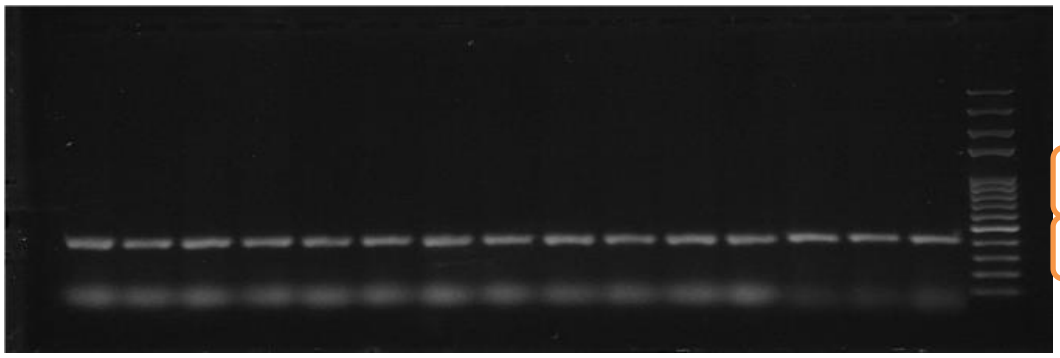


Figure 3.3. The amplification of *bla*$_{CTX}$ gene of Salmonella samples were fractionated on 1.5% agarose gel electrophoresis stained with Eth. Br. M: 100bp ladder marker. 754bp PCR products, *aadA* gene527bp PCR products, *qnrB* gene 466bp PCR products, *bla*$_{TEM}$ gene 800bp PCR products, *qnrA* gene500bp PCR products, *dfr* gene 400bp PCR products, *intI1* gene 400bp PCR products.

Table 3.3. Comparison between molecular and antibiotics resistance findings. Note in our antibiotics resistance study we didn't use trimethoprim alone against to the bacterial isolates as well as IntI 1 gene is multiple antibiotics resistance gene because of that such genes we did not include them to the comparisons table

Molecular results	**Antibiotics results**
Aminoglycosides resistance gene100%	Aminoglycosides 100 %
β-lactams resistance gene 92%	Penicillin TIC, Pi 100% Pencillin TIM 70% Cephalosporins 100% Monobactam 100%
Fluoroquinolone 52.5 %	Fluoroquinolone 70%

2.4.16s rRNA results

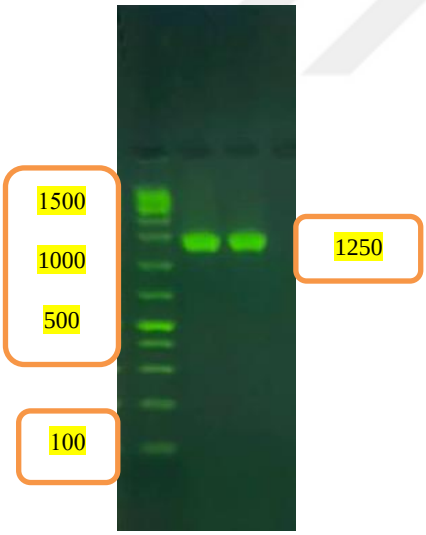


Figure 3.4. PCR product of 16s rRNA the band size 1250 bp. The product was electrophoresis on 1.5% agarose at 5 volt/cm2. 1x TBE buffer for 1:30 hours. N: DNA ladder (1000 plus).

By selected the isolate randomly as a confirmation step the result shown that the isolate is 100% is *salmonella typhi* (GenBank: MZ456232.1)

3.4. Proteomic analysis

3.4.1. Colony SDS-PAGE screening

In order to further diagnose *Salmonella* isolates, colony SDS-PAGE experiment was carried out by culturing first each isolates on *SS*-agar separately, followed by picking up a single colony from each plate that belongs to a particular isolate. Each colony was transferred first into Eppendorf tube, washed three times with 1X phosphate buffer (PBS), and suspended then with PBS (20ul), SDS-loading dye (3ul), and Betameracptoethanol (2ul.) Each tube was then boiled at 95°C for 5 min and spined for 15000 rpm for 5 min. The supernatant which represents the total soluble protein contents of each isolates was run on SDS-PAGE. Results of protein migration on SDS-PAGE shows a similar pattern of protein bands of all isolates (Figure 3.5). This suggests that all isolates are indeed belong to the same species of bacteria due to have similar contents of proteins.

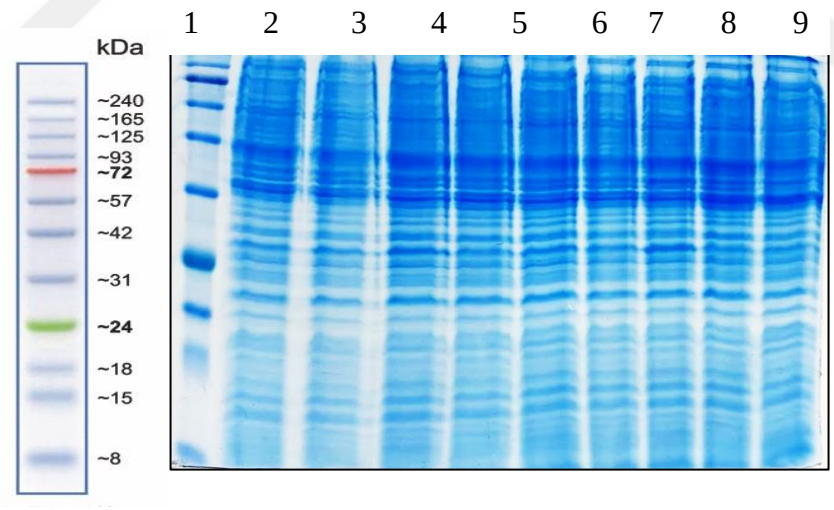


Figure 3.5 Colony SDS-PAGE analysis of total soluble fractions contents of *Salmonella* isolates. Lane 1, protein ladder size in kDa is as indicated in lift side inserted image; lane 2-9 represents isolates from (13-21), each line represents total soluble protein contents of a particular isolates.

3.4.2. Analysis of protein secretion

Based on several studies conducted on *Salmonella* secreted proteins via SDS-PAGE analysis and amino acid sequencing, Flagellin (FliC) (>50 kDa) is the main protein found in the cell free media that were analyzed on SDS-PAGE [67].

To investigate the ability of the isolates used in this study to show similar phenomena, all isolates were cultured on BHI broth for 9 hours on 37°C with agitation of 250 rpm, and the cell-free secreted proteins were obtained by centrifuging the culture media first and then filtering the media through 0.2 μm membrane filter. A 100 ul of each cell free media that used to culture each isolate were analyzed on SDS-PAGE. All cultured isolates showed clearly the presence of FliC on the media at the expected size suggesting that all isolates showed the same ability (Figure 3.6), which add another level of bacterial diagnosis based on recreated proteins. To get more insight regarding the secreted proteins, the cell-free secreted proteins of cultured media were cultured with less agitation at 100 rpm, and for 6h to avoid protein oxidation and degradation, protein concentration using acetone precipitation methods described by thermofisher (method) were also applied. At least six faint bands corresponding to effectors of type III secretion system (T3SS), SipA, SipB, SipC/FlgE, SipD, in addition to flagellin (FliC and FliD) can be recognized (Figure 3.7), which is consistence with several other studies. *Salmonella* is known to infect phagocytic cells and non-phagocytes, in the case of phagocytes it must face the harsh environments such as the hypoxia, high level of nitric oxides, and acidic pH. Thus, in the current study it was assumed that lowering the pH of growth media could effects on ability of effectors secretion, it may enforce the bacteria to enter in dormant state so all activities included secretion will inhabited, or it may secrete more virulence factors to interact with host immune cells during active infections so that more proteins could be secreted. To test this hypothesis, one isolate of *Salmonella* that shows previously the ability to produce T3SS effectors, have been cultured on nutrient broth instead of BHI broth at three different pH: 5.5, 6.5, and 7.5. Nutrient broth is not a rich media like BHI media; however, it is not a minimal media. Thus it may reduce the nutritional overabundance provided in the case of BHI that may limit the effect of pH changes.

Investigating the secretion was done by exploring the cell free secreted proteins in the media after 4, and 6 hours of growth for each pH condition. Although there was no change in the number of detect bands on SDS-PAGE; however, there was a dramatic change in the level of secreted proteins on pH 5.5 in comparing with 7.5. Low concentration of overall secreted proteins and missing of some bands (SipB) in acidic environment can be clearly seen (Figure 3.8), suggesting that assembly and secretion of

effectors may decline due to harsh environments that force the bacteria to slow down their activity.

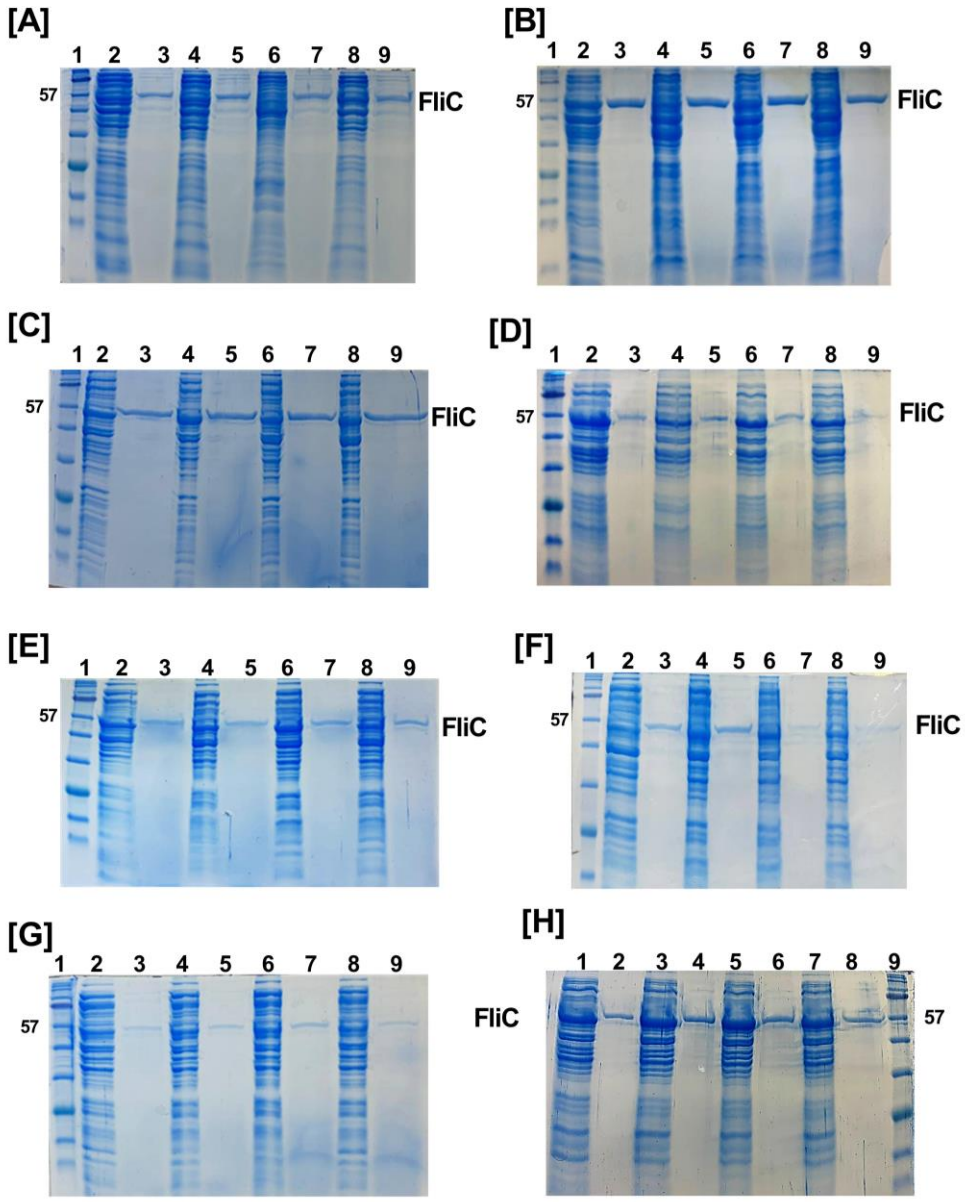


Figure 3.6. SDS-PAGE analysis of Salmonella cell lysate and cell free secreted proteins of 32 different isolates out of 40. In panela A-G; 1st lane is protein marker, size is indicated in kDa, the full details of Mwt are as indicated in Figure 1. Panel A: Lane 2 and 3 represent the cell lysate and cell free secreted proteins of isolate 1, lane 4 and 5, lane 6 and 7, lane 8 and 9 are for isolates #2, #3, #4. Panel B; for isolates #5, #6, #7, and #8 and so for all other panels except for Panel [H], were the marker ladders is lane 9 instead of 1. Flagellin (FliC) protein is indicated. Pic (A) represents the isolates from(1-4), Pic (B) represents the isolates from(5-8), Pic (C) represents the isolates from(9-12), Pic (D) represents the isolates from(13-16), Pic (E) represents the isolates from(17-20), Pic (F) represents the isolates from(21-24), Pic (G) represents the isolates from(25-28), Pic (H) represents the isolates from(29-32)

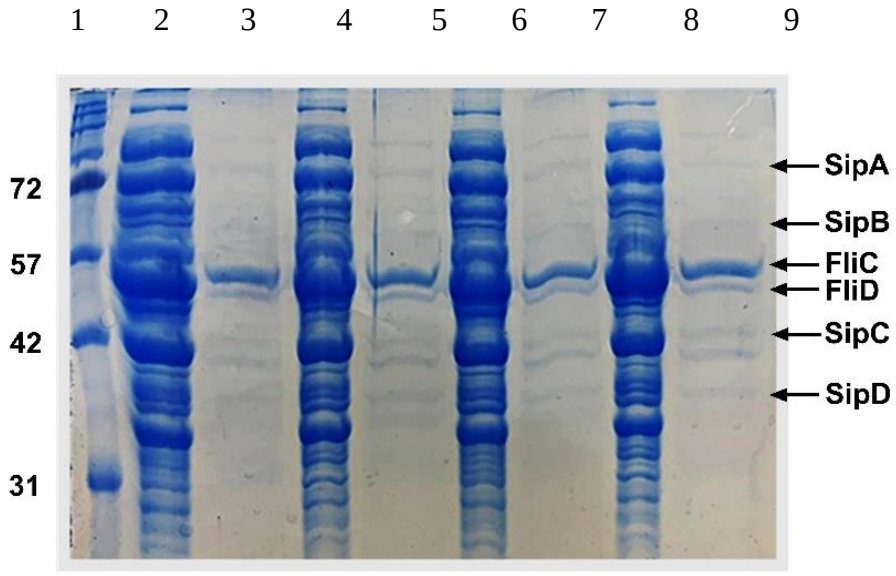


Figure 3.7. SDS-PAGE analysis of *Salmonella* cell lysate and cell free secreted proteins precipitated by cold-acetone. Lane 1, protein ladder size in kDa is indicated; lanes 2 and 3, lanes 4 and 5, lanes 6 and 7, lanes 8 and 9; cell lysate and cell free secreted proteins respectively of 4 different isolates of Salmonella enterica serotype typhi. Pic represents the isolates from (33-36)

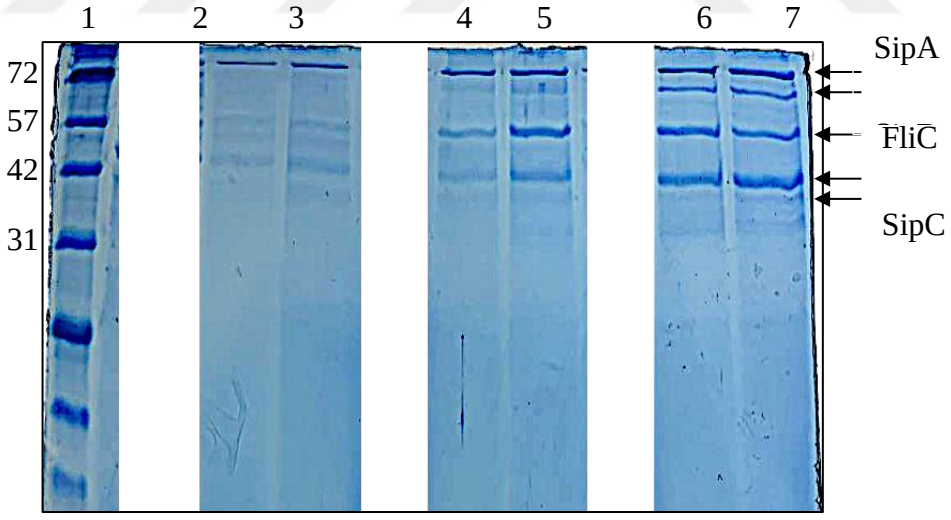


Figure 3.8. SDS-PAGE analysis of *Salmonella* cell free effector proteins secreted in the Nutrient media. Lane 1, protein marker (size in kDa is indicated); lane 2 and 3, secreted proteins after 4 and 6 h of growth in nutrient broth (pH 5.5); Lane 4 and 5, secreted proteins after 4 and 6 h of growth in nutrient broth (pH 6.5); lane 6 and 7, secreted proteins after 4 and 6 h of growth in nutrient broth (pH 7.5).
Pic represents the isolates from(37-40)

CHAPTER 4
DISCUSSION

A large number of studies have recently been published to approve various tools for the detection of *Salmonella* spp. As the globe battles antibiotic resistance, achieving this goal will necessitate an accurate, quick, and early diagnosis. Typhoid fever and other bacterial illnesses are among Iraq's most common infections. However, there were just a few papers published in Iraq for determining antimicrobial susceptibility testing. As a result, this project was started with the goal of detecting *S. typhi* clinical strains and determining their antibiotic susceptibility patterns. Because of the generation of H_2S on *SS* agar, black colour colonies were thought to be *S. typhi* colonies [64]. As a result, identification based on culture media is inaccurate, as previously reported [65]. Based on biochemical testing, 40 out of 100 isolates were identified as *S. typhi* within the total sample size(Table 3.1). Because of its accuracy, identification based on biochemical tests using Vitek 2 compact was highly recommended for Gram-negative bacteria [66,67]. In addition, one isolate was taken randomly and identified as *S. typhi* using 16s rRNA. As shown in the results chapter. The results of the Vitek 2 compact identification were quite similar to the conventional method as all isolates was *S. typhi* except 10 out of 50 isolates detected as *Salmonella* spp. only. The current study discovered MDR *S. typhi* from patients in Baghdad, Iraq (Table 3.1).. These findings are congruent with previous data from Iraq [64], and Kenya, where 68 % of *S. typhi* infections were MDR [68]. But our findings conflict with a study from India, which found that only a tiny percentage of *S. typhi* had MDR [73]. In Nepal, however, no MDR has been identified [68]. The high incidence of MDR could be due to the widespread use of antibiotics without visiting a doctor, which is prevalent in developing countries [64], especially in Iraq. 100% of *S. typhi* strains were resistant to Ticarcillin and Piperacillin, which had the highest frequency of resistance with MIC more than 128 among the resistant drugs (Table 3.1)., Such antibiotics are beta-lactams with a broad spectrum of action. *Salmonella* spp. isolated from poultry showed similar results [70, 71].

Salmonella spp. have been found to be resistant to Ticarcillin in several studies [64]. The current finding contradicts a case report by Perera et al. [72], who found that *S. typhi* is sensitive to Piperacillin. Piperacillin is frequently combined with tazobactam, which enhances piperacillin efficiency by blocking beta-lactamase enzymes in several species. Piperacillin/tazobactam sensitivity was found in all (40 n) of *S. typhi* strains in the present study (Table 3.1). Perera et al. [72] had comparable findings. Piperacillin/tazobactam, according to Lowman, is still an effective antibiotic towards Gram-negative bacteria [73]. To treat Gram-negative bacteria, ticarcillin is frequently combined with clavulanic acid. Ticarcillin/clavulanic acid resistance was found in 30 % (12 n) of *S. typhi* strains. This goes hand with hand with previous findings [64] that showed Ticarcillin/clavulanic acid efficiency against a variety of Gram-negative bacteria. 100 % (40n) of *S. Typhi* strains had moderate resistance with MIC about 64 to ceftazidime, cefepime and aztreonam among the isolated strains (Table 3.1). This contradicts with the results reported by Yang et al [74], which found that *Salmonella* spp. are susceptible to ceftazidime and cefepime. The current data is comparable to a previous study that found *S. enterica* serotype Typhimurium to be resistant to aztreonam [75]. Recent research has found that combining aztreonam and ceftazidime-avibactam improves the medication's effectiveness towards Gram-negative bacteria [76]. A family of antibiotics known as aminoglycosides includes tobramycin, amikacin and gentamicin. In present study 100 % of *S. typhi* strains indicated resistance to all aminoglycoside antibiotics (Table 2). Aminoglycosides have poor action against *S. enterica* serovar Typhimurium according to Lo et al. [77]. Moreover, amikacin resistance was discovered in 100% of *S. typhi* strains. *S. typhi* susceptibility to amikacin has been demonstrated in studies in India and Ethiopia, which contradicts with our findings. *S. typhi* strains were shown to be 100% resistant to gentamicin, which contradicts with previous results that revealed *S. typhi* was susceptibility to gentamicin [78]. Furthermore, recent research has found that Gram-negative bacteria are susceptible to gentamicin [76]. Tobramycin resistance was found in 100 % of *S. typhi* strains in this study .This is in stark contrast to a survey performed in Baghdad, Iraq, that found tobramycin sensitivity in 80 % of *S. typhi* strains [79]. This is strong evidence that *S. typhi* is becoming more resistant to tobramycin. Furthermore, ciprofloxacin sensitivity has been observed in 30% of *S. typhi* strains.

S. typhi resistance to ciprofloxacin has been documented in previous research [80]. Another study in Ethiopia, however, discovered that *S. typhi* is susceptible to ciprofloxacin [78], contradicting the current findings. Ciprofloxacin is a replacement antibiotic for treating MDR-*Salmonella* spp., according to a review. Reduced ciprofloxacin sensitivity puts typhoid fever treatment at risk, especially in underdeveloped countries. Iraq had the largest ciprofloxacin sensitivity reduction among Middle Eastern countries [81], which is consistent with the findings of this investigation. Furthermore, the current result is similar to the study carried out in Baghdad which found *S. typhi* was to be resistant to ciprofloxacin [79]. The study, on the other hand, found that 100% of *S. typhi* is susceptible to Imipenem, Meropenem, Piperacillin/Tazobactam and Trimethoprim/Sulfamethoxazole. In contrast, *S. typhi* was shown to be resistant to imipenem, meropenem, and trimethoprim/sulfamethoxazole in an Indonesian investigation [82], whereas it was found to be sensitive to trimethoprim/sulfamethoxazole in a Nepalese study [68]. In Pakistan, however, *S. typhi* has a high resistance rate to trimethoprim/sulfamethoxazole [83]. For nearly 30 years, minocycline has been a successful anti-MDR antibacterial [64]. In contrast to our findings, a South Korean researchers found that 30 strains of *Salmonella* spp. were resistant to minocycline [84]. *S. typhi* isolates had the most resistance to Ticarcillin, Piperacillin, Ceftazidime, Cefepime, Aztreonam, Amikacin, Gentamicin and Tobramycin, according to the data. As a result, we strongly advise against using such medicines to treat typhoid. Besides, meropenem and imipenem were shown to have the strongest effect. Molecular studies on *S. typhi* strains revealed that $bla_{CTX\text{-}M}$28 (70%) was the most frequently found gene, followed by bla_{TEM} 9 (22.5%), and none of the isolates carried bla_{SHV} gene. Other findings in Iraq [85], Pakistan [86], and Bangladesh [87] are close to our findings. Infections caused by extended spectrum beta-lactamase (ESBL) -producing organisms are becoming more common in various parts of the world, 3rd generation of Cephalosporin resistance is provided to these enzymes.

Several monitoring studies in Iraq revealed a significant prevalence of Beta -lactamases, particularly ESBL-producing bacteria [88, 89, 90, 91]. Exposure of these microbes to various types of β-lactam medicines result in increased production and mutation in -lactamases enzymes. By hydrolysing these medications, such as cephalosporins and penicillins, these microbes will be able to resist them [92].

Several Gram-negative bacteria can resist quinolones by changing drug targets, decreasing drugs, accumulating drugs, shielding drug sites, and modifying enzymatic drug targets [91]. There are other chromosomal mutations to consider. This is mostly due to genes that code for topoisomerase IV and DNA gyrase, as well as genes that code for outer membrane and efflux proteins. Quinolone resistance, qnr proteins that protect topoisomerase-IV and DNA gyrase from quinolones [93]. Touati et al in 2008 discovered determinants of qnr in North and South America, Europe and Asia on a large scale [94]. According to the current findings, 70 % of the isolates tested were positive for ciprofloxacin resistance. So, the study was carried out to clarify the existence of qnr genes in *S. typhi* and discovered 12 positive findings for *qnrB* and 9 positive results for *qnrA*. The dominance of the *qnrB* genes in this investigation is similar to that seen in Thi-Qar province/Iraq [93] and Poirel et al in EU (2006) [95]. Similarly, quinolone resistance genes in Gram-negative bacteria were shown to be abundant in clinical isolates in a research by Mohammad in 2017 [93]. Furthermore, in 2013, Le Hello et al indicated that quinolone resistance in *Salmonella* was caused via plasmid-mediated genes that code for DNA topoisomerase protective proteins [96]. Our findings in analysing the molecular mechanisms of ciprofloxacin (CIP) resistance was that *qnrB* is the most common indicator for CIP resistance, as it was found in 12 (30%) of all CIP-resistant isolates. The second most common indicator is *qnrA*, which was found in 9 (22.5%) of all CIP-resistant isolates. In all, the *qnr* gene was carried by 52.5 % of the isolates, our findings go hand with hand with study held in China [97]. The trimethoprim gene was amplified in 12 (30%) of the *S. typhi* isolates. *dfr* genes are the most common source of trimethoprim resistance. Trimethoprim resistance in Gram-negative and Gram-positive bacteria is related to its interference with folate production. After binding to dihydrofolate reductase (DHFR), which catalyses the production of tetrahydrofolate from dihydrofolate, it has bacteriostatic properties [98]. DHFRs from eukaryotic cells can bind trimethoprim as well, although the drug has a greater affinity for bacterial enzymes. Our finding of the trimethoprim gene distance from another study in Iran [99] and Bangkok [100],

The difference between our results and such other studies may be due to the differences in geographical location and dietary habits. In present study, 15 (37.5%) of the 40 *Salmonella* isolates were confirmed to have Class 1 integron by PCR analysis.

At the same time our results were similar to the results of Gupta et al [101]. There was no significant difference in antibiotic resistance between Class 1 integron bearing isolates and Class 1 integron negative isolates in our investigation. Molecular features of 18 MDR *S. typhi* isolates in an Indian study held between 2009 and 2013 showed all isolates carried class1 integron gene [102], this study is distance from our finding that showed only 15 out of 40 isolates carried intl1 gene this differences may be is related to the isolates number in this study is less than our isolates number [102]. All of the *Salmonella* isolates exposed to a PCR test to assess *aadA* gene and confirmed that they have this gene. Our findings differ from an Indian research conducted between 2009 and 2013, in which just one isolate out of 18 was positive for the *aadA* gene [106].When we compared the molecular results with the antibiotic resistance results, as shown in the (Table 3.3), most of the results were similar to each other except for some difference, as 30% of the isolates carried the *dfr* gene. Seven of the isolates that do not carry the fluroquinolone resistance gene but at the same time are carriers of the *intI1* gene, which confers multiple resistance to antibiotics and this explains the conflicting results between fluroquinolone resistance gene and fluroquinolone antibiotic. Our results revealed that the percentage of gene results is lower than the antibiotics resistance percentage to such antibiotics (fluroquinolone). Our findings for the aminoglycoside resistance gene and resistance activity were compatible with each other.

It was seen that according to the results of SDS-PAGE, although not definite, the *Salmonella* strains could not be distinguished based on whole cell protein profiles [103]. Our findings indicate that the serotyped Salmonella strains seen in reference labs are closely linked. However, alternative molecular methods like plasmid analysis, PFGE, or RFLP should be used to distinguish and characterize *Salmonella* serovars with reliability. Invasion of *Salmonella* to the host cells requires attachment firstly and thereafter releasing the virulence invasion factors [104,105]. *Salmonella* infections trigger innate immune system through several pathways. Innate immune response is the first defense line, the phagocytic cells of both macrophages and dendritic cells are primarily involved [106]. Recognizing the pathogen associated patterns is however detected via host Toll-like receptors (TLR), which are conserved receptors spread on the plasma membrane and endosomal membranes of immune and non-immune cells [106; 107]. Antimicrobial effector genes are triggered through the activated receptors which

leads to stimulates macrophage to face engulfed pathogens via acidifying phagosomes, generation of reactive oxygen species (ROS), reactive nitrogen species (RNS), and stimulates antimicrobial agents that eliminate pathogens [108, 109]. Despite the immune response that *Salmonella* can face, however it has several mechanisms to break host defenses. One of the most important strategy is the presence of several secretion systems that are essential to delivery invasion factors which helps the pathogen eventually to colonize, survive and reproduce within hosts [110]. Four secretion systems that have fundamental roles in the pathogenicity are known including the type I secretion system (T1SS), the type III secretion system (T3SS), the type IV secretion system (T4SS) and the type VI secretion system (T6SS) [111, 112, 113, 114]. For pathogenicity of most bacteria, multiple virulence genes should be expressed simultaneously at the correct time in a correct location. In *S. typhimurium* it is estimated that 4% of genome, that encodes over 200 virulence genes, is essential to assure fatal infections of mice's [115]. These genes are found on chromosome or on plasmids, they are present either as a single gene, islets, or as pathogenicity islands which are large cassettes consisting of series of genes and operons. Pathogenicity islands are typically responsible for a particular virulence phenotype during the course of infection, thus harboring a single pathogenicity island can convert non-pathogenic enteric flora to a pathogen with a certain pathogenicity.

Five pathogenicity islands (SPIs) have been identified so far in *Salmonella* chromosome required for bacterial pathogenicity and host-microbial interactions.

Salmonella pathogenicity island 1 (SPI1) is responsible for invasion due to its role in bacterial penetration of intestine epithelial cells, however, SPI2, 3, and 4, are essential for growth and survive within the host which reflects its role during the bacterial systemic phase of disease.

Horizontal gene transfer via phage infection or via acquiring plasmids are the main methods for SPIs harboring. SPIs are highly conserved across different serotypes of *Salmonella* [116]. T3SS encodes genes of both SPI1 and SPI2, thus it allows *Salmonella* to translocate effectors into the host cell cytosol [117]. SPI1 was found to encode 29 genes of regulators and secreted effectors of T3SS [118].

Effectors translocation via SPI1 of T3SS allows *Salmonella* to penetrate non-phagocytic cells, such as intestinal epithelial microfold cells [117, 119]. Among these effectors are SipA (Salmonella invasion protein, which is also known as *Salmonella* secreted protein Ssp), SipB, SipC, and AvrA (a virulence factor A) [116]. Results of the current study confirms that all *S. enterica* serotype typhi isolates studied here have the ability to produce effectors of T3SS-SpI1 and proteins of flagella which are consistent to a molecular weight of SipA, SipB, SipC, and SipD in addition to FliC and FliD. Secretion of T3SS-SpI1 and flagellar proteins of *S. typhimurium* was firstly well-studied in Komoriya's study. Based on comparing between mutant and wild strains using direct and unprejudiced experiments of protein precipitation by trichloroacetic acid followed by SDS-PAGE analysis and protein sequencing technique, the identity of secreted proteins were identified [120]. Analysis of *Salmonella* secretion ability to secrete these effectors via SDS-PAGE analysis to explore the effectiveness of different factors on the secretion activity were investigated by several researchers [121]. However, there are some limitations regarding this technique in exploring the secretion of proteins, the aeration could cause oxidizing of several proteins which could explain the disappearing of several bands after long time of growth; in addition, SDS-PAGE is not the optimum experiments to screen the low concentrations of secreted proteins, unlike the western blotting technique for example. In order to establish a successful infections, enteric bacteria including *Salmonella* must firstly tolerate and survive in the acidic pH of stomach, which then it can pass down and colonize intestine, which is relatively basic. The alteration in the pH of the environment can switch on/off of several genes involved in the virulence secretion systems.

Similarly, the acidification of bacteria-containing vacuoles (BCV) of immune cells that engulphed bacteria can lead to same stress. Therefore, studding the effect of pH factor can provide key answers. Here, screening the *Salmonella* secreted proteins via SDS-PAGE shows a dramatic decrease in the level of secreted proteins under acidic conditions of culture media unlike the natural pH. Indeed, several studies found that the changes in the extracellular pH effects on the component and effectors of T3SS. For example, in *Shigella flexneri*, it was found that invasiveness of bacteria decreased 10 folds when the extracellular pH decreased from 7.4 to 6.0 [103].

Moreover, the alkaline pH leads to increase the expression of T3SS genes in *Pseudomonas syringae* and *Erwinia amylovora* [122, 123, 124].

Results of current study are also consistent with several studies that found that the gene expression of most SPI-1 proteins and effectors are suppressed in the stomach but induced when the pH levels shifted to alkaline conditions in the intestine [125,126]. Results of the current study is consistent with what was found by Sedillo-Torres and co-authors, where it is indicated that *Salmonella enterica* serovars Typhi and Typhimurium were able to secrete T3SS-SPI1 and FliC, and when the culture media of bacteria were treated with the Hibiscus acid extracted from Hibiscus sabdariffa L. It dramatically reduce the secretion of both flagellin and T3SS-SpI1 effectors, inhabit bacterial motility, and reduced the *Salmonella* invasion ability to tissue-cultured eukaryotic cells. The effectors proteins were diagnosed based on molecular weight indicated in SDS-PAGE and then after confirmed by western blot [127]. However, and based on my results of the current study, acidic pH is behind the inhibition; thus, there is a conclusion that the virulence activity inhibition was due to particular effect of Hibiscus acid is inaccurate especially that their experiments did not investigate other acids effects as controls. Unlike SPI-1, SPI-2 were found to be upregulated in the acidic conditions. The acidification of *Salmonella*-containing vacuoles (SCV) was shown to reach a range of pH between 4.0 and 5.0 within 60 min after formation [128]. Several T3SS-SPI2, which their gene expressions were studied using transcriptional fusion encoding with the green fluorescent protein (GFP), were found that their gene expression inside the host cell is downregulated when the SCV acidification was abolished [129].

Although several studies showed that induction of T3SS-SPI2-mediated secretion *in vitro* is occurred due to acidification, however, induction of T3SS-SPI2 gene expression due to the effect of acidic pH has been controversial [130; 131; 132; 133].

If there is a real variation between SPI-1 and SPI-2 in response to extracellular pH level, it could be due to the specificity of each one. The SPI-1 secretion system's main function is to enable *Salmonella* to invade the epithelial cells, while SPI-2 is essential for facilitating, surviving, replicating and disseminating the bacteria from SCVs [134] ,[135].

CONCLUSION

According to the study's findings, using the Vitek 2 compact and PCR to identify *S. typhi* produces accurate results that are crucial for correctly diagnosing typhoid. According to the aforementioned data, we have seen an increase in the number of antibiotic-resistant *S. typhi*, necessitating a greater awareness of the need for antibiotic use. To prevent the spread of multidrug resistance, antibacterial sensitivity tests should be continuously monitored, and an appropriate antibiotic dosage should be kept up. SDS-PAGE whole cell protein profiles could not be used to discriminate between the *Salmonella* strains. Our data suggest a close relationship between the serotyped *Salmonella* strains found in reference labs. However, alternative molecular techniques, such as plasmid analysis, PFGE, or RFLP, should be employed to accurately identify and classify *Salmonella* serovars.

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