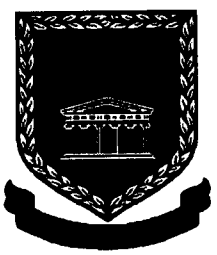
BIOINFORMATICS AND POLYMERASE CHAIN REACTION : TOOLS TO DETERMINE THE HOST SPECIFICITY OF SALMONELLA TYPHIMURIUM



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DELCLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and has not previously in its entirety, or in part, been submitted at any university for a degree.

Signature: ..... Date : .....

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LIST OF ABBREVIATIONS

ATP	adenosine triphosphate
bp	base pairs
BLAST	Basic Local Alignment Search Tool
BME	Bacterium-mediated endocytosis
BPW	Buffered Peptone Water
DNA	Deoxyribonucleic acid
eg.	example
et al.	and others
fig.	figure
g	gram
kb	kilobase
h	hour
KCl	Potassium chloride
ml	milliliter
MSA	Multiple sequence alignment
MSP	Maximal segment pair
PCR	Polymerase Chain Reaction
sig	*Salmonella* invasion gene
SPI	*Salmonella* Pathogenicity Island
spp.	species
T_a	annealing temperature
Tris-HCl	Tris(hydroxymethyl)aminomethane hydrochloric acid
TSB	Tryptone soya broth
μ	micro
°C	degrees Celsius
μg	micro gram
μl	microliter

INTRODUCTION

The increase in human illness resulting from bacterial contamination of food, and reports of the occasional severe cases of foodborne illness, have increased the concerns of consumers, food safety researchers, and regulatory agencies. Raw poultry products are perceived to be responsible for a significant amount of illness because of the relatively high frequency of contamination of poultry with *Salmonella* species. *Salmonella* serovar Typhimurium has drawn attention as one of the major causative agents of food poisoning. Therefore the control of *Salmonella* serovar Typhimurium has become the subject of emergence disease.

Standard culture methods for detecting *Salmonella* species in poultry involve whole-carcass rinses, enrichment in selective broth, followed by culturing the enriched culture on selective agar. The completion time for these assays is typically 48-96 hours. Thus there is a need for novel methods that can detect close to 'real time', small numbers of viable bacterial cells within a given volume of food or rinse to facilitate the removal of contaminated food immediately from the food chain.

The development of better techniques is crucial to accurately assess the safety of a food product, the effectiveness of new control measures to minimize the pathogens in a production or processing environment, and the basic biology and ecology of pathogens in the food environment.

The Polymerase Chain Reaction (PCR) technique has been described as one of the most promising microbiological methods for the detection of many foodborne pathogens. PCR-based methods for the detection of *Salmonella* spp. which contaminate poultry have been developed with the aims of increasing sensitivity and speed of the assay.

This study looked at the development of a better technique, ie. Polymerase Chain Reaction (PCR), for the detection of the foodborne pathogen *Salmonella typhimurium*. The study was undertaken with the aim of designing a primer pair that would solely detect *Salmonella typhimurium,* which is fast becoming one of the rather bothersome pathogenic organisms in the poultry industry. Instead of detecting this particular serotype, several other *Salmonella* serotypes were detected as well. Reinforcing the notion that pathogenic salmonellae species seem to share conserved genes and sequences, which have notably been identified in the invasion of the host's epithelial cells. In the process of designing the elusive primer it was found that *S.typhimurium* and *E.coli* share yet another nucleotide sequence. Namely that which codes for an ATP-dependent protease binding subunit (clpB) gene. Antibiotic resistance in pathogenic bacteria is a problem in industrialized and developing countries. This is especially evident in *Salmonella typhimurium*. The effects of ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracycline are evaluated on several *Salmonella* and non-*Salmonella* serotypes to reiterate the need to address this problem and to seek alternative therapies, such as herbal poultry feed supplements.

Chapter 1

Literature Review

1.1 What is *Salmonella*?

Salmonella is one of the most important food-contaminating pathogenic bacteria. Infection by *Salmonella typhimurium* is an important cause of morbidity and mortality in poultry and infection with *Salmonella enteritidis* is a major cause of foodborne illness (Cohen *et al.,* 1994). The increase in human illness resulting from bacterial contamination of food (Bean *et al.*, 1996), and reports of the occasional severe cases of foodborne illness (Smith *et al.*, 1993; Smith, 1994), have increased the concern of consumers, food processors, food safety researchers, and regulatory agencies. Raw poultry products are perceived to be responsible for a significant amount of illness because of the relatively high frequency of contamination of poultry with *Salmonella* spp. (Bailey, 1998; Geilhausen *et al.*, 1996; Gouws *et al.*, 1998). The examination of food and samples of animal origin for their presence has become routine all over the world. *Salmonella* infections are a principle source of gastroenteritis and enteric fever in a variety of animals, including humans (McCormick *et al.*, 1996).

Salmonella belong to the Enterobacteriaceae family. Enteric bacteria (Greek *entereikos,* pertaining to the intestine), all degrade sugars and cleave pyruvic acid to yield formic acid fermentation. The family can be divided into two groups

based on their fermentation products. The majority (eg. The genera *Escherichia, Proteus, Salmonella* and *Shigella*) carry out mixed acid fermentation and produce mainly lactate; acetate; succinate; formate and ethanol. In butanediol fermentation the major products are butanediol, ethanol and carbon dioxide. *Enterobacter, Serratia, Erwinia* and *Klebsiella* are butanediol fermenters (Prescott *et al.,* 1996).

Salmonellosis (S*amonella* gastroenteritis) is caused by over 2 300 *Salmonella* serotypes (Schaechter *et al.*, 1989) and most of these are human pathogens (Lee and Falkow, 1990). In order for salmonellae to cause disease via an oral route of infection, the bacteria must penetrate the epithelial cells of the intestine (Carter and Collins, 1974; Hohmann *et al.*, 1978; Ozama *et al.,* 1973; Takeuchi, 1967). Several studies have shown that salmonellae preferentially attach and enter phagocytic M cells of the Peyer's patches (Hohmann *et al.*, 1978; Jones *et al.*, 1994; Penheiter *et al.*, 1997). However, studies have also shown that bacteria can be found in nonphagocytic enterocytes and can also cause a systemic infection when the small intestine is bypassed (Carter an Collins, 1974). In addition, salmonellosis causes substantial losses in livestock. There is a wide spread occurrence in animals, especially poultry and swine. Environmental sources of the organism include water, soil, insects, factory surfaces, animal feces, raw meats, raw poultry and raw seafood, to name only a few (Anonymous 1991).

1.2 The characteristics of *Salmonella*

Salmonella is a rod-shaped, motile bacterium- nonmotile exceptions *S.gallinarum* and *S.pullorum*, non-sporeforming and Gram-negative. Salmonellae are chemoorganotrophic, with an ability to metabolize nutrients by the respiratory and fermentative pathways (Le Minor, 1981; Brenner, 1984). They grow optimally at 37°C and are unable to ferment lactose, sucrose or salicin, although glucose and certain monosaccharides are fermented with the production of gas. However, *Salmonella typhimurium* can ferment lactose with the production of gas (Jay, 1996). They grow on citrate as the sole carbon source, generally produce hydrogen sulfide, decarboxylate lysine and ornithine, and do not hydrolyse urea. The methyl-red reaction is positive, the Voges-Proskrauer test is negative and the indole is negative. The G+C content of DNA is 50-53 mole% (Andrews, 1985).

1.3 Structure, Classification and Antigenic Types

Based on the Kauffmann-White scheme, *Salmonella* was classified into serotypes by the combination of antigens namely : H or flagellar antigen; O or somatic antigen; and Vi antigen (possessed by only a few serovars). H antigen may occur in either or both of two forms, called phase 1 or phase 2. The organisms tend to change from one phase to the other. O antigens occur on the surface of the outer membrane and are determined by specific sugar sequences on the cell surface. Vi antigens is a superficial antigen overlying the O antigen; it is present in a few serovars, the most important being *Salmonella typhi* (Salyers and Whitt, 1994).

Antigenic analysis of salmonellae by using specific antisera offers clinical and epidemiological advantages. Determination of antigenic structure permits one to identify the organisms clinically and assign them to one of nine serogroups (A-I), each containing many serovars (Table 1.1). H antigen also provides a useful epidemiological tool with which to determine the source of infection and its mode of spread (http://gsbs.utmb.edu/microbook/ch021.htm August 2001).

Table 1.1 Ecological classification of Salmonellae

Species	Representative Serovar(s)*	Reservoir (Host preferences)
S.choleraesuis	One only	Animals (swine)
S.typhi	One only	Humans
S.enteritidis	Paratyphi-A	Humans
	Schottmuelleri	
	Pullorum	Animals (fowl)
	Dublin	Animals (cattle)
	Typhimurium	
	Derby	
	Enteritidis	Humans and many animals
	Heidelberg and hundreds of related serovars	

* It is now accepted practice to refer to the 1800 serovars of *Salmonella* as though they constituted separate species (eg. *S.pullorum*).

(Adapted from Grady G.F., Keusch G.T., New Engl. J Med 285:831, 1971.)

As with other Gram-negative bacilli, the cell envelope of salmonellae contains a complex lipopolysaccharide (LPS) structure that is liberated on lysis of the cell and, to some extent, during culture. The lipopolysaccharide moiety may function

as an endotoxin, and may be important in determining virulence of the organisms. This macromolecular endotoxin complex consists of three components, an outer O-polysaccharide coat, a middle portion (the R core), and an inner lipid A coat. Lipopolysaccharide structure is important for several reasons. First, the nature of the repeating sugar units in the outer O-polysaccharide chains is responsible for O antigen specificity; it may also help determine the virulence of the organism. Salmonellae lacking the complete sequence of O-sugar repeat units are called rough because of the rough appearance of the colonies; they are usually avirulent or less virulent than the smooth strains which possess a full complement of O-sugar repeat units. Second, antibodies directed against the R core (common enterobacterial antigen) may protect against infection by a wide variety of Gram-negative bacteria sharing a common core structure or may moderate their lethal effects. Third, the endotoxin component of the cell wall may play an important role in the pathogenesis of many clinical manifestations and Gram-negative infections. Endotoxins evoke fever, activate the serum complement, kinin, and clotting systems, depress myocardial function, and alter lymphocyte function (Chopra *et al.*, 1994).

1.4 Pathogenesis

Salmonellosis includes several syndromes (gastroenteritis, enteric fever, septicemia, focal infections and an asymptomatic carrier) (Fig. 1.1). Particular serovars show a strong propensity to produce a particular syndrome (*S.typhi*,

S.paratyphi-A, and *S.schottmuelleri* produce enteric fever; *S.choleraesuis* produces septicemia or focal infections; *S.typhimurium* and *S.enteritidis* produce gastroenteritis); however, on occasion, any serotype can produce any of the syndromes (Rubin and Weinstein, 1977).

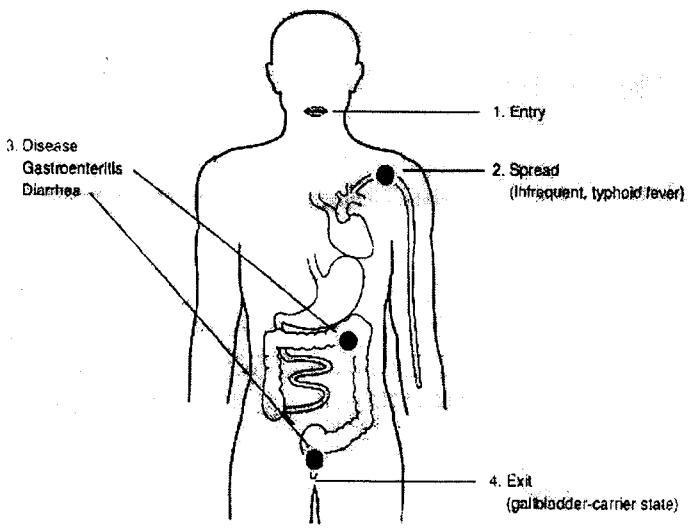

Fig 1.1 Pathogenesis of salmonellosis

(Taken from www.http://gsbs.utmb.edu.microbook.htm)

The septicemic form of salmonella infection can be an intermediate stage of infection in which the patient is not experiencing intestinal symptoms and the bacteria cannot be isolated from fecal specimens. The severity of the infection and whether it remains localized in the intestine or disseminates to the bloodstream may depend on the resistance of the patient and the virulence of the *Salmonella* isolate (http://gsbs.utmb.edu.microbook/ch021.htm August 2001).

The incubation period for *Salmonella* gastroenteritis (food-poisoning) depends on the dose of bacteria. Symptoms usually begin 6 to 48 hours after ingestion of

contaminated food or water and usually take the form of nausea, vomiting, diarrhea, and abdominal pain. Myalgia and headache are common; however, the cardinal manifestation is diarrhea. Fever (38°C to 39°C) and chills are also common. At least two-thirds of patients complain of abdominal cramps. The duration of fever and diarrhea varies, but is usually 2 to 7 days (http://www.oxylit.com/salmonella.html August 2001). Chronic consequences such as arthritic symptoms may follow 3-4 weeks after onset of acute symptoms. During the acute phase of the disease, as many as 1 billion *Salmonella* can be found per gram of feces. Most adult patients recover, but the loss of fluids can cause problems for children and elderly people (http://vm.cfsan.fda.gov March 2001).

Enteric fevers are severe systemic forms of salmonellosis. The best studied fever is typhoid fever, the form caused by *Salmonella typhi*, but any species of *Salmonella* may cause this type of disease. *Salmonella typhi* and paratyphoid bacteria normally cause septicemia and produce typhoid or typhoid-like fever in humans. *Salmonella* septicemia has been associated with subsequent infection of virtually every organ system. The symptoms begin after an incubation period of 10 to 14 days. The symptoms of enteric fevers are nonspecific and include fever, anorexia, headache, myalgias, and constipation. The fatality rate of typhoid fever is 10% compared to less than 1% for most forms of salmonellosis. Postenteritis reactive arthritis and Reiter's syndrome have also been reported to occur with a frequency of about 2% of culture-proven cases. Septic arthritis,

subsequent or coincident with septicemia, also occurs and can be difficult to treat. Enteric fevers may be preceded by gastroenteritis, which usually resolves before the onset of systemic disease. Enteric fevers are severe infections and may be fatal if antibiotics are not promptly administered (http://vm.cfsan.fda.gov March 2001).

Most non-typhoidal salmonellae enter the body when contaminated food is ingested (Fig. 1.2). Person-to-person spread of salmonellae also occurs. To be fully pathogenic, salmonellae must possess a variety of attributes called virulence factors. These include (1) the ability to invade cells, (2) a complete lipopolysaccharide coat, (3) the ability to replicate intracellularly, and (4) possibly the elaboration of toxin(s). After ingestion, the organism colonize the ileum and colon, invade the intestinal epithelium, and proliferate within the epithelium and lymphoid follicles (Galan and Curtiss, 1990). The mechanism by which salmonellae invade the epithelium is partially understood and involves an initial binding to specific receptors on the epithelial cell surface followed by invasion. Invasion occurs by the organism inducing the enterocyte membrane to undergo "ruffling' and thereby to stimulate pinocytosis of the organisms (Fig. 1.3). Invasion is dependent on rearrangement of the cell cytoskeleton and probably involves increase in cellular inositol phosphate and calcium. Attachments and invasion are under distinct genetic control and involve multiple genes in both chromosomes and plasmids (McCormick *et al.*, 1996).

Ingestion of organisms

↓

Colonization of lower intestine (ileum and cecum)

↓

Mucosal invasion

↓ Cytotoxin

Acute inflammation

↓ Ulceration, Prostaglandin synthesis, Enterotoxins, Cytokines

Activation of adenyl cyclase

↓ ↑Cyclic AMP

Fluid production (large and small bowel)

↓

Diarrhea

Fig.1.2 Scheme of the pathogenesis of *Salmonella enterocolitis* and diarrhea

(Taken from http://gsbs.utmb.edu.microbook.htm August 2001)

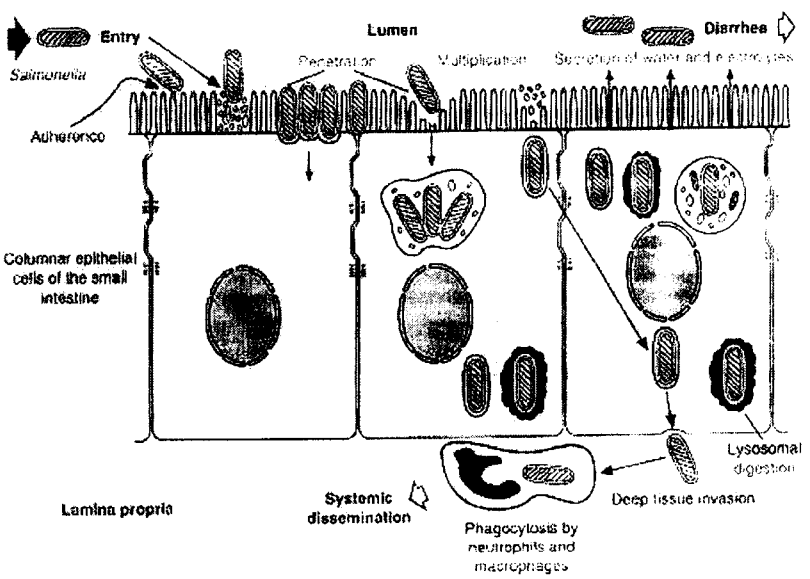

Fig.1.3 Invasion of intestinal mucosa by *Salmonella*

(Taken from http://gsbs.utmb.edu.microbook.htm August 2001)

After invading the epithelium, the organisms multiply intracellularly and then spread to mesenteric lymph nodes and throughout the body via the systemic circulation; they are taken up by the reticuloendothelial cells. The reticuloendothelial system confines and controls spread of the organism. However, depending on the serotype and the effectiveness of the host defenses against that serotype, some organisms may infect tissues like liver and spleen (Conlan and North, 1992), gallbladder, bones, meninges, and other organs (Fig. 1). Fortunately, most serovars are killed promptly in extraintestinal sites, and most common human *Salmonella* infection, gastroenteritis, remains confined to the intestine (Finlay and Falkow, 1989).

After invading the intestine, most salmonellae induce an acute inflammatory response, which can cause ulceration. They may elaborate cytotoxins that inhibit protein synthesis. Whether these cytotoxins contribute to the inflammatory response or to ulceration is not known. However, invasion of the mucosa causes the epithelial cells to synthesize and release various proinflammatory cytokines, including: Interleukin (IL)-1, IL-6, IL-8, Tumor Necrosis Factor (TNF)-2, IFN-U, Monocyte Chemotactic Protein (MCP)-1, and GM-CSF. These evoke an acute inflammatory response and may also be responsible for damage to the intestine. Because of the intestinal inflammatory reaction, symptoms of inflammation such as fever, chills, abdominal pain, leukocytosis, and diarrhea are common. The stools may contain polymorphonuclear leukocytes, blood and mucus (Takeuchi, 1967).

Figures 1.2 and 1.3 summarize the pathogenesis of *Salmonella* eneterocolitis and diarrhea. Only strains that penetrate the intestinal mucosa are associated with the appearance of an acute inflammatory reaction (Fig. 1.4); the diarrhea is due to secretion of fluid and electrolytes by the small and large intestines. The mechanisms of secretion are unclear, but the secretion in not merely a manifestation of tissue destruction and ulceration. *Salmonella* penetrate the epithelial cells but, unlike *Shigella* and invasive *E.coli*, do not escape the phagosome. Thus, the extent of intercellular spread and ulceration of the epithelium is minimal. *Salmonella* escape from the basal side of epithelial cells into the lamina propria (Foster and Spector, 1995). Systemic spread of the organism can occur, giving rise to enteric fever. Invasion of the intestinal mucosa is followed by activation of mucosal adenylate cyclase; the resultant increase in cyclic AMP induces secretion. The mechanism by which adenylate cyclase is stimulated is not understood; it may involve local production of prostglandins or other components of the substances which may stimulate intestinal secretion. However, the precise role of these toxins in the pathogenesis of *Salmonella* enterocolitis and diarrhea has not been established (Giannella, 1979).

Fig 1.4 Electron photomicrograph demonstrating invasion of guinea pig ileal epithelial cells by *Salmonella typhimurium.* Arrows point to invading *Salmonella* organisms. (Taken from Akio Takeuki, Walter Reed Army Institute of Research, Washington, D.C.).

1.5 Host defenses

Various host defenses are important in resisting intestinal colonization and invasion by *Salmonella.* Normal gastric acidity (pH<3.5) is lethal to salmonellae (Giannella *et al.*, 1973). In healthy individuals, the number of ingested salmonellae is reduced in the stomach, so that fever or no organisms enter the intestine. Normal small intestinal motility also protects the bowel by sweeping ingested salmonellae through quickly. The normal intestinal microflora protects against salmonellae, probably through anaerobes, which liberate short-chain fatty acids that are thought to be toxic to salmonellae. Alteration of the anaerobic intestinal flora by antibiotics also renders the host more susceptible to

salmonellosis. Secretory or mucosal antibodies also protect the intestine against salmonellae. Animal strains genetically resistant to intestinal invasion by salmonellae have been described. When these host defenses are absent or blunted, the host becomes more susceptible to salmonellosis. For example, in AIDS, *Salmonella* infection is common, frequently persistent and bacteremic, and often resistant to even prolonged antibiotic treatment. Relapses are common. The role of host defenses in salmonellosis is extremely important, and much remains to be learned (http://gsbs.utmb.edu/microbook/ch021.htm August 2001).

1.6 Epidemiology

Contaminated food is the major mode of transmission for non-typhiodal salmonellae because salmonellosis is a zoonosis and has an enormous animal reservoir. The most common animal reservoirs are chickens, turkeys, pigs, and cows; dozens of other domestic and wild animals also harbor these organisms. Because of the ability of salmonellae to survive in meats and animal products that are thoroughly cooked, animal products are the main vehicle of transmission. The magnitude of the problem is demonstrated by the following fields of salmonellae: 41% of turkeys examined in California, 50% of chickens cultured in Massachusetts, and 21% of commercial frozen egg whites examined in Spokane, WA (http://gsbs.utmb.edu/microbook/ch021.htm August 2001).

The epidemiology of typhoid fever and other enteric fevers primarily involves person-to-person spread because these organisms lack a significant animal

reservoir. Contamination with human feces is the major mode of spread, and the usual vehicle is contaminate water. Occassionally, contaminated food (usually handled by an individual who harbors *S.typhi*) may be the vehicle. Plasmid DNA fingerprinting and bacteria phage lysotyping of *Salmonella* isolates are powerful epidemiologic tools for studying outbreaks of salmonellosis and tracing the spread of the organism in the environment (Mishu *et al.*, 1994).

In typhoid fever and non-typhoidal salmonellosis, two other factors have epidemiologic significance. First, an asymptomatic human carrier exists for the agents of either form of the disease. Approximately 3% of persons infected with *S.typhi* and 0.1% of those infected with non-typhoidal salmonellae become chronic carriers. The carrier state may last from many weeks to years. Thus, human as well as animal reservoirs exist. Interestingly, children rarely become chronic typhoid carriers. Second, use of antibiotics in animal feeds and indiscriminant use of antibiotics in humans increase antibiotic resistance in salmonellae by promoting transfer of R factors. Salmonellosis is a major public health problem because of its large and varied animal reservoir, the existence of human and animal carrier states, and the lack of a concerted nationwide program to control salmonellae (http://gsbs.utmb.edu/microbook/ch021.htm August 2001)

1.7 Diagnosis

The diagnosis of salmonellosis requires bacteriophage isolation of the organisms from appropriate clinical specimens. Laboratory identification of the genus

Salmonella is done by biochemical tests; the serological type is confirmed testing. Feces, blood, or other specimens should be plated on several nonselective and selective agar media (blood, MacConkey, eosin-methylene blue, bismuth sulfite, *Salmonella-Shigella*, and brilliant green agars) as well as into enrichment broth such as selenite or tetrathionate. Any growth in enrichment broth is subsequently subcultured onto various agars. The biochemical reactions of suspicious colonies are then determined on triple sugar iron agar and lysine-iron agar, and a presumptive identification is made. Biochemical identification of salmonellae has been simplified by systems that permit the rapid testing of 10-20 different biochemical parameters simultaneously. The presumptive biochemical identification of *Salmonella* then can be confirmed by antigen analysis of O and H antigens using polyvalent and specific antisera. Fortunately, approximately 95% of all clinical isolates can be identified with the available group A-E typing antisera. *Salmonella* isolates then should be sent to a central or reference laboratory for more comprehensive serological testing and confirmation (Stephen *et al.*, 1985)

1.8 Control

Salmonellae are difficult to eradicate from the environment. However, because the major reservoir for human infection is poultry and livestock, reducing the number of salmonellae harbored in these animals would significantly reduce human exposure. In Denmark, for example, all animal feeds are treated to kill salmonellae before distribution, resulting in a marked reduction in salmonellosis.

Other helpful measures include changing animal slaughterhouses, food processing plants, and restaurants; cooking and refrigerating foods adequately in food processing plants, restaurants, and homes; and expanding of government enteric disease surveillance programs (http://gsbs.utmb.edu.microbook.htm August 2001).

Vaccines are available for typhoid fever and partially effective, especially in children. No vaccines are available for non-typhoidal salmonellosis. Continued research in this area and increased understanding of the mechanisms of immunity to enteric infections are of great importance (http://gsbs.utmb.edu.microbook.htm August 2001).

General salmonellosis treatment measures include replacing fluid loss by oral and intravenous routes, and controlling pain, nausea, and vomiting. Specific therapy consists of antibiotic administration. Typhoid fever and enteric fevers should be treated with antibiotics. Antibiotic therapy of non-typhoidal salmonellosis should be reserved for the septicemic, enteric fever, and focal infection syndromes. Antibiotics are not recommended for uncomplicated *Salmonella* gastroenteritis because they do not shorten the illness and they significantly prolong the fecal excretion of the organisms and increase the number of antibiotic-resistant strains (http://gsbs.utmb.edu.microbook.htm August 2001).

1.9 Antibiotic resistance

The emergence of pathogens possessing multiple-antibiotic resistance genes has become a major concern in recent years. The routine use of antibiotics in medical and agricultural circles has resulted in widespread antibiotic resistance and in the development of genetic mechanisms efficient for the dissemination of antibiotic gene cassettes, especially within and between species of gram-negative organisms (Briggs and Fratamico, 1999).

Modern medicine is dependent on chemotherapeutic agents, which are chemical agents that are used to treat disease. Chemotherapeutic agents are pathogenic microorganisms that kill or inhibit their growth at concentrations low enough to avoid undesirable damage to the host. Most of these agents are antibiotics [Greek *anti*, against, and *bios*, life], microbial products or their derivatives that can kill susceptible microorganisms or inhibit their growth. Drugs such as sulfonamides are sometimes called antibiotics although they are synthetic chemotherapeutic agents, not microbially synthesized (Prescott *et al.*, 1996).

Chemotherapeutic agents can be synthesized by microorganisms or manufactured by chemical procedures independent of microbial activity. A number of the most commonly employed antibiotics are natural- that is, is totally synthesized by one or a few bacteria or fungi. In contrast, several important chemotherapeutic agents are completely synthetic. The synthetic antibacterial drugs are the sulfonamides, trimethoprim, chloramphenicol, ciprofloxacin,

isoniazid, and dapsone. Many antiviral and antiprotozoan drugs are synthetic. An increasing number of antibiotics are semisynthetic. Semisynthetic antibiotics are natural antibiotics that have been chemically modified by the addition of extra chemical groups to make them less susceptible to inactivation by pathogens. Ampicillin, carbenicillin, methicillin are good examples (Prescott *et al.*, 1996).

Chemotherapeutic agents, like disinfectants, can either be static or cidal (Table 1.2). Static agents reversibly inhibit growth; if the agent is removed, the microorganisms will recover and grow again.

TABLE 1.2: PROPERTIES OF SOME COMMON ANTIBACTERIAL DRUGS

Drug	Primary effect	Spectrum	Side effects*
Ampicillin	Cidal	Broad (gram +, some –)	Allergic responses (diarrhea, anemia)
Chloramphenicol	Static	Broad (gram +, –; rickettsia and chlamydia)	Depressed bone marrow functio, allergic reactions
Streptomycin	Cidal	Broad (gram +,–; mycobacteria)	Allergic responses, loss of hearing, nausea, renal damage
Sulfonamides	Static	Broad (gram +,–)	Allergic responses (renal and hepatic injury, anemia)
Tetracycline	Static	Broad (gram +,–; rickettsia and chlamydia)	Gastrointestinal upset, teeth discoloration (renal and hepatic injury)

* Occasional side effects are in parentheses. Other side effects not listed may also arise. Taken from Prescott *et al.* 1996.

Researchers often use antibiotics as instruments to dissect metabolic processes by inhibiting or blocking specific steps and observing the consequences. In practice, the antibiotic is administered and changes in cell function is monitored. If one desired to study the dependence of bacterial flagella synthesis on RNA transcription, the flagella could be removed by high-speed mixing in a blender, followed by actinomycin D addition to the incubation mixture. The bacterial culture would then be observed for flagella regeneration in the absence of RNA synthesis. The results of such experiments must be interpreted with caution. Flagella synthesis may have been blocked because actinomycin D inhibited some other processes, thus affecting flagella regeneration indirectly rather than simply inhibiting transcription of a gene required for flagella synthesis. Furthermore, not all microorganisms respond in the same way to a particular drug (Prescott *et al.*, 1996).

Antibiotics are known to inhibit a variety of essentially biological processes, including DNA replication (e.g., novobiocin), transcription (e.g., rifamycin B) and bacteria cell wall synthesis (e.g., penicillin). However, the majority of known antibiotics, including a great variety of medically useful substances, block translation. This situation is presumably a consequence of the translational machinery's enormous complexity, which makes it vulnerable to disruption in many ways. Antibiotics have also been useful in analyzing ribosomal mechanisms because the blockade of a specific function permits its biochemical dissection into its component steps (Lewin, 1997).

1.9.1 Ampicillin

The powerful antibiotic penicillin was discovered by Alexander Fleming in 1928 when he observed, by chance, that bacterial growth was inhibited by a contaminating mold (Penicillium). Since then, many synthetic derivatives of penicillin have been made and used for a wide spectrum of applications. Ampicillin is one of the most useful of these derivatives and serves as a highly effective medication to quench many bacterial infections. Side effects include fever, joint pain, swelling, skin rash, hives, and itching (Lewin, 1997).

1.9.2 Chloramphenicol

Chloramphenicol, the first of the 'broad spectrum" antibiotics, inhibits the peptidyl transferase activity on the large subunit of prokaryotic ribosomes. However, its clinical uses are only limited to severe infection because of its toxic side effects, which are caused, at least in part, by the chloramphenicol sensitivity of mitochondrial ribosomes (Lewin, 1997).

1.9.3 Fluoroquinolones

Fluoroquinolones account for about 11% of antimicrobial prescriptions in human medicine worldwide and represent the drug of choice for the treatment of a wide range of human infectious diseases. They were introduced into veterinary medicine in Europe in the late 1980s and early 1990s and in the USA in 1995. Following their introduction, resistant strains of bacteria, including *Salmonella*, started to emerge. Resistance to quinolones depends on chromosomal mutations

and the subsequent spread of resistant clones. While the selective pressure caused by the use of quinolones facilitates their epidemic transmission, the resistant mutants may spread independently of quinolone use. The public health hazard posed by quinolone-resistant zoonotic *Salmonella* serovars has been a subject of concern. The fluoroquinolones are on the World Health Organization (WHO) list of drugs that should be reserved for human use. Considering the mounting evidence that quinolone-resistant zoonotic *Salmonella* are the cause of severe, sometimes fatal, infections in humans, the use of fluoroquinolones in food animals should be discontinued or severely restricted. Such an intervention should be accompanied by prudent use measures involving all other groups of antimicrobials to reduce the need for fluoroquinlones in veterinary medicine (Bager and Helmuth, 2001).

1.9.4 Streptomycin

Streptomycin, which was first discovered in 1944 by Selman Waksman, is a medically important member of a family of antibiotics known as aminoglycosides that inhibit prokaryotic ribosomes in a variety of ways. At low concentrations, streptomycin induces the ribosomes to characteristically misread mRNA: One pyrimidine may be mistaken for the other in the first place and second codon positions and either pyrimidine may be mistaken for adenine in the first position. This inhibits the growth of susceptible cells but does not kill them. At higher concentrations, however, streptomycin prevents proper chain initiation and thereby causes cell death (Lewin, 1997).

1.9.5 Sulfonamides

Structural analogues are molecules that are structurally similar to metabolic intermediates. The similarity of these analogues allow them to compete with the metabolites' metabolic processes. These analogues are just different enough to interfere with the normal cellular metabolism. An example of an antimetabolite used as a chemotherapeutic agent, are the sulfonamides. Sulfonamides are structurally related to sulfanilamide, an analogue of p-aminobenzoic acid. When sulfanilamide or another sulfonamide enters a bacterial cell, it competes with p-aminobenzoic acid for the active site of an enzyme involved in folic acid synthesis, and the folate concentration decreases. The decline in folic acid is detrimental to the bacterium because folic acid is essential to the synthesis of purines and pyrimidines, the bases used in the construction of DNA, RNA, and other important cell constituents. The resulting inhibition of purine and pyrimidine synthesis lead to cessation of bacterial growth or death of the pathogen. Sulfonamides are selectively toxic for many pathogens because these bacteria manufacture their own folate and cannot effectively take up the cofactor. In contrast, in humans cannot synthesize folate and must obtain it in their diet; therefore sulfonamides will not effect the host (Prescott *et al.*, 1996).

1.9.6 Tetracycline

Tetracycline and its derivatives are broad-spectrum antibiotics that bind to the small subunit of prokaryotic ribosomes, where they inhibit aminoacyl-tRNA binding. Tetracycline also blocks the stringent response by inhibiting ppGpp

synthesis. This indicates that de-acylated tRNA must bind to the A site in order to activate stringent factor. Tetracycline-resistant bacterial strains have become quite common, thereby precipitating a serious clinical problem. Most often, however, resistance is conferred by a decrease in bacterial cell membrane permeability to tetracycline rather than any alteration of ribosomal components (Lewin, 1997).

1.10 Virulence Genes

At least 60 genes are required for virulence in *Salmonella enterica*. The requirement for so many virulence determinants is thought to reflect the complex life cycle of this pathogen in infected animals (Groisman and Ochman, 1997). Many of the genes implicated in *Salmonella* virulence are also present in nonpathogenic strains of *E.coli*. These genes encode enzymes responsible for the biosynthesis of nutrients that are scarce within host tissues, transcriptional and post-transcriptional regulatory factors, proteins necessary for the repair of damaged DNA, and products necessary for defense against host microbial mechanisms. The presence of these genes in nonpathogenic species suggests that they promote survival within nutritionally deprived and/or potentially lethal environments that microorganisms encounter inside and outside animal hosts (Groisman and Ochman, 1997).

Apart from the requirements for loci that are also present in nonpathogenic species, *Salmonella* virulence demands several genes that are absent from

benign microorganisms. Although some of these genes reside on a plasmid common to many *Salmonella* serovars, the vast majority is encoded within pathogenicity islands- large clusters of virulence genes not found in related species. *Salmonella*-specific virulence genes reside in several regions of the chromosome and often encode determinants responsible for establishing specific interactions with the host. Therefore, these *Salmonella*-specific genes help to define the molecular basis for pathogenicity in this intracellular pathogen (Groisman and Ochman, 1997).

1.10.1 The multiple virulence features of the SPI-1

The best characterized pathogenicity island of *Salmonella* is SPI-1, a 40kb region harboring some 25 genes and mapping to the 63'region of the *Salmonella enterica* sv. *Typhimurium* chromosome (Galan and Ginocchio, 1994). Genes within the SPI-1 were originally identified because their inactivation prevented *Salmonella* from invading epithelial cells *in vitro* (Galan and Curtis, 1989).

This system mediates the translocation of a battery of bacterial proteins into host cells which stimulate or interfere with host cellular functions (Galan, 1998). These effector proteins include an exchange factor for *Rho* GTPases (SopE) (Hardt *et al.*, 1998), a tyrosine phosphatase (SptP) (Fu and Galan, 1998; Kaniga *et al.*, 1996), an actin-binding protein (SipA) (Zhou *et al.*, 1999), and an inositol phosphate phosphatase (SopB) (Norris *et al.*, 1998). The concerted action of these effector proteins results in host cell actin cytoskeleton rearrangements and

nuclear responses that ultimately lead to bacterial internalization and the production of proinflammatory cytokines (Chen *et al.*, 1996; Hobbie *et al.*, 1997). In addition, this type III secretion system is involved in the initiation of programmed cell death in macrophages (Chen *et al.*, 1996; Monack *et al.*, 1996), the stimulation of neutrophil migration across the intestinal epithelium (McCormick *et al.*, 1993), and fluid accumulation in ligated intestinal loops and the generation of diarrhea (Eckmann *et al.*, 1997; Galyov *et al.*, 1997).

Functionally, proteins associated with the centisome 63 type III protein secretion system can be divided into at least three categories (Collazo and Galan, 1996): (i) proteins that are components of the type III secretion machinery (e.g., *InvA*, *InvC*, *InvG*, and *PrgH*), (ii) proteins that are involved in the translocation of effector molecules into the cytoplasm of the host cell (e.g., *SipB*, *SipC*, and *SipD*), and (iii) proteins that upon translocation modulate host cell functions (e.g., *SopE*, *SipA*, *SopB*, *SptP*, and *AvrA*). Although most of the proteins associated with the invasion-associated type III secretion system are encoded within SPI-1, at least two effector molecules delivered by this system are encoded elsewhere in the bacterial chromosome. *SopB* is encoded within a pathogenicity island (SPI-5) at centisome 25 (20 in S. dublin) (Wood *et al.*, 1998), and *SopE* is encoded within the genome of a cryptic bacteriophage located at centisome 60 (Hardt *et al.*, 1998).

The SPI-1 encodes two distinct regulatory proteins, *InvF* and *HilA*, as well as the components of a type III secretion system, termed *Inv/Spa* system. The *Inv/Spa* secretion system is necessary for the transient formation of cell-surface appendages whose appearance is induced upon contact with host cells. Proteins secreted by the *Inv/Spa* system stimulate a signal transduction that elicits the internalization of bacteria into the host cell (Galan and Ginocchio, 1994). Strains defective in *inv* or *spa* genes are not invasive, and nonpathogenic *E.coli* can be passively internalized into host cells by co-infection with wild-type *Salmonella* (Francis *et al.*, 1993).

The expression of components and substrates of this type III secretion system is subject to complex regulatory mechanisms (Hueck, 1998). A number of environmental cues are known to affect type III secretion-associated gene expression (Bajaj *et al.*, 1995; Bajaj *et al.*,1996; Ernst *et al.*, 1990; Galan and Curtiss, 1990; Lee and Falkow, 1990; Schiemann and Shope, 1991; Wood *et al.*, 1998). Thus, growth under high-osmolarity and low-oxygen conditions stimulates the expression of type III secretion-associated proteins, resulting in increased levels of bacterial internalization into host cells. Bacterial internalization is influenced by the bacterial growth state as well as by carbohydrate utilization. The actual mechanisms by which these environmental signals influence gene expression are not understood.

At least two transcriptional regulatory proteins are encoded within SPI-1 (Bajaj *et al.*, 1995; Kaniga *et al.*, 1996). These are *HilA*, a member of the *OmpR/ToxR* family of transcriptional regulators (Bajaj *et al.*, 1995), and *InvF*, which belongs to the AraC family of regulatory proteins (Kaniga *et al.*, 1994). Although both of these proteins influence the expression of the invasion phenotype, their actual regulatory target genes and their functional relationship with each other are poorly understood. *HilA* presumably directly activates the transcription of the *invF* and *prgH* promoters, but its direct role in the regulation of expression of genes encoding effector proteins delivered through the type III secretion system has not been rigorously investigated (Bajaj *et al.*, 1995; Bajaj *et al.*, 1996). *InvF* is required for efficient entry into host cells, but its regulatory target genes have not been identified (Kaniga *et al.*, 1994). In addition to the specific regulatory proteins encoded within SPI-1, the expression of the invasion-associated type III secretion system is influenced by several global regulatory networks. A growing list of loci have various degrees of influence on the expression of the centisome 63 type III secretion system. This includes the PhoP-PhoQ and RcsB-RcsC two-component regulatory systems (Arricau *et al.*, 1998; Belhau and Miller, 1993; Pegues *et al.*, 1995), the flagellum-associated sigma factor FliA (σ^{28}) (Eichelberg *et al.*, 1995), the UvrY (SirA) response regulator system (Johnston *et al.*, 1996), and DNA topoisomerase I (Galan and Curtiss, 1990).

Besides conferring the ability to enter the nonphagocytic cells, an additional property has recently been ascribed to the SPI-1 island: the induction of

apoptosis in *Salmonella* infected macrophages (Chen *et al.*, 1996, Monack *et al.* 1996). Surprisingly, apoptosis and host cell invasion exhibit several of the same phenotypic and genotypic requirements. First, macrophage cytotoxicity occurs under growth conditions that promote *Salmonella* invasion. Second, the SPI-1 encoded *hil*A, *org*A, *spa*N, *spa*O, *sip*B, *sip*C, and *sip*D genes are essential for entry into nonphagocytic cells and for killing of infected macrophages (Chen *et al.*, 1996; Monack *et al.*, 1996).

In summary, the SPI-1 island encodes determinants that mediate: (1) the invasion of nonphagocytic host cells, (2) macrophage apoptosis *in vitro* and (3) an as yet unknown function mediated by the SipA and SptP proteins, which are also secreted by the Inv/Spa secretion apparatus but are dispensable for invasion. The role of SPI-1 in invasion is supported by both *in vitro* and *in vivo* analyses; however, its putative role in bacteria-mediated macrophage apoptosis requires further investigation into the pattern of expression of SPI-1-encode genes in host tissues during infection (Groisman and Ochman, 1997).

1.10.2 The SPI-2 island confers distinct virulence attributes in *Salmonella*

A second 40kb pathogenicity island, designated SPI-2, has been mapped to 31' on the *Salmonella enetrica* sv *Typhimurium* chromosome (Ochman *et al.*, 1996; Shea *et al.*, 1996). SPI-2 contains at least 17 genes that code for a two-component regulatory system and a type III secretion system, designated Spi/Ssa (Ochman *et al.*, 1996; Shea *et al.*, 1996), that are distinct in structure

and function from the SPI-1-encoded Inv/Spa system and the type III secretion system that mediated the export and assembly of flagellar components in Gram-negative and Gram-positive bacteria.

Although the role of the SPI-2 island has not been fully elucidated, the virulence properties of mutants defective in SPI-2-encoded genes suggests that this pathogenicity island is required for systemic disease. However, not all genes contained in SPI-2 appear to be required for intramacrophage survival, and some SPI-2 mutants are slightly reduced in their ability to invade epithelial cells (Hensel *et al.*, 1997).

The phylogenetic distribution of SPI-2 differs from that of SPI-1 in two respects: first SPI-2 sequences are restricted to the genus *Salmonella* (Hensel *et al.*, 1997; Ochman *et al.*, 1996), whereas SPI-1 hybridising sequences have been detected in other bacterial pathogens (Groisman and Ochman, 1993). Second, several from within SPI-2 are not present in strains of *Salmonella bongori*, but they are found in all seven remaining subspecific groups of *Salmonella enterica* (Hensel *et al*, 1997; Ochman *et al.,* 1996).

1.10.3 Pathogenicity islets of *Salmonella*

Several other *Salmonella*-specific sequences are essential for virulence in this facultative intracellular pathogen. These regions are much smaller than SPI-1 and SPI-2 and may be referred to as 'pathogenicity islets'.

1.10.3.1 The *sifA* gene

At 27' on the *S.enterica* sv typhimurium chromosome, there is a 1.6-kb segment that harbors a gene, *sifA*, required for the formation of filamentous structures in the lysosomal vacuoles of infected epithelial cells (Stone *et al.*, 1992). The *sifA*-containing segment is situated between the *potB* and *potC* genes, which correspond to the *potABCD* operon of *Escherichia coli.* The housekeeping *potABCD* operon of nonpathogenic bacteria mediates polyamine uptake, whereas the *sifA* gene has no homologs in the sequence databases and is apparently restricted to *Salmonella.*

Additional features of the *sifA* gene support the view that this locus was integrated into the *Salmonella* chromosome and is not ancestral to the Enterobacteriacea. First, the base composition of the *sifA* gene is only 41% G+C, whereas the adjoining *pot* genes have a G+C contents typical of the *Salmonella* genome. Second, the *sifA*-containing fragment is flanked by 14-bp direct repeats, suggesting that the incorporation of the *sifA* fragment into the *Salmonella* genome occurred by a site-specific recombination mechanism similar to those mediating the integration of phage genomes and the insertion of transposable elements (Groisman and Ochman, 1997).

1.10.3.2 *PagC* and *msgA* genes

Two virulence genes, *pagC* and *msgA*, have been localized to a low G+C region at 25' on the *S.enterica* sv Typhimurium chromosome. Of the four additional

genes that have been localized to the region harboring *pagC*, only *msgA* is required for intra-macrophage survival and mouse virulence. Despite the similarity in their virulence phenotypes, the *pagC* and *msgA* genes differ in their regulation and phylogenetic distribution: expression of *pagC*, but not *msgA*, is dependent on the PhoP regulatory protein, and *msgA*-hybridizing sequences are detected in enteric species that lack the *pagC* gene (Hohmann *et al.*, 1978).

1.10.3.3 Fimbrial genes

At least five fimbrial operons, *fim*, *agfA*, *lpf*, *ser* and *pef*, have been identified in *Salmonella*, the majority of which have not been detected in other enteric species (Baumler *et al.*, 1997). Phylogenetic analysis of these sequences indicates that the *fim* operon and *agfA* gene are ancestral to *Salmonella*. In contrast, the *lpf*, *pef* and *ser* operons are each marked by events of acquisition and/or loss by lineages or subspecific groups of *Salmonella*. The specific combination of fimbrial genes and, hence, adhesive properties of the cell appear to be associated with the host range of certain serovars and subspecies (Baumler *et al.*, 1997). However, the mechanism by which the presence or absence of a fimbrial operon affects the colonization of a particular host is unknown, and the observed relationship could have arisen by chance (Groisman and Ochman, 1997).

1.10.3.4 *iviVI-A* and *iviVI-B* genes

Two genes of low G+C content were recently recovered by *in vivo* expression technology (IVET), a procedure that allows the identification of promoter

sequences induced during the course of infection (Mahan *et al.*, 1996). These genes, termed *iviVI-A* and *iviVI-B*, are organized in an operon that is regulated by the PhoP protein, and they may mediate the adhesion and/or invasion of host cells because the products of both genes exhibit sequence similarity to proteins that specify these traits in other pathogens. The *iviVi-AB* operon maps to 7' on the *S.enterica* sv Typhimurium chromosome, a region that contains another *Salmonella*-specific gene of low G+C content, *sinR* (Groisman and Ochman, 1993). However, it is unknown whether the *sinR* region is part of the *iviVI* virulence gene cluster.

1.10.4 Plasmids

Large plasmids of 50-100kb have shown to play important roles in the ability of specific *Salmonella* serovars to progressive disseminated infection (Gulig and Doyle, 1993). Although earlier literature often referred to these plasmids as 'cryptic', they are now designated *Salmonella* virulence plasmids (Gulig *et al.*, 1994).

The distribution of virulence plasmids in the genus *Salmonella* is neither uniform or random, but is rather highly serovar specific. A striking association exists between virulence plasmids and serovars that are host adapted to domestic animals. *S.dublin* (cattle), *S.choleraesuis* (pigs), *S.gallinarum-pullorum* (fowl), and *S.abortusovis* (sheep) (Gulig and Doyle, 1993; Barrow *et al.*, 1987; Colombo *et al.*, 1992). Virtually all wild-type isolates of these serovars contain virulence

plasmids. In addition, virulence plasmids are found in the broad host-range serovars of *S.typhimurium* and *S.enteritidis*, although only a variable proportion of isolates contain such a plasmid (Gulig *et al.*, 1994).

Homology between virulence plasmids from different serovars have been examined using region-specific probes and by hetero-duplex analysis. Within a particular serovar, virulence plasmids from separate isolates are remarkably similar. However, considerable differences exist between plasmid from different serovars. The *S.typhimurium* (100kb) contains virtually all of the smaller *S.enteritidis* (60kb) and nearly al the *S.choleraesuis* plasmid (50kb). In contrast, the *S.dublin* plasmid (80kb) has extensive regions that do not hybridize to any of the other plasmids tested (Montenegro *et al.*, 1991).

1.10.4.1 *Salmonella* plasmid virulence (*spv*) genes

Sequence and functional relationships. All *Salmonella* virulence plasmids share a highly conserved region of approximately 8kb which encodes the *spv* regulon, consisting of the positive regulator *spv*R and four structural *spv*ABCD (Gulig and Doyle, 1993). Molecular genetic analysis has shown that the *spv* region is required for the expression of the plasmid virulence function. DNA sequences from *S.typhimurium*, *S.dublin* and *S.choleraesuis* have been determined and show less than 0.5% divergence at the nucleotide level. The regions appear functionally equivalent in the mouse model even when introduced into a different

serovar. The virulence plasmids from *S.enteritidis* restores virulence to a cured *S.dublin* strain (Beninger *et al.*, 1988).

1.10.4.2 The function of *spv* genes in virulence

The role of the *spv* region during *Salmonella* infection has been characterized at the pathological level but is not understood in mechanistic terms. Early experiments in the mouse model showed that the virulence plasmids is not required for the invasion through the intestinal mucosa, nor for the dissemination and persistence in deep tissues such as lymph nodes, liver and spleen (Heffernan *et al.*,1987). Instead, strains containing virulence plasmids grow faster at these sites and rapidly overwhelm mice that are genetically susceptible. The ability to grow inside host cells has been shown *in vivo* to correlate with the phenotype (Fierer *et al.*, 1992; Gulig and Doyle, 1993). Molecular epidemiology studies in livestock and humans indicate that the *S.typhimurium* plasmid is an important virulence trait in extra-intestinal disease (Barrow and Lovell, 1989; Fierer *et al.*, 1992). In the host-adapted serovars that infect humans (*S.dublin* and *S.cholerasuis*) and particularly associated septicemia (Blaser and Feldman, 1981; Fang and Fierer, 1991). Therefore, the virulence plasmids appear to enhance the ability of certain serovars to multiply in tissues outside the intestinal tract (Gulig *et al.*, 1994).

1.11 Target populations

All age groups are susceptible, but symptoms are most severe in the elderly, infants and the infirm. AIDS patients suffer salmonellosis frequently (estimated 20-fold more than general population) and suffer from recurrent episodes. It is estimated that from 2 to 4 million cases of salmonellosis occur in the U.S. annually. Based on reports form the Centers for Disease Control and Prevention (CDC), *Salmonella* was the leading cause of foodborne illness outbreaks in the US between 1973 and 1987 (Bean and Griffith, 1990).

1.12 *Salmonella* outbreak reports

In recent years, there has been a perception that the frequency of *Salmonella* infections in both humans and animals is increasing. This may be due in part to a growing awareness among producers and veterinarians, improved recovery and identification techniques for *Salmonella*, and the media publicity given human outbreaks of salmonellosis associated with food animal products (Robinson, *et al.*, 1992).

Based on reports from the Centers for Disease Control and Prevention (CDC), *Salmonella* was the leading cause of foodborne illness outbreaks in the US between 1973 and 1987 (Bean and Griffin, 1990). Financial losses resulting from *Salmonella* infections are considerable (Hogue *et al.*, 1997). A 1996 Economic Research Service (ERS) report of foodborne *Salmonella* infections in humans estimated a total of $0.6 to 3.5 billion dollars annually in medical expenses and

productivity losses (Buzby *et al.*, 1996). Other costs to producers include treatment costs and livestock deaths resulting from *Salmonella* infections, increased cull rates, reduced feed efficiency and decreased weight gain (Robinson *et al.*, 1992).

Public and health agencies are becoming increasingly concerned about the occurrence of *Salmonella typhimurium* (definitive type [DT] or phage type) 104 that is resistant to at least five antimicrobial drugs: ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracycline (R-type ACSSuT). One of the concerns regarding this pathogen is that it is associated with higher hospitalization and mortality rates among people than for other *Salmonella* infections (Wall *et al.*, 1994).

The pattern of DT104, including multidrug resistant (mr) DT104 (mrDT104), occurrence in the US is not well established and requires further investigation. Based on the evaluation of cattle *Salmonella* isolates which have been banked over time, it appears that mrDT104 has been present in the US since at least the early 1990's. According to data accumulated by the National Veterinary Services Laboratories (NVSL) and reported by the US Health Association, the percentage of *Salmonella typhimurium* isolates from clinically ill animals has increased slightly since 1990 (Robinson *et al.*, 1992).

During October 1996, the Nebraska Department of Health was notified about an outbreak of diarrheal illness among elementary school children in Cass County, a farming community in east central Nebraska. During October 12-14, a total of 19 (52%) of 32 children attending an elementary school developed diarrhea (100%), fever (89%), headache (89%), nausea (89%), and vomiting (58%); three reported bloody diarrhea. None required hospitalization, and all recovered (Hosek *et al.*, 1997).

On October 10, during lunch at the school, children had been served cold chocolate milk poured from cartons. Of the 22 children who drank the milk, 18 (82%) developed diarrhea, compared with one (10%) of 10 children who did not drink it (risk ratio {RR} = 8.2; 95% confidence interval {CI} = 1.3-53.1). Inspection of the school refrigerator detected numerous cartons of milk with expiration dates predating October 10, but cultures of samples obtained from these remaining cartons were negative for enteropathogens. In addition, some children had handled a turtle brought to the school for "show-and-tell' and reportedly ill kitten during October 7-9. However, neither the turtle nor kitten were available for testing. Culture of stool samples obtained from seven children all yielded *Salmonella typhimurium* R-type ACSSuT. Phage-typing at CDC confirmed the isolates as DT104 (Hosek *et al.*, 1997).

Salmonella typhimurium DT104 was first reported in the UK in 1984; this organism is now the second most prevalent strain of *Salmonella* isolates from

humans in the UK after *Salmonella* serotype *Enteritidis* phage type 4 (Threlfall, *et al.*, 1996). Contact with ill farm animals and consumption of chicken, pork sausages, and meat paste were identified as risk factors for DT104 infection in England and Wales (Wall *et al.*, 1994). The organism has been isolated from several species (poultry, sheep, pigs {Anonymous, 1993}, cats, wild birds, rodents, foxes, and badgers {Evans and Davies, 1996} and has been transmitted from cattle and sheep to humans (Fone and Barker, 1994). Although R-type ACSSuT is the most common Antimicrobial resistance pattern of DT104 isolates (present in 54%-67% of DT104 isolates in the UK during 1992-1995), resistance of DT104 isolates to trimethoprim and fluoroquinolones is emerging. In the UK, from 1993 to 1995, trimethoprim-resistant DT104 (R-type ACSSuTTm) increased from 1% to 27% of isolates, and ciprofloxacin-resistant DT104.

1.13 Detection of *Salmonella*

1.13.1 General aspects

Traditional biochemical and immunochemical methods for the detection of microorganisms in foods have been supplemented by a number of DNA-based methods during the last decade. The conventional methods lack the required speed for analysis of food products (D'Aoust, 1989). Rapid detection methods like enzyme-linked immunosorbent assay, motility enrichment, DNA hybridization (Blackburn, 1993), antibodies (Toresma *et al.*, 1992) suffer from lack of specificity, which limits their acceptance. Besides the development of direct hybridization techniques, emphasis has been laid on *in vitro* amplification

methods. The most developed *in vitro* amplification method is the Polymerase Chain Reaction (PCR) that allows rapid and selective identification microorganisms. Although the PCR method has advantages (particularly specificity, sensitivity) it also has limitations, one of which is its inability to allow differentiation between viable and non-viable microorganisms (Scheu *et al.*, 1998).

The development of quick and accurate procedures for detecting and characterizing microorganisms is needed in many areas of research and areas in biological safety is a major issue. Along with medical microbiological control is increasingly being applied in the food industry. Traditional morphological and physiological criteria are fundamental parameters contributing to the identification of microorganisms and pathogens in food. These criteria may be influenced by environmental conditions (Scheu *et al.*, 1998).

1.13.2 General aspects of genotypic detection

Genetic characteristics of microorganisms as opposed to biochemical and immunological properties, can be employed for their identification and typing. A genotypic detection system of microorganisms does not depend on either growth state nor on environmental influences and is, therefore more precise (Scheu *et al.*, 1998).

1.13.3 *In vitro* amplification methods

Sensitivity can be greatly improved through the use of the different *in vitro* amplification methods (Polymerase Chain Reaction, Q-beta replicase amplification, Ligase chain reaction, Self-sustained sequence replication or Nucleic acid amplification).

1.14 Polymerase Chain Reaction (PCR) technique

The PCR technique allows rapid and selective identification and/or detection of microorganisms in different matrices by amplifying specific gene fragments. The reaction cycle consists of three steps: (1) denaturation of the double-stranded DNA; (2) annealing of short DNA fragments (primers) to single DNA strands; (3) extension of the primers with the key enzyme, a thermostable DNA-polymerase. Following the completion of one cycle, the sample is denatured for the next annealing and extension steps during which not only the original target region amplified, but also the amplification product of the cycle. As long as there is excess in primer and nucleotide concentration, this process leads to an exponential increase of the number of copies of the target DNA. The detection of amplification products is possible through gel electrophoresis, ethidium bromide staining and visual examination of the gel using ultraviolet light. To increase the sensitivity, and more importantly, to confirm the identity of the amplification product. Southern blotting and hybridization with a specific probe should follow. Colourmetric or fluorimetric hybridisation of amplification products may also be

carried out in a micro-titre plate. The advantage of this method is that it may be automated to reduce time and cost (Scheu *et al.*, 1998).

Since its discovery (Mullis *et al.*, 1986; Mullis and Faloona, 1987) PCR has been used for many applications in molecular biology and medical diagnostics (Jones and Bej, 1994), but it has only recently raised interest as a detection method in food hygiene and control.

Major criteria for the detection of microorganisms in food using PCR-systems are their specificity and sensitivity. To a large extent specificity is determined by the sequences of primers (oligonucleotides) that are unique to the target microorganism, and the annealing conditions that have to be optimized in order to minimize non-specific priming. Specificity can be increased by using probes that exclusively recognize the correct amplified target DNA (Scheu *et al.*, 1998).

1.14.1 Limitations of PCR

Differentiation between viable and nonviable microorganisms. Amplification products using any set of primers only demonstrate that the appropriate target nucleic acid sequences are present in the sample. As long as intact nucleic acid sequences are present in a sample, they will be amplified by PCR. Therefore, DNA from dead microorganisms may lead to false-positive results.

As dead, viable but not culturable, and culturable microorganisms can be present in a sample, it is necessary to have a method that can distinguish between these. One way to prevent the detection of dead microorganisms is a propagation step prior to the PCR analysis. In this way it is possible not only to increase the sensitivity of the test, but to restrict the detection to culturable cells (Josephson *et al.*, 1993). According to literature, the selection of living microorganisms with the aid of antibody-coated paramagnetic beads should be possible (Kapperud *et al.*, 1993). However, Hornes *et al.* (1991) showed that non-viable bacteria with intact cell surface antigens could also be enriched through immunomagnetic separation. Using PCR-based detection without prior culture enrichment, one would expect that false positive results will frequently be obtained.

1.14.2 Inhibition of PCR reaction

A major problem in using PCR methods with food is the presence of PCR inhibitors. False negative results can occur for various reasons: (i) the presence of substances chelating divalent magnesium-cations necessary for PCR; (ii) degradation of nucleic acids targets and/or primers through nucleases (Rnase, Dnase); (iii) direct inhibition of the target DNA polymerase. Therefore, to reduce false negative results the inclusion of amplification controls is essential to indicate the presence of PCR inhibitors in the sample to be tested. For this purpose an exogenous DNA standard is added to the sample and amplified simultaneously in a single PCR reaction mixture (Cave *et al.*, 1994; Jin *et al.*, 1994; Kolk *et al.*, 1994).

1.14.2.1 Substances Inhibiting PCR

Several research groups have demonstrated that many substances can directly inhibit the activity of the DNA polymerase (Demeke and Adams, 1992; Ahokas and Erkkila, 1993; Katcher and Schwartz, 1994; Wiedbrauk *et al.* 1995). Studies relating to the amounts of food components that were added PCR mixtures showed that relatively high levels of oil, salt , carbohydrate and amino acids have no inhibitory effect. In addition, calcium ions could be identified as a source of PCR inhibition, when applied to the detection of *Listeria monocytogenes* in milk (Bickley *et al.*, 1996). Furthermore, different enrichment broths and their components as well as DNA extraction solutions can be tolerated even at levels corresponding to running PCR directly in the media. Ionic detergents (eg. Sodium dodecyl sulfate, sodium deoxycholate and sarkosyl) have also been shown to have inhibitory effects (Weyant *et al.*, 1990). Moreover, unspecific DNA (0,4mg unrelated DNA added to 100ul PCR mixture) is able to completely inhibit the PCR (Rossen *et al.*, 1992).

1.14.2.2 Prevention of PCR inhibition

The removal of contaminating substances from DNA is an important step in many applications. One of the easiest procedures to circumvent the inhibition of PCR is to extensively dilute the food samples (Weyant *et al.*, 1990). However, besides attenuating, dilution of the samples also decreases sensitivity when the amount of the DNA template is a limiting factor. In addition, PCR inhibition can be prevented by separating bacteria from the food matrix prior to DNA extraction.

This can be done by preparing a subculture of food samples on selective agar media and using the bacterial colonies for PCR (Wiedbrauk *et al.,* 1995; Wood *et al.*, 1996).

Another possibility is the purification of DNA by gel filtration or anion exchange columns. The latter can be supplemented by silica-based systems (eg. Glassmilk). A common principle of these purification methods is the binding of DNA to a matrix in order to wash away the contaminating substances and subsequently to elute the purified DNA. Fluids with potential PCR inhibitors may be processed by analysis or ultrafiltration prior to PCR. Ultrafiltration systems which are commercially available may be used, as dialysis is a very time-consuming process (Khan *et al.*, 2000).

A simple method for eliminating DNA polymerase inhibitors is the use of Chelex 100, a chelating resin which has a high affinity for polyvalent metal ions. Chelex 100 also has a protective effect against the degradation of DNA by chelating metal ions which catalyse the breakdown of DNA (Singer-Sam *et al.,* 1989). In addition, an improvement in the lysis of gram-positive bacteria has been observed when Chelex 100 is used (Walsh *et al.*, 1991). The chelating effect may be increased by heating under alkaline conditions to denature the template DNA and increase the PCR signal (Poli *et al.*, 1993).

To relieve inhibition in samples containing endogenous protease activity the addition of bovine serum albumin (BSA) could be useful (Woodward and Kirwan, 1996). Several of the substances whose inhibition is relieved by the addition of BSA in the PCR contain phenolic groups. Since phenols bind to proteins by forming hydrogen bonds with the peptide bond oxygen, it can be conclude that BSA is able to scavenge such substances and thus their binding to and subsequent inactivation of DNA polymerase (Kreader, 1996).

Studies using several thermostable DNA polymerases indicated that the extent of PCR inhibition can also depend upon the type of DNA polymerase used. For example DNA polymerase from *Thermus thermophilus* and *Thermus flavus* were completely resistant to substances that effectively inhibited Taq DNA polymerase (Martin *et al.*, 1994).

1.14.3 Factors affecting the PCR

1.14.3.1 Denaturing temperature and time

The specific complementary association due to hydrogen bonding of single-stranded nucleic acids is referred to as "annealing": two complimentary sequences will form hydrogen bonds between their complimentary bases (G to C, and A to T or U) and form a double-stranded, anti-parallel "hybrid" molecule. If the Nucleic Acid (NA) is not single-stranded, like most RNA viruses, it may be changed to a single-stranded NA by heating it to a point above the "melting temperature" of the double-or partially-double-stranded form, and then flash

cooling it: this ensures the "denatured' or separated strands do not re-anneal. Additionally, if the NA is heated in buffers of ionic strength lower than 150mM NaCl, the melting temperature is generally less than 100°C- which is why PCR works with denaturing temperatures of 91-97°C (Innis and Gelfand, 1990).

Taq polymerase is given as having a half-life of 30min at 95°C, which is partly the reason for no more than 30 amplification cycles: however, it is possible to reduce the denaturing temperature after about 10 rounds of amplification, as the mean length of target DNA is decreased: for templates of 300bp or less, denaturation temperature may reduced to as low as 88°C for 50% (G+C) templates which means one may do as many as 40 cycles without much decrease in enzyme efficiency (Innis and Gelfand, 1990).

1.14.3.2 Annealing temperature and primer design

1.14.3.2.1 Annealing temperature and Time

Primer length and sequence are of critical importance in designing the parameters of a successful amplification: the melting temperature (Tm) of Nucleic Acid (NA) increases both with its length, and with increasing (G+C) content. A simple formula for calculation of the Tm is :

$$Tm = 4 (G+C) + 2 (A+T)\ ^{o}C$$

Thus, the annealing temperature chosen for a PCR depends directly on length and composition of the primer(s). The aim should be an annealing temperature (Ta) of about 5°C below the lowest Tm of the primers used (Innis and Gelfand, 1990). One consequence of using too low a Ta is that one or both primers will anneal to sequences other than the true target. As internal single-base mismatches may be tolerated: however, it can lead to "non-specific' amplification and consequent reduction in yield of the desired product, if the 3'-most base is paired with a target. A consequence of too high Ta is that too little product will be made, as the likelihood of primer annealing is reduced; another and important consideration is that a pair of primers with very different Ta's may never give appreciable yields of a unique product, and may also result in advertent "asymmetric" or single-strand amplification of the most efficiently primed product strand (Innis and Gelfand, 1990).

A simple set of rules for primer sequence design is as follows (adapted from Innis and Gelfand, 1990) :

- primers should be 17-28 bases in length;
- base composition should be 50-60% (G+C);
- primers should end (3') in a G or C, or CG or GC: this prevents "breathing" of ends and increases efficiency of priming;
- Tm's between 55-80°C are preferred;

- 3'-ends of primers should not be complimentary (ie. base pair), as otherwise primer dimmers will be synthesized preferentially to any other product;
- primer complimentarity (ability to form secondary structures such as hairpins) should be avoided;
- runs of three or more Cs or Gs at the 3'-ends of primers may promote mispriming at G or C-rich sequences (because of stability of annealing), and should be avoided.

1.14.3.3 Cycle number

The number of amplification cycles necessary to produce a band visible on a gel depends largely on the starting concentration of the target DNA. Innis and Gelfand (1990) recommended from 40-45 cycles to amplify 50 target molecules and 25-30 to amplify 3×10^5 molecules to the same concentration. This is the so-called plateau effect, which is the attenuation in the exponential rate of product accumulation in late stages of PCR, when product reaches 0.3-1.0nM. This may be caused by degradation of reactants (dNTPs, enzyme); reactant depletion (primers, dNTPs-former problem with short products, latter for longer products); end-product inhibition (pyrophosphate formation); competition for reactants by non-specific products; competition for primer binding by re-annealing of concentrated (10nM) product (Innis and Gelfand, 1990).

1.15 Bioinformatics

Currently there is a trend to design serotype specific primers, which will target a unique, conserved region in a serotype (Soumet *et al.*, 1999; Zhu *et al.*, 1996). Entrez is a retrieval system for searching several linked databases and is provided by the National Center for Biotechnology Information (NCBI). It provides access to several avenues related to gene sequences. Namely, the Biomedical literature (PUBMED), the nucleotide sequence database (Genbank), three-dimensional macromolecular structures, complete genome assemblies, population study data sets and Online Mendelian Inheritance in Man (OMIM) (http://www3.ncbi.nlm.nih.gov/Entrez/index.html March 2001). Using information found in the Gene Bank database, a conserved region can be found for the chosen serotype. This can be achieved by using the Bioinformatics tool, BLAST (Basic Local Alignment Search Tool) (Altschul *et al.*, 1990).

1.15.1 BLAST

BLAST (Basic Local Alignment Search Tool) is a set of similarity search programs designed to explore all of the available sequence databases regardless of whether the query is protein or DNA. The BLAST programs have been designed for speed, with a minimal sacrifice of sensitivity to distant sequence relationships. The scores assigned in a BLAST search have a well-defined statistical interpretation, making real matches easier to distinguish from random background hits. BLAST uses a heuristic algorithm which seeks local as opposed

to global alignments and is therefore able to detect relationships among sequences which share only isolated regions of similarity (Altschul *et al.*, 1990).

1.15.2 Expect (E) value

The Expect value (E) is a parameter that describes the number of hits one can "expect" to see just searching a database of a particular size. It decreases exponentially with the Score (S) that is assigned to a match between two sequences. Essentially, the E value describes the random background noise that exists for matches between sequences. The Expect value is used as a convenient way to create a significance threshold for reporting results. When the Expect value is increased from the default value of 10, a larger list with more low-scoring hits can be reported (http://www.ncbi.nlm.nih.gov/BLAST/.html March 2001).

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Chapter 2

Polymerase Chain Reaction detection of *Salmonella typhimurium* using nucleotide sequences coding for invasion (sigD and sigE) and ATP dependent protease binding subunit (clpB) genes

The style of this chapter is in accordance with that of the *Journal of Applied Microbiology*.

2.1 ABSTRACT

Salmonella are a major cause of foodborne infections and there is great interest in understanding the pathogenesis of *Salmonella* infection. The serovar *Salmonella typhimurium* has emerged as one of the most important foodborne pathogens. Humans acquire the bacteria from contaminated foods such as beef products, poultry, eggs, egg products or water. Current protocols for the detection of *Salmonella* are time-consuming and rather inefficient. The development of Polymerase Chain Reaction (PCR) technology has the potential to solve these problems. **AIMS** : Search the National Center for Biotechnology Information (NCBI) database for *Salmonella typhimurium* nucleotide sequences involved in host invasion. Design a primer pair unique to the chosen *Salmonella typhimurium* nucleotide sequence. **MATERIALS AND METHODS** : From the nucleotide sequence coding for *Salmonella typhimurium* invasion gene D protein (sigD) and invasion gene E protein (sigE), a primer set (Red1 and Red2) was

designed to detect a 350bp PCR product. From a second nucleotide sequence coding for *Salmonella typhumurium* ATP dependent protease binding subunit (clpB) gene, Dav1 and Dav2 was designed to detect a 1024bp PCR product. A total of 11 *Salmonella* strains and 10 non-*Salmonella* samples were used in this experiment. The Promega Genomic Wizard and the QIAGEN Miniprep Kits were used to extract genomic and plasmid DNA, respectively. The extracted products (1.5µl) was added to the PCR reactional mixture (23.5µl). **RESULTS AND DISCUSSION :** Both the primer sets, Red1+Red2 and Dav1+Dav2, amplified the expected *Salmonella typhimurium* as well as several other *Salmonella* strains. With the exception of *E.coli,* none of the other non-*Salmonella* strains were amplified. These results would suggest a closer serology between the *Salmonella* strains amplified. The technique proved to be sensitive and efficient.

2.2 INTRODUCTION

Salmonella spp. are responsible for the highest number of documented cases of food poisoning in the developed world (Dickinson *et al.* 1995). These gram-negative bacteria are responsible for 16 million annual cases of typhoid fever and 1.3 billion annual cases of gastroenteritis, which together result in more than 3 million deaths annually. The serovar *Salmonella typhimurium* has emerged as one of the most important foodborne pathogens. In addition, the salmonellae cause disease in a number of animal species (Groisman and Ochman 2000). *Salmonella* are typically acquired through the consumption of contaminated water or food and must endure the acid pH of the stomach before they adhere to and enter the cells lining the intestinal epithelium (Giannella *et al.* 1973). These invasive microorganisms destined to cause systemic disease must also survive in blood and replicate in the macrophages of the liver and spleen (Groisman and Ochman 1997).

At least 60 genes are required for virulence in *Salmonella enterica*. The requirement for so many virulence determinants is thought to reflect the complex life cycle of this pathogen in infected animals. Although some of these genes reside on a plasmid common to many *Salmonella* serovars, the vast majority is encoded within pathogenicity islands – large clusters of virulence genes not found in related species. *Salmonella*-specific virulence genes reside in several regions of the chromosome and often encode determinants responsible for establishing specific interactions with the host (Groisman and Ochman 1997).

Genes essential for *Salmonella typhimurium* invasion have been localized to *Salmonella* pathogenicity island 1 (SPI1) on the chromosome. However, it is clear that other genes are required for the invasion process (Hong and Miller 1998). Some of these genes include *Salmonella typhimurium* invasion gene D protein (*sigD*) and invasion gene E protein (*sigE*). *SigD* appears to be secreted through the SPI1-encoded type III secretion apparatus and requires *SigE* for this process to occur. Several other proteins secreted by the SPI1-encoded apparatus that are required for *Salmonella* invasion into cultured epithelial cells have been identified. It has been proposed that these proteins act by stimulating endocytosis of the bacteria by the host cell (bacterium-mediated endocytosis or BME) (Galan 1996; Portnoy and Smith 1992). How these proteins stimulate the host cells has yet to be determined, but it seems that many proteins may be involved in this process (Hong and Miller 1998). Other genes may have a function different to those mentioned above. Such as the *Salmonella typhimurium* ATP dependent protease binding subunit (*clpB*) gene. A stress response gene, specifically heat-response, found to be involved in the virulence and colonization of the chicken alimentary tract (Turner *et al.* 1997). Another would be the *Salmonella typhimurium* invasion gene D protein (*sigD*) and invasion gene E protein (*sigE*) genes. Genes essential for *Salmonella typhimurium* invasion into the host cell (Hong and Miller 1998).

Conventional methods for the detection of *Salmonella* spp. in foods require a minimum of 4 days to obtain presumptive results after initiation of sample

analysis (Nishihara *et al.* 1990). The need for more rapid methods for the detection of *Salmonella* spp. are growing in food industries and government agencies. In recent years new methods have been developed to increase the efficiency and speed of food microorganism detection while reducing labour (Konuma 1989).

Many methods, such as culture methods (D'Aoust 1981; De Smedt *et al.* 1986), enzyme immunoassay (Ibrahim *et al.* 1985) and DNA-DNA hybridization (Knight *et al.* 1990), are now available for detecting *Salmonella* spp. in foods. An increasing number of reports indicate that application of the Polymerase Chain Reaction (PCR) technique to reliably and quickly detect pathogens in foods, is a promising new diagnostic tool to monitor food safety (Takahisa *et al.* 1998).

PCR technology is one of the most promising of the rapid microbiological methods for the detection and identification of bacteria in a wide variety of samples. The high sensitivity and specificity of the PCR makes it an attractive means for achieving this purpose (Cohen et al. 1994).

A new approach to rapid sequencing comparison, BLAST, directly approximates alignments that optimize a measure of local similarity, which is known as the maximal segment pair (MSP) score. Mathematical results on the stochastic properties of MSP scores allow an analysis of the performance of this method as well as the statistical significance of alignments it generates. The basic algorithm

is simple and robust; it can be implemented in a number of ways and applied in a variety of contexts including straightforward DNA and protein sequence database searches, motif searches, gene identification searches and in the analysis of multiple regions of similarity in long DNA sequences. In addition to its flexibility and tractability to mathematical analysis, BLAST is an order of magnitude faster than existing sequence comparison tools of comparable sensitivity (Altschul *et al.*,1990).

The aim of this chapter was to develop a PCR technique to detect *Salmonella typhimurium*. This would be accomplished through several steps. The first being to search the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov, March 2001) nucleotide database for relevant nucleotide *Salmonella typhimurium* genes assumed to be essential in invasion and/or virulence of the host. The chosen nucleotide sequences would then be subjected to a computer program, BLAST, to determine the similarity sequences with other nucleotide sequences present in the database. Followed by a Multiple Sequence Alignment to identify a conserved region unique to the chosen nucleotide sequence from which a primer pair for *Salmonella typhimurium* may be designed.

2.3 MATERIALS AND METHODS

2.3.1 Primer Design

2.3.1.1 NCBI nucleotide search

The National Center for Biotechnology Information (**NCBI**) (www.ncbi.nlm.nih.gov, March 2001) nucleotide database was searched for relevant nucleotide *Salmonella typhimurium* gene sequences that were assumed to be essential in invasion and/or virulence of the host. This was achieved by typing in relevant key words in the "Search" area provided, bringing up a list of all nucleotide sequences with the relevant key words. The Medline link to the particular nucleotide sequence chosen diverts to the abstract of the article on which research had been done on the genes involved. Most times the abstract links to a site where the full article can be viewed and more literature on the gene involved will be available.

2.3.1.2 BLAST

The BLAST algorithm of Altschul *et al.* (1990) was used to compare the *Salmonella typhimurium* with the sequences generated in this study. A list and an alignment of all the similar nucleotide sequences will be made available. Each nucleotide sequence would be assigned an E-value, a parameter that describes the number of hits one could "expect" to see. As well as a Score value, which is a value assigned to match between two nucleotide sequences. Attention would be paid to all those nucleotide sequences to which E-values zero had been

assigned, as these would be the nucleotide sequences with the most sequence similarities.

2.3.1.3 Multiple Sequence Alignment (MSA)

Once the nucleotide sequence was chosen, it was subjected to a MSA by using the CLUSTAL W program of Thomson *et al.* (1994). This is a computer program that compares the chosen nucleotide sequence to those sequences that have E-values=0. The MSA program automatically aligns the nucleotide sequences against one another. Thus making it easy to identify regions of nucleotide sequence similarities/conservation as well as regions where the chosen nucleotide sequence may differ. Particular attention is paid to those regions where the nucleotide sequence may differ to the rest of the nucleotide sequences, as this region could be the site for primer design.

2.3.2 Isolates

Isolates were obtained from Onderstepoort Research Institute, Pretoria. Glycerol stocks (25%) were made by inoculating 100µl of stock into 5ml of TSB, grown overnight at 37°C. Thereafter, 1ml of the overnight culture and 1ml of a 25% Glycerol solution was mixed and stored at -70°C. Glycerol working stock (25%) was made as well and stored at -4°C.

Table 2.1 List of *Salmonella* and non-*Salmonella* serotypes used in this experiment

***Salmonella* serotypes**	**Non-*Salmonella* serotypes**
Salmonella berta	*Escherichia coli*
Salmonella braenderup	*Staphylococcus aureus*
Salmonella choleraesuis	*Listeria monocytogenes*
Salmonella dublin	*Klebsiella pneumoniae*
Salmonella enteritidis	*Pseudomonas aueriginosa*
Salmonella gallinarum	*Micrococcus luteus*
Salmonella infantis	*Staphylococcus epidermis*
Salmonella java	*Bacillus subtilus*
Salmonella paratyphi	*Shigella flexeneri*
Salmonella typhi	
Salmonella typhimurium	

2.3.3 Culture conditions

Tryptone Soya Broth (TSB) (Oxoid) (CM1219) was prepared. An aliquot of 100µl of each culture was inoculated into 5ml of TSB grown aerobically overnight at 37°C in a shaking incubator (200rpm).

2.3.4 DNA extraction

2.3.4.1 Genomic DNA

Bacterial genomic DNA was prepared from 2ml of the overnight culture with Promega Genomic DNA Purification Kit (Catalogue number 3139) as recommended by manufacturer.

2.3.4.2 Plasmid DNA

Bacterial plasmid DNA was prepared from 2ml of the overnight culture with QIAGEN Miniprep KIT50 (Catalogue number 27104) as recommended by manufacturer.

2.3.5 PCR amplification conditions

PCR amplification was conducted using a Perkin-Elmer 2400. The PCR mixture contained 3µl of 10× Reaction Buffer (500mM KCl; 100mM Tris-HCl pH9.0), 1µl (250nmoles) each of oligonucleotide primer Red1 and Red2 or Dav1 and Dav2, 0.25µl of each dNTP (0.625mM), 2µl of 25mM $MgCl_2$, 14.3µl dH_2O and 1U/µl of Taq polymerase. Total reactional mixture equals 25µl. Samples were denatured for 2min at 95°C. Thirty cycles of amplification was run for 1min at 94°C, 30s at 54°C and 1min at 72°C. The reaction was completed by a final 5min extension at 72°C.

2.3.6 Detection of amplified DNA

Amplified products were analyzed by gel electrophoresis. Eleven microliters of each sample was loaded onto a 1.2% electrophoresis-grade agarose gel, and was run in 1×TBE buffer at 85V for 2hrs. Gels were stained with ethidium bromide and visualized and photographed under UV light with a UV transilluminator.

2.4 RESULTS

2.4.1 NCBI nucleotide database search

Table 2.2 Results of NCBI nucleotide database sequence search

ACCESSION NUMBER	FUNCTION
AF021817	codes for Salmonella typhimurium invasion gene D protein (sigD) and invasion gene E protein (sigE) genes
AF010250	codes for Salmonella typhimurium ATP dependent protease binding subunit (clpB) gene

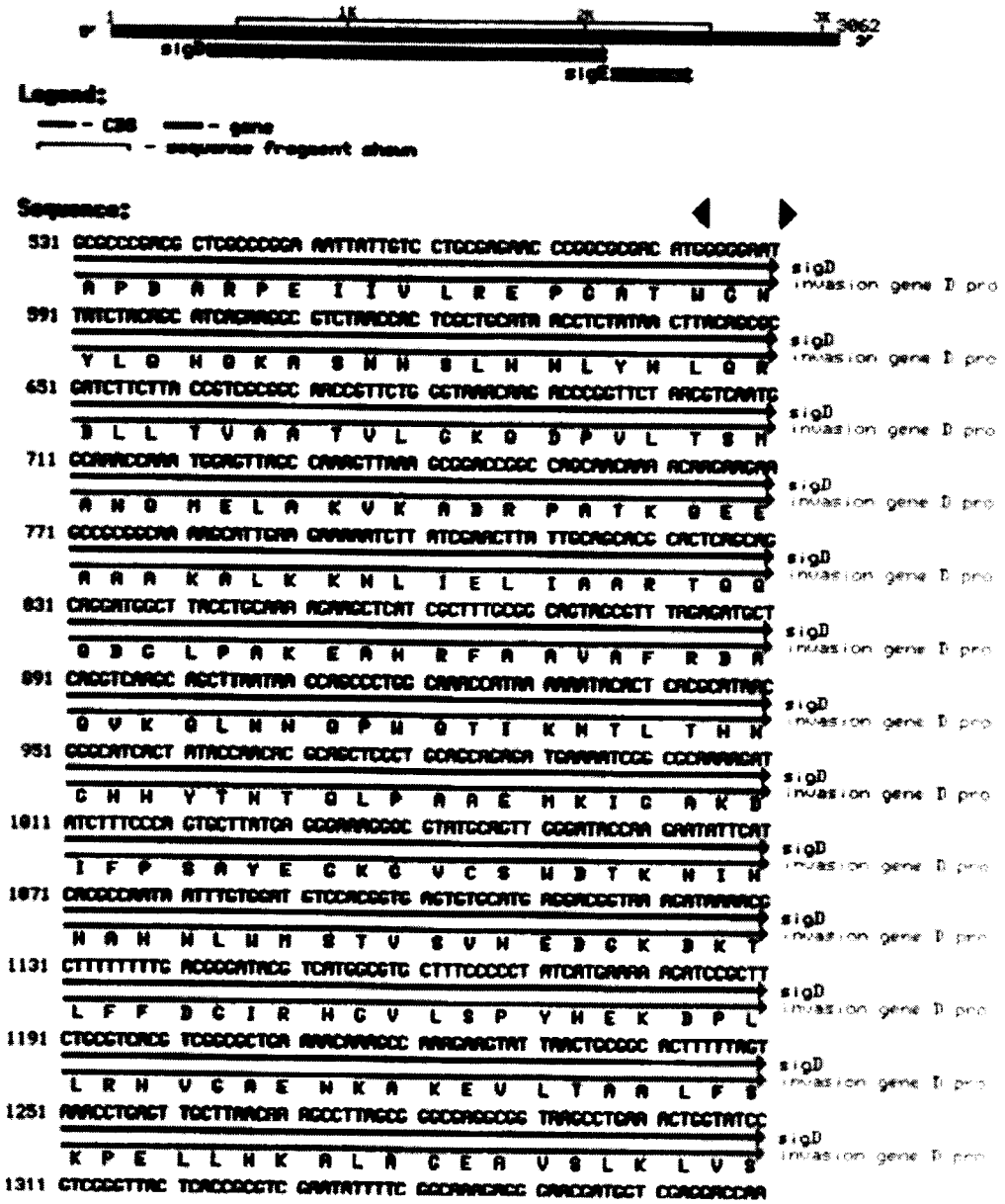

Figure 2.1 Graphics nucleotide sequence of AF021817 taken from the NCBI database (http://www.ncbi.nlm.nih.gov/entrez/viewer ,March 2001)

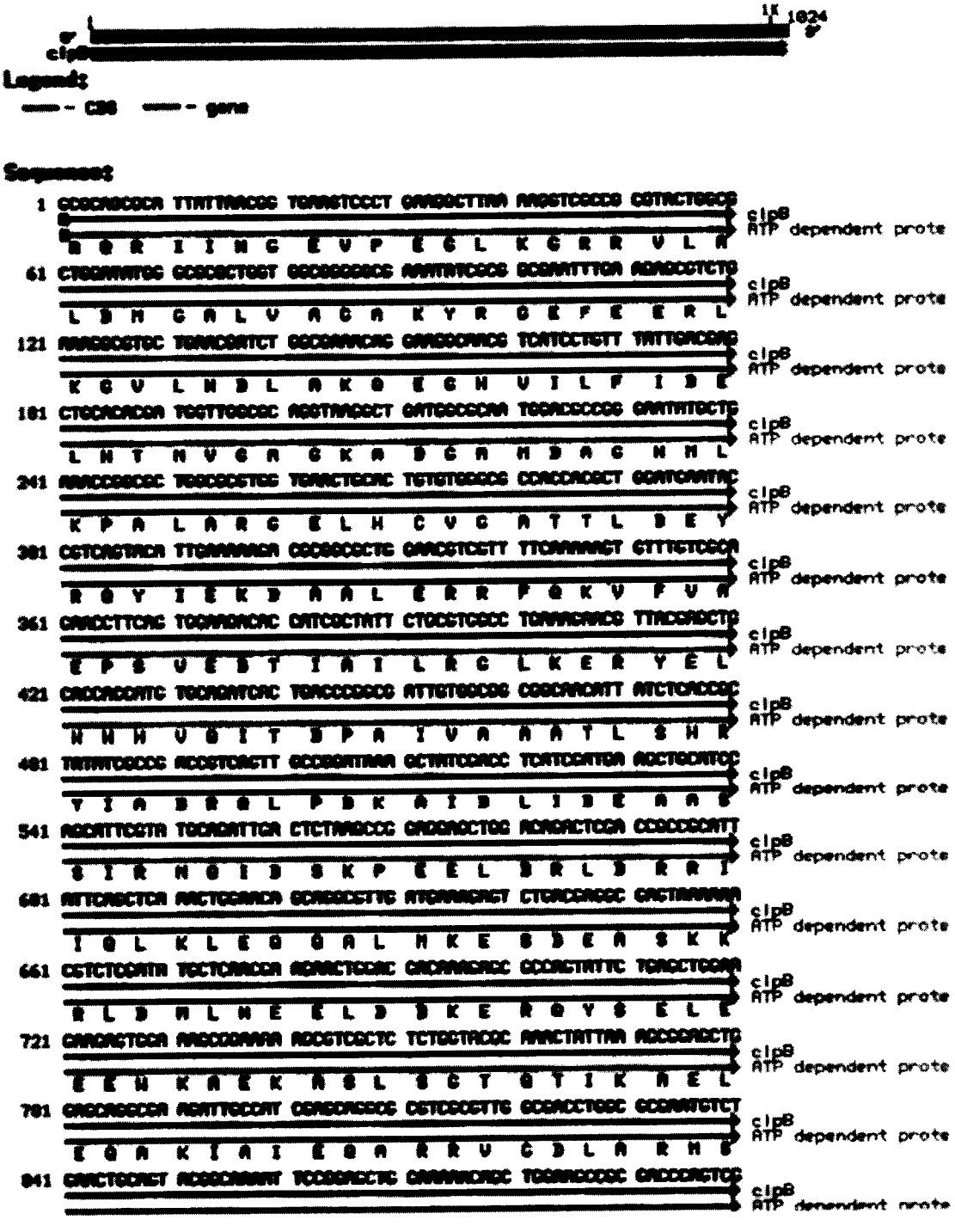

Figure 2.2 Graphics nucleotide sequence of AF010250 taken from the NCBI database (http://www.ncbi.nlm.nih.gov/entrez/viewer, March 2001)

2.4.2 BLAST

Sequences AF021817 and AF010250 was subjected to the BLAST algorithm of Altschul *et al.* (1990).

Sequences producing significant alignments:	(bits)	Value
gi|2582384|gb|AF021817.1|AF021817 Salmonella typhimurium in...	5939	0.0
gi|16763390|ref|NC_003197.1| Salmonella typhimurium LT2, co...	5822	0.0
gi|16419592|gb|AE008747.1|AE008747 Salmonella typhimurium L...	5822	0.0
gi|3511127|gb|AF060858.1|AF060858 Salmonella dublin regulat...	5438	0.0
gi|16758993|ref|NC_003198.1| Salmonella enterica subsp. ent...	5426	0.0
gi|16502231|emb|AL627269.1|AL627269 Salmonella enterica ser...	5426	0.0
gi|2351769|gb|U90203.1|SDU90203 Salmonella dublin secreted ...	3291	0.0
gi|6862590|gb|AF213335.2|AF213335 Salmonella typhimurium in...	2476	0.0
gi|7248842|gb|AF231141.1|AF231141 Salmonella dublin outer p...	2413	0.0
gi|6644415|gb|AF213334.1|AF213334 Salmonella blockley outer...	2413	0.0
gi|6644413|gb|AF213333.1|AF213333 Salmonella typhi outer pr...	2288	0.0
gi|13469833|gb|AF323077.1|AF323077 Salmonella enterica subs...	1499	0.0
gi|13469835|gb|AF323078.1|AF323078 Salmonella enterica subs...	910	0.0

Figure 2.3 BLAST results of sequence AF021817 producing significant alignments.

Sequences producing significant alignments:	(bits)	Value
gi|16763390|ref|NC_003197.1| Salmonella typhimurium LT2, co...	2030	0.0
gi|16421206|gb|AE008821.1|AE008821 Salmonella typhimurium L...	2030	0.0
gi|4102205|gb|AF010250.1|AF010250 Salmonella typhimurium AT...	2030	0.0
gi|16758993|ref|NC_003198.1| Salmonella enterica subsp. ent...	1586	0.0
gi|16503805|emb|AL627276.1|AL627276 Salmonella enterica ser...	1586	0.0
gi|16445223|ref|NC_002655.2| Escherichia coli O157:H7 EDL93...	864	0.0
gi|16127994|ref|NC_000913.1| Escherichia coli K12, complete...	864	0.0
gi|15829254|ref|NC_002695.1| Escherichia coli O157:H7, comp...	864	0.0
gi|147363|gb|M29364.1|ECOPROT Escherichia coli ATP-dependen...	864	0.0
gi|12517009|gb|AE005489.1|AE005489 Escherichia coli O157:H7...	864	0.0
gi|1788939|gb|AE000345.1|AE000345 Escherichia coli K12 MG16...	864	0.0
gi|13362858|dbj|AP002562.1|AP002562 Escherichia coli O157:H...	864	0.0
gi|1799991|dbj|D90887.1|D90887 E.coli genomic DNA, Kohara c...	864	0.0
gi|41113|emb|X57620.1|ECCLPB E.coli ClpB gene for an analog...	864	0.0

Figure 2.4 BLAST results of sequence AF010250 producing significant alignments.

2.4.3 Multiple Sequence Alignment

Nucleotide sequences that were found to have E-values = 0, were subjected to a Multiple Sequence Alignment. The Multiple Sequence Alignment was analyzed for both conserved regions as well as distinctions amongst the sequences.

2.4.4 Primer Design

Table 2.3. **Nucleotide sequences of the primers designed for the specific detection of *Salmonella typhimurium***

PRIMERS	NUCLEOTIDE SEQUENCE (5'-3')	
Red1	5' TGA CGG GAT ACG TCA TGG 3'	18mer
Red2	5' AAT GCG GCG ACG ACG TCC 3'	18mer
Dav1	5' GCA GCG CAT TAT TAA CGG TG 3'	20mer
Dav2	5' AAC AGT TTT TCA CGT TCG CCT 3'	21mer

Accession Number : AF010250

gi : 4102205 Salmonella typhimurium ATP dependent protease binding subunit (clpB) gene, partial cds, 1024bp

GCGCAGCGCATTATTAACGGTGAAGTCCCTGAAGGCTTAAAAGGTCGCCGCG
TACTGGCGCTGGATATGGGCGCGCTGGTGGCGGGGGCGAAATATCGCGGCGA
ATTTGAAGAGCGTCTGAAAGGCGTGCTGAACGATCTGGCGAAACAGGAAGG
CAACGTCATCCTGTTTATTGACGAGCTGCACACGATGGTTGGCGCAGGTAAG
GCTGATGGCGCAATGGACGCCGGGAATATGCTGAAACCGGCGCTGGCGCGTG
GTGAACTGCACTGTGTGGGCGCCACCACGCTGGATGAATACCGTCAGTACAT
TGAAAAAGACGCGGCGCTGGAACGTCGTTTTCAAAAAGTGTTTGTCGCAGAA
CCTTCAGTGGAAGACACCATCGCTATTCTGCGTGGCCTGAAAGAACGTTACG
AGCTGCACCACCATGTGCAGATCACTGACCCGGCGATTGTGGCGGCGGCAAC
ATTATCTCACCGCTATATCGCCGACCGTCAGTTGCCGGATAAAGCTATCGACC
TCATCGATGAAGCTGCATCCAGCATTCGTATGCAGATTGACTCTAAGCCGGA
GGAGCTGGACAGACTCGACCGCCGCATTATTCAGCTCAAACTGGAACAGCAG
GCGTTGATGAAAGAGTCTGACGAGGCGAGTAAAAAACGTCTCGATATGCTCA
ACGAAGAACTGGACGACAAAGAGCGCCAGTATTCTGAGCTGGAAGAAGAGT
GGAAAGCGGAAAAAGCGTCGCTCTCTGGTACGCAAACTATTAAAGCGGAGCT
GGAGCAGGCGAAGATTGCCATCGAGCAGGCGCGTCGCGTTGGCGACCTGGC
GCGAATGTCTGAACTGCAGTACGGCAAAATTCCGGAGCTGGAAAAACAGCTG
GAAGCCGCGACCCAGTCGGAAGGTAAAACCATGCGTCTGTTACGTAACAAAG
TAACGGATGCGGAAATTGCCGAAGTGCTAGCGCGCTGGACCGGTATTCCGGT
TTCCAGAATGCTGGAAGGCGAACGTGAAAAACTGTTGC

Figure 2.5 Nucleotide sequence of *Salmonella typhimurium* ATP dependent protease binding subunit (clpB) gene

Forward primer : Dav1 5' GCA GCG CAT TAT TAA CGG TG 3'

Reverse primer : Dav2 5' AAC AGT TTT TCA CGT TCG CCT 3'

Accession number : AF021817

gi : 2582384 Salmonella typhimurium invasion gene D protein (sigD) and invasion gene E protein (sigE) genes, complete cds, 3062bp

TATCTGTTCAAGCATGGAATAGGAAAAACGAATATTCTTCGTCACGGTCTTACTTGTCCGGGGCTTTGCT
GGCATACACACACCTGTATAACATTTGATGTAACGCCGTTACTTTACGCAGGAGTAAATCGGTGAATTTG
ATCTGAGTCAAGAAGGTGGGTTTTCAATAAAAGTTGTGCCATAAATTGTGAAGTTTGTAGATTTTATGAA
CATTTGATGTACCGATCTCCCCCATGATCGCCACTACGCTATGGACGTCAGGATGCCTCCCCGCCTGATC
AGAAGCGTTTCCTCATTAAAAAGGACATTTTTTTAAAGTTCCTGGTGCATAAAAGTCACATCCTTTTAAA
GGGTTGTTAACCCTGTTGAATGTTCCCACTCCCCTATTCAGGAATATTAAAAACGCTATGCAAATACAGA
GCTTCTATCACTCAGCTTCACTAAAAACCCAGGAGGCTTTTAAAAGCCTACAAAAAACCTTATACAACGG
AATGCAGATTCTCTCAGGCCAGGGCAAAGCGCCGGCTAAAGCGCCCGACGCTCGCCCGGAAATTATTGTC
CTGCGAGAACCCGGCGCGACATGGGGGAATTATCTACAGCATCAGAAGGCGTCTAACCACTCGCTGCATA
ACCTCTATAACTTACAGCGCGATCTTCTTACCGTCGCGGCAACCGTTCTGGGTAAACAAGACCCGGTTCT
AACGTCAATGGCAAACCAAATGGAGTTAGCCAAAGTTAAAGCGGACCGGCCAGCAACAAAACAAGAAGAA
GCCGCGGCAAAAGCATTGAAGAAAAATCTTATCGAACTTATTGCAGCACGCACTCAGCAGCAGGATGGCT
TACCTGCAAAAGAAGCTCATCGCTTTGCGGCAGTAGCGTTTAGAGATGCTCAGGTCAAGCAGCTTAATAA
CCAGCCCTGGCAAACCATAAAAAATACACTCACGCATAACGGGCATCACTATACCAACACGCAGCTCCCT
GCAGCAGAGATGAAAATCGGCGCAAAAGATATCTTTCCCAGTGCTTATGAGGGAAAGGGCGTATGCAGTT
GGGATACCAAGAATATTCATCACGCCAATAATTTGTGGATGTCCACGGTGAGTGTGCATGAGGACGGTAA
AGATAAAACGCTTTTTTT[redacted]CGTGCTTTCCCCCTATCATGAAAAAGATCCGCTT
CTGCGTCACGTCGGCGCTGAAAACAAAGCCAAAGAAGTATTAACTGCGGCACTTTTTAGTAAACCTGAGT
TGCTTAACAAAGCCTTAGCGGGCGAGGCGGTAAGCCTGAAACTGGTATCCGTCGGGTTACTCACCGCGTC
GAATATTTTCGGCAAAGAGGGAACGATGGTCGAGGACCAAATGCGCGCATGGCAATCGTTGACCCAGCCG
GGAAAAATGATTCATTTAAAAATCCGCAATAAAGATGGCGATCTACAGACGGTAAAAATAAAACC[redacted]
[redacted]TAATGTGGGTGTTAATGAGCTGGCGCTCAAGCTCGGCTTTGGCCTTAAGGCATCGGA
TAGCTATAATGCCGAGGCGCTACATCAGTTATTAGGCAATGATTTACGCCCTGAAGCCAGACCAGGTGGC
TGGGTTGGCGAATGGCTGGCGCAATACCCGGATAATTATGAGGTCGTCAATACATTAGCGCGCCAGATTA
AGGATATATGGAAAAATAACCAACATCATAAAGATGGCGGCGAACCCTATAAACTCGCACAACGCCTTGC
CATGTTAGCCCATGAAATTGACGCGGTACCCGCCTGGAATTGTAAAAGCGGCAAAGATCGTACAGGGATG
ATGGATTCAGAAATCAAGGGAGAGATCATTTCCTTACATCAGACCCATATGTTAAGTGCCCCTGGTAGTC
TTCCGGATAGCGGTGGACAGAAAATTTTCCAAAAAGTATTACTGAATAGCGGTAACCTGGAGATTCAGAA
ACAAAATACGGGCGGGGCGGGAAACAAAGTAATGAAAAATTTATCGCCAGAGGTGCTCAATCTTTCCTAT
CAAAAACGAGTTGGGGATGAAAATATTTGGCAGTCAGTAAAAGGCATTTCTTCATTAATCACATCTTGAG
TCTTGAGGTAACTATATGGAAAGTCTATTAAATCGTTTATATGACGCGTTAGGCCTGGATGCGCCAGAAG
ATGAGCCACTGCTTATCATTGATGATGGGATACAGGTTTATTTTAATGAATCCGATCATACACTGGAAAT
GTGCTGTCCCTTTATGCCATTGCCTGACGACATCCTGACTTTGCAGCATTTTTTACGTCTGAACTACACC
AGCGCCGTCACTATCGGCGCTGACGCAGACAATACTGCTTTAGTGGCGCTTTATCGCTTGCCGCAAACCA
GTACCGAAGAAGAGGCGCTCACTGGTTTTGAATTATTCATTTCAAACGTGAAGCAATTGAAAGAGCATTA
TGCATAATTTAATACGTCAACATACTTTCTTAATGAGATAAAACGCGATACGTATGCCCTTTACAAGAGA
CAAGACCAGAATCTTTGGTGGAAATGTAAGGGGCAAACGTTCATCTCTCTCATTTTGCTCTGTTTGCGGG
AGCATTTTTAGTGTGTAAGTATTCCTGCTCATCAGGTTTTTACGCCATCACGCGCATTTATTCTGGTATA
AGTTGAAATACTGCAAAAAATATTGGTGCTTATTATTTTTTCTTTAAGTAAATTTTCGCTGAACAAACTT
AATTGTTTATTCAATGATGATGAAGCGTAAGCTATGCTGGAAATGAAGGAATCAATAGCAAGGATAATCT
TATTATTCACGGGTGATATTACTTCTGCTTCACCGTTATGGCAGATATCATCGCCTCTTGTCAGATGCCA
GACACCTACTCATACTCAACCAAAGCTCTAAATACAAAAATCACCTTATATCTTTTTTTATTATTCCTTG
TATAAATGTGACTTGACTCACACCTATAAGGAGTCGGCTCACTTCCATAAGAAGGAATCAAAATGCCAAT
AACTAACGCGTCCCCAGAAAATATATTAAGATATTTGCATGCGGCCGGTACC

Figure 2.6 Nucleotide sequence of *Salmonella typhimurium* invasion gene D protein (sigD) and invasion gene E protein (sigE) genes

Forward primer : Red1 5' TGA CGG GAT ACG TCA TGG 3'

Reverse primer : Red2 5' AAT GCG GCG ACG ACG TCC 3'

Table 2.4: Detection of PCR amplification products using primer pair Red1+Red2 (Plasmid DNA)

SEROVAR	PLASMID DNA (Red1+Red2)			
	T_a = 53°C	T_a = 54°C	T_a = 55°C	T_a = 56°C
S.berta	–	–	–	–
S.braenderup	+	+	–	+
S.choleraesuis	–	–	–	–
S.dublin	–	–	–	–
S.enteritidis	–	–	–	–
S.gallinarum	+	–	+	+
S.infantis	–	–	–	–
S.java	–	–	–	–
S.paratyphi	–	–	–	–
S.typhi	–	–	–	–
S.typhimurium	–	-	–	–
E.coli	–	–	–	–

\+ : Amplified PCR product detected

\- : Amplified PCR product NOT detected

Table 2.5: Detection of PCR amplification products using primer pair Red1+Red2 (Genomic DNA)

SEROVAR	GENOMIC DNA (Red1+Red2)			
	T_a = 53°C	T_a = 54°C	T_a = 55°C	T_a = 56°C
S.berta	–	–	–	+
S.braenderup	+	+	+	+
S.choleraesuis	–	–	–	–
S.dublin	–	–	–	+
S.enteritidis	–	–	–	+
S.gallinarum	–	+	+	+
S.infantis	+	+	+	+
S.java	+	+	+	+
S.paratyphi	+	+	+	–
S.typhi	+	+	–	+
S.typhimurium	–	+	–	–
E.coli	+	+	+	+

\+ : Amplified PCR product detected

\- : Amplified PCR product NOT detected

Table 2.6: Detection of PCR amplification products using primer pair Dav1+Dav2 (Plasmid DNA and Genomic DNA)

SEROVAR	PLASMID DNA		GENOMIC DNA	
	T_a = 55°C	T_a = 56°C	T_a = 55°C	T_a = 56°C
S.berta	–	–	+	–
S.braenderup	+	+	+	+
S.choleraesuis	–	–	–	–
S.dublin	–	–	–	–
S.enteritidis	–	–	+	–
S.gallinarum	+	+	–	–
S.infantis		–	+	–
S.java	– –	–	–	+
S.paratyphi	–	–	–	–
S.typhi	–	–	+	–
S.typhimurium	–	-	+	–
E.coli	–	–	+	–

\+ : Amplified PCR product detected

\- : Amplified PCR product NOT detected

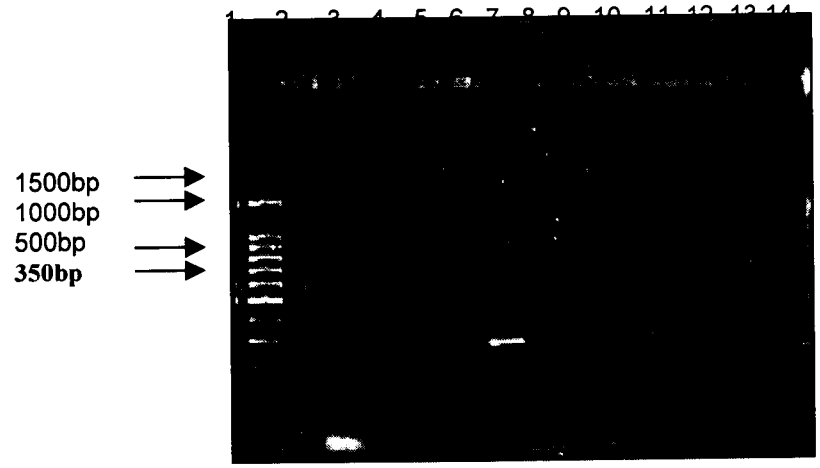

Figure 2.7 . PCR detection of *Salmonella typhimurium* by the Red1+Red2 primer pair using plasmid DNA: Agarose gel electrophoresis of PCR amplified products (Ta = 53°C) from artificially inoculated samples after 18hrs enrichment in TSB. Lane 1 carries a molecular sized marker (DNA 100bp ladder). Lane 2 - *S.berta*, lane 3 - *S.braenderup*, lane 4 – *S.choleraesuis*, lane 5 – *S.dublin*, lane 6 – *S.enteritidis*, lane 7 – *S.gallinarum*, lane 8 – *S. infantis*, lane 9 – *S.java*, lane 10 – *S.paratyphi*, lane 11 – *S.typhi* , lane 12 – *S.typhimurium*, lane 13 – *S.typhimurium* (control), lane 14 - *E.coli*.

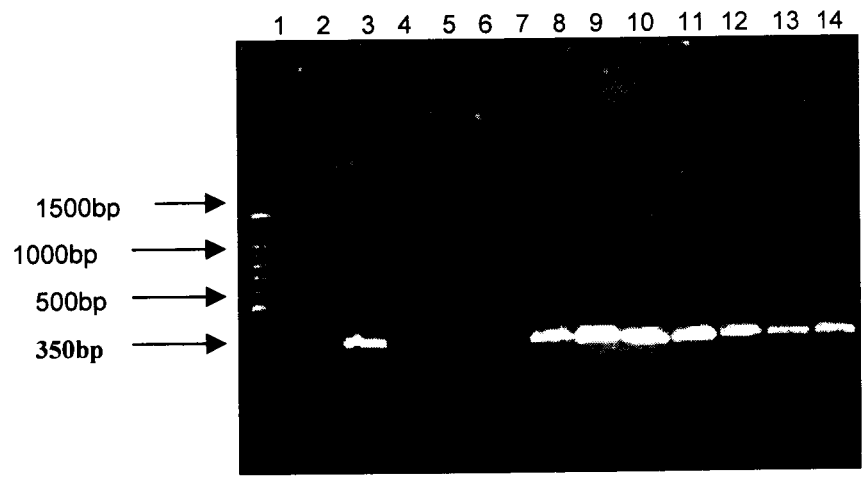

Figure 2.8. PCR detection of *Salmonella typhimurium* by the primer pair Red1+Red2 using Genomic DNA : Agarose gel electrophoresis of PCR amplified products (Ta = 54°C) from artificially inoculated samples after 18hrs enrichment in TSB. Lane 1 carries a molecular sized marker (DNA 100bp ladder). Lane 2 - *S.berta*, lane 3 - *S.braenderup*, lane 4 – *S.choleraesuis*, lane 5 – *S.dublin*, lane 6 – *S.enteritidis*, lane 7 – *S.gallinarum*, lane 8 – *S. infantis*, lane 9 – *S.java*, lane 10 – *S.paratyphi*, lane 11 – *S.typhi* , lane 12 – *S.typhimurium*, lane 13 – *S.typhimurium* (control), lane 14 - *E.coli*.

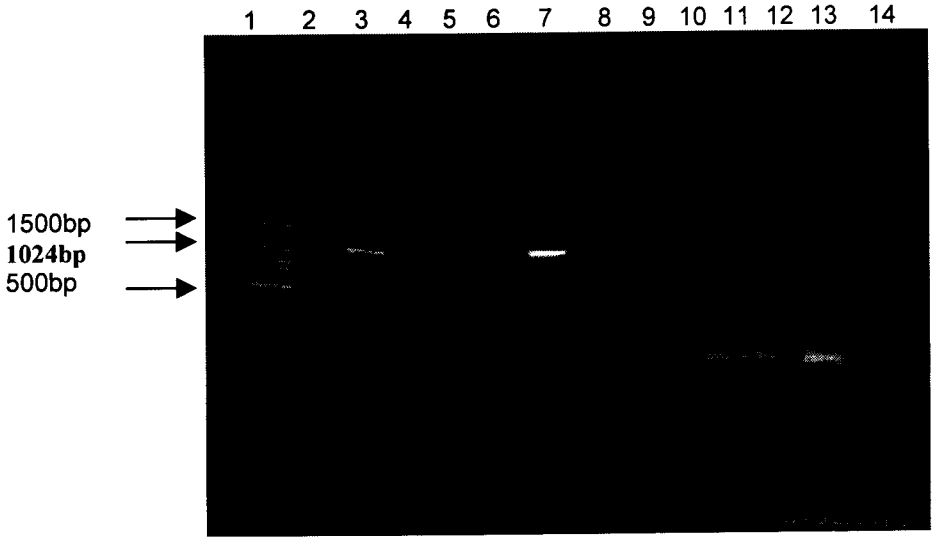

Figure 2.9. PCR detection of *Salmonella typhimurium* by the primer pair Dav1+Dav2 using Plasmid DNA: Agarose gel electrophoresis of PCR amplified products (Ta = 55°C) from artificially inoculated samples after 18hrs enrichment in TSB. Lane 1 carries a molecular sized marker (DNA 100bp ladder). Lane 2 - *S.berta*, lane 3 - *S.braenderup*, lane 4 – *S.choleraesuis*, lane 5 – *S.dublin*, lane 6 – *S.enteritidis*, lane 7 – *S.gallinarum*, lane 8 – *S. infantis*, lane 9 – *S.java*, lane 10 – *S.paratyphi*, lane 11 – *S.typhi* , lane 12 – *S.typhimurium*, lane 13 – *E.coli*, lane 14 – Blank.

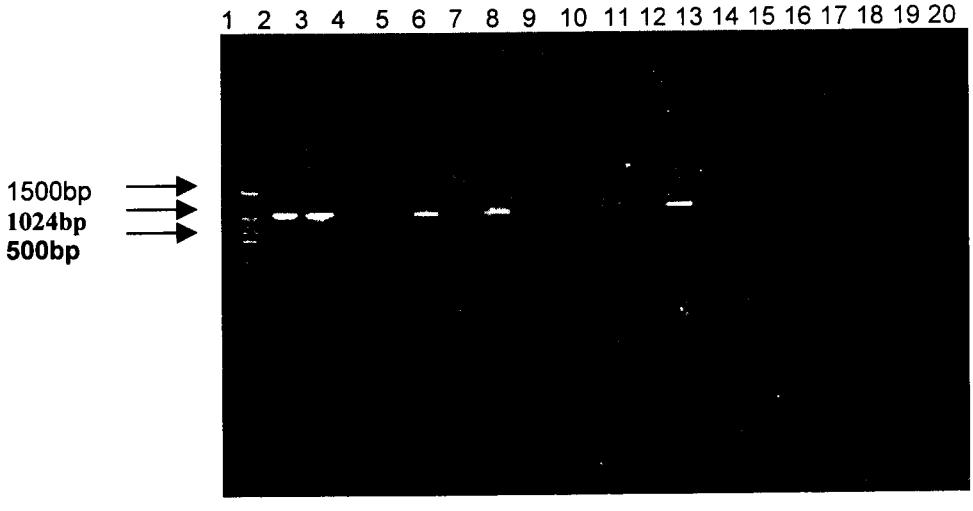

Figure 2.10. PCR detection of *Salmonella typhimurium* by the primer pair Dav1+Dav2 using Genomic DNA: Agarose gel electrophoresis of PCR amplified products (Ta = 55°C) from artificially inoculated samples after 18hrs enrichment in TSB. Lane 1 carries a molecular sized marker (DNA 100bp ladder). Lane 2 - *S.berta*, lane 3 - *S.braenderup*, lane 4 – *S.choleraesuis*, lane 5 – *S.dublin*, lane 6 – *S.enteritidis*, lane 7 – *S.gallinarum*, lane 8 – *S. infantis*, lane 9 – *S.java*, lane 10 – *S.paratyphi*, lane 11 – *S.typhi* , lane 12 – *S.typhimurium*, lane 13 – *E.coli*, lanes 14-20 – blank.

2.5 DISCUSSION

The nucleotide primer pair, Red1 and Red2, was designed from the nucleotide sequence obtained from the NCBI database (Fig. 2.1). This nucleotide sequence encodes for the *Salmonella typhimurium* invasion gene D protein (sigD) and invasion gene E protein (sigE) genes. The *Salmonella* and the non-*Salmonella* serovars used were subjected to PCR conditions at various annealing temperatures (T_a) (Tables 2.4, 2.5, 2.6). This was performed in order to optimize the PCR. Plasmid DNA PCR amplicons (350bp) detected *S.braenderup* and *S.gallinarum* at 53°C (Fig. 2.7) and at 56°C, *S.braenderup* at 54°C and *S.gallinarum* at 55°C. PCR of the plasmid DNA did however not detect *Salmonella typhimurium,* the serotype it was designed to detect. Genomic DNA proved to be more specific (Table 2.5) to this serovar. As shown in Fig. 2.8 several other *Salmonella* serotypes were also detected, namely *S.braenderup, S. gallinarum, S.infantis, S. java, S.paratyphi, S.typhi* and *S.typhimurium.* With the exception of *E.coli*, none of the other non-*Salmonella* serotypes tested detected the 350bp PCR amplicon (results not shown). From this it can be concluded that those *Salmonella* serotypes, detected by primer pair Red1+Red2, and *E.coli* must share a conserved region on their genomic chromosome.

The second primer pair, Dav1 and Dav2, was designed from the nucleotide sequence obtained from the NCBI database (Fig. 2.2). This nucleotide sequence encodes for an ATP dependent protease subunit (clpB). These are associated with virulence and colonization of chicken alimentary tracts. Subjected to the

BLAST program, both Dav1 and Dav2 primers had E-values = 0 for the serotype *Salmonella typhimurium*. Suggesting that the 1024bp PCR amplicon should detect the *Salmonella typhimurium* serotype alone. This was however not the case, where several serotypes other than the chosen serotype that was also detected. Figure 2.10 shows these serotypes to be *S.berta*, *S.braenderup*, *S.enteritidis*, *S.infantis* and *S.typhi*, as well as *E.coli*. These results would suggest that these *Salmonella* serotypes, together with *E.coli*, share a conserved region and thus may share the ATP dependent protease binding subunit (clpB) gene.

Plasmid and genomic DNA extractions were used and tested the same serotypes. What was interesting about with the Red primer pair was that, if the 350bp amplicon was detected in the plasmid DNA, it would most likely be detected in the genomic DNA. Such was the case for *Salmonella braenderup* and *Salmonella gallinarum* (Figures 2.7 and 2.8). With the Dav primer pair, the 1024bp amplicon was detected by *Salmonella braenderup* and *Salmonella gallinarum* (Fig. 2.9), but only *Salmonella braenderup* was detected when using genomic DNA (Fig. 2.10).

Nucleotide sequences are chosen from databases. Primers are then designed from the statistics reviewed. Yet, it seems that when the theoretical data is put into practice, the expected results does not concur. There are several possibilities as to why this may be happening. The sequences which are in the

database may not be correct. This seems highly unlikely, since stringent rules apply to those who intend to submit sequences to any database which will be available to the public. Secondly, the databases are updated at such a rapid rate that from the time the chosen sequence is downloaded, to the time it is being tested in the laboratory, the nucleotide sequence has already changed in some way. Even single base pair corrections, additions or deletions could affect the expected results in the laboratory. Thirdly, serotypes have been known to differ from place to place as a result of their environmental conditions. It may be that the serotypes used to perform the experiments in this laboratory differs by some degree to those downloaded from the nucleotide databases. Possible solutions to this problem experienced, would be to have more stringent rules when submitting a nucleotide sequence to a database. To have a waiting period of some sort upon submission of the new nucleotide sequence, while it is being compared to what is already available in the database. If similarities are found between the new submitted sequence and a relevant nucleotide sequence in the database, then the more updated will be replaced.

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Chapter 3

Salmonella typhimurium encoding for an ATP dependent protease binding subunit (clpB) gene found to be common in *Escherichia coli*

The style of this chapter is in accordance with that of the *Journal of Applied Microbiology*.

3.1 ABSTRACT

A previous study dealt with the development of a molecular technique, namely Polymerase Chain Reaction (PCR), to detect *Salmonella typhimurium*. This being one of the major serotypes in the *Salmonella* family responsible for food-poisoning. A sequence coding for *Salmonella typhimurium* ATP dependent protease subunit (clpB) gene, had previously been used to design a primer pair, Dav1 and Dav2. *Salmonella typhimurium* as well as several other serotypes, including *Escherichia coli*, detected the 1024bp PCR amplicon using Dav1 and Dav2. The detection of *E.coli* thus reinforces the notion that the genus *Salmonella* and *Escherichia* did indeed share a common ancestor 120 million years ago. Consequently they possess similar genomic structures. **AIMS:** To sequence several *Salmonella* sequences, as well as *Escherichia coli,* which had previously been detected by primer pair Dav1 and Dav2. To subject these *Salmonella* sequences and *E.coli*, to a BLAST program. To compare the Multiple Sequence Alignment for sequences similarities. **MATERIALS AND METHODS:**

Sequences were sent to the Department of Genetics at the University of Stellenbosch for sequencing. Each sequence was then subjected to a BLAST program. Sequences were also subjected to a Multiple Sequence Alignment. **RESULTS AND DISCUSSION:** In the BLAST program, the sequences which had been sent for sequencing came back as *Salmonella typhimurium.* Even the *Escherichia coli* serotype which had been sent for sequencing as well. The *E.coli* serotype used in this experiment was subjected to biochemical tests by using an API 20E strip. The results confirmed the *E.coli* genus. The Multiple Sequence Alignment of the sequences showed a definite conservation of sequences. From the results it can be concluded that these sequences share the same ATP dependent protease binding subunit (*clpB*) gene. That the *Salmonella* serotypes detected probably share a closer serology and that the *clpB* gene share the same function in both *Salmonella* and *Escherichia coli*.

3.2 INTRODUCTION

The genus *Salmonella* is a member of the family Enterobacteraceae. Its closest known relative is *Citrobacter* and other close relatives include *Escherichia/Shigella*, *Klebsiella* and Enterobacter (Le Minor 1992). It is estimated that *Salmonella* and *Escherichia/Shigella* shared their most recent common ancestor 120-160 million years ago (Doolittle *et al.*, 1996; Feng *et al.*, 1997; Ochman and Wilson 1987).

Despite this long history of divergence, recent physical and genetic maps of several *Salmonella enterica* serovars and of *E.coli* K12 suggest that they all possess similar genomic structures (Liu and Sanderson 1995). Many of the genes implicated in *Salmonella* virulence are also present in nonpathogenic strains of *E.coli*. These genes encode enzymes responsible for the biosynthesis of nutrients that are scarce within host tissues, transcriptional and post-transcriptional regulatory factors, proteins necessary for the repair of damaged DNA, and products necessary for defense against host microbial mechanisms (Groisman and Ochman 1997).

There are few differences between the genomes of *E.coli* K12 and *Salmonella typhimurium* Latent Type 2 (LT2), including: an inversion encompassing around 10% of the genome (Riley and Krawiec 1987); several segments of DNA, amounting to around 14% of the genome, exclusive to one or other species (Riley and Krawiec 1987) (of particular interest is the so-called 40Kb

"pathogenicity island" (Mills *et al.* 1995) at around 59' on the LT2 chromosome); and many small differences in the fine structure of genes, operons and intergenic regions. However, the broad message is one of conservation of genome structure between *E.coli* and *S.enterica.*

Currently there is a trend to design serotype specific primers, which will target a unique, conserved region in a serotype (Soumet *et al.* 1999; Zhu *et al.*, 1996). Entrez is a retrieval system for searching several linked databases and is provided by the National Center for Biotechnology Information (NCBI). It provides access to several avenues related to gene sequences. Namely, the Biomedical literature (PUBMED), the nucleotide sequence database (Genbank), three-dimensional macromolecular structures, complete genome assemblies, population study data sets and Online Mendelian Inheritance in Man (OMIM). Using information found in the Gene Bank database, a conserved region can be found for the chosen serotype. This can be achieved by using the Bioinformatics tool, BLAST (Basic Local Alignment Search Tool) (Altschul *et al.*, 1990).

BLAST (Basic Local Alignment Search Tool) is a set of similarity search programs designed to explore all of the available sequence databases regardless of whether the query is protein or DNA. The BLAST programs have been designed for speed, with a minimal sacrifice of sensitivity to distant sequence relationships. The scores assigned in a BLAST search have a well-defined statistical interpretation, making real matches easier to distinguish from random

background hits. BLAST uses a heuristic algorithm which seeks local as opposed to global alignments and is therefore able to detect relationships among sequences which share only isolated regions of similarity (Altschul *et al.*, 1990).

The Expect value (E) is a parameter that describes the number of hits one can "expect" to see just searching a database of a particular size. It decreases exponentially with the Score (S) that is assigned to a match between two sequences. Essentially, the E value describes the random background noise that exists for matches between sequences. The Expect value is used as a convenient way to create a significance threshold for reporting results. When the Expect value is increased from the default value of 10, a larger list with more low-scoring hits can be reported (Altschul *et al.*, 1990).

A primer pair designated, Dav1 and Dav2, had previously been designed to detect the serotype *Salmonella typhimurium*. The sequence, from which the primer pair had been designed, had been selected from the National Center for Biotechnology Information (NCBI) database. Accession number AF010250 codes for a *Salmonella typhimurium* ATP dependent protease binding subunit (clpB) gene. Performing the Polymerase Chain Reaction (PCR) using the Dav primers, specific for *Salmonella typhimurium*, showed some unexpected results (Refer Fig. 2.10). Several *Salmonella* serotypes other than the target serotype, detected the 1024bp PCR amplicon. Of the non-*Salmonella* serotype tested, only

E.coli was detected. Based on these results the *Salmonella* serotypes which had been used in this experiment were sequenced and the results analysed.

3.3 MATERIALS AND METHODS

3.3.1 Isolates

The serotypes used had been obtained from the Onderstepoort Research Institute, Pretoria. Glycerol stocks (25%) were made by inoculating 100µl of stock into 5ml of TSB, grown overnight at 37°C. Thereafter, 1ml of the overnight culture and 1ml of a 25% Glycerol solution was mixed and stored at -70°C. Glycerol working stock (25%) was made as well and stored at -4°C. Bacterial genomic DNA was prepared from 2ml of the overnight culture with Promega Genomic DNA Purification Kit (Catalogue number 3139) as recommended by manufacturer. The extracted genomic DNA was then subjected to Polymerase Chain Reaction technique using Dav1+Dav2, which had previously been designed to detect *Salmonella typhimurium*.

Table 3.1 Isolates sent to the Department of Genetics at the University of Stellenbosch for nucleotide sequencing

ISOLATES	*PCR detection
Salmonella berta	+
Salmonella braenderup	+
Salmonella enteritidis	+
Salmonella infantis	+
Salmonella typhi	+
Salmonella typhimurium	+
Escherichia coli	+

* PCR detection using Dav1+Dav2 primers coding for *S.tyhphimurium* ATP dependent protease subunit (clpB) gene

3.3.2 Sequencing

Isolates were sent for nucleotide sequencing to the Department of Genetics, Central DNA Sequencing Facility at the University of Stellenbosch, Cape Town, South Africa. The process involves the following three steps:

3.3.2.1 Construction of a genome map

Mapping of genome is the first step of the genome sequencing. The mapping includes digestion of the genomic DNA to shorter fragments, multiplied in bacteria, and then each fragment is aligned along with original genomic DNA .

3.3.2.2 Determination of the genomic base sequences

Through the use of an aligned DNA map, the base sequence of sequential DNA fragment is determined. The sample DNAs are further fragmented into smaller pieces so that their base sequences can be determined by an automatic DNA sequencer. The resulting data will be assembled to determine the base sequence of the entire DNA fragment.

3.3.2.3 Analysis of sequence data

Computational analysis of the genomic sequences enables us to find biologically important information including genes, regulatory signals and other functional regions in the genomic sequences. Furthermore, comparison of the sequences also provides the evolutionary conserved regions which may retain common

biological importance between them. These signals and regions are then experimentally analyzed to understand the biological roles.

3.3.3 BIOCHEMICAL TESTS

Biochemical tests were performed on the *E.coli* used in this experiment to verify its genus. The BioMerieux API 20E (Catalogue Number 20100) was used to test for the Enterobacteriacea as recommended by the manufacturers.

3.4 RESULTS

3.4.1 Isolate Sequencing

S.berta, *S.braenderup*, *S.enteritidis*, *S.infantis*, *S.typhi*, *S.typhimurium* and *E.coli* had each detected the 1024bp PCR amplicon. It was for this reason that these seven sequences were to be sequenced.

3.4.2 BLAST

Of the sequences sent for sequencing, each one returned as that of *Salmonella typhimurium* ATP dependent protease binding subunit (*clpB*) gene. Nucleotide sequences taken from www.ncbi.nlm.nih.gov/blast/Blast.cgi. (Nucleotide sequence highlighted in red codes for *Salmonella typhimurium* ATP dependent protease binding subunit (*clpB*) gene).

Table 3.2 BLAST results of sequences producing significant alignments