Faculty of Pharmacy Nursing and Health Professions
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Determination of the rate of *Salmonella enteritidis* in layer farms by polymerase chain reaction

تحديد معدل السالمونيلا المعوية في الدجاج البيّاض عن طريق تفاعل البلمرة المتسلسل

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Dedication

All challenging work requires personal efforts, as well as the encouragement of others, especially those who are very close to our heart. I dedicate my dissertation work to my family. A special feeling of gratitude to my loving parents whose words of encourages me. My brothers & sisters have never left my side and are very special. I also dedicate this dissertation to my lovely wife who have supported me throughout the study. I will always appreciate all they have done.

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الإقرار

أنا الموقع أدناه مقدم الرسالة التي تحمل العنوان:

Determination of the rate of *Salmonella enteritidis* in layer farms by polymerase chain reaction

تحديد معدل السالمونيلا المعوية في الدجاج البيّاض عن طريق تفاعل البلمرة المتسلسل

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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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List of Abbreviation

Symbol	Abbreviation
ASM	American Society for Microbiology
aw	water activity
bp	Base pair
CDC	Center of Disease Control and Prevention
DNA	Deoxyribonucleic acid
EEU	EU/European Economic Area
EU	European Union
g	Gram
H antigen	Flagellar antigen
H_2S	Hydrogen sulfide
hrs.	Hours
M	Molar
mg	Milligram
ml	Milliliter
MS	Member States
nm	Nanometer
µg	Microgram
µl	Micro liter
µm	Micrometer
O antigen	Somatic antigen
°C	Degree Celsius
PCBS	Palestinian Central Bureau of Statistics
PCR	Polymerase chain reaction
pH	a scale of acidity from 0 to 14
PT	phage type
rate/100K	Rate per 100000
rpm	Round Per Minute
ser.	serotype

Spp.	Species
SS agar	*Salmonella Shigella* agar
Subsp.	Subspecies
TSI agar	Triple sugar iron agar
U.S.A	United States of America
UK	United Kingdom
US	United States
USD	United States Dollar
USDA	U.S. Department of Agriculture
UV	Ultraviolet
V	Volt
Vi antigen	Virulence antigen
WHO	World Health Organization
XLD agar	Xylose Lysine Deoxycolate agar
XLD agar	Xylose lysine desoxycholate agar

Abstract

Salmonella is one of the most frequently isolated foodborne pathogen. It is of major public health concern worldwide. Human illness with *Salmonella enteritidis* is often due to the consumption of contaminated eggs or eggs products. The prevalence of this pathogen in egg-laying poultry is an important public health risk factor. Data on the prevalence of *Salmonella* spp. in Palestinian poultry flocks is lacking. The objective of this study was to investigate the prevalence of *Salmonella* in local layers flocks in general and the rate of *Salmonella enterica* serovar *Enteritidis* infecting the oviduct of the chicken layers.

This study was conducted on 415 samples collected from several layer chicken farms in Northern West Bank provinces, Palestine. *Salmonella* species were identified by conventional biochemical testing, serology and confirmed by PCR

The rate of *Salmonella* prevalence in the sample tested was 12% (52/415). However, *Salmonella enterica* serovar *Enteritidis* was not recovered. This serovar has been the most frequently isolated from the oviduct of chicken layers. Our negative results can be due to the sample size tested.

Enteritidis serovar has tropism to the ovary of the chicken layers. In addition, the eggs can be internally and persistently infected with serovar. We recommend that further studies should be conducted taking into consideration to increase the sample size and to test the chicken layers and their eggs for the presence of *Salmonella* with emphasis on the *S. enteritidis* serovar.

ملخص الدراسة

السالمونيلا هو أكثر مسببات الأمراض المنقولة عن طريق الأغذية. هو مصدر قلق كبير للصحة العامة في جميع أنحاء العالم. مرض الإنسان التي تسببه السالمونيلا المعوية غالباً ما يكون بسبب استهلاك البيض أو لمنتجات البيض الملوثة. كثرة انتشار هذه الجرثومة الممرضة في دواجن وضع البيض يعتبر عاملاً مهما من المخاطر التي تهدد الصحة العامة. لا تتوفر معلومات عن انتشار السالمونيلا في الدواجن في فلسطين . الهدف من هذه الدراسة هو البحث في انتشار السالمونيلا في قطعان الدواجن المحلية بشكل عام ومعدل السالمونيلا المعوية serovar Enteritidis التي تصيب قناة المبيض في الدجاج البياض.

أجريت هذه الدراسة على 415 عينة تم جمعها من عدة مزارع دواجن للدجاج البياض في محافظات شمال الضفة الغربية في فلسطين. تم التعرف على أنواع السالمونيلا من خلال الاختبارات الكيميائية الحيوية التقليدية والأمصال والتأكد من صحة النتائج بواسطة PCR .

وكان معدل السالمونيلا في العينة التي تم اختبارها 12 ٪ (415/52). ومع ذلك، فان *Salmonella enterica* serovar Enteritidis. لم تكن موجودة بين عزلات السالمونيلا مع انها الأكثر في التسبب في التهاب قناة المبيض في الدجاج البياض. نتائجنا السلبية هذه يمكن أن تكون بسبب حجم العينة الذي تم اختباره.

منذ ان ال Enteritidis serovar لديه النزعة للتواجد في المبيض في الدجاج البياض، بالإضافة الى امكانية أن تصاب البيضة من داخلها وباستمرار بهذه الجرثومة. نوصي بإجراء مزيد من الدراسات مع مراعاة زيادة حجم العينة واختبار الدجاج البياض والبيض للحصول على نتائج مفيدة حول وجود السالمونيلا مع التركيز على.*S. enteritidis* serovar.

Chapter One

Introduction

1.1 Overview

Salmonellosis is one of the common and vastly spread food-borne illnesses. It constitute a big common health load, and represents a considerable cost in many states. It can cause a variety of illnesses in several hosts whereas in others; it can exist without symptoms (Nakhala, 2013).

Salmonella keeps being a major source of foodborne gastric or enteric illnesses and is responsible for considerable human sufferance, mortality and represents a significant cost in several countries (Herikstad & et al, 2002). Salmonellosis is one of the most common foodborne infections in the United States, resulting in an estimated 1.2 million human cases and $365 million in direct medical costs annually (CDC, 2013). *Salmonella* exists commonly in domestic animal, including the poultry, and can be isolated from wild animals, birds, creepers, and rodents (Evira, 2018). Poultry meat is an important intermediary of foodborne *Salmonella* contagions in humans. The true proportion of all salmonellosis cases are related with poultry consuming (Salehi & et al, 2005). In Palestine, control of *Salmonella* and preventing foodborne diseases are due to the tremendous efforts carried out by the environmental health inspectors in all districts (Issa & et al, 2017).

Poultry, turkey and chicken in particular are extremely colonized with *Salmonella* without remarkable symptoms (carriers) at incipient production scale. The existence of *Salmonella* in healthy poultry is considered as the major risk factor that permits bacteria to readily transport in poultry meat and table eggs to humans (Antunes & et al, 2016).

Poultry ranching, whether for meat or eggs productions, forms the most important agricultural industry in Palestine. Poultry farming is primarily limited to small private companies owned by individuals and not exclusive to big companies as seen in neighboring countries. Therefore, the large number of farms distributed throughout the country makes controlling of health status of the flocks by the Ministry of Agriculture extremely difficult. Eggs and egg products are nutritious foods that constitute important part of the people's diet (Ministry of Agriculture, 2016). Consuming eggs, however, has been associated with negative health impact when handled improperly and can be a major cause of foodborne disease (EFSA & ECDC, 2016). *Salmonella enteritidis* has been considered as important pathogen that infects poultry, eggs, fresh meat, milk, fruits and vegetables causing salmonellosis or gastroenteritis manifested by fulminant diarrhea with low grade fever, septicemia, and nausea and vomiting in affected humans (MFMER, 2018).

1.2 Objectives of this work

1.2.1 General Objectives

- To determine the rate of *Salmonella enteritidis* infections in layer farm by polymerase chain reaction (PCR).
- To investigate the occurrence of *Salmonella* spp. and *Salmonella enteritidis* in layers farms distributed in Palestine.
- To estimate the prevalence of *Salmonella* in different poultry layers farms distributed in Palestine.

1.2.2 Specific objectives

- To compare between *Salmonella* species and other pathogens.
- The findings of this research regarding prevalence of *Salmonella* spp. and *Salmonella enteritidis* in Palestine will be reported to the Ministry of Agriculture and Ministry of Health to carry the necessary measures to curb the spread of salmonellosis by adopting and implementing strict guidelines on the poultry farms.

1.2.3 The specified problems and research question

The fundamental questions handled in this thesis are:

- What is the prevalence of *Salmonella* in different poultry layers farms throughout Palestine?
- What are the efforts and measures taken to curb the spread of the salmonellosis in Palestine? What preventive measures have been proposed?

1.3 Significance of the study

Food-borne illnesses have featured as a serious public health trouble in many countries in the recent decade. Gastric or enteric pathogens such as *Salmonella enteritidis* and similar diseases carried by food can be a challenge to many countries at the same time. Understanding the global nature of these problems is essential to develop monitoring and control strategies (Herikstad & et al, 2002).

Salmonella is one of the most frequently isolated food-borne pathogens. It is one of the world's top public health concerns, causing 93.8 million food-borne diseases and 155,000 deaths yearly (Eng et al, 2015).

Human contagions with *S. enteritidis* are often related to the consuming of contaminated eggs, so the spread of this pathogen in laying poultry is an important risk factor for public health. Numerous and complex environmental influences on *Salmonella* persistence and transmission are exerted by management practices and housing facilities used in commercial egg production (Gast & et al, 2017)

There is no doubt that agriculture constitutes on of the main components of Palestinian culture as well as economy and social life (Ministry of Agriculture, 2010). In addition, the Palestinians are strongly attached to their land making it part of their national identity and an important element of income, food export, jobs and economy. Unfortunately, and for many reasons where Israeli occupation is the most important, the utilization and cultivation of land has not been operating well to its full potential (UNCTAD, 2015). To emphasize, the value of optimal utilization of Palestinian land reflects positively on food security for the population as well as it contributing to a certain extent to the limited job sector and economy. Data from the agricultural census published by the Palestinian Central Bureau of Statistics (PCBS) for 2010/ 2011 indicates the presence of 85,885 agricultural holdings in the West Bank. About 68.2% are plant holdings, 10% are livestock holdings, and 21.8% are mixed holdings. The ownership of Palestinian land has been notably increasing due to agricultural land inheritance (ARIJ, 2015).

Traditionally, Palestinian farmers usually raise and breed their livestock to generate additional income and provide food security for their families. A major setback for these practices is the various diseases that inflect livestock. This has been associated with negative impact on the economy and social aspects of Palestinian farmers, thus

negatively affecting their abilities to safely breed livestock in this country (Adra & et al, 2016).

According to Ministry of Health in Palestine, many cases of foodborne illness were reported between 2009 and 2017 as shown Table 1.1.

Table 1.1: Number and rate/100K of food poisoning cases in Palestine reported from 2009 -2017.

Year	No of cases	Rate per 100000
2009	598	15.2
2010	442	11.6
2011	288	6.9
2012	320	7.3
2014	221	4.9
2015	156	3.33
2017	132	3

The number of cases and the rate of foodborne illness caused by *Salmonella* species has been monitored and reported annually in the Annual Report of Health published by the Ministry of Health in Palestine. As shown in Table 1.2, the numbers of cases reported ranged from 64 in 2017 to 406 in 2009. However, the number has tripled in 2017 as compared to 2015.

Table 1.2: Number and rate/100K of *Salmonella* cases in Palestine reported from 2009 -2017

Year	No of cases	Rate per 100000
2009	406	10.7
2010	319	8.3
2011	223	5.5
2012	198	4.7
2014	55	1.2
2015	20	0.8
2017	64	1.44

Data on the prevalence of *Salmonella* spp. in poultry flocks is lacking. Therefore, the objective of this study was to investigate the prevalence of *Salmonella* spp. in general and *Salmonella enteritidis* in particular among local layers flocks in the West Bank, Palestine.

Chapter Two

Background and Literature review

2.1 Food Poisoning

The phrase “food poisoning” is limited only to severe inflammation of the stomach and intestines as a result of bacterial contamination of drink or food. The “food-borne” sickness is generally either toxic or contagious in nature, caused by agents which enter the body through the intake of food. Food-borne illness is a disease caused by ingestion of food polluted by any agent, biological or chemical (Ramanathan, 2010).

Food poisoning is known as a disease caused by the consumption of beverages or food contaminated with bacteria and / or their toxins, viruses, parasites or chemical materials. Symptoms, which vary in degree and distinction, include headache, vomiting, abdominal pain, and diarrhea; more severe cases can lead to life-threatening conditions leading to disability or death. Most cases are moderate and get better without any specified medication. Some patients have serious illness and need hospitalization and antibiotic treatment (Friday and Ogori, 2014).

Contagions that require the ingestion of living cell can be considered food borne poisoning. It is estimated that more than eighty-one million people are influenced by foodborne diseases yearly. Diseases such as food poisoning are becoming more popular as our lifestyles change; On the one hand, we eat more catered meals that being prepared in advance. On the other hands, there are no precise figures on how much food poisoning is the result of mishandling by the consumer, but it is believed to be between 12 and 20% (Ramanathan, 2010).

Salmonellosis is a type of food contagion resulting from the consumption of beverages or foods contaminated with *Salmonella.* The *Salmonella* family involves more than 2300 serotypes, but two types, *Salmonella enteritidis* and *Salmonella typhimurium* are the most common in the United States and account for half of the infections (USDA & FSIS, 2011). Once ingested, bacteria can continue to live and grow in the intestine, creating an infection and causing disease. The potential and severity of the disease depends to a large extent on the size of the dose, the host's resistance and the specific strain of *Salmonella* that causes the disease. *Salmonella* bacteria are spread by direct or indirect contact with the intestinal contents or excreta of animals, including humans (Kendall, 2012). *Salmonella* is mostly associated with contamination of poultry, table eggs, raw meats, and dairy product. Many other foods have been involved in outbreaks caused by *Salmonella*, which include salads, mayonnaise, orange juice, milk and dairy products (Figure 2.1). *Salmonella* enters other foods through cross contamination by contact with utensils, raw foods, equipment and hands (Ramanathan, 2010). *Salmonella enteritidis* is the dominant serovar correlated with *Salmonella* outbreaks (Eng & et al, 2015). In 2015, *Salmonella enteritidis* accounted for 60.3% of all *Salmonella* outbreaks and 61.1% human cases of all cases in *Salmonella* outbreaks in the EU (Jansa & et al, 2017). The evidence supporting the relationship with the suspect food was reported as strong. Eggs and egg products were the most frequently associated with *Salmonella enteritidis* outbreaks with strong evidence (Moffatt & Musto, 2013). The proportion of outbreaks with strong evidence decreased from 46.1% in 2014 to 29.3% in 2015 (ECDC & EFSA, 2017, March 7). Despite the decrease in the number of cases of salmonellosis in the European Union (EU), it represents the main

cause of outbreaks transmitted by food and the second most frequently reported zoonosis (Mascaro & et al, 2017).

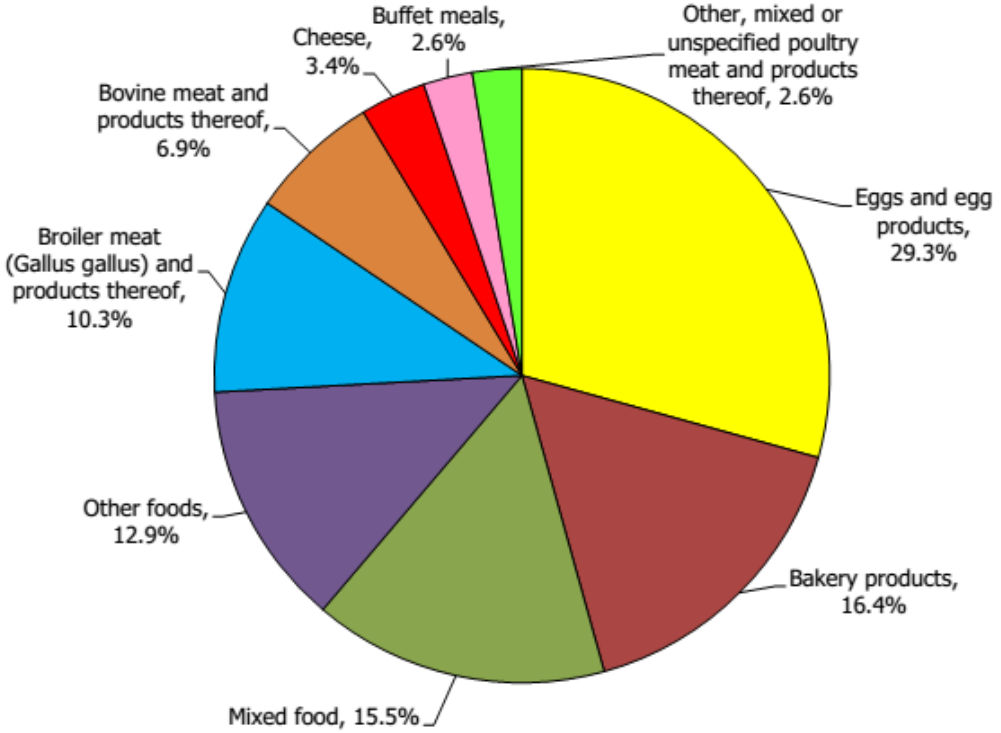


Figure 2.1: Distribution of food vehicles in strong-evidence foodborne outbreaks caused by *Salmonella enteritidis*, EU, 2015(n=116) (ECDC & EFSA, 2017, March 7).

The main reservoir of *Salmonella* is the gastrointestinal tract of a wide range of domestic and wild animals, which can result in the contamination of a variety of animal and plant foods. The 2012 report showed that, *S.enteritidis*, *S.typhimurium* and monophasic *S.typhimurium* were the serovars most related to the human disease (Figure 2.2), followed by *S.infantis* and *S.stanley* was the causative pathogen in 0.8% and 1.4% of human cases in 2011 and 2012, respectively. The cases of *S.enteritidis* in humans are more commonly related to the consumption of contaminated poultry meat and eggs, while cases of *S.typhimurium* are generally related to the consumption of contaminated poultry, bovine and pork meat (Hugas & Beloeil, 2014).

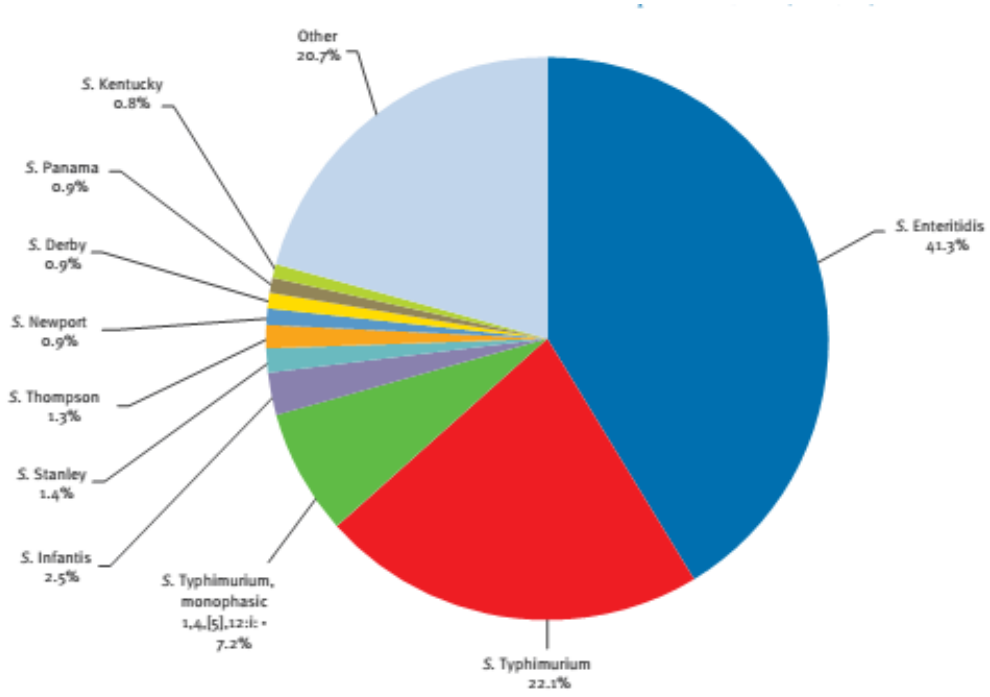


Figure 2.2: Distribution of the 10 most common *Salmonella* serovars in humans in the European Union, 2012 (N=82,409) (Hugas & Beloeil, 2014).

2.2 *Salmonella* infections (Salmonellosis)

Salmonellosis is a common bacterial disease that affects the gastrointestinal tract (Crum-Cianflone, 2008). *Salmonella* bacteria commonly live in human and animal intestines and are disseminated through the stool. Humans become infected more frequently through contaminated food or water (CDC, 2013). In general, people with *Salmonella* infection have no symptoms. Others suffer from fever, abdominal cramps and diarrhea within 8 to 72 hours. Most healthy people recover within a few days without specific treatment (Nakhala, 2013). In some cases, the diarrhea associated with *Salmonella* infection can be so dehydrating as to require prompt medical attention. Life-threatening complications also may develop if the infection spreads beyond your intestines. Your risk of acquiring *Salmonella* infection is higher if you travel to countries with poor sanitation (MFMER, 2018).

Numerous outbreaks of *S.enteritidis* infections in humans have been associated with consumption of eggs or foods containing eggs. Egg contents may become contaminated with *Salmonella* from soiling of the shell with feces of infected hens, either by direct penetration of the shell or on breaking the eggs (Long & et al, 2017). There is evidence that *S.enteritidis* also gains access to egg contents by migrating from the cloaca to the reproductive organs, trans ovarian migration following systemic infection and localization in the ovaries, oviducts or peritoneum of laying hens, can also cause infection to the eggs (Whiley & Ross, 2015). Indeed, hens naturally infected with *S. enteritidis* have been found to carry the organism in the reproductive tract as well as in the ceca (Poppe & et al, 1992). *Salmonellae* are isolated more often from poultry and poultry products than from any other food animals. Chickens can be infected with many different serovars of paratyphoid *Salmonella*. Among these paratyphoid Salmonellae, infections due to *S. typhimurium, S. enteritidis* and *S. heidelberg*, are of worldwide in distribution with wide host range and are of major economic and public health significance (Mengistu & et al, 2011). *Salmonella enteritidis* has been the major cause of food-borne salmonellosis pandemic in humans over last 20 years, during which contaminated hen's eggs were the most important transporter of the infection (Legesse, 2017). This because it has the unique ability to contaminate eggs without causing discernible illness in the birds infected. The infection route to humans involves colonization, survival and multiplication of the pathogen in the hen house environment, the bird and, finally, the egg. Epidemiological investigations in Hungary, the United Kingdom, the United States, and Germany confirmed that the food most associated with increased illness in people was the egg (Guard-Petter, 2001). The transmission of *Salmonella enteritidis* infections to humans by contaminated eggs has been a prominent

food safety issue throughout much of the world for more than a decade. Diverse risk reduction strategies have been recommended and incorporated into regional and national microbial quality assurance programs for eggs. Prospective targets for S. *enteritidis* control programs exist along the entire egg production and utilization continuum (Gast. and Holt, 2001).

In 2015, a total of 94,625 confirmed salmonellosis cases were reported by 28 EU MS, resulting in an EU notification rate of 21.2 cases per 100,000 population (Visscher & et al, 2018). This represented a 1.9% increase in the EU notification rate compared with 2014. There was a statistically significant decreasing trend of salmonellosis in the 8-year period between 2008 and 2015. Ten MS reported 126 fatal cases among the 16 MS that provided data on the outcome of their cases. This gives an EU case fatality rate of 0.24% among the 52,605 confirmed cases for which this information was available (Table 2.1) (EFSA and ECDC, 2016).

Table 2.1: Reported hospitalization and case-fatality rates due to zoonosis in confirmed human cases in the EU, 2015 (EFSA and ECDC, 2016).

Disease	Number of confirmed[a]	Hospitalisation				Deaths			
	Human cases	Status available (%)	Number of reporting MS[b]	Reported hospitalised cases	Proportion hospitalised (%)	Outcome available (%)	Number of reporting MS[b]	Reported Deaths	Case Fatality (%)
Campylobacteriosis	229,213	27.0	17	19,302	31.2	73.7	16	59	0.03
Salmonellosis	94,625	34.0	16	12,353	38.4	55.6	16	126	0.24
Yersiniosis	7,202	23.9	14	530	30.9	59.8	14	0	0.0
STEC infections	5,901	39.4	14	853	36.3	56.2	15	8	0.24
Listeriosis	2,206	44.9	18	964	97.4	69.1	20	270	17.7
Tularaemia	1,079	14.9	9	89	55.6	15.6	10	0	0.0
Echinococcosis	872	20.5	13	107	59.8	23.5	13	1	0.49
Q fever	833	NA[c]	NA	NA	NA	47.7	12	3	0.36
Brucellosis	437	42.8	8	130	69.5	31.1	8	1	0.74
Trichinellosis	156	72.5	7	30	34.5	75.0	8	0	0.0
West Nile fever[a]	127	51.2	7	54	83.1	51.2	5	2	1.57
Rabies	0	NA[c]	NA	NA	NA	0.0	0	0	0.0

(a): Exception made for West Nile fever where the total number of cases was included.
(b): Not all countries observed cases for all diseases.
(c): NA-not applicable as the information is not collected for this disease.

From 2005 to 2010 the CDC reported an unusually high number of cases in the US as a result of several independent *Salmonella* outbreaks. Frequent factors associated with outbreaks included raw ingredients in contact with contaminated animal or people, improper storage or incomplete cooking of food products (CDC, 2010). The most recent outbreak leading to significant concern among health authorities, consumers and farms owners, was the January 2010 multistate outbreak that lasted one entire year. This outbreak caused by *S.enteritidis* contaminated eggs affected 16 states and resulted in an estimated number of 1939 cases. The estimated 380 million contaminated chicken eggs shipped across the US lead to a massive egg recall recommended by the US Food and Drug Administration to prevent further spread of the infection (Sanchez-Vargas & et al, 2011). The major worldwide incidences of foodborne salmonellosis are given in (Table 2.2) (Kumar, 2009).

Table 2.2: Worldwide major foodborne salmonellosis outbreaks (Kumar, 2009).

Sl No.	Country	Source	*Salmonella* serovar	No. of cases	References
1.	Holland (1981)	Salad	Indiana	600	Beckers et al., 1985
2.	Scotland (1981)	Raw milk	Typhimurium PT204	654	Cohen et al., 1983
3.	Canada (1984)	Cheese	Typhimurium PT10	2700	D'Aoust et al., 1985
4.	USA (1985)	Pasteurized milk	Typhimurium	16284	Lecos, 1986
5.	China (1987)	Egg drink	Typhimurium	1113	Ye et al., 1990
6.	Japan (1988)	Cuttlefish	Champaign	330	Ogawa et al., 1991
7.	USA (1991)	Cantaloupes	Poona	400	Francis et al., 1991
8.	France (1993)	Mayonnaise	Enteritidis	600	Geiss et al., 1993
9.	Germany (1993)	Paprika chips	Saint-paul, Javiana	670	Lehmacher et al., 1995
10.	Finland (1994)	Alfalfa sprouts	Bovismorbificans	492	Ponka et al, 1995.
11.	USA (1995)	Orange Juice	Hartford	62	Parish, 1998
12.	Canada (1998)	Cheddar cheese	Enteritidis PT8	700	Ratman et al.1999
13.	Japan (1999)	Cuttlefish chips	Oranienburg, Chester	1500	Tsujii and Hamada, 1999
14.	Australia (1999)	Orange Juice	Typhimurium	427	Anon. 1999
15.	USA (1999)	Orange Juice	Muenchen	220	Boase et al., 1999
16.	USA (2000)	Orange Juice	Enteritidis	74	Butler, 2000
17.	Germany (2004)	Fermented sousage	Goldcoast	24	Bremer et al., 2004

2.3 Salmonellosis outbreaks related to eggs

Prior to the 1980s, *S.enteritidis* was rarely isolated from poultry, and most isolates may have been derived from contaminated feed. Recently, however, the incidence of *S. enteritidis* infection in poultry flocks has been increasing in Britain, the United States and other countries. At the same time, a dramatic increase in the number of outbreaks of food poisoning due to *S.enteritidis* in man has been reported (Suzuki, 1994). Epidemiological studies have attributed the outbreaks of *S.enteritidis* food poisoning to the consumption of contaminated eggs or egg products (Moffatt & Musto, 2013). Thus, *S.enteritidis* has become the most serious pathogen for man and the poultry, industry (Suzuki, 1994).

Salmonella enteritidis is the most commonly detected serovar in humans of non-typhoidal salmonellosis in the European Union/European Economic Area (EU/EEA). The number of human cases of *S.* Enteritidis has declined substantially in recent years (Fig. 2.4), most likely as a result of successful control measures implemented in poultry populations, and measures in food businesses to improve hygiene implemented under EU Food law (ECDC & EFSA, 2017, Feb. 3). A multi-country outbreak of *Salmonella enteritidis* phage type (PT) 8 linked to eggs is ongoing in the European Union/ European Economic Area (EU/EEA) (ECDC & EFSA, 2016, Oct. 27). Outbreak cases, both confirmed and probable, have been reported by Belgium, Croatia, Denmark, Finland, France, Greece, Hungary, Italy, Luxembourg, the Netherlands, Norway, Slovenia, Sweden and the United Kingdom. Eleven confirmed cases are reported to have travelled to Poland during the incubation period. Poland is therefore likely to be affected by this outbreak as well. Croatia and Hungary reported a fatal case each. The outbreak peaked at the end of September 2016, shortly before the vehicle of infection

was identified and control measures were implemented at the farm and distribution level. Since then, the number of cases caused by the outbreak types of S. Enteritidis has steadily decreased (ECDC and EFSA, 2017, March 7).

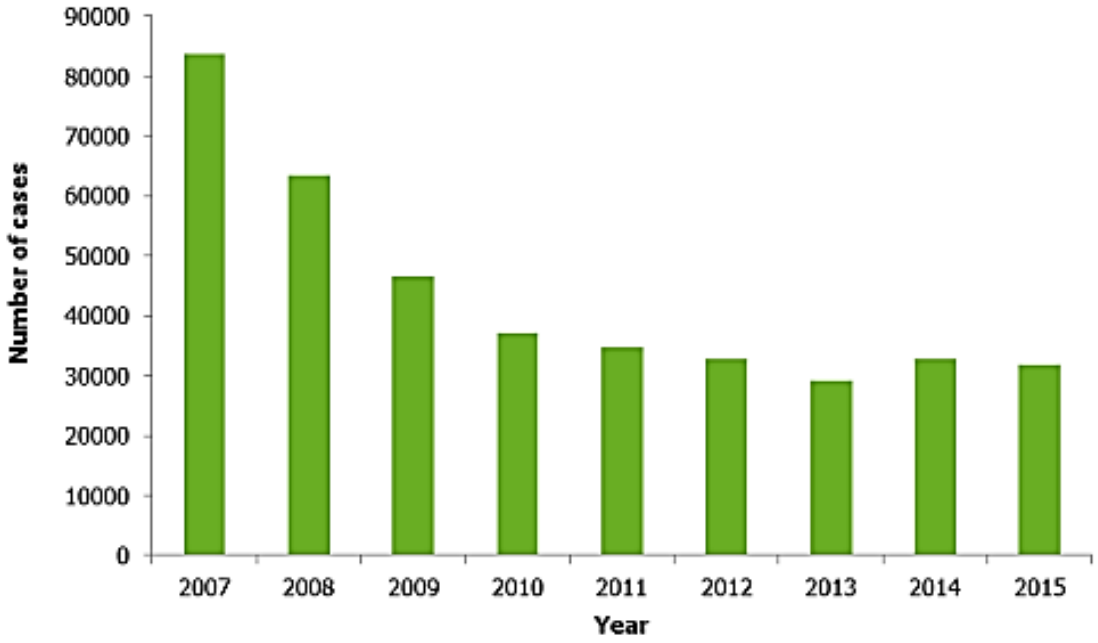


Figure 2.3: Number of confirmed cases of *Salmonella enteritidis* by year, EU/EEA, 2007–2015 (ECDC & EFSA, 2017, March 7).

Two most commonly reported *Salmonella* serovars in 2013 were *S.enteritidis* and *S. typhimurium*, representing 39.5 % and 20.2 %, respectively, of all reported serovars in confirmed human cases. *S. Enteritidis* continued to decrease, with 4,720 fewer cases (14.1 % less) reported in the EU in 2013 than in 2012. In the two year period from 2011 to 2013, cases of *S.typhimurium* decreased by 11.1 %, while cases of *S.infantis*, the fourth most common serovar, increased by 26.5 %. The increase observed in S. Derby, the fifth most common serovar in 2013, could be partly explained by a local outbreak in one EU MS (EFSA and ECDC, 2015).

A total of 997 outbreaks of *S.enteritidis* were reported in the United States between 1985 and 2003 (Figure 2.4), resulting in 33,687 illnesses, 3,281 hospitalizations and 82 deaths (Braden, 2006). In the U.S.A, the number of reported *Salmonella enteritidis* outbreaks increased from 26 in 1985 to 85 in 1990, then gradually decreased to 34 in 2003. In addition, the number of reported SE outbreaks decreased from a high of 2656 in 1990 to a low of 578 in 2003 (Msallam, 2008). The one exception was 1994, a year in which 14000 cases were attributed to an outbreak of SE infection caused by consumption of a contaminated, nationally distributed ice cream product. A food vehicle has been confirmed in approximately 44% of the *Salmonella enteritidis* outbreaks. About 75% of outbreaks had vehicles that were either primarily egg-based or that contained egg ingredients (Braden, 2006).

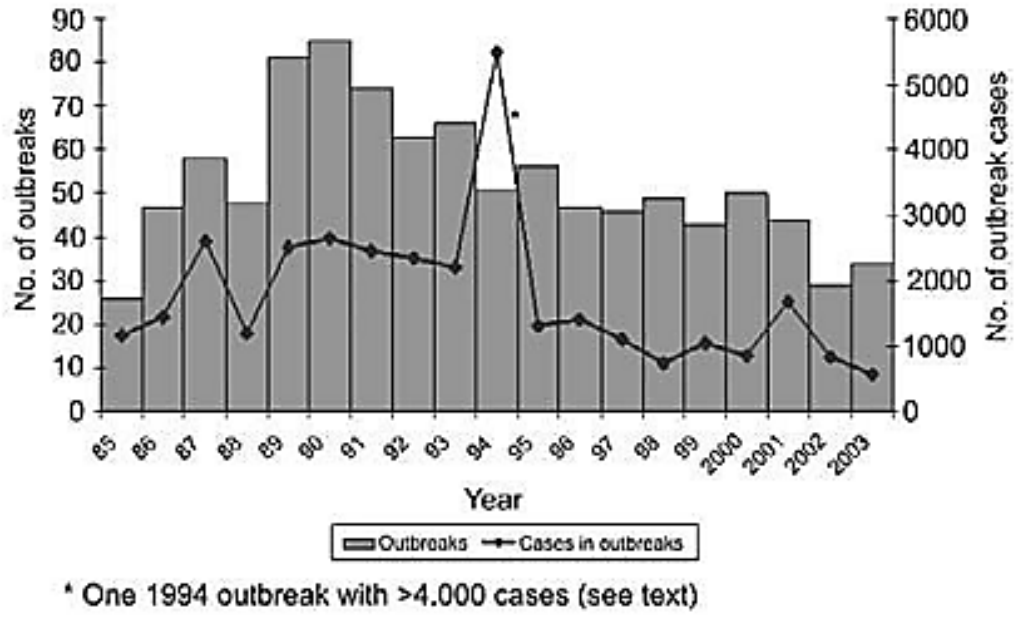


Figure 2.4: Number of outbreaks of *Salmonella enterica* serotype *Enteritidis* infection and associated cases in the United States, 1985–2003 (Braden, 2006).

2.4 Economic cost due to salmonellosis

Food-borne illness is among the most widespread public health problems creating social and economic burdens as well as human suffering that makes it a concern that all countries need to address (WHO and FAO, 2002). The social and economic impact of food-borne disease is considerable. It imposes costs upon the public sector, on industry, in particular the wholesale and retail food industry, and very importantly upon the infected person and their family. The disease can cause hospitalization; in a small proportion of cases can cause death (Sockett, and Roberts, 1991). In the United States, foodborne illness costs billions of dollars every year. Pathogens are microorganisms that cause disease which include bacteria, fungi, parasites and viruses. Researchers at the Economic Research Service (ERS) of the U.S. Department of Agriculture (USDA) estimate that the annual cost of human illnesses for seven of these foodborne pathogens (six bacteria and one parasite) from all food sources is $5.6-$9.4 billion (Buzby & et al, 1996). Typhoid and non-typhoid salmonellosis remain major public health problems and are clearly the most economically important food-borne disease (Akpabio, (2015). *Salmonella* is a leading cause of foodborne illness worldwide with an estimated annual economic loss of 3.7 billion dollars (SGS, 2015).

Salmonella species are Gram-negative bacilli associated with animal and human infections. *Salmonella spp*. infections lead to high morbidity rates not only in the developing world but also in industrialized countries and high mortality mainly in the poorest nations of the developing world. It is believed that epidemics caused by *Salmonella* spp. may have significantly affected the history of humankind, even at present, the effect of *Salmonella* infections on entire communities can result in

economic burden to developing and also industrialized nations (Sanchez-Vargas & et al, 2011).

2.5 Taxonomy and Nomenclature of *Salmonella*

The nomenclature for *Salmonella* is still evolving and the debate on the name for the type species is not likely to be settled any time soon. In the meantime, the work of isolating, identifying, and reporting on *Salmonella* serotypes must go on for diagnostic, therapeutic, and public health purposes. We believe that the nomenclature system used at CDC, essentially based on the recommendations established by the WHO Collaborating Centre, adequately addresses the concerns and requirements of clinical and public health microbiologists. Because the type species name has not been officially approved and in order to shorten reports, *Salmonella enterica* subsp. enterica serotype *Typhimurium*, for example, is shortened to *Salmonella* serotype (ser.) *Typhimurium* or *Salmonella typhimurium*. To ensure backward compatibility with literature searches on *Salmonella* serotypes from electronic databases, both versions of the serotype name should be listed as key words in manuscripts. In 1999, at the American Society for Microbiology (ASM) Publications Board Meeting, a proposal that relevant ASM journals adopt the *Salmonella* nomenclature currently used at Centers for Disease Control and Prevention (CDC) was unanimously endorsed by the board, with plans to update 2000 ASM Instructions to the Authors (Brenner & et al, 2000).

The nomenclature of the genus *Salmonella* has reached an unsatisfactory state of affairs, with two systems of nomenclature in circulation. One system, proposed in the 1980s by Le Minor and Popoff, has received wide acceptance, although it does not conform to the rules of the Bacteriological Code. The other system, which conforms to

the rules of the Bacteriological Code, is being used by an ever-decreasing minority. As a result of a number of recent Requests for an Opinion, the Judicial Commission of the International Committee on the Systematics of Prokaryotes has issued an Opinion with the intention that it should solve these discrepancies. However, like all Opinions, it is limited to matters of nomenclature and does not help to interpret the taxonomic consequences. The Judicial Commission has therefore asked experts in the field of nomenclature and taxonomy to write a commentary on the nomenclatural and taxonomic consequences of Opinion. The current widely accepted interpretation that the genus *Salmonella* currently includes only two species (Tindall & et al, 2005).

The Salmonellae are a heterogeneous group of bacteria in the genus *Salmonella* of the family Enterobacteriaceae. The taxonomy and nomenclature of *Salmonella* have changed over the years and are still evolving. Currently, the CDC recognizes two species which are divided into seven subspecies: *S.enterica* (six subspecies) and *S.bongori* (one subspecies). The subspecies are divided into over 50 serogroups based on somatic (O) antigens present. The serogroups are further divided into over 2300 serotypes based on flagellar (H) antigens (Fig. 2.5). The CDC now recommends that all organisms identified as *Salmonella* be reported by genus and serotype (or serogroup) omitting the reference to species. *Salmonella* serotypes are recognized with antigenic formulas listed in the document called the Kauffman-White Scheme. Updating this scheme is the responsibility of the WHO Collaborating Centre for Reference and Research on *Salmonella*, which is located at the Pasteur Institute, Paris, France (Harrison & Gay, 2011).

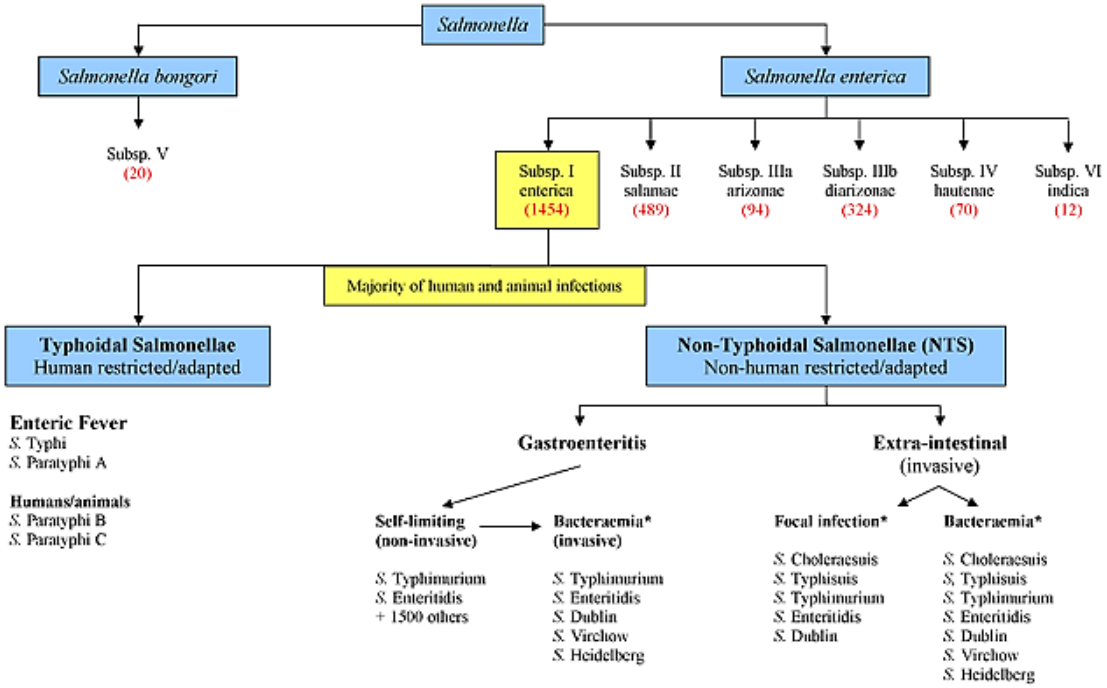


Figure 2.5: Classification of the genus *Salmonella* (Akyala & Alsam, 2015)

Salmonella have many different serotypes. Some serotypes are only found in one kind of animal or in a single place. Others are found in many different animals and all over the world. Some can cause especially severe illnesses when they infect people; while others cause milder illnesses. More than 2,500 serotypes have been described for *Salmonella* in Table 2.3 but, because they are rare, scientists know very little about most of them. Less than 100 serotypes account for most human infections. What we learn about the more common serotypes can help us better understand illness and the natural history of all the *Salmonella* strains (CDC, 2019).

Table 2.3: Present number of serovars in each species and subspecies (Kumar, 2009).

***Salmonella* species and subspecies**	**No. of serovars** (Source Popoff et al., 2004)
Salmonella enterica	
subsp. *enteria* (I)	1504
susbsp.*salmae* (II)	502
subsp. *arizoane* (IIIa)	95
subsp. *diarizonae* (IIIb)	333
subsp. *houtene* (IV)	72
subsp. *indica* (VI)	13
Salmonella bongori	22
Total	2541

Salmonella bacteria look alike under the microscope but can be separated into many serotypes based on two structures on their surface:

1. The outermost portion of the bacteria's surface covering, called the O antigen; and
2. A slender threadlike structure, called the H antigen that is part of the flagella.

The O antigens are distinguished by their different chemical make-up. The H antigens are distinguished by the protein content of the flagella. Each O and H antigen has a unique code number. Scientists determine the serotype based on the distinct combination of O and H antigens (CDC, 2019).

The exact reasons for this high level of surface-antigen diversity are still unknown. In order to avoid confusion between *Salmonella* serovars and species, the name of the serovar starts with a capital letter and is not italicized. The former *S.*

typhimurium is now written in the form *S.enterica* supsp. *enterica* serovar *Typhimurium* or briefly *S.typhimurium*. *Salmonella* species belong to the same proteobacterial family as e.g. *Escherichia coli, Shigella, Yersinia* and others in the family of *Enterobacteriaceae* (Figure 2.6). *Salmonella* diverged from *E. coli* approximately 100-160 million years ago and acquired the ability to invade host cells (Lienemann, 2015)..

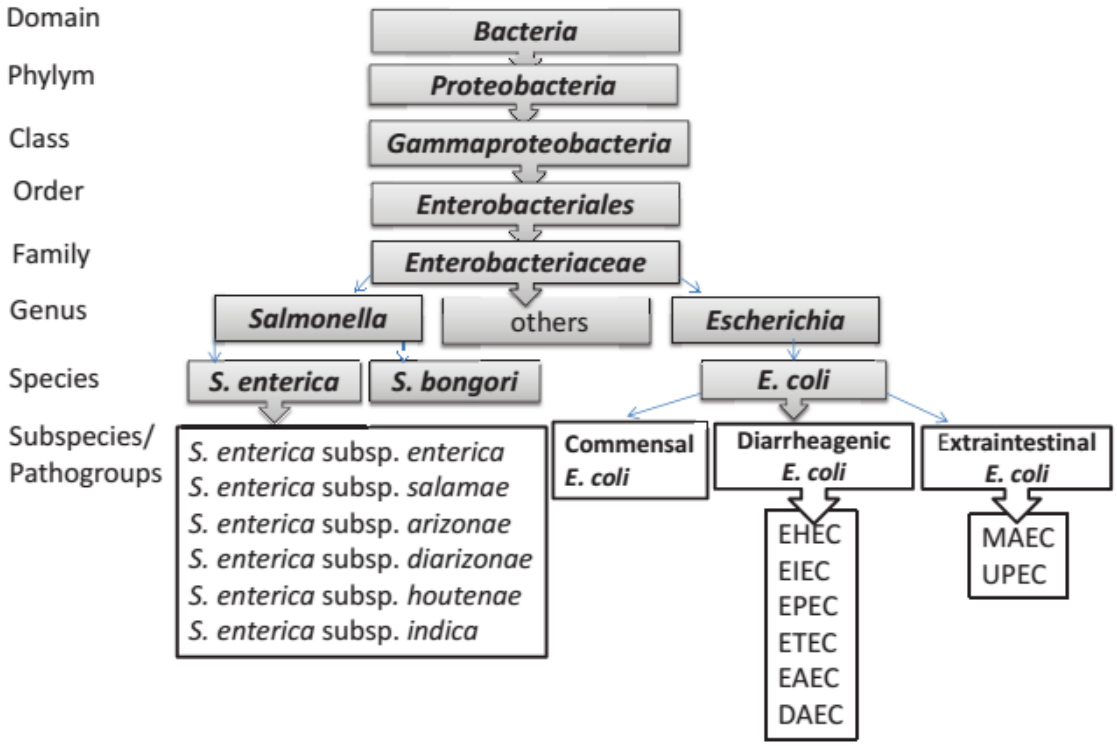


Figure 2.6: Taxonomy of *Salmonella* and *Escherichia coli* species (Lienemann, 2015).

2.6 General characteristics of *Salmonella*

Salmonella are 0.2 to 1.5 by 2 to 5 µm in size, gram negative, facultative anaerobic, rod shaped bacteria belonging to family *Enterobacteriaceae*. Members of this genus are motile by peritrichous flagella, except, *Salmonella* Pullorum and *Salmonella* Gallinarum. *Salmonella* are chemoorganotrophic, with ability to metabobolize nutrients by both respiratory and fermentative pathways. Hydrogen sulphide is produced by most Salmonellae but a few serovars like *Salmonella* paratyphi

A and *Salmonella* choleraesuis do not produce H_2S. Most Salmonellae are aerogenic, however, *Salmonella typhi* does not produce gas (Sanchez-Vargas & et al, 2011).

The genus *Salmonella* are ubiquitous pathogens found in humans and livestock, wild animals, reptiles, birds, insects and can multiply under various environmental conditions outside the living hosts. They do not require sodium chloride for growth, but can grow in the presence of 0.4 to 4%. Most *Salmonella* serotypes grow at temperature range of 5 to 47°C with optimum temperature of 35 to 37°C but some can grow at temperature as low as 2 to 4°C or as high as 54°C . They are sensitive to heat and often killed at temperature of 70°C or above. *Salmonella* grow in a pH range of 4 to 9 with the optimum between 6.5 and 7.5. They require high water activity (aw) between 0.99 and 0.94 (pure water aw=1.0) yet can survive at water activity less than 0.2 such as in dried foods. Complete inhibition of growth occurs at temperatures less than 7°C, pH less than 3.8 or water activity less than 0.94 (Ferede, 2014).

These organisms are facultative an aerobic bacilli, non-spore forming that produce acid during glucose fermentation and reduce nitrates. *Salmonella typhi* is a fastidious organism that can grow in basal media, such as nutrient agar. On MacConkey agar, these organisms produce colorless colonies since *S. typhi* is a lactose-free fermenter. Low selective media such as MacConkey and deoxycholate agar and selective intermediate agar plates such as *Salmonella-Shigella* agar are widely used in the laboratory for the culture of the suspected sample. Highly selective media such as bright green agar and bismuth sulfite agar may also be used for the detection of *Salmonella typhi*. Strontium selenite and selenite F broth were enrichment broths for the body (Eng & et al, 2015).

Most of the *Salmonellae* do not ferment lactose and this property has been the basis for the development of numerous selective and differential media for the culture and presumptive identification of *Salmonella spp*. Such media includes xylose lysine decarboxycholate agar, *Salmonella-Shigella* agar, brilliant green agar, Hektoen enteric agar, MacConkey's agar, lysine iron agar and triple sugar iron agar. Isolation of *Salmonella* from food and environmental samples with culture method utilizes the multiple steps of pre-enrichment and enrichment on the selective and differential media in order to increase the sensitivity of the detection assay. A few *Salmonella* serovars do not exhibit the typical biochemical characteristics of the genus and these strains pose problem diagnostically because they may not easily be recovered on the commonly used differential media. About 1% of the *Salmonella* serovars submitted to Centers for Disease Control (CDC) ferment lactose; hydrogen sulphide production too was quite variable (Kumar, 2009). The recently developed *Salmonella* chromagar medium has been described as a very promising mean for detection of both lactose positive and lactose negative *Salmonella* isolated from food samples. This medium represents an approved alternative to current standard reference plated media for the foods tested (McNamara & Schultz, 2005).

2.7 Mechanisms of egg contamination by *Salmonella enteritidis*

The eggs may be contaminated on the outer surface of the shell and internally. Internal contamination can be the result of penetration of bacteria through the egg shell or by direct contamination of the egg content before oviposition, caused by infection of the reproductive organs. Once inside the egg, the bacteria must cope with the antimicrobial factors in albumin and the vitelline membrane before migration to the yolk can occur. It seems that the *Enteritidis* serotype has an intrinsic characteristic that

allows an epidemiological association with chicken eggs that are not yet defined (Gantois & et al, 2009).

2.7.1 Internal contamination of the egg after the penetration of the shell

After oviposition, any contaminated environment in the area of the egg laid, such as the nest box, the hatchery environment or the hatchery truck, may lead to contamination of the outer shell. The presence of chicken manure and other moist organic materials facilitates the survival and growth of *Salmonella* by providing the necessary nutrients and a degree of physical protection. When the eggs are artificially contaminated in the shell with faeces containing *Salmonella* and subsequently stored at 25^{o}C, the numbers increase by 1-2 logs during the first day and 4-5 logs after three days (Schoeni & et al, 1995)

Salmonella probably survive for longer at lower temperatures due to the slower metabolism of *Salmonella* that lives in unfavorable conditions on the surface of the dried egg shell (Radkowski, 2002). It is possible that penetration occurred before testing and washing the eggs. The egg shell seems to allow penetration more easily immediately after laying the egg. It is suggested that during the first minutes after oviposition, the cuticle is immature and some pores are open. In addition, when the egg is exposed to environment temperature lower than hens' body temperature, a negative pressure can develop and the bacteria migrate more easily through the shell and membranes. In addition, the cuticle in older eggs dehydrates, resulting in their contraction, and the pores are more exposed to bacterial penetration. It is believed that a positive temperature differential, combined with the presence of moisture, provides an ideal opportunity for the bacteria to penetrate the eggshell (Gantois & et al, 2009).

Salmonella on shell surfaces survive better as either the temperature (range 10°C to 23°C) or relative humidity (range 45%-75%) (Messens & et al, 2006). Penetration of the eggshell and shell membranes by *Salmonella* are not specific characteristic for SE only in the addition to *Salmonella* other bacteria can pass through the barrier. In a comparative study, the penetration of seven selected bacterial species originally isolated from egg contents was assessed using an agar model filling eggs with agar and dipping in bacterial suspension ,differences in the bacterial strains used and differences in the number of bacteria inoculated (Gantois & et al, 2009). The results indicate that Gram-negative, motile and non-clustering. The results of the agar approach indicates that the Gram-negative, motile and non-clustering bacteria penetrated the eggshell most frequently; *Pseudomonas* sp. (60%) and *Alcaligenes* sp. (58%) were primary invaders followed by *S.enteritidis* (43%). All selected strains were able to penetrate; penetration was observed most frequently after 4–5 days. Particularly *S.enteritidis* was a primary invader of whole eggs: the membranes and/or the content of 32% of the whole eggs were contaminated (De Reu & et al, 2006).

2.7.2 Contamination of eggs during egg formation

Salmonella is taken orally by the hens and enters the intestinal tract. Bacteria that colonize in intestinal lumen can invade intestinal epithelial cells (intestinal colonization). As a consequence, immune cells, more specifically macrophages, are attracted to the site of invasion and enclose the bacterium *Salmonella*. This allows the bacteria to survive and multiply in the intracellular environment of the macrophage. These infected macrophages migrate to internal organs, such as the reproductive organs (systemic spread). In addition to systemic spread, bacteria can also access the oviduct through an ascending infection of the cloaca. A possible route of contamination of the

egg is the penetration of *Salmonella* through the shell of the egg and the shell membranes after the contamination of the outer shell. The contamination of the surface can be the result of an infection of the vagina or fecal contamination. The second possible way is the direct contamination of the yolk, the membranes of the yolk, the albumin, the shell membranes and the egg shell originated by the infection of the ovary, the infundibulum, the magnum, the isthmus and the gland of the shell, respectively. Salmonella bacteria deposited in the albumin and vitelline membrane can survive and grow in the antibacterial environment. They are also able to migrate and penetrate the yolk membrane to reach the yolk. After reaching this rich environment, they can grow widely, see Figure 2.7 (Gantois & et al, 2009).

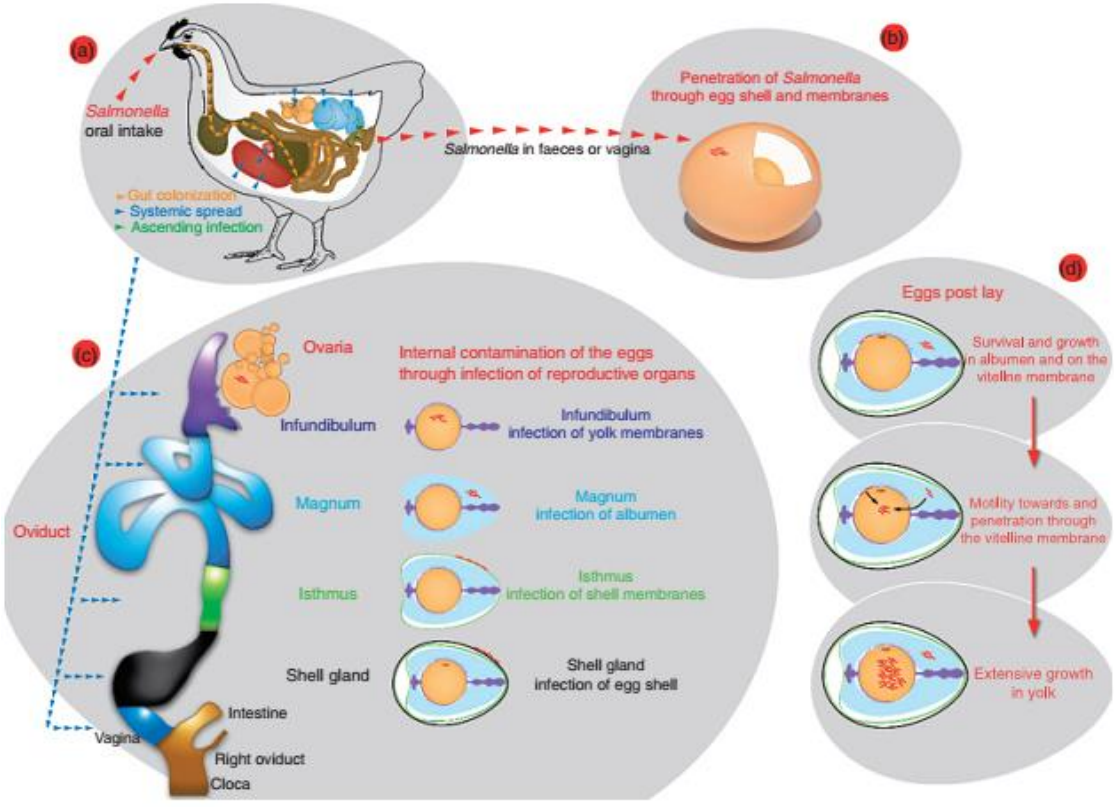


Figure 2.7: Pathogenesis of egg contamination by *Salmonella* (Gantois & et al, 2009).

Chapter Three

Materials and Method

3.1 Study Area

Three of the Northern governorates of the West Bank in Palestine were selected as the study area for this project. These governorates are Jenin, Tulkarm and Qalqilya (Figure 3.1).

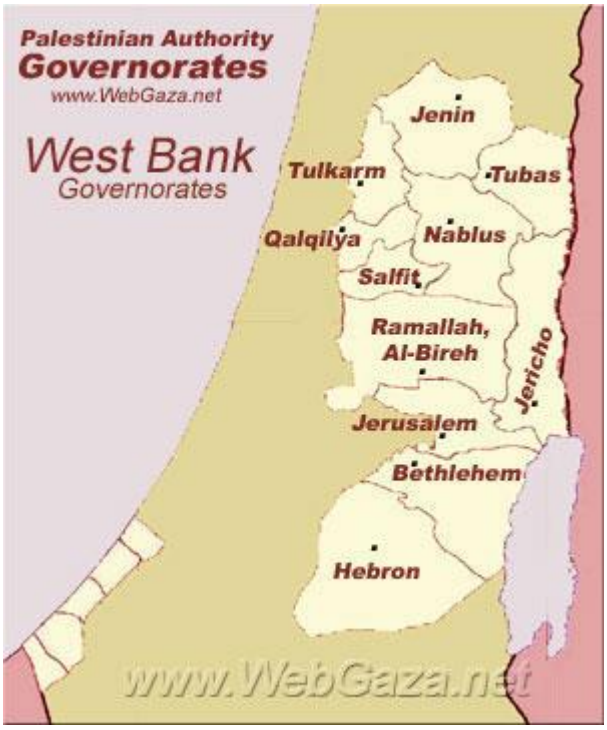


Figure 3.1: Palestinian Authority Governorates in West Bank

According to the publications of the Palestinian Central Bureau of Statistics (PCBS, 2010, 2011 and 2014), the total area of these three governorates is 995 square kilometers; which include about 17.1% of the total land area in the West Bank. The population of these governorates was estimated to be 604,084 by mid-2015, 54.9% of the Palestinian population in the West Bank. The number of animal and mixed holdings in the Northern West Bank governorates was 12,393, about 48.3% of the total animals

and mixed holdings in the West Bank during the agricultural year 2012/ 2013. The justification for the selected study area was based on the population growth rate of people, where it is high in these areas compared to other parts of the West Bank. Therefore, the requirement for animal proteins such as eggs will be higher. There is higher chicken eggs consumption in these areas compared to other areas of the West Bank (Palestinian Central Bureau of Statistics, 2015).

3.2 Materials

3.2.1 PCR primers

In this study, the primers to conduct the specific molecular identification of the *Salmonella* isolates (particularly *S.enteretidis*) were obtained from previous studies using the PCR methodology for *Salmonella enteritidis* detection. The sequences of the primers are shown in Table 3.1.

Table 3.1: Oligonucleotide primers used for verifying *Salmonella enteritidis* detection

Primer name	Sequence	Reference
Inv-A forward	5'CGGTGGTTTTAAGCGTACTCTT-3'	Fratamico, (2003)
Inv-A reverse	5'CGAATATGCTCCACAAGGTTA-3'	
IE1L forward	5'AGTGCCATACTTTTAATGAC-3'	Wang & Yeh (2002)
IE1R reverse	5'ACTATGTCGATACGGTGGG-3'	

3.2.2 Media for Bacterial Culture

Various types of media were used to provide specific information regarding the identification, enumeration, and segregation of bacterial isolates. The types of media used throughout this project are shown in Table 3.2.

Table 3.2: The bacterial culture media used to isolate and identify *Salmonella enteritidis* in layer farm.

Culture media	Manufacturer
Nutrient agar	Oxoid (UK)
Salmonella Shigella agar	Oxoid (UK)
Selenite F-broth	Oxoid (UK)
Triple sugar iron agar	Oxoid (UK)
Urea broth	Oxoid (UK)

3.2.3 Reagents and Materials

Chemicals and Reagents used in this study are listed in Table 3.3

Table 3.3: List of Chemicals and Reagents used in the study

Reagents and Materials	Source
Gram stain	Oxoid
Oxidase test	Oxoid
Anti-*Salmonella* agglutination sera	Remel

3.2.4 Equipment

- Incubator
- Water Bath
- Autoclave
- Microbiological Hood Olympus microscope
- Vortex Mixer
- Petri dishes
- Centrifuge
- Hot plate
- Disposable inoculating loops
- Eppendorf Tubes
- Swabs with transport medium.
- Glassware, Pipettes, Funnels, Beakers
- Disposable inoculating loops
- Eppendorf Tubes
- Swabs with transport medium.
- Glassware, Pipettes, Funnels, Beakers
- Spectrophotometer

3.3 Methods

3.3.1 Collecting samples

Samples were collected randomly from layer chicken farms in the selected study area at the West Bank. Sampling were collected between January 2018 and November

2018. The sample was obtained from cloacae of layers chickens using a sterile swab. The swabs were taken to the laboratory on the same day and cultured.

3.3.2 Isolation of *Salmonella*

For isolation of *Salmonella*, the following procedures were carried out:

- The swab samples were cultivated on Selenite broth enrichment medium and incubated at 37^{o} C for 24 hours.
- Cells from selenite broth culture were inoculated on SS and incubated at 37^{o} C for 24 hours.
- Typical black colonies were selected from the *Salmonella Shigella* agar (SS) agar, streaked onto the surface of nutrient agar dishes and incubated at 37^{o} C for 24 hours.
- The colonies were then examined and *Salmonella* identification was confirmed.

3.3.3 Identification of *Salmonella*

The isolates were analyzed to determine their morphology and biochemical characteristics according to the standard protocol described in the laboratory manual for the isolation and identification of foodborne pathogens.

3.3.3.1 Colony morphology

Colony morphology of *Salmonella* is presented in Table 3.4.

Table 3.4: Colony morphology of *Salmonella*

Medium	Colony appearance
Salmonella Shigella agar	Colorless colonies with or without black centers owing to H_2S production

3.3.3.2 Biochemical confirmation

All Suspected *Salmonella* isolates were subjected to biochemical testing for identification. The biochemical characterization included two tests: triple sugar iron test (TSI) and urease test. The noticeable changes in the different mediums are listed in Table 3.5.

a) TSI agar slant surface was streaked and the butt was stabbed, and was incubated for 24 h at 37ºC.
b) Urea broth was stabbed by sterile loop and was incubated for 24 h at 37ºC

Table 3.5: Biochemical responses of *Salmonella* on TSI and Urea tests

Test or substrate	Result
Lactose/sucrose	-
Glucose (TSI)	+
H_2S (TSI)	+
Urease	-

3.3.3.3 Serological confirmation

Salmonella spp. can be separated into many serotypes depending on two cell wall structures on the surface of the outer membrane. Scholars define the serotype based on the various combinations of somatic "O" and flagella "H" antigens. The antigenic formula of *Salmonella* serotypes are listed in the Kauffman-White scheme and are expressed as follows: O antigens; Vi antigen when existing; H antigens phase 1; H antigens phase 2 when existing. In the present study the used polyvalent antisera to test for the presence of O and H Antisera.

A small amount of bacteria, from an 18-hour culture on nutrient agar, was gradually mixed with a drop of antiserum on a clean glass slide to form a uniform and light suspension using a platinum loop

3.3.4 Molecular testing

3.3.4.1 Molecular identification

Identification of *Salmonella* was carried out using PCR and conventional culture, biochemical testing and serological methods. PCR is used for rapid identification and confirmation of all *Salmonella* serotypes. The genomic DNA of all *Salmonella* isolates was purified, quantitated and amplified. The specificity of the primers was checked by the supplier using live attenuated vaccine (VIR SALE) for *Salmonella enteritidis* (Bivouac Company) and used as positive control. *E. coli* ATCC25922 was used as negative control.

3.3.4.2 DNA Preparation

Sample processing is a critical step in the PCR screening. It is necessary to remove or deactivate substances that may block the PCR procedure. A washing step was used to decrease the concentration of PCR inhibitors.

The amount of 0.5 ml of sterile distilled water was added to sterile Eppendorf tube. A loopful of fresh bacteria taken from nutrient agar was suspended in the Eppendorf tube containing the distilled water. The sample was mixed gently for about 30 seconds by vortex. The tube was then centrifuged at 400 rpm for 3 minutes. The supernatant was transferred to a new tube. An amount of 0.5 ml of sterile distilled water was added to sterile Eppendorf tube and were mixed again by vortex. All samples were

put in a water bath for 10 minutes at 100^{o} C, and then transferred immediately to an ice containing beaker for 8 minutes.

3.3.4.3 Determination of DNA concentration and purity

The purified DNA was quantitated by using the Nano drop measuring the absorbance at 260 and 280 nm. At 260 nm, the concentration of the purified DNA was determined. To check for the purity of the extracted DNA, the absorbance was measured at 280 nm. The ratio between the two absorbance (A260/280) should be from 1.4 to 1.9. The information obtained are listed in Table 3.6.

Table 3.6: The ratio between the two absorbance (A260/280)

Number	Concentration (ng/uL)	Ratio
1	1267	1.9
2	537	1.8
3	1172	1.9
4	844	1.7
5	824	1.9
6	1302	1.8
7	1532	1.9
8	1763	1.9
9	1814	1.9
10	352	1.8
11	988	1.9
12	563	1.9
13	1401	1.8
14	716	1.9
15	991	1.9
16	862	1.9
17	1071	2
18	2076	1.8
19	1248	1.7
20	698	1.8
21	831	1.7
22	1081	1.8
23	1062	1.7
24	1749	1.80
25	1337	1.8
26	1198	1.9
27	1208	1.9
28	1321	1.7

29	1326	1.9
30	340	1.9
31	1030	1.9
32	492	1.8
33	416	1.9
34	456	1.7
35	860	1.8
36	550	1.8
37	895	1.7
38	920	1.7
39	1030	1.9
40	1125	1.9
41	1200	1.8
42	540	1.8
43	380	1.9
44	985	1.9
45	982	1.8
46	870	1.8
47	905	1.7
48	1070	1.8
49	1043	1.9
50	1084	1.7
51	1113	1.8
52	1307	1.7

3.3.4.4 Determination of the DNA quality by Agarose gel electrophoresis

The integrity and purity of the extracted DNA was also evaluated by agarose electrophoresis. An agarose gel of 2 % was prepared containing ethidium bromide. The extracted DNA samples from *Salmonella* isolates were loaded with a loading dye on the gel, electrophoresed for 45 minutes and viewed under the UV light and photographed using the Gel Documentation System (Biorad, CA, USA).

3.3.4.5 PCR Cycling Condition

PCR was performed in a total of 25 µl reaction mixture containing 12.5 µl of PCR master mix (Promega), 0.5µl (0.2 micromol) of forward primer, 0.5µl (0.2 micromol) of reverse primer, 6.5µl of PCR grade water and 5µl (125 nanogram/ milliliter) of the

template. The entire PCR reaction was performed under the following condition; initial denaturation: 95˚C / 3 min., denaturation: 95˚C / 30 sec., annealing: 56˚C / 40 sec., extension: 72˚C / 45 sec. No. of cycles: 34 and final extension: 72˚C / 7 min. These conditions are described in Table 3.6.

Table 3.7: Conditions of PCR confirmation test

Condition	Temp. (°C)	Time	Number of Cycles
Primary Denaturation	95	3min	1
Secondary Denaturation	94	30 sec	34
Annealing	58	40 sec	34
Extension	72	45 sec	34
Final Extension	72	7	1
Hold at	4	-	-

3.3.4.6 Molecular identification

Amplified PCR products were electrophoresed in 2% agarose gel. The samples and a 50 bp DNA ladder are loaded in the wells in the amount of 5µl per sample. A current of 60 V for 1 hour was passed on horizontal electrophoresis unit. Specific amplicons were observed under ultraviolet trans-illumination compared with the marker. The gel was photographed by a gel documentation system and the data are analyzed.

Chapter Four

Results

4.1 Description of the study sample

Four hundred and fifty samples were collected from forty five layer farms in the Northern west bank, Palestine and were distributed according to governorates as shown in the Table 4.1.

Table 4.1: Geographical distribution of layer chicken farms and samples according to governorates

Governorates	Number of farms	Number of samples	Total sample (%)
Tulkarm	25	215	51.8
Qalqylia	8	80	19.2
Jenin	12	120	28.9

Result of culture by biochemical testing and serology

- *Salmonella* plated on *Salmonella Shigella* agar produced colonies that appeared black in color or have black center. The colonies were flat with transparent borders and H_2S production as shown in the Figure 4.1.

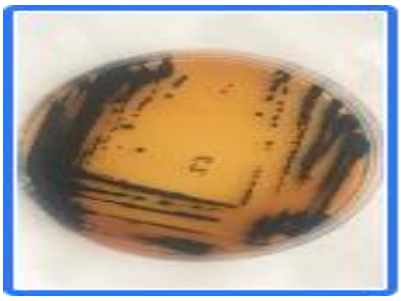


Figure 4.1: *Salmonella* on *Salmonella Shigellla* agar

4.2 Growth of *Salmonella* on TSI agar

Salmonella on TSI will produce alkaline (red) slant and alkaline butt (red/black) indicating non-lactose fermentation with the red alkaline color masked by the black color due to hydrogen sulfide (black) production (K/K +). The Figure 4.2 shows this reaction.

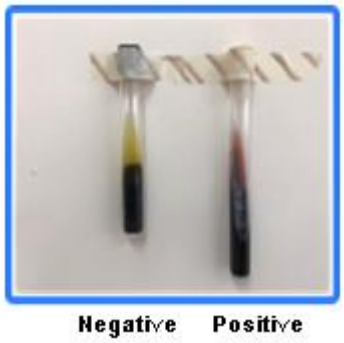


Figure 4.2: Reaction of *Salmonella* on TSI agar

4.3 Urease Test

A loopful of the *Salmonella* grown on culture media was transferred to a urea broth containing tube and incubated at 35-37°C for 22-26 hrs. *Salmonella* does not produce the urease enzyme and therefore the urea broth will remain yellow while the positive control tube will turn pink in color as shown in the Figure 4.3.

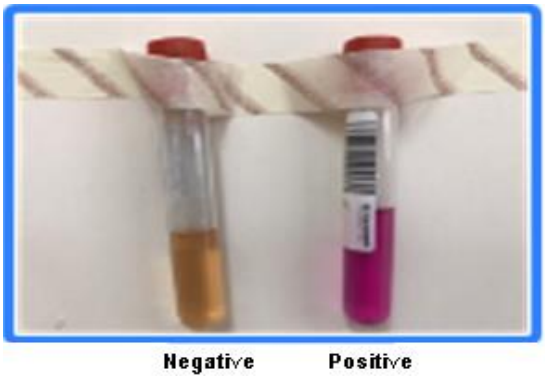


Figure 4.3: Reaction of *Salmonella* on urea media

4.4 Serological confirmation

Serology was performed on a clean glass slide by mixing one drop from each polyvalent antisera with one drop taken from broth culture or suspension from an agar growth in 0.9% sterile saline. The two drops were carefully and thoroughly mixed and any agglutination after two minutes for both the somatic "O" and flagella "H" antisera was considered positive for the tested *Salmonella* strain. Table 4.2 summarizes the serological evaluation of the tested isolates

Figure 4.4: Serological confirmation result

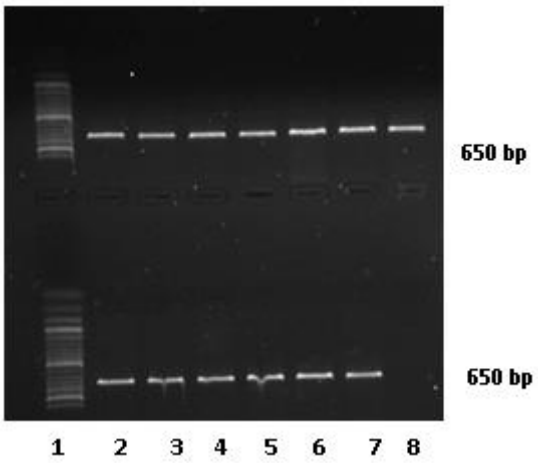
Table 4.2: Characteristics of *Salmonella* isolates tested

Governorate	SS Agar	TSI	Urease	Polyvalent sera	Number Positive	Percent n=52
Tulkarem	B[1]	K/K+[2]	N[3]	P[4]	30	57.5
Qalqylia	B[1]	K/K+[2]	N[3]	P[4]	4	7.5
Jenin	B[1]	K/K+[2]	N[3]	P[4]	18	34.6
				Total	52	100

1: B = black colonies, 2: K/K+= alk/alk+H2s, 3: N=Negative, 4: P= positive.

4.5 PCR Results

The following gel represents the PCR results for the *Salmonella* isolates tested. It is clear that all isolates gave a 650 bp band as expected.



Lane 1: Molecular weight marker. Lanes 2: Positive control.
Lanes 3 to 7: *Salmonella* isolate. Lane 8: Negative control

Figure 4.5: Identification of *Salmonella* species by PCR

PCR was also applied to detect the possible presence of *Salmonella enteritidis* among the recovered isolates. Figure 4.6 below shows that none of the tested isolates was *Salmonella enteritidis*.

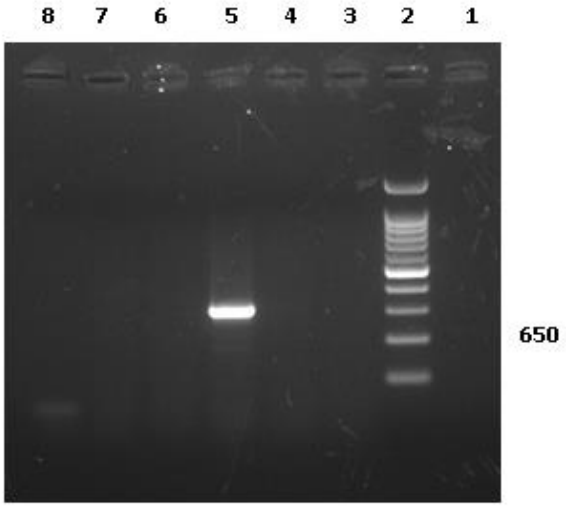


Figure 4.6: Gel electrophoresis to detect the presence of *S.enteritidis* among the isolated tested. Lane 1: Negative Control. Lane 5: *S.enteritidis* positive control. The remaining lanes are negative for *S.enteritidis*.

4.6 Discussion

Foodborne salmonellosis in humans has been consistently traced to the consumption of infected poultry and products. Salmonellosis is of major concern in many countries worldwide for causing public health problems. In addition, it causes major economic losses despite improved hygienic processing of poultry and products (Suresh et al. 2011)

This study was conducted on 450 samples collected from 45 layer farms in the Northern west bank, Palestine. The aim of the study was to evaluate the level rate of *Salmonella* infections in the layer chickens in general and the rate of *S.enteritidis* in particular.

The rate of infection of the layer chickens tested was 11.6% (52.450). *Salmonella* that has been recovered and identified by conventional biochemical tests, was further confirmed by serology and molecular (PCR) methods. However, *S. enteritidis* has not been recovered by PCR using specific primers and reference organism as positive control from the sample tested.

In a study conducted in Jordan, investigation of an outbreak followed similar methodologies as conducted in this project. Investigating the presence of *Salmonella* in seven outbreaks in Jordan showed a presumptive identification of 200 isolates. Confirmation of the identification using universal primers by PCR showed that out of the 200 isolates presumptively identified, only 180 isolates were true *Salmonella* spp. The presence of *S. enteritidis* among the *Salmonella* isolates was investigated using specific primers by PCR. The rate of *S. enteritidis* was (45/180) or 25% (Jaradat, ZW, 2014).

Another study conducted in Jordan to determine the presence of *Salmonella* in chicken feeds. A total of 1546 samples were collected. The rate of *Salmonella* recovered was 2.3% (36/1546). A total of 11 different serotypes of *Salmonella* was identified at various rates. However, *S. enteritidis* (22.2%) was the most frequent serotype recovered from chicken feed. Therefore, the predominance of *Salmonella* in chicken feeds makes it necessary to implement more stringent regulations to control the spread of *Salmonella* in poultry and subsequent transmission to humans (Alshawabka, KM, 2006).

A study conducted in Egypt to investigate the presence of *Salmonella* in egg contents found that *Salmonella* are predominant bacteria inside the eggs than. The rate of *Salmonella* was the least of 4.0% (3/75) (Awny C, et al., 2018). In our study we didn't examine egg contents, but the rate of *Salmonella* in the sample tested was 12.0%. However, all our samples were negative for *S. enteritidis* which is considered the most frequent in infected poultry.

Another Egyptian study was conducted to isolate *S.enterica* from raw chicken meat (Tarabees et al., 2017). Out of 100 samples tested two *S.enteritidis* (2.0%) and three *S.typhimurium* (3.0%) were isolated. Isolation of these two serovars even at low incidence has important implications.

A study conducted in Jordan (Malkawi, HI, 2003) to identify the presence of *Salmonella* in meat and poultry products used conventional and molecular techniques. The results obtained showed that the rate of *Salmonella* was 81.0% (172/212) by conventional method as compared to the more sensitive and specific PCR method of 87.0% (185/212). In the current project, we didn't rely solely on the conventional

biochemical methods to identify *Salmonella* in the samples tested, but we further included serology and PCR as well to get an accurate profile on *Salmonella* infecting chicken layers which will contribute to implementing guidelines to better handling and processing of chicken layers and how to prevent or reduce ovarian infection and the possibility of transmission the infections to the eggs.

It has been repeatedly confirmed that *Salmonella enteritidis* is the predominant serovar associated with salmonellosis worldwide through internal contamination of the egg. Studies have shown that *S.enteritidis* has tropism and ability to persist in the reproductive tract of poultry (Gantois I. et al., 2008). Research showed that the same clones of *S.enteritidis* infecting the oviduct of poultry are isolated from the eggs laid by the same Chicken layer. Further studies were conducted to determine the persistence of the *Enteritidis* serovar in chicken eggs making this serovar the only one transmitted to humans through eggs. The ability of the *S.enteritidis* to survive in and transmit through eggs has been correlated to genes important for the association of this serovar with chicken eggs (Clavijo & et al, 2008).

It is important to point here that our main objective in this project was to determine that *S.enteritidis* is the predominant serovar infecting the oviduct of chicken layers in Palestine and subsequently investigate the rate of passage of this serovar to the eggs. Unfortunately, none of the *Salmonella* isolated was the *S. enteritidis* serovar.

The only limitation in this study would be the sample size. Sampling cannot be the reason for not recovering *S.enterica* serovar *Enteritidis* from the sample tested. The samples were collected by a veterinary doctor who has the knowledge and experience to properly collect the samples. The techniques used in this project could not be blamed

for not detecting the presence of this serovar since we used sensitive and specific molecular techniques (PCR with specific primers). Furthermore, the PCR technique applied in this research has been optimized using a reference strain of *S.enterica* serovar *enteritidis* using the same steps and reagents as the sampled tested.

Finally, I recommend that this project must be continued to determine the presence of contamination of the reproductive organs of chicken layers and to determine the contamination of the eggs produced by the same chicken layer. In addition, it is recommended to get adequate representative sample size.

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