An-Najah National University
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Molecular Characterization of *Salmonella Enterica* Serotype Typhimurium and Enteritidis Isolates from Food Samples in West Bank / Palestine

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Dedication

I would dedicate my sincere appreciation to my precious mother and father who have always encouraged me to move forward and always were there for me when I needed them. I also dedicate this dissertation to my loving sisters and brothers who were always standing by my side. I will always appreciate all what they have done. And, to my dear friends who always supported me and were always there for me, great love and thanks to them. And special thanks to my work colleagues in Qalqelyia who always supported me and gave me convenience while working on this research.

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الاقرار

أنا الموقعة أدناه مقدمة الرسالة التي تحمل العنوان:

Molecular Characterization of *Salmonella Enterica* Serotype Typhimurium and Enteritidis Isolates from Food Samples in West Bank / Palestine

التوصيف الجزيئي لعزلات السالمونيلا المعوية من النمط المصلي Typhimurium و Enteritidis من عينات الغذاء في الضفة الغربية / فلسطين

أقر بأن ما اشتملت عليه هذه الرسالة إنما هي نتاج جهدي الخاص ،باستثناء ما تمت الإشارة إليه حيث ما ورد، وإن هذه الرسالة ككل، أو أي جزء منها لم يقدم من قبل لنيل أية درجة عملية أو لقب علميا وبحثي لدى أية مؤسسة تعليمية أو بحثية أخرى.

Declaration

The work provided in this thesis, unless otherwise referenced, is the researcher's own work, and has not been submitted elsewhere for any other degree or qualification.

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Date: التاريخ: 30/12/2021

Table of Contents

List of Tables

List of Figures

List of Abbreviations

Symbol	Abbreviation
PCR	Polymerase Chain Reaction
mPCR	Multiplex- Polymerase Chain Reaction
REP-PCR	Repetitive Extragenic Palindromic Sequences-Polymerase Chain Reaction
RAPD-PCR	Random Amplification of Polymorphic DNA-Polymerase Chain Reaction
ERIC-PCR	Enterobacterial Repetitive Intergenic Consensus-Polymerase Chain Reaction
BOXAIR-PCR	A primer corresponding to the BOXA subunit of the BOX element PCR
WHO	World Health Organization
EFSA	European Food Safety Authority
ECDC	European Centre for Disease Prevention and Control
S.	*Salmonella*
V	Virulotype
Spp.	Species
LPS	Lipopolysaccharides
C	Cluster or Clone
n	Number
No.	Number
L	Lane
var	Serovar
H_2S	Hydrogen Sulfide
NTS	Non Typhoidal *Salmonella*
antisera	Antiserum
EU	European Union
Subsp.	Subspecies
MS	Mass Spectrometry
DNA	Deoxyribonucleic acid

AFLP	Amplified Fragment Length Polymorphism
PFGE	Pulsed-Field Gel Electrophoresis
PCR-RFLP	Polymerase Chain Reaction-Restriction Fragment Length Polymorphism
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
PU	Palindromic Units
MLST	Multilocus Sequence Typing
BOX	Box Element PCR
REP	Repetitive Extragenic Palindromes
SE	*Salmonella* Enteritidis
ST	*Salmonella* Typhimurium
MgCl2	Magnesium Chloride
Mg2+	Magnesium ion
TSI	Triple Sugar Iron
SIM	Sulfide-Indole-Motility test
ATCC25922	Nonpathogenic Strain of Escherichia Coli
pH	A scale of acidity from 0 to 14
Tris-HCl	(hydroxymethyl) aminomethane (THAM) hydrochloride
mM	Millimole
min.	Minutes
g	Gram
s	Seconds
µM	Micro Molar
µl	Micro liter
bp	Base pair
µg/ml	Microgram per Milliliter
U	Unite
ng	Nanogram
ºC	Degree Celsius

et al.	and others
DMSO	Dimethyl sulfoxide
rDNA	Ribosomal DNA
dNTPs	Deoxynucleoside triphosphate
TM	Melting Temperature
STMO159	A putative restriction endonuclease for *S.* Typhimurium
SEN1383	A hypothetical protein for *S.* Enteritidis
Taq	Thermus Aquaticus DNA Polymerase
UV	Ultraviolet
E. coli	*Escherichia Coli*
P.aeruginosa	*Pseudomonas aeruginosa*
X	Times
Tris-EDTA	Ethylenediamine Tetraacetic Acid; buffered solution
GTG	Giemsa-Trypsin-Giemsa /poly-trinucleotide
T3SS	Type III secretion systems
SSCP	Single-Strand Conformation Polymorphism
SPIs	*Salmonella* Pathogenicity Islands
PAIs	Pathogenicity Islands or Pathogenicity Islets
S.1,4,[5],12:i:	A Monophasic Variant of *Salmonella* Typhimurium
H antigen	Flagellar antigen
O antigen	Somatic antigen
K antigen	Capsular polysaccharide antigen
TTSS	Type III Secretion System
SPSS	Statistical Package for the Social Sciences
UPGMA	Unweighted Pair Group Method for Arithmetic Averages
SPI	*Salmonella* Pathogenicity Island
ESBLs	Extended-spectrum beta-lactamases
MBLs	Metallo-β-Lactamases
β-lactamases	Beta-lactamases

MLVA	Multiple locus variable number of tandem repeats analysis
MSC	Masters of Sciences
pUO-StVR2.	Virulence-resistance plasmid which originated from pSLT of *Salmonella enterica* serovar Typhimurium
Vi antigen	Capsular protein antigens / Virulence antigen
invA	Invasion gene A
IBM	International Business Machines Corporation
OPP-16	RAPD Primer/Genetic marker
F	Forward
R	Reverse
spv	*Salmonella* plasmid virulence
pefA	Plasmid encoded fimbriae A
sitC	*Salmonella* iron transport

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Abstract

Salmonellae is one of the most frequently isolated foodborne pathogens. It is of major public health concern worldwide. Poultry meat and eggs represent an important source of Salmonellae organism for consumer health. The occurrence of virulence factors among Salmonellae Typhimurium (*S.* Typhimurium) appears to be lacking in Palestine. This study aimed to evaluate the occurrence of *S.* Typhimurium and *S.* Enteritidis using multiplex PCR (mPCR) among isolates collected from the local market, and to assess genetic relationships between strains of *S.* Typhimurium using virulence factors profiling and fingerprint profiling by RAPD-PCR and repetitive sequence PCR (REP-PCR) using ERIC-PCR and BOXAIR-PCR.

The overall occurrence percentage of *S.* Typhimurium and *S.* Enteritidis was 54.9% and 0.0%, respectively. Only 13 out of 17 virulence genes were detected in these 28 isolates. The occurrence of the detected genes among these isolates was 100%, 50%, 46.4%, 39.3%, 35.7%, 35.7%, 32.1%, 25%, 25%, 17.6%, 14.3%, 14.3%, 3.6% for *invA*, *sopB*, *prgH*, *sitC*, *pefA*, *tolC*, *cdtB*, *msgA*, *sifA*, *iroN*, *spiA*, *ipfC* and *pagC*, respectively. The remaining

virulence genes were absent in all of the isolates. Based on the combination of presence and absence of virulence genes, eight profiles were detected among these isolates, the most common genetic profile was V5 (each 32.1%). In the present study, on the basis of their genetic profile at cut-off point 96%, both ERIC and BOX primers allowed for discrimination into 4 and 6 clusters or clones of 16 *S.* Typhimurium isolates, respectively. Results of PCR typing methods showed that, strains S83 (chicken wings), S86 (chicken), and S87 (chicken) are clustered together using both ERIC-PCR and BOX-PCR typing methods and they had the same virulotype (V1) and strains S53 (chicken), S73 (chicken), S78 (beef burgher) and S80 (beef burgher) also clustered together by both typing methods and had the same virulotype (V8).

The following conclusion with potential implication for the isolation and identification of Salmonellae from food sources were drawn; Contamination of food with Salmonellae especially with *S.* Typhimurium was high and indicated a bad microbiological quality of food. In addition, the data presented were considered the first attempt to identify a wide range of virulence genes of the *S.* Typhimurium isolates recovered from different food types in the Palestinian market. This emphasizes the need for rigorous public health and food safety methods to lower the human health hazard and risk associated with Salmonellae infection.

Chapter One

Introduction

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1.1 General background

1.1.1 *Salmonella* infection (Salmonellosis)

Foodborne microorganisms are major pathogens affecting food safety and causing human illness worldwide. These foodborne infections and intoxications result from the consumption of various foodstuffs, mainly animal products contaminated with vegetative pathogens or their toxins. Most of these microorganisms have zoonotic nature, resulting in a significant impact on both human public health and the economic sector (Abebe *et al*., 2020).

According to the World Health Organization (WHO), foodborne diseases are defined as diseases of infectious or toxic nature which are caused by the consumption of food or water (Abebe *et al*., 2020). Approximately, 250 known causative agents can cause foodborne diseases; these include bacteria, parasites, viruses, prions, toxins, and metals. The symptoms and severity of these foodborne illnesses vary, ranging from mild gastroenteritis to life-threatening neurologic, hepatic, and renal infections (Argaw and Addis, 2015). WHO has reported that 1.8 million childhood deaths were due to acute diarrheal diseases, predominantly in the developing countries, and a high proportion of these cases were due to contamination of food products and potable water (WHO, 2008). Although large numbers of bacterial strains have been identified to be involved in foodborne diseases,

many other new emerging strains were also reported (WHO, 2008). In the developed counties, the annual incidence of microbiological foodborne illnesses is estimated to be around 30% of the population (De Guisti *et al*., 2007).

Approximately, 60% of human illnesses are zoonotic diseases that are mainly transmitted to humans from animals and about 75% of new emerging human infectious diseases are transferred from vertebrate animals to humans (Abebe *et al*., 2020). Bacteria are the causative agents of two-thirds of human foodborne diseases worldwide with a high burden in the developing countries. The most frequent bacterial pathogens that can cause foodborne diseases and deaths in the world including *Campylobacter* species, *Salmonella* spp., *Staphylococcus aureus* (*S. aureus*), *Listeria monocytogenes* (*L. monocytogenes*), and *Escherichia coli* (*E. coli*) (Abebe *et al*., 2020).Animal-based food particularly dairy products (milk, cheese, yogurt, and ice cream), meat (beef, mutton, and pork), poultry and eggs are the main reservoirs by which humans are exposed to the pathogenic bacteria including *Salmonella* spp. (Abebe *et al.*, 2020).

According to the WHO, *Salmonella* spp. are among the 31 pathogenic agents showing the highest ability of provoking intestinal or systemic disease in humans among diarrheal and/or invasive pathogens, and the third causative agent of death among food-borne diseases (Ferrari *et al*., 2019). *Salmonella* are considered one of the most frequently isolated foodborne pathogens worldwide (Abebe *et al*., 2020; Eng *et al*., 2015). Foodborne

illnesses including salmonellosis have become serious public health problems in many countries in the recent decade (Abuseir *et al*.,2020). Non-typhoid *Salmonella* accounts for 93.8 million foodborne infections and 155,000 deaths per year (Eng *et al*., 2015). In China, 70%-80% of foodborne bacterial outbreaks are attributed to *Salmonella* infection (Li *et al.,* 2020).

There are more than 2,600 serotypes for the genus *Salmonella*, most of these serotypes have the ability to adapt within different types of animal hosts, including humans. In addition, more than half of *Salmonella* serotypes belong to *Salmonella enterica* subsp. *enterica*, which is associated with the majority of *Salmonella* infections in humans (Eng *et al*., 2015).

Salmonellosis is an important zoonotic infection seen in all animal species (Seifi *et al*., 2019). It is considered the second major cause of foodborne disease worldwide, which may lead to severe symptoms and death (Scallan *et al*., 2011; EFSA and ECDC, 2019; Abuseir *et al*., 2020; Jeníková *et al*., 2000). *Salmonella* serotype Typhimurium (*S.* Typhimurium) and *Salmonella* serotype Enteritidis (*S*. Enteritidis) are considered the most common serotypes that can cause infections in both humans and animals (Kaushik *et al*., 2014). Clinically, *Salmonella* spp. have been categorized into 2 groups based on their ability to develop specific pathologies in humans; these are invasive (typhoidal) or noninvasive (non-typhoidal *Salmonella*) (Okoro *et al*., 2012). In humans, *Salmonella* spp. can cause

gastroenteritis and enteric fever with bacteremia, resulting from foodborne infection (Eng *et al*., 2015). Typhoidal serovars (*S.* Typhi and *S.* Paratyphi A) do not infect animals but they can cause typhoid fever to humans. So typhoid fever is not considered a zoonotic disease, and it can display several symptoms to humans, such as high fever, diarrhea, vomiting, headaches, and, in extreme cases, death. Therefore, the presence of typhoidal serovars indicates contamination from sick individuals or chronic carriers through poor hygiene management during food and water handling (Ferrari *et al.,* 2019; Abebe *et al*., 2020). Non-typhoidal *Salmonella* is considered one of the most important zoonotic bacterial foodborne pathogens. The most common non-typhoidal *Salmonella* reservoir is the intestinal tract of a large number of domestic and wild animals and a variety of food matrices that can serve as vehicles for transmission of *Salmonella* spp. to humans through fecal contamination (Ferrari *et al*., 2019).Animal products are considered the main vehicles of salmonellosis due to the ability of *Salmonellae* to survive in meat and animal products that are not thoroughly cooked or not properly handled (Akoachere *et al*., 2009).A wide range of animal-origin food products such as milk, eggs, poultry, beef, and pork are considered the major source for the transmission of non-typhoidal *Salmonella*. Raw poultry products including eggs are considered a significant reservoir for *Salmonella* and are often and consistently implicated in human salmonellosis sporadic cases and outbreaks (Abebe *et al*., 2020). Eggs may be contaminated on the outer surface of the shell and internally (Abuseir *et al*., 2020). The existence of

Salmonella in healthy poultry is considered a major risk factor, that is responsible for transporting the infection from poultry products such as meat and table eggs to humans. Poultry can be infected fundamentally with *S.* Enteritidis, *S.* Typhimurium, and *S.* Heidelberg, these serotypes are distributed worldwide and they are of major economic and public health significance (Abuseir *et al*., 2020).

Past studies have reported that chopping boards, butchers' hands, and knives used for retail chicken processing constitute potential sources for *Salmonella* cross-contamination (Li *et al*., 2020). The cross-contamination between meats and personnel and equipment used during the day in the processing of meats due to improper and ineffective cleaning and disinfection particularly with chopping boards, knives, and tables were the risk factors for *Salmonella* contamination (Dhanalakshmi *et al*., 2018; Issa *et al.,*2017).

1.1.2 Nomenclature/Taxonomy

The genus of *Salmonella* contains two species, *Salmonella bongori* and *S. enterica*, the latter is further subdivided into six subspecies: *S. enterica* subsp. *enterica*, *S. enterica* subsp. *salamae*, *S. enterica* subsp. *arizonae*, *S. enterica* subsp. *diarizonae*, *S. enterica* subsp. *indica*, and *S. enterica* subsp. *houtenae*, or I, II, IIIa, IIIb, IV, and VI, respectively. Of all the subspecies of *Salmonella*, the *S. enterica* subsp. *enterica* (I) is the most common and is found predominantly associated with most infections in human and worm-blooded animals. On the other hand, the other five subspecies of *S. enterica*

and *S. bongori* are mainly found in cold-blooded animals and the environment and rarely in humans (Porwollik *et al.,* 2004; Jajere, 2019). Serotyping is considered the first step to characterize *Salmonella* isolates although it does not provide sufficient discriminatory subtyping for outbreaks investigation. The conventional method to define a *Salmonella* serotype is a phenotypic method, based on the standard Kauffman-White-Le Minor scheme. The serotype is based on the agglutination of the bacteria with specific antibodies to identify three groups of surface structures expressed on the bacterial lipopolysaccharide (LPS) somatic (O), flagella (H), and capsular polysaccharide (K) antigens (Ferrari *et al.,* 2019). This provides the antigenic formula of the strain associated with the name and subspecies of the serotype. Until now, there are 46 different serotypes of O antigens and 114 different serotypes of H antigens identified in *Salmonella* spp., different combinations between these antigens, more than 2600 serotypes were detected (Diep *et al.,* 2019). The surface K antigens are rarely found among the majority of *Salmonella* serotypes and are heat-sensitive polysaccharides mainly located at the bacterial capsular surface (Jajere, 2019).

1.1.3 Morphology, Bacteriological Culture, and Isolation Procedures

The bacterial genus *Salmonella* is 0.2 to 1.5 by 2 to 5 μm in size, Gram-negative bacillus, facultative anaerobe, non−spore former that belongs to the family Enterobacteriaceae (Okoro *et al*., 2012; Abed Al-Daym, 2019; Jajere, 2019). *Salmonella* spp. grow in a pH range of 4 to 9 with the

optimum pH between 6.5 and 7.5. Members of this genus are motile by the means of flagella, with the exception of *Salmonella* Gallinarum (*S.* Gallinarum) and *Salmonella* Pullorum (*S.* Pullorum). Most of the *Salmonella* serotypes have the ability to produce hydrogen sulfide (H_2S) with the exception of a few serotypes such as *Salmonella* Paratyphi A (*S.* Paratyphi A), and *Salmonella* Choleraesuis (*S.* Choleraesuis). This pathogen is considered a non-fastidious bacterium that can grow in a simple nutrient medium and multiply under various environmental conditions outside the living hosts. Enrichment broths for *Salmonella* such as Strontium selenite and selenite F broth and selective and differential media such as MacConkey, deoxycholate agar, and *Salmonella-Shigella* agar are widely used in the laboratory for the culture of the suspected sample. Most of the *Salmonella* strains are non-lactose fermenting bacteria and this property has been used for the development of many differentials and selective media for the isolation and identification and diagnosis of *Salmonella* isolates. These media include xylose lysine decarboxylate agar, *Salmonella-Shigella* agar, brilliant green agar, Hektoen enteric agar, MacConkey's agar, lysine iron agar, and triple sugar iron agar. Generally, isolation of *Salmonella* using culture method from different types of food and environment sample needs the multiple steps of pre-enrichment and selective enrichment and growth on the selective and differential media to increase the sensitivity of the detection assays (Abed Al-Daym, 2019; Jajere, 2019). After isolation, identification of the genus *Salmonella* is carried out by certain biochemical tests. The presumptive biochemical

identification of *Salmonella* then can be confirmed by antigenic analysis of both O and H antigens using polyvalent and specific antisera. Now, various *Salmonella* serotypes can be identified by polymerase chain reaction (PCR) technique using specific primers (Kaushik *et al*., 2014; Malorny *et al*., 2003). Although, isolation of *Salmonella* by conventional methods, such as growth in a culture medium followed by serotyping is considered the gold standard method for confirmation of *Salmonella*. However, conventional *Salmonella* serotyping is laborious and time-consuming. Conventional bacterial culture methods are still used most often to detect and identify *Salmonella*, these methods require at least several days including selective enrichment and plating followed by biochemical tests. Recently, PCR-based techniques are used effectively for rapid detection of *Salmonella* serovars using specific primers for a target gene. However, effective surveillance of foodborne pathogens can be achieved through a combination of conventional and PCR-based techniques (Kaushik *et al*., 2014; Seifi *et al*., 2019).

1.1.4 *Salmonella* Typhimurium and *Salmonella* Enteritidis

Salmonella Typhimurium, *S.* Enteritidis, *S.* Heidelberg, and *S.* Newport are the epidemiologically important non-typhoidal *Salmonella* serotypes, which have been responsible for the majority of human *Salmonella* disease burden worldwide (Jajere, 2019).In the European Union, the second most frequently bacterial genus involved in gastrointestinal outbreaks in humans is *Salmonella* and more particularly the species *S.* Enteritidis and *S.*

Typhimurium (Paniel *et al.,* 2019).*S.* Enteritidis and *S*. Typhimurium are prevalent in poultry, and about 95% of cases are caused by the consumption of contaminated food, especially meat and eggs. Poultry are considered one of the most important reservoirs of *Salmonella* that can transmit these non-typhoidal *Salmonella* serotypes to humans through the food chain. *S.* Typhimurium is the most frequently isolated serovar from broilers (Dhanalakshmi *et al.,* 2018). The gastrointestinal tract is considered the main reservoir of *Salmonella* in mammals (cattle and pigs) and poultry (Paniel *et al.,* 2019). Farm animals carrying these microorganisms barely develop symptoms, making it almost impossible to notice these infections (Paniel *et al.,* 2019).Contaminated poultry products such as meat and eggs continue to play a central role in the spreading the infection of the *S*. Enteritidis and *S*. Typhimurium serovars to humans (Ferrari *et al.,* 2019; Wang *et al.,* 2019; Paniel *et al.,* 2019).Epidemiological studies showed that, unlike other non-host adapted *Salmonella* serotypes such as *S*. Typhimurium, which is isolated from a variety of food animal sources, *S*. Enteritidis is predominantly recovered from poultry, suggesting that serovar has likely developed to acquisition a significant tendency to the poultry host (Shah *et al*., 2017). In addition, *S*. Typhimurium has also been detected in a wide range of poultry- and animal-derived foods such as retail chickens and pigs from various market types i.e. wet markets and supermarkets and animal products stored at various temperatures i.e. ambient, chilled, and frozen (Li *et al.,* 2020).

In 2015, *S.* Enteritidis was representing 45.7% of all reported serovars in confirmed human cases (EFSA and ECDC, 2016) and accounted for 60.3% of all *Salmonella* outbreaks and 61.1% human cases in all *Salmonella* outbreaks in the EU countries (EFSA and ECDC, 2017). The prevalence of *S.* Enteritidis among *Salmonella* isolates recovered from different types of samples including food has been reported. The prevalence ranged between 1.3% and 67.8% (Busani *et al.,*2005; White *et al.,* 2007; Jalali *et al.,* 2008; Moussa *et al.,* 2010; Harsha *et al.,* 2011; Ramya *et al.,* 2012; Hassanin *et al.,* 2014; Magwedere *et al.,* 2015;Thung*et al.,* 2016; El-Tayeb *et al.,* 2017; Amajoud *et al.,* 2017; Proroga *et al.,* 2018; Tegegne, 2019; Elkenany *et al.,* 2019; Siriken *et al.,* 2020). Among more than 2,500 serovars of *Salmonella enterica*, *S.* Typhimurium was one of the most frequently isolated worldwide (Medeiros *et al.,* 2015), and it is one of the leading serovars that cause salmonellosis worldwide (Medeiros *et al.,* 2015).

A study conducted in India showed that out of 370 samples, 23.7% chicken meat and 7.7% milk samples were found positive for *Salmonella* based on biochemical reactions. The serotyping of *Salmonella* isolates showed an incidence of 6.1% of *S.* Typhimurium, 2.6% of *S*. Newport, 1.7% of *S*. Gallinarum, and 0.4% each of *S*. Enteritidis, *S*. Infantis, and *S*. Worthington in broilers; and 2.1% of *S*. Typhimurium and 1.4% of *S*. Newportin market milk samples (Kaushik *et al.,* 2014).

A study conducted in Egypt showed that the occurrence of *S*. Enteritidis and *S*. Typhimurium in raw chicken meat was 2.0% and 3.0%, respectively (Tarabees *et al.,* 2017; Abuseir *et al.,* 2020)

A systematic review from Ethiopia revealed that *S*. Typhimurium (prevalence of 9.4%) was ranked third most common serotype. In the United States, *S*. Enteritidis and Typhimurium are among the top five most common serotypes reported (Scallan *et al.,* 2011; Al-Rifai *et al.,* 2019).

In Shaanxi, among the 406 *Salmonella* isolates that belonged to 39 serotypes, *S*. Typhimurium was the most prevalent. This serotype, one of the most important worldwide, contributes to deaths in young broiler chickens and salmonellosis in humans (Li *et al.,* 2020).

Prior studies conducted in Africa and North America have revealed that *S*. Typhimurium is the most common serotype in cattle and chickens (Li *et al.,* 2020). Also, a study conducted in Shaanxi Province, China in 2020 revealed that *S*. Typhimurium was the predominant serotype in retail raw chickens, followed by *S*. Thompson, *S*. Essen, *S*. Infantis, *S*. Riseen, and *S*. Enteritidis (Li *et al.,* 2020).

Between 2001 and 2007 in the USA, Canada, Australia, and New Zealand, *S*. Typhimurium was the leading isolated serovar. In the same period, *S*. Typhimurium appeared as the second most isolated serovar in Africa, Asia, Europe, and Latin America, exceeded only by *S.* Enteritidis (Medeiros *et al.,* 2015). Foodborne outbreaks of salmonellosis have been most frequently associated with *S.* Enteritidis and *S.* Typhimurium in India (Dhanalakshmi *et al.,* 2018). The prevalence of *S.* Typhimurium among *Salmonella* isolates recovered from different types of samples including food has been reported. The prevalence had a range 3.6%-52.9% (Busani *et*

al., 2005; Moussa *et al.,* 2010; Hassanin *et al.,* 2014; Magwedere *et al.,* 2015; Ammar *et al.,* 2016; Amajoud *et al.,* 2017; El-Tayeb *et al.,* 2017; Proroga *et al.,* 2018; Nouichi *et al.,* 2018; Elkenany *et al.,* 2019; Issa *et al.,*2017; Al-Dawodi *et al.*,2012; Habib *et al.,*2021).

1.1.5 PCR-based typing methods

Identifying and typing *Salmonella* isolates are crucial for diagnosis, treatment, epidemiological surveillance, and tracking the source of an outbreak. Multiple typing methods, including phenotypic and genotypic, are still being used to differentiate microorganisms at the strain level. Bacterial isolates can be characterized based on phenotypic traits, by using biotyping, serotyping, phage typing, antibiotic susceptibility testing, mass spectrometry (MS) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of cellular-extracellular components, and based on nucleic acid, by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), pulsed-field gel electrophoresis (PFGE), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), ribotyping, multilocus sequence typing and mPCR (Karatuğ *et al.,* 2018).

An effective typing method to differentiate *Salmonella* strains is required for epidemiological studies and to track the source of *Salmonella* outbreaks. *Salmonella enterica* is divided into serovars, depending on the O and H antigens, but the serotyping method needs experts and reagents. Using the serotyping method is limited to reference laboratories. This

technique has a low power of discrimination and alone is of restricted use as an epidemiological method (Hashemi and Baghbani-Arani, 2015).

The application of different molecular techniques for detecting and typing foodborne pathogens in surveillance studies provides reliable epidemiological data for tracing the source of infections in humans. A wide range of different molecular typing methods for identification, speciation, typing, classifying, and/or characterizing foodborne pathogens have been used (Adzitey *et al*., 2013). These include PFGE, amplified fragment length polymorphism, ribotyping, repetitive DNA sequence-PCR (REP-PCR), multilocus sequence typing (MLST), enterobacterial repetitive intergenic consensus sequences-based PCR (ERIC-PCR), plasmid profiling, and insertion sequence fingerprint (Ross and Heuzenroeder, 2008).

The gold standard molecular typing technique is PFGE. However, the disadvantage of using this method is that it does not show the same discriminatory power among different *Salmonella* serotypes, laborious, and time-consuming (Winokur, 2003).So, an effective, easy, rapid, and reproducible method that has the ability to differentiate among genetically unrelated strains with similar phenotypes, is needed (Wattiau *et al.,* 2011).Several other DNA-based typing methods have been developed for rapid, easy, and simple applicable typing methods that are possible to be available to any laboratory and have high discriminatory power for typing the various *Salmonella* isolates. These molecular typing methods include

RAPD-PCR, BOXAIR, and repetitive extragenic palindromic sequences (REP) and enterobacterial repetitive intergenic consensus (ERIC-PCR), which capture variation on a genomic scale (Hashemi and Baghbani-Arani, 2015). The extensive spread distribution of these repetitive DNA elements in the various microorganism genomes is useful for rapid identification of bacterial species and strains, and analysis of bacterial genomes (Suh and Song, 2006). The combined use of RAPD-fingerprinting and REP-fingerprinting offers an excellent means that can be applied for the discrimination of *Salmonella* strains (Hashemi and Baghbani-Arani, 2015).

In RAPD, genomic DNA is amplified by PCR with short arbitrary primer sequences to generate distinctive patterns of PCR amplicons with various lengths. Regardless of an observed deficiency of reproducibility and sometimes unacceptable sensitivity to reaction conditions, RAPD fingerprinting has been used to study the diversity of organisms' genomes (Khoodoo *et al.,* 2002). The Palindromic Units (PU), which are also called Repetitive Extragenic Palindromes (REP) are present in multiple copies (about 500-1000), dispersed throughout the genomes of many different bacterial species such as the chromosome of *Salmonella* spp. and *Escherichia coli*. The REP primers used to identify Repetitive Extragenic Palindromes which are scattered over many bacterial genomes producing amplicons differ in their size depending on the site of the REP sequences. Multiple copies of repeated units of REP sequence have been targeted by this method; these sequences include an inverted repeat of 35-40 nucleotides long, found in clusters in which successive copies (up to six)

are arranged in alternate orientation (Martin *et al.,* 1992; Hashemi and Baghbani-Arani, 2015).

The ERIC-PCR sequences are 124-127 nucleotide long, highly conserved at nucleotide level include central core inverted repeats and are present in about 150 copies in *S*. Typhimurium and 30-50 copies in *E. coli*. The ERIC-PCR sequences, contrary to REP, appear to occur singly (Martin *et al.,* 1992). The ERIC-PCR is a PCR-fingerprinting technique but the primers are not arbitrary because the primers for ERIC-PCR were designed to specific known target sequences. The banding pattern in ERIC-PCR is achieved by amplification of the genomic DNA segments that are located between the ERIC elements or between the ERIC elements and other repetitive DNA sequences (Martin *et al.*, 1992; Zulkifli *et al*., 2009).

The consensus BOX elements are mosaic repetitive sequences, composed of boxA (59-bp) subunit, boxB (45-bp) subunit, and boxC (50-bp) subunit, and is thus 154-bp long. The boxB subunit was present alone as a single copy or as a variable number of direct tandem repeats flanked by boxA and boxC. The DNA sequences of the BOX elements are entirely different from the prokaryotic interspersed repetitive DNA sequences REP and ERIC, although there are similarities to REP and ERIC concerning size, copy number, and potential to form stable stem-loop structures (Martin *et al.,* 1992). The BOXAIR elements are inverted repeat sequences present in a certain bacterial species, including *Salmonella* (Hashemi and Baghbani-Arani, 2015).

The PCR-based typing methods were used to assess genetic relationships between strains of *Salmonella* spp. (Del Cerro *et al.,* 2002; Weigel *et al.,* 2004; Suh and Song, 2006; Elemfareji and Thong, 2013; Hashemi and Baghbani-Arani, 2015; Pooncharеon *et al.,* 2019; Sedeik *et al.,* 2019).

1.1.6 Virulence gene typing

Salmonella spp. can establish an infection and cause illness through the expression of several virulence genes that interact with host cells. These virulence genes play very important roles in a broad spectrum of pathogenic mechanisms. These mechanisms including invasion, adhesion, toxin production, systemic infection, antibiotic resistance, fimbrial expression, intracellular survival, and iron and Mg^{2+} uptake (Hensel, 2004). The genes *prgH*, *invA*, *spaN* (*invJ*), *spiA*, *tolC*, *orgA*, *sipB*, *pagC*, *pefA*, *msgA*, *sopB, spvB*, *lpfC* and *sifA* are expressed to produce certain proteins associated with invasiveness traits, such as cellular invasion/survival and adhesin or pili production. Other genes encode certain proteins thought to be very important to virulence. These factors including *iroN* and *sitC*, both these genes are involved in iron acquisition, and *cdtB* gene is considered as a putative toxin-encoding gene (Skyberg *et al.,* 2006). The functions of these genes are presented in Table 1.1.

Table (1.1): Function of virulence factors of *S.* Typhimurium used in virulence genotyping in this study (Skyberg *et al.,* 2006).

Virulence factor	Virulence-related function
invA, orgA, prgH, tolC, sopB, lpfC, cdtB, pefA	Host recognition/invasion
spaN	Entry into non-phagocytic cells, killing of macrophages
sipB	Entry into non-phagocytic cells, killing of macrophages
iroN, sitC	Iron acquisition
pagC, msgA, spiA	Survival within macrophage
sifA	Filamentous structure formation
spvB	Growth within-host

There are several studies showed that *Salmonella* strains contain a wide range of virulence factors associated with pathogenesis (Skyberg *et al.,* 2006; Huehn *et al.,* 2010; Elemfareji and Thong, 2013; Borges *et al.,* 2013; Mezal *et al.,*2014; Rowlands *et al.,* 2014; Gharieb *et al.,* 2015; Tarabees *et al.,* 2017; Srisanga *et al.,* 2017; Thung *et al.,* 2018; Liaquat *et al.,*2018; Elkenany *et al.,* 2019).

1.2 Aims of the Study

The prevalence and molecular characterization of *S.* Typhimurium and *S.* Enteritidis isolates recovered from food samples have not been examined previously in the West Bank-Palestine. The current study aimed to:

1. characterize and document the prevalence and distribution of *S.* Typhimurium and *S*. Enteritidis isolates in food samples.

2. *S.* Typhimurium isolates recovered from different types of food were fingerprinted by RAPD-PCR and REP-PCR, using ERIC-PCR and

BOXAIR-PCR to assess genetic relationships between strains of *S.* Typhimurium.

3. Also, the pathogenic potential of recovered *S.* Typhimurium in the present study was assessed using virulotyping PCR assay, targeting 17 virulence gene sequences. To the best of our knowledge, this is the first study that determines the occurrence of *S.* Typhimurium and the distribution of virulence genes in isolates recovered from food samples in Palestine.

Chapter Two

Materials and Methods

Chapter Two
Materials and Methods

2.1 Samples collection

A total of 51 *Salmonella* isolates were recovered from different types of food samples, which were collected from the local market in different governorates and areas in the West Bank, Palestine during 2019. These samples were Chicken (18), Chicken breast (1), Kebab (1), Turkey (2), Cheese (1), Beef burger (11), Chicken wings (4), Hummus (1), Turkey meat (1), Boneless chicken (1), Parsley (1), Tahini (4), Restaurant's salad (1), Halawa (1), Meat (1), Fillet-fish (1) and Beef meat (1). All *Salmonella* isolates were collected and identified by Dr. Amjad Hussein **(**Chemical, Biological and Drugs Analysis Center, An-Najah National University, Palestine**).** Identification of these isolates was done by conventional methods using enrichment, selective and differential media, Gram staining, biochemical tests (Motility test (SIM), and Triple Sugar Iron test (TSI)). All cultures that were negative for Lactose/Sucrose, positive for Glucose, produce H_2S, and motile were kept for serological confirmation. The serological confirmation used is genus-specific, to confirm the *Salmonella* isolates using specific antisera (Biorad). The agglutination test was carried out on a glass slide. One drop from specific antiserum was mixed with one drop from suspected *Salmonella* broth culture or 0.9% sterile saline suspected *Salmonella* suspension. Any agglutination after two minutes for both the somatic “O” and flagella “H” antisera was considered a positive reaction for the tested *Salmonella* spp. These isolates are stored in the

Chemical, Biological, and Drugs Analysis Center (Nablus, Palestine) at -70ºC.

2.2 DNA isolation and PCR technique

2.2.1 DNA extraction

The DNA genome of *Salmonella* spp. was prepared for PCR according to the method described previously (Adwan *et al*., 2013). Briefly, cells were scraped off an overnight Mueller Hinton agar plate, re-suspended in 800 µl of 1X Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8]), centrifuged for 5 minutes at 14,000 x g; after that, the supernatant was discarded. Then the pellet was re-suspended in 400 µl of sterile distilled water, and boiled for 10-15 min. Then, the cells were incubated on ice for 10 min. The debris was pelleted by centrifugation at 14,000 x g for 5 min, and sample supernatant was transferred into a new Eppendorf tube. The concentration of the DNA sample was determined using a nanodrop spectrophotometer (Genova Nano, Jenway), and the DNA samples were stored at -20ºC for further analysis.

2.2.2 *Salmonellae* spp. confirmation and *S.* Typhimurium and *S.* Enteritidis identification by multiplex PCR (mPCR)

For mPCR detection, three primer pairs were used in this study. These primer pairs were used to identify specific target genes, included *invA* for *Salmonella* spp. identification, *STMO159* (a putative restriction endonuclease) for *S.* Typhimurium identification, and *SEN1383* (a

hypothetical protein) for *S.* Enteritidis identification. Target gene, primer sequence, and amplicon size for these primer pairs are presented in Table 2.1. The mPCR reaction mix was carried according to the method described previously (Ranjbar *et al*., 2017) with some modifications. A final volume of 25 µl mPCR reaction mix was performed as follows: 12.5 µl of PCR premix (ReadyMix TM Taq PCR Reaction Mix with MgCl2, Sigma), 0.3µM of each primer and 3 µl (50-70 ng) of target DNA template. DNA amplification was carried out using a thermal cycler (Mastercycler personal, Eppendorf, Germany) according to the following conditions: initial denaturation at 94ºC for 3 min; followed by 30 cycles of denaturation at 94ºC for 1 min, annealing at 57ºC for 1 min and extension at 72ºC for 1 min. Then, these cycles were followed by a single final extension step at 72ºC for 5 min. The PCR amplicons were then detected by electrophoresis through 1.5% agarose gels to determine the size of amplicons after staining with a final concentration of 0.5µg/ml of ethidium bromide dye. The sizes of the PCR products were determined by comparing them with a 100-bp DNA ladder. Live attenuated vaccine for *S.* Typhimurium and *S.* Enteritidis (Biovac Company) was used as positive control and *E. coli* ATCC25922 strain was used as a negative control.

Table (2.1): Oligonucleotide primers used for *Salmonella* spp. confirmation, *S.* Typhimurium, and *S.* Enteritidis detection.

Target gene	Primer Sequence 5'→3′	Amplicon size (bp)	Reference
invA- secretory protein (*Salmonella* spp.)	*invA* F: GTATTGTTGATTAATGA GATCCG *invA* R: ATATTACGCACGGAAA CACGTT	404	Ranjbar *et al*., 2017
SEN1383-a hypothetical protein (*S.* Enteritidis)	SEN1383 F: TGTGTTTTATCTGATGC AAGAGG SEN1383 R: TGAACTACGTTCGTTCT TCTGG′	304	Ranjbar *et al*., 2017
STM0159–a putative restriction endonuclease (*S.* Typhimurium)	STM0159 F: ATG ATG CCT TTT GCT GCT TT' STM0159 R: TCC CAG CTC ATC CAA AAA	224	Ranjbar *et al.*, 2017
enterobacterial repetitive intergenic consensus	ERIC1: ATG TAA GCT CCT GGG GAT TCAC ERIC2: AAG TAA GTG ACTGGG GTG AGC G	-	Versalovic*et al.* 1991
interspersed repetitive DNA sequence (BOX)	BOXAIR CTACGGCAAGGCGACG CTGACG	-	Dombek *et al.* 2000
random amplification of polymorphic DNA (RAPD)	OPP-16CCA AGC TGC C	-	Albufera *et al*. 2009

2.2.3 Molecular typing of *S.* Typhimurium by ERIC- PCR, and BOXAIR-PCR

Salmonella Typhimurium isolates recovered from different food samples were fingerprinted by ERIC-PCR and BOXAIR-PCR using ERIC-PCR primers and interspersed repetitive DNA sequence (BOX) primers, respectively, to assess genetic relationships between the strains of *S.*

Typhimurium from these different sources. The primers for ERIC-PCR and BOXAIR-PCR are presented in Table 2.1. Each PCR reaction mix (25 µl) was composed of 10 mM PCR buffer pH8.3; 3 mM $MgCl_2$; 0.4 mM of each dNTP; 0.8 µM of each primer; 1.5U of Taq DNA polymerase and 3 µl of DNA template. The DNA amplification for ERIC-PCR was carried out using a thermal cycler (Mastercycler personal, Eppendorf, Germany) according to the following conditions: initial denaturation for 2 min at 94ºC, followed by 40 cycles of denaturation at 94ºC for 50 s, annealing at 50ºCfor 40 s and extension at 72ºC for 1 min. Then, these cycles were followed with a final extension step at 72ºC for 5 min. For BOXAIR-PCR, the thermal conditions were: initial denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 50 s, annealing at 50°C for 40 s and extension at 72°C for 2 min. After that, these cycles were followed with a final extension step at 72°C for 5 min.

The PCR products were analyzed by electrophoresis on1.5% agarose gels. The bands in gel images were analyzed using a binary scoring system, which recorded the absence and presence of bands as 0 and 1, respectively. The binary matrix was analyzed by the unweighted pair group method for arithmetic averages (UPGMA), using SPSS statistical software version 20 (IBM). The clusters of the fingerprints in the constructed dendrogram were described at a 96% similarity level. The number of different bands in each fingerprint was considered for comparison between *S.* Typhimurium strains as previously described (Adwan *et al.,* 2016; Adwan *et al.,* 2016; Adwan *et al.,* 2016; Adwan *et al.,* 2018), based on the following criteria: identical

clones (no different bands), "closely related clones" (have 1 different band),"possibility different clones" (have two different bands), "different clones" (have three or more different bands).

2.2.4 Molecular typing of *S.* Typhimurium by RAPD-PCR

Salmonella Typhimurium isolates recovered from different food samples were fingerprinted by RAPD-PCR using the RAPD primer OPP-16 to assess genetic relationships between the strains of *S.* Typhimurium from these sources. The primer sequence for RAPD-PCR is presented in Table 2.1. RAPD-PCR was carried as described previously with some modification (Hashemi and Baghbani-arani, 2015). Each PCR reaction mix was carried out as well as ERIC- PCR mix and BOXAIR-PCR mix. DNA amplification for RAPD-PCR was carried out using a thermal cycler (Mastercycler personal, Eppendorf, Germany) according to the following thermal conditions: initial denaturation for 3 min at 94ºC, followed by 40 cycles of denaturation at 94ºC for 1 min, annealing at 35ºC for 1 min and extension at 72ºC for 2 min. Then, these cycles were followed with a final extension step at 72ºC for 5 min. The PCR products were analyzed as well as the ERIC- PCR, and BOXAIR-PCR.

2.2.5 Virulotyping of *S.* Typhimurium isolates by multiplex PCR (mPCR)

Three mPCR reactions were used to amplify the seventeen virulence genes. Pools of reaction, target gene, primer sequence, amplicon size for these primers are presented in Table 2.2.

The mPCR was carried as described previously with some modification (Skyberg *et al.,* 2006). Each PCR reaction mix (25 µl) was composed of 10 mM PCR buffer pH 8.3; 6 mM MgCl2; 0.3 mM of each dNTP; 0.3µM of each primer; 1.5U of Taq DNA polymerase, 3% DMSO, and 3 µl of target DNA template. DNA amplification for mPCR was carried out using a thermal cycler (Mastercycler personal, Eppendorf, Germany) according to the following thermal conditions: initial denaturation for 3 min at 94ºC, followed by 25 cycles of denaturation at 94ºC for 40 s, annealing at 62ºC for 40 s and extension at 72ºC for 2 min. After that, these cycles were followed with a final extension step at 72ºC for 5 min. The PCR products were then detected by electrophoresis through 1.5% agarose gels to determine the size of amplified fragments after staining with a final concentration of 0.5 µg/ml of ethidium bromide dye. The sizes of the amplicons of these genes were determined by comparing them with a 100-bp DNA ladder.

Table (2.2): Virulence gene primers used in this study (Skyberg *et al.*, 2006).

Target gene	Primer sequence 5′→3′	Amplicon size	pool
spvB	spvB F: CTA TCA GCC CCG CAC GGA GAG CAG TTT TTA spvB R: GGA GGA GGC GGT GGC GGT GGC ATC ATA	717	1
spiA	spiA F: CCAGGGGTCGTTAGTGTATTGCGTG AGATG spiA R: CGCGTAACAAAGAACCCGTAGTGA TGGATT	550	1
pagC	pagC F: CGCCTTTTCCGTGGGGTATGC pagC R: GAAGCCGTTTATTTTTGTAGAGGAG ATGTT	454	1
cdtB	cdtB F: ACAACTGTCGCATCTCGCCCCGTCA TT cdtB R: CAATTTGCGTGGGTTCTGTAGGTGC GAGT	268	1
msgA	msgA F: GCC AGG CGC ACG CGA AAT CAT CC msgA R: GCG ACC AGC CAC ATA TCA GCC TCT TCA AAC	189	1
invA	invA F: CTG GCG GTG GGT TTT GTT GTC TTC TCT ATT invA R: GTT TCT CCC CCT CTT CAT GCG TTA CCC	1070	2
sipB	sipB F: GGA CGC CGC CCG GGA AAA ACT CTC sipB R: ACA CTC CCG TCG CCG CCT TCA CAA	875	2
prgH	prgH F: GCC CGA GCA GCC TGA GAA GTT AGA AA prgH R: TGA AAT GAG CGC CCC TTG AGC CAG TC	756	2

Target gene	Primer sequence 5′→3′	Amplicon size	pool
spaN	Span F: AAA AGC CGT GGA ATC CGT TAG TGA AGT span R: CAG CGC TGG GGA TTA CCG TTT TG	504	2
orgA	orgA F: TTT TTG GCA ATG CAT CAG GGA ACA orgA R: GGC GAA AGC GGG GAC GGT ATT	255	2
tolC	tolC F: TAC CCA GGC GCA AAA AGA GGC TAT C tolC R: CCG CGT TAT CCA GGT TGT TGC	161	2
iroN	Iron F: ACT GGC ACG GCT CGC TGT CGC TCT AT iron R: CGC TTT ACC GCC GTT CTG CCA CTG C	1205	3
sitC	sitC F: CAG TAT ATG CTC AAC GCG ATG TGG GTC TCC sitC R: CGG GGC GAA AAT AAA GGC TGT GAT GAA C	768	3
lpfC	lpfC F: GCC CCG CCT GAA GCC TGT GTT GC lpfC R: AGG TCG CCG CTG TTT GAG GTT GGA TA	641	3
sifA	sifA F: TTT GCC GAA CGC GCC CCC ACA CG sifA R: GTT GCC TTT TCT TGC GCT TTC CAC CCA TC	449	3
sopB	sopB F: CGG ACC GGC CAG CAA CAA AAC AAG AAGAAG sopB R: TAG TGA TGC CCG TTA TGC GTG AGT GTA TT	220	3
pefA	pefA F: GCG CCG CTC AGC CGA ACC AG pefA R: GCA GCA GAA GCC CAG GAA ACA GTG	157	3

Chapter Three

Results

Chapter Three
Results

3.1 *Salmonellae* spp. confirmation and *S.* Typhimurium and *S.* Enteritidis detection

A total of 51 *Salmonella* isolates were recovered from different types of food samples, which were collected from local markets in different areas in the West Bank, Palestine during 2019. These isolates were identified using conventional methods and specific antisera to a genus level by Dr. Amjad Hussein **(**Chemical, Biological and Drugs Analysis Center, An-Najah National University, Palestine**)**. All these *Salmonella* isolates were subjected to mPCR using specific primers to confirm that these isolates belonged to a *Salmonella* genus and to determine the occurrence of *S.* Typhimurium and *S.* Enteritidis serotypes among these isolates. As expected, PCR confirmation of conventional and serological methods positive strains was documented by the appearance of the amplified DNA fragment of 404-bp for the *invA* gene, a target for *Salmonella* genus, in all 51 (100%) *Salmonella* isolates examined. In addition, 28 (54.9%) isolates were *S.* Typhimurium serotype and produced amplified DNA fragment of 224-bp for*STMO159* gene (a putative restriction endonuclease), while amplified DNA fragment of 304-bp for *SEN1383* gene (a hypothetical protein) for *S.* Enteritidis serotype was not detected in all *Salmonella* isolates. Multiplex PCR profile specific for genes responsible for detection *Salmonella* genus (*invA* gene; 404-bp), *S.* Typhimurium serotype (STMO159, a putative restriction endonuclease; 224-bp), and *S.* Enteritidis

serotype (SEN1383, a hypothetical protein; 304-bp) is shown in Figure 3.1. Occurrence of *S.* Typhimurium serotype among *Salmonella* spp. isolated from different types of food samples is presented in Table 3.1

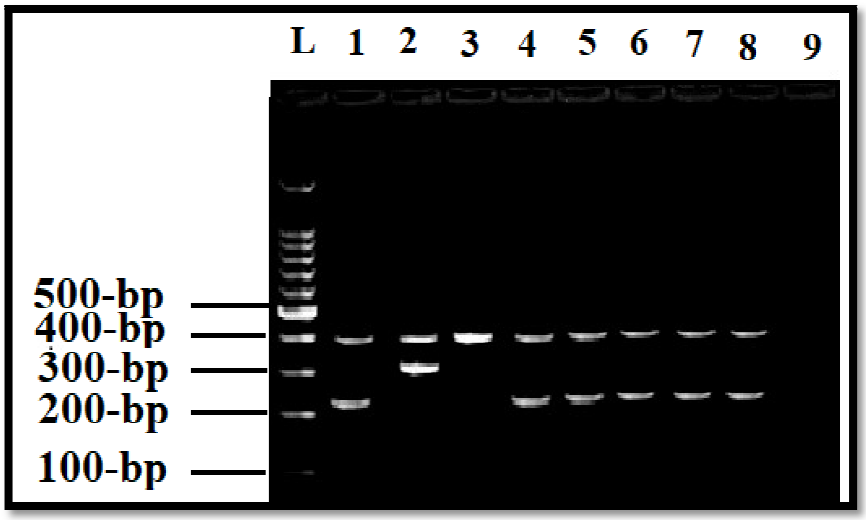

Figure (3.1): Multiplex PCR profile specific for genes responsible for detection isolates of *Salmonella* genus, (*invA* gene; 404-bp) *S.* Typhimurium serotype (STMO159, a putative restriction endonuclease; 224-bp) and *S.* Enteritidis (SEN1383, a hypothetical protein; 304-bp).

Lanes: L represents 100 bp ladder; lanes 4, 5, 6, 7, and 8represent *S.* Typhimurium serotype: lane 3 represents *Salmonella* genus. lane 9 represents *E. coli* as a negative control; Lanes1 and 2 represent live attenuated vaccine for *S.* Typhimurium and *S.* Enteritidis serotypes (Biovac Company) as a positive control, respectively.

Table (3.1): Occurrence of *S.* Typhimurium serotype among *Salmonella* spp. isolated from different types of food samples.

Source	*Salmonella* spp. n (%)	*S.* Typhimurium n *(%)**
Chicken	18(35.3%)	9 (50%)
Beef Burger	11(21.5%)	9 (81.8%)
Beef Meat	1(1.96%)	0 (0.0%)
Chicken wings	4(7.84%)	4 (100%)
Chicken Breast	1(1.96%)	1 (100%)
Boneless Chicken	1(1.96%)	0 (0.0%)
Kebab	1(1.96%)	1 (100%)
Meat	1(1.96%)	0 (0.0%)
Turkey Meat	1(1.96%)	1 (100%)
Turkey	2(3.92%)	1 (100%)
Fillet Fish	1(1.96%)	1 (100%)
Parsley	1(1.96%)	0 (0.0%)
Tahinia	4(7.84%)	0 (0.0%)
Hummus restaurants	1(1.96%)	1 (100%)
Halawa	1(1.96%)	0 (0.0%)
Cheese	1(1.96%)	0 (0.0%)
Restaurant Salad	1(1.96%)	0 (0.0%)
Total	51 (100%)	28 (54.9%)

***: number of isolates**

3.2 Virulotyping of *S.* Typhimurium serotype isolates by multiplex PCR (mPCR)

PCR targeting 17 virulence genes (*invA, orgA, prgH, tolC, sopB, lpfC, cdtB, pefA*, *spaN*, *sipB*, *iroN, sitC*, *pagC, msgA, spiA*, *sifA*, *spvB*) were conducted in the current study to characterize28*S.* Typhimurium isolates virulence. Only 13 genes were detected in these 28 *S.* Typhimurium isolates. The occurrence of the detected genes among these isolates was 100%,50%,46.4%, 39.3%, 35.7%, 35.7%, 32.1%, 25%,25%, 17.6%, 14.3%, 14.3%, 3.6% for *invA*,*sopB*,*prgH*,*sitC*,*pefA*,*tolC*, *cdtB*, *msgA*, *sifA*, *iroN*, *spiA*,*ipfC* and *pagC*, respectively. The remaining virulence genes

were absent in all the *S.* Typhimurium isolates. Based on the combination of presence and absence of virulence genes, 8 profiles were detected among these isolates, the most common genetic profilewasV5 (each 32.1%). The *invA* gene which is genus-specific gene was detected in all isolates. Figure 3.2 and Table 3.2 showed data about virulence gene profiles detected in this study.

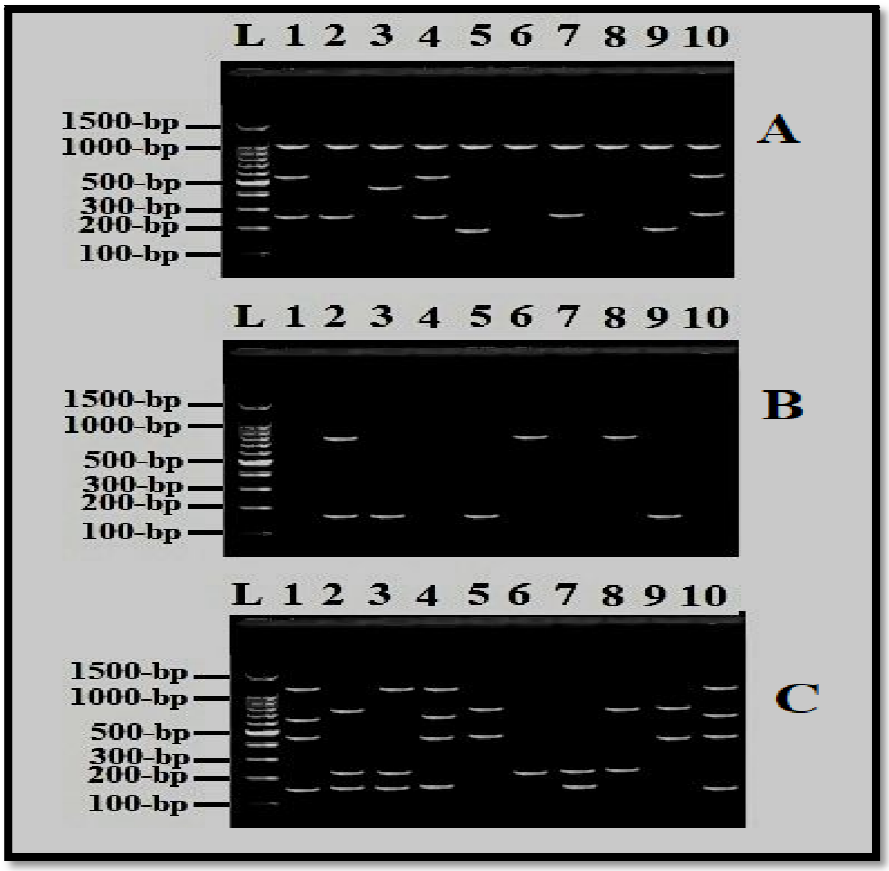

Figure (3.2): Multiplex PCR profiles specific for *S.* Typhimurium virulence factors.

Figure A: *spiA* gene (550-bp), *pagC* gene (454-bp), *cdtB* gene (268-bp) and *msgA* gene (189-bp). Figure B: *invA* gene (1070-bp), *prgH* gene (756-bp), *tolC* gene (161-bp). Figure C: *iroN* gene (1205-bp), *sitC* gene (768-bp), *ipfC* gene (641-bp), *sifA* gene (449-bp), *sopB* gene (220-bp) and *pefA* gene (157-bp).

Table (3.2): Virulence gene profile of 28*S.* Typhimurium isolated from different types of food samples

Virulotypes (V)	Gene combinations	No. of Strains (%)
V1	*invA, spiA,cdtB, iroN, ipfC, sifA, pefA*	4 (14.3)
V2	*invA, cdtB, prgH,tolC, sitC, sopB, pefA*	2 (7.1)
V3	*invA, pagC, tolC, iroN, sopB, pefA*	1 (3.6)
V4	*invA, msgA, tolC, sitC, sifA*	5 (17.9)
V5	*invA, prgH, sopB,*	9 (32.1)
V6	*invA, cdtB,sopB, pefA*	3 (10.7)
V7	*invA, prgH, sitC, sopB,*	2 (7.1)
V8	*invA, msgA, tolC, sitC, sifA,*	2 (7.1)

3.3 Genotyping of *S.* Typhimurium serotype by PCR-based methods

In the present study, ERIC and BOX primers allowed for discrimination into 4 and 6 clusters or clones of 16 *S.* Typhimurium serotype isolates, respectively, based on their genetic profile at cut-off point 96%. RAPD-PCR using the RAPD primer OPP-16 did not allow for discrimination between *S.* Typhimurium isolates. The RAPD OPP-16primer did not produce any amplified fragments during PCR amplification.

According to the ERIC-PCR profile, strains of cluster C1 and C2, C3 and C4, and C3 and C2 are closely related clones. Strains of C4 and C1, and C3 and C1 are different clones, while strains of C4 and C2 are possibly different clones. ERIC-PCR DNA fingerprint pattern, dendrogram, and the relationship between the clones of 16 *S.* Typhimurium strains recovered from different food samples are presented in (Figure 3.3, Figure 3.4, and Table 3.3).

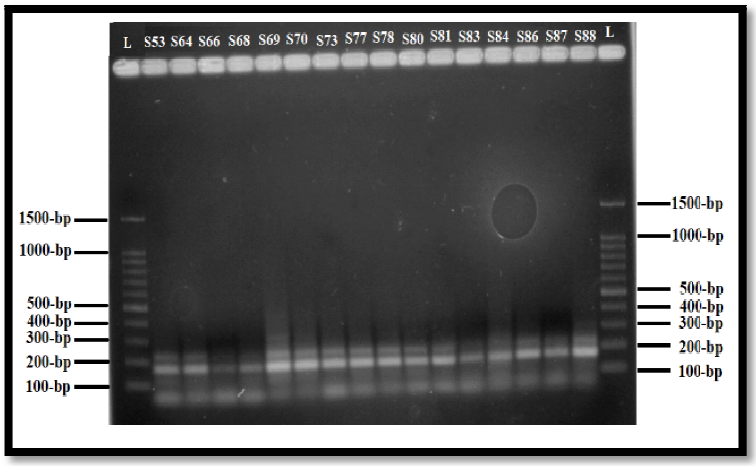

Figure (3.3): DNA fingerprint patterns generated by ERIC-PCR typing of 16 *S.* Typhimurium serotype isolates recovered from different food samples electrophoresed in a 1.5% agarose.

Lanes L:100-bp ladder; other lanes referring to *S.* Typhimurium serotype isolates.

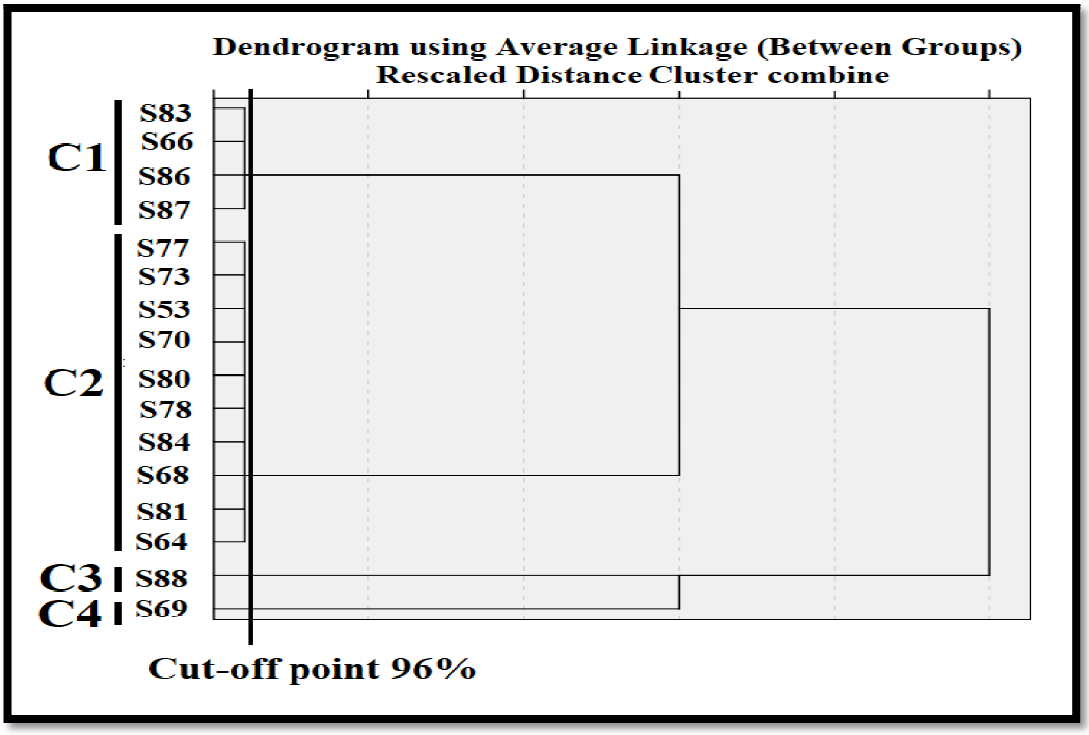

Figure 3.4 Dendrogram of 16 *S.* Typhimurium serotype isolates based on the UPGMA method using Average linkage (between groups)/Squared Euclidean Distance by SPSS software version 20ز

derived from analysis of the ERIC-PCR-profiles at a 96% similarity level. C: Cluster.

Table (3.3): Relationship between the clones or the clusters depending on the number of different bands based on ERIC-PCR profile of 16 *S.* Typhimurium serotype isolates.

Cluster or clone	Cluster relationship			
	C1	**C2**	**C3**	**C4**
C1	1	2	4	4
C2		1	2	3
C3			1	2
C4				1

1. identical clones,

2. closely related clones,

3. possibility different clones,

4. different clones.

C: cluster or clone

According to the BOX-PCR profile, Strains of cluster C1 and C2, C3 and C4, C4 and C5, and C4 and C6 are closely related clones. Strains of cluster C2 and C3, C5 and C2, C5 and C3, C6 and C2, C6 and C3, and C6 and C5are possibility different clones, while strains of C3 and C1, C4 and C1, C4 and C2, C5 and C1, and C6 and C1 are different clones. BOX-PCR DNA fingerprint pattern, dendrogram, and the relationship between the clusters or clones of 16 *S.* Typhimurium serotype isolates recovered from different food samples are presented in (Figure 3.5, Figure 3.6, and Table 3.4).

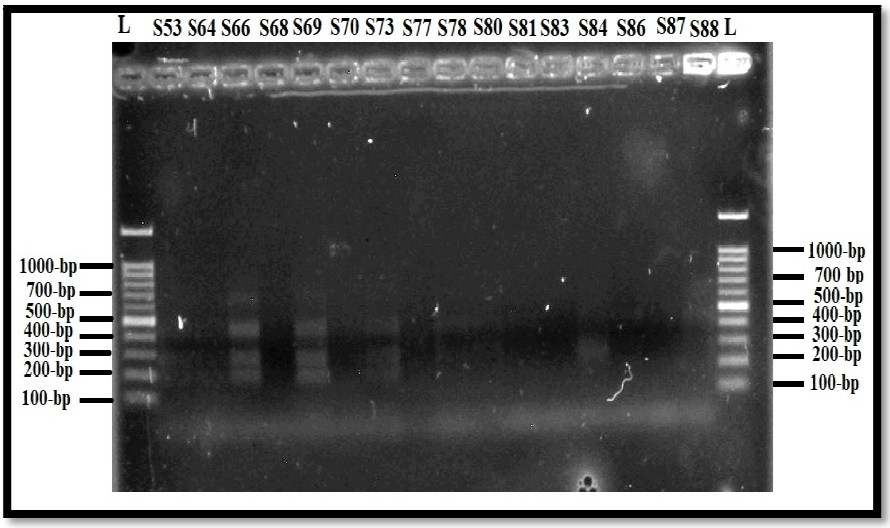

Figure (3.5): DNA fingerprint patterns generated by BOX-PCR typing of 16 *S.* Typhimurium serotype isolates recovered from different food samples electrophoresed in a 1.5% agarose.

Lanes L: 100-bp ladder; other lanes referring to *S.* Typhimurium isolates.

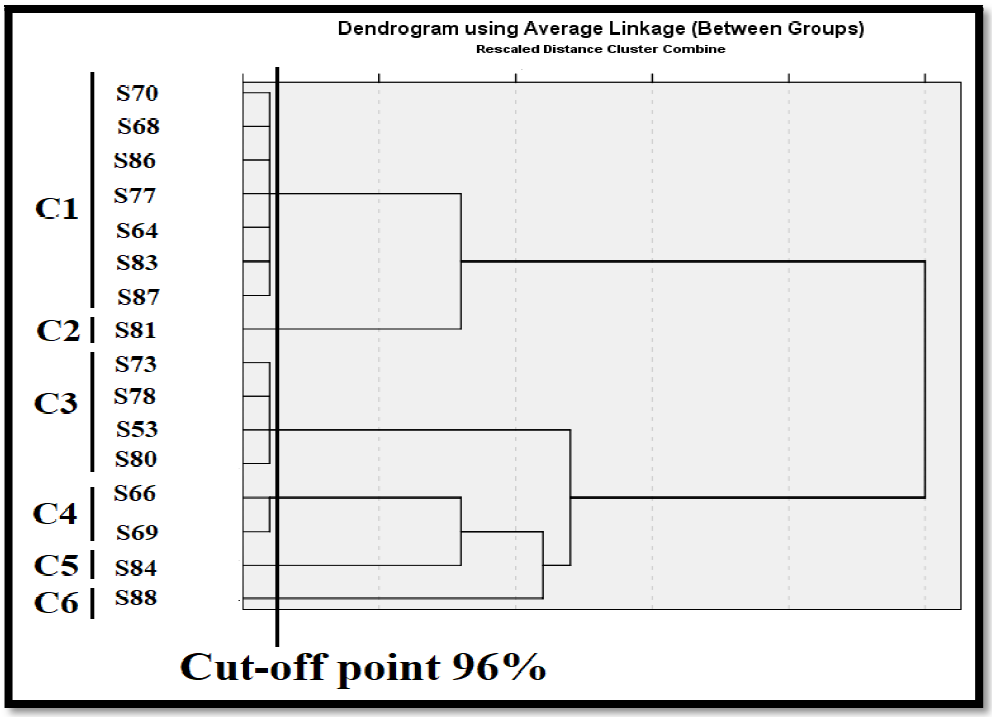

Figure (3.6) Dendrogram of 16 *S.* Typhimurium serotype isolates based on the UPGMA method using Average linkage (between groups)/Squared Euclidean Distance by SPSS software version 20.

derived from analysis of the BOX-PCR-profiles at a 96% similarity level. C: Cluster.

Table (3.4) Relationship between the clones or the clusters depending on the number of different bands based on BOX-PCR profile of 16 *S.* Typhimurium serotype isolates.

Cluster or clone	Cluster relationship					
	C1	**C2**	**C3**	**C4**	**C5**	**C6**
C1	1	2	4	4	4	4
C2		1	3	4	3	3
C3			1	2	3	3
C4				1	2	2
C5					1	3
C6						1

1. identical clones,

2. closely related clones,

3. possibility different clones,

4. different clones.

C: cluster or clone

Results of PCR typing methods showed that strain S83 (chicken wings) and strains S86and S87 (chicken) are clustered together using both ERIC-PCR and BOX-PCR typing methods and they had the same virulotype pattern (V1). However, strainsS78 and S80 (beef burgher) and strains S53 and S73 (chicken) also clustered together by both typing methods and had the same virulotype pattern (V4).

Chapter Four

Discussion

Chapter Four
Discussion

Salmonellosis remains a significant public health problem causing food poisoning in humans. Poultry, its products and eggs, represent an important source of *Salmonella* organism for consumer health (Jinu *et al.,* 2014). Most infections result from the ingestion of foods of animal origin contaminated with *Salmonella* species such as chicken, eggs, beef, shellfish, and milk (Ahmed *et al.,* 2014). *Salmonella enterica* is highly diverse, containing over 2,500 different serovars. The representative serovars from this species are the most commonly isolated serovars during outbreaks of foodborne salmonellosis, including *S.* Enteritidis, *S.* Typhimurium, *S.* Virchow, and *S.* Infantis (Tarabees *et al.,* 2017).

S. Enteritidis and *S.* Typhimurium are the most predominant isolated organisms in most *Salmonella* cases associated with the consumption of contaminated poultry, pork, and beef products. Contamination with *Salmonella* in poultry products can occur at multiple steps along the food chain, including production, processing, distribution, retail marketing, handling, and preparation (Tarabees *et al.,* 2017). Monitoring of *Salmonella* spp. along the food chain is conducted during pre-harvest (farm animals and their feed), processing (cutting plants and slaughterhouses), and post-harvest (retail and catering) stages (EFSA, 2018). Kotova *et al.* (1988) observed that humans develop the *Salmonella* carrier state after acute salmonellosis, which is the result of occupational exposure to poultry (6.1% -8.8%). To the best of our knowledge, this work is the first to

molecularly characterize *S.* Typhimurium strains in the West Bank, Palestine, and assessing their distribution of virulence genes of the recovered *Salmonella* isolates.

The present preliminary screening study was conducted to shed light on the occurrence of *S.* Typhimurium and *S.* Enteritidis, genotyping the detected isolates using PCR-based methods, and the virulence genotyping of these isolates that were recovered from different types of food. Results of this study were that all the isolates identified as *Salmonella* spp. by conventional and specific antisera to a genus level had had *invA* gene, which is a target for *Salmonella* genus. Detection of the *invA* gene with specific PCR primers is a rapid, sensitive, and specific method for the identification of *Salmonella* at the genus level in a variety of food samples. The current study supported the ability of specific primer sets for detection *invA* gene can confirm the isolates as *Salmonella* spp. The protein encoded by the *invA* gene is essential for the invasion of host epithelial cells (Darwin and Miller, 1999). As anticipated, PCR confirmation of *Salmonella* isolates diagnosed by conventional methods and specific antisera was documented by the appearance of amplified DNA fragments of 404-bp length for the *invA* gene in all 51 (100%) *Salmonella* strains tested, regardless of the serotype or the type of food sample. Several studies had also proven the effective detection of all *Salmonella* isolates using specific primers for the *invA* gene (Malorny *et al.,* 2003; Helmy *et al.,* 2009; Moussa *et al.,* 2010; Shanmugasamy *et al.,* 2011; Borges *et al.,* 2013; Ammar *et al.,* 2016; Ranjbar *et al.,*2017; Srisanga *et al.,* 2017;

Proroga *et al.,* 2018), which was used as a target gene in PCR assays and a confirmatory test for *Salmonella* detection (Malorny *et al.,* 2003; Helmy *et al.,* 2009; Shanmugasamy *et al.,* 2011;Proroga *et al.,* 2018).In contrary to our result of the current study, a published report showed that the *invA* gene was detected in 96.43% of *Salmonella* isolates (Nouichi *et al.,* 2018).

Identifying *Salmonella* serovars using serotyping method is highly expensive and time-consuming. For these reasons, the use of other techniques such as PCR techniques for recognition and identification of *S.* Typhimurium and *S.* Enteritidis as described in this study is an alternative method to the conventional techniques. In the current study, PCR technique used for the identification of *S.* Typhimurium was very specific and produced an amplified DNA fragment of 224-bp for *STMO159* gene (a putative restriction endonuclease), while amplified DNA fragment of 304-bp for *SEN1383* gene (a hypothetical protein) for *S.* Enteritidis. Results of this research study showed that the occurrence of *S.* Typhimurium and *S.* Enteritidis was 54.9% and 0%, respectively. These results indicated the health hazard of these food types as a source of *Salmonella* foodborne pathogens.

A study conducted in Egypt showed that *S.* Enteritidis (33.3%) was the most common among *Salmonella* isolates recovered from bulk milk, raw market milk, followed by *S.* Typhimurium (25.9%), *S.* Heidelberg (14.8%), and others (El-Baz *et al.,* 2017).In another study carried out in the same previous country, 7 *Salmonella* serovars were isolated from freshly dead

and diseased broiler chickens, in which the most common serovars identified were *S*. Typhimurium (52.9%), followed by *S*. Enteritidis and *S*. Arizona, each had occurrence rate 11.8%. Other *Salmonella* serotypes included *S*. Kentucky, *S*. Montevideo, *S*. Birkenhead, and *S*. Virchow were 23.5% (Ammar *et al.,* 2016). Another Previous study carried out in Egypt showed that 50% of *Salmonella* isolates recovered from poultry meat was *S.* Typhimurium, while *S.* Rubislaw, *S.* Kiel, and *S.* Derby (10% each) and 20% were Untypable *Salmonella* spp. (Gharieb *et al.,* 2015). A study carried out by Hassanin *et al.,* (2014), showed the occurrence of *S.* Enteritidis and *S.* Typhimurium recovered from ready-to-eat meat samples and ready-to-eat chicken samples were 37.5% and 29.2%, respectively (Hassanin *et al.,* 2014). Rabie *et al.,* (2012) found that the *Salmonella* isolates collected from diarrheic broiler chickens, raw frozen chickens' meat, and diarrheic patients with food poisoning signs, were serologically identified as 58.3% and 41.6% for *S*. Enteritidis and *S.* Typhimurium respectively (Rabie *et al.,* 2012). In a new study carried out in Saudi Arabia, *Salmonella* strains recovered from clinical and environmental samples showed that the *S.* Enteritidis serotype had the highest prevalence (39.4%), followed by *S*. Paratyphi (21.2%), *S*. Typhimurium (15.2%), *S*. Typhi and *S*. Arizona (12.1%) (El-Tayeb *et al.,* 2017). Another study carried out in Saudi Arabia showed that the occurrence of *S*. Enteritidis and *S.* Typhimurium was the most common serotypes recovered among the *Salmonella* serotypes 55.6% and 22.2%, respectively, isolated from frozen chickens and chicken cuts (Moussa *et al.,* 2010). In Morocco, it has been

shown that *S.* Kentucky was the most common serotype (22.9%) isolated from food products, while the occurrence of *S.* Typhimurium and *S.* Enteritidis was lower than the other serovars, they were 6.2% and 4.2%, respectively (Amajoud *et al.,* 2017).In Algeria, a recent study showed that the most predominant *Salmonella* isolates collected from carcasses and feces of cattle and sheep was *S*. Muenster (39.3%), while *S*. Typhimurium was (3.6%) (Nouichi *et al.,* 2018), even this serotype is scarcely identified from humans, foods, or animals (Van Cauteren *et al.,* 2009).

According to the European Food Safety Authority, it was mentioned that the most prevalent serovar continues to be *Salmonella* Enteritidis (*S*E) and *Salmonella* Typhimurium (*S*T) and monophasic *S*. Typhimurium (1,4,[5],12: i:-), these serotypes representing 49.1%, 13.4%, and 8%, respectively, of all reported serovars confirmed in human cases (EFSA and ECDC, 2018). A study conducted in Italy, France and Switzerland from 2000 to 2011, showed that the occurrence of *S*. Enteritidis and *S*. Infantis reduced significantly, *S*. Typhimurium existed stable, while other serovars, including *S*. (4,[5],12:i:-) and *S*. Napoli raised significantly (Graziani *et al.,* 2013). In Italy, A series of studies has been carried out (Busani *et al.,* 2005; Capuano *et al.,* 2013; Proroga *et al.,* 2018). The most common *S. enterica* serotypes of human origin were *S.* Typhimurium (32.7%), *S.* Enteritidis (26.7%), the monophasic variant of *S.* Typhimurium (*S.* 4,[5],12: i:-) (24.7%), and *S.* Napoli (4.7%) (Proroga *et al.,* 2018). A study carried out by Capuano *et al.,* (2013) in Italy showed that the prevalence of *S*. 4,[5],12: i: -, *S*. Enteritidis and *S*. Typhimurium, in food samples was 37.2%, 32.5%,

and 30.2%, respectively (Capuano *et al.,* 2013). Another study in the same country reported that *S.* Typhimurium was the most common serotype in foods of animal origin (18.8%), followed by *S.* Derby and *S.* Enteritidis for 10.5% and 9.9%, respectively (Busani *et al.,* 2005). In addition, Anumolu and Lakkineni (2012) revealed a wide variation in the detection of *Salmonella* Typhimurium in poultry samples, out of 50 chicken samples, 3 samples were positive for *Salmonella* Typhimurium. Nearly similar results were also obtained by Shaltout *et al*., (2019), El-Kader *et al.,* (2015), and Rao *et al.,* (1977), where they could isolate *S.* Typhi, *S.* Typhimurium, and *S.* Enteritidis from different meat samples with a percentage of 3.3 % for each strain.

In Mexico, *S.* Typhimurium was the most common serotype (23.9%) isolated from vegetables, while *S.* Enteritidis was 2.81% (Quiroz-Santiago *et al.,* 2009). In Malaysia, the occurrence of *S.* Enteritidis and *S.* Typhimurium among *Salmonella* isolates recovered from beef meat samples was 16.7% and 11.1%, respectively (Thung *et al.,* 2018). In South Africa, 2012-2014; *Salmonella* strains recovered from food-producing animals, meat, animal feed, the environment, and other non-human sources, showed that the prevalence of *S.* Enteritidis and *S.* Typhimurium was21.5% and 4.0%, respectively (Magwedere *et al.,* 2015).

The previous studies showed that *Salmonella* serovars vary geographically, at the global level. *S.* Enteritidis and *S.* Typhimurium were considered the most common serovars recorded and clinically significant (EFSA and

ECDC, 2015; Ammar *et al.,* 2016). The differences in occurrence rates of *Salmonella* serotypes may be affected by different factors such as differences in the sampling method, sample types, *Salmonella* detection protocol, geographic region, and the housing and husbandry conditions (Busani *et al.,* 2005; Nouichi *et al.,* 2018).

Different molecular techniques have been used to distinguish the strains of *Salmonella* isolates including PFGE, ERIC-PCR, RAPD-PCR, Single Strand Conformation Polymorphism (SSCP), hybridization, and ribotyping-PCR. Results of PCR typing methods showed that strains S83 (chicken wings), S86 (chicken), and S87 (chicken) are clustered together using both ERIC-PCR and BOX-PCR typing methods and they had the same virulotype (V1), and strains S53 (chicken), S73 (chicken), S78 (beef burgher) and S80 (beef burgher) also clustered together by both typing methods and had the same virulotype (V4). Results showed that using more than one molecular method is useful in an epidemiological study of *S.* Typhimurium. In the present study, both ERIC and BOX primers allowed for discrimination into 4 and 6 clusters or clones of 16 *S.* Typhimurium isolates, respectively, based on their genetic profile at cut-off point 96%. RAPD-PCR using the RAPD primer OPP-16 did not allow for discrimination between *S.* Typhimurium isolates. On the other hand, a study conducted in Colombia (Lozano-Villegas *et al.,* 2019) revealed that genotyping of *Salmonella* spp. using RAPD primers allowed the typing of 34 of 49 strains of *Salmonella* spp. The best discriminatory index was

observed when GTG 5 (0.92) and OPP 16 (0.85) primers were used alone or combined with RAPD-PCR and BOX-PCR (0.99).

PCR-based fingerprinting methods are considered as a simple and easily applicable typing technique and potentially available to any molecular laboratory. ERIC-PCR is a useful method for bacterial DNA typing for analysis and evaluation of fingerprinting. It is used in the epidemiology of *Salmonella spp.* (Sedeik *et al.,* 2019). It was reported that the Rep-PCR fingerprinting technique has been used as an epidemiological tool for several bacterial pathogens (Suh and Song, 2006). ERIC and BOX-PCR amplification had the ability to detect a highly genetic homogeneity among *S*. Enteritidis serotype isolates from both chicken and human except one isolate, which originated from chicken and showed a different DNA band pattern from other isolates (Suh and Song, 2006). The greater ability of rep-PCR to discriminate for genotyping of *Salmonella* subspecies when compared with PFGE, given the equally high reliability of both genotyping methods, was previously reported (Weigel *et al.,* 2004). It was suggested that RAPD, ERIC-PCR, REP-PCR, BOXAIR-PCR are good discriminatory techniques to type the different clinical *Salmonella* isolates and these methods are sufficient to determine genetic relationships among *Salmonella* strains for epidemiological purposes when different techniques were combined (Hashemi and Baghbani-arani, 2015).

It was reported that BOX-A1R-based repetitive extragenic palindromic-PCR (BOX-PCR) is considered to be the best method referring to other

repetitive element-based PCR typing methods, specifically, (ERIC)-, poly-trinucleotide (GTG)5-, and repetitive extragenic palindromic (REP-PCR). BOX-PCR provides a convenient molecular typing method to distinguish *Salmonella* spp. of the same and different serotypes according to genetic relatedness and should be proper for application in typing and tracking route of transmission in outbreaks. Similar results were reported by Pooncharoen *et al.* (2019) and Lozano-Villegas *et al.* (2019) who showed that BOX-PCR can differentiate the genetic relationship between *Salmonella* isolates as well as grouping them into different clusters according to their origin. However, both ERIC-PCR and REP-PCR placed all *Salmonella* isolates of the same type into one group (Pooncharoen *et al.,* 2019). Previously, it was shown that the PCR-ribotyping technique had a very low discrimination power. However, the RAPD-PCR typing technique using specific primers which it was proposed as a simple and useful method for discriminating isolates between and within *Salmonella* serotypes (Del Cerro *et al.,* 2002).

The virulence of *Salmonella* is linked to a combination of chromosomal and plasmid factors (Yehia *et al.,* 2020). Many virulence factors proved to play different roles in the pathogenesis of *Salmonella* infections. These virulence factors can be referred to as a virulence-associated plasmid gene or to a group of virulence factors located on chromosomes such as flagella, capsule, adhesion systems, and type III secretion systems (T3SS) encoded on the *Salmonella* pathogenicity islands (SPIs) (Jajere, 2019). The T3SS is regarded as the most important virulence factor of *Salmonella* (Lou *et*

*al.,*2019). Other studies showed that *S. enterica* as well as other enteropathogenic pathogens produce different virulence factors, which play a role in adhesion systems including adhesins, invasins, fimbriae, hemagglutinins, exotoxins, and endotoxins. These virulence factors assist the pathogenic *Salmonella* serotypes to colonize its host through the process of attachment, invasion, survival, and evasion of the defense mechanisms of the host (Jajere, 2019). Information about the virulence factors among *Salmonella* serovars appears to be lacking in Palestine. Virulotyping techniques are useful approaches to study *Salmonella* epidemiology. Several studies on *Salmonella* spp. virulotyping (Herrero *et al.,* 2006; Soto *et al.,* 2006; Capuano *et al.,* 2013) have been conducted, little information is known about the relationship between the strains detected in food and their pathogenicity in human hosts.

The studies showed that there are many virulence factors detected in *Salmonella* genus, certain of these factors are limited and exclusive to specific serotypes. Some of these virulence factors can be expressed and activated during the time of infection process inside the host cells (Elemfareji and Thong, 2013). *Salmonella* Typhimurium isolates have a range of virulence factors that play a role in *Salmonella* infection, diseases and interact with their host cells (Tarabees *et al.,* 2017). In the current study, 17 virulence genes were targeted by mPCR to characterize 28 *S.* Typhimurium isolates virulence, these factors include *invA, orgA, prgH, tolC, sopB, lpfC, cdtB, pefA*, *spaN*, *sipB*, *iroN, sitC*, *pagC, msgA, spiA*, *sifA* and *spvB*. Only 13 virulence genes were detected in these 28 *S.*

Typhimurium isolates. The remaining virulence factors were not detected in all the *S.* Typhimurium isolates. Based on the combination of presence and absence of virulence genes, 8 profiles were detected among these isolates, the most common genetic profile was V5 (each 32.1%).

Of these 17 *Salmonella* genes assayed by mPCR in the current study, only 12 of these genes are located in pathogenicity islands (PAIs) or pathogenicity islets, these included *invA*, *orgA*, *prgH*, *spaN*, *sipB*, *sitC*, *pagC*, *msgA*, *spiA*, *sopB*, *lpfC*, and *sifA*. The other2 genes included *pefA* and *spvB* are found on plasmids, while the remaining 3 genes (*iroN*, *tolC*, and *cdtB*) reside somewhere in the *Salmonella* genome (Skyberg *et al.,* 2006).The following 14 genes that targeted by mPCR in the current study, included *invA*, *orgA*, *prgH*, *spaN*, *tolC*, *sipB*, *pagC*, *msgA*, *spiA*, *sopB*, *lpfC*, *pefA*, *spvB*, and *sifA* encode products that play an important role in a pathogenesis process, such as a cellular invasion, survival within a cell, and adhesin or pili production. The *invA*, *prgH*, *spaN*, *sipB*, *spiA*, and *sifA* genes are also associated with type III secretion system. Other remaining virulence genes, included *iroN* and *sitC* are linked with iron acquisition and *cdtB* virulence gene is connected with toxin synthesis (Skyberg *et al.,* 2006).

Results of the current study are in contrast to a previous study from Egypt, which reported that only 9 genes *sitC*, *iroN*, *sopB*, *sifA*, *lpfC*, *span*, *sipB*, *invA*, and *tolC* were successfully amplified in cases of *S.* Typhimurium isolated from chicken meat (Tarabees *et al.,* 2017). PCR for the *invA* gene

is a rapid and reliable technique with a possible diagnostic application for the identification of *Salmonella* spp. The *invA* virulence gene is the most common and clinically significant genetic marker for the serovar that causes salmonellosis globally. The marker is found in both *S.* Typhimurium and *S.* Enteritidis (Yehia *et al.,* 2020). The *invA* gene is used because it contains sequences specific to the genus, *Salmonella* (Yehia *et al.,* 2020). Therefore, the *invA* gene which isa genus-specific gene was detected in all isolates. The protein product of this gene is necessary for the invasion of intestinal epithelial cells in hosts (Darwin and Miller, 1999).Our report corroborates many recent studies in Egypt (Awadallah and Abd-Elall, 2015) and Nigeria (Smith *et al.,* 2015) conducted on *Salmonella* isolated from humans, animals, food, and water samples in which *invA* gene (284 bp) was prevalent at 96%.A wide prevalence of this gene (100%) had also been recorded earlier among *Salmonella* isolates, irrespective of their serovars or sample source by previously published works (Helmy *et al.,* 2009; Moussa *et al.,* 2010; Shanmugasamy *et al.,* 2011; Fazl *et al.,* 2013; Rowlands *et al.,* 2014; Mohamed *et al.,* 2014; Ammar *et al.,* 2016; Ranjbar *et al*., 2017, Proroga *et al.,* 2018; Thung *et al.,* 2018; Elkenany *et al.,* 2019). The *invA* gene is considered a useful marker or target gene for molecular investigation of *Salmonella* serotypes by PCR technique (Rowlands *et al.,* 2014).

The *Salmonella* outer protein encoded by *sopB* gene was found in 50% of *S.* Typhimurium isolates. This factor is located in SPI-5, associated with TTSS-1, and it is required for full virulence in a murine model (Elemfareji

and Thong, 2013). A study from Malaysia reported that 50% of the *S.* Typhimurium isolates harbored *sopB* virulence gene (Thung *et al.,* 2018). The obtained percentage was approximately similar to that reported from *S.* Typhimurium (44.4%) isolated from broilers in Egypt (Ammar *et al.,* 2016). The occurrence of *sopB* factor in this study was less than that reported from *S.* Typhimurium isolated in India, which showed that all tested *S.* Typhimurium isolates carried *sopB* gene (Rahman, 2006).

Fimbriae in *Salmonella* spp. play a significant role in the pathogenicity, because they contribute to the attachment of these pathogens to the host epithelial cells. The plasmid-encoded fimbriae are encoded by the *pef* operon (Murugkar *et al.,* 2003). Among the *S.* Typhimurium isolates tested, the *pef*A gene was detected in 35.7% of the isolates. The obtained percentage was about similar to that reported previously from *S.* Typhimurium isolates (44.4%) recovered from broilers in Egypt (Ammar *et al.,* 2016), and was in contrast to another study from Malaysia where all *S.* Typhimurium isolates obtained from beef meat usually carried *pef*A gene (Thung *et al.,* 2018).In addition, the results of this study were in contrast to another recent study from Egypt, which showed that all *S.* Typhimurium isolates recovered from cloacal swabs, farm environment, and whole chicken carcasses samples did not carry *pef*A gene (Elkenany *et al.,* 2019). Also, the result of this study was in contrast to another recent study from Italy, which showed that the occurrence of pefA gene among *S.* Typhimurium isolates of human origin was 8.2% (Proroga *et al.,* 2018). In Brazil, the occurrence of the virulence gene *pefA* was more than that in

Palestine; it was 66.7% among *S.* Typhimurium isolates, associated or not with foodborne salmonellosis (Rowlands *et al.,* 2014). Virulotyping of *S.* Typhimurium serotype in this study showed that the occurrence of *prgH* gene was 46.4%. This result was, in contrast, to a study conducted by Srisanga *et al.,* (2017), which showed that the occurrence of this gene among different *Salmonella enterica* including *S.* Typhimurium serotype recovered from dogs and cats was 91.8% (Srisanga *et al.,* 2017). The presence of virulence genes in *S.* Typhimurium isolates recovered from different types of food samples may play an important role in infection. Pathogenicity of *Salmonella* strains included *S.* Typhimurium is controlled by a set of factors encoded by specific virulence genes that assist these types of pathogens to express the virulence in the host cells, at the end this led to the appearance of typical symptoms of infection in an infected individual (Gharieb *et al.,* 2015).

Conclusion

The preliminary data from this study have considerable epidemiological implications. Molecular assays using PCR-based methods for identification, virulotyping, and genotyping of *S.* Typhimurium is a useful approach for drawing up a group of genes to use in the epidemiological characterization of *S.* Typhimurium isolates. Contamination of food with *Salmonella* especially with *S.* Typhimurium indicates the bad microbiological quality of food. This serotype of *Salmonella* may act as a source of human infection. The present study emphasizes the need for

rigorous public health and hygienic measures during food preparation to lower the human health hazard risk associated with *Salmonella* diseases. Moreover, the recovered *S*. Typhimurium isolates exhibiting multiple virulence genes, which constitute a possible risk to humans from consumption of these products. Early detection of the virulence gene provides many benefits for public health, especially for rapid diagnosis and control of contamination and infection.

Recommendations

- Strict hygiene and control measures should be applied in order to avoid contamination that could occur from the production phase to consumption.
- Increase monitoring and surveillance efforts to improve knowledge of the incidence and seriousness of these food borne diseases and related hazards.
- Increase awareness among individuals behaviors related to safe food-handling practices and commitment to hygienic practices.
- Improve the inspection activities including periodic meat inspection and regular sampling from different slaughterhouses and local markets.

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الملخص

السالمونيلا هي واحدة من أكثر مسببات الأمراض البكتيرية التي تنتقل عن طريق الأغذية، وهي مصدر قلق رئيسي للصحة العامة في جميع أنحاء العالم. تمثل الدواجن ومنتجاتها وكذلك البيض، مصدرًا مهمًا لهذه البكتيريا، ونقلها للإنسان مما له تأثير كبير على الصحة العامة. ان عوامل الخطورة والضراوة لهذه البكتيريا وخاصة السالمونيلا التيفيميوريوم غير مدروسة في فلسطين.

حيث هدفت هذه الدراسة الى التحقق من حدوث السالمونيلا انتريتيديس والسالمونيلا التيفيميوريوم باستخدام تقنية تفاعل البوليمراز المتسلسل المتعدد Multiplex PCRبين عزلات السالمونيلا التي تم جمعها من السوق المحلي. بالإضافة إلى ذلك، فقد تم عمل تنميط لعوامل الخطورة والضراوة وتنميط لبصمات الحمض النووي بواسطة تقنية RAPD-PCR والتسلسل المتكرر (Rep-PCR)، عن طريق استخدام ERIC-PCR و BOXAIR-PCR لتقييم العلاقات الوراثية بين سلالات السالمونيلا التيفيميوريوم باستخدام تفاعل البوليمراز المتسلسل mPCR.

حيث اظهرت نتائج هذه الدراسة بأن النسبة الإجمالية لحدوث السالمونيلا التيفيميوريوم والسالمونيلا انتريتيديس قد كانت 54.9٪ و 0.0٪ على التوالي. وكذلك فقد تم تمييز وتوصيف 28 عزلة من السالمونيلا التيفيميوريوم لجينات الضراوة. حيث تم اكتشاف 13 جيناً فقط من أصل 17 في هذه العزلات الـ 28 من السالمونيلا التيفيميوريوم. وكانت نسبة حدوث الجينات

المكتشفة بين هذه العزلات 100٪ ، 50٪ ، 46.4٪ ، 39.3٪ ، 35.7٪ ، 35.7٪ ، 32.1٪ ، 25٪ ، 25٪ ، 17.6٪ ، 14.3٪ ، 14.3٪ ، 3.6٪ لـــ *invA* و *sopB* و *prgH* و *sitC* و *pefA* و *tolC* و *cdtB* و *msgA* و *sifA* و *iroN* و *spiA* و *ipfC* و *pagC* على التـــوالي. وبالنسبة لجينات الضراوة المتبقية فكانت غائبة في جميع عزلات الســـالمونيلا التيفيميوريـــوم. وبناء عليه، واستنادا الى الجمع بين وجود وغياب جينات الضراوة فقد تم الكشف عن 8 ملامح بين هذه العزلات، وكان المظهر الجيني الأكثر شيوعًا هو V5 (كل 32.1٪). في هذه الدراسة، كل من بادئات ERIC و BOX قد سمحت بالتمييز في 4 و 6 مجموعات أو نســـخ مـــن 16 عزلة من السالمونيلا التيفيميوريوم على التوالي، وذلك على أساس خصائصهم الجينية عند نقطة الفصل 96٪. وقد أظهرت نتائج طرق تصنيف تفاعل البلمرة المتسلســـل PCR أن الســـلالات S83 (أجنحة الدجاج) و S86 (الدجاج) و S87 (الدجاج) تتجمع معًا باستخدام كل من طـــرق التنميط ERIC-PCR و ,BOX-PCRوكان لديهم نفس نوع الضـــراوة (V1). وكـــذلك فـــإن السلالات S53 (دجاج) ،S73 (دجاج) ، S78 (برغر لحم بقري) و S80 (برغر لحم بقري) قد تم تجميعهم معًا أيضًا من خلال طرق التنميط وكان لديهم نفس نوع الضراوة (V8).

من خلال هذه الدراسة، فقد تم التوصل إلى بعض الاستنتاجات مع الآثار المحتملة لعزل وتحديد السالمونيلا من مصادر الغذاء. حيث أن نسبة تلوث الطعام بالسالمونيلا خاصةً مع الســـالمونيلا التيفيميوريوم كانت عالية ويشير ذلك إلى جودة ونوعية ميكروبيولوجية ســـيئة مـــن الأغذيـــة. بالإضافة إلى ذلك، تعتبر البيانات المقدمة هي المحاولة الأولى لتحديد مجموعـــة واســـعة مـــن جينات الضراوة لعزلات السالمونيلا التيفيميوريوم الفلسطينية المستخلصة مـــن أنـــواع غذائيـــة مختلفة. وتؤكد الدراسة الحالية على ضرورة اتباع طرق وأساليب صـــارمة للصـــحة العامـــة وسلامة الأغذية لتقليل المخاطر المحتملة لهذه البكتيريا على صحة الإنسان.