Republic of Iraq
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Thesis

Isolation and Identification of salmonella spp from beef and broiler chicken at Kerbala province

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1444 A.H **2022 A.D**

بسم ٱللهِ الرَّحْمٰنِ الرَّحِيمِ

﴿ قَالُواْ سُبْحَٰنَكَ لَا عِلْمَ لَنَا إِلَّا مَا عَلَّمْتَنَا إِنَّكَ أَنتَ ٱلْعَلِيمُ ٱلْحَكِيمُ ﴾

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Declaration

I hereby declare that this thesis is my origin work except for equations and citations which have been fully acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at University of Kerbala or other institutions.

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/ / 2022

Dedication

To

To my leader on whom my difficulties based, Imam Hussein (AlayhiAlsalam)

For those who strive to console me and make me happy.... My father

For whom is that paradise under her feet...My mother

To my wife who supported me and encouraged me in every step and my beloved daughter Qosem

To those whom gave me the strength and support… "My sisters and my brothers"

To the person who paved our way of science and knowledge … "My Supervisor, Asst.prof. Hikmat Sahib Al-Nassir"

Acknowledgments

First and foremost, I should deeply thank to my Lord Allah without whom none of this would have beenpossible.

I would like to thank the Dean of the College of Veterinary Medicine ***Prof. Dr. Wifaq J. Albazi*** and my esteemed professors who preferred of me for their advice.

I would like to express my sincerely grateful and deep appreciation to my supervisor**. Assist. Prof.** ***Hikmat sahib*** **and Assist. Prof Dr.** ***Ali jasim jafer*** for his guidance and advisement through the period of the study

My great appreciation to **Assist. Prof. Dr.** ***hayder al karawy*** College of Veterinary Medicine in University of Kerbala for his help in his statistical analysis.

I also wish to thank my friends ***saif muhannad*** and a ***Amjad Abdul-Hassan*** for supportive throughout study and research period.

Sajad Adnan khudair

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List of abbreviations

Abbreviations	Meaning
*agf*A	Aggregative fimbriae A
AMR	Antimicrobial Resistance
AST	Antimicrobial Susceptibility Testing
BLAST	Basic Local Alignment Search Tool
C	Citrate
CLSI	Clinical and Laboratory Standards Institute
CRISPR	Clustered Regularly Interspaced Palindromic Repeats
ECDC	European Centre for Disease Prevention and Control
EFSA	European Food Safety Authority
ERIC	Enterobacterial repetitive intergenic consensus sequence
F	Frequency
Hila	Hyperinvasive locus A
I	Indole
ICEF	Integrative conjugative elements
*inv*A	Virulence genes invasion A
ISRs	Intergenic Sequence Regions
ITS	Internal Transcribed Spacer
MDR	Multidrug resistance

MLEE	Multi-Locus Enzyme Electrophoresis
MLST	Multi-locus sequence typing
MLVA	Multi-Locus Variable number of tandem repeats Analysis
MR	Methyl red
NCBI	National Center for Biotechnology Information
NGS	Next Generation Sequencing
NTS	Non typhoidal Salmonella
PFGE	Pulsed-field gel electrophoresis
Rrn	Ribosomal RNA
SC	Simmons Citrate
*sir*A	Sporulation inhibitor of replication protein A
SNPs	Single Nucleotide Polymorphisms
SS	Salmonella Shigella
STs	Sequence types
TBE	Tris-borate-EDTA
TSI	Triple Sugar Iron
Ttr	Transthyretin
*via*B	Vi capsular antigen B
VP	Voges Proskauer
WGS	Whole Genome Sequences
XLD	Xylose-Lysine-Desoxycholate

Abstract

This study was conducted at kerbala province during the period from November 2021 to March 2022. A total of 310 samples were collected from local and imported chicken meat and red meat.

These samples were gathered from different locations in Kerbala province and cultured on appropriate media for *Salmonella* spp cultivation and identification .

Then it followed by the initial bacterial isolation process on the special and distinctive culture media for *Salmonella spp.* Results showed that rate of contamination in all examined samples was 54 (17.4 %) from the total samples 310, furthermore the isolation rate of red meat was 23 (14.3%) from the total samples 160, as well as the isolation rate of broilers samples was 31 (20.6%) from the total samples 150.

Moreover, the contamination rate of *Salmonella* among red meat were 4 (20%) meat cut, 9 (45%) minced meat, 1 (5%) sausage, and 1 (5%) burger for the local red meat samples, and the contamination rate of *Salmonella* for the imported samples were 2 (10%) meat cut, 6 (20%) minced meat, 0 sausage, and 0 burger.

On the other hand the contamination rate of *Salmonella* among broilers samples were 9 (36%) skin, 10 (40%) carcass wash, and 4 (16%) liver, for the local broilers samples, and the contamination rate of *Salmonella* for the imported samples were 5 (20%) skin, 1 (4%) carcass wash, 2 (8%) liver.

The statistical model of the contamination rate in the current study was performed to show the differences by applying the Chi-square test, where the significance was measured at P value level ($p<0.05$). The outcome revealed that there were significant differences between the collected samples and the positive isolation from these samples.

The antibiotic resistance among Salmonella isolates from both red meat and broilers was evaluated through the current study, and results revealed that resistance was reported in 50 (92%) of *Salmonella* Spp isolates to tetracycline and 52 (96%) Ampicillin .

Study was carried out to identify 10 isolation of *Salmonella* spp previously detected from red meat and broilers by using conventional PCR and Real Time PCR as well as 5 isolation were used for sequencing and specific genetics virulence gene type of salmonella spp . Current results revealed that 10 isolation (100%) were identified on salmonella Spp by conventional PCR . Current finding also found out 5 isolation which were subjected to the detection of *inv*A by using RT -PCR were reported salmonella *enterica* concerning sequencing and sequence analysis of *Salmonella* 2 red meat isolation (out of 2 examined) were reported as S. Agona , which 3 broilers isolates (out of 3 examined) were reported S.Infantis .it could be concluded from this study that minced meat was more contamin than the other product.salmonella enterica show resistance to the tetracycline,ampicillin.

Chapter One: Introduction

1. Introduction

Salmonellae are consistently reported to be among the leading international sources of foodborne human disease. *Salmonella* is a true potential pathogen for both humans and animals and cause significant economic losses worldwide, due to variety of diseases ranging from mild diarrhea to severe systemic infections like typhoid fever (Gast and Porter, 2020).

Salmonella belongs to the family Enterobacteriaceae. There are only 2 species: *Salmonella enterica* (subdivided into the 6 subspecies *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae*, and *indica*) and *Salmonella bongori* , *salmonellae* ferment glucose, dulcitol, mannitol, and maltose, but do not ferment lactose, and sucrose, *salmonella* enterica is one of the most ubiquitous enteropathogenic bacterial species on earth, and comprises more than 2500 serovars (Nair., 2018).

The pathogenicity of each *Salmonella* strain is determined by a set of genes associated with the bacterial ability to colonize mucosa of the intestinal tract, invading host cells, replicate within these cells, and to survive by destroying the phagocytic components (Elder *et al*., 2016).

The attachment ability of Salmonella has also been associated with the moisture content of meat; when carcasses are still fresh and the moisture of the skin is high, the transference from carcasses to other surfaces is more marked (Carrasco *et al.*, 2012).

The burden of foodborne diseases is substantial, almost 1 in 10 people fall ill and 33 million of healthy life year are lost every year. Foodborne diseases can be severe, especially for young children. Diarrhoeal diseases are the most common illnesses resulting from unsafe food, 550 million people falling ill each year, including 220 million children under the age of 5 years. Salmonella is 1 of the 4 key global causes of diarrhoeal diseases (WHO, 2019).

Antimicrobial resistance is an increasing global problem, and the emerging antimicrobial resistance has become a public health issue worldwide. A variety of foods and environmental sources harbor bacteria that are resistant to one or more

antimicrobial drugs used in human or veterinary medicine and in food-animal production (Rossi ,2011).

The control of foodborne pathogens such as *Salmonella* enterica is difficult because of their ability to survive during food production, processing, storage and improper cooking. Therefore, it is important to understand the ecology of Salmonella enterica and the genetic variation of different strains, in order to design specific management practices to reduce risks associated with this pathogen, several molecular typing methods are used to differentiate Salmonella enterica isolates, including multilocus variable number tandem-repeat analysis, multilocus sequence typing or multiplex-PCR-based methods and whole genome sequencing (Malorny *et al*., 2008).

The current study aimed to the detection of ***Salmonella*** in red meat and broiler chicken and this can be achieved by the following

1. Investigating the contamination rates of red meat and broilers meat as well as their products with food borne-Salmonella spp at Kerbala Province.

2. Determining the resistance of bacteria for different antibiotics through sensitivity test.

3. Conducting a molecular study by using PCR for rapid isolation and identification of the bacteria.

4. Study the purity (sequencing) and specific genetic virulence gene type of this bacteria .

Chapter Two: Review Of The Related Literature

2. literature Review

2.1. Meat contamination by *Salmonella*:

Foodborne illness remains a global public health concern. In Canada, foodborne pathogens cause an estimated 4 million cases of gastrointestinal illness annually from known and unspecified agents and serotypes of Campylobacter, Escherichia coli and Salmonella are commonly implicated. (Smith *et al*., 2019).

The presence of food pathogens such as non-typhoidal Salmonella (NTS) in meat is also a concern, since this organism is a major cause of food-borne gastroenteritis worldwide (Nhung *et al*., 2010).

Meat production is central to livelihoods in many countries, with meat from livestock and poultry being a key protein source in subsistence communities (OECD/FAO, 2016). In many low-resource settings, industrialization, urbanization, and the shift from planned to market economies are leading to rapid changes in the way that food is produced, distributed, sold, and consumed (Carron *et al.*, 2018; Grace, 2017). Such market-driven changes within agricultural production towards wider distribution networks, centralised processing, larger-scale and more intensive systems, have been linked to the emergence of zoonotic diseases (Jones *et al*., 2013).

2.2. Incidence of Salmonella in broiler :

Food safety is one of the most important concerns of humans, and closely related to health, economic development, and social stability. *Salmonella* is a potential pathogen for humans, most foodborne salmonellosis is caused by non-host-adapted serotypes. **(**Ahmed,2018)

Salmonellosis is caused by microorganisms of the genus *Salmonella* and is one of the most problematic zoonosis affecting the health of men and animals In the poultry industry, day-old chicks may get infected at hatchery through contact with fomites and eggshells with the presence of contaminated feces, which may trigger the animal infection through penetration and multiplication of the paratyphoid *salmonella* inside the egg Such process may harm the embryonic development and become a source of

infection to other chicks as well as several animals, including man **(** de Albuquerque *et al*.,2014).

Salmonellosis is one of the main infections affecting commercial poultry, causing losses to poultry production, and posing a public health concern Salmonella, the causative agent for salmonellosis, are gram negative, rod shaped, facultative anaerobic bacteria causing gastroenteritis , Fowl typhoid and pullorum disease, are widely distributed septicemic diseases, caused by S. gallinarum and S. pullorum, respectively and infect primarily chickens and turkeys. These bacteria are transmitted mainly transovarially. Feces of infected birds, contaminated feed, water and litter can also be sources for infection. Clinical signs in chicks and poults include anorexia, dehydration, weakness, diarrhea and high mortality. Decreased egg production, fertility and hatchability are the most important clinical signs in mature birds. Gross and microscopic lesions include hepatitis, typhlitis, omphalitis, pneumonia, ophthalmitis salpingitis, synovitis and peritonitis. (Nabil *et al*.,2018).

Poultry gastrointestinal tract is considered as a major reservoir for various pathogenic bacteria that can cause cross-contamination of poultry meat and egg products ,for example, Salmonella can invade the intestinal epithelial cells and survive intracellularly within macrophages (Nabil *et al*.,2018) and these intracellular Salmonella are not easily controlled by antibiotics. Bacteriophage control has received much attention as a potential treatment approach for bacterial infections **(**Golkar et al.,2014). due to the emergence of antibiotic-resistant bacteria (Agada *et al*.,2014).

2.3.Historical Review:

Salmonella was first visualized in 1880 by Karl Eberth in the Peyer's patches and spleens of typhoid patients, four years later, Georg Theodor Gaffky was able to successfully grow the pathogen in pure culture, a year after that, medical research scientist Theobald Smith discovered what would be later known as *Salmonella* enterica (Vañó Sempere and lourdes, 2020).

At the same time, Smith was working as a research laboratory assistant in the veterinary division of the United States Department of Agriculture. The division was under the administration of Daniel Elmer Salmon, a veterinary pathologist. Initially,

Salmonella Choleraesuis was thought to be the causative agent of hog cholera, so Salmon and Smith named it as "Hog-cholerabacillus" (Adhikari, 2017).

The name *Salmonella* was not used until 1900, when Joseph Leon Lignières proposed that the pathogen discovered by Salmon's group was called as *Salmonella* in his honor (Rafiullah *et al*., 2018).

The typhoid bacillus was first isolated in 1884, when the German microbiologist Gaffkey obtained *S*. Typhi from human spleens (Rogers *et al*., 2021). In 1892 Loeffler described the causative agent of murine typhoid, (then known as bacillus typhi) that caused an epidemic typhoid fever–like disease in mice (Cohen *et al*., 2021)**.**

Salmonella belongs to the family Enterobacteriaceae.

These are 3 µm long and 0.6-0.7 µm in diameter, often motile, growing on ordinary media, facultative anaerobic (García *et al*., 2019).

The peptidoglycan was surrounded by a periplasmic membrane and an outer membrane. This is in direct contact with the external environment and bears part of the antigenic characteristics of the bacteria, it is also involved in the phenomena of virulence. This membrane contains in particular the lipopolysaccharide (LPS) whose region hydrophilic, in contact with the external medium, corresponds to the “O” antigenic region (Han *et al*., 2021).

The flagella found in most salmonellae, except *S. Gallinarum*, consist of a quaternary assembly of several thousand copies of a single protein, "flagellin" and correspond to the "H" antigenic region (Wu *et al*., 2013).

2.4. Classification of Salmonella

The genus *Salmonella* is a member of the family Enterobacteriaceae which consists of only two species, *Salmonella* enterica and *Salmonella* bongori, S. enterica is divided into six subspecies: S. enterica subsp. enterica, S. enterica subsp. salamae, S. enterica subsp. arizonae, S. enterica subsp. diarizonae, S. enterica subsp. houtenae and S. enterica subsp. Indica as shown in Figure (2-1) (Guibourdenche *et al*. 2010).

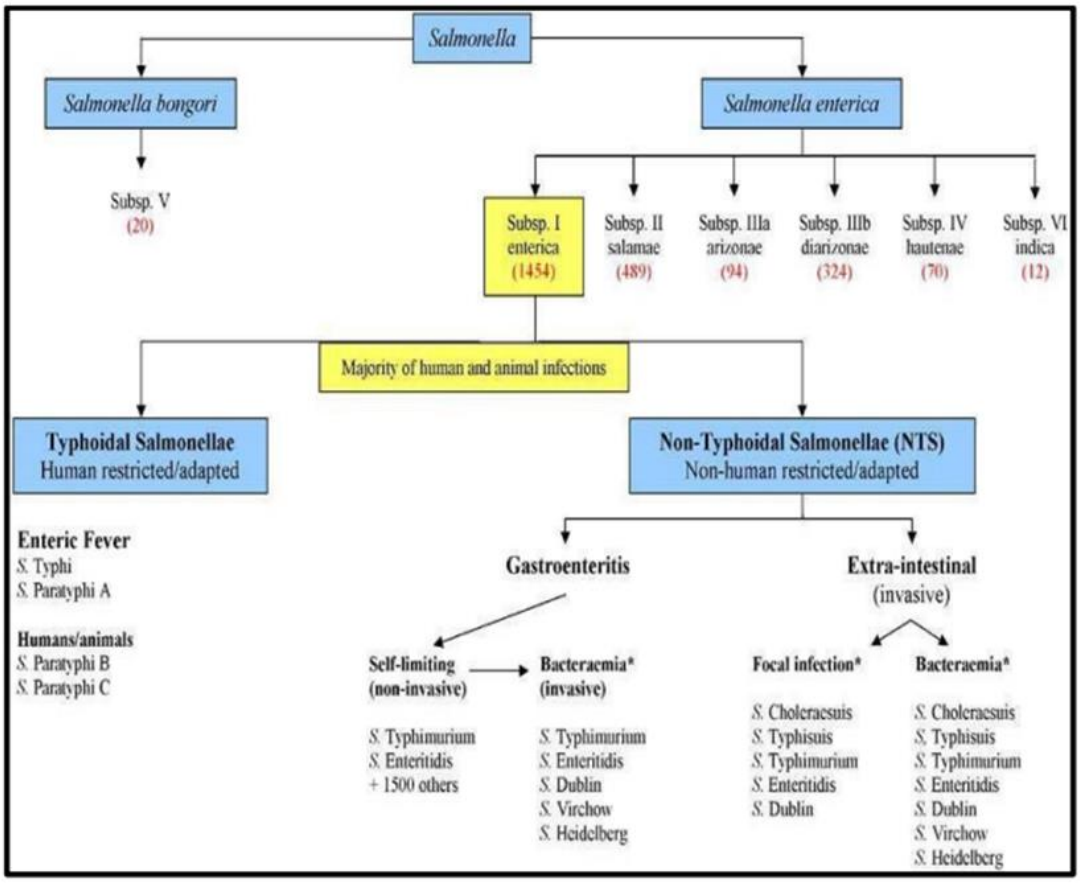


Figure (2-1): Classification of the Genus Salmonella (Akyala and Alsam, 2015).

Salmonella was classified using the Kauffmann-White scheme based on three major antigen determinants: somatic (O), flagellar (H), and virulence (Vi) or capsular (K) antigens. This approach was endorsed by the International Association of Microbiologists in 1934 (Sarker, 2018).

Salmonella are gram-negative, oxidase negative, catalase positive, nonspore forming rods. It is also considered as facultative anaerobes. Almost all Salmonella species are motile via peritrichous flagella except S. pullorum and S. gallinarum, Salmonella are extensively represented within the environment and can cause a wide range of illnesses in both humans and animals (Lopes *et al*. 2016).

The optimal growth temperature of Salmonella is 37celsius ; however, growth has been recorded between 2 and 4 Celsius and as high as 45celsius , Salmonella can live in a wide pH range from as low as pH 3.8 to as high as pH 9.5 with an optimum pH of 6.5–7.5 (Ryan *et al*., 2017).

It can ferment glucose, mannitol, arabinose, maltose, dulcitol and sorbitol, forming acid and gas except for S. Typhi, S. Gallinarum and rare an aerogenic variants in other

subtypes form only acid and no gas. Generally, Salmonella dos not fermented Lactose, sucrose, salicin or adonitol. It is indole negative, Methyl Red positive (MR), Voges Proskauer negative (VP), and citrate positive (IMViC – + – +) except for S. Typhi and S. Paratyphi A which are citrate negative as it needs tryptophan as the growth factor, Hydrogen sulfide is produced except for S. Paratyphi A, S. Choleraesuis, S. Typhisuis and S. Sendai. Urease is not hydrolyzed by Salmonella (Kuma, 2016).

2.5.Pathogenesis of Salmonella

Salmonella is a facultative intracellular pathogen capable of infecting a variety of host cells, resulting in several manifestations of disease, including enteric fever, bacteremia, and gastroenteritis. The most common clinical manifestation of salmonellosis is diarrhea. In certain instances (defined by host factors, the strain of *Salmonella*, and its dose) septicemia occurs, host factors including age, immune status, concurrent disease, and composition of the normal flora (Sastry *et al*., 2016).

Once swallowed, a mouthful of *Salmonella* enters the small intestine spreading all of the *Salmonella* that just multiplied to surrounding cells, and the process starts over again. *Salmonella* enters the rest of the intestinal tract and it is then excreted in the stool. As this cycle of invasion and cell destruction repeats, millions of bacteria are produced in the intestine, and their numbers continue to grow exponentially (Brands, 2010).

After that, *Salmonella* penetrates the mucosal epithelium of the small intestine, interacting with columnar epithelial cells and microfold cells overlying the Peyer's patches. Interaction between *Salmonella* and the epithelium triggers the chemotaxis of phagocytic cells to the infected site (Gillespie and Hawkey, 2006).

This cellular response involves both neutrophils and macrophagesmigrating to the luminal surface where they begin eradicating the bacterial pathogen. Penetration of microfold cells results in the presentation of *Salmonella* to macrophages residing in the lymphoid follicles, *Salmonella* has been shown to survive and replicate within macrophages from many hosts. It has been demonstrated that macrophages play a role in the dissemination of *Salmonella* to the organs of the reticuloendothelial system, such as mesenteric lymph nodes, liver, and spleen. Survival within

macrophages is essential for the full expression of *Salmonella* virulence (Elder *et al*., 2016**)**

2.6.Epidemiology

Salmonella enterica infections are estimated globally to cause illnesses of about 5 million cases, and the mortality rate may increase for about 50% in some conditions (Naghavi *et al*., 2015). Most cases are confined to the developing world, where the disease is endemic with the greatest burden being in Asia (EFSA ,2019).

Salmonella enterica are prevalent in domestic animals such as poultry, pigs, and cattle, and can be transmitted through the food chain by the animal origin food products, slaughter is considered as an important step causing *Salmonella* contamination in meat products (Gu *et al*., 2020).

Salmonella enterica has been recognized as a major and important foodborne pathogen for humans and animals over more than a century, causing human foodborne illness as well as high medical and economic cost. Salmonella infections may cause gastroenteritis, Typhoid fever, Bacillus enters with ingestion of fecal contaminated food or water (Witkowska *et al*., 2018).

Because of the importance of Salmonella enterica in food-borne diseases, numerous typing methodologies have been developed and used to trace salmonellosis outbreaks to the contaminated source and to delineate the epidemiology of Salmonella enterica infections (Kotetishvili *et al*., 2002).

2.6.1.Epidemiology of *Salmonella enterica* serovar Infantis

Salmonella enterica serovar Infantis (*S*. Infantis) was reported in March 1968 (Ewing, 1968), *S*. Infantis is the most frequent serovar in broilers flocks and broiler meat in Europe. In humans, *S*. Infantis has been classified as the fourth most prevalent serovar in Non-Typhoidal *Salmonella* (NTS) (Alba et al., 2020).

The host nonspecific serovar *S*. Infantis is the most recurrence strain in various countries, involving Asian countries, it was detected and isolated from veterinary and human hospitals, foods such as vegetables, meat and animal products (Almeida et al., 2013).

S. Infantis has been among the top 10 serotypes that causing human illness in the

United States every year since 2010, and Poultry is the most common source of *S.* Infantis (WHO, 2019), EFSA and ECDC reporting on antimicrobial resistance among pathogens and indicator bacteria in humans, animals and food, stated that *S.* Infantis showed resistance to more than 90% of the tested antimicrobials (EFSA and ECDC ,2014).

In 2014 first report of a multidrug resistant emergent *S.* Infantis strain was detected (Tyson *et al,.* 2021). Thus, resistant *Salmonella enterica* serovars are not only undermines medical treatment, but can also serve as a vehicle to transfer antimicrobial resistance AMR genes along the food chain (Hooton *et al., 2019).*

2.6.2.Epidemiology of *Salmonella enterica* serovar Agona

Salmonella Agona was first isolated from cattle in Ghana in 1952 (Guinée *et al*., 1964). Within the last several years, Salmonella Agona has been one of the top 20 most commonly reported serotypes causing human infections (CDC, 2014). Salmonella enterica serovar Agona is an important zoonotic pathogen, and in 2010 it became the 10th most frequently reported nontyphoidal Salmonella serovar in humans in the European Union, increasing 15% on 2009 (Chen *et al*., 2009)(Cooke *et al*., 2009). It has caused a number of human disease outbreaks in the European Union, as well as internationally, involving a range of foodstuffs, including ready-to-eat savory snacks, cereal , air-dried raw beef , infant milk formula, and fennel-aniseed-caraway infusion (ECDC, 2013)(Rabsch *et al*., 2005).

2.7.Resistance Gen In Salmonella And Antibiotic

Antimicrobial medications are used for three main purposes: to treat infected human and animal, prophylactic use in human and animal, and sub-therapeutically as growth boosters in food animals (Vuthy *et al*. 2017). One of the biggest factors to the development and spread of antimicrobial resistance (AMR) is the extensive and random use of antibiotics in veterinary medicine, including food animal production (Marshall and Levy ,2011).

Salmonella has developed high resistance to a broad spectrum of antibacterial agents, resulting in increasing healthcare expenses and treatment failure, antimicrobials with

a broad spectrum action raise bacteria's selective burden and stimulate the emergence of multidrug-resistant pathogens (Burjaq and Abu-Romman, 2020).

Single or multiple resistance to Salmonella spp. isolates from chicken meet, as well as the risk of these resistant isolates being transmitted to human, are of major concerns (Vidayanti *et al., 2021).*

Salmonella multidrug resistance (MDR) is a serious public health concern across the world, and antibiotics are being used more often in both humans and animals (Talebi Bezmin Abadi *et al*., 2019).

These MDR salmonellae can be transferred to humans at any point in the manufacturing process (Afshari et al. 2018). many problems are associated with antibiotic resistance, such as bacteremia, treatment failure, and poor clinical outcomes. Drug-resistant infections are more likely than susceptible infections to cause *Salmonella* bacteremia (Krueger *et al*., 2014).

A major problem in Salmonella infections is the genetic transmission of multiple drug resistance genes by plasmids among enteric bacteria. Thus, susceptibility testing is essential and assists in choosing the appropriate antibiotic (Carroll and Hobden ,2016).

2.8.Molecular Identification of *Salmonella*

A comparison of the genomes of numerous sequenced enteric bacteria reveals some striking similarities. All have a single chromosome with a size of 4.3–5.0 Mb (Yang *et al*., 2005). When the chromosomes of different enteric bacteria are compared, a common set of so-called "core genes" emerges that are shared by all enteric species (Anjum *et al*., 2005).

These core genes can be considered as genes that carry out "household" activities related to the common shared lifestyle of intestinal colonization and transmission (environmental survival). Although a complete characterization of the core gene set is difficult to achieve, the mutual genome can be recognized by comparing DNA sequences and studying common gene function. These core genes might be involved in central metabolism or polysaccharide biosynthesis or may encode for structural proteins (Baker and Dougan, 2007).

The DNA sequences of core genomes of Escherichia coli and Salmonella differ by 10% only. According to phylogenetic analyses, Salmonella and E. coli diverged from a common ancestor 120–150 million years ago (Bäumler *et al*., 1998).

The capacity of Salmonella to colonize the host and produce virulence is found in virulence plasmids (pSLT) and *Salmonella* pathogenicity islands (SPIs), which are gene 9 clusters situated on chromosomes. Salmonella has gained several SPIs as a result of its evolution. Salmonella has five major SPIs (1–5) that are related to pathogenicity, with SPI-1 and SPI-2 being the most studied (Fabrega and Vila, 2013). It was assessed that 5–7% of the genes in the Salmonella genome are involved in pathogenicity (Jarvik *et al*., 2010).

Salmonella virulence is thought to have developed in a multistep process, beginning with the acquisition of SPI-1 by all Salmonella spp., and the acquisition of other SPIs differentiating S. enterica from S. bongori, followed by the extension of the host range to warm-blooded vertebrates (Bäumler *et al*., 1998).

Except for a few, the SPIs are substantially conserved throughout members of S. enterica, but are missing from closely related species such as S. bongori and E. coli (Jacobsen *et al*., 2011).

While most SPIs are unlikely to retain their mobility, certain Salmonella genomic islands have been shown to excise and transfer. This excision is also induced in specific locations by host circumstances, such as macrophage survival and oxidative stress (Quiroz *et al*., 2011).

Genetic variations among SPI-1, SPI-3, and SPI-5 have been reported, whereas SPI2 and SPI-4 were shown to be highly conserved among 13 distinct Salmonella serovars isolated from warm blooded animals (bovine, porcine, avian, and horse), the environment, and human patients (Amavisit *et al*., 2003). With the exception of S. Typhimurium, all isolates within the same serovar were similar in terms of the five SPIs that were investigated. Salmonella persistence and enteritis in chickens have been linked to SPI-1 and SPI-2 (Morgan *et al*., 2004; Dieye *et al*., 2009).

SPI-1 encodes multiple proteins involved in epithelial cell invasion (Fàbrega and Vila, 2013). This pathogenicity island has the invasion invA gene, which is found in the

majority of Salmonella strains. SPI-2 is necessary for encoding of proteins required for intracellular survival and replication in host cells, such as epithelial cells and macrophages. It also helps Salmonella propagate throughout the body. SPI-2 contains the spiC gene, which codes for structural components and secretion and helps in the formation of mediators that are crucial for Salmonella pathogenicity.

The aforementioned gene is also needed for the expression of flagella filament components and has been linked to Salmonella infection (Brenner *et al*., 2000). Concerning SPI-3, it can be found in all lineages and is required for survival in macrophages and the ability of Salmonella to grow in low-magnesium environments. In addition, Salmonella colonization is aided by fibronectin-binding proteins, which are encoded by SPI-3, for example, MisL increases Salmonella colonization in chickens (Carnell *et al*., 2007). On the other hand, SPI-4 and SPI-5 distributions have not been established, 10 despite the fact that their importance is understood. SPI-4 has a role in the first contact with the intestinal epithelium and helps to maintain long-term persistence (Bonny *et al*., 2011).

SPI-4 has the orfL gene, which is required for intra-macrophage survival, as well as a mechanism involved in toxin secretion and apoptosis (Boko et al., 2013). SPI-5 has been found to cluster genes that encode multiple T3SS effector proteins. During infection, SPI-5 is involved in the execution of various pathogenic processes (Bonny *et al*., 2011).

Its initial gene, pipD SPI5, has a target on the host cell's surface or within the cell (Cristian *et al*., 2008). SPI-6 has been found to transport proteins into the environment or host cells in response to external stimuli (Stevens *et al*., 2009; Leung *et al*., 2011). Whole genome sequences are available to numerous Salmonella strains (Baker et al., 2011). Multilocus sequence typing (MLST), which is a sequence-based approach, provides a more accurate suggestion of the genomic link among various Salmonella isolates, and could eventually replace serotyping (Achtman *et al*., 2020).

Different S. Typhi isolates were compared and determined to be closely related (clonal) and to have originated about 30,000– 50,000 years ago from a single source (Baker and Dougan, 2007). More than 200 out of 4,000 genes in S. Typhi are functionally impaired or inactive. Whereas most of these homologs in S.

Typhimurium are still fully functional (De Jong *et al*., 2012).

For instance, the S. Typhi type strain Ty2 had 4.79 mega bases, the multidrug-resistant (MDR) isolate CT18 possessed 4.86 mega bases, and the S. Typhimurium strain LT2 4.81 mega bases (Deng et al., 2003).

Other virulence factors that are not included in the SPI are found in Salmonella. Extrachromosomal DNA in the form of motile genetic elements known as plasmids can also be carried by different Salmonella strains. Plasmids are a rapidly evolving gene pool that frequently contain genes related with virulence or drug resistance (Anjum *et al*., 2005).

The Salmonella virulence plasmid, which is made up of five genes (spvRABCD), aids in the pathogen's systemic dissemination and replication outside the intestine (Brisabois *et al*., 2001). Salmonella plasmids are present in a variety of serovars, including S. Typhimurium, S. Enteritidis, S. Dublin, S. Choleraesuis, and S. Gallinarum, and they range in size from 50 to 100 kb depending on the serovar (Rychlik *et al*., 2009). Plasmid transfer and plasmid gene transposition have played a significant influence in Salmonella evolution (Brilli *et al*., 2008).

The plasmids that circulate among Salmonella populations in people and animals have gained a lot of attention, and obviously these plasmids are native to Salmonella (Williams *et al*., 2013).

Serotype-specific virulence plasmids may be found in numerous strains of Salmonella serovars (Maurer, 2017). These plasmids are of Low-copy-number (one to two copies per 11 cell) (van Asten and van Dijk, 2005).

The Salmonella plasmid virulence (spv) locus is found on each of the plasmids, and its expression has been shown to be critical for Salmonella multiplication in the reticulo-endothelial system, including the liver and spleen (van Asten and van Dijk, 2005).

Salmonellae have a range of plasmid types in addition to virulence plasmids. In contrast to the Salmonella virulence plasmids, it appears that such plasmids spread through horizontal gene transfer (Folster *et al*., 2011).

Resistance plasmids have been shown to occur in multiple Salmonella serovars, in different sizes and shapes (García-Quintanilla and Casadesús, 2011;

Folster *et al*., 2012). Dynamics of genetic loss, acquisition, and preservation that participate in S. enterica subsp. enterica evolution have been demonstrated through the presence and absence of individual virulence genes, phages, and plasmid replicons (Worley *et al*., 2018). In addition to be mobile themselves, plasmids carry various mobile genetic elements, such as integrons and conjugative transposons. Class 1 integrons, which can be found on the bacterial chromosome or plasmids, are the most prevalent kind found in Salmonella isolates. Among Salmonella's class 1 integrons, a considerable number of unique gene cassette arrays have been discovered, some of which appear to have been originated from other bacterial species (Krauland *et al*., 2010).

Salmonella also contains integrative conjugative elements (ICEs). These elements appear to be key participants in the horizontal gene transfer between bacterial species because they harbor genes essential for their own excision and transfer to recipient bacteria (Wozniak *et al*., 2009). This is represented in Salmonella by SPI-7, which is an ICE found not only in Salmonella but also in a range of other Enterobacteriaceae (Seth-Smith *et al*., 2012). SPI-7 ICEs have been reported in S. enterica, S. bongori, and other bacterial species, and they appear to have evolutionary relationship, suggesting that ICE transfer happens both interand intra- species, increasing ICE diversity (Switt *et al.,* 2012).

Numerous examples of genetic transfer have been reported to occur between Salmonella and other bacterial species. For example, the transfer of heavy metal resistance operons from environmental bacteria and Klebsiella spp. to Salmonella has also been proposed (Reva and Bezuidt, 2012). Similar effects of low-molecular-weight plasmids have also been exerted on S. Enteritidis (Rychlik *et al*., 2001). Obtaining some plasmids, on the other hand, might boost phage susceptibility (Smarda *et al*., 1990). Other factors in Salmonella might be regarded as virulence agents. Fimbriae (pili) are filamentous surface structures that assist Salmonella in colonizing the epithelium (Collinson *et al*., 1996).

Salmonella has been shown to have many fimbrial operons ranging in size from 7 to 9 kb. S. Enteritidis PT4, for example, possesses 13 fimbrial operons (Betancor *et al*., 2012), and the 13 main fimbrial subunits of this bacterium have been shown to have a role in bacterial adhesion and colonization in chicken gut (Clayton *et al*., 2008). The loci where these subunits were identified were also conserved in S. Paratyphi and S. Gallinarum (van Asten and van Dijk, 2005). However, the specific involvement of flagella (motility and rotational orientation) in Salmonella pathogenicity, as well as their putative function in mammalian cell attachment and invasion, are uncertain (van Asten and van Dijk, 2005).

Salmonella persistence in the intestine may be aided by other virulence factors, such as surface polysaccharides. Many mutants affecting the biosynthesis of lipopolysaccharide (LPS) have been detected in Salmonella strains recovered from chickens (Stevens et al., 2009). The capability of S. Enteritidis to live in egg albumen has been attributed to the LPS (Gantois *et al*., 2006).

The use of next-generation sequencing (NGS) has risen in the last decades as the cost has fallen. By using NGS to sequence the genomes of two Salmonella species and six S. enterica subspecies, researchers were able to explore the evolutionary diversity of the genus Salmonella (Fookes *et al*., 2011; Desai *et al*., 2013).

Depending on the phylogenetic studies of Salmonella species using NGS, differences between S. enterica and S. bongori have been shown, putting the last in a distinct evolutionary position between E. coli and S. enterica (Fookes *et al*., 2011). The two Salmonella species diverged 40–63 million years ago, and their evolutionary history has resulted in significant variations between them (McQuiston et al., 2008). The separation of these two species is due to the gain of the SPIs, particularly SPI2, by S. enterica (Wallis and Galyov, 2000). Furthermore, S. enterica has more G + C content than S. bongori where three of the 22 SPIs are present. This divergence might be attributed to S. bongori's adaptation to cold-blooded hosts (Fookes *et al*., 2011). S. enterica subsp. arizonae is found between S. bongori and the other S. enterica subspecies, according to Whole-Genome Sequencing (WGS) and microarray

investigations (Desai *et al*., 2013). Microarray data also revealed that subspecies arizonae shared only 77% of its genes with S. Typhimurium, and that the virulence plasmid genes pSLT and SPI-2 genes between subspecies arizonae and S. enterica subsp. enterica serotypes differed significantly.

These findings support the fact that gaining of virulence genes was important 13 in the divergence of various Salmonella subspecies (Chan *et al*., 2003). Both subspecies houtenae and diarizonae diverged 30 million years ago from subspecies enterica, indica, and salamae (Franzin and Sircili, 2015).

The divergence between subspecies arizonae and diarizonae is due to the acquisition of SPI-18, β-glucuronide use or some Salmonella fimbriae gain by subspecies diarizonae (Desai *et al*., 2013; Franzin and Sircili ,2015). The divergence of subspecies indica occurred due to SPI-5 loss (Desai *et al*., 2013).

2.8.1. The Virulence Gene Invasion A

Salmonella spp. can be detected rapidly and accurately by including primer sequences in the molecular techniques specific for their invA gene. The invA gene is considered to be standard for the identification of infections caused by Salmonella in foods of animal origin (Malorny *et al*., 2003).

This gene is important due to its ability to invade cells and survive in macrophages (Gole *et al*., 2013). The World Health Organization (WHO) mentioned that S. Typhimurium and S. Enteritidis are the most significant serovars harboring the virulent invA gene causing salmonellosis globally (WHO, 2006).

The Polymerase Chain Reaction (PCR) method has emerged as a powerful, rapid and a reliable tool for detection and identification of food-borne pathogens such as Salmonella (Baumgartner *et al*.,1992), where several chromosomal genes including invA are target genes for PCR amplification of Salmonella species (Darwin and Miller ,1999).

The invA gene of Salmonella contains those sequences that are unique to this genus and has been proved as a suitable PCR target with potential diagnostic applications (Jamshidi, 2009).

The virulence of Salmonella is linked to a combination of chromosomal and plasmid factors. Different genes, such as invA, spv, fimA, and stn, are known as major virulence genes responsible for salmonellosis. The chromosomally located invA gene codes for a protein in the inner membrane of bacteria necessary for invasion of epithelial cells (Sharma and Das, 2016). In addition, the invA gene of enteric bacteria contains sequences distinctive to the current genus and has been established as an appropriate PCR target with potential diagnostic application (Mohamed, 2013); (Shanmugasamy et *al*., 2011).

2.9.Isolation and Identification of Salmonella

A broad assortment of media has been developed and evaluated for isolating and identifying *Salmonella enterica*. Although some evidence suggests that proper selection of culture media is contingent on the type of sample being tested, several commercially available formulations have been consistently effective for a variety of applications. (Gast and Porter, 2019).

Many techniques were used for the identification of *Salmonella* genus, for example, the culture media that supplemented by specific reagents that detect *Salmonella enterica*, like, *Salmonella Shigella* medium that recommended as differential and selective medium for the isolation of *Salmonella* and *Shigella* species, gram-positive bacteria are inhibited by bile salts, growth of *Salmonella* species is uninhibited and appears as colourless colonies with black centres resulting from H_2S production, *Shigella* species also grow as colourless colonies which do not produce H_2S (National Infection Service Food, 2017).

Furthermore, the Xylose-Lysine- Deoxycholate medium (XLD) was originally formulated for the isolation and identification of both *Salmonellae* and *Shigellae*, it relies on xylose fermentation, lysine decarboxylation and production of hydrogen sulphide for the primary differentiation of *Shigellae* and *Salmonellae* from non-pathogenic bacteria, these were the most common selective and differential medium used for the identification of *Salmonella*, also there was more specific medium for this purpose like chrome medium, and Brilliant Green culture media and many other culture technique used over the years (Salm-surv and June, 2010).

However, these techniques were not enough for the specific and reliable identification of *Salmonella enterica*. So the biochemical tests are critical for distinguishing the *Salmonella* genus from other members of *Enterobacteriaceae*, the Biochemical tests were used for differentiation of colonies suspected as being *Salmonella* before sending them for specific typing (HPA, 2008).

Wide variety of protocols were used for the identification of *Salmonella* genus like Urease test, was considered to be a major criterion for the differentiation of *Salmonella* from *Proteus* species, while *Proteus* species are capable of hydrolyzing urea to produce ammonia and carbon dioxide, that allows for Proteus to be distinguished from non-lactose- fermenting members of the *Enterobacteriaceae* (Brink, 2010).

Another important biochemical test, is the Triple Sugar Iron test (TSI) needed for a differentiation of bacteria based on their fermentation of lactose, glucose and sucrose, as well as the formation of hydrogen sulfide, and Gas production from carbohydrate metabolism. TSI is most frequently used in the identification of the *Enterobacteriaceae* (Vadhani, 2000).

Simmons Citrate test (SC) is recommended for the differentiation of the family *Enterobacteriaceae* based on whether or not citrate is utilized as the sole source of carbon (MacFaddin, 2000).

Many other tests were used for the detection of *Salmonella* species, and a variety of automated tests and commercial kits that are also available for this purpose such as VITEK1, and VITEK2 in addition to the serological tests, but the molecular methods remain the gold standard in all fields of identification (Eyigor *et al*., 2012).

2.9.1. Molecular Identification and Typing Methods

A number of molecular methods for the detection of *Salmonella enterica* in clinical and food samples have been developed including immunoassays, nucleic acid hybridization, and polymerase chain reaction techniques (Li *et al*., 2000).

2.9.1.1. Polymerase Chain Reaction PCR

In vitro amplification of DNA by the polymerase chain reaction (PCR) has become potential of a powerful alternative in microbiological diagnostics due to its

promptness and accuracy, PCR technique has been widely applied for the detection of food- borne pathogens such as *Salmonella enterica*, *Staphylococcus aureus*, *Bacillus cereus* and other pathogens because of its high specificity and sensitivity, fast response, and low cost. However, single PCR can only detect a single pathogen at a time (Tao *et al*., 2020).

Another molecular approach is the Real-time fluorescence quantitative PCR, which has a detection flux of less than six due to the limitation of fluorescence detection channels. These methods are still difficult to meet the needs of non-directional screening of food-borne pathogens. Multiplex PCR, a method which can detect multiple targets in a single reactor tube and another type of PCR technique has been also used (Tao *et al*., 2020).

However, due to lack of international validationand standard protocols, as well as, variable quality of reagents and equipment, the methodology has difficulties to move from expert to the end user laboratories. For example, many PCR-based methods published for the detection of *Salmonella* differ in specificity, detection limit and sample treatment (Malorny *et al*., 2003).

Recently, many DNA-based techniques have been developed for the differentiation of pathogenic bacteria including *Salmonella enterica*. Enterobacterial repetitive intergenic consensus sequence (ERIC) polymerase chain reaction (PCR), PCR-ribotyping and pulsed-field gel electrophoresis (PFGE) techniques have been extensively used for detection of genetic lineages among *Salmonella* serovars (Kumar *et al*., 2009), PCR has been the most reliable and precise method to rapid, sensitive, and specific detection of *Salmonella* from poultry samples within a relatively short time. Several PCR assays have been conducted by targeting various *Salmonella* genes, such as *inv*A, 16S rRNA, *agf*A, *via*B, *hil*A, *sir*A, ttr, virulence-associated plasmids (Halatsi et al., 2006), and evaluate the nucleotide diversity of the ISRs in rRNA operons (*rrn*H) for the assignment of *Salmonella* serotypes (Kipper *et al*., 2019). Another advantage of the molecular based techniques, they are not dependent on the utilization of a substrate or the expression of antigens, thereby circumventing the phenotypic variations in biochemical patterns and lack of detectable antigens (Mirhosseini *et al*., 2009).

2.9.1.2. Real-Time PCR

Real-time PCR has become the prominent tool for the DNA and RNA detection and quantification throughout the last decades. By using these tools, one can obtain accurate detection within a two-fold range, with a dynamic range of input material reaching 6 to 8 orders of magnitude (Fraga *et al*., 2014). This technique is characterized by its ability to track the PCR reaction progress in real time (RT), its ability to precisely measure the amplicon amount at each cycle allowing for highly accurate quantification of the amount of starting material in samples, an increased dynamic range of detection, occurrence of amplification and detection in a single tube, avoiding the need for post-PCR manipulations (Bonab *et al*., 20 2015).

Real-time PCR is done in a thermal cycler that can illuminate each sample with a light beam of at least one specific wavelength and measure the fluorescence generated by the excited fluorophore. The thermal cycler can also quickly heat and cool samples, allowing researchers to take use of the nucleic acids' and DNA polymerase's physicochemical features (Zeybek *et al*., 2020).

Optimization to guarantee that all reaction parameters are properly set for reliable results is a part of obtaining an optimal and accurate real-time PCR experiment. While optimization takes time, it is definitely worth the effort. The obtained results will have the maximum sensitivity, dynamic range, high efficiency in parallel with high accuracy, and excellent reproducibility. All of these criteria contribute to data confidence and, eventually, outcomes that are accepted by the scientific community (Bonab *et al*., 2015).

In quantitative real time PCR, the PCR product is assessed at each cycle with fluorescent dyes that provide a high fluorescent signal in direct proportion to the amount of PCR product molecules (amplicons) produced (Kashani and Malau-Aduli, 2014). Users may determine the starting quantity of target with remarkable accuracy by monitoring reactions during the exponential-amplification phase of the reaction (Kralik and Ricchi, 2017).

When it was initially improved, scientists reasoned that the cycle numbers and the PCR end-product amount can be used to estimate the original quantity of genetic material by comparison with a known standard. The data gathered during the exponential phase of the reaction provide information about the initial quantity of the amplified target. Fluorescent reporters applied in real-time PCR include dyes that bind

to double-stranded DNA (dsDNA), or dye molecules attach to PCR primers or probes that hybridize with PCR amplicon during amplification (Saini *et al*., 2017).

The real-time PCR apparatus creates an amplification plot, which signifies the product accumulation over the whole PCR reaction time by plotting fluorescence against cycle number (Navarro *et al*., 2015).

2.9.1.3.Genotyping methods of *Salmonella*

The ultimate goal for the detection of pathogens is the ability to not only detect specific pathogens occurring in low numbers in samples but also to be able to distinguish subtle diversity or genetic differences for improving tracking to original sources either during outbreak investigations or more routine analysis during food processing. As improvements have been made in sequencing technologies, these newly developed Next Generation Sequencing methods have started to be applied more routinely to microbial detection (Gilbert *et al*., 2011).

Salmonella enterica is the confirmed causative agent of numerous foodborne outbreaks throughout the world. Successful monitoring of the outbreaks and effective implementation of control programs essentially requires the accurate identification of the infection source and the transmission pathways throughout the food chain. Traditional typing relied on phenotypic characteristics such as serotyping, phage typing and antimicrobial susceptibility testing. More recently, a wide range of methods relying on differences at genome level, such as Pulsed-Field Gel Electrophoresis (PFGE), Multi-Locus Variable number of tandem repeats analysis (MLVA),

Multi Locus Sequence Typing (MLST), Single Nucleotide Polymorphisms (SNPs) of Whole Genome Sequences (WGS) and Clustered Regularly Interspaced Palindromic Repeats (CRISPR) have been introduced, thoroughly studied and extensively applied. All these techniques are characterized by certain advantages and drawbacks that should be taken into consideration before any conclusion of epidemiological nature is drawn(Hackett, 2015).

Next generation sequencing technologies provide lots of information about species, serovar, virulence, population genetics, pathogenicity, antimicrobial resistance, and subtype of bacteria in just one sequencing approach (Oakeson *et al*., 2017).

2.9.1.3.1.Multi-Locus Sequence Typing (MLST)

Multi-locus sequence typing is an improved version of a previously commonly applied method called Multi-Locus Enzyme Electrophoresis (MLEE). In the latter, differences between isolates were assessed on the basis of enzyme polymorphisms detected through different electrophoretic mobilities. In MLST these differences are assessed directly in the nucleotide sequence of seven housekeeping genes between isolates. Selection of proper number and type of genes as markers allows the adjustment of the discriminatory power, this method was used for the evaluation of some schemes that have taken place and the discriminatory power is close to the respective of classical serotyping, which in no case is adequate for outbreak analysis. However, it may accurately depict the genetic relatedness in long-term epidemiological studies (Kotetishvili *et al*., 2002).

MLST and the classification of the *Salmonella* in sequence types (STs) were proposed, since it provides the genetic background of each bacterial isolate with the analysis of the DNA sequences of informative genes. However, the discriminatory power and other advantages of whole genome sequencing has overcome this method (Achtman *et al*., 2012).

2.9.1.3.2.Sanger sequencing approach and second-generation sequencing methods

Sanger and Coulson for DNA sequencing was called `plus and minus' in 1975 (França *et al*., 2002).

DNA sequencing is an increasingly affordable tool for *Salmonella enterica* analysis. Traditional sequencing (with Sanger technology) is still the most used DNA sequencing procedure in veterinary laboratories. Molecular methods are also becoming user friendly and less labor intensive. Therefore, the analysis of ISRs is a fast and practical way of evaluating *Salmonella* isolates. It is easier and cheaper than performing MLST analysis or the in-silico analysis after WGS (Achtman *et al*., 2012; Feasey *et al*., 2016), this methodology is suitable to be used as a diagnostic tool for *Salmonella*serotypes by the analysis of the ISRs in *rrn*H operon (Kipper *et al*., 2019).

As New Generation Sequence (NGS) technologies have improved their deployment has enabled the rapid assembly of microbial sequences encoding complete genomes

within a day rather than weeks or months with conventional Sanger sequencing methods (Ricke *et al*., 2015), and was an important advancement in genomic research for over 40 years because DNA fragments for sequencing could be generated fairly easily, far more cheaper and were relatively accurate compared with other methods (Park *et al*.,2018).

2.9.1.3.3.Whole Genome Sequencing (WGS)

Whole Genome Sequencing (WGS) is a quite promising genomic tool regarding epidemiological genomic surveillance, for nearly 100 years serotyping has been the gold standard for the identification of *Salmonella* serovars. Despite the increasing adoption of DNA-based subtyping approaches, serotype information remains a cornerstone in food safety and public health activities that aimed at reducing the burden of salmonellosis, at the same time, recent advances in whole-genome sequencing (WGS) promise to revolutionize our ability to perform advanced pathogen characterization in support of improved source attribution and outbreak analysis, as it decoding the microorganisms' genetic code and with development of bioinformatics tools scientist now able to understand many biology related to pathogens like; virulence, antigen targets and antibiotic resistant genes (Yoshida *et al*., 2016), WGS still remains to be fully harnessed conceptually and fine-tuned technologically. This promising technology currently faces three major challenges: speed, data analysis and interpretation, and cost (Struelens and Brisse, 2013).

2.9.1.3.4.The 16S rRNA sequencing

In 1996, 16S rRNA gene-based PCR primers have been described, which should make the specific detection of *Salmonella* possible (Trkov and Avguštin, 2003).

In bacteria, the 5S, 16S, and 23S rRNA genes are organized into a gene cluster linked together by internal transcribed spacer (ITS) regions containing tRNAs and conserved adjacent regions, the cluster is expressed as a single operon, and the individual RNA molecules transcribed are processed by at least three different RNAases into rRNAs and tRNAs, the number and location of rRNA operons (*rrn*) is very diverse: they may be present in 1–15 copies, and over 80% of bacterial genomes

sequenced have more than one operon in prokaryotic genomes (Espejo and Plaza, 2018).

The 16S rRNA gene (*rrn*) has become the most commonly used molecular marker in microbial ecology, it has long been used for phylogenetic classification of bacteria (Trkov and Avguštin, 2003).

The 16S ribosomal RNA gene became a single criterion on which order would be created to reflect bacterial diversity, further study of diversity within microbial groups was also aided by other molecular based techniques which traversed beyond the limitations of studying morphology to classify microorganisms. However, the 16S rRNA gene remained the primary reference for bacterial classification. Various laboratories in the world have generated and deposited 16S rRNA gene sequences in web-based databases. So rapid was this process that DNA sequence databases have over a short period been flooded with 16S rRNA gene sequences to afford researchersthe opportunity to search these databases to classify bacteria (Khayalethu, 2013),

However, this method does not always allow to identify bacteria to the species or subspecies level due to high sequence similarities between some species or strains (Deurenberg *et al*., 2017), this method also fails to distinguish certain species, as described for *E. coli* and *Shigella* spp. The 16S rRNA genes of which share >99% sequence identity (Devanga Ragupathi *et al*., 2018). Furthermore, Sanger sequencing, which is generally used for 16S rRNA gene sequencing, is challenging in complex, polymicrobial samples (Deurenberg *et al*., 2017)

2.10.Bioinformatics

Sequence similarity searching is a very important bioinformatics task, the *Salmonella* genome size ranged from 4.6 to 5.1 megabases (Mbs), and it is organized into several operons (Dhanani *et al*., 2015).

For this purpose many softwares have been used for nucleotide editing and sequence alignment such as SnapGene software (www.snapgene.com), and BLAST of NCBI(blast.ncbi.nlm.nih.gov/Blast.cgi) and many more.

Basic Local Alignment Search Tool (BLAST) is one of the most popular

bioinformatics tools, it is a sequence similarity search program that can be used to quickly search a sequence database for matches to a query sequence. Several variants of BLAST have been existed to compare all combinations of nucleotide or protein queries (is called BLASTp) againsta nucleotide or protein database (Camacho *et al*., 2009).

Chapter Three: Methodology

3.Materials and Methods

3.1.Materials

3.1.1.Equipment and Instruments

Table (3-1): All the Equipment and Instruments Used in This Study

Type of Equipment	Manufacturer, origin
Autoclave	Hirayama, Japan
Bunsen burner	Labgard, USA
Class II biological safety cabinet	Labgard
Cryogenic tubes	Ultra-Cruz, Germany
Conventional PCR thermal cycler	Bio base, China
Centrifuge	Hettich, Germany
Deep freezer	Samsung, Korea
Drying oven	Bio base
Eppendorf tube (different size)	Ultra-Cruz
Electronic balance	Bio base
Electrophoresis Unit	Bio base
Flat Bottom glass Tube with a Screw cap	Germany
High-speed refrigerated centrifuge	Bio base
Incubator	Bio base
Inoculation loop	Lab-tech, Italy
Magnetic stirrer with a hot plate	Wise stir, Belgium
Micropipette sets from 0.5µl to 1000µl	CYAN
Micropipette tips (different sizes)	Citotest, China
Microwave oven	Samsung, Korea
Mini centrifuge	Gusto, China
Petri plates (Disposable plastic)	Sunvian, China
Plain tube	Sunvian
Plastic rack	Sunvian
Refrigerator	Kelon, Japan
Sterile swap	Sunvian
Steel rack	Citotest, China
UV trans-illuminator	Bio base
Vortex mixer	Bio base
Water bath	FALC BI, Italy
Water distiller	K&K, Korea

3.1.2.Biological and Chemical substance

Table (3-2): Biological Chemical and Materials

Substances	Manufacturer, origin
10xTBE buffer	Promega, USA
Absolute Ethanol	Bio world, USA
Glycerol	Bio world
Agarose	Intron, Korea
Ethidium bromide	BDH, England
Sulfuric acid (H2So4)	BDH
Barium chloride dehydrate	BDH

3.1.3.Culture media

Table (3-3): Culture Media Used During The Study

Culture Media	Company and Origin of Suppliers
Salmonella Shigella Agar	Himedia, India
Selenite F Broth	Himedia
Brain heart infusion Agar	Himedia
Nutrient broth	Himedia
Triple Sugar Iron Agar	Oxoid, England
Xylose Lysine Deoxycholate agar	Oxoid
Urea Agar	Oxoid

3.1.4.Antibiotics discs

Table (3-4): Antibiotic Discs (LD/Italy)

Antibiotic class	Antibiotic name and content	code	Inhibition zone diameter (mm)(CLSI 2020)	
			S	R
Penicillins	**Ampicillin (10µg)**	AMP	≥ 17	≤ 13
Aminoglycosides	Gentamicin (10 µg)	CN	≥15	≤12
	Amikacin (30 µg)	AK	≥ 17	≤14
Folate pathway antagonists	sulfamethoxazole23.75 µg	SMX	≥16	≤10
Cephalosporin	Ceftriaxone (30 µg)	CRO	≥ 23	≤19
Macrolides	Erythromycin (15 µg)	E	≥23	≤13
Tetracyclines	Tetracycline (30 µg)	TE	≥19	≤14
Fluoroquinolones	Ciprofloxacin (5 µg)	CIP	≥21	≤15
Phenicols	Chloramphenicol (30 µg)	C	≥18	≤12
Quinolone	Nalidixic acid (30 µg)	NA	≥ 19	≤13

R= Resistance, S=Sensitive

3.1.5.DNA Amplification Materials

3.1.5.1.DNA Polymerase and Molecular Weight Marker

Table (3-5): DNA Extraction Materials

DNA amplification materials and Content		
1. DNA Extraction Kit (G-spin ™ Genomic DNA) / Intron, Korea		
G-buffer	Pre buffer	Washing buffer A
Washing buffer B	Binding buffer	Elution buffer
Lysozyme powder	Ribonuclease A powder	Proteinase K powder
2. GoTaq® G2 Green Master Mix, 2X / Promega, USA		
Taq DNA polymerase	$MgCl_2$	dNTPs
Reaction buffer	DNA Polymerase,	(blue and yellow) loading dyes
3. SiZer-100 DNA Marker Solution / Intron, Korea		
100 to 1,500 bp		

3.1.5.2.Conventional PCR Primers

Table (3-6): Primers Used for the Detection of *Salmonella* Isolates,Integrated DNA Technologies, USA.

Primer		Sequence 5'-3'	Amplicon size	References
ITR 1–2 NF	F	GTGAAATTATCGCCACGTTCGGGCAA	ranged 284bp	(Sunar et al. 2014)
	R	TCATCGCACCGTCAAAGGAACC		

3.2.The Methods

3.2.1.Study Design and Specimens Collection

A Cross-sectional study was performed to collect a total of 310 samples, figure (3-1) summarized the number of all information of the samples, *Salmonella enterica* was isolated from local and imported broilers and red meat.

The swab were collect by take 25 cm^2 and the organ samples were collect by take 100gm from the different type of samples and the samples were collected during the period from November 2021 to March 2022, these samples were gathered from different locations in Kerbala province and cultured in appropriate media according to internationally known protocols for bacterial cultivation and identification (MacFaddin,2000). Then it was followed by the initial bacterial isolation process on the special and distinctive culture media of *salmonella* such as SS agar and the followed propagation (NHS ,2017).

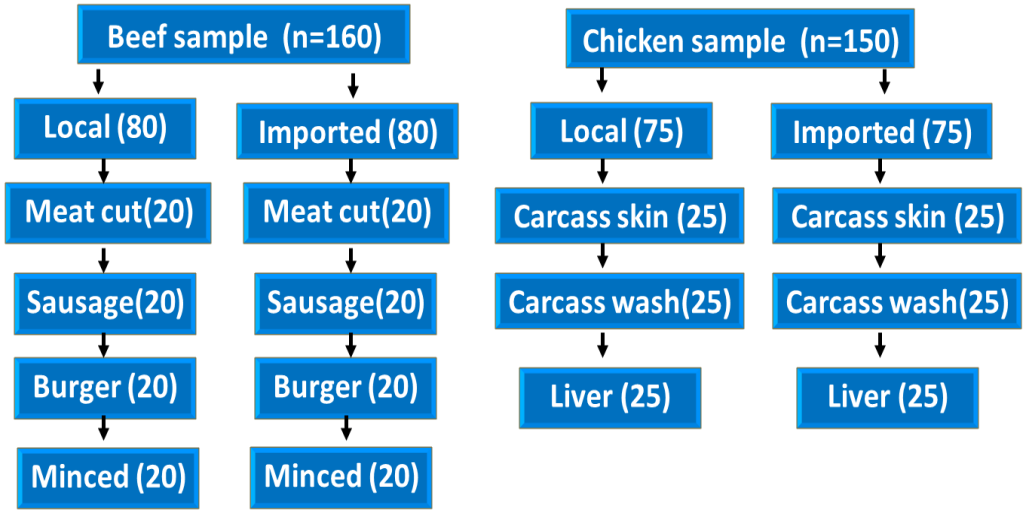


Figure (3-1) Shows the all Samples Collected From Beef and Chicken

The nucleic acid materials of the germ were then collected, in order to target a specific genomic gene of the *Salmonella enterica* genome (*invA* gene) to be amplified. The whole DNA was extracted from isolated bacteria, then the PCR products were subjected to gel electrophoresis, DNA bands were illuminated using a gel documentation system (Salm-surv and June 2010).

The five sample isolated amplicons were sent to MACROGEN® for sequencing using the Sanger sequencer. The resulted sequences were cleaned and quality trimmed using quality threshold of more than 20. High quality sequences (reverse or forward) of nucleotides were identified and compared with archives available in the National Center for Biotechnology Information (NCBI) and classified through the use of bioinformatics algorithms and programs specified for this type of analysis, these steps of isolation and identification of *Salmonella enterica* were summarized in Figure (3-2) (HPA, 2008)**.**

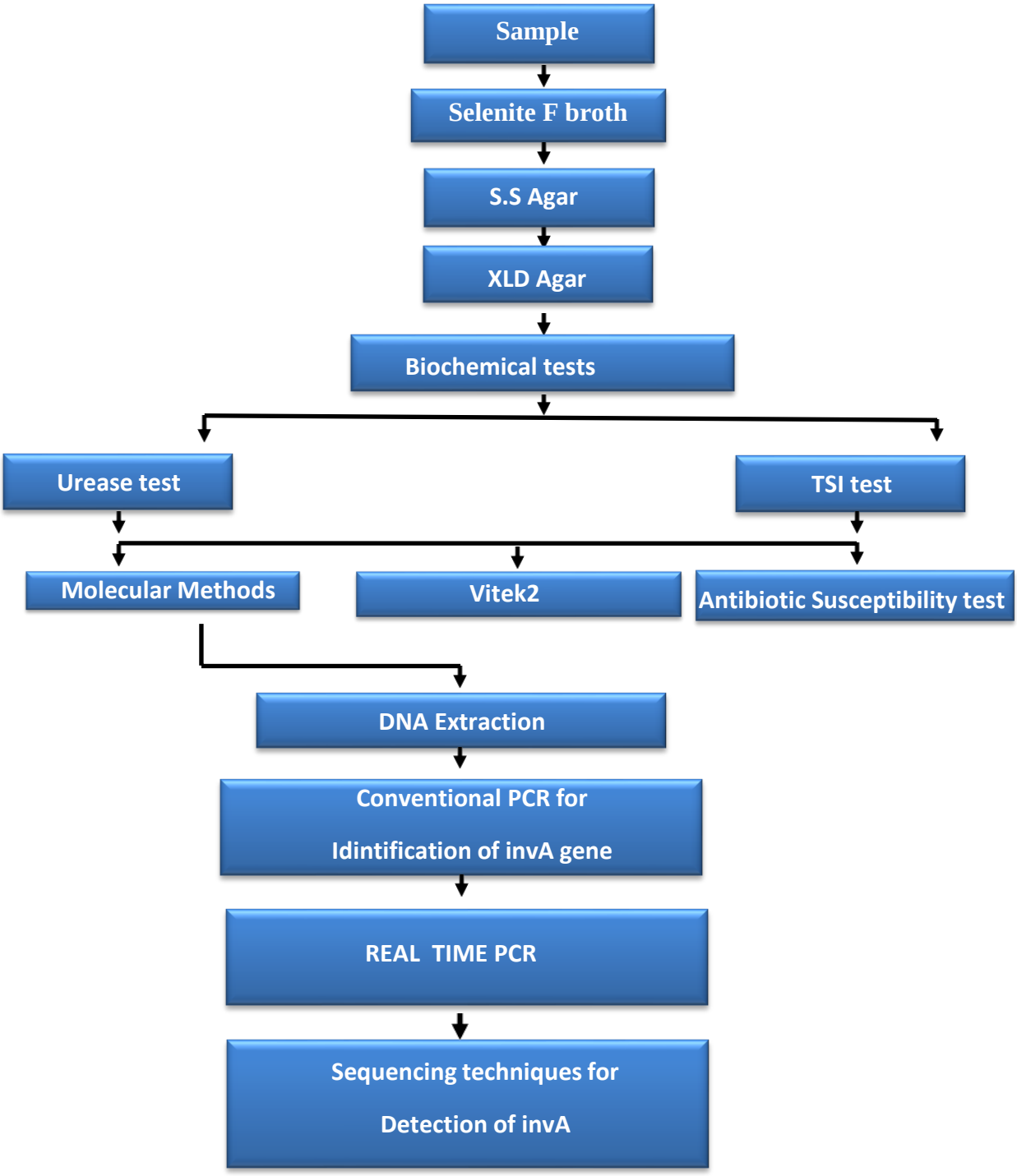


Figure 3-2: Schematic Diagram of Isolation and Identificationprocedures of *Salmonella* Serotypes.

3.2.2. *Salmonella* Isolation and Identification

The *Salmonella* isolation and identification were based on the morphological examination on the culture media and microscope, as well as the biochemical tests, and molecular detection (Salm-surv and June 2010).

3.2.2.1. Characteristics of Bacterial Culture

All samples were inoculated for 24 hours at 37°C on *Salmonella* Shigella agar and Xylose Lysine Deoxycholate agar. Colonies on *Salmonella* Shigella agar were shown Colorless colonies with black centers if H2S is produced. However, on Xylose Lysine Deoxycholate agar the colonies show clear colonies with black centers (Salm-surv and June, 2010).

3.2.3. Solutions and Reagents Preparation

3.2.3.1. Preparation of culture media

The media and reagents that used in this study were ready to use medium available from several companies including Oxoid and Himedia formulated and prepared according to the manufacturer's instructions.

3.2.3.1.1. Selenite F Broth

Selenite Broth is recommended as enrichment media for the isolation of *Salmonella* from feces, urine or other pathological materials. Selenite was inhibitory for coliforms and certain other microbial species, such as fecal *streptococci*, present in fecal specimens and, thus, was beneficial in the initial recovery of the *Salmonella* species**.** This media was prepared according to manufacturer company instructions by Suspending 4.0 grams of Part B in 1000 ml distilled water, then, add 19.0 grams of Part A, mix well and warm to dissolve the medium completely. After that distributed in sterile test tubes, sterilize in a boiling water bath or free flowing steam for 10 minutes without an autoclave, excessive heating is detrimental. Discard the prepared medium if a large amount of selenite is reduced (indicated by red precipitate at the bottom of tube/bottle) (MacFaddin, 2000).

3.2.3.1.2. Salmonella Shigella Agar

Salmonella Shigella medium that is recommended as a differential and selective medium for the isolation of *Salmonella* and *Shigella* species, gram- positive bacteria are inhibited by bile salts. This media was prepared according to manufacturer company instructions by Suspending 60.00 grams in 1000 ml distilled water. Boil

with frequent agitation to dissolve the medium completely, without autoclave or overheat, then cool to about 50°C. Mix and pour into sterile Petri culture plates, growth of *Salmonella* species is uninhibited and appears as colorless colonies with black centers resulting from H2S production. Although *Shigella* species on the same media show colorless colonies, it do not produce H2S (National Infection Service Food ,2017).

3.2.3.1.3. Xylose-Lysine-Desoxycholate Agar

Xylose-Lysine-Desoxycholate Agar (XLD) was originally formulated for the isolation and identification of both *Salmonella* and *Shigella*, it relies on xylose fermentation, lysine decarboxylation and production of hydrogen sulfide**.** This media is prepared according to manufacturer company instructions by Suspend XLD Agar (53.0 grams / liter) in de-ionized water. Heat to 100C to dissolve. Cool and aseptically dispense into Petri dishes, *Salmonella* suspect colonies grow as red colonies with a black center. Other bacteria that may grow on XLD agar are usually yellow and the agar will also turn to yellow (Salm-surv and June, 2010).

3.2.3.1.4. Triple Sugar Iron Agar (TSI)

Triple Sugar Iron test (TSI) is needed to differentiate bacteria based on their fermentation of lactose, glucose and sucrose, as well as the formation of hydrogen sulfide, and gas production from carbohydrate metabolism. This media was prepared according to manufacturer company instructions by Suspending 64.42 grams (the equivalent weight of dehydrated medium per liter) in 1000 ml purified distilled water. Heat to boiling to dissolve the medium completely and mix well and distribute into test tubes. Sterilize by maintaining at 15 pis pressure (121°C) for 30 minutes or as per validated cycle, strain of *Salmonella* typically produce an acid (yellow) bottom with gas bubbles and an alkaline (deep pink) slope, with blackening due to hydrogen sulfide production (Salm-surv and June ,2010).

3.2.3.1.5. Urease Test

Urease test, was considered to be a major criterion to differentiate Proteus from non-lactose-fermenting members of the Enterobacteriaceae. This media was prepared

according to manufacturer company instructions by Suspendiing about 24.52 grams of the dehydrated medium is dissolved in 950 ml distilled water in a beaker. The solution is heated to bring it to a boil in order to dissolve the medium completely. The prepared suspension is sterilized by autoclaving at 15 lbs pressure, 121°C for 15 minutes. The beaker is taken out following the autoclaving and cooled to 50°C. To the beaker, 50 ml of sterile 40% urea solution is added and mixed well. The medium is dispensed into tubes and set in a position to obtain agar slants (Brink ,2010).

3.2.3.1.6.Gram´s stain

Smears were prepared from the culture by emulsifying a part of a colony in a drop of normal saline on a glass slide, dried and fixed by heating. Then the slides were flooded by crystal violet for 1 minute and then washed with tap water. Iodine solution was applied for 1 minute, and thenthe slide was washed with tape water. The smear was then decolorized with few drops of acetone for seconds and washed immediately with water. Then the smear was flooded with dilute carbol Fuchsine for 30 seconds and washed with tap water. Slides were then blotted with filter paper and examined under oil immersion lens. Gram-positive bacterialcells appeared violet in color while that of Gramnegative bacteria appeared red.

3.2.3.1.7. Nutrient Broth

Nutrient media are basic culture media used for maintaining microorganisms, cultivating fastidious organisms by enriching with serum or blood. They are also used for purity checking prior to biochemical or serological testing. This media was prepared according to manufacturer company instructions by Suspend 13.0 grams in 1000 ml purified / distilled water. Heat, if necessary, to dissolve the medium completely. Dispense into tubes or flasks as desired. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes(Salm-surv and June, 2010).

3.2.3.1.8. Mueller-Hinton Agar

The major use of Mueller-Hinton Agar is for Antimicrobial Susceptibility Testing (AST), This media was prepared according to manufacturer company instructions by Adding 38g to 1 litre of distilled water. Bring to the boil in order to dissolve the

medium completely. Sterilize by autoclaving at 121°C for 15 minutes (Das, Tiwari, and Shrivastava, 2010).

3.2.3.1.9. McFarland Standard Solution

McFarland's 0.5 is the standard turbidity solution and it is the most commonly used in the inoculum preparation process, that has a specific optical density to provide turbidity equal to 1.5×108 CFU/ml bacterial suspension. This solution was prepared by dissolving 1.175g of barium chloride dehydrate in 100 ml of distilled water (wt/vol), then 0.5 ml of this solution was added to 99.5 ml of 1% (vol/vol) Sulfuric acid. The solution was stored in Para-filmed test tube for 6 months at room temperature (Benson, 2002)**.**

3.2.3.1.10. Lysozyme Enzyme

This solution was prepared by suspending 20 mg of lysozyme powder in 200 µl distilled water according to manufacturer company instructions and stored at -20°C. This enzyme was used to break the bacterial cell wall by the degradation of polymer cell substances responsible for cell rigidity.

3.2.3.1.11. Ribonuclease (A) Enzyme

It was prepared by dissolving 3 mg of RNase A lyophilized powder in 300 µl distilled water and stored at -20°C according to the directions of the manufacturer company, this solution was used for degrading the RNA of the bacterial cell.

3.2.3.1.12. Proteinase K Enzyme

It was prepared by adding 1.76 mg of proteinase K powder to 88 µl distilled water and processed at -20 C° according to the directions of the manufacturer company, this enzyme was used for the digestion of any kind of proteins, therefore to purify the extracted DNA.

3.2.3.1.13. Ethidium Bromide (EB) stock

This solution was prepared by suspending 5 mg of EB into 100 ml of distilled water and preserved in a dark tube, used for stain the DNA on the gel, and visualized under UV light (Pospiech and Neumann., 1995).

3.2.3.1.14. Tris-borate-EDTA (TBE) buffer stock

Tris-borate-EDTA buffer was used at a concentration of 1 X (1: 10 dilution of the concentration stock). The stock solution was diluted by distilled water and stored at room temperature.

3.2.4. Preservation and maintaining the *Salmonella* isolates

3.2.4.1.Short-term storage method

It was a storage method to maintain the pure culture for one month by preparing a slant of brain heart infusion agar in screw-capped tubes and streaked by a charged loop of a single colony of *Salmonella* bacteria then wrapped up with parafilm, held at 4°C (Vandepitte *et al*., 2003).

3.2.4.2. Long-term storage method

Using this maintaining method, for storing the pure isolates for more than 6 months in brain heart infusion agar supplemented with 20% glycerol,
in brain heart infusion agar was added into cryogenic tubes and inoculated by a single colony of *Salmonella* bacteria and stored in the freezer at -20°C (Vandepitte *et al*., 2003).

3.2.4.3. Vitek2 Diagnostic Method (BCL Identification Card)

The isolates suspected to be Salmonella were identified by the automated Vitek2 system with its identification card at Imam Al-Hijjah Hospital, located in Kerbala, Iraq. The 64-well card contained 43 colorimetric substrates for the phenotypic identification of bacterial species. For detecting of the bacterial identity using Vitek2, the isolate was plated onto XLD agar and incubated overnight at 37°C. The next day, a suspension of the organism was prepared in saline (0.45-0.50% NaCl) inside a polystyrene tube to a density equivalent 36 to a McFarland tube number 0.5. The density was determined using a Vitek2 DensiChek spectrophotometer. Subsequently, the tube and the card were inserted into the Vitek2 cassette, and the card was auto-inoculated within the Vitek2 instrument via a vacuum-release method. The wells of the card were optically scanned and read each 15 min, with a total incubation time of approximately 8 hr (Gardner and Altman, 1995).

3.2.4.4.Real-Time PCR

Detection of *Salmonella SPP* by RT-PCR:

3.2.4.4.1. Intended use:

The Real-TM Kit as mentioned **(2.1.3.2. and 2.1.3.3.)** is a test for the qualitative detection of *Salmonella spp.* in the Chicken and meat animal product.

3.2.4.4.2. Protocol :

Reaction mixture PCR tube were prepared in 25 µl of RT-PCR mix, the reagent of the mix was illustrated under this ratio Table .

Table(3-7): Content of the Reaction Mixture of Real Time-PCR assay

No.	Content of reaction mixture	Volume
1.	Amplification Mix	10 µl
2.	Oligo Mix	10 µl
4.	Template DNA Final Concentration ≥ 10 ng/ml)	5 µl
Total volume		25 µl

Once, the mixture was ready, aliquot 21ul of Master Mix in the tubes for RT-PCR and add in each tube 5ul of extracted DNA, after that, RT- PCR PreMix were added into PCR PreMix tube. Then, real-time PCR tubes were sealed by the optical adhesive film and mixed by vigorous vortexing for resuspension of PreMix pellet. The tubes were centrifuged at 3000 rpm for 2 min , then Exicycler™ 96 Real-Time Thermal Block was started, and the tubes were set inside the instrument and started the program of amplification according to kit instruction (Table 2-9, 2-10):

Table (3-8) Thermal profile for *Salmonella spp.* (according to the manufacturer's instructions)

Time	Temperature (^{o}C)	Cycle
10 minute	**95**	**1**
15 second	**95**	**50**
60 second	**52**	

3.2.5. Molecular investigation procedure

The DNA of all isolates was extracted directly from colonies aged 24 hours, as instructed by the DNA extraction kit manufacturing company that mentioned in Table (3-8), The preparation of the primers achieved according to the procedure of each primer depending on the manufacturer's instruction by suspending the lyophilized product with nuclease-free water. Moreover, the PCR design and amplification conditions were as in Table (3-9), the conventional PCR was achieved according to the manufacturing company of the master mix and the reaction mixture was prepared in a total volume of 25µl. All *Salmonella*- positive isolates were subjected to detection of the invA gene (Sunar *et al*. 2014).

Table (3-9). PCR Design and Amplification Conditions.

Primers	Phase	Temperature	Time	Cycle
ITR 1–2 NF	Initial denaturation phase	95°C	5 Min	1X
	Denaturation phase	95°C	30 Sec	X35
	Annealing phase	60 ºC	35 Sec	
	Extension phase	72 ºC	55 Sec	
	Final extension phase	72 ºC	5 Min	1X
	Hold	4 ºC	Infinite	

3.2.5.1. Preparation of Agarose Gel and DNA loading

The procedure of gel electrophoresis was conducted according to. The preparation of the gel was by dissolving one gram of agarose in 100 ml of 1X (TBE) buffer. The

mixture then was applied in a boiling water bath until all the powder melts and clarified, then allowed to cool down to about 50 Celsius, add 5µl of ethidium bromide to the combination, and the gel was then poured in a balanced gel electrophoresis tank with two combs at the end and middle of it. The two ends of the gel template were sealed. After half an hour, the gel hardens, the combs were lifted, and the seals were released (Rabilloud *et al*. 2010).

The comb created wells were used to load sample DNA. Five µl of DNA ladder marker was loaded in one well of each row for confirming PCR product size of 11 fragments between 100 and 1,500 bp, followed by the same amount of PCR product loaded into other wells. The gel template was then set in the chamber of the electrophoresis and poured with a TBE buffer. The procedure was conducted at 75 volts for one hour (Matsumoto *et al*. 2005).

3.2.5.2. Sequencing and Analysis

3.2.5.2.1. Identity Analysis of the invA gene.

All positive isolates were sent and sequenced by Sanger method to Macrogen. The nucleotide sequences of the invA gene were analyzed and edited manually for quality trimming, using SnapGene version (5.2.5) (www.snapgene.com).

InvA gene were aligned separately using MUSCLE a multiple sequence alignment tool version 2.1 (Edgar and Edgar, 2004). InvA gene sequences of the *Salmonella* isolates were submitted to the BLASTn program (available from NCBI) in order to assign a closer serotype, then the closest available reference was chosen and its sequence was downloaded. Afterward all sequences were included in the previous alignment matrices. Nucleotide sequences were evaluated in a pairwise comparison for identity analysis.

3.2.6. Susceptibility Test for Antimicrobials Using Disk Diffusion (DD) Method (CLSI 2020)

Step 1: Preparation of Inoculum

Few colonies of the fresh isolate were selected from XLD agar and suspended with BHI broth medium to make direct colony suspension and compared visually with McFarland standard 0.5%.

Step 2: Culturing of Petri-Dishes

A sterile cotton swab was inserted into the direct suspension and squeezed well on the inner wall of the tube in order to remove excess fluid. Then Muller-Hinton agar plate was then inoculated using the streaking method across the whole agar surface more than three times.

Step 3: Application of the Antibiotic Discs

The discs were placed over equal distances between each disc on the agar plate with a size of 90mm and 200mm. Then, incubated in an inverted position at 37°C.

Step 4: Reading the Results

The inhibition zone diameter was calculated after incubation for 18 hours.

3.2.7. Statistical analysis

Statistical Package for the Social Sciences (version 21) was used to analyze the current data. Differences were obtained by applying the Chi- square test. Differences were set as significant at ($p<0.01$) (Sahu, 2016).

3.2.8. Ethical Approval

This study did not include the use of genetically changed organisms or biological materials and was carried out under the supervision and recommendations of the Faculty of Veterinary Medicine, University of Kerbala, according to the controls approved by it. All samples used in this study were collected according to the research protocols for each type, without additional materials or manipulation.

Chapter Four: Results

4. Results

4.1. Isolation of Salmonella spp from red meat

Current result illustrated in table 4.1 indicate that total of 80 samples of local fresh red meat were collected from different location of Karbala province and as follow 20 sample of meat,20 samples from minced meat ,20 samples of sausage and 20 samples of burger, and that salmonella spp were isolated from 4(20%),9(45%),1(5%) and1(5%) samples respectively. The contamination rate of minced meat with salmonella spp (45%) was significantly high($p<0.05$) from other sources of examined meat sample.Similarly salmonella spp were isolated from 2(10%) , 6(20%) , 0(0%) and 0(0%) of imported meat collected from 20 meat cut, 20 minced ,20 sausage and 20 burger samples respectively. Once again ,that contamination rate of minced meat with salmonella spp 30% was found significantly high ($p<0.01$) than other source

Table(4-1): Isolation of *Salmonella* spp .from local and imported beef

Type of meat	No.Sample	No.Sample examined for each Type	Meat cut	Minced	Sausage	Burger	Total positive No (%)	Chi-Square (χ^2)
			No.Sample positive	No.Sample positive	No.Sample positive	No.Sample positive		
Local	80	20	4 (20.00%)	9 (45.00%)	1 (5.00%)	1 (5.00%)	15 (18.75%)	12.78**
Imported	80	20	2 (10.00%)	6 (20.00%)	0 (0.00%)	0 (0.00%)	8 (10.00%)	8.261**
, ** (P≤0.01).								

4.2.isolation of Salmonella spp from chicken meats.

Current study results illustrated in table 4.2 indicate that total of 75 samples of local chicken meat were collected from different location of Karbala province and as follow 25 sample of skin ,25 sample from carcass wash and 25 samples of liver and that salmonella spp were isolated from 9(36 %),10(40%) and 4(16%) samples respectively. The contamination rate of carcass wash with salmonella spp (40%) was significantly high($p<0.05$) from other source of examined chicken samples Similarly salmonella spp were isolated from 5(20%) , 1(4%) , and 2(8.0%) of imported chicken meat collected from 25 carcass skin , 25 carcass wash and 25 liver sample respectively. The high contamination rates of with salmonella spp 20% was found in carcass skin which is significantly high ($p<0.01$) than other sources

Table (4-2): Isolation of Salmonella spp .from local and imported chicken meat sold at Kerbala markets.

Type of meat	No .Sample	No.sample examined for each Type	Carcass skin chicken	Carcass wash	Liver	Total positive No (%)	Chi-Square ($\chi 2$)
			No.sample positive	No.sample positive	No.sample positive		
Local	75	25	9 (36.00%)	10 (40.00%)	4 (16.00%)	23 (30.66%)	9.316 **
Imported	75	25	5 (20.00%)	(4.00%) 1	2 (8.00%)	8 (10.66%)	4.973 *
** (P≤0.01).							

4.3.Convetional identification

4.3.1.Culture characteristics

All samples were cultured on Salmonella-Shigella agar, and the colonies were circular, smooth, convex and pale in color with a black center. Then sub-cultured by using selective media Xylose-Lysine-DeoxychTIONAolate (XLD) agar for the confirmation of the xylose fermentation, lysine decarboxylation and production of hydrogen sulfide, while this bacterium appeared on XLD agar as a small red colony with black center Figure (4-1).

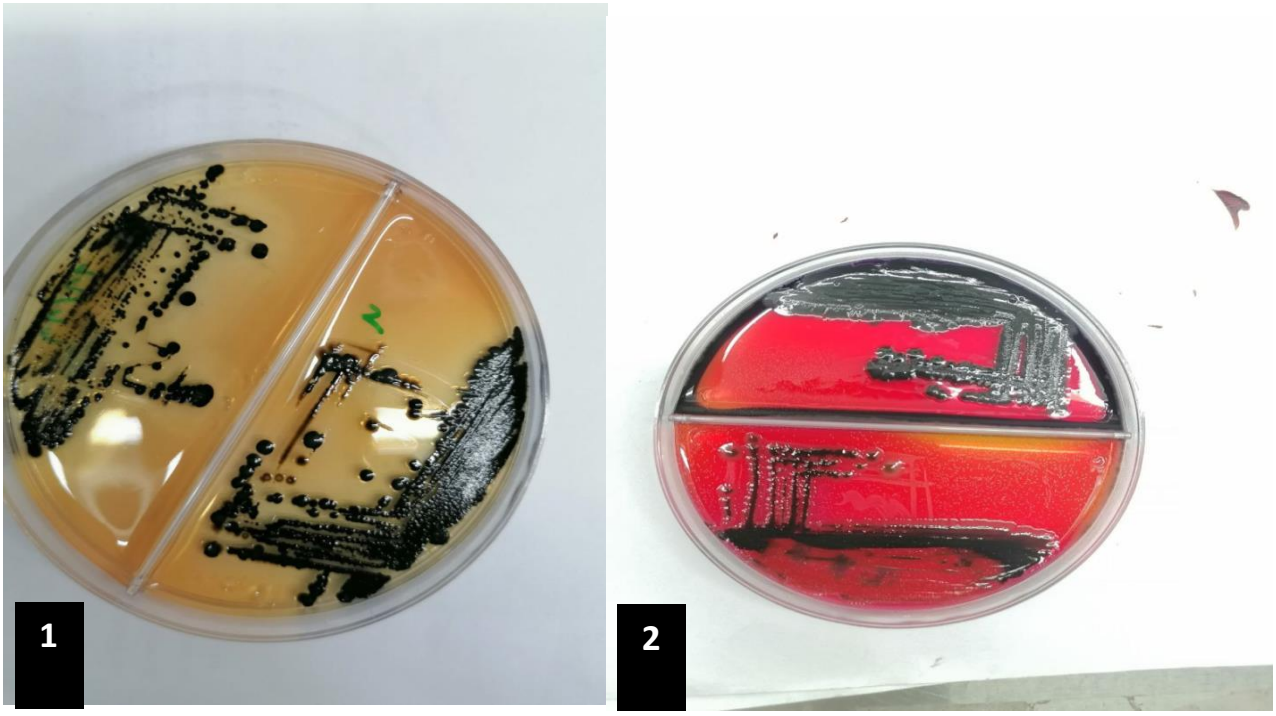
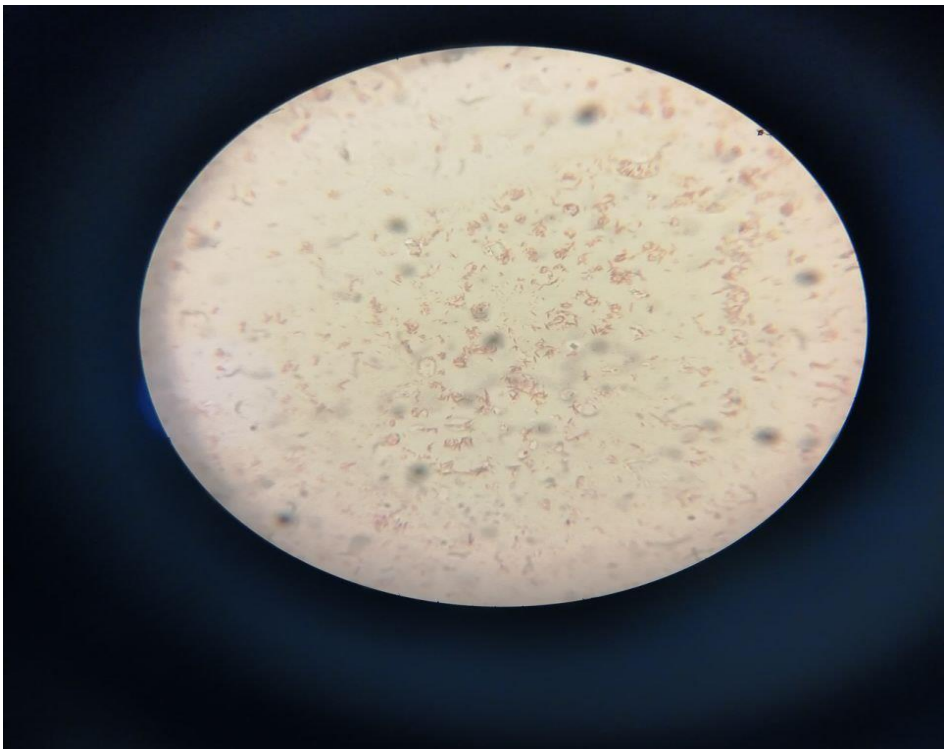


Figure 4-1. Isolated *Salmonella* on SS agar and XLD agar. Figure 1.Isolated *Salmonella* on SS agar and XLD agar.(1) shows the positive result of *Salmonella* isolates on an SS agar plate. (2) Shows the positive .*Salmonella* isolation XLD agar plate.

4.3.2.Microscopic characteristic

Gram negative salmonella with rod shape and red color in gram stain as shown as in figure (4-2).



Figure(4-2). Microscopic Examination of Salmonella

4.3.3.Biochemical characteristic

Then Biochemical tests were implemented such as the Urease testthat revealed the inability of *Salmonella* to urea hydrolysis. In addition to that, the ability of*Salmonella* to ferment certain sugars and produce the hydrogen sulfide and gas was tested by using the Triple sugar iron (Tsi) test Figure (4-3)

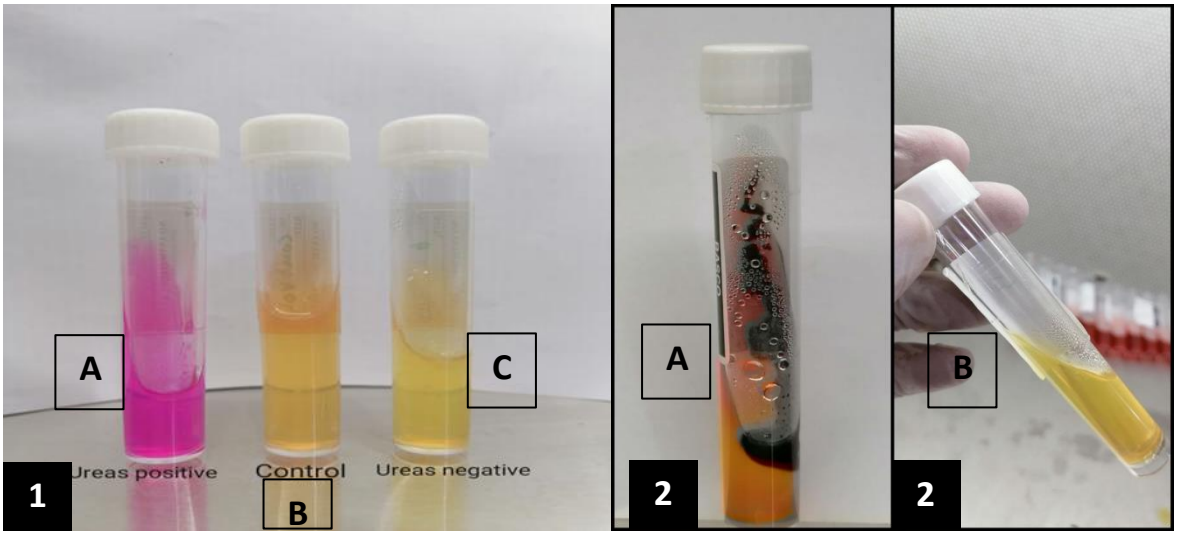


Figure 4-3. Isolation result of *Salmonella* on Urease test, , and TSItest. (1. A) show the positive result of the Urease test,(1.B) show control (1. C) show the negative result.(2. A) showing the positive result of the TSI test, and (2. B) negative result.

4.4. Diagnosis using the Vitek2 system

Fifty four isolates of suspected Salmonella collected randomly, were confirmed by the Vitek2 system as S. enterica subsp. enterica (Table 4-3) and (Table 4-4). Detection inconsistencies were observed at the serovar level, the red meat and poultry samples were diagnosed as S. enterica subsp. enterica, S. Enteritidis, S. Typhimurium, S. Paratyphi B, and S. Paratyphi C.

Table (4-3): Identification of Salmonella spp of beef. by the Vitek2 system.

Sample	No. of samples	Bacterial species	Probability
Meat cut	4	*S. Typhimurium*	99%
Minced	9	*S. Typhimurium*	99%
Sausage	1	*S. Paratyphi B*	99%
Burger	1	*S. Paratyphi B*	99%
Meat cut(im)	2	*S. Paratyphi C*	99%
Minced(im)	6	*S. Typhimurium*	99%

Im= imported

Table (4-4): Identification of Salmonella spp in chiken meat by the vitek2 system.

4.5. Molecular identification of *Salmonella*

Sample	No. of samples	Bacterial species	Probability
Carcass skin	9	*S. Enteritidis*	99%
Carcass wash	10	*S. Enteritidis*	99%
Liver	4	*S. Paratyphi B*	99%
Carcass skin (im)	5	*S. Typhimurium*	99%
Carcass wash (im)	1	*S. Paratyphi B*	99%
Liver (im)	2	S. Enteritidis	99%

4.5.1. Polymerase chain reaction results of *Salmonella* isolates

All the isolates 5 red meat and 5 broilers were subjected to the conventional PCR by using specific primers with amplicon size ranged from 284bp. The electrophoresis results are demonstrated in Figure (4-4).

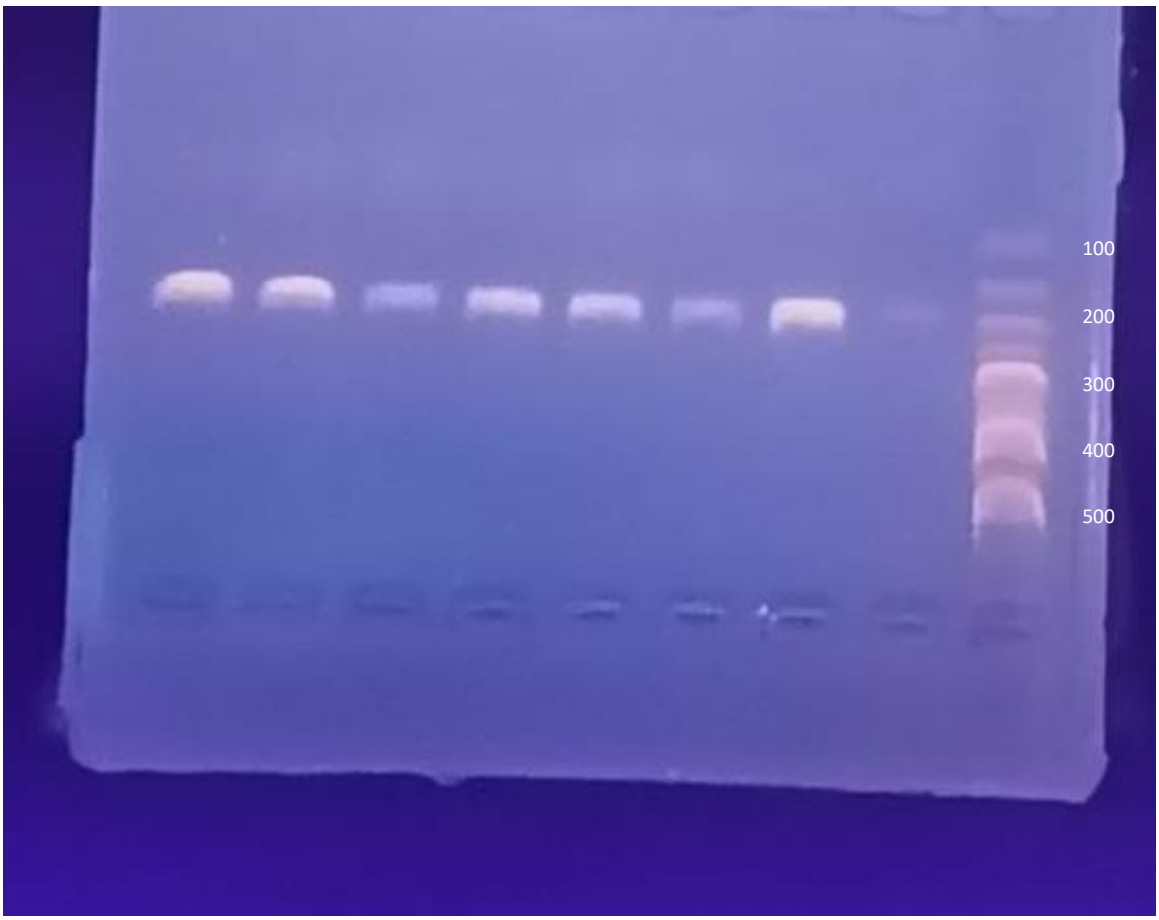


Figure 4-4: Gel electrophoresis results of *Salmonella*isolated from Red meat and Chicken.

4.5.2. Real Time PCR results of *Salmonella* isolates.

The next step was the real time identification method for the final diagnosis of five PCR diagnosed Salmonella isolates, as a final step before the sequencing; all five isolates was subjected to the detection of invA by using RT-PCR, the figure (4-5) demonstrate the cycle threshold of the invA amplification result.

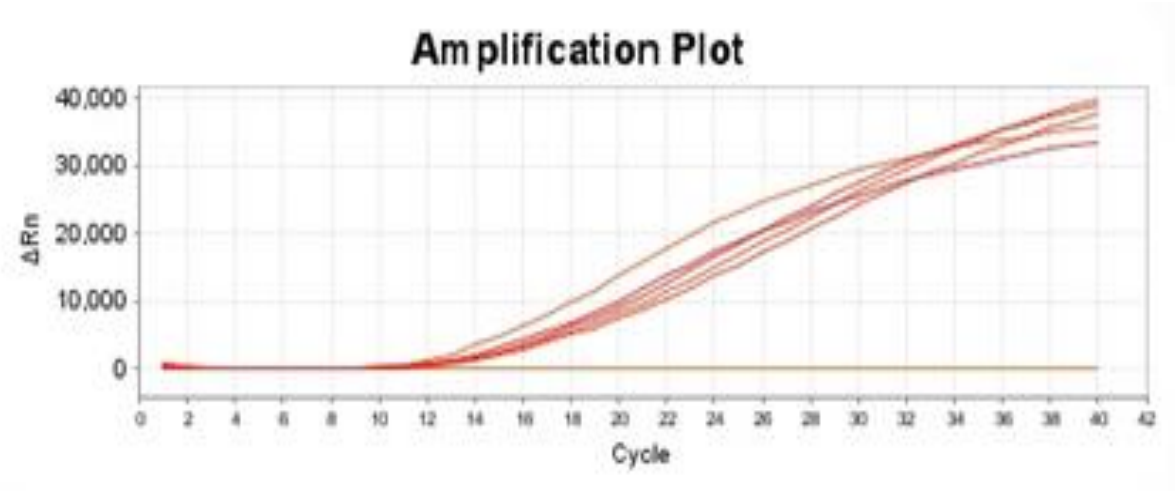


Figure 4-5: cycle threshold of RT-PCR

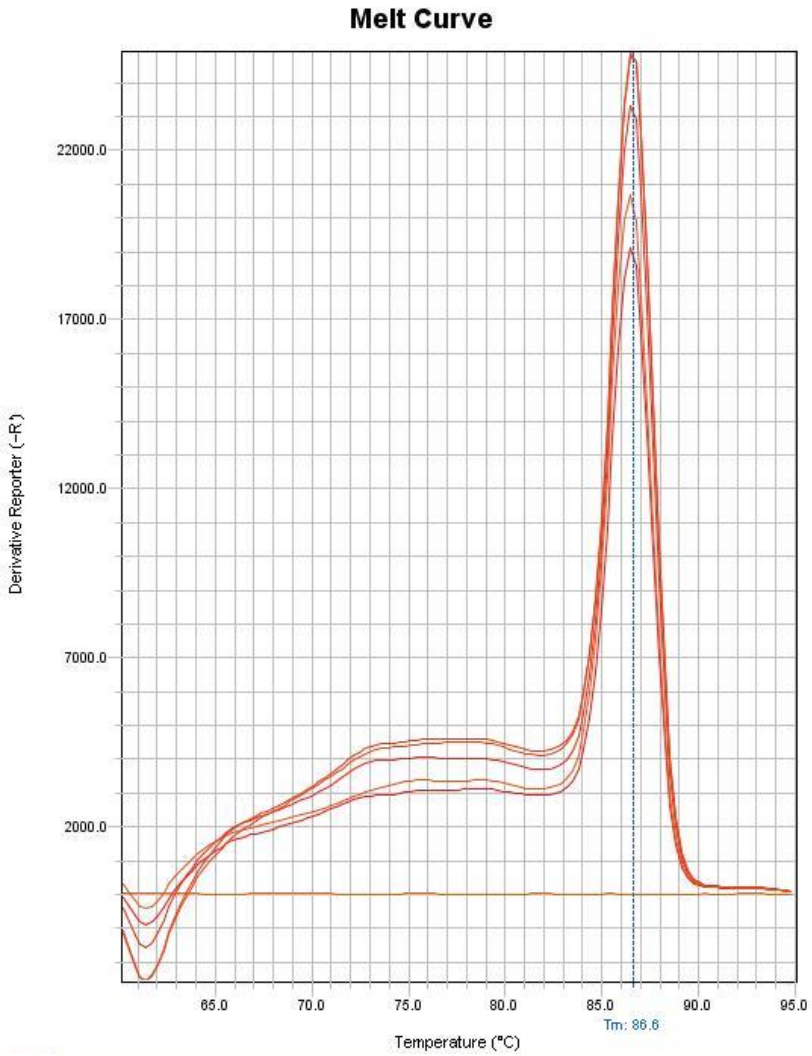


Figure 4-6: RT-PCR the Amplification Carve of invA Gene Expression start in 86 C.

4.5.3.Sequencing and sequence analysis of *Salmonella* isolates

Among the 54 positive isolates of the *Salmonella* genus five isolates selected for sequencing, only two serotypes were identified by using DNA based sequence search. All sequences of the isolates were submitted to the BLASTn search tool of National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov). The two strains *S.* Infantis (3) isolates and *S.* Agona (2) isolates were identified. The significance was measured at P value level ($p<0.01$ and $p<0.05$).

4.6. Characterization of *Salmonella* sequencing .

Two strains were detected among the 5 isolates of the *Salmonella* genus that isolated from red meat and chicken. The BLASTn search hits to NCBI records were as mentioned in Table (4-5).

Table(4-5): NCBI database search results using BLASTn search tool.

Number	Scientific Name	No. of isolates	Percent Identity
1.	*Salmonella* enterica subsp.enterica serovar Infantis strain	1	100%
2.	*Salmonella* enterica subsp.enterica serovar Infantis strain	1	99.05%
3.	*Salmonella* enterica subsp.enterica serovar Infantis strain	1	98.75%
4.	*Salmonella* enterica subsp. enterica serovar Agona	2	100%

4.7.Evaluation of antibiotic susceptibility test

All 54 isolates were tested for their susceptibility to 10 antimicrobial drugs and classified as resistant, and susceptible, Figure (4-7).

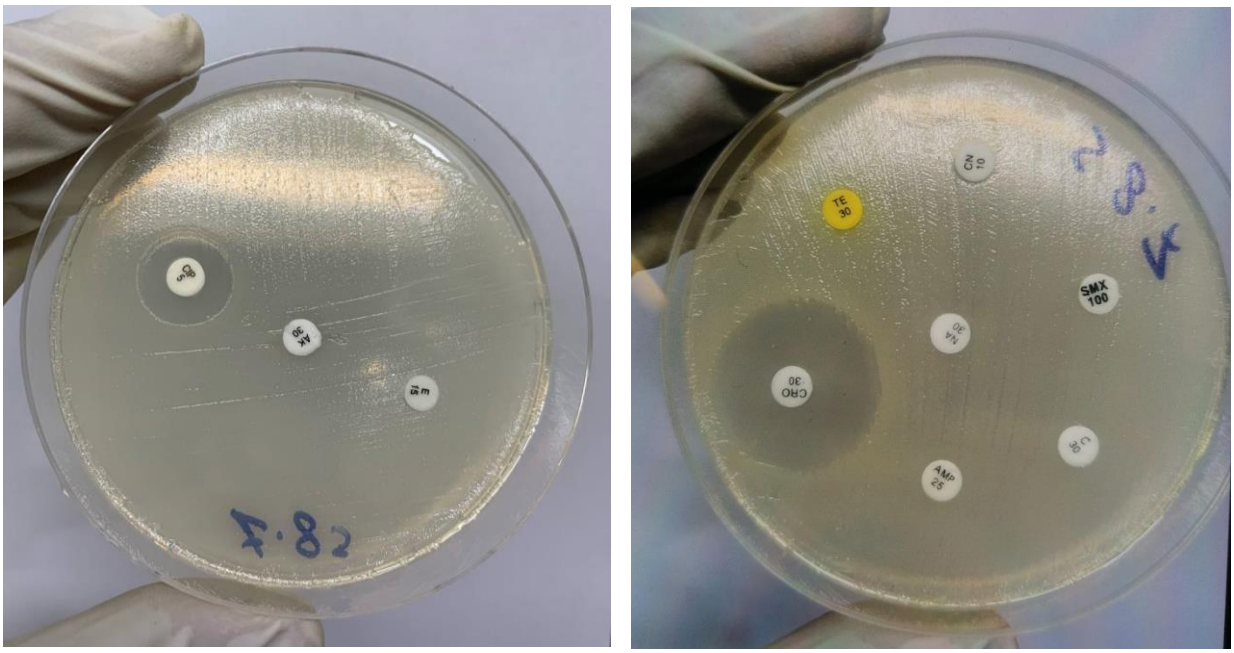


Figure 4-7:This figure demonstratesthe extreme resistance of some *Salmonella* isolates according to the antibiotic susceptibility test.

The prevalence of susceptibility to each antibiotic tested is presented in Table (4-6 and 4-7). From the total positive isolates 54 (17.4%) the resistant pattern is as follows: tetracycline 50 (92.6%), ampicillin 52 (96.3%), nalidixic acid 46 (85.2%), erythromycin 44 (81.4%), sulfamethoxazole 39(72.3%), ciprofloxacin 47 (87%), chloramphenicol 16 (29.6%), and susceptible for ceftriaxone 52 (96.2%), showed significant differences ($p<0.01$) as summarized in Table (4-7).

The results of disc diffusion methods reveled the evaluation of antibiotic resistance among salmonella isolated from red meat samples, the resistant pattern as follows: the highest resistance rate of isolates was (91%) for Ampicillin, and resistance rate for Tetracycline was (87 %), while all salmonella isolates was susaptable for ceftriaxone as shown in table (4-6).

Table (4-6): Evaluation of antibiotic resistance among *Salmonella* isolated from beef samples.

Antibiotic	S	R	P-value
	F (%)	F (%)	
Ampicillin	2 (8.6%)	21(91.4%)	
Gentamicin	3 (13%)	20 (87%)	
Erythromycin	7(30.4%)	16(69.6%)	
Tetracycline	3 (13%)	20(87%)	
Ciprofloxacin	5(21.7%)	18(78.3%)	
Amikacin	17(74%)	6(26%)	
Sulfamethaoxazole	9(39.1%)	14(60.9%)	
Chloramphenicol	22(95.6%)	1(4.4%)	
Nalidixic acid	6 (26%)	17(74%)	P<0.01
Ceftriaxone	23(100%)	0(0%)	

F=Frequency,R=resistant,S=susceptible,Chi-squaredtest.

reveled chicken samples, the resistant pattern as follows: the highest resistance rate of isolates was (100%) for Ampicillin, and resistance rate for Tetracycline was (96%) as shown in table (4-7).

Table (4-7): Evaluation of antibiotic resistance among *Salmonella* isolatedfrom chicken samples.

Antibiotic	S	R	P-value
	F (%)	F (%)	
Ampicillin	0 (0%)	31 (100%)	P<0.01
Gentamicin	10 (32.2%)	21 (67.8%)	
Erythromycin	3(9.6%)	28(90.4%)	
Tetracycline	1 (3.2%)	30(96.8%)	
Ciprofloxacin	2(6.4%)	29(93.6%)	
Amikacin	22(71%)	9(29%)	
Sulfamethaoxazole	6(19.3%)	25(80.7%)	
Chloramphenicol	16(51.6%)	15(48.4%)	
Nalidixic acid	2 (6.4%)	29(93.6%)	
Ceftriaxone	29(93.6%)	2(6.4%)	

Chapter five: Discussion

5. Discussion

5.1.Isolation of Salmonella spp from Beef and Chicken Meat

Percent's of infected samples with Salmonella spp that collected from local markets in the current study table(4-1) were (20%) meat cut, (45%) minced, (5%) sausage, (5%) burger, respectively. Contamination rate of meat cut was similar to (Saad *et al*., 2011) who reported (8%) in Egypt, and was higher than (Mezali and Hamdi 2012) who reported (5.5%) in Algeria local markets. Number of salmonella isolates in minced meat was lower than (Zaiko *et al*.,2021) who documented (18.8%) in minced meat in Moscow, but it's a higher than what found by (Terentjeva *et al*., 2017) in Latvia. The reason for the high contamination rate in minced than other products can be due to mixing of flesh with low quality parts of the carcass such as mouth muscle ,tongue, lymph nodes..et. these parts of carcass are actually contaminated with bacteria by The contamination of the meat mincing machine and the poor storage of meat . Salmonellosis' rate in sausage was lower than what found by (Ed-dra *et al*., 2017) and (Abd El Tawab *et al*., 2015) who report's (15%) and (2.5 %) in Morocco and Egypt respectively. Frequency of salmonella in burger was higher than (El-tawab *et al*., 2015) who found (0%) in Egypt, while it's lower than (Ejo et al. 2016) who revealed (2.9%) in Ethiopia.

Current study data about salmonella isolation from imported meat reporting (10%) meat cut, (30%) minced, (0%) sausage, (0%) burger, respectively. And these of meat cut isolation were a higher than (Sehgal apoorva, 2018) who found (2%) in Egypt, and were lower than (Abdal *et al.*, 2016) who found (40%) in Al- Diwaniyia City, and these of minced meat was comparable with (Kusumaningrum *et al*., 2012) who found (12.5%) of minced meat in Indonesia, and were a higher than what found by (Moustafa *et al*., 2014) in Egypt, and were in sausage lower than what found by (Ertaş *et al*., 2014) and (Hegazy, 2016) who found (4%) and (10 %) in Turkey and Egypt respectively. As well as in burger was similar with (Shaltout *et al*., 2017) who found (0%) in Egypt, but was lower than (Shaltout *et al*., 2017)who found (23.3%) in Assiut City.

Salmonella's Isolates broiler which collected from local markets in the current study were; (36%) skin, (40%) carcass wash and (16%) liver, respectively in table (4-2) , Skin contamination was lower than (Taha *et al*., 2015) who found (19%) in Kurdistan,

Iraq. But in carcass wash were higher than (Rivera-Pérez *et al*., 2014) that found (10%) in California, United States. As well as the isolation rate of liver was higher than (Taib *et al*., 2019) who found (4%) in Duhok, Iraq. The reason is due to the contamination of the carcass from touching the secretions of the internal organs during slaughter, as well as the lack of cleanliness in the place of slaughter and the use of contaminated slaughter tools.

Salmonella Isolates which collected from imported broilers in the current study were: (20%) skin, (4%) carcass wash and (8%) liver respectively in table (4-2), skin contamination was higher than (Taib *et al*., 2019) who found (8%) in Duhok, Iraq, and in carcass wash were a higher than (Naik *et al*., 2015) report's (10%) in India, and in liver was lower than (Ahmed and Khudor, 2019) who found (80%) in Basra, Iraq, the reason behind the high contamination rates of chicken carcasses than beef can be attributed to the contamination from the intestine during evisceration and the lack of cleanliness in the place of slaughter and the use of contaminated slaughter tools.and contaminated slaughter hands and contemined knives and utensils and Isolation rate difference might come from many reasons such as the difference in prevalence between the different geographical regions, study design and the meat and poultry industry being poorly managed in many parts of Iraq, where biosecurity and disease prevention are still lagging and do not receive valuable attention from breeders and slaughters. The causes of contamination in the skin of imported chickens are many, including transportation and poor storage, in addition to contamination from the source and contaminated people.

5.2.Diagnosis using the Vitek2 system

VITEK-2 system was used to confirm the diagnosis of the *Salmonella* isolates depending on the phenotypic characterization by testing a set of biochemical reactions; the technique is based on colourimetric changes and usually requires 18 to 24 h to identify organisms (O'Hara and Miller, 2003)

Vitek-2 system confirms the identification of 54 (100%) of salmonella spp.

Results of the VITEK-2 system indicated that this technique is more accurate than the SS agar and XLD agar. It is a rapid method if we compare it to the traditional isolation and identification procedures, which is consistent with Sariguzel and his colleagues (Wani et al., 2016).

In comparison with other studies, the Vitek2 compact system used in these studies was able to confirm the species S. enterica, but not to the serotype level (Ahmed and Marmar 2015).

5.3. Molecular identification of *Salmonella*

5.3.1.Polymerase Chain Reaction Result of Salmonella Isolates

The invA gene was chosen in current study because it involves sequences distinctive to the genus Salmonella, and has been agreed as an appropriate target for the polymerase chain reaction (PCR) with diagnostic capability (Shanmugasamy *et al*,. 2011). This gene encodes for a protein found in the inner membrane of bacteria and is responsible for the invasion of the host's epithelial cells (Wang *et al*., 2009). This gene is also necessary for complete virulence of Salmonella, and is believed to initiate the internalization needed for deeper tissue invasion (Oladapo *et al*., 2013). Furthermore, the use of the invA gene can significantly reduce the false-negative reports faced by most laboratories (Salehi *et al*., 2005). Accordingly, rapid detection of Salmonella spp. by PCR targeting the invA gene is recommended (Ifeanyi Smith, 2015).

In the current study, the optimization trial for PCR showed successful amplification for the specific invA gene at different annealing temperatures. However, the temperature of 55°C was used later in the next experiments as it showed slightly sharp bands (on the agarose gel) than the other temperatures. The

findings of the current study were in accordance with (Sunar *et al*. 2014) who found (100%) *Salmonella* isolate carrying this gene among 25 samples.

5.3.2. Real Time PCR results of *Salmonella* isolates.

Five isolates were confirmed as Salmonella based on RT-PCR figure (4-5). The same level of accuracy was observed in the previous reports (Monteiro *et al*., 2016). In a recent study, the identification of S. enterica by RT-PCR was considered rapid but high expensive compared with conventional methods (Kasturi, 2020). The current data are in accordance with a report presented by (González-Escalona *et al*., 2009), stating that RT-PCR gives accurate detection of S. enterica and saves time with high efficiency, making it an ideal method of diagnosis (Salman *et al*., 2021).

Many animals can be infected with non-typhoidal *Salmonella* with public health concerns since the ingestion, colonization, and shedding events typically cause no harm to the animals and *Salmonella* that is ubiquitous in the environment. *Salmonella* can therefore contaminate poultry meat (from fecal shedding) or during processing from intestinal leakage and meat slicer, resulting in one of the leading causes of *Salmonella* infections in humans (Najmin *et al*., 2018).

5.3.3.Sequencing of salmonella isolates

Despite the serious clinical consequences recently caused by *S.* Agona and *S.* Infantis, there is a lack of information about the prevalence of these to *Salmonella* or the antimicrobial resistance profile of these serotypes in Iraq. Generally, according to our knowledge, this is the first report of *S.* Agona serotype isolated from minced meat-derived samples in Iraq.

The results of this study showed that 60% of the isolates were *S.* Infantis strain, and the prevalence rate was agreed with (Rahmani *et al*., 2013) that reported the prevalence of this strain was about (75%) among chickens samples in Iran. Meanwhile, (Tirziu *et al.*, 2015) found 18 (42.9%) isolates of S. Infantis isolates among the poultry samples collected from different poultry farms in Romania. Furthermore, in Egypt, (Fekry *et al*., 2018) reported (43.3%) among broiler samples that found a lower prevalence rate of *S.* Infantis.

The results of the current study showed that the prevalence of *S. Agona* was 40% of the isolates, this result was in accordance with (Modarressi and Thong, 2010) in Malaysia that reported 40% of the isolated strain were *S.* Agona from meat samples. In Senegal, (o, Sow *et al.*, 2021) reported (4%) among broiler samples that found a lower prevalence rate of *S.* Agona.

These findings supported that *S.* Infantis was the predominant strain among the samples of the current study, this distribution may be due to inhibition of this serovar the colonization of the other *Salmonella* serovars, and probably its ability to resist antibiotics.

Generally, the results of the current study revealed that the prevalence rate of *S.* Infantis isolates was higher than *S.* Agona among the poultry and meat derived samples, in fact, the differences in the rates can be explained by several causes such as antimicrobial resistance are enzymes designated as "extended-spectrum beta-lactamases" (ESBL) and "ampC beta-lactamases" (AmpC). The bacteria require certain "resistance genes" to produce these enzymes, these genetic properties can be passed on from one bacterial generation to the next through cell division during propagation. However, it can also be passed on from one bacterial cell to another on transmissible gene sections such as plasmids (Franco *et al*., 2015).

This study shed light on an important finding, which is the increasing rate of isolated non-typhoidal *Salmonella* with a public health concern. currently the global increase in the number of isolated *S.* Infantis from broiler, was due to acquiring adaptive chromosomal mutations and a novel mega-plasmid pESI (plasmid for emerging *S.* Infantis), which confers resistance to multiple drugs, heavy metals, and disinfectants, but also enhances its virulence-associated phenotypes and its pathogenicity (Aviv *et al*., 2014).

5.4.Evaluation of Antibiotic Susceptibility Test

The resistance rate of tetracycline(87%), ampicillin(91.4%), nalidixic acid(74%), sulfamethoxazole(60.9%), and ciprofloxacin(78.3%) in the current study table (4-6) was agreed with the results of (Hameed *et al*,. 2014); (Harb *et al*., 2018); (Hassan and Alhatami, 2019) among poultry and meat samples in Al-Hilla province (85%), the Middle Euphrates, and in Thi-Qar governorate (100%) respectively. The high percentage of tetracycline and ampicillin is due to improper use as well as long-term use in raising and treating poultry and livestock.

Furthermore, the resistance rate of erythromycin (90.4%) among *Salmonella* isolates in the current study was compatible with the results of studies in other countries such as Turkey and Egypt that(89.7%)(90%) (Yildirim *et al*., 2011); (Abd-Elghany *et al*., 2015). While the resistance rate of ceftriaxone (6.4%) in this study was in accordance with (Sodagari *et al*., 2015) results in Iran.

These findings support that the increased antibiotic resistance in zoonotic bacteria could be due to indiscriminate and unrestricted use of antimicrobial agent whether in treatment or prevention in a poultry and cattle farms due to the lack of a definitive diagnosis. Moreover as a growth promoter in the poultry industry, this means that these rates of resistance changed according to the source of isolate and the antibiotic selective pressure and many other factors affecting the antibiotic resistance pattern among poultry and cattle beyond the geographic factor (Ja *et al*., 2017).

5.5. Salmonella Sources of Infection

Salmonella spp. mainly occurs as animals sources and transmitted to human through environmental processing as travelling ; food industry ; animals un hygiene managements.((Kareem , 2018) and (Mahdi , 2019). Salmonella experimentally induced sever pathological changes and cause to elevated value of apoptosis in infected tissues.(Maha and Muna., 2020).

Chapter Six: Conclusion and Recommendation

6.1. Conclusion:

1) The fresh and imported meat can be infected by *salmonella*, the prevalence percentage of *salmonella* in poultry was 23 , red meat was 31 isolate
2) Minced meat and skin of chicken was more predictive host for salmonella SPP infection was more predictive host for *Salmonella SPP* infection
3) The prevalence rate *S. Infantis* strain was 60% and 40% for *S. Agona,* in red meat and poultry sample
4) The number of isolated *S. Infantis* from broiler was higher than other samples
5) Multi drug resistance salmonella was detected in red meat at kerbala province
6) The finding also found out that 5 isolation were subjected to the detection of InvA gene by usig Rt-Pcr were reported as Salmonella enterica

6.2. Recommendation.

1. Make newly trains to investigate about different types of *Salmonella Spp* on others food industry samples and prevalence rate of *Salmonella* in iraq
2. Specific identification on special gene that causes seriously diseases in concern with animals and human health
3. Put critical orders for meat hygiene for protection from food born n's diseases pathogens
4. Put disinfectant management before and after brushed meat from local market and Prevent handling of local and important meat as possible to prevent contamination
5. Studying technological methods to preservation of food or prevent contamination such as nanomaterial (bacteriocin) that produced by LAB, antioxidant agent,plant traction and some organic acid.

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الخلاصة

أجريت هذه الدراسة في محافظة كربلاء خلال الفترة من تشرين الثاني (نوفمبر) 2021 إلى آذار (مارس) 2022. تم جمع 310 عينة من لحوم الدجاج واللحوم الحمراء المحلية والمستوردة.

جمعت هذه العينات من مواقع مختلفة في محافظة كربلاء وزرعت على وسط مناسب لزراعة السالمونيلا وتحديد هويتها.

ثم تلتها عملية العزل البكتيري الأولي على وسط الاستزراع الخاص والمميز لبكتيريا Salmonella spp.

أظهرت النتائج أن معدل التلوث في جميع العينات المفحوصة كان 54 (17.4٪) من مجموع العينات 310 ، كما بلغ معدل عزل اللحوم الحمراء 23 (14.3٪) من مجموع 160 عينة ، وكذلك معدل عزل عينات الفراريج. كان 31 (20.6٪) من مجموع العينات 150.

علاوة على ذلك ، كان معدل تلوث السالمونيلا بين اللحوم الحمراء 4 (20٪) لحم مفروم ، 9 (45٪) لحم مفروم ، 1 (5٪) سجق ، 1 (5٪) برجر لعينات اللحوم الحمراء المحلية ، و معدل تلوث السالمونيلا للعينات المستوردة كان 2 (10٪) لحم مفروم ، 6 (20٪) لحم مفروم ، 0 سجق ، 0 برجر.

من ناحية أخرى بلغ معدل تلوث السالمونيلا بين عينات الدجاج اللاحم 9 (36٪) جلود ، 10 (40٪) غسل ذبيحة ، 4 (16٪) كبد لعينات الفراريج المحلية ، ومعدل تلوث بكتيريا السالمونيلا للفروج. العينات المستوردة كانت 5 (20٪) جلد ، 1 (4٪) غسيل ذبيحة ، 2 (8٪) كبد.

تم إجراء النموذج الإحصائي لمعدل التلوث في الدراسة الحالية لبيان الفروق من خلال تطبيق اختبار Chi-square ، حيث تم قياس الدلالة عند مستوى القيمة p) P <0.05). أظهرت النتائج وجود فروق ذات دلالة إحصائية بين العينات التي تم جمعها والعزلة الإيجابية من هذه العينات.

تم تقييم مقاومة المضادات الحيوية بين عزلات السالمونيلا من كل من اللحوم الحمراء والفروج من خلال الدراسة الحالية ، وأظهرت النتائج وجود مقاومة في 50 (92٪) من عزلات Salmonella Spp للتتراسيكلين و 52 (96٪) أمبيسلين.

تم إجراء دراسة لتحديد 10 عزلات من Salmonella spp تم اكتشافها سابقًا من اللحوم الحمراء والفروج باستخدام PCR التقليدي و Real Time PCR بالإضافة إلى 5 عزل تم استخدامها لتسلسل الجينات الوراثية ونوع الفوعة الجينية من Salmonella spp. أظهرت النتائج الحالية أن 10 عزلة (100٪) تم تحديدها على Salmonella Spp بواسطة تفاعل البوليميراز المتسلسل التقليدي. وجدت النتائج الحالية أيضًا أن 5 عزل تعرضوا للكشف عن الغزو باستخدام RT -PCR تم الإبلاغ عن السالمونيلا المعوية فيما يتعلق بالتسلسل والتحليل المتسلسل لعزل اللحوم الحمراء 2 Salmonella (من أصل 2 تم فحصها) تم الإبلاغ عنها على أنها S. Agona ، والتي 3 تم الإبلاغ عن عزلات من دجاج التسمين (من أصل 3 تم فحصها) S.Infantis ، ويمكن الاستنتاج من هذه الدراسة أن اللحوم المفرومة كانت ملوثة أكثر من المنتج الآخر ، وأظهرت السالمونيلا المعوية مقاومة للتتراسيكلين ، الأمبيسلين.

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة كربلاء

كلية الطب البيطري

عزل وتحديد اصناف السالمونيلا من لحوم الابقار و الدواجن في محافظه كربلاء

رسالة

مقدمة إلى مجلس كلية الطب البيطري في جامعة كربلاء كجزء من متطلبات نيل درجة الماجستير في فرع الصحة العامة

كتبت بواسطة

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2022 م