Methods for serological and PCR detection of *Salmonella enteritidis* in chickens

By

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Preface

The experimental work described in this dissertation was carried out in the Department of Biochemistry, University of Natal, Pietermaritzburg, from January 1999 to December 2001 under the supervision of Prof. Theresa H.T. Coetzer and co-supervision of Dr Roger F. Horner.

These studies represent original work by the author and have not been submitted in any other form to any other university. Where use has been made of the work of others, it has been duly acknowledged in the text.

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Abstract

Salmonella enteritidis (*S. enteritidis*) is a bacterial pathogen of chickens, and is currently one of the leading causes of human food poisoning in the world. It is believed that contaminated poultry products, especially eggs and egg products, have been responsible for the dramatic increase in the incidence of this *Salmonella* serotype. Detection of *S. entertidis* has conventionally involved bacteriological examination of samples, yet these procedures are time-consuming which could lead to the rapid spread of *S. enteritidis* through commercial flocks and potentially cause a human health risk. A number of alternative detection techniques, mostly based on serological methods, have been reported as effective diagnostic assays. However, some of these reports have not been supported by representations of SDS-PAGE gels or Western blots. The objective of this study was the evaluation of these serological techniques as well as a PCR amplification technique, which has been reported to show promising results as a diagnostic method. The techniques discussed in these reports were evaluated with regards to how rapid they were, their specificity and their potential for use in local diagnostic laboratories.

Antigens from the outer surface of *S. enteritidis* were purified by several methods and their antigenicity was tested by separating the antigens by means of SDS-PAGE, followed by Western blotting using sera of chickens infected with *S. enteritidis*. A high degree of cross reactivity was observed with many of the antigens tested, especially the lipopolysaccharides (LPSs) and outer membrane proteins (OMPs) which had previously been reported as containing antigens which could be used for specific detection of *S. enteritidis*. This cross-reactivity could be explained by the conserved nature of many of the LPS and OMP antigens among the *Salmonella* serotypes tested. A fimbrial antigen, SEF14, which has been reported as a novel antigen, was seen as a prominent band at 14.3 kDa and was found to react with antibodies against *S. enteritidis*, yet not to the specificity levels described in previous reports.

PCR amplification of the *sefA* gene sequence, which encodes for the SEF14 fimbrial antigen, was found to give a predicted product of 310 bp when using a previously described oligonucleotide primer pair. This amplified product was found to be specific for *S. enteritidis* and other serogroup D *Salmonella* serotypes that are not poultry pathogens.

The cross-reactivity observed with many of the serological techniques used in this study, meant that detection of *S. enteritidis* infection in chickens was considerably hindered. However, the identification of further novel antigens by serological means, could result in the development of new vaccines. The specificity and speed afforded by PCR amplification indicated that this technique showed excellent potential for use in local diagnostic laboratories.

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Abbreviations

A_{280}	absorbance at 280 nm
ABTS	2,2'-azino-di-(3-ethyl)-benzthiozoline sulfonic acid
AP	alkaline phosphatase
ARVL	Allerton Regional Veterinary Laboratories
ATCC	American Type Culture Collection
BCA	bicinchoninic acid
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
Bis	*N, N'*-methylenebisacrylamide
Bp	base pair(s)
BSA	bovine serum albumin
BSA-TBS	bovine serum albumin dissolved in Tris-buffered saline
c	concentration
CFA	colony factor antigen
Dist. H_2O	distilled water
DMF	dimethylformamide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
g	relative centrifugal force
HEPES	*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid
HPLC	high-performance liquid chromatogaraphy
HRPO	horseradish peroxidase
IEC	ion exchange chromatography
IgG	immunoglobulin G
IMS	immunomagnetic separation
Kb	kilobase
kDa	kilodalton(s)
l	litre
LPF	long polar fimbriae
LPS(s)	lipopolysaccharide(s)

MEC	molecular exclusion chromatography
M_r	relative molecular mass
mRNA	messenger ribonucleic acid
NBT	nitroblue tetrazolium
OMP(s)	outer membrane protein(s)
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PBS-Tween	Tween 20 diluted in phophate buffered saline
PCR	polymerase chain reaction
PEF	plasmid encoded fimbriae
PT	phage type
PEG	polyethylene glycol
PMB	Pietermaritzburg
RNA	ribonucleic acid
rpm	revolutions per minute
RT	room temperature
SA	South Africa
SAPA	South African Poultry Association
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SPF	specific pathogen-free
spp.	species/serotypes
TBS	Tris-buffered saline
TEMED	*N, N, N', N'*-tetramethylethylenediamine
tricine	*N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine
Tris	2-amino-2-(hydroxymethyl)-1,3-propandiol
UK	United Kingdom
USA	United States of America
UV	ultraviolet
WCP	whole cell protein(s)
WHO	World Health Organisation
XLD	xylose-lysine-deoxycholate
XLT-4	xylose-lysine-tergitol 4

CHAPTER 1

General Introduction

Salmonella is one of the most extensively studied bacterial genera in terms of cell structure, physiology and genetics, yet *Salmonella* remains the second-leading cause of food-borne illness in most developed countries in the world (Darwin & Miller, 1999). *Salmonella* is part of a larger family called Enterobacteriacae. This bacterial family is responsible for a large number of diseases in animals and man and comprises of Gram-negative bacilli, which are antigenically related and biochemically similar. Genera in this family include *Escherichia, Citrobacter, Klebsiella, Proteus, Hafnia, Shigella* as well as *Salmonella* (Jordan, 1990).

Taxonomically, the species concept has been applied with great difficulty to members of the genus *Salmonella*. Initially, strains from different clinical conditions were considered to be different species, but it was later recognised that a large number of these so-called species were ubiquitous and classified as the genus *Salmonella*. Through much of the 1970's and 1980's, a three-species concept was applied by many, with the recognition of *S. typhi, S. choleraesuis* and *S. enteritidis*. Currently, however the more than 2400 members of the genus *Salmonella* can be recognised as separate serovars or serotypes, belonging to seven genetically and biochemically-distinct subspecies under the species name of *S. enterica*. The nomenclature that will be followed in this dissertation will be that described by Le Minor & Popoff (1987) for the taxonomy of the genus *Salmonella*. Based on this taxonomy, members of the nontyphoidal salmonellae are designated as serotypes of the *Salmonella enterica* subspecies *enterica*. A simplified nomenclature is often preferred and according to this simplification, *Salmonella enterica* subspecies *enterica* serovar Enteritidis will be referred to as *Salmonella enteritidis (S. enteritidis)*.

All *Salmonella* species (*spp.*) are facultative anaerobes, meaning they can live without oxygen but prefer environments with oxygen. *Salmonella* are ubiquitous organisms, residing in the intestinal tracts of birds, farm animals, reptiles and humans. The majority of infected animals become subclinical excretors. However, some *Salmonella* are able to survive for up to 9 months in the environment, in sites such as moist soil, water, faecal

particles and animal feeds, especially blood-and-bone and fish meals (Quinn *et al.,* 1994). Transmission to humans, however, is usually food borne and results from eating raw or undercooked meat, eggs or milk. Cross-contamination also occurs via other foods, which are consumed without cooking. All species of *Salmonella* are potential pathogens for man (Kwang *et al.,* 1996), yet of the more than 2400 serotypes identified in the genus, only about a dozen account for about 75% of all isolates from animals and humans. This makes these few serotypes incredibly important from both economic and health standpoints. *Salmonella enteritidis* is one of these serotypes as it is the most frequently isolated of the food-borne *Salmonella spp.* (Hilton & Penn, 1998; Maré *et al.*, 2001). Approximately 40 000 cases of salmonellosis are reported every year in the USA alone (Darwin & Miller, 1999), yet only 1% of *Salmonella*-related infections are reported (Chalker & Blaser, 1988). Thus there are actually 2 to 4 million cases in the USA each year with an estimated annual cost of over $2 billion (Darwin & Miller, 1999).

The infective dose of *Salmonella* in humans has been reported to range from as few as 15-20 cells to as many as 10^9 cells (Todd, 1996). The infective dose is dependent on the age and health status of the host, with the young, the old and the immune-compromised being the most susceptible to infection. Acute symptoms of *S. enteritidis* infection in humans include diarrhoea, abdominal cramps, vomiting, and often fever (Favrin *et al.,* 2001). If the infection is severe, it may lead to dehydration and septicemia meaning that the infection has spread to the organs and blood, and can be fatal. Infection may last several days, depending on ingested dose, strain characteristics and host factors. Chronic symptoms may include several arthritic symptoms that may follow three to four weeks after acute symptoms (Todd, 1996). *S. enteritidis* infection also occurs concurrently with human immunodeficiency virus (HIV) infection and is one of the common complications of acquired immunodeficiency syndrome (AIDS) (Levine *et al.,* 1991). Control of *S. enteritidis* infection is, therefore, extremely important in South Africa, where HIV infection has reached epidemic proportions.

1.1 *Salmonella enteritidis*

In many countries, the dominant *Salmonella* serotype associated with cases of food poisoning has been *S. enteritidis* (Rabsch *et al.*, 2000; Baileys *et al.*, 2000). This is widely thought to be due to the increased number of poultry products infected with this serotype (Fadl *et al.*, 2002; Wang & Yeh, 2002).

In the early 20th century, serotype *S. gallinarum* (which includes two biovars, *gallinarum* and *pullorum*) (Section 1.3) was endemic in poultry flocks in Europe and the Americas (Bullis, 1977). However, the inception of national surveillance programs in the 1930's, and their method of test-and-slaughter, led to the eradication of *S. gallinarum* from commercial poultry flocks in the USA, England and Wales, by the 1970's (Rodrigue *et al.*, 1990). This is due to *S. gallinarum* having no animal reservoir other than domestic and aquatic fowl (Rabsch *et al.*, 2000). At the same time, the number of human cases of infection with *S. enteritidis* began to increase in these countries. Results submitted by 50 countries to the World Health Organisation (WHO) salmonella surveillance system between 1979 and 1987 showed a substantial increase in *S. enteritidis* on several continents (Rodrigue *et al.*, 1990). By the 1980's, *S. enteritidis* had emerged as a major concern for food safety and public health and by 1990, *S. enteritidis* had become the most frequently reported *Salmonella* serotype in the USA and United Kingdom (Mishu *et al.*, 1994).

Before 1970, human outbreaks of salmonellosis were primarily associated with eggshell contamination by the non-host-adapted serotype, *S. typhimurium*, which led to a number of control programs being implemented (Faddoul & Fellows, 1966). As the frequency of *S. typhimurium*-associated cases stabilised, the incidence of *S. enteritidis* outbreaks began to increase in the 1970's (Keller *et al.*, 1997). By the mid-1980's, *S. enteritidis* had risen to the predominant position in man and animals as a result of vertical and horizontal transmission within and between large poultry organisations in many parts of the world (O' Brien, 1990). Between 1972 and 1996, the increase in *S. enteritidis* isolates in the USA was rated at 459 percent, while all other serotypes increased by only 18 percent (Saeed, 1998). Epidemiological studies showed that contaminated eggs or egg products were the major source of human infections, which led to disruptions of the egg industries in Europe, the USA and other countries, including South Africa to a lesser extent.

Slaughter policies were introduced into these parts of the world in an attempt to decrease the incidence of human cases, but with no effect (Le Bacq *et al.*, 1994; Bayer Health Group, 1994). By 1998, over 46 billion eggs were distributed, sold and used as shell eggs in the USA alone. Based upon surveillance studies, the United States Food Safety and Inspection Service (FSIS) assessed and calculated that 2.3 million of these eggs contained *S. enteritidis* when laid. United States public health surveillance data also estimated 637 000 cases of human illness per year directly related to the consumption of *S. enteritidis*-contaminated raw or undercooked eggs (Whiting *et al.*, 2000).

1.2 *S. enteritidis* in South Africa

Non-poultry hosts, like cattle, pigs, a dog and a cat, were the first animals from which *S. enteritidis* was isolated in South Africa, from the period of 1977 to 1991 (Bayer Health Group, 1994). However, since the first poultry associated outbreak in 1991, the incidence of *S. enteritidis* has increased drastically in South Africa (Maré *et al.*, 2001). Poultry infections were first observed as acute deaths in young broiler chickens followed by confirmed isolates from limited numbers of laying flocks. From 1991, the most affected area in South Africa was the Western Cape, and it was only in August or September of 1993 that *S. enteritidis* was first detected in the commercial poultry flocks of KwaZulu-Natal. However, by the time the first isolate was confirmed, a prototype control system was already in place in KwaZulu-Natal, reducing the potential spread of infection (Horner, 1996).

Based on recommendations from the European community and the USA approach to the problem of *S. enteritidis*, a *S. enteritidis* reduction plan was accepted by the South African Poultry Association (SAPA) and approved by the Directorate of Animal Health. This was introduced to replace the slaughter policy, which had proven unsuccessful in Britain, Europe and the USA (Bayer Health Group, 1994).

On a national level, the reduction plan involved five key areas:

(1) Strict control ensuring *S. enteritidis*-free pure line breeding stock.

(2) Application of bio-security measures at all levels of the poultry production system.

(3) A monitoring system for poultry production systems and poultry products.

(4) Application of sound kitchen hygiene and cooking practices, to ensure safe food handling in all households.
(5) Effective communication between poultry producers, consumers and state authorities, to ensure success of the *S. enteritidis* reduction plan (Bayer Health Group, 1994).

The South African poultry industry is composed of a number of elements. The grand parent flocks are the pureline breeding stocks from which parent flocks are produced, which in turn produce both commercial broiler and commercial egg producing flocks. Since 1994, there has been a steady increase in commercial layers in the South African poultry industry. In 1994, there were 12.5 million layers in the industry producing approximately 193 000 cases of eggs per week. By the end of 2002, the industry comprised over 17 million layers, producing approximately 260 000 cases of eggs per week (Coetzee, 2003).

A primary aim of the *S. enteritidis* reduction plan was to maintain a negative *S. enteritidis* status in the grand parent and parent flocks, thereby preventing vertical transmission through the entire poultry organisation in South Africa. Other aims included maintaining a negative *S. enteritidis* status in commercial flocks, use of vaccines, antibiotic drugs, modern hygiene measures and bio-security steps as well as ensuring sound production systems. Producers of grand parent and parent flocks are required to have flocks monitored by both bacteriological and serological means. Hatcheries are required to test eggs and fluff regularly during production. Commercial broiler and layer producers are recommended to have bacteriological monitoring of meat and eggs respectively as well as bacteriological monitoring of mortalities and the production environment (Bayer Health Group, 1994).

Although the prototype *S. enteritidis* control system was already in place at this time in KwaZulu-Natal, the more extensive programme was suggested to the KwaZulu-Natal poultry breeding establishment, resulting in a voluntary monitoring scheme being applied. Once set up, the scheme was effective in quickly identifying and confirming isolates, resulting in only 31 confirmed field cases of *S. enteritidis* in commercial chickens, two cases in parrots and one in ducks, between August 1993 and the end of 1996 (Horner, 1996). The scheme allowed for early detection of *S. enteritidis* infection in newly placed parent stocks and the subsequent monitoring of these stocks during their production cycle.

This has ensured a *S. enteritidis*-free commercial flock in Kwazulu-Natal. To maintain the current low levels of *S. enteritidis* in KwaZulu-Natal, a continued monitoring programme was required. A central project of SAPA has been the constant upgrading of guidelines to the poultry industry, regarding the monitoring of *S. enteritidis,* with constant input from the industry (Coetzee, 2003). The development of potential rapid, simple and inexpensive techniques for detecting *S. enteritidis* in poultry remains an important means of controlling *S. enteritidis*.

1.3 Morphology of *S. enteritidis*

S. enteritidis are Gram-negative non-sporulating rods that are 2 to 3 μm long and motile due to the presence of long flagellae. Like all *Salmonella spp., S. enteritidis* are facultative anaerobes and like many *Salmonella spp., S. enteritidis* are quite resilient to heat, and able to adapt to extremes in environmental conditions (Humphrey *et al.,* 1995). This adaptability is, in part, thought due to the expression of fimbriae on the surface of the bacterium (Section 3.5). They are resistant to freezing and drying and are able to grow within a wide temperature range, from extremes as low as 2 to 4°C to as high as 54°C (Palumbo *et al.,* 1995). They have also been reported to grow within a pH range of 4.5 to 9.5, with the optimum pH between 6.5 and 7.5. Preconditioning to thermal and acid stress has been shown to allow strains to adapt to greater extremes (D'Aoust, 1997). However, a temperature of 130°C applied for one second will kill *S. enteritidis* and at 63°C, all *S. enteritidis* will be killed after 30 min (Bayer, 1994).

When compared to colonies of the avian-adapted *S. gallinarum*, that appear as flat, grey-coloured and tiny (up to 1 mm), colonies of *S. enteritidis* appear similar, being flat and grey-coloured, yet slightly larger, growing up to 3 mm in diameter, after 12 h incubation at 37°C (Figure 1.1). Colonies of other *Salmonella* serotypes used in this investigation, as well as *E. coli*, were similar in appearance.

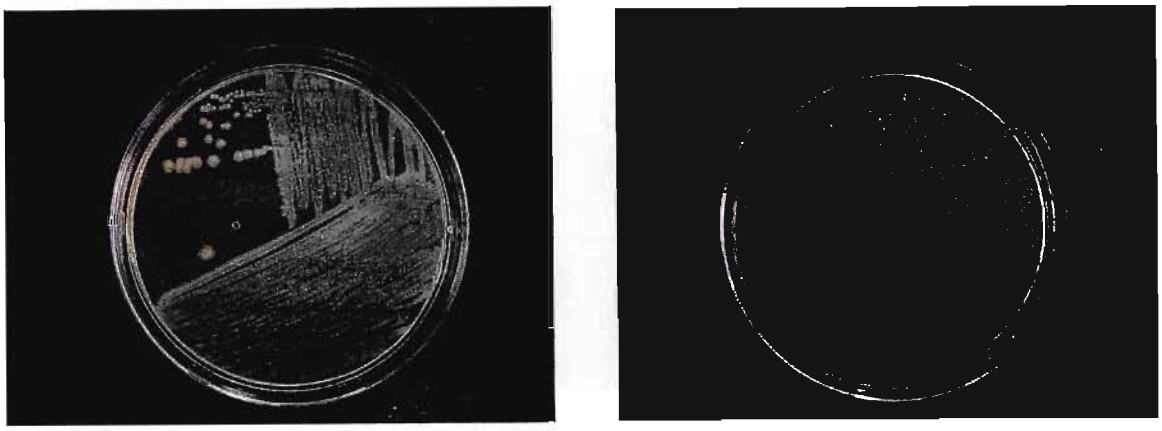

Fig. 1.1 *S. enteritidis* (left) and *S. gallinarum* (right) grown on nutrient agar for 12 h at 37°C

The biochemistry of *S. enteritidis* and how it relates to the detection of the serotype is discussed in Section 1.5.1.

1.4 *S. enteritidis* infection in chickens

There are many sources of *S. enteritidis* infection for the domestic fowl. Poultry, like many other animals, are often unapparent carriers, latently infected or less frequently, clinically ill. They may also excrete *S. enteritidis* into the intestine and thus into their faeces (known as bacterial shedding), forming a large *S. enteritidis* reservoir and source of contamination for other animals, humans and the environment (Gast *et al.*, 1989). Infection in poultry often occurs via horizontal transmission by faeces, feed, water, litter, fluff, dust, shavings, straw, insects, equipment and other fomites contaminated with *S. enteritidis*. Horizontal transmission also occurs by contact with other chicks or poults, rodents, pets, wild birds, other wild or domestic animals or even personnel contaminated with *S. enteritidis*. Vertical transmission occurs when follicles in the ovary of a hen are infected, resulting in the direct contamination of the yolk, follicular membrane surrounding the yolk or albumin of the developing egg (Miyamoto *et al.,* 1997; Okamura *et al.*, 2001).

1.4.1 *S. enteritidis* and eggs

The major source of *S. enteritidis* infection in humans is eggs or egg products and it is therefore important to understand how eggs relate to these forms of transmission. Horizontal transmission in eggs is caused by penetration through the egg shell by *S. enteritidis* and vertical transmission occurs by the direct contamination of the egg

contents from the infection of reproductive organs with *S. enteritidis*. This occurs before the egg is covered by the shell (Timoney *et al.*, 1989; Berrang *et al.*, 1998). More salmonellae have been shown to spread and contaminate chicks in the hatcher, after pip, than through the eggshells or in the incubator (Bailey *et al.*, 1994). This report found that only one contaminated egg in a hatching cabinet will lead to substantial *Salmonella* spread throughout the hatching cabinet. Young birds are extremely vulnerable to infection because of a poorly developed competitive intestinal flora and immune system (Corrier *et al.*, 1992) compounded by the effects of stress (Arnold & Holt, 1995). Cross contamination resulted in the production of seeder birds, which led to colonisation of other birds during maturation (Cason *et al.*, 1993; Bailey *et al.*, 1994).

The problem with the *S. enteritidis* serotype is its unique ability to asymptomatically colonise the reproductive tract of a hen and contaminate the contents of every egg as they are forming (Whiting *et al.*, 2000). On average, 26 h is required for the formation of a completed egg. The ovum spends approximately 5 h in the magnum at the top of the oviduct, where it is surrounded by thick albumin. This is followed by the addition of two shell membranes in the isthmus. The remaining 21 h are required for shell deposition in the uterus, after which the completed egg is moved through the vagina to pass through the cloaca as it is laid (Keller *et al.*, 1995). Keller *et al.*, (1995) suggested that there was a dramatic drop in the incidence of *S. enteritidis* colonisation of freshly laid eggs compared with that of forming eggs. Once the egg shell and its cuticle were in place, the possibility of bacterial contamination from internal and external sources was greatly diminished. Results indicated that the vast majority of *S. enteritidis*-positive forming eggs were associated with either colonised ovarian tissue or with tissues of the upper ovarian oviduct and not tissues of the vagina or cloaca. This indicates that infection of the forming egg occurs in the upper oviduct prior to eggshell deposition. However, as the 41.9°C egg is laid into the room temperature environment and the egg rapidly cools, a pressure gradient is created, which could be enough to draw bacteria into the egg. Contamination of freshly laid eggs could therefore indeed occur as they pass through a heavily colonised cloaca or vagina (Berrang *et al.*, 1999).

Poultry are commonly infected with a number of *Salmonella* serotypes. Infection is mostly confined to the gastrointestinal tract and the birds often excrete *Salmonella* in their faeces (Poppe, 2000). However, egg contamination with *Salmonella* serotypes other than

S. enteritidis has been reported. *S. gallinarum* and *S. pullorum* are avian-adapted serotypes that infect the hen's ovary and cause transovarian transmission into the egg. These serovars cause pullorum disease and fowl typhoid respectively, which, unlike *S. enteritidis* infection, are overt diseases in chickens. *S. gallinarum*- and *S. pullorum*-infected flocks can be slaughtered quickly to prevent the spread of the disease (Cooper & Thorns, 1996). Other vague-adapted serotypes, including *S. typhimurium, S. kentucky, S. heidelberg, S. hadar, S. saintpaul* and *S. thompson,* have been isolated from the reproductive organs of hens or the contents of eggs (Okamura *et al.*, 2001).

1.4.2 Pathogenesis of *S. enteritidis* in chickens

When adult laying hens were experimentally infected by oral inoculation with 10^8 *S. enteritidis*, the microorganisms were isolated two days post-infection from the intestinal tissues, heart, liver, spleen, gall-bladder and from various sections of the ovary and oviduct (Keller *et al.,* 1995). Forming eggs were found to be positive for *S. enteritidis* at a rate of 30%. Freshly laid eggs in the same experiment were positive at a rate of less than 0.6%, indicating that colonisation of forming eggs takes place in the reproductive tract, but that factors within the eggs significantly control the pathogen before the eggs are laid. These factors included antibodies, antibacterial enzymes, and iron-sequestering and bacterial protease-inhibiting proteins within the egg yolk and albumin (Keller *et al.*, 1995).

It is the rapid systemic infection of hens that can cause an outbreak of *S. enteritidis* (Cooper *et al.*, 1989). Studies on the pathogenesis of experimentally infected chickens, showed that after invasion of a host via the caeca and crop, the bacteria multiply in the liver and spleen and then spread to other organs, producing a systemic infection (Barrow, 1991; Gast, 1993; Poppe, 2000). Environmental conditions such as osmolarity, oxygen tension, pH and the stimuli of short-chain fatty acids regulate *Salmonella* virulence by modulating the expression of invasion genes in *Salmonella* in epithelial cell invasion. Since the crop is the first host environment encountered by *S. enteritidis* after ingestion, it is able to influence the survival and virulence of *S. enteritidis*. The crop is a non-secretory organ, which functions to store food before passage into the gizzard. Physiological changes in the crop, such as those that take place after feed withdrawal, a practice used in

the poultry industry to stimulate multiple egg-laying cycles in laying hens, can be more favourable for the survival and colonisation by *S. enteritidis* (Durant *et al.,* 1999).

The age of hens at the time of infection with *S. enteritidis* influences intestinal colonisation of the hens, egg production, and the isolation rates of *S. enteritidis* from egg shells and egg albumin. When compared to hens of 37 and 27 weeks of age respectively, hens at 62 weeks of age were more often colonised intestinally, produced fewer eggs and eggs that were produced had a higher percentage of contamination. Hens at 20 weeks of age were less susceptible to *S. enteritidis* infection than mature birds at 55 weeks of age and showed no clinical signs, while hens at 60 weeks of age showed clinical septicaemia and associated mortality (Gast & Beard, 1990b).

To understand the pathogenesis of *S. enteritidis* infection in chickens, investigators have studied the consequences following the experimental, oral inoculations of chickens with *S. enteritidis* (Gast & Beard, 1991b; Barrow & Lovell, 1991; Thiagarajan *et al.,* 1996b). Prior to invasion of any cell type, the bacteria must encounter and attach to one or more cell types found in the host. Attachment of bacteria to host cell surfaces is considered an essential step in pathogenesis (Darwin & Miller, 1999). Bacterial surface hydrophobicity, charge, cell-density and exopolysaccharides have been cited as important factors for adherence to surfaces and cells (Woodward *et al.,* 2000). Another strategy used by bacterial pathogens, including *S. enteritidis*, for the colonisation and adherence to specific host target tissues such as the gastrointestinal mucosa, involves bacterial fimbriae (Rajashekara *et al.,* 2000). Fimbriae are bacterial lectin-like adhesive appendages, which bind glycoprotein or glycolipid receptors on epithelial cells (Section 3.5) (Isberg, 1991; Collinson *et al.,* 1993). The majority of *Enterobacteriaceae* express flagella, which are considered to assist in directional motility (Dibb-Fuller *et al.*, 1999). The possession of active flagella combined with chemotaxis is also an important factor in the pathogenicity of *Salmonella* (Robertson *et al.*, 2000). Allen-Vercoe *et al.,* (1999) showed that *S. enteritidis* flagella play an important role in the pathogenesis of infection in the chick gut. *S. enteritidis* flagella are long (5-10 μm), rigid structures, thought to promote interaction between bacteria and host by increasing surface area for initial contact and overcoming repulsive forces (Allen-Vercoe & Woodward, 1999). Worton *et al.,* (1989) suggested that the early association of *S. typhimurium* with host tissues could have involved a loose non-specific association with superficial mucus. Motility would therefore

have been an important virulence factor in enabling salmonellae to penetrate the mucus layer and attach specifically to epithelial cells. This was supported by the findings of McHan *et al.,* (1988), which demonstrated greater adhesion of *S. typhimurium* to chicken cecal epithelia in the absence of an intact mucus layer.

Like other salmonellae, once attached to epithelial cells, *S. enteritidis* is able to gain access to these cells that are normally not able to carry out phagocytic functions. This is an important pathogenic property as it allows the microorganism access to deeper tissues and to avoid host defense mechanisms. This is facilitated by a complex set of biochemical interactions between the host and bacteria. The bacteria are believed to secrete a number of effector proteins that trigger a set of signaling events in the host cell, leading to marked cytoskeletal rearrangements, membrane ruffling and bacterial uptake by micropinocytosis (Darwin & Miller, 1999; Galán, 1996). In addition to invasion, programmed cell death (apoptosis) of infected macrophages and recruitment of polymorphonuclear neutrophils (PMN) seems to occur (Chen *et al.*, 1996). Following invasion of intestinal epithelial cells, *S. enteritidis* was found in macrophages in the lamina propria of the mucous membrane (Popiel & Turnbull, 1985). The *S. enteritidis* infection thus becomes systemic, with the microorganism transported through the host's vascular system. The colonisation of preovulatory follicles by blood-borne *S. enteritidis* could involve interactions with the cellular components in the ovarian follicular wall (Thiagarajan *et al.,* 1994). The highly vascularised structure of the follicular theca is composed of vessels of increased permeability and this anatomical feature may facilitate the transport of *S. enteritidis* from the blood of the hen to the developing preovulatory follicle (Barrow & Lovell, 1991). Blood-borne *S. enteritidis* may be deposited near the basement membrane itself since many of the blood vessels terminate here. From this point the bacteria may penetrate the basement membrane and enter the yolk, after invading and multiplying in the granulosa cell layer of the preovulatory follicle, which is located close to the egg yolk mass (Thiagarajan *et al.,* 1996). This process would lead to the production of a *Salmonella*-contaminated egg.

1.5 Detection and control of *S. enteritidis*

Preharvest food safety is the most important component of an effective detection and control strategy for *S. enteritidis* infection. A number of control strategies have been implemented to reduce the prevalence of *S. enteritidis* in poultry and thus effectively, the reduction of human outbreaks. These strategies rely on the most rapid, inexpensive and specific methods of detection of *S. enteritidis* infection available.

1.5.1 Conventional detection of *S. enteritidis*

Conventional testing methods for the detection of *S. enteritidis,* are based on standard bacteriological culturing, followed by biochemical confirmation to determine the genus and specific serological tests to determine the serotype. The testing can take from 4 to 7 days to complete, sometimes providing only a presumptive diagnosis (Rajashekara *et al.,* 1999; Van der Zee & Huis in't Veld, 2000).

The procedure for identifying *Salmonella* generally consists of four phases (Figure 1.2). The first phase is pre-enrichment in a non-selective medium, such as buffered peptone water (BPW), to allow multiplication of the target organism and any others present in the sample, as well as allowing the resuscitation of any injured cells. Phase two is selective enrichment, for the survival or growth of *Salmonella* in the sample and inhibition of other organisms, using media such as xylose-lysine-tergitol 4 (XLT-4), a modification of xylose-lysine-deoxycholate (XLD). The third phase is isolation, in which selective agar media are used to restrict the growth of bacteria other than *Salmonella.* This produces presumptive isolates. Use of semi-solid diagnostic *Salmonella* medium (DIASALM) containing nitrofurantoin (DIASALM-N) results in the specific suppression of serovars other than *S. enteritidis* (van der Zee & van Netten, 1992). The final phase is confirmation, in which the isolates are subjected to biochemical testing and serological assays, to confirm that the isolates are *Salmonella* and to determine whether the serotype is *S. enteritidis* (Van der Zee & Huis in't Veld, 2000).

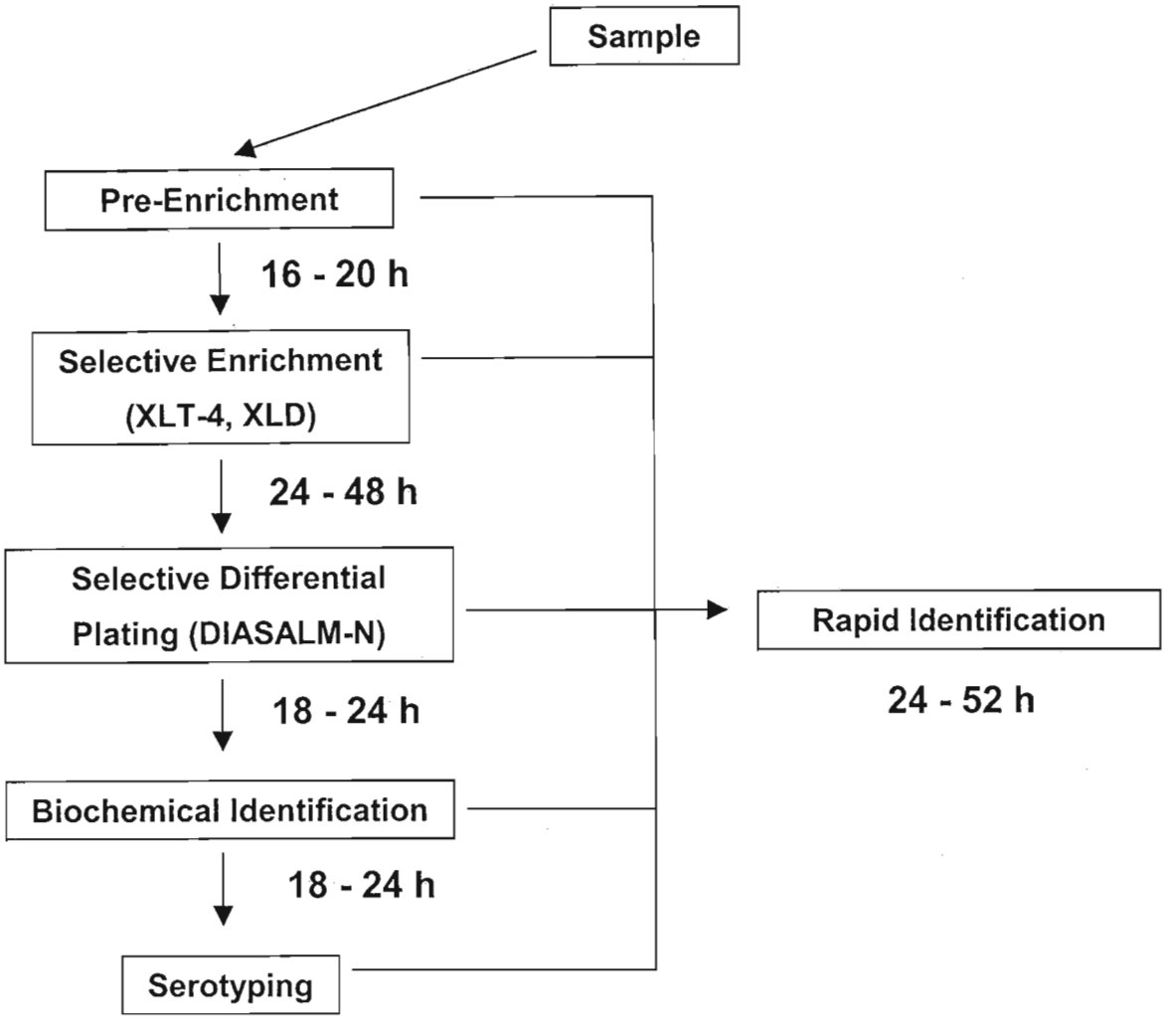

Figure 1.2 Flow chart showing the steps required for the isolation and identification of *S. enteritidis* (Swaminathan & Feng, 1994)

Most enteric bacteria, including many *Salmonella* serotypes, are similar in microscopic and culture morphology appearance (Fig. 1.1). Biochemical tests have conventionally been used to identify them after a preliminary examination of their morphology, motility and growth responses (Fig. 1.2). Like all other members of the *Enterobacteriaceae*, *S. enteritidis* degrades sugars by means of glycolysis and cleaves pyruvic acid in formic acid fermentations. This can be determined by the methyl red and Voge-Proskauer tests. Like all other *Salmonella*, *S. enteritidis* carries out mixed acid fermentation and produces mainly lactate, acetate, succinate, formate (or hydrogen gas and carbon dioxide), and ethanol. In butanediol fermentation, the major products are butanediol, ethanol and carbon dioxide. Some of the other more commonly used tests are those for lactose and citrate utilisation, indole production from tryptophan, urea hydrolysis, and hydrogen sulfide production (Table 1.1).

Table 1.1 Characteristics of selected genera in the *Enterobacteriaceae* (Quinn *et al.*, 1994).

Characteristics	*Salmonella*	*Escherichia*	*Shigella*
Methyl Red	+	+	+
Voges-Proskauer	absent	absent	Absent
Indole production	absent	(+)	D
Citrate use	(+)	absent	Absent
H_2S production	(+)	absent	absent
Urease	absent	absent	absent
β-galactosidase	absent	(+)	absent
Gas from glucose	(+)	+	absent
Acid from lactose	( )	+	absent
Phenylalanine deaminase	absent	absent	absent
Lysine decarboxylase	(+)	(+)	absent
Ornithine decarboxylase	(+)	(+)	D
Motility	(+)	d	absent
Gelatin liquifaction	absent	absent	absent
% G + C	50-53	48-52	49-53

Note: d = strains vary in possession of characteristics; + = present; (+) = usually present; ( ) = usually absent

This series of tests has conventionally been effective in identifying the genus of a bacterial isolate. However, a positive *Salmonella* isolate requires serotyping before a full identification is complete. Bacterial growth for serotyping should be taken from a TSI agar slant, or from nutrient agar, as cultures from selective media are often unsuitable for serotyping. Serotyping is based on the O (somatic) and H (flagellar) antigens and a slide agglutination test is generally used. Rapid slide agglutination involves placing a drop containing polyvalent crystal violet-stained bacteria (antigen) on a microscopic slide. An equal amount of serum or whole blood from a suspected animal is mixed with the bacterial sample and examined. Agglutination of the bacterial cells occurs when blood containing homologous antibodies is used (Barrow, 2000).

A serogroup comprises serotypes with similar O antigens. Certain serotypes share an almost identical antigenic formula. These serotypes must be distinguished by biochemical

tests (Table 1.1) and are known as biotypes. Examples of two biovars are *S. pullorum* (antigen formula O9,12:-,-) and *S. gallinarum* (antigen formula O1,9,12:-,-). The antigen formula of *S. enteritidis* is 09,12;g,m:1,7. The antigens of some of the *Salmonella* serotypes used in this investigation are included in Table 1.2.

Isolates of a certain serotype can be further characterised according to the sensitivity of the isolates to a series of bacteriophages at appropriate dilutions, known as phage typing. A bacteriophage is an obligate intracellular parasite that multiplies inside bacteria by making use of some or all of the host biosynthetic machinery. Phage conversion in *Salmonella* is characterised by a change in phenotype of the bacterial cell as a consequence of the modification of O antigens. This conversion is catalysed by a phage-encoded enzyme (usually a glycosyl transferase) that modifies the side chains of O antigens. This important mechanism is believed to help the micro-organism escape neutralising activities of antibodies produced by animal hosts (Davis *et al.*, 1990). Phage type (PT) 4 was responsible for a large proportion of the human food poisoning cases in the UK and since 1987, has become the predominant isolate in broilers and laying hens in the UK (Quinn *et al.*, 1994). However, since 1996, a sharp increase in *S. enteritidis* PT6 has been reported (Evans, 1998). In the USA, PT8 and PT13 were the most common phage types, yet no single phage type was more likely to be associated with eggborne infection (Mishu *et al.*, 1994). By 1996, 82 percent of *S. enteritidis* cases isolated in KwaZulu-Natal region of South Africa had been PT 4, whereas 83 percent of cases in the Western Cape region were PT 34, which had not occurred in KwaZulu-Natal (Horner, 1996).

Table 1.2 Antigens of some *Salmonella* serotypes (Quinn *et al.*, 1994).

Serotype	Serogroup	Somatic (O) antigens	Flagella (H) antigens	
			Phase 1	Phase 2
S. enteritidis	D_1	1, 9, 12	g, m	[1,7]
S. dublin	D_1	1, 9, 12, [Vi]	g, p	
S. gallinarum	D_1	1, 9, 12		
S. pullorum	D_1	9, 12		
S. berta	D_1	9, 12	f, g, t	
S. typhimurium	B	1, 4, [5], 12	I	1, 2

Note:[ ] = antigen may be present or absent; 1 = O factor whose presence is due to phage conversion

Each phase in a conventional detection procedure requires at least 16 h, up to a maximum of 48 h. One or more steps can be omitted depending on the type of sample, the infection level and reason for investigation. Clinical samples such as organ samples (from post mortem) or rectal and cloacal swabs would be expected to contain higher numbers of *S. enteritidis* and direct incubation in/on selective media could be used. In contrast, samples expected to contain low numbers of *S. enteritidis*, such as eggs, environmental swabs, feed and litter, would require all four phases of the identification procedure to be performed. Low levels of *S. enteritidis* would be expected in these samples due to factors such as uneven distribution, heat and pH stress, and antimicrobial substances (Humphrey *et al.*, 1996).

Regardless of which combination of conventional methods is used, the time span that is required to obtain a negative result for clinical methods is at least 24 h and can be delayed by up to 96 h if the whole cultural method has to be used. Presumptive positive results, serotyping not included, require a minimum time of 48-96 h depending on the method used (Van der Zee & Huis in't Veld, 2000).

The development of alternative methods aimed not only at reducing the analytical time, but also increasing specificity of the test procedures. Most efforts in this regard consisted of media or procedural modifications that shortened microbiological assays by a limited extent. However, due to the intermittent shedding nature of *S. enteritidis* (Section 1.4), not all infected flocks would be detected using conventional bacteriological isolation

techniques (Yap *et al.*, 2001). To address this problem, a new generation of rapid diagnostic methods was developed, some of which have been commercialised. Figure 1.2 illustrates how rapid methods can be utilised in conjunction with, or replace, the four phases of conventional procedures for *S. enteritidis* detection, reducing the total time required for identification. These rapid methods no longer rely solely on agar media and biochemical identification, and include assays that measure bacterial metabolites, enzyme activity and composition of bacterial cells, and highly specific DNA- and antibody-based reactions.

1.5.2 Serological detection of *S. enteritidis*

Exposure of chickens to *S. enteritidis* normally leads to rapid systemic infection, accompanied by the production of circulating immunoglobulins, normally of the IgG class (Barrow, 1994). Like mammals, hen's immunoglobulins consist of heavy (H) and light (L) chains, bridged by disulfide bonds and are made up of variable domains, which contain the antigen-binding site, and constant domains. There are three predominant immunoglobulins in the hen, namely the immunoglobulins M (IgM), G (IgG) and A (IgA) (Ivanyi, 1981). Present in all vertebrates, IgM delivers the first immune response, being a good agglutinator because of its pentameric structure. As in mammals, the IgA of hens is found in the fluids of the gall bladder and other secretions of the body. Avian IgG, like IgG of mammals, delivers the second immune response. Avian IgG is structurally different to mammalian IgG in that avian IgG has an additional constant domain in place of the hinge region found in mammalian IgG. The molecular mass of avian IgG is thus about 190 kilodaltons (kDa), unlike the approximate 150 kDa of mammalian IgG. Unlike mammalian IgG, avian IgG also takes part in the anaphylactic reactions of the immune system (Erhard & Schade, 2001).

The predominant antibody isotype of egg yolk is IgY. The concentrations of IgM, which contributes significantly to the agglutination reactions in serum (Nicholas & Cullen, 1991), are low in egg yolks (Erhard & Schade, 2001). This is due to IgM and IgA being transferred together with other proteins in the oviduct into the egg white. However, so little is transferred that only trace amounts are detectable in the finished egg and only in the egg white. In the egg follicle, IgG is passed on by receptors, hence selectively, but in

large quantities, into the egg yolk, where it becomes known as IgY. Concentrations and contents of IgY in the serum and egg yolk are comparable when taking time of egg-formation into account. This means that egg yolk can be used as ready supply of IgY as well as a means of titration after immunization of a hen (Erhard & Schade, 2001). Egg yolk offers a source of IgY that can be detected by the same methods applied to serum, yet which can be collected by less labour, jeopardises flock biosecurity less and is less stressful to the birds than bleeding methods to obtain serum IgG. Studies by Gast and Beard (1990 a,b,c) showed that IgY from egg yolks could be used to detect *S. enteritidis*-exposed hens, for longer intervals after infection and at higher frequencies than bacteriological sampling methods. In a study involving laying flocks, suspected as sources of a human *S. enteritidis* outbreak, the presence of specific antibodies in egg yolks was directly correlated with the presence of *S. enteritidis* in tissue samples (Gast & Beard, 1991a).

A humoral response against *Salmonella* antigens can be detected in chickens one week post-infection and persists for at least ten weeks even if the bird is no longer culture-positive (Holt, 2000). The antigenic determinants of *Salmonella* are composed of somatic (O), flagellar (H) and surface antigens (Vi). Variations in these antigens correlate with different *Salmonella* serotypes (Jongerius-Gortemaker *et al.,* 2002). For example, serogroup D *Salmonella*, including *S. enteritidis,* can be identified using antibodies against their O9 somatic antigens. The O9 antigen is the immunodominant epitope of the lipopolysaccharide of the serogroup D *Salmonella* serotypes and is a tyvelose residue of the O antigen repeat (Rabsch, 2000). However, the test does not differentiate between *S. enteritidis* and other serogroup D *Salmonella* serotypes.

In studies carried out to investigate the serology of *S. enteritidis* infection, serum IgG, IgM and IgA antibody isotypes were detected following infection of specific pathogen-free (SPF) chickens. Serum IgM titres appeared before IgG, as expected, and disappeared earlier. Serum IgG titres were raised two weeks post-infection, reached a peak at five weeks and remained high for a further four weeks. IgA was the predominant isotype in bile and gut washings (Hassan *et al.,* 1990; Wysocki *et al.*, 2002), as expected.

A number of assays based on detecting these circulating antibodies were developed. A major advantage of these serological methods over bacteriological methods is that whereas *S. enteritidis* organisms are excreted intermittently, serum IgG concentrations are persistent. However, soon after infection serum IgG concentrations will be low (although rapidly increasing), whereas bacterial excretion will be at a maximum (Barrow, 1994). Complement fixation and slide agglutination (Section 1.5.1), using either serum or whole blood, have been used for many years, and were especially effective for detecting the poultry carriers of *S. pullorum* and *S. gallinarum*. Although relatively crude, application of the results of slide agglutination testing was successful in largely eradicating *S. pullorum* from the intensive poultry industry in many countries (Barrow, 1994). This test was adapted for use with *S. enteritidis*, yet slide-agglutination was found to preferentially identify IgM-producing birds, at the early stages of infection. The proportion of birds producing IgM would be relatively low, and this isotype does not persist at high titre, but it could help in identifying recent infections. The practical difficulties of employing agglutination-based systems for large-scale testing, needed for the modern poultry industry, also far outweighed any potential advantages (Yap *et al.*, 2001).

Immunoblotting and the enzyme linked immunosorbent assay (ELISA) were found to be sensitive, reliable and applicable for large-scale screening of flocks and largely superceded other methods of *S. enteritidis* detection in poultry flocks in many countries (Barrow, 2000). Although equally specific and sensitive, the ELISA was far more applicable than immunoblotting, especially when developments in ELISA such as miniaturisation and mechanisation, together with the availability of commercially prepared high-quality reagents, were taken into account (Cooper & Thorns, 1996).

The efficiency of ELISA results is heavily dependent on the choice of antigen and source of antibodies. Crude surface-extracted antigens and lipopolysaccharides (LPSs) are the most commonly used discriminating antigens in *Salmonella* detection. LPSs are a large group of substances consisting of a lipid (Lipid A) buried in the outer membrane of the walls of Gram-negative bacteria. Attached to Lipid A is the core polysaccharide, containing unique sugars. In *Salmonella,* the core polysaccharide is constructed of ten sugars, many of them unusual in structure. LPSs are immensely antigenic and are used as the O-somatic antigen in Gram-negative bacteria (Guard-Petter *et al.*, 1995; Hitchcock &

Brown, 1983; Westphal & Jann, 1965). Chickens infected with *S. enteritidis* produce a sustained antibody response to the LPS antigen, and commercial test kits have been produced for large scale screening of flocks for LPS antigens. However, varying degrees of cross-reactivity has been demonstrated with sera of chickens infected with different *Salmonella* serotypes (Barrow *et al.*, 1992, Barrow, 1992).

The outer membrane proteins of *Salmonella* are known to play an important role in evoking an immune response. Kim *et al.*, (1991) reported using an antigen identified from the outer membrane proteins (OMP) of *S. enteritidis* to develop an ELISA-based test, shown to be more sensitive than standard detection methods.

ELISAs that use flagellar antigens for detecting salmonellae were also successfully developed (Timoney *et al.*, 1990; van Zijderveld *et al.*, 1992). Early work was unable to detect flagella-specific IgY or IgM in chickens infected with *S. enteritidis* (Humphrey *et al.*, 1989; Chart *et al.*, 1990). Flagellar antigens also showed confusing cross-reactivity and flagella-specific antibodies were not as persistent as LPS-specific antibodies. However, flagellar antigens can be used successfully to differentiate infection caused by flagellate and non-flagellate serotypes, particularly *S. enteritidis* from *S. gallinarum* and *S. pullorum* (Timoney *et al.*, 1990). These three serotypes are all serogroup D *Salmonella*. In the United States poultry industry, the *S. gallinarum* and *S. pullorum* eradication program makes use of a whole-cell antigen from *S. pullorum.* This antigen does not distinguish between *S. enteritidis*, *S. gallinarum* and *S. pullorum* (Rajashekara *et al.*, 1998). The ability to identify *S. enteritidis* infection is therefore very important to avoid confusion before declaring a flock positive for *S. gallinarum* or *S. pullorum.*

A novel fimbrial antigen SEF14, was first described on strains of *S. enteritidis* (Thorns *et al.*, 1990). The *sefA* gene, encoding SEF14, has been shown to have a limited distribution among serogroup D *Salmonella* serotypes (Rajashekara *et al.*, 1998). SEF14 fimbriae are produced by *S. enteritidis*, *S. dublin, S. moscow* and *S. blegdam* strains. Though many of the remaining serogroup D *Salmonella* serotypes such as *S. typhi, S. gallinarum* and *S. pullorum* possess the intact gene, they fail to express SEF14 fimbriae (Turcotte & Woodward, 1993; Thorns *et al.*, 1994). Of the approximately 7 500 isolates of *S. enteritidis* tested, representing all phage types, all possessed the *sefA* gene sequence and all expressed SEF14 fimbriae. Ninety percent of *S. dublin* isolates also expressed SEF14

at the cell surface (Wooodward & Kirwan, 1996), but *S. dublin* is a host-adapted serotype that is primarily a cattle pathogen. *S. enteritidis* is thus the only serotype isolated from poultry that expresses SEF14 fimbriae (Thorns *et al.,* 1996a). This made SEF14 an obvious candidate antigen for use in serological detection of flocks infected with *S. enteritidis* as well as eggs and egg products. Results indicated that birds infected with *S. enteritidis* readily seroconvert within 10 days of infection and IgG response persists for at least four weeks thereafter (Thorns *et al.,* 1996a). The production of SEF14 antibodies following infection was the first specific anti-fimbrial response to *S. enteritidis* infection (Thorns, 1995).

A study by Thorns *et al.*, (1996) involved using SEF14 for both indirect and competitive ELISAs and no cross-reactions were observed with *S. typhimurium*- or *S. gallinarum*-infected birds. A dot-blot test (utilising the same principle as immunoblotting and ELISA) was also evaluated using the SEF14 antigen, yielding highly specific results. The only cross-reaction seen was with antisera against *S. blegdam,* an extremely rare serotype, which is not found in poultry (Cooper& Thorns, 1996).

Due to its relatively high IgY concentrations, egg yolk, rather than serum, was considered a possible source of anti-*S. enteritidis* antibodies. A standard indirect ELISA was considered more appropriate for analysing egg yolk than a competitive ELISA. These techniques are discussed in Section 2.10.2. The low dilution of the samples used in the competitive ELISA may have lead to interference and a loss of sensitivity, with false negative reactions. There have also been some affinity problems and it may be less sensitive than the indirect ELISA. In the field, both systems have been known to produce false positive results (Barrow, 2000). However, several ELISAs, using both serum and egg yolk, have been used to study field infections of chickens with *S. enteritidis* (Gast & Beard, 1991a; Brigmon *et al.*, 1995; Wysocki *et al.*, 2002;).

The ELISA is relatively robust and can accommodate poor quality sera. However, much cross-reactivity has been observed between *S. enteritidis* and other *Salmonella* serotypes, using many of the ELISA techniques. Moreover, 24 to 48 h preenrichment or selective enrichment steps (Fig. 1.1) are often required before the organism can be detected by ELISA (Tsen, 1994). As for most other serological tests, the ELISA should be regarded as a flock test, to be accompanied by bacteriological testing.

1.5.3 Nucleic acid detection of *S. enteritidis*

With both rapid and traditional approaches to the detection of *S. enteritidis*, long analysis times have been used due to poor sensitivity. Enrichment (Section 1.5.1) has been an essential step, because single potential *Salmonella* cells present in a sample must be enriched to reach the threshold sensitivity of the detection method. This enrichment has been achieved by cell multiplication, which is a lengthy process. With rapid detection methods, multiplication must typically result in a target cell concentration of 10^4-10^6 cells ml^{-1} to give a positive result (Bennet *et al.*, 1998).

The target concentration can be increased more rapidly by use of an alternative target, nucleic acid, and the use of the polymerase chain reaction (PCR), which amplifies a target sequence exponentially (approximately 10^7-fold) in just 2-3 h. Detection of amplified product indicates the presence of the target nucleic acid sequence and hence the target organism (Bennet *et al.*, 1998).

The sensitivity, specificity and speed of the technique makes PCR potentially one of the most valuable tools for detection of *S. enteritidis*. One major disadvantage of DNA-based techniques such as PCR, is that the test is unable to distinguish between DNA from viable and dead organisms. The inclusion of an enrichment step prior to PCR was suggested to overcome this. Large numbers of dead cells in a sample prior to enrichment could, however, still result in a positive result (Szabo & Mackey, 1999). Szabo & Mackey (1999) also suggested amplifying mRNA by reverse transcription PCR (RT-PCR) to distinguish between viable and dead *S. enteritidis* cells, as mRNA has a short half-life and would only be expected to be found in samples with viable cells.

A number of target sequences have been chosen for PCR detection of *S. enteritidis*. Wood *et al.* (1994) developed a PCR assay using a target sequence from a 2 kilobase (kb) DNA fragment unique to the serotype-specific virulence plasmid of *S. enteritidis*. Their results showed specificity between *S. enteritidis* and *S. typhimurium* in the absence of cross reactions such as those seen using serological techniques such as the ELISA (Van Zijderveld *et al.*, 1992). However, many isolates of *S. enteritidis* do not have the virulence plasmid, and the plasmids that many isolates have, are genetically variable (Woodward & Kirwan, 1996; Lampel *et al.*, 1996). Primers have also been used that were found to be

specific to all members of the genus *Salmonella*, but not only exclusively to *S. enteritidis* (Cohen *et al.,* 1994). Multiplex PCR-based assays use more than one set of primers and have been developed for the simultaneous detection of *S. enteritidis* and all *Salmonella* serovars (Mahon *et al.,* 1994; Soumet *et al.,* 1999a, b). However, relatively few serovars of *Salmonella* were used to test the specificities of the primers and the technique is relatively time-consuming.

SEF14 is the fimbrial antigen found to have major potential for serological detection of *S. enteritidis* (Section 1.5.2). The distribution of *sefA*, the gene sequence coding for SEF14, is limited to serotypes of the serogroup D *Salmonella*. The recorded incidence of these serotypes, including avian-adapted *S. gallinarum* and *S. pullorum,* is very low (Woodward & Kirwan, 1996). The *sefA* gene sequence therefore seemed a logical target sequence for PCR detection of *S. enteritidis*. Woodward & Kirwan (1996) designed an oligonucleotide primer pair in accordance with the basic principles of primer design (Innis & Gelfand, 1990), expecting to produce an amplified PCR product of 310 base pairs (bp). The PCR amplified the target sequence successfully and *S. enteritidis* was detected in all experimentally infected eggs. Samples were only taken for PCR after 16 h of growth in medium, because of partial PCR inhibition by factors in the egg. In spite of this, results were still obtained in less than 24 h.

Woodward & Kirwan (1996) speculated that immunoseparation of *S. enteritidis* from the sample mixture could concentrate the bacteria and possibly remove potential inhibitors, reducing time taken to obtain results. Immunomagnetic separation (IMS) can replace a selective enrichment step with a 10 min immunocapture procedure using commercially-available antibody-coated paramagnetic beads (Safarik *et al.,* 1995). The paramagnetic beads are coated with polyclonal antibodies, which can target and concentrate *Salmonella* cells in a mixed suspension with no loss of viability (Favrin *et al.*, 2001). IMS has previously been combined with ELISA for the detection of *S. enteritidis* (Holt *et al.,* 1995; Málková *et al.,* 1998; Mansfield & Forsythe, 2000), and IMS has also been incorporated into commercial *S. enteritidis* detection kits that report being able to test for *S. enteritidis* within 24 h (Radlo, 1994). Combining IMS with PCR could prove to be a rapid and specific method of *S. enteritidis* detection in poultry products.

1.5.4 Control of *S. enteritidis*

The eradication of *S. enteritidis* isolates from the environment is practically impossible. In fact, the widespread nature of infection in many countries has resulted in the suspension of attempts to eliminate infection from many breeder and layer flocks, with the focus largely shifting to attempts to control infection (Wilks *et al.*, 2000). Control of *S. enteritidis* is difficult due to its widespread distribution and capacity to survive in the environment (Doran *et al.*, 1993). An easy diagnosis of *S. enteritidis* infection is not always possible, with the most advanced detection tests not always providing exact results. The poultry industry is therefore constantly looking at feasible ways of at least reducing *S. enteritidis* in poultry products (Gashe & Mpuchane, 2000).

Modern poultry husbandry practices include breeding livestock in overcrowded batteries at optimal temperature and low light intensity to enhance growth rates and mass increases. Birds are also fed meal, which can be heavily contaminated with *S. enteritidis* and other infectious bacteria. For this reason, several antimicrobial agents such as antibiotics, high temperatures and pressures, organic acids, bacteriocins and oxidising agents have been used individually or in combination in attempts to suppress the outbreak of epidemics in flocks (Gouws & Brözel, 2000; Manie *et al.*, 1998; Mokgatla *et al.*, 1998; Goodnough & Johnson, 1991).

The use of subtherapeutic doses of antibiotics in feed promotes bacterial resistance by decimating the less hardy microorganisms normally present in the bird, leaving the drug-resistant strains to flourish (Boonmar *et al.*, 1998). The *S. enteritidis* reduction plan adopted by SAPA in 1993 has taken into account that excessive use of antimicrobials may lead to problems of antibiotic resistance (Bayer, 1994). The reduction plan suggested the use of newer generation broad-spectrum drugs to be used in conjunction with all aspects of the control program, and if used, antimicrobial therapy should be used at the correct dose and for the correct period of time. However, a study undertaken by Manie *et al.*, (1998) in the Western Cape region of South Africa showed that a large proportion of salmonellae found on fresh poultry at abattoirs were showing signs of multiple antibiotic resistance. In a report by Bedson Africa (2001), findings indicated that strains of *S. enteritidis* isolated in Southern America were resistant to several antibiotics (Table 1.3). These findings correspond to what was observed in the Southern African poultry industry (Bedson Africa,

2001). Selective pressure exerted by widespread antimicrobial use is presumed to be the driving force in the development of this antibiotic resistance in *S. enteritidis* and other infectious microorganisms. The use of medically important drugs as supplements in poultry feed should be reassessed in terms of the potential negative effects on food safety and public health.

Table 1.3 *Salmonella enteritidis in vitro* sensitivity to several antibiotics (Bedson Africa, 2001).

Antibiotic	Number of Strains Tested	Number of Resistant Strains	% Resistant
Oxytetracycline	48	28	58
Furazolidone	48	23	48
Neomycin	48	18	37.5
Gentamycin	48	11	23
Norfloxacin	48	10	21.9
Ampicillin	48	9	18.8
Enrofloxacin	48	8	16.7
Sulfas + TMP	48	8	16.7
Fosfomycin	48	0	0

The South African *S. enteritidis* reduction plan has suggested the use of probiotics (Bayer Health Group, 1994). A probiotic is a culture of competitive non-pathogenic microflora, intended to exclude *S. enteritidis* from the intestine or to suppress the establishment of *S. enteritidis* infection in the intestine. Because commercially-reared poultry are slow to develop an intestinal microflora, introduction of this culture of microflora was intended to enhance the rate at which such a flora becomes established (Mead & Barrow, 1990). Colonisation of native intestinal microflora of broiler chickens not only minimised infection in chicks, but also significantly reduced the period of infection even after infection was established in chicks (Baba *et al.*, 1991). This strategy, although effective in broilers, does not address asymptomatic adult layers and the production of *S. enteritidis*-infected eggs.

Application of modern vaccines to poultry in grand parent, parent and commercial laying flocks has also been recommended as part of the *S. enteritidis* reduction plan in South Africa (Bayer, 1994). Vaccination is aimed at protecting chickens against invasion and

infection by *S. enteritidis* throughout the laying period. This leads to reduced persistence and bacterial shedding in an immunised flock, thereby disrupting the chain of infection. *Salmonella* vaccines are generally of two types, either live or inactivated (also known as killed), each having specific uses and advantages. The potential of genetically engineered vaccines has also been realised, with a number of approaches being assessed in order to select the most effective vaccines (Griffin, 1991).

It is generally accepted that a live attenuated strain of *Salmonella* constitutes a better vaccine against systemic infection than a vaccine made from inactivated virulent *Salmonella* (Griffin, 1991). Live vaccines are especially effective against *S. enteritidis* infection, due to the intracellular parasitism of *S. enteritidis* and the sustained stimulation of cell-mediated immune reactions by a live vaccine (Hahn, 2000). A live *S. enteritidis* vaccine is a *S. enteritidis* strain of extremely low virulence, which maintains good invasiveness, immunogenicity and viability. Unlike live vaccines, inactivated vaccines contain killed antigen and an immune response-enhancing adjuvant. Although inactivated vaccines pose a lower epidemiological risk than live vaccines, they were observed as having a lower efficacy than live vaccines in *S. enteritidis* infection (Hahn, 2000). Inactivated vaccines can only be administered by injection, giving live vaccines a further advantage in that live vaccines can be administered by a number of methods. The most convenient method of administration is via the drinking water, which involves less stress for the birds and less labour intensive. The oral administration of vaccines also simulates the natural route of *S. enteritidis* infection (Hahn, 2000).

S. enteritidis is unlikely to be eliminated from poultry by relying solely on the test-and-slaughter method used to eradicate *S. gallinarum* in England because, unlike *S. gallinarum*, *S. enteritidis* can be reintroduced into flocks from other animal reservoirs, such as rodents. Instead, vaccination seems to be the most effective means of controlling *S. enteritidis* in domestic fowl. In fact, the rapid decline in human cases of *S. enteritidis* in Europe has been attributed to the ongoing use of *S. enteritidis* vaccines in poultry (Rabsch, 2000) and with continued use, this decline may continue.

1.6 Objectives of the current study

Although *S. enteritidis* control guidelines for South African poultry producers are in place, *S. enteritidis* remains a potential problem in South Africa. Commercial poultry producers are still required to test poultry and poultry products for *S. enteritidis* at an approved laboratory. A number of techniques discussed in this chapter are currently being used to isolate and identify *S. enteritidis* from a number of poultry sources. Although novel techniques for rapid isolation of *S. enteritidis* are currently available, when taking cost into account, cultural enrichment seems to be the technique most commonly used prior to bacteriological identification of *S. enteritidis*. The objective of this study was the identification of rapid and specific techniques, which could be used as alternative to bacteriological examination for the diagnosis of *S. enteritidis* infection of chickens.

Many approaches to identify *S. enteritidis* infection in chickens rely heavily on slight conformational and structural differences between proteins of *S. enteritidis* and other Gram-negative bacteria. By establishing which of these proteins produce an immunological response in chickens, novel antigenic markers for *S. enteritidis* infection in chickens can be identified. Proteins involved in attachment of *S. enteritidis* are known to be strongly immunogenic (Darwin & Miller, 1999). These structures are found on the cell surface and play a vital role in bacterial adherence as well as avoiding host defences, and include capsules, surface layers, OMPs, lipopolysaccharide, flagella and fimbriae (Finlay & Falkow, 1989; Charles *et al.*, 1996). Information gathered particularly on the OMPs and fimbriae, in particular SEF14 of *S. enteritidis*, indicates that the protein subunits of these structures could be useful antigenic determinants of *S. enteritidis* (Thorns *et al.*, 1996b).

A number of OMPs of *S. enteritidis* have been identified as novel antigens and have provided the basis for serological assays for *S. enteritidis* (Kim *et al.*, 1991), yet their cross-reactivity to other *Salmonella* serotypes was unclear (Thorns *et al.*, 1996). Part of this study intended to identify the presence of antigens from the OMPs of *S. enteritidis*, that may be specific for this micro-organism. This will be discussed in Chapter 3. OMPs were isolated from *S. enteritidis* and other *Salmonella* serotypes and their use in serological detection of *S. enteritidis* infection of chickens examined by probing with antibodies raised in chickens.

As *S. enteritidis* is the only *Salmonella* serotype isolated from poultry that expresses SEF14 fimbriae (Turcotte & Woodward, 1993), SEF14 is an obvious candidate antigen for detection of *S. enteritidis* in chickens. SEF14 responses were highly specific to birds infected with *S. enteritidis* (Thorns *et al.*, 1996a). This study also intended to measure these responses by isolating SEF14 from *S. enteritidis* and probing the SEF14 preparation with chicken anti-*S. enteritidis*, anti-*S. gallinarum* and anti-*S. berta* antibodies, and antibodies raised in chickens against surface-extracted OMPs of *S. enteritidis*, *S. gallinarum* and *S. berta*, by means of Western blotting. Affinity-separated antibodies, specific to SEF14, were also characterised by Western blotting. These results will be discussed in Chapter 3.

Although SEF14 is a novel antigen of *S. enteritidis*, a lack of reproducible SEF14 responses in experimentally infected layers has been observed (Thorns *et al.*, 1996). Consequently, new techniques were developed based on detection of the *sefA* gene, encoding SEF14. The primer pair designed by Woodward & Kirwan (1996) and modified by Szabo & Mackey (1999) was used in order to amplify a section of the *sefA* gene. Amplification of the target sequence would indicate the presence of a limited number of *Salmonella* serotypes. Positive amplification results from a poultry source would more than likely indicate the presence of *S. enteritidis*. The findings of these studies will be discussed in Chapter 4.

CHAPTER 2

General Materials and Methods

The methods described in this chapter were essentially used throughout the experimental part of this research. Most of these techniques are considered as fundamental in biochemistry and protein research. However, other techniques such as microbial cultivation and immunochemical procedures were also included in this chapter. More specialised procedures have been included in the appropriate chapters.

2.1 Materials

Most of the chemicals used in this study were from Sigma (St. Louis, USA), Roche (Mannheim, Germany), BDH (Poole, England) or Merck (Darmstadt, Germany), and were of the highest purity available (AR grade). Tris and N, N'-methylenebisacrylamide were from ICN Biochemicals (Aurora, USA). Acrylamide, N,N,N',N'-tetramethyl-ethylenediamine (TEMED) and silver nitrate were from BDH. Sodium thiosulfate was from Riedel de Haën (Hannover, Germany); sodium carbonate was from Orion Chemicals (SA); peptone was from Saarchem (SA) and bacto-agar, yeast extract and 2-mercaptoethanol were from Merck. Snakeskin pleated dialysis tubing was from Pierce (Rockford, Il, USA). Q-Sepharose, Sephadex-G25, standard molecular exclusion chromatography proteins, sodium N-lauroyl sarcosinate detergent, Freund's incomplete adjuvant and horse-radish peroxidase- and alkaline phosphatase-labelled rabbit anti-chicken IgG were from Sigma. Sephadex G-50 and molecular mass marker proteins were from Pharmacia (Uppsala, Sweden); BSA (fraction V), 4-nitro blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) were from Roche; Elite fat-free milk powder was from NCD (SA), and Nunc-Immuno F96 Maxisorp plates were from Nalge Nunc International (Denmark). Poly-Prep® chromatography columns were from Bio-Rad (Hercules, CA, USA) and non-immune chicken serum was provided by Prof. T.H.T. Coetzer (Department of Biochemistry, University of Natal, PMB, SA).

Oligonucleotide primers for PCR were synthesised by the University of Cape Town, Biochemistry Department synthetic laboratory.

When required, reagents were diluted with distilled water (dist.H_2O), or where necessary, with ultrapure Milli-Q^+ deionised water. Distilled water was obtained with a Milli-RO® 15 Water Purification System (Millipore, Marlboro, USA) and deionised water, with a minimum resistivity, was obtained with a Milli-Q plus Ultra-Pure Water System (Millipore, Marlboro, USA).

2.2 Growth and isolation of bacteria

For general growth of Gram-negative bacteria, media such as Luria Bertani (LB) medium have long been used. LB medium is a nutritionally complex medium with no selective agents, generally used as a broth or by the addition of agar, as a plating medium (Sambrook, 1989). Buffered peptone water (BPW) is one of the most widely used pre-enrichment broths for *Salmonella* and is often the medium of choice in many published reference methods. In addition to providing conditions for the growth of bacterial cells, BPW also buffers against pH changes brought about by the growth and metabolism of the micro-organisms (Baylis *et al.*, 2000). This investigation required the growth of a number of *Salmonella* serotypes as well as other micro-organisms. However, no pre-enrichment or selective enrichment of the bacterial samples was needed as they were obtained as working cultures of high purity. Therefore, either LB medium or BPW were used to grow the bacterial cultures. Contamination of samples was reduced by the use of working cultures grown from frozen cultures kept at -70°C.

2.2.1 Materials

Luria Bertani (LB) broth. Bacto-tryptone (10 g), bacto-yeast extract (5 g) and NaCl (10 g) were dissolved in 950 ml dist.H_2O. The pH was adjusted to 7.0 with NaOH and the volume was adjusted to 1 litre with dist.H_2O. The solution was sterilised by autoclaving.

Luria Bertani (LB) plating medium. Bacto-tryptone (10 g), bacto-yeast extract (5 g) and NaCl (10 g) were dissolved in 950 ml dist.H_2O. The pH was adjusted to 7.0 with NaOH and the volume was adjusted to 1 litre with dist.H_2O. Bacto-agar (15 g) was added to the

medium before sterilising by autoclaving. The solution was allowed to cool to 60°C before pouring into sterile petri dishes.

Peptone buffered water. Bacto-peptone (10 g) and NaCl (5 g) were dissolved in 950 ml dist.H_2O. The pH was adjusted to pH 7.2 with HCl and the volume was adjusted to 1 litre with dist.H_2O. The solution was sterilised by autoclaving.

2.2.2 Procedure

Cultures of *S. enteritidis* American Type Culture Collection (ATCC) strain 13076, *S. typhimurium* ATCC 14028, *S. gallinarum* ATCC 9182, *S. berta* ATCC 8392, *S. blegdam* K68, *S. dublin* ATCC 39184 were obtained from ARVL and *Escherichia coli* JM103 was obtained from the School of Molecular and Cellular Biology, University of Natal. *Staphylococcus aureus* ATCC 25923 and *Pseudomonas aeruginosa* ATCC 27853 were obtained from the laboratory of Vetdiagnostix Veterinary Pathology Services, Pietermaritzburg, South Africa. These strains were used throughout the investigation. Bacteria were stored frozen at -70°C in LB broth supplemented with glycerol [15% (w/v)] and working cultures were grown on LB agar plates and stored at 4°C.

When large quantities of bacteria were needed, cultures of each bacterial type were grown overnight in 5 ml LB broth at 37°C, with shaking on an orbital shaker. An aliquot (1 ml) of this culture was transferred to 1 litre of either LB broth or peptone buffered water and this culture was grown overnight at 37°C with shaking on an orbital shaker.

2.3 Bradford dye-binding protein assay

This assay is based on the binding of the dye, Coomassie Brilliant blue G-250 to proteins (Bradford, 1976). Under the pH conditions of the assay, the dye is present in its red cationic form and has an absorbance maximum at 465 nm. When the dye binds to a protein, there is a stabilisation of the doubly protonated blue anionic form of the dye, which absorbs maximally at 595 nm. It is believed that peptides of nine residues or more that contain basic or aromatic residues are required to observe the colour development

upon interaction with the dye (Copeland, 1994). Determination of the increase in absorbance at 595 nm as a function of protein added provides a sensitive protein assay.

Advantages of this assay are that, unlike the Biuret and Lowry assays, the Bradford assay is relatively free from interference from other cellular components, commonly used salts and other reagents found in protein solutions, as well as being about four times as sensitive as the Lowry assay (Switzer & Garrity, 1999). A disadvantage of the assay is significant variation in colour response of different proteins. This variation was reduced by substituting Coomassie Brilliant blue G-250 with Serva Blue G dye and increasing the amount of dye and decreasing the phosphoric acid concentrations (Read & Northcote, 1981). Sensitivity at higher protein concentration was however lost by doing this.

The assay can be performed using two methods. A macro-assay can be used for proteins in the 10-25 μg range. This investigation made use of the micro-assay, for proteins in the 1-5 μg range, due to the small amounts of protein obtained following purification procedures.

2.3.1 Materials

Dye Reagent. Serva Blue G dye (50 mg) was dissolved in a mixture of 88% phosphoric acid (50 ml) and 99.5% ethanol (23.5 ml). The solution was made up to 500 ml with dist.H_2O and stirred for 30 min on a magnetic stirrer. The resulting solution was filtered through Whatman No. 1 filter paper and stored in an amber-coloured bottle. This solution could be stored for up to 6 months although visual checks were made for precipitation before use. If precipitation was observed, the solution was filtered and recalibrated.

Standard protein solution. Ovalbumin (1 mg) was dissolved in dist.H_2O (1 ml). This solution was diluted in dist.H_2O to 100 μg/ml for the assay.

2.3.2 Procedure

Assays for the standard curve were carried out in quintuplicate at five concentrations of ovalbumin. Ovalbumin standard (0-50 µl of the 100 µg/ml solution, i.e. 1-5 µg) or sample was diluted to 50 µl with dist.H_2O, or buffer respectively, in 1.5 ml polyethylene microfuge tubes. Dye reagent (950 µl) was added and the mixture was mixed immediately by inversion of the tube. The colour was allowed to develop for 2 min after mixing, and the absorbance at 595 nm was measured in 1 ml plastic microcuvettes. Concentrated samples were diluted in buffer and dye reagent was added as above. Cuvettes were cleaned after use with 25% (v/v) sodium hypochlorite or 70% (v/v) ethanol. Linear regression analysis of the standard curve produced by the data allowed protein concentrations of unknown samples to be calculated.

2.4 Tris-tricine SDS-PAGE

Modern polyacrylamide gel electrophoresis (PAGE) is based on the discontinuous system used by Ornstein (1964) and Davis (1964). This system makes use of two different buffer and gel systems to resolve protein bands. The gel buffers are composed of Tris-HCl, the stacking gel buffer at pH 6.8 and the separating gel buffer at pH 8.8, with the tank buffer composed of Tris-glycine, pH 8.3. In the stacking gel at pH 6.8, proteins have an intermediate mobility between chloride ions, which have a high mobility, and the weakly dissociated glycinate ions, which have a low mobility. When a current is applied, proteins stack at a sharp interface between the leading chloride ions and trailing glycinate ions. Upon reaching the separating gel at pH 8.8, the glycinate ions are increasingly dissociated and mobile, overtaking the proteins and forming a front with the chloride ions and tracking dye. Using this system, proteins are separated according to molecular size, shape and charge.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was introduced by Laemmli (1970) and was the first system to separate proteins purely according to their size. Currently, SDS-PAGE is one of the most extensively used techniques for the analytical separation of proteins and peptides.

The denaturing detergent SDS binds most proteins in a constant weight ratio (approximately one molecule of SDS per two amino acid residues), so that they end up possessing similar charge densities. Therefore the rate at which a protein migrates through the polyacrylamide is no longer dependent on the intrinsic charge of the protein, but rather is determined solely on the basis of its molecular size (i.e., smaller proteins migrating more rapidly through a gel of defined pore size than larger proteins). As an inverse relationship exists between distance migrated by a protein in a gel and the logarithm of the molecular mass of the protein, a standard curve can be constructed relating distance migrated to logarithm of the molecular mass of a number of standard proteins.

To obtain a more accurate estimation of molecular mass, the protein preparation can treated with an excess of soluble thiol (usually 2-mercaptoethanol) and SDS before entering the electric field. Under these conditions, the thiol reduces all disulfide bonds (-S-S-) present within and/or between peptide units, while the anionic SDS binds to all regions of the proteins, disrupting most noncovalent intermolecular and intramolecular protein interactions.

Although the Laemmli system has advantages for separation of large proteins, an alternative to this system that improved resolution in the range of 5-100 kDa was developed (Schägger & von Jagow, 1987). This system, using tricine as the trailing ion, enhances electrophoretic separation of proteins by allowing stacking and separation to occur at the same pH and at low acrylamide concentrations.

This investigation made use of Tris-tricine SDS-PAGE to examine various bacterial protein preparations. The resultant protein bands were visualised via Coomassie blue staining as well as silver staining. SDS-PAGE was also used in conjunction with Western blotting to examine the immunogenicity of a number of these preparations.

2.4.1 Materials

Solution A [3 M Tris-HCl, 3.3% (w/v) SDS, pH 8.45]. Tris (72.7 g) was dissolved in approximately 180 ml of dist.H_2O [6 ml of a 10% (w/v) solution] added, adjusted to pH 8.45 with HCl, and made up to 250 ml with dist.H_20.

Solution B [49.5% (w/v) acrylamide, 3 % (w/v) Bis-acrylamide]. Acrylamide (48 g) and Bis-acrylamide (3 g) were dissolved and made up to 100 ml with dist. H_2O and stored in an amber coloured bottle at 4°C.

Solution C [500 mM Tris-HCl, pH 6.8]. Tris (3 g) was dissolved in 40 ml dist.H_2O, adjusted to pH 6.8 with HCl and made up to 50 ml.

Solution D [10% (w/v) ammonium persulfate]. Ammonium persulfate (0.1 g) was made up to 1 ml just before use.

Gel buffer [3.0 M Tris-HCl, 0.3% (w/v) SDS, pH 8.45]. Tris (72.7 g) and 10% (w/v) SDS (6 ml) were dissolved in 150 ml dist.H_2O. The pH was adjusted to 8.45 with HCl and the volume was made up to 200 ml with dist.H_2O. The buffer was filtered through Whatman No. 1 filter paper and stored at 4°C.

Anode buffer [200 mM Tris-HCl pH 8.9]. Tris (24.22 g) was dissolved in approximately 950 ml of dist. H_2O, adjusted to pH 8.9 with HCl and made up to 1 litre.

Cathode buffer [100 mM Tris-HCl, 100 mM Tricine, 0.1% (w/v) SDS, pH 8.25]. Tris (12.2 g) and Tricine (17.9 g) was dissolved in approximately 950 ml of dist. H_2O, SDS [10 ml of a 10% (w/v) solution] added, adjusted to pH 8.25 with HCl, and made up to 1 litre.

Non-reducing treatment buffer [3 M Tris-HCl, 4% (w/v) SDS, 20% (w/v) glycerol, pH 8.45]. Gel buffer (2.5 ml), 10% (w/v) SDS (4 ml) and glycerol (2 ml) were made up to 10 ml with dist.H_2O. Aliquots (1 ml) were stored frozen for up to 3 months.

Reducing treatment buffer [125 mM Tris-HCl, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, pH 8.45]. Prepared in the same manner as non-reducing treatment buffer, except for the inclusion of 10% (v/v) 2-mercaptoethanol (1 ml).

Molecular mass standards. Standard proteins used for molecular mass determination were: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic

anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa). Lyophilised markers were reconstituted in reducing treatment buffer (100 µl) and heated at 100°C for 5 min for Coomassie staining and immunoblot analysis, and stored at 4°C. For silver staining, the reconstituted markers were diluted 1:50 in reducing treatment buffer, and stored at 4°C.

Table 2.1 Reagents for two Tris-tricine electrophoresis gels, using a Bio-Rad Mini PROTEAN II® vertical slab cell.

	Solution A (ml)	Solution B (ml)	Solution D (µl)	TEMED (µl)	Ultrapure H_2O (ml)
Separating gel (10%)	6	3.6	50	5	8.4
Stacking gel (4%)	1.5	0.5	30	12	4

2.4.2 Procedure

For SDS-PAGE, the electrophoresis cell was assembled as described in the Bio-Rad Mini PROTEAN II® vertical slab electrophoresis unit manufacturer's manual. Prior to use, all gel casting equipment was washed with soap water and cleaned with alcohol. The outer (8.3 cm × 10.2 cm) and inner (7.3 cm × 10.2 cm) glass plates were clamped into the sandwich assembly, with two 1.5 mm polyethylene spacers separating them at the edges. The separating gel solution was run into the space between the plates, to a depth approximately 3 cm from the top of the outer glass plate, and overlaid with dist.H_2O. The water was removed with a syringe once the gel had set (as evidenced by the formation of the interface between the gel and water, usually about 45 min). The stacking gel solution was poured into up to the top of the inner glass plate and a 10- or 15-well comb was inserted to form the sample application wells. The comb was removed once the stacking gel had set (approximately 20 min) and the wells were rinsed with dist. H_2O.

Cathode buffer (containing SDS) was poured into the upper chamber and anode buffer into the lower chamber of the electrophoresis cell. Samples were combined with an equal volume of non-reducing or reducing treatment buffer and incubated in a boiling water bath for 2 min, to ensure even binding of SDS to the sample proteins. A marker dye, bromophenol blue (1-2 µl), which migrates with the buffer front, was added to each sample prior to loading onto the gel. The electrophoresis cell was connected to a power pack and

set at 80 V (maximum current) until the bromophenol blue dye had migrated to the bottom of the stacking gel. The voltage was increased to 100 V (maximum current) and electrophoresis was continued until the bromophenol blue dye reached the bottom of the separating gel. The gel was removed and prepared for either staining or imunoblotting. Relative molecular mass values of protein bands were determined by measuring the relative mobility (distance migrated by protein/distance migrated by bromophenol blue dye) of the protein molecular mass standards and sample proteins. The $\log_{10}$ of the molecular mass of the protein standards was plotted against their relative mobility and the standard curve was used to determine the sizes of proteins in the samples.

2.5 Staining of proteins in polyacrylamide gels

Staining of a gel is necessary to visualise the proteins separated therein. Two visualisation methods are most often used. The choice between them depends largely on the sensitivity of detection required. The first method involves the binding of Coomassie Brilliant Blue R-250 dye to proteins within the gel matrix. Although this method is capable of detecting a protein band containing as little as 0.1 to 0.5 μg of protein, a second method, silver staining, is sensitive enough to detect from 2 to 5 ng of protein per band on the gel. Silver staining is a technique that utilises the reduction of ionic silver to its insoluble metallic form, which precipitates on the proteins in the gel (Copeland, 1994). Of the two staining methods, Coomassie staining is the most often used method, yet the choice between them depends largely on the sensitivity required for the application.

In both cases, stained proteins can be difficult or impossible to elute from a gel. Copper staining is a rapid technique, which was found to be threefold more sensitive than Coomassie staining yet approximately 10-fold less sensitive than silver staining. The technique also fixes proteins within a polyacrylamide gel in a reversible manner allowing polypeptides to be excised and eluted from the gel at any time. When using a denaturing gel, the method yields negatively stained gels with protein bands appearing clear against an opaque whitish-blue background. When applied to non-denaturing gels, containing no SDS, the pattern was reversed, giving whitish-blue proteins bands against a clear background. The sensitivity of the stain in non-denaturing gels is also reduced and appears equal to that of Coomassie staining (Lee *et al.*, 1987).

2.5.1 Coomassie blue R-250 staining of proteins

This technique involves saturation of a gel with a solution of methanol, acetic acid, and water containing Coomassie Brilliant Blue R-250 dye. The methanol and acetic acid in this solution serve to fix the proteins within the gel matrix, while the Coomassie Brilliant blue binds to the proteins in the gel. The Coomassie dye interacts primarily with proteins through arginine residues, although weak interactions also occur with tryptophan, tyrosine, phenylalanine, histidine and lysine residues (Switzer & Garrity, 1999). Destaining involves washing the gel with the same aqueous methanol / acetic acid solution without the dye. This removes the dye from the areas of the gel that do not contain protein, leaving a transparent gel background on which proteins are visible as dark blue bands. Well-hydrated gels are stable and can be kept for long periods of time.

Coomassie staining is considerably less complicated than silver staining and because different proteins tend to stain to the same extent with this dye, relative amounts of different proteins can be quantitated using this method (Hames & Rickwood, 1990).

2.5.1.1 Materials

Stain stock solution [1% (w/v) Coomassie blue R-250]. Coomasie blue R-250 (1 g) was dissolved in 100 ml of dist.H_2O by stirring for 1 hr at room temperature. The solution was filtered through Whatman No. 1 filter paper.

Staining solution [0.125% (w/v) Coomassie blue R-250, 50% (v/v) methanol, 10% (v/v) acetic acid]. Stain stock solution (62.5 ml) was mixed with methanol (250 ml) and acetic acid (50 ml), and made up to 500 ml with dist.H_2O.

Destaining solution I [50% (v/v) methanol, 10% (v/v) acetic acid]. Methanol (500 ml) was mixed with acetic acid (100 ml) and made up to 1 litre with dist.H_2O.

Destaining solution II [7% (v/v) acetic acid, 5% (v/v) methanol]. Acetic acid (70 ml) was mixed with methanol (50 ml), and made up to 1 litre with dist.H_2O.

2.5.1.2 Procedure

The gel was removed from the electrophoresis apparatus using gloves, and placed into Coomassie blue R-250 staining solution for 4 h. The gel was rinsed in dist.H_2O and placed into destain solution I overnight, and into destain solution II to effect complete destaining. Gels were stored in polythene zip-seal bags and kept well hydrated.

2.5.2 Silver staining of proteins.

This technique is based on the reduction of silver salts to metallic silver. When this method of staining was initially used, problems were experienced with reproducibility and high background staining. To prevent a high background of non-specific silver deposition, Blum *et al.,* (1987) introduced a modification of the procedure, whereby sodium thiosulfate was used to treat the gel before staining. Sodium thiosulfate was found to chemically dissolve silver salts by complexation, thus enhancing the contrast between proteins on the transparent background. By doing this, the sensitivity of the procedure was also greatly increased. High background staining was also prevented during silver staining by maintaining scrupulously clean glass containers.

Silver staining begins by fixing the proteins in the gel matrix by means of an aqueous methanol/acetic acid solution. This is followed by pre-treatment with sodium thiosulfate and saturation of the gel with a solution of silver nitrate. Charged side chains of proteins interact with silver ions and under high pH and reducing conditions (facilitated by the addition of the reducing agent sodium carbonate), the silver ions are reduced to metallic silver. The metallic silver precipitates and causes the appearance of protein bands that are black in colour on a transparent background (Switzer & Garrity, 1999).

Besides being 10 to 100 times more sensitive, silver staining is also far more rapid than Coomassie staining. This is mainly due to the removal of the destaining step in the silver stain procedure. Coomassie destaining is usually an overnight step but can take up to a number of days. A disadvantage of silver staining is that unlike Coomassie reagents, the solutions required for silver staining need to be freshly prepared, to reduce unnecessary backround.

2.5.2.1 Materials

30% (v/v) Nitric acid. Nitric acid [545 ml of a 55% (v/v) solution] was diluted to 1 litre with ultrapure H_2O.

Fixing solution [50% (v/v) methanol, 12% (v/v) acetic acid, 0.5% (v/v) formaldehyde. Methanol (50 ml), acetic acid (12 ml) and formaldehyde [50 µl of a 37% (v/v) solution] were diluted to 100 ml with ultrapure H_2O.

50% (v/v) ethanol. Absolute ethanol (50 ml) was diluted to 100 ml with ultrapure H_2O.

Pre-treatment solution (0.02% (w/v) $Na_2S_2O_3.5\ H_2O$). $Na_2S_2O_3.5H_2O$ (20 mg) was dissolved in 100 ml ultrapure H_2O.

Impregnation solution [0.2% (w/v) $AgNO_3$, 0.75% (v/v) formaldehyde]. $AgNO_3$ (200 mg) was dissolved in 100 ml of ultrapure H_2O and formaldehyde [75 µl of a 37% (v/v) solution] was added.

Developing solution [60 g/l Na_2CO_3, 0.5% (v/v) formaldehyde, $Na_2S_2O_3.5\ H_2O$]. Na_2CO_3 (6 g) was dissolved in 90 ml of deionised H_2O and $Na_2S_2O_3.5H_2O$ (2 ml of the pre-treatment solution) and formaldehyde [50 µl of a 37% (v/v) solution] were added and the volume made up to 100 ml with ultrapure H_2O.

Stopping solution [50% (v/v) methanol, 12% (v/v) acetic acid]. Methanol (50 ml) and acetic acid (12 ml) were diluted to 100 ml with ultrapure H_2O.

Washing solution [50% (v/v) methanol]. Methanol (50 ml) was diluted to 100 ml with ultrapure H_2O.

2.5.2.2 Procedure

All steps were carried out at room temperature on an orbital shaker, in glass containers scrupulously cleaned with 30% (v/v) nitric acid. On completing electrophoresis, the gel was soaked in fixing solution for a minimum of 1 h, followed by washing in 50% (v/v) ethanol (3 × 20 min) to remove acetic acid, and incubation in pre-treatment solution

(1 min). The gel was rinsed in ultrapure H_2O (3 × 20 sec) and soaked in impregnation solution (25 min). After rinsing in ultrapure H_2O (3 × 20 sec), the gel was incubated in developing solution. Once the first protein bands were visible, the gel was washed in ultrapure water until the bands were sufficiently developed. Development was stopped by soaking the gel in stopping solution (20 min) and the gel was washed in washing solution and stored in polythene zip-seal bags in the dark.

2.5.3 Copper staining of proteins

Copper staining is rapid as it involves a single 5 min incubation step in copper choride ($CuCl_2$). The technique depends on two competing reactions, the precipitation of a Cu^{2+}-Tris-SDS complex and the complexation of copper by proteins. Tris and SDS complex with Cu^{2+} to form the background precipitate and the interaction of Cu^{2+} and/or SDS with protein appears to inhibit precipitation in the region of the protein bands. Once copper-stained, polypeptides separated by electrophoresis can be excised and restained with Coomassie blue or silver, without a destaining step. This is possible as the initial steps of both Coomassie and silver staining effectively solubilise the background in the copper-stained gel. Polypeptides can therefore be further characterised (Lee *et al.*, 1987).

2.5.3.1 Materials

Staining solution [0.3 M $CuCl_2$]. $CuCl_2.2H_2O$ (5.15 g) was made up to 100 ml in dist.H_2O.

2.5.3.2 Procedure

On completing electrophoresis, the gel was dipped for several seconds in dist.H_2O and then immersed in staining solution, in a plastic tray. The gel was rocked on an orbital shaker for 5 min at RT, and washed in dist.H_2O for 2 to 3 min, to remove excess staining solution. The gel was stored in dist.H_2O. Polypeptide bands of interest were excised with a scalpel and placed in the wells of a SDS-polyacrylamide gel for further electrophoresis.

2.6 Ion exchange chromatography

Ion exchange chromatography (IEC) is a form of chromatography based on the attraction between oppositely charged particles and is routinely used for the purification of biological

materials such as proteins and amino acids. These materials have ionisable groups and may carry a net positive or negative charge. The net charge is dependent on their pK_a and the pH of the solution (Wilson, 1986).

Ion exchange is usually carried out in a column packed with an ion-exchanger. The solute is applied to the column, interacting with the charged groups of the ion-exchanger, followed by elution with an aqueous buffer of higher ionic strength. There are two types of ion-exchangers, namely cation and anion exchangers. Cation-exchangers possess negatively charged groups and attract positively charge molecules, whereas anion exchangers possess positively charged groups and will attract negatively charged molecules (Wilson, 1986).

Q-Sepharose, is an anion exchanger found to have higher selectivity than the widely-used DEAE-Sepharose. The quaternary amino ethyl- (Q-) substituent was found to have higher selectivity than diethylaminoethyl- (DEAE-) and is effective in a wide variety of applications, including purification of proteins, membrane proteins, polysaccharides, nucleic acids and high-molecular mass compounds when linked to the agarose gel, Sepharose (Supelco, 1997).

Gradient elution from IEC columns is far more common than isocratic elution. A continuous gradient tends to give better resolution with less peak tailing. Generally with an anion exchanger, such as Q-Sepharose, the pH gradient of the elution buffer is decreased or the ionic strength, increased, to affect elution of the solute (Wilson, 1986). This study made use of a sodium chloride gradient, increasing from an ionic strength of 0 M to 0.8 M. Relative protein concentration of eluted fractions was determined by measuring the absorbance of the samples at 280 nm, as proteins absorb strongly at this wavelength (Sambrook *et al.*, 1989).

2.6.1 Materials

0.02 M Phosphate buffer, pH 7.5 [0.02 M NaH_2PO_4]. NaH_2PO_4 (2.76 g) was dissolved in approximately 800 ml dist.H_2O. The pH was adjusted to 7.5 and the volume was made up to 1 litre with dist.H_2O.

Elution buffer [0.8 M NaCl]. NaCl (4.65 g) was dissolved in approximately 80 ml 0.02 M phosphate buffer. The pH was adjusted to 7.5 and the volume was made up to 100 ml with 0.02 M phosphate buffer, pH 7.5.

2.6.2 Procedure

A Q-Sepharose ion-exchange column (25 × 115 mm) (Section 2.6) was equilibrated with two volumes of 0.02 M phosphate buffer, pH 7.5 at a flow rate of 50 ml/h. The sample was applied to the column at a flow rate of 1 ml/h and washed through at a flow rate of 5 ml/h, until an absorbance (280 nm) baseline was reached on plot of the eluant fractions. Fractions (1 ml) were eluted from the column using a salt gradient, increasing from 0 M NaCl (0.02 M phosphate buffer, pH 7.5) to 0.8 M NaCl (elution buffer) at a flow rate of 5 ml/h. The absorbance was monitored continuously at 280 nm and protein peaks were collected using a fraction collector.

2.7 Molecular exclusion chromatography

Molecular exclusion chromatography (MEC) or gel filtration chromatography utilises the principle of molecular sieving, whereby molecules are separated according to their molecular size and shape. MEC is usually carried out on a long narrow column containing gel media, which consists of spherical beads containing pores of a specific size distribution. The column is in equilibrium with a suitable solvent for the molecules to be separated. When a sample is applied to the column, separation occurs when molecules of different sizes are either included or excluded from the pores within the gel matrix. Large molecules that are completely excluded from the pores, will pass through the interstitial spaces and are rapidly eluted in the column's void volume. Small molecules diffuse into the gel pores and distribute between the solvent inside and outside the gel matrix. They pass through the column at a slower rate and their elution is therefore retarded. Consequently, molecules separate based on their size and are eluted in order of decreasing molecular mass (Wilson, 1986).

MEC gel media are generally selected according to their fractionation ranges and the type of investigation being conducted, namely preparative or analytical. Sephadex G-100 is a

dextran, cross-linked with epichlorohydrin, which has an effective fractionation range of 4 to 150 kDa, proving suitable for the range of proteins investigated in this study. Sephadex G-100 also exhibits very low non-specific interactions with solutes (Supelco, 1997).

2.7.1 Materials

0.02 M Tris buffer, pH 7.6. Tris (4.85 g) was dissolved in approximately 1.5 litres of dist.H_2O. The pH was adjusted to 7.6 and the volume was made up to 2 litres with dist.H_2O.

Standard molecular mass proteins. Blue Dextran (M_r > 2 000 000) (2 mg/ml), BSA (M_r 68 000) (5 mg/ml), ovalbumin (M_r 45 000) (5 mg/ml), myoglobin (M_r 18,800) and α-lactalbumin (M_r 14 400) were dissolved with heating, in 0.02 M Tris buffer, pH 7.6 (3 ml).

2.7.2 Procedure

A Sephadex G-100 molecular exchange column (25 × 705 mm), maintained at 4°C and at a flow rate of 25 ml/h, was equilibrated with two volumes of 0.02 M Tris buffer, pH 7.6. Standard molecular mass proteins were applied to the column and eluted with 0.02 M Tris buffer, pH 7.6. The absorbance was monitored continuously at 280 nm to determine the elution volume of each standard protein.

Once the standard proteins were eluted, the column was washed with two volumes of 0.02 M Tris buffer, pH 7.6 and a standard curve was constructed, relating the $\log_{10}$ of the molecular mass and Kav of each standard protein eluted from the column. Kav for each protein could be calculated by the following formula:

$$Kav = \frac{Ve - Vo}{Vt - Vo}$$

Where Ve = elution volume of protein; Vo = void volume of column and Vt = total volume of column.

Sample (volume at approximately 2% of the total bed volume of the column) was applied to the column and protein peaks were collected using a fraction collector, with the absorbance monitored continuously at 280 nm. The molecular mass of proteins eluted at each absorbance peak was determined by calculation of the Kav of each protein. The $\log_{10}$ of the molecular mass of the protein was calculated with the following formula, calculated from the standard curve constructed during calibration of the column:

$$\text{Log}_{10}\ \text{molecular mass} = \frac{\text{Kav} - 1.56086}{-0.25516}$$

2.8 Desalting of protein samples

It was often necessary to desalt a protein sample, i.e. to change the buffer in which a protein was dissolved, to reduce the ionic strength of the solution. This took place before application of a sample to an IEC column (Section 2.6) to prevent non-specific elution from the column, or before electrophoresis using SDS-PAGE (Section 2.4), to prevent smearing of bands.

The most common method of desalting a sample solution was to dialyse the sample against the new buffer, but if the volume was small enough, MEC with Sephadex G-25 was used instead. The advantage of using Sephadex G-25 (fractionation range 1 to 5 kDa) is that all proteins larger than 5 kDa are completely excluded by the small pore gel while the original sample buffer ions are included into, and thus retarded by, the gel. In this way, the protein is eluted immediately at the void volume of the column and the original sample buffer ions (including salts) elute later at the total volume of the column. Therefore, a rapid and complete change in sample can take place, as long as the protein does not interact non-specifically with the gel and the volume of sample applied to the column does not exceed one third of the column volume (Bio-Rad, 1995).

2.8.1 Desalting by dialysis

Dialysis tubing was cut to a length that accommodated the protein solution but that still left about a third of the tube unoccupied. This was done in order to accommodate an increase in sample volume that might have caused the dialysis tube to leak. For buffer changes, the

dialysis tubing was placed into at least ten volumes of buffer and stirred at 4°C, with 3 to 4 changes of buffer over a period of 12 to 24 h.

2.8.2 Desalting by molecular exclusion chromatography

A Sephadex G-25 column (25 × 260 mm) was equilibrated with the desired sample buffer and the sample was applied to the column and eluted with the same buffer at a flow rate of 50 ml/h. The absorbance was monitored continuously at 280 nm and the protein peak was collected using a fraction collector.

2.9 Protein concentration techniques

Protein samples were often either too dilute for visualisation on SDS-PAGE gels or of too great a volume for direct application to an MEC column. Two techniques for protein concentration were used in this study, one being dialysis of the protein sample against polyethylene glycol (PEG), M_r 20 000, and the second being precipitation of the sample with SDS and KCl.

Dialysis of the sample against sucrose or PEG 20 000 is a simple technique that is based on the principle of concentration gradients. A concentration gradient is set up between the concentrated solute (sucrose or PEG) outside the dialysis tube and the solvent (water) inside the tube, causing water to move out of the dialysis tube, into the sucrose or PEG. Sucrose can be used because of its low cost and ready availability, but the disadvantage of sucrose is that it is small enough to move through the pores of the dialysis tube, contaminating the sample and making it more viscous. Generally, this would not be a problem, but when the sample needs to be sucrose-free, the more expensive solute, PEG, is used. This is a high molecular mass (M_r 20 000) solute that cannot pass through the pores of the dialysis tube. Salt ions are also able to move freely through the pores, preventing the need for removal of salt after dialysis of proteins in low salt buffers. Dialysis against sucrose and PEG was found to be effective for the concentration of large volumes of sample. However, PEG is more efficient than sucrose, as samples were found to become more concentrated against PEG than against sucrose. Therefore, dialysis against PEG

20 000, at 4°C, was often used for the concentration of dilute protein samples from affinity chromatography.

When maintaining protein conformation was not necessary, i.e. for reducing SDS-PAGE, or where larger sample numbers and smaller sample volumes required concentration, precipitation with SDS-KCl was used. SDS-KCl precipitation is quick and efficient as proteins are precipitated out of solution by KCl after complexing to SDS. This method is more effective on smaller samples, as the concentration factor is approximately five-fold.

2.9.1 Dialysis against sucrose or PEG

A sample was placed in a Pierce's Snakeskin® pleated dialysis tubing (molecular weight cut-off M_r 10 000), the ends sealed either with knots or dialysis clips. The bag was placed into granular commercial white sugar or PEG (M_r 20 000) in a plastic tray and the sample allowed to concentrate at 4°C. After the sample had concentrated (5 to 10-fold after 2 to 4 h), the bag was rinsed briefly in dist.H_2O to remove excess external sucrose or PEG and the sample was squeezed out. Gloves were worn throughout the process to prevent contamination with keratin that shows as a contaminant that is approximately 68 kDa in size on SDS-PAGE gels (Osborn & Wu, 1980).

2.9.2 SDS/KCl precipitation

2.9.2.1 Materials

5% (w/v) SDS. SDS (0.5 g) was dissolved in 10ml dist.H_2O.

3 M KCl. KCl (2.24 g) was dissolved in 10 ml dist.H_2O.

2.9.2.2 Procedure

Protein sample (100 µl) and 5% (w/v) SDS (10 µl) were mixed by inversion in a 1.5 ml polyethylene microfuge tube. 3 M KCl (10 µl) was added and the sample was again mixed. The SDS-protein precipitate was sedimented by centrifugation (2 min, 12 000 × g, RT) and the supernatant discarded. The pellet was redissolved in sample buffer (20 µl).

2.10 Immunochemical techniques

2.10.1 Production of antibodies in chickens

Exposing the immune system to a novel antigen triggers a rapid immune response in poultry. The primary response occurs when an animal is first injected with an antigen to which it has never been exposed before. Low titres of antibody are formed, mostly of the IgM class. A booster injection of the same antigen induces the secondary response. This response is usually stronger, with high titres of IgG produced, usually with higher affinity for the antigen. A series of booster injections are generally used to induce the secondary response.

Before an animal can be immunised with an antigen, an adjuvant needs to be emulsified with the antigen. An adjuvant is a substance that increases the specific immune response of an animal to an antigen. Freund's complete adjuvant (FCA) is the most commonly used adjuvant, consisting of mineral oil containing killed *Mycobacterium tuberculosis*, which induces both antibody and cell-mediated immune reactions. Freund's incomplete adjuvant (FIA) lacks *M. tuberculosis* and induces only an antibody response. Freund's adjuvants also contain Arlacel A, as an emulsifier. Once emulsifed with the adjuvant, the antigen is resistant to dispersion and, as a stable depot, effects a prolonged antigen stimulation (Allison & Byars, 1994; Schwarzkopf *et al.*, 2001).

Although injection is often the route used for the immunisation of poultry with peptides, inoculation of live bacteria is usually via a route that simulates natural infection, such as oral or intranasal (Barrett, 1994).

In order to evaluate when antibody titres are high enough for a high yield upon isolation, a means of monitoring the immune response is required. The ELISA is the technique most commonly used for this purpose and was used in this investigation to monitor the titre of anti-*Salmonella* OMP IgG (Section 2.8.1). Once the peak of antibody production has been reached, the IgG can be isolated. Avian IgG is found in the serum, and can be isolated from blood. However, as avian IgY is transferred through the egg follicle to the yolk, the amount of IgY available from eggs is enormous. A single egg can yield 100-250 mg of

IgY. This can be isolated from the yolk with ease, and thereby avoiding bleeding, which from an animal welfare point of view is preferred (Erhard & Schade, 2001).

Chicken serum IgG and egg yolk IgY can be isolated using the water-soluble polymer PEG M_r 6 000. PEG precipitiation occurs by steric exclusion, whereby proteins are concentrated in the extrapolymer space, eventually exceeding their solubility limit, and precipitating from solution. Proteins can be precipitated differentially by different concentrations of PEG 6 000, according to the nett charge on the molecules. This charge is determined by the pH of the surrounding solution (Ingham, 1990). However, this study did not require the purification of IgG from the immune sera of inoculated birds. The nitrocellulse-based immunoassay, Western blotting (Section 2.10.3), used throughout this study, is sensitive enough to detect antigens with the direct use of serum obtained from inoculated chickens (Rajashekara *et al.*, 1998).

2.10.1.1 Production of anti-*Salmonella* antibodies in chickens

Single-comb White Leghorn hens (*Gallus gallus*) obtained from the ARVL specific-pathogen free (SPF) flock received a 1 ml oral dose of overnight cultures of *S. enteritidis* (ATCC 13076), *S. gallinarum* (ATCC 9182) and *S. typhimurium* (ATCC 14028) in dist.H_2O respectively. Experimental oral infection of SPF chickens with *S. enteritidis,* produces serum IgG titres against whole *S. enteritidis* bacteria that are high within 2 weeks and peak at 5 weeks (Section 1.5.2). However, due to time constraints in this investigation, IgG was isolated from orally-infected hens after 3 weeks. Blood was drawn from chickens into vacuum tubes by cardiac puncture, and serum was collected by centrifugation (1 000 × g, 10 min, 4°C).

2.10.1.2 Production of anti-*Salmonella* outer membrane protein (OMP) antibodies in chickens

Antibodies against *S. enteritidis* OMPs, *S. gallinarum* OMPs, and *S. berta* OMPs were raised in three groups of SPF single-comb White Leghorn chickens (*Gallus gallus*) respectively, by intramuscular injections at a site in both breast muscles of each chicken. Primary immunisation was with 250 µg of OMP fraction in PBS (750 µl), isolated from *S. enteritidis* (ATCC 13076), *S. gallinarum* (ATCC 9182) or *S. berta* (ATCC 8392) respectively (Section 3.3.1). These antigens were emulsified in a 1:1 ratio with Freund's

incomplete adjuvant. Booster immunisations were administered using a 1:1 ratio with Freund's incomplete adjuvant bi-weekly for a total of 6 weeks. Each inoculated chicken's immune response to the *Salmonella* OMP's was monitored weekly by ELISA analysis (Section 1.5.2) of antibodies in the serum, using OMP's of the respective *Salmonella* serotypes as antigens. Blood was drawn from the wing vein and a serum supernatant was obtained by centrifugation (1 000 × g, 10 min, 4°C) of clotted blood. IgY could not be isolated from egg yolk because egg production in all chickens ceased directly after primary immunisation. Therefore, when serum IgG titres were seen to peak, at approximately 4 weeks after the primary immunisation, blood was drawn from chickens into vacuum tubes by cardiac puncture, and serum was collected by centrifugation (1 000 × g, 10 min, 4°C).

2.10.2 Enzyme-linked immunosorbent assay (ELISA)

The ELISA, like the radioimmunoassay, is based on the specific interaction between antigen and antibody, and resulted from an aggressive search for alternatives to labelling of antigens or antibodies with radioisotopes for use in radioimmunoassays (Engvall & Perlmann, 1971). There are two basic systems available; the two-site ELISA, commonly known as competitive ELISA (van Zijderveld *et al.*, 1992) and the indirect ELISA (Barrow, 1992).

The competitive ELISA makes use of a specific mechanism of coating antigen to wells followed by blocking of all remaining sites. Blocking of residual sites in the wells is achieved with protein solutions that do not participate in the antigen-antibody reaction. This is vital as antibodies used in detection steps are prone to non-specific binding. One percent bovine serum albumin (BSA), one percent non-fat milk, or another non-reactive protein, are common choices for blocking reagents. The incubation time of the blocking step (2 h to 30 min) depends on the temperature used (4°C, 21°C or 37°C). Antigen-specific primary antibodies and free antigen are incubated in a separate well, which is then transferred to the ELISA well, where free primary antibodies bind to immobilised antigen. The primary antibody-antigen complex is then detected using secondary antibodies labelled with an enzyme. The enzyme catalyses a colour reaction, which can be recorded spectrophotometrically (Barrow, 2000).

The indirect, non-competitive, solid phase ELISA is a technique commonly used to measure the titre and specificity of an antibody preparation. The indirect ELISA involves the coating of a detection antigen onto the wells of a 96 well polyvinyl microtitre plate. The antigen is adsorbed to the sides of the wells by non-specific hydrophobic interactions. Residual sites in the wells are blocked with non-reactive proteins and the antigen-specific primary antibody is then added to the wells, allowing the antigen to interact directly with the primary antibody. The amount of interaction between the primary antibody and antigen is quantitated by the secondary antibody-enzyme conjugate as the secondary antibody binds to the primary antibody-antigen complex and the conjugated enzyme catalyses a colour reaction, which can be read and recorded by an ELISA microplate reader.

A washing step is included between each step in the ELISA procedure. Tween-20 (0.05%-0.2%) or a similar non-ionic detergent is usually used in a washing buffer, to block hydrophobic interactions and act as a wetting agent. Washing buffers sometimes contain 0.1-0.5 M NaCl to limit ionic interactions of proteins with the plate (Thorpe & Kerr, 1994).

The current study required the production of chicken anti-*Salmonella* OMP antibodies and an indirect ELISA was used to monitor titre of the antibodies produced. The system used in this study utilised rabbit anti-chicken IgY antibodies conjugated to the enzyme horseradish peroxidase (HRPO). The enzyme HRPO has a high specificity for hydrogen peroxide (H_2O_2), which together catalyse the oxidation of 2,2'-azino-di-(3-ethyl)-benzthiozoline sulfonic acid (ABTS), a hydrogen donor, yielding a soluble blue-green coloured complex which can be measured spectrophotometrically at 405 nm (Clark & Engvall, 1980). Plots of A_{405} versus log antibody concentration were constructed. The titre was taken as the mean highest dilution at which the primary antibody response was greater than that of the pre-immune antibody control sample.

2.10.2.1 Materials

Phosphate buffered saline (PBS), pH 7.2. NaCl (8 g), KCl (0.2 g), $Na_2HPO4.2H_2O$ (1.15 g) and KH_2PO_4 (0.2 g) were dissolved in dist.H_2O (1 litre).

Fixing solution [70% (v/v) methanol]. Methanol (70 ml) was diluted to 100 ml with dist.H_2O.

0.5% (w/v) Bovine serum albumin-PBS (BSA-PBS). BSA (0.5 g) was dissolved in PBS (100 ml).

0.1% (v/v) PBS-Tween. Tween-20 (1 ml) was diluted to 1 litre with PBS.

0.15 M Citrate-phosphate buffer, pH 5.0. A citric acid.H_2O (21.1 g/l) solution was titrated with a solution of $Na_2HPO_4.2H_2O$ (35.6 g/l) to pH 5.0.

Substrate solution [0.05% (w/v) ABTS and 0.0015% (v/v) H_2O_2 in citrate-phosphate buffer. ABTS (7.5 mg) and H_2O_2 (7.5 µl) were dissolved in citrate-phosphate buffer, (15 ml) for one ELISA plate.

Stopping buffer [citrate-phosphate-0.1% (w/v) NaN_3]. NaN_3 (0.1 g) was dissolved in citrate-phosphate buffer (100 ml).

2.10.2.2 Procedure

The wells of microtitre plates (Nunc Immuno Maxisorp F96 plates) were coated with antigen at a concentration of 1 µg/ml in PBS (150 µl, overnight). The antigen was fixed to the wells with 70% (v/v) methanol for 10 min at room temperature. Non-specific binding of antibody was prevented by blocking the wells with 0.5% BSA-PBS (200 µl, 1 h at 37°C), and the wells were washed three times with 0.1% (v/v) PBS-Tween. Serial doubling dilutions, starting from 1/10 serum dilution, in 0.5% BSA-PBS, were prepared on the plate and incubated (100 µl, 2h at 37°C), and the wells were washed three times with 0.1% (v/v) PBS-Tween. The HRPO-linked secondary antibody (diluted to 1:300 in 0.5% BSA-PBS) was added to each well and incubated (120 µl, 1 h at 37°C), followed by washing three times with 0.1% (v/v) PBS-Tween. Finally, the ABTS-H_2O_2 substrate was added (150 µl) and the colour was allowed to develop in the dark. The reaction was stopped after 25 min by the addition of NaN_3-citrate phosphate buffer (50 µl) to each well, and the absorbance at 405 nm was measured in a Bio-Tek EL 312 ELISA microplate reader. The spectrophotometric data was used to construct titration curves.

2.10.3 Western blotting

Specific components in complex mixtures of proteins may be identified using antibodies following separation of the components by electrophoresis. Combination of these two techniques with the sensitivity of an enzyme assay produces the three-step procedure known as immunoblotting or Western blotting. In addition to its use in qualitative analysis of antigens, Western blotting can be used to quantify the amount of protein present in a pure or impure solution and qualitatively analyse the antibodies raised against specific proteins. Together with other nitrocellulose-based assays, Western blotting has been shown to possess higher specificity for detection of antibodies to soluble proteins, than microtitre plate-based ELISA (Borden & Kabat, 1986)

In this technique, as described by Towbin *et al.*, (1979), proteins separated by SDS-PAGE, are transferred to a sheet of nitrocellulose membrane, which binds most proteins strongly. Subsequently, the unoccupied binding sites on the nitrocellulose sheet are saturated (blocked) with a non-antigenic protein (non-fat milk). This prevents non-specific binding of antibodies to the nitrocellulose. A primary antibody, specific for the protein of interest (antigen) is applied to the membrane to allow the antigen-antibody interaction to occur. This immunochemical reaction is detected by incubating the membrane with an enzyme-conjugated detection antibody, which has a strong affinity for the primary antibody as well as catalysing a coloured reaction. A rabbit anti-chicken IgG–alkaline phosphatase conjugate was used throughout this study. The substrate for the reaction catalysed by alkaline phosphatase (AP) is a combination of 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and nitroblue tetrazolium (NBT). AP hydrolyses BCIP to form an intermediate, that undergoes dimerisation, producing an indigo dye. The NBT is reduced to NBT-formazan by the two reducing equivalents produced by the dimerisation. When together, these substrates form an intense blue-purple precipitate that is insoluble in water and resists fading on exposure to light (Moe & Kirkeby, 1982; Thorpe & Kerr, 1994), unlike the soluble blue-green precipitate formed when using the HRPO system (Section 2.8.2).

Potential outer membrane protein antigens and fimbrial protein antigens of *S. enteritidis* and other closely-related bacteria were identified by Western blotting in this investigation. These may possibly be used for identification of *S. enteritidis*.

2.10.3.1 Materials

Blotting buffer [25 mM Tris, 192 mM glycine, pH 8.3]. Tris (9.08 g) and glycine (43.2 g) were dissolved in 1.4 litres of dist. H_2O and methanol (600 ml) was added. Prior to use, 10% (w/v) SDS (2 ml) was added.

0.1% (w/v) Ponceau S. Ponceau S (0.1 g) was dissolved in 1% (v/v) glacial acetic acid (100 ml).

Tris buffered saline (TBS) [20 mM Tris, 200 mM NaCl, pH 7.4]. Tris (4.84 g) and NaCl (23.38 g) were dissolved in 1900 ml of deionised H_2O, adjusted to pH 7.4 with HCl, and made up to 2 litres with deionised H_2O.

Blocking solution [5% (w/v) low fat milk powder in TBS]. Low fat milk powder (5 g) was dissolved in TBS (100 ml).

Substrate buffer [50 mM Tris-HCl, 5 mM $MgCl_2$, pH 9.5]. Tris (0.61 g) and $MgCl_2$ (0.05 g) were dissolved in 90 ml of dist. H_2O, adjusted to pH 9.5, and made up to 100 ml with dist. H_2O.

Alkaline phosphatase substrate solution [3% (w/v) 5-bromo-4-chloro-3-indolyl-phosphate (BCIP), 1.5% (w/v) nitroblue tetrazolium (NBT) in substrate buffer. NBT (30 mg) was dissolved in DMF (1 ml) and BCIP (15 mg) was dissolved in 50% (v/v) DMF (1 ml). These solutions were then mixed and made up to 100 ml with substrate buffer.

2.10.3.2 Procedure

Nitrocellulose was cut to a suitable size and totally immersed in blotting buffer. The immersed nitrocellulose membrane was sandwiched, with the gel lying squarely on top of it, between 3 pieces of Whatman No. 4 filter paper and two pieces of Scotchbrite foam, also totally immersed in blotting buffer, with care being taken to avoid entrapment of air. The sandwich was placed into the Western blotting apparatus, which was placed in the buffer tank. The apparatus was connected to a power supply so that the nitrocellulose membrane was on the anodal side of the chamber, and the blotting was effected overnight at 30 V (maximum current). A magnetic stirrer bar was used to ensure even distribution of

cooling in the buffer tank and the entire apparatus was immersed in a tank of cold water, kept at 10°C by a refrigerated circulator. Once blotting was complete, the sandwich was removed and the filter paper was peeled off the gel. The gel was carefully removed, and stained to assess the efficiency of the blotting step.

The nitrocellulose membrane was air dried for about 1.5 h followed by staining in Ponceau S (1 min). The membrane was rinsed in dist. H_2O and the positions of the molecular weight markers were marked with a pencil. The membrane was destained by addition of a drop of 500 mM NaOH to the dist. H_2O. Unoccupied sites on the membrane were blocked for 1 h with blocking solution, washed in TBS (3 × 5 min), and incubated for 2 h in primary antibody diluted in 0.5% BSA-TBS. Following washing in TBS (3 × 5 min), the membrane was incubated for 1 h in alkaline phosphatase-linked secondary antibody, diluted in 0.5% BSA-TBS, and washed again in TBS (3 × 5 min). The membrane was immersed in substrate solution and reacted in the dark until purple-coloured bands were clearly evident against the lightly stained background. The membrane was removed from the substrate solution, washed in dist. H_2O, and dried between filter paper discs to ensure preservation of the bands.

2.10.4 Immunoaffinity chromatography

Protein purification by immunoaffinity chromatography is based on the binding interactions of antigens and antibodies, and is a highly specific and reversible process. An insoluble matrix is packed into a chromatographic column, in the form of a packed bed, for the purification process. By covalently immobilising an antibody or antigen ligand onto the matrix, a target antigen (by attaching to immobilised antibody) or antibody (by attaching to immobilised antigen) can be separated from a sample mixture in a single pass through the column. Contaminants are washed off and the bound antigen or antibody can be eluted from the column by lowering the pH of the buffer. Lowering the pH disrupts the antibody-antigen complex, but not the covalent ligand-matrix linkage. This investigation made use of Pierce's AminoLink™ support system (Pierce, Rockford, IL, USA). This support matrix is composed of 4% cross-linked beaded agarose containing aldehyde functional groups. Reductive amination is used to immobilise amine-containing proteins and peptides to the support matrix (Hermanson, 1996). Primary amines react with the

aldehyde groups to form labile Schiff bases. Reduction of the Schiff base intermediate with sodium cyanoborohydride ($NaCNBH_3$) results in the formation of a stable secondary amine bond. $NaCNBH_3$ is the preferred reducing agent for two reasons. It is not only specific for the Schiff base structure but is a gentler reagent, especially for immobilising antibodies. Unreacted aldehydes on the support are quenched with Tris, followed by a second addition of $NaCNBH_3$, to form secondary amines that are stable over a wide pH range (Hermanson, 1996). The Aminolink® support also allows coupling of proteins of diverse molecular mass and isoelectric points (Pierce, 1988).

2.10.4.1 Materials

0.1 M Sodium phosphate buffer, 0.05% sodium azide, pH 7.0. $NaH_2PO_4.2H_2O$ (7.8 g) and sodium azide (0.25 g) were dissolved in 450 ml of dist.H_2O. The pH was adjusted to 7.0 with NaOH and the volume was made up to 500 ml with dist.H_2O.

1 M Tris-HCl, pH 7.4. Tris (60.75 g) was dissolved in 450 ml of dist.H_2O, and the pH was adjusted to 7.4 with HCl. The volume was made up to 500 ml with dist.H_2O.

1.0 M NaCl. NaCl (29.22 g) was dissolved in 500 ml dist.H_2O.

0.05% NaN_3. NaN_3 (0.25 g) was dissolved in 500 ml dist.H_2O.

0.1 M $NaCNBH_3$. $NaCNBH_3$ (0.06284 g) was dissolved in 1 ml dist.H_2O 1h before use.

0.1 M Glycine, pH 2.8. Glycine (3.7535 g) was dissolved in 450 ml dist.H_2O. The pH was adjusted to 2.8 with HCl and the volume was made up to 500 ml with dist.H_2O.

2.10.4.2 Procedure for immobilisation of a protein to a gel matrix

The protein to be immobilised to the gel matrix was SEF 14 fimbrial protein purified from *S. enteritidis* (Section 3.5). The SEF14 preparation was diluted in 0.1 M sodium phosphate buffer, pH 7.0, to a concentration of approximately 1 mg/ml. The solution was dialysed extensively against 0.1 M sodium phosphate buffer, pH 7.0, to remove the 10 mM Tris-HCl buffer used during the SEF14 purification procedure. This prevents blocking of the support coupling sites by the amine groups of the Tris-HCl buffer.

AminoLink® gel was supplied as a 50% slurry in 0.05% sodium azide. A volume of gel slurry, double the volume of gel needed, was thus transferred into a Büchner funnel and the liquid drained, leaving only a wet cake remaining. The wet cake was washed with an equal volume of 0.1 M sodium phosphate buffer, pH 7.0. This was repeated 3 times and the drained gel cake was transferred to a 0.8 × 4 cm Poly-Prep® chromatography column.

A volume of SEF14 protein solution, equivalent to the volume of the gel cake, was added to the gel. 1.0 M $NaCNBH_3$ (50µl per ml of slurry) was added in a fume hood. The column was capped and mixed by end-over-end rotation in a Heidolph REAX 2 mixer for 2 h at RT, to facilitate immobilisation of protein to the gel. The column was removed from the mixer and the immobilisation reaction was allowed to proceed for an additional 4 h at RT.

The gel cake was washed with one volume of 1.0 M Tris-HCl, pH 7.4. This washing step was repeated once. A volume of 1.0 M Tris-HCl, pH 7.4, equivalent to the volume of the gel cake, was added, and 1.0 M $NaCNBH_3$ (50 µl per ml of slurry) was added in a fume hood to block unreacted sites on the gel. The tube was mixed for 30 min at RT. The gel was drained and washed with 10 bed volumes of 1.0 M sodium chloride, followed by 10 bed volumes of 0.05% sodium azide. The gel was refrigerated as a 50% (v/v) slurry in 0.1 M sodium phosphate buffer, pH 7.0, containing 0.05 % sodium azide.

2.10.4.3 Procedure for immunoaffinity purification

Before use, the column was brought to RT and equilibrated with 10 bed volumes of 0.1 M sodium phosphate buffer containing 0.05 % sodium azide, pH 7.0, at a flow rate of 11.5 ml/h. Chicken serum, containing chicken anti-*S. enteritidis* IgG (purification procedure described in Section 2.10.1.2) was applied to the column and cycled through the column overnight. The column was washed with 10 bed volumes of 0.1 M sodium phosphate buffer containing 0.05 % sodium azide, pH 7.0 and purified chicken anti-*S. enteritidis* SEF14 antibodies were eluted from the column using 0.1 M glycine, pH 2.8. Fractions (1 ml) were collected in microfuge tubes containing 40 µl of 1 M Tris-HCl, pH 9.5, to neutralize the pH of eluted samples. Measuring the absorbance of the fractions at 280 nm monitored the elution of protein. Protein concentrations were determined using the method described by Bradford (Section 2.3).

CHAPTER 3

Analysis of Salmonella protein profiles for identification of *Salmonella enteritidis*

3.1 Introduction

For a bacterial pathogen to successfully infect a susceptible host, the bacterium needs to present a cell surface that contains components that allow it to associate with and adhere to specific host tissues (Isberg, 1991; Thiagarajan *et al.*, 1996a). Bacterial cell adherence to animate and inanimate surfaces is probably one of the most important factors contributing to the transmission of *S. enteritidis*, and factors such as mucosal surface hydrophobicity, charge and cell density are thought to be critical in accomplishing this (Woodward *et al.*, 2000). However, it is the components and appendages on the bacterial cell surface that play the most vital role in bacterial adherence as well as avoiding host defences. These virulence-associated surface structures include capsules, surface layers, outer membrane proteins, lipopolysaccharide, flagella and fimbriae (Finlay & Falkow, 1989).

The cell envelope of Gram-negative bacteria is made up of an inner and outer membrane separated by a layer of peptidoglycan (Fig. 3.1). The inner, cytoplasmic membrane is the site of biochemical reactions involved in respiration and oxidative phosphorylation, and the synthesis of structural membrane components. The outer membrane is a highly specialised structure that lies outside of the peptidoglycan layer, thus forming a physical barrier between the cell and its external environment. The peptidoglycan layer between the two membranes consists of a network of amino sugars and amino acids. The amino sugars (*N*-acetylglucosaminyl-*N*-acetylmuramyl dimers) form long linear strands that are covalently bound together between two muramyl residues by short tetrapeptides. The components of the outer membrane are either covalently linked to the peptidoglycan or involved with it through ionic bonds in such a way that a tight network is produced. The outer membrane acts as a barrier to antibiotics, detergents and other toxic chemicals, and has selective permeability, which allows the entry of nutrient solutes from the medium in which the cell is located (Osborn & Wu, 1980; Benz, 1988).

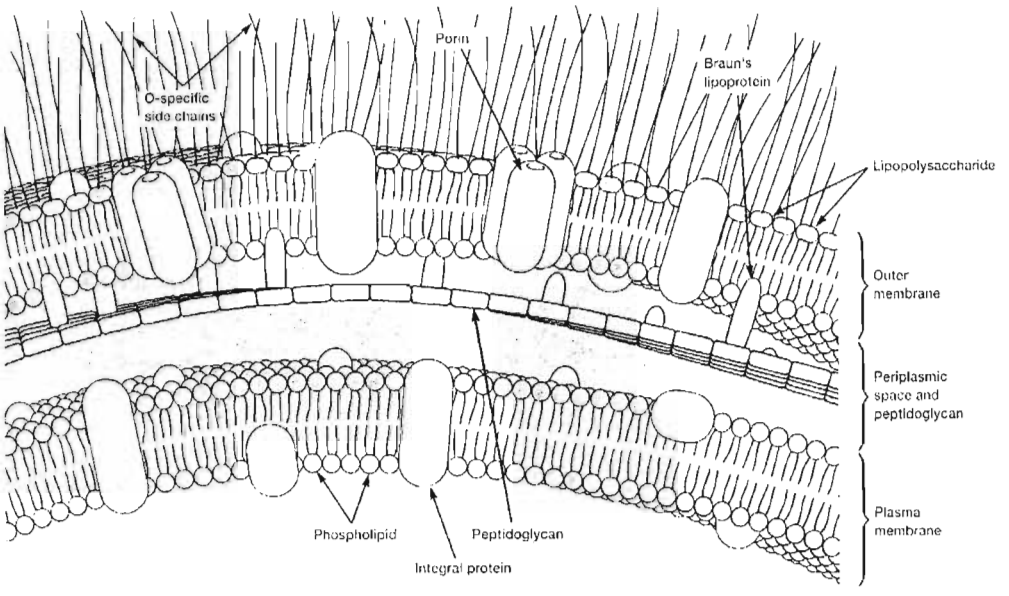

Figure 3.1 Diagram of the envelope of a generalised Gram-negative bacterium (Thorns & Woodward, 2000)

A common feature of the envelope of Gram-negative bacteria, including *Salmonellae*, is that they contain lipopolysaccharides (LPSs). These LPSs are commonly known as endotoxins because they are an endogenous component of the envelope and because of the wide variety of adverse reactions they cause in host animals. The structure of LPS is divided into three distinct elements, the core oligosaccharide, Lipid A, and the O-antigen side chain. Both the core oligosaccharide and Lipid A are highly conserved among Gram-negative bacteria, including *Salmonellae.* It is the O-antigen side chains that provide variability in LPS and are major antigenic domains of *Salmonellae.* There are over 2 000 different O-antigens side chain types in *Salmonellae* and it is these O-antigen side chains that could have potential for *S. enteritidis* specificity (Hauschildt *et al.*, 2000).

The majority of *Enterobacteriaceae* have flagella, which are considered to assist bacteria in motility, while their role in pathogenesis is equivocal. It has been suggested that flagella of *S. enteritidis* assist colonisation of epithelial cells by enabling motility rather than providing an adhesin. Motility would enable *S. enteritidis* to penetrate the mucus layer and attach specifically to epithelial cells (Robertson *et al.*, 2000). Flagella extend outward from the cytoplasmic membrane, and are slender, rigid structures, about 20 nm across and up to 15 or 20 μm long (Dibb-Fuller *et al.*, 1999). As discussed in Section 1.5.2, flagellar antigens were used for differentiation of flagellate *S. enteritidis* from *S. gallinarum*, *S. pullorum* and other aflagellate serotypes.

Fimbriae are short, fine surface appendages found on many Gram-negative bacteria. Fimbriae are thinner than flagella and not involved in motility. A cell may be covered by as many as 1 000 fimbriae, yet they are only visible under electron microscopy, due to their size. They appear as slender tubes about 3 to 10 nm in diameter and up to several μm in length. Like flagella, fimbriae extend outward from the cytoplasmic membrane. Sex pili are similar to fimbriae, yet are larger than fimbriae (around 9 to 10 nm in diameter). About 1 to 10 are found per cell and they are genetically determined by sex factors or conjugative plasmids. They are required for bacterial mating (Thorns & Woodward, 2000).

The transmission of *S. enteritidis* relies heavily on the components of the outer surface of the bacterium. In the last ten years, there has been an interest in the molecular and antigenic characterisation and functions of these surface components, with the focus on potential use in diagnostic tests and as active components in vaccines.

Proteins can be typed by numerous characteristics, including molecular mass, charge, shape and antigenicity. Using these characteristics, a genus- or serotype-specific fingerprint can be obtained using whole cell protein profiles or fractionated cell protein profiles, e.g. OMP or fimbrial fraction. In this way, inter-genus and intra-genus comparisons can be made. Identification of serotype-specific markers on fingerprints, has been the basis for numerous diagnostic tests, including those for *S. enteritidis* (Cooper & Thorns, 1996; Rajashekara *et al.*, 1998; Rajashekara *et al.*, 1999).

This study involved the purification and characterisation of components of *S. enteritidis,* particularly the structures of the outer surface of the bacterial cell. These purified cellular components, including outer membrane proteins, lipopolysaccharides, fimbrial proteins and proteins of the whole cell, were characterised by SDS-PAGE analysis (Section 2.4) to determine their composition, followed by Western blot analysis (Section 2.10.3) to determine the relative antigenicity of each component.

3.2 Whole cell protein typing

The electrophoretic banding patterns of the whole cell proteins (WCPs) of most Gram-negative bacteria would be expected to be similar as their general bacterial composition is relatively conserved. This is especially true of serotypes within a genus (Guard-Petter *et al.*, 1995). However, should reproducible differences be observed, these could be used as potential markers for an identification assay. Some forms of bacterial taxonomy have even been based on the electrophoretic WCP patterns (Jackson, 1985). Visual comparison of WCPs fingerprints, together with identification and molecular mass determination of prominent proteins has allowed the allocation of *Haemophilus paragallinarum* field isolates to the appropriate serotype (Taylor, 1998). This part of the study involved the comparison of WCP fingerprints of *S. enteritidis* with those of other *Salmonella* serotypes and another Gram-negative bacteria, *E. coli*. WCP samples of most bacteria are relatively easy to obtain, and characterisation of novel protein markers would prove valuable for identification of *S. enteritidis*.

3.2.1 Materials

Phosphate buffered saline (PBS), pH 7.4. NaCl (8 g), KCl (0.2 g), $NaHPO_4.2H_2O$ (1.15 g) and KH_2PO_4 (0.2 g) were dissolved in dist.H_2O (950 ml), the pH was adjusted to 7.4 and the volume was made up to 1 litre with dist.H_2O.

3.2.2 Procedure

Cells from cultures of *S. enteritidis*, *S. typhimurium*, *S. gallinarum*, *S. berta*, *S blegdam*, *S. dublin* and *E. coli* were suspended in PBS. The protein concentration of bacterial cells was determined using the method of Bradford (1976) (Section 2.3). Aliquots containing 25 µg of protein per sample were mixed with an equal volume of non-reducing treatment buffer (Section 2.4.1) and heated at 100°C for 5 min to effect complete denaturation of the samples and allow uniform binding of SDS molecules. The whole cell proteins were then loaded onto a Tris-tricine gel and separated by electrophoresis (Section 2.4), after which proteins bands were visualised by silver staining (Section 2.5.2) or Western blot analysis (Section 2.10.3).

3.2.3 Results and discussion

Non-reducing SDS-PAGE, in conjunction with Coomassie blue R-250 staining of proteins, revealed similar protein profiles of the six *Salmonella* serotypes, including *S. enteritidis*, as well as *E. coli* (Fig. 3.2). These protein profiles were complex and quantitatively appeared very similar. No unique bands, which could have been used as serotype-specifying protein, were identified in the *S. enteritidis* preparation.

The complex nature of the preparations was to be expected, when considering the source. Whole bacterial cells contain numerous proteins, many of which are common to most Gram-negative bacteria (Guard-Petter, 1995). It would therefore be expected that isolation of particular proteins, such as OMPs and fimbrial proteins of *S. enteritidis*, both known to produce immunogenic reactions (Cooper & Thorns, 1996), would provide simpler protein banding patterns. This could facilitate the identification of serotype-determining proteins.

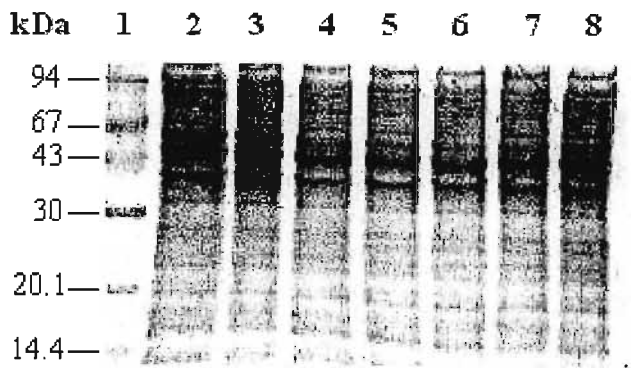

Figure 3.2 Non-reducing SDS-PAGE of the WCPs of Gram-negative bacteria. Samples were boiled in non-reducing treatment buffer, loaded onto a 10% Tris-tricine SDS-PAGE gel and stained with Coomassie blue R-250. Lane 1, Molecular mass markers (phosphorylase b, 94 kDa; BSA, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; soybean trypsin inhibitor, 20.1 kDa; α-lactalbumin, 14.4 kDa); **Lane 2**, *S. enteritidis* ATCC 13076; **Lane 3**, *S. gallinarum* ATCC 9182; **Lane 4**, *S. typhimurium* ATCC 14028; **Lane 5**, *S. berta* ATCC 8392; **Lane 6**, *S. blegdam* K68; **Lane 7**, *S. dublin* ATCC 39184; **Lane 8**, *E. coli* JM103.

3.3 Outer membrane proteins (OMPs)

A typical feature of the outer membrane structure is a phospholipid bilayer with lipopolysaccharide (LPS) molecules tightly packed on the outer surface of the membrane, forming an extremely hydrophilic layer, for interaction with the environment. Proteins

also play vital roles in the structure and function of the outer membrane and constitute roughly half of the mass of the outer membrane (Van Iterson, 1984).

Outer membrane proteins (OMPs) are important determinants of pathogenicity in many bacteria, including *Salmonella*. Analysis of OMPs from several Gram-negative pathogens has resulted in the identification of at least twenty proteins that have proven to be useful epidemiologic and virulence markers (Choi *et al.*, 1989).

Certain proteins are always present in the outer membrane, regardless of the environment. These are called major proteins, and include the pore-forming proteins (porins) that make up the general water-filled pores involved in the passage of small hydrophilic molecules of molecular mass less than 600 Da, such as glucose, across the outer membrane (Arockiasamy & Krishnaswamy, 1999, Tokunaga *et al.*, 1979a,b). The most abundant major protein of the outer membrane is a small lipoprotein (7.2 kDa) called murein lipoprotein (Braun & Rehn, 1969). Murein lipoprotein covalently anchors the outer membrane to the peptidoglycan layer and is embedded in the outer membrane by its hydrophobic end. A number of OMPs, termed minor proteins, are synthesised and inserted into the outer membrane only under certain conditions, known as induction. These proteins protrude from the outer surface of the membrane and serve as receptors for bacteriophages and bacteriocins and are also active in the first step of high affinity iron-sequestering and the uptake of phosphate and vitamin B_{12} (Chart, 1995; Hancock, 1991).

In comparison to the cytoplasmic and other orthodox membranes, the outer membrane contains a small variety of proteins in rather large quantities. Therefore, it is relatively easy to purify and characterise these major proteins. When the protein components of orthodox membranes were separated by SDS-PAGE, thirty to forty bands, stained to different intensities, were resolved. Although SDS-PAGE analysis of OMPs of Gram-negative bacteria has shown a large number of protein bands, only four or five were present in overwhelmingly large amounts. Unless rather large amounts of the protein preparation were used, it appeared that these are the only proteins present (Osborn & Wu, 1980). These four or five bands correspond to the major proteins of the outer membrane.

The universal nomenclature for the OMPs of Gram-negative bacteria was initially complicated, as the designations were not agreed upon. However, it has since been

rationalised in accordance with the nomenclature established for structural genes in current linkage maps of *E. coli* and *S. typhimurium* (Table 3.1) (Osborn & Wu, 1980).

Although the *ompB, ompC* and *ompF* loci of *E. coli*, *S. typhimurium* and *S. enteritidis* are equivalent both in terms of map location and function, only about half of the peptides of either the OmpC or OmpF proteins are similar or identical between the species. Thus there has been considerable evolution of these genes since they diverged from common ancestral genes. This is in contrast to the OmpA protein, which was highly conserved between the species (Lee & Schnaitman, 1980).

Table 3.1 Recommended genetic nomenclature for major outer membrane proteins of *Salmonella typhimurium* (Osborn & Wu, 1980).

Specifying gene	Molecular Mass (kDa)	Function	Nomenclature
ompA	33	Structural	OmpA
ompC	36	Structural	OmpC
ompD	34	Structural	OmpD
ompF	35	Structural	OmpF
lamB	44	Structural	LamB
lpp	7.2	Structural	Murein lipoprotein

The OmpC, D and F proteins act as constitutively expressed porins and are arranged in small oligomers, presumed to be triplets of identical subunits (Palva & Randall, 1979). In addition, these proteins serve as receptors for various bacteriophages (Misra, 1993) and OmpC and OmpF are linked to the peptidoglycan layer (Chart, 1995). OmpA is a receptor protein that affects the envelope morphology and maintains the outer membrane integrity (Lounatmaa, 1979).

There are ten to twenty proteins present in lesser amounts in the outer membrane of Gram-negative bacteria and these were likewise designated according to the specifying genes (Table 3.2).

A number of the OMPs of the Gram-negative bacteria have been reported as heat-modifiable. This behaviour was characterised by an observable shift in molecular mass of

a protein when the membrane was heated in the presence of an anionic detergent, such as SDS, at 50°C or higher. The major heat-modifiable protein in the *E. coli* outer membrane was found to be OmpA (Beher *et al.*, 1980). When heated to 100°C in the presence of SDS, OmpA underwent a characteristic conformational change, raising the apparent molecular mass from 28 kDa to 33.5 kDa.

Table 3.2 Properties of some proteins present in lesser amounts in the outer membrane of Gram-negative bacteria (Osborn & Wu, 1980).

Gene specifying protein	**Molecular mass (kDa)**	Function
feuB	81	Fe^{3+}-enterochelin uptake
cit	80.5	Fe^{3+}-citrate uptake
tonB	78	Fe^{3+}-ferrichrome uptake
cir	74	Not determine
fbe	60	Vitamin B12 uptake
lamB	55	Maltose uptake
ompR	27.4	Not determined
tsx	27	Nucleoside uptake
ompH	17.9	DNA-binding

The OmpA protein of a number of other Gram-negative bacteria, including *S. typhimurium*, was heated and corresponding changes in molecular masses were observed. When heated, the molecular mass of *S. typhimurium* OmpA increased from 28 kDa to 33 kDa (Beher *et al.*, 1980). This was indicative of the OmpA subunit denaturing to a higher molecular mass structure only upon heating. These results indicate that the protein probably existed in the membrane in a highly ordered configuration (Frasch & Mocca, 1978; Hancock & Carey, 1979).

The OMP profile of *S. enteritidis* has shown three major heat-modifiable OMPs, namely OmpA and the porins OmpC and OmpF. The three OMPs were observed when outer membranes were incubated at 100°C prior to SDS-PAGE, whereas these OMPs did not enter the separation gel when pre-incubation was carried out at room temperature (Chart, 1995).

OMPs of Gram-negative bacteria can be isolated by a combination of preparative techniques, which generally include sonication, centrifugation and a step that exploits the selective solubility of the membrane-bound proteins in a particular detergent (Carlone, 1986). Sonication produces high frequency sound waves, which disrupt and break cell walls and centrifugation is used to isolate cellular fractions of differing density. Detergents are soluble amphiphiles, having both hydrophobic and hydrophilic moieties. Interaction of the hydrophobic regions of a detergent with hydrophobic regions of OMPs and membrane lipids results in solubilisation of the outer membrane and formation of mixed detergent-lipid-protein complexes. Detergent-lipid-protein complexes are further solubilised to give detergent-protein complexes and detergent-lipid complexes (Neugebauer, 1988). Once released from the membrane, the protein-detergent samples are dialysed to remove detergent. Protein profiles of these detergent-solubilised membranes can then be compared after SDS-PAGE and staining.

The mild anionic detergent sodium *N*-lauroyl sarcosinate has been used repeatedly for the preparation of insoluble OMP-enriched fractions of Gram-negative bacteria, including *S. enteritidis* (Carlone *et al.*, 1986; Kim *et al.*, 1991; Charles *et al.*, 1996). Sodium *N*-lauroyl sarcosinate (N-Dodecanoyl-N-methylglycine sodium salt) was found to be more effective for outer membrane solubilisation than Triton X-100, which has been used to extract the inner membrane proteins of *E. coli.* It is also milder than the strong anionic detergent SDS, which affects all membrane proteins. Brij 59 is another commonly used detergent, yet cannot solubilise the inner membrane proteins (Choi *et al.*, 1989). Sodium *N*-lauroyl sarcosinate was therefore used for the preparation of OMPs throughout this study.

Because of the aggregative behaviour of OMPs, a clear and reproducible polypeptide pattern could only be obtained during SDS-PAGE if the samples were boiled for several minutes in buffer containing SDS and 2-mercaptoethanol, i.e. under reducing conditions (Benz, 1988). Therefore, the OMPs used throughout this study were separated by electrophoresis under reducing conditions. Kim *et al.*, (1991) found that after SDS-PAGE analysis of the detergent-insoluble OMPs of *S. enteritidis*, the protein bands were distributed between 14 kDa and 68 kDa.

OMPs of *Salmonella* are potential surface antigens and evoke a detectable immune response (Charles *et al.*, 1996; Muthakarruppan *et al.*, 1992), making them possible target antigens and important in terms of diagnosis and possible vaccine design. Adjuvantal OMPs from *S. enteritidis* have been used to vaccinate turkeys. The OMPs used were either positively- or negatively-charged liposomes and lipid-conjugated immunostimulating complexes. Bacterial shedding was reduced in all vaccinated birds and colonisation of all tissues was extremely low (Charles *et al.*, 1994). This technique of an adjuvanted OMP vaccination was applied to chickens with similar results (Meenakshi *et al.*, 1999).

Salmonella porins induce both humoral and cell mediated immunity in animal models (Arockiasamy & Krishnaswamy, 1999). The identification of OMP antigens from *S. enteritidis* that might be specific for the micro-organism would contribute to the development of serological tests for the detection of *S. enteritidis*. The immunogenicity of these OMPs can be characterised by testing the ability of the OMPs to bind immunoglobulins. One of the simplest means of accomplishing this is by Western blot analysis after SDS-PAGE. Utilising this technique, Kim *et al.*, (1991) and Charles *et al.*, (1996) identified two protein bands at 43 kDa and 46 kDa, which had specific antigenic properties for *S. enteritidis* when Western blotted. Charles *et al.*, (1996) concentrated these proteins and used them as the coating antigen in an immunobinding assay for screening antibodies against *S. enteritidis* in chicken sera.

However, there is relatively limited information concerning the immune responses of chickens against purified OMPs. The objective of this part of the study was to evaluate the role of OMPs in protection against *S. enteritidis* infection by characterisation of the physical and antigenic differences between the OMPs of *S. enteritidis* and other *Salmonella* serotypes.

3.3.1 OMP isolation procedure

3.3.1.1 Materials

10 mM HEPES buffer, pH 7.4. HEPES (2.383 g) was dissolved in 950 ml of dist.H_2O and the pH was adjusted to 7.4 with NaOH. The volume was made up to 1 litre with dist.H_2O.

2% (w/v) Sodium *N*-lauroyl sarcosinate. Sodium *N*-lauroyl sarcosinate (2 g) was dissolved in dist.H_2O (100 ml).

3.3.1.2 Procedure

Bacteria were grown on LB-agar plates overnight at 37°C (Section 2.2) and harvested in HEPES buffer (pH 7.4). OMP were isolated essentially as described by Choi *et al.*(1989), with a few modifications. While being held on ice, the bacterial suspension was sonicated (three bursts of one min at 75% power) with a Virtis Virsonic 60 sonicator (New York, USA) set at a power of 15 Watts, to disrupt the bacterial cells. Any unbroken cells and large debris were removed by centrifugation (1,700 × g, 20 min, 4°C) and the resultant supernatant was centrifuged (100 000 × g, 60 min, 4°C) to obtain the whole membrane fraction. The resulting pellet was resuspended in 10 mM HEPES buffer, pH 7.4, to which an equal amount of 2% sodium *N*-lauroyl sarcosinate detergent was added and the mixture was held overnight at 4°C. The mixture was centrifuged (100 000 × g, 60 min, 4°C), and the resultant detergent-insoluble pellet was resuspended in PBS, pH 7.4 (2 ml) (Section 2.10.2.1), and dialysed against PBS for 24 h (three changes). The protein concentration of the suspension was determined using the method of Bradford (1976) as described in Section 2.3, and purified OMPs were subjected to SDS-PAGE (Section 2.4) and Western blot analysis (Section 2.10.3).

3.3.1.3 Results and discussion

Reducing SDS-PAGE of the OMPs of *Salmonella* serotypes showed profiles containing several, common bands in each lane (Fig. 3.3). Several bands unique to *S. enteritidis* were also identified. These bands were in the range of 14.4 kDa to 30 kDa.

It should be noted that procedures used for the examination of OMPS might also isolate outer membrane-associated protein structures such as flagella, fimbriae, and surface protein layers. The residual protein constituents of these structures could appear on SDS-PAGE gels as bands that could be mistakenly considered true OMPs. The isolation and examination of these structures could elucidate the extent to which this occurs (Chart,1995).

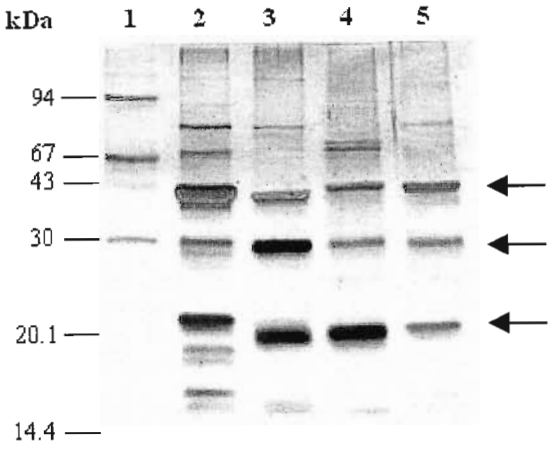

Figure 3.3 Reducing SDS-PAGE of OMPs isolated from *Salmonella* serotypes. Samples were boiled in reducing treatment buffer, loaded onto a 10% Tris-tricine gel and silver stained. **Lane 1**, Molecular mass markers (as in Fig. 3.2); **Lane 2**, *S. enteritidis* ATCC 13076; **Lane 3**, *S. gallinarum* ATCC 9182; **Lane 4**, *S. berta* ATCC 8392; **Lane 5**, *E. coli* JM103. Arrows indicate major outer membrane proteins.

The profiles in Fig. 3.3 show the OMP profiles of strains of *S. enteritidis*, *S. gallinarum*, *S. berta* and *E. coli*. These profiles revealed that, unlike Kim *et al.*, (1991), who found that the protein bands of the OMPs of *S. enteritidis* were distributed between 14 kDa and 68 kDa, protein bands of the *S. enteritidis* OMPs investigated were distributed between 15.7 kDa and 103 kDa (lane 2). However, Kim *et al.*, (1991) had stained the SDS-PAGE gels with Coomassie blue R-250. Silver staining is approximately 10-fold more sensitive than Coomassie staining (Section 2.5). Fig. 3.3 does show the prominent bands to be in the range described by Kim *et al.*, (1991). The faint bands of 103 kDa and 81.4 kDa could have been residual proteins from outer membrane-associated structures, described by Chart (1995), or merely OMPs found in quantities not detectable with Coomassie staining.

Three prominent bands were seen in all samples tested, with estimated molecular masses of 19.8 kDa, 30 kDa and 40.8 kDa. Two of these could correspond to the major OMP porins, OmpA (between 28 kDa and 33 kDa) and the precursor of OmpC (41.3 kDa) respectively (Pattery *et al.*, 1999; Osborn & Wu, 1980). Lee & Schnaitman (1980) reported slight variations in the structure of OmpC and OmpF between *Salmonella* serotypes and *E. coli*. This could account for the slight differences in molecular mass of the band denoted as 40.8 kDa, between the serotypes. Fadl *et al.*, (2002) found that the expression of a 19.3 kDa OMP was up-regulated when *S. enteritidis* was grown on LB

medium, as was the case in this study. This OMP could represent the prominent 19.8 kDa band seen in Fig. 3.3, however, contamination by fimbriae would appear to be the most likely explanation for this band. Type 1 fimbriae have ubiquitous distribution among *Salmonella* serotypes and have been described as protein subunits of between 20 and 22 kDa (Thorns, 1995).

Table 3.3 Analysis of the OMPs of Gram-negative bacteria for the identification of bands specific to *S. enteritidis*.

Molecular mass of protein band (kDa)	*S. enteritidis*	*S. gallinarum*	*S. berta*	*E. coli*
103	+	+	-	-
81.4	+	+	-	+
71.3	-	-	+	-
67.9	+	+	+	-
40.8	+	+	+	+
30	+	+	+	+
29.3	+	-	-	-
21.4	+	-	-	-
19.8	+	+	+	+
18.8	+	-	-	-
18.5	+	-	-	-
17.0	-	-	+	-
16.1	+	-	+	+
15.7	+	+	+	+
3	-	+	-	-

Note: + = protein band present; - = protein band absent; +, = prominent protein band present

Protein bands found to be unique to *S. enteritidis* corresponded to molecular masses of 18.5 kDa, 18.8 kDa, 21.4 kDa and 29.3 kDa (Table 3.3). The 29.3 kDa band could represent the transcriptional regulatory protein OmpR (27.4kDa) or Tsx, the OMP involved in nucleoside uptake (27 kDa) (Osborn & Wu, 1980). Either of the 18.5 kDa or 18.8 kDa bands could represent OmpH (17.9 kDa) which is similar to an *E. coli* DNA-binding OMP.

The antigenic properties of OMPs resolved by reducing SDS-PAGE (Fig. 3.3) were investigated by Western blotting (Fig. 3.4).

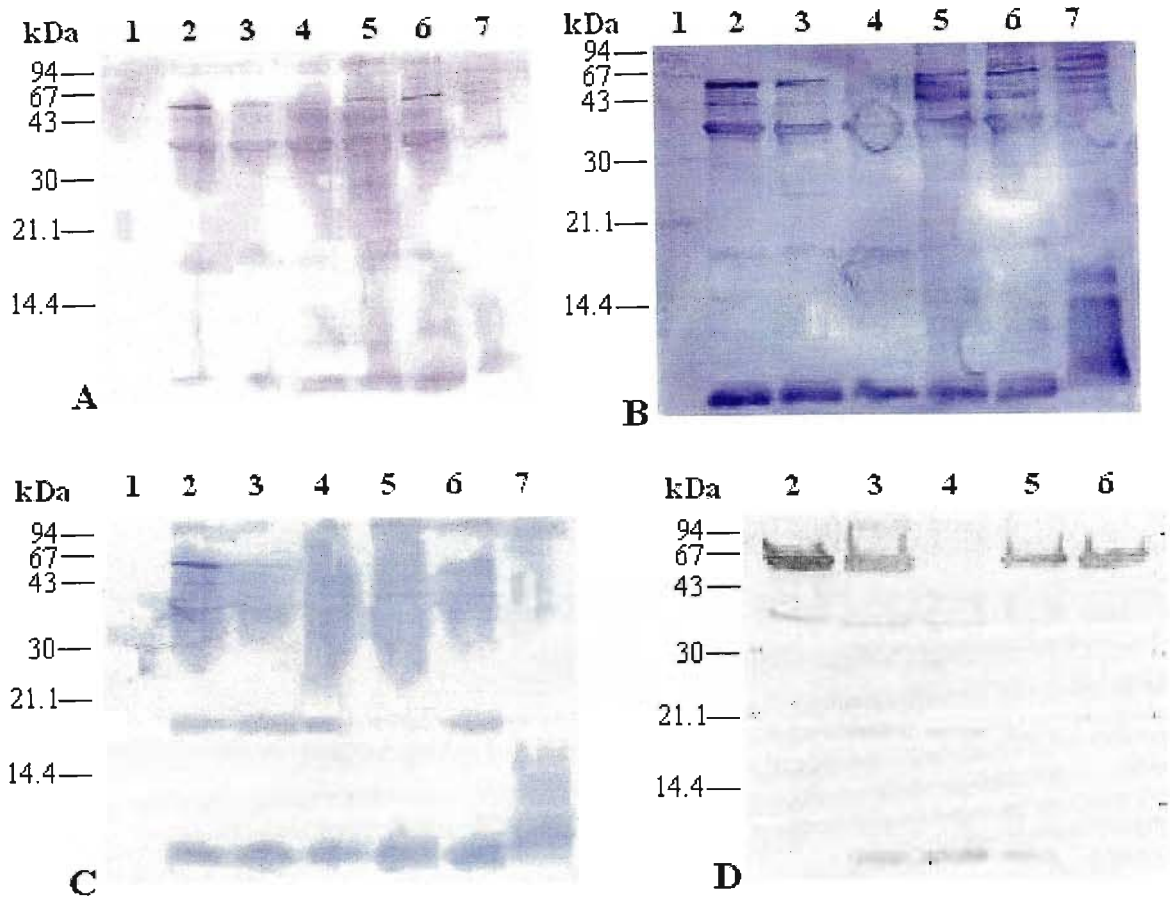

Figure 3.4 Western blot analysis of OMPs of Gram-negative bacteria. Samples were resolved by reducing Tris-tricine SDS-PAGE, electroblotted onto nitrocellulose and probed with chicken serum containing antibodies against *Salmonella* serotypes: A, *S. enteritidis*; B, *S. enteritidis* OMPs; C, *S. gallinarum*; D, *S. berta*. **Lane 1**, Molecular mass markers (as in Fig. 3.2); **Lane 2**, *S. enteritidis* ATCC 13076 OMPs; **Lane 3**, *S. typhimurium* ATCC 14028 OMPs; **Lane 4**, *S. gallinarum* ATCC 9182 OMPs; **Lane 5**, *S. berta* ATCC 8392 OMPs; **Lane 6**, *S. blegdam* K68 OMPs; **Lane 7**, *E. coli* JM103 OMPs (Insufficient material prevented inclusion of lane 7 in D).

The result of Western blotting of the OMPs of *Salmonella* serotypes as well as *E. coli*, indicated large amounts of cross-reactivity between the *Salmonella* serotypes and with other Gram-negative bacteria. A number of prominent bands in each lane showed no specificity in their binding of anti-Salmonella antibodies.

The most prominent of the cross-reactive bands were 7 kDa, 18.1 kDa, and 36.3 kDa in size. The 7 kDa band probably represented the 7.2 kDa murein lipoprotein, a major protein component of the outer membrane of *Enterobacteriaceae*. The lipoprotein appears to be

highly conserved among members of the *Enterobacteriaceae* (Osborn & Wu, 1980), which explains the cross-reactivity of this band. The cross-reactive bands at 18.1 kDa and 36.3 kDa could represent the 20 kDa type 1 fimbriae isolated as part of the OMP fraction, and one of the porins, OmpA or OmpC, respectively. Thorns *et al.*, (1995) have described type 1 fimbriae as immunogenic and having widespread distribution among *Salmonellae.* Fimbriae will be discussed in Section 3.5. OmpA would be the more likely porin representing the 36.3 kDa band, as the ubiquitous nature and cross-reactivity with antisera to the different serotypes, exhibited by the band, corresponded to the high immunogenicity and conserved nature of OmpA among *Enterobacteriaceae* (Lee & Schnaitman, 1980).

Another strongly antigenic band was present at 58.4 kDa in all of the OMP profiles of serotypes tested, except *S. gallinarum*. This OMP of *S. gallinarum* did not react with any antibodies except with those against *S. gallinarum*. This was the only serotype-specific reaction seen amongst the prominent, antigenic bands.

Although cross-reactivity to the antisera was seen in all of the *Salmonella* serotypes, the OMPs appeared to have stronger immunogenic binding to chicken antisera raised against *S. enteritidis* OMPs, than to chicken antisera raised against whole *S. enteritidis*.

Kim *et al.*, (1991) and Charles *et al.*, (1996) had previously identified two OMPs at 43 kDa and 46 kDa, which had specific antigenic properties for *S. enteritidis*. In their studies, chicken antiserum against *S. enteritidis* reacted with both of these polypeptides, yet antisera against *S. typhimurium*, *S. pullorum* and *S. arizonae* failed to react with either of them. Fig. 3.4 shows that neither of these polypeptides reacted specifically with antiserum against *S. enteritidis*. A 43 kDa band of *S. enteritidis* did react strongly with antiserum against *S. enteritidis* OMPs. However corresponding reactions were seen with 43 kDa bands of *S. berta* and *S. blegdam*. This indicated that this reaction was not specific for *S. enteritidis*.

Fadl *et al.*, (2002) identified two OMPs at 82.3 kDa and 75.6 kDa, which were more immunogenic and abundantly expressed than the other OMPs of *S. enteritidis*. These OMPs reacted specifically with chicken antiserum against *S. enteritidis*. However, the two bands were not detected in this study. This was possibly due to Fadl *et al.*, (2002) finding that the two proteins of interest were only up-regulated and synthesised when *S. enteritidis*

was incubated with human intestinal epithelial cells. They were therefore only expressed during attachment to host epithelial cells and their use as diagnostic markers, was limited.

Western blotting of the OMPs of *S. enteritidis* did not reveal novel antigens for serological identification of *S. enteritidis*. Had a novel antigen been identified, the protein band could have been eluted from the gel and either used as a coating antigen for a rapid dot immunobinding assay (Charles *et al.*, 1996), using the same principle as Western blotting, or for the production of antibodies against the specific protein. The results of this study showed the cross-reactive nature of the OMPs of *Salmonella*, even when reacted with antibodies raised against a purified OMP fraction, meaning that a more selective approach needs to be taken to detect *S. enteritidis* in chickens.

3.3.2 Identification of heat-modifiable OMPs

The OMP profiles of *S. enteritidis* and other *Salmonella* serotypes were examined for possible identification of heat-modifiable OMPs. The effect of heating on the antigenicity of heat-modifiable and heat-stable OMPs was also determined by Western blotting.

3.3.2.1 Procedure

OMPs were extracted from *Salmonella* serotypes *S. enteritidis, S. typhimurium, S. gallinarum, S. berta* and *S. dublin* (Section 3.3.1). Samples containing 2.5 µg OMP were aliquoted into 1.5 ml microfuge tubes and mixed with equal volumes of non-reducing treatment buffer (Section 2.4.1). OMP samples from *S. enteritidis* were incubated at temperatures of 0°C, 25°C, 37°C, 60°C and 100°C respectively, for 20 min, and analysed by SDS-PAGE (Section 2.4). OMP profiles were also examined by Western blotting after incubation at 37°C and 100°C respectively.

3.3.2.2 Results and discussion

Non-denaturing SDS-PAGE, followed by silver staining, of the heat-treated OMPs of *S. enteritidis*, showed a single obvious heat-modifiable OMP (Fig. 3.5). Preparations of *S. enteritidis* OMPs incubated at 0°C, 25°C, 37°C and 60°C appeared to have no differences in their profiles, when resolved by SDS-PAGE. However, when the preparation was heated to 100°C, a prominent band appeared at 33.8 kDa (Fig. 3.5, lane 6).

Although three major OMPs of *S. enteritidis* are known to be heat-modifiable, namely OmpA, OmpC and OmpF, only a single band indicated heat-modification in this study. This heat-modifiable protein was identified as OmpA of *S. enteritidis*, which when heated to 100°C prior to SDS-PAGE, can be observed at 33 kDa, but when pre-incubation is carried out at lower temperatures, does not enter the separation gel (Chart, 1995)

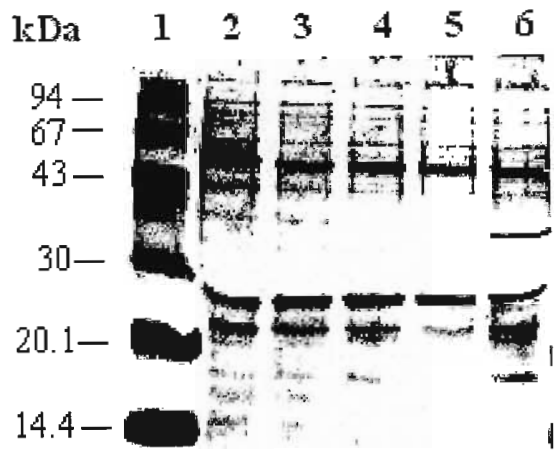

Figure 3.5 Non-reducing SDS-PAGE of heat-modifiable OMPs of *S. enteritidis*. OMP samples from *S. enteritidis* ATCC 13076 were incubated at 0°C, 25°C, 37°C, 60°C or 100°C in non-reducing treatment buffer for 20 min, loaded onto a 10% Tris-tricine gel and silver stained. **Lane 1**, Molecular mass markers (as in Fig. 3.2); **Lanes 2-7**, *S. enteritidis* OMPs incubated at: **Lane 2**, 0°C; **Lane 3**, 25°C; **Lane 4**, 37°C; **Lane 5**, 60°C; **Lane 6**, 100°C.

As with SDS-PAGE, Western blot analysis of the OMP profiles seemed to indicate that a single heat-modifiable protein was apparent in the OMP preparation. Western blotting was expected to detect the same modification in the molecular mass of OmpA, as seen with SDS-PAGE. This would have corresponded to the appearance of a prominent band of approximately 33 kDa after incubation at 100°C. However, a prominent band of 28.3 kDa was observed in each of the OMP preparations that had been heated to 100°C. This band was not observed in the case of OMP preparations incubated at 37°C prior to SDS-PAGE. It would appear that OmpA, when heated at 100°C, had formed a band at 28.3 kDa. When OmpA of *S. typhimurium* and *E. coli* is heated, the molecular mass increases from 28 kDa to 33.5 kDa, due to denaturing of the protein subunit to a higher molecular mass (Beher *et al.*, 1980). It was thought that, in this study, the OmpA protein had been modified by heat to form the 28 kDa configuration, but denaturation had not occurred to the extent that the 33.5 kDa configuration, seen in Fig. 3.5, was formed.

The increase in incubation temperature from 37°C to 100°C appeared to reduce the number of faint bands between 40 kDa and 70 kDa, which were likely caused by the proteins in this region being variably denatured by SDS in the treatment buffer. Incubation of the OMPs at 100°C would have caused the complete denaturation of the proteins in this range, allowing them to form more prominent bands of slightly larger molecular mass at 58.7 kDa. This was noticeably clear in the case of the bands of *S. berta* within this range. Heating of the sample to 100°C resulted in the formation of a prominent band of 58.7 kDa, while heating at 37°C resulted in the formation of a faint band of 58.7°C, with fainter bands of slightly lower molecular mass, also apparent.

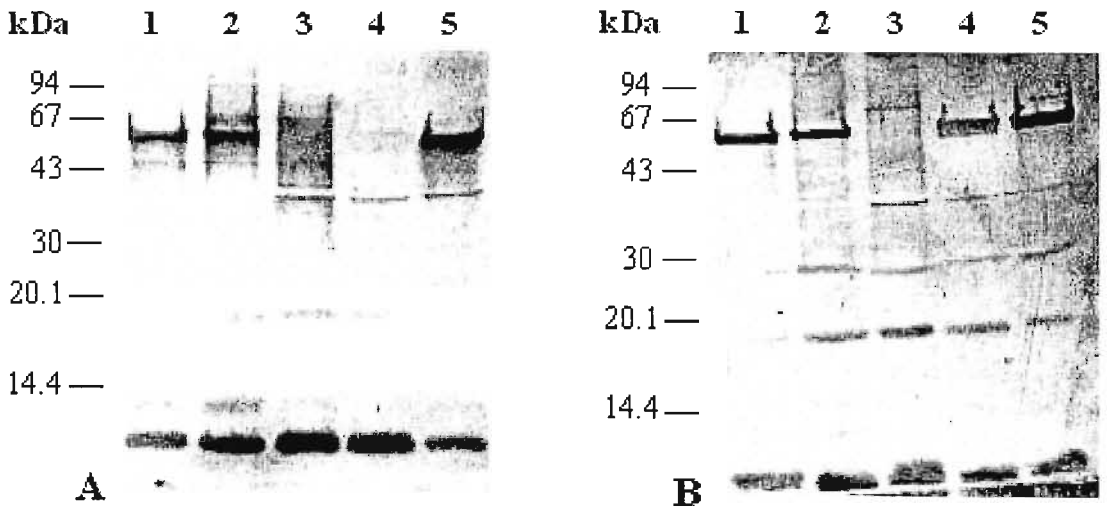

Figure 3.6 Western blot analysis of the heat-modifiable OMPs of *Salmonella* serotypes. Samples were incubated at: A, 37°C, or B, 100°C, resolved by reducing Tris-tricine SDS-PAGE, electroblotted onto nitrocellulose and probed with chicken serum containing antibodies against *S. enteritidis* OMPs. **Lane 1**, *S. enteritidis* ATCC 13076 OMPs; **Lane 2**, *S. typhimurium* ATCC 14028 OMPs; **Lane 3**, *S. gallinarum* ATCC 9182 OMPs; **Lane 4**, *S. berta* ATCC 8392 OMPs; **Lane 5**, *S. dublin* ATCC 39184 OMPs.

3.4 Typing of lipopolysaccharides

Endotoxic LPS is a major component of the outer membrane of *S. enteritidis* and other Gram-negative bacteria. The O side chains of LPS represent the somatic antigens of Gram-negative bacteria and are readily recognised by host antibodies, yet the structure of the O side chains could rapidly be changed to possibly avoid detection (Section 1.5.1). Antibody interaction with the LPS before reaching the outer membrane proper may also protect the cell wall from direct attack (Guard-Petter *et al.*, 1995).

LPS are prepared from whole bacteria by the proteolytic digestion of cellular proteins with proteinase K (Guard-Petter *et al.*, 1995). SDS-PAGE is commonly used for characterisation of LPS of Gram-negative bacteria. As LPS does not stain well with carbohydrate stains (periodic acid-Schiff or alcian blue methods), SDS-PAGE-separated LPS profiles are stained with silver (Hitchcock & Brown, 1983) or subjected to immunological analysis (Chart *et al.*, 1991).

SDS-PAGE and silver staining has often been used as a simple method to detect quantitative changes in O-antigen/core linkage ratios, by interpreting differences in migration patterns of discrete sugar moieties (Guard-Petter *et al.*, 1995; Chart *et al.*, 1989; Chart *et al.*, 1991). This study investigated whether SDS-PAGE and Western blot techniques would show differences in LPS structure and antigenicity between *S. enteritidis* and other *Salmonella* serotypes.

3.4.1 Procedure

The procedure used for LPS isolation was that described by Chart *et al.* (1989). Cultures of *S. enteritidis* ATCC 13076, *S. gallinarum* ATCC 9182, *S. typhimurium* ATCC 14028 and *S. berta* ATCC 8392 were scraped from agar plates and placed into pre-weighed microfuge tubes containing 30 µl reducing treatment buffer (Section 2.4.1) until the bacterial mass reached 300 µg (wet mass). After heating at 100°C for 10 min, 30 µl of each of the bacterial suspensions was mixed with reducing treatment buffer (30 µl) (Section 2.4.1) containing proteinase K (100 µg) before incubation at 60°C for 1 h. An aliquot (30 µl) of the preparation, comprising 150 µg total bacterial cell mass, was loaded onto a reducing 5% Tris-tricine SDS-PAGE gel (Section 2.4). The antigenicity of the preparation was tested by Western blot analysis using sera of chickens infected with *S. enteritidis*, to probe for unique markers with which to identify *S. enteritidis* (Section 2.8.3).

3.4.2 Results and discussion

SDS-PAGE analysis of the LPS in proteinase K-digested whole cell lysates of all four *Salmonella* serotypes tested revealed identical LPS profiles (Fig. 3.7). Similar amounts of

LPS with characteristic O-polysaccharide chains of heterogenous chain length were observed among all 4 serotypes tested. The expression of long-chain LPS gives a characteristic "ladder" pattern after silver staining of *Salmonella* LPS after SDS-PAGE (Cox & Woolcock, 1994), which was apparent with the serotypes analysed. Although SDS-PAGE has revealed that the O-specific polysaccharides, core oligosaccharides and lipid-A content contribute to the morphological heterogeneity of SDS-PAGE LPS profiles of different serotypes (Hitchcock & Brown, 1983), no heterogeneity was shown between the serotypes examined (Fig. 3.7).

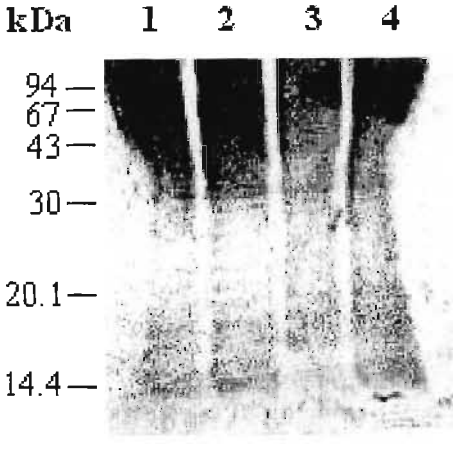

Figure 3.7 Reducing SDS-PAGE of LPS of *Salmonella* serotypes. Samples were loaded onto a reducing 10% Tris-tricine gel and silver stained. **Lane 1**, *S. enteritidis* ATCC 13076; **Lane 2**, *S. gallinarum* ATCC 9182; **Lane 3**, *S. typhimurium* ATCC 14028; **Lane 4**, *S. berta* ATCC 8392.

By probing the sample resolved with SDS-PAGE, with antibodies against *S. enteritidis*, antigenic cross-reactivity between LPS of the *Salmonella* serotypes was investigated. Results of the Western blot showed that a high degree of cross-reactivity had occurred (Fig. 3.8). Of the four serotypes investigated, all showed LPS of molecular mass larger than 30 kDa, which were strongly recognised by chicken anti-*S. enteritidis* antibodies. LPS of molecular mass below 30 kDa, which were found to produce the "ladder" pattern, were only slightly recognised by antibodies in chicken sera, producing very weakly coloured bands (not visible in Fig. 3.8). However, this weakly coloured banding pattern was similar for the four serotypes tested .

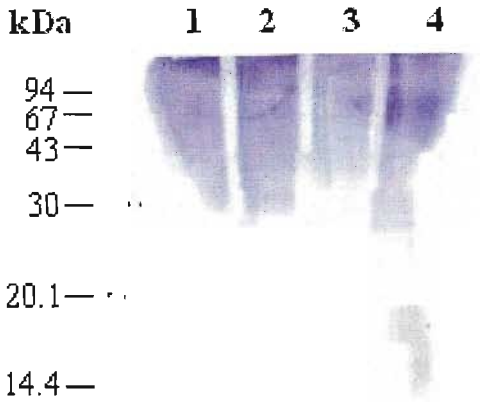

Figure 3.8 Western blot of LPS of *Salmonella* serotypes. Samples were resolved by reducing Tris-tricine SDS-PAGE, electroblotted onto nitrocellulose and probed with chicken serum containing antibodies against *S. enteritidis*. **Lane 1**, *S. enteritidis* ATCC 13076; **Lane 2**, *S. gallinarum* ATCC 9182; **Lane 3**, *S. typhimurium* ATCC 14028; **Lane 4**, *S. berta* ATCC 8392.

Immunisation of chickens with Gram-negative bacteria leads to sustained production of antibodies specific to LPS (Sunwoo *et al.*, 1996). This has resulted in the production of diagnostic tests to detect these antibodies, although a high degree of cross-reactivity has been demonstrated with sera of chickens infected with different *Salmonella* serotypes. Chart *et al.*, (1990) demonstrated by immunoblotting that antibodies against the common 12 antigens present in *Salmonella* serogroups B and D (Section 1.5.1) caused the cross-reactions. Of the serotypes investigated in this part of the study, *S. enteritidis*, *S. gallinarum* and *S. berta* are members of serogroup D and *S. typhimurium* is a member of serogroup B.

The banding patterns of the LPS of *Salmonella* serotypes showing great complexity and similarity and a large amount of cross-reactivity was seen when LPS were probed with sera of chickens infected with *S. enteritidis*. LPS therefore seemed an unlikely antigen candidate for simple visual differentiation of *S. enteritidis* from other *Salmonella* serotypes, using Western blotting. In future studies, the O-antigen side chains that are generally serotype-specific should be isolated and subjected to Western blot analysis outlined here.

3.5 Fimbrial proteins

Fimbriae (or pili) are fibrillar, proteinaceous, polymeric surface appendages, each composed of about 10^3 helically-arranged protein monomers (fimbrin), common to several members of the *Enterobacteriaceae*, including most of the salmonellas (Müller *et al.*, 1991; Thorns, 1995; Clouthier *et al.*, 1998). It is currently believed that fimbriae function by mediating interaction of bacteria with surfaces of other cells, and by allowing bacteria to interact with host cells and other solid substrates. Fimbriae are thus thought to play a crucial role in virulence (Strom & Lory, 1993).

Expression of fimbriae by strains of *Salmonella* was first described over 40 year ago (Duguid & Gillies, 1958), yet very little insight has been gained into the variety and function of *Salmonella* fimbriae. Only in the last ten years has work focused on the potential role of *Salmonella* fimbriae as diagnostic and protective antigens (Thorns, 1995).

Fimbriae of the *Enterobacteriaceae* are broadly classified on the basis of the ability of the monosaccharide mannose to inhibit the adhesion of fimbriae to, and agglutination of erythrocytes (haemagglutination). According to this classification the two major classes were named mannose-sensitive and mannose-resistant (Müller *et al.*, 1991). In 1966, a study of over 1400 *Salmonella* strains led to the further classification of fimbriae according to their morphology and ability to mediate erythrocyte agglutination in the presence or absence of D-mannose (Duguid *et al.*, 1966). This classification was used successfully for many years, yet with the description of a number of novel fimbriae that are morphologically similar and do not mediate haemagglutination reactions of any type, it is now less appropriate. A large number of *Salmonella* fimbriae have since been described (Table 3.4).

Table 3.4 Summary of the characteristics of *Salmonella* fimbriae (Thorns & Woodward, 2000).

Fimbrial class	Fimbrial name	Morphology[a]	Diameter (nm)	Subunit mass (kDa)	Haemagglutination reaction[b]	*Salmonella* serotype	Genetic determinant	
							Operon	Location
Type 1	F1	R	7-8	21	MS	*S. typhimurium*	*fim*	chromosome
	SEF21	R	7-8	21	MS	*S. enteritidis*	*fim*	chromosome
Type 2		R	7-8	nd	NH	*S. pullorum*	nd	nd
		nd	nd	nd	NH	*S. dublin*	nd	nd
		nd	nd	nd	NH	*S. gallinarum*	nd	nd
Type 3		F	3-5	22	MR	*S. enteritidis*	nd	nd
		nd	nd	nd	MR	*S. typhimurium*	nd	nd
Type 4		F	3	nd	MR	*S. typhimurium*	nd	nd
Type 4-like	Bundle-forming fimbria	F	7	18.5-21	nd	*S. dublin*	nd	nd
Unclassified	SEF14	F	3	14.3	NH	*S. enteritidis*	*sef*	chromosome
		nd	nd	nd	NH	*S. dublin*	nd	nd
GVVPQ	SEF17	F	3	17	NH	*S. enteritidis*	*agf*	chromosome
	Thin aggregative fimbriae	F	3	17	NH	*S. typhimurium*	nd	nd
Unclassified	Long polar fimbriae	R	7-8	nd	nd	*S. typhimurium*	*lpf*	chromosome
Unclassified	Plasmid-encoding fimbriae	nd	Nd	nd	nd	*S. enteritidis*	*pef*	plasmid
						S. typhimurium	nd	nd

Note: nd = not determined; [a] R = rigid and rod shaped; F = fibrillar; [b] MS = mannose-sensitive; NH = no haemagglutination; MR = mannose resistant

Type 1 fimbriae of *Salmonella* are rigid structures, 7 nm in diameter and up to 100 nm in length. As many as 300 type 1 fimbriae are expressed per cell, although only about 10 percent of cultured bacteria appear to express them at any one time. They consist of a number of identical protein subunits non-covalently linked around a hollow core. Protein subunits of molecular masses of 20-22 kDa have been reported for type 1 fimbriae of *S. typhimurium* and *S. enteritidis* (Müller *et al.*, 1991) (Table 3.4). Type 1 fimbriae mediate mannose-sensitive haemagglutination and lectin-based binding to mannoside glycoprotein receptors situated on a variety of epithelial cells (Table 3.4) (Kukkonen *et al.*, 1993). Most *Salmonella* serotypes express type 1 fimbriae, with conservation at both molecular and structural levels across the genera. This was confirmed by the extensive cross reactivity seen against antibodies raised against type 1 SEF21 fimbriae of *S. enteritidis* (Sojka *et al.*, 1996).

Type 2 fimbriae are morphologically similar to type 1, but lack agglutination ability. They were first observed on strains of *S. pullorum* and *S. gallinarum* and have since been described on *S. paratyphi B* and *S. dublin* (Table 3.4). The close molecular and antigenic relationship between type 1 and type 2 fimbriae could indicate that type 2 fimbriae are non-agglutinating variants of type 1 fimbriae. This is plausible, as there have been suggestions that there are separate genes for the structural and haemagglutinating components of type 1 fimbriae in *E. coli* (Minion *et al.*, 1986).

Type 3 fimbriae are thinner, more flexible structures with a diameter of 3-5 nm, which mediate the agglutination of tannic acid-treated erythrocytes in the presence of α-D-mannose (mannose resistant). The subunits of type 3 fimbriae of *S. enteritidis* have a molecular mass of approximately 22 kDa (Table 3.4). As with type 1 fimbriae, there is a strong antigenic cross-reactivity between type 3 fimbriae expressed by *Salmonella,* as well with those expressed by *Klebsiella* and *Yersinia.*

Type 4 fimbriae were originally defined as thin, flexible fimbriae of *S. typhimurium*, 4 nm in diameter and able to mediate erythrocyte agglutination of fresh erythrocytes in the presence of mannose. Type 4-like fimbriae, termed bundle-forming pili (bfp) and composed of 18.5-21 kDa fimbrial subunits, have also been expressed by *S. dublin* (Table 3.4).

The designation of the GVVPQ fimbriae refers to the short amino acid sequence conserved at the N terminus of the major fimbrial subunit of certain fimbriae. SEF17 fimbriae were first described on *S. enteritidis* (Collinson *et al.*, 1991) and are structurally and functionally similar to the fimbriae of certain strains of diarrhoeagenic *E. coli* (Robertson *et al.*, 2000). SEF17 fimbriae comprise protein subunits of molecular mass 17 kDa (Table 3.3), and contain a receptor for the tissue-matrix protein fibronectin (Collinson *et al.* 1993). These fimbriae are typically expressed by certain strains of *S. enteritidis* cultured on solid media such as colony factor antigen (CFA) at temperatures up to 30°C, although a few strains of *S. enteritidis* express SEF17 at temperatures up to 42°C (Dibb-Fuller *et al.*, 1997). These strains spontaneously aggregate in a suspension prepared from organisms cultured on solid media (Table 3.4) (Collinson *et al.*, 1991). Austin *et al.*, (1998) suggested that SEF17 present on adherent, but killed bacteria, may act as sites for recolonisation due to the aggregative properties of the fimbriae.

SEF14 fimbriae were first described on strains of *S. enteritidis* (Thorns *et al.*, 1990; Müller *et al.*, 1991). These fimbriae were identified and characterised, but did not fit into the existing classification due to the size (less than 3 nm in diameter) and non-haemagglutination characteristics. The fimbriae are composed of repeating subunit proteins of 14.3 kDa (Table 3.4) and are specific to certain serotypes of *Salmonella* serogroup D, including *S. enteritidis* (Section 1.5.2). SEF14 is expressed on organisms cultured above 30°C and expression is subject to catabolite repression (Thorns & Woodward, 2000). Peralta *et al.*, (1994) showed a role for SEF14 fimbriae in the *in vitro* adhesion of *S. enteritidis* to mouse epithelial cells. It has also been speculated that SEF14 fimbriae may contribute to tropism towards reproductive tissues in poultry (Table 3.4) (Woodward *et al.*, 2000). This would prove important in the production of *S. enteritidis*-contaminated eggs.

The plasmid-encoded fimbriae (PEF) and long polar fimbriae (LPF) of *S. typhimurium* are considered to be involved in the adhesion to villus small intestine cells and Peyer's patches, respectively. *S. enteritidis* expresses PEF during infection in chickens, yet its role in the infection process remains unclear (Robertson *et al.*, 2000). PEF is encoded by a gene on the *S. enteritidis*-associated virulence plasmid, although plasmid-free organisms remain fully virulent in chickens (Dibb-Fuller *et al.*, 1999). *S. enteritidis* also has the

genetic potential to express LPF, but expression of the structure has not been confirmed (Allen-Vercoe & Woodward, 1999).

Table 3.5 Properties of *Salmonella enteritidis* fimbriae (Thorns, 1995).

Fimbria	Properties
Type 1	Binding to undamaged host intestinal epithelial cells.
	Binding to tissue matrix proteins, e.g. laminin, fibronectin and collagen.
SEF17	Binding to inanimate objects.
	Binding to tissue matrix proteins, e.g. laminin, fibronectin and collagen.
	Auto-aggregation.
SEF14	Binding to host epithelial cells.
	Possible tropism towards reproductive tissue of host.
PEC	Possible adhesion to small intestine villus cells.
LPF	Unknown.

S. enteritidis is unique in its ability to express SEF14 and SEF17, both of which contribute to adherence, although under different conditions (Woodward *et al.*, 2000). Adherent wild-type strains of *S. enteritidis* expressd large quantities of SEF14 at 37°C, but not at ambient temperature (25°C), when grown on media of low osmolarity. Their expression was enhanced by growth on agar surfaces. This prompted the hypothesis that surface contact was an environmental signal for SEF14 regulation. SEF17 were expressd copiously at ambient temperature, when grown on low osmotic, and nutritionally poor media, yet not at 37°C (Woodward *et al.*, 2000; Walker *et al.*, 1999). SEF17 has also been implicated in adherence to inanimate surfaces (Rajashekara *et al.*, 2000).

The ongoing epidemic of *S. enteritidis* may relate in part to the success of the bacterium passing down the food chain with adherence to inanimate and non-living objects. *S. enteritidis* may adhere to surfaces such as eggs, food-processing equipment, animal carcasses and farm-yard implements, over a wider range of environmental conditions than other *Salmonella* serotypes. SEF17 could be specifically expressd by *S. enteritidis*, under cooler, nutritionally poor conditions, typical of an environment external to a host. Binding to an inanimate object by SEF17, together with the aggregative properties of SEF17, would increase the chance of transmission to a host. Once in the warmer, nutritionally rich

environment of a host, surface contact would trigger the expression of SEF14 by *S. enteritidis*, allowing the bacterium to adhere to and infect the host.

3.5.1 Purification of SEF14 from *S. enteritidis*

Large-scale purification of fimbriae has traditionally relied on a number of partial purification steps. Sonication has been used for the disruption and breaking of bacterial cells, before further purification of SEF14 (Collinson *et al.*, 1991; Dibb-Fuller *et al.*, 1997). Following fractionation of cells, ammonium sulfate precipitation has been used as a preliminary step in SEF14 purification (Feutrier *et al.*, 1986). Ammonium sulfate operates on a steric exclusion mechanism, whereby proteins are concentrated, until they eventually exceed their solubility limit, and are precipitated (Ingham, 1990).

These techniques, together with an anion exchange chromatography (Section 2.6), to selectively elute fimbriae based on their charged side chains, and molecular exclusion chromatography (Section 2.7), to elute SEF14 from the fimbrial preparation according to its molecular mass, have been used in a stepwise purification procedure. The result of the procedure was purified SEF14 (Thorns *et al.*, 1996a,b). This technique, with modifications made as to the types of columns used for chromatography, was employed for the purification of SEF14 from *S. enteritidis*.

3.5.1.1 Procedure

S. enteritidis was grown in 1 litre of BPW (Section 2.2.1) for 18 h at 37°C, and harvested by centrifugation (4 000 × g, 10 min, 4°C). The pellet was resuspended in PBS, pH 7.2 (10 ml, Section 2.10.2.1). SEF14 was removed from the bacterial surface by heating the suspension (30 min, 60°C). The bacterial cells were partially disrupted by mild sonication (three bursts of one min, at 25% power) with a Virtis Virsonic 60 sonicator (New York, USA) set at 5 Watts (RMS). Cells and cellular debris were sedimented by centrifugation (3 000 × g, 20 min, 4°C) and 25% (w/v) dry ammonium sulfate was slowly added to the supernatant, with stirring. The mixture was stirred for a further 10 min at RT, to dissolve the ammonium sulfate. Protein precipitate was collected by centrifugation (10 000 × g, 20 min, 20°C). A further 35% (w/v) dry ammonium sulfate was slowly added to the supernatant and dissolved by stirring (10 min, RT). Precipitated protein was collected by

centrifugation (10 000 × g, 20 min, 20°C) and the SEF14-containing pellet was resuspended in 0.02 M phosphate buffer, pH 7.5 (4 ml) (Section 2.8.2) and applied to a Sephadex G-25 column, in order to effect desalting. The sample was eluted from the column with 0.02 M phosphate buffer, pH 7.5. Samples of the eluant were separated on a reducing SDS-PAGE gel, to determine the approximate molecular mass of the constituent proteins in the sample (Fig. 3.9).

The desalted sample was applied to a Q-Sepharose column (Fig 3.10) and eluted by an increasing ionic strength gradient (Section 2.6). Samples of the fractions corresponding to absorbance peaks were separated on a reducing SDS-PAGE gel (Fig. 3.11) and fractions suspected of containing SEF14, based on molecular mass, were combined. The combined fractions were concentrated by dialysis against PEG 20 000 (Section 2.9.1) and applied to a Sephadex G-100 MEC column (Fig 3.11) as outlined in Section 2.7. Samples of fractions corresponding to absorbance peaks were separated on a reducing SDS-PAGE gel (Fig. 3.13), to determine whether SEF14 had eluted from the column as an absorbance peak.

3.5.1.2 Results and discussion

SDS-PAGE of the eluant from the Sephadex G-25 column, showed a relatively heterogenous preparation, with bands ranging from approximately 14 kDa to over 100 kDa (Fig. 3.9), with a prominent band at 14 kDa, corresponding to the molecular mass of SEF14 subunit. Smearing of the bands would have been indicative of high ionic strength of the buffer, which would not be optimal for IEC, the next step in the purification procedure. However, no obvious smearing of the bands was apparent, indicating that desalting of the sample had proven effective.

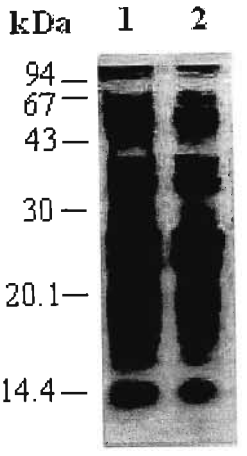

Figure 3.9 Reducing SDS-PAGE of SEF14 fimbrial protein preparation, after elution from a Sephadex G-25 column. Eluant; **Lane 1**, 40 µg; **Lane 2**, 30 µg, from the column was boiled in reducing treatment buffer, loaded onto a 10% Tris-tricince gel and stained with Coomassie blue R-250.

Desalted sample was applied to, and eluted from, a Q-Sepharose column. An elution profile is shown in Fig. 3.10.

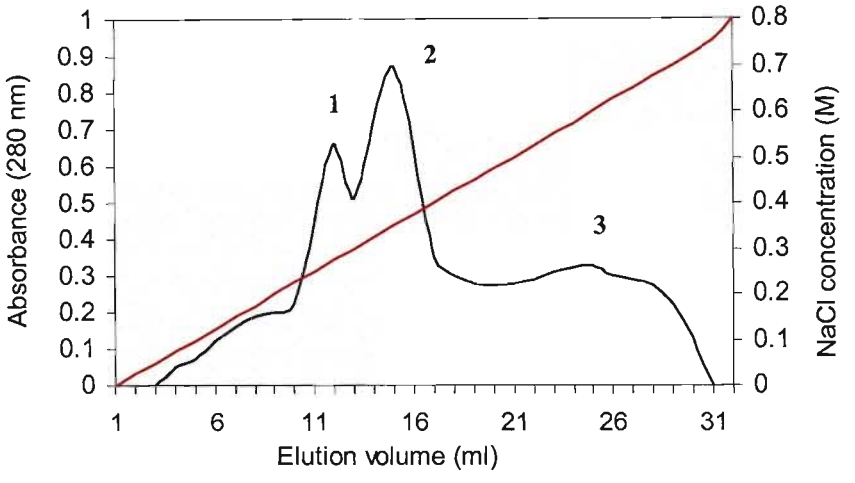

Fig. 3.10 Elution profile of a Q-Sepharose column during purification of SEF14 fimbrial protein from *S. enteritidis*. Column: (25 × 115 mm) bed volume 56 ml; buffer 0.02 M phosphate buffer, pH 7.5; flow rate, 50 ml/h(—) indicates the plot of the absorbance versus the elution volume and (—) indicates the plot of NaCl concentration in the buffer, versus the elution volume.

From Fig. 3.10, it appeared that protein eluted from the Q-Sepharose column in three peaks (Table 3.6).

Table 3.6 Elution volume of absorbance peaks from a Q-Sepharose column during purification of SEF14 fimbrial protein from *S. enteritidis*.

Absorbance peak	Elution volume of peak (ml)	Absorbance (280 nm)
1	12	0.66
2	15	0.87
3	25	0.33

Samples in the fractions corresponding to peaks were subjected to reducing SDS-PAGE (Fig. 3.11). These results showed that absorbance peaks 1 and 2 of Table 3.6 appeared to have identical protein profiles, with bands ranging from approximately 5 kDa to over 95 kDa. However, the reason for differential elution of the proteins in peaks 1 and 2, was unclear. Peak 3 appeared to have a protein profile consisting of bands of over 50 kDa. As denatured subunits of SEF14 have a molecular mass of 14.3 kDa (Table 3.4), fractions corresponding to elution volumes between 10 and 17 ml, were combined for further purification. These fractions included elution peaks 1 and 2, which contained prominent bands corresponding to this approximate size (Fig. 3.11).

Combined samples from the Q-Sepharose column (peaks 1 and 2) were concentrated and applied to a Sephadex G-100 MEC column. The elution profile is shown in Fig. 3.12. From Fig. 3.12, it appeared that protein eluted from the Sephadex G-100 column in two absorbance peaks. The Kav and corresponding estimated molecular mass of proteins corresponding to both peaks, was calculated as described in Section 2.7.2 and shown in Table 3.7.

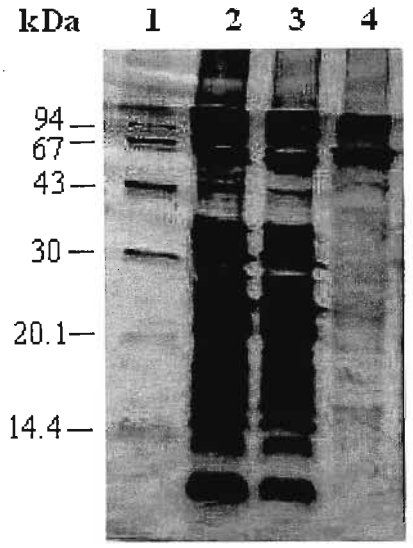

Figure 3.11 Reducing SDS-PAGE of fractions eluted from a Q-Sepharose column during isolation of SEF14 from *S. enteritidis*. Samples were boiled in reducing treatment buffer, loaded onto a 10% Tris-tricine gel and silver stained. **Lane 1**, Molecular mass markers (as in Fig. 3.2); **Lane 2**, Absorbance peak 1; **Lane 3**, Absorbance peak 2; **Lane 4**, Absorbance peak 3.

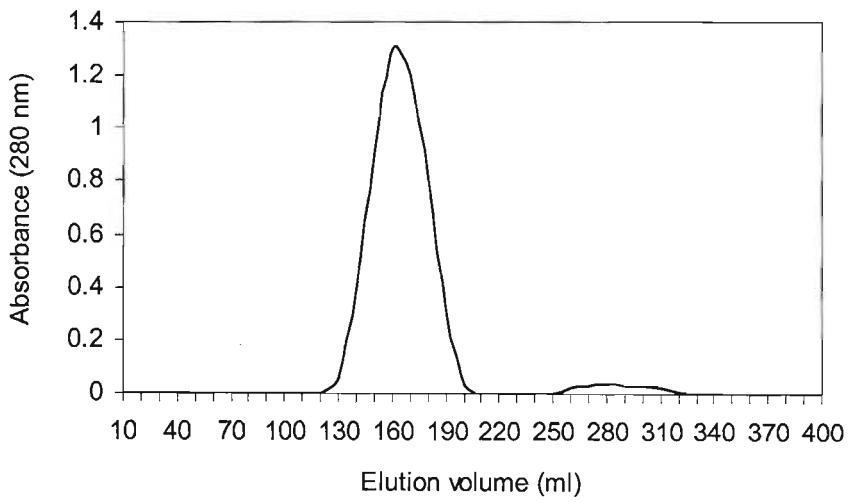

Fig.ure 3.12 Elution profile from a Sephadex G-100 column during SEF14 fimbrial protein purification from *S. enteritidis*. Column: (25 × 705 mm) bed volume 352 ml; buffer, 0.02 M Tris buffer, pH 7.6; flow rate, 25 ml/h.

Table 3.7 Analysis of absorbance peaks from a Sephadex G-100 column during purification of SEF14 fimbrial protein from *S. enteritidis*.

Absorbance peak	Kav of eluted protein	Molecular mass of protein (kDa)
1	0.122	468
2	0.653	3.6

From the results of Table 3.7, it appeared that the SEF14 fimbrial subunits of expected molecular mass of 14.3 kDa was not eluted from the Sephadex G-100 column as one of the absorbance peaks. However, reducing SDS-PAGE was used for verification of this. Samples from peaks 1 and 2 of Fig. 3.11 were prepared as described in Section 2.4.2 and loaded onto a reducing SDS-PAGE gel (Fig. 3.13).

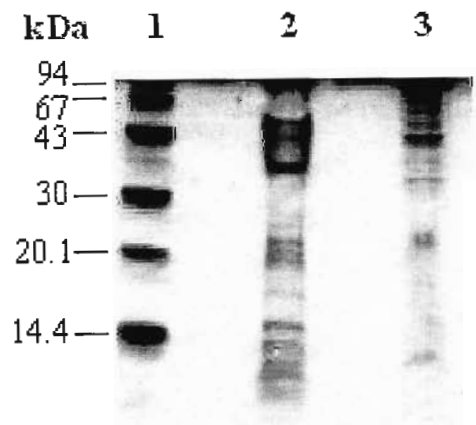

Figure 3.13 Reducing SDS-PAGE of fractions eluted from a Sephadex G-100 column during purification of SEF14 from *S. enteritidis*. Samples were boiled in reducing treatment buffer, loaded onto a 10% Tris-tricine gel and silver stained. **Lane 1**, Molecular mass markers (as in Fig. 3.2); **Lane 2**, Protein peak 1, corresponding to an estimated M_r of 468 kDa by MEC; **Lane 3**, Protein peak 2, corresponding to an estimated M_r of 3.6 kDa by MEC.

SDS-PAGE confirmed calculations based on the Kav of the peaks, that neither of the two peaks contained significant quantities of SEF14, corresponding to a molecular mass of 14.3 kDa. From calculations based on the Kav of peak 1, protein eluted in peak 1 was estimated to be approximately 468 kDa in size. Reducing SDS-PAGE analysis showed a protein banding pattern ranging from below 10 kDa up to approximately 50 kDa. Fimbriae such as SEF17 are known to be auto-aggregative (Collinson *et al.*, 1991) and involved in the binding of *S. enteritidis* to inanimate objects and tissue matrix proteins (Table 3.5). Small numbers of these fimbriae could possibly have caused the aggregation of fimbriae

and other proteins during the preparation, resulting in the elution of a protein aggregate of 468 kDa. Reducing conditions of SDS-PAGE could have caused separation of the aggregates, resulting in the banding pattern seen. A minor band was visible at 14-15 kDa.

From calculations based on the Kav of absorbance peak 2, protein eluted in peak 2 was estimated to be approximately 3.6 kDa in size. The banding pattern of absorbance peak 2 after reducing SDS-PAGE, showed a prominent band at 40 kDa and minor bands at 23 kDa and between 40 kDa and 95 kDa. No bands smaller than 23 kDa were visible.

Reducing SDS-PAGE gels of the samples eluted from the Sephadex G-25 desalting column and the Q-Sepharose column showed a prominent band of approximately 14 kDa, which could have been SEF14 (Fig. 3.9 and 3.11). However, MEC purification resulted in samples of heterogenous nature, with prominent bands not corresponding to the molecular mass of the fimbrin subunit of SEF14, of 14.3 kDa. This stepwise procedure proved unsuccessful at purifying SEF14, as well as time-consuming. A simple and efficient technique for purifying SEF14 was required.

3.5.2 Simultaneous purification of SEF21 and SEF14 from *S. enteritidis*

Fimbriae can be sheared from bacteria by blending. However, if the bacteria are flagellated, as well as bearing both sex pili and fimbriae, as is *S. enteritidis*, methods must be found for separating these appendages. Short periods of blending at low speeds would remove flagella and sex pili, leaving the fimbriae attached. These can then be removed by more vigorous blending. After shearing the fimbriae, the whole organism can be removed by low speed centrifugation and the fimbriae can be subsequently deposited at high speed (Thorns & Woodward, 2000).

S. enteritidis SEF14 fimbriae have been purified by using a simple, rapid procedure involving manipulations of ionic strength along with differential centrifugation and ultracentrifugation in the presence of 0.2% SDS (Feutreier *et al.*, 1986). The procedure produced high yields of fimbriae of high purity. By SDS-PAGE, the molecular mass of the fimbrin subunit was found to be 14.3 kDa, which agreed with that calculated from the amino acid composition of SEF14. Modifications to this procedure by Müller *et al.* (1991)

were based on the hydrophobicity of SEF14 and the insolubility of SEF21 in boiling SDS. This modified procedure allowed the simultaneous purification of SEF14 and SEF21 and was used in this study.

3.5.2.1 Materials

0.15 M Ethanolamine, pH 10.5. Ethanolamine (4.49 ml) was made up to 500 ml with dist.H_2O.

10 mM Tris HCl, 0.2% (v/v) SDS, pH 7.5. Tris (1.211 g) was dissolved in approximately 800 ml dist.H_2O and the pH was adjusted to 7.5. SDS (2 g) was added and dissolved with stirring, and the volume was made up to 1 litre with dist.H_2O.

Laemmli sample buffer [0.125 M Tris-HCl (pH 6.8), 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 2% (w/v) SDS]. Tris (1.52 g), glycerol (10 ml) and 2-mercaptoethanol (5 ml) were dissolved in approximately 70 ml dist.H_2O. The pH was adjusted to 6.8 and SDS (2g) was added and mixed with stirring. The volume was made up to 100 ml with dist.H_2O.

3.5.2.2 Procedure

S. enteritidis was grown in 1 litre of BPW (Section 2.2) for 18 h at 37°C, harvested by centrifugation (4 000 × g, 10 min, 4°C) and the pellet was resuspended in 60 ml of 0.15 M ethanolamine, pH 10.5, which was found to maintain SEF14 and SEF21 in solution (Müller, *et al.* 1991). Fimbriae were separated from the cells by shearing them in a Dounce homogeniser at RT. (3 × 1 min) after which cells and cellular debris were removed by centrifugation (1,200 × g, 15 min, 4°C). Only trace amounts of fimbriae were found to sediment under the centrifugation conditions used to sediment cellular debris (Müller, *et al.* 1991). The supernatant was centrifuged (100 000 × g, 1 h, 4°C) to remove membrane vesicles and the clarified supernatant was dialysed overnight against 10 mM Tris-HCl (pH 7.5) containing 0.2% SDS, in Snakeskin pleated dialysis tubing (molecular mass cut-off M_r 10 000), in order to precipitate the more hydrophobic SEF14. Precipitated SEF14 was pelleted by centrifugation (15 000 × g, 15 min, 4°C) to separate it from SEF21, which remained in the supernatant fraction.

The supernatant fraction was concentrated approximately three-fold by dialysis against PEG (M_r 20 000) as described in Section 2.9.1. SEF21 was precipitated by addition of 50 ml ice-cold acetone. The precipitated SE21 was recovered by centrifugation (15 000 × g, 20 min, 4°C). The pellet was resuspended in Laemmli sample buffer (4 ml) and boiled (5 min) to solubilise contaminants. The insoluble SEF21 fimbriae were recovered by centrifugation (250 000 × g, 2 h, 4°C), boiled once more with Laemmli sample buffer (4 ml) and centrifuged again (250 000 × g, 2 h, 4°C) to yield pure SEF21.

The SEF14 and SEF21 preparations were subjected to SDS-PAGE and Western blot analysis.

3.5.2.3 Results and discussion

Reducing SDS-PAGE, in conjunction with silver staining, of the precipitated fraction after dialysis against 10 mM Tris-HCl, pH 7.5, containing 0.2% SDS, revealed a protein profile containing two prominent bands, of approximately 21 kDa and 14.5 kDa (Fig. 3.14). These bands correspond with the subunit masses of SEF21 (21.5 kDa) and SEF14 (14.3 kDa) respectively (Thorns & Woodward, 2000).

Partial precipitation of SEF21 during dialysis was speculated to have caused SEF21 contamination of the SEF14 preparation. The SEF14 preparation was again dialysed overnight against 10 mM Tris-HCl (pH 7.5) containing 0.2% SDS, in an attempt to remove the SEF21 contamination. SDS-PAGE and silver staining indicated that the repeated dialysis step had removed the majority of contaminating SEF21, producing a high yield of SEF14 fimbrial subunit (Fig. 3.15). A faint band at 21.2 kDa, indicated the presence of the contaminating SEF21 fimbrial subunit.

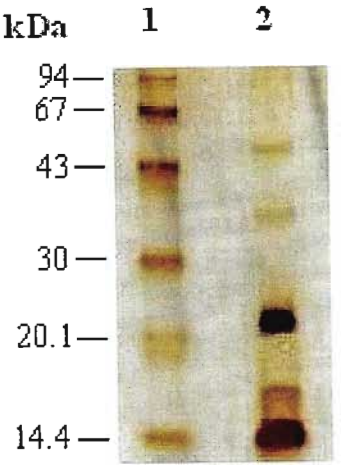

Figure 3.14 Reducing SDS-PAGE of a SEF14 fimbrial protein preparation from *S. enteritidis*. Samples were boiled in reducing buffer, loaded onto a 10% Tris-tricine gel and silver stained. **Lane 1**, Molecular mass markers (as in Fig. 3.2); **Lane 2**, SEF14 fimbrial protein preparation.

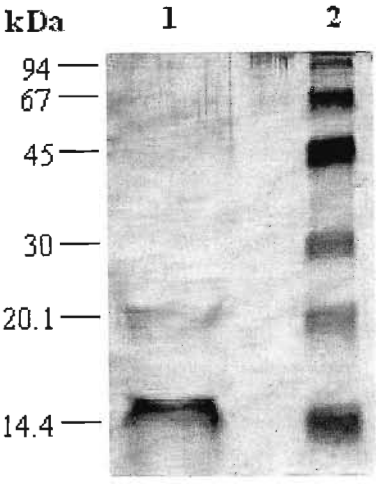

Figure 3.15 Reducing SDS-PAGE of a SEF14 preparation from *S. enteritidis*, after repeated dialysis against 10 mM Tris-HCl (pH 7.5) containing 0.2% SDS. Samples were boiled in reducing buffer, loaded onto a 10% Tris-tricine gel and silver stained. **Lane 1**, SEF14 fimbrial protein preparation; **Lane 2**, Molecular mass markers (as in Fig. 3.2).

The SEF14 and SEF21 preparations were resolved by SDS-PAGE and probed with chicken antibodies raised against *S. enteritidis* (Fig. 3.16), in order to determine whether antibodies specific to the fimbrial subunits of SEF14 or SEF21 were produced by *S. enteritidis*-infected chickens. Most *Salmonella* serotypes express SEF21 fimbriae, with conservation at both molecular and structural levels across the genera. Extensive cross reactivity is seen with antibodies raised against SEF21 fimbriae of *S. enteritidis* (Sojka *et*

al., 1996). SEF21, commonly found to be strongly immunogenic, was surprisingly not visible as a band at 21.5 kDa in the SEF21 preparation (Fig. 3.16). A band that could have been the fimbrial subunit of SEF21, was seen when antibodies raised against *S. berta*, were used to probe the SDS-PAGE-resolved SEF14 preparation (Fig. 3.18, lane 4). SDS-PAGE of the SEF14 preparation did indicate residual SEF21 contamination (Fig. 3.14 and 3.15). The estimated molecular mass of this protein was 18.5 kDa though.

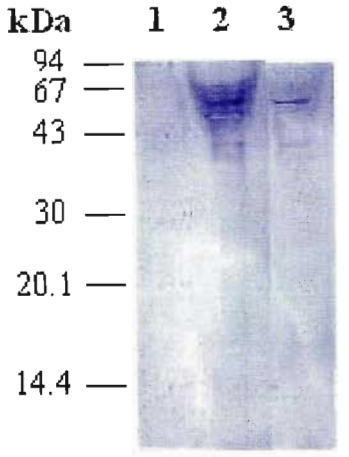

Figure 3.16 Western blot analysis of SEF14 and SEF21 fimbrial proteins from *S. enteritidis* using chicken anti-*S. enteritidis* antibodies. Sample was resolved by reducing Tris-tricine SDS-PAGE, electroblotted onto nitrocellulose and probed with chicken serum containing antibodies against *S. enteritidis*. **Lane 1**, Molecular mass markers (as in Fig. 3.2). **Lane 2**, SEF14 preparation; **Lane 3**, SEF21 preparation.

Chicken antibodies against *S. enteritidis* did produce strong immunogenic reactions with a band at 59 kDa, and to a lesser extent with a band at 40 kDa, in both preparations (Fig. 3.16). Similar reactions were seen when the SEF14 preparation was probed with chicken antibodies raised against *S. gallinarum* and *S. berta* respectively (Fig. 3.18). Like, *S. enteritidis*, S. *gallinarum and S. berta* are serogroup D serotypes. S. *gallinarum* has the intact gene sequence for SEF14, yet does not express the fimbriae, and *S. berta* does not possess the gene sequence (Thorns *et al.*, 1994). The 59 kDa band was suspected to be the same strongly antigenic band that was present at 58.4 kDa in all of the OMP profiles of serotypes tested, except *S. gallinarum* (Fig. 3.4). A faint 50 kDa band was shown to be part of the SEF14 preparation (Fig. 3.14). Repeated dialysis may have reduced the amount of this protein to levels at which it was not visible on a SDS-PAGE gel after dialysis, but it may not have been removed completely from the preparation.

The distribution of SEF14 is restricted to certain serogroup D *Salmonella* (Rajashekara *et al.,* 1998) and specific reactivity to antibodies raised against *S. enteritidis* and SEF14 of *S. enteritidis*, has been reported (Thorns *et al.*, 1990). In this study, a small amount of reactivity was seen between the SEF14 fimbrial subunits (14.3 kDa) and antibodies raised against *S. enteritidis*. A faint band of 14.4 kDa in the SEF14 preparation indicated this reactivity, but not to the extent that was anticipated. When considering the purity of the SEF14 preparation (Fig. 3.15) and the immunogenic nature of SEF14 (Thorns, 1995), a dominant band at approximately 14 kDa would have been expected when probing with anti-*S. enteritidis* antibodies. The serum used in this experiment was obtained from chickens three weeks post-infection (Section 2.10.1.2). As chickens readily seroconvert within ten days of *S. enteritidis* infection and the antibody response persists for at least four weeks post-infection (Thorns *et al.*, 1993), this is well within the range for effective isolation of serum anti-*S. enteritidis* antibodies. Expression of SEF14 during infection of the birds could have been minimal, resulting in a low titre of SEF14-specific antibodies in the sera of infected birds.

Due to this low titre of specific antibodies, SEF14 was immobilised on an affinity column and the sera of *S. enteritidis*-infected chickens were passed through the column (Section 2.10.4). This purification step was used to obtain higher concentrations of SEF14-specific antibodies. However, a Western blot using these antibodies to probe for SEF14 (Fig. 3.17) showed little difference from results previously obtained with anti-*S. enteritidis* antibodies (Fig. 3.16). Once again, SEF14 fimbrial subunits (14.3 kDa) indicated immunogenicity, but not to the extent shown by the band at 58 kDa. It would appear that although this 58 kDa protein was present in the SEF14 preparation in small quantities, the protein was highly immunogenic, probably more than SEF14, and led to the purification of antibodies specific for this protein when the sera of birds infected with *S. enteritidis* was passed through the affinity purification column. This 58 kDa protein, although highly immunogenic, showed no diagnostic potential for *S. enteritidis* as it appeared common to the *Salmonella* serotypes tested.

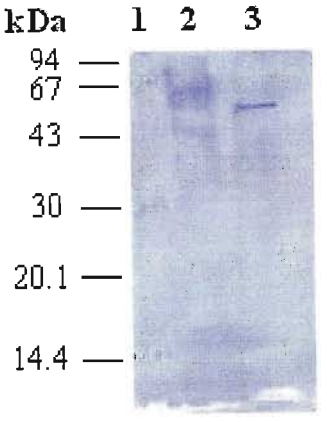

Figure 3.17 Western blot analysis of SEF14 and SEF21 fimbrial proteins from *S. enteritidis* using chicken anti-SEF14 antibodies. Sample was resolved by reducing Tris-tricine SDS-PAGE, electroblotted onto nitrocellulose and probed with chicken serum containing antibodies against *S. enteritidis* SEF14 fimbrial protein. **Lane 1**, Molecular mass markers (as in Fig. 3.2). **Lane 2**, SEF14 preparation; **Lane 3**, SEF21 preparation.

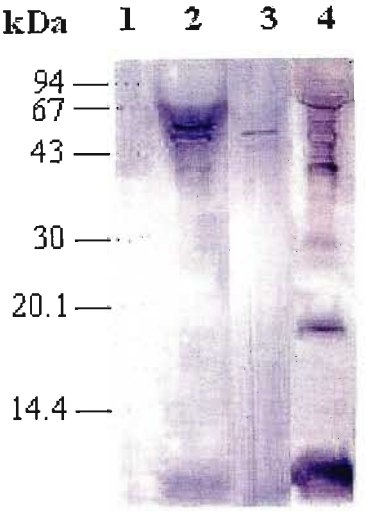

Figure 3.18 Western blot analysis of SEF14 fimbrial protein from *S. enteritidis*. Sample was resolved by reducing Tris-tricine SDS-PAGE, electroblotted onto nitrocellulose and probed with chicken serum containing antibodies against: **Lane 2**, *S. enteritidis* ATCC 13076; **Lane 3**, *S. gallinarum* ATCC 9182; Lane 4, *S. berta* ATCC 8392. **Lane 1**, Molecular mass markers (as in Fig. 3.2).

Murein lipoprotein (7.2 kDa) appeared to have been isolated in the SEF14 preparation. This protein appeared as a band of 7 kDa when the SEF14 preparation was probed with antibodies against *S. enteritidis* and other *Salmonella serotypes* (Fig. 3.17 and Fig. 3.18). The antigenicity of this protein has been shown when resolved by SDS-PAGE, as part of the OMP fraction, and probed with antibodies against *Salmonella* serotypes (Fig. 3.4).

3.6 Conclusions

There is strong evidence that SEF14 is a good candidate antigen for serological detection of *S. enteritidis*. Most techniques described in earlier reports have involved qualitative analysis of the detection *S. enteritidis* (Thorns *et al.*, 1993, Thorns *et al.*, 1996). The techniques discussed in this study required the quantitative analysis of the protein composition of SEF14 preparations. The results discussed here have indicated that these techniques might need a relatively pure preparation of SEF14. Although SDS-PAGE analysis of SEF14 preparations showed slight contamination, Western blot analysis revealed cross-reactive proteins present in the preparation. It would appear that the procedure described by Müller *et al.* (1991), which involves differential precipitation of fimbriae from cellular debris by ammonium sulfate precipitation, produces a crude preparation that is highly contaminated with proteins of varying molecular mass. The method described by Thorns *et al.* (1996), which involved a step-wise purification and extracts SEF14 by means of charged side chains and the molecular size of its subunits, seemed an appropriate means of overcoming this, yet was unsuccessful in this study.

CHAPTER 4

Polymerase chain reaction amplification of the *sefA* gene of *Salmonella enteritidis*

4.1 Introduction

The polymerase chain reaction (PCR) has caused the rapid transformation of many scientific fields. PCR was introduced by Saiki and colleagues in 1985, and has since replaced many standard techniques for DNA and RNA analysis and enabled the development of new techniques, which would not otherwise have been possible (Taylor, 1991).

PCR is based on the DNA polymerase-catalysed extension of a DNA molecule. DNA polymerases are enzymes that catalyse the 5' to 3' synthesis of a complementary strand of DNA, using a single-stranded DNA template, but requiring a double-stranded starting region. For PCR, an oligonucleotide primer bound to a specific sequence on the template DNA supplies this double-stranded starting region. Two primers of different sequence are used and each oligonucleotide in the primer pair is complementary to sequences on both ends of a target DNA sequence, yet complementary to opposite strands of the double-stranded DNA.

The first step in the cyclic PCR amplification procedure is the denaturing of the template DNA, which includes the target sequence, by heat. The DNA is then cooled in the presence of the primers, allowing the primers to anneal to their complementary sequences. DNA polymerase is then added, together with magnesium and the four free deoxyribonucleic acid bases (dATP, dCTP, dGTP and dTTP). The DNA polymerase catalyses primer extension in the direction of the other primer, by adding the free nucleotides one-by-one, to the 3' end of the DNA strand. The cycle is repeated until amplification becomes limited, usually between 30 and 40 cycles. The first few cycles of amplification will yield DNA products with sequences that go beyond the sequence complementary to the other primer. However, further cycles yield a product of defined size, consisting of DNA regions flanked by each primer. This product will accumulate

exponentially, unlike the longer products, which will accumulate in a linear fashion (Sambrook *et al.*, 1989; Giovanonni, 1991).

Originally, Klenow DNA polymerase was used for PCR. However, the enzyme needed replacement after every cycle, as it was not stable at the high temperatures (usually between 90°C and 95°C) needed for denaturing double-stranded DNA. The use of thermostable *Taq* DNA polymerase, isolated from the thermophilic bacterium *Thermus aquaticus* (and currently produced recombinantly), meant that replacement of the DNA polymerase was not required and that annealing and extension of the primers was more specific, due to activity of the DNA polymerase at higher temperatures. The cycling sequence could also be automated, saving considerable time. *Taq* DNA polymerase can synthesise DNA at a theoretical rate of about 150 nucleotides per second per enzyme molecule at around 75-80°C, although polymerisation normally takes place at around 72-75°C in most amplification procedures (Gu, 1995). *Taq* polymerase is, however, limiting after the amplification factor has reached about 10^7, which is why PCR amplification is generally restricted to about 40 cycles (Sambrook *et al.*, 1989; Taylor, 1991).

PCR has been used for the detection of pathogenic micro-organisms for some time (Hance *et al.*, 1989) and several papers have been published regarding the PCR detection of *Salmonella* using a number of target sequences in genomic and plasmid DNA of the bacteria. PCR has also been investigated as a technique for the specific detection of *S. enteritidis* (Section 1.5.3).

The template DNA to be used in this study was genomic DNA from *S. enteritidis* strain ATCC 13078. The genes that encode for the expression of the SEF14 fimbriae of *S. enteritidis* have been characterised more fully than that of any other *Salmonella* fimbriae. These genes are located on a small pathogenicity island of the *S. enteritidis* genome, known as the *sef* operon.

The *sefABCD* genes make up part of the complex *sef* operon, which is responsible for the biogenesis, translocation and expression of SEF14 by *S. enteritidis*. The nucleotide sequence of the *sef* operon represents the first sequence of a fimbrial operon from *S. enteritidis* (Clouthier *et al.*, 1993, 1994; Collighan & Woodward, 2002).

The *sefA* gene encodes the SEF14 fimbrial subunit, which when compared to the amino acid sequences of proteins listed on Genbank, SWISS-PROT and GENPEPT databases, was shown to be unique (Clouthier *et al.*, 1993). The *sefB* gene encodes subunit transport proteins homologous to *E. coli* and *Klebsiella pneumoniae* fimbrial periplasmic chaperone proteins. This made the *sefB* gene the first chaperone protein gene to be characterised from *Salmonella* species (Clouthier *et al.*, 1993). The *sefC* gene encodes fimbrial usher proteins located in the outer membrane and the *sefD* gene codes a fimbrial adhesin distinct from SEF14, given the name SEF18 (Clouthier, 1994, Edwards *et al.*, 2000). Adjacent to *sefD*, there is a regulatory protein, encoded by *sef*R, which activates transcription of the *sef* genes (Fig. 4.1).

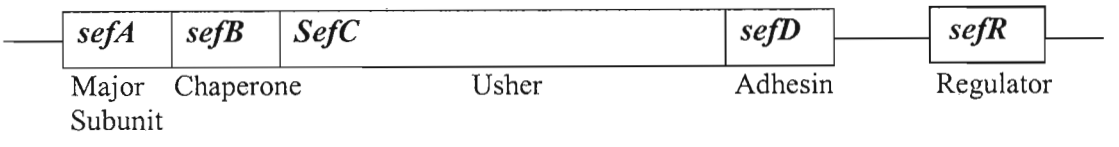

Figure 4.1 The *sef* pathogenicity island showing organisation of the *sef* genes (Clouthier *et al.*, 1993)

The *sefABC* genes are transcribed as part of a single mRNA transcript. However, *sefB* and *sefC* were not expressed in the absence of *sefA*, and no transcription sites were found immediately upstream of *sefB* or *sefC*. Transcription of *sefB* and *sefC* was initiated from the *sefA* promoter region (Clouthier *et al.*, 1993).

Turcotte & Woodward (1993) used a radiolabelled probe and colony hybridisation to test 74 serotypes, representing 17 serogroups of *Salmonella*, for the presence of the *sefA* gene sequence. As discussed in Section 1.5.2, the distribution of the *sefA* gene sequence was restricted to serotypes of serogroup D *Salmonella*. Of these serotypes, *S. enteritidis* was the only poultry pathogen that expressd the gene product, a novel fimbrin, which is the structural subunit of the SEF14 fimbrial protein (Turcotte & Woodward, 1993). The *sefA* gene sequence was therefore used as a target sequence for the detection of *S. enteritidis* in poultry and poultry products.

Clouthier *et al.* (1993) determined the DNA sequence of the *sefA* gene (Fig. 4.4), allowing the development of oligonucleotide primer pairs for specific detection of the *sefA* gene sequence and potentially *S. enteritidis*, if detected in poultry samples.

4.2 Oligonucleotide primers

When designing oligonucleotide primers, a number of factors are typically considered. Oligonucleotide primers should be at least 16 nucleotides in length and are generally synthesised in the range 18-30 nucleotides. Nucleotides of this length are too short to form stable hybrids at the temperature used for polymerisation and extension (72°C). Primer sequences should have a similar G+C content, minimal secondary structure (self-complementarity) and low complementarity to each other, particularly in the 3' region (Taylor, 1991; Innis & Gelfand, 1993). Woodward & Kirwan (1996) designed an oligonucleotide primer pair (Fig. 4.2) used for specific detection of a 310 bp region of the *sefA* gene of *S. enteritidis,* in accordance with these basic principles of primer design.

SEFA-2 5'- GCA GCG GTT ACT ATT GCA GC -3' (Forward)
SEFA-4 5'- CTG TGA CAG GGA CAT TTA GCG -3' (Reverse)

Figure 4.2 Oligonucleotide primer pair designed by Woodward & Kirwan (1996) for the detection of the *sefA* gene sequence of *S. enteritidis.*

However, Szabo & Mackey (1999) modified this primer pair by adding a cytosine base to the 3' end of the reverse primer in order to balance the G+C content of each primer (Fig. 4.3). This ensures that oligonucleotide primers have similar denaturation or "melting" (T_m) temperatures.

SEFA-1 5'- GCA GCG GTT ACT ATT GCA GC -3' (Forward)
SEFA-2 5' CTG TGA CAG GGA CAT TTA GCG -3' (Reverse)

Figure 4.3 Oligonucleotide primer pair designed by Szabo & Mackey (1999) for the detection of the *sefA* gene sequence of *S. enteritidis.*

Sequence analysis using the Net Primer® software available from Premier Biosoft International (Palo Alto, CA, USA) showed that the G+C content of the forward and reverse primers was 55% and 50% respectively and the T_m of the primers was 58.78°C and 60°C respectively.

Szabo & Mackey (1999) also modified the PCR amplification procedure used by Woodward & Kirwan (1996). When compared with the procedure used by Woodward & Kirwan (1996), the modified procedure of Szabo & Mackey (1999) had dropped the denaturing and annealing temperatures by 1°C each, to 94°C and 60°C respectively reflecting the T_m of the modified primer pair. The orientation and position of the primer pair SEFA-1 and SEFA-2 (Szabo & Mackey, 1999) with respect to the *sefA* gene sequence data is shown in Fig. 4.4.

The modified oligonucleotide primer pair described by Szabo & Mackey (1999) was used in this investigation. However, before use, the primers were analysed for their potential to form secondary structures using the Net Primer® software (Fig. 4.5, Fig. 4.6 and Fig. 4.7).

Sequence analysis showed that a number of secondary structures could potentially have formed when using the primers. Self-complementarity was possible with each of the primers. The SEFA-1 primer could form a 3' hairpin as well as two types of 3' dimers (Fig. 4.5) and the SEFA-2 primer was able to form a single 5' dimer (Fig. 4.6). However, other secondary structures, including palindromes and repeats were not found to be possible for each of the primers. When used in conjunction, the primer pair could possibly form two types of 3' cross-dimers (Fig. 4.7).

```
LOCUS       STYSEFA                  498 bp    DNA     linear   BCT 26-JUL-1993
DEFINITION  Salmonella enteritidis fimbrial protein (sefA) gene, complete cds.
ACCESSION   L11008
VERSION     L11008.1  GI:310645
KEYWORDS    fimbrial protein; sefA gene.
SOURCE      Salmonella enteritidis
  ORGANISM  Salmonella enteritidis
            Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales;
            Enterobacteriaceae; Salmonella.
REFERENCE   1  (bases 1 to 498)
  AUTHORS   Clouthier,S.C., Muller,K.H., Doran,J.L., Collinson,S.K. and
            Kay,W.W.
  TITLE     Characterization of three fimbrial genes, sefABC, of Salmonella
            enteritidis
  JOURNAL   J. Bacteriol. 175 (9), 2523-2533 (1993)
  MEDLINE   93239677
   PUBMED   8097515
COMMENT     Original source text: Salmonella enteritidis (individual_isolate 3B) DNA.
FEATURES             Location/Qualifiers
     source          1..498
                     /organism="Salmonella enteritidis"
                     /mol_type="genomic DNA"
                     /isolate="3B"
     gene            1..498
                     /gene="sefA"
     CDS             1..498
                     /gene="sefA"
                     /codon_start=1
                     /transl_table=11
                     /product="fimbrial protein"
                     /protein_id="AAA27219.1"
                     /db_xref="GI:310646"
                     /translation="MRKSASAVAVLALIACGSAHAAGFVGNKAVVQAAVTIAAQNTTS
                     ANWSQDPGFTGPAVAAGQKVGTLSITATGPHNSVSIAGKGASVSGGVATVPFVDGQGQ
                     PVFRGRIQGANINDQANTGIDGLAGWRVASSQETLNVPVTTFGKSTLPAGTFTATFYV
                     QQYQN"

BASE COUNT      121 a    118 c    126 g    133 t
ORIGIN
        1 atgcgtaaat cagcatctgc agtagcagtt cttgctttaa ttgcatgtgg cagtgcccac
       61 gcagctggct ttgttggtaa caaagcagtg gttcaggcag cggttactat tgcagctcag
                                          Primer SEFA-1 ---->
      121 aatacaacat cagccaactg gagtcaggat cctggcttta cagggcctgc tgttgctgct
      181 ggtcagaaag ttggtactct cagcattact gctactggtc cacataactc agtatctatt
      241 gcaggtaaag gggcttcggt atctggtggt gtagccactg tcccgttcgt tgatggacaa
      301 ggacagcctg ttttccgtgg gcgtattcag ggagccaata ttaatgacca agcaaatact
      361 ggaattgacg ggcttgcagg ttggcgagtt gccagctctc aagaaacgct aaatgtccct
                                          <---- Primer SEFA-2
      421 gtcacaacct ttggtaaatc gaccctgcca gcaggtactt tcactgcgac cttctacgtt

      481 cagcagtatc aaaactaa
//
```

Figure 4.4 Complete Genbank sequence entry encompassing the *sefA* gene (Genbank accession number L11008). The position of the oligonucleotide primers SEFA-1 and SEFA-2 are underlined. Arrows show the orientation of each primer.

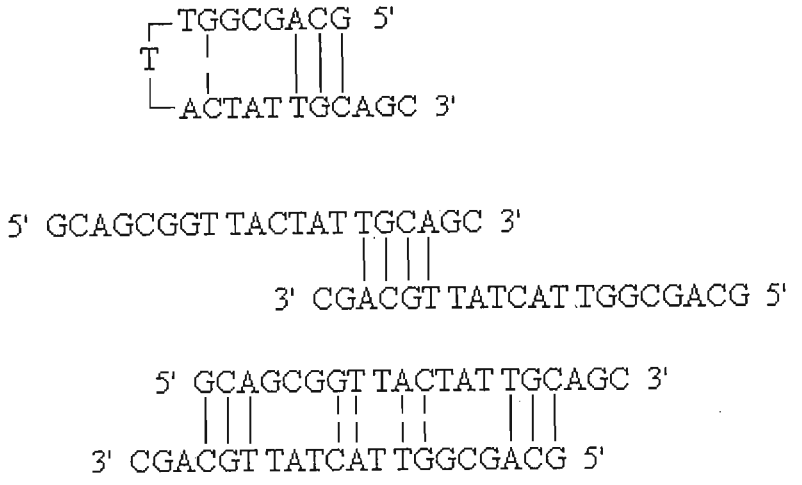

Figure 4.5 Potential secondary structures of the SEFA-1 oligonuceotide primer.

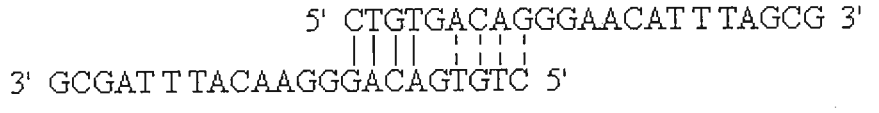

Figure 4.6 Potential secondary structure of the SEFA-2 oligonucleotide primer.

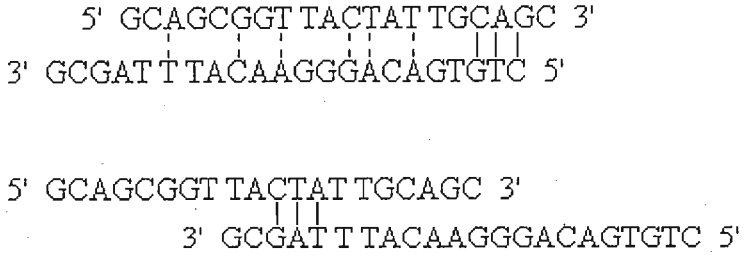

Figure 4.7 Potential secondary structures of the SEFA-1 and SEFA-2 oligonucleotide primer pair.

Although the oligonucleotide primer pair could have formed potentially problematic secondary structures upon annealing (especially 3' cross dimers of the primer pair), the primers were synthesised for use. Each of the oligonucleotide primers is generally used in large molar excess (Sambrook *et al.,* 1989). A large proportion of oligonucleotide primers would therefore still bind to their target sequences in the event of some oligonucleotides forming dimers or hairpins, and amplification of the target sequence would still occur.

However, using oligonucleotides at an excessive concentration can cause random priming, leading to amplification of undesirable non-target sequences (Sambrook *et al.*, 1989).

4.3 Purification of bacterial genomic DNA using phenol:chloroform:isoamyl alcohol

To determine whether the target sequence within the *sefA* gene of *S. enteritidis* could be amplified, genomic (chromosomal) DNA of *S. enteritidis* was purified for use as template DNA. Genomic bacterial DNA has conventionally been purified by means of phenol:chloroform:isoamyl alcohol extraction.

Bacterial cells are mixed with the detergent SDS and Proteinase K. The denaturing anionic detergent SDS binds to all proteins within the bacterial cells, disrupting most noncovalent intermolecular and intramolecular protein interactions. Proteinase K is a non-specific serine protease, which together with SDS causes the cells to lyse. Proteinase K has strong proteolytic activity even in the presence of SDS and rapidly inactivates endogenous nucleases in the lysed cells (Maloy, 1990). Phenol:chloroform:isoamyl alcohol (25:24:1) is added to the mixture resulting in the separation of DNA and protein. Liquid phenol is known to be an excellent solvent for many proteins. The partition coefficient in biphasic phenol-water mixtures very often allows an almost complete extraction of proteins from aqueous solutions. In contrast, nucleic acids are generally water-soluble but phenol-insoluble. Phenol is a weak acid, the dissociation constant at 18-19°C in water being $1.1\text{-}1.2 \times 10^{-10}$. Both the favourable partition coefficient and the dissociation power of phenol-water mixtures often affect separation of proteins from nucleic acids by phenol-water (Westphal & Jann, 1965). The phenol-protein phase can then be discarded and isopropanol used to precipitate the DNA from the aqueous phase.

Although this method does yield genomic DNA from bacteria, the quality of the DNA preparation is generally low. This is due to contamination with residual protein as well as phenol, which has an inhibitory effect on PCR amplification (Giovanonni, 1991).

4.3.1 Materials

TE buffer [10 mM Tris-HCl, 1 mM EDTA, pH 8.0]. Tris (0.303 g) and EDTA (0.093 g) were dissolved in approximately 180 ml of dist.H_2O. The pH was adjusted to 8.0 with HCl and the volume was made up to 250 ml with dist.H_2O. The buffer was autoclaved (121°C, 15 min).

500 mM Tris-HCl, pH 8.0. Tris (30.28 g) was dissolved in approximately 450 ml of dist.H_2O, the pH was adjusted to 8.0, and the volume was made up to 500 ml with dist.H_2O. The buffer was autoclaved (121°C, 15 min).

100 mM Tris-HCl, pH 8.0. Tris (6.06 g) was dissolved in approximately 450 ml of dist.H_2O, the pH was adjusted to 8.0, and the volume was made up to 500 ml with dist.H_2O. The buffer was autoclaved (121°C, 15 min).

10% (w/v) SDS. SDS (1 g) was dissolved in 10 ml dist.H_2O.

Phenol equilibrated to pH 7.0. Phenol crystals were melted at 68°C in a fume hood and hydroxyquinoline (0.1% w/v) was added. An equal volume of 500 mM Tris-HCl, pH 8.0 was added and stirred for 15 min, after which the solution was allowed to settle. As much of the aqueous phase as possible, was removed. An equal volume of 100 mM Tris-HCl, pH 8.0 was added, stirred for 15 min, and the aqueous layer removed. This was repeated until the pH of the phenol reached pH 7.0. When equilibrated, 0.1 vol of 100 mM Tris-HCl was added and the phenol was stored in an amber bottle at 4°C for up to a month.

Chloroform:isoamyl alcohol [24:1]. Chloroform (120 ml) and isoamyl alcohol (5 ml) were added to an amber bottle, mixed and stored at RT.

Proteinase K [20 mg/ml]. Proteinase K (2 mg) was dissolved in 100 µl sterile dist.H_2O. Aliquots (20 µl) were stored at -20°C.

4.3.2 Procedure

The procedure used to purify bacterial genomic DNA was essentially that described by Maloy (1990). Separate cultures of *S. enteritidis* ATTC 13076, *S. typhimurium* ATTC 14028, *S. gallinarum* ATTC 9182, *S. blegdam* ATTC 8392 and *E. coli* JM103, *Staphylococcus aureus* ATCC 25923, *Psuedomonas aeruginosa* ATCC 27853 and *Bacillus subtilis* ATCC 6051 were grown overnight in 5 ml LB broth (Section 2.2.1). An aliquot (1.5 ml) of each culture was added to a microfuge tube and centrifuged (30 sec, 13 000 × g, RT). The supernatant was poured off and the tubes refilled and centrifuged again. The pellet was resuspended in 567 µl TE buffer and vortexed thoroughly to mix. SDS (10 % (w/v), 30 µl) and Proteinase K (3 µl) were added to each tube, which were mixed by inverting, and incubated at 37°C for 1 hr. Phenol:chloroform:isoamyl alcohol (25:24:1) (0.7 ml) was added. This was mixed thoroughly by inverting the tube until an emulsion had formed and then spun for 5 min in a microfuge. The supernatant was carefully removed using a Pasteur pipette and transferred to a clean microfuge tube. Isopropanol (0.6 vol.) was added to the supernatant to precipitate the DNA. The tube was gently inverted until stringy white DNA fibres became apparent. The tubes were centrifuged in a microfuge for 10 min at 13 000 rpm after which the supernatant was poured off and 1 ml of ice cold 70 % ethanol was added. The tubes were spun in a microfuge for 5 min. The supernatant was poured off, the tubes drained, and the DNA dried completely. The DNA was resuspended in 100 µl TE buffer by gently inverting the tubes overnight a 4°C. The quantity and quality of the DNA preparation was determined using ultraviolet light absorption (Section 4.5), following 1% (w/v) agarose gel electrophoresis (Section 4.7). The DNA samples were stored at -20°C.

4.4 Kit purification of bacterial genomic DNA

For comparison with the conventional method of DNA purification (Section 4.3), bacterial genomic DNA was isolated using the Nucleospin Tissue kit from Macherey-Nagel, Dűren, Germany. This kit was designed for rapid, small-scale preparation of genomic DNA from many sources, including bacteria, and removes the need for phenol during the extraction procedure (Macherey-Nagel, 2001).

4.4.1 Materials

Buffers, T1, B1, BW, B5 concentrate, BE, reagent B2, lyophilised Proteinase K, Nucleospin Tissue columns and 2 ml collecting tubes were provided in the Nucleospin Tissue Kit.

Buffer B3, Proteinase K solution and buffer B5 were prepared according to the manufacturer's instructions (Macherey-Nagel, 2001). Buffer B3 was prepared by transferring the total contents of buffer B1 to reagent B2 and mixing well. Proteinase K solution was prepared by adding 1.35 ml dist.H_2O to 30 mg lyophilised Proteinase K and mixing well. This solution was stored at -20°C. Buffer B5 working solution was prepared by adding 96-100% ethanol to the buffer B5 concentrate. For 10 preparations, 16 ml ethanol was added to 4 ml buffer B5 concentrate and mixed well.

4.4.2 Procedure

DNA was isolated according to the manufacturer's instructions (Macherey-Nagel, 2001). Before starting the procedure, water baths were set at 56°C and 70°C respectively and before elution, elution buffer BE was equilibrated to 70°C.

Separate cultures of *S. enteritidis* ATTC 13076, *S. typhimurium* ATTC 14028, *S. gallinarum* ATTC 9182, *S. blegdam* ATTC 8392 and *E. coli* JM103 were grown overnight and 1 ml of each of these cultures was centrifuged for 5 min at 7 500 rpm. The supernatant was discarded and the pellet resuspended in 170 µl buffer T1. Proteinase K (25 µl) solution was added, the solution was vortexed vigorously and incubated, with occasional vortexing, at 56°C until complete lysis was obtained (1-3 h). The sample could also be incubated overnight. After vortexing the samples, 200 µl B3 was added and mixed by vortexing vigourously. The sample was incubated at 70°C for 10 min, vortexed, followed by addition of 210 µl ethanol and immediate vortexing. The addition of ethanol and the chaotropic salts in buffer B3 to the lysate provided the appropriate conditions for the binding of DNA to the silica membrane in the Nucleospin Tissue columns in the following step of the procedure (Macherey-Nagel, 2001).

For each sample, one Nucleospin Tissue column was placed into a 2 ml collecting tube and the sample was applied to the column. The tube was centrifuged (10 000 rpm, 1 min) in a microfuge, the flowthrough was discarded and the column was placed back in the collecting tube. Buffer BW (500 µl) was added to the column and the column was centrifuged (10 000 rpm, 1 min), the flowthrough was discarded and the column placed back in the collecting tube. Buffer B5 (600 µl) was added to the column and the column was centrifuged (10 000 rpm, 1 min), the flow-through was discarded and the column placed back in the collecting tube. By passing buffers BW and B5 through the matrix of the column, contaminants were removed, leaving pure DNA bound to the silica membrane of the column. Residual ethanol was removed by centrifuging the column for 3 min at full speed.

DNA was eluted from the column by placing the column into a 1.5 ml microfuge tube and adding 200 µl prewarmed elution buffer BE (70°C). The column was closed, incubated for 2 min at 70°C and centrifuged at full speed for 1 min. The quantity and quality of the DNA preparation was determined using ultraviolet light absorption (Section 4.5).

4.5 DNA quantification by ultraviolet absorption

245 µl dist.H_2O and 5 µl of prepared DNA were mixed in a clean microfuge tube and transferred to a quartz cuvette. The absorbance was measured at 260 nm and 280nm. The DNA concentration was calculated by using the following equation:

$$\text{Concentration of DNA } (\mu g/\mu l) = A_{260} \times 0.05 \times \text{dilution factor}$$

The ratio between the absorbance readings at 260 nm and 280 nm (A_{260}/A_{280}) provides a quantitative estimate of the purity of the DNA preparation. An A_{260}/A_{280} ratio of 1.8 is indicative of a pure DNA preparation, with contamination by proteins or phenol resulting in an A_{260}/A_{280} ratio of less than 1.8, due to the increased absorbance at 280 nm, caused by these contaminants (Sambrook *et al.*, 1989).

4.6 PCR amplification of the *sefA* gene

S. enteritidis is the only poultry pathogen that expressd the gene product, a novel fimbrin, which is the structural subunit of the SEF14 fimbrial protein (Turcotte & Woodward, 1993). The objective of this part of the study was the PCR amplification of part of the *sefA* gene sequence, which encodes this fimbrin using oligonucleotide primers described by Szabo & Mackey, 1999).

4.6.1 Materials

All reagents were kept on ice until use. Recombinant *Taq* DNA polymerase, 10 × PCR buffer and the dNTP mixture for PCR were provided in the *TaKaRa Taq*™ kit from Takara Biomedical Co., Ltd. Shiga, Japan.

Recombinant *Taq* DNA polymerase [1unit/µl]. Recombinant *Taq* polymerase from the *TaKaRa Taq*™ kit (2 µl) was made up to 10 µl with sterilised dist.H_2O.

Supplied 10 × PCR buffer [100 mM Tris HCl (pH 8.3), 500mM KCl, 15 mM $MgCl_2$].

Supplied dNTP mixture [2.5 mM of each dNTP (pH 7-8)].

Template DNA [0.1 µg/µl]. Template DNA purified using the methods described in Sections 4.2 and 4.3 was diluted to a concentration of 0.1 µg/µl with sterilised dist.H_2O.

Oligonucleotide primers [10 pmol/µl]. Oligonucleotide primers were diluted to a concentration of 10 pmol/µl with sterilised dist.H_2O.

4.6.2 Procedure

The reaction mixture was prepared on ice, by mixing the components indicated in Table 4.1, in 0.5 ml thin wall PCR tubes. A microprocessor-controlled thermal cycler (Perkin Elmer Gene Amp PCR system 2400; Perkin Elmer, Norwalk, CT, USA) was used for automated temperature control during the PCR process. The tubes were placed in the thermal cycler and thermal cycling was started immediately. The DNA was denatured at 94°C for 5 min, followed by amplification through 40 cycles of denaturing, annealing and

extension. The amplification cycle included subjecting the samples to 94°C for 45 sec (denaturation of DNA), 60°C for 45 sec (annealing of primers) and 72°C for 1 min (DNA synthesis and extension). A final DNA extension took place at 72°C for 5 min followed by storage of the samples at 4°C. Identification of the resultant DNA bands was accomplished by comparison with 100 bp ladders of molecular mass markers after agarose gel electrophoresis and staining with ethidium bromide (Sambrook *et al.*, 1989).

Table 4.1 Reaction mixture for PCR amplification of the *sefA* gene of *Salmonella enteritidis*.

Component	Volume (µl)
Sterile dist.H_2O	37
10 × PCR buffer	5
dNTP mixture	4
Forward primer	1
Reverse primer	1
Template DNA	1
Taq DNA polymerase	1

4.7 Agarose gel analysis of PCR amplification products

Agarose is a purified form of a linear galactose-containing aerogel-xerogel hybrid colloid isolated from agar or recovered directly from agar-bearing marine algae. The primary structure of agarose is a repeating agarobiose unit consisting of alternating 1,3-linked β-D-galactopyranose and 2,4-linked 3,6-anhydro-α-L-galactopyranose moieties. The secondary structure of agarose consists of linear polysaccharide chains linked by non-covalent bonds (hydrogen bonds) (Dean *et al.*, 1985).

Agarose has considerable gel strength and relative biological inertness and is therefore suitable for gel filtration. Agaroses with varying characteristics were initially used for protein separation. The basis for these separations was the sieving of charged molecules according to their sizes, with electrophoretic movement of larger molecules being restricted more than that of smaller molecules. This concept was applied to the separation of DNA, with a number of new specialised agaroses developed for DNA ranging from greater than 10 megabases to as small as 8 bp (Allen & Budowle, 1994). On application of

an electric field, negatively-charged DNA molecules migrate toward the positively charged anode at a rate determined by factors such as the molecular size and conformation of the DNA molecules, concentration of the agarose and the composition of the electrophoresis buffer (Sambrook *et al.*, 1989).

The concentration and molecular mass of electrophoretically-separated double-stranded DNA fragments can be determined by visual comparison with DNA fragments of known molecular mass and concentration. A 123 bp DNA ladder (Invitrogen Corp, USA) was used for this purpose during this investigation. A 123 bp ladder is suitable for sizing double-stranded DNA from 123 to 3 075 bp. The ladder is prepared from a plasmid containing repeats of a 123 bp DNA fragment and consists of 34 fragments ranging in length from 123 to 4 182 bp. Up to 25 separate bands can be clearly visualised on 0.6% to 1.5% agarose gels after ethidium bromide staining (Invitrogen Products, 2003)

The most common means of visualising electrophoretically-separated DNA fragments is by ultraviolet illumination after staining with the fluorescent dye ethidium bromide. This dye contains a planar group capable of intercalating between the stacked bases of DNA. This intercalated dye has an increased fluorescent yield in comparison to free dye in solution. The dye-DNA complex is capable of absorbing ultraviolet radiation at 302 and re-emitting at 590 nm in the orange-red range of the visible spectrum. Absorption at 366 as well as 254 nm produce less fluorescence and additionally cause photonicking and photobleaching. The sensitivity of this technique is limited to approximately 5 ng of double-stranded DNA (Sambrook *et al.*, 1989).

4.7.1 Materials

50 × Tris-acetate stock solution (TAE buffer) [2 M Tris-acetate, 1 mM EDTA, pH 8.0]. Tris (242 g) was dissolved in 800 ml dist.H_2O, glacial acetic acid (57.1 ml) was added, and made up to 900 ml with dist.H_2O. EDTA (18.612 g) was dissolved in 90 ml dist.H_2O, the pH was adjusted to 8.0 with glacial acetic acid and the solution was made up to 100 ml with dist.H_2O. The EDTA and Tris solutions were combined and autoclaved (121°C, 15 min).

1 × TAE working buffer. 50 × TAE buffer (20 ml) was diluted to 1 litre with dist.H_2O.

1.5 % (w/v) Agarose. Agarose (0.45 g) was mixed in TAE working solution (30 ml). The agarose was dissolved as described in Section 4.7.2.

Ethidium bromide solution (10 mg/ml). Ethidium bromide (0.05 g) was dissolved in dist.H_2O (5 ml) and stored in a bottle wrapped in aluminium foil.

Gel-loading buffer [1 mM EDTA, 0.5 % (w/v) bromophenol blue, 50 % (v/v glycerol]. EDTA (0.007 g) and bromophenol blue (0.1 g) were dissolved in dist.H_2O (10 ml) and glycerol (10 ml) was added and mixed in thoroughly.

123 bp Molecular mass markers. 123 bp DNA ladder (5 µl) was mixed with gel-loading buffer (2 µl).

4.7.2 Procedure

The gel casting apparatus was prepared by sealing the open ends of a clean, dry perspex casting tray (100 × 66 mm) with masking tape and placing the tray on a horizontal surface. The agarose solution was placed in a beaker and the beaker was weighed. The solution was heated in a microwave oven, with intermittent swirling, until the agarose was dissolved. The beaker was weighed again and hot water was added to the original weight. The solution was cooled to 60°C and 2.5 µl of ethidium bromide solution was added to the hot agarose. The hot agarose was poured into the gel casting apparatus, the 8 well comb was placed in position, and the gel was allowed to cool for approximately 15 min.

Once the gel had set completely, the comb and masking tape were removed and the tray was positioned in the electrophoresis tank. TAE working solution was added to the tank to cover the gel and 1.5 µl of ethidium bromide solution was added to the anodic side of the tank, as ethidium bromide migrates in the opposite direction to DNA, in an electric field (Sharp *et al.*, 1973).

Gel loading buffer (2 µl) was added to the PCR amplification product (5 µl) prior to loading, increasing the density of the sample to permit accurate loading, as well as providing a visual marker (bromophenol blue) for observation of electrophoretic progress. Electrophoresis was carried out at 100 V for 5 min to allow the sample to migrate from the wells into the gel. The voltage was adjusted to 60 V and electrophoresis was carried out for approximately 45 min. The casting tray and gel were removed from the electrophoresis tank and the gel was examined on an ultraviolet transilluminator, prior to examination using a Kodak 1DL.E35 scientific imaging system and photographing with a Kodak DC 290 digital camera.

4.8 Results and discussion

Examination of the PCR products showed that amplification of a single 320 bp product had taken place when using the genomic DNA of *S. enteritidis* and *S. gallinarum* (Fig. 4.8). This was visualised as a single prominent band in each lane corresponding to these serotypes. As agarose gel electrophoresis can only give an estimate of molecular mass, the values cannot be considered as absolute. These bands correspond almost exactly to the 310 bp size of the expected PCR product, and were therefore considered to be the 310 bp product resulting from the amplification of the target sequence of the *sefA* gene, using the primer pair designed by Woodward & Kirwan (1996).

The distribution of the *sefA* gene is limited to certain serogroup D *Salmonella*. These include *S. enteritidis*, *S. gallinarum*, *S. pullorum* *S. dublin*, *S. rostock*, *S. serenban*, *S. moscow*, *S. blegdam* and *S. typhi* strains (Turcotte & Woodward, 1993). This was confirmed by the results of this study. Amplification of this product was not seen with *Salmonella* serotypes other than *S. enteritidis or S. gallinarum*, or with the other non-*Salmonella* bacteria, *E. coli*, *S. aureus* and *P. aeruginosa* (Fig. 4.8). The three controls included in the experiment, also showed that the primers described by Woodward & Kirwan (1996) were specific for the target sequence of the genomic template DNA and that amplification could only occur when *Taq* polymerase was added to the reaction mixture.

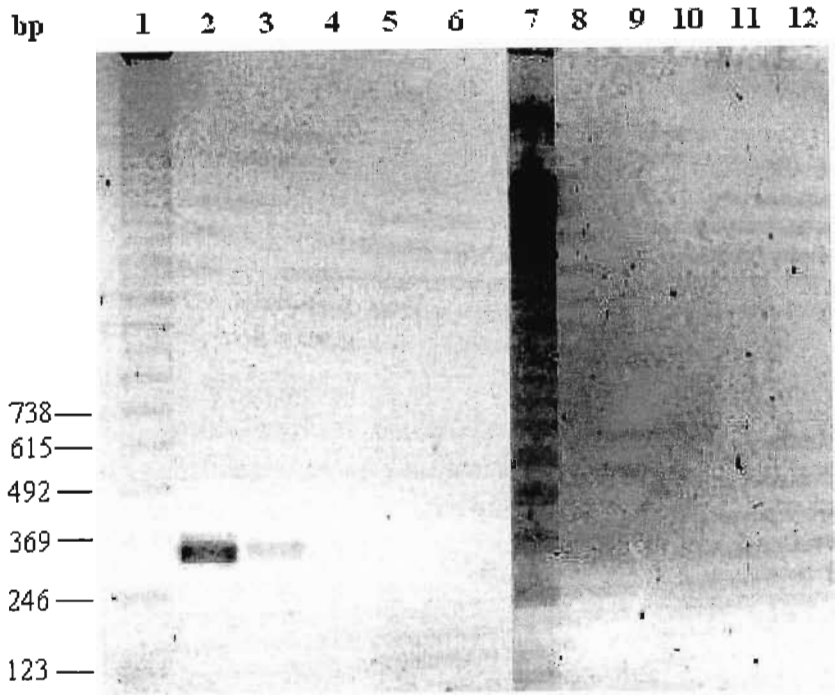

Figure 4.8 Agarose gel from PCR of genomic DNA from bacteria using primers for the specific detection of the *sefA* gene. Lanes 1 and 7, Molecular mass markers (123 bp DNA ladder); **Lane 2**, *S. enteritidis* ATCC 13076; **Lane 3**, *S. gallinarum* ATCC 9182; **Lane 4**, *S. typhimurium* ATCC 14028; **Lane 5**, *S. berta* ATCC 8392, **Lane 6**, *Staphylococcus aureus* ATCC 25923; **Lane 8**, *Pseudomonas aeruginosa* ATCC 27853; **Lane 9**, *E. coli* JM103; **Lane 10**, Control reaction mixture with no primers; **Lane 11**, Control reaction mixture with no template DNA; **Lane 12**, Control reaction mixture with no *Taq* polymerase.

This part of the study also included the comparison of conventional phenol:chloroform:isoamyl alcohol purification with a rapid, kit-based technique for the purification of genomic DNA from *S. enteritidis* (Fig. 4.9). Although both methods purified DNA, as determined by DNA quantification using ultraviolet light absorption (Section 4.5), a visible difference was seen when comparable amounts of the purified genomic DNA from each technique was used as template DNA for PCR amplification of the *sefA* gene. The PCR product of the amplification reaction that used DNA purified with phenol:chloroform:isoamyl alcohol was seen in the expected region of 310 bp, yet a number of fainter bands appeared in positions above and below the band (Fig. 4.9, B). Phenol is known to have an inhibitory effect on PCR amplification, by affecting the function of *Taq* polymerase (Giovanni, 1991). This could have affected the PCR amplification reaction in such as way that resulted in amplification products of variable length. Phenol:chloroform:isoamyl alcohol purification of DNA from *S. enteritidis* also resulted in DNA preparations that were highly contaminated with protein (result not shown). The kit-purified DNA was found to be of high purity, with less protein

contaminants than the DNA purified by conventional means. Visual inspection of the resultant PCR amplification product suggests that the kit-purified DNA produces a singular, sharp band at the expected molecular mass of 310 bp (Fig. 4.9, A).

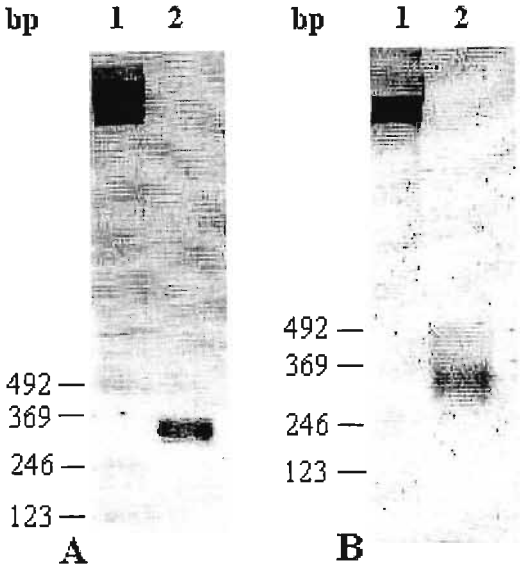

Figure 4.9 Agarose gels from PCR of *S. enteritidis* genomic DNA using primers for the specific detection of the *sefA* gene. Genomic DNA was purified using: A, DNA purification kit; B, Phenol:chloroform:isoamyl alcohol. **Lane 1**, Molecular mass markers (123 bp DNA ladder); **Lane 2**, *S. enteritidis* ATCC 13076.

PCR amplification of the 310 bp region of the *sefA* gene appeared to be specific for the poultry pathogens *S. enteritidis* and *S. gallinarum*. However, worldwide levels of *S. gallinarum* in the poultry industry have been drastically reduced, allowing PCR amplification of this region to potentially be used as a means of detecting *S. enteritidis* in poultry. The method described is simple and rapid, with high levels of specificity. Unlike serological techniques, cross reactivity is virtually eliminated, allowing rapid qualitative analysis of results.

CHAPTER 5

General Discussion

Since the mid-1980s, *Salmonella enteritidis* has become the predominant clinical isolate in human cases of illness, in many countries worldwide (Rajashekara *et al.*, 1999). This is believed to be due to the increased number of poultry products, especially chicken eggs, infected with *S. enteritidis* (Fadl *et al.*, 2002), which has had a significant impact on the economic well-being of the poultry industry worldwide, including South Africa. Unrecognised vertical transmission from foundation stocks to commercial flocks has been suggested to at least be partly responsible for the increased incidence of infection by this serotype (O'Brien, 1990). Based on recommendations from the international community, a *S. enteritidis* reduction plan was introduced to South Africa (Bayer Health Group, 1994). This plan was aimed at keeping foundation stocks free from *S. enteritidis* infection, thus preventing the transmission of *S. enteritidis* through the entire South African poultry industry.

An essential component of the *S. enteritidis* reduction plan was the accurate diagnosis of *S. enteritidis* infection in foundation and commercial flocks. Traditional differentiation amongst the *Salmonella* serotypes and strains has relied on bacteriological examinations, followed by biochemical identification and serotyping. The testing can take up to 7 days yet sometimes not provide a conclusive result (Rajashekara, 1999). Due to the demand for rapid detection of *Salmonella*, there has been a move toward alternative methods, aimed at reducing analytical time and increasing specificity (Swaminathan & Feng, 1994). The majority of these assays are immunological techniques, yet DNA-based assays are rapidly becoming common (Van der Zee & Huis in't Veld, 2000).

Serological assays, such as immunoblotting and enzyme-linked immunosorbent assay (ELISA), are based on the production of circulating immunoglobulins when a host becomes infected with *S. enteritidis*. In studies carried out to investigate the serology of *S. enteritidis* infection in chickens, serum antibodies against *S. enteritidis* antigens were detected for up to nine weeks post-infection (Wysocki *et al.*, 2002). Most successful techniques for the detection of *S. enteritidis* have been ELISA-based, using a number of *S. enteritidis* antigens. These antigens include flagella, lipopolysaccharides, outer

membrane proteins (OMPs) and fimbriae (Barrow, 2000; Thorns, 1995). These are all antigens of the outer surface, which are known to evoke a strong immune response in infected chickens (Cooper & Thorns, 1996). Although ELISA tests are imminently suitable for large-scale screening, nitrocellulose-based assays such as Western blotting have also been shown to possess higher specificity for detection of antibodies to soluble proteins than microtitre plate-based ELISA. In addition, nitrocellulose has a higher binding capacity for proteins, ensuring that the concentration of protein remains constant for the duration of the test (Borden & Kabat, 1986). Cross reactivity of unknown origin was also reported with a number of antigens used in ELISA tests (Tsen, 1994) and bacteriological examination has usually accompanied ELISA testing.

The aim of this study was to evaluate published diagnostic assays for use in local diagnostic laboratories. These assays are concerned with the rapid detection of *S. enteritidis* in chickens, using methods other than conventional bacteriological examination. Many commercially used assays are currently based on the detection of *S. enteritidis* antigens, using specific antibodies. Recently, however, polymerase chain reaction (PCR)-based assays have been used for the rapid detection of pathogenic micro-organisms. These assays are based on the amplification of a sequence of DNA, known to be specific for a micro-organism. The first main objective of this study was the purification of surface exposed *S. enteritidis* antigens and to determine by SDS-PAGE and Western blotting, the specificity of these antigens, when exposed to antibodies raised in chickens against *S. enteritidis* and other *Salmonella* serotypes. The second main objective was to determine whether PCR amplification of a target sequence could be used to detect *S. enteritidis*. These types of detection systems could then be compared according to their specificity, simplicity and how rapidly results were obtained.

As an initial study into the protein components of *Salmonella* serotypes, the whole cell proteins (WCPs) were resolved by SDS-PAGE to determine whether reproducible differences in the electrophoretic banding patterns could be observed. Visual comparison of the WCP patterns, together with identification of prominent proteins, revealed no bands specific to *S. enteritidis*. The WCP fingerprints of each serotype appeared complex and very similar. Therefore, to reduce the complexity of the banding patterns, an attempt was made to purify potential *S. enteritidis*-specific antigens prior to SDS-PAGE and Western blot analysis.

The banding profile of the long-chain lipopolysaccharides (LPS) of *S. enteritidis* and other *Salmonella* serotypes appeared as the characteristic "ladder" patterns described by Cox & Woolcock (1994). However, no distinguishable antigenic markers were observed, either with silver staining after SDS-PAGE, or by means of Western blotting, using sera containing antibodies raised against *S. enteritidis*. A high degree of cross-reactivity, demonstrated by Chart *et al.*, (1990) to be caused by antibodies against common antigens present in *Salmonella* serogroups D and B, was observed with all of the serotypes tested.

OMPs of *Salmonella* are known to evoke an immune response, which we sought to use as a means to differentiate *S. enteritidis* from other *Salmonella* serotypes and non-*Salmonella* bacteria (Section 3.3). There have been reports of protein components of OMP fractions of *S. enteritidis*, which were uniquely antigenic to *S. enteritidis*. When analysing the OMP fraction of *S. enteritidis*, Kim *et al.*, (1991) and Charles *et al.*, (1996) described two protein bands with molecular masses of 43 kDa and 46 kDa which reacted with antibodies to *S. enteritidis*, but not with antibodies to other *Salmonella* serotypes. Surprisingly, these results were not accompanied by figures showing these Western blot results. In this study these protein bands could not be identified using Western blotting of OMPs resolved by reducing SDS-PAGE.

Fadl *et al.*, (2002) identified two OMPs of 75.6 kDa and 82.3 kDa that were more immunogenic and abundantly expressed than the other OMPs of *S. enteritidis*. These two bands were also not detected in this study. However, a number of bands common to all of the *Salmonella* serotypes, as well as *E. coli*, were found to be highly antigenic when exposed to chicken sera containing antibodies against *S. enteritidis*, *S. gallinarum* and *S. berta*. The cross reactivity of the proteins in these bands could be explained by the presence of major OMPs, such as murein lipoprotein and OmpA, which have relatively conserved structures among the *Enterobacteriaceae* (Braun & Rehn, 1969; Lee & Schnaitman, 1980). The molecular masses of some these cross-reactive bands, calculated from the results of SDS-PAGE, corresponded approximately with the molecular masses of known major OMPs, namely OmpA (33 kDa), murein lipoprotein (7.2 kDa) and the precursor of *S. enteritidis* OmpC (41.3 kDa) (Pattery *et al.*, 1999). It also appeared that type 1 fimbriae, which are highly conserved among the *Enterobacteriaceae,* of 20 kDa, could have been isolated with the OMP fraction, seen on SDS-PAGE gels and Western blots as bands at 18.1 kDa.

Protein bands found to be unique on SDS-PAGE gels of *S. enteritidis* OMPs, corresponded to molecular masses of 18.5 kDa, 18.8 kDa and 29.3 kDa, possibly representing OMPs present in lesser, varying amounts, namely OmpH (17.9 kDa), OmpR (27.4 kDa) or Tsx (27 kDa) (Osborn & Wu, 1980). However, none of these bands were seen to be specifically antigenic when reacted with sera containing antibodies to *S. enteritidis.*

To potentially increase the specificity of the Western blotting procedure, with regards to detecting OMP antigens specific to *S. enteritidis*, antibodies against the OMPs of *S. enteritidis* were raised in chickens. Although a 43 kDa band which had not been detected by anti-*S. enteritidis* antibodies was apparent, this band was common to every *Salmonella* serotype tested. Besides visibly stronger antigenicity displayed by the OMPs when blotted with antisera against *S. enteritidis* OMPs, a large amount of cross-reactivity between the serotypes was still apparent.

SDS-PAGE identification of heat-modifiable *Salmonella* OMPs showed a single band at 33.8 kDa when the OMP preparation was heated to 100°C, but not when the preparation was incubated at 0°C, 25°C, 37°C or 60°C. This heat-modifiable protein was identified as OmpA, a protein which when incubated at temperatures lower than 100°C, does not enter the separating gel. When heated to 100°C, OmpA is seen as a band of 33.5 kDa (Chart, 1995). However, Western blot analysis of OMPs of *S. enteritidis* and other *Salmonella* serotypes at 37°C and 100°C did not show OmpA at 33.5 kDa, but rather the presence of a common band at 28.3 kDa, which is not useful for diagnostics. This band was thought to be OmpA, as Beher *et al.*, (1980) have reported that OmpA of *S. typhimurium* and *E. coli* increases in molecular mass from 28 kDa to 33.5 kDa, as the protein denatures upon heating to 100°C.

Western blotting of *S. enteritidis* OMPs did not reveal novel antigens for possible serodiagnosis of *S. enteritidis* infection in chickens. The large number of common antigens among the OMPs *Enterobacteriacae*, especially the porin proteins, showed much cross-reactivity. This prevented the further isolation and characterisation of antigens from the OMP fraction of *S. enteritidis*. However, use of Tris-Tricine SDS-PAGE in conjunction with Western Blot analysis proved to be an effective method of separating and testing OMPs for their reactivity with antibodies raised against *Salmonella* serotypes and their OMP antigens.

Unlike many of the cross-reactive OMPs, the SEF14 fimbrial antigen is a specific structure expressed predominantly by *S. enteritidis*, and is thought to play a role in the tropism of *S. enteritidis* towards reproductive tissues in poultry (Woodward *et al.*, 2000). Though other *Salmonella* serotypes have also been shown to express the SEF14 antigen (Turcotte & Woodward, 1993), many of these serotypes are extremely rare in chickens (Thorns *et al.*, 1992; Rajashekara *et al.*, 1998). These features have made SEF14 an attractive candidate for diagnosing *S. enteritidis* infection in chickens and have led to the development of assays for specific detection of *S. enteritidis* infection in chickens (Thorns *et al.*, 1996; Cooper & Thorns, 1996; Rajashekara *et al.*, 1999).

This study described the comparison of techniques for the purification of SEF14 from *S. enteritidis*. A purification procedure that was based on charges of the side chains, as well as the molecular mass of SEF14 was found to be time consuming and yielded very little SEF14. Aggregation of proteins, which might have been due to the auto-aggregative nature of fimbriae such as SEF17 (Austin *et al.*, 1998), could have led to premature elution of SEF14, from the Sephadex G-100 column used for molecular exclusion chromatography. SDS-PAGE analysis of the eluant of the column did, however, show small amounts of a protein of about 14 kDa, which could have been SEF14. The bands of this size were very faint though, indicating that the yield of this protein was very low.

Another purification technique was based on the shearing of fimbriae from the bacterial surface, prior to manipulations of ionic strength, differential centrifugation and ultracentrifugation in the presence of 0.2% SDS (Feutrier *et al.*, 1986). This relatively rapid technique produced a high yield of a protein at 14.5 kDa, presumed to be the SEF14 fimbrial subunit. Western blotting of the 14.5 kDa band, showed very little binding to chicken anti-*S. enteritidis* antibodies. A faint band at 14.4 kDa was visible though. Minimal expression of SEF14 during infection could have resulted in low titres of SEF14-specific antibodies. SEF14 was therefore immobilised on an affinity column and the sera of chickens infected with *S. enteritidis* were passed through the column in order to concentrate antibodies specific to SEF14. Faint bands at 14.3 kDa indicated the binding of antibodies to the SEF14 antigen, but not to the extent that was anticipated.

The extensive cross-reactivity seen with serological assays used in this study is indicative of the structural and antigenic similarities of the Gram-negative bacteria and in particular,

Salmonella serotyes. A means of overcoming this cross-reactivity is the highly specific DNA-based technique of PCR amplification. PCR has been used for the detection of micro-organisms, including *Salmonella*, for some time (Mahon *et al.*, 1994; Soumet *et al.*, 1999a,b). Although a number of target sequences have been chosen for PCR detection of *S. enteritidis*, none have proven effective in every situation.

The limited distribution of the SEF14 antigen among *Salmonella* serotypes has made the *sefA* and other genes of the *sef* operon, which code for the expression of SEF14, the targets of much research. The distribution of the *sefA* gene sequence is restricted to the serogroup D *Salmonella* serotypes. Of these serotypes, *S. enteritidis* is the only poultry pathogen that expresses the gene product, the fimbrin subunit of SEF14 fimbriae. The *sefA* gene sequence has therefore been used as a target sequence for the detection of *S. enteritidis* in poultry and poultry products (Turcotte & Woodward, 1993).

This study used the oligonucleotide primer pair designed by Woodward & Kirwan (1996) and modified by Szabo & Mackey (1999) for the specific detection of *S. enteritidis* (Chapter 4). The objective of this was the comparison of the PCR amplification technique with the previously described serological assays. The PCR amplification procedure required both positive and negative control reactions in the form of a reaction which contains template DNA from a strain of *S. enteritidis,* and reactions which do not contain vital elements required for amplification, respectively. The positive control reaction showed that amplification of the expected 310 bp fragment had taken place. This was observed as a single prominent band at 310 bp. Amplification of the 310 bp fragment had not occurred in the negative controls, proving that the oligonucleotide primers were specific for the target gene sequence on the template DNA and that amplification had occurred only when *Taq* polymerase was present in the reaction mixture. Except for *S. gallinarum,* amplification of the 310 bp product was not seen with other *Salmonella* serotypes tested or the non-*Salmonella* bacteria tested, namely the Gram-negative rods *Escherichia coli* and *Pseudomonas aeruginosa* and Gram-positive cocci *Staphylococcus aureus*.

Although *S. gallinarum* produced a positive result for PCR amplification of the 310 bp DNA fragment, this was expected, as *S. gallinarum*, like *S. enteritidis*, is a serogroup D serotype. However, as *S. gallinarum* has been eradicated from commercial poultry flocks

and other serogroup D serotypes are not found in poultry (Rodrigue *et al.*, 1990), a positive result in the PCR amplification obtained from a poultry source, could more than likely be considered positive for *S. enteritidis*. This can prove valuable for diagnosis of *S. enteritidis* in samples obtained from the field.

The PCR amplification technique was highly specific for *S. enteritidis* and *S. gallinarum*, with a single prominent band observed. This was in contrast to the serological assays, which had shown numerous bands, with excessive cross-reactivity. Comparison of means of isolating DNA from samples, also showed that kit purification of template DNA had produced clearer, more conclusive results, when compared to template DNA purified with phenol:chloroform:isoamyl alchohol. Kit purification was also more rapid.

Although reports have shown that PCR can be inhibited by certain biological samples (Woodward & Kirwan, 1996), techniques such as immunomagnetic separation (IMS) could concentrate bacteria rapidly, thereby removing potential inhibitors and the need for selective enrichment procedures. Combination of IMS with PCR amplification, based on the detection of the *sefA* gene, could result in a technique able to diagnose *S. enteritidis* infection in chickens within 24 h. This would prove useful in South Africa, where rapid diagnosis of infection could prevent further spread of *S. enteritidis* into the intensive poultry industry, thereby reducing the potentially health risk of *S. enteritidis* to human consumers of poultry products.

The results of this study indicate that the cross-reactive nature of the outer surface antigens of *S. enteritidis* could result in limitations to the published serological assays. The limited specificity of these techniques was shown by the high number of constituent proteins in the purified OMP, LPS and fimbrial fractions, as well as the cross-reactivity of these antigens. This meant that identification of prominent, antigenic proteins, which were used in published assays was not possible. Successful serological assays require identification of antigens and antibodies that show specificity for one another in such a way, that the assay is reproducible. This was not the case for the serological assays evaluated in this study.

Although the serological identification of antigens of *S. enteritidis* is primarily concerned with the detection of bacteria, antigens identified as specific to *S. enteritidis* have also been used in vaccine development (Rabsch, 2000). The use of *S. enteritidis* vaccines in poultry

has contributed to the decline of human cases of *S. enteritidis* infection in Europe (Thorns, 1995). However, it is important to develop new, more effective vaccines and the continued search for novel *S. enteritidis* antigens could facilitate this. Of the antigens examined in this study, SEF14 appeared to provide the most promising results for use in vaccine development. As SEF14 is a not a prerequisite for virulence, *sefA* gene deletion mutants from live vaccine strains were constructed to distinguish vaccinal antibody responses from those resulting from field infections (Thorns *et al.*, 1996b).

The published method of PCR amplification of the gene that encodes the fimbrial structure, SEF14, was evaluated in this study and found to give reproducible positive results for *S. enteritidis*. The specificity of the DNA-based PCR technique was found to be higher than that of the immunological techniques evaluated as a positive result for PCR could be determined by the presence of a single prominent band. PCR detection of *S. enteritidis*, therefore, showed good potential for use in diagnostic laboratories and will be tested on field samples in a follow-on study.

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