MOLECULAR EPIDEMIOLOGY OF *SALMONELLA* TYPHIMURIUM DT160 IN NEW ZEALAND

A thesis presented in partial fulfilment of the requirements for the degree of

MASTER OF VETERINARY STUDIES

IN

PUBLIC HEALTH

AT MASSEY UNIVERSITY, PALMERSTON NORTH,

NEW ZEALAND

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2010

Abstract

Salmonellosis is a zoonotic bacterial disease of national and international importance. In New Zealand (NZ), the most common foodborne notifiable disease is campylobacteriosis, which is followed by salmonellosis. In 1998, *Salmonella enterica* subsp. *enterica* serotype Typhimurium Definitive Type 160 (DT160) was identified in NZ. Since first reported, *S.* Typhimurium DT160 has caused several epidemics in the country but has not produced significant illness worldwide.

Therefore, the objectives of the project were to investigate the molecular epidemiology of *S.* Typhimurium DT160 and the association between isolates from human and animal origin. Ninety *Salmonella* isolates obtained in the period between 1999 and 2009 from the Institute of Environmental Science and Research, NZ were assessed for colony morphology, serotype, susceptibility to 11 antimicrobials, virulotyped using Polymerase Chain Reaction (PCR) and the Pulsed Field Gel Electrophoresis (PFGE) patterns were also determined. In addition, 4 isolates were further assessed with Triple Sugar Agar, API20E biochemical and motility tests.

All 90 isolates were confirmed as *Salmonella* spp. with no indications for resistance to multiple antimicrobials. All isolates were susceptible to the antimicrobials used in this study with the exception of 26 and 8 isolates that had intermediate susceptibility against tetracycline and oxytetracycline, respectively. In an attempt to discriminate between potentially pathogenic and pathogenic *Salmonella* isolates, PCR-based virulotyping was performed based on 12 potential virulence genes. Results revealed that all isolates were positive for at least 10 of the 12 virulence genes. Two of the six isolates negative for one of the virulence genes (*invA*, *iroN*, *pefA* or *sifA*) were of human origin and the remaining four were sparrow

isolates. The PFGE patterns determined with restriction enzymes *Xba*I and *Spe*I demonstrated that the genotype profile AA1 accounted for 78/90 (86.7%) of the isolates, whilst the second most common profile, AA2, was found in only three isolates (3.3%), comprising two isolates from sparrows and one from a human. The remaining nine profiles were found in single isolates. All isolates of AA2 profile were PCR negative for *sifA.*

In conclusion, no obvious correlation was observed between the phenol- and geno-type and the isolates, year and month of isolation, and source of the samples. There was no obvious evidence for multidrug resistance among DT160 isolates. The PFGE and virulotyping profiles suggest close relation among majority of isolates with predominant and epidemiologically important genotype persistent in multiple hosts. Finally, the few genotypes with low prevalence in multiple hosts may indicate emergence of sporadic genomic variants in the population.

Acknowledgements

The acknowledgement is the last part of my thesis that I have to write but it is definitely not the least important, as I would not have been able to accomplish this work without the help of my supervisors, family and friends.

It has been a great opportunity for me to have been able to further advance my post graduate education at Massey University. This experience has nourished me with knowledge and I have gained vast experience working with people from a number of different backgrounds.

First of all, I would like to express my deepest gratitude to the Vice-Chancellor of University Putra Malaysia, the Dean and Head of the Department of Pathology and Microbiology at the Faculty of Veterinary Medicine for having confidence in me to carry out this responsibility and supporting me during this process.

I am too much indebted to the Malaysian Government for funding my two years of education. Without this help, it would be impossible for me to pursue my scientific training.

My special thank you goes to my enthusiastic main supervisor, Dr. Daniel Petkov who has patiently provided me with knowledge in molecular biology by sharing his experiences in this field throughout the course of the study. Thank you for the guidance particularly on academic writing skills and giving me the chance to explore in details the demanding molecular techniques that I will need for a strong scientific career. It was a pleasure as well to work with my two co-supervisors, Prof. Nigel French and Dr. Julie Collin-Emerson who have given me the opportunity to work on this project. Their advice and assistance is much appreciated.

In addition to my supervisors, I am also grateful and lucky to have met Errol Kwan, Lynn Rogers, Rebecca Pattison, Rukhshana Akhter and Angie Reynolds who were always there for me when I needed excellent technical assistance. It was wonderful working with all of you! And also to Shoukai Yu and Hamid Irshad who have spent their precious time in assisting me in the Kappa analysis and the pulsed-field gels and PFGE analysis respectively.

Furthermore, I would like to acknowledge Dr. Phil Carter and Dr. Muriel Dufour from the Institute of Environmental Science and Research Limited, New Zealand (NZ) for supplying the *Salmonella* isolates used in this study. This work was supported by grant 11406 from the Ministry of Agriculture and Forestry Biosecurity, NZ.

To my family and friends, in New Zealand and overseas, Thank you so much for all the love, care and moral support and making my daily life fun all the way through. God bless.

"So, verily, with every difficulty, there is relief. Verily, with every difficulty there is relief"

(HQ 94:5-6)

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List of Abbreviations

ACSSuT	AMPICILLIN, CHLORAMPHENICOL, STREPTOMYCIN, SULPHONAMIDES, TETRACYCLINE
AK	AMIKACIN
AMC	AMOXICILLIN-CLAVULANIC ACID
AMP	AMPICILLIN
ATCC	AMERICA TYPE CELL CULTURES
BD	BECTON DICKINSON
bp	BASE PAIRS
C	CHLORAMPHENICOL
CDC	CENTERS FOR DISEASE CONTROL AND PREVENTION
CIP	CIPROFLOXACIN
CLB	CELL LYSIS BUFFER
CLSI	CLINICAL AND LABORATORY STANDARDS INSTITUTE
CPD	CEFPODOXIME
CSB	CELL SUSPENSION BUFFER
DNA	DEOXYRIBONUCLEIC ACID
DT	DEFINITIVE PHAGE TYPE
EDTA	ETHYLENEDIAMINETETRAACETIC ACID
ESR	INSTITUTE OF ENVIRONMENTAL SCIENCE AND RESEARCH
EU	EUROPEAN UNION
FOX	CEFOXITIN
kbp	KILOBASE PAIRS
LPS	LIPOPOLYSACCHARIDE
MDR	MULTI DRUG RESISTANT
$MgCl_2$	MAGNESIUM CHLORIDE
MIN	MINUTES
mL	MILLILITRES
MLVA	MULTILOCUS VARIABLE NUMBER TANDEM REPEAT ANALYSIS
mM	MILLIMOLAR
mm	MILLIMETERS
NA	NALIDIXIC ACID
NCTC	NATIONAL COLLECTION OF TYPE CULTURES
NTP	NUCLEOTIDE TRIPHOSPHATE
NZ	NEW ZEALAND
OT	OXYTETRACYCLINE
PCR	POLYMERASE CHAIN REACTION
PFGE	PULSED FIELD GEL ELECTROPHORESIS
PT	PHAGE TYPE
SKG	SEAKEM GOLD

SPI	*SALMONELLA* PATHOGENIC ISLAND
SXT	TRIMETHOPRIM-SULFAMETHOXAZOLE
TBE	TRIS-BORATE EDTA
TE	TRIS-EDTA
TET	TETRACYCLINE
TTSS	TYPE III SECRETION SYSTEM
TSI	TRIPLE SUGAR IRON
USA	UNITED STATE OF AMERICA
WHO	WORLD HEALTH ORGANISATION

Chapter 1: Literature review

1.1. Introduction

Infection with the bacterial species *Salmonella*, is called salmonellosis. *Salmonella* was discovered by Theobald Smith and named after the scientist Daniel Salmon who contributed to the identification and characterisation of Salmonella (Schultz 2008). *Salmonella* species (spp.) are distributed worldwide and can remain viable for months in soil, water and faeces (Connor and Schwartz 2005; Moore *et al.* 2003; You *et al.* 2006). *Salmonella* can cause disease in almost all animal species including horses, pigs, cattle (Schaer *et al.* 2010), birds (Shivaprasad 2000; Tizard 2004), sheep (Baker *et al.* 2007) and humans (Callaghan and Simmons 2001). Human infections are commonly caused by the nontyphoidal *Salmonella* spp. serotypes (predominantly *Salmonella enterica* subspecies *enterica* serotype Typhimurium (*S.* Typhimurium) and *S.* Enteritidis), and rarely by typhoidal *S. enterica* serotype Typhi (Connor and Schwartz 2005; Parry *et al.* 2002). Most individuals with nontyphoidal salmonellosis will develop diarrhoea, fever and abdominal cramps lasting between a few hours to several days. The clinical form of the disease is usually last between four and seven days and in majority of the cases affected individuals recovers without treatment. In some patients bacteria may spread from intestines to the other organs resulting in death (Crum-Cianflone 2008; Molyneux *et al.* 2009). In addition, the host may become a persistently infected asymptomatic carrier and intermittently shed bacteria (Todd *et al.* 2008). Rheumatologic arthritis (Reiter's syndrome) is a rare clinical form in humans which presents as joint pain, conjunctivitis, and painful urination that may last for years (Townes 2010). Salmonellosis occurs predominantly during the summer months and higher risk groups are infants, immunodeficient and elderly individuals (Crum-Cianflone 2008; Hohmann 2001).

Approximately 40 000 cases of salmonellosis are reported annually in the United States of America (USA) but as milder cases are either not diagnosed or reported the true number is estimated to be 30 times higher. The rate of diagnosed salmonellosis in children less than 5 years of age is higher in comparison to all other groups (Crum-Cianflone 2008). Outbreaks of salmonellosis in animals are a zoonotic threat, but due to the limited surveillance performed in animals it is difficult to identify a human outbreak that corresponds closely to an outbreak in a single animal species (Baker *et al.* 2007).

Nontyphoid salmonellosis is one of the leading causes of diarrhoea in developed countries (Crum-Cianfione, 2008; Mead *et al.* 1999). In the USA, nontyphoid salmonellosis accounts for 30% of all deaths associated with foodborne diseases (Mead *et al.* 1999). In the U.S. approximately 1.4 million infections annually as presumed to originate from consumption of contaminated food products, predominantly of from poultry, beef or pork origin (Crum-Cianfione 2008; Gilbert *et al.* 2010). Since 1997 in the USA, approximately 45% of all *Salmonella* isolates of both human and animal origin were serotype *S.* Typhimurium followed by *S.* Enteritidis, *S.* Newport and *S.* Heidelberg [Centers for Disease Control and Prevention (CDC), 2006]. Serotypes *S.* Typhimurium and *S.* Enteritidis have different epidemiology in comparison to the other *Salmonella* spp. and are often targeted with control legislation. In the UK in the 1980s, there was an increase of salmonellosis cases caused by *S.* Enteritidis then followed by a decrease a number during the 1990s. The number of human infections caused by *S.* Enteritidis related consumption of poultry products has also declined since 1998, coincident with the introduction of vaccination programs for egg-laying hens (Cogan and Humphrey 2003).

In 2009, approximately 39% of the human enteric disease outbreaks in New Zealand (NZ) were caused by *Salmonella* spp., followed by *Campylobacter* spp., with the most common routes of transmission being person-to-person, foodborne, and environmental

[Institute of Environmental Science and Research Limited (ESR), 2010]. Since 1996 the number of infections in humans caused by *S.* Brandernburg has also increased in NZ (Clarke and Tomlinson 2004), and some of the epidemics have been associated with close contact between humans and sheep (Baker *et al.* 2007). DT160 has a broad host range that includes humans (Callaghan and Simmons 2001), birds (Connolly *et al.* 2006; Piccirillo *et al.* 2010) and other species. In birds, salmonellosis epidemics caused by *S.* Typhimurium DT160 have caused high mortality mostly in passerines [sparrows (*Passer domesticus*), finches (*Lonchura domestrica*), and blackbirds (*Turdus merula*)], and to a lesser extent in psittacines [sulphur-crested cockatoos (*Cacatua galerita*) and kakas (*Nestor meridionalis*)] (Alley *et al.* 2002). In 1998, *S.* Typhimurium DT160 was identified as a human pathogen in NZ (Callaghan and Simmons 2001), and the following year the incidence of DT160 isolates from birds such as sparrows have increased significantly and the geographical distribution expanded from the South to the North Island (Callaghan and Simmons 2001; ESR 2010). While human epidemics caused by *S.* Typhimurium DT160 in NZ between 1998 and 2000 have paralleled episodes of epizootic fatality in sparrows and other birds (Connolly *et al.* 2006; ESR 2010; Thornley *et al.* 2003) there is no clear evidence that DT160 is transmitted between birds and human population (Alley *et al.* 2002; Callaghan and Simmons 2001). Between 2006 and 2010 in New Zealand *S.* Typhimurium DT160 was the predominant phage type followed closely by DT1, DT101, and DT156 (ESR 2010).

In contrast to the predominant *Salmonella* Typhimurium Definitive types in NZ one of the most important types in Canada, Europe, and USA is DT104 (Fisher 2004). There are several reports of DT104 Multidrug Resistant (MDR) type ACSSuT (Ampicillin, Chloramphenicol, Streptomycin, Sulphonamides and Tetracycline) infecting humans as well as animals (CDC 2006; Lawson *et al.* 2004; Graziani *et al.* 2008; Helms *et al.* 2002). The range of the resistance profile for *S.* Typhimurium DT104 has recently been expanded to

include other major antibiotic classes fluoroquinolone (ciprofloxacin), nalidixic acid, and trimethoprim, and the range of animal species infected has widened to include cattle, swine, sheep, and poultry (Poppe *et al.* 1998; Threlfall *et al.* 2003).

Salmonellosis is a major public health concern with a significant socioeconomic impact and a noticeable cost for the society of every country. It is estimated that in the USA alone, the total annual cost associated with 15 000 salmonellosis hospitalizations, 160 000 visits to physician and more than 580 deaths has reached three billion dollars (USD). In addition, the cost for each case of human salmonellosis can vary significantly between 40 USD and 4.6 million USD depending on severity and complications of the infection (WHO 2005). Between 1994 and 1999, Denmark spent approximately 40 million USD for control measures related to foodborne salmonellosis and of this; 15.5 million went to compensation of farmers, with the remainder for establishing surveillance measures. In 2001, the broiler, layer hens, and swine industries in Denmark invested 14.1 million USD for control of *Salmonella* on farms and in food processing plants. These measures have lead to a considerable savings for the Danish society of 25.5 million USD in reduction of salmonellosis (Wegener *et al.* 2003; WHO 2005).

1.2. *Salmonella* nomenclature

Salmonella is a gram negative (-), non-spore forming, predominantly motile, facultative anaerobic bacterium that belongs to the *Enterobacteriaceae* family (Edwards and Ewing's 1986).

To ensure comprehensible communication among scientists, health officials and the public, *Salmonella* nomenclature has been used worldwide since 1987 (Le Minor and Popoff 1987). Taxonomically, *Salmonellae* are divided into 2 species; *S. bongori* and *S. enterica*. *S. enterica* is further subdivided into 6 subspecies: *S. enterica* subsp. *enterica* (I), *S. enterica* subsp. *salamae* (II), *S. enterica* subsp. *arizonae* (IIIa), *S. enterica* subsp. *diarizonae* (IIIb), *S. enterica* subsp. *houtenae* (IV) and *S. enterica* subsp. *indica* (VI). In addition, the symbol "V" denotes *S. bongori* (Grimont and Weill 2007). The antigenic formulae of serotypes are defined by the WHO Collaborating Centre for Reference and Research on *Salmonella* at the Pasteur Institute, France, and new serotypes are listed in the updates of the Kauffmann-White scheme (Brenner *et al.* 2000). As the complete names of *Salmonellae* species, subspecies, and serotypes are long, they are often cited as abbreviations in the literature. For example, the abbreviated designation for *Salmonella enterica* subspecies *enterica* serotype Typhimurium is *S.* Typhimurium (Grimont and Weill 2007) and it will be used throughout this Thesis.

1.3. *Salmonella* disease and pathogenicity

There is a complex interaction between the *Salmonella* spp., environment, different animal species and humans. The course and outcome of infection caused by *Salmonellae* depends on many factors including the genetic makeup of the host and the age, gender, diet, behaviour, immune status, and genetic makeup of the bacteria. Susceptibility of the host to certain *Salmonella* strains can also have a significant impact (Bueno *et al.* 2008). Finally, stress can influence severity of the infection (Soderholm and Perdue 2001), recovery and possible development of the asymptomatic carrier state resulting in the shed of *Salmonella* for extended periods. When recently weaned piglets of different farm origin were inoculated with *S.* Typhimurium and were introduced to a social stress factor by co-mingling, significantly higher colony forming units of *Salmonella* were counted from stool samples taken from the rectum in comparison to cecum (Callaway *et al.* 2006). Therefore, infected animals might not show clinical signs but still shedd bacteria that represent an epidemiological challenge and risk for the rest of the animals as well as for humans. Additional major risk factors are consumption of inappropriately cooked and contaminated animal products or close proximity between animal species (Tizard *et al.* 1979; Wong *et al.* 2007). Commensal microflora, bacterial virulence factors encoded within *Salmonella* Pathogenic Islands (SPI), and infectious dose have further effect on the interaction between the animal host and *Salmonella* (Groisman and Ochman 1996; Lawley *et a.l* 2008). A study of the prevalence of *S.* Typhimurium in birds in Scotland, UK for the period between January and December of 2004 has reported that *S.* Typhimurium DT56 was highly prevalent in greenfinches between January to February of the same year (Grant *et al.* 2007). Although the above study did not identify the source of *Salmonella*, it has raised the issue of the complex relationship between factors that could influence the disease prevalence during different seasons of the year. The primary route of infection with *Salmonella*, oral and aerosol, are

especially effective in overcrowded confined environments during transportation, and when animals are held in abattoir pens before slaughter (Rostagno *et al.* 2003).

While many members of the family *Enterobacteriaceae* are normal part of the intestinal flora, others such as *Salmonella* are not usually found in the gastrointestinal tract and when present they might become pathogenic. The intestinal epithelium has several functions including digestion and absorption of nutrients and acts as a barrier between the host's internal and external environment (Soderholm and Perdue 2001). A framework of tight junctions between the continuous epithelial cell layers is a principal component of the intestinal barrier that restricts migration of molecules. Thus, in order for *Salmonella* to cause enteritis and septicaemia, the bacterium has to adhere to and penetrate through the intestinal mucosa (Tsolis *et al.* 1999) by rearranging the cytoskeleton of the enterocytes (Galan *et al.* 1992). The lipopolysaccharide (LPS), a major cell wall component of *Salmonella* is composed of lipid A, core oligosaccharide and O antigenic polysaccharide chain (Luk and Lindberg 1991; Luderitz *et al.* 1966). While the polysaccharides are major determinants for the serological specificity and immunogenicity, endotoxicity is due mostly to the lipid A component (Bayston and Cohen 1990; Neter *et al.* 1956). *Salmonella* is a facultative intracellular bacterium that can survive within and use phagocytic cells to disseminate itself to the mesenteric lymph nodes, spleen, liver (Jones and Falkow 1996) and other internal organs. The primary sites of invasion for these bacteria are the aggregated lymphoid nodules (Peyer's patches) in the ileum (Reynolds and Kirk 1989), which are covered by a specialised Follicle Associated Epithelium (FAE) containing approximately 50% microfold (M) cells, the function of which is to sample antigens from the intestinal lumen and deliver them to the antigen-presenting cells (Libby *et al*. 2004). The M cells enhance the host susceptibility towards bacterial infection by facilitating the internalisation of *Salmonella* by

macropinocytosis and inclusion of the bacterium into membrane-bound vacuoles (Libby *et al*. 2004).

1.4. *Salmonella* typing

Currently, the total number of *Salmonella* serotypes is 2579, including 2557 and 22 for *S. enterica* and *S. bongori*, respectively (Grimont and Weill 2007). In the USA most cases of salmonellosis in animals, as well as in humans, are caused by the few serotypes *S.* Enteritidis, *S.* Newport, and *S.* Typhimurium which are also serotypes of *S. enterica* subsp. *enterica* (CDC 2006). While serotypes *S.* Enteritidis and *S.* Typhimurium can infect a wide range of hosts including birds, pigs, sheep and cattle and humans, others such as *S.* Cholerasuis, *S.* Dublin and *S.* Typhi infect primarily pigs, cattle and humans, respectively (Kingsley and Baumler 2000; WHO 2005).

In order to find the mode of transmission and a reservoir for an outbreak, the epidemiological investigations of *Salmonella* outbreaks have commonly relied on characterisation of the bacterial isolates with bacteriophages (Anderson *et al.* 1977 and 1978), phenotypic criteria obtained with biochemical tests (hydrogen sulphide, lysine decarboxylase, citrate, mannitol, inositol, sorbitol, trehalose, and other reactions), and serological methods (Quinn *et al.* 1994). The molecular techniques Pulsed Field Gel Electrophoresis (PFGE) and Polymerase Chain Reaction (PCR) are also utilized for *Salmonella* epidemiological studies. These two techniques explore genomic differences between bacterial isolates and can provide additional information for an epidemiological study and a possible relatedness between isolates (Foley *et al.* 2006).

1.4.1. Bacteriophages (phage) typing

Bacteriophages are 2 major groups of viruses that can infect and kill bacteria. While the lytic phages will kill the host bacterium immediately following infection, lysogenic

phages may first integrate their genome within the genome of the host bacterium and kill the host at a later stage (Felix 1956). While most phages are specific for a particular bacterial species, some can only infect certain substrains within the same species (Anderson *et al.* 1977 and 1978). Categorising *Salmonellae* spp. with the help of phages was introduced by Callow and has been employed since approximately 1943 (Anderson *et al.* 1978; Callow 1959) to further discriminate isolates of *S.* Enteritidis and *S.* Typhimurium into different subtypes. This technique relies on a battery of phages to determine whether isolates obtained from different places and times are related to a set of phages. In general, a petri plate of nutrient agar is overlaid with a liquid medium containing the bacterial isolate of interest. Once the culture overlay has dried a set of phage suspensions are inoculated onto the surface of the agar and plates are incubated overnight at 37^{o}C. On following day, phage lysis reactions are recorded and compared to standards (Anderson *et al.* 1978; Callow 1959). Each isolate is assigned a Definitive (Phage), or Untypable Types [DT, (PT) or UT] (Anderson *et al.* 1977). *Salmonella* isolates within each serotype may be differentiated into a number of phage types based on their pattern of susceptibility to the set of phages (Rabsch 2007). Phage typing can show possible linkages between outbreaks and help identify and track a common source of an outbreak (Baggesen *et al.* 2010).

1.4.2. Serotyping

Serotyping is a phenotypic technique used for characterisation of *Salmonella* isolates that is based on immunologic reactivity of specific antibodies against various combinations of the surface thermostable O somatic (cell wall) and thermolabile H (flagella) antigens (Grimont and Weill 2007; Luderitz *et al.* 1966). Currently, more than 2579 *Salmonella* serotypes have been identified using the Kauffmann-White scheme that is updated every 5 years (Grimont and Weill 2007; Kauffman 1964). The antigenic formulae of serotypes are

defined by the WHO Collaborating Centre for Reference and Research on *Salmonella* at the Pasteur Institute, France, and the new serotypes are listed in the annual updates of the Kauffmann-White scheme. Isolates from different subspecies can have the same O and H antigens. The outer portion of the LPS contains 2 groups of O antigens with the first group present in all members of *Enterobacteriacae* family and the second group consisting of less important ancillary antigens that can vary within a given serotype. Previously designated with letters, the 46 O-serogroups have recently been replaced by numbers. Approximately 97% of the isolates that might cause disease in humans fall within the following serogroups: A (1), B (4), C1 (7), C2 (8), D1 (9), E1 (3, 10), and G (13). The H antigen is presented as phase 1 and 2 and while phase 1 is similar within different enteric bacteria, phase 2 is specific for *Salmonella*. There are 119 H antigens that are designated with numbers and the letter "z" (Grimont and Weill 2007; Luk and Lindberg 1991).

Serotyping is an essential tool that can be used for investigating of *Salmonella* outbreaks (Clark *et al.* 2009; Threlfall and Frost 1990). The commercially available and commonly used slide agglutination test is based on specific O and H anti-*Salmonella* sera. The antiserum is either a mixture of mono- or poly-clonal antibodies produced *in vitro* or *in vivo* in different animal species including the mouse and rabbit. In principle, the serum and the isolate of interest are mixed on a glass slide and a visible agglutination representing an antibody-antigen complex will be formed if matching antigens are present (Quinn *et al.* 1994).

Recently, a study conducted with the most prevalent strains of *S.* enterica in Malaysia has demonstrated that multiplex PCR based on genes coding for O and H antigens could be used successfully for PCR-based serogrouping and this approach has the potential to one day replace the use of sera when serogroups have to be determined (Lim and Thong 2009).

1.4.3. Virulotyping

Most bacterial pathogens including pathogenic *Salmonella* spp. have multiple virulence properties that enable them to invade, survive within the host and ultimately cause a disease (Bowe *et al.* 1998; Groisman and Ochman 1997; Marcus *et al.* 2000). A study investigating fatal infections caused by *S.* Typhimurium using mice as an animal model found that potentially about 4% of the bacterial genome or more than 200 virulence genes contribute to virulence (Bowe *et al.* 1998). Similar to other intracellular bacteria, *Salmonella* has evolved to survive within the phagosome of the macrophages that entrap and digest the bacterial pathogens (Sano *et al.* 2007; Sansonetti 2001). Virulence genes are often located within the *Salmonella* Pathogenic Islands (SPI), which are large, unstable chromosomal segments absent in related non-pathogenic organisms (Blum *et al.* 1994; Groisman and Ochman 1996). The SPIs contribute to invasion and survival of the microorganism within the host intestinal epithelium and macrophages. These genomic islands are categorised into five groups designated SPI-1 through SPI-5. Most of the virulence genes cluster within SPI-1 and SPI-2 which are approximately 40 000 base pairs (bp) long each (Marcus *et al.* 2000). The virulence genes within the SPIs may be acquired from other organisms through horizontal transfer (Marcus *et al.* 2000). The two *Salmonella* species *S. enterica* and *S. bongori* have specific invasion genes located within SPI-1 but, while *S. enterica* has SPI-2 that contributes to pathogenicity, (Groisman and Ochman 1997; Hensel 2000) *S. bongori* does not have most of the genes present in SPI-1 (Ochman and Groisman 1996). Furthermore, while the genomic sequences of SPI-2 are found in the genus *Salmonella* including the serotype *S.* Typhimurium (Hensel 2000) SPI-1 is present in *S.* Typhimurium and also in other bacterial pathogens (Groisman *et al.* 1993).

SPI-1 is located at the 63 centisome location on the *Salmonella* chromosome and contains at least 29 genes (Groisman and Ochman 1996; Marcus *et al.* 2000) and the

specialised Type III Secretion System (TTSS) that is essential for the gut-associated stages of the bacterial infection (Mirold *et al.* 1999). Experiments performed with mice co-infected with wild-type as well as a non-invasive *S.* Typhimurium *invA* mutant have shown that TTSS within the SPI-1 is vital for invasion, colonisation and replication of *Salmonella* within the intestinal epithelium (Marcus *et al.* 2000). One of the functions of TTSS is to deliver the so called "translocated effector proteins" to the host cells. Although the TTSS is well conserved among Gram (-) bacteria, the effector proteins are diverse and vary between related strains (Galan and Bliska 1996). The *invA, prgH, sopB, sitC* and *sopE* genes are located within the SPI-1 (Marcus *et al.* 2000; Prager *et al.* 2000; Zhou *et al.* 1999). The *prgH* gene is part of the *prg* locus located at 59 min on the *Salmonella* chromosome promotes virulence and survival within the macrophages (Behlau and Miller 1993). The *sopE* gene encodes for an effector protein that enables *Salmonella* to invade the host cell by rearranging the cell's actin cytoskeletal and membranes. Only a few strains of *S.* Typhimurium have been identified to have this gene and all of the strains positive for *sopE* are either precursors of epidemic strains or identified as part of epidemics. The *sopE* gene was most frequently identified in serotypes *S.* Enteritidis, *S.* Hadar, *S.* Newport and *S.* Virchow in England and Wales during 2001 (Hopkins and Threfall 2004). The *inv/spa* gene cluster encodes for proteins with complementary function that facilitate translocation of *Salmonella* through the intestinal epithelial cells. The *inv/spa* genes have also been identified in *Shigella* and other invasive bacterial pathogens (Ginocchio and Galán 1995). Interestingly, while it was shown that *invA* and *orgA* genes are required for the *S.* Typhimurium to enter the intestinal mucosa and to cause disease, strains with these mutated genes were still able to produce a similar form of disease when bacteria were administered intraperitoneally in a murine model (Galan *et al.* 1992; Jones and Falkow 1994). However, the movement of the bacteria can only occur if *sipB* is also present suggesting that the TTSS within SPI-1 assists with delivery of effector

proteins to the host cells (Marcus *et al.* 2000) that allows *Salmonella* to penetrate into intestinal epithelium (Galan and Curtiss, 1989; Jones and Falkow 1994).

SPI-2 is located at 31 centisome of *Salmonella* chromosome and comprises more than 40 genes. This island and the PhoP-PhoQ regulatory system are 2 multifunctional virulence sytems that control intracellular survival and replication of *S.* Typhimurium. The TTSS of SPI-2 is structurally and functionally different from the one within SPI-1. In general, TTSS's form cylindrical like structures that export proteins across the membranes of the bacterium. Some of these proteins are needed for formation of pores in the host cell membrane that facilitates injection of bacterial effector proteins into the host cell cytosol (Holden 2001). These effector proteins modify the physiology of the host cell enabling *S. enterica* to survive and replicate within the cell (Hansen-Wester *et al.* 2004). The *sifA* gene is present only in *Salmonella* spp. and encodes for a protein that can induce the formation of lysosomal membrane glycoprotein with filamentous structures. These filamentous structures are used by *Salmonella* spp. to bind and transfer the protein to the vacuoles where the intracellular bacteria can survive (Stein *et al.* 1996). The *sifA* protein helps bacteria survive and colonise murine macrophages (Brumell *et al.* 2001). When macrophages were infected with *S.* Typhimurium, the *spiC* protein that is secreted into the cytosol of the macrophages contributes to the inhibition of the endosomal trafficking by disrupting the endosome fusion which helps bacteria to survive within the phagocyte (Shotland *et al.* 2003; Uchiya *et al.* 1999). In comparison to the wild type, *spi* mutant *Salmonella* spp. do not have a modified form of flagellin in the supernatant which suggests that the *spi* secretory system encodes for a protein that can change the activity of secreted proteases (Ochman *et al.* 1996). The *sitC* gene of *S.* Typhimurium encodes for proteins enhancing the bacterial growth in an iron-restricted environment. The expression of *sit* operon can increase 18-fold in environment low in iron, thus supporting the uptake of iron by bacteria (Zhou *et al.* 1999). Iron is an important factor

for infectivity of *S.* Typhimurium and as part of non-specific defence mechanisms; the host can reduce the availability of this nutrient for the bacteria (Janakiraman and Slauch 2000).

The SPI-3 is 17 000 bp long and although the function of the genes encoded within SPI-3 of *S.* Typhimurium have been investigated (Blanc-Potard 1999; Marcus *et al.* 2000) the contribution of this island to virulence is not entirely clear. The *misL* gene encodes for a surface protein with binding specificity for fibronectin thus promoting bacterial adhesion and invasion into the intestinal epithelial cells (Dorsey *et al.* 2005).

Although little is known about SPI-4 this island is approximately 25 000 bps long, located at the 92 centisome of *Salmonella* chromosome and similarly to SPI-3 has a mosaic structure indicating that both islands have evolved in a multistep process with distinct transfer events (Marcus *et al.* 2000). Some of the genes within SPI-4 encode for proteins that may induce apoptosis in the host immune cells and help bacteria survive within the macrophages (Schmidt and Hensel 2004). It was recently reported that all 9 strains from human and avian origin of the newly identified serotype Keurmassar of *S. enterica* did not have the *orfL* gene which is part of the SPI-4 (Gassama-Sow 2006).

Using the Southern hybridization technique, it was shown that the SPI-5 is present only in the tested *Salmonella* serotypes *S.* Cholerasuis, *S.* Enteritidis, *S.* Gallinarum, *S.* Pullorum and *S.* Typhimurium but not in enteropathogenic *Escherichia coli* (*E. coli*), *Shigella sonnei, Yersinia pseudotuberculosis* (Wood *et al.* 1998). Therefore, it was suggested that SPI-5 is conserved in *Salmonella* spp. but not in other enteropathogenic bacteria. The *sopB* gene encodes for an outer effector protein that changes the host cytosol leading to inflammation, enhanced chlorine secretion leading to fluid accumulation within the intestines and profuse diarrhoea (Galyov *et al.* 1997; Norris *et al.* 1998). The *pipD* gene of *S.* Typhimurium is located within this island and might contribute to the enteric but not to the systemic form of salmonellosis (Galyov *et al.* 1997; Wood *et al.* 1998).

Within the host, *Salmonella* encounters an environment with a low concentration of iron and bacteria have to compete with the host for this element (Janakiraman and Slauch 2000). The *iroN* gene encodes for an iron uptake protein that gives a competitive advantage to the bacteria. The gene has been identified in *S. enterica* but not in *S. bongori* and *E. coli* (Baumler *et al.* 1998). It was suggested that *iroN* protein is located in the outer bacterial membrane and exposed to the host immune system. Because some of the utilised substrate for the *iroN* receptor was also identified in *S. enterica* isolates obtained from soil samples it was thought that this protein might provide advantages for *S. enterica* to survive in the environment (Baumler *et al.* 1998).

The gene *pefA* is a plasmid encoded and involved in a fimbrial associated virulence, *Salmonella* attachment, colonisation of the intestinal epithelial surfaces, determination of the tissue tropism and possibly influences the inflammatory response (Baumler *et al.* 1997; Friedrich *et al.* 1993). The *pefA* gene is involved also in the activation of PhoA fusion which contributes to the formation of the fimbrial structures (Friedrich *et al.* 1993). When 158 isolates from different *Salmonella* serotypes obtained from healthy and sick avian species were analysed for the presence of 17 potential virulence genes it was found that the *pefA* gene was present less frequently than the other genes and in fewer serovars but in a higher percentage of isolates from sick, rather than healthy birds. In addition, *sopB* gene was present in isolates of all sick and in all but one isolate from healthy birds (Skyberg *et al.* 2006).

1.4.4. Pulsed Field Gel Electrophoresis (PFGE)

Characterisation and determination of possible relatedness between bacterial isolates obtained from outbreaks are two basic elements for a successful epidemiological investigation (Maslow and Mulligan 1996; Trujillo *et al.* 2010). Conventional phenotyping techniques such as serotyping and bacteriophage susceptibility (Farmer 1988) are time consuming with some inherent disadvantages. As different phenotypic traits are often inconsistently expressed some bacterial strains might not be distinguishable, making the data obtained with these techniques inconclusive. Therefore, there is a need for rapid, accurate and repetitive genotyping techniques to assist public health authorities in detecting and tracking a source of a disease. One approach is to analyse large genomic bacterial DNA. For example, *S.* Typhimurium LT2 comprises a 4,857 kilo bp chromosome (McClelland *et al.* 2001). Conventional agarose and polyacrylamide gel electrophoresis could only be used for analyses of fragments up to 20-50 000 bps (Herschleb *et al.* 2007) as the resolution for a larger DNA fragments becomes progressively worse with the size of the fragment.

PFGE genotyping technique allows for analyses of DNA fragments up to 10 mega bps (Anand 1986) and it is based on digestion of DNA with restriction enzymes with rare restriction sites generating between 10 and 20 high molecular weight DNA fragments. In principle, DNA samples from bacteria can be prepared by immobilizing the bacterial cells in plugs formed from a low gelling and melting temperature agarose. The DNA fragments are separated by gel matrixes that are subjected to a programmed variation in both the direction and duration of electrical field. Although maximum separation between the DNA fragments obtained by restriction digestion can be achieved by adjusting the variable pulse-time based on the size range of the DNA fragments, this may lead to compromising the resolution between the fragments (Maule 1998). The PFGE electrophoretic banding patterns (profiles) produced are highly reproducible and specific for a variety of bacterial organisms (Gautom

1997). However, the sensitivity of PFGE depends on the choice of restriction enzyme. The nucleotides (GA), for example, are part of the 3' restriction site (TCTAGA) for *Xba*I enzyme and digestion might be affected by DNA methylation (Luo *et al.* 2003). The (GA) are also the first 2 nucleotides within the methylation site (GATC) where DNA Adenine Methylase (DAM) could methylate (A) nucleotide. The likelihood for such methylation to occur at the *Xba*I site is 1/16 which means that 1 of every 16 sites will not be cleaved (Luo *et al.* 2003). The considerable number of *Xba*I restriction patterns that have been identified within *Salmonella* spp. over the years can be used for comparative analysis. In addition, methylation of the *Xba*I sites is usually complete with a lower probability for introducing variability into the restriction patterns which makes *Xba*I suitable to use for surveillance of *Salmonella* outbreaks (Luo *et al.* 2003). Due to the expected high genetic similarity among *Salmonella* isolates and, to increase the overall discrimination power of PFGE, a second restriction digestion of the bacterial DNA can be performed with *Spe*I enzyme with (ACTAGT) restriction site (CDC 2009). The PFGE is a robust method that could be used for short- as well as for long-term epidemiological surveillance as it was demonstrated with 142 isolates of *S.* Typhimurium obtained from multiple sources within a 30 year period (Refsum *et al.* 2002).

Although, it is generally accepted that PFGE is one of the most discriminatory subtyping method for *S.* Typhimurium (Foley *et al.* 2006) different laboratories could use slightly modified protocols for preparation of plugs, restriction digestions and electrophoretic separation of DNA fragments. Therefore, inter-laboratory comparisons could be challenging and standardised guidelines published by PulseNet should be followed when PFGE profile comparisons are needed for epidemiological surveillance related to *Salmonella* (CDC 2009; Ribot *et al.* 2001 and 2006). In addition, the laboratory performing the PFGE technique can seek PulseNet certification from CDC and assistance from the International Molecular

Subtyping Network for Foodborne Disease Surveillance that has participants from Africa, Asia Pacific, Caribbean, China, Europe, Latin America, Middle East and USA (Pulsenet international, CDC, USA). This system is considered vital for comparing the data obtained from different locations over long period of time.

1.5. Antibiotic susceptibility

Antibiotic sensitivity test is an important step following the isolation and identification of *Salmonella*. Salmonellosis can be a self-limiting disease and treatment with antibiotics might prolong the shedding of bacteria and recovery of the patient (Weir *et al.* 2008). Over the years, some *Salmonella* spp. have developed resistance towards a variety of antimicrobials commonly used as a first line of defence for treatment against infections in humans as well as in animals including tetracycline (Weir *et al.* 2008) and fluoroquinolones (Hohmann 2001; Rowe *et al*. 1997).

Antimicrobials are naturally occurring, semi-synthetic or synthetic compounds that can be administered orally, parenterally or topically. They are used for treatment and as prophylactic agents for humans and animals and as a growth promoters in food producing animals (Phillips *et al.* 2004). Indiscriminate use of antibiotics has created an opportunity for a number of pathogenic as well as commensal bacterial to become resistant to certain antimicrobials (Davis *et al.* 1999; WHO 2002 and 2005). Fortunately, development of antimicrobial resistance is not uniform among different bacteria species as some, for example, may become resistant in a very short period of time against fluoroquinolones and tetracycline (Hooper 2001; Giguere *et al.* 2006; Li and Wang 2010) while others such as *Streptococcus uberis* may remain susceptible to β-lactams for a very long time (Haenni *et al.* 2010). In general, diminished antimicrobial potency within the clinically susceptible range may also lead to development of clinical resistance (Phillips *et al.* 2004). Discovery of the

antimicrobial agents in combination with improvement of sanitation, housing, nutrition and immunisation programmes have led to a dramatic decrease in morbidities and mortalities caused by some of the infectious diseases (Hemminki and Paakulainen 1976). However, these recent achievements have been seriously jeopardised by the emergence and spread of antibiotic resistant bacteria such as multidrug resistant *S.* Typhimurium DT104, penicillin-resistant *Streptococcus*, vancomycin resistant enterococci, Methicillin Resistant *Staphylococcus aureus* (MRSA), multi-resistant *Mycobacterium tuberculosi*s (Graziani *et al.* 2008; Poppe *et al.* 1998; Rattan *et al.* 1998; Rowe *et al.* 1997; Taj *et al.* 2010). When infections become resistant to first line antimicrobials such as tetracycline (Weir *et al.* 2008) and ciprofloxacin (Rowe *et al.* 1997) treatment has to be continue with the more expensive and potentially more toxic second (ceftriazone) (Bhutta 1996) and third line (aztreonam and imipenem) antimicrobials (Basnyat *et al.* 2005).

Ampicillin and amoxicillin-clavulanic acid are examples for broad spectrum, beta (β) lactam bactericidal antibiotics [Clinical and Laboratory Standards Institute (CLSI), 2008]. This antibiotic class was discovered in 1928 by Alexander Fleming (Ligon 2004) and has main activity against non-β-lactamase producing Gram positive (+) and some Gram (-) bacteria. Clavulanic acid, sulbactam and tazobactam are β-lactamase inhibitors that can be given simultaneously with the members of this antibiotic class (Bush 1988). Some of the bacterial isolates should be tested against drug combination with β-lactamase inhibitors as the susceptibility to penicillin alone might not be a reliable indicator (CLSI 2008). Amoxicillin, for instance, is often used in combinations with clavulanic acid to treat infections caused by penicillinase-producing bacterial strains such as *Haemophilus influenza*. Based on their antimicrobial properties cephalosporins have been classified into four generations with each new generation covering a greater spectrum of Gram (-) bacteria than the preceding generation. The 4^{th} generation has a truly broad activity spectrum (Murray *et al.* 2003). While

there is a good correlation between the fourth generation cephalosporin cephalothin and the members of the same group, the correlation between representatives from the other 3 generations is not as clear and results should be interpreted with care (CLSI 2008). It is recommended to include representatives from each generation of cephalosporins, such as cefoxitin, as representatives for the second and cefpodoxime for the third generation (CLSI 2008; Murray *et al.* 2003) when routine antimicrobial susceptibility tests are performed.

Amikacin is an aminoglycoside bactericidal class antibiotic and members of this class are often used in the human medicine for treatment of aerobic, Gram (-) bacteria such as *Psedomonas aeruginosa, Leptospira* spp. and Gram (+) *Staphylococci* spp. (Giguere *et al.* 2006). The antimicrobial mode of action inhibits protein synthesis by binding to the bacterial 30S ribosomal subunit causing misreading of the mRNA (Carter 2000) leading to eventual death of the bacterial cell. The reported adverse side effects are nephro- and vestibular toxicity and neuromuscular paralysis (Murray *et al.* 2003). Amikacin, in comparison to other aminoglycosides drugs such as kanamycin, gentamicin and neomycin, has broader antimicrobial activity and it is also more resistant towards bacterial enzymatic inactivation. This antibiotic is less toxic to the nephrons of the kidney (Giguere *et al.* 2006). Resistance toward members of the aminoglycosides has been reported for *S.* Typhiumurium PT204c and DT104 in UK and a few other areas of the world (Graziani *et al.* 2008; Poppe *et al.* 1998; Threlfall *et al.* 1985).

Ciprofloxacin and nalidixic acid belong to the broad spectrum fluoroquinolones and are well absorbed when administered orally (Giguere *et al.* 2006). Fluoroquinolone's mechanism of action is interference with DNA replication (Murray *et al.* 2003). Based on its antibacterial spectrum this class is divided into four generations with an increased antibacterial spectrum for each generation. In comparison to the other antimicrobial classes, the uses of fluoroquinolones have been shown to induce a higher incidence of MRSA and

resistant *Clostridium difficile* (Kuijper *et al.* 2006). Nalidixic acid is a first generation fluoroquinolone which is still used predominantly for treatment of urinary tract infections. The broad-spectrum ciprofloxacin is a second generation fluoroquinolone with activities against Gram (+) and (-) bacteria, and while it is approved for use in the human medicine, it can also be used in small animal medicine as an extra-label use (Giguere *et al.* 2006). *Salmonella* resistance against ciprofloxacin has been reported in isolates obtained from human patients (Piddock *et al.* 1993).

Chloramphenicol is a bacteriostatic that was originally derived from *Streptomyces venezuelae* and has broad spectrum activity including many Gram (+) and (-) bacteria. The mode of action for this antibiotic is by binding reversibly to the peptidyltransferase of the 50S ribosomal subunit preventing elongation of the peptide chain. Bone marrow toxicity is a major adverse effect. The use of chloramphenicol as an antibiotic growth promoter was banned in the US and EU partly because of its overuse in animal production and the likely contribution to increased resistance in *Salmonella* Typhi (Hughes and Heritage 2004). Several reports have documented *S.* Typhimurium DT104 resistance type ACSSuT isolated from humans and animals (Lawson *et al.* 2004; Graziani *et al.* 2008; Helms *et al.* 2002).

Tetracycline is a broad spectrum bacteriostatic class discovered in the 1940's (Sader *et al.* 2007). This antibiotic binds reversibly to the ribosomal 30S subunit and disrupts protein synthesis by preventing aminoacyl-tRNA binding to the messenger RNA (Brodersen *et al.* 2000; Carter 2000). Resistance towards the tetracycline group has already been reported in Taiwan for both human and animal isolates (Tsen *et al.* 2002). Although there are several reports for *S.* Typhimurium DT104 obtained from humans and animals that are resistant to multiple drugs including tetracycline (Graziani *et al.* 2008; Poppe *et al.* 1998; Rowe *et al.* 1997), no reports were found for DT160 isolates resistant to tetracycline.

Sulfonamides were introduced around 1930 and they prevent synthesis of bacterial DNA by inhibition of folate metabolism. Sulfonamides competitively inhibit bacterial modification of *p*-aminobenzoic acid (essential factor for folic acid synthesis) into dihydrofolate. Trimethoprim is a pyrimidine that inhibits the enzyme dihydrofolate reductase and also interferes with folic acid metabolism. Potentiated sulfonamides such as trimethoprim-sulfadiazine have a synergistic effect as they block the synthesis of bacterial folic acid at different sites and are active against a broader range of microorganisms (CLSI 2008; (Murray *et al.* 2003). There are reports for *S.* Typhimurium DT104 as well as other serotypes with different multidrug resistant profiles that include sulfonamides (Lawson *et al.* 2004; Graziani *et al.* 2008; Guerra *et al.* 2004).

1.6. *Salmonella* surveillance

Human patients suspected to have "foodborne disease" might exhibit clinical signs such as diarrhoea, pyrexia, headache, nausea, abdominal pain, vomiting and, in severe cases, malena (Broide *et al.* 2005; CDC 2010). Correct diagnosis and identification of the causative microorganism are important for proper treatment but the above clinical signs are rarely helpful. As the presence of infectious diarrhoea caused by enteric pathogens such as *Campylobacter*, *Clostridium difficile* and *Salmonella* is often associated with morbidity and mortality (Guerrant *et al.* 2001), it is essential that optimal diagnostic procedures, appropriate treatment plans and preventive measures are implemented (Woolhouse *et al.* 1997).

The main purpose for the surveillance is to provide information for monitoring of the prevalence and distribution of a disease, detection of outbreaks, and prediction of emerging infectious agents and development of preventative measures. To achieve higher probability for successful control of the foodborne salmonellosis, it is important for an epidemiological investigation to include the entire food chain from the farm to the final consumer. The global

increase of *Salmonella* prevalence is also driven partly by changes in human demographics (Altekruse *et al.* 1997) and preference toward consumption of semi-cooked and pre-packaged food (Thornley *et al.* 2002).

Salmonellosis is a serious national and international threat, particularly for countries with export-oriented economies with a heavy reliance on products from animal origin. In the last decade as a consequence of the globalization, often the end food product that consists of ingredients from multiple sources can be distributed rapidly to a large number of people that are geographically separated. The emergence and spread of antimicrobial resistant bacteria was recognised in September 2001 by the WHO as a global threat. This organization has launched a Global Strategy for Containment of Antimicrobial Resistance with a goal to involve all countries and to reduce the spread of resistant bacteria (WHO 2002).

Several international and national surveillance systems have also been set up to help monitor for potential outbreaks of *Salmonella*. The WHO Global Salm-Surv is a worldwide network of participants involved in *Salmonella* surveillance, serotyping and antimicrobial resistance. This network was initiated in 2000 by the WHO, Danish Veterinary Laboratory and CDC. Surveillance data for antimicrobial resistance of *Salmonella* are also available at the National Food Institute, Technical University of Denmark (www.antimicrobialresistance.dk).

In the EU, results from different surveillance programs are published in *Eurosurveillance*, a European scientific journal focused on issues related to epidemiology, surveillance, prevention and control of communicable diseases.

The National *Salmonella* Surveillance System in the USA, for example, collects reports for human *Salmonella* isolates from within the USA and this information is reported through the Public Health Laboratory Information System (CDC 2006). The Foodborne Disease Active Surveillance Network (FoodNet) is another major component of the

Emerging Infectious Program of the CDC in the USA. According to FoodNet in 2007, most foodborne infections were due to *Salmonella* (41%) followed by *Campylobacter* (35%). The greatest number of fatalities was due to *Salmonella* and 5% of the mortalities were due to outbreaks the majority of which were food related (CDC 2007).

In New Zealand one of the earliest measures taken for control of *Salmonella* was the establishment in 1963 of the *Salmonella* Investigation Unit, Department of Agriculture. The Unit was able to show that very young calves are especially susceptible to clinical and subclinical infection with *Salmonella*, especially after transportation and temporary housing in lairages (Clark *et al.* 2002). Currently the public health surveillance for infectious diseases is performed by the ESR and results are published on annual basis in the form of Public Health Surveillance Reports (www.surv.esr.cri.nz/surveillance/NZPHSR.php). The Enteric Reference Laboratory has an essential role in this process and publishes the results monthly for human and quarterly for non-human *Salmonella* sero- and phage-typing. Finally, the ERL is also a reference and surveillance laboratory for other members of the *Enterobacteriaceae* family and *Campylobacter* spp. from human, animal and environmental sources.

Chapter 2: Colony morphology, Serology and Antibiotic Sensitivity Testing

2.1. Introduction

Even though patients infected with *Salmonella* can recover without treatment with antibiotics, in certain situations when antimicrobials are used they might prolong the shedding of bacteria and recovery of the patient (Weir *et al.* 2008). Some *Salmonella* spp. have developed resistance towards a variety of first line antimicrobials, such as tetracycline and fluoroquinolones, that are used for treatment of infections in humans as well as in animals (Hohmann 2001; Rowe *et al*. 1997; Weir *et al.* 2008). Antibiotic sensitivity testing is an *in vitro* phenotyping method performed following the isolation and identification of *Salmonella* and the results from these tests can be used as guidelines for treatment of patients as well as epidemiological surveillance.

The naturally occurring, semi-synthetic or synthetic antibiotics can be administered orally, parentally or topically. They are usually prescribed by medical practitioners for treatment of patients but can also be used as a prophylactic agents for humans and animals and as a growth promoters in food producing animals (Phillips *et al.* 2004). However, their indiscriminate use has occasionally led to the emergence of pathogenic and commensal bacteria resistant to certain antimicrobials (Davis *et al.* 1999; WHO 2002 and 2005). Fortunately, the mechanism of antimicrobial resistance is not an universal phenomenon that is used by all bacterial species. For instance, *Staphylococcus aureus* may become resistant in a short period of time against fluoroquinolones and tetracycline (Giguere *et al.* 2006; Hooper 2001; Li and Wang 2010) but *Streptococcus uberis* may remain susceptible to β-lactams for a very long time (Haenni *et al.* 2010).

2.2. Materials and methods

Bacterial strains

Ninety randomly selected isolates of *S.* Typhimurium DT160 were obtained on Dorset egg slopes from ESR, NZ. The sources of these isolates were avian species, poultry-related environmental samples and human clinical isolates that had been obtained between the years 1999 and 2009. Thirty isolates were of human origin and 60 were isolated either from avian species or from environments related to poultry. Freeze-dried *S.* Menston [National Collection of Type Cultures (NCTC7836)] and *Escherichia coli* (*E. coli*) [America Type Cell Cultures (ATCC 25922)] were purchased from the New Zealand Reference Culture Collection, ESR. *S.* Typhimurium F98 phage type 14 (NCTC12190) and *S.* Typhimurium SL1344 with unknown phage type (NCTC 13347) cultures were purchased in freeze-dried form from the Health Protection Agency, United Kingdom. Prior to further testing, all *Salmonella* strains were sub-cultured on Columbia Horse Blood agar (Fort Richard Laboratories Ltd., Auckland, NZ) and nutrient agar (Merck, Germany), incubated at 37^{o}C for 24 hours and used for the downstream procedures.

Colony morphology

All *Salmonella* isolates were streaked onto nutrient agar and onto blood agar plates. Their size and morphological characteristics were observed and recorded.

Serotyping

All 90 *S.* Typhimurium DT160 isolates and the *S.* Menston positive control were serotyped by a slide agglutination serological test using a polyclonal mixture of monoclonal mouse antibodies (Enteroclon Anti-*Salmonella* A-67, omnivalent, specific for O and Vi antigens and

Enteroclon Anti-*Salmonella* poly-H phase 1 & 2 sera (SIFIN, Berlin, Germany) as recommended by the manufacturer. Briefly, using a wire loop, a small amount of bacteria was picked from a well-isolated colony, transferred onto a glass slide and mixed with a drop of either poly-H or poly-O antiserum. The homogenous, slightly milky suspension was tilted back and forth for less than 20 times on the glass slide and the reaction read on a dark surface by the naked eye within 1 minute (min) from the time of mixing. A drop of sterile physiological saline (0.85% NaCl) instead of the serum was used as a negative control and the test performed simultaneously with the test sample.

The *Salmonella* isolates that were negative for either poly-H or poly-O anti-*Salmonella* serum from SIFIN were retested using *Salmonella* H antiserum polyclonal rabbit a-z and *Salmonella* O antiserum polyA-I and Vi from BD (Becton Dickinson, NZ) as recommended by the manufacturer. The biochemical profiles of the same isolates were also tested with the API20E bacterial identification test kit (Biomerieux, I'Etoile, France), Triple Sugar Iron (TSI) agar and motility confirmed using the motility test. A well-isolated colony was used for inoculation of the TSI agar taking care to ensure that the bottom portion of the agar had also been inoculated. The cap of the tube was placed on loosely, and the tubes were incubated for 18 to 24 hours (hrs) at 37^{o}C. The motility test was conducted by observing the growth of the bacteria in a semi-solid Bacto motility test medium [Becton Dickinson, United States of America (USA)]. The centre of the medium was inoculated with well-isolated colony and the medium incubated for 24 hrs at 37^{o}C. The API20E test consisting of 20 separate biochemical reactions and an additional oxidase test were used as per manufacturer's instructions. Briefly, inocula from the isolates were prepared by mixing a single bacterial colony with 5 millilitres (ml) of sterile distilled water until homogenous suspensions were formed. The biochemical reactions were inoculated with 200 µl suspensions, incubated at for 18-24 hrs at 37ºC and the results read based on colour changes for each reaction. The tests were separated into 3 groups

and on each positive reaction a specific numerical value was assigned "1" for a positive reaction in the first test of the group, "2" for a positive reaction in the second test of the group and "4" for a positive reaction in the third test of the group. A 7 digit number (e.g. 4305542) was obtained after the values corresponding to positive reactions in each group were added and the analytical profile index was analysed using APILab Plus software (Biomerieux, I'Etoile, France).

Antimicrobial susceptibility testing

All 90 *Salmonella* isolates and the control *E. coli* ATCC25922 were tested using the Kirby Bauer Disc Diffusion method following the "M31-A3 Performance Standards For Antimicrobial Disk And Dilution Susceptibility Tests For Bacteria Isolated From Animals; Approved Standards" (Clinical and Laboratory Standards Institute (CLSI), 2008) guidelines. Isolates were tested for susceptibility to the following antibiotics with the respective disc concentrations: 10 µg ampicillin (AMP), 30 µg amikacin (AK), 30 µg amoxicillin-clavulanic acid (AMC), 30 µg chloramphenicol (C), 30 µg cefoxitin (FOX), 10 µg cefpodoxime (CPD), 5 µg ciprofloxacin (CIP), 30 µg nalidixic acid (NA), 30 µg oxytetracycline, 25 µg trimethoprim-sulfamethoxazole (SXT), and 30 µg tetracycline (TET) antibiotic discs (Oxoid, NZ Ltd.). Briefly, a sterile, non-toxic swab was used to collect between 4 to 5 well-separated colonies from an overnight culture streaked on a nutrient agar plate. The bacteria were suspended into 4 to 5 ml of sterile, normal saline and the turbidity of the suspension was adjusted with sterile saline to obtain a suspension visually similar to that of a 0.5 McFarland standard. The turbidity was read against a standard card with black lines on a white background. A new swab was dipped into the suspension and, following removal of excess inoculum by pressing the swab gently against the wall of the tube, bacteria were spread evenly

on the Mueller Hinton agar plate (Fort Richard Laboratory Ltd., Auckland, NZ). No more than 6 drug-impregnated 6 mm discs were applied to the agar surface. An automatic dispenser was employed to ensure discs were no closer than 24 mm from centre to centre. Each disc was pressed gently with sterile forceps to ensure complete contact with the agar and the inoculated plates were incubated for 16 to 18 hrs at 37^{o}C. The zones of inhibition were measured using a digital calliper and end points determined based on the areas showing no bacterial growth visible to the naked eye. The interpretation of the inhibition zones for AK, AMC, AMP, C, CPD, SXT and TET and *E. coli* ATCC25922 was based on M31-A3, CLSI 2008 and for NA and CIP on those described in M100-S20, CLSI 2010 (Appendix 4 and 5).

2.3. Statistical analysis

To assess concordance, an inter-rater agreement Kappa quadratic weighted analysis was performed for repeat antimicrobial susceptibility using MedCalc Software bvba version 11.4.20 software (www.medcalc.be/manual/kappa.php, Belgium). Twenty five percent (25%) of the total isolates were examined. Kappa analysis evaluates the agreement between two classifications on ordinal or nominal scales (Fleiss *et al.* 2003).

2.4. Results

2.4.1. Colony morphology and Serology

The colonies of all *Salmonella* isolates were approximately 2-3 mm in size and appeared moist and raised on nutrient agar and grey, round and non-haemolytic on blood agar regardless of their species of origin.

At the time of initial isolation, all isolates were serotyped by ESR using the Kauffmann-White Scheme. Serological confirmation was undertaken in this laboratory using poly-O and poly-H sera. Eighty six isolates showed agglutination to poly-O and poly-H sera from SIFIN. Three of the isolates when tested with sera from SIFIN, were negative for agglutination when tested with poly-H but positive for poly-O and one isolate was negative when tested with poly-O but positive with poly-H (Table 1). These four isolates agglutinated with poly-H and poly-O sera from BD and were also confirmed to be *Salmonella* spp. with the API20E biochemical, TSI and motility test (Appendix 1, 2 and 3). The API20E profile index for the four isolates indicated 76% probability that the isolates are indeed *Salmonella* spp. On TSI agar the four isolates produced red (alkaline) slant, yellow (acid) butt with blackening of the medium as a result of hydrogen sulphide production that is indicative for members of *Enterobacteriaceae* family.

2.4.2. Antimicrobial susceptibility

Due to the growing concerns related to antimicrobial susceptibility of *S.* Typhimurium DT160, all isolates were tested against 11 antimicrobials that are often used for treatment of human patients or animals. Except for tetracycline and oxytetracycline, all isolates were susceptible to the tested antibiotics. Based on the reference standards for tetracycline described in CLSI M31-A3, 26 isolates fell within the intermediate range of susceptibility to tetracycline and eight isolates within the same range for susceptibility to oxytetracycline (Appendix 6 and Table 2). The selection of the antimicrobials used in this study was based on the commonly-prescribed antibiotics in human as well as in veterinary medicine. Susceptibility to tetracycline was also evaluated. This antibiotic can be used as a representative for the tetracycline group of antibiotics which includes the oxytetracycline. However, the results for tetracycline and oxytetracycline may not similar.

2.4.3. Statistical analysis

Kappa analysis for the antimicrobial susceptibility test of the 25% of the overall isolates indicated good agreement between the two technicians performing the tests for all antimicrobials excepting for ciprofloxacin for which the borders of the inhibition zones were not clear and therefore not easily measured (Appendix 7).

Table 1: Results for the 4 isolates tested negative for either poly-H or poly-O anti-*Salmonella* sera from SIFIN and further evaluated with sera from BD, API20E, TSI, and mobility tests.

Internal laboratory number	ESR isolate number	Sources	Serotyping				API20E test	TSI agar	Motility
			SIFIN		BD				
			poly-H	poly-O	poly-H	poly-O			
DT22	ER004560	Human	N	P	P	P	4305542=SSP*	R/Y/H_2S+**	NM
DT50	ER104613	Avian White eye	P	N	P	P	4305542=SSP	R/Y/H_2S+	NM
DT86	ERL042332	Sparrow	N	P	P	P	4305542=SSP	R/Y/H_2S+	NM
DT89	ERL07266	Human	N	P	P	P	4305542=SSP	R/Y/H_2S+	NM

*4305542=SSP is an API20E analytical profile index
** R, Alkaline, Y, Acid, H_2S hydrogen sulphide production
ERL, Enteric Reference Laboratory
NM, Non-motile
P, positive; N, negative
TSI, Triple Sugar Iron agar

Table 2: Summary of the 34 isolates identified to be within the intermediate range (15-18 mm diameter**) for sensitivity to either tetracycline or oxytetracycline.

Internal laboratory number	ESR isolate Number	TET	Internal laboratory number	ESR isolate Number	TET	Internal laboratory number	ESR isolate Number	OT
DT27	ERL084557	18*	DT63	ERL072196	17	DT2	ERL052816	18
DT36	ERL0812664	18	DT64	ERL030011	18	DT3	ER003370	16
DT43	ERL02182	18	DT69	ERL04205	18	DT5	ERL091067	18
DT44	ERL050427	18	DT70	ER003038	18	DT6	ERL091081	18
DT47	ERL084152	18	DT72	ER004952	17	DT7	ERL042607	16
DT51	ERL081730	18	DT73	ER011172	18	DT8	ERL063767	17
DT52	ERL083326	18	DT75	ERL03695	16	DT10	ER016216	18
DT54	ERL072372	18	DT76	ERL08372	17	DT14	ERL091904	18
DT57	ERL052818	18	DT77	ERL062557	16			
DT58	ERL061789	18	DT78	ER005001	18			
DT60	ERL033073	18	DT80	ERL041935	18			
DT61	ER005391	18	DT82	ERL033068	17			
DT62	ERL082846	18	DT88	ERL034404	18			

* millimetres -diameter of zone inhibition

**based on CLSI M31-A3

ERL, Enteric Reference Laboratory; ESR, Environmental Science and Research

OT, oxytetracycline; TET, tetracycline

µg, microgram; mm, millimetres

Chapter 3: Virulotyping - screening for potentially virulence genes with Polymerase Chain Reaction (PCR).

3.1. Introduction

While *Salmonella* Typhimurium DT160 has not been reported to infect humans and animals in NZ before the 1998, sporadic cases of mortality in sparrows and other passerines were noticed during winter months following the initial identification of DT160 in NZ (Alley *et al*. 2002). An investigation of contaminated feed and other environmental samples used in poultry has identified the presence of DT160 in the poultry environment. *S.* Typhimurium DT160 was also isolated from the intestinal content, liver and other internal organs of sparrows (Tizzard *et al*. 1979). In England, cases of gastroenteritis in humans were linked to sparrows harbouring the same *Salmonella* type that have contributed to the contaminatation of food used in a human hospital (Penfold *et al*. 1979). This finding has shown that excreta from birds can be one of the mechanisms for spreading DT160 and should be taken seriously when preventive measures are considered.

A study investigating mortality caused by *S.* Typhimurium using mice as an animal model has suggested that more than 200 genes are involved in the infection (Bowe *et al.* 1998). Multiple virulence properties are also needed for the bacteria to be able to invade, survive within the host and eventually cause a disease (Bowe *et al.* 1998; Groisman and Ochman 1997; Marcus *et al.* 2000). In order to survive within the phagocytic macrophages, *Salmonella* similarly to other intracellular bacteria, has evolved different genetic regulatory mechanisms (Sano *et al.* 2007; Sansonetti 2001). Some of the virulence genes of *Salmonella* are positioned within the SPIs. The SPIs are unstable chromosomal segments that may not be present in related non-pathogenic

organisms (Blum *et al.* 1994; Groisman and Ochman 1996). Some of the pathogenic islands give survival advantages to the bacteria present in the intestinal epithelial and macrophage cells. One of the mechanisms, for example, helps *Salmonella* avoid degradation by the lysosomal enzymes produced after the phagocytosis has taken place in the macrophages (Jones and Falkow 1996). Alghotugh, in *Salmonella*, currently five pathogenic islands are known indicated as SPI-1 through SPI-5 most of the virulent genes are clustered within SPI-1 and SPI-2 (Marcus *et al.* 2000).

In our study, we have focused on identification of 12 potentially virulence genes (*iroN, invA, misL, orfL, pefA, pipD, prgH, sifA, sitC, sopB, sopE* and *spiC*) located within different SPIs and a plasmid. The location of the genes is as follows: *sopB, sopE, prgH, sitC* and *invA* are in SPI-1, *spiC* and *sifA* are in SPI-2, *misL* in SPI-3; *orfL* in SPI-4, and *pipD* in SPI-5. Finally, while the *pefA* has been identified in a *Salmonella* plasmid; there was no information for the location of the *iroN* gene.

3.2. Materials and methods

DNA extraction

DNA of *S.* Typhimurium was extracted by boiling bacteria in water as previously described by Hughes *et al.,* 2008, with some modifications. Briefly, genomic DNA was extracted from an overnight culture grown on a nutrient agar plate. Using a disposable wire loop, three to four colonies were picked up and suspended in 1 ml of 2% Chelex (BioRad Laboratories, USA) and mixed by vortexing. The negatively-charged Chelex resin binds divalent ions in the samples and may improve the efficiency of the PCR (Walsh *et al.* 1991). The mixture was heated at 95^{o}C for 10 min using a heating block and the tube was cooled at room temperature and centrifuged at

12,470 *g* for 3 min. Approximately 800 µl of the supernatant containing extracted DNA was aliquoted and stored at -20^{o}C until further used.

PCR

The PCR reaction was synthesised with primers, the sequences of which were previously published (Table 3), in a 25 µl mixture containing 5 µl crude DNA (Appendix 9), 1X PCR reaction buffer, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, and 0.2 µM of each of the primers and, 0.5 U Platinum *Taq* polymerase (Invitrogen NZ Ltd., NZ). The PCR cycle parameters were as follows: 96^{o}C for 2 min followed by 32 cycles at 94^{o}C for 30 sec for denaturation, 55^{o}C for 30 sec (annealing) and at 72^{o}C for 30 sec (extension) applied for the *orfL, pefA, pipD, sopB, spiC,* and *sifA* genes and 1 min (extension) for the *invA, iroN, misL, prgH, sitC, and sopE* genes. A final extension at 72^{o}C for 2 min was used for all genes.

Gel electrophoresis and documentation

Three microlitres of PCR products from *misL, orfL, pipD, pefA, sopB, spiC, and sifA* were electrophoresed on a 1.3% agarose gel (AppliChem GmbH, Germany) and run at a constant 70 V for 105 min. The PCR products for the remaining genes were electrophoresed on a 1.8% agarose gel and run at constant 70 V for 120 min. A 1 kb plus DNA ladder (Invitrogen NZ Ltd., NZ) was used as the molecular marker. The PCR products were visualised with ethidium bromide as per the manufacturer's recommendations and documented using Gel Doc with Quantity One 4.6.2 (Bio-Rad, Segrate, Milan, Italy) software (Table 4 and Appendix 10). Isolates found to be PCR negative were retested one more time.

PCR sequence analysis

To confirm that the PCR products were the targeted sequences, PCR products obtained from the 2 control strains, *S.* Typhimurium SL1344 and F98 were sequenced. The PCR products were purified using an ethanol precipitation technique and products quantified using a NanoDrop Spectrophotometer ND-1000 (Biolab, USA). Separate stocks were prepared for forward and reverse primers at a concentration of 3.2 pmol/15 µl. The templates were prepared to a concentration of 2 ng/100 bp/15 µl then premixed with the forward primer in a total reaction volume of 15 µl by adding sterile distilled water. The same method was applied for the reverse primer mixture. Sequencing was performed by capillary separation on an ABI3730 DNA Analyzer at the Allan Wilson Centre Genome Services, Massey University, Palmerston North, NZ. The nucleotide sequences from all targeted genes were aligned with the previously published sequences of *S.* Typhimurium LT2 and SL1344 (Wellcome Trust Sanger Institute, United Kingdom (UK) using the BLAST nucleotide algorithm at the National Center for Biotechnology Information, United States National Library of Medicine (Appendix 8).

Table 3: Potentially virulent genes with the expected PCR product sizes used to virulotype *Salmonella* isolates. The nucleotide length of the predicted PCR products is based on *S.* Typhimurium SL1344.

Gene	Salmonella Pathogenicity Island (SPI)	Gene function	Primers (5' to 3')	PCR product	Reference
iroN	na	Siderophore Iron acquisition	F: CGCTTTACCGCCGTTCTGCCACTGC* R: ACTGGCACGGCTCGCTGTCGCTCTAT**	1205	Skyberg *et al.* 2006
invA	SPI-1	Type III secretion system	F: AGTTTCTCCCCCTCTTCATGCGTTACCC R: CTGGCGGTGGGTTTTGTTGTCTTCTCTATT	1070	Skyberg *et al.* 2006
sitC	SPI-1	Iron transport	F: CAGTATATGCTCAACGCGATGTGGGTCTCC R: CGGGGCGAAAATAAAGGCTGTGATGAAC	768	Skyberg *et al.* 2006
prgH	SPI-1	Type III secretion system	F: TGAAATGAGCGCCCCTTGAGCCAGTC R: GCCCGAGCAGCCTGAGAAGTTAGAAA	756	Skyberg *et al.* 2006
sopE	SPI-1	Type III secreted protein	F: TCCAAAAACAGGAAACCACAC R:TCAGTTGGAATTGCTGTGGA	652	Hopkins and Threfall 2004
misL	SPI-3	Survival in macrophages	F:GTCGGCGAATGCCGCGAATA R:GCGCTGTTAACGCTAATAGT	561	Gassama-Sow *et al.* 2006
sifA	SPI-2	Type III secretion system	F: GTTGCCTTTTCTTGCGCTTTCCACCCATCT R: TTTGCCGAACGCGCCCCCACACG	449	Skyberg *et al.* 2006
pipD	SPI-5	Type III secreted effector protein	F: CGTTATCATTCGGATCGTAA R: CGGCGATTCATGACTTTGAT	399	Gassama-Sow *et al.* 2006
orfL	SPI-4	Adhesin/Autotransporter	F:GGAGTATCGATAAAGATGTT R:GCGCGTAACGTCAGAATCAA	332	Gassama-Sow *et al.* 2006
spiC	SPI-2	Type III secretion system	F:CCTGGATAATGACTATTGAT R:AGTTTATGGTGATTGCGTAT	301	Gassama-Sow *et al.* 2006
sopB	SPI-1	Type III secreted effector protein	F: TAGTGATGCCCGTTATGCGTGAGTGTATT R: CGGACCGGCCAGCAACAAAACAAGAAGAAG	220	Skyberg *et al.* 2006
pefA	plasmid	Fimbriae	F:GCGCCGCTCAGCCGAACCAG R:CAGCAGAAGCCCAGGAAACAGTG	156	Skyberg *et al.* 2006

*F, Forward; **R, Reverse; na, not available

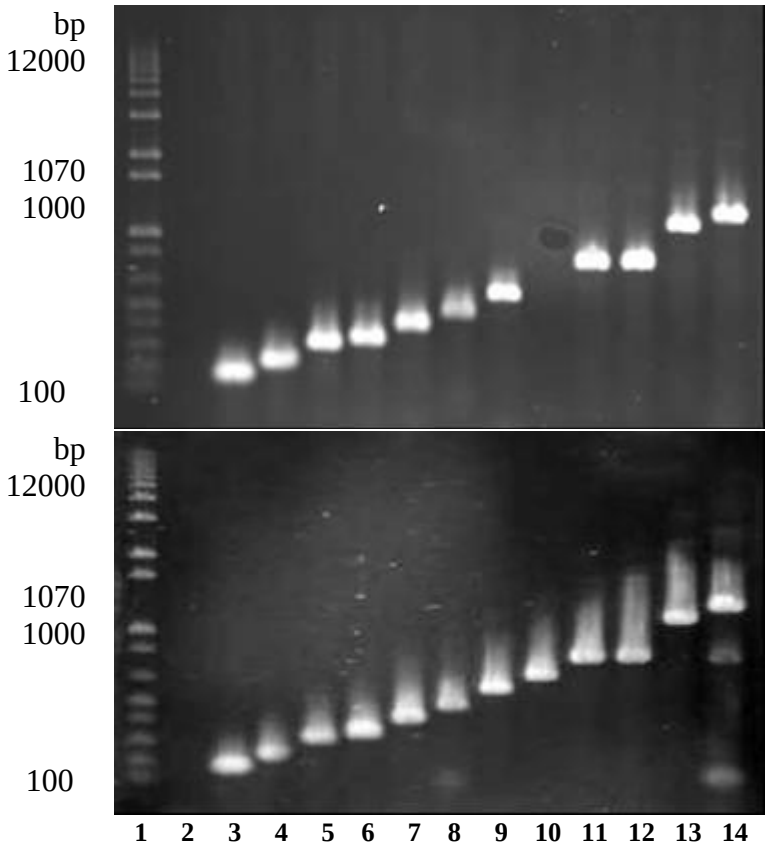
3.3. Results

Optimization of the conditions for the PCR

Initially the PCR reactions were performed on a wide range of temperatures and as 55^{o}C consistently produced single and well distinguishable bands for the respected genes this temperature was chosen as an optimum annealing temperature.

Virulotyping

S. Typhimurium SL1344 was chosen as one of the two control strains as this strain is well characterised and the genome is completely sequenced and available for analysis. As expected, SL1344 was positive for the *sopE* gene whilst F98 was PCR negative. The analysis of the nucleotide sequence of the 12 PCR products confirmed that all sequences had not only the expected length of the product but also shared a high degree of similarity with the respective published gene sequences of *S.* Typhimurium LT2 and SL1344 strains. The reference strain SL1344 was the only strain in which *sopE* was identified and most of the tested isolates carried 11 of the 12 potential virulence genes. The genes *misL, orfL, pipD, prgH, sitC, spiC* and *sopB* were identified in every isolate. In addition to the *sopE* gene, six isolates were PCR negative for one of the following genes; *invA, iroN, pefA* and *sifA* (Figure 1) and of these isolates, four were sparrow and two human origin. To verify the PCR patterns these six isolates were retested.



Salmonella enterica Typhimurium F98 (top gel)
Salmonella enterica Typhimurium SL1344 (bottom gel)

Lane	Gene	Size (bp)	Results (-/+)		Lane	Gene	Size (bp)	Results (-/+)	
			F98	SL1344				F98	SL1344
1	DNA marker				**8**	*sifA*	449	+	+
2	PCR mix (-) DNA		-	-	**9**	*misL*	561	+	+
3	*pefA*	156	+	+	**10**	*sopE*	642	-	+
4	*sopB*	220	+	+	**11**	*prgH*	756	+	+
5	*spiC*	301	+	+	**12**	*sitC*	768	+	+
6	*orfL*	332	+	+	**13**	*invA*	1070	+	+
7	*pipD*	399	+	+	**14**	*iroN*	1205	+	+

bp, base pairs

Table 4: Representative gels for the PCR-based virulotyping using 12 potentially virulence genes. Two *S.* Typhimurium control strains SL1344 and F98 were used.

Chapter 4: Molecular epidemiology analysis using PFGE

4.1 Introduction

A successful epidemiological investigation requires at least two basic elements such as characterisation and determination of relatedness between bacterial isolates (Maslow and Mulligan 1996; Trujillo *et al.* 2010). The conventional phenotypic techniques such as serotyping and bacterial susceptibility to bacteriophages can be time consuming, expensive and in addition phage typing can also be technically demanding (Farmer 1988). Results may also be inconclusive as phenotypic traits are often inconsistently expressed in bacterial strains leading to the inability to differentiate bacterial isolates. Therefore, a rapid and specific genotyping technique is needed to assist the public health authorities in detecting and tracking the source of a disease.

The genome of *S.* Typhimurium LT2 has been well characterised. The large genomic bacterial DNA, comprised of 4, 857 kilo bps chromosome (McClelland *et al.* 2001) is a real analytical challenge. The introduction of PFGE genotyping technique has allowed for analyses of DNA fragments up to 10 mega bps (Anand 1986). This method is based on the use of restriction enzymes with rare DNA restriction sites generating between 10 and 20 high molecular weight DNA fragments. These DNA fragments are separated using gel and programmed variations in both the direction and duration of electrical field. The banding patterns (profiles) produced from PFGE electrophoresis can be reproducible and specific for a variety of bacterial organisms (Gautom 1997). Nonetheless, the sensitivity of PFGE is highly dependent on the choice of restriction enzyme. The *Xba*I enzyme is one of the suitable enzymes for surveillance of *Salmonella* outbreaks as the methylation of the sites is usually complete with lower probability for

introducing variability into the banding patterns (Luo *et al.* 2003). Over the years considerable numbers of *Xba*I restriction patterns have been identified for *Salmonella* spp. that can be used for comparative analysis. In cases in which high genetic similarity is expected among the *Salmonella* isolates, a second restriction digestion of the bacterial DNA can be performed using *Spe*I enzyme which leads to the increase of the overall discrimination power of the PFGE (CDC 2009).

4.2 Materials and methods

Preparation of plugs

All 90 DT160 isolates and the 3 control strains, *S.* Braenderup H9812, *S.* Typhimurium SL1344 and F98, were placed on Columbia Horse Blood agar (Fort Richard) and incubated for 24 hrs at 37ºC. Two millilitres of a cell suspension buffer (CSB) (100 mM Tris, 100 mM EDTA, pH 8.0) was transferred into a 12 mm x 75 mm Falcon tube and a small portion of a bacterial colony from an agar plate was transferred to the tube with a cotton swab moistened with CSB and then mixed until an even suspension was formed. The concentration of the cells was adjusted to between 0.40 and 0.45 optical density using a Dade Microscan Turbidity Meter and read at 610 nanometres. Four hundred microlitres of the adjusted suspension was transferred into a 1.5 ml microcentrifuge Eppendorf tube and gently mixed with 20 mg/ml Proteinase K (Bioline GmbH, Germany). One percent SeaKem Gold (SKG) agarose (Cambrex Bio Science, Inc. Rockland, USA) in 0.5 X Tris-Borate EDTA (TBE) buffer was melted in a microwave oven on a low-medium power for 20-30 sec and 400 µl of the molten agarose mixed with the cell suspension in a microcentrifuge tube. The mixture was dispensed into the reusable plug mould where it was solidified at room temperature for 10-15 min.

Lysis and washing of cells in agarose plugs

Five millilitres of cell lysis buffer (CLB) (50 mM Tris, 50 mM EDTA, pH 8.0, 1% Sarcosyl), 0.5 mg/µl of Proteinase K, and solidified plugs were mixed into a 50 ml tube and incubated in a water bath for 120 min between 54 and 55ºC with constant agitation at 150-175 revolutions per minute (rpm). Following incubation, the supernatant was decanted, between 10 and 15 ml of pre-heated sterile water was added and the mixture incubated at the above conditions. This washing step was repeated one more time. Preheated TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) was added to the tube and the mixture incubated at the above conditions. This step was repeated three times and the plugs were transferred into a microcentrifuge tube and stored at 4ºC in 5-10 ml of TE buffer until further use.

*Restriction digestion of DNA in agarose plugs with Xba*I *and Spe*I

Plugs were removed from the TE buffer, placed on a glass slide and cut into 2.0 to 2.5 mm lengths. Each plug was transferred to a 1.5 ml Eppendorf tube and 175 µl of sterile water, 20 µl of restriction buffer, and 50 U of *Xba*I restriction enzyme (Hoffmann-La Roche Ltd., Germany) were added as recommended by the PulseNet PFGE manual (CDC 2009). The above mixture was incubated in a water bath for 120 min at 37ºC. The same procedure was applied for *Spe*I 50 U (New England Biolabs Inc.) restriction enzyme. The standard marker, *S.* Braenderup H9812, was digested with *Xba*I using the above conditions.

Electrophoresis and gel documentation

Electrophoresis was performed on a contour-clamped, homogenous electric field (CHEF) Mapper electrophoresis system (BioRad, USA), the cooling module of which was set at 14ºC, with the pump turned on 30 min prior to the beginning of a gel run. The plugs, digested with either *Xba*I or *Spe*I, were loaded into the wells in an agarose gel (1% SKG agarose in 0.5 X TBE), ensuring an absence of air bubbles, and run for 18 hrs at electrophoresis conditions recommended for *Salmonella* restriction with *Xba*I by the PulseNet PFGE manual. Finally, the gel was stained with ethidium bromide for approximately 10 min followed by de-staining with distilled water for approximately 30 min. The DNA banding patterns were visualised with a Gel Doc system (BioRad, Milan, Italy) and analysis performed with BioNumerics Version 2.0 software (Applied Maths NV, USA). PFGE analysis is based on a comparison between selected isolates and the percentage similarity between banding patterns. The percentage similarity was calculated using the Dice similarity coefficient method with a 1.5% optimisation and 2% band filtering tolerance window. A dendrogram was constructed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA).

The PFGE standard marker

Salmonella Braenderup H9812 was adopted by PulseNet International as a universal standard strain due to its evenly distributed restriction bands over the entire range of band sizes that are normally seen in foodborne pathogens tracked by PulseNet. This characteristic assists in PFGE normalization for an accurate comparison of gel images that require the use of a well-characterized size standard in at least three lanes of each gel (Hunter *et al*. 2005).

4.2.1 List of solutions used for PFGE.

Cell lysis buffer

50 mM Tris

50 mM EDTA

pH 8.0

1% Sarcosyl

Cell suspension buffer

100 mM Tris

100 mM EDTA

pH 8.0

TE buffer

10 mM Tris

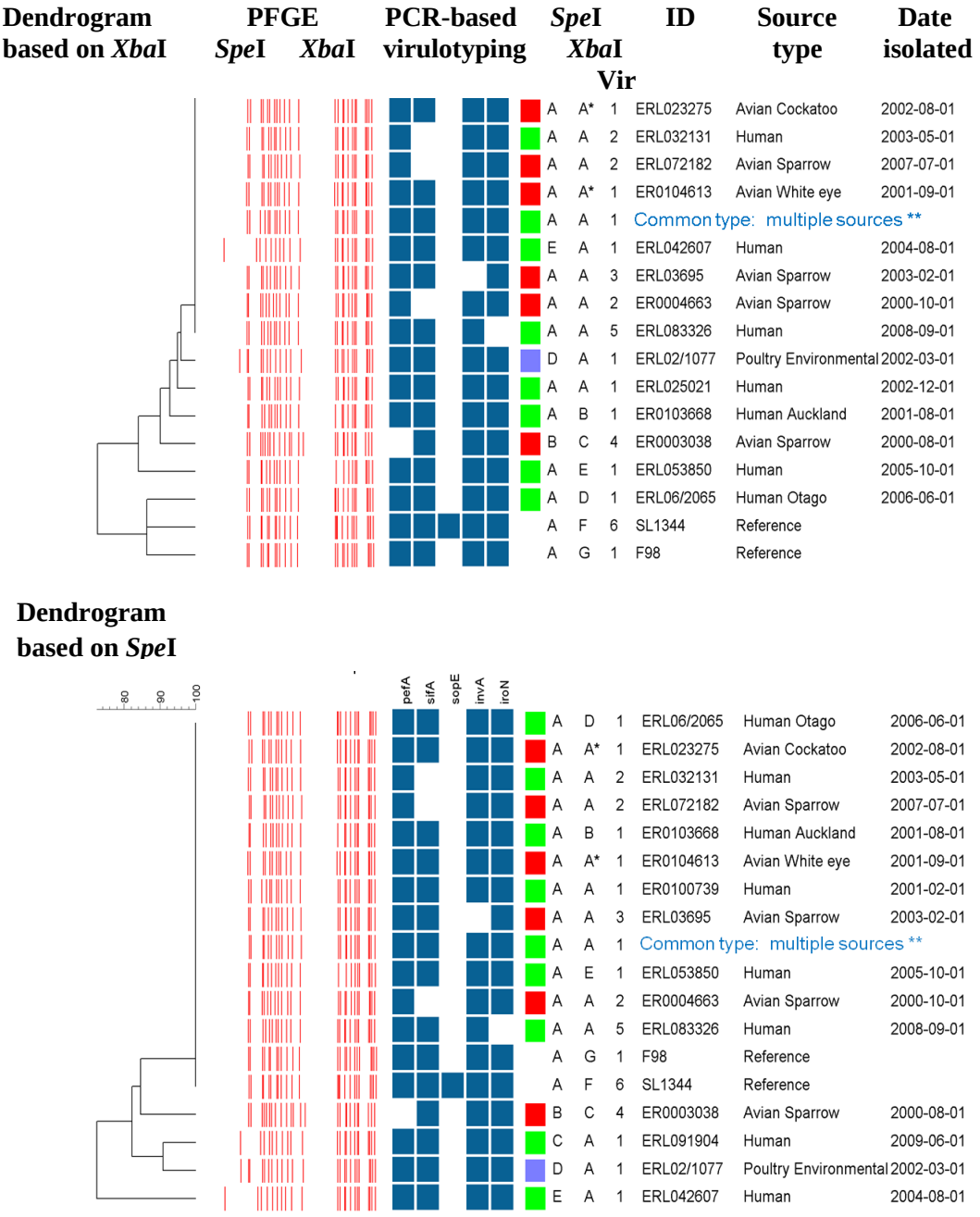
1 mM EDTA

pH 8.0

4.3 Results

The pulsed field profiles based on *Xba*I and *Spe*I restriction enzymes for all 90 isolates were visualized with a Gel Doc (Appendix 11) and analysed using Bionumerics software. Visual inspection of the PFGE gels revealed at least 5 different *Spe*I and 6 different *Xba*I patterns, but there was evidence of a single common two-enzyme profile that accounted for the majority of isolates (Figure 1). When the isolates with the unique PFGE banding patterns were retested, the identified unique restriction patterns were similar to the one generated earlier (Appendix 12).

Cluster analysis, using a cut-off value of 95% similarity based on the Dice index, identified five *Spe*I and five *Xba*I clusters. When combined with the PCR-based virulotyping there were a total of 11 different genotype profiles among the 90 isolates. However, genotype profile AA1 accounted for 78/90 (86.7%) of isolates while the second most common was genotype profile AA2 which accounted for 3/90 (3.3%). These isolates were PCR negative for *sifA* and comprised two sparrow isolates and one human isolate. The remaining nine profiles were represented by single isolates (Figure 1).



*Notable band shift detected on gels, but within tolerance in cluster analysis
**Total of 76 isolates comprising 23 human, 24 avian, 29 poultry environment

Figure 1: Genotypes of *Salmonella* DT160 based on two-enzyme PFGE and virulence gene PCR. The two UPGMA dendrograms are based on the *Spe*I band pattern, using a Dice similarity coefficient calculated using 1.5% optimisation and 2% band filtering tolerance. Combined profile AA1 (which includes A*A1) was the most common, accounting for 78/90 (87%) of isolates (only 4 representatives of this profile are provided in the diagram.

Chapter 5: General discussion and conclusion

In an attempt to identify possible relatedness between *S.* Typhimurium DT160 isolates from avian and human origin, we have compared 90 isolates from poultry environments, several avian species and human patients. The degree of relatedness was determined through a combination of techniques such as PCR-based virulotyping, PFGE banding patterns and antibiotic susceptibility.

Proper characterization of *Salmonella* isolates is essential for investigations of *Salmonella* outbreaks as non-typhoidal *Salmonella* strains have reached epidemic proportions in many countries despite improvements in sanitation and hygiene. Hence, precise strain identification is a prerequisite for implementing efficient surveillance and effective preventative measures aimed at combating the spread and eradication of the source of infection (Threlfall and Frost 1990). Outbreaks of *Salmonella* are a potential zoonotic hazard but it is difficult to find an outbreak of human infection that mirrors so closely a corresponding outbreak in a single animal species due to the limited surveillance performed for salmonellosis in animals in comparison to humans (Baker *et al*. 2007).

Identification and characterisation of *Salmonella* is crucial for surveillance, improvement of preventative measures and control of foodborne diseases. The widely used conventional serotyping method based on the Kauffmann-White scheme uses surface expressed antigens: it is time consuming and subjectively dependent on the level of technical experience and quality of the antisera. Therefore, the availability of well-trained personnel and high quality anti-sera are essential, but both are very expensive in resource limited settings.

While four of the tested *Salmonella* isolates were negative for either poly-H or poly-O sera from SIFIN, all of them were positive when tested with poly-H and poly-O sera from BD. The ambiguity of the results could be due to the different preparations of the antisera. Enteroclon mouse antisera from SIFIN are a mixture of monoclonal antibodies that may have been specific for certain antigens present on the targeted protein. These antigens might not have been present on the tested *Salmonella* isolates leading to the negative result. Furthermore, the anti-*Salmonella* rabbit sera from BD were prepared from polyclonal antibodies with a likely broader range of antigens leading to the positive results. In addition, the nature of the antibodies might have been affected by the different animal species in which the antibodies were generated. To confirm their species these four isolates were also tested against 20 biochemical reactions (API20E strip), TSI and motility. The combined result from the above tests has shown that these isolates are from *Enterobacteriaceae* family with a high degree of probability for *Salmonella* spp. This conclusion was further supported from the data obtained with the PCR-based virulotyping and PFGE banding patterns.

In the recent years, the prevalence of antibiotic resistance among *Salmonella* spp. including an alarming number of reports for MDR strains has increased significantly. *S.* Typhimurium DT104 has developed resistance to a number of antibiotics that are used frequently for treatment of humans and animals. The results from our investigation did not identify indications for multidrug resistance among the DT160 isolates we tested. However, a total of 34 DT160 isolates characterised in this study fell within the intermediate range of susceptibility to either tetracycline or oxytetracycline. This result is slightly different to the one reported in 2003 for the same definitive type in NZ (Thornley *et al.* 2003) where the isolates were found to be susceptible to all antimicrobials tested, including tetracycline. This may be of concern since resistance

towards the tetracycline group has already been reported in Taiwan for both human and animal isolates (Tsen *et al.* 2002). Due to the rapid development of MDR among *Salmonella* spp., it is possible for DT160 to acquire resistance to a number of antibiotics used frequently to treat gastrointestinal infections both in humans and in animals. There is a considerable concern that once MDR develops in some of the bacterial species the resistant genes are retained in the population even when antimicrobial drugs are withdrawn (WHO 2005). It was reported, for example, that outbreaks in Mexico, the Indian subcontinent and Southeast Asia caused by *Salmonella* strains with different Vi phage types were resistant to chloramphenicol and that this resistance was encoded by a plasmid of the H1 incompatibility group seen most frequently in combination with resistance towards streptomycin, sulfonamides and tetracyclines (Rowe *et al*. 1997). It was also suggested that emergence of MDR could occur by exchange of resistant genes among *Salmonella* spp. It is our hope that the findings in this investigation will contribute to the national and international database for antimicrobial susceptibility of *Salmonella*.

To become pathogenic, *Salmonella* spp. might need up to 200 pathogenicity genes (Bowe *et al.* 1998). A panel of 12 potential virulence genes were selected and the 90 isolates were screened with PCR for the presence of the gene sequences. The PCR products from the 12 targeted genes were sequenced and aligned to the published sequences of *S.* Typhimurium LT2 and SL1344 (Wellcome Trust Sanger Institute, UK) using the BLAST program (National Center for Biotechnology Information, United States National Library of Medicine). A high degree of sequence and length similarity was demonstrated for all genes. All 11 potential virulence genes were present in the isolates from poultry environment and isolates from avian species other than sparrow. Interestingly, no more than one gene was missing per single isolate. Two of the six

isolates that were negative for one of the genes was of human and four were of sparrow origin. One human and two sparrow isolates were PCR negative for the *sifA* gene that is responsible for invasion and survival of the *Salmonella* in macrophages (Brumell *et al.* 2001; Galan *et al.* 1992). However, the apparent absence of either *invA* or *sifA* genes does not necessarily mean that *Salmonella* would not be able to either invade or survive within the host cells as other genes might have complementary function. The *iroN* gene is associated with iron acquisition and, while present in all phylogenetic lineages of *S. enterica,* it is absent in other closely related species such as *S. bongori* and *E. coli* (Baumler *et al.* 1998). In this study, only one isolate was PCR negative for the *pefA* gene. This plasmid-encoded gene is essential for *Salmonella* attachment to intestinal epithelial surfaces (Baumler *et al.* 1997). The absence of targeted genes has to be interpreted with caution as a nucleotide mismatch between the primers and the target genomic sequence may lead to a false negative result. The *sopE* gene encoding for translocator effector protein can be present in *Salmonella* isolates related to clinical disease in human (Hopkins and Threlfall 2004). This gene appeared to be missing from all tested isolates except the control SL1344 strain. In conclusion, it appears that there was no correlation between the presence or lack of a particular virulence gene with the origin of the isolates.

PFGE sub-typing can be used to trace the clonal relationship between bacterial isolates. To minimise the potential variability between different laboratories, certification for PFGE from the PulseNet Aotearoa NZ, which is recognised by CDC, was obtained for this laboratory. The *Xba*I enzyme was selected due to its infrequent-cutting pattern and ability to produced well defined and reliable restriction DNA banding patterns for the *Salmonella* spp. Due to the expected high genetic similarity among the isolates, a second restriction digestion, run in parallel, was performed with

*Spe*I enzyme to increase the overall discriminatory power of PFGE (Ribot *et al.* 2006). Analyses of the isolates digested with either *Xba*I or *Spe*I enzymes revealed five *Spe*I and five *Xba*I clusters that, when combined with the results from the PCR-based virulotyping has produced 11 genotype profiles. However, as 78/90 (86.7%) of isolates grouped to the AA1 genotype profile, it suggests that the majority of isolates were genetically very similar and that this pattern has remained relatively stable over the time period sampled. These findings are consistent with a recent report (Dyet *et al*. 2010) that used multilocus variable-number tandem-repeat analysis (MLVA) on isolates of *S.* Typhimurium DT160. Although, most of the uncommon genotypes were of human and avian origin, there was no obvious correlation between genotype profile, virulotype, source or date of isolation (Figure 1). Genotype profile AA2 accounted for 3/90 (3.3%) of isolates. These isolates were PCR negative for *sifA* and comprised two sparrows and one human isolate. The remaining nine profiles were represented by single isolates.

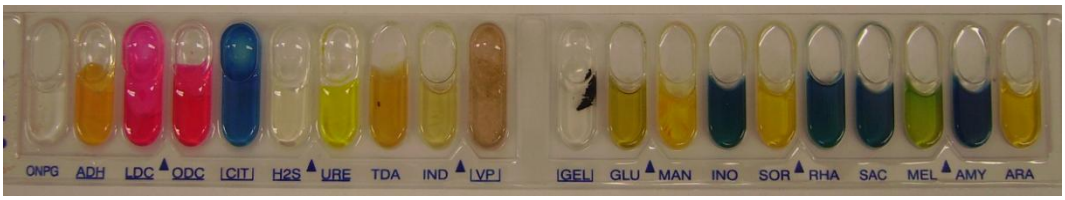
These data suggest that there are no indications for multi-drug resistance among the DT160 isolates examined in this study. In addition, there was no obvious correlation between the intermediate range of sensitivity of the 34 isolates to tetracycline and oxytetracycline, virulotyping, PFGE and seasonality. The data from PFGE profiles, PCR-based virulotyping and sensitivity to the 11 antimicrobials has indicated that majority of the DT160 isolates were very closely related and that isolates grouping in the genotype profile AA1 were predominant (87% of isolates), representing a dominant epidemiologically-important clone that has persisted in multiple hosts, including avian wildlife and poultry. The small proportion of isolates that were distinguishable from genotype AA1 each occurred at low prevalence and in multiple hosts, indicating that genomic variants arose in the population, but this has not resulted in the evolution and emergence of a new dominant, host-associated clone. No obvious correlation was found

between the source and the date of isolation and any of the other characteristics examined.

For the conclusion, all the 90 isolates examined in this study provided no evidence of multidrug resistance among DT160 isolates. The PFGE and virulotyping profiles also indicated that the majority of isolates were very closely related. Thus this suggests that a dominant epidemiologically-important genotype persisted in multiple hosts, including avian wildlife and poultry. In the other hand, other genotypes occurred at a low prevalence, in multiple hosts, indicating that some genomic variants arose sporadically in the population. Overall, there is no obvious correlation was found between either phenotype or genotype and the variables- the source and time period. Finally, the genetic similarity between the majority of isolates originated from avian, human and poultry environments in NZ between 1999 and 2009 suggests that they were very closely related strains that freely circulated between ranges of animal species.

Appendices

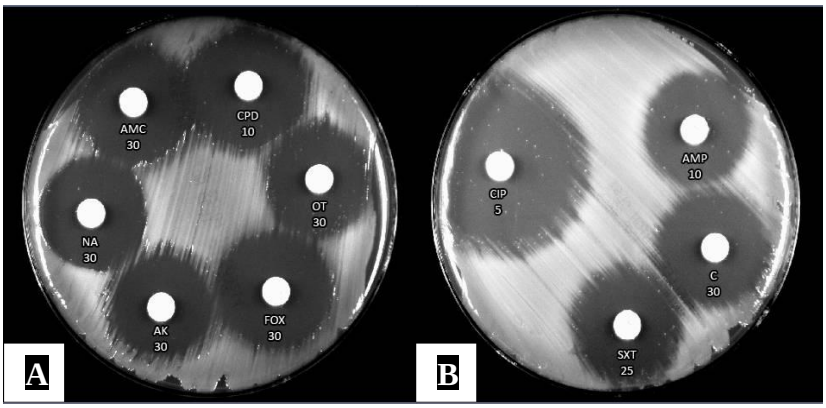
Appendix 1. The *Salmonella* isolates (ER004560, ER104613, ERL042332, and ERL07266) with ambiguous serology were tested with API20E strip that includes 20 biochemical reactions. Based on positive and negative reactions the genus and species of the isolates were determined with 7 digit profile.



Appendix 2. The *Salmonella* isolates (ER004560, ER104613, ERL042332, and ERL07266) with ambiguous serology were found to be non-motile when tested in semi-solid Bacto motility medium.

Appendix 3. When tested in Triple sugar iron agar the *Salmonella* isolates (ER004560, ER104613, ERL042332, and ERL07266) with ambiguous serology produced characteristic features for the Enterobacteriaceae family - acid butt, alkaline slant, hydrogen sulphide without gas formation.

Appendix 4. Two representative photographs (A and B) of the antimicrobial disc diffusion test used to test susceptibility of the *Salmonella* isolates against 11 antimicrobials. Photographs show the control *Escherichia coli* (*E. coli*) grown on a petri plate with Mueller Hinton agar. Six (A) and four (B) discs saturated with different antibiotics were placed on the surface of the agar.



AK, Amikacin; AMC, Amoxicillin-clavulanic acid; AMP, Ampicillin;
C, Chloramphenicol; FOX, Cefoxitin; CIP, Ciprofloxacin; CPD, Cefpodoxime;
OT, Oxytetracycline; NA, Nalidixic acid; SXT, Trimethoprim-sulfamethazole

Appendix 5. Zone diameter interpretive standard for Enterobacteriaceae family and the quality control ranges of antimicrobial disc diffusion susceptibility test for the reference strain *E. coli* ATCC25922 on Mueller Hinton Agar.

Antimicrobial agent	**Disc content (µg)**	**Zone diameter (mm)**			***E. coli***
		S	**I**	**R**	
Amikacin, AK*	30	≥17	15-16	≤14	19-26
Amoxicillin-clavulanic acid, AMC	20/10	≥18	14-17	≤13	18-24
Ampicillin, AMP	10	≥17	14-16	≤13	16-22
Cefoxitin, FOX**	30	≥18	15-17	≤14	23-29
Cefpodoxime, CPD	10	≥21	18-20	≤17	23-28
Chloramphenicol, C	30	≥18	13-17	≤12	21-27
Ciprofloxacin, CIP**	5	≥21	16-20	≤15	32-40
Nalidixic acid, NA**	30	≥19	14-18	≤13	na
Tetracycline	30	≥19	15-18	≤14	18-25
Trimethoprim-sulfamethoxazole, SXT	1.25/23.75	≥16	11 15	≤10	23-29

S, susceptible; I, Intermediate; R, resistant
*CLSI M31-A3; **CLSI M100-S20
µg, microgram; mm, millimetres
na, not available

Appendix 6. Results from the assessment of antibiotic susceptibility of the *Salmonella* isolates against 11 antimicrobials.

Internal laboratory number	ESR isolate number	Antibiotics										
		AK	AMC	AMP	C	FOX	CIP	CPD	NA	OT	SXT	TET
DT1	ERL062065	*22	27	24	29	27	28	26	21	21	23	21
DT2	ERL052816	25	24	23	27	24	36	26	22	18***	27	19
DT3	ER003370	24	27	26	25	27	34	26	22	16	27	19
DT4	ERL021077	25	28	27	26	28	37	28	24	19	27	21
DT5	ERL091067	24	27	25	25	26	36	27	25	18	25	19
DT6	ERL091081	24	27	25	26	26	39	25	24	18	26	20
DT7	ERL042607	21	22	22	23	23	34	23	19	16	20	20
DT8	ERL063767	21	25	24	26	26	33	26	24	17	23	20
DT9	ERL053052	22	23	20	27	26	36	26	22	19	23	20
DT10	ER016216	21	21	22	25	23	33	24	20	18	21	21
DT11	ERL063449	24	27	24	28	25	37	26	24	23	26	21
DT12	ERL06257	23	25	24	26	25	35	26	24	23	24	22
DT13	ERL033614	19	23	22	24	23	33	22	22	19	22	21
DT14	ERL091904	21	24	21	27	23	34	25	23	18	21	20
DT15	ERL06292	22	25	26	22	25	34	25	23	21	26	20
DT16	ERL073108	21	24	24	29	24	37	26	25	21	24	20
DT17	ERL073368	22	28	24	27	25	37	29	24	22	25	19
DT18	ERL05998	23	27	24	29	26	38	27	23	22	25	20
DT19	ERL052645	22	27	24	26	27	37	28	26	21	25	21
DT20	ERL050172	23	27	24	26	26	38	28	26	21	25	20
DT21	ERL064025	24	25	24	26	28	35	29	25	23	24	20
DT22	ER004560	25	26	23	29	26	37	28	25	24	21	21
DT23	ER012226	24	26	23	26	26	37	27	25	23	25	20
DT24	ER015215	26	28	26	29	28	39	28	26	25	28	19
DT25	ER015709	22	28	23	26	26	36	28	23	23	27	19
DT26	ERL023783	21	24	21	25	22	34	23	22	21	21	19
DT27	ERL084557	22	24	21	25	23	34	25	23	22	22	18
DT28	ERL021519	22	23	23	25	25	34	25	24	22	21	20
DT29	ERL091580	23	23	23	26	24	34	24	22	21	23	20
DT30	ERL022680	22	24	22	25	24	34	25	24	21	21	21
DT31	ERL023275	20	24	21	23	23	32	23	22	20	20	19
DT32	ERL043701	22	23	22	25	22	33	24	22	20	22	21
DT33	ERL09880	23	24	23	26	24	35	25	24	21	23	19
DT34	ERL022681	21	23	22	24	24	33	24	22	21	22	19
DT35	ERL005619	20	24	21	23	23	32	23	22	20	20	20
DT36	ERL082664	24	25	24	27	25	38	28	25	23	27	18
DT37	ERL064109	25	27	24	29	27	39	28	27	24	29	20
DT38	ERL053554	24	26	24	27	26	37	27	25	22	27	22
DT39	ERL072176	23	26	23	27	27	36	27	24	22	25	19
DT40	ERL082092	23	25	23	26	26	36	27	25	23	24	19

Appendix 6: (cont.) Results from the assessment of antibiotic susceptibility of the *Salmonella* isolates against 11 antimicrobials.

Internal laboratory number	ESR isolate number	Antibiotics										
		AK	AMC	AMP	C	FOX	CIP	CPD	NA	OT	SXT	TET
DT41	ERL032131	25	28	24	27	26	36	30	27	23	27	19
DT42	ERL0919	23	26	24	27	26	37	27	25	24	26	20
DT43	ERL072182	24	26	25	27	26	36	28	25	24	27	18
DT44	ERL050427	26	29	26	29	27	39	30	27	24	28	18
DT45	ER013668	24	27	23	27	27	39	28	27	24	26	20
DT46	ERL032893	26	29	26	29	27	37	29	26	25	28	19
DT47	ERL084152	24	27	25	26	26	36	27	25	25	26	18
DT48	ERL09395	24	26	24	27	26	35	27	25	24	25	19
DT49	ERL063325	23	28	24	28	27	36	28	26	25	26	19
DT50	ER014613	23	27	23	26	27	36	25	24	24	23	19
DT51	ERL081730	24	26	25	29	26	36	28	24	23	29	18
DT52	ERL083326	24	27	24	27	26	36	29	26	25	26	18
DT53	ERL052925	22	28	25	27	27	37	28	26	25	25	19
DT54	ERL072372	24	26	24	26	26	35	26	26	23	26	18
DT55	ERL081168	24	26	25	27	26	35	28	26	24	25	19
DT56	ERL082847	23	26	25	27	26	37	29	26	25	26	19
DT57	ERL052818	24	26	26	27	27	37	28	26	25	26	18
DT58	ERL061789	23	26	25	28	27	38	27	25	25	27	18
DT59	ERL042806	24	26	25	28	27	38	29	25	25	26	19
DT60	ERL033073	24	26	25	26	27	35	27	24	25	26	18
DT61	ER005391	24	28	25	24	28	38	29	27	24	28	18
DT62	ERL082846	24	26	25	26	27	38	28	26	25	27	18
DT63	ERL072196	24	28	25	29	27	37	28	27	25	28	17
DT64	ERL030011	24	27	24	25	27	36	24	25	23	26	18
DT65	ER010739	25	26	25	26	27	38	27	26	26	26	20
DT66	ERL043592	25	28	26	27	28	38	28	26	26	27	19
DT67	ER012224	24	27	24	26	28	36	29	25	25	27	20
DT68	ERL043718	24	26	25	25	28	37	27	26	25	26	19
DT69	ERL04205	25	27	25	25	27	34	27	25	25	26	18
DT70	ER003038	24	27	24	25	27	36	28	24	23	27	18
DT71	ERL033362	25	27	25	26	28	39	26	28	24	29	20
DT72	ER004952	25	27	24	27	28	37	29	26	24	27	17
DT73	ER011172	24	26	23	27	27	36	28	26	25	28	18
DT74	ERL043779	23	25	25	28	26	35	27	25	24	19	19
DT75	ERL03695	24	27	25	26	26	38	27	25	25	29	16
DT76	ERL08372	23	26	25	27	25	37	26	24	21	24	17
DT77	ERL062557	23	26	23	26	25	35	28	23	23	20	16
DT78	ER005001	24	27	22	25	27	35	27	25	24	23	18
DT79	ERL025021	25	27	24	24	26	36	28	24	24	24	19
DT80	ERL041935	23	26	26	29	27	36	27	25	23	26	18

Appendix 6: (cont.) Results from the assessment of antibiotic susceptibility of the *Salmonella* isolates against 11 antimicrobials.

Internal laboratory number	ESR isolate number	Antibiotics										
		AK	**AMC**	**AMP**	**C**	**FOX**	**CIP**	**CPD**	**NA**	**OT**	**SXT**	**TET**
DT81	ERL02312	24	25	23	26	25	35	26	24	23	23	19
DT82	ERL033068	23	27	23	24	27	35	27	25	25	22	17
DT83	ERL072197	24	27	25	24	27	36	27	26	25	24	19
DT84	ER004783	23	27	23	28	27	37	28	25	25	26	19
DT85	ERL053850	23	26	23	28	27	35	28	25	24	24	19
DT86	ERL042332	24	27	24	26	27	37	26	25	25	26	19
DT87	ERL025008	23	26	23	25	25	36	28	26	25	25	19
DT88	ERL034404	23	25	22	26	25	36	26	23	23	25	18
DT89	ERL07266	23	25	22	25	25	35	26	25	23	22	19
DT90	ER004663	23	25	22	25	25	35	26	24	24	21	19
***E coli* ATCC 25922**		23-25	20-23	16-21	23-25	26-28	33-39	25-28	23-27	22-25	24-29	20-23

* millimetres

** *E. coli* ATCC 25922 quality control for conducting the antibiograms

*** The numbers highlighted with yellow are within the intermediate range of susceptibility against OT and TET

ERL, Enteric Reference Laboratory; ESR, Environmental Science and Research

AK, Amikacin; AMC, Amoxicillin-clavulanic acid; AMP, Ampicillin; C, Chloramphenicol; FOX, Cefoxitin; CIP, Ciprofloxacin; CPD, Cefpodoxime; OT, Oxytetracycline; NA, Nalidixic acid; SXT, Trimethoprim-sulfamethazole; TET, Tetracycline

Appendix 7. Twenty five percent randomly selected *Salmonella* isolates were retested for antibiotic sensitivity against the 11 antibiotics and inter-rater agreement was evaluated with Kappa analysis.

Internal laboratory number	ESR isolate number	Antibiotics																			
		AK		AMC		AMP		C		FOX		CIP		CPD		NA		OT		SXT	
		1*	2**	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
DT1	ERL062065	21***	21	26	25	19	18	29	29	24	23	33	35	26	26	24	23	22	21	26	26
DT3	ER003370	24	24	26	25	19	18	27	26	25	26	24	37	26	26	25	24	21	18	24	24
DT10	ER016216	24	23	25	26	20	20	25	26	25	26	35	37	27	27	25	24	23	24	26	25
DT12	ERL06257	23	23	27	27	21	19	26	25	26	26	36	35	26	24	25	25	25	24	24	24
DT14	ERL091904	22	22	24	23	20	19	25	27	22	23	33	37	25	25	22	23	21	21	23	23
DT15	ERL06292	22	22	25	25	20	19	25	26	25	24	33	36	26	26	23	22	21	20	23	24
DT27	ERL084557	22	23	25	25	20	18	22	24	24	25	32	35	24	24	24	23	25	24	23	22
DT32	ERL043701	22	22	24	23	19	19	26	26	24	23	34	35	25	25	23	22	22	22	23	22
DT38	ERL053554	21	21	25	25	20	20	24	24	22	23	29	34	26	24	23	23	22	21	23	22
DT43	ERL072182	21	22	23	24	19	19	24	26	22	22	34	36	25	24	22	22	21	21	24	23
DT46	ERL032893	21	23	23	24	19	17	24	25	24	23	33	36	26	26	23	23	22	20	24	25
DT47	ERL084152	22	22	24	23	19	17	23	26	21	23	32	36	23	25	22	21	20	20	22	23
DT50	ER014613	24	24	27	27	19	18	24	25	25	25	31	34	23	25	24	25	23	23	24	25
DT54	ERL072372	22	23	22	25	17	18	23	25	22	23	33	35	24	25	24	23	22	21	22	23
DT56	ERL082847	22	22	25	25	19	17	26	26	25	24	31	36	23	24	24	23	22	22	23	23
DT62	ERL082846	24	24	25	25	19	18	26	26	25	24	34	36	24	25	25	24	24	22	24	24
DT68	ERL043718	21	22	28	28	21	20	26	26	24	25	35	37	26	25	26	25	25	23	24	23
DT72	ER004952	22	23	25	24	19	18	26	25	24	24	33	36	26	26	24	23	19	21	23	23
DT79	ERL025021	24	24	27	26	20	18	26	26	25	26	36	37	27	27	24	24	25	22	26	26
DT85	ERL053850	23	23	25	25	18	18	23	27	24	25	35	36	27	26	24	24	22	21	24	24
DT87	ERL025008	23	22	24	24	18	17	23	23	24	23	32	36	25	26	24	24	21	21	23	23
DT90	ER004663	24	24	25	25	18	16	24	24	24	24	35	34	24	24	24	23	22	22	21	22
E.coli ATCC 25922		20	21	19	19	16	16	23	24	24	25	30	32	23	24	22	22	19	20	21	22

* Technician 1; **Technician 2; *** millimetres; ERL, Enteric Reference Laboratory; ESR, Environmental Science and Research
AK, Amikacin; AMC, Amoxicillin-clavulanic acid; AMP, Ampicillin; C, Chloramphenicol; FOX, Cefoxitin; CIP, Ciprofloxacin;
CPD, Cefpodoxime; OT, Oxytetracycline; NA, Nalidixic acid; SXT, Trimethoprim-sulfamethazole; TET, Tetracycline

Appendix 8. Nucleotide alignments for the 12 targeted potential virulence genes. PCR products for the various genes were obtained from the *S.* Typhimurium F98 and SL1344 control strains were sequenced and aligned with published sequences of *S.* Typhimurium LT2 and SL1344 strains. The ambiguity characters within the sequences were not removed.

iroN

CGACCCRGGCCGGGTAGCAGCAAGAAGGTGACACCCGGCGGGGTAACTTTAGCCTTAGCGGTCCGTTGGCTGGTGATACGTTGACGATGCGCCTGTACGGTAATCTCAACAGAACCGATGCCGATAG
CTGGGATATTAACTCCTCCGCCGGTACCAAAAATGCCGCCGGACGGGAGGGGGTCACCAATAAAGATATTAATAGCGTCTTCTCATGGAAAATGACCCCGCAGCAAATCCTTGATTTTGAAGCGGGA
TATAGTCGGCAGGGGAATATTTATGCTGGCGATACGCAAAACAGTACTTCAAACGCGGTTACCAAAAGTCTGGCGCAGTCCGGGCGGGAGACCAATCGCCTGTACCGGCAGAATTACGGTCTAACTC
ATAACGGCATCTGGGACTGGGGGCAAAGCCGCCTGGGGTTCTACTATGAAAAAACCGATAACACCCGCATGAATGAGGGGCTCTCCGGCGGCGGCGAGGGGCGTATTACCAACGACCAGACGTTCA
CCACTAACCGCCTGACCTCGTATCGCACCAGCGGCGAGGTGAATGTCCCGGTGATATGGCTATTTGAGCAAACGCTCACCGTCGGCGCGGAGTGGAACCGCGATGAGCTGAACGATCCTTCCTCTAC
CAGTCTGACGGTTAAGGATAGCAATATCGCCGGGATTCCCGGGTTCGGCGGCTAACCCGKARSCMRSCMAAAAAAWWAAAWYKGGRAAAAWTCWMRGTKGSCGSYYKKTWWCGGTTTKRAAAR
AATAWAAWWWTYKSAAAMCCYTWWKKGGSCCCSGGKWMMCCAAAWWWWTTWWWTTYCCSGGGGGYYYYKGSSSYTTTTTTKRKWTTTWTTTKRRRSRSRAAWYYYCSGGGGGRRRAWAWW
TTTTTRWKKSYYCCMSMRKYYTTTWAMMMWYYTTTTTYCCCSSGRGRGRAKWTMRKRSGTGRRRAAAAYMYMYYCTCCMSGGGGTRARAAAAMMSCSSGSGRWWTWAYTGKCGCCCGYGTTCT
CTCTYTWWRAGRSYSCSYCCGCAMAAYAWKTKTKTTMTMTMMMMMCACCRYRTGWGGGACGGGGGWTTATWTCTTCACTTCTYCYACAACKSARAGACRSACARCGTGTGKTSTYCWWCRCAG
AAAAAWATATA

ref|NC 003197.1| *Salmonella enterica* subsp. *enterica* serovar Typhimurium str., LT2 chromosome, complete genome, Length=4857432, Score = 1240 bits (671), Expect = 0.0, Identities = 680/684 (99%), Gaps = 2/684 (0%), Strand=Plus/Minus. Part of Features in this part of subject sequence TonB-dependent siderophore receptor protein

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Query  9        GCCGGGTAGCAGC-AAGAAGGTGACACCCGGCGGGGTAACTTTAGCCTTAGCGGTCCGTTGGCTGGTGATACGTTGACGATGCGCCTGTACGGTAATCTCAACAGAACCGATGCCGATAG  127
                 |||||  ||||||  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2925209  GCCGGAAAGCAGCAAAGAAGGTGACACCCGGCGGGGTAACTTTAGCCTTAGCGGTCCGTTGGCTGGTGATACGTTGACGATGCGCCTGTACGGTAATCTCAACAGAACCGATGCCGATAG  2925090

Query  128      CTGGGATATTAACTCCTCCGCCGGTACCAAAAATGCCGCCGGACGGGAGGGGGTCACCAATAAAGATATTAATAGCGTCTTCTCATGGAAAATGACCCCGCAGCAAATCCTTGATTTTGA  247
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2925089  CTGGGATATTAACTCCTCCGCCGGTACCAAAAATGCCGCCGGACGGGAGGGGGTCACCAATAAAGATATTAATAGCGTCTTCTCATGGAAAATGACCCCGCAGCAAATCCTTGATTTTGA  2924970

Query  248      AGCGGGATATAGTCGGCAGGGGAATATTTATGCTGGCGATACGCAAAACAGTACTTCAAACGCGGTTACCAAAAGTCTGGCGCAGTCCGGGCGGGAGACCAATCGCCTGTACCGGCAGAA  367
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2924969  AGCGGGATATAGTCGGCAGGGGAATATTTATGCTGGCGATACGCAAAACAGTACTTCAAACGCGGTTACCAAAAGTCTGGCGCAGTCCGGGCGGGAGACCAATCGCCTGTACCGGCAGAA  2924850

Query  368      TTACGGTCTAACTCATAACGGCATCTGGGACTGGGGGCAAAGCCGCCTGGGGTTCTACTATGAAAAAACCGATAACACCCGCATGAATGAGGGGCTCTCCGGCGGCGGCGAGGGGCGTAT  487
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2924849  TTACGGTCTAACTCATAACGGCATCTGGGACTGGGGGCAAAGCCGCCTGGGGTTCTACTATGAAAAAACCGATAACACCCGCATGAATGAGGGGCTCTCCGGCGGCGGCGAGGGGCGTAT  2924730

Query  488      TACCAACGACCAGACGTTCACCACTAACCGCCTGACCTCGTATCGCACCAGCGGCGAGGTGAATGTCCCGGTGATATGGCTATTTGAGCAAACGCTCACCGTCGGCGCGGAGTGGAACCG  607
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Sbjct  2924729  TACCAACGACCAGACGTTCACCACTAACCGCCTGACCTCGTATCGCACCAGCGGCGAGGTGAATGTCCCGGTGATATGGCTATTTGAGCAAACGCTCACCGTCGGCGCGGAGTGGAACCG  2924610
```

Appendix 8: (cont.) Nucleotide alignments for the 12 targeted potentially virulence genes. PCR products for the respected gene obtained from the *S.* Typhimurium F98 and SL1344 control strains were sequenced and aligned with published sequences of *S.* Typhimurium LT2 and SL1344 strains. The ambiguity characters within the sequences were not removed.

```
Query  608      CGATGAGCTGAACGATCCTTCCTCTACCAGTCTGACGGTTAAGGATAGCAATATCGCCGGGATTCCCGGGTTCGGCGGCTAACC  691
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||
Sbjct  2924609  CGATGAGCTGAACGATCCTTCCTCTACCAGTCTGACGGTTAAGGATAGCAATATCGCCGGGATTCCCGG-TTCGGCGGCTAACC  2924527
```

gb|U97227.1|SEU97227 *Salmonella enterica* ferric enterochelin esterase homolog (iroD), IroE (iroE) and TonB dependent outer membrane siderophore , receptor protein (iroN) genes, complete cds, Length=4837, Score = 1173 bits (635), Expect = 0.0, Identities = 666/680 (97%), Gaps = 5/680 (0%) Strand=Plus/Minus

```
Query  16    AGCAGC-AAGAAGGTGACACCCGGCGGGGTAACTTTAGCCTTAGCGGTCCGTTGGCTGGTGATACG-T-T-GACGATGCGCCTGTACGGTAATCTCAACAGAACCGATGCCGATAGCTGG  131
             |||||| |||||||||||||||||||||||||||||||||| |||||||||||||||||| | | |||||||||||||||||||||||||||||||||||||||||| ||||||
Sbjct  3905  AGCAGCAAAGAAGGTGACACCCGGCGGGGTAACTTTAGCCTTAGCGGCCCGTTGGCTGGTGATACGTTATCGACGATGCGCCTGTACGGTAATCTCAACAGAACCGATGCCGACAGCTGG  3786

Query  132   GATATTAACTCCTCCGCCGGTACCAAAAATGCCGCCGGACGGGAGGGGGTCACCAATAAAGATATTAATAGCGTCTTCTCATGGAAAATGACCCCGCAGCAAATCCTTGATTTTGAAGCG  251
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3785  GATATTAACTCCTCCGCCGGTACCAAAAATGCCGCCGGACGGGAGGGGGTCACCAATAAAGATATTAATAGCGTCTTCTCATGGAAAATGACCCCGCAGCAAATCCTTGATTTTGAAGCG  3666

Query  252   GGATATAGTCGGCAGGGGAATATTTATGCTGGCGATACGCAAAACAGTACTTCAAACGCGGTTACCAAAAGTCTGGCGCAGTCCGGGCGGGAGACCAATCGCCTGTACCGGCAGAATTAC  371
             ||||| |||||||||||||||| ||||| |||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3665  GGATACAGTCGGCAGGGGAATATCTATGCCGGCGATACGCAAAACAGTAATTCAAACGCGGTTACCAAAAGTCTGGCGCAGTCCGGGCGGGAGACCAATCGCCTGTACCGGCAGAATTAC  3546

Query  372   GGTCTAACTCATAACGGCATCTGGGACTGGGGGCAAAGCCGCCTGGGGTTCTACTATGAAAAAACCGATAACACCCGCATGAATGAGGGGCTCTCCGGCGGCGGCGAGGGGCGTATTACC  491
             |||||||||||||||||||||||| |||||||||||||||||||||||||||||||||||||||||||||||| |||||||||||||||| ||||||||||||||||||||||||||||||
Sbjct  3545  GGTCTAACTCATAACGGCATCTGGGGCTGGGGGCAAAGCCGCCTGGGGTTCTACTATGAAAAAACCGATAATACCCGCATGAATGAGGGACTCTCCGGCGGCGGCGAGGGGCGTATTACC  3426

Query  492   AACGACCAGACGTTCACCACTAACCGCCTGACCTCGTATCGCACCAGCGGCGAGGTGAATGTCCCGGTGATATGGCTATTTGAGCAAACGCTCACCGTCGGCGCGGAGTGGAACCGCGAT  611
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3425  AACGACCAGACGTTCACCACTAACCGCCTGACCTCGTATCGCACCAGCGGCGAGGTGAATGTCCCGGTGATATGGCTATTTGAGCAAACGCTCACCGTCGGCGCGGAGTGGAACCGCGAT  3306

Query  612   GAGCTGAACGATCCTTCCTCTACCAGTCTGACGGTTAAGGATAGCAATATCGCCGGGATTCCCGGGTTCGGCGGCTAACC  691
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||
Sbjct  3305  GAGCTGAACGATCCTTCCTCTACCAGTCTGACGGTTAAGGATAGCAATATCGCCGGGATTCCCGG-TTCGGCGGCTAACC  3227
```

Appendix 8: (cont.) Nucleotide alignments for the 12 targeted potentially virulence genes. PCR products for the respected gene obtained from the *S.* Typhimurium F98 and SL1344 control strains were sequenced and aligned with published sequences of *S.* Typhimurium LT2 and SL1344 strains. The ambiguity characters within the sequences were not removed.

invA

```
GCGACGATGGTCAGTTTATCGTTATTACCAAAGGTTCAGAACGTGTCGCGGAAGTCGCGGCCCGATTTTCTCTGGATGGTATGCCCGGTAAACAGATGAGTATTGATGCCGATTTGAAGGCCGGTATTATTGATGCGGATGCCGC
GCGCGAACGGCGAAGCGTACTGGAAAGGGAAAGCCAGCTTTACGGTTCCTTTGACGGTGCGATGAAGTTTATCAAAGGTGACGCTATTGCCGGCATCATTATTATCTTTGTGAACTTTATTGGCGGTATTTCGGTGGGGATGACT
CGCCATGGTATGGATTTGTCCTCCGCCCTGTCTACTTATACCATGCTGACCATTGGTGATGGTCTTGTCGCCCAGATCCCCGCATTGTTGATTGCGATTAGTGCCGGTTTTATCGTGACCCGCGTAAATGGCGATAGCGATAATA
TGGGGCGGAATATCATGACGCAGCTGTTGAACAACCCATTTGTATTGGTTGTTACGGCTATTTTGACCATTTCAATGGGAACTCTGCCGGGATTCCCACTGCCGGTTTTTGTTATTTTATCGGTGGTTTTAAGCGTACTCTTCTA
TTTTAAATTCCGTGAAGCAAAACGTAGCGCCGCCAAACCTAAAACCAGCAAAGGCGAGCAGCCGCTCAGTATTGAGGAAAAAGAAGGGTCGTCGTTAGGACTGATTGGCGATCTCGATAAAGTCTCTACAGAGACCGTACCGTTG
ATATTACTTGTGCCGAAGAGCCGGCGTGAAGATCTGGAAAAAGCTCAACTTGCGGAGCGTCTACGTAGTCAGTTCTTTATTGATTATGGCGTGCGCCTGCCGGAAGTATTGTTACGAGATGGCGAGGGCCTGGACGATAACAGCA
TCGTATTGTTGATTAATGAGATCCGTGTTGAACAATTTACGGTCTATTTTGATTTGATGCGAGTGGTAAATTATTCCGATGAAGTCGTGTCCTTTGGTATTAATCCAACAATCCATCAGCAAGGTAGCAGTCAGTATTTCTGGGT
ACGMTGCAGAGAGAGARAAAAMAC
```

ref|NC_003197.1| *Salmonella enterica* subsp. *enterica* serovar Typhimurium str. LT2 chromosome, complete genome, Length=4857432, Score = 1853 bits (1003), Expect = 0.0, Identities = 1008/1010 (99%), Gaps = 1/1010 (0%), Strand=Plus/Minus. Features in this part of subject sequence invasion protein

```
Query  8        TGGT-CAGTTTATCGTTATTACCAAAGGTTCAGAACGTGTCGCGGAAGTCGCGGCCCGATTTTCTCTGGATGGTATGCCCGGTAAACAGATGAGTATTGATGCCGATTTGAAGGCCGGTA  126
               |||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3040090  TGGTCCAGTTTATCGTTATTACCAAAGGTTCAGAACGTGTCGCGGAAGTCGCGGCCCGATTTTCTCTGGATGGTATGCCCGGTAAACAGATGAGTATTGATGCCGATTTGAAGGCCGGTA  3039971

Query  127      TTATTGATGCGGATGCCGCGCGCGAACGGCGAAGCGTACTGGAAAGGGAAAGCCAGCTTTACGGTTCCTTTGACGGTGCGATGAAGTTTATCAAAGGTGACGCTATTGCCGGCATCATTA  246
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3039970  TTATTGATGCGGATGCCGCGCGCGAACGGCGAAGCGTACTGGAAAGGGAAAGCCAGCTTTACGGTTCCTTTGACGGTGCGATGAAGTTTATCAAAGGTGACGCTATTGCCGGCATCATTA  3039851

Query  247      TTATCTTTGTGAACTTTATTGGCGGTATTTCGGTGGGGATGACTCGCCATGGTATGGATTTGTCCTCCGCCCTGTCTACTTATACCATGCTGACCATTGGTGATGGTCTTGTCGCCCAGA  366
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3039850  TTATCTTTGTGAACTTTATTGGCGGTATTTCGGTGGGGATGACTCGCCATGGTATGGATTTGTCCTCCGCCCTGTCTACTTATACCATGCTGACCATTGGTGATGGTCTTGTCGCCCAGA  3039731

Query  367      TCCCCGCATTGTTGATTGCGATTAGTGCCGGTTTTATCGTGACCCGCGTAAATGGCGATAGCGATAATATGGGGCGGAATATCATGACGCAGCTGTTGAACAACCCATTTGTATTGGTTG  486
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3039730  TCCCCGCATTGTTGATTGCGATTAGTGCCGGTTTTATCGTGACCCGCGTAAATGGCGATAGCGATAATATGGGGCGGAATATCATGACGCAGCTGTTGAACAACCCATTTGTATTGGTTG  3039611

Query  487      TTACGGCTATTTTGACCATTTCAATGGGAACTCTGCCGGGATTCCCACTGCCGGTTTTTGTTATTTTATCGGTGGTTTTAAGCGTACTCTTCTATTTTAAATTCCGTGAAGCAAAACGTA  606
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3039610  TTACGGCTATTTTGACCATTTCAATGGGAACTCTGCCGGGATTCCCACTGCCGGTTTTTGTTATTTTATCGGTGGTTTTAAGCGTACTCTTCTATTTTAAATTCCGTGAAGCAAAACGTA  3039491

Query  607      GCGCCGCCAAACCTAAAACCAGCAAAGGCGAGCAGCCGCTCAGTATTGAGGAAAAAGAAGGGTCGTCGTTAGGACTGATTGGCGATCTCGATAAAGTCTCTACAGAGACCGTACCGTTGA  726
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3039490  GCGCCGCCAAACCTAAAACCAGCAAAGGCGAGCAGCCGCTCAGTATTGAGGAAAAAGAAGGGTCGTCGTTAGGACTGATTGGCGATCTCGATAAAGTCTCTACAGAGACCGTACCGTTGA  3039371
```

Appendix 8: (cont.) Nucleotide alignments for the 12 targeted potentially virulence genes. PCR products for the respected gene obtained from the *S.* Typhimurium F98 and SL1344 control strains were sequenced and aligned with published sequences of *S.* Typhimurium LT2 and SL1344 strains. The ambiguity characters within the sequences were not removed.

```
Query  727      TATTACTTGTGCCGAAGAGCCGGCGTGAAGATCTGGAAAAAGCTCAACTTGCGGAGCGTCTACGTAGTCAGTTCTTTATTGATTATGGCGTGCGCCTGCCGGAAGTATTGTTACGAGATG  846
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||||
Sbjct  3039370  TATTACTTGTGCCGAAGAGCCGGCGTGAAGATCTGGAAAAAGCTCAACTTGCGGAGCGTCTACGTAGTCAGTTCTTTATTGATTATGGCGTGCGCCTGCCGGAAGTATTGTTACGCGATG  3039251

Query  847      GCGAGGGCCTGGACGATAACAGCATCGTATTGTTGATTAATGAGATCCGTGTTGAACAATTTACGGTCTATTTTGATTTGATGCGAGTGGTAAATTATTCCGATGAAGTCGTGTCCTTTG  966
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3039250  GCGAGGGCCTGGACGATAACAGCATCGTATTGTTGATTAATGAGATCCGTGTTGAACAATTTACGGTCTATTTTGATTTGATGCGAGTGGTAAATTATTCCGATGAAGTCGTGTCCTTTG  3039131

Query  967      GTATTAATCCAACAATCCATCAGCAAGGTAGCAGTCAGTATTTCTGGGTA  1016
                ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3039130  GTATTAATCCAACAATCCATCAGCAAGGTAGCAGTCAGTATTTCTGGGTA  3039081
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sitC

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ATGGCCAAKGTGGGCGGTCTGTGCGCGTTTCTCTCCTGCTATTTGATGCTCAAAGGCTGGTCGCTCATTGGCGATGCGCTATCTCACTCTATTGTGCCTGGCGTCGCTGGCGCCTGGATGTTAGGGCTGCCCTTCTCGCTCGGCG
CGTTTCTTTCCGGCGGACTGGCGGCAGGCAGTATGCTCTTTCTTAACCAACGCTCACGCCTGAAAGAARACGCGATTATCGGGYTTATCTTCTCCTCTTTTTTTGGCGTCGGCCTTTTTATGGTGTCGCTCAATCCGATGTCGGT
GAATATCCARACRATTATTCTCGGCAACGTGCTGGCRATCGCACCAGCGGATATTGCGCAACTGGCGATTATCGGCGCAKTCTCGCTGACGATTCTGCTGTTAAAGTGGAAAGATCTGATGGTCGTCTTTTTCGATGAAACCCAT
GCGCGTTCTATCGGGCTTAATCCGGGTCGACTAAAGCTGTTGTTTTTCACTCTATTGTCCGTCTCTACCGTGGCGGCCCTGCAAACCGTTGGGGCGTTCCTGGTGATCTGTCTCGTCGTCACGCCGGGCGCGACGGCCTGGTTAC
TCACCGACCGTTTTCCACGTCTGCTCATGATTGCCGTCGTGATTGGCAGCCTGACCAGTTTTCTGGGCGCATGGCTTAGCTACTGGCTGGATGGCGCCACGGGTGGAATTATTGTCGTCATGCAAACCTTACTGTTCATCACAGC
CTTTATTKTCGCCCCGA
```

ref|NC 003197.1| *Salmonella enterica* subsp. *enterica* serovar Typhimurium str. LT2 chromosome, complete genome, Length=4857432, Score = 1328 bits (719), Expect = 0.0, Identities = 726/733 (99%), Gaps = 0/733 (0%), Strand=Plus/Plus. Features in this part of subject sequence fur regulated Salmonella iron transporter

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Query  10       GTGGGCGGTCTGTGCGCGTTTCTCTCCTGCTATTTGATGCTCAAAGGCTGGTCGCTCATTGGCGATGCGCTATCTCACTCTATTGTGCCTGGCGTCGCTGGCGCCTGGATGTTAGGGCTG  129
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3008166  GTGGGCGGTCTGTGCGCGTTTCTCTCCTGCTATTTGATGCTCAAAGGCTGGTCGCTCATTGGCGATGCGCTATCTCACTCTATTGTGCCTGGCGTCGCTGGCGCCTGGATGTTAGGGCTG  3008285

Query  130      CCCTTCTCGCTCGGCGCGTTTCTTTCCGGCGGACTGGCGGCAGGCAGTATGCTCTTTCTTAACCAACGCTCACGCCTGAAAGAARACGCGATTATCGGGYTTATCTTCTCCTCttttttt  249
                |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3008286  CCCTTCTCGCTCGGCGCGTTTCTTTCCGGCGGACTGGCGGCAGGCAGTATGCTCTTTCTTAACCAACGCTCACGCCTGAAAGAAGACGCGATTATCGGGCTTATCTTCTCCTCTTTTTTT  3008405

Query  250      GGCGTCGGCCTTTTTATGGTGTCGCTCAATCCGATGTCGGTGAATATCCARACRATTATTCTCGGCAACGTGCTGGCRATCGCACCAGCGGATATTGCGCAACTGGCGATTATCGGCGCA  369
                |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3008406  GGCGTCGGCCTTTTTATGGTGTCGCTCAATCCGATGTCGGTGAATATCCAGACGATTATTCTCGGCAACGTGCTGGCGATCGCACCAGCGGATATTGCGCAACTGGCGATTATCGGCGCA  3008525
```

Appendix 8: (cont.) Nucleotide alignments for the 12 targeted potentially virulence genes. PCR products for the respected gene obtained from the *S.* Typhimurium F98 and SL1344 control strains were sequenced and aligned with published sequences of *S.* Typhimurium LT2 and SL1344 strains. The ambiguity characters within the sequences were not removed.

```
Query  370      KTCTCGCTGACGATTCTGCTGTTAAAGTGGAAAGATCTGATGGTCGTCTTTTTCGATGAAACCCATGCGCGTTCTATCGGGCTTAATCCGGGTCGACTAAAGCTGTTGTTTTTCACTCTA 609
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3008526  GTCTCGCTGACGATTCTGCTGTTAAAGTGGAAAGATCTGATGGTCGTCTTTTTCGATGAAACCCATGCGCGTTCTATCGGGCTTAATCCGGGTCGACTAAAGCTGTTGTTTTTCACTCTA 3008765

Query  490      TTGTCCGTCTCTACCGTGGCGGCCCTGCAAACCGTTGGGGCGTTCCTGGTGATCTGTCTCGTCGTCACGCCGGGCGCGACGGCCTGGTTACTCACCGACCGTTTTCCACGTCTGCTCATG 669
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3008646  TTGTCCGTCTCTACCGTGGCGGCCCTGCAAACCGTTGGGGCGTTCCTGGTGATCTGTCTCGTCGTCACGCCGGGCGCGACGGCCTGGTTACTCACCGACCGTTTTCCACGTCTGCTCATG 3008825
Query  610      ATTGCCGTCGTGATTGGCAGCCTGACCAGTTTTCTGGGCGCATGGCTTAGCTACTGGCTGGATGGCGCCACGGGTGGAATTATTGTCGTCATGCAAACCTTACTGTTCATCACAGCCTTT  729
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3008766  ATTGCCGTCGTGATTGGCAGCCTGACCAGTTTTCTGGGCGCATGGCTTAGCTACTGGCTGGATGGCGCCACGGGTGGAATTATTGTCGTCATGCAAACCTTACTGTTCATCACAGCCTTT  3008885

Query  730      ATTKTCGCCCCGA  742
                 ||| |||||||||
Sbjct  3008886  ATTTTCGCCCCGA  3008898
```

gb|AF128999.1|AF128999 *Salmonella* typhimurium *SitA (sitA), SitB (sitB), SitC (sitC),* and *SitD (sitD)* genes, complete cds, Length=4000, Score = 1328 bits (719), Expect = 0.0, Identities = 726/733 (99%), Gaps = 0/733 (0%, Strand=Plus/Plus

```
Query  10    GTGGGCGGTCTGTGCGCGTTTCTCTCCTGCTATTTGATGCTCAAAGGCTGGTCGCTCATTGGCGATGCGCTATCTCACTCTATTGTGCCTGGCGTCGCTGGCGCCTGGATGTTAGGGCTG  129
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2108  GTGGGCGGTCTGTGCGCGTTTCTCTCCTGCTATTTGATGCTCAAAGGCTGGTCGCTCATTGGCGATGCGCTATCTCACTCTATTGTGCCTGGCGTCGCTGGCGCCTGGATGTTAGGGCTG  2227

Query  130   CCCTTCTCGCTCGGCGCGTTTCTTTCCGGCGGACTGGCGGCAGGCAGTATGCTCTTTCTTAACCAACGCTCACGCCTGAAAGAAARACGCGATTATCGGGYTTATCTTCTCCTCttttttt  249
              |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||| |||||||||||||| ||||||||||||||||||||
Sbjct  2228  CCCTTCTCGCTCGGCGCGTTTCTTTCCGGCGGACTGGCGGCAGGCAGTATGCTCTTTCTTAACCAACGCTCACGCCTGAAAGAAGACGCGATTATCGGGCTTATCTTCTCCTCTTTTTTT  2347

Query  250   GGCGTCGGCCTTTTTATGGTGTCGCTCAATCCGATGTCGGTGAATATCCARACRATTATTCTCGGCAACGTGCTGGCRATCGCACCAGCGGATATTGCGCAACTGGCGATTATCGGCGCA  369
              |||||||||||||||||||||||||||||||||||||||||||||||||||| || |||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct  2348  GGCGTCGGCCTTTTTATGGTGTCGCTCAATCCGATGTCGGTGAATATCCAGACGATTATTCTCGGCAACGTGCTGGCGATCGCACCAGCGGATATTGCGCAACTGGCGATTATCGGCGCA  2467

Query  370   KTCTCGCTGACGATTCTGCTGTTAAAGTGGAAAGATCTGATGGTCGTCTTTTTCGATGAAACCCATGCGCGTTCTATCGGGCTTAATCCGGGTCGACTAAAGCTGTTGTTTTTCACTCTA  489
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2468  GTCTCGCTGACGATTCTGCTGTTAAAGTGGAAAGATCTGATGGTCGTCTTTTTCGATGAAACCCATGCGCGTTCTATCGGGCTTAATCCGGGTCGACTAAAGCTGTTGTTTTTCACTCTA  2587

Query  490   TTGTCCGTCTCTACCGTGGCGGCCCTGCAAACCGTTGGGGCGTTCCTGGTGATCTGTCTCGTCGTCACGCCGGGCGCGACGGCCTGGTTACTCACCGACCGTTTTCCACGTCTGCTCATG  609
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2588  TTGTCCGTCTCTACCGTGGCGGCCCTGCAAACCGTTGGGGCGTTCCTGGTGATCTGTCTCGTCGTCACGCCGGGCGCGACGGCCTGGTTACTCACCGACCGTTTTCCACGTCTGCTCATG  2707
```

Appendix 8: (cont.) Nucleotide alignments for the 12 targeted potentially virulence genes. PCR products for the respected gene obtained from the *S.* Typhimurium F98 and SL1344 control strains were sequenced and aligned with published sequences of *S.* Typhimurium LT2 and SL1344 strains. The ambiguity characters within the sequences were not removed.

```
Query  610   ATTGCCGTCGTGATTGGCAGCCTGACCAGTTTTCTGGGCGCATGGCTTAGCTACTGGCTGGATGGCGCCACGGGTGGAATTATTGTCGTCATGCAAACCTTACTGTTCATCACAGCCTTT  729
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2708  ATTGCCGTCGTGATTGGCAGCCTGACCAGTTTTCTGGGCGCATGGCTTAGCTACTGGCTGGATGGCGCCACGGGTGGAATTATTGTCGTCATGCAAACCTTACTGTTCATCACAGCCTTT  2827

Query  730   ATTKTCGCCCCGA  742
              ||| |||||||||
Sbjct  2828  ATTTTCGCCCCGA  2840
```

prgH

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CCGAYTGCAAGACGAGCCGCGTTTTAAAACGGAATTGTAGCAGCACTGGCCGGGTTTTTTATATTGGGAATTGGGACTGTGGGGACGTTATGGATACTTAACTCGCCGCAGCGGCAGGCCGCAGAGCTCGATTCGTTATTGGGGC
AGGAGAAGGAGCGTTTTCAGGTGTTGCCAGGCCGGGACAAAATGCTCTATGTCGCTGCGCAAAATGAAAGAGATACGTTGTGGGCTCGTCAGGTTTTAGCGAGGGGCGATTATGATAAAAATGCGCGAGTGATTAACGAAAACGA
AGAAAATAAGCGTATCTCTATCTGGCTGGATACCTATTATCCGCAGCTGGCTTATTATCGGATTCATTTCGATGAGCCGCGTAAACCCGTTTTCTGGCTAAGCCGCCAGCGAAACACGATGAGCAAGAAAGAGCTCGAGGTGTTA
AGTCAAAAGCTGAGAGCGCTAATGCCTTACGCGGATTCGGTTAACATCACGTTGATGGACGATGTTACCGCAGCAGGCCAGGCGGAAGCGGGGCTAAAACAGCAGGCGTTACCTTATTCCCGCAGGAATCATAAGGGGGGCGTAA
CGTTTGTTATTCAGGGGGCGCTCGATGATGTAGAAATACTCAGAGCCCGTCAATTTGTCGATAGCTATTACCGCACATGGGGAGGACGCTATGTGCAGTTTGCGATCGAATTAAAAGATGACTGGCTCAAGGGSC
```

ref|NC_003197.1| *Salmonella enterica* subsp. *enterica* serovar Typhimurium str., LT2 chromosome, complete genome, Length=4857432, Score = 1293 bits (700), Expect = 0.0, Identities = 705/707 (99%), Gaps = 2/707 (0%), Strand=Plus/Minus

```
Query  9        AAG-ACGAGCCGCGTTTT-AAAACGGAATTGTAGCAGCACTGGCCGGGTTTTTTATATTGGGAATTGGGACTGTGGGGACGTTATGGATACTTAACTCGCCGCAGCGGCAGGCCGCAGAG  126
                 ||| |||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3017124  AAGAACGAGCCGCGTTTTAAAAACGGAATTGTAGCAGCACTGGCCGGGTTTTTTATATTGGGAATTGGGACTGTGGGGACGTTATGGATACTTAACTCGCCGCAGCGGCAGGCCGCAGAG  3017005

Query  127      CTCGATTCGTTATTGGGGCAGGAGAAGGAGCGTTTTCAGGTGTTGCCAGGCCGGGACAAAATGCTCTATGTCGCTGCGCAAAATGAAAGAGATACGTTGTGGGCTCGTCAGGTTTTAGCG  246
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3017004  CTCGATTCGTTATTGGGGCAGGAGAAGGAGCGTTTTCAGGTGTTGCCAGGCCGGGACAAAATGCTCTATGTCGCTGCGCAAAATGAAAGAGATACGTTGTGGGCTCGTCAGGTTTTAGCG  3016885

Query  247      AGGGGCGATTATGATAAAAATGCGCGAGTGATTAACGAAAACGAAGAAAATAAGCGTATCTCTATCTGGCTGGATACCTATTATCCGCAGCTGGCTTATTATCGGATTCATTTCGATGAG  366
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3016884  AGGGGCGATTATGATAAAAATGCGCGAGTGATTAACGAAAACGAAGAAAATAAGCGTATCTCTATCTGGCTGGATACCTATTATCCGCAGCTGGCTTATTATCGGATTCATTTCGATGAG  3016765

Query  367      CCGCGTAAACCCGTTTTCTGGCTAAGCCGCCAGCGAAACACGATGAGCAAGAAAGAGCTCGAGGTGTTAAGTCAAAAGCTGAGAGCGCTAATGCCTTACGCGGATTCGGTTAACATCACG  486
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3016764  CCGCGTAAACCCGTTTTCTGGCTAAGCCGCCAGCGAAACACGATGAGCAAGAAAGAGCTCGAGGTGTTAAGTCAAAAGCTGAGAGCGCTAATGCCTTACGCGGATTCGGTTAACATCACG  3016645

Query  487      TTGATGGACGATGTTACCGCAGCAGGCCAGGCGGAAGCGGGGCTAAAACAGCAGGCGTTACCTTATTCCCGCAGGAATCATAAGGGGGGCGTAACGTTTGTTATTCAGGGGGCGCTCGAT  606
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3016644  TTGATGGACGATGTTACCGCAGCAGGCCAGGCGGAAGCGGGGCTAAAACAGCAGGCGTTACCTTATTCCCGCAGGAATCATAAGGGGGGCGTAACGTTTGTTATTCAGGGGGCGCTCGAT  3016525
```

Appendix 8: (cont.) Nucleotide alignments for the 12 targeted potentially virulence genes. PCR products for the respected gene obtained from the *S.* Typhimurium F98 and SL1344 control strains were sequenced and aligned with published sequences of *S.* Typhimurium LT2 and SL1344 strains. The ambiguity characters within the sequences were not removed.

```
Query  607      GATGTAGAAATACTCAGAGCCCGTCAATTTGTCGATAGCTATTACCGCACATGGGGAGGACGCTATGTGCAGTTTGCGATCGAATTAAAAGATGACTGGCTCAAGGG  713
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3016524  GATGTAGAAATACTCAGAGCCCGTCAATTTGTCGATAGCTATTACCGCACATGGGGAGGACGCTATGTGCAGTTTGCGATCGAATTAAAAGATGACTGGCTCAAGGG  3016418
```

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gb|U21676.1|STU21676  Salmonella typhimurium PrgH protein (prgH), PrgI protein (prgI), PrgJ protein (prgJ), and PrgK protein (prgK) genes complete , cds, and OrgA protein (orgA) partial cds, Length=4035, Score = 1293 bits (700),  Expect = 0.0, Identities = 705/707 (99%), Gaps = 2/707 (0%),Strand=Plus/Plus

Query  9     AAG-ACGAGCCGCGTTTT-AAAACGGAATTGTAGCAGCACTGGCCGGGTTTTTTATATTGGGAATTGGGACTGTGGGGACGTTATGGATACTTAACTCGCCGCAGCGGCAGGCCGCAGAG  126
              ||| |||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1377  AAGAACGAGCCGCGTTTTAAAAACGGAATTGTAGCAGCACTGGCCGGGTTTTTTATATTGGGAATTGGGACTGTGGGGACGTTATGGATACTTAACTCGCCGCAGCGGCAGGCCGCAGAG  1496

Query  127   CTCGATTCGTTATTGGGGCAGGAGAAGGAGCGTTTTCAGGTGTTGCCAGGCCGGGACAAAATGCTCTATGTCGCTGCGCAAAATGAAAGAGATACGTTGTGGGCTCGTCAGGTTTTAGCG  246
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1497  CTCGATTCGTTATTGGGGCAGGAGAAGGAGCGTTTTCAGGTGTTGCCAGGCCGGGACAAAATGCTCTATGTCGCTGCGCAAAATGAAAGAGATACGTTGTGGGCTCGTCAGGTTTTAGCG  1616

Query  247   AGGGGCGATTATGATAAAAATGCGCGAGTGATTAACGAAAACGAAGAAAATAAGCGTATCTCTATCTGGCTGGATACCTATTATCCGCAGCTGGCTTATTATCGGATTCATTTCGATGAG  366
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1617  AGGGGCGATTATGATAAAAATGCGCGAGTGATTAACGAAAACGAAGAAAATAAGCGTATCTCTATCTGGCTGGATACCTATTATCCGCAGCTGGCTTATTATCGGATTCATTTCGATGAG  1736

Query  367   CCGCGTAAACCCGTTTTCTGGCTAAGCCGCCAGCGAAACACGATGAGCAAGAAAGAGCTCGAGGTGTTAAGTCAAAAGCTGAGAGCGCTAATGCCTTACGCGGATTCGGTTAACATCACG  486
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1737  CCGCGTAAACCCGTTTTCTGGCTAAGCCGCCAGCGAAACACGATGAGCAAGAAAGAGCTCGAGGTGTTAAGTCAAAAGCTGAGAGCGCTAATGCCTTACGCGGATTCGGTTAACATCACG  1856

Query  487   TTGATGGACGATGTTACCGCAGCAGGCCAGGCGGAAGCGGGGCTAAAACAGCAGGCGTTACCTTATTCCCGCAGGAATCATAAGGGGGGCGTAACGTTTGTTATTCAGGGGGCGCTCGAT  606
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1857  TTGATGGACGATGTTACCGCAGCAGGCCAGGCGGAAGCGGGGCTAAAACAGCAGGCGTTACCTTATTCCCGCAGGAATCATAAGGGGGGCGTAACGTTTGTTATTCAGGGGGCGCTCGAT  1976

Query  607   GATGTAGAAATACTCAGAGCCCGTCAATTTGTCGATAGCTATTACCGCACATGGGGAGGACGCTATGTGCAGTTTGCGATCGAATTAAAAGATGACTGGCTCAAGGG  713
              |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1977  GATGTAGAAATACTCAGAGCCCGTCAATTTGTCGATAGCTATTACCGCACATGGGGAGGACGCTATGTGCAGTTTGCGATCGAATTAAAAGATGACTGGCTCAAGGG  2083
```

Appendix 8: (cont.) Nucleotide alignments for the 12 targeted potentially virulence genes. PCR products for the respected gene obtained from the *S.* Typhimurium F98 and SL1344 control strains were sequenced and aligned with published sequences of *S.* Typhimurium LT2 and SL1344 strains. The ambiguity characters within the sequences were not removed.

sopE

```
AASGMWCCATMATTAAAGTGGTGCACTTCTGTCATAATGATCTTCTCCGCATAGATTTTAAAGGATGCCTGCTGATGTTGWTTGATAAACATACGTGGATACTTACTGTTTGCTGATGAAATCAACGGAGTTATAAAAGGATTGG
CCCCTGCGCCGCTTGGCGTAAAAACGTCATTTTTGGTTGTTCCGGGCAGACCTGCATTTTTCGCTGCTTCGCCAATTTCCTGAAGAAAAGGCGCTATGTTGATCCCTTTGCTGATGAGCAAATTACAACACTGATCTTTATTCTT
GCTGTAAACTGCCGATAGTATAGCTTCACGGGTCTGGCTGGCGTATGCGGGGTCTTTACTCGCACTACCTCTAATATCTATATCATTGAGCGTTTGAAGCATAAAATCTTTAACGACTTTATTTGTCAACACTGCCCGGCCCTCA
GATGCGCTTCCTCGGTGAAAGTGTGTTGCAGAAGACTCAGTGTTCTTATGCGAAATAAAACGTTCCGATAATTTTGACCTTAATTCGATGAAGTGATTTTTTACTGCGAGAATACTTTTTGCTAAAGAATTTTTCTCGGTTGATT
TTTCTTTTAGTAGTGTGGTTTCYGGTTTTTTGGAACAAKACCMCACTMYWAAARAAAAAWCAMCSAWMAAAATTCTTTAACMMAAARTATTCTSCRKWAAAAATCMTTCTTSAAATTARGGKMWWATTWTSGGAASKTTTTAWTT
TCSWKAASAACWCTGARTCYTYYGCMMMMCTTTCCCARKAASGCAYYTAKGGCSGGGCRYKGTTKMMMWATYAARGCWKYAARGATTTTTWKGCTYTCAACSYYWKTATMTTRRWTTTYAKAGGKRGTGSSRKTAAKAMCCCMWM
CCRCCA
```

gb|AF043239.1|AF043239 *Salmonella enterica* subsp. *enterica* serovar Typhimurium str. SL1344. *SopE (sopE)* gene, complete cds, Length=6125, Score = 1081 bits (585), Expect = 0.0, Identities = 594/599 (99%), Gaps = 2/599 (0%), Strand=Plus/Plus

```
Query  17    AGTGGTGC-ACTTCTGTCATAATGATCTTCTCCGCATAGATTTTAAAGGATGCCTGCTGATGTTGWTTGATAAACATACGTGGATACTTACTGTTTGCTGATGAAATCAACGGAGTTATA  135
             |||||||| |||||||||||||||||||||||||||||||||||||||||||||||||||||||| |||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1648  AGTGGTGCAACTTCTGTCATAATGATCTTCTCCGCATAGATTTTAAAGGATGCCTGCTGATGTTGATTGATAAACATACGTGGATACTTACTGTTTGCTGATGAAATCAACGGAGTTATA  1767

Query  136   AAAGGATTGGCCCCTGCGCCGCTTGGCGTAAAAACGTCATTTTTGGTTGTTCCGGGCAGACCTGCATTTTTCGCTGCTTCGCCAATTTCCTGAAGAAAAGGCGCTATGTTGATCCCTTTG  255
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1768  AAAGGATTGGCCCCTGCGCCGCTTGGCGTAAAAACGTCATTTTTGGTTGTTCCGGGCAGACCTGCATTTTTCGCTGCTTCGCCAATTTCCTGAAGAAAAGGCGCTATGTTGATCCCTTTG  1887

Query  256   CTGATGAGCAAATTACAACACTGATCTTTATTCTTGCTGTAAACTGCCGATAGTATAGCTTCACGGGTCTGGCTGGCGTATGCGGGGTCTTTACTCGCACTACCTCTAATATCTATATCA  375
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1888  CTGATGAGCAAATTACAACACTGATCTTTATTCTTGCTGTAAACTGCCGATAGTATAGCTTCACGGGTCTGGCTGGCGTATGCGGGGTCTTTACTCGCACTACCTCTAATATCTATATCA  2007

Query  376   TTGAGCGTTTGAAGCATAAAATCTTTAACGACTTTATTTGTCAACACTGCCCGGCCCTCAGATGCGCTTCCTCGGTGAAAGTGTGTTGCAGAAGACTCAGTGTTCTTATGCGAAATAAAA  495
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2008  TTGAGCGTTTGAAGCATAAAATCTTTAACGACTTTATTTGTCAACACTGCCCGGCCCTCAGATGCGCTTCCTCGGTGAAAGTGTGTTGCAGAAGACTCAGTGTTCTTATGCGAAATAAAA  2127

Query  496   CGTTCCGATAATTTTGACCTTAATTCGATGAAGTGAttttttactgcgagaatacttttgctaaagaatttttctcggttgatttttctttttagtagtgtggtttcyggttttttGGA  614
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||  |||||| |||
Sbjct  2128  CGTTCCGATAATTTTGACCTTAATTCGATGAAGTGATTTTTTACTGCGAGAATACTTTTTGCTAAAGAATTTTTCTCGGTTGATTTTTCTTTTAGTAGTGTGGTTTCCTGTTTTT-GGA  2245
```

Appendix 8: (cont.) Nucleotide alignments for the 12 targeted potentially virulence genes. PCR products for the respected gene obtained from the *S.* Typhimurium F98 and SL1344 control strains were sequenced and aligned with published sequences of *S.* Typhimurium LT2 and SL1344 strains. The ambiguity characters within the sequences were not removed.

misL

```
ARRTRRRRMSTGATGGCATCCGTACTCCGGCGCGATACTCCGGATAACCCGGTATTTATTATTACCGGTGACCGTACCCSCMTCTACGTGGATGGTCAGGATGGKGACGGTATTAACGCCGGATATAACTCACTGGGSCAGGGTT
GGACCGGATCGGCCAATATTTACGTCGGCGACGATCTGTATATCAAAAMSACCGGTAGCCAGGGACGTGGCATTACAGCCAACGCCATGCGGGATGCGTCACGTGCCAAAAATACKATTGTCGTCGGCAATCSTGCGCATATTGT
TACTACCGGCGACAGTTCKGAGGGGCTGCGTACCGGACAAAGCGGTTCGTTGATTCGTCTGGGTGACGATGCAACTATCGAAACATCCGGCGCATCCTCAACGGGGATTTACGCAGCATCCTCCTCCARAACCSAGCTTGGCAAC
AACGCTACTATTACGGTGAACGGTGCCAGCGCACACSCGGWATATGCAACTAACGCTACCGKTAATTTGGGAGARAATGCCMCTWTTWGCGTKAACASCGCSAM
```

```
ref|NC 003197.1| Salmonella enterica subsp. enterica serovar Typhimurium str. LT2 chromosome, complete genome, Length=4857432, Score =  887 bits
(480),  Expect = 0.0,  Identities = 505/527 (95%), Gaps = 2/527 (0%),  Strand=Plus/Plus

Query  12       GATGGCATCCGTACTC-CGGCGCG-ATACTCCGGATAACCCGGTATTTATTATTACCGGTGACCGTACCCSCMTCTACGTGGATGGTCAGGATGGKGACGGTATTAACGCCGGATATAAC  129
                ||||||||||||||| ||||||| |||||||||||||||||||||||||||||||||||||||||||||||||| | |||||||||||||||||||| ||||||||||||||||||||||||
Sbjct  3955163  GATGGCATCCGTACTCACGGCGCGAATACTCCGGATAACCCGGTATTTATTATTACCGGTGACCGTACCCGCATCTACGTGGATGGTCAGGATGGTGACGGTATTAACGCCGGATATAAC  3955282

Query  130      TCACTGGGSCAGGGTTGGACCGGATCGGCCAATATTTACGTCGGCGACGATCTGTATATCaaaamsaCCGGTAGCCAGGGACGTGGCATTACAGCCAACGCCATGCGGGATGCGTCACGT  249
                ||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||  |||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3955283  TCACTGGGCCAGGGTTGGACCGGATCGGCCAATATTTACGTCGGCGACGATCTGTATATCAAAACGACCGGTAGCCAGGGACGTGGCATTACAGCCAACGCCATGCGGGATGCGTCACGT  3955402

Query  250      GCCAAAAATACKATTGTCGTCGGCAATCSTGCGCATATTGTTACTACCGGCGACAGTTCKGAGGGGCTGCGTACCGGACAAAGCGGTTCGTTGATTCGTCTGGGTGACGATGCAACTATC  369
                |||||||||||| ||||||||||||||| |||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3955403  GCCAAAAATACGATTGTCGTCGGCAATCGTGCGCATATTGTTACTACCGGCGACAGTTCGGAGGGGCTGCGTACCGGACAAAGCGGTTCGTTGATTCGTCTGGGTGACGATGCAACTATC  3955522

Query  370      GAAACATCCGGCGCATCCTCAACGGGGATTTACGCAGCATCCTCCTCCARAACCSAGCTTGGCAACAACGCTACTATTACGGTGAACGGTGCCAGCGCACACSCGGWATATGCAACTAAC  489
                |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||| |||| |||||||||||||||||||||||||||||||||||||||| ||| ||||||||||||||
Sbjct  3955523  GAAACATCCGGCGCATCCTCAACGGGGATTTACGCAGCATCCTCCTCCAGAACCGAGCTTGGCAACAACGCTACTATTACGGTGAACGGTGCCAGCGCACACGCGGTATATGCAACTAAC  3955642

Query  490      GCTACCGKTAATTTGGGAGARAATGCCMCTWTTWGCGTKAACASCGC  536
                ||||||| |||||||||||| |||||| || || |||| |||| |||
Sbjct  3955643  GCTACCGTTAATTTGGGAGAGAATGCCACTATTAGCGTTAACAGCGC  3955689
```

Appendix 8: (cont.) Nucleotide alignments for the 12 targeted potentially virulence genes. PCR products for the respected gene obtained from the *S.* Typhimurium F98 and SL1344 control strains were sequenced and aligned with published sequences of *S.* Typhimurium LT2 and SL1344 strains. The ambiguity characters within the sequences were not removed.

sifA

```
GRASGSCCTMGGAGATTTTTTTTGAGTTGAAGAGTTAGCMTGCGCATCGCAAAGAGATAGATTTCAGGTTCATAATCCTCATGAAAATGATGCCACCATTATTCTTCGCATCATGGATCAAAACGAAGAGAACGAATTGTTACGT
ATCACTCAAAATACCGATACCTTTAGCTGTGAAGTCATGGGGAATCTTTATTTTTTAATGAAAGATCGCCCGGATATTTTAAAATCGCATCCACAAATGACGGCCATGATTAARAGAAGATATAGCGAAATCGTARACTACCCCC
TCCCTTCGACATTATGTCTCAATCCTGCTGGCGCGCCGATATTATCGGTTCCATTAGACAACATAGAGGGGTATTTATATACTGAATTGAGAAAAGGACATTTAGATGGGTGGAAAGCGCAAGAAAAGGCAAMAA

gb|U51867.1|STU51867  Salmonella enterica subsp. enterica serovar Typhimurium str. SL1344. PotB (potB) gene, partial cds, SifA (sifA), complete
cds, and PotC (potC) gene, partial cds, Length=1705,  Score =  743 bits (402),  Expect = 0.0, Identities = 408/412 (99%), Gaps = 1/412 (0%,
Strand=Plus/Plus

Query  12   GAGAtttttttttGAGTTG-AAGAGTTAGCMTGCGCATCGCAAAGAGATAGATTTCAGGTTCATAATCCTCATGAAAATGATGCCACCATTATTCTTCGCATCATGGATCAAAACGAAGAG  130
            |||||||||||||||||| |||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  566  GAGATTTTTTTTGAGTTGAAAGAGTTAGCCTGCGCATCGCAAAGAGATAGATTTCAGGTTCATAATCCTCATGAAAATGATGCCACCATTATTCTTCGCATCATGGATCAAAACGAAGAG  685

Query  131  AACGAATTGTTACGTATCACTCAAAATACCGATACCTTTAGCTGTGAAGTCATGGGGAATCTTTATTTTTTAATGAAAGATCGCCCGGATATTTTAAAATCGCATCCACAAATGACGGCC  250
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  686  AACGAATTGTTACGTATCACTCAAAATACCGATACCTTTAGCTGTGAAGTCATGGGGAATCTTTATTTTTTAATGAAAGATCGCCCGGATATTTTAAAATCGCATCCACAAATGACGGCC  805

Query  251  ATGATTAARAGAAGATATAGCGAAATCGTARACTACCCCCTCCCTTCGACATTATGTCTCAATCCTGCTGGCGCGCCGATATTATCGGTTCCATTAGACAACATAGAGGGGTATTTATAT  370
            |||||||| |||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  806  ATGATTAAGAGAAGATATAGCGAAATCGTAGACTACCCCCTCCCTTCGACATTATGTCTCAATCCTGCTGGCGCGCCGATATTATCGGTTCCATTAGACAACATAGAGGGGTATTTATAT  925

Query  371  ACTGAATTGAGAAAAGGACATTTAGATGGGTGGAAAGCGCAAGAAAAGGCAA  422
            ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  926  ACTGAATTGAGAAAAGGACATTTAGATGGGTGGAAAGCGCAAGAAAAGGCAA  977
```

Appendix 8: (cont.) Nucleotide alignments for the 12 targeted potentially virulence genes. PCR products for the respected gene obtained from the *S.* Typhimurium F98 and SL1344 control strains were sequenced and aligned with published sequences of *S.* Typhimurium LT2 and SL1344 strains. The ambiguity characters within the sequences were not removed.

pipD

```
CSSYRGMATGMGTGAGCCGGTTTCATTCGGCGGGCGTCGGAATGAGCGCAACGGAAACCATTTATAACGGCAGAGCGGCGCTGGCTGCCGATCCTTACGTGACAAAAACGGGAATCACGGAAGACGCCATTGAGTCCGTGATCCT
GCCAGTGGCGCAATCGGCGCGTCAGGGCGCCAAATTACTGGGAGATATTATTGAACAAAAAGGCGCGGGCGAAGGTTTCGGCGTCGCGTTTATTGATAGCAAAGAGATATGGTATCTGGAGACGGGAAGCGGTCATCAATGGCTG
GCAGTACGACTTCCGGCAGATAGCTATTTCGTTTCCGCCAATCAGGGACGTTTACGCCATTACGATCCRATGATAACGA
```

```
ref|NC 003197.1| Salmonella enterica subsp. enterica serovar Typhimurium str. LT2 chromosome, complete genome, Length=4857432, Score =  643 bits
(348),  Expect = 0.0, Identities = 359/364 (98%), Gaps = 3/364 (0%), Strand=Plus/Minus

Query  8        ATGMGTG-AGCCGGTTTC-ATTCGGCGGGCGTCGGAATGAGCGCAACGGAAACCATTTATAACGGCAGAGCGGCGCTGGCTGCCGATCCTTACGTGACAAAAACGGGAATCACGGAAGAC  125
                 ||| ||| |||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1181825  ATGGGTGAAGCCGGTTTCAATTCGGCGGGCGTCGGAATGAGCGCAACGGAAACCATTTATAACGGCAGAGCGGCGCTGGCTGCCGATCCTTACGTGACAAAAACGGGAATCACGGAAGAC  1181706

Query  126      GCCATTGAGTCCGTGATCCTGCCAGTGGCGCAATCGGCGCGTCAGGGCGCCAAATTACTGGGAGATATTATTGAACAAAAAGGCGCGGGCGAAGGTTTCGGCGTCGCGTTTATTGATAGC  245
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1181705  GCCATTGAGTCCGTGATCCTGCCAGTGGCGCAATCGGCGCGTCAGGGCGCCAAATTACTGGGAGATATTATTGAACAAAAAGGCGCGGGCGAAGGTTTCGGCGTCGCGTTTATTGATAGC  1181586

Query  246      AAAGAGATATGGTATCTGGAGACGGGAAGCGGTCATCAATGGCTGGCAGTACGACTTCCGGCAGATAGCTATTTCGTTTCCGCCAATCAGGGACGTTTACGCCATTACGATCCRA-TGAT  364
                 |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||| | ||||
Sbjct  1181585  AAAGAGATATGGTATCTGGAGACGGGAAGCGGTCATCAATGGCTGGCAGTACGACTTCCGGCAGATAGCTATTTCGTTTCCGCCAATCAGGGACGTTTACGCCATTACGATCCGAATGAT  1181466

Query  365      AACG  368
                 ||||
Sbjct  1181465  AACG  1181462
```

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gb|AF060858.1|  Salmonella dublin regulatory protein CopR (copR), histidine kinase, (copS), SPI-4 pathogenicity island containing dipeptidase
homolog (pipD), SopB (sopB), PipC (pipC), PipB (pipB), and PipA (pipA) genes, complete cds; and tRNA-Ser gene, complete, sequence; and unknown
genes, Length=9739,  Score =  632 bits (342),  Expect = 4e-178, Identities = 357/364 (98%), Gaps = 3/364 (0%), Strand=Plus/Plus

Query  8     ATGMGTG-AGCCGGTTTC-ATTCGGCGGGCGTCGGAATGAGCGCAACGGAAACCATTTATAACGGCAGAGCGGCGCTGGCTGCCGATCCTTACGTGACAAAAACGGGAATCACGGAAGAC  125
              ||| ||| |||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3147  ATGGGTGAAGCCGGTTTCAATTCGGCGGGCGTCGGAATGAGCGCAACGGAAACCATTTATAACGGCAGAGCGGCGCTGGCTGCCGATCCTTACGTGACAAAAACAGGAATCACGGAAGAC  3266

Query  126   GCCATTGAGTCCGTGATCCTGCCAGTGGCGCAATCGGCGCGTCAGGGCGCCAAATTACTGGGAGATATTATTGAACAAAAAGGCGCGGGCGAAGGTTTCGGCGTCGCGTTTATTGATAGC  245
              |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3267  GCCATTGAGTCCGTGATCCTGCCAGTGGCGCAATCGGCGCGTCAGGGCGCCAAATTACTGGGAGACATTATTGAACAAAAAGGCGCGGGCGAAGGTTTCGGCGTCGCGTTTATTGATAGC  3386
```

Appendix 8: (cont.) Nucleotide alignments for the 12 targeted potentially virulence genes. PCR products for the respected gene obtained from the *S.* Typhimurium F98 and SL1344 control strains were sequenced and aligned with published sequences of *S.* Typhimurium LT2 and SL1344 strains. The ambiguity characters within the sequences were not removed.

```
Query  246   AAAGAGATATGGTATCTGGAGACGGGAAGCGGTCATCAATGGCTGGCAGTACGACTTCCGGCAGATAGCTATTTCGTTTCCGCCAATCAGGGACGTTTACGCCATTACGATCCRA-TGATAACG  368
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||| | ||||||||
Sbjct  3387  AAAGAGATATGGTATCTGGAGACGGGAAGCGGTCATCAATGGCTGGCAGTACGACTTCCGGCAGATAGCTATTTCGTTTCCGCCAATCAGGGACGTTTACGCCATTACGATCCGAATGATAACG  3510
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orfL

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CTCAARGCTGAGTTTGAGCATACGGCACGTTTAACGGTGGTACTCACCGAATCAGCCGACGGCTGGCGCTATCGACCGGATTCTGCTTTGGCGGACGGTAGCTACACATTCACCGTGACGGTAACAGATGTGGCAGGCAACCAGC
AAACATCCGCGCCTTTAAAGGTGACGATAGACGGTACGTTGACTACGCCGGTGATTGAACTGGCAGCTGGCGAAGATAGCGGTACTGTTGGCGATCGCCTCACCAATCACGATCGGCCTGTGTTCGACATACATCAGGTTGATTC
TGMGTTACGCGCA
```

gi|16763390|ref|NC_003197.1| *Salmonella enterica* subsp. *enterica* serovar Typhimurium str. LT2 chromosome, complete genome lcl|19363, Length=303,
Score = 516 bits (279), Expect = 5e-147, Identities = 293/299 (98%), Gaps = 5/299 (1%), Strand=Plus/Plus

```
Query  4491961  TGAGTATTGAGCATAACGGCACGTTTAAAACGGTGGTACTCACCGAATCAGCCGACGGCTGGCGCTATCGACCGGATTCTGCTTTGGCGGACGGTAGCTACACATTCACCGTGACGGTAA  4492080
                |||||  ||||||||  ||||||||||  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  9        TGAGT-TTGAGCAT-ACGGCACGTTT--AACGGTGGTACTCACCGAATCAGCCGACGGCTGGCGCTATCGACCGGATTCTGCTTTGGCGGACGGTAGCTACACATTCACCGTGACGGTAA  124

Query  4492081  CAGATGTGGCAGGCAACCAGCAAACATCCGCGCCTTTAAAGGTGACGATAGACGGTACGTTGACTACGCCGGTGATTGAACTGGCAGCTGGCGAAGATAGCGGTACTGTTGGCGATCGCC  4492200
                |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  125      CAGATGTGGCAGGCAACCAGCAAACATCCGCGCCTTTAAAGGTGACGATAGACGGTACGTTGACTACGCCGGTGATTGAACTGGCAGCTGGCGAAGATAGCGGTACTGTTGGCGATCGCC  244

Query  4492201  TCACCAATCACGATCGGCCTGTGTTCGACATACATCAGGTTGATTCTGACGTTACGCGC  4492259
                |||||||||||||||||||||||||||||||||||||||||||||||||||  |||||||||
Sbjct  245      TCACCAATCACGATCGGCCTGTGTTCGACATACATCAGGTTGATTCTGM-GTTACGCGC  302
```

emb|AJ576316.1| *Salmonella* typhimurium ST4/74 Salmonella Pathogenicity Island 4 (siiABCDEF genes), Length=24660, Score = 516 bits (279), Expect =
3e-149, Identities = 293/299 (97%), Gaps = 5/299 (1%), Strand=Plus/Plus

```
Query  9      TGAGT-TTGAGCAT-ACGGCACGTTT--AACGGTGGTACTCACCGAATCAGCCGACGGCTGGCGCTATCGACCGGATTCTGCTTTGGCGGACGGTAGCTACACATTCACCGTGACGGTAA  124
              |||||  ||||||||  ||||||||||  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  15049  TGAGTATTGAGCATAACGGCACGTTTAAAACGGTGGTACTCACCGAATCAGCCGACGGCTGGCGCTATCGACCGGATTCTGCTTTGGCGGACGGTAGCTACACATTCACCGTGACGGTAA  15168

Query  125    CAGATGTGGCAGGCAACCAGCAAACATCCGCGCCTTTAAAGGTGACGATAGACGGTACGTTGACTACGCCGGTGATTGAACTGGCAGCTGGCGAAGATAGCGGTACTGTTGGCGATCGCC  244
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  15169  CAGATGTGGCAGGCAACCAGCAAACATCCGCGCCTTTAAAGGTGACGATAGACGGTACGTTGACTACGCCGGTGATTGAACTGGCAGCTGGCGAAGATAGCGGTACTGTTGGCGATCGCC  15288

Query  245    TCACCAATCACGATCGGCCTGTGTTCGACATACATCAGGTTGATTCTGM-GTTACGCGC  302
              ||||||||||||||||||||||||||||||||||||||||||||||||||  |||||||||
Sbjct  15289  TCACCAATCACGATCGGCCTGTGTTCGACATACATCAGGTTGATTCTGACGTTACGCGC  15347
```

Appendix 8: (cont.) Nucleotide alignments for the 12 targeted potentially virulence genes. PCR products for the respected gene obtained from the *S.* Typhimurium F98 and SL1344 control strains were sequenced and aligned with published sequences of *S.* Typhimurium LT2 and SL1344 strains. The ambiguity characters within the sequences were not removed.

spiC

```
AGMWGCGAGTRATTTTCTMGAGCATTTTCTATTTCTTTGTTCTTAAATGACCTGGAAAGCTTACCTAAGCCTTGTCTTGCCTATGTGACACTACTGCTTGCAGCACACCCGGACGTCCATGATTATGCTATACAGCTCACAGCGG
ATGGGGGATGGTTAAACGGTTATTATACCACAAGTAGTAGCTCTGAGCTTATTGCTATTGAGATAGAAAAACACCTGGCTTTAACTTGCATTTTAAAAAATGTAATACGCAATACCATAAACT
gi|16763390|ref|NC_003197.1| Salmonella enterica subsp. enterica serovar Typhimurium str. LT2 chromosome, complete genome, Length=268, Score =
457 bits (247),  Expect = 3e-129 Identities = 256/260 (99%), Gaps = 3/260 (1%), Strand=Plus/Plus


Query  1480122  ATTTTCTCAGAAGCATTTTCTATTTCTTTGTTCTTAAATGACCTGGAAAGCTTACCTAAGCCTTGTCTTGCCTATGTGACACTACTGCTTGCAGCACACCCGGACGTCCATGATTATGCT  1480241
                |||||||  | ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  12       ATTTTCT-MG-AGCATTTTCTATTTCTTTGTTCTTAAATGACCTGGAAAGCTTACCTAAGCCTTGTCTTGCCTATGTGACACTACTGCTTGCAGCACACCCGGACGTCCATGATTATGCT  129

Query  1480242  ATACAGCTCACAGCGGATGGGGGATGGTTAAACGGTTATTATACCACAAGTAGTAGCTCTGAGCTTATTGCTATTGAGATAGAAAAACACCTGGCTTTAACTTGCATTTTAAAAAATGTA  1480361
                |||||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  130      ATACAGCTCACAGCGGATGGGGGATGGTTAAACGGTTATTATACCACAAGTAGTAGCTCTGAGCTTATTGCTATTGAGATAGAAAAACACCTGGCTTTAACTTGCATTTTAAAAAATGTA  249

Query  1480362  ATACGCAATCACCATAAACT  1480381
                ||||||||| ||||||||||
Sbjct  250      ATACGCAAT-ACCATAAACT  268


gb|U51927.1|STU51927  Salmonella typhimurium mouse-virulent wild-type strain 14028s. SpiR and SpiB genes, partial cds, and SpiC and SpiA genes,
complete cds, Length=5715, Score =  457 bits (247),  Expect = 2e-125, Identities = 256/260 (98%), Gaps = 3/260 (1%)Strand=Plus/Plus

Query  12    ATTTTCT-MG-AGCATTTTCTATTTCTTTGTTCTTAAATGACCTGGAAAGCTTACCTAAGCCTTGTCTTGCCTATGTGACACTACTGCTTGCAGCACACCCGGACGTCCATGATTATGCT  129
              |||||||  | ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2991  ATTTTCTCAGAAGCATTTTCTATTTCTTTGTTCTTAAATGACCTGGAAAGCTTACCTAAGCCTTGTCTTGCCTATGTGACACTACTGCTTGCAGCACACCCGGACGTCCATGATTATGCT  3110

Query  130   ATACAGCTCACAGCGGATGGGGGATGGTTAAACGGTTATTATACCACAAGTAGTAGCTCTGAGCTTATTGCTATTGAGATAGAAAAACACCTGGCTTTAACTTGCATTTTAAAAAATGTA  249
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3111  ATACAGCTCACAGCGGATGGGGGATGGTTAAACGGTTATTATACCACAAGTAGTAGCTCTGAGCTTATTGCTATTGAGATAGAAAAACACCTGGCTTTAACTTGCATTTTAAAAAATGTA  3230

Query  250   ATACGCAAT-ACCATAAACT  268
              ||||||||| ||||||||||
Sbjct  3231  ATACGCAATCACCATAAACT  3250
```

Appendix 8: (cont.) Nucleotide alignments for the 12 targeted potentially virulence genes. PCR products for the respected gene obtained from the *S.* Typhimurium F98 and SL1344 control strains were sequenced and aligned with published sequences of *S.* Typhimurium LT2 and SL1344 strains. The ambiguity characters within the sequences were not removed.

sopB

```
CCGGCSGGGGCAAAGMWTTGTAGAAATCTTATCGAACTTAWTGCAGCACGCACTCAGCAGCAGGATGGCTTACCTGCAAAAGAAGCTCATCGCTTTGCGGCAGTAGCGTTTAGAGATGCTCAGGTCAAGCAGCTTAATAACCAGC
CCTGGCAAACCATAAAAAATACACTCACGCATAACGGGCATCACTAAACGMTRCTGMCGSAAAGYGAWRAGCTWCTTTTGCAGWTWWSCCMTCKMGCTGCTMAGTGCGTGCTSCAMTAARTTSRATWAMTWTTTYCTYCAKTGCT
TTTGCCSSKYCTTCTYYTKGTTTTRTTGSKRGCCGSYCCGAMMKC

ref|NC 003197.1| Salmonella enterica subsp. enterica serovar Typhimurium str.LT2 chromosome, complete genome, Length=4857432, Score = 307 bits
(166),  Expect = 4e-84, Identities = 167/168 (99%), Gaps = 0/168 (0%), Strand=Plus/Minus

Query  24       AAATCTTATCGAACTTAWTGCAGCACGCACTCAGCAGCAGGATGGCTTACCTGCAAAAGAAGCTCATCGCTTTGCGGCAGTAGCGTTTAGAGATGCTCAGGTCAAGCAGCTTAATAACCA  143
                ||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1179530  AAATCTTATCGAACTTATTGCAGCACGCACTCAGCAGCAGGATGGCTTACCTGCAAAAGAAGCTCATCGCTTTGCGGCAGTAGCGTTTAGAGATGCTCAGGTCAAGCAGCTTAATAACCA  1179411

Query  144      GCCCTGGCAAACCATAAAAAATACACTCACGCATAACGGGCATCACTA  191
                ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1179410  GCCCTGGCAAACCATAAAAAATACACTCACGCATAACGGGCATCACTA  1179363


gb|AF213335.2|AF213335 Salmonella typhimurium invasion protein D (sopB) gene, partial, cds, Length=1300, Score =  307 bits (166),  Expect = 2e-
80, Identities = 167/168 (99%), Gaps = 0/168 (0%), Strand=Plus/Plus

Query  24   AAATCTTATCGAACTTAWTGCAGCACGCACTCAGCAGCAGGATGGCTTACCTGCAAAAGAAGCTCATCGCTTTGCGGCAGTAGCGTTTAGAGATGCTCAGGTCAAGCAGCTTAATAACCA  143
            ||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  99   AAATCTTATCGAACTTATTGCAGCACGCACTCAGCAGCAGGATGGCTTACCTGCAAAAGAAGCTCATCGCTTTGCGGCAGTAGCGTTTAGAGATGCTCAGGTCAAGCAGCTTAATAACCA  218

Query  144  GCCCTGGCAAACCATAAAAAATACACTCACGCATAACGGGCATCACTA  191
            ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  219  GCCCTGGCAAACCATAAAAAATACACTCACGCATAACGGGCATCACTA  266
```

Appendix 8: (cont.) Nucleotide alignments for the 12 targeted potentially virulence genes. PCR products for the respected gene obtained from the *S.* Typhimurium F98 and SL1344 control strains were sequenced and aligned with published sequences of *S.* Typhimurium LT2 and SL1344 strains. The ambiguity characters within the sequences were not removed.

pefA

```
GGRACTGGATCGTTAGCTACCAGCCGGGTATTTTGTTGACTTCGCCATGAAACCGGTTGACCCGAATGCACAGGGTTGTGCAAATCTGGCTCAGAAAACGGCCACTGTTTCCTGGGCTTCTGCTGAATRRMGCATAACAARTG

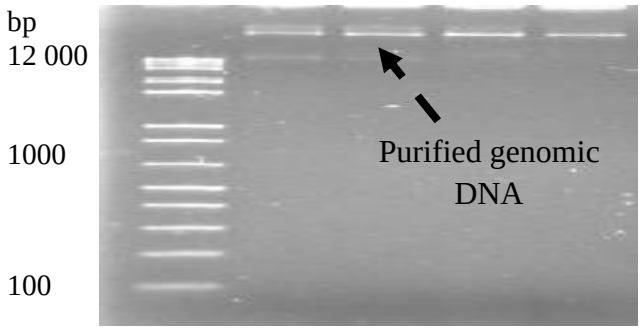
ref|NC_003277.1| Salmonella typhimurium LT2 plasmid pSLT, complete sequence, Length=93939, Score =  195 bits (105),  Expect = 2e-52, Identities =
110/112 (98%), Gaps = 2/112 (1%), Strand=Plus/Minus

Query  16     GCT-ACCAGCCGGGT-ATTTTGTTGACTTCGCCATGAAACCGGTTGACCCGAATGCACAGGGTTGTGCAAATCTGGCTCAGAAAACGGCCACTGTTTCCTGGGCTTCTGCTG  125
               ||| |||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  13913  GCTAACCAGCCGGGTAATTTTGTTGACTTCGCCATGAAACCGGTTGACCCGAATGCACAGGGTTGTGCAAATCTGGCTCAGAAAACGGCCACTGTTTCCTGGGCTTCTGCTG  13802

gb|L08613.1|STYPEFABCD Salmonella typhimurium ORF1, partial cds; pefB, pefA, pefC, pefD, ORF5, ORF6, pefI, ORF7, ORF8, ORF9, rck, ORF11,
complete,  cds's; 13900 base-pairs, Length=13900,  Score =  195 bits (105),  Expect = 7e-47, Identities = 110/112 (98%), Gaps = 2/112 (1%),
Strand=Plus/Plus

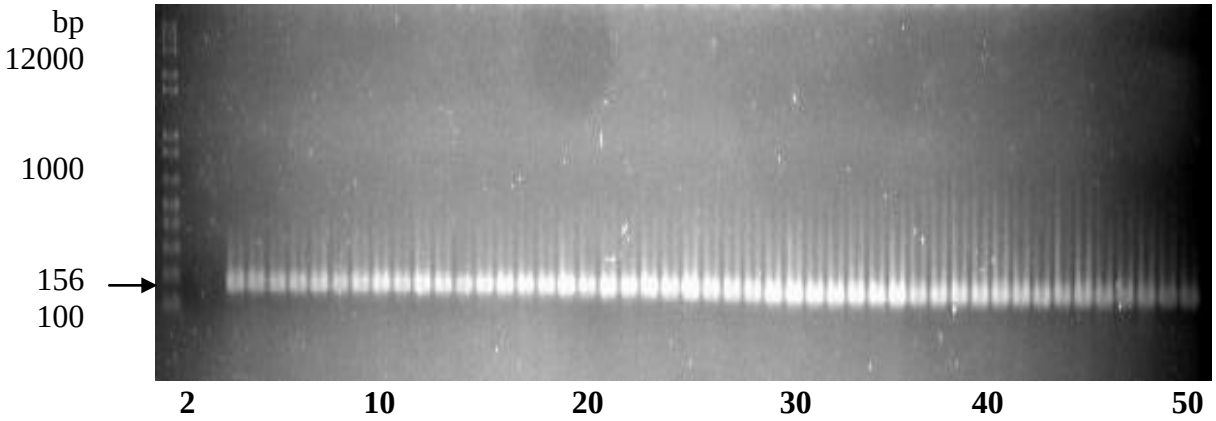
Query  16    GCT-ACCAGCCGGGT-ATTTTGTTGACTTCGCCATGAAACCGGTTGACCCGAATGCACAGGGTTGTGCAAATCTGGCTCAGAAAACGGCCACTGTTTCCTGGGCTTCTGCTG  125
              ||| |||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3052  GCTAACCAGCCGGGTAATTTTGTTGACTTCGCCATGAAACCGGTTGACCCGAATGCACAGGGTTGTGCAAATCTGGCTCAGAAAACGGCCACTGTTTCCTGGGCTTCTGCTG  31
```

Appendix 9. High molecular weight DNA purified from the *Salmonella* isolates was visualised on agarose gels (arrow).



Appendix 10. PCR products from the 12 targeted potentially virulence genes from all 90 *Salmonella* isolates and F98 and SL1344 control strains were visualised on agarose gel.

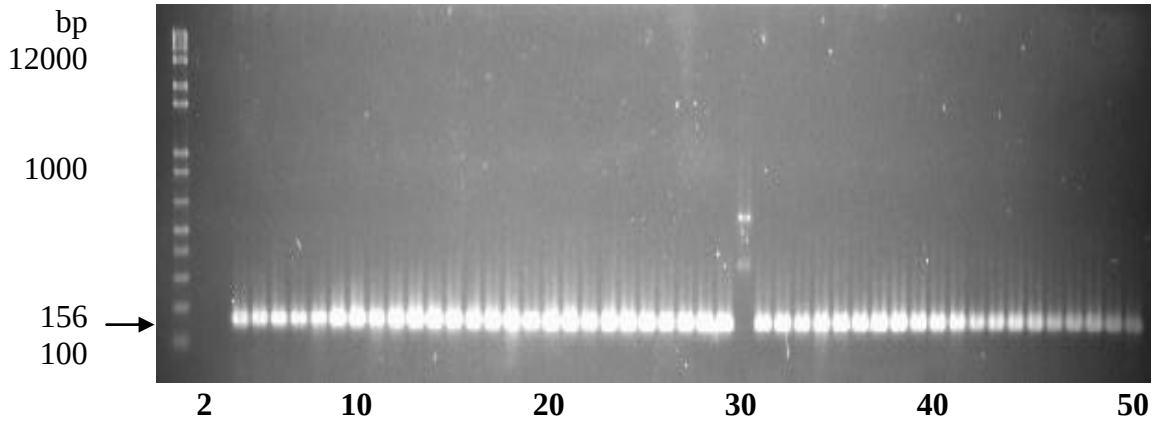
Example: *pefA* 156 bp



Lane	Sample	Results	Lane	Sample	Results
1	DNA marker	+	**26**	DT21	+
2	PCR mix (-) DNA	-	**27**	DT22	+
3	PCR mix (-) *Taq*	-	**28**	DT23	+
4	(+) control F98	+	**29**	DT24	+
5	(+) control SL1344	+	**30**	DT25	+
6	DT1	+	**31**	DT26	+
7	DT2	+	**32**	DT27	+
8	DT3	+	**33**	DT28	+
9	DT4	+	**34**	DT29	+
10	DT5	+	**35**	DT30	+
11	DT6	+	**36**	DT31	+
12	DT7	+	**37**	DT32	+
13	DT8	+	**38**	DT33	+
14	DT9	+	**39**	DT34	+
15	DT10	+	**40**	DT35	+
16	DT11	+	**41**	DT36	+
17	DT12	+	**42**	DT37	+
18	DT13	+	**43**	DT38	+
19	DT14	+	**44**	DT39	+
20	DT15	+	**45**	DT40	+
21	DT16	+	**46**	DT41	+
22	DT17	+	**47**	DT42	+
23	DT18	+	**48**	DT43	+
24	DT19	+	**49**	DT44	+
25	DT20	+	**50**	DT45	+

Appendix 10. (cont.) PCR products from the 12 targeted potentially virulence genes from all 90 *Salmonella* isolates and F98 and SL1344 control strains were visualised on agarose gel.

Example: *pefA* 156 bp



Lane	Sample	Results	Lane	Sample	Results
1	DNA marker	+	**26**	DT66	+
2	PCR mix (-) DNA	-	**27**	DT67	+
3	PCR mix (-) *Taq*	-	**28**	DT68	+
4	(+) control F98	+	**29**	DT69	+
5	(+) control SL1344	+	**30**	DT70	-
6	DT46	+	**31**	DT71	+
7	DT47	+	**32**	DT72	+
8	DT48	+	**33**	DT73	+
9	DT49	+	**34**	DT74	+
10	DT50	+	**35**	DT75	+
11	DT51	+	**36**	DT76	+
12	DT52	+	**37**	DT77	+
13	DT53	+	**38**	DT78	+
14	DT54	+	**39**	DT79	+
15	DT55	+	**40**	DT80	+
16	DT56	+	**41**	DT81	+
17	DT57	+	**42**	DT82	+
18	DT58	+	**43**	DT83	+
19	DT59	+	**44**	DT84	+
20	DT60	+	**45**	DT85	+
21	DT61	+	**46**	DT86	+
22	DT62	+	**47**	DT87	+
23	DT63	+	**48**	DT88	+
24	DT64	+	**49**	DT89	+
25	DT65	+	**50**	DT90	+

Appendix 11. Conditions for the PFGE performed using either *Xba*I or *Spe*I restriction enzymes with all 90 *Salmonella* isolates and control strains *S.* Typhimurium F98 and SL1344 and *S.* Braenderup. The PFGE banding patterns were visualised on an agarose gel.

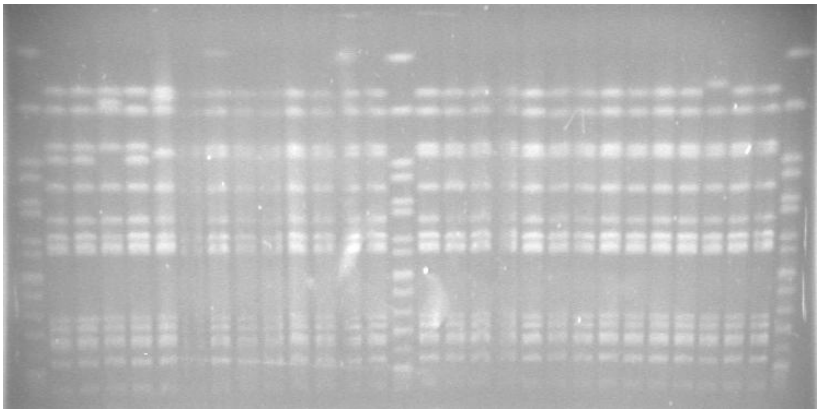
Example PFGE worksheet: 11

Machine DR-11	**Agarose concentration:** 1.0% Seakem gold	**Enzyme** *Xba*I + BSA 2 hours (hrs)		
Run conditions	**volts/cm** 6.0	18.5 **hr**	14 **°C**	**Included angle** 120°
Ramping	2.2 **sec to** 63.8 **sec in**	linear **factor**		**Date** 17 June, 2010

Lane	Sample	Comments
1	*S.* Braenderup ladder	
2	*S. enterica* (1)	control F98 had too many plugs for 1 tube so making sure all the same
3	S. *enterica* (2)	control F98
4	*S.* Typhimurium	control SL1344
5	*S. enterica* (3)	control F98
6	DT1	odd shaped plug (possibly from the end)
7	DT2	plug too soft-repeat
8	DT3	
9	DT4	small plug
10	DT5	end of plug
11	DT6	soft plug
12	DT7	
13	DT8	
14	DT9	
15	*S.* Braenderup ladder	
16	DT10	
17	DT11	
18	DT12	
19	DT13	well slightly damaged- repeat
20	DT14	
21	DT15	
22	DT24	
23	DT27	
24	DT28	
25	DT29	
26	DT30	
27	DT31	
28	DT32	
29	DT67	
30	*S.* Braenderup ladder	

Appendix 11. (cont.) Conditions for the PFGE performed using either *Xba*I or *Spe*I restriction enzymes with all 90 *Salmonella* isolates and control strains *S.* Typhimurium F98 and SL1344 and *S.* Branderup. The PFGE banding patterns were visualised on agarose gel.

PFGE worksheet: 11

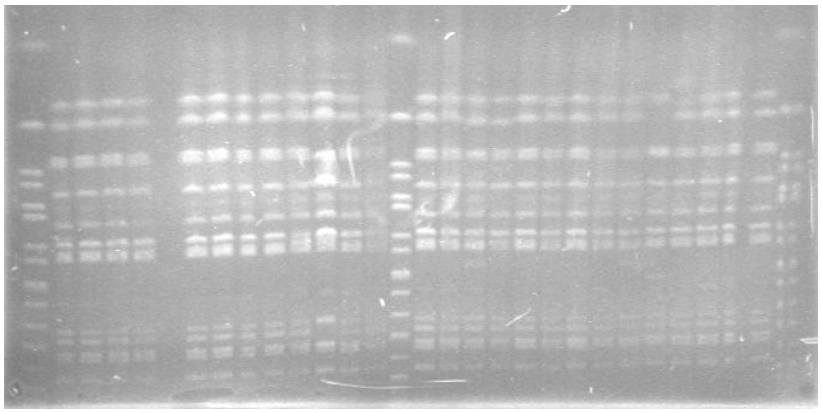


1 5 10 15 20 25 30

Example PFGE worksheet: 18

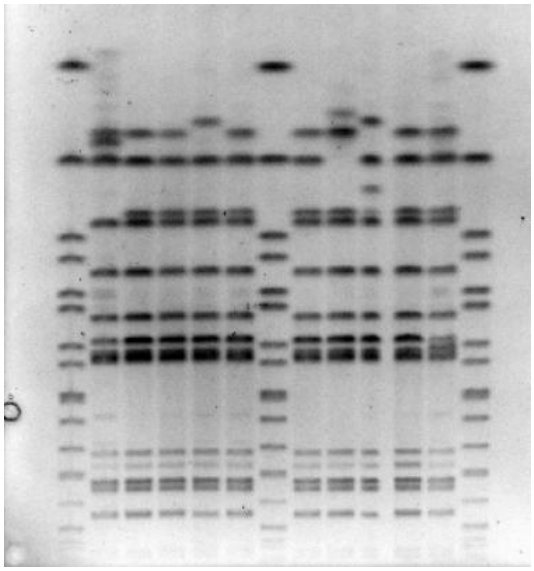
Machine DR-11	**Agarose concentration** 1.0% Seakem gold	**Enzyme** *Xba*I + BSA for 2 hours		
Run conditions	**volts/cm** 6.0	19 **hr**	14 **°C**	**Included angle** 120°
Ramping	2.2 **sec to** 63.8 **sec in**	linear **factor**		**Date** 2 August, 2010

Lane	Sample	Comments	Lane	Sample	Comments
1	*S.* Braenderup ladder		**16**	DT75	
2	DT2		**17**	DT78	
3	DT60		**18**	DT79	
4	DT18		**19**	DT80	
5	DT26		**20**	DT81	
6	DT40	repeat	**21**	DT82	
7	DT57		**22**	DT83	
8	DT58		**23**	DT84	
9	DT59		**24**	DT86	
10	DT61		**25**	DT85	
11	DT65		**26**	DT87	
12	DT70		**27**	DT88	
13	DT71		**28**	DT89	repeat
14	DT74	repeat	**29**	DT90	
15	*S.* Braenderup ladder		**30**	*S.* Braenderup ladder	



1 5 10 15 20 25 30

Appendix 12. The isolates with the unique PFGE banding patterns were retested by using *Xba*I. The banding patterns are more clearly visualised confirming the results generated earlier.



Lane 1, 7 and 13 are the *Salmonella* Braenderup size ladder. Lanes 3,4,6,8,11 and 12 are *Xba*I pattern A, lane 5 is *Xba*I pattern A*, lane 2 is *Xba*I pattern D, lane 9 is *Xba*I pattern B.

Appendix 13. Key table showing the corresponding Environmental Science and Research (ESR) isolate number from the Enteric Reference Laboratory (ERL), to our internal laboratory identifier number (DT).

Internal laboratory number	ESR isolate number	Internal laboratory number	ESR isolate number	Internal laboratory number	ESR isolate number
DT1	ERL062065	DT31	ERL023275	DT61	ER005391
DT2	ERL052816	DT32	ERL043701	DT62	ERL082846
DT3	ER003370	DT33	ERL09880	DT63	ERL072196
DT4	ERL021077	DT34	ERL022681	DT64	ERL030011
DT5	ERL091067	DT35	ERL005619	DT65	ER0100739
DT6	ERL091081	DT36	ERL082664	DT66	ERL043592
DT7	ERL042607	DT37	ERL064109	DT67	ER012224
DT8	ERL063767	DT38	ERL053554	DT68	ERL043718
DT9	ERL053052	DT39	ERL072176	DT69	ERL04205
DT10	ER016216	DT40	ERL082092	DT70	ER0003038
DT11	ERL063449	DT41	ERL032131	DT71	ERL033362
DT12	ERL06257	DT42	ERL0919	DT72	ER004952
DT13	ERL033614	DT43	ERL072182	DT73	ER011172
DT14	ERL091904	DT44	ERL050427	DT74	ERL043779
DT15	ERL06292	DT45	ER013668	DT75	ERL03695
DT16	ERL073108	DT46	ERL032893	DT76	ERL08372
DT17	ERL073368	DT47	ERL084152	DT77	ERL062557
DT18	ERL05998	DT48	ERL09395	DT78	ER005001
DT19	ERL052645	DT49	ERL063325	DT79	ERL025021
DT20	ERL050172	DT50	ER0104613	DT80	ERL041935
DT21	ERL064025	DT51	ERL081730	DT81	ERL02312
DT22	ER004560	DT52	ERL083326	DT82	ERL033068
DT23	ER012226	DT53	ERL052925	DT83	ERL072197
DT24	ER015215	DT54	ERL072372	DT84	ER004783
DT25	ER015709	DT55	ERL081168	DT85	ERL053850
DT26	ERL023783	DT56	ERL082847	DT86	ERL042332
DT27	ERL084557	DT57	ERL052818	DT87	ERL025008
DT28	ERL021519	DT58	ERL061789	DT88	ERL034404
DT29	ERL091580	DT59	ERL042806	DT89	ERL07266
DT30	ERL022680	DT60	ERL033073	DT90	ER0004663

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