THE PREVALENCE AND PUBLIC HEALTH IMPLICATIONS OF *Salmonella brandenburg*, AN EMERGING PATHOGEN OF SHEEP IN NEW ZEALAND

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DEDICATED TO THE MEMORY OF OUR LATE SISTER, KGALALELO

ABSTRACT

Since the first case of *Salmonella brandenburg* abortion was recorded from a single sheep farm in the South Island of New Zealand in 1996, the outbreak had spread to more than 300 farms by the lambing season of 2000. This study, which was funded by Meat New Zealand and other stakeholders, is a pilot project to estimate the prevalence of *Salmonella brandenburg* and consider its potential foodborne and occupational health risk implications.

Eight farms, four control farms and four affected farms were chosen from known infected areas. Control farms were those which had not experienced *Salmonella brandenburg* outbreaks, while the four affected properties had clinical outbreaks during the 2000 lambing season which had either been laboratory confirmed or not. At each farm faecal samples were collected from 50 lambs and 50 ewes at drafting and at slaughter. Therefore a total of 200 samples were taken from each group of animals sent for slaughter. The sampling was done in two phases to determine the effect of time interval on the prevalence of *Salmonella brandenburg* in sheep between abortion outbreaks and slaughter. The same sampling routine was followed for the November-December (Phase I) and February-March (Phase II) periods. The isolates from faecal cultures that had been confirmed as *Salmonella* spp. by slide agglutination test were sent to the Institute of Environmental Science and Research (ESR) for serotyping. All the 133 samples sent for serotyping turned out as *S. brandenburg.*

The primary comparison of the study was done between the prevalence of *Salmonella brandenburg* in animals from affected farms and control farms. In addition the study was also interested in comparing *S. brandenburg* prevalence **within** class i.e. in lambs or ewes from the same farm at drafting and at slaughter. Comparison was also made **between** classes i.e. the prevalence of the organism in lambs and ewes from the same farm at drafting and at slaughter.

During phase I of the on-farm sampling the prevalence of *Salmonella brandenburg* in tested lambs and ewes from affected farms was 12.0.% and 18.7% respectively. The on-farm prevalence for the control farms was 4.0% for lambs and 3.5% for ewes. During phase I of abattoir sampling the overall prevalence for the affected farms was 9.0 % for lambs and 22.0 % for ewes compared to 0.0% for lambs and 1.0% for ewes from control farms. The high prevalence of *Salmonella brandenburg* in animals from affected farms as compared to control farms showed that affected farms were associated with high excretion rates and therefore high levels of environmental contamination.

During phase II of the on-farm sampling the prevalence of *S. brandenburg* in tested lambs and ewes from affected farms was 2.5% and 2.7% respectively. The phase II on-farm prevalence of *S. brandenburg* from control farms was 0.0% for lambs and 0.8% for ewes. During the same phase, abattoir prevalence of *S. brandenburg* in lambs and ewes from affected farms was 0.0% and 2.7% respectively compared to 0.5% for lambs and 0.0% for ewes from control farms. Like in phase I the overall prevalence of *S. brandenburg* was higher in animals from affected farms as compared to animals from

control farms. The study also showed that the prevalence of the organism was very high during phase I compared to phase II irrespective of class of animal or site of sampling. This could have been due to the high number of animals still excreting the organisms closer to the outbreak period or the high level of environmental contamination. Both factors would have contributed to a higher prevalence of positive cultures. The higher prevalence of positive cultures during the November-December period as compared to the February-March period showed that the risk of infection and product contamination was greatest at commencement of the season (November-December) but was greatly reduced by February. Therefore further research is required to find the production and processing methods that might reduce the risk of infection and product contamination during the period of November-December.

However the positive cultures of *Salmonella brandeburg* in control farms suggested a spreading disease outbreak and that the absence of clinical outbreaks of the disease did not mean an absence of infected animals on-farm. It is very important to do further investigations to find on-farm risk factors that might result in the absence or presence of clinical outbreaks.

The study did not show any obvious differences in the *S. brandenburg* prevalence **within** class between on-farm and slaughter samples. There was also no obvious difference in the prevalence of the organism **between** ewes and lambs from the same farm, either during on-farm or abattoir sampling.

Pulsed-field gel electrophoresis (PFGE) of the 24 isolates, which were a representative sample of the study, gave an identical profile. The PFGE and the serotyping suggested that the outbreak strain had become the dominant serotype in the sampled farms in the outbreak regions of the South Island of New Zealand. Therefore factors that gave rise to this dominance should be further investigated.

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LIST OF ABBREVIATIONS

BGA	brilliant green agar
BHI	brain heart infusion
BPW	buffered peptone waters
EDTA	ethylene diamine tetra-acetic acid
ELISA	enzyme linked immunosorbent assay
ESR	Institute of Environmental Science and Research
FAE	follicle associated epithelium
LIA	lysine iron agar
MQ	Milli-Q
MUCAP	4-methylumbel-liferyl caprylate
PCR	polymerase chain reaction
PFC	pulsed-field certified
PFGE	pulsed-field gel electrophoresis
RFLP	restriction fragment length polymorphism
RVS	Rappaport-Vassiliadis
SPI	Salmonella pathogenicity island
TBE	trisbase, boric acid, EDTA
TE	tris-HCl, EDTA
TSA	trypticase soy agar
TSI	triple sugar iron
XLD	xylose lysine desoxycholate

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CHAPTER ONE – GENERAL INTRODUCTION

1.1 Introduction

Historically disease in sheep in New Zealand caused by *Salmonella* spp. was associated with management practices in summer and autumn (from early January to May). These management activities included change in nutrition, transport to slaughter plants, mustering and yarding (Marchant, 1999; Fenwick, 2000). Salmonellosis manifested clinically as diarrhoea and death, with *S. hindmarsh* and *S. typhimurium* the commonly isolated serotypes (Marchant, 1999). Cases of *Salmonella* spp. abortion in sheep in New Zealand were sporadic and usually caused by the serotypes *hindmarsh, typhimurium* and *oranienburg* (Clark *et al.*, 1999). Overseas ovine abortion had been mainly caused by the host adapted *Salmonella abortusovis* and the non-host adapted *Salmonella montevideo* (Marchant, 1999). However, the picture of *Salmonella* spp. abortion in sheep changed in 1996 after the first case of *Salmonella brandenburg* was reported in the South Island. Previously as reported by Hosie (1991) *Campylobacter* spp. and *Toxoplasma gondii* accounted for 42% and 33% respectively of ovine abortions in New Zealand.

Since 1996 there has been an increase in the number of reports of *Salmonella brandenburg* abortions in ewes in late pregnancy in the South Island of New Zealand. The abortion storms were reported to affect at least 30 % of the ewes in affected flocks with up to 100 % mortality in aborting ewes. By the winter of the 2000 lambing season

the abortion storms had occurred in the regions of Canterbury, Otago and Southland (Clark, 1999; Clark 2001b). Since the first case was reported in a merino flock in Canterbury in 1996, the disease has progressed so that by the 2000 lambing season there were 337 farms with laboratory confirmed cases. The cases were from both sheep and cattle farms (Table 1).

Table 1.1: The number sheep and cattle farms with laboratory confirmed cases of *Salmonella brandenburg* infections

Lambing season	Canterbury farms	Otago farms	Southland farms
1996	1 (0)*	0 (0)	0 (0)
1997	17 (0)	0 (0)	1 (1)
1998	31 (3)	55 (2)	67 (0)
1999	45 (5)	71 (4)	162 (10)
2000	36 (4)	62 (16)	233 (40)

* cattle farms in brackets
Table from Clark (2001b)

The economic loss due to abortions, ewe mortality, a possible loss of access to the export markets and public health issues posed by outbreaks of *Salmonella brandenburg* abortion, prompted the stakeholders in the sheep industry to propose a pilot study of the problem. The key aspect of the pilot study was to evaluate the implications of the outbreaks of *S. brandenburg* in ewes on the risk of infection of ewes and lambs at the time of slaughter.

The general hypothesis of the study was that the occurrence of abortion outbreaks was associated with the risk of *Salmonella* spp. contamination of meat at slaughter and that the contamination was highest at the commencement of the slaughter season (November-

December), closer to the abortion outbreaks. The other hypothesis was that the prevalence of *Salmonella* spp. was high in animals at slaughter compared to that of the same animals on farm. This hypothesis was based on the fact that with stress of congregation on farm, transportation and lairage the animals will be induced to excrete the pathogen. Also during drafting, transportation and lairaging the close contact between the animals would facilitate the spread of salmonellae to previously unexposed animals (Robinson 1967; Grau *et al.*, 1968; Grau *et al.*, 1969; Grau & Smith, 1974; Wray *et al.*, 1991; Gough & McEwen, 2000). The prevalence of *Salmonella* spp. in animals on-farm and at slaughter in the pilot study was estimated by culture of rectal faeces collected on farm and culture of caecal contents at slaughter. Descriptive analysis was then applied on the data collected.

Salmonella brandenburg isolates from the study were subtyped using pulsed-field gel electrophoresis (PFGE). This enabled the determination of the genetic relatedness between the isolates on farm and those at the abattoir compared to the original isolates from the 1996 outbreaks. PFGE has been shown to have a higher discriminatory power than other genomic typing methods like IS 200, ribotyping and restriction fragment length polymorphism (RFLP) in the typing of *Salmonellla brandenburg* isolates (Baquar *et al.*, 1994; Olsen *et al.*, 1993). PFGE is based on the embedding of the microbe in agarose, lysis of the embedded microbe, cutting the genome of the lysed microbe with a rare cutter, transferring the agarose embedded DNA fragments into agarose gel wells and separating the fragments using electricity. Since the mid 1980's PFGE has been replacing serotyping as method of choice for microbial typing (Tenover *et al.*, 1995).

1.2 Objectives Of The Research

The specific objective of the study was to evaluate the implications of the outbreaks of *S. brandenburg* disease in ewes on the risk of infection of ewes and lambs at the time of slaughter. This was achieved by comparing the prevalence of *Salmonella brandenburg* in lambs and sheep from case and control farms, on-farm and at slaughter. The animals were sourced from the outbreak area of Southland, in the South Island of New Zealand.

The secondary objectives were to determine:

- The influence of time interval between ewe abortions and slaughter on prevalence of *Salmonella brandenburg* in animals from affected and control farms presented for slaughter
- The influence of class of animal (lambs compared with ewes) on the prevalence of *Salmonella brandenburg* in animals presented for slaughter
- The influence of transport and lairage on the prevalence of *Salmonella brandenburg* in animals at slaughter i.e. the on farm compared with the slaughter prevalence

CHAPTER TWO: LITERATURE REVIEW

2.1 Microbiology of Salmonella spp.

The typhoid bacillus that later became a member of the genus *Salmonella* was first identified in 1881 from sections of a spleen and mesenteric lymph nodes (Grimont *et al.*, 2000). From the 1880's to 1914 only four serotypes or species as they were then called had been isolated. However, after 1928 there was an explosion in the isolation and naming of new serotypes. The Kauffmann-White scheme that was started in 1934, published names and antigenic formulae of members of the genus. If the Kauffmann-White scheme were maintained in its original format of naming serotypes as if they were species, there would have been more than 9000 *Salmonella* species by the 1960's (Kelterborn, 1967).

The genus *Salmonella* belongs to the family enterobacteriaceae. Like other members of the family, salmonellae are gram negative, non-spore forming and usually motile rods. They are facultative anaerobes, oxidase negative, catalase positive, ferment glucose, convert thiosulphate to hydrogen sulphate and grow readily on basic media (Le Minor & Popoff, 1987; Quinn *et al.*, 1994; Grimont *et al.*, 2000). The genus has three antigens, somatic (O), flagellae (H) and capsular (Vi) and is subdivided into serogroups characterised by the dominant O and H antigens. The antigenic formulae that are based on the O and H antigens are used to describe the different serotypes of the genus. Sometimes identification of *Salmonella spp.* has to be done beyond the serotype level in the investigation of disease outbreaks. This is done using genotypic and other phenotypic based techniques.

The taxonomy of the genus has always been controversial because of the different approaches taken by different workers in the field. *Salmonella* serotypes have been named according to their clinical role, geographical area of first isolation, antigenic specificity, biochemical properties (subgenera I-IV) and DNA relatedness (subgroups I-VII). It was this confusion in taxonomy that prompted Le Minor & Popoff (1987) to propose a one species for sub-group I. They proposed that *Salmonella cholerasuis* be named *Salmonella enterica* and become the sole species for the sub-group with seven subspecies: *salamae, arizonae, enterica, diarizonae, houtenai, bongori* and *indica*. However scientific evidence using DNA hybridisation has suggested that *Salmonella* spp. is a two species genus. The two species are *bongori* and *enterica* with the latter, in which all the pathogenic serotypes are grouped, being divided into six subspecies; *enterica, salamae, arizonae, diarizonae, houtenae* and *indica* (Gonzalez, 2000; Grimont *et al.*, 2000). Therefore using this system a serotype like *brandenburg* will be known as Salmonella *enterica* subspecies *enterica* serovar Brandenburg. But conventionally serotypes are still written as if they were species for example *Salmonella enterica* subspecies *enterica* serovar Brandenburg is still written as *Salmonella Brandenburg*. For simplicity, this conventional nomenclature will be adopted in this thesis.

Molecular typing is indicated for disease surveillance, identification of source, investigation of persistence, treatment failure (new or recurring infection), diagnostics and food production quality assurance (Maslow *et al.*, 1993). The main molecular typing methods used in the identification of *Salmonella* spp. include pulsed-field gel electrophoresis (PFGE), plasmid profiling and polymerase chain reaction (PCR)

based methods like random amplification of polymorphic DNA (RAPD) (Olsen, 2000). The phenotypic variations within a serotype are expressed through identification of biotypes, phagetypes and plasmid content of the different strains of a serotype (Maslow *et al.*, 1993; Ekperigin & Nagaraja, 1998).

Phenotypically salmonellae are differentiated from other enterobacteriaceae by being non- lactose fermenters, adinotol, sucrose, salicin and 2-ketogluconate fermenters, their inability to hydrolyse urea and for being able to hydrolyse 4-methylumbelliferyl caprylate (MUCAP). It is these phenotypic characteristics of the *Salmonella* spp. that are used in the isolation of the genus using selective media, indicators and substrates (Grimont *et al.*, 2000).

Members of the genus readily grow in regular media like blood agar (BA), deoxycholate citrate agar (DCA) and McConkey agar. Some selective media like brillian green agar (BGA) and xylose lysine desoxycholate (XLD) whose selectivity is based on a dye and bile salts respectively, are used in the isolation of salmonellae (Harvey & Price, 1979). The ideal growth requirements of *Salmonella* spp. are a temperature range of 35-37 degrees Celsius (^{0}C), a pH range 6.5-7.5 and a water activity of greater than 0.930 (Ekperigin & Nagaraja, 1998). The high isolation temperature at 42 ^{0}C is used for the inhibition of other competing enterobacteriaceae while incubation at 37 ^{0}C is indicated for host adapted serotypes and uncontaminated samples (Waltman, 2000). Although the high temperature aids in the isolation of pure cultures it is unsuitable for certain serotypes. It can also inhibit the growth of colonies if the selective media being used has a high concentration of inhibitory substances. Therefore it is important to use the high incubation temperatures with the correct type

of selective media (Harvey & Price, 1979). Laboratory methods and the type of sample being processed also influence the sensitivity of salmonellae isolation.

Of all bacteria, salmonellae probably have the widest array of isolation media and enrichment broths. This variety of isolation methods is because there are different requirements for isolating salmonellae from clinical disease cases, food, food animals and the environment (Waltman, 2000; Harvey & Price, 1979). This is a factor that makes the standardisation of its isolation methods very hard.

Experiments have shown that of the three commonly collected sample materials of animal origin for the isolation of salmonellae mesenteric lymph nodes have the highest proportion of positive samples. Caecal contents have a moderate sensitivity while faecal swabs have the least sensitivity for the isolation of salmonellae from animal samples (Tay *et al.*, 1989; Gay *et al.*, 1994). Funk *et al.* (2000) also estimated the effect of weight of the sample material on the relative sensitivity of salmonellae isolation. Their study showed that the relative sensitivity of isolation increased with sample weight with rectal swabs being the least sensitive and the 1 gram, 10 grams and 25 grams faecal samples had an increasing relative sensitivity of isolation of salmonellae in that order.

Highly contaminated samples like faecal swabs normally do not require pre-enrichment because this will result in overgrowth of other enterobacteriaceae. Usually the highly contaminated samples are put directly into selective enrichment broths like selenite broth. Although the caecal samples and other lower intestinal tract samples

can be regarded as contaminated they require pre-enrichment. This aids in the resuscitation of the sub-lethally injured cells (Rings, 1985; Aho, 1992).

The salmonellae in caecal contents and the lower gastro-intestinal tract have been exposed to the bactericidal abomasal pH (< 4.8), bacterial interference from the resident flora, the bactericidal bile acids and in the ruminants, inhibitory volatile fatty acids (Baumler *et al.*, 2000). The other stressors for bacterial cells are exposure to heat, desiccation, irradiation and high molar concentrations. An attempt to directly expose the already stressed cells to a selective enrichment medium might reduce the sensitivity of both the isolating medium and sample material. This is because of the inhibiting ingredients of selective enrichment broths. Therefore pre-enrichment using buffered peptone water (BPW) would be indicated for highly contaminated samples if the cells in the sample material are suspected of having been exposed to hostile conditions, while direct selective enrichment would be recommended for raw food and highly contaminated samples containing suspected non-stressed cells (Harvey & Price, 1979; Wray *et al.*, 1990; Aho, 1992; Waltman, 2000).

Different isolation protocols also influence the sensitivity of isolation of salmonellae from different samples. The sensitivity of the enrichment broths can be increased by multiple subcultures onto growth media. The broths used in the selective growth of *Salmonella* spp. include tetrathionate broth, selenite broth and Rappaport-Vassiliadis (RVS) broth. The multiple cultures can be done either at the same time or at different times. The sensitivity is increased because cells are not uniformly distributed in the enrichment broth thus multiple cultures increases the probability of picking the cells in the media. During different periods of incubation the different serotypes have

different growth rates due to changes in the inhibition-growth balance. Therefore multiple sub-cultures can be used to increase the sensitivity of isolation from samples that are suspected to have multiple *Salmonella* serotypes (Jameson, 1962; Harvey & Price, 1967; Harvey & Price, 1979).

The use of high volume of inoculum of the sample material into the enrichment broth can also increase the sensitivity of isolation of salmonellae. But because this overloads the enrichment broth and reduces its selectivity this approach is rarely used (Harvey & Price, 1979). Sensitivity of the enrichment methods can also be increased by the so-called delayed secondary enrichment procedure.

Delayed secondary enrichment involves incubating an inoculum in a broth like RVS at 37-42 ^{0}C for 24 hours and subculturing the inoculum-broth mixture onto a solid medium, and standing the remaining inoculum-selective broth mixture at room temperature for up to five days. After this period an inoculum of the mixture is transferred into a fresh broth and incubated at 37-42 ^{0}C for 24 hours and sub-cultured onto solid media. Rigby & Pettit (1980) showed that delayed secondary enrichment has the highest sensitivity in the isolation of salmonellae from broiler chicken faeces as compared to pre-enrichment and direct enrichment. They also reported that pre-enrichment was more sensitive than direct enrichment. These results were similar to those reported by Davies *et al.* (2000) whose experiments with swine faeces showed that delayed secondary enrichment increased *Salmonella spp.* detection by 25% from culture negative samples.

O' Carroll *et al.* (1999) showed that delayed secondary enrichment increased detection of salmonellae in both same day processed faecal samples and faecal samples that had been stored before processing. However, because delayed secondary enrichment is more time and resource intensive it will be more appropriate for samples, which have been stored before processing because it would compensate for the damaging effect of storage on salmonellae organisms.

Delayed secondary enrichment has the highest sensitivity for the isolation of salmonellae because it gives cells more time for multiplication as compared to pre-enrichment and direct enrichment. It also allows for rapid growth during the second incubation after the delay period. Compared to direct enrichment, pre-enrichment allows resuscitation of injured cells and increased multiplication of the initially low number of cells. This gives pre-enrichment better sensitivity than direct enrichment in the isolation of *Salmonella* spp. (Rigby & Pettit, 1980).

The isolation of salmonellae from the different samples also has some epidemiological importance as the point of infection can be inferred. Isolation from the mesenteric lymph nodes is usually associated with long term infection and bacteraemia, while isolation from caecal contents only, is usually associated with recent infection. The animals could have been recently infected on-farm, in transit or during holding in the meat plant pens. Rapid dissemination is also possible as illustrated by experiments in oesophagotomised, 6-8 week old pigs from which *Salmonella* spp. organisms were detected in their caecal contents three hours after intra-nasal challenge (Fedorka-Cray *et al.,* 1995). Similarly Hurd *et al.* (2001a) showed in a study of pigs challenged in lairage like conditions that *Salmonella* spp.

organisms could be isolated from autopsy samples of pigs within two hours of exposure. However, due to the amount of time stock spend during transportation and in the lairages, caecal isolation of *Salmonella* spp. at slaughter is most likely associated with exposure on the farm (Tay *et al.*, 1989).

2.2 Pathogenesis of *Salmonella* spp.

Infection of a susceptible host by the pathogenic serotypes of *Salmonella spp.* has two clinical outcomes. These are a localised infection that results in enteritis or a generalised infection that results in systemic manifestation of the disease. The generalised infection or acute salmonellosis results from the breaching of the small intestines integrity by the pathogenic serotype (Ekperigin & Nagaraja, 1998). However, oral exposure can also result in failure of infection with a resultant transient excretion as organisms are shed in the faeces. This is a result of a failure to successfully colonise the gastrointestinal tract. The clinical manifestation of salmonellosis is due to an interaction of agent, host and environmental factors. The mechanisms by which these interactions result in disease are still not well understood.

Some of the virulence mechanisms of salmonellae are coded for in the virulence plasmid genes. It is the presence of these plasmids that results in the expression of virulence by the pathogenic salmonellae. However, experiments have shown that the virulence plasmids are not the only requirement for virulence and instead are part of a multifactorial complex that results in systemic disease (Baumler *et al.*, 1998). Colonization, adhesion and invasion are achieved by endocytosis that is a requirement for infection. Part of the ability of the *Salmonella* spp. to invade the gastrointestinal tract is encoded for in the Salmonella Pathogenicity Island 1 (SPI 1). This is part of the salmonellae chromosome with a cluster of genes encoding for intestinal penetration. The mechanisms of cell damage by *Salmonella* spp. are not well understood but it is thought both enterotoxins and cytotoxins play a role (Bauer & Hormansdorfer, 1996).

Enteritis results in villi degeneration, enterocyte degeneration, increased secretory activity by the Goblet cells, inflammation in the lamina propria and increased leukocyte count in the intestinal lumen. The above factors result in malabsorption and when coupled with increased intestinal permeability due to vascular damage and with stimulation of the adenylate cyclase system result in diarrhoea (Bauer & Hormansdorfer, 1996). The adenylate cyclase system that is stimulated by prostaglandins produced during the inflammatory process induces increased intestinal fluid secretion, while malabsorption is due to fusion of villi surfaces during healing. The blockade of lymph and blood vessels due to cellular infiltration of the intestinal wall during inflammation also contributes to malabsorption. The maldigestion that also contributes to diarrhoea is due to the loss of intestinal derived enzymes following inflammatory enterocyte necrosis (Rings, 1985). The *Salmonella* genus has virulence factors that contribute to the above enteric changes. These include endotoxins, enterotoxins, cytotoxins, lipopolysaccharides, siderophores, flagellae and fimbriae (Bauer & Hormansdorfer, 1996).

The protective mechanisms of the host during the intestinal phase of *Salmonella spp.* infection include the acidic pH of the abomasum, bactericidal compounds in the small intestines, peristalsis and bacterial interference from the resident flora. The low pH in the abomasum and the bile salts secreted into the small intestines are bactericidal. Peristalsis is also an important defence mechanism because 6-10 hours after infection of mice with salmonellae, 80% of the cells can be detected in the faeces, 15% in the gut lumen and only 5% in the gut associated lymphoid tissue

(Baumler *et al.*, 2000). The resident flora of the gastro intestinal tract gives protection against infection by competing for adhesion sites, producing inhibitory substances and competing for nutrients. The *Salmonella* spp. evade the above mechanisms through the stress response gene, rpoS which is triggered by change in environment resulting in production of heat stress proteins, expression of acid tolerance etc (Humphrey, 2001). Salmonellae also evade the hostile gastro-intestinal environment by their tropism for Peyers patches through the special M cells that are only found on the follicle-associated epithelium (FAE) of the small intestines. The FAE also has enterocytes with special surface receptors that are thought to contribute to the salmonellae tropism for Peyers patches. Agent adhesins, the long polar (LP) fimbrae are also thought to play a role in this tropism for the Peyers patches (Baumler *et al.*, 2000). Also genetic changes acquired through mutations result in changes in tolerance, antibiotic resistance and virulence, which enable some *Salmonella* serotypes to evade initial local defences against infection and to ultimately cause systemic disease (Humphrey, 2001).

The ability of salmonellae to cause systemic disease is facilitated by their ability to survive inside macrophages, which transport the viable bacterial cells to hepatocytes in the liver and to cells of other organs making up the reticulo-endothelial system. Part of this ability to cause systemic disease is coded for in the Salmonella Pathogenicity Island 2 (SPI 2). This is part of the salmonellae chromosome with a group of genes encoding for systemic disease. It is thought that the emergence of the SPI 2 in the evolution of *Salmonella spp.* pathogenicity resulted in the divergence into the two species *Salmonella bongori* and *Salmonella enterica* with *S. enterica* being the species with SPI 2 (Lax *et al.*, 1995; Baumler *et al.*, 1998). The extra-intestinal

manifestations of disease are due to the production of endotoxins that cause fever, vascular damage and thrombosis. Failure of host mechanism to contain the pathogen result in septicaemia with pathogen multiplication in target organs like the central nervous system, lungs, musculoskeletal system and placenta. These result in clinical signs associated with meningitis, pneumonia, arthritis (joint ill) and abortion or reduced fertility respectively (Bauer & Hormansdorfer, 1996).

It is generally accepted that salmonellae enter systemic circulation in the lower small intestines or upper large intestines where they are transported to the reticulo-endothelial system organs. A study by Samuel *et al.* (1981) in cattle and sheep showed that the lymph nodes draining the small intestines had a significantly higher prevalence of *Salmonella* spp. than those draining the abomasums. This confirmed that the small intestines are the main point of entry for salmonellae into systemic circulation. From the reticulo-endothelial system they are transported to target organs for multiplication. During this phase of infection faecal excretion of salmonellae can be present or absent. From the target organs the cells are then transported back into general circulation and it is during this phase that faecal excretion, diarrhoea and pathological changes are observed (Lax *et al.*, 1995; Baumler *et al.*, 2000). However it should be emphasised that most of the present knowledge of the pathogenesis of *Salmonella* spp. is based on the mouse typhoid model which will not necessarily be true for other species (Baumler *et al.*, 2000).

The lymphoid system acts as a barrier to systemic disease caused by salmonellae in vertebrates. Only a small percentage of non-typhoidal *Salmonella* spp. infections in vertebrates result in bacteraemia with the main limiting factor being the inability to

multiply in host macrophages (Baumler *et al.*, 1998). It is the ability of some of the serotypes belonging to *Salmonella enterica* subspecies *enterica* to multiply inside macrophages that gives the ability to cause systemic disease. The infected macrophages also inhibit the cell-mediated immunity mounted by the infected host (Baumler *et al.*, 2000). This means macrophages act as a limiting factor of the host range of pathogenic *Salmonella* serotypes. The host-adapted serotypes usually cause systemic disease in both young and old. In contrast the non-host adapted serotypes like *Salmonella typhimurium* usually cause disease in the young with a naïve immune system and in the old of most vertebrates if their immune system is impaired (Baumler *et al.*, 1998).

The disease causing serotypes of *Salmonella* can be classified as host adapted, primary pathogens, opportunistic pathogens and non-host adapted. Those serotypes that are primary pathogens in one host and opportunists in other hosts include *S. dublin* (cattle), *S. choleraesuis* (swine), *S. typhi* (human), *S. paratyphi* (human), *S. schottmuelleri* (human), *S. pullorum* (chickens), *S. abortusovis* (sheep), *S. abortusequi* (horses), *S. typhisuis* (swine) and *S. abortusbovis* (cattle). For a serotype to be regarded as host adapted it has to cause disease and be able to circulate within a specific host population. This means the most important characteristics of a host adapted serotype is the ability to be invasive, persist in the host and generate more than one secondary cases from a primary case (Kingsley & Baumler, 2000). Therefore host adaptation is not associated with virulence as shown by *Salmonella dublin* and *Salmonella choleraesuis* that are highly invasive in humans. But because they do not cause secondary cases in people they are not regarded as host adapted to us.

Host adaptation could be facilitated by selective pressure due to the host immune response or human interventions like antimicrobial use and test and slaughter policies. It is thought that the present *Salmonella enteritidis* pandemic could have been facilitated by the eradication of *Salmonella gallinarum*, which like *S. enteritidis* is a group D_1 serotype. This could have created a niche for *Salmonella enteritidis* to out-compete other serotypes and become the dominant D_1 serotype (Kingsley & Baumler, 2000). Agent factors like host specific adhesions, multiple horizontal transfer of plasmids and point mutations, narrow the pathogens host range thus resulting in host adaptation (Baumler *et al.*, 1998).

The carrier status confers to a serotype the ability to survive during the inter-epidemic periods when there are not enough numbers of susceptible hosts to perpetuate infection. The host adapted serotypes survival in the host is due to its ability to evade the hosts immunity. *Salmonella typhi,* a human adapted serotype, evades the hosts' immune system by residing in the gall bladder of human carriers between inter-epidemic periods. The virulence of a serotype is also associated with its ability to cause carrier status in the host. Non-typhoid serotypes are associated with carrier status rates of 0.2-0.6% in humans while the more virulent *Salmonella typhi* is associated with a carrier status rate of 1-4% in humans (Kingsley & Baumler, 2000).

The different serotypes have different propensities to cause either systemic or enteric disease in different hosts. However the distribution of serotypes causing either form of disease will depend on the host affected and the geographical location. In Germany the systemic disease in sheep is usually associated with *S. abortusovis, S. dublin, S.*

montevideo and *S. typhumurium* while enteric disease is usually associated with *S. dublin, S. typhimurium, S. derby, S. newport* and *S. agona* (Bauer & Hormansdorfer, 1996). The capacity of the different serotypes to cause systemic disease is thought to be due to their invasiveness. Brackelsberg *et al.* (1997) showed using a tissue culture that *Salmonella dublin* had high intracellular count compared to *Salmonella typhimurium* when the same tissue culture is incubated with the two serotypes.

The difference in invasiveness between *S. dublin* and *S. typhimurium* may also be an indication of the difference in disease severity when hosts are infected in nature. This can in part also explain why the host adapted *Salmonella dublin* takes a long time to be cleared from cattle post infection while the non-host adapted *Salmonella typhimurium* is cleared within a relatively short time interval (Brackelsberg *et al.*, 1997). The severity of salmonellosis is also influenced by the infective dose, host factors and environmental factors (Hunter & Izsak, 1990; Higgs *et al.*, 1993).

The infective dose of salmonellae is estimated to range from 10^3-10^7 organisms depending on a number of factors. The dose can be lowered by a fatty diet which protects *Salmonella spp.* organisms from the acid pH in the stomach. The infective dose can also be lowered by increased rate of stomach emptying, hypochlorhydria and disturbances to the normal flora of the gastro-intestinal tract (Lax *et al.*, 1995; Newton- Clarke, 1995). However, the route of infection can also influence whether a challenge results in clinical manifestation irrespective of the dose. It has been shown experimentally using similar doses of *Salmonella abortusovis* in sheep that both sub-cutaneous and conjuctival challenges consistently cause abortion if given at mid-gestation compared to oral and intragastric challenges(Pardon *et al.*, 1990; Sanchis *et*

al., 1991). The age, immune and health status of the host also can influence both infective dose and severity of *Salmonella spp.* infection. A previously exposed, vaccinated, unstressed animal is likely to have a less severe disease compared to a naïve, stressed and animal with a concurrent disease.

It has been shown experimentally that both cell mediated and humoral immune response are required to protect against infection by *Salmonella spp.* Whether the body mounts cell-mediated immunity or humoral immunity is determined by cytokines. The cytokines, which include interferon gamma, tumour necrosis factor alpha and interlukins, also influence the activity of the effector cells and the intensity of the immune response (Holt, 2000). It is through this knowledge that both killed and attenuated *Salmonella spp.* vaccines are used in different parts of the world.

Despite their safety, the killed parenteral vaccines do not illicit any cell mediated immunity, they do not stimulate the mucosal defence through IgA and they are based on antigens which are elicited in in-vitro conditions. IgA gives protection against infection through increasing the sequestration of iron from the pathogens, inhibiting bacterial adherence, neutralising bacterial toxins, acting as an opsonis for mucosal phagocytes and involvement in the antibody-mediated cytotoxicity (Holt, 2000).

Salmonella spp. serotypes belonging to the same serogroup show some cross protection because of shared surface antigens. The five most important serogroups associated with disease causing serotypes in man and livestock are B, C_1, C_2, D_1 and E_1 (Kingsley & Baumler, 2000). This should have assisted in preparation of a universal killed *Salmonella spp.* vaccine but the huge number of serotypes and the

possible interference between antigens make this hard to achieve. The immunity elicited by cross protection between serotypes is also reported to be weak and short-lived.

Unlike killed vaccines, live attenuated vaccines elicit strong cell mediated immunity and they confer a better protection to infection. However the live vaccines can induce disease and *Salmonella spp.* shedding in the host. This makes their use, particularly in food animals an important public health issue.

Presently the most viable option for both killed and live vaccines is the use of mutants whose virulence genes have been deleted. Care should be taken because deletion of certain virulence factors can render the mutants ineffective due to reduced immunogenicity. However, the use of mutants is presently limited by their variable results in field trials and public perception towards the use of genetically modified organisms in food animals (Lax *et al.*, 1995).

2.3 Epidemiology of *Salmonella* spp. with special reference to sheep

The genus *Salmonella* consists of more than 2000 serotypes, with only a small proportion of them causing diseases in humans and livestock (Lax *et al.*, 1995; Bauer & Hormansdorfer, 1996; House & Smith, 1998). The genus *Salmonella* is ubiquitous with a worldwide distribution. The occurrence of its serotypes is determined by the presence of susceptible hosts. The presence of certain serotypes at times also shows seasonality in those geographical areas in which they occur (Ekperigin & Nagaraja, 1998; Davies, 2001). The importance of the salmonellae as pathogens is made even more important by the increase in international trade and travel. This means some serotypes that might have been restricted to certain geographical areas are now of international importance. Most of the serotypes cause disease in a wide range of hosts with only a few serotypes being host adapted. In New Zealand, salmonellae were first recognised as a cause of disease in sheep in 1949 (Salisbury, 1958). Animals play an amplification role in the spread of salmonellae through environmental contamination, contaminated feed of animal origin and the infection of humans by eating food of animal origin (House & Smith, 1998). However, people can also infect animals causing sporadic cases or epidemics. Sporadic cases of salmonellosis in animals acquired from humans are due to infection from handlers while epidemics are usually caused by human effluent contaminating animal pastures (House & Smith, 1998).

The occurrence of salmonellae in sheep in New Zealand has traditionally been associated with the mid-summer to early autumn period with a peak in January. The outbreaks were mainly associated with checking i.e. yarding, transportation and changes of nutrition. In the 115 outbreaks studied by Salisbury (1958) the most

susceptible classes of sheep were two tooths, followed by hoggets and the least susceptible were lambs. These classes accounted for 82%, 12% and 5.2% of the outbreaks respectively. In a study of ovine mortality on 141 farms in the North Island Robinson & Royal (1971) found that salmonellosis was the main cause of mortality in sheep with 437 out 1437 rib samples submitted from dead animals positive for salmonellae. The samples were mainly from two and four tooths. A study by Kane (1979) in a New Zealand abattoir showed that most of the carcasses, which were positive for *Salmonella* spp. were from two and four tooths. These results could have been a reflection of the high proportion of infected live two and four tooths as compared to the other classes of animals presented for slaughter. The highest number of mortalities caused by salmonellae was found during the December-May period during the Robinson & Royal (1971) study. The deaths coincided with the yarding of animals for shearing, dipping, confinement for facial eczema and selling or buying. Robinson & Royal (1971) also showed in their study that 66 of the 141 farms had a degree of environmental contamination with debris and soil samples yielding salmonellae.

The ability of *Salmonella* spp. to survive for weeks or even months in the environment makes environmental contamination a very important way of maintaining infection within a susceptible host population. Environmental contamination by salmonellae is achieved through faecal excretion, aborted foetuses, placentae and vaginal discharges (Clark, 2001b). A Ministry of Food and Agriculture (MAF) study in New Zealand showed that *Salmonella brandenburg* can remain viable in the environment for up to six months (Clark, 1999). This result is consistent with the result of a study in which experimental seeding of the environment resulted in the

recovery of viable *S. typhimurium* organisms after 12 weeks (Tannock & Smith, 1971). Salmonellae are also reported to survive in horse manure for up to 12 months (Newton-Clarke, 1995). Water analysis in the two rivers of Matuara and Otaki in Southland region of New Zealand showed that during the lambing season (August-September) the water content was 110 salmonellae per litre of water compared with 1.2 per litre of water during the rest of the year. This is an indication of the high environmental contamination with infected materials during this period of the year as illustrated by the isolation of *Salmonella brandenburg* from some of the water samples (Clark, 2001a).

Despite the high survivability of *Salmonella* spp. in the environment and their ability to multiply in a temperature range of 7-45 ^{0}C the cells are still susceptible to direct sunlight and are also inactivated by the commonly used disinfectants like iodophores, sodium hypochloride and formaldehyde (Rings, 1985). The high survivability of salmonellae in the environment means that it is possible that some of the *Salmonella brandenburg* outbreaks in sheep in the South Island are a result of the persistence of the organisms in yards. A study in the South Island of New Zealand showed that 2-3% aborted sheep were still excreting *Salmonella brandenburg* 6 months after the abortion storms (Clark, 2001a). With *Salmonella brandenburg* being a non-host adapted serotype this prolonged excretion is unlikely to be associated with a carrier status of the aborted ewes but due to re-infection from environment, despite Clark (2001a) suspecting some carrier status in the aborting ewes. Non-host adapted serotypes are usually cleared from the hosts bodies 2-16 weeks after infection (Anderson & Blanchard, 1989; Pelzer, 1989).

Different geographical areas have different serotypes causing morbidity within their sheep populations. In Europe the serotypes that infect sheep include *S. abortusovis, S. typhimurium, S. arizonae* and *S. dublin* (Ekperigin & Nagaraja, 1998). In Australia the most important serotypes are *typhimurium, bovismorbificans, derby* and *havana* (Murray, 1994). A study of salmonellosis in sheep in England and Wales during the period 1975-1981 showed that the important serotypes were *typhimurium, dublin, montevideo* and *arizonae.* However, in the period prior to 1975 the sheep adapted serotype, *S. abortusovis* was dominant. In the period of the study S. abortusovis was isolated in the 1975-1976 season from only nine cases (Sojka *et al.*, 1983). The understanding of the geographical distribution of the different serotypes is important in outbreak investigation because diagnosis can be made based on the clinical presentation unique to the different serotypes (Rings, 1985).

The reasons for predominance of certain serotypes within a geographical area are still not well understood. However, predominance may be associated with the host building strong immunity to the serotype. This results in the serotype becoming endemic because it co-exists with host without killing off its host. This is due to a relative reduction in the serotypes pathogenicity, which the host achieved by building up some protective immunity (Sojka *et al.*, 1983).

Attainment of dominance by a serotype could also be due to the narrowing of the niche width. Niche narrowing can be facilitated by factors like the use of antibiotics, eradication of certain serotypes and the increased homogeneity of the host population through breeding. The above factors can result in a previously unimportant serotype within a population out-competing other serotypes and multiplying to levels that make

an epidemic possible (Hunter & Iszak, 1990). This could explain the current pandemic of *Salmonella enteritidis* in poultry (Barrow, 1993) and the increasing importance of *Salmonella brandenburg* as a sheep pathogen in the South Island of New Zealand.

In a study of *Salmonella brandenburg* isolates in New Zealand between 1985-1994 Wright *et al.* (1998) found that the serotype had been isolated from humans, pigs, cows, goats, poultry, meat, meat and bone meal and the environment. The serotype made up only 1 % of the 14 000 human isolates studied. The incidence in humans was found to increase during the December-March period and in 1994-5 there was an increase in human cases in the upper North Island. This increase in human cases preceded the first abortion storms in sheep in the South Island. Whether there was a link between the two incidents is not yet known. However scavenging wild birds could have recycled *Salmonella brandenburg* from human sewage to animal pastures.

The introduction of a new serotype to a susceptible host population could result in persistence within the population. However, this is not always the case as failure to find a suitable niche can result in an initial rise of cases followed by a rapid decline to pre-outbreak levels as illustrated by *Salmonella eastbourne*. This serotype was introduced to The United States and Canada in 1973 through milk chocolate and the number of human cases rapidly peaked. But after 1974 the number of cases rapidly fell to the pre-outbreak levels (D'Aoust, 1994). This has not been the case with *S. brandenburg* in the South Island because the number of affected sheep farms has increased from only one in 1996 to 331 in 2001 (Clark, 2001b). Therefore the host has not been able to adapt to the serotype as it is continuing to cause abortions and ewe

mortalities in both properties that had previously had abortion storms and those that did not.

The *Salmonella brandenburg* abortion storms in the South Island of New Zealand are reported to affect up to 30 % of ewes in a flock with a mortality rate of up to 50%. Most deaths occur in ewes that failed to expel the foetus or foetuses (Smart, 2000). A survey of the 1998 lambing season by Boxall *et al.* (1999) showed that the two main categories of ewes affected were carrying multiple foetuses (63% of affected ewes) and they were mainly two tooths or mixed age (76% of the affected ewes). The high numbers of aborting two tooths in the survey above could be due to their naïve immune system. This, when compounded by the metabolic stress of carrying multiple foetuses, makes the two tooths more susceptible to abortion as compared to the older ewes.

The most important source of host-adapted strains to a susceptible host population is the introduction of asymptomatic carriers into the herd or flock. The host-adapted serotypes of like *S. dublin* in cattle are often associated with a lifelong carrier status. This is in contrast to the non-host adapted serotypes in cattle, which are usually cleared in 2-16 weeks after infection. Usually isolations of non-host adapted *Salmonella* serotypes in animals beyond this time period is due to reinfection from the contaminated environment (Anderson & Blanchard, 1989; Pelzer, 1989).

The on-farm spread of host-adapted serotypes is perpetuated by factors like contamination of pasture, water and feed by faeces, uterine and vaginal discharges and birth materials like placentae. However, the assumption that salmonellae in feed

is an important source of salmonellosis in animals is questionable. In most cases the serotypes isolated in feed are different from those isolated in animals that were exposed to that feed (Murray, 1994; Davies & Hinton, 2000). *Salmonella* spp. in feed is mostly due to post-processing contamination and failure of rendering of animal derived feed. The other factors that are important in relation to spread of salmonellae on farms are associated with general husbandry and management practices. These include high stocking densities, failure to isolate sick animals, use of the same equipment in both clean and dirty areas without disinfection, ineffective vermin control, failure in waste management, contamination of water sources by effluent and agricultural runoff and failure to quarantine replacement animals before introduction into a flock or herd (Murray, 2000). The above factors are also important in the introduction and or spread of non-host adapted *Salmonella spp.* serotypes in farms.

The non-host adapted serotypes show no predilection of any kind of host as illustrated by *Salmonella typhimurium, Salmonella brandenburg* etc (Lax *et al.*, 1995; Clark 2001a). The *Salmonella* serotypes that infect a wide range of hosts can be introduced into a susceptible population through a wide array of sources. Unlike the host adapted serotypes the non-host adapted serotypes do not necessarily require an asymptomatic carrier to be introduced into a susceptible herd or flock. The non-host adapted serotypes can transmitted between farms in a number of ways including pet movements, stock movements, wildlife, delivery and stock vehicles, feed and possibly wind (Wray *et al*, 1991; Bauer & Hormansdorfer, 1996; Fedorka-Cray *et al.*, 1998; Fenwick, 2000; Smart 2000; Davies, 2001). In New Zealand *Salmonella brandenburg,* which has assumed importance as a cause of abortion and ewe mortality in the South Island is suspected to be spread by contaminated waterways, blackbacked

gulls, other scavengers and prolonged survival of the pathogen in sheep yards (Clark, 2001a; Clark, 2001b).

The role of wildlife in the spread of *Salmonella* spp. seems to be very important worldwide, particularly in sheep. In the outbreaks of *Salmonella montevideo* abortions in sheep in Scotland, wild birds were suspected of spreading the organism during lambing (Caldow & Graham, 1998). A study in The South Island of New Zealand by Clark *et al.* (1999) to investigate the spread of *Salmonella brandenburg* showed that 42% and 52% of captured black backed gulls in case and control farms respectively were positive for the organism. Some of the captured birds had bacterial counts of up to 25 x 10^6 per gram of gastro-intestinal contents. All the birds with *Salmonella brandenburg* positive gut contents were captured during the months of September, December and July while all the birds captured during the period March-June were negative. This has very important epidemiological importance because it showed that the gulls only carried *Salmonella brandenburg* organisms during the period of high environmental contamination i.e. the lambing season. It can be concluded that gulls are not a source of *S. Brandenburg,* but play only a possible transmission role during the outbreak periods. Therefore the gulls are only transient excretors of *Salmonella brandenburg* and would not harbour *Salmonella* spp. and spread it for more than one lambing season (Clark *et al.,* 2000; Clark, 2001a).

A study by Fenlon (1981) on seagulls showed that 12.9% of the 1242 faecal samples processed were positive for *Salmonella spp.* with faecal counts ranging from 0.18-191 colony-forming units(cfu) per gram. The serotypes isolated from the study included; *agona, panama, typhimurium, heidelberg, brandenburg* and *give*. These

serotypes are also commonly isolated from humans and sewage sludge (Fenlon, 1981). The above study showed the potential of seagulls recycling salmonellae from sewage effluent from sea to pasture (Reilly *et al.*, 1981). Since the infective dose of *Salmonella* spp. in animals and humans is dependent on several factors the bacterial counts in seagulls' faeces could be high enough to cause an infection. Immune status of host, nutrition, physiological changes and other environmental stresses are important in determining the infective dose of *Salmonella* spp..

Seagulls have also been suspected of playing a mechanical role in the transmission of *Salmonella montevideo* abortion outbreaks sheep in Scotland (Sharp *et al.*, 1983). An independent study by Coulson *et al.* (1983) in Eastern Scotland found that ovine abortion outbreaks in February were associated with massive movement of herring gulls into the area for breeding. Eight out of the nine gulls sampled in the area during this period had faecal samples positive for *Salmonella montevideo*. It is during this period that there is high mobility of sea gulls between coast and in land for feeding. After feeding on pasture, stubble and refuse tips the birds usually roost in large groups which means large deposition of faeces in pastures thus an increased risk for sheep infection from grazing contaminated pastures (Coulson *et al.*, 1983).

The black backed gulls are known to scavenge on aborted foetuses and have a habit of congregating into feeding parties. They are also known to feed within a radius of up to 50 kilometres from their nesting areas (Clark *et al.*, 1999). It is the feeding behaviour of wild birds, low prevalence of *Salmonella* spp. in the wild bird population and the isolation of salmonellae from the wild birds without clinical signs that shows that although birds are not necessarily reservoirs they have the potential to mechanically

transmit salmonellae between farms (Wilson & MacDonald, 1967; Mikaelian *et al.*, 1997). Birds are not only implicated in the spread of salmonellae to domestic animals. A study by Hart *et al.* (1987) showed that swallows and gulls were important in the *Salmonella* spp. cycle in an Australian marsupial, the quokka.

Transportation is an important source of infection for animals. It is also a very significant stress factor. Any form of stress is generally accepted to be a contributing factor in the development of salmonellosis. In a study of mortalities in live sheep export trade between Western Australia and the Middle East, salmonellosis was found to be a common cause of death in sheep at receival and on board transport ships (Richards *et al.*, 1989; Higgs *et al.*, 1993). To study the role played by stress in the sheep mortalities Higgs *et al.* (1993) measured the weights of adrenal glands in autopsied sheep in a study to reproduce the intensive handling associated with live sheep trade. They found out that sheep that died of salmonellosis and inanition had heavier and enlarged adrenal glands compared to the control animals. Enlarged adrenal glands are a physiological indicator of stress because during chronic stress there is hyper activity associated with production of corticosteroids. Gough & McEwen (2000) have also reported a large number of lambs developing salmonellosis five days after transportation with a mortality of 47 out of a total 640 lambs.

Vehicles are also an important source of infection for animals being transported to slaughter plants. A study by Wray *et al.* (1991) showed that 20.5% and 6.5% of calf transporting vehicles were positive for *Salmonella typhimurium* before and after washing respectively. Presence of salmonellae in vehicles used for the transport of livestock to the abattoirs means that the animals are being continuously exposed to

infection and this contributes to the prevalence of *Salmonella* spp. in animals presented for slaughter (Grau *et al.*, 1968).

The treatment of animals before slaughter and the time interval between leaving the farm and slaughter also influences the prevalence of *Salmonella* spp. as determined through faecal culture. A study by Grau *et al.* (1968) and Grau *et al.* (1969) showed that the longer the time spent in lairage the higher the prevalence of salmonellae in faeces of sheep and cattle. High environmental contamination and long periods in lairage are also associated with increased faecal shedding. The importance of environmental contamination is shown by the fact that the serotypes isolated from the environment are very similar to those from the fleece and carcases as shown by Grau & Smith (1974) in their experiments on pre-slaughter treatment of sheep.

Sheep that had been experimentally exposed to salmonellae from a seeded yard for 24 hours had positive samples isolated from nasal swabs, rumen and faeces on removal from the yard. Slaughtered sheep from the same experiment had positive samples from the caecum and rectum 12 hours after removal and the last positive samples were received from their faeces 5-7 days after removal from the experimental yard (Robinson, 1967).

It has also been shown that withholding feed for three days prior to slaughter also resulted in increased rumen counts of salmonellae in both cattle and sheep (Grau *et al.*, 1968; Grau *et al.* 1969) . Grau and co-workers also showed experimental inoculation with *Salmonella* spp. resulted in quick clearance from the rumen and faeces when the animals were being fed. The results of the epidemiological studies

based on faecal culture are also influenced by the number of animals sampled, the nature of the sample, time interval between collection and processing, sample storage, culture methods used and inter-laboratory variation (O'Carroll *et al.,* 1999).

The prevalence of *Salmonella* spp. in livestock as determined by faecal culture is also influenced by commingling and the nature of samples taken i.e. the site of sampling, the volume of sample and the number of animals sampled. In a study on swine by Hurd *et al.* (2001b) on-farm samples yielded only one serotype while the samples at slaughter yielded 17 serotypes. This experiment showed that commingling resulted in the infection of animals with different serotypes during transportation and holding in meat plant pens. In the same study Hurd *et al.* (2001b) showed that different sample sites yielded different prevalences of *Salmonella spp.* infection in swine presented for slaughter e.g. 21.2%of the caecal contents, 43.6% of the lymph nodes adjacent to the ileocecal junction and 52% of the distal colon contents sampled were positive.

It is reported that the prevalence of *Salmonella* spp. in dairy cattle, sheep, calves and beef cattle presented for slaughter in New Zealand ranges from 13-15% (Ekperigin & Nagaraja, 1998). However the prevalence of salmonellae in animals when determined by faecal culture is underestimated because faecal shedding in carrier animals occurs only 3-4% of the time, it is cyclical, it is a one time episode and not all animals which are seropositive for salmonellae are also shedding the organism through their faeces (Smith *et al.*, 1994). To overcome this shortcoming of faecal culture there must be multiple faecal sampling of animals being studied for at least three consecutive days. This is not logistically and economically feasible. Serology has also been

recommended as an alternative to faecal culture because it can also identify non-faecal excreting animals.

However, serology on its own also has limitations. Its use can result in either the overestimation or underestimation of *Salmonella* carriage in domestic animals. The lag period between infection and expression of antibodies in circulation means the recently infected animals will be missed resulting in underestimation of the prevalence of *Salmonella* spp. in animals. However, maternal antibodies, cross reactivity between serotypes and interference from antibodies due to vaccination might result in the overestimation of the prevalence of previous exposure to *Salmonella* spp. in animals (Hollinger, 2000).

The other alternative is the combined use of serological methods and faecal sampling to determine the prevalence of *Salmonella* spp. in livestock. However, this method will only increase the sensitivity for detecting the more invasive serotypes like *Salmonella typhimurium* because they induce high enough antibody titres to be detected by serological methods like ELISA (Galland *et al.*, 2000; Stege *et al.* 2000). The molecular epidemiological methods can also be used in tandem with traditional faecal culture and typing methods to explain the behaviour of *Salmonella* spp. within host populations.

Pulsed-field gel electrophoresis (PFGE) has been used to study the epidemiology of *Salmonella brandenburg* in the South Island of New Zealand. The PFGE profiles of the isolates from outbreaks of *Salmonella brandenburg* in the South Island of New Zealand have so far yielded one pattern. This pattern, which is different from that of

other isolates analysed before the onset of abortion outbreaks in sheep in 1996, has remained stable since the first profile of the 1996 isolates. This shows that the abortion outbreaks in the affected regions are due to a strain of *Salmonella brandenburg* with a common ancestor although the original source has not been identified. This stable pattern could be a reflection of the genetic stability of the outbreak strain i.e. it has a long molecular clock (Boxall *et al.*, 1999; Clark, 2001a). However, Clark (2001a) reported the isolation of an isolate of *Salmonella brandenburg* from a sheep yard dust sample that had lost one large molecular weight band and gained three lower molecular weight bands. According to a criteria described by Tenover *et al.* (1995) this could have been due to a single independent genetic event like a point mutation, insertion or deletion of a DNA sequence which still make this isolate closely related to the outbreak strain.

2.4 *Salmonella brandenburg* infections in sheep and other animals

Salmonella brandenburg being a non-host specific serotype has been associated with disease in a wide range of animals and human beings. In the Kauffman-White scheme *S. brandenburg* is considered as one of the frequently isolated serotypes, being in the top 50 of the more than half a million cultures analysed. A serotype is categorised as common if it was isolated more than 500 times in the study. (Kelterborn, 1967). Other than sheep *S. brandenburg* has been isolated from cases of cattle, horses, cats, dogs and people in New Zealand (Clark, 1999; Clark *et al.*, 2000).

The clinical manifestations of *Salmonella brandenburg* infection in pregnant ewes are lethargy followed by abortion and up to 30% may die. Abortion storms occur typically in the second half of gestation. The manifestation of clinical signs in ewes makes *Salmonella brandenburg* different from other abortion causing serotypes like *Salmonella montevideo, Salmonella abortusovis* and *Salmonella typhimurium* in Europe and *Salmonella arizonae*, serotype O: 61/ H:1,5,7 in the North Americas (Long *et al.*, 1978; Linklater, 2000). *Salmonella abortusovis* and *Salmonella montevideo* are rarely associated with clinical signs in the ewe. The only clinical manifestation in pregnant ewes associated with the two serotypes is abortion in the last half of gestation. Although *Salmonella typhimurium* and *Salmonella dublin* maybe associated with scours and anorexia in the ewes before abortion usually the infected animals die of septicaemia before aborting their lambs (Linklater, 2000; Wray & Linklater, 2000).

The aborted foetuses rapidly undergo autolysis and on *post mortem* have swollen livers, bloody-putrid stomach contents, and their lungs may show changes consistent with pneumonitis (Bailey, 1997; Roe, 1999). These *post mortem* changes are different from those associated with aborted foetus due to *Salmonella montevideo* infection. The absence of post mortem changes in abortion due to this serotype means that there is sudden foetal death after the organism crosses the placental barrier (Caldow & Graham, 1998). *Post mortem* examination of aborted ewes infected by *S. brandenburg* show changes consistent with acute enteritis, septicaemia and placentitis (Bailey, 1997).These changes except for placentitis are similar to those reported by Richards *et al.* (1993) in a study of sheep found dead in ships in journeys between Australia and the Middle East. The commonly isolated serotypes in this study were *S. typhimurium*, *S. bovismorbificans* and *S. havana*. *Salmonella brandenburg* has also been isolated in a non-pregnant hogget with metritis (Clark, 1999; Clark *et al.*, 2000).

In New Zealand *Salmonella brandenburg* has been isolated from dairy cattle with bloody diarrhoea, dysentery, sporadic abortions, reduced production and death in calves (Clark *et al.*, 2000; Clark, 2001a; Clark, 2001b). The serotype has also been recovered from dogs with diarrhoea, bitches with uterine infections and a male dog with a reproductive tract infection. Isolation of the serotype has also been recorded from a case of diarrhoea in foals (Clark *et al.*, 2000; Clark, 2001a).

2.5 The public health implications of the *Salmonella brandenburg* abortion storms in sheep in the South Island of New Zealand

Non-typhoidal salmonellosis in humans due to *Salmonella typhimurium* was first reported in Germany in 1888. People fell ill after eating ground meat derived from a moribund cow (Tauxe, 1991). Since then non-typhoidal salmonellosis in humans has replaced the host adopted *Salmonella typhi* as the most important cause of human morbidity. Non-typhoidal salmonellosis in people typically manifest itself as diarrhoea, abdominal pain, myalgia, and fever 8-72 hours after ingestion of the agent (D'Aoust, 1994). The 1970's and 1980's in the United States were marked by an explosion of non-typhoidal salmonellosis in humans with the dominant serotypes being *typhimurim, enteritidis, heidelberg* and *newport*. Beef, chicken, turkey and eggs were the most common foods of animal origin associated with cases where a source was identified (Tauxe, 1991). Due to lack of host specificity *Salmonella brandenburg* is of public health importance. Like other non-host adapted serotypes it poses a risk as a foodborne pathogen and an occupational hazard to farmers, veterinarians, animal handlers and meat plant workers as a zoonoses. It has already been isolated from cases involving humans, food animals, other animals, foodstuffs, feeds, water, sewage and other sources (Kelterborn, 1967).

Salmonella brandenburg as a human pathogen was first isolated in Germany in the 1930's. In New Zealand *Salmonella brandenburg* was first isolated from a human case in 1985 and until 1996 this serotype accounted for 1-2% of the annual cases of human salmonellosis (Fenwick, 2000). However from 1997 the human cases of *S. brandenburg* increased to 2.8% followed by further increases to 7.4% in 1998 and to

7.6% in 1999. The temporal pattern of occurrence of this serotype in people in the South Island of New Zealand corresponds with that seen in sheep. The human cases reach a peak during the lambing period which is in the third quarter of the year (Fenwick, 2000; Smart, 2000). These cases of *S. brandenburg* are mainly in rural males, average age of 28.2 years and 47% of the affected people are agricultural workers or their families (Fenwick, 2000; Clark, 2001b). This illustrates the importance of *Salmonella brandenburg* as an occupational hazard in farm workers.

The handling of infected materials and lack of personal hygiene increases the risk of this occupational hazard to the farm workers. This is shown by the fact that the number of human cases in the South Island increase during the lambing season when the high-risk material is being handled (Clark; 2001b). The above trend is similar to the one in the Scotland where the number of animal and human cases of *Salmonella typhimurium* DT 104 increases in spring-autumn when there is a high human-animal contact during the calving season (Calvert *et al.*, 1998). Although reported human cases of *Salmonella brandenburg* infection in the South Island so far have been due to handling of high-risk material, the potential for contamination of meat and subsequent disease in both consumers and meat plant workers must be acknowledged.

Human salmonellosis is usually associated with fever, abdominal cramps, nausea, vomiting and diarrhoea. There can also be extra intestinal sequelae to bacteraemia due to the bacteria localising in the different tissues and organs. The sequelae includes pneumonia, thrombosis, renal failure, meningitis, encephalitis and polyarthritis.

The severity of both the enteric and systemic forms of human salmonellosis is influenced by factors like extremes of age, immune status, antibiotic or antacid use and cancer therapy (D'Aoust, 1994; Gomez *et al.*, 1997; Pelzer, 1999). Salmonellosis is usually severe in the very young and old, in people with concurrent disease and those on immunosuppressive drugs leading to a weak immune response to infection. Antibiotics interfere with the normal flora thus increasing the likelihood of a successful infection outcome by enteric pathogens. Antacids protect enteric pathogens from the hostile acidic pH of the stomach and like antibiotics increase the likelihood of a successful infection by salmonellae and other enteric pathogens.

The heterogeneity of consumer susceptibility which is influenced by immune status of the host, anomalies of the gastro-intestinal tract and presence of concurrent disease has very important implications for infective dose and control measures of salmonellae infections. The infective dose of salmonellae in people has been reported to be in the range of 10 to 10^7 organisms. The infective dose can be influenced by virulence of the serotype and type of food. Fatty foods are associated with lower infective doses because the organisms are protected in a micelle from the hostile acidic environment of the stomach. Water is also associated with lower infective doses because unlike solid food it does not stimulate the production of acid in the stomach. The wide range of infective dose of *Salmonella* spp. for humans means that there should be stringent measures of control throughout the production chain (D'Aoust, 1994).

A study in the United States showed a prevalence of salmonellae of 1.5% on lamb carcasses. Similar studies in Australia showed a *Salmonella spp.* prevalence of 0.7%

in lamb carcasses and a 1.3% prevalence in frozen lamb meat (Duffy *et al.*, 2000; Phillips *et al.*, 2001). This low prevalence maybe an underestimation of the actual prevalence, because carcass samples are taken from a restricted swabbed area compared with broilers where a whole carcass sample is taken. The fact that there is *Salmonella* spp. isolated from lamb carcasses in other countries means that there is a possibility of *Salmonella brandenburg* becoming an important foodborne pathogen associated with lamb.

The first case where sheep meat was reported to have been a source of foodborne salmonellosis was in 1919-1920 in Germany after people had eaten sausages made from sheep meat. The animals had developed dysentery during transportation to the meat plant, and had to undergo emergency slaughter on arrival (Wray & Linklater, 2000). In the United Kingdom there has been reports of sheep meat in kebabs being associated with foodborne outbreaks of salmonellosis in people (Synnott *et al.*, 1993; Evans *et al.*, 1999). Although cross contamination at kebab manufacturing establishments or restaurants where the kebabs were served cannot be ruled out, it was possible that the lamb meat became infected at processing or on the farm.

Although in New Zealand so far the human cases of *Salmonella brandenburg* infection have been associated with the handling of high-risk materials, in other parts of the world this serotype has been associated with foodborne illness. The cases of human salmonellosis associated with *S. brandenburg* have been reported in Germany in 1930 and United Kingdom in 1945. In both countries contaminated pork or pork products were implicated as sources of human infections. In the United Kingdom the early 1960's saw an increase in the number of human *S. brandenburg* cases. The

number of cases peaked at 220 in 1963. Epidemiological studies showed that the outbreaks were associated with pork sausages, with contamination traced back to the retail premises, carcases and lairages (Jones *et al.*, 1964). Outbreaks of human salmonellosis associated with *S. brandenburg* have also been reported in Switzerland in 1992 (Baquar et al., 1994). The Swiss outbreaks were associated with eating of meat and meat products (Baquar *et al.,* 1994). These reported outbreaks illustrate that although in New Zealand an outbreak associated with lamb has not be reported there should be increased vigilance. Lamb is a potential high-risk meat source for *S. brandenburg* infection in New Zealand.

The abortion storms and the associated mortalities in ewes due to metritis have resulted in veterinarians recommending the use of antibiotics to reduce ewe mortality. This is of public health concern because the overuse of antibiotics has been linked with the emergence of multiple drug resistant strains of salmonellae such as *Salmonella typhimurium* DT 104. This serotype is resistant to commonly used antibiotics like ampicillin, chloramphenicol, streptomycin, sulphonamides and tetracyclines (Dargatz *et al.*, 1998; Akkina *et al.*, 1999). Therefore, extreme caution is required because selection pressure due to antibiotic use may result in emergence of antibiotic resistant strains of *Salmonella brandenburg* in the South Island of New Zealand. This could create an undesirable public health dimension to the already serious animal health problem. The multi drug resistant strain of *Salmonella typhimurium* is reported to cause higher mortalities and hospitalisation rates as compared to other zoonotic *Salmonella* serotypes (Dargatz *et al.*, 1998; Akkina *et al.*, 1999). However, to reduce mortality in ewes and address animal welfare concerns associated with the *S. brandenburg* outbreaks, the use of antimicrobials is inevitable.

Antimicrobials should be used prudently whereby the correct antibiotic, correct dose and correct duration of treatment is followed. This would reduce the risk of inducing antibiotic resistance in *Salmonella brandenburg* and other bacterial pathogens through uncontrolled use of antibiotics (Anon, 2001b).

2.6 Control and prevention of salmonellosis in animals and humans

The control of *Salmonella* spp. involves the prevention of colonisation and neutralisation after colonisation. The three main control levels of infection in poultry are flock level, slaughter hygiene and public education (Barrow, 1993). These three levels can be similarly applied for control of salmonellae infections in other food animals. In order to put in place any control measures the on-farm factors that expose hosts to infection must be identified. The on-farm factors associated with salmonellosis include introducing untested replacements into a 'clean' flock without quarantine, contaminated water sources, contaminated feed, failure to control vermin, presence of undetected carrier animals in the flock, unhygienic farm practices and absence of isolation facilities (Smith *et al.*, 1994). At the harvesting phase of production the main sources of exposure for live animals and carcases, are transport, lairages, storage, dirty skin, abattoir environment, visceral contamination, butchering and miscellaneous sources (Patterson, 1969). Prevention in contrast deals with measures that prevent contact between the agent and host (Ekperigin & Nagaraja, 1998), this would include putting in place measures that prevent the animals from coming into contact with the agent. However, due to the present production systems, exposure to the disease causing agents can only be minimised and not eliminated. Minimising animal-host contact would in turn reduce the foodborne risk of infection to consumers. At farm level prevention should be used in conjunction with control measures like vaccination and competitive inhibition (Ekperigin & Nagaraja, 1998).

Vaccination is one of the most important methods used to control salmonellosis in livestock. It can be achieved through the use of killed bacteria and adjuvants or

attenuated live vaccines. The killed vaccines are preferred for vaccination against non-host adapted strains because of the safety issue. Live vaccines may induce shedding of the pathogen in faeces and this will create a public health hazard associated with food animals. However for host adapted strains like *Salmonella abortusovis* that has never been isolated in human beings it is better to use live vaccines. Live vaccines induce a stronger and longer lasting immune response as compared to killed vaccines (Pardon *et al.*, 1990).

Vaccination against *Salmonella* spp. infection in domestic animals has evoked a lot of debate because of the conflicting experimental results to prove or disprove the effectiveness of vaccines. Despite previous reports of unsuccessful attempts at eliciting protection from vaccines Jonas (1967) reported that an inactivated *Salmonella typhimurium* vaccine tested in mice resulted in resistance to a challenge. In New Zealand the efficacy of the bivalent *Salmonella typhimurium* and *Salmonella bovismorbificans* vaccine has been shown in case control studies. Wallace & Murch (1967) reported resistance to *Salmonella* spp. infection in vaccinated sheep flocks as compared to unvaccinated flocks and reported that the maximum protection to infection was attained 5-10 days after vaccination. This result therefore suggested that the vaccine could be used in face of and effectively change the course of an outbreak. Beckett (1967) in a study of summer- autumn outbreaks in the North Island of New Zealand reported that unvaccinated flocks experienced high mortalities compared to vaccinated flocks and that vaccination changed the course of the disease after 14 days. Dewes (1979) also reported that the use of vaccine resulted in lower mortalities, no clinical disease and reduced environmental contamination. Due to the experimental results Wallace & Murch (1967) suggested that primary vaccination

should be carried out at weaning with a booster before the anticipated time of the outbreaks. They also suggested that all replacement stock should be vaccinated on arrival.

The *Salmonella brandenburg* strain that causes abortion outbreaks in the South Island of New Zealand is suitable for control by vaccination because of several factors.It is genetically stable as shown by the PFGE profiles that have not changed since 1996. It also shares O and H antigens with vaccine strain of *Salmonella typhimurium* being used in New Zealand. The somatic antigen formulae for *Salmonella typhimurium* and *Salmonella brandenburg* are O: 1,4, 5, 12 and O: 1, 4, 12 respectively (Marchant, 1999; Marchant, 2000; Marchant *et al.*, 2000). Mouse challenge results from Salvexin™- a licensed vaccine of killed whole cell strains of *Salmonella typhimurium* (2) and one strain each of *Salmonella hindmarsh* and *Salmonella bovismorbificans* showed cross protection to *Salmonella brandenburg* challenge. Therefore the licensing authorities allowed labelling claims of protection against *Salmonella brandenburg* in sheep. Preliminary data from farms that vaccinated with Salvexin™ ,compared to those that did not vaccinate, showed some protection in two tooths but mixed results for the mixed age ewes. This maybe due to the fact that immunity after exposure in mixed age sheep interfered with immunity induced through vaccination. Schering-Plough has also developed an inactivated whole cell vaccine with outbreak *Salmonella brandenburg* strain. However, the sheep challenge using killed vaccines Salvexin + Brandenburg™ antigens, and Salvexin™ did not show any major differences in efficacies of the two vaccines. All the experimental animals aborted within seven days (vaccinated and unvaccinated) i.e. both were ineffective in face of a high challenge. The only useful finding was that the onset of abortion was quick in

unvaccinated ewes, followed by those vaccinated with Salvexin™ only, while those vaccinated with Salvexin + Brandenburg™ antigens had a delayed onset of abortions compared to the unvaccinated ewes and those vaccinated with Salvexin™ only (Marchant *et al.*, 2000).

Vaccines are indicated for both the protection of animal health and public health. However, salmonellosis in animals and food poisoning are mostly associated with contamination by non-host adapted serotypes. This makes the use of vaccines in animals to control food poisoning serotypes questionable because of the huge number of serotypes involved. This is particularly so because mostly single or bivalent antigen vaccines are used in the control of salmonellosis. Therefore these vaccines play a limited role in the prevention of human salmonellosis acquired from food of animal origin. It is reported that the use of combined vaccines might result in interference between antigens of the different serotypes which will result in decreased efficacy, and that cross protection between serotypes is both weak and short lived, lasting not more six months (Barrow & Wallis, 2000; Wallis, 2000). It is these shortcomings associated with cross protection and polyvalent vaccines which are an indication for the future use of herd specific vaccines, particularly for rare serotypes (Wallis, 2000).

Killed vaccines have several shortcomings that include failure to elicit a cell-mediated immunity, failure to elicit immunoglobin A and being prepared based on the antigens elicited in the *in-vitro* environment. Although the live attenuated vaccines can overcome the above shortcomings they have a potential to revert back to virulence and the organisms could be cultured from the product resulting in the rejection by importing countries, which conduct *Salmonella* spp. testing at the port of entry. This

reversion to virulence also has public health implications because potential pathogens are being introduced into animals that are ultimately going to be eaten by human beings (Barrow & Wallis, 2000; Holt, 2000; Wallis, 2000). However, there are other molecular techniques that are presently being investigated to make live attenuated vaccines safe (Smith *et al.,* 1984; Khalil *et al.*, 2001).

The understanding of *Salmonella* spp. virulence means there is a possibility of inducing a mutation in Salmonella Pathogenicity Island 1 (SPI 1), which will delete the salmonellae factors, associated with invasiveness thus giving some safety to the live vaccines. The other molecular based technique is to use aromatic dependent (auxotrophic) strains of *Salmonella* spp. in live vaccines. Since there are no aromatic metabolites in animals these strains will not be able to revert to virulence in animals (Smith *et al.*, 1984). An experiment on two-week-old calves using an aromatic dependent strain of *Salmonella dublin* showed that with a live challenge the animals gave no positive blood cultures. This meant the vaccine enabled the animals to clear the *Salmonella* spp. at mesenteric lymph node level or in the blood stream. This ability to clear the organisms could have been achieved through the antigen specific antibodies acting as an opsonin and the non-specific intracellular killing.

The non-specific intracellular killing of *Salmonella spp.* conferred to a host by a vaccine is very important because it protects the host against the multiple serotypes that the host is exposed to (Smith *et al.*, 1984). Work in New Zealand by Khalil *et al.* (2001) on a strain of *Salmonella typhimurium* with a deleted adenyl cyclase and cAMP receptor protein showed it had a reduced virulence in pregnant sheep compared to a wild strain. Therefore this genetically modified strain has a potential to be used as

a live attenuated vaccine against *Salmonella typhimurium* and other group B salmonellae serotypes like *Salmonella brandenburg*.

Even then these possibilities still have to face public perception, for the public is not yet ready to accept genetically modified organisms in their food although it can accept antimicrobial use in animals destined for the food chain. It is this public resistance to live vaccines which makes registration of live vaccines very long and tedious. This in effect may mean that by the time they are registered the epidemiology of the *Salmonella* serotype(s) has changed, rendering the vaccine useless (Wallis, 2000).

However, the effectiveness of vaccines for the protection against *Salmonella* infections should be treated with caution because it is known that animals acquire some form of protection against future infections after exposure. There is an opinion in the scientific community that the post exposure protection in sheep is only overcome if the animals are stressed (Barrow & Wallis, 2000; Wray & Linklater, 2000). In the United Kingdom endemicity is only associated with the host adapted serotype *Salmonella abortusovis* while the non-host specific serotypes like *Salmonella montevideo* are usually associated with one abortion episode. In the following lambing seasons abortion due to non-host adapted serotypes affects two tooths and replacement animals only because of the naïve immunity (Linklater, 2000).

The problems associated with both killed and live vaccines mean an ideal vaccine for *Salmonella* spp. control is yet to be found. An ideal vaccine would elicit strong gastro-intestinal and cell mediated immunity, be avirulent to humans, be easy to administer, evoke long lasting immunity, and be compatible with both competitive

inhibition organisms and growth promoting antimicrobials in countries where their use is allowed (Barrow & Wallis, 2000). While still searching for an ideal vaccine other methods of prevention particularly at farm level should be put in place because this would increase the microbiological quality of meat at slaughter (Galland *et al.*, 2000).

Since the nature of the present production methods is such that animals are continuously being exposed to *Salmonella* spp. infection, resources should be made available to identify and control those factors that increase risk of transmission and infection (Mallinson *et al.*, 2001). Replacement animals are one of the most important sources of *Salmonella* spp. infection to naïve animals. In order to reduce the role of replacement animals as a source of infection, animals should be quarantined for four weeks before they are introduced into the flock. This precaution means animals that might show signs of salmonellosis will be identified and not be introduced into the flock.

There should also be isolation facilities on-farm so that sick animals can be isolated from the rest of the flock or herd so that the oral faecal route of transmission that is important for *Salmonella* spp. is cut. Although not economically feasible for production level flocks, animals at the top of the production pyramid i.e. the breeding flock should be tested serologically and by faecal culture. Animals that are positive should be culled to reduce the risk of transmission of salmonellae to the commercial flocks (Rings, 1985; Mallinson *et al.*, 2001). However, this approach is more applicable to poultry and pig production systems and less so for sheep production systems.

Feed and water sources can also be an important source of *Salmonella spp.* to animals. The importance of feed as a source of salmonellae for livestock was illustrated by a study in the United States. A 10 months study by Williams *et al.* (1969) found that 211 of 311 samples of animal and fish protein meals tested were positive for *Salmonella* spp. Sourcing of feed from manufacturers who use approved processes could prevent the exposure of animals to *Salmonella* serotypes associated with feed. Feed decontamination at processing could be achieved through either heat, chemical treatment or irradiation as recommended by the World Health Organisation (Anon, 1994; Headrick & Tollefson, 1998). Feed should also be stored away from vermin on-farm and if troughs are used they should be cleaned regularly. Troughs should also not be over filled to avoid feed remaining for a long time without being utilised. This practice exposes feed to vermin and other contaminants like faecal matter. To reduce the risk of water source contamination, effluent, manure and other farm waste like straw should be disposed away from water sources (Pelzer, 1989; Dargatz *et al.*, 1998; Akkina *et al.*, 1999).

Vermin should be controlled by trapping of rodents, use of flytraps and scare dummies for gulls. Gulls have been implicated in the spread of *Salmonella* spp. in Europe, Australia and New Zealand. These measures would help prevent the persistence of the newly introduced serotypes like *S. brandenburg* by controlling new infections and the transmission of the pathogen from already infected farms to uninfected farms (Jones *et al.*, 1964).

The specific measures for the control of *Salmonella brandenburg* abortions in sheep in the South Island of New Zealand include the burning or burying of foetal material *post partum*. This would reduce environmental contamination and the spread of this microorganism from farm to farm by gulls, rodents and other vermin. The aborting ewes should be isolated from the rest of the flock to reduce the transmission to other animals. The ewes should also be culled because they are a potential reservoir for the infection. Despite the results of vaccination being questionable, vaccination of two tooths at tupping and a booster at scanning is recommended. Alternatively there should be a time interval of 4-6 weeks between primary and secondary vaccination. There should be a reduction in the frequency of yarding and the periods that the animals stay yarded. This will reduce stress to the animals and thus the associated excretion of salmonellae. Avoiding unnecessary yarding will also assist in reducing the animal-to-animal transmission facilitated by the close proximity during yarding. There should also be personal hygiene and disinfection to reduce the spread of infection from animal to animal through contaminated equipment, hands and protective clothing. Scavengers particularly black backed gulls should be controlled through the use of scare dummies, poisoning with alphachloralose, egg and nests destruction, and shooting (Clark *et al.*, 1999; Fenwick, 2000; Smart, 2000; Clark, 2001a).

Foodborne salmonellosis can be controlled by the use of both control and prevention measures listed above for food animals. Hygienic slaughter and the use of processes that reduce carcass contamination are also important in the control of foodborne salmonellosis. A study by Grau & Smith (1974) showed that long waits in the lairages resulted in increased environmental contamination due to increased shedding. This in

turn resulted in high fleece contamination which resulted in a high carcase microbial count. Despite contrasting results from a study by Duffy *et al* (2000) it is obvious that reduced time in the lairages, short fleece and clean lairages will result in clean animals presented for slaughter. This will in turn reduce the risk of carcase contamination.

The fleece and gastro-intestinal contents are the most important sources of enteric pathogens on carcase surfaces (Hadley *et al.*, 1997). A study by Hadley *et al.* (1997) showed that changes in slaughter procedures to compensate for wet soiled, long fleeced animals presented for slaughter did not decrease the microbial load of carcases. This means if dirty animals are presented for slaughter they should be either cleaned and given time to dry, or rejected. Penalising farmers for presenting dirty animals for slaughter could discourage them from presenting dirty animals for slaughter. Alternatively farmers could be encouraged to present clean animals for slaughter by a paying a premium for clean animals. A standardised soiling scale should be used by the regulatory authorities in the acceptance or rejection of animals for slaughter, so that the farmers know exactly what is required (Hadley *et al.*, 1997).

The cleaning and disinfection between loads of trucks that transport animals can also reduce the bacterial load on animals presented for slaughter. This coupled with the cleaning and disinfection of lairages between mobs would reduce the risk of environmental cross contamination of animals presented for slaughter (McGrath & Patterson, 1969). This would reduce the environmental load of salmonellae because the genus is susceptible to the commonly used disinfectants (Rings, 1985).

The other harvest-associated measures to reduce the risk of carcase contamination with enteric pathogens include hygienic dressing, improved methods of pelt removal and carcase decontamination. Carcase decontamination can be done using physical, chemical and microbial methods. However, most of these methods except temperature based decontamination and acid rinses are still not widely used due to reluctance of bodies like the European Union, which fears that their acceptance will result in over reliance in carcase decontamination at the expense of general plant hygiene (Mead, 2000). An automated inverted method of pelt removal and steam vacuuming of carcases are known to reduce the bacterial counts on lamb carcases. In order for steam vacuuming to be effective the Steamvac™ should be located at a point in the process where all possible contamination procedures have been completed. Also all carcases should be steam vacuumed with special attention to leg and breast which are the primary sites for soiling in sheep (Hadley *et al.*, 1997; Duffy *et al.*, 2000).

Temperature abuse of carcases that results in increased growth rate of enteric spoilage and pathogenic organisms should also be prevented. The measures of prevention include never leaving carcases on line during breaks, continuous monitoring of temperatures, adequate spacing of carcases in chillers to ensure uniform cooling and practicing the first in first out principle on carcase release from the chillers.

In addition to intervention at primary source and slaughter, resources should be put into public education to reduce foodborne salmonellosis due to poor handling of foods of animal origin at home (Samuel *et al.*, 1981; Dargatz *et al.*, 1998; Akkina *et al.*, 1999). Food and water are the primary sources of non-typhoidal salmonellosis to people with person-person and pet-human transmission playing only a secondary role.

Data from 39 countries which submit reports to the World Health Organisation showed that between 1985 and 1995 there was an increased incidence of foodborne human salmonellosis (Gomez *et al.*, 1997). The prevention of foodborne salmonellosis at consumer level should include education in safe handling, storage and preparation of food of animal origin.

The public should also be educated about the safety of low doses of irradiation that can be used to disinfect food. However, not only consumer resistance makes the use irradiation uncommon it also still expensive and not readily available particularly for meat (D'Aoust, 1994; Gomez *et al.,* 1997). Food safety legislation should be reviewed from time to time to keep up with changes in production methods and emergence of new pathogens of public health importance.

Legislation on labelling of foods of animal origin to prescribe minimum information required for high-risk food products can also assist in increasing public awareness about the possible risks associated with mishandling foods of animal origin at home (Headrick & Tollefson, 1998).

2.7 Conclusion

Salmonella brandenburg as cause of ewe mortalities and abortions in the South Island of New Zealand does not only pose a welfare problem to sheep but it is also a threat to the lamb export industry. Although the importance of this serotype as a foodborne pathogen is unclear, indications are that a high dose taken orally can cause disease in man. Therefore it is important that the pathogenesis and epidemiology of the pathogen is understood in order to put into place control and preventive measures (Logtestijn van, *et al.*, 1993). The potential for the use of *Salmonella brandenburg* infection in New Zealand lamb as a trade barrier cannot be ignored. This should act as a stimulus to do further research into the development of vaccines and into identification of risk factors contributing to infection (Clark *et al.*, 2000).

Despite 100 years since its first isolation a lot is still not understood about the genus *Salmonella*. Mechanisms for host adaptation, serotype dominance within a geographical area or population, ecology in the gastro-intestinal tract and role of humoral and cell mediated in the resistance of hosts to infection are still not well understood (Wray, 1995).

The wide array of methods used at present are a hindrance to the full understanding of the epidemiology of the genus. The results from different studies can only be compared with confidence if the different researchers use standardised methods for isolation of *Salmonella* spp. (Harvey & Price, 1979).

Public education is very important and should involve the media because food scares generated by the ill informed media lower public confidence both in the meat industry and in the regulatory bodies (Humphrey, 2001). The cost of 'Salmonella in egg scares' in Europe particularly in the United Kingdom nearly crippled the industry. It is estimated that this particular food scare cost the egg industry in the United Kingdom 70 million British pounds. The cost to the industry was broken down into surveillance costs, regulatory measures costs, reduced demand and the slaughter of infected flocks costs (Persson & Jendtey, 1992). But if the media and public are involved from the start such damage to the lamb industry in New Zealand might be avoided if the '*Salmonella brandenburg* in lamb scare' was to occur.

Although salmonellae are ubiquitous and cause disease in man and livestock, there have been cases where a certain serotype suddenly assumed importance. This phenomena might be related to narrowing of the serotypes niche width. The niche width can be narrowed by increased production intensification, reduced genetic diversity of the host population and standardization of the food type. It might be this narrowing of niche width which resulted in the *Salmonella enteritidis* epidemic in chickens and the emergence of *Salmonella brandenburg* as a sheep pathogen in the South Island of New Zealand (Hunter & Izsak, 1990). Even though *S. brandenburg* is a short-term epidemic unlike *Salmonella enteritidis*, which has been there since the1970's, it is imperative that the contributing husbandry methods are investigated so that the *Salmonella brandenburg* abortion outbreaks in sheep are controlled and prevented.

A further investigation of the pathogenesis of *S. brandenburg* is required so that the period of greatest vulnerability in the animals is identified. This would assist in putting into place measures that remove risk factors at this period. Smart (2000) observed that *Salmonella brandenburg* abortions peaked 4-6 weeks before lambing.

Salmonella spp. could be regarded as primarily human pathogens and it is through human's activities that the members of the genus are spread in the environment. The increased incidence of *Salmonella* spp. infections could be due to the decrease in the genetic base of the hosts because of the breeding methods that promote homogeneity of the host e.g. poultry breeding and the *Salmonella enteritidis* pandemic. The different husbandry methods like high stocking densities, housing of food animals, and standardisation of food types for both humans and animals, have resulted in the increased incidence of human foodborne salmonellosis (Murray, 2000).

The control and prevention of *Salmonella* spp. infections in humans and domestic animals requires an integrated approach with all stakeholders playing a role. These are farmers, feed and food producers, veterinarians, medical practitioners, environmental health scientists and the general public. The general public could be reached by putting resources into public education (Dargatz *et al.*, 1998). It is also very important to involve the feed and meat industry and deal effectively with issues of commercial confidentiality (Anon, 1994). In the past the hesitance of the industry to release commercially sensitive information may have hindered efforts to control foodborne diseases, including salmonellosis.

CHAPTER THREE: DETERMINING THE PREVALENCE OF *Salmonella spp.* IN SHEEP AT DRAFTING ON SELECTED CASE AND CONTROL FARMS IN THE SOUTH ISLAND OF NEW ZEALAND

3.1 Introduction

Salmonella brandenburg was first isolated from the faeces and urine samples of a nurse with gastro enteritis in Berlin, in 1929. Shortly afterwards it was isolated from another patient in a sanitorium. The microorganism was initially put in the paratyphus group, but later Kauffmann and Mitsui described its antigenic formula and named it *Salmonella brandenburg* because Berlin is situated in the county bearing the same name. By the mid 1960's the serotype had been isolated worldwide from a number of sources. These included human, food animals, other animals, foodstuffs, feedstuffs, water, sewage and other unspecified samples (Kelterborn, 1967).

A World Health Organisation consultation on control of *Salmonella* spp. infection in animals and human foodborne infections has recommended the standardisation of diagnostic methods for *Salmonella* spp. This would enable the comparison and sharing of data on research activities (Harvey & Price, 1979; Anon, 1994). The use of faecal culture as the method for the estimation of prevalence of salmonellae infection in animals has its shortcomings because animals that are infected but not shedding the organisms are not detected. These include both the seropositive and carrier animals. It is reported that animals infected with the host adapted serotypes shed salmonellae organisms only 3-4% of the time (Smith *et al.*, 1994). In order to reduce the underestimation associated with faecal sampling and intermittent nature of faecal excretion of *Salmonella* spp. organisms

there should be multiple sampling of animals tested over several days (Corrier *et al.*, 1990).

The combination of faecal culture and serology can also assist in correcting some of the underestimation associated with the use of faecal culture alone (Galland *et al.*, 2000; Hollinger, 2000; Stege *et al.*, 2000). However, for the purposes of this study serology was not suitable because the study was interested in all salmonellae in control and affected farms, and there is no specific *S. brandenburg* serological test. Serology was also not suitable because the study was interested in the risk of contamination at slaughter not previous the history of exposure. The ewes from affected farms would have had antibodies from previous exposure but they may or may not have been shedding hence only faecal cultures would have met the objectives of the study. Also in this pilot study, time constraints, costs and interference with on farm activities meant multiple faecal samples on-farm or a serological study could not be undertaken.

The specific objective of this chapter was to compare the prevalence of faecal samples positive *Salmonella* spp. on sheep farms with or without the history of recent *Salmonella brandenburg* disease using rectal faecal samples taken at drafting before slaughter.

3.2 Materials and Methods

3.2.1 Flock selection

Four control farms and four case farms were purposively selected by Gary Clark (**Labnet, Invermay**) on the basis of the disease history of the individual farms and willingness to cooperate in the study. The choice of the case farm depended on the clinical occurrence of abortions storms on the farm during the 2000 lambing season with laboratory confirmation of *Salmonella brandenburg* as the cause of the abortions. To get the worst-case scenario regarding the implications of abortion outbreaks with respect to potential carcass contamination, case farms with severe abortion outbreaks were selected. Sheep veterinary practitioners in the Southland region provided this information. Control farms were chosen on the basis of absence of abortion outbreaks in the 2000 season and no clinical history of *Salmonella brandenburg* disease on the farm since the abortions were first identified as being caused by this serotype in the 1996/97 lambing seasons.

3.2.2 Sampling

During drafting 50 ewes and 50 lambs on each farm were randomly chosen for faecal sampling. 50 animals were chosen to give a 90% confidence interval and an error of 10% for the expected prevalence of 50%. Using a non-bactericidal lubricant on a sterile glove approximately one gram of faecal material was collected from the rectum of each animal

sampled. The faecal samples were then put into labelled plastic bottles and transported to the laboratory (**LabNet, Invermay**) in cooler boxes where they were processed using standardised methods.

The study was conducted in two phases, phase I (November-December) and phase II (February-March) with no changes in sampling and culture methods. The November-December period was chosen because it is closer to the *Salmonella brandenburg* abortion outbreaks, while the February-March period is a longer time after the outbreaks. This difference in time interval would indicate if there is a difference in the risk of infection in animals presented for slaughter closer to the abortion outbreaks as compared to animals presented for slaughter after a longer time since the abortion outbreaks.

3.2.3 Isolation techniques

The faeces were added to buffered peptone water (BPW) in a 1:10 dilution ratio. The BPW-faeces mixture was then incubated at 37 degrees Celsius (^{0}C) for 16 – 24 hours. After this period 0.1 ml of the BPW-faeces mixture was transferred to 10 ml of Rappaport-Vassiliadis (RVS) broth and incubated for 22-26 hours at 42 ^{0}C . This was followed by the subculture onto xylose lysine desoxycholate (XLD) agar and modified brilliant green agar (BGA- with Novobiocin to inhibit *Proteus* spp.) and incubated at 37 ^{0}C for 18-24 hours.

One suspect colony was transferred from either XLD or BGA and inoculated into triple sugar iron (TSI) and lysine iron agar (LIA) tubes, and incubated for 18-24 hours at 37 ^{0}C. Suspect *Salmonella* spp. from TSI and LIA were subcultured onto trypticase soy agar (TSA) and MacConkey and incubated at 37 ^{0}C for 18-24 hours.

3.2.4 Identification (Presumptive and confirmative)

If the colonies on MacConkey were pure non-lactose fermenting, polyvalent O and H standard slide agglutination tests were performed on the TSA colonies to confirm that the colonies were *Salmonella* spp..If the colonies were not confirmed as Salmonellae another two suspect colonies were taken from the XLD and BGA plates and the above steps repeated.

The confirmed *Salmonella* species colonies were then subcultured onto two nutrient agar slopes with one sample being kept in the field laboratory while the other one was sent to Massey University at the end of the trials. At Massey University the colonies were subcultured onto blood agar (BA) at 37 ^{0}C for 18-24 hours twice. One pure colony was then subcultured onto a nutrient agar slope and sent to The Institute of Environmental Science and Research (ESR), the national reference laboratory for serotyping. The rest of the colonies were then transferred to glycerol broth and frozen at minus 70 ^{0}C for storage.

3.2.5 Descriptive analysis

Statistical hypothesis tests could not be applied on data from this study because it was a descriptive pilot study where farm selection was done purposively to give the worst-case scenario with regard to the implication of abortion outbreak on product contamination. There was lack of independence of samples within farm i.e. if there was an infected animal on farm the other animals were likely to be infected too. Therefore comparison could be only be done at farm level but the number of farms was too low to make any statistical analysis of the data (four control and four affected farms). There was also no information on power i.e. the probability of effect.

3.3 Results

Table 3.1: Summary of the proportion of animals positive for *Salmonella* spp. by faecal culture of on-farm samples during phases I and II

	Phase I		Phase II	
	Lambs (n/N)*	Ewes	Lambs	Ewes
Affected Farms	Lambs 24/200	Ewes 28/150	Lambs 5/200	Ewes 4/150
Control Farms	Lambs 8/200	Ewes 7/200	Lambs 0/200	Ewes 1/129
Totals	32/400	35/350	5/400	5/279

* n is the number of samples positive for *Salmonella* spp. and N is the total number of samples cultured

In phase I a total of 750 samples were cultured with 67 of them giving a positive culture for *Salmonella* spp.. In phase II 679 samples were cultured and only 10 gave positive culture for *Salmonella* spp. (Table 3.1). All the isolates yielded *S. brandenburg* on serotyping.

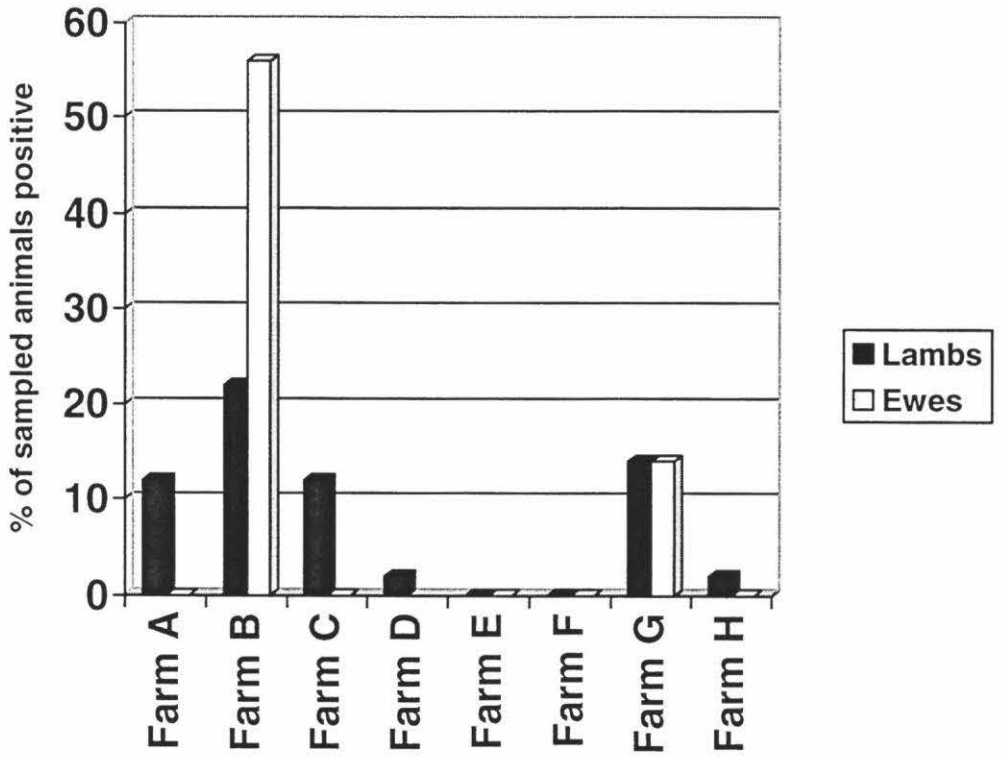
The phase I (November-December) on-farm prevalence of *Salmonella* spp. in sampled animals from affected farms (A-D) ranged from 2-22% in lambs and 0-56% in ewes (Table 3.2; Figure 1).

Table 3.2: The number and proportion of faecal samples positive for *Salmonella* spp. in lambs and ewes from affected farms during Phase I (November-December) of the on-farm sampling

Sampling site	Farm A n	%	Farm B n	%	Farm C n	%	Farm D n	%	Total positive	Total sampled	Total % positive
Lambs	6	12%	11	22%	6	12%	1	2%	24	200	12.0
Ewes	0	0%	28	56%	0	0%	X*	-	28	150	18.7

X*- No animals submitted at drafting
n- Number of animals positive for *Salmonella* spp. out of 50 animals tested unless indicated

Figure 1: On farm Prevalence of *Salmonella* spp. in lambs and ewes from case and control farms during phase I



However, the phase I on farm prevalence of *Salmonella* spp. in sampled animals from control farms (E-H) ranged from 0-14% for both lambs and ewes as illustrated in table 3.3 and figure 1.

Table 3.3: The number and proportion of faecal samples positive for *Salmonella* spp. in lambs and ewes from control farms during Phase I (November-December) of the on-farm sampling

Sampling site	Farm E n	Farm E %	Farm F n	Farm F %	Farm G n	Farm G %	Farm H n	Farm H %	Total positive	Total sampled	Total % positive
Lambs	0	0%	0	0%	7	14%	1	2%	8	200	4.0
Ewes	0	0%	0	0%	7	14%	0	0%	7	200	3.5

Table 3.4, table 3.5 and figure 2 show a lower prevalence of *Salmonella* spp. in both lambs and ewes during phase II of the on farm sampling. The prevalence of *Salmonella* spp. in sampled lambs and ewes for both classes of animals from affected farms ranged from 0-6%. The prevalence was similarly low for animals from control farms with 0% for lambs and 0-2% for ewes.

Table 3.4: The number and proportion of faecal samples positive for *Salmonella* spp. in lambs and ewes from affected farms during Phase II (February-March) of the on-farm sampling

Sampling site	Farm A n	%	Farm B n	%	Farm C n	%	Farm D n	%	Total positive	Total sampled	Total % positive
Lambs	2	4%	0	0%	3	6%	0	0%	5	200	2.5
Ewes	3	6%	1	2%	0	0%	X	-	4	150	2.7

Table 3.5: The number and proportion of faecal samples positive for *Salmonella* spp. in lambs and ewes from control farms during Phase II (February-March) of the on-farm sampling

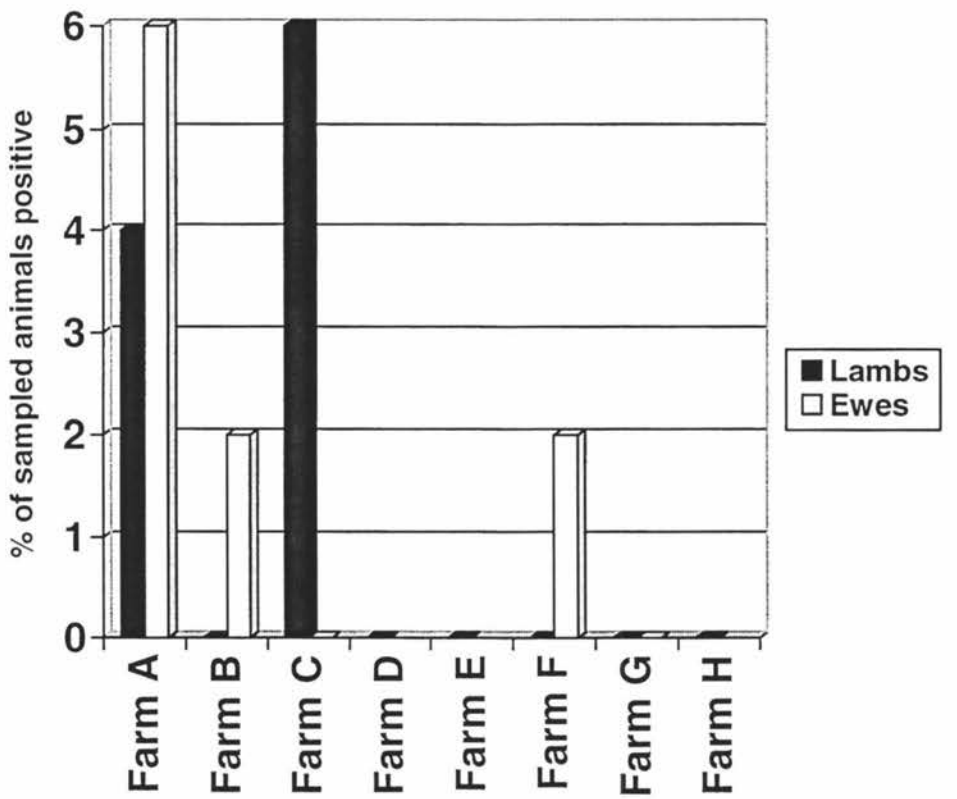
Sampling site	Farm E n	%	Farm F n	%	Farm G n	%	Farm H n	%	Total positive	Total sampled	Total % positive
Lambs	0	0%	0	0%	0	0%	0	0%	0	200	0.0
Ewes	X	-	1	2%	0	0%	0 (29)	0%	1	129	0.8

All positive figures shown in these tables are out of 50 animals sampled unless indicated by brackets ()

The overall percentage of the positive animals from the total sampled in affected farms during phase I was not uniformly distributed among the farms. Farm B had 22% and 56% of its lambs and ewes respectively testing positive for *Salmonella* spp.. All the affected farms during phase I had at least one positive animal while for control farms only farm E and F did not have a positive animal as shown in table 3.2, table 3.3 and figure 1.

During phase II three of the affected farms had at least one positive animal while only farm D did not have a single positive animal. However, only 50 animals were tested for *Salmonella* spp. at farm D while on each of the other three farms 100 animals were tested (Table 3.4). During the same phase only control farm F had a single positive animal. However, farm E did not submit ewes for testing and only 29 ewes were submitted for testing from farm H (Table 3.5).

Figure 2: On farm Prevalence of *Salmonella* spp. in lambs and ewes from case and control farms during phase II



During phase I of the on farm sampling 12.0% of the lambs and 18.7% of the ewes sampled from affected farms were positive for *Salmonella* spp. as compared to 4.0% and 3.5% of lambs and ewes respectively from control farms (Table 3.2; Table 3.3; Figure 1). In phase II 2.5% of lambs and 2.7% of ewes from affected farms were positive for *Salmonella* spp. as compared to 0.0% of lambs and 0.8% ewes from control farms (Table 3.4; Table 3.5; Figure 2).

All the laboratory isolates confirmed as *Salmonella* spp. in the field laboratory were confirmed as *Salmonella brandenburg* by The Institute of Environmental Science and Research (ESR), the national reference laboratory in New Zealand as shown in table 3.6 below.

Table 3.6: The summary of animals positive for *Salmonella* spp. during phases I and II of the on farm Sampling

Farm	Lambs +ve		Serotypes	Ewes +ve		Serotypes
A	6	(2)**	All SB#	0	(3)	All SB
B	11	(0)	All SB	28	(1)	All SB
C	6	(3)	All SB	0	(0)	-
D	1	(0)	SB	X	(X)	-
E	0	(0)	-	0	(X)	-
F	0	(0)	-	0	(1)	SB
G	7	(0)	All SB	7	(0)	All SB
H	1	(0)	SB	0	(0)	-

+ve means a confirmed *Salmonella* spp. culture

**Phase II figures are in brackets ()

#SB represents *Salmonella brandenburg*

X means animals not submitted

3.4 Discussion

During phase I of the on farm study 12.0% of sampled lambs and 18.7% of sampled ewes from affected farms were positive for *Salmonella brandenburg*. During the same phase 4.0% of sampled lambs and 3.5% of sampled ewes from control farms were positive for *Salmonella brandenburg*. The results show a major difference in prevalence within animal class between control and affected farms. This difference could have been due to high environmental contamination and the high numbers of excreting animals in affected farms compared to control farms. Also all four affected farms during phase I and three out of four affected farms during phase II had at least one positive animal compared to two out of four and one out of four control farms during phases I & II respectively. These figures showed that there is a high likelihood of infection in affected farms compared to control farms due to either the presence of high number of excreting animals post abortion storms or the high environmental contamination which occurred during the abortion outbreaks. Salmonellae organisms are known to survive for months in the environment (Tannock & Smith, 1971; Newton-Clarke, 1995; Clark 1999). The presence of *S. brandenburg* positive animals in control farms suggested that the organism may be widely disseminated in Southland region of New Zealand.

Most of the positive animals from affected farms during phase I were contributed by farm B which had 22% of its lambs and 56% of its ewes testing positive. This indicated that

the proportion of infected animals was highly variable between farms with the history of outbreaks of *S. brandenburg* abortions. This variability in the proportion of positive animals maybe a reflection of the environmental contamination on this particular farm due to poor hygiene and other husbandry methods that favoured exposure of the animals to the pathogen (Murray, 2000).

Despite farms E-H being classified as control farms because of no previous clinical or bacteriological diagnosis farms G and H had animals testing positive for *Salmonella brandenburg*. This might be an indication of *Salmonella brandenburg* being a dominant and endemic serotype of salmonellae in the outbreak areas. Therefore this means for clinical outbreaks to occur in the other farms but not others there should be a certain threshold of infection among animals on farm or different management practices between the affected and unaffected farms. This is because the presence of infection on farm is not necessarily associated with *Salmonella brandenburg* disease outbreaks as shown by the results of the on-farm study. Management practices, which could encourage a disease outbreak include various stressors that are known to precipitate infection caused by other salmonellae (Kane, 1979; Gough & McEwen, 2000; Wray & Linklater, 2000).

All four affected farms had positive lambs while only one out of three affected farms had positive ewes at on-farm sampling during phase I. Since ewes were exposed to infection during the abortion outbreaks it was expected that more ewes would test positive and that if farms had positive animals those animals were more likely to be ewes. This result meant the previously exposed ewes could have developed protective immunity to new

Salmonella brandenburg infection and that they were no longer excreting the organisms. Meanwhile the immunologically naïve lambs which had acquired the infection from the environment were either still excreting the organisms because of lack of protective immunity or because at the time of testing the infection had been recently acquired.

Since the *Salmonella brandenburg* outbreaks were first reported in 1996 very high percentages of aborting ewes in affected flocks were recorded. These were as high as 30% of the pregnant ewes in affected flocks (Clark 1999; Clark *et al.*, 2000). However, the prevalence of *Salmonella brandenburg* in sampled animals in the on farm study was not high compared to previously published figures with the exception of affected farm B. This might have been due to the interval between abortions during July-September and the time of sampling in November-December. During this time interval there could have been a reduction in the level of environmental contamination and the infected animals could also have acquired protective immunity and cleared the organisms from their systems. A study in New Zealand of the Southland region waterways showed a high salmonellae organisms count during lambing season (August-September) and much lower counts during the rest of the year (Clark, 2001b).

During phase II of the on farm sampling three of the four affected farms had at least one positive animal compared to only one out of four control farms. The control farm that tested positive was not farm G or H meaning, in the two phases of the on-farm sampling three of the four control farms had animals that tested positive for *Salmonella brandenburg*. With farm F testing positive this meant that only farm E did not have

animals testing positive for *Salmonella brandenburg*. However, during this phase only 9 out of 350 animals from affected farms and 1 out of 329 animals from control farms tested positive for *Salmonella brandenburg*. This as explained above could have been due to a reduction in environmental contamination levels and a reduction in the numbers of animals excreting the organism as compared to Phase I.

The variability mentioned above is also illustrated in phase II by the sharp decline in the proportion of positive animals in farm B compared to phase I. This could be a reflection of the dynamic nature of salmonellae infection in populations. There could also have been certain management practices introduced in farm B that could have resulted in reduced environmental contamination and thus a reduction in post abortion prevalence of *Salmonella brandenburg* in animals. The reduction in environmental contamination could also have been due to a reduction in the number of animals excreting the organisms because with time more animals developed protective immunity to infection by *Salmonella brandenburg*.

The serotyping of all submitted on farm isolates from affected and control farms yielded *Salmonella brandenburg*. This was very surprising because there are more than 2500 serotypes of *Salmonella spp.*. This might be a reflection of the extent to which the outbreak strain of *Salmonella brandenburg* has established itself as a dominant serotype in the affected regions of the South Island. A serotype can become dominant through factors like the presence of host specific adhesins, acquisition of pathogenicity plasmids and point mutations that give it an advantage over competitors (Baumler *et al.*, 1998).

However, there are also non-agent factors like environmental factors and host factors that can lead to dominance of a certain serotype within a susceptible host population.

A previously unimportant serotype can assume dominance because of selective pressure imposed on it by factors associated with the host population or the environment. If a host mounts a strong immunity the severity of disease will be reduced resulting in the serotype becoming more prevalent in the susceptible population. The other factors that can cause a serotype to become dominant are antibiotic use, eradication of competitors and the homogeneity of host population achieved through breeding methods (Hunter & Iszak, 1990; Barrow, 1993; Kingsley & Baumler, 2000). A recently introduced serotype can also become dominant if it finds a suitable niche within a previously unexposed host population. All or some of the above factors could have played a role in the establishment of *Salmonella brandenburg* as a dominant serotype in sheep in the affected regions of the South Island of New Zealand.

The estimation of prevalence using faecal culture is an underestimation considering the intermittent nature of excretion and insensitivity of the culture methods (Corrier *et al.*, 1990). Serology could be more accurate in estimating the presence of salmonellae infection in a population. But serology too has shortcomings, which include; missing recent infections, inability to differentiate between long term and recent infection if not repeated, and the limited number of serotypes that can be detected by methods like ELISA (Wolf van der *et al.*, 2001).

The results of the prevalence of *Salmonella* spp. on farm can also be influenced by the study design. In this study rectal samples were chosen over wool samples because faecal contamination of the anal area may result in environmental contamination having a major influence on the faecal culture results . Therefore it is very hard to compare data from different studies if different experimental designs are employed (Egan *et al.*, 1997). The study also has a limitation due to the absence of data on the sensitivity of the isolation methods used. The sensitivity of isolation methods has been investigated for poultry and swine (Rigby & Petit, 1980; Davies *et al.*, 2000; O'Carroll *et al.*, 1999).

3.5 Conclusions

The affected farms had a higher number of positive animals as compared to control farms, the pattern being similar for both phases I and II. There was also a high likelihood of an affected farm having at least one positive animal compared to control farms as illustrated by four out four and three out of four affected farms having at least one positive animal during phases I and II respectively. Only two control farms had positive animals in phase I and one control farm had a positive animal in phase II.

Lambs and ewes had an equal chance of infection by *Salmonella brandenburg* during the post abortion outbreak period of the study. Therefore the class of animal did not influence the prevalence.

The time interval between sampling and occurrence of *Salmonella brandenburg* abortion outbreaks had an effect on the prevalence of infection in sampled animals as illustrated by the high numbers of positives in phase I as compared to phase II.

The data in the study was consistent with *Salmonella brandenburg* being a predominant seroype in the outbreak areas of the South Island of New Zealand.

CHAPTER FOUR: DETERMINING THE PREVALENCE OF *Salmonella spp.* IN SHEEP FROM SELECTED CASE AND CONTROL FARMS AT SLAUGHTER IN THE SOUTH ISLAND OF NEW ZEALAND

4.1 Introduction

The presence of salmonellae in the caecum of slaughtered animals is likely to be due to on farm exposure, if the period spent in lairage is short and if the animals have been transported from farms close to the meat plant (Tay *et al.*, 1989). Despite this, rapid dissemination of salmonellae is suspected in pigs, with reported detection of the organism in the gastro-intestinal tract of piglets 2-3 hours after experimental infection (Fedorka-Cray *et al.*, 1995; Hurd *et al.,* 2001a). However, sheep in this study were held overnight in the lairages thus the *Salmonella spp.* organisms isolated from their caeca could have been due to infection on the farm, during transportation or during overnight holding in the lairages.

Previous work on the faecal prevalence of *Salmonella* spp. has shown that older animals had higher rate of infection than lambs. This could have been due to the longer period of environmental exposure to the salmonellae (Kumar *et al.*, 1973). Stressors like transportation also increase the faecal prevalence of salmonellae in animals (Davies *et al.*, 1997; Gough & McEwen, 2000). Actually the first reported case of salmonellosis in sheep in 1919/20 was a post transportation dysentery in the animals (Wray & Linklater, 2000). In a study by Corrier *et al.* (1990) an on farm prevalence of salmonellosis in calves of 0% increased to 1.5% after transportation. Transportation does not only contribute as a stressor but the vehicles can also act as a source of *Salmonella* spp.

infection if they are not properly cleaned between consignments. A study which yielded 12 different serotypes, by Wray *et al.* (1991) showed that 22 out of 107 vehicles were positive for *Salmonella spp.* before washing compared to 4 out of 62 after washing.

The environment is also a very important source of salmonellae to animals. A study by Wray *et al.* (1991) on market environments showed that 7 out of 14 animal markets were positive for salmonellae and out of 838 samples taken from these markets 31 yielded positive results for *Salmonella* spp. Since the animal market environment is similar to that of the lairage at meat plants because of the congregation of animals from different sources, lairages could also be potential sources of infection for animals destined for slaughter.

The sensitivity of isolation of salmonellae differs for different sites in a carcase. The mesenteric lymph nodes, faecal samples and faecal swabs have a decreasing sensitivity in that order (Gay *et al.,* 1994). However, in this study the site of sampling was determined by the ease with which the samples could be obtained, and by non-interference with the production line and the processing procedures. The sensitivity of isolation is also influenced by the methodology employed. The use of buffered peptone water (BPW) and Rappaport-Vassiliadis (RVS) broth for pre-enrichment and selective enrichment respectively in raw foods and heavily contaminated samples for the isolation of salmonellae as used in this study has been reported to be more sensitive than both tetrathionate and selenite broths (Waltman, 2000).

The main aim of this chapter was to estimate the abattoir prevalence of *Salmonella* spp. in animals sourced from farms with or without a history of recent *S. brandenburg* disease and to compare the results with those of the on-farm study.

4.2 Materials and Methods

4.2.1 Flock selection

Flock selection is as described in chapter three.

4.2.2 Sampling

In the abattoir the viscera of the first and the last animal of the mob to be sampled were tagged on the killing floor so that they could be identified and retrieved in the gut room. In the gut room the caeca were collected into individual buckets to minimise the risk of cross contamination. The caeca and the large intestines were collected after removal of the small intestines that are used for making casings. The caecal samples were collected in the gut room.

The surface of the caecum was then singed with a hot, size 24 scalpel blade that had been dipped in methylated spirits and passed through a Bunsen burner flame. An incision was then made in the singed area of the caecum to scoop out approximately one gram of the caecal contents. The use of sample bottles with scoops minimised the chance of spillage and cross contamination because after scooping the caecal material the lid was screwed onto the bottle at the same time as the sample was being deposited into the bottle. The

samples were then placed in a cooler box and taken to the on-site laboratory for processing.

4.2.3 Isolation techniques

10 ml of buffered peptone water (BPW) was then poured into the container with the sample to give an approximate dilution factor of 1: 10. The faeces-BPW mixture was then incubated at 37 ^{0}C for 16-24 hours. After this period 0.1 ml of the mixture was transferred to Rappaport-Vassiliadis (RVS) broth and incubated at 42 ^{o}C for 24 hours.

A loopful of the RVS broth-culture mixture was then streaked onto a half xylose lysine desoxycholate (XLD)/brilliant green agar (BGA) plate after 24 hours. The plates were incubated at 37 ^{0}C for 18-24 hours.

4.2.4 Identification (presumptive and confirmative)

The plates were read after 18-24 hours incubation and a suspect *Salmonella* spp. colony on either BGA or XLD was subjected to slide agglutination with polyvalent antigen H and O using Serobact^{TM} for confirmation as *Salmonella* species. Suspect salmonellae colonies on BGA are pink surrounded by red media while on XLD, suspect colonies are either red with black centres or just red for the non-sulphur producing strains.

A single colony from the XLD/BGA plate that had a positive slide agglutination test was then streaked onto a Dorset egg slope and incubated at 37 ^{0}C for 24 hours and stored at 4 ^{0}C. At the end of the trial the isolates on the Dorset egg slopes were then sent to Massey University where they were subcultured twice on blood agar (BA) as above. Pure colonies were transferred to nutrient agar and sent to The Institute of Environmental Science and Research (ESR), the national reference laboratory for serotyping. The rest of the culture was then transferred to glycerol broth and frozen at minus 70 ^{0}C.

4.2.5 Descriptive analysis

As described in chapter three the testing of hypothesis could not be done for the pilot study.

4.3 Results

Table 4.1: Summary of the proportion of animals positive for *Salmonella* spp. by culture of caecal contents collected at the abattoir during phases I and II

	Phase I		Phase II	
	Lambs	Ewes	Lambs	Ewes
Affected Farms	Lambs 18/200	Ewes 31/141	Lambs 0/200	Ewes 4/150
Control Farms	Lambs 0/194	Ewes 2/206	Lambs 1/198	Ewes 0/126
Totals	18/394	33/347	1/398	4/276

In phase I a total of 741 samples were cultured with 51 of them giving a positive culture for *Salmonella* spp.. In phase II 674 samples were sampled and 5 only gave positive culture for *Salmonella* spp. (Table 4.1). All the isolates yielded *S. brandenburg* on serotyping.

The prevalence of *Salmonella* spp. in sampled animals sourced from affected farms at slaughter ranged from 0-32% for lambs and 4-61% for ewes in phase I (Table 4.2; Figure 3). The prevalence of *Salmonella* spp. in lambs and ewes sourced from control farms was 0% and 0-2% respectively (Table 4.3; Figure 3).

Table 4.2: The number and proportion of caecal samples positive for *Salmonella* spp. in lambs and ewes from affected farms during Phase I (November-December) of the abattoir sampling

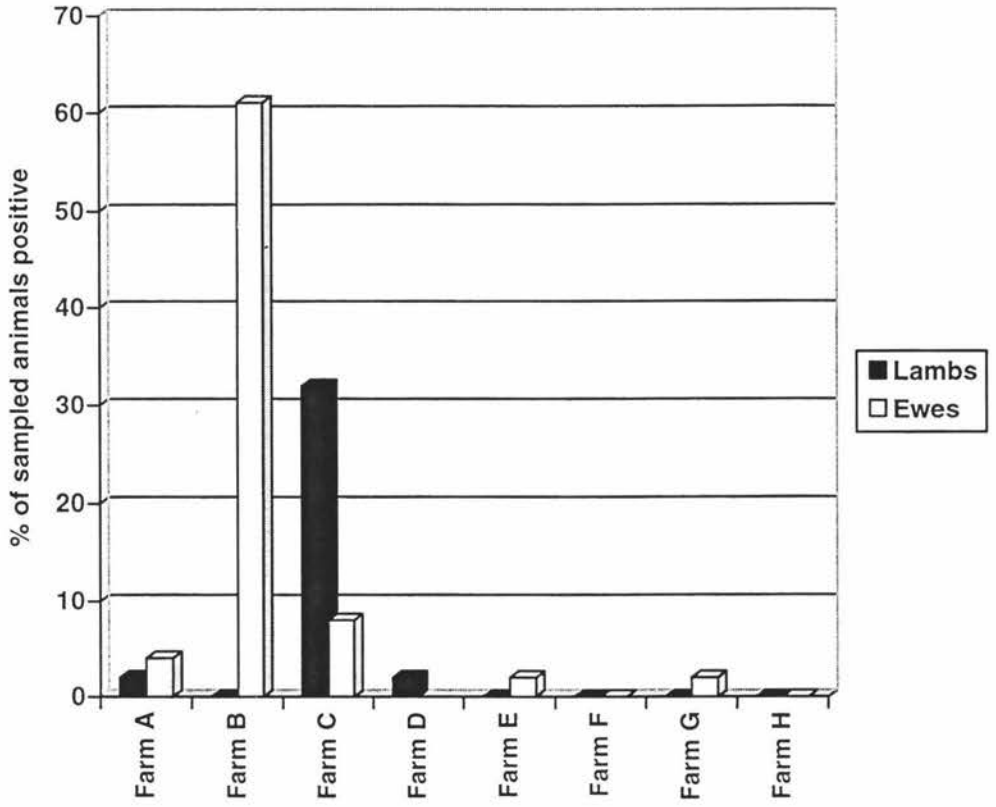
Sampling site	Farm A n %	Farm B n %	Farm C n %	Farm D n %	Total positive	Total sampled	% positive
Lambs	1 2%	0 0%	16 32%	1 2%	18	200	9.0
Ewes	2 4%	25 (41) 61%	4 8%	X -	31	141	22.0

All positive figures shown in these tables are out of 50 animals sampled unless indicated by brackets ()
X- No animals submitted for slaughter
n- Number of animals positive for *Salmonella* spp. out of 50 animals tested unless indicated

Table 4.3: The number and proportion of caecal samples positive for *Salmonella* spp. in lambs and ewes from control farms during Phase I (November-December) of the abattoir sampling

Sampling site	Farm E n %	Farm F n %	Farm G n %	Farm H n %	Total positive	Total sampled	% positive
Lambs	0 (47) 0%	0 (49) 0%	0 (52) 0%	0 (46) 0%	0	194	0.0
Ewes	1 (53) 2%	0 (51) 0%	1 (54) 2%	0 (48) 0%	2	206	1.0

Figure 3: Abattoir Prevalence of *Salmonella* spp. in lambs and ewes from case and control farms during phase I



During phase II of the abattoir sampling the affected farms had a *Salmonella* spp. prevalence of 0% for lambs and a range of 0-4% for ewes as shown in Table 4.4 and figure 4.

Table 4.4: The number and proportion of caecal samples positive for *Salmonella* spp. in lambs and ewes from affected farms during Phase II (February-March) of the abattoir sampling

Sampling site	Farm A n %	Farm B n %	Farm C n %	Farm D n %	Total positive	Total sampled	% positive
Lambs	0 0%	0 0%	0 0%	0 0%	0	200	0.0
Ewes	2 4%	2 4%	0 0%	X -	4	150	2.7

The range of *Salmonella* spp. prevalence in lambs and ewes from control farms during phase II was 0-2% and 0% respectively as illustrated by table 4.5 and figure 4.

Table 4.5: The number and proportion of caecal samples positive for *Salmonella* spp. in lambs and ewes from control farms during Phase II (February-March) of the abattoir sampling

Sampling site	Farm E n %	Farm F n %	Farm G n %	Farm H n %	Total positive	Total sampled	% positive
Lambs	0 0%	1 2%	0 0%	0 (48) 0%	1	198	0.5
Ewes	X -	0 0%	0 (45) 0%	0 (26) 0%	0	126	0.0

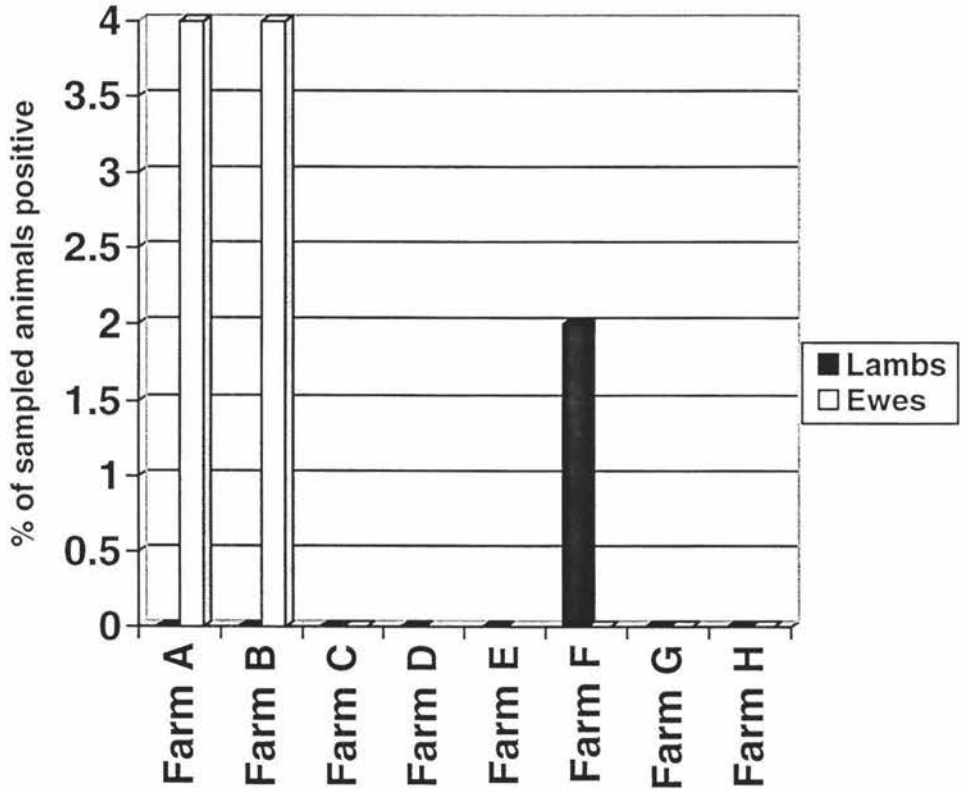
During phase I of abattoir sampling all affected farms had at least one *Salmonella* spp. positive animal while for control farms only farms E and G had animals positive for *Salmonella brandenburg* at slaughter (Table 4.2; Table 4.3).

The total number of *Salmonella* spp. positive animals from affected farms during phase I was not uniformly distributed. Farm C contributed 16 out of 18 positive lambs while farm B contributed 25 out of 31 positive ewes (Table 4.2).

During phase II of the abattoir sampling none of the lambs from the affected farms were positive for *Salmonella* spp. while farms A and B each had two positive ewes (Table 4.4). Of the four control farms only farm F had a positive animal. No ewes were positive for *Salmonella* spp., however farm E did not submit ewes for slaughter and farms G and H submitted only 45 and 26 ewes respectively of a requested 50 per farm (Table 4.5).

During phase I of the abattoir sampling 9.0% of the lambs and 22.0% of the ewes sampled from affected farms were positive for *Salmonella* spp. as compared to 0.0% and 1.0% of lambs and ewes respectively from control farms (Table 4.2; Table 4.3; Figure 3). In phase II 0.0% of lambs and 2.7% of ewes from affected farms were positive for *Salmonella* spp. as compared to 0.5% of lambs and 0.0% ewes from control farms (Table 4.4; Table 4.5; Figure 4).

Figure 4: Abattoir Prevalence of *Salmonella spp.* in lambs and ewes from case and control farms during phase II



All but one of the field laboratory confirmed isolates of *Salmonella spp.* from phases I and II of the abattoir study sent for serotyping at the ESR, the national reference laboratory were *Salmonella brandenburg*. The one isolate from the affected farm C was a mixed culture of *Salmonella brandenburg* and *Salmonella enteritidis* PT 4 (Table 4.6).

Table 4.6: The summary of animals positive for *Salmonella* spp. during phases I and II of the abattoir Sampling

Farm	Lambs +ve		Serotypes	Ewes +ve		Serotypes
A	1	(0)**	SB#	2	(2)	All SB
B	0	(0)	-	25	(2)	All SB
C	16	(0)	15 SB & 1SE*-SB	4	(0)	All SB
D	1	(0)	SB	X	(X)	-
E	0	(0)	-	1	(X)	SB
F	0	(1)	SB	0	(0)	-
G	0	(0)	-	1	(0)	SB
H	0	(0)	-	0	(0)	-

+ve means a confirmed *Salmonella spp.* culture
**Phase II figures are in brackets ()
#SB represents *Salmonella brandenburg*
X means animals not submitted
* SE-SB is mixed colony of *Salmonella enteritidis* PT4 and *Salmonella brandenburg*

4.4 Discussion

During phase I of the abattoir study 9 % of sampled lambs and 22% of sampled ewes from affected farms were positive for *Salmonella brandenburg*. In comparison 0% of lambs and 1.0% of ewes sourced from control farms were positive for *Salmonella brandenburg*. Therefore as in the on-farm study there was a large difference between lambs and ewes from affected farms and control farms (**within** class comparison). Also during phase I of the abattoir sampling all affected farms had at least one animal testing

positive for *Salmonella brandenburg,* while only two of the four control farms had animals that tested positive for *Salmonella brandenburg*. As discussed in chapter three, this reflected the number of *Salmonella brandenburg* excreting animals and a higher environmental contamination in affected farms compared to control farms. However, what was important was that four out four affected farms had positive animals compared to two out of four control farms. This was because the total number of positive animals was not evenly distributed among the four farms. During this phase of the abattoir sampling farms B and C contributed 45 of the 49 positive animals. As explained for the on-farm sampling this could have been a reflection of the number of infected animals on farm or the level of environmental contamination. A highly contaminated environment would result in a high proportion of positive animals due to a high number of transiently excreting animals.

Also during phase I two affected farms had both ewes and lambs testing positive for *Salmonella brandenburg* while the other two farms had either class positive for *Salmonella brandenburg*. This result illustrates that either class has comparable chance of being infected with *Salmonella brandenburg* after an abortion outbreak. This result which was similar to the on farm study contradicted previous reports that ewes at slaughter had a higher prevalence of *Salmonella spp.* infection because of their long period of environmental exposure compared to lambs (Kumar *et al.*, 1973). In comparison the two control farms that had positive animals, each had one ewe testing positive for *Salmonella brandenburg*.

The fact that the positive animals on the control farms were ewes illustrates that there is more to abortion storms than just infected animals. These factors that precipitate the *Salmonella brandenburg* infection in pregnant ewes in South Island of New Zealand into abortion outbreaks are still not yet understood. However the infected ewes could have been infected after the abortion outbreaks in the affected farms. This would illustrate that *Salmonella brandenburg* is being spread to previously unexposed ewes. The study results suggest that the serotype is dominant in the affected regions. Therefore the farm to farm spread would ultimately lead to the serotype's endemicity, and the presence of subclinical excretors and latent carriers.

During phase II of the abattoir sampling 4 out of 350 animals from affected farms and 1 out of 324 animals from control farms were positive for *Salmonella brandenburg*. Therefore the total number of positive animals was very low compared to that the of phase I sampling where 49 out of 341 animals from affected farms and 2 out of 400 animals from control farms were positive for *Salmonella brandenburg*. This was similar to the on-farm pattern and confirmed that the longer the time interval between sampling and abortion outbreak the lower the number of *Salmonella brandenburg* positive animals. This as explained in chapter three could be due to reduced environmental contamination or a lower number of animals excreting the organisms thus reducing the infection transmission. All the four positive animals from the affected farms during phase II were ewes, the importance of which is unclear. However, the four ewes like the single positive lamb from the control farms were unlikely to be persistently infected. Excretion of non-host specific serotypes for long periods after salmonellosis outbreaks is usually

associated with reinfection from the environment (Anderson & Blanchard, 1989; Pelzer, 1989).

During phase I of the on-farm sampling all affected farms had positive lambs but on sampling the same flocks at slaughter farm B did not have any positive lambs. This could have been due to the differences in the sampling methods used at slaughter and on-farm. Due to the logistics of collection and processing of specimens, sampling was handled by two different study groups and the samples were processed at different laboratories using standardised methods. It was also statistically possible to get major differences in the on-farm and abattoir sampling results because the animals tested on-farm were not necessarily those tested at slaughter although they were in the same group destined for slaughter.

There was no obvious pattern between the numbers of *Salmonella brandenburg* positive animals on-farm and at slaughter. The total number of infected animals on-farm and at slaughter were approximately the same for both sites. This finding was different from other work on *Salmonella spp.* where the prevalence at slaughter was higher than the on-farm prevalence probably due to stress associated with transport and lairages. Environmental contamination either in transit or in lairages is also thought to contribute to the higher prevalence at slaughter compared to the on-farm prevalence (Grau *et al.*, 1968; Wray *et al.*, 1991; Gough & McEwen, 2000; Hurd *et al.*, 2001b). Cross contamination during transport and holding in lairages might render the slaughter results of *Salmonella* spp. prevalence unreliable but for the purposes of this study the prevalence

at slaughter gave a better indication of the risk of product contamination. However, the results from this study suggested that the contribution of cross contamination during transportation and lairaging was minimal as illustrated by the low prevalence of *S. brandenburg* in animals from control farms in the study.

Like during the on-farm sampling, some control farms tested positive during the abattoir sampling. This meant absence of disease was not necessarily associated with absence of infection. It also meant the infection was present in previously unaffected farms as defined by bacteriological and clinical diagnosis in this study. The non-host adapted serotypes can be introduced into previously unaffected farms through a wide array of routes which include pet movements, stock movements, wildlife, stock or delivery vehicles, feed and possibly wind (Wray *et al*, 1991; Bauer & Hormansdorfer, 1996; Fedorka-Cray *et al.*, 1998; Fenwick, 2000; Smart, 2000; Davies, 2001).

Like the on-farm results the serotyping of abattoir cultures gave *Salmonella brandenburg* except for one isolate that gave a mixed culture of *Salmonella brandenburg* and *Salmonella enteritidis* PT4. As explained in chapter three this might be a reflection of the dominance of the outbreak strain in the outbreak areas. However the cause of this dominance cannot be easily identified because it is multifactorial. The presence of *Salmonella enteritidis* PT4 which is primarily an avian serotype might be an indication of the role played by avian species like the black backed gull in the epidemiology of the *Salmonella brandenburg* infection in sheep in the South Island of New Zealand. However, in Europe particularly the United Kingdom *S. enteritidis* PT4 is an important

cause of foodborne salmonellosis associated with eating of eggs and other egg products (Rodrigue *et al.,* 1990). The rapid increase of human salmonellosis in the late 1970's to mid 1980's associated with *S. enteritidis* PT 4 in Europe and phage types 8 and 13a in the Americas led to Rodrigue *et al.* (1990) calling *S. enteritidis* a pandemic.

4.5 Conclusion

There was a big difference between the number of infected animals from affected and control farms. The study therefore showed that farms experiencing clinical disease presented a greater risk of *Salmonella brandenburg* contamination at slaughter.

The number of positive animals during the November-December sampling was very high compared to those of February-March sampling. Therefore time interval between abortion outbreaks and sampling had a big influence on the prevalence of *Salmonella brandenburg* irrespective of class of animals, farm's *Salmonella brandenburg* infection status and whether samples were taken on-farm or at slaughter.

The number of lambs or ewes positive for *Salmonella brandenburg* on-farm and at slaughter was approximately the same. Therefore within class there was no a significant difference in prevalence of *Salmonella brandenburg* for both on-farm and abattoir sampling.

There was no obvious pattern of *Salmonella brandenburg* prevalence between ewes and lambs from the same farm either during on-farm or abattoir sampling. However, farm B contributed 25 of 31 positive ewes during phase I of the abattoir sampling and similarly farm C contributed 16 of 18 positive lambs during the same phase. This is mainly indicative of the variation of proportion of positive animals between farms. Therefore it can be concluded that on average there was no difference in the prevalence of *S. brandenburg* **between** classes of stock.

CHAPTER FIVE: TYPING OF *Salmonella Brandenburg* ISOLATES FROM SHEEP BY PULSED-FIELD GEL ELECTROPHORESIS (PFGE)

5.1 Introduction

Traditionally serotyping, phagetyping and biotyping have been used for *Salmonella spp.* typing because of their good reproducibility, typerability and stability. However, the reproducibility of the traditional methods is questionable because of the possible acquisition of plasmids. The development of pulsed-field gel electrophoresis (PFGE), which is based on the restriction analysis of the full genome of an organism gave a more stable typing method with a very high reproducibility. PFGE is indicated for determining relatedness of outbreak strains, the identification of transmission routes and reservoirs, differentiation of relapse and new infections, identification of factors responsible for persistence, diagnostic purposes and in hazard analysis critical control point in food production. The identification of transmission routes and reservoirs can aid in the eradication of disease agents within a population (Threlfall & Frost, 1990; Olsen *et al.*, 1993; Hollinger, 2000; Olsen, 2000). PFGE has been previously used to study the epidemiology of bacterial disease outbreaks in both animal and human populations (Baquar *et al.*, 1994; Suzuki *et al.*, 1995; Wegener & Baggensen, 1996; Ahmed *et al.*, 2000).

Since the first reported cases of the *Salmonella brandenburg* outbreak in sheep in the South Island of New Zealand all the PFGE profiles of the isolates have been identical

(Clark *et al.*, 1999). However, an isolate from yard dust reported by Clark (2001a) differed from previous profiles by three bands. This difference in three bands is not significant because according to a scheme proposed by Tenover *et al.* (1995) a difference of at least three bands indicates an independent single genetic event which means the isolate reported by Clark (2001a) is closely related to the other outbreak isolates. Although the isolates from sheep show only a single profile the typing of 115 human isolates of *Salmonella brandenburg* isolates collected between 1990 to1995 in New Zealand showed 13 different profiles (Wright *et al.*, 1998), none of which corresponds to the sheep strain.

The main aim of this chapter was to examine the diversity of PFGE profiles of isolates in 2000 and compare them with the original outbreak strain profile. The PFGE of the isolates was also done to investigate if there has been any genetic changes in the outbreak isolates as reported previously by Clark (2001a), and investigate if there is any difference between the on-farm and abattoir isolates for the control and affected farms. The PFGE method used in the study was a modification of the previously used methods (Bohm & Karch, 1992; Cameron *et al.*, 1994; Nauerby *et al.*, 2000).

5.2 Materials and methods

5.2.1. Bacterial isolates

Isolates to be processed were selected purposively to represent all the possible combination of events that could have been represented in the study. There were 64 possible combinations of events in the study (Tables 2-10). These were 8 farms, 2 sample types (farm/abattoir), 2 classes of animals, and 2 time points (8*2*2*2=64). Since no isolates were obtained at some of the events (Tables 2-10), less than 64 isolates were selected. One isolate was randomly selected from each event so that an isolate from each class of stock (lamb and ewe), on-farm sampling, abattoir sampling, early season and late season was chosen. Where there were more than one isolates for the event the isolates were numbered in ascending order and using Excel 2000 ^{TM} the integers were randomised and the first one selected for PFGE. Using the above criteria of selecting a single isolate for each event 24 isolates were chosen from a total of 133. Ideally all the isolates could have been analysed but financial resources, facilities limitations and time constraints meant the above approach was the most efficient to screen for diversity.

The isolates that had previously been stored at –70 ^{0}C were transferred to blood agar (BA) plates. The frozen culture-broth mixture was scraped on the surface with a loop and transferred to a blood agar plate. After allowing the mixture to thaw it was streaked

onto the plate and incubated at 37 ^{0}C for 18-24 hours. A single colony was then transferred to another BA plate and incubated as previously to give a pure colony of *Salmonella brandenburg*. Single colonies harvested from the second blood agar plates were transferred with a loop to three millilitres of brain heart infusion broth (BHI) and incubated at 37 ^{0}C overnight.

5.2.2 DNA preparation

After the incubation period the BHI broth mixture was put into a spectrometer and the optical density of the cells adjusted to 1.4 read at 610 nm. Eppendorf tubes were filled with 200 micro litres of cells from each sample that was then centrifuged at 13000 revolutions per minute for five minutes. The supernatant was decanted and the cells resuspended in 150 micro litres cold Pett IV buffer (10mM Tris-HCl, pH 8, 1M NaCl). The Pett IV buffer-cell suspension was again centrifuged at 13000 revolutions per minute for five minutes. After centrifuging the supernatant was decanted and the cell pellet resuspended in 50 micro litres of Pett IV buffer. The cell-Pett IV suspension was then mixed with 100 micro litres of molten 1% low melt agarose. 100 micro litres of the cell agarose suspension was then dispensed into plug molds and left to cool for one hour in an ice bath.

The plugs were then placed into Eppendorf tubes with one millilitre of the lysis buffer containing 1% proteinase K and incubated at 56 ^{0}C overnight. After incubation the plugs were transferred to plastic universal bottles containing 10 millilitres of Tris-HCl,

EDTA (TE) buffer. The universal bottles were placed in ice and placed on a rocking machine (shaker) for one hour. After one hour the TE buffer was decanted and replaced with another fresh 10 millilitres, placed on ice and treated as above. The process was repeated three more times so that the plugs had been rinsed with 10 millilitres of TE buffer five times over an incubation time of five hours in ice on a rocking machine. After the fifth rinsing the plugs were transferred to Eppendorf tubes containing 1 millilitre of TE buffer and stored at 4 ^{0}C until needed.

5.2.3 Restriction endonuclease digestion of plug-incorporated DNA

A glass slide that had been dipped into ethanol and flamed was used as a cutting surface for the plugs. Each plug was placed on the glass slide and one third of it cut with a scalpel blade which was flamed between each plug. The two thirds of the plug remaining were returned to the Eppendorf tubes, suspended in 1 millilitre of TE buffer and stored at 4 ^{0}C. The other third of the plug was placed in Eppendorf tube containing 100 microlitres of the restriction buffer and equilibrated in ice for 45 minutes. After the equilibration period the restriction buffer was decanted and replaced with 100 microlitres of the cutting buffer. The plugs and the 30 units of XbaI containing buffer were equilibrated in ice for 45 minutes followed by incubation at 37 ^{0}C for 12-24 hours.

5.2.4 Pulsed-field gel electrophoresis

To make a 30 well gel, 1 % pulsed-field certified (PFC) agarose was prepared and poured into a gel mold. The gel was allowed to solidify for one hour after which the gel tank was filled with 2.5 litres of Tris-base, Boric Acid, EDTA (TBE) buffer. The TBE buffer was circulated at 14 ^{0}C for one hour and then pre-electrophorised at 6 Volts per centimetre for 5.5 seconds for 1.5 hours. The gel that was still on gel tank black plate was removed from the electrophoresis chamber, placed on a clean bench and the buffer pipetted out of the 30 wells.

The plugs were tipped out of the cutting buffer on a flamed glass slide. Using the flamed glass slide and a 'hockey stick' glass rod the 24 plugs from the study isolates, two plugs from isolates of known profiles and four lambda DNA molecular weight markers were tipped and pushed into the 30 empty wells. Molten 1% agarose was then used to fill up the wells and allowed to set. With plugs and agarose filled wells the gel was put back into the electrophoresis chamber and run at 6 Volts per centimetre for 22 hours with an initial switch time of 5 seconds and final switch time of 50 seconds.

After 22 hours the plug was removed from the electrophoresis chamber and placed in a plastic chamber containing a solution of ethidium bromide prepared from 800 millilitres of Milli-Q pure (MQ) water and 80 microlitres of 10 milligrams per millilitre of ethidium bromide solution (10%). The plug was left in the Ethidium solution to stain for 10 minutes followed by brief rinse in another plastic container filled with MQ water.

After the rinse the gel was taken out of the plastic container and placed under ultra violet light and photographed with the image being screened and saved in the computer.

5.2.5 Interpretation of the pulsed-field profiles

The profiles were interpreted using the scheme recommended by Tenover *et al.* (1995) that classified the profiles into four categories. The categories are: indistinguishable profiles, closely related profiles, possibly related profiles and unrelated profiles. The 'indistinguishable profiles' are identical, the 'closely related profiles' differ by 2-3 bands, the 'possibly related profiles' differ by 4-6 bands and 'unrelated profiles' differ by 7 or more bands. The difference in bands could be due to one independent genetic event for the closely related profiles, two independent genetic events for the possibly related profiles and more than three genetic events for the unrelated profiles. The genetic event maybe a point mutation and insertion or deletion of genetic material in the genome of the microbe. Although the Tenover scheme of interpretation of PFGE profiles is widely used the authors recommend that it should be used in a restricted population e.g. a hospital and over narrow time interval of 1-3 months (Tenover *et al.*, 1995). In this study the profiles were compared using only the presence or absence of bands ignoring their intensity as reported by Cameron *et al.* (1994).

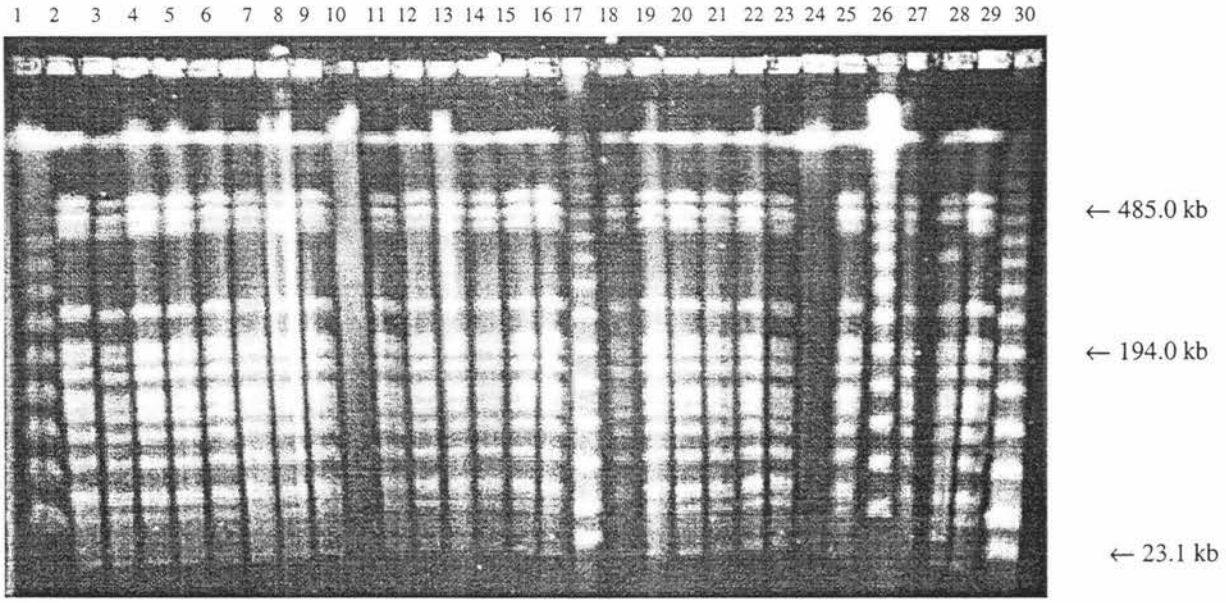
5.3 Results

The different isolates were put in lanes 1-30 as shown in Table 5.1 below.

Table 5.1: The sources, sites and lanes of the representative isolates as they appear on the PFGE profiles

Farm-Status	Phase	Sample ID	Source	Site	**Lane**
-	-	PFG marker	-	-	**1**
A-Affected	1	L2/12142	Lamb	Farm	**2**
A-Affected	1	E18/1380	Ewe	Meat Plant	**3**
A-Affected	1	L8/1379	Lamb	Meat Plant	**4**
A-Affected	2	E2/927	Ewe	Farm	**5**
A-Affected	2	L1/927	Lamb	Farm	**6**
A-Affected	2	E19/1780	Ewe	Meat Plant	**7**
B-Affected	1	E25/13571	Ewe	Farm	**8**
B-Affected	1	L10/13571	Lamb	Farm	**9**
B-Affected	1	E47/1592	Ewe	Meat Plant	**10**
B-Affected	2	E1/1617	Ewe	Farm	**11**
B-Affected	2	E27/1873	Ewe	Meat Plant	**12**
C-Affected	1	L5/13572	Lamb	Farm	**13**
C-Affected	1	E45/1612	Ewe	Meat Plant	**14**
C-Affected	1	L49/1611	Lamb	Meat Plant	**15**
C-Affected	2	L1/1831	Lamb	Farm	**16**
-	-	PFG marker	-	-	**17**
D-Affected	1	L1/13856	Lamb	Farm	**18**
D-Affected	1	L32/1953	Lamb	Meat Plant	**19**
E-Control	1	E18/1978	Ewe	Meat Plant	**20**
F-Control	2	E1/928	Ewe	Farm	**21**
F-Control	2	L44/1795	Lamb	Meat Plant	**22**
G-Control	1	E6/12504	Ewe	Farm	**23**
G-Control	1	L4/12504	Lamb	Farm	**24**
G-Control	1	E31/1511	Ewe	Meat Plant	**25**
-	-	PFG marker	-	-	**26**
H-Control	1	L1/12503	Lamb	Farm	**27**
New strain	-	-	Collection	-	**28**
Old strain	-	-	Collection	-	**29**
-	-	PFG marker	-	-	**30**

Figure 5: PFGE profiles of representative isolates from lambs and ewes



The profiles of isolates that produced readable profiles were identical to that of the original strain in lane 29. None of the 24 readable profiles from the 26 isolates gave a profile identical to the new strain reported by Clark (2001a) that was run in lane 28. The two isolates in lanes 10 and 24 did not give readable profiles.

5.4 Discussion

Of the 26 representative isolates 24 gave readable profiles which were identical to the original strain. This illustrated that the outbreak isolate has become endemic in the outbreak areas and that it has become the dominant serotype. The fact that the genotypic profile of the outbreak has not changed since 1996 shows the conditions in the outbreak

areas favour its survival and that it is not under selection pressure. It is known that environmental pressures like the use of antibiotics can result in changes in dominance of certain serotypes of *Salmonella* spp. through selection pressure.

However this interpretation should be treated with caution because it has been reported that PFGE has limitations in differentiating within type for closely related strains in an outbreak (Bohm & Karch, 1992). The interpretation of PFGE profiles therefore requires a multifaceted approach i.e. in addition to the genotyping results, other phenotyping methods and sound epidemiological data should also be used to make a conclusive interpretation (Eisenstein, 1990; Maslow *et al.*, 1993; Ahmed, 2000; Amavisit *et al.*, 2001).

5.5 Conclusion

The lack of diversity in the 2000 season *S. brandenburg* isolates PFGE profiles suggests remarkable stability over a period of four years. This would suggest that *S. brandenburg* in the outbreak areas is under no selection pressure and might have attained some dominance over other competing salmonellae serotypes.

CHAPTER SIX: THE POTENTIAL PUBLIC HEALTH AND SOCIO-ECONOMIC IMPACT OF SALMONELLA BRANDENBURG INFECTION IN LAMB

It is very important for the New Zealand meat industry to put into place measures that will ensure continued access of its product to international markets. The effect of the 'brandenburg in lamb scare' on the New Zealand economy could be even bigger than the 'Salmonella in eggs scare' of the late 1980's in Europe because of the dependency of New Zealand on primary produce, particularly meat. Therefore New Zealand, as the world largest exporter of lamb, must be proactive in the control of *Salmonella brandenburg*. Although the importance of *S. brandenburg* as a foodborne pathogen is not yet known, it has already shown its potential as a human pathogen through the disease it causes in agricultural workers in the South Island of New Zealand (Fenwick, 2000). The cases of agricultural workers are not only a cause for concern for the industry but they also impose a cost burden on the public health system. The potential costs of the *Salmonella brandenburg* due to the contaminated product would include costs to the public sector, food industry, other industries and individuals and their families (Sockett & Roberts, 1991; Buzby & Roberts, 1997).

The public sector costs associated with foodborne infections include resources spent on tests, disease investigations, man-hours, hospitalisation, outpatients costs and medicines. In 1992 the costs of human salmonellosis were estimated to be US$3.99 billion in The United States and 350-502 million British pounds in the United Kingdom (Gomez *et al.*, 1997). The estimates for the cost of human salmonellosis in New Zealand for 1995 was NZ$59 million (Gilbert *et al.*, 1996). However it is very

hard to compare the estimates from different countries or studies because different methodologies are employed to arrive at the figures (Todd, 1989). The individuals and their families experience costs associated with travel, general practitioners fees and other costs including communications, cancellations of engagements etc (Todd, 1989; Sockett & Roberts, 1991).

The losses to the industry due to *Salmonella brandenburg* would include costs to the primary producer, additional costs to the processor and costs associated with loss of sales and recall of product. The loss of consumer confidence results in both domestic and international demand falling resulting in economic hardships to the primary producer (Mallinson *et al.,* 1989; Ashworth & Mainland, 1995). In a survey undertaken by Boxall *et al.* (1999) in the South Island of New Zealand on average farmers lost 132 lambs (range, 23-500) and 128 ewes (range, 15-350). In monetary terms this is very substantial. Smart (2000) attempted to convert these losses into dollars, and estimated costs to individual farmers were as high as NZ$ 115 600. The additional costs incurred by the processor are mainly associated with taking measures to reassure the public. These include putting into place new systems to minimise contamination, product decontamination and extra cleaning of the working surfaces. The other potential costs to the processor include rejection of products and or litigation. Food scares would also have far reaching consequence for international trade in lamb and lamb products as shown by the near collapse of the beef industry in The United Kingdom in the 1990's after the 'mad cow disease' food scares (Ashworth & Mainland, 1995).

International trade and travel have been implicated in the spread of the so-called exotic *Salmonella* serotypes. In the post world war II period dried egg imports from the United States were implicated in increased dominance of *S. typhimurium* and appearance of newer serotypes like *S.anatum,, S. virchow* and *S. montevideo* in the United Kingdom (Barrow, 1993). International trade has also been implicated in outbreaks of salmonellosis associated with crimson red dye used for gastro-intestinal motility tests, chocolate and fish meal (Steele, 1983). With the growth of the tourism industry and increased volume of international travel, there is an increased risk that travellers could carry back home the serotypes that they got infected with outside their country of residence. However, the trans-frontier spread of *Salmonella* serotypes associated with international trade will be of concern to the sheep industry because this can result in technical barriers to trade in an attempt by importing countries to reduce introduction and spread of exotic serotypes.

New Zealand is the largest exporter of sheep meat in the world. The provisional results for the year 2001 ending in June show that the sheep meat export was worth NZ$ 2.12 billion (Anon, 2001a). In 1996, 55 % of the country's exports were to the European Union with the bulk of the meat going into the United Kingdom. The other markets that include Asia, North America and the Middle East usually offer prices that are 5% lower than those offered by the European Union (Cunningham, 2000). The presence of *Salmonella brandenburg* in meat would therefore pose a threat to the continued access to the lucrative European markets. Importing countries in their attempts to reduce the risk of introduction of pathogens will either resort to testing at port of entry and subsequent rejection of positive consignments or impose technical food safety barriers like the lowering of the tolerance for *Salmonella* spp. This

practice in the 1970's and 1980's affected both Australian and the New Zealand meat industries (Eyles, 1994; Buzby & Roberts, 1997). It is the imposition of these scientifically questionable restrictions that gave birth to the General Agreement on Tariffs and Trade (GATT) and the World Trade Organisation (WTO), the intention of which is to open the international markets (Zepeda *et al.,* 2001).

The GATT agreement came about as an attempt to harmonize international trade and it gave birth to the WTO. The WTO's main aim is to settle international trade disputes (Buzby & Roberts, 1997). In order to harmonize international trade in animals and animal products the WTO, through the Sanitary and Phytosanitary (SPS) measures of 1995, gave the responsibility of setting standards to the Office Internationale Epizootique (OIE) (Zepeda *et al.,* 2001). In order to remove unscientific based trade barriers it was agreed that any country that imposes standards that are more stringent than those set by the OIE must justify its decision using scientific methods and risk analysis (Zepeda *et al.*, 2001). Despite the efforts of the OIE and the Codex Alimentarius commission with the advice of the International Committee for Microbiological Standards of Food (ICMSF) there is still a lot of subjectivity associated with determination of microbiological hazards and microbiological criteria used. The issue is further complicated by the fact that trade in agricultural products is still essentially based on sampling. Therefore trade between countries is based on memoranda of agreement with intensity of testing based on history of compliance and new information concerning pathogens and hazards associated with the food being tested.

Therefore the responsibility is still on the New Zealand meat industry to ensure that *Salmonella brandenburg* is controlled. The successful control of this microorganism will mean a reduced risk to the consumer and an unimpeded access to the international markets by the New Zealand sheep meat (Eyles, 1994; Anon, 1997).

CHAPTER SEVEN: GENERAL DISCUSSION AND CONCLUSIONS

The understanding of pathogenesis and epidemiology of tuberculosis and brucellosis has resulted in the eradication of these diseases in most of the developed countries. However, limitations in the understanding of the more complex epidemiology and host range of enteropathogens like *Campylobacter* spp. and *Salmonella* spp. makes their eradication elusive (Logtestijn *et al.,* 1993). Further research should be done on the pathogenesis and epidemiology of *Salmonella brandenburg* in sheep in the South Island of New Zealand. The study should focus on factors that could have favoured the emergence of this serotype including host-pathogen relationship and environmental factors (Lax *et al.*, 1995). The emergence of a new serotype in an animal population has several possible origins including mutations of the previously non-pathogenic strain, transfer from humans to animal through sewage or animal handlers, introduction through feed and introduction through wildlife, including birds (Davies, 2001). The role of environmental sources like carrier animals, fomites and vehicles used for transporting animals particularly to the meat plants should also be investigated (Davies, 2001).

The emergence of an apparently new kind of *Salmonella* serotype is not unique. In the United Kingdom *Salmonella abortusovis* used to be a dominant serotype in sheep until the mid 1970's when its importance diminished. The reduced importance of *S. abortusovis* coincided with an increase in the variety of serotypes isolated in sheep (Sojka *et al.*, 1993). The research on factors that influenced the dynamics and emergence of Salmonella *brandenburg* as a sheep pathogen should be compared with those of serotypes like *S. montevideo* in the United Kingdom. However, extrapolation

of risk factors from other studies should be avoided as shown by a study in The Netherlands. Van der Wolf *et al.* (2001) showed that an increase in herd size of finishing pigs was associated with a decrease in the risk of salmonellosis due to the improved management as compared to smaller herds. This went against the widely held wisdom that the larger the herd size the higher the risk of salmonellosis.

Salmonellosis in sheep is usually associated with congregation of animals (Richards *et al.*, 1993). The outbreaks of ovine abortion due to *S. brandenburg* should be investigated to show if any of the predisposing factors associated with high stocking density are involved. These include disrupted social settings, unusual noises and interrupted feeding patterns. Other factors like peri-parturient weather patterns, humidity and inappetance should also be considered in the study (Higgs *et al.,* 1993). The peaking of abortion cases in late gestation means risk factors associated with this period must be investigated. These include high stocking density at 2-3 months gestation, yarding and other peri-parturient stresses like poor nutrition and multiple lambs (Davies & Renton, 1992; Clark 2001a). A survey by Boxall *et al.* (1999) showed that 63% of aborting animals were carrying multiple lambs, 76% of aborting animals were either two tooths or of mixed age while the abortion within the four tooths was only 14%. Therefore in addition to the stress of multiple lambs, age had an influence on susceptibility to *S. brandenburg* abortions with the younger ewes being more susceptible to the outbreak strain.

The increased isolation of *Salmonella brandenburg* could be due to the competitive advantage that it has over other serotypes in the field. The serotype could have acquired this dominance through host adaptation (Wray, 1995; Akkina *et al.*, 1999;

Baumler *et al.*, 2000). Factors associated with the narrowing of the niche width like intensification, reduced genetic diversity of livestock and uniform husbandry methods should be investigated. These could be possible causes for *Salmonella brandenburg* gaining a competitive advantage over other serotypes in the South Island. The gaining of a competitive advantage by a host adapted serotype results in the serotype overcoming competitors setting up conditions suitable for an epidemic (Hunter & Izsak, 1990). Further studies in host adaptation of the *Salmonella brandenburg* outbreak strain in sheep in New Zealand will also help elucidate why the outbreaks are confined to the southern regions of the South Island of New Zealand. At present *Salmonella brandenburg* has not been reported as a cause of abortion in the sheep farming areas of the North Island of New Zealand.

Wright *et al.* (1998) reported that there was an apparent increase in the cases of human *Salmonella brandenburg* infections in New Zealand during the period 1985 to 1995. Collaboration between the veterinary services and human public health authorities is required to investigate the possible link between apparent rise of human cases of *Salmonella brandenburg* prior to the emergence of the serotype as an important ovine pathogen in the South Island of New Zealand. The fact that *Salmonella brandenburg* is one of the common serotypes associated with human sewage sludge makes this a credible path to follow (Fenlon, 1981). Gulls are known to recycle sewage disposed into the sea back to land (Reilly *et al.*, 1981)

A study of the occurrence of *Salmonella montevideo* as a cause of abortion in sheep and cattle in Scotland showed that a previously unimportant serotype became an important cause of abortion in sheep with 87% of the abortions occurring during the

lambing months. The same serotype also caused abortion in cattle and scour in calves. The clinical picture associated with this serotype is very similar to that of *Salmonella brandenburg* in the South Island of New Zealand. Epidemiologically there are similarities because like *S. montevideo, S. brandenburg* has been isolated from multiple sources like birds, other animals, effluent and humans (Sharp *et al.*, 1983). The role of birds, sewage from human waste and farms should be further investigated in the South Island of New Zealand. A study done by Coulson *et al.* (1983) on *Salmonella montevideo* abortion in sheep in Scotland showed that the abortion pattern in sheep were associated with the simultaneous migrations of herring gulls into Scotland for breeding. A similar study should be done on black backed gulls in New Zealand. This should help elucidate the significance of black backed gulls in the epidemiology of *Salmonella brandenburg.* Trapped black backed gulls in the outbreak regions of the South Island of New Zealand have been reported to have *Salmonella brandenburg* counts of up to 25 million organisms per gram of faeces (Clark, 1999).

Due to the worldwide consumer concern over the safety of red meat it is also important to study how ewe to lamb transmission impacts on product quality. Food safety crises place a heavy economic burden on farmers and countries through increased surveillance, new regulatory measures, sale losses and slaughter of infected animals. 'The *Salmonella* in egg crisis' of 1988 to 1989 cost the United Kingdom poultry industry close to 70 million British pounds (Persson & Jendtey, 1992). Therefore it is important for the industry to address the problem of *Salmonella brandenburg* in the product before it is turned into food scare by the media.

The other important issues of public health importance that need to be investigated are the role of ewe to human, product to human and human to human transmission of *Salmonella brandenburg* in New Zealand. Presently animals from a known *Salmonella brandenburg* case farm are confined for more than 28 days before they are transported out of the farm for slaughter or any other purpose (Davies, 2001). However, it is important to investigate if the length of this period has any bearing on the transmission of *Salmonella brandenburg* to other farms or to product if animals are sent for slaughter. Product from known case farms should not be used in high-risk products like comminuted meats (Humphrey, 2001).

The present study showed that the clinically affected flocks presented an elevated risk of infection in slaughtered animals and that the risk was elevated early in the killing season, the November-December period. The study also showed that *S. brandenburg* was present in clinically **unaffected** farms and that the proportions of infected ewes and lambs were similar. The above findings have an important bearing on the measures that can be taken at the abattoir to reduce the risk of cross contamination. This means early in the killing season any measures that are taken to minimise the risk of cross contamination should be applied to both classes of stock and to both unaffected and affected flocks. However, this study suggested that transportation and lairaging did not have a major impact on the prevalence of *Salmonella* spp. because the proportions of infected animals on-farm were similar to those at slaughter. The serotyping of all the 133 isolates in the study gave *S. brandenburg* and the subtyping of 24 representative isolates using PFGE yielded a single profile identical to that of the original isolates of the 1996 outbreak.

Since the first reported outbreaks of *Salmonella brandenburg* in sheep in 1996 the pulsed-field gel electrophoresis profiles (PFGE) of the isolates have shown an unusual genetic stability of the outbreak strain. The host, pathogen and environmental factors responsible for this stability should be looked into. A study by Wright *et al.* (1998) showed 13 different profiles of 115 human isolates of *S. brandenburg* collected between 1990 and 1995 none of which were similar to that of the sheep outbreak. The discriminatory power of PFGE as a *Salmonella brandenburg* typing method is therefore not responsible for the non-detection of changes in the genetic make up of the outbreak strain in sheep in the South Island of New Zealand.

Further work on the efficacy of vaccination as a control measure of *Salmonella brandenburg* needs to be explored. The organism is a good candidate for control by vaccination because it is stable as shown by a single PFGE profile of the outbreak strain over a five year period and the possibility of cross protection with the Salvexin™ vaccine antigens as shown by the mouse challenge trials. The high economic losses to farmers with an average loss of NZ$ 10 000 for affected farms should also been an added incentive to find an effective vaccine. However, field trials of the Salvexin + Brandenburg ™ vaccine on sheep have so far given equivocal results (Marchant, 1999; Marchant *et al.*, 2000).

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APPENDICES

APPENDIX I- MEDIA PREPARATION

1. BACTO BRAIN HEART INFUSION (BHI) Broth

   Dissolve 37 grams in 1 litre distilled water
   Dispense in 3 millilitres Bijoux bottles
   Sterilise in the autoclave for 15 minutes at 15 pounds pressure (121^0C)

2. 15 % BACTO GLYCEROL BROTH

   Add 2.4 grams nutrient broth (BBL)
   Add 45 millilitres glycerol
   Dissolve in 300 millilitres distilled water
   Dispense in 3 millilitres Bijoux bottles
   Autoclave for 15 minutes at 15 pounds pressure 121^0C)

3. BLOOD AGAR

   Salt base

   Bacto agar (DIFCO) 15.0 grams
   Sodium chloride 5.0 grams
   Add 1.0 litre of distilled water and dissolve by heating
   Autoclave for 15 minutes at 121 ^{0}C
   Cool to 45-50 ^{0}C
   Dispense 10 millilitres in petri dishes and allow to cool and dry

   Blood base

   Add 44.0 grams Columbia agar (DIFCO) to 1.0 litres distilled water
   Dissolve by heating
   Autoclave and cool as above
   Add 5% v/v of sheep blood
   Pour 10-15 millilitres over the salt base
   Allow to cool and dry
   Store at 4 ^{0}C

4. BACTO NUTRIENT AGAR (SLOPE)

   3.0 grams Bacto beef extract
   5.0 grams Bacto peptone
   5.0 grams NaCl
   15.0 Bacto agar

Make up to 1.0 litres with distilled water
Heat to dissolve
Cool to 50-60 ^{0}C and dispense into Bijoux bottle
Autoclave at 121 ^{0}C for 15 minutes
Lay racks on their sides on a slant maker

APPENDIX II- BUFFER PREPARATION

1. Pett IV buffer

   20 millilitres of 5M NaCl
   1 millilitre of 1M Tris-HCl
   2 millilitres of 0.5M EDTA
   Make up mixture to 100 millilitres with MQ water
   Autoclave

2. 10:1 TE buffer

   1millitre 1M Tris-HCl
   200 microlitres of 0.5M EDTA
   Make up to 100 millitres with MQ water
   Autoclave

3. 5X TBE buffer

   108 grams Tris
   55 grams boric acid
   40 millilitres 0.5 EDTA
   Make up to 2 litres with MQ water
   Autoclave

4. 0.5X TBE buffer

   200 millitres 5X TBE
   1800 millilitres MQ water
   Mix and autoclave

5. 1M Tris-HCl pH 8.0

   Dissolve 12.1 grams Tris base in 80 millilitres MQ water
   Adjust to pH 8.0 with concentrated HCl
   Equilibrate for at least 5 minutes
   Make up to 100 millilitres with MQ water
   Autoclave

6. 0.5 EDTA pH 8.0

   Dissolve 18.6 grams of disodium EDTA in 70 millilitres MQ water
   Adjust to pH 8.0 with NaOH pellets
   Equilibrate for at least 5 minutes
   Make up to 100 millilitres with MQ water
   Autoclave