In Silico-Based Development of a Sensitive PCR Assay to Detect and Differentiate three major *Salmonella enterica* Serovars

by

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ABSTRACT

Serovars of the bacterium *Salmonella enterica* are important worldwide causative agents of bacterial food poisoning. Of more than 1500 serovars of this species; *Salmonella* Enteritidis, *Salmonella* Typhimurium and *Salmonella* Gallinarum are the most important serovars affecting human health and poultry industry. They cause great economic losses both due to their implication in animal welfare and their ability to contaminate poultry food products forming a serious food safety problem. Early detection and typing of these serovars is essential for food safety regulations and poultry industry. The current techniques to detect and serotype these serovars are time consuming and require technical expertise. The aim of this work was to develop a practical PCR-based method that can detect and distinguish the above mentioned 3 serovars. Genomic sequence data of *Salmonella* Gallinarum, *Salmonella* Enteritidis and *Salmonella* Typhimurium was used through NCBI database (as of Month 2015). Intensive comparative genomic analysis was conducted to identify unique segments for each serovar. The identified unique segments were used as specific markers to design 3 close nested PCR assays that can detect and distinguish the three serovars. The detection specificity of the PCR assays were tested using a wide range of *Salmonella enterica* serovars. The detection sensitivity of the three PCR tests was examined using 10 fold serial dilutions of the control DNAs. The analysis revealed unique segments for each serovar that can serve as specific genetic markers. PCR-primers for each unique segment produced the specific amplicon for each serovar. The developed close nested PCR techniques showed amplification signal of as low as (0.03 ng- 3 pg) DNA, which is 100-fold more sensitive than traditional PCR protocols. Three of the most important *Salmonella enterica* serovars, which are associated with poultry health and human food poisoning were detected and typed by a very sensitive PCR technique. The developed PCR can be used as a routine test to manage and control the poultry health and safety of poultry products.

تطوير فحصٍ حسّاس للكشف عن ثلاثة أنواع من بكتيريا السّالمونيلّا بالاعتماد على التّحليل الحاسوبيّ

بواسطة

أمل نافذ شرباتي

الملخّص

تختلف أنواع بكتيريا السّالمونيلّا المعروفة لتتوزع على 1500 نوعٍ تقريباً، وتعتبر بعض أنواعها -لا سيما المعويّة- من أهم العوامل المسبّبة للتسمّم الغذائيّ الجرثوميّ عالمياً. تعتبر بعض أنواع السّالمونيلّا المعويّة كالسّالمونيلّا الملهبة للأمعاء، والسّالمونيلّا التيفيّة الفأريّة، والسّالمونيلّا الدّجاجيّة من أهم الأنواع الّتي تؤثر على صحّة الإنسان والدّواجن، مما يسبّب خسائر اقتصاديّة كبيرة؛ لما تسببه من تلويثٍ لمنتجات الدّواجن الغذائيّة، مما يشكّل مشكلة خطيرة على سلامة الأغذية والصّحة الغذائيّة.

يعدّ الكشف المبكر عن أنواع السّالمونيلّا الثّلاثة السابقة أمراً ضرورياً لأنظمة السّلامة الغذائيّة وسلامة منتجات الدّواجن، وتتطلّب التّقنيّات المستخدمة حالياً في الكشف عنها الكثير من الوقت والمال والخبرة الفنيّة والتّقنيّة، وهو ما دفعنا للقيام بهذا العمل أملاً في تطوير فحص PCR عمليّ، يُمَكّننا من الكشف عن الأنواع الثّلاثة والتّمييز بينها. استُخرجت لهذه الغاية بيانات تسلسل الجينوم من الأنواع الثلاثة (الملهبة للأمعاء، والتيفيّة الفأريّة، والدّجاجيّة) من قاعدة بيانات NCBI (آذار- 2015)، وأُجريَ تحليل مكثّف للجينوم لتحديد مناطق فريدة لكل نوع منها، وبعد إيجاد القطع الفريدة والمميزة لكل نوع على المستوى الحاسوبي، تم استخدامها لتصميم ثلاثة فحوص (PCR المغلق المتداخل) يمكن من خلالها الكشف والتمييز بين هذه الأنواع الثّلاثة.

تم اختبار خصوصيّة الفحص بتطبيقه على مجموعةٍ واسعةٍ من أنواع السّالمونيلا المعويّة؛ تم التّأكد بواسطته أنّ المناطق الّتي تم إيجادها هي مناطق فريدة ونادرة لكل نوع، ما يمَكّننا من اعتبارها علاماتٍ وراثيّةٍ محدِّدَةٍ لها. أما بالإشارة إلى فحص الحساسيّة؛ فقد تم اختباره باستخدام تراكيز مخفّفة متسلسة من الحمض النّووي ذو تركيز محدّد مسبقاً، وأعطى إشارةً منخفضةً تصل إلى (0.03 نانوغرام – 3 بيكوغرام) من تركيز الحمض النّووي، وتعتبر بذلك أكثر حساسية بما نسبته 100 ضعف من فحوص PCR التقليديّة.

مكننا هذا الفحص من الكشف عن ثلاثة من أهم أنواع السّالمونيلّا المعويّة الّتي ترتبط بصحة الدّواجن والتّسمم الغذائيّ البشريّ، وتمييزها بواسطة تقنيّة PCR حسّاسة. ويمكن استخدام هذا الفحص كاختبار روتينيّ لإدارة ومراقبة صحّة الدّواجن وسلامة منتجاتها.

Declaration

I declare that the Master Thesis entitled "***In Silico*-Based Development of a Sensitive PCR Assay to Detect and Differentiate three major *Salmonella enterica* Serovars**" is my own original work, and hereby certify that unless stated, all work contained within this thesis is my own independent research and has not been submitted for the award of any other degree of any institution, except where due acknowledgment is made in the text.

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Dedication

This thesis work is dedicated to my husband, Alaa, for your patience, love, friendship and support.

Also to my children, Abed and Omar, for letting me experience the kind of love that people freely die for.

Also to my mother, who has been a constant source of support and encouragement during the challenges of study and life. You has helped me along all of my life.

Also to my brothers and sisters, Ahmad, Ayman, Amani, and Iman, who have always loved me unconditionally and whose good examples have taught me to work hard for the things that I aspire to achieve.

Also to my husband's mother, my friends, and to anyone how help me and pray for me.

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Abbreviation

BPW	Buffered Peptone Water
CDC	Centers for Disease Control and Prevention
NTS	Non Typhoidal *Salmonella*
FDA	Food and Drug Administration
BLAST	Basic Local Alignment Search Tool
NCBI	National Center for Biotechnology Information
EU	European Union
FSIS	Food Safety and Inspection Service
AOAC	Association of Official Analytical Chemists
ISO	International Organization for Standardization
PCR	Polymerase Chain Reaction
FT	Fowl Typhoid

List of Figures

1.1 The taxonomy of *Salmonella* 2
1.2 Features of host adaptation in *Salmonella* and effects on clinical syndrome in the host. 4
1.3 Infection routes of *Salmonella enterica* 7
1.4 *Salmonella* in food 8
1.5 Distribution of the 10 most common *Salmonella* serovars in humans in the EU, 2012. 9
1.6 Scheme of Food Emergency Response Network for *Salmonella* culture method. 11

2.1 Window sliding approach 16
2.2 The close nested PCR program 19

3.1 PCR products for marker specificity of *Salmonella* Gallinarum. 22
3.2 PCR products for marker specificity of *salmonella* Enteritides. 23
3.3 PCR products for marker specificity of *Salmonella* Typhimurium. . . 24
3.4 The sensitivity of close nested PCR and conventional PCR for *Salmonella* Gallinarum. 24
3.5 The sensitivity of close nested PCR and conventional PCR for *Salmonella* Enteritides. 25
3.6 The sensitivity of close nested PCR and conventional PCR for *Salmonella* Typhimurium. 26

List of Tables

1.1 Examples of natural hosts for *Salmonella* serovars 3
1.2 Variations review among non typhoidal *Salmonella* (NTS) and typhoidal serovars linked with disorder in humans, from (Gal-Mor et al., 2014). 6
1.3 Comparison between culture, ELISA, and PCR detection methods. The source of table from (Park et al., 2014). 13

2.1 *Salmonella* strains used in this study 15
2.2 Close nested PCR primers 18

Contents

Acknowledgement **viii**

1 Introduction **1**
1.1 Taxonomy of *Salmonella* . . . 2
1.2 Hosts of *Salmonella entarica* subsp. *enterica* . . . 3
1.3 Pathogenicity of *Salmonella* . . . 3
1.3.1 Typhoidal *Salmonella* . . . 4
1.3.2 Non typhoidal *Salmonella* (NTS) . . . 5
1.3.2.1 *Salmonella* infection mechanism . . . 5
1.4 Transmission of *Salmonella* . . . 7
1.5 *Salmonella* and food . . . 8
1.6 Economic impact of *Salmonella* infection . . . 10
1.7 Diagnosis and detection of *Salmonella* . . . 10
1.7.1 Culture method . . . 10
1.7.2 Rapid *Salmonella* detection methods . . . 11
1.7.2.1 Immunology-based assay . . . 12
1.7.2.1.1 Enzyme-linked immunosorbent assay (ELISA) 12
1.7.2.2 Nucleic acid-based assay . . . 12
1.7.2.2.1 Polymerase chain reaction (PCR) . . . 13
1.8 The need for a practical method . . . 14
1.9 Objectives . . . 14

2 Materials and methods **15**
2.1 Bacterial strains . . . 15
2.2 Genomic DNA extraction . . . 16
2.3 Marker screening . . . 16
2.4 Primer design . . . 18
2.5 Marker specificity validation . . . 18
2.6 Close nested PCR . . . 19
2.7 Sensitivity of close nested PCR . . . 20

3 Results **21**
3.1 Marker screening . . . 21
3.2 Markers validation . . . 21
3.3 Sensitivity of close nested PCR . . . 23

4 Discussion **27**

CHAPTER 1

Introduction

Salmonellosis is one of the major public health problems across the world. This disease is caused by a group of bacteria known as *Salmonella* that are typically associated with food contamination. *Salmonella* is a Gram-negative bacterial genus that belong to the Enterobacteriaceae family. The first member of this genus, namely *Salmonella* Typhi, was discovered in 1880s by Karl Joseph Eberth and later the genus was given its name after Daniel Salmon, whose group played an important role in describing this bacterium (Schultz, 2008). *Salmonella* is a facultative anaerobic rod shape bacterium that grows optimally at 37 °C and pH between 4 to 9. Under laboratory conditions, this bacterium is typically grown in the same culture medium of the Enterobacteria producing colonies that have 2-4 mm diameter with smooth and rough edges (Coburn et al., 2007).

Biochemically, *Salmonella* strains are catalase positive and oxidase negative, similar to most genera of the Enterobacteriaceae family. In addition, *Salmonella* ferment D-glucose with the production of acid and frequently gas. Other carbohydrates are usually fermented by *Salmonella* such as L-arabinose, disaccharide, D-mannitol, D-mannose, L-rhamnose, D-sorbitol (except subspecies *indica* VI), trehalose, D-xylose and dulcitol. *Salmonella* produce negative reaction for indole, Voges Proskauer (VP), citrate utilization, and urea hydrolysis. On the other hand, *Salmonella* are methyl red and simmons citrate positive. Many of these characteristics can be used for biochemical validation of *Salmonella*, like the capability of *Salmonella* to grow on citrate as a sole carbon source, H_2S production and lysine decarboxylation (Jensen and Hoorfar, 2000, Abulreesh, 2012).

Salmonella is widely distributed through the environment and it is ubiquitous in natural water and sediments (Abulreesh, 2012). *Salmonella* causes major infections in domestic and wild animals birds, like chickens, geese, turkeys and ducks, which are considered as the most important reservoirs of this bacteria (Padron, 1990). Domesticated and free-living animals can carry *Salmonella* without noticeable symptoms. Therefore, poultry products, undercooked meat, and leafy greens can be potential sources of infection (Abulreesh, 2012).

1.1 Taxonomy of *Salmonella*

The genus *Salmonella* is divided into two species, namely, *Salmonella* enterica and *Salmonella* bongori (Virlogeux et al., 1996). *Salmonella enterica* is divided into seven different subspecies (subsp.): *Salmonella enterica* subsp. *enterica* (subspecies I), *Salmonella enterica* subsp. *salamae* (subspecies II), *Salmonella enterica* subsp. *arizonae* (subspecies IIIa), *Salmonella enterica* subsp. *diarizonae* (subspecies IIIb), *Salmonella enterica* subsp. *houtenae* (subspecies IV), *Salmonrella enterica* subsp. *indica* (subspecies VI) and (subspecies VII) (Grimont et al., 2007), see figure 1.1.

Currently, there are about 2,500 serovars of *Salmonella*. Classification of *Salmonella* is defined based on White-Kauffmann scheme, which classifies *Salmonella* serovars based on their outer (O) antigen, flagellar (H) and capsular (Vi) antigens (Corrêa et al., 1992).

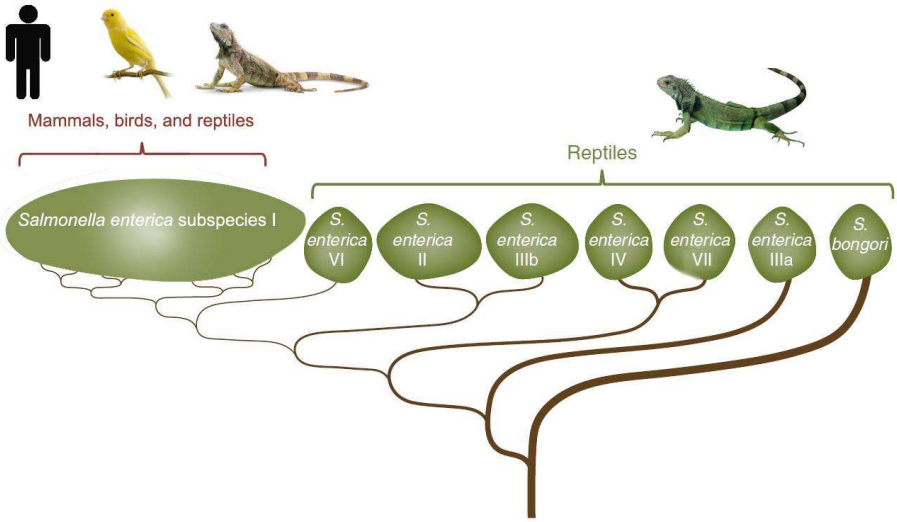

Figure 1.1: The taxonomy of *Salmonella*. The genus *Salmonella* includes two species, *Salmonella enterica* and *Salmonella bongori*. Serovars of *Salmonella bongori* and *Salmonella enterica* subspecies II, IIIa, IIIb, IV, VI, and VII are largely linked to reptile and can be sometimes transmitted from this reservoir to humans. Most of serovars belonging to *Salmonella enterica* subspecies I are generalists reservoirs in mammalian, avian, and reptilian species. A small group of specialist serovars with a more restricted host range have separately evolved from this group many times. Source (Bäumler and Fang, 2013).

Currently, there are twenty two serovars that belong to *Salmonella bongori* species, and approximately 1400 serovars are identified as serovars for *Salmonella enterica* subsp., which lead to a lot of *Salmonella* infections in human and warm blooded animals (Porwollik et al., 2004). On the other hand, *Salmonella bongori* and other *Salmonella* subsp. have mainly inhabited in the cold blooded animals and environment (Pui et al., 2011).

1.2 Hosts of *Salmonella entarica* subsp. *enterica*

The important serovars of the *enterica* subsp. I can be divided into three groups depending on their host specificity (Table 1.1):

1. *Salmonella* serovars exclusively related to a particular host species, called host restricted serovars, like Typhi (restricted to human) and Gallinarum (restricted to poultry).

2. *Salmonella* serovars prevalent in one unique host species, but can occasionally infect other species. these are called host adapted serovars, like Choleraesuis (pig is preferred host) and Dublin (cattle is the preferred host) (Uzzau et al., 2000).

3. The third category includes the majority of serovars that can affect a broad range of unrelated host species and therefore they are definitely zoonotic bacteria (Tessari et al., 2012). Some serovars of this category like Typhimurium and Enteritidis are on the top list of the major causes of food borne infections (Gantois et al., 2009).

Table 1.1: Examples of natural hosts for *Salmonella* serovars

Natural host	**Serovars**	**Host(s) infected**	**Other host(s) Rarely infected**
Host restricted serovars	Typhi	Human	
	Paratyphi A,C	Human	
	Gallinarum	Poultry	
	Abrtusovis	Ovine	
	Typhisuis	Swine	
Host adapted serovars	Choleraesuis	Swine	Human
	Dublin	Bovine	Human, Bovine
Unrestricted serovars	Typhimurium		
	Enteritidis		

1.3 Pathogenicity of *Salmonella*

Salmonella serovars with broad and narrow host range appear to be different in clinical symptoms. Infections with generalist serovars (broad host range) generally cause gastrointestinal disease with high morbidity and low mortality (Uzzau et al., 2000, Gyles et al., 2008). In contrast, infections with host adapted or restricted serovars, such as Typhi, Choleraesuis, Abortusequi, and Gallinarum typically cause systemic disease with high rate of mortality (Uzzau et al., 2000, Gyles et al., 2008) (Figure 1.2).

Based on associated pathogenicity, the different serovars of *Salmonella enterica* are generally divided into two major groups: typhoidal and non-typhoidal *Salmonella* (NTS) serovars. Regardless of their genetic similarity, these two groups produce the following different diseases.

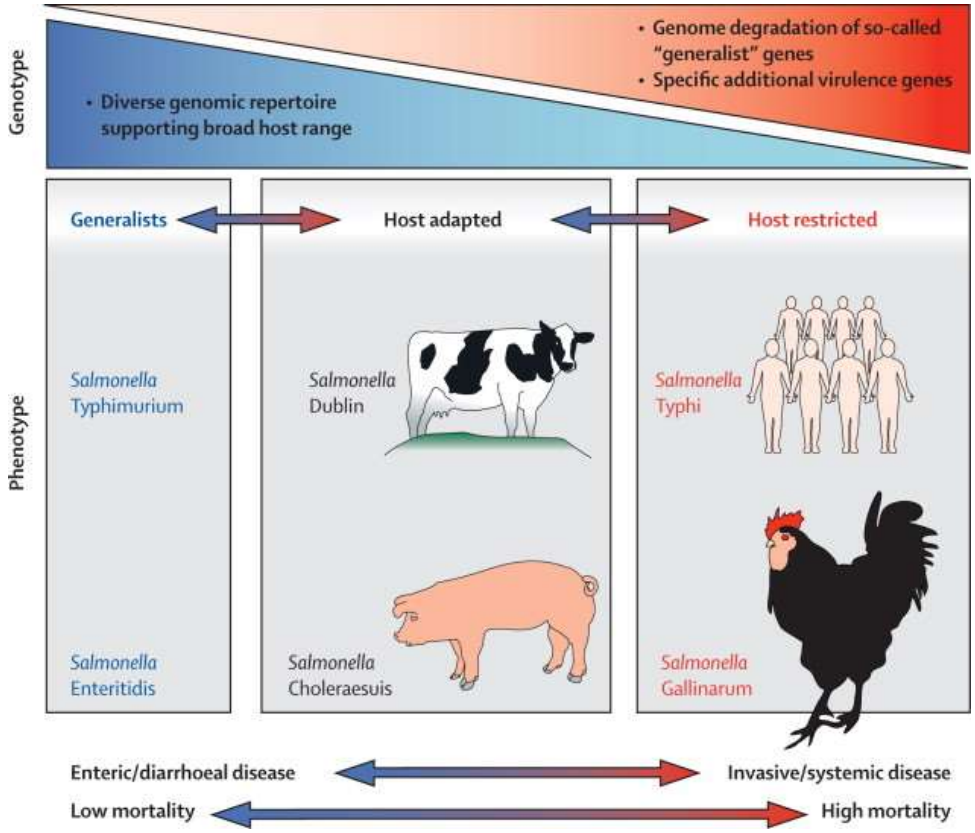

Figure 1.2: Features of host adaptation in *Salmonella* and effects on clinical syndrome in the host. From (Feasey et al., 2012).

1.3.1 Typhoidal *Salmonella*

A small number of *Salmonella enterica* serovars are restricted to specific host including human host or animal hosts. These specialist pathogens named typhoidal *Salmonella* serovars, which are the causative agents of typhoid fever (also known as enteric fever). Typhoid fever in human is an invasive, life-threatening, and systemic disease with an approximated global yearly burden in excess of 27 million cases, resulting in more than 200,000 deaths (Buckle et al., 2012), and it is considered as endemic in the developing countries, and in the regions that suffer from lack of clean water and adequate sanitation, which facilitate the spread of these kinds of pathogens through the fecal-oral route.

Fowl typhoid (FT) which is caused by *Salmonella* Gallinarum is an example of typhoid fever in animal (Priyantha, 2009). The FT is a sever systemic disease in poultry that causes sever hepatomegaly and splenomegaly accompanied by liver with bronzing aspect, anemia, and septicemia (Shivaprasad, 2000). The mortality and morbidity may reach up to 80 % (de Paiva et al., 2009). The FT may affect all chicken age groups, but the growing and mature birds are more susceptible (Shivaprasad, 2000).

1.3.2 Non typhoidal *Salmonella* (NTS)

In comparison with typhoid fever, that is more common in the developing world, the non typhoid salmonellosis occurs worldwide, and there are about 93.8 million cases of gastroenteritis as a result of NTS infection annually, which leads to approximately 155,000 deaths (Majowicz et al., 2010).

In contrast to typhoid fever, many people infected with NTS resolved by themselves (McGovern and Slavutin, 1979). After 6-12 hours of ingestion the pathogen, the symptoms of NTS infection will appear, like acute gastroenteritis, watery diarrhea, nausea, vomiting, abdominal pain, and fever which don't remain more than 10 days (Glynn and Palmer, 1992).

Some NTS serovars can cause an invasive, extra-intestinal disease forming bacteremia and focal systemic infections, which called invasive NTS (iNTS) (Mandal and Brennand, 1988). Interestingly, some NTS serovars (for example, Typhimurium, Dublin, and Choleraesuis) have higher probability to produce extra-intestinal infections than others (Marzel et al., 2014) (Table 1.2).

The most important source of NTS transmission is the contaminated food products like poultry meat, eggs, and dairy products. (Haeusler and Curtis, 2013). In addition, there are also a non-animal sources of infection, like sprouts, tomatoes, fruits, peanuts, and spinach of contaminated and not cooked (Bayer et al., 2014).

1.3.2.1 *Salmonella* infection mechanism

Both typhoidal and NTS serovars adhere to the epithelium of the small intestine after ingestion of bacteria (Liu et al., 1988). In this stage, the infection by typhoidal serovars doesn't stimulate a high inflammatory response (Chanh et al., 2004), so, it is associated with minimal neutrophil transmigration through the intestinal epithelium. When compared with NTS serovars, they produce a huge neutrophil recruitment during intestinal inflammation (McCormick et al., 1995).

The lack of effective intestinal inflammation and the insufficient neutrophil transmigration help the invasion of typhoidal serovars to the deeper tissues of the gut to move to systemic sites (House et al., 2001).

Table 1.2: Variations review among non typhoidal *Salmonella* (NTS) and typhoidal serovars linked with disorder in humans, from (Gal-Mor et al., 2014).

	NTS serovars	**Typhoidal serovars**
Serovars	Represented by the ubiquitous serovars Typhimurium and, Enteritidis, but 1500 other serovars of *Salmonella enterica* sp.I, are known	Typhi, Paratyphi, and Sendai
Host range	Broad	Human-restricted
Epidemiology	Worldwide	Endemic in developing countries especially Southeast Asia, Africa, and South America
Reservoirs	Farm animals, produce, pets	None, human to human transmission
Clinical manifestations	Self-limiting gastroenteritis, individuals (diarrhea, vomiting, cramps). In immunocompromised patients, disease is associated with invasive extraintestinal infections.	Invasive, systemic disease in immunocompetent individuals (fever, chills, abdominal pain, rash, nausea, anorexia, hepatosplenomegaly, diarrhea or constipation, headache, dry cough)
Disease course	Short incubation period (6–24 h). Brief duration of symptoms (less than 10 days). Long-term carriage has not been observed	Long incubation period (7–21 days), Extended duration of symptoms (up to 3 weeks), One to four percent of infected individuals
Human immune response	Robust intestinal inflammation, neutrophil recruitment, Th1 response	Minimal intestinal inflammation, leukopenia, Th1 response
Genetic basis of disease differences and host specificity	Low degree of genome degradation, able to use terminal electron acceptors for anaerobic, respiration in the inflamed gut, Unique virulence factors	5 % of the genome is degraded (e.g., inactivated metabolic and virulence factor genes) Unique virulence factors and pathogenicity islands (e.g., Vi antigen, SPIs 7, 15, 17, and 18).

1.4 Transmission of *Salmonella*

Salmonella serovars are transmitted through the fecal-oral route. Animals and human can be infected through ingestion of contaminated food, drinking of contaminated water or by direct contact with infected animals (Modarressi and Thong, 2010) (Figure 1.3). The vertical transmission is known as a very important transmission route in birds, with contamination of the vitelline membrane, albumen and maybe the egg yolk. *Salmonella* can be also transmitted within uterus in mammals (Liljebjelke et al., 2005).

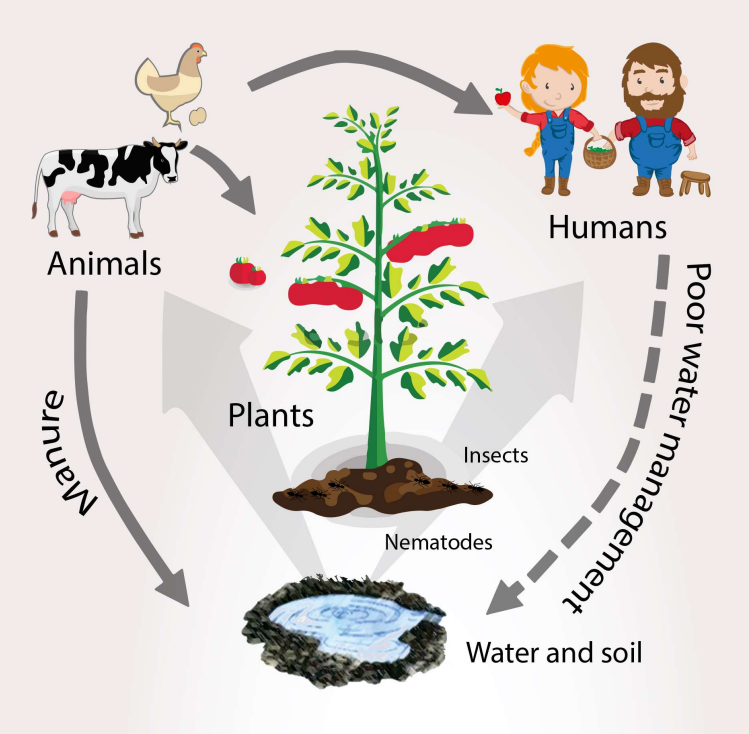

Figure 1.3: Infection routes of *Salmonella enterica*. The infection by *Salmonella* could be transmitted from contaminated water or from infected animals through the direct contacts or from contaminated animals' products, or from contaminated fruit and vegetables.

The indirect transmission route of *Salmonella* can be through the asymptomatic carrier of the pathogen. In this condition, *Salmonella* is carried asymptomatically inside the intestines or gall bladder of numerous animals, which are continuously or occasionally shedding it in their feces. In addition, it was found that fomites and insects can transmit *Salmonella* (Hoelzer et al., 2011).

1.5 *Salmonella* and food

Food borne pathogens remain on the top list of the public health and food industry problems. In this regard, *Salmonella* species are considered as the main cause of the sever cases of food borne illness leading to 35% of the hospitalisations and 28% of the deaths (Scallan et al., 2011).

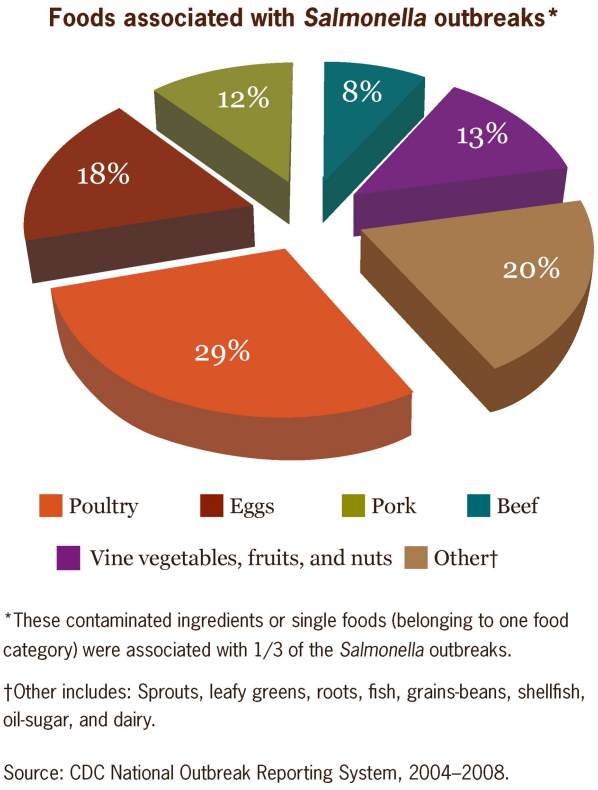

Figure 1.4: Food associated with *Salmonella*.

The typical way for *Salmonella* transmission to human occurs when consuming contaminated foods, which might be contaminated directly by animal feces or indirectly by other sources. The contaminated poultry products such as egg, poultry meet, and the pork, beef and dairy products, vegetables, juices in addition to other type of foods are considered as major source of salmonellosis (Sahar et al., 2009, Linam and Gerber, 2007). Poultry meat and eggs are among the most important sources from which *Salmonella* enters into the food chain (Howard et al., 2012), see figure 1.4. The poultry environments may be contaminated with *Salmonella* through contaminated poultry feed or water. The pathogen can also find its way to the poultry farms by carrier animals, including rodents, wild animals, birds and insects (Park et al., 2008).

A number of *Salmonella* serovars are recognised to cause extra-intestinal infections in poultry (Poppe, 2000), and the major serovars that lead to infections in chickens are *Salmonella* Typhimurium, *Salmonella* Enteritidis, and *Salmonella* Gallinarum. The first two serovars can infect humans and they are the primary reasons for food contamination (Carvajal et al., 2008). While these kinds of infections do

not produce severe symptoms in poultry, but the eggs and meat of infected animals can become a reservoir for infection of consumers. These types of asymptomatic bird carriers play a big part in *Salmonella* distribution and in food contamination (Carvajal et al., 2008).

Salmonella Typhimurium, *Salmonella* Enteritidis infect chickens through fecal-oral route, colonise the alimentary tract, attack internal organs like the liver and spleen, and at last distribute towards the reproductive tract (Chappell et al., 2009). *Salmonella* Enteritidis bacteria can be transmitted to the eggs, which are often transmitted to humans by intake of these contaminated eggs, which is actually a significant public health problem (Philippe Velge et al., 2005). In Europe, the serovars Enteritidis and Typhimurium are the second and third most common causes of bacterial gastroenteritis, which come after *Campylobacter* species (AEFS, 2010), see figure 1.5.

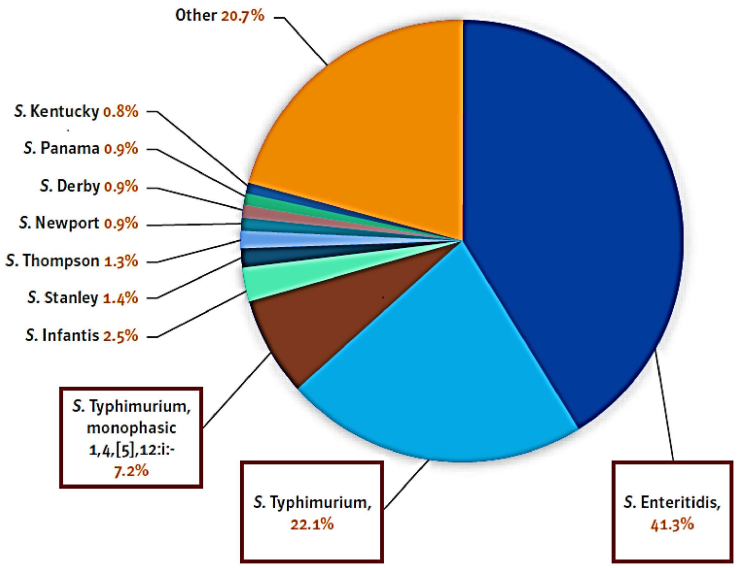

Figure 1.5: Distribution of the 10 most common *Salmonella* serovars in humans in the EU, 2012. Source: (EUSR) on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in 2012. EFSA Journal 2014;12(2):3547, 312 pp.doi:10.2903/j.efsa.2014.3547.

In comparison, *Salmonella* Gallinarum infection is host specific and leads to fowl typhoid (FT), an acute disease that results in septicemia in poultry (McGruder et al., 1993). Although *Salmonella* Gallinarum is restricted to poultry, it has a major impact on this very important industry. FT outbreaks result in severe economic loses worldwide (Wigley et al., 2005).

The world's food consumption has risen dramatically as a result of increase of population, we need to increase the production of animals in large farms. So, when *Salmonella* serovars- which have non-specific hosts- introduced on farms may easily become distributed among animals. In this situation, it becomes very difficult to eliminate the pathogens from the infected farms, also to remove them from products used as food.

1.6 Economic impact of *Salmonella* infection

According to Centers for Disease Control and Prevention (CDC), viruses are still the main causative agents for food borne diseases with a prevalence of 59% , followed by bacteria with a prevalence of 39%, and finally parasites 2%. However, it is important to highlight that bacterial agents are responsible for the majority of hospitalisations 63.9% and deaths 63.7%. Of the different bacterial agents, *Salmonella* species were regarded as the main causes of most severe cases leading to 35% of the hospitalisations and 28% of the deaths (Scallan et al., 2011).

Salmonella is a food borne pathogen in many countries for at least over 100 years (Coburn et al., 2006). Recently, it was estimated that all over the world *Salmonella* infection causes an annual 93.8 million human cases of gastroenteritis and 155000 deaths (Majowicz et al., 2010). In the United States the approximated total cost related to *Salmonella* cases may reach up to several billion dollars yearly (WHO et al., 2015). Therefore, there is a great global interest to improving *Salmonella* detection and typing methodologies.

1.7 Diagnosis and detection of *Salmonella*

Salmonella can be detected by culture method, Immunology-based assays, or nucleic acid-based assays.

1.7.1 Culture method

The isolation of *Salmonella* species by culture method includes a non selective pre-enrichment for a predefined weight or volume of the tested sample, followed by a selective enrichment step using selective agars. Finally, the candidates colonies are confirmed by several biochemical and serological tests.

The pre-enrichment step is used to improve the sensitivity of *Salmonella* detection. The aim of the pre-enrichment step is to promote cell growth in a non-selective medium (Tietjen and Fung, 1995). The Buffered Peptone Water (BPW) and lactose broth are most frequently used in this step.

The incubated pre-enrichment media are mixed into enrichment selective media containing several inhibitory reagents such as bile salts, brilliant green, novobiocin, sulphacetamide, thiosulphate, deoxycholate, malachite green, tetrathionate, and cycloheximide (Arroyo and Arroyo, 1995, Maciorowski et al., 2006). The inhibitors are used to allow *Salmonella* to keep proliferating, and to inhibit other bacteria (Tietjen and Fung, 1995).

Some regulatory agencies such as the Food and Drug Administration (FDA), the Bacteriological Analytical Manual (BAM) and the Food Emergency Response Network (FERN) approved Rappaporte Vassiliadis (RV) medium and tetrathionate (TT) broth to be used as an official *Salmonella* enrichment media, Figure (1.6). Enrichment media continue to be modified, to improve *Salmonella* growth, and to increase the sensitivity and selectivity.

At the end of the enrichment steps, the amount of *Salmonella* reaches more than 10^4 cells per ml^{-1} contained in the selective enrichment media, which will be streaked on a solid selective media to isolate positive *Salmonella* colonies, and to suppress other bacteria (Carrique-Mas and Davies, 2008, Tietjen and Fung, 1995).

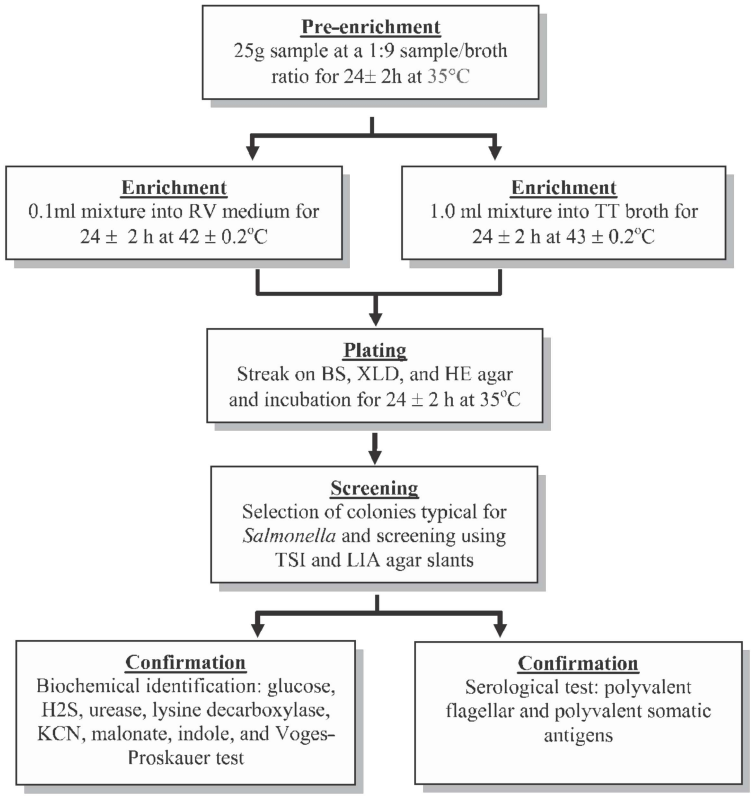

Figure 1.6: Scheme of the Food Emergency Response Network (FERN), and the Food and Drug Administration (FDA) for *Salmonella* culture method. RV: Rappaporte Vassiliadis medium; BS: bismuth sulfite agar; HE: Hektoen enteric agar; XLD: xylose lysine desoxycholate agar; TT: tetrathionate broth, TSI: Triple sugar iron agar, and LIA: Lysine iron agar

The xyloselysine-deoxycholate agar (XLD), Salmonella-Shigella agar (SS), bismuth-sulfite agar (BSA), brilliant green agar (BGA), and Hektoen enteric (HE) are widely used as selective plating media for *Salmonella* serovars, which are differentiated by colors from other coliforms on these media (Mallinson et al., 2000).

After the plating step, lysine iron agar (LIA) and triple sugar iron agar (TSI) are used to screen the expected *Salmonella* colonies after isolating them from the plating media. In these screening media, when the culture produces typical reactions for *Salmonella*, its tested using the biochemical and serological tests (Sørensen et al., 2004).

1.7.2 Rapid *Salmonella* detection methods

The rapid methods are classified into immunology-based assays and nucleic acid-based assays groups (Alakomi and Saarela, 2009), aiming to enable the *Salmonella* species detection in the samples with reliable results in less than 24 hours (Ferretti et al., 2001).

1.7.2.1 Immunology-based assay

Immunology-based assays are widely used to detect *Salmonella* in different sample types, depending on the binding of mono- or polyclonal antibodies with *Salmonella* antigens (Maciorowski et al., 2006).

The immunology-based assays include immunochromatography, latex agglutination tests, enzyme-linked immunosorbent assay (ELISA), and immunodiffusion. These assays are still considered to be a useful choice for routine analysis; because of their ability to detect viable non-culturable *Salmonella* cells (Maciorowski et al., 2006).

1.7.2.1.1 Enzyme-linked immunosorbent assay (ELISA)

ELISA is one of the most popular assays for the detection of antigens or products of *Salmonella* species. Various ELISA systems have been developed to detect *Salmonella* species. The detection strategies in ELISA assay depend on the specific *Salmonella* antibody or antigens linked on the solid phase of ELISA. When the sample contains *Salmonella* antigens or antibody against *Salmonella*, they bind to the antibody or to the antigens on the solid matrix and form a complex. When the sample contains *Salmonella* antigens or antibodies, the concentration of antigens or antibodies measured through the signal like colour alteration because of enzymatic cleavage of a chromogenic substrate (Blivet et al., 1998, Wiuff et al., 2000).

ELISA assays are not used as screening tests, but it is considered as confirmatory tests with specific and reliable results (Eijkelkamp et al., 2009). The detection sensitivity in the ELISA reaches 10^4 and 10^5 CFU/ml^{-1}, depending on the presence of non-culturable cells, inhibitory substances (e.g. heavy metals, antibiotics, polysaccharides, fats, organic compounds, and proteins), and sample matrix (Alakomi and Saarela, 2009). The sample purification and preparation methods are very important to improve the sensitivity of ELISA, in addition to some other modifications like centrifugation, organic solvent extract, dilution, and enrichment media (Kutter et al., 2006).

1.7.2.2 Nucleic acid-based assay

The nucleic acid-based assays depend on the detection of any fragment of *Salmonella* DNA directly, and they can recognise *Salmonella* species without using pure cultures (Glynn et al., 2006).

The two main methods that use nucleic acid (DNA) detection are:

1. Detection the target DNA fragment of *Salmonella* through the hybridisation on a specific DNA probe, which is called DNA probe hybridisation assay (Mozola, 2006).

2. Amplification of the target DNA fragment of *Salmonella*, which is called Polymerase chain reaction (PCR) assay.

Several improvements have been suggested to enhance the molecular-based detection of small numbers of bacteria, and they can be used as routine analysis for large number of samples (Mozola, 2006).

1.7.2.2.1 Polymerase chain reaction (PCR)

The PCR assays are *in vitro* amplification of a specific fragment of DNA to one million fold in 2 to 3 hours (McKillip and Drake, 2004). Different detection ways for amplified products are used, like real-time PCR and gel-based systems (Eijkelkamp et al., 2009).

Many developments on the basic PCR have enhanced the specificity and the sensitivity for discovering a very low concentration of the target DNA, which also affect the enrichment times to be shorter than other detection methods (Eijkelkamp et al., 2009). Some important PCR techniques are used for *Salmonella* detection are real-time PCR and multiplex PCR (Eijkelkamp et al., 2009), see (Table 1.3) for comparison between ELISA, PCR, and culture method.

Several studies have been published to detect *Salmonella* in poultry products and feeds using PCR techniques, by targeting selected antibiotic resistance or virulence genes along *Salmonella* species or serovars (Cohen et al., 1993, 1996, Maciorowski et al., 2000, 2005, Oliveira et al., 2002, Löfström et al., 2004, Seo et al., 2004, Salomonsson et al., 2005, Bansal et al., 2006, Eyigör et al., 2007, Jarquin et al., 2009, Wise et al., 2009, Levin, 2009, Melendez et al., 2010).

Table 1.3: Comparison between culture, ELISA, and PCR detection methods. The source of table from (Park et al., 2014).

Aspect	**Culture**	**ELISA**	**PCR**
Require Time	5-7 day	2 day	1 day
Automation	No	Yes	Yes
Number of Samples	Small	Large	Large
Choices	Small	Large	Large

1.8 The need for a practical method

The conventional culture based methods are unable to recognise non-culturable infectious agents, and they are time-consuming, work intensive, and not specific enough to detect and define *Salmonella* at the strain level, specially while working with several samples (Maciorowski et al., 2006).

Even-though the majority of molecular methods involve some sort of pre-enrichment phase, the total assay time of of these techniques is very short in comparison with the culture-based methods. In addition, the molecular methods are considered more sensitive as they include exponential amplification of the target DNA (Maciorowski et al., 2005).

The *Salmonella* Gallinarum, and *Salmonella* Enteritidis, and *Salmonella* Typhimurium were considered as the most important *Salmonella enterica* serovars that cause poultry infection (Lee, 2015). The control of poultry health and *Salmonella* infection management are critical issues to reduce the losses in poultry industry, and to protect humans from contaminated poultry products. So, the detection and rapid identification of these serovars in poultry and poultry products are very important in *Salmonella* control process.

1.9 Objectives

The aim of this work is to develop a close nested PCR (single-tube PCR) that can detect and distinguish *Salmonella* Gallinarum, *Salmonella* Enteritidis, and *Salmonella* Typhimurium from other *Salmonella* serovars.

CHAPTER 2

Materials and methods

2.1 Bacterial strains

A total of forty four samples of *Salmonella enterica*, from 16 different *Salmonella enterica* serovars, were used in this study. These samples were generously provided by Jovac Company, Jordan Food and Drug Administration (JFDA), Central Health Laboratory Ramallah, and Konkuk University (KU)-Korea. The samples from the first three sources were stored in 500 μl of 50 % glycerol mixed with 500 μl of bacterial broth at -80°C, and the samples from KU were stored at -18°C since they are DNA samples, Table (2.1).

Table 2.1: *Salmonella* strains used in this study

Strains	**Number**	**Source**
SG9R vaccine	3	JOVAC Company
Gallinarum/Pullorum	3	JOVAC Company
Gallinarum	3	KU-Korea
Typhimurim	14	JFDA
Enteritides	3	JOVAC Company
Infantis	1	Central Health Lab (CHL)
Newport	1	CHL
Bredeney	1	CHL
Muenchen	1	CHL
Hadar	1	CHL
Typhimurium	1	CHL
Anatum	1	CHL
Hadar	1	KU-Korea
Montevideo	1	KU-Korea
Enteritides	2	KU-Korea
Typhimurim	1	KU-Korea
Mbandaka	1	KU-Korea
Virchow	1	KU-Korea
Senftenberg	1	KU-Korea
Infantis	1	KU-Korea
London	1	KU-Korea
Newport	1	KU-Korea

2.2 Genomic DNA extraction

The glycerol stored samples were cultured in XLD selective medium overnight at 37°C, and the DNA extraction was made using the boiling method. About 4 to 5 isolated colonies were taken from XLD plate by tip touch, and were put in 200 μl of distilled water in a 2-ml microfuge tube. Then, they were mixed carefully by pipetting up and down several times. The result mixture was centrifuged at 3000 rpm for 5 min, the supernatant was discarded carefully to preserve the pellet. The centrifugation step was repeated again after adding 200 μl of distilled water, and mixed gently. The supernatant was discarded, and 100 μl of distilled water was added again. Then, the tube that contains the mixture was boiled in water path for 15 min. Finally, the tube was cooled using ice, and the supernatant was collected, and stored at -18°C.

The concentrations of collected DNA from *Salmonella* Gallinarum, Enteritidis and Typhimurium serovars were measured by Eppendorf Biophotometer spectrophotometry with serial number (6131 25073), and serial dilutions to 3 ng of DNA concentration were performed for later use.

2.3 Marker screening

The complete genome sequence *Salmonella* Gallinarum strain (287\91) with accession number (NC_011274.1) was downloaded from National Center for Biotechnology Information (NCBI) in Fasta format. Then, a BLASTN-based window sliding approach was used to perform a multiple genome comparison in order to identify the specific marker for each serovar. The query sequence of *Salmonella* Gallinarum strain (287\91) was divided into windows of 5000 bp (fragments), Figure (2.1).

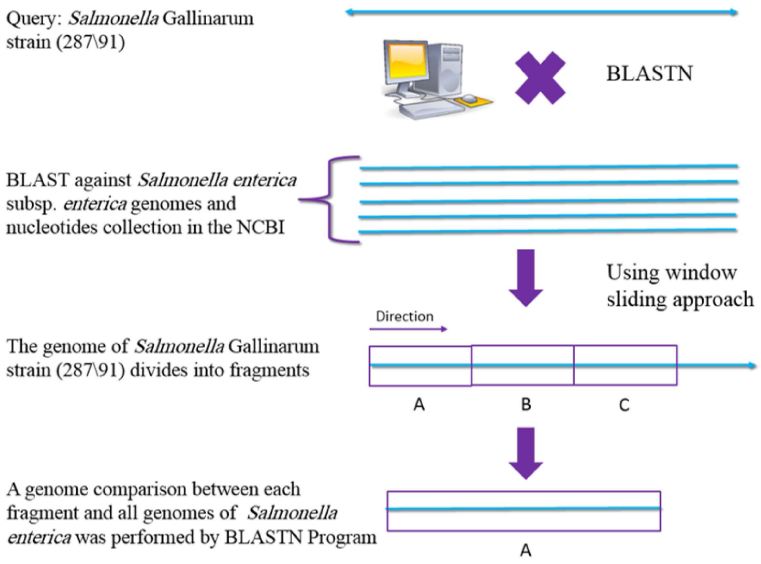

Figure 2.1: Window sliding approach

Then, a genome comparison between each fragment and all genomes of *Salmonella enterica* was performed by BLASTN Program (`http://blast.ncbi.nlm.nih.gov/Blast.cgi`) according to the following criteria:

1. Query: *Salmonella* Gallinarum strain (287\91).

2. Database: Nucleotide collection (nr/nt).

3. Organism determined as: *Salmonella enterica* subsp. *enterica* (taxid:59201).

4. The program selection is optimized to a highly similar sequences.

Then, selected segments were tested against *Salmonella* Gallinarum, Enteritidis and Typhimurium serovars to confirm the specificity, and the test was performed using BLASTN according to the following criteria:

1. Query: *Salmonella* Gallinarum strain (287\91) with determined range of the segment.

2. Database: Reference genomic sequences.

3. Organism determined as: *Salmonella* Typhimurium (taxid:90371), *Salmonella* Enteritidis (taxid:149539), *Salmonella* Gallinarum (taxid:594), *Salmonella* gallinarum/pullorum (taxid:604).

4. The program selection is optimized to a highly similar sequences.

Akiba et al. (2011) found several distinctive segments for *Salmonella* Enteritidis strain P125109 (PT4) (NC_011294.1) and *Salmonella* Typhimurium strain LT2 (NC_003197.1) within seven selected serovars of *Salmonella enterica* subsp. *enterica*. In this research, these segments were tested using BLASTN in order to find a specific segment that can identify them within the rest of the *Salmonella enterica* subsp. *enterica* serovars. These segments were tested following the previous criteria, but with changing the query according to the tested serovar.

2.4 Primer design

The oligo 7 software was used to design the external and internal primers for the close nested PCR for each *Salmonella* Gallinarum (SG), *Salmonella* Enteritidis (SE), and *Salmonella* Typhimurium (ST) marker. The inner primers were designed to have annealing temperature at 54 ± 2 °C, and 65 ± 2 °C annealing temperature for the outer primer, which allows the specific, unaccompanied annealing of each primer set.

Primers specificity was tested by BLASTN program. Firstly, against *Salmonella*, then, against chicken genome, which is called in the BLAST Gallus gallus (taxid:9031). The primers were synthesised in Hy-Labs Ltd., (Table 2.2).

Table 2.2: Close nested PCR primers

Primer name	Sequence from 5' to 3'	Length	Amplicon size (bp)	Tm
SG_EXT_F-334163	GAAGATACTTGCAGGTGACCAA	22	802	58
SG_EXT_R-334964	GCACTACCACCATAACGTGATA	22		57
SG_INT_F-334326	GACTTAGGTGTTGAGGAATA	20	511	52
SG_INT_R-334836	GTAAGCTATATCGGTCAAGAGA	22		54
SE_EXT_F-1471624	CGTAGGGGCAGGGCAAAACGCAT	23	1096	68
SE_EXT_R-1472719	GTGGTGGCTGGCGAATGGTGAGCA	24		67
SE_INT_F-1471761	GATGCTCTGGTTAAGGCA	18	914	54
SE_INT_R-1472674	GATATACTCCCTGAATCTGAGA	22		53
ST_EXT_F-4744487	GGAGTGCACATTAATCCCGCAGCGTA	26	708	66
ST_EXT_R-4745194	GGGAGCTCGGGCTTTTGGGCA	21		67
ST_INT_F-4744577	GGACGATATCTCACGCA	17	295	53
ST_INT_R-4744871	GATATTGGTACGAGGTTTCA	20		52

2.5 Marker specificity validation

Conventional PCR reaction was used to validate the marker specificity for *Salmonella* Gallinarum, *Salmonella* Enteritidis, and *Salmonella* Typhimurium. In separate PCR reactions for each *Salmonella* strain, the internal pair of close nested PCR primers- which are shown in (Table 2.2)- for *Salmonella* Gallinarum marker one, *Salmonella* Enteritidis, and *Salmonella* Typhimurium were used for validation of marker specificity. Also, the specificity for marker two of *Salmonella* Gallinarum was tested using this pair of primers: SG-594-F-1141799:5'-CGGTGATGATTCAACAGAGGAT-3', and SG-594-R-1142392:5'-CACCTTCATCAGTGACACGAA-3'

The specificity of the markers was examined by testing the reaction against a panel of serovars that came out from the serovars of interest, which are usually recovered from poultry such as Enteritidis, Typhimurium, Hadar, Infantis, and Bredeney, together with other *Salmonella* serovars.

Each 25 μl of PCR mixtures contained 1 μl of each primers (forward and reverse), which were 10 μM solutions, in addition to 2.5 μl of 10x buffer, and 2.5 μl of 20 μM MgSO4, 0.6 μl of 10 μM dNTPs, and 0.75 U of *Taq* DNA polymerase from Hy-Labs Ltd., item number (HTD0078), were used in the PCR mixture. Finally, 1

μl of the 60 ng of the DNA template was used. These concentrations were calculated according to *Taq* polymerase products information from Hy-Labs Ltd. The PCR reaction was done in (MJ mini BioRAD) PCR machine, and the program was started at an initial denaturation step at 94 °C for 4 minutes. Then, 30 cycles were started at 94 °C for 45 seconds, 57 °C for 45 seconds, and 72 °C for 1 minute. Then, a final extension step at 72 °C for 7 minutes. After that, the gel electrophoresis was done to separate the PCR products on 1 % of agarose gel in 0.5x TBE buffer, and the PCR products were observed under ultraviolet light by staining the bands with (0.5 mg/ml) of ethiduim bromide.

2.6 Close nested PCR

In the close nested PCR, the reagents of both PCR rounds were included in a single tube to avoid the possibility of contamination in two-tube PCR in the nesting step. Optimization of the PCR was performed to the annealing temperature, combination of cycles, and concentration of the primers. Firstly, the perfect annealing temperature of the primers was determined by doing a gradient PCR. Then, the optimization of the primer concentration was done by changing the concentrations of two primer pairs (pairs of the inner primer and outer primer). To optimize the PCR cycles; different combinations were tested: 15 plus 40; 20 plus 45; and 25 plus 40 to the first and second amplifications (Figure 2.2).

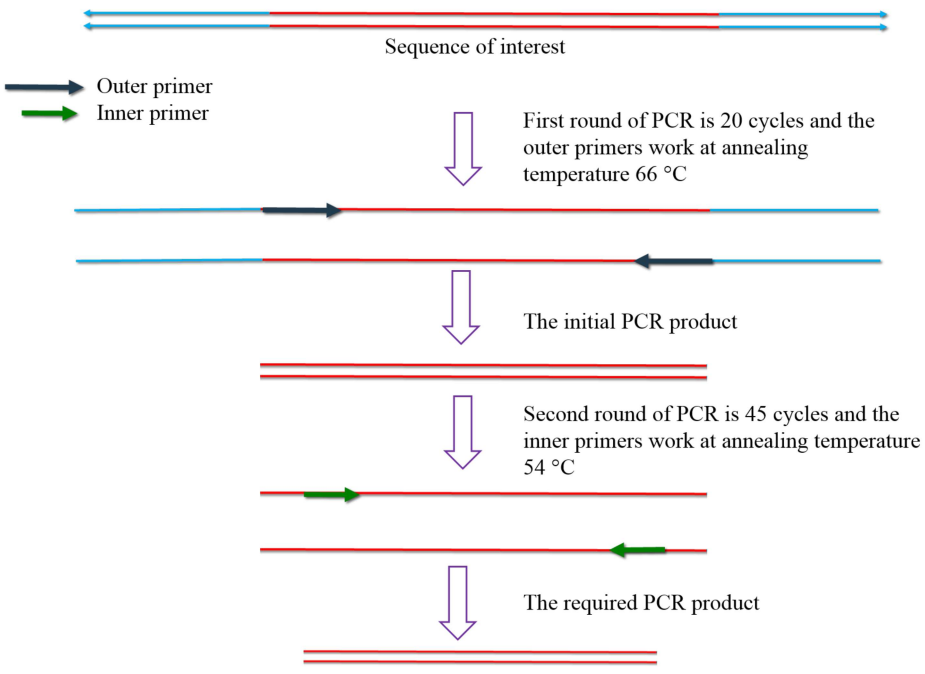

Figure 2.2: The close nested PCR program

Each 25 μl of PCR mixture contains 1 μl of each pairs of primers (outer and inner pair), with 2 μM concentration for the inner and 0.125 μM concentration for the outer primers, in addition to other PRC components as what mentioned in PCR reaction in previous section (3.5).

The PCR reaction was done in (MJ mini BioRAD) PCR machine, and the program was started at an initial denaturation step at 94 °C for 5 minutes, then 20 cycles at 94 °C for 1 minute, the annealing temperature for the outer primers was 66 °C for 1 minute, and 72 °C for 1 minute, followed by another 45 cycles of 94 °C for 45 seconds, 54 °C (annealing temperature for inner primers) for 45 seconds, and 72 °C for 1 minute. The final extension was performed at 72 °C for 10 minutes. After that, the gel electrophoresis was done to separate the PCR products, on 1% of agarose gel in 0.5x TBE buffer, and the PCR products were observed under ultraviolet light by staining the bands with (0.5 mg/ml) of ethiduim bromide.

2.7 Sensitivity of close nested PCR

The lowest detection limit of the close nested PCR was determined by a serial dilution for 3 ng concentration of genomic DNA of *Salmonella* Gallinarum, *Salmonella* Enteritidis, and *Salmonella* Typhimurium. So, four to six 10-fold dilutions were prepared from 3 ng of DNA.

The sensitivity of the close nested PCR was compared with a conventional PCR. The conventional PCR was performed using internal primers for each *Salmonella* serovars, and using the same serially diluted DNA samples.

CHAPTER 3

Results

3.1 Marker screening

The complete genomic sequence of *Salmonella* Gallinarum, Enteritidis, and Typhimurium were compared with other reference genomes of *Salmonella enterica* serovars in the NCBI databanks using BLASTN program.

The strain (287\91) was used as a reference genome of *Salmonella* Gallinarum. Two different specific segments (markers) were identified by the window sliding approach. These segments are common between *Salmonella* Pullorum and Gallinarum biotypes, and no specific region was found for *Salmonella* Gallinarum biotype, because these two biotypes are closely related. The first segment (marker one) is 1016 bp and its coordinates are (334041) to (335056) according to the reference genome (287\91). The second segments (marker two) is 1286 bp and its coordinates are from (1141638) to (1142922) according to the reference genome (287\91). Using the window sliding approach and the *Salmonella* Enteritidis strain (PT4) as a reference genome, one marker was identified for this serovar. The length of this marker is 1671 bp and its coordinates are from (1013381) to (1015051) according to the reference genome. For the *Salmonella* Typhimurium, the genome of the strain LT2 was used as a reference. One unique marker was found that is 1299 bp and its coordinates from (4744000) to (4745299).

3.2 Markers validation

The markers specificity was validated both *in silico* and *in vitro*. In the *in silico* analysis, each marker was compared against all the *Salmonella enterica* subsp, both finished and unfinished genomes of the NCBI database (as of March 2015). The analysis showed that each marker is highly specific to its serovar and does not exist in any other analysed genomes. On the other hand, the *in vitro* validation was made by using specific PCR reaction to screen an extensive panel of *Salmonella* serovars. Each marker of the three studied serovars shows only a specific band for its corresponding serovar.

The results for *Salmonella* Gallinarum in figure 3.1-(A) shows that only *Salmonella* Gallinarum serovar can generate specific target band (802 bp) for marker one. Whereas the other 15 *Salmonella* serovars and non-Salmonella strains did not show any band. In figure 3.1-(B), the *Salmonella* Gallinarum marker two also revealed that *Salmonella* Gallinarum serovar can generate specific target band (594 bp) for this one. However, a faint band with same size of target band (594bp) in lane 3 was

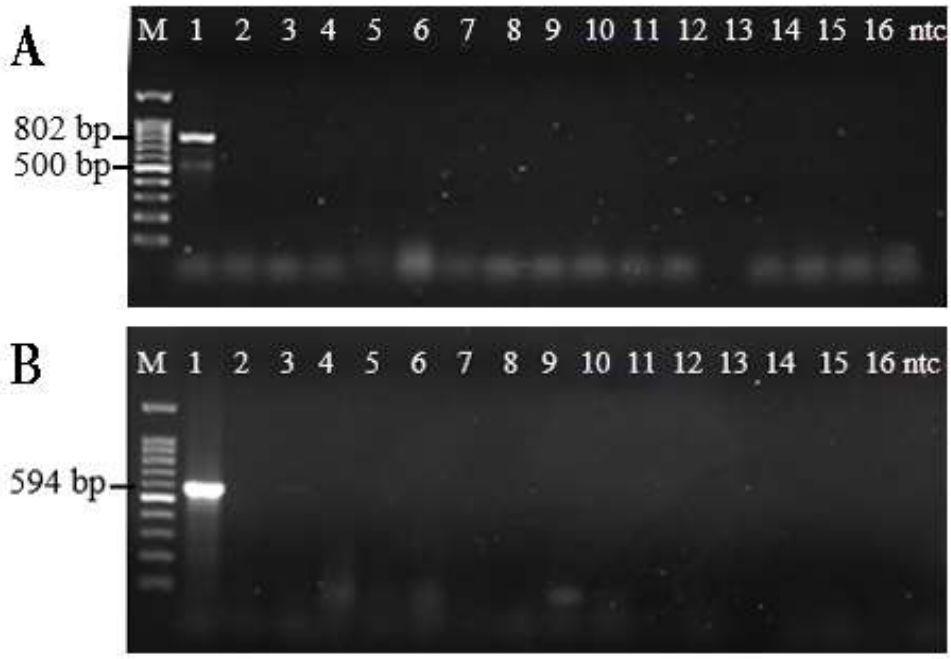

Figure 3.1: PCR products for markers specificity of *Salmonella* Gallinarum serovar. (A) The result for *Salmonella* Gallinarum segment one. (B) The result for *Salmonella* Gallinarum segment two. Lane M: 100 bp DNA ladder, lane 1: *Salmonella* Gallinarum, lane 2 : *Salmonella* Enteritides, lane 3: *Salmonella* Anatum, lane 4: *Salmonella* Bredeney, lane 5: *Salmonella* Muenchen, lane 6: *Salmonella* Hader, lane 7: *Salmonella* Infantis, lane 8: *Salmonella* Virchow, lane 9: *Salmonella* Newport, lane 10: *Salmonella* Hader, lane 11: *Salmonella* Montevideo, lane 12: *Salmonella* Mbandaka, lane 13: *Salmonella* Typhimurium, lane 14: *Salmonella* Senftenberg, lane 15: *Salmonella* London, lane 16: Not *Salmonella*, lane NTC: no template control.

also detected from *Salmonella* Anatum. Therefore, this marker (marker two) was excluded from further experiments to develop the close nested PCR assay *Salmonella* Gallinarum.

For *Salmonella* Enteritidis, the result revealed that only *Salmonella* Enteritidis serovar can generate specific target band (914 bp) for its marker. Whereas other *Salmonella* serovars and non- *Salmonella* strains didn't show any bands (Figure 3.2). Finally, the result for *Salmonella* Typhimurium revealed that only *Salmonella* Typhimurium serovar can generate specific target band (294 bp) for it's marker. In the gel result for *Salmonella* Typhimurium marker, an unspecific band with approximately 200 bp appeared with *Salmonella* Virchow serovar (Figure 3.3).

Figure 3.2: PCR products for marker specificity of *Salmonella* Enteritides. Lane M: 100 bp DNA ladder, lane 1: *Salmonella* Enteritides, lane 2: *Salmonella* Enteritides, lane 3: *Salmonella* Typhimurium, lane 4: *Salmonella* Gallinarum, lane 5: *Salmonella* Bredeney, lane 6: *Salmonella* Muenchen, lane 7: *Salmonella* Hader, lane 8: *Salmonella* Anatum, lane 9: *Salmonella* Infantis, lane 10: *Salmonella* Newport, lane 11: *Salmonella* Montevideo, lane 12: *Salmonella* Mbandaka, lane 13: *Salmonella* Virchow, lane 14: *Salmonella* Senftenberg, lane 15: *Salmonella* London, lane NTC: no template control.

3.3 Sensitivity of close nested PCR

The close nested PCR reactions were used for the detection and identification of *Salmonella* Gallinarum, *Salmonella* Enteritides, and *Salmonella* Typhimurium. So, three separated reactions, using pairs of outer and inner primers for each marker, were performed.

The close nested PCR produced the specific bands: 914 bp, 511 bp, and 294 bp for *Salmonella* Enteritides, *Salmonella* Gallinarum, and *Salmonella* Typhimurium, respectively. The primers used to performe the conventional PCR for each marker were selected based on empirical comparison between the outer and the inner pair of primers. According to that, the inner primer pairs for *Salmonella* Gallinarum, *Salmonella* Enteritides, and *Salmonella* Typhimurium were selected as they produced better results.

The detection sensitivity lower limit of the close nested PCR versus the conventional PCR assays was tested on serially diluted DNA sample and was as follows: for *Salmonella* Gallinarum (close nested PCR: 3 pg; conventional PCR: 0.3 ng) Figure 3.4, for *Salmonella* Enteritides (close nested PCR: 0.03 ng; conventional PCR: 0.3 ng) Figure 3.5, and for *Salmonella* Typhimurium (close nested PCR: 0.03 ng; conventional PCR: 0.3 ng) Figure 3.6.

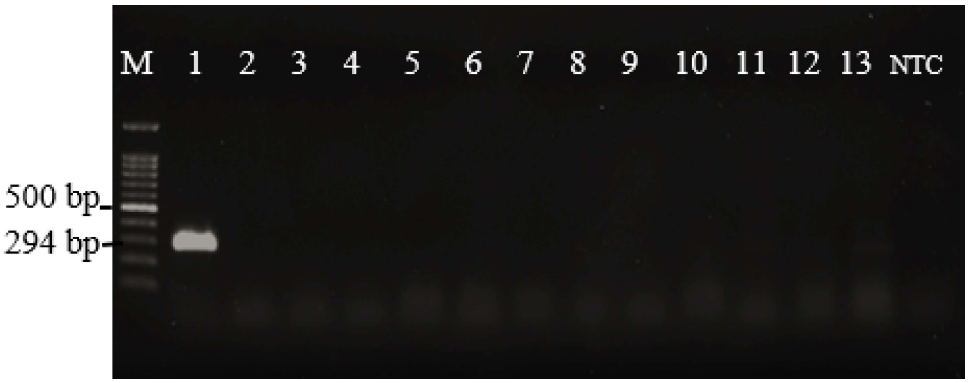

Figure 3.3: PCR products for marker specificity of *Salmonella* Typhimurium. Lane M: 100 bp DNA ladder, lane 1: *Salmonella* Typhimurium, lane 2: *Salmonella* Enteritides, lane 3: *Salmonella* Hader, lane 4: *Salmonella* Gallinarum, lane 5: *Salmonella* Bredeney, lane 6: *Salmonella* Muenchen, lane 7: *Salmonella* Newport, lane 8: *Salmonella* Anatum, lane 9: *Salmonella* Infantis, lane 10: *Salmonella* London, lane 11: *Salmonella* Montevideo, lane 12: *Salmonella* Mbandaka, lane 13: *Salmonella* Virchow, lane NTC: no template control.

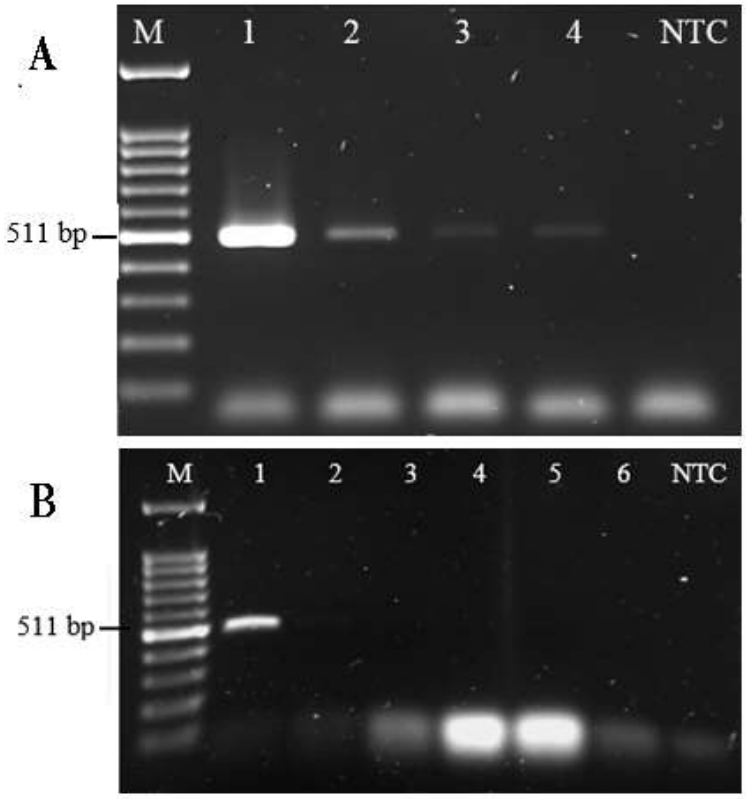

Figure 3.4: The sensitivity of close nested PCR and conventional PCR for *Salmonella* Gallinarum. (A) The close nested PCR, lane M: 100 bp DNA ladder, lane 1: 3 ng, lane 2: 0.3 ng, lane 3: 0.03 ng, lane 4: 3 pg, lane NTC: no template control. (B) The conventional PCR, lane 1: 3 ng, lane 2: 0.3 ng, lane 3: 0.03 ng, lane 4: 3 pg, lane 5: 0.3 pg, lane 6: 0.03 pg, lane 6: 3 fg, NTC: no template control.

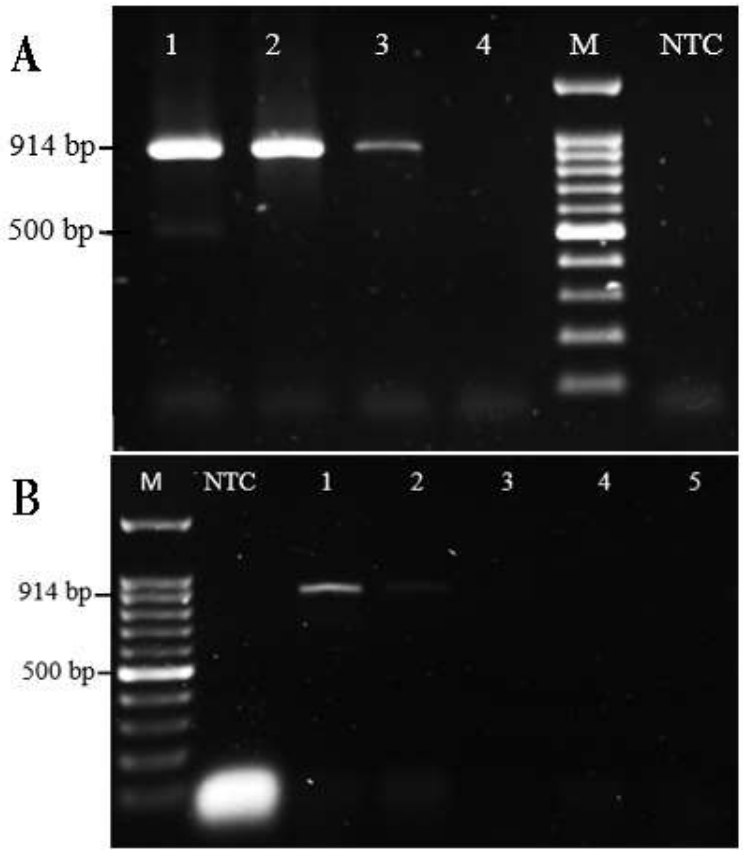

Figure 3.5: The sensitivity of close nested PCR and conventional PCR for *Salmonella* Enteritides. (A) The close nested PCR, lane M: 100 bp DNA ladder, lane 1: 3 ng, lane 2: 0.3 ng, lane 3: 0.03 ng, lane 4: 3 pg, lane NTC: no template control. (B) The conventional PCR, lane 1: 3 ng, lane 2: 0.3 ng, lane 3: 0.03 ng, lane 4: 3 pg, lane 5: 0.3 pg, NTC: no template control.

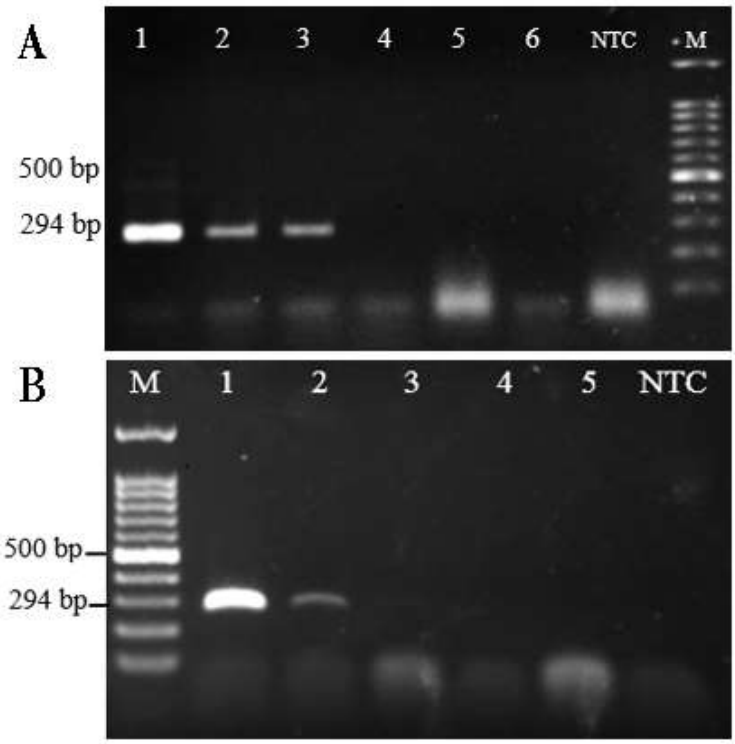

Figure 3.6: The sensitivity of close nested PCR and conventional PCR for *Salmonella* Typhimurium. (A) The close nested PCR, lane M: 100 bp DNA ladder, lane 1: 3 ng, lane 2: 0.3 ng, lane 3: 0.03 ng, lane 4: 3 pg, lane 5: 0.3 pg, lane 6: 0.03 pg, lane NTC: no template control. (B) The conventional PCR, lane 1: 3 ng, lane 2: 0.3 ng, lane 3: 0.03 ng, lane 4: 3 pg, lane 5: 0.3 pg, NTC: no template control

CHAPTER 4

Discussion

In this work, different specific segments (markers) for *Salmonella* Gallinarum, *Salmonella* Enteritides, and *Salmonella* Typhimurium were identified and used to develop three rapid and sensitive close nested PCR assays. The specificity for each segment was validated by *in silico* and *in vitro*. Both validation methods have confirmed the specificity of the selected segments. Earlier reports identified markers by different genome comparison approaches (Pugliese et al., 2011, Akiba et al., 2011). However, in the present work we used a window sliding approach which can be preformed on large number of genomes that are publicly available. This approach had the advantage of allowing *in silico* validation on an extensive set of Salmonella genomes in contrast to other genome comparison tools that are limited in terms of the number of the genomes that can be simultaneously compared.

Using genomic comparison method, two unique DNA segments were identified for *Salmonella* Gallinarum. The first DNA segment (marker) length was 1016 bp; a part of the *Salmonella* Pathogenicity Island 6 (SPI-6). A few previous reports have studied the genomic variations between the different *Salmonella* serovars like *Salmonella* Gallinarum, *Salmonella* Enteritidis, and *Salmonella* Typhimurium in the SPI-6 structure, size, and genetic variations. It was demonstrated that some of these variations can be specific for these serovars (Blondel et al., 2009, Thomson et al., 2008). The second specific segment that was found in the *Salmonella* Gallinarum genome is a part of ROD9 region that contains a number of gene clusters (Thomson et al., 2008); this fragment length was 1286 bp.

The genomic analysis of the specific segment of the *Salmonella* Enteritidis shows that it is a region that contains three genes (SEN-RS04705, SEN-RS04710, and SEN-RS04715). The product of (SEN-RS04705) gene is enterohemolysin, and hypothetical proteins are proposed from (SEN-RS04710) and (SEN-RS04715). For *Salmonella* Typhimurium, the specific segment was 1299 bp, and it is located in the locus (STM4495), which produce the type II restriction enzyme methylase subunit.

Three close nested PCR reactions using two pairs of primers for each (outer and inner) were developed to identify most of the important serovars that contaminate the poultry and poultry products. In comparison with the traditional culture method; the close nested PCR is faster, more efficient and economical for routine screening and monitoring, which is important in food industry and public health (Sachse, 2003, Jeon et al., 2007). This PCR approach is highly specific, particularly when a large number of other *Salmonella* serovars are tested. The detection sensitivity of the developed close nested PCR was approximately 100 folds more sensitive than the conventional PCR.

Many nucleic acid-based assays for *Salmonella* serovars detection and differenti-

ation were developed (Jeon et al., 2007, Kisiela et al., 2005, O'Regan et al., 2008, Hadjinicolaou et al., 2009, de Freitas et al., 2010, Pugliese et al., 2011, Akiba et al., 2011, Park et al., 2009). Most of these assays are not highly specific and sensitive to detect and differentiate *Salmonella* serovars. Hadjinicolaou et al. (2009) developed a multiplex real-time PCR assay was developed for the detection of *Salmonella* Enteritides, and *Salmonella* Typhimurium. Although this methods shows a good level of sensitivity, it requires a real time PCR machine, which is typically not available in many routine microbiology labs with resource limiting settings.

To meet the needs of most developing countries, the majority of *Salmonella* PCR-based detection method use the conventional gel electrophoresis-based approach. (de Freitas et al., 2010, Zhai et al., 2014, Yukawa et al., 2015, Park et al., 2009). The conventional PCR (single or multiplex) is rapid, and it's specificity depends on the specificity of the target DNA, but its sensitivity is low in comparison with the real-time PCR. (Park et al., 2013). In order to overcome this limitation, developed a conventional gel electrophoresis-based approach (close nested PCR) that might reach the sensitivity of real-time PCR.

A few previous research groups have used the typical nested or semi-nested PCR for *Salmonella* detection (Pugliese et al., 2011, Hashimoto et al., 1995, Pratap et al., 2013). In contrast to the typical nested PCR that is prone to false positive results due the high risk of contamination, the close nested PCR is considered very accurate since the two rounds of PCR are performed in the same tube (single tube); without the need to open the tube in between the rounds (Park et al., 2013).

In conclusion, the developed close nested PCR assays can serve as a useful tool to identify the contaminated and infected poultry products as well as environmental samples that are associated with poultry farms. The approach described in this work may represent a suitable alternative to culture-based methods for *Salmonella* serovars detection, since it shows high specificity and good level of sensitivity. Therefore, it may be suitable for routine detection of *Salmonella* Gallinarum, *Salmonella* Enteritidis and *Salmonella* Typhimurium directly from contaminated samples.

As a future work, we plan to developed a multiplex close nested PCR assay for the three serovars. In addition, we plan to optimise the multiplex PCR on contaminated samples from poultry and poultry products.

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