SALMONELLA BRANDENBURG IN NEW ZEALAND SHEEP: THE DEVELOPMENT OF A SEROLOGICAL DIAGNOSTIC TEST AND A CASE CONTROL STUDY

A THESIS PRESENTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF VETERINARY STUDIES AT MASSEY UNIVERSITY

JOANNE ISABEL KERSLAKE

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ABSTRACT

Salmonella Brandenburg causes acute diarrhoea and severe illness in a variety of animals and was first isolated in New Zealand in 1986. Since 1996 *Salmonella* Brandenburg has been associated with an emerging epidemic of abortions and deaths in sheep in the southern regions of the South Island. Little is known about the specific epidemiology of *Salmonella* Brandenburg in sheep and as a result control to date has been largely based on anecdotal evidence and general principles. This study focused on the following aims:

- To develop a serological test for use in epidemiological studies and for monitoring future control efforts targeting *Salmonella* Brandenburg in New Zealand sheep.
- To identify factors associated with the occurrence and severity of *Salmonella* Brandenburg outbreaks in New Zealand sheep.

Traditionally *Salmonella* diagnosis has depended on bacteriological culture. Such tests are time consuming, labour and equipment intensive, and may lack sensitivity. ELISA (Enzyme-Linked Immunosorbent Assay) methodologies offer an alternative for the diagnosis of *Salmonella* infection. Therefore the development of an ELISA test for detecting antibodies to *Salmonella* Brandenburg organism in sheep plasma was undertaken. Expression of common antigens has resulted in a high level of antibody cross-reactivity between different serovars in serological tests. Lipopolysaccharides (LPS) (O Antigens) are the primary cause of these cross-reactions. Cross-reactivity with two common sheep serovars (*Salmonella* Typhimurium and *Salmonella* Brandenburg) was of major concern for the development of a *Salmonella* Brandenburg ELISA. This was overcome by preparing an antigen mainly composed of flagella and fimbria proteins (LPS free). The antigen preparation was of a relatively crude and non-characterised nature and could only produce a reasonable optical density response at a high concentration. Unfortunately, while the ELISA was responsive, the specificity of the ELISA for *Salmonella* Brandenburg antibodies remained poor. Further investigation of the specificity of the antigen preparation, through the use of different sera, or through the development of a more pure and specific antigen, is needed for the successful development of a sensitive and specific serological test for determining *Salmonella* Brandenburg exposure in New Zealand sheep.

A case control study was performed as part of a large-scale ongoing investigation aimed at identifying factors associated with *Salmonella* Brandenburg disease in New Zealand sheep. Details of disease prevalence and farm management methods were collected from two affected regions in southern New Zealand. Associations between possible risk factors and *Salmonella* Brandenburg were evaluated using odds ratios, with analyses being performed at two different levels:

- farm level analysis to compare affected vs. unaffected farms using a case-control approach.
- within farm analysis restricted only to affected farms to evaluate risk factors associated with severity of reported disease on affected farms.

Data were collected from 405 farms containing a total of 1, 170,737 ewes. Of the 175 case farms, 97% had diseased mixed age (MA) ewes, 45% had diseased two-tooth (TT) ewes, and 5% had diseased hogget (H) ewes.

Salmonella Brandenburg appeared to occur in better performing flocks, which are often associated with intensive farming methods. At the farm level, factors such as increased total number of ewes, feeding of hay, and controlled winter grazing appeared to increase the risk of disease. Farming methods such as controlled winter grazing may result in higher stress levels and increase the shedding of *Salmonella* Brandenburg organisms. This may create a higher risk of exposure in sheep yards and on pasture, resulting in a higher risk of disease. Feeding crop and having hilly terrain decreased the risk of a farm having disease. A protective effect of hilly terrain could be due to less intensive farm management, with a subsequent reduction in stress associated disease risk. Within affected farms, disease appeared to be more severe with the removal of rams after July, feeding of hay, and the practices of strop grazing. Shearing after July, increasing the total number of pre-lamb yardings, and vaccinating for *Salmonella* appeared to be protective. Therefore reducing stress and vaccinating ewes appear to reduce the risk of a *Salmonella* Brandenburg outbreak.

DEDICATION

To Isabella Kerslake

Always wishing that I could have been closer.

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I arrived at the Epicentre as a bit of a lost soul, unsure in what I wanted to do in the year 2000, let alone what I wanted to do in life. It was at the Epicentre where that all changed.

I met and knew so many amazing and interesting people during my Masters that influenced and supported me in a number of fantastic different ways. I am grateful to everyone; in whatever little or huge parts that you played in the past couple of years of my life, I could not have grown or become half the person that I am today without all of you.

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To my ma, pa and brother, there is not a time in this life that you have not been there for me. You have seen and received the worst and the best of me during my Masters and you have taken every little bit in your stride and offered me nothing but love and amazing support. I love and thank you for that.

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CHAPTER ONE

Review of the Literature

Salmonella

Salmonella are facultatively anaerobic gram-negative rods of the Enterobacteriaceae family. They can be non-motile or motile with peritrichous flagella, and generally have simple nutritional requirements. *Salmonella* can grow in temperatures ranging from 5 °C to 47°C, and are able to tolerate pH levels ranging from 4 to 9. They are durable and versatile organisms that have the ability to survive in harsh environments. Since 1918, the Kauffmann and White scheme has classified 2249 *Salmonella* serovars. The combined profile of somatic (O) antigens and flagella (H) antigens make up the antigenic profile of the *Salmonella* serovar, forming the basis of the Kauffman and White classification scheme.

Salmonella organisms are pathogenic to both humans and warm-blooded animals, where they can cause diseases such as typhoid and gastroenteritis. Salmonellosis is a common infectious disease, where the main sources of infection are *Salmonella* excretion from domesticated animals or contaminated food of animal origin. Disease primarily occurs in the intestinal tract where the organisms establish infection by attaching themselves to the wall of the intestinal epithelium. Organisms then invade intestinal cells of the ileum and colon (intracellular parasitism) and multiply. When the cell is destroyed, the organism spreads and causes inflammation often resulting in enteritis.

Extra-intestinal manifestations can also occur. If *Salmonella*e break through the intestinal barrier, organisms may then spread through the body via the lymph and blood vessels. If the immune response does not overcome this infection, septicaemia may result. Organ specific problems such as pneumonia, meningitis, septic arthritis and abortions may also follow bacteraemia (Wray and Wray, 2000).

Salmonella in sheep

World-wide, the prevalence of ovine salmonellosis is relatively low. However, when

outbreaks occur they often have a severe effect on individual farms where extensive stock and financial losses occur. *Salmonella* serovars, such as *Salmonella* Abortus-ovis, *Salmonella* Montevideo, *Salmonella* Dublin and *Salmonella* Typhimurium have all been recognised as common causative agents of ovine salmonellosis (Wray and Wray, 2000).

Salmonella Abortus-ovis

Salmonella Abortus-ovis was first recognised in Germany in 1921, and in the late 1950s to early 1960s was endemic in the Southwest of England. It is host specific to sheep and causes abortion in the last 4-6 weeks of pregnancy, which is sometimes followed by ewe death. Clinically affected ewes do not generally discharge organisms more than a few days after abortion. In addition, ewes that have recovered from clinical infection often return to normal fertility the following season.

Salmonella Montevideo

Salmonella Montevideo emerged as a sporadic cause of abortion and death amongst ewes in Southeast Scotland between 1970 and 1981. A total of 67 outbreaks were reported in this period with another outbreak occurring in 1982, involving 37 farms. In this region of Scotland, 20-30 farms are still affected annually. *Salmonella* Montevideo showed similarities to *Salmonella* Abortus-ovis with abortion being the predominant clinical symptom. Most affected ewes did not scour and only showed transient mild illness. Approximately 10% of aborting ewes died due to systemic illness.

Salmonella Typhimurium

Salmonella Typhimurium is the most common *Salmonella* serovar in sheep. The predominant phage type can differ between countries. For example, in Australia the predominat phage type is DT135/9, while in the UK it is DT104. Infection with *Salmonella* Typhimurium often results in both enteric and systemic symptoms. Affected animals usually develop a high temperature and scour profusely and usually die from septicaemia or dehydration. However, sudden death without prior signs of illness may also occur.

Salmonella Dublin

In the late 1960's and early 1970's *Salmonella* Dublin was seen as the predominant serotype of the UK. *Salmonella* Dublin infection produces similar clinical signs to *Salmonella*

Typhimurium.

Treatment and prevention of *Salmonella* associated disease

To reduce the spread of infection affected animals are usually isolated and the rest of the flock spread out. This controls the disease to a certain point, although by the time Salmonellosis is detected it has often already spread widely through the flock. Unfortunately, no treatment is consistently effective. Treatment of animals with clinical symptoms associated with *Salmonella* infection usually comprises of antibiotic and fluid therapy and other various treatments aimed at alleviating the symptoms associated with gastro-enteritis and septicaemia.

Vaccination

Josland (1954), was the first to investigate the use of a vaccine to control Salmonellosis in sheep. He discovered an injection of formalised alum-precipitated *Salmonella* Typhimurium vaccine resulted in low and inconsistent antibody response. Even though a greater number of vaccinated animals survived following challenge, compared to unvaccinated animals, he concluded that prophylactic vaccination was of little use.

Cooper (1967), decided to investigate the efficacy of a monovalent vaccine for protecting sheep against *Salmonella* Typhimurium. The vaccine gave significant protection against death at the 5% level in both an experimental challenge trial and a field trial. While this vaccine provided significant protection against *Salmonella* Typhimurium, *Salmonella* Bovis-morbificans still proved to be a significant problem.

Wallace and Murch (1967) and Beckett (1967) performed a number of vaccination trials on numerous farms with a non-viable bivalent *Salmonella* Typhimurium and *Salmonella* Bovis-morbificans vaccine. Both investigated the same bivalent vaccine, but Wallace and Murch took a different approach to Beckett and vaccinated sheep prior to challenge, while Beckett vaccinated sheep during challenge. Both groups found more deaths occurred in the control group compared to the vaccinated group. Beckett also found the vaccine offered protection for a period of 14 days after administration. Both concluded that the non-viable vaccine was a useful method for stimulating resistance in sheep flocks that have been previously challenged with *Salmonella*.

In 1968, Rudge *et al.* and Davies (1969) investigated the same bivalent vaccine in experimentally infected sheep. They found that vaccination produced a significant level of immunity, and vaccinated sheep showed a lower incidence of infection following challenge.

In 1974, Cooper and MacFarlane investigated sheep that had received either one or two doses of a New Zealand manufactured bivalent vaccine. Interestingly, they found that there were no deaths amongst vaccinated sheep; and that sheep infected with *Salmonella* Typhimurium had significantly fewer organisms in their blood if they had been vaccinated twice (once with a sensitiser, once with a booster). Therefore, the use of a bivalent vaccine was considered to provide some level of protection to the animal.

In the 1980s, Schering Plough released a *Salmonella* vaccine called Salvexin®. This is an inactivated whole cell vaccine containing four *Salmonella* strains - *Salmonella* Typhimurium (2), *Salmonella* Bovismorbificans and *Salmonella* Hindmarsh. At the time of writing, Salvexin® was the only available *Salmonella* vaccine in New Zealand.

Salmonella history and isolation in New Zealand sheep

Since 1949, when *Salmonella* was first diagnosed in New Zealand (Salisbury, 1958), a number of *Salmonella* serotypes have been identified as causative organisms of ovine illness (Table 1.).

Table 1. *Salmonella* serotypes isolated from sheep in New Zealand (Clark *et al.*, 1999).

S. Adelaide	*S*. Infantis
S. Anatum	*S*. Mbandaka
S. Bovismorbificans	*S*. Oranienberg
S. Brandenburg	*S*. Saintpaul
S. Dublin	*S*. Tennessee
S. Enteritidis	*S*. Typhimurium
S. Heidelberg	Group B 4,12:-:1,2
S. Hindmarsh	Species rough:r:1,5

The most common *Salmonella* serotypes of sheep recognised in New Zealand are *Salmonella* Hindmarsh and *Salmonella* Typhimurium. Clinical symptoms usually consist of acute diarrhoea and severe illness (Clark *et al.*, 1999). Outbreaks usually occur at the beginning of the year and are often associated with factors such as high stocking rates, facial eczema control, transport and change in nutrition. Carrier animals and intermittent faecal excretion from animals are thought to be important in the transmission of *Salmonella*.

Overseas, abortion storms have often been associated with *Salmonella*. Fortunately, New Zealand has not experienced severe abortion storms, although sporadic outbreaks of abortions have occurred. The majority of ovine abortions in New Zealand are a result of infection of host animals by organisms such as *Campylobacter* or *Toxoplasma gondii*. Because of the development and widespread usage of vaccines against *Campylobacter* and *Toxoplasma*, sheep abortions have not been a major problem in the New Zealand sheep industry, until recently, with the appearance of *Salmonella* Brandenburg.

Salmonella Brandenburg disease outbreaks in New Zealand sheep.

In 1996 a Canterbury (New Zealand) sheep farm experienced an outbreak of abortions, and a number of deaths amongst pregnant ewes (Bailey, 1997). The causative organism was identified as *Salmonella* Brandenburg, an uncommon *Salmonella* isolate amongst New Zealand sheep. As seen below, further outbreaks of *Salmonella* Brandenburg were seen in 1997 - 2001 throughout the southern regions of the South Island (Table 2.).

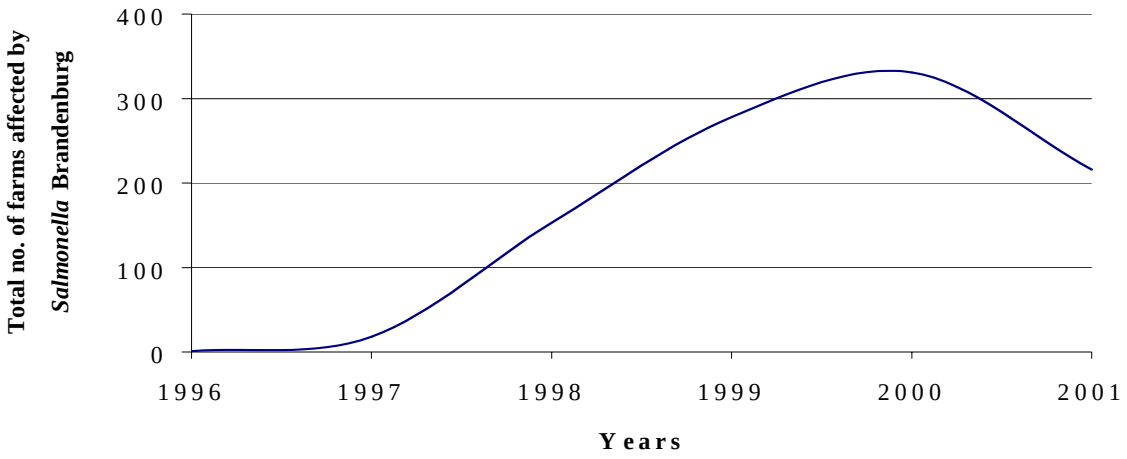
Table 2. Number of ovine *Salmonella* Brandenburg confirmed lab submissions (Clark, 2000).

Years	Canterbury	Otago	Southland
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 (14)	62 (16)	233 (40)
2001	8 (12)	21 (19)	187 (42)

( ) Cattle

Animal Health Laboratory records clearly show that in 1997 17 farms in mid Canterbury and one in Southland experienced an epidemic of abortions and deaths in ewes due to *Salmonella* Brandenburg (Bailey, 1997). In 1998, over 100 farms were affected and for the first time, cases were reported in Otago. From 1999 to 2001, a total of 297 (1999), 401 (2000) and 216 (2001) infected farms were reported throughout Canterbury, Otago and Southland. *Salmonella* Brandenburg is now recognised as a common ovine isolate in the South Island. Figure 1 shows the total number of farms infected by *Salmonella* Brandenburg per year. It shows an increasing trend, signalling an emerging epidemic of *Salmonella* Brandenburg in the South Island over this period.

Figure 1. Annual increase of *Salmonella* Brandenburg affected farms in Canterbury, Otago and Southland.

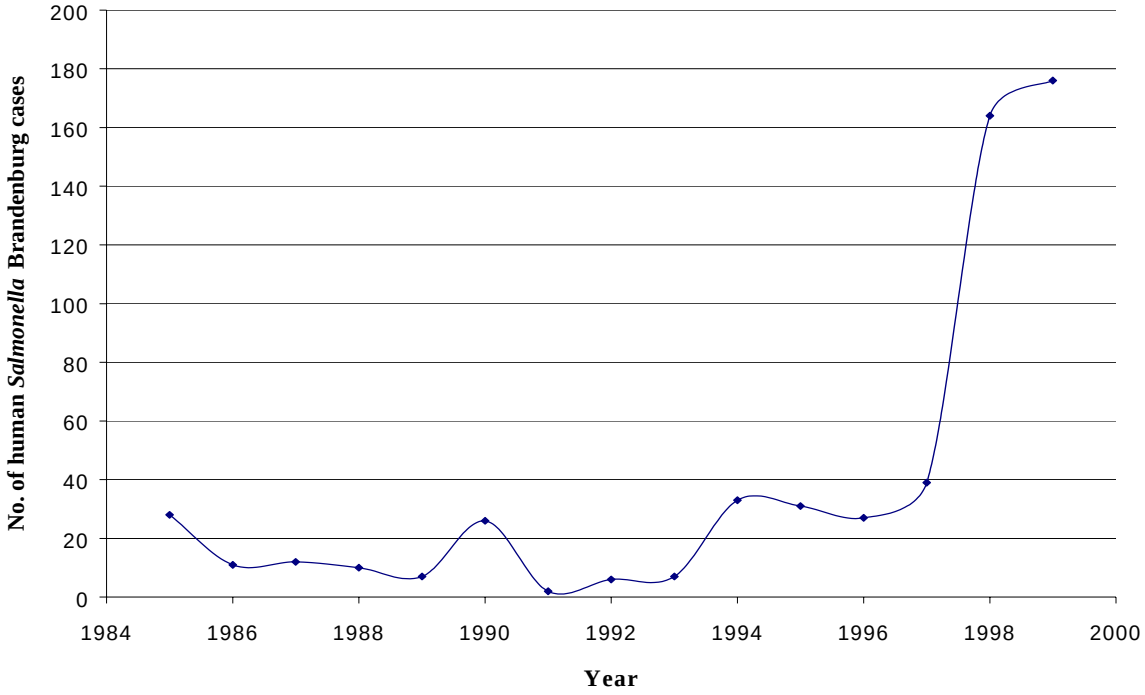


Salmonella Brandenburg causes major financial losses. An average lambing loss of 17% on affected farms has been observed throughout the Southern region, where most farmers have experienced a loss of lambs ranging from 23-500, and a loss of ewes ranging from 15-350 (Boxall *et al.*, 1999). There is a definite need to develop strategies to control this disease, as an estimation of the potential financial costs to farmers averages $10,400 per farm per annum (Roe, 1999). Currently farmers are under intense economic pressure; therefore it is essential to retain high performing stock. A disease such as *Salmonella* Brandenburg cannot be left uncontrolled if farms are to remain financially viable.

Salmonella Brandenburg has also been recognised as a zoonosis. It can cause severe diarrhoea and stomach cramps in people, with recovery taking up to 6 weeks (Clark *et al.*,

1999). In New Zealand, *Salmonella* Brandenburg has been an infrequent human pathogen, accounting for 1% of human Salmonellosis cases (Wright *et al.*, 1998). Averages of approximately 33 human cases per annum were diagnosed with *Salmonella* Brandenburg from 1994 - 1997 (Figure 2). In 1998 and 1999, 168-178 human cases were diagnosed (Smart, 1999). All cases appear to have work related exposure to the organism, for example, farmers and abattoir staff.

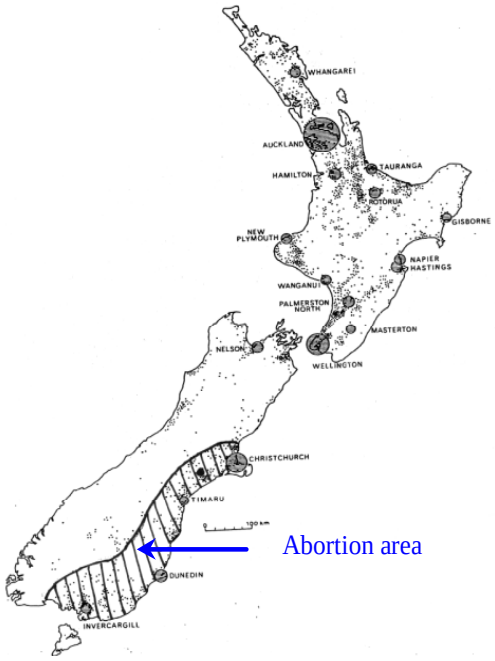
Figure 2. The total number of human *Salmonella* Brandenburg cases reported from 1985 - 1999 (Smart, 1999).



The Current Situation

Currently, the disease in sheep has not spread north of Oxford, Canterbury (Figure 3.), and the number of infected farms in Canterbury has appeared relatively constant for the past three years. In Otago, after the initial outbreak, the number of infected farms seems to have levelled off, with the only subsequent spread being from the Milton-Balclutha area to West Otago. However, in Southland the number of infected farms is still increasing annually, with the number nearly doubling from 1998 to 1999, and increasing another 27% in 2000. In 1998 the disease was reported in the Winton-Otautau area, and by 2000 it had spread to the Northern Southland, Gore, Edendale and Tokonui areas (Clark, 2000).

Figure 3. Location of farms affected with *Salmonella* Brandenburg from 1996-2001 throughout New Zealand.



Although *Salmonella* Brandenburg infection has been epidemic in sheep since 1997, in New Zealand *Salmonella* Brandenburg infection has occurred sporadically in cattle, pigs, dogs, sheep, and birds for years (Table 3).

Table 3. *Salmonella* Brandenburg infection in other species (Bailey, 1997).

	Bird	Cat/dog	Cattle	Deer	Goats	Horses	Pigs	Sheep
1990	0	0	0	0	0	0	2	0
1991/92	0	0	0	0	0	0	0	0
1993	0	1	0	0	0	0	2	0
1994	0	0	1	0	0	0	0	0
1995	0	0	0	0	2	0	1	1
1996	1	1	0	0	0	0	0	0
1997	0	1	0	1	0	0	3	27
1999	1	4	18	1	1	2	1	264
2000	0	4	106	0	1	4	2	545
2001	1	8	137	5	1	1	1	344

Since 1999, there has been increasing concern about the number of cattle being affected by *Salmonella* Brandenburg. In 1999, there were 18 farms that reported infected cattle. In 2000, this number increased to 70, indicating that *Salmonella* Brandenburg infection in cattle is escalating, which is probably related to the increased contamination of the southern environment (Clark, 2000; Keller, M. *per comm.*).

Salmonella Brandenburg infection is believed to occur mainly in the autumn/spring period, and primarily in pregnant ewes. Abortion usually occurs after 3 months of gestation and is more likely to occur in multiple bearing ewes. In an affected flock, the disease has been shown to take a course of about 16-20 days, with the number of cases peaking around 8-10 days after identification of the first case (Smart, 1999). In a new area, the abortion rate and death rate of clinically infected ewes can be reasonably high, with 5-20% of the ewes aborting and 10-100% of the aborting ewes dying. However in subsequent years of infection, both abortion and ewe death numbers appear to drop (Clark, 2000).

It is not known how or when this bacterium infects sheep. However, it is believed that infection may occur primarily through excretion and ingestion of *Salmonella* organisms. It has been shown by (Clark *et al.*, 2000) that ewes can excrete *Salmonella* for up to 6 months and that *Salmonella* can survive in the environment for up to 3 months (Tannock and Smith, 1971). Furthermore *Salmonella* is able to survive in dust (Robinson, 1967). Therefore, infection may occur through licking and smelling of aborted foetuses, through the ingestion of faecal contaminated pasture and water, or through such things as sheep yards where ingestion of dust occurs.

This information implicates many different factors when considering how *Salmonella* Brandenburg may be transmitted. *Salmonella* transmission has been shown to occur through foodstuff (Al-Hindawi and Taha, 1979), pasture and water (Hunter and Izsak, 1990; Robinson, 1970), dust (Robinson, 1967) and scavengers.

In Scotland, scavengers such as seagulls have been implicated in the spread of *Salmonella* serotypes. For example in the 1970s, gulls were implicated in the spread of *Salmonella* Montevideo in cases of sheep abortions (Coulson *et al.*, 1983; Reilly *et al.*, 1985). The

outbreak of *Salmonella* Montevideo in Scotland has paralleled the *Salmonella* Brandenburg situation in New Zealand in many ways. Common links such as infection of cattle, dogs and humans have been seen in both situations.

In 1998/99 researchers from Ministry of Agriculture and Forestry (MAF) and Massey University showed that black backed gulls sourced from *Salmonella* Brandenburg infected, and non-infected farms, carried *Salmonella* Brandenburg organisms in their intestinal contents (Clark *et al.*, 1999). Therefore, it has been suggested that, as in Scotland, these gulls have the potential to act as reservoirs of infection, and are capable of spreading the disease.

Farming practices also have to be taken into consideration when considering how *Salmonella* may be transmitted. Interestingly, *Salmonella* Brandenburg appears to be occurring in better performing flocks, where there is a higher stock rate, rotational grazing and a high percentage of multiple births. Currently, better performing flocks are often associated with intensive farming methods, such as higher stock numbers, controlled winter grazing and pre-lamb yarding, which may result in higher stress levels for pregnant ewes. Environmental stress factors such as poor weather conditions and poor feeding conditions also place a lot of stress on stock. In Scotland, the number of *Salmonella* Montevideo cases reached epidemic proportions in the winter of 1982, when ewes were subjected to prolonged cold stress (Coulson *et al.*, 1983). Activation of latent infection from poor feeding or starvation has also been suggested as a possible cause of salmonellosis (Cooper, 1967). In another study, higher feed intakes in *Salmonella* Dublin infected sheep appeared to reduce the disease (Baker *et al*, 1971). It has been shown by Barham, *et al*. (2002), that animals under stress tend to excrete more frequently. This potentially creates higher risks of infection in yards or on pasture. Intensive farming is therefore likely to result in a higher risk of outbreaks of contagious diseases.

Salmonella Brandenburg - preventative measures

Factors such as nutrition, health, immune responses, stress and environment all play a role in the occurrence and severity of a disease. A number of preventative and control measures have been suggested in an attempt to reduce both the risk of occurrence and severity of

disease (Clark *et al.*, 1999):

- Rapid disposal of aborted foetuses,
- Rapid removal of aborted ewes into quarantine mobs,
- Reduce stress and overcrowding,
- Cull aborted ewes,
- Control of scavengers,
- Practise good hygiene measures,
- Clean and disinfect vehicles,
- Care in buying sheep and
- Vaccination of susceptible sheep.

Salmonella Brandenburg Vaccine development in New Zealand

Because *Salmonella* Brandenburg was an uncommon isolate in New Zealand, there was no available vaccine when the *Salmonella* Brandenburg epidemic broke out. Researchers undertook a strain type investigation, using DNA based methods, to investigate *Salmonella* Brandenburg isolates. Cultured samples were sent from Animal Health Laboratories, and isolates showed the same RFLP (Restriction Fragment Length Polymorphism). This showed that the sheep were all infected with the same strain of *Salmonella* bacteria (Boxall *et al*, 1999), an important discovery in determining the likely role of a vaccine.

Trials were performed in 1998 to investigate the possible role of vaccination in controlling and preventing *Salmonella* Brandenburg. Due to the common somatic antigens shared between *Salmonella* Brandenburg and *Salmonella* Typhimurium, a mouse challenge trial was conducted to investigate whether Salvexin® might provide some cross protection against *Salmonella* Brandenburg (Marchant, 1999). Although some degree of cross-reaction was demonstrated, the researchers could not be confident that Salvexin® would protect against clinical *Salmonella* Brandenburg disease in sheep. A sheep challenge trial was also carried out. In this trial, groups of pregnant sheep were given no vaccine (control group), Salvexin ®, or Salvexin® with added *Salmonella* Brandenburg antigens. The three groups of sheep were then challenged with *Salmonella* Brandenburg organisms (Marchant, 1999). It was found that the addition of *Salmonella* Brandenburg antigens to Salvexin was beneficial for the protection of the sheep against infection with *Salmonella* Brandenburg.

At the beginning of 2000, Salvexin B+® a new *Salmonella* vaccine containing antigens against five *Salmonella* strains (*Salmonella* Typhimurium (2), *Salmonella* Hindmarsh, *Salmonella* Bovis-morbificans and *Salmonella* Brandenburg.) was released. The recommendation from the manufacturer was that all breeding sheep should be vaccinated. The recommended vaccination program consisted of two doses of Salvexin B+ (a sensitiser and a booster) given four to eight weeks apart, with one vaccination given at least two weeks before the challenge period. Further vaccination surveys are needed in the field to evaluate the efficiency of this new vaccine.

The specific epidemiology of *Salmonella* Brandenburg

Little is known about the specific epidemiology of *Salmonella* Brandenburg disease. There is a definite need for research in this area to understand disease infection and transmission routes so effective control and prevention practices can be developed.

The immunology of *Salmonella* infection

The immune response

The immune system reacts to *Salmonella* infection in two different ways. It produces either innate or specific immune responses. When a microorganism invades, it is the innate response that usually occurs as the primary line of defence. The innate response consists of various physical barriers, such as skin, and simple biochemical reactions that destroy bacteria. This immune response is non-specific and at times insufficient. If the innate response is insufficient the host will respond to the invasion via specific immunity. Specific immunity is split into two different categories; humoral immunity and cellular immunity. In humoral immunity, B-lymphocyte cells produce antibodies, which bind to the surface of a foreign protein (antigen), and enhance the engulfment of foreign proteins through phagocytic cells. Cellular immunity is mediated by various T-lymphocytes, which either directly kill host cells or causes the activation of phagocytic defence. Both humoral and cellular immunity play an important role in protection against *Salmonella* infection (Mastroeni *et al*, 1993).

Antibody production

If *Salmonella* organisms are ingested and pass successfully through the stomach they enter

the intestine. From here *Salmonellae* may invade the bowel wall through specialised epithelial cells, which overlay intestinal lymphoid tissue. It is here that they encounter the first line of specific immune defence. As soon as the host is infected, the immune system produces a rapid humoral response. Antibody production in every host differs depending on the individual antigens of the microorganism. Antigens can produce an immuno-dominant response early on, or a delayed immune response depending on how invasive the microorganism is. After two weeks of infection with *Salmonella*, an antibody response has been detected in chickens, (Chart *et al.*, 1990; Gast and Beard, 1990; Hassan *et al.*, 1991a; Humphrey *et al.*, 1991b; Kim *et al.*, 1991) pigs, (Gray *et al.*, 1996) cattle, and sheep (Brennan *et al.*, 1994). ImmunoglobulinM (IgM) is often the first antibody class detected, followed by immunoglobulinG (IgG) and immunoglobulinA (IgA). IgG concentrations rise, peak, and persist for about 2-3 months after infection, while IgA and IgM concentrations decrease (Chart *et al.*, 1992; Hassan *et al.*, 1991a).

Factors affecting the immune response

The humoral response can be affected by the following factors: dose of challenge organisms (Gray *et al.*, 1996; Humphrey *et al.*, 1991a), virulence of organism (Gray *et al.*, 1995), route of administration (Chart *et al.*, 1992; Gray *et al.*, 1995), genetic background of the host (Barrow, 1992) and the age of the host (Humphrey *et al.*, 1991b; Gast and Beard, 1988; Thorns *et al.*, 1996)

Diagnostic techniques for *Salmonella* infection

Bacteriological methods

The diagnosis of *Salmonella* serovar infections is primarily achieved through bacteriological culturing methods, and depends on the isolation of a specific organism. In the case of abortions, direct samples from the foetal, stomach or placenta tissues are needed, while in the case of enteric septicaemia, the organisms are isolated through cultures from internal organs, faeces and intestinal lymph nodes. Once the sample has been collected, there are a large variety of media and methods available for both isolation and identification of *Salmonella*. Bacteriological culturing generally includes the following 5 steps;

1. Direct culture
2. Non-selective pre enrichment - allows resuscitation and multiplication of bacteria.
3. Selective enrichment - survival and growth of only *Salmonella* organisms
4. Isolation - using selective agar media, restricts growth of bacteria other than *Salmonella*
5. Biochemical and serological confirmation - isolates are subjected to a variety of biochemical and serological tests to confirm and identify the *Salmonella* serovar.

Serological methods

Various alternative serological methods have been utilised for the diagnosis of *Salmonella* infections. There are many different kinds of serological tests that have been developed over the years, all of which exhibit widely variable results in terms of test performance. The most common serological technique that has been utilised in the past for diagnosis of *Salmonella* is agglutination-based serology.

The slide agglutination test is a crude test that utilises serum or whole blood. It is easy to conduct but requires a high level of skill to interpret. In the past it has been successfully used in the poultry industry in the regional eradication of *Salmonella* Pullorum and *Salmonella* Gallinarum. Unfortunately, the test does have a number of disadvantages such as cross-reactions, antigen quality and requirements. It has also been suggested that slide agglutination may potentially be able to identify IgM, which is relatively low in chronically infected animals but relatively high in newly infected animals. Thus, the slide agglutination test may be more sensitive in the earlier stage of infections. Unfortunately, this conventional serological agglutination test has produced poor results in detecting *Salmonella* infection. Using the Micro-agglutination Test (MT), the sensitivity of the agglutination test can be increased. However, this test also usually detects IgM concentrations, which rise and fall rapidly in response to infection. Therefore, while this test may increase the sensitivity, detection is still more difficult. Overall agglutination-based assays suffer from limited sensitivity and a tendency to produce both false-negative and false positive results (Kim *et al*, 1991).

Another serological test that has been developed is the Micro-antiglobulin Test (MAT). The

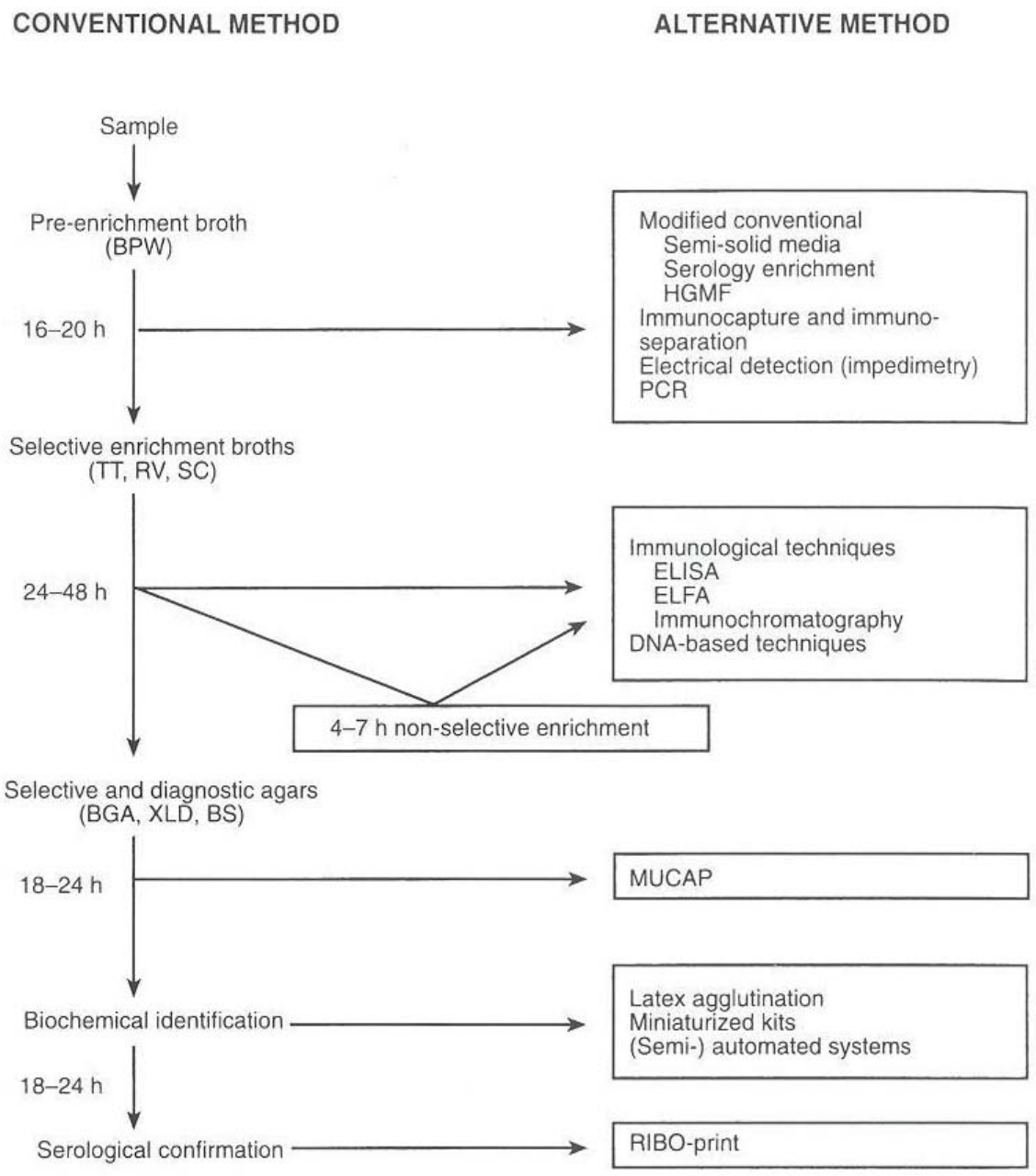
MAT has been used in the poultry industry and has been found to be more reliable than other agglutination assays (Williams and Whittemore, 1976). This test has been able to detect antibody early and persistently throughout infection. The MAT is reported to be more efficient than the MT or Rapid Slide Test (RST), and results often correlate well with the Enzyme Linked Immunosorbent Assay (ELISA) results. Unfortunately, the practical difficulties in performing this test on a large scale outweigh any advantages that it may offer over the ELISA (Nicholas and Cullen, 1991).

A number of ELISAs have been independently developed throughout the world, and are now recognised as a useful serological technique for detection of *Salmonella* infections in populations. It is a quick, sensitive and reproducible assay that is able to cope with large numbers of samples. Compared to other serological tests, the ELISA has been shown to be more sensitive and specific than agglutination tests, such as the RST Test and MT (Kim *et al.*, 1991). Good correlation has been seen between the MAT and the ELISA (Cooper *et al.*, 1989; Nicholas and Cullen, 1991). While ELISA's have been shown to be more sensitive, they are not necessarily more specific than the MAT (Cooper *et al.*, 1989).

Serological methods vs. bacteriological methods

Bacteriological sampling does not always provide an accurate indication of infection, though modification of conventional direct culture by the addition of enrichment and selective media, may improve the likelihood of recovering organisms. Such improvements to conventional culture are costly in terms of labour, equipment and time. Alternative, more rapid diagnostic techniques, offer considerable promise in the diagnosis of *Salmonella*, particularly when large numbers of samples are being screened (Figure 4).

Figure 4. Alternative methods for diagnosis of *Salmonella* infection (Wray CA and WA, 2000).



Key: BPW, Buffered Peptone Water; RV, Rappaport-Vassiliadis; SC, Selinite Cystine; BGA, brilliant green agar; XLD, xylose-lysine-deoxycholate; BS, bismuth sulphate; HGMF, hydrophobic grid membrane filter; PCR, polymerase chain reaction; ELISA enzyme-linked immunosorbent assay; ELFA, enzyme-linked fluroscent assay; MUCAP, 4-methylumbelliferyl caprylate.

Serological methods have a number of advantages over bacteriological culturing. However, problems also exist in this area (Wray and Davis, 1994). For example:

- Intestinal colonisation of *Salmonella* may not stimulate an antibody response that can be detected by conventional serological tests,
- Serological agglutination tests rely on agglutination of bacteria and therefore are biased towards IgM responses (Kim *et al*, 1991),
- Serological methods should only be used to identify infected populations, rather than infected individuals, because of low sensitivity at the individual level,
- Animals with a positive serological response may not still be infected with *Salmonella* organisms,
- The need to be able to differentiate between a vaccine response and a natural infection response,
- The effect of antibiotic therapy on serological response is still unclear and
- More than 2000 different *Salmonella* serovars exist, and therefore serological cross-reactions between different serovars may occur.

ELISA : Rapid specific serological test?

Because bacteriological testing has been shown to be unreliable, and due to the relative insensitivity of conventional serological tests, there remains a need for a rapid, specific serological test. The ELISA has overcome many of the problems associated with bacteriological tests and conventional serological tests. Due to the high titre of IgG that persists for months after initial infection, ELISA may be able to detect the infection of an intermittent excretor of *Salmonella* and serum samples can also be easily collected. As already mentioned, the ELISA has been shown to be more sensitive and specific than agglutination tests, such as the RST Test and MT (Kim *et al.*, 1991), where good correlation has been shown between MAT and the ELISA (Cooper *et al.*, 1989; Nicholas and Cullen, 1991). However, while the ELISA has been shown to be more sensitive, it is not necessarily more specific than the MAT (Cooper *et al.*, 1989), and problems still exist. While the ELISA may be an appropriate method for diagnosing previous exposure to infection and for detecting *Salmonella* antigens, it cannot differentiate between active infection and previous infection. The presence of detectable antibodies does not imply active infection, only that the animal has been infected at some stage in the past. Another

problem that occurs in ELISAs are discrepancies in the interpretation of results. Caution needs to be taken when determining the cut-off optical density as it has a great effect on the number of positive or negative results. A number of different ELISA techniques are being used throughout the world, which could potentially cause problems when comparing results from different studies (Barrow *et al*, 1996).

Although the ELISA is more specific than other serological tests, there are still problems with cross-reactivity. The specificity of the assay has been one of the largest problems encountered in the development of a suitable ELISA test. More than 2000 different *Salmonella* serovars exist, hence it is not surprising that serological cross-reactions between serovars possessing the same somatic antigens, have been encountered. The antigens used in an ELISA are a crucial element in attempting to optimise the specificity of the assay.

Antigens used in ELISA

Development of an effective ELISA depends on the isolation of specific antigenic components from the organism of interest. In the past, a variety of antigenic components have been used in an ELISA to detect specific antibodies to various *Salmonella* serovars. These include:

- Lipopolysaccharides (LPS) (Hassan *et al.*, 1991a; Nicholas and Cullen, 1991),
- Whole flagella (Timoney *et al.*, 1990b),
- Recombinant flagella protein containing serotype specific flagellin fragment (Baay and Huis in't Veld, 1993),
- SEF14 fimbrial fragment (Hoorfar *et al.*, 1996; Thorns *et al.*, 1990),
- Outer membrane proteins (Hassan *et al.*, 1991b; Kim *et al.*, 1991),
- Disrupted whole bacterial cell proteins (Hassan *et al*, 1991a) and
- Flagella proteins (Barrow *et al*, 1991; Gast and Holt, 1998)

Overall, LPS have been the most commonly used antigens for ELISA's. This is the reason for the high number of cross-reactions between different *Salmonella* groups. This has particularly been seen between *Salmonella* groups B and D on a number of occasions (Barrow, 1992; Hassan *et al.*, 1991a; Nicholas and Cullen, 1991), where Chart *et al.* (1990) have found that both groups B and D share a common predominant O antigen epitope 12.

A variety of techniques have previously been explored in an attempt to overcome the problem of cross-reactions. For example:

a) Periodate treatment of group D LPS is believed to destroy the two cross-reacting epitopes O antigen 1 and O antigen 12 of group D, while leaving the specific epitopes untouched (House, 1993),
b) The use of different antigens from other specific groups. In 1993, (Baay and Huis in't Veld) used *Salmonella* group D2 antigens, which contain the specific O antigens 9 and 46 but not the cross-reacting O antigen 12, to try to capture *Salmonella* enteritidis (group D1) antibodies. This affinity method did not work because O antigen 9 was not recognised by *Salmonella* Enteritidis antibodies,
c) The use of a blocking ELISA. If a monoclonal antibody is used and the reaction is blocked with the reactive antigen, the specificity of the reactions can be measured. Hoorfar *et al.* (1996) used a monoclonal antibody specific for O9 LPS to confirm infection by *Salmonella* Dublin, a group D (O9) serovar. Confirmation was also backed up by bacteriological evidence,
d) Chemical modification of the antigen (Konrad, 1994),
e) Zamora *et al* (1999) increased the specificity of his ELISA by removing the common LPS factor O: 12 from the antigen preparation. The end result was an antigen mixture consisting of essentially fimbriae and flagella and outer membrane proteins (Zamora *et al*, 1999).
f) The use of more sensitive solid phase immunoassays, for example radioimmunoassays and chemiluminiscence. Compared to an ELISA, the chemiluminscent immunoassay has a wider measurement spectrum, takes less time and has improved test performance. Compared to the operating costs of a CLIA (Chemiluminisent Immuno-assay), the ELISA is less expensive.

While LPS is believed by many to play a dominating role in the immune response, others believe that the immune response is mainly directed against other membrane proteins. There are numerous reports of improved specificity and sensitivity for the detection of specific serotypes, when using outer membrane and flagella proteins as antigens in ELISA tests (Kim *et al.*, 1991; Nicholas, 1992; Nicholas and Cullen, 1991; Timoney *et al.*, 1990a).

Specific immune responses have been demonstrated using flagellar antigens for both *Salmonella* Enteritidis and *Salmonella* Typhimurium (Baay and Huis in't Veld, 1993).

It would appear that use of antigens derived from outer membrane proteins, flagella and fimbriae fractions may make it possible to avoid cross-reactions between different serovars. A better understanding and identification of the surface structures of *Salmonella* serotypes, would help the future development of diagnostic tests through the identification of new, more effective antigens for use in vaccines. Flagella antigens are more numerous within *Salmonella* groups and therefore potentially have a greater antigenic diversity compared to LPS. However, while a mixture of antigens may increase the sensitivity of the assay, it can also make them more broadly cross-reactive. Baay and Huis in't Veld (1993) suggested that flagella antigens might be a better candidate in an ELISA than LPS. This was due to the fact that flagella-specific IgG concentrations had been shown to peak earlier after infection compared to LPS-specific concentrations, and because flagella-specific IgG concentrations only persist for four months compared to the LPS-specific IgG concentrations which persist for several months (Baay and Huis in't Veld, 1993; Hassan *et al.*, 1991a). Using flagella antigens therefore may result in fewer false negatives and fewer false positives compared to using LPS antigens. Flagellar antigens have already been used on a number of occasions to differentiate between the flagella serovar *Salmonella* Enteritidis and the non-flagella serovar *Salmonella* Pullorum and *Salmonella* Gallinarumin in the poultry industry (Timoney *et al.*, 1990b).

ELISA Development and Application

There are a number of factors that need to be taken into consideration when developing and using ELISAs to detect particular antigens. The primary concern is to obtain a capturing antigen specific to the *Salmonella* serovar being investigated. Cross-reactivity is one of the biggest concerns, as it can cause numerous problems in sero-diagnosis and epidemiological studies, where it is important to identify a particular serovar. As the ELISA technique has improved, its possible applications have also increased. A whole range of test samples can now be checked for the presence of antibodies. These include sera, milk, egg yolk and meat juice. ELISAs can be used to investigate patterns of serological responses and monitor the effectiveness of control measures.

ELISA Development and Application in the Poultry Field

The two most common *Salmonella* serovars affecting the Poultry industry are *Salmonella* Enteritidis and *Salmonella* Typhimurium. These serovars are not easily detected by conventional agglutination tests, so a more specific and sensitive serological test is needed. A number of ELISAs have been developed and used successfully in the detection of *Salmonella* Enteritidis and *Salmonella* Typhimurium in the poultry industry. In fact, these tests have been so successful that their use has been suggested as a flock-monitoring tool.

In 1989, Cooper *et al*. investigated chicken flocks that had been naturally infected by *Salmonella* Enteritidis, in order to evaluate a number of serological tests for the detection of *Salmonella* Enteritidis. An indirect ELISA was used to measure O antibody response, as an indication that *Salmonella* had infected the host. It was shown that flock infection could be detected using this test, and that apart from the micro-antiglobulin (MAT) test, it was more sensitive than other conventional serological tests.

In 1991, Hassan *et al.* also developed and used an indirect ELISA for detection of *Salmonella* Typhimurium antibodies in chicken sera. High titres of IgG were detected through the use of whole cell, flagella and LPS antigens, with the highest titres arising from the use of whole cell antigens. It was suggested, however, that these high titres might have been due to various cross-reactions with other enterobacteriae or normal gut flora.

Chart *et al.* (1990) also investigated chickens that were naturally infected by *Salmonella* Enteritidis, using an indirect LPS ELISA. Positive responses were observed for 43 out of 58 sera. Further investigations showed that the antibodies were bound to the *Salmonella* Enteritidis antigen O12, a predominant LPS epitope of *Salmonella* Enteritidis. It was suggested that with bacteriological back up of positive results, the ELISA could be used as a rapid means of screening chicken sera for indication of *Salmonella* infection.

For the poultry industry, an ideal ELISA would contain a capturing antigen that is specific for *Salmonella* Enteritidis and *Salmonella* Typhimurium, but is not LPS based as these are known to cross-react.

In 1990, Timoney *et al.* used flagella in an indirect ELISA to investigate detection of antibodies in sera from chickens naturally or experimentally infected with *Salmonella* Enteritidis.

Nicholas and Cullen (1991) also developed an indirect ELISA for detection of *Salmonella* Enteritidis antibodies in chickens, and compared LPS and heat extracted antigens as capturing agents. The heat extracted ELISA detected antibody in nearly all birds, while the LPS ELISA detected antibody in only 60% of the birds. This suggested the use of heat extracted proteins may be more efficient.

In 1991, Kim *et al.* used an antigen from the outer membrane specific to *Salmonella* Enteritidis, to develop an ELISA for detection of *Salmonella* Enteritidis antibodies in chickens. This ELISA was specific and sensitive in detection of *Salmonella* Enteritidis.

Baay and Huis in't Veld (1993) used both LPS and Flagella antigens for detection of *Salmonella* Enteritidis in chickens. High antibody titres were observed in early stages of infection using flagella antigens. However, after the initial infection, flagella antibody levels tended to fall, whereas LPS antibody titres persisted.

An alternative to screening of chicken flock serum is to sample the egg yolk, which can be a good source of antibody. However, the antibody concentration in an egg is often too low to create a response in a conventional serological test, which are usually ineffective in detecting IgG concentrations (the predominant antibody class in egg yolk). Because of the greater sensitivity and specificity of the ELISAs, which are capable of detecting IgG, indirect ELISA is capable of detecting antibodies in yolk.

In 1990, Dadrast *et al*. developed an indirect LPS ELISA for the purpose of identifying *Salmonella* infected flocks, using antibodies from egg yolks from infected birds. Egg yolk usually contains antibodies from the bird, which can allow the identification of infected birds. Higher levels of antibody to both *Salmonella* Typhimurium and *Salmonella* Enteritidis were found in eggs compared to those found in serum.

Nicholas and Andrews (1991) also investigated an ELISA, for detection of antibodies to *Salmonella* Enteritidis and *Salmonella* Typhimurium in eggs from naturally and experimentally infected chickens. This work extended that of Dadrast, who had also detected *Salmonella* antibodies in eggs under experimental conditions.

Another ELISA based on a different antigen was also developed to detect antibodies to *Salmonella* Enteritidis and *Salmonella* Typhimurium in the yolk of hen eggs. Four flocks of chickens that had previously been exposed to *Salmonella* Enteritidis were investigated. Large proportions of eggs from the infected flocks were found to contain antibodies to *Salmonella* Entertidis (Thorns *et al.*, 1996).

ELISA Development and Application in the Bovine Field

A number of ELISAs have been developed and applied to monitor infections of *Salmonella* Typhimurium and *Salmonella* Dublin in cows. These are the two most common *Salmonella* serotypes that currently affect the cattle industry around the world though *Salmonella* Dublin is not a common isolate in the NZ cattle industry. Serological data may be useful for identification of carrier animals, and removal of these could have a major impact on reducing foodborne infections in man and reducing the impact of disease.

Smith *et al.* (1989) evaluated the use of an indirect LPS ELISA for detecting antibodies to *Salmonella* Dublin in serum and milk samples. The ELISA was used to identify *Salmonella* Dublin in the mammary gland of carrier animals, and to monitor the shedding pattern of the organism from a group of infected cattle. The assay showed promise in its ability to differentiate uninfected, recently infected, recovered, and carrier cows. Therefore, specific serum IgG concentrations could be useful as an indicator of carrier animal status.

Spier *et al.* (1990) also evaluated the use of an indirect ELISA to identify *Salmonella* Dublin carriers, by determining immunoglobulin reactions to *Salmonella* Dublin LPS in either milk or serum. Again, serum IgG specific for *Salmonella* Dublin was the most indicative parameter of carrier status. It was concluded that ELISA based detection of immunoglobulins might prove to be useful in a screening test for *Salmonella* Dublin carriers.

An indirect ELISA was used by House (1993) to detect *Salmonella* antibodies in a milking herd. They also concluded that specific serum IgG could be used for identification of *Salmonella* Dublin carrier animal.

In 1996, Hoorfar *et al.* investigated herds with clinical outbreaks of *Salmonella* Dublin and *Salmonella* Typhimurium, using an O antigen ELISA. As previously mentioned, there have been promising results and it has been suggested that serological data may provide information about carrier animals. Hoorfar's results however, did not support this. The study involved sequential testing of blood samples after the outbreak, followed by post mortem to determine true infection status. They found that, although serology based on O antigens is useful for the identification of a herd infected with *Salmonella*, it is not sufficient for identification of individual carrier animals.

In Denmark, indirect LPS-based ELISAs combined with blocking assays for the confirmation of specific LPS, have been used in the serological detection of *Salmonella Dublin* in cattle herds. Hoorfar and Bitsch (1995) reported promising results using this method, with positive optical densities always being found in endemic areas and negative optical densities in *Salmonella* free areas. Hoorfar and Wedderkopp (1995) have also achieved interesting results in the choice of specimen area. Informative results have been provided by using both milk and serum samples, with significant correlation between serum and meat juice samples. Overall, the indirect LPS-based ELISA can be considered very useful for herd testing.

ELISA Development and Application in the Swine field

Due to an increase of human salmonellosis cases over the years, common swine *Salmonella* serovars such as Typhimurium and Infantis, have provided problems throughout the pig industry. In Denmark, an increase of outbreaks of clinical salmonellosis in pigs between 1987–1992, saw the development and utilisation of an indirect ELISA in the pig industry. ELISA is utilised in a nation wide control program, which eventually will aid in controlling Salmonellosis in pigs (Mousing *et al.*, 1997).

The ELISA used is a standard indirect ELISA which contains several O antigens. Serum samples are used to monitor breeder and multiplier herds, and meat juice samples to monitor slaughter pigs. Slaughter herds are broken into three groups, depending on results of the ELISA. The first group is considered to have a low incidence of infection, the second group an increased incidence of infection, and the third group an unacceptably high incidence of infection. The control program is designed to place restrictions on the second and third group, in an attempt to reduce the extent of the problem.

Potential application of a specific *Salmonella* Brandenburg ELISA in sheep

ELISAs have been used, and are being used in detection of antibodies for *Salmonella* serovars in a variety of farm animals. ELISA has been used extensively in the Poultry industry, for detection of *Salmonella* Enteritidis antibodies (Zamora *et al*, 1999), and in the Swine industry for the detection of *Salmonella* Infantis antibodies (Christensen *et al*, 1999). Experiments have also been performed on cattle for the detection of *Salmonella* Typhimurium and *Salmonella* Dublin antibodies (Hoorfar and Bitsch, 1995; Hoorfar *et al*, 1997). However, there appears to have been little use of ELISA in sheep. *Salmonella* Brandenburg is responsible for an emerging disease epidemic in the southern regions of the South Island and little is known of the specific epidemiology of this *Salmonella* serotype. Currently, there is no control program aimed at reducing the chance of infection or development of the disease. The development of a suitable ELISA could provide a sero-epidemiology approach, to assist in the following:

- Determination of when animals have been exposed to and infected with the organism.
- The proportion of a flock that has been infected or exposed.
- Information on recovery and carrier status.

Information such as this could be used to monitor control and prevention methods that may reduce the impact *Salmonella* Brandenburg is having on the New Zealand sheep industry.

In many countries where *Salmonella* has been a problem, different methods have been developed to monitor this disease. Bacteriological testing of animal populations has been applied in an attempt to monitor and control *Salmonella* infection. ELISA is now recognised as a useful monitoring method that can be utilised as a management tool in slaughter plants. Such a test could be used to monitor the level of exposure/infection of *Salmonella* Brandenburg within the New Zealand sheep industry.

Study Aims

To identify factors associated with the occurrence and severity of Salmonella Brandenburg outbreaks.

The research project was designed to focus on the identification of environmental, management and animal risk factors associated with *Salmonella* Brandenburg in sheep. A case control study was developed based on a retrospective survey sent to affected and unaffected farms within Otago and Southland to compare associations between possible risk factors and *Salmonella* Brandenburg.

Development of serological test for Salmonella Brandenburg.

The research project undertaken focussed on the development of an indirect ELISA for use in epidemiological studies and/or monitoring of *Salmonella* Brandenburg exposure in New Zealand sheep. The indirect ELISA involves the use of a capturing antigen, which is coated onto the wells of a microtitre plate. Firstly, blocking reagent is added to the wells to reduce non-specific binding, which is followed by addition of serum. If there are any specific antibodies in the sample then they bind to the capturing antigen, detected by a host-specific antibody conjugate. These ELISAs are extensively used in the detection of specific antibodies from serum samples.

The specificity and efficiency of a *Salmonella* Brandenburg ELISA depends on the isolation and specificity of its antigenic components. *Salmonella* Brandenburg belongs to serogroup B, and is known to have somatic O antigens 1, 4 and 12, and specific H antigens (bacterial flagellae) e, n, l and v. Unfortunately, *Salmonella* Typhimurium, which is also a common *Salmonella* serotype in New Zealand sheep, also belongs to group B. *Salmonella* Typhimurium is known to have somatic O antigen 1, 4, 5 and 12. There were reservations in using a LPS-based ELISA as a serological test for *Salmonella* Brandenburg exposure. This is because the various O antigens that make up the polysaccharide portion of a LPS, means that there is a possibility of cross-reaction between *Salmonella* Brandenburg and *Salmonella* Typhimurium antibodies. Because of this, an approach was taken based on results published by (Zamora B.M. *et al*, 1999). This paper focused on the preparation of a specific *Salmonella* Enteritidis antigen for serological detection of *Salmonella* Enteritidis infections in chicken flocks. The method used includes a purification step (filtration and concentration) which excludes cross-reacting LPS antigens from the antigen preparation.

Thus, the final product of the antigen preparation is composed of mainly fimbria and H (flagella) antigens. The author then performed an indirect ELISA to determine the specificity of the test. Cross reactions between *Salmonella* Enteritidis (group D) and *Salmonella* Typhimurium (group B) which share common O antigens, were not seen in this antigen preparation. Therefore, the specificity and efficiency of an indirect ELISA for both control studies, and/or monitoring depends on the successful isolation of a specific *Salmonella* Brandenburg antigen.

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CHAPTER TWO

THE DEVELOPMENT OF AN INDIRECT ELISA

MATERIALS AND METHODS

Antigen preparation

A stock culture of *Salmonella* Brandenburg held frozen at Massey University was thawed and the culture was streaked onto a blood agar plate using a sterile loop. The plate was incubated at 37ºC for 12 to 24 hours. Six colonies of *Salmonella* Brandenburg were used to inoculate 1L of nutrient broth, which was then shaken overnight at 37ºC. The cells were pelleted by centrifugation (1500g for 30 minutes) and the supernatant removed by decanting. The pellet was re-suspended in 4mL of PBS (Phosphate Buffered Saline pH 6.8) and heated at 60ºC for 30 minutes to kill the bacteria. The resuspended cells were centrifuged at 1100g for another 20 minutes, and the supernatant removed and stored.

Removal of LPS (Lipo-polysaccharide) from supernatant

1mL of air was drawn into a 10mL syringe followed by the supernatant. A 0.2µm 32mm Acrodisc® Supor ® Membrane (PALL Gelman Laboratory) syringe filter was attached to the 10mL syringe and the supernatant was pushed through the filter by applying gentle thumb pressure. The filtrate was then filtered through a 0.2µm Acrodisc® Posidyne® Membrane Positively charged Nylon 6,6 (PALL Gelman Laboratory) syringe filter to remove the LPS. The filtered supernatant was tested for LPS presence using a polyvalent O agglutination test. 10µL of polyvalent O antisera was added to 20µL of supernatant and observed for agglutination.

Concentration and de-salting of the supernatant

The supernatant was concentrated 1250 times in a 10mL stir cell ultrafiltration cell (Amicon) using a 25mm YM-3 ultra-filtration membrane (Diaflo®) followed by a YM-3 Centricon (Millipore). The supernatant was then de-salted by dialysis (500 MW cut of dialysis tubing, (Spectra/Por®) against 1mM BTP (Bis-triphosphate). The concentrated and de-salted supernatant will now be referred to as crude antigen preparation.

Sera

Several sources of serum were collected for development and testing the ELISA.

Serum samples from slaughter plants

From the 5th of February to the 2nd of March 2001 blood was collected at the Mataura Alliance Freezing Works from 8 Southland farms. Four of the farms had sheep exhibiting clinical *Salmonella* Brandenburg disease within the previous 12 months (case farms), the other 4 had not (control farms).

Blood samples were taken from sheep and lambs sourced from case (n=30 per farm) and control (n=20 per farm) farms at the time of slaughter. A total of 400 samples were collected. Samples were allowed to clot overnight at 4^0C, then centrifuged at 2500g and the serum was stored in labelled tubes at -70^0C.

Serum samples from a previous Salmonella vaccine trial

In 1999 Schering Plough® undertook a clinical trial investigating efficacy of a *Salmonella* vaccine product, Salvexin B+. In the trial, vaccinated and non-vaccinated pregnant ewes were exposed to *Salmonella* Brandenburg. A total of 45 pregnant ewes were included in the trial. The vaccine Salvexin®, which contained *Salmonella* Typhimurium and Hindmarsh antigens, was administered to 15 animals. Another 15 received Salvexin B+®, a new *Salmonella* vaccine that contained *Salmonella* Typhimurium, Hindmarsh, and Brandenburg antigens. The remaining 15 ewes were negative controls and received no vaccine. All sheep were experimentally challenged with *Salmonella* Brandenburg. Blood samples were taken prior to vaccination, after vaccination, and every day for one week following the challenge. All blood samples were prepared as before and the serum frozen at -70ºC. These serum samples were available for use in the current project.

Serum samples from experimentally infected animals

Rabbits were experimentally infected with *Salmonella* Typhimurium, *Salmonella* Hindmarsh, or *Salmonella* Brandenburg to produce specific *Salmonella* antibodies. Approval was obtained from the Massey University Animal Ethics Committee for this experiment.

Serum samples from the previous *Salmonella* vaccine trial were used in the development of the ELISA. Anti-sera from experimentally infected rabbits was used to check the specificity of the ELISA and serum samples collected from slaughter plants were used for validation of the ELISA.

Preparation of antigen for immunising rabbits

Salmonella Brandenbrug, *Salmonella* Typhimurium and *Salmonella* Hindmarsh stock cultures were streaked onto blood agar plates and incubated at 37ºC overnight. Three single colonies from each plate were suspended separately into 3mL of PBS and heat-killed at 60ºC for 1hour. 100µL of each suspension was then streaked onto a second blood agar plate and incubated at 37ºC overnight to ensure that the sample contained no viable cells. The suspension was adjusted to give turbidity equal to McFarland Standard 3 with PBS (phosphate buffered saline) and stored at 4 ºC.

Purchase and storage of rabbits

Eight rabbits were purchased from and housed at the Small Animal Production Unit (SAPU) at Massey University from the 23rd of July to the 5th of November 2001 (Appendix I). Of the eight rabbits, 2 were inoculated with *Salmonella* Brandenburg, 2 were inoculated with *Salmonella* Hindmarsh, 2 were inoculated with *Salmonella* Typhimurium and 2 were negative controls and thus not inoculated. Prior to being inoculated, up to 5mL of blood were collected from the marginal ear vein of each of the rabbits (Appendix II).

Inoculation schedule for rabbits

1mL of suspension and 1mL of Freund's Incomplete Adjuvant were mixed to form a water-in-oil emulsion. 1mL of the emulsion was inoculated subcutaneously at multiple sites on the neck of each rabbit. After 10-14 days a second injection of the emulsion was administered. Ten to fourteen days later a maximum of 5mL of blood was collected from the marginal vein of the ear of each rabbit. If the rabbit serum had a sufficient antibody titre, the rabbit was anaesthesised and exsanguinated. If not, the rabbit was left for another 10-14 days and the process repeated.

Serum samples from 2001 outbreak

Blood samples and cultures were collected from ewes on Southland farms during

August/September 2001. The Central Southland Veterinary Clinic was contacted and asked to provide blood samples from ewes that had aborted (vaccinated and unvaccinated) and from vaccinated and unvaccinated ewes that had not aborted. Vaginal swabs were taken from each ewe that had aborted and cultures were grown to confirm presence of *Salmonella* Brandenburg. Three to four weeks later blood samples from the same ewes were collected to provide follow up samples for monitoring the change in antibody titre. Blood samples from non-diseased ewes (vaccinated and unvaccinated) provided negative controls for this part of the study.

Protein Concentration

Determination of the protein concentration of the antigen preparation was carried out using the Bradford Protein Assay.

For equipment and materials of this method, and all following methods, please refer to Appendix III.

Methodology

100µL of four-protein standards and crude antigen preparation were mixed with 1mL of Bradford reagent and incubated for 20-30 minutes at room temperature. Optical density (OD) was measured at 590nm and translated into protein concentration.

Electrophoresis

Electrophoresis methodology

Proteins in the crude antigen preparation were separated by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. Resolving gel was poured into the glass plate sandwich and left at room temperature for 1 hour. The stacking gel was placed on top and an 8 well 0.75mm comb was used for well formation. The gel was then placed in an electrophoresis chamber and 300mL of fresh electrode buffer was prepared and added to the upper buffer chamber to cover the gel. The remainder of the buffer was poured into the lower buffer chamber. 10-20 µL samples of crude antigen preparation were mixed with sample buffer and heated to 100ºC for 2 minutes before being loaded into the gel. Molecular weight marker was prepared and as directed by the maker (Biorad®). Electrophoresis was carried out at 200V for 45min, after which time, the gel was removed and stained with Comassie

Blue G-250. After 20 minutes the gel was then placed in de-staining solution and left for 1 hour on an orbital shaker.

Western Blot

Western Blot methodology

Following electrophoresis the gel was rinsed and equilibrated in transfer buffer for 10 minutes to remove salts and detergents.

Electro-transfer

0.2µm nitro-cellulose membrane and 4 filter papers were cut to the dimension of the gel. The membrane, filter papers and fibre pads were soaked in transfer buffer for 20-30 minutes until completely saturated with buffer. The buffer tank was half filled with transfer buffer and a frozen bio-ice cooling unit placed next to the electrode. The gel holder cassette was assembled by placing a pre-soaked fibre pad on the cathode side, followed by 2 pre-soaked filter papers, the gel, the nitro-cellulose membrane, 2 pre-soaked filter papers, and lastly a second pre-soaked fibre pad. All components were centred and air bubbles removed. The gel holder cassette was placed in the buffer tank with the cathode end facing the black cathode panel. The buffer tank was filled with transfer buffer and a constant voltage of 90V was applied for 45min.

Blocking

Following transfer the membrane was “blocked” by placing it in blocking solution for 2 hours at room temperature while gently shaking.

Immuno-detection

The membrane was washed 3 times in washing buffer (10 minutes each wash) before being incubated in a 1:50 dilution of serum for 1 hour with shaking at room temperature. It was then washed three times with washing buffer as before. Following this, the membrane was incubated with a 1:10,000 dilution of anti-sheep antibody for one hour at room temperature. It was then washed again as before, before being incubated with 4-chloro-napthol for 20min. The development of a blue/black colour indicated the presence of an antigen.

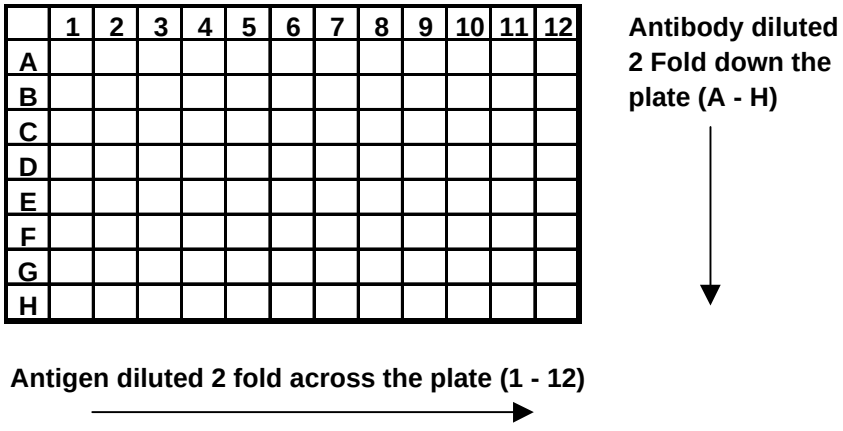
The western blot procedure was optimised using ovalbumin an anti-pdg serum as a positive control.

ELISA Development

ELISA Methodology

100µL of crude antigen preparation was administered to each well of column one and diluted two-fold across all columns (Figure 5). The plate was covered and incubated overnight at 4ºC. Excess antigen was removed by adding 250µL of washing buffer to all wells and allowing them to stand for 3 minutes, after which time, the plate was inverted and shaken. Excess liquid was removed by banging the plate three times on the bench over a paper towel. 250µL of blocking buffer was then added to all wells and incubated for 1 hour at room temperature. The plate was washed again as described before. 100µL of diluted primary antibody was added to row A and diluted 2-fold down all columns (Figure 5). The plate was incubated for one hour at room temperature. The plate was again washed. 50µL of diluted secondary antibody was added to all wells and incubated for 1 hour at room temperature followed by a final wash. 50µL of substrate was added to all wells and left for 20 minutes. After 20 minutes, 50µL of stopping solution was added to the wells and the optical density of the solution in the wells was measured at 450nm.

Figure 5. Diagram of the 96-well ELISA plate.



ELISA Development Problems

A series of modifications to the above ELISA protocol were made in an attempt to increase

the amount of specific binding between the crude antigen preparation and *Salmonella* Brandenburg antibodies. Each of the following experiments examined the effect of altering one component of the procedure. All experiments included a control consisting of the original protocol in order to compare the effects of any modification.

Step 1

Negative control wells within each plate were returning high background optical densities. A blocking step was added after the washing of the plate. After washing, 250µL of blocking buffer was added to all wells and left for one hour.

Step 2

In response to repeated low optical density readings, the following modifications were tried.

a) 100µL of serum from *Salmonella* Brandenburg affected ewes were added to all wells and incubated overnight at 4ºC. The plate was then washed and incubated with secondary antibody to ensure that the colour change reaction was occurring.
b) The Tween-20 detergent in the washing buffer was replaced with Thesit, to determine the effect of a different detergent.
c) Washing buffer containing no detergent was used.
d) The plate was washed once instead of three times in each washing step.
e) Two different blocking buffer concentrations of gelatine were used (0.25% and 0.5%)
f) Skim milk was used as a blocking agent rather than gelatine.
g) Higher concentrations of antigen were used.
h) A crude antigen preparation of ovalbumin and a known ovalbumin specific anti-serum (pdg anti-sera), were prepared to generate a positive control for the indirect ELISA.
i) A second antigen preparation was prepared in order to generate a more concentrated antigen.

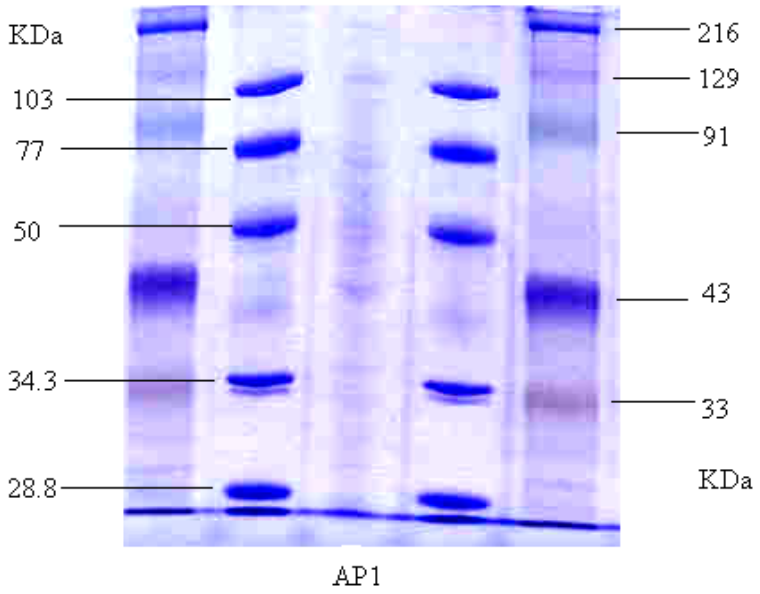
CHAPTER THREE

RESULTS

Antigen Preparation One

Results of agglutination tests indicated that there were little or no LPS in the antigen preparation. The protein concentration of antigen preparation one was determined to be 0.25µg/mL. Gel electrophoresis of concentrated samples confirmed the presence of multiple protein bands between 28.8 and 216 KDa (Figure 6).

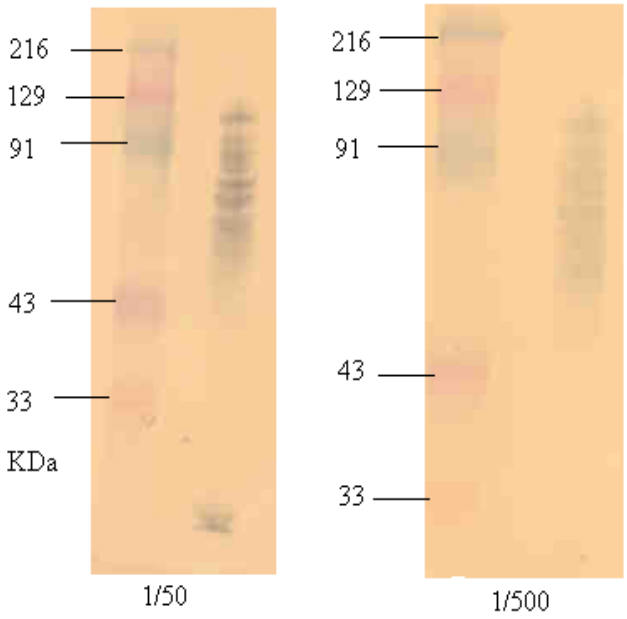
Figure 6. Gel Electrophoresis of the protein components of Antigen Preparation One (AP1).



Western Blot of antigen

Membranes were incubated with either a 1/50 or 1/500 dilution of serum from a previous *Salmonella* vaccine trial. The serum contained *Salmonella* Brandenburg, *Salmonella* Typhimurium and *Salmonella* Hindmarsh antibodies. Protein bands between 43 and 91 KDa, identified in electrophoresis, showed binding to *Salmonella* antibodies (Figure 7).

Figure 7. Western Blot of antigen preparation one incubated in 1/50 and 1/500 dilution of serum from a previous vaccine trial.



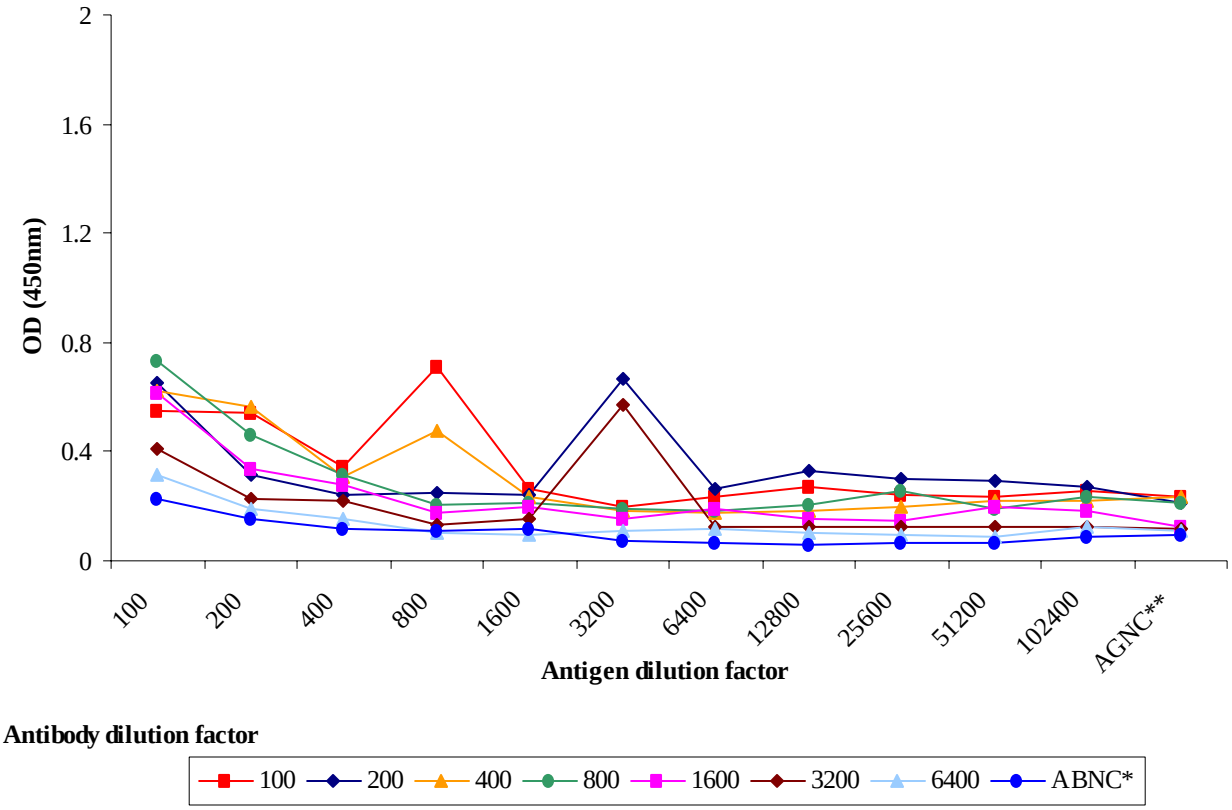
Specificity of antigen

One membrane was incubated in a 1/50 dilution of serum containing *Salmonella* Brandenburg, *Salmonella* Typhimurium and *Salmonella* Hindamarsh antibodies. Ten protein bands between 28.8 and 129 KDa indicated binding of *Salmonella* antibodies. No protein bands were seen on membranes incubated in serum containing *Salmonella* Typhimurium and Hindmarsh antibodies alone or in serum containing no *Salmonella* antibodies (Appendix IVa).

Indirect ELISA of Antigen Preparation One

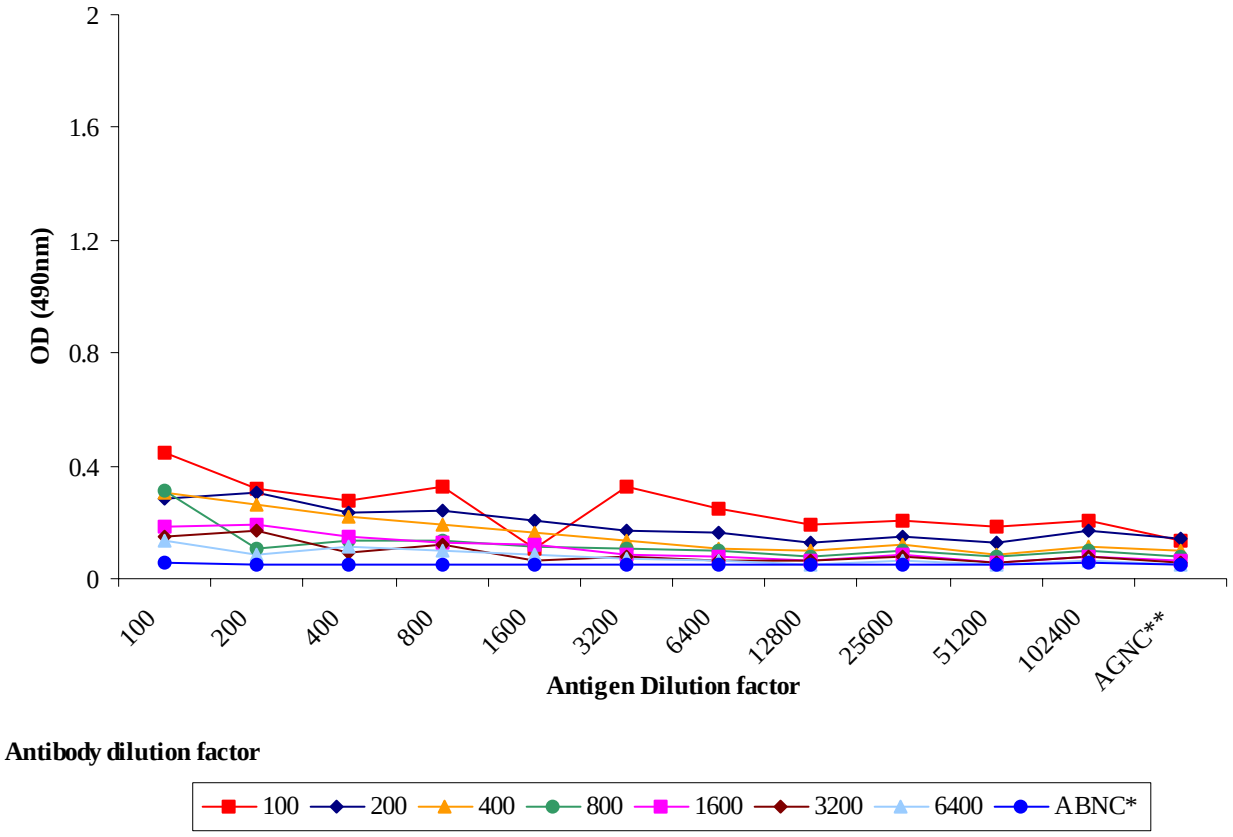
Optical densities at varying antigen and antibody concentrations are shown in figures 8-16 and 18. The first figure of each pair (e.g. Figure 8a) represents a base comparison and the second of each pair (e.g. Figure 8b) shows the effect of one modification of the ELISA methodology.

Figure 8a. Optical Density values of serial dilutions of antigen preparation one and anti-sera. Antigen preparation concentration is represented by a serial two-fold dilution of a stock solution of 0.25µgmL. Antibody concentrations are unknown but each line represents a different dilution of anti-sera.



*ABNC = Antibody Negative Control **AGNC = Antigen Negative Control

Figure 8b. Optical Density values of serial dilutions of antigen preparation one and anti-sera, with a blocking step added to the original ELISA methodology (ELISA methodology one).



*ABNC = Antibody Negative Control **AGNC = Antigen Negative Control

Figure 9. Optical Density values of serial dilutions of anti-sera (direct ELISA), (ELISA methodology 2a). Antibody concentrations are represented by a serial two-fold dilution of unknown concentration. The red line represents anti-sera from a previous vaccine trial and the blue line represents anti-sera from a *Salmonella* Brandenburg affected ewe. Both sera were used in the development of the indirect ELISA.

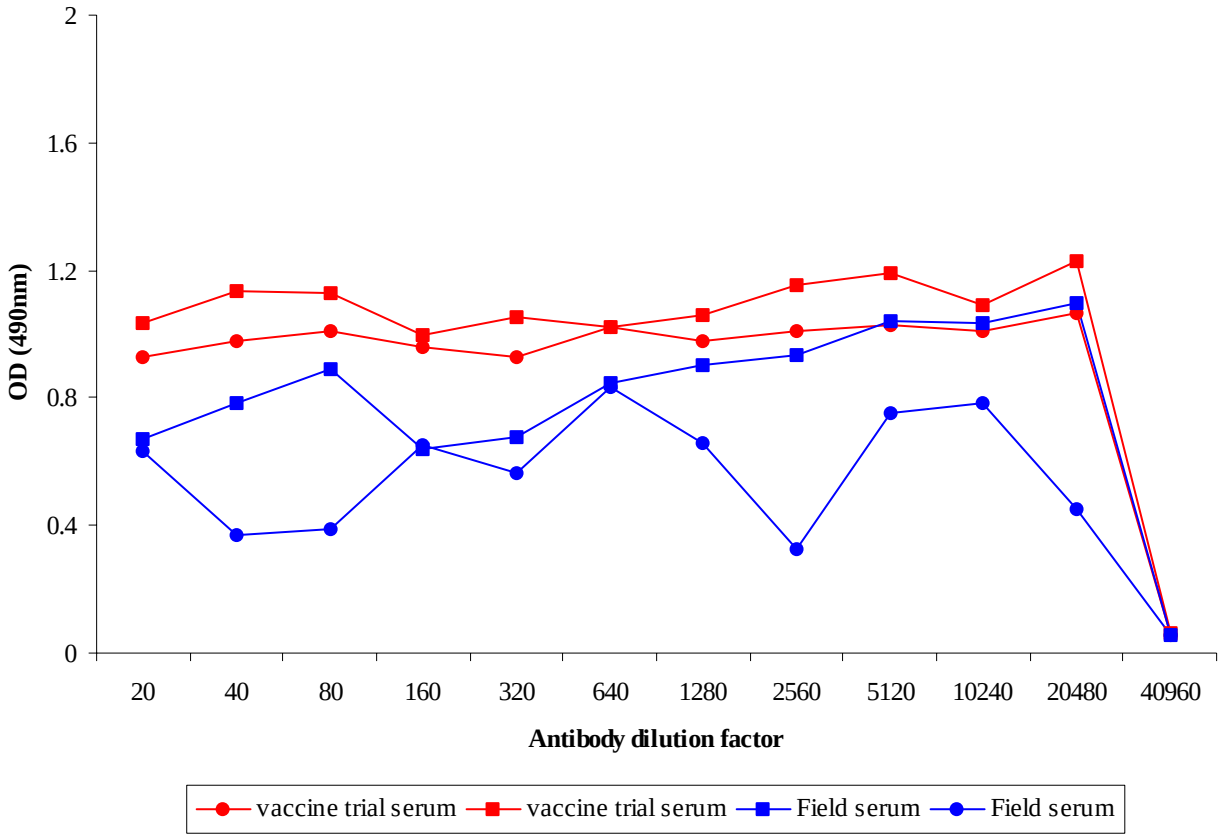
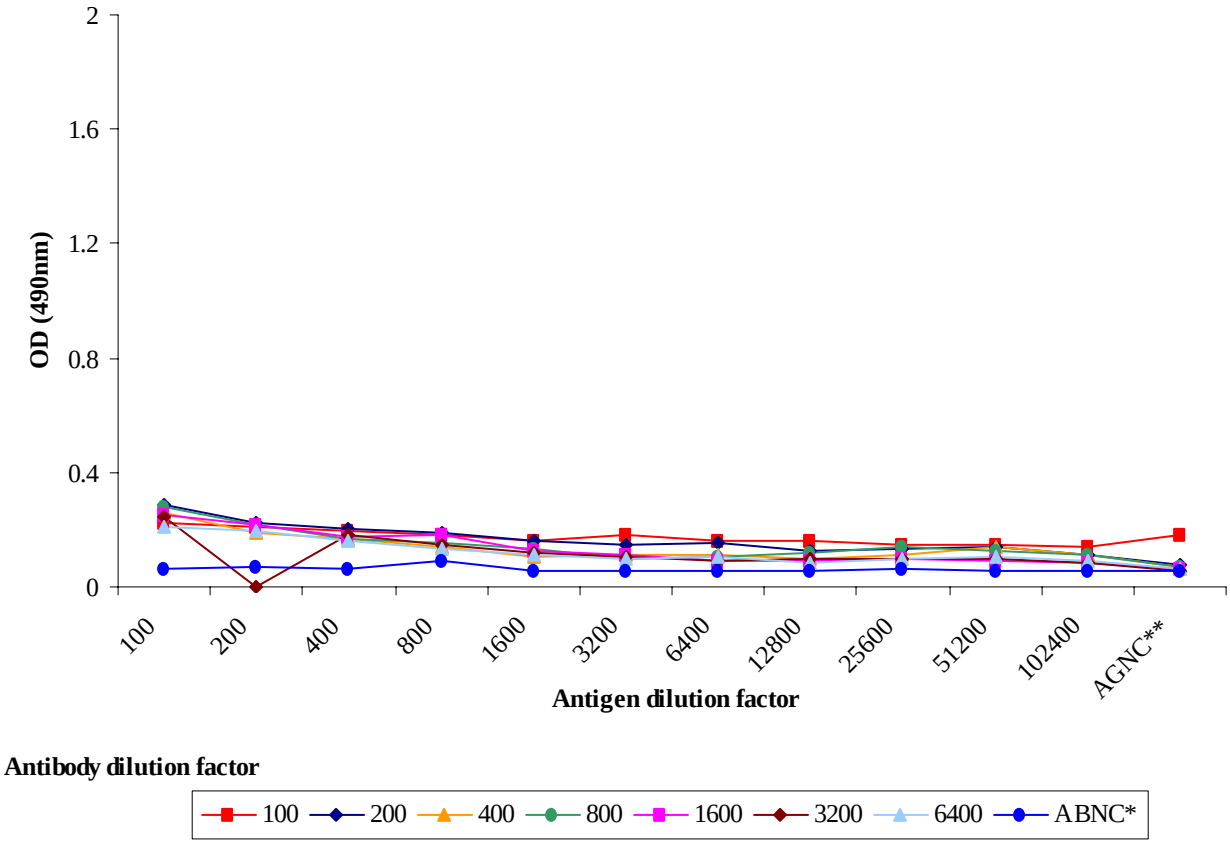
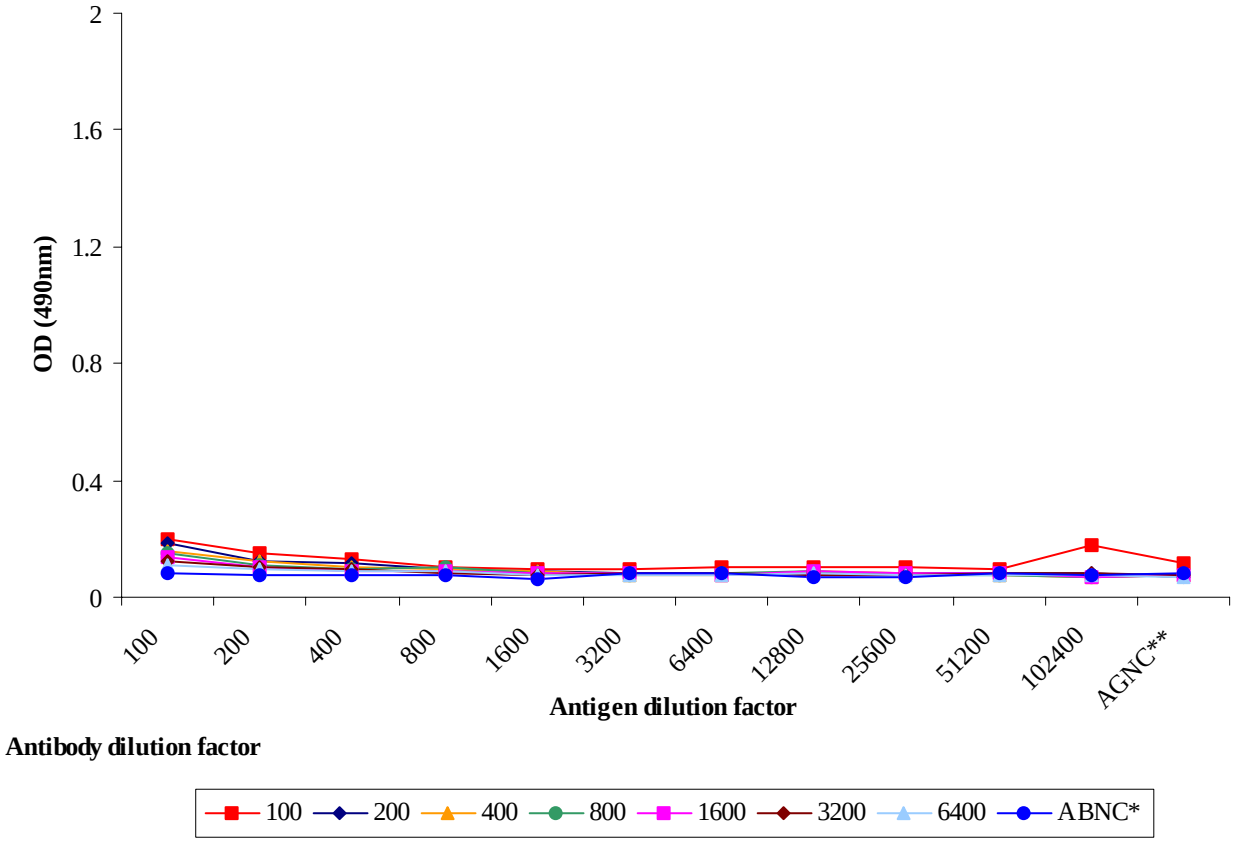


Figure 10a. Optical density values of serial dilutions of antigen preparation one and anti-sera (ELISA methodology one). Antigen concentration is represented by the serial two-fold dilution of a stock solution of 0.25µgmL. Antibody concentrations are unknown but each line represents a different dilution of anti-sera.



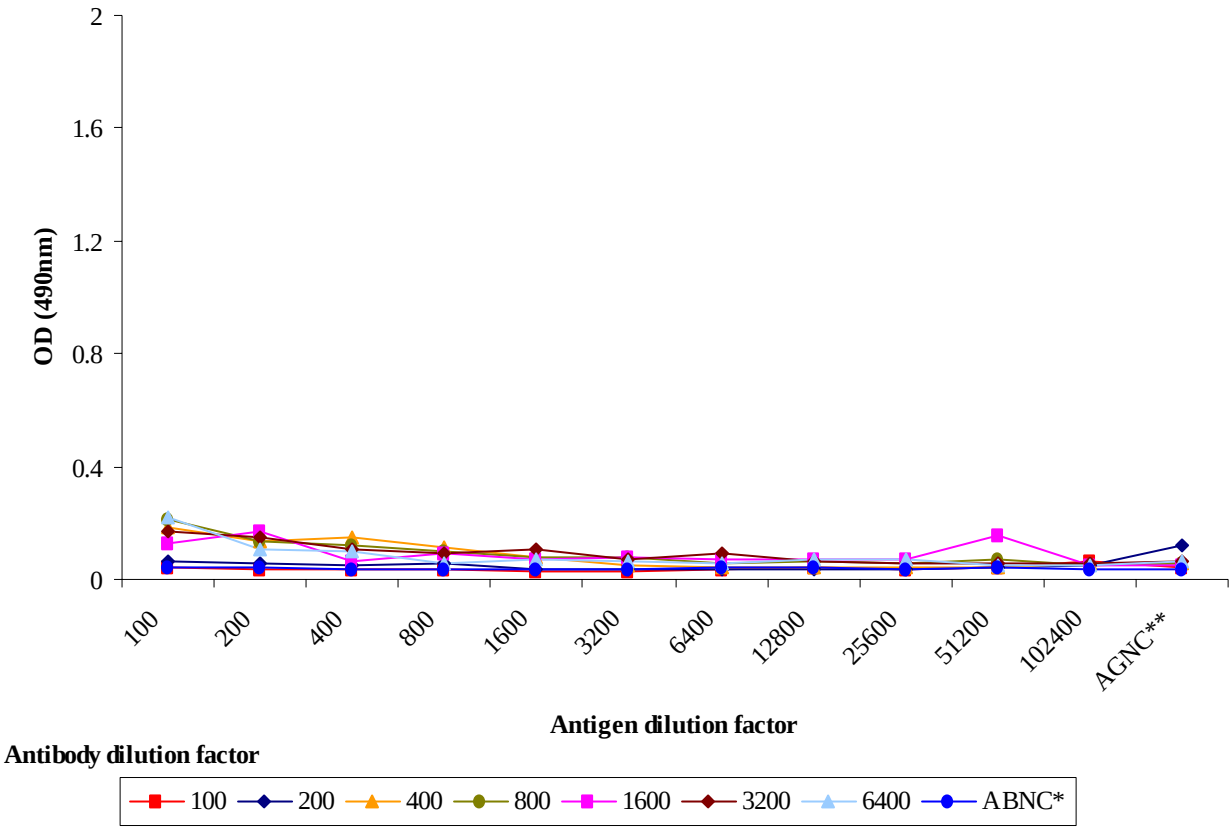
*ABNC = Antibody Negative Control **AGNC = Antigen Negative Control

Figure 10b. Optical density values of serial dilutions of antigen preparation one and anti-sera, using a different detergent (Thesit) in the washing buffer of ELISA methodology one (ELISA methodology 2b.)



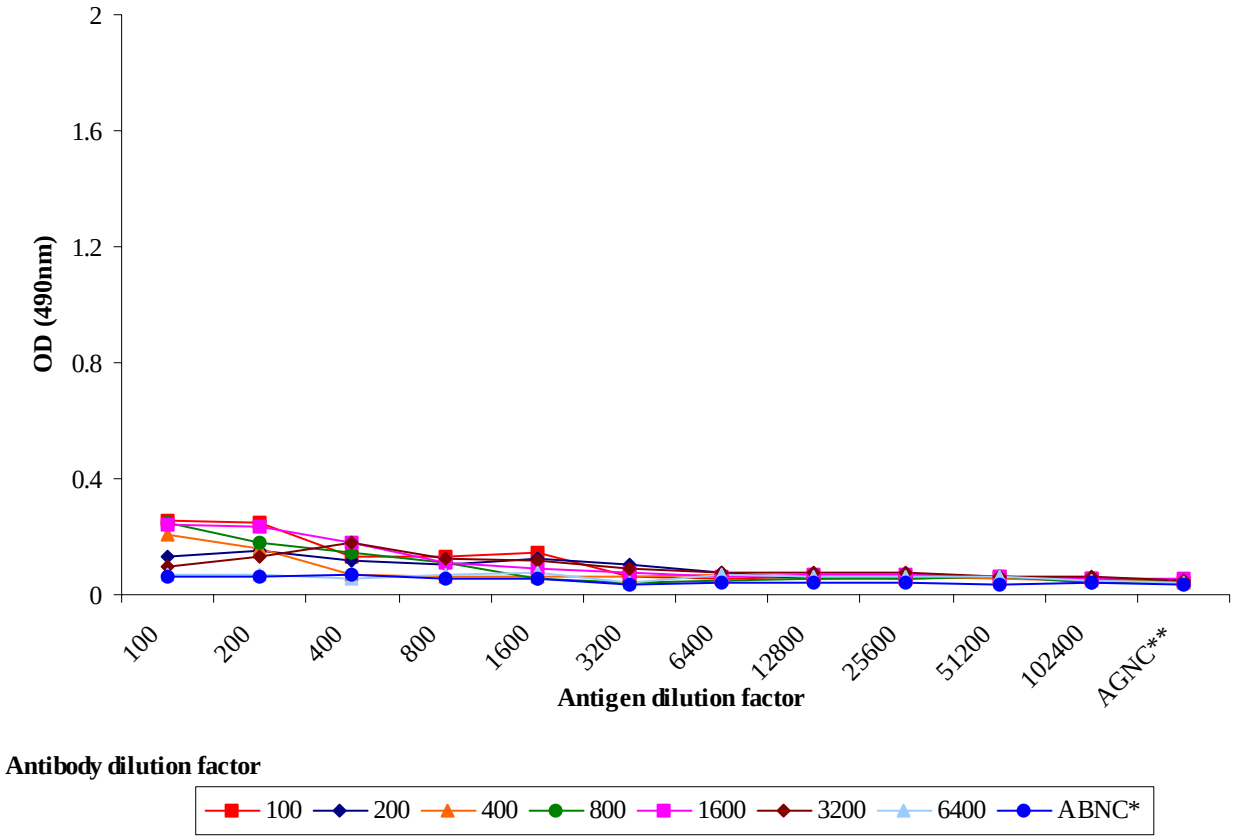
*ABNC = Antibody Negative Control **AGNC = Antigen Negative Control

Figure 11a. Optical density values of serial dilutions of antigen preparation one and anti-sera (ELISA methodology one). Antigen concentration is represented by the serial two-fold dilution of a stock solution of 0.25µgmL. Antibody concentrations are unknown but each line represents a different dilution of anti-sera.



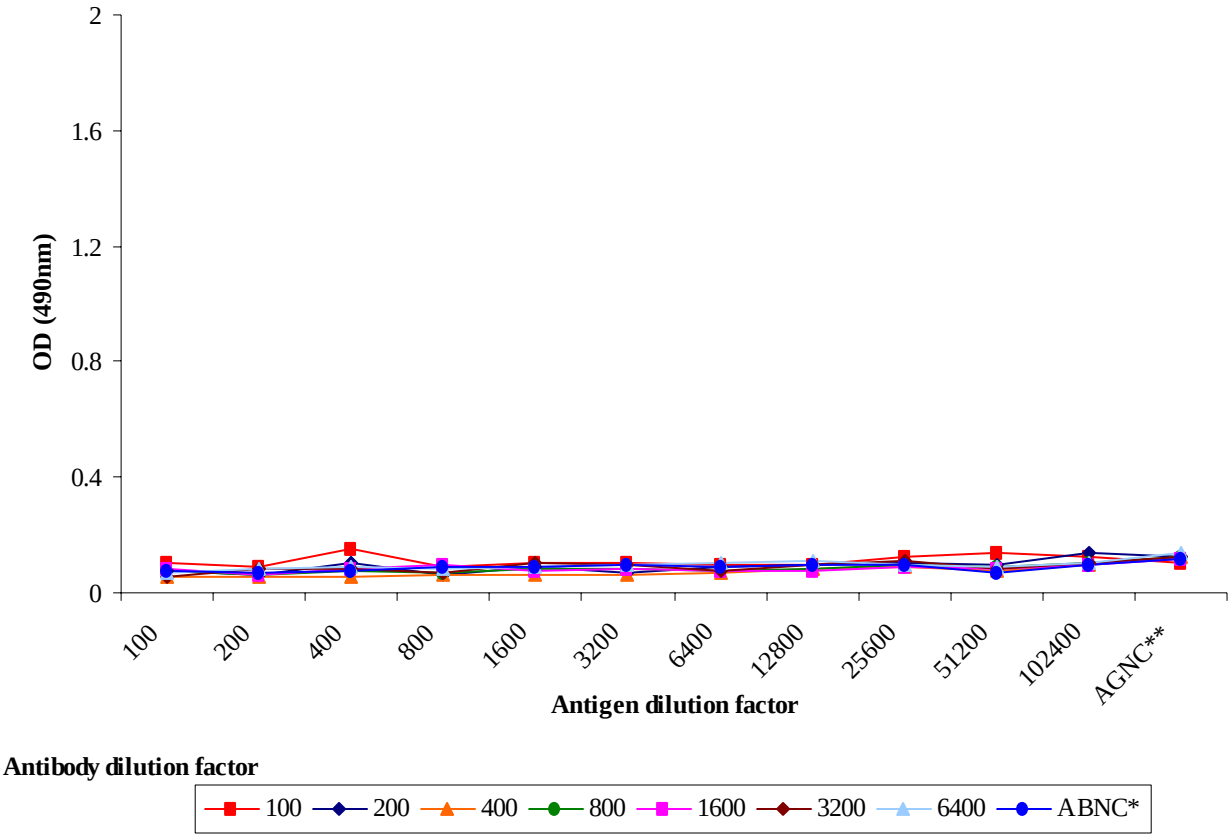
* ABNC = Antibody Negative Control **AGNC = Antigen Negative Control

Figure 11b. Optical density values of serial dilutions of antigen preparation one and anti-sera, using no detergent in the washing buffer of ELISA methodology one. ELISA methodology 2c.)



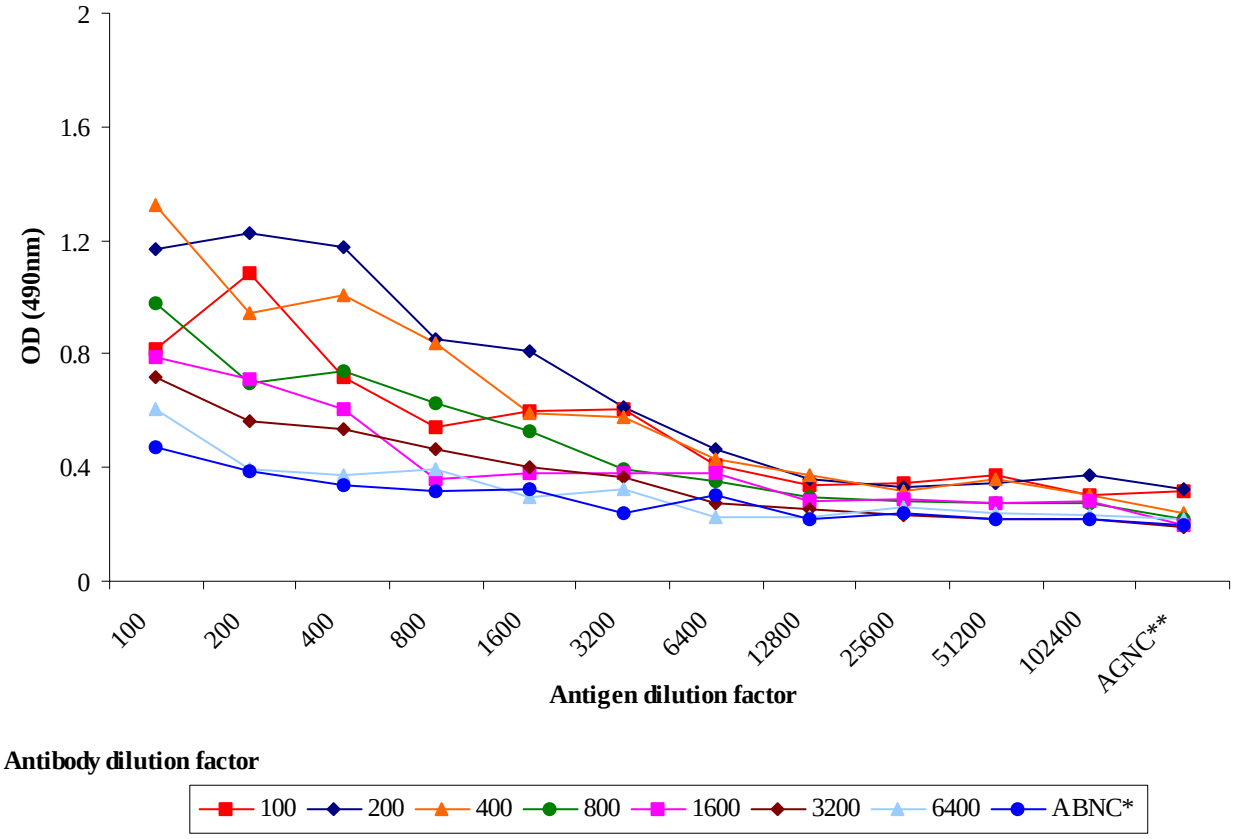
* ABNC = Antibody Negative Control **AGNC = Antigen Negative Control

Figure 12a. Optical density values of serial dilutions of antigen preparation one and anti-sera (ELISA methodology one). Antigen concentration is represented by the serial two-fold dilution of a stock solution of 0.25µgmL. Antibody concentrations are unknown but each line represents a different dilution of anti-sera.



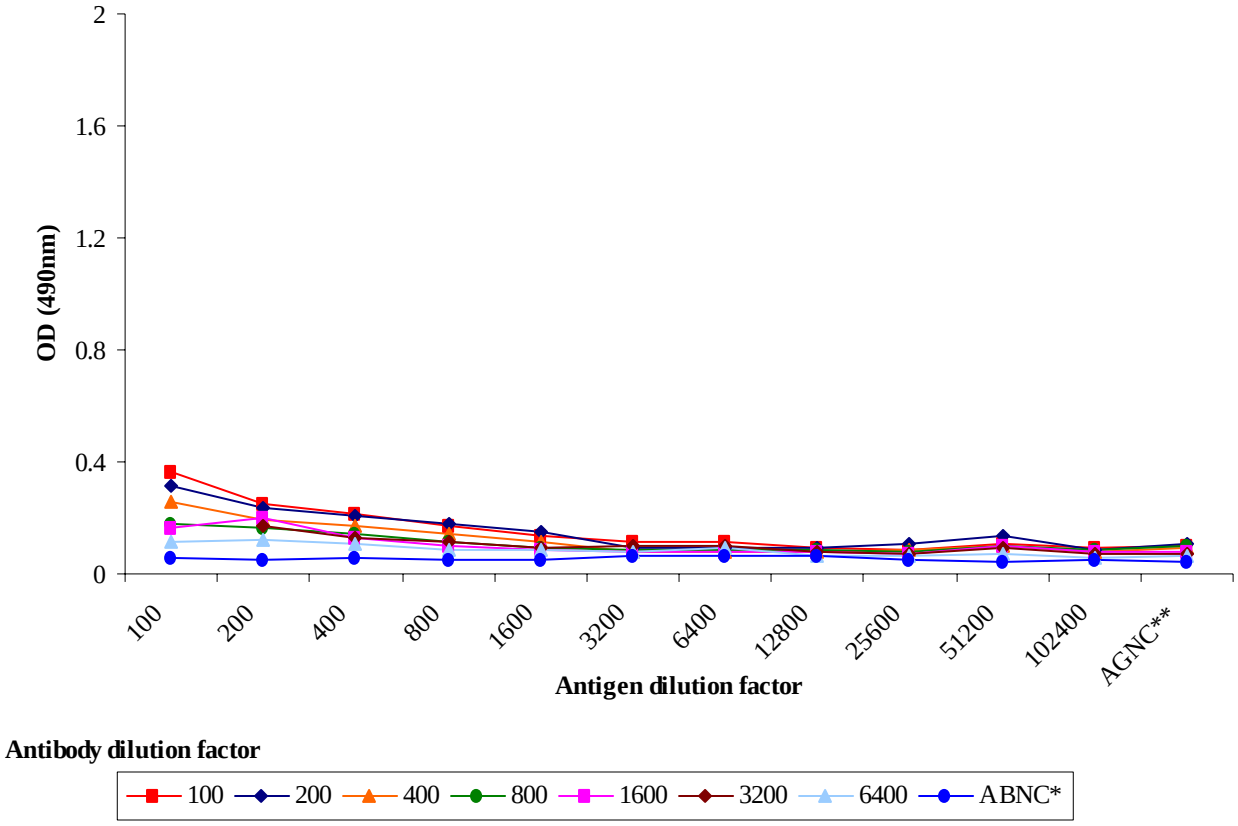
* ABNC = Antibody Negative Control **AGNC = Antigen Negative Control

Figure 12b. Optical density values of serial dilutions of antigen preparation one and anti-sera, using one wash instead of three in ELISA methodology one (ELISA methodology 2d.)



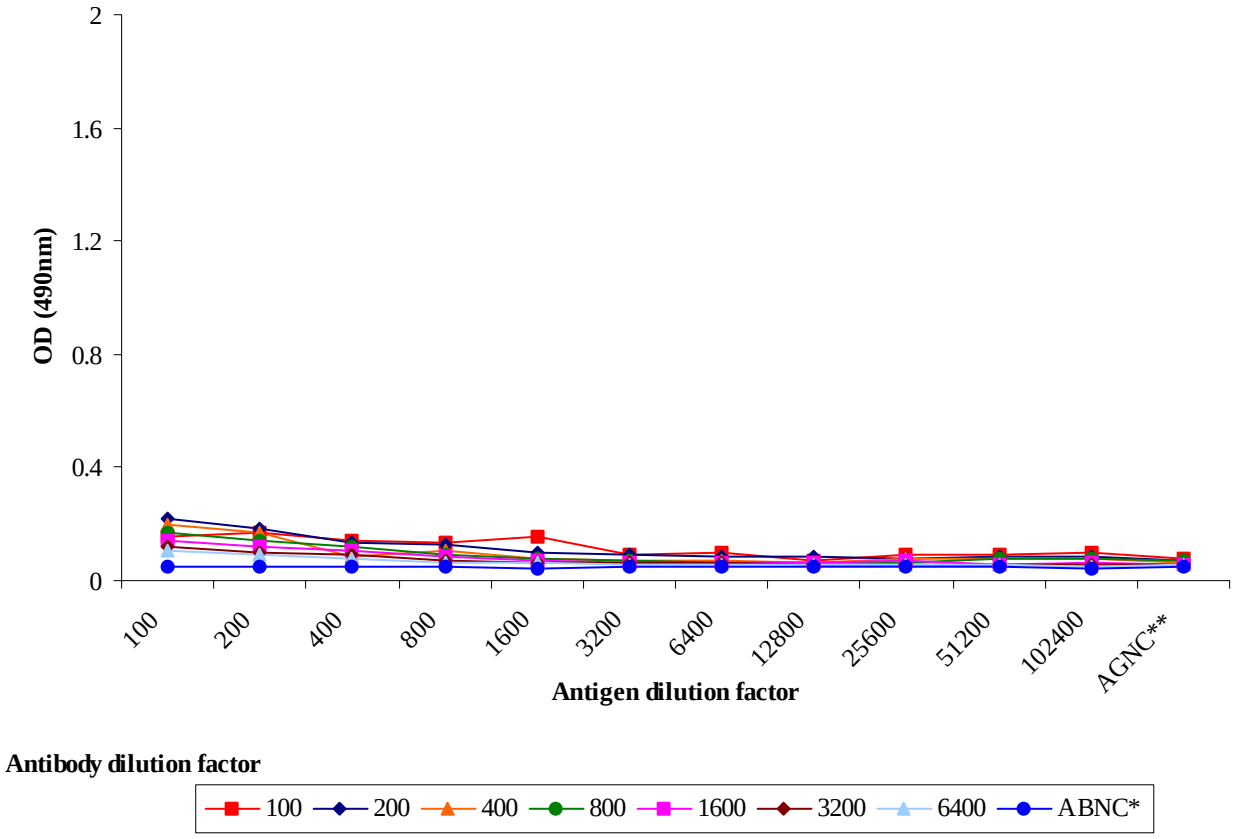
*ABNC = Antibody Negative Control **AGNC = Antigen Negative Control

Figure 13a. Optical density values of serial dilutions of antigen preparation one and anti-sera (ELISA methodology one). Antigen concentration is represented by the serial two-fold dilution of a stock solution of 0.25µgmL. Antibody concentrations are unknown but each line represents a different dilution of anti-sera.



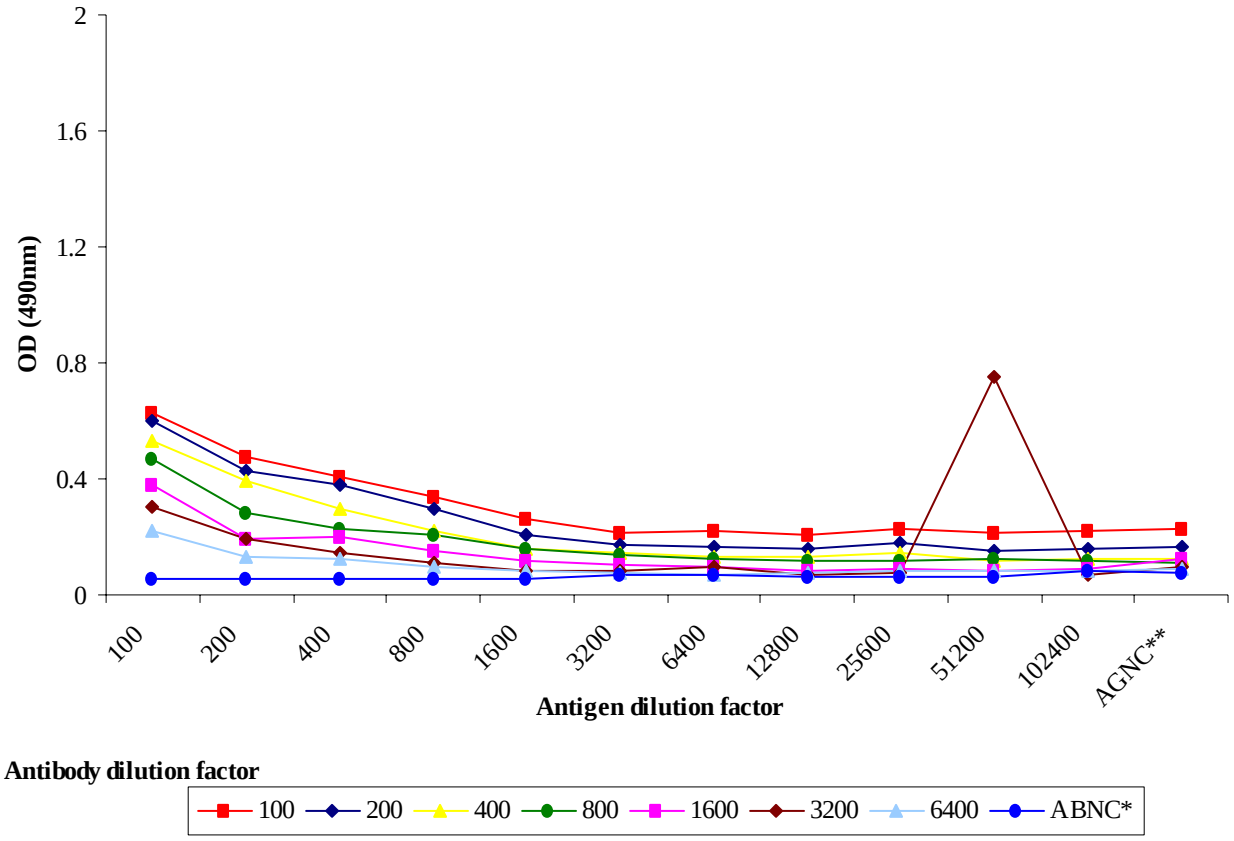
*ABNC = Antibody Negative Control **AGNC = Antigen Negative Control

Figure 13b. Optical density values of serial dilutions of antigen preparation one and anti-sera, using a blocking buffer of 0.25% in ELISA methodology one (ELISA methodology 2e.)



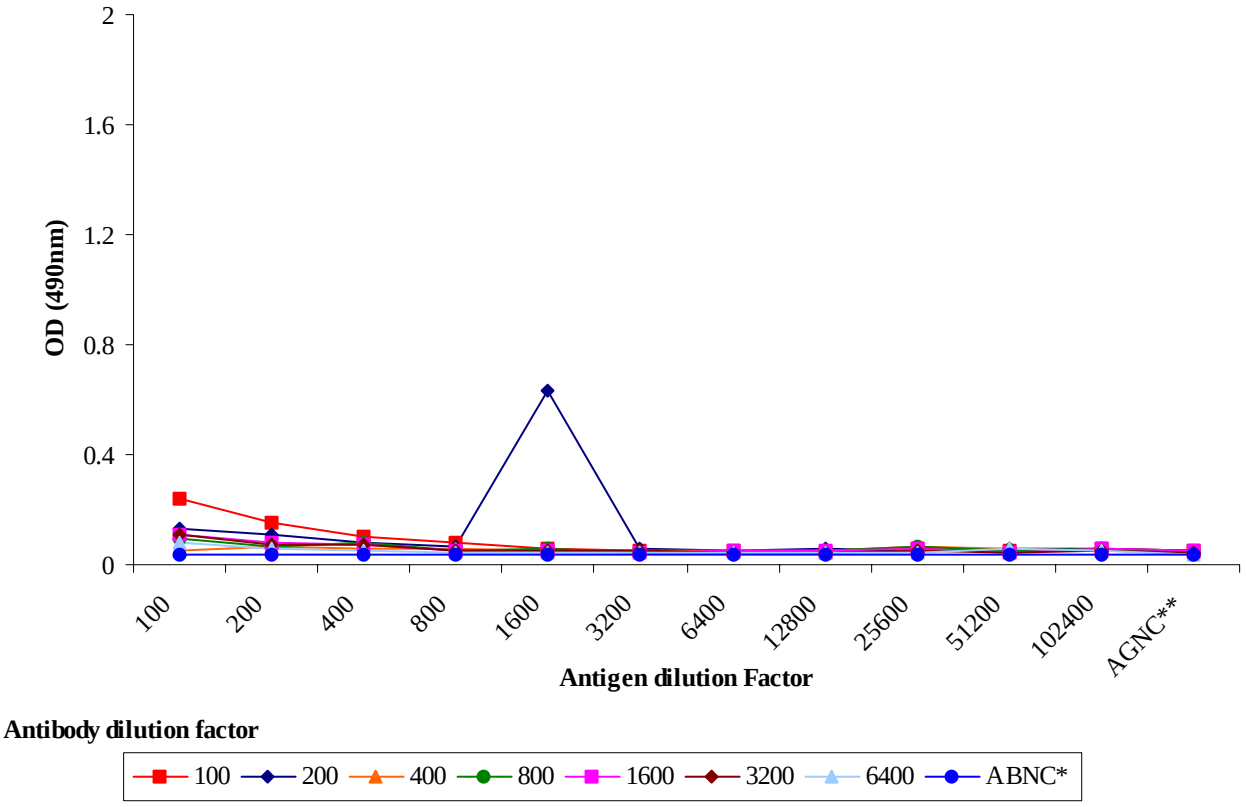
*ABNC = Antibody Negative Control **AGNC = Antigen Negative Control

Figure 14a. Optical density values of serial dilutions of antigen preparation one and anti-sera (ELISA methodology one). Antigen concentration is represented by the serial two-fold dilution of a stock solution of 0.25µgmL. Antibody concentrations are unknown but each line represents a different dilution of anti-sera.



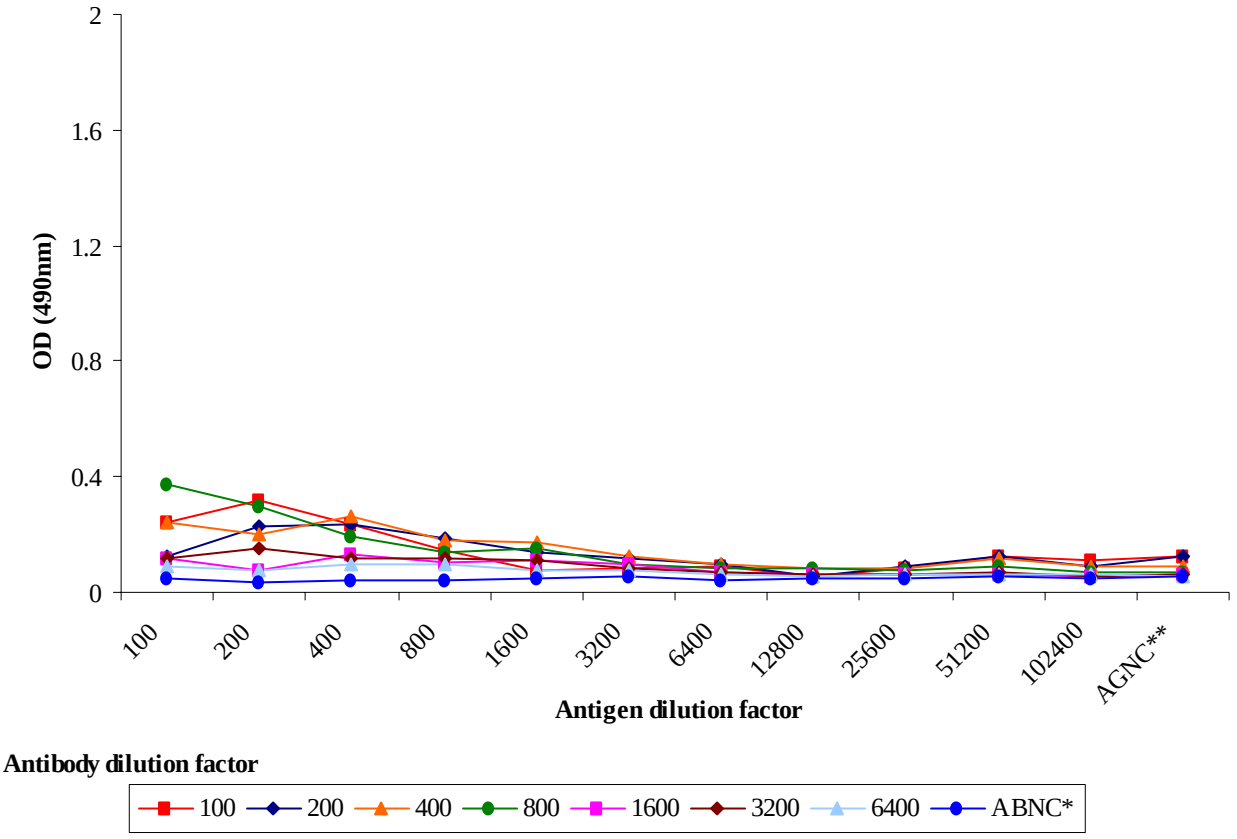
*ABNC = Antibody Negative Control **AGNC = Antigen Negative Control

Figure 14b. Optical density values of serial dilutions of antigen preparation one and anti-sera, using a different type of blocking buffer (skim milk) in ELISA methodology one (ELISA methodology 2f.).



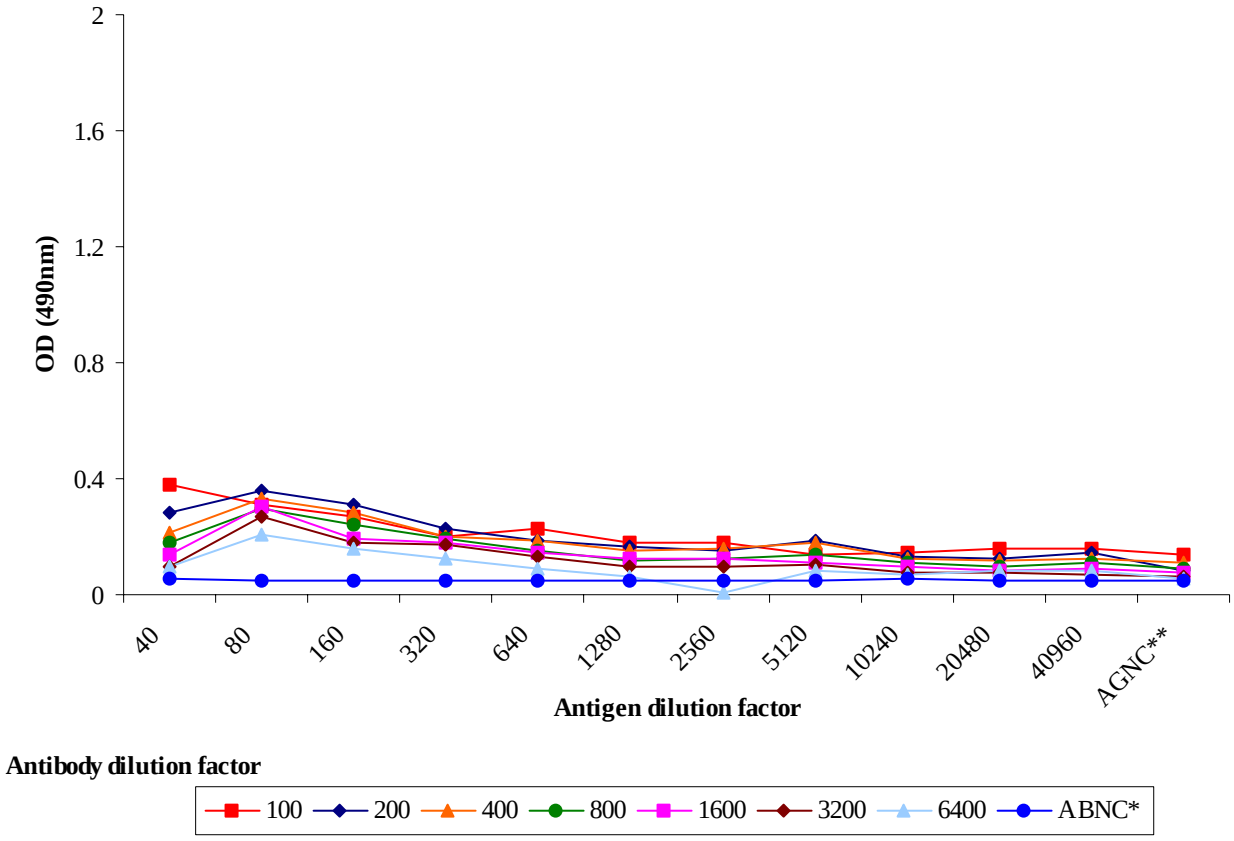
*ABNC = Antibody Negative Control **AGNC = Antigen Negative Control

Figure 15a. Optical density values of serial dilutions of antigen preparation one and anti-sera (ELISA methodology one). Antigen concentration is represented by the serial two-fold dilution of a stock solution of 0.25µgmL. Antibody concentrations are unknown but each line represents a different dilution of anti-sera.



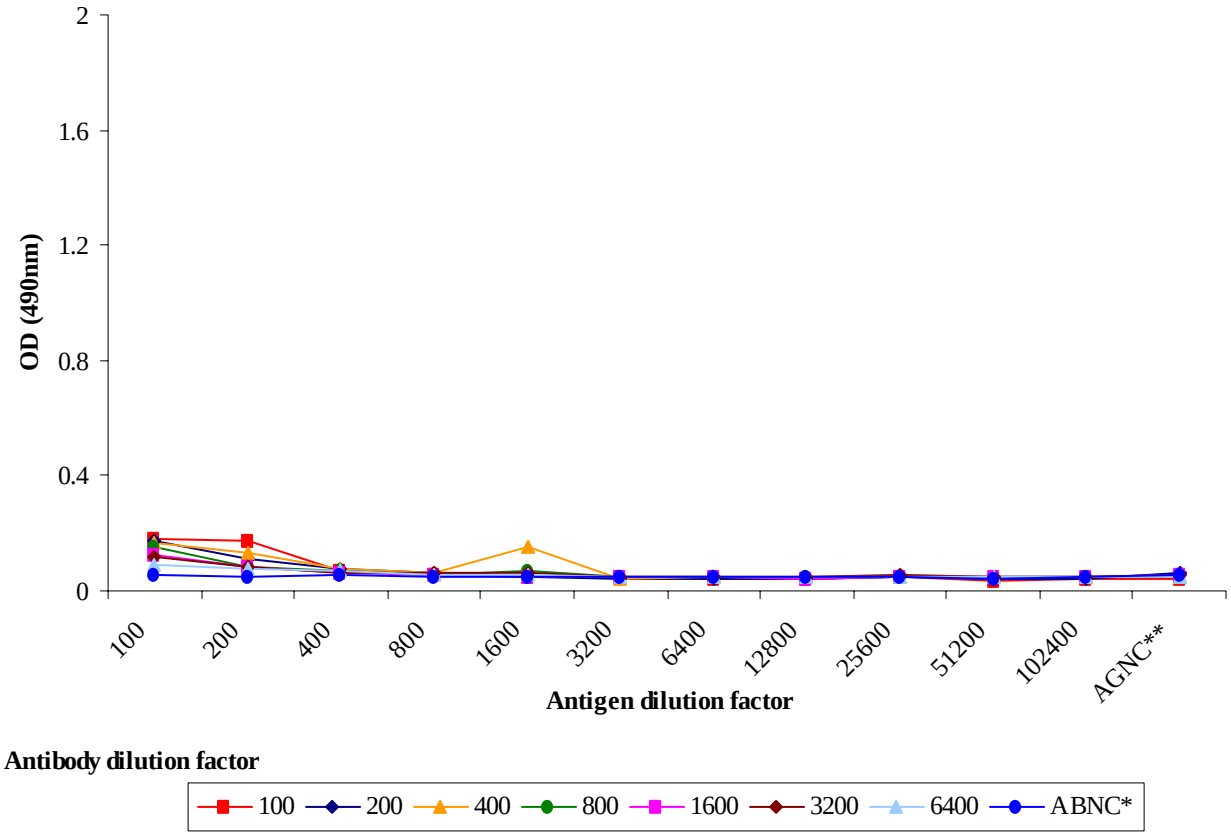
* ABNC = Antibody Negative Control ** AGNC = Antigen Negative Control

Figure 15b. Optical density values of serial dilutions of antigen preparation one and anti-sera, using an antigen concentration of 1/40 instead of 1/100. (ELISA methodology 2g.).



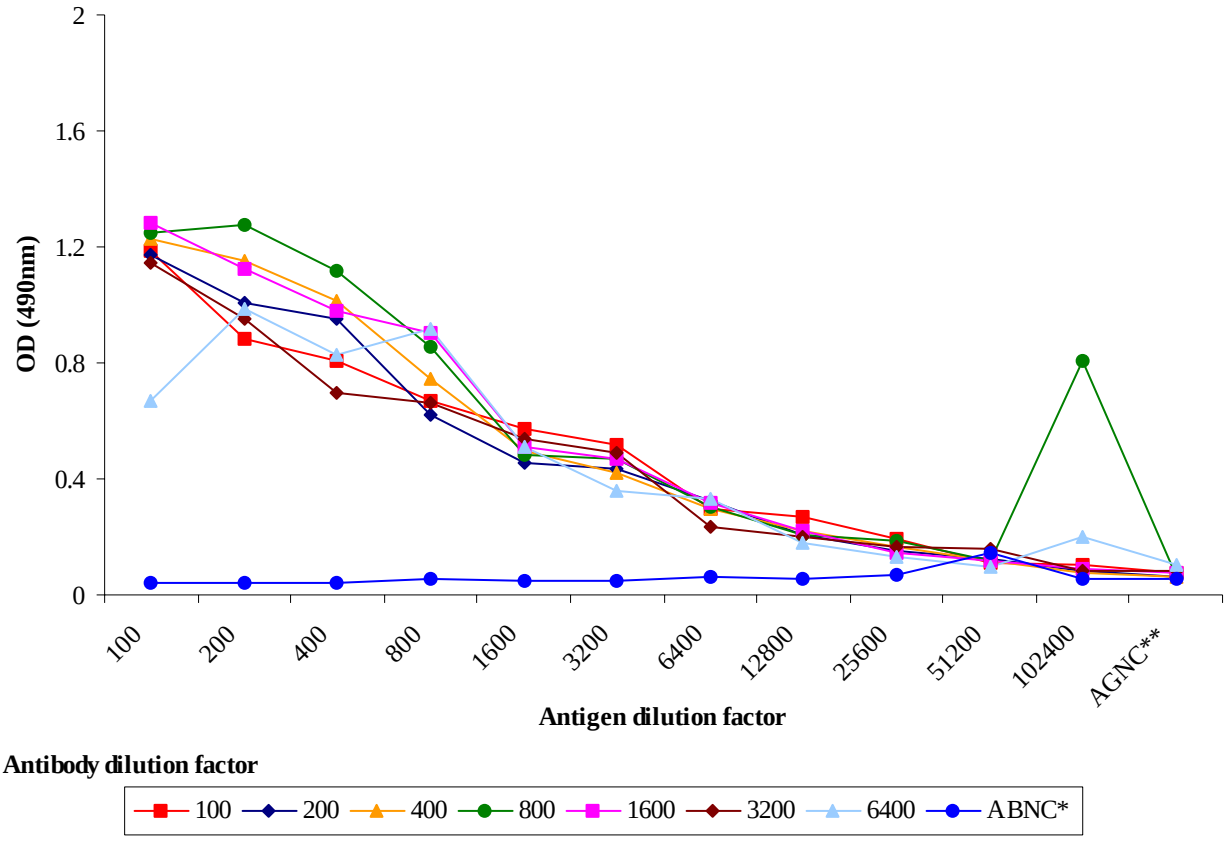
* ABNC = Antibody Negative Control ** AGNC = Antigen Negative Control

Figure 16a. Optical density values of serial dilutions of antigen preparation one and anti-sera (ELISA methodology one). Antigen concentration is represented by the serial two-fold dilution of a stock solution of 0.25µgmL. Antibody concentrations are unknown but each line represents a different dilution of anti-sera.



* ABNC = Antibody Negative Control ** AGNC = Antigen Negative Control

Figure 16b. Optical density values of serial dilutions of a crude preparation of ovalbumin and pdg anti-sera (ELISA methodology 2h.). Ovalbumin concentration is represented by a serial two-fold dilution of a solution of 0.25µgml. Antibody concentrations are unknown but each line represents a different dilution of anti-sera.

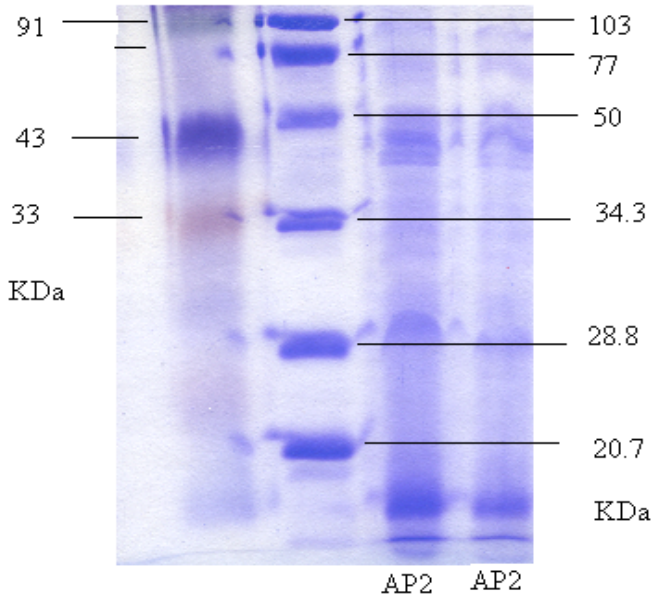


* ABNC = Antibody Negative Control **AGNC = Antigen Negative Control

Antigen Preparation Two

Results of agglutination tests indicated that there were little or no LPS in the antigen preparation. The protein concentration of antigen preparation two was determined to be 0.70µg/mL. Gel electrophoresis confirmed the presence of multiple protein bands between 20.7 and 103 KDa.

Figure 17. Gel electrophoresis of protein components of Antigen Preparation Two (AP2).



Western Blot of antigen preparation

Specificity of antigen preparation two

Three membranes were incubated in a 1/50 dilution of specific anti-serum from experimentally infected rabbits. Membranes were incubated in anti-serum containing either *Salmonella* Brandenburg, *Salmonella* Typhimurium or *Salmonella* Hindmarsh antibodies. Protein bands were seen between 20.7 and 103 KDa on two membranes, where one was incubated in anti-*Salmonella* Brandenburg serum and the other was incubated in anti-*Salmonella* Typhmurium serum. No protein bands were seen on the membrane incubated in serum containing *Salmonella* Hindmarsh antibodies or in serum containing no *Salmonella* antibodies (negative control) (Appendix IVb).

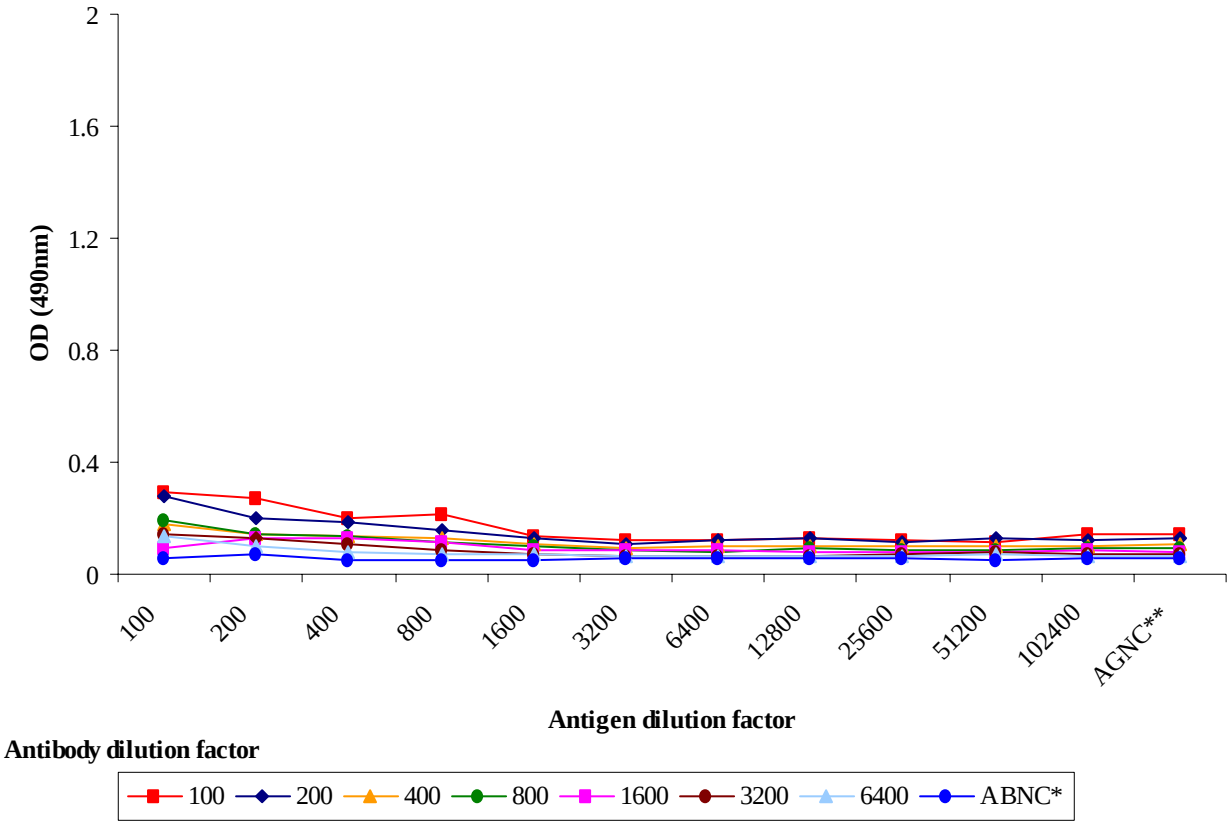
Specificity of antigen preparation one and two

Three membranes containing both antigen preparation one and two were incubated in 1/50 dilution of specific serum from experimentally infected rabbits. Membranes were incubated in anti-serum containing either *Salmonella* Brandenburg, *Salmonella* Typhimurium or *Salmonella* Hindamarsh antibodies. Protein bands were seen between 20.7 and 103KDa for both antigen preparation one and two when incubated in serum containing either *Salmonella* Brandenburg or *Salmonella* Typhimurium antibodies. No protein bands were seen in the membranes incubated in serum containing *Salmonella* Hindamarsh antibodies or in serum containing no *Salmonella* antibodies (negative control) (Appendix IVc).

Two membranes containing both antigen preparation one and two were incubated in 1/50 dilution of specific serum from a previous vaccine trial. Membranes were incubated in anti-serum containing *Salmonella* Brandenburg, *Salmonella* Typhimurium and *Salmonella* Hindamarsh antibodies or in serum containing only *Salmonella* Typhimurium and *Salmonella* Hindmarsh antibodies. Protein bands were seen between 20.7 and 103KDa for both antigen preparation one and two when incubated in serum containing all three *Salmonella* serovars; Brandenburg, Typhmurium and Hindmarsh. No protein bands were seen in the membranes incubated in serum containing just the two *Salmonella* serovars; Typhimurium and Hindmarsh (Appendix IVd).

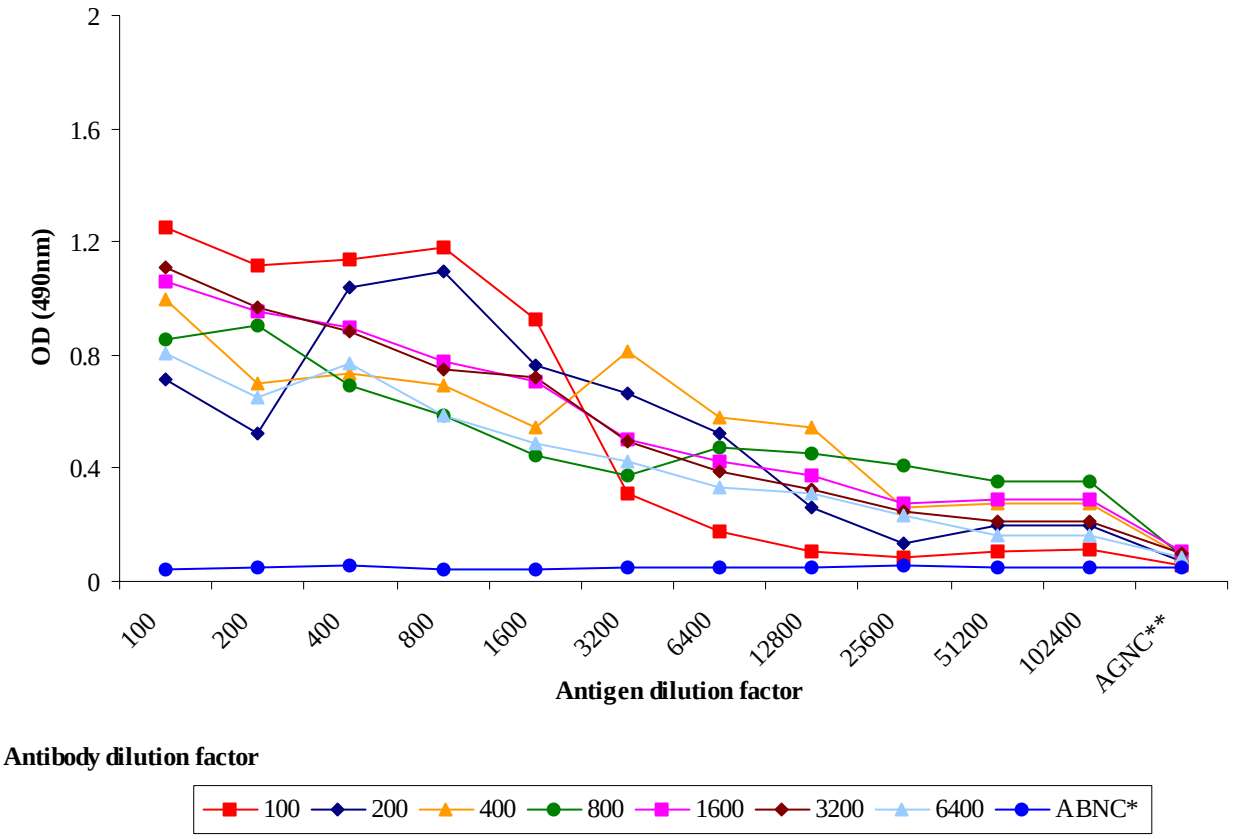
Indirect ELISA of Antigen Preparation Two.

Figure 18a. Optical density values of serial dilutions of antigen preparation one and anti-sera (ELISA methodology one). Antigen preparation concentration is represented by a serial two-fold dilution of a stock solution of 0.25µgmL. Antibody concentrations are unknown but each line represents a different dilution of anti-sera.



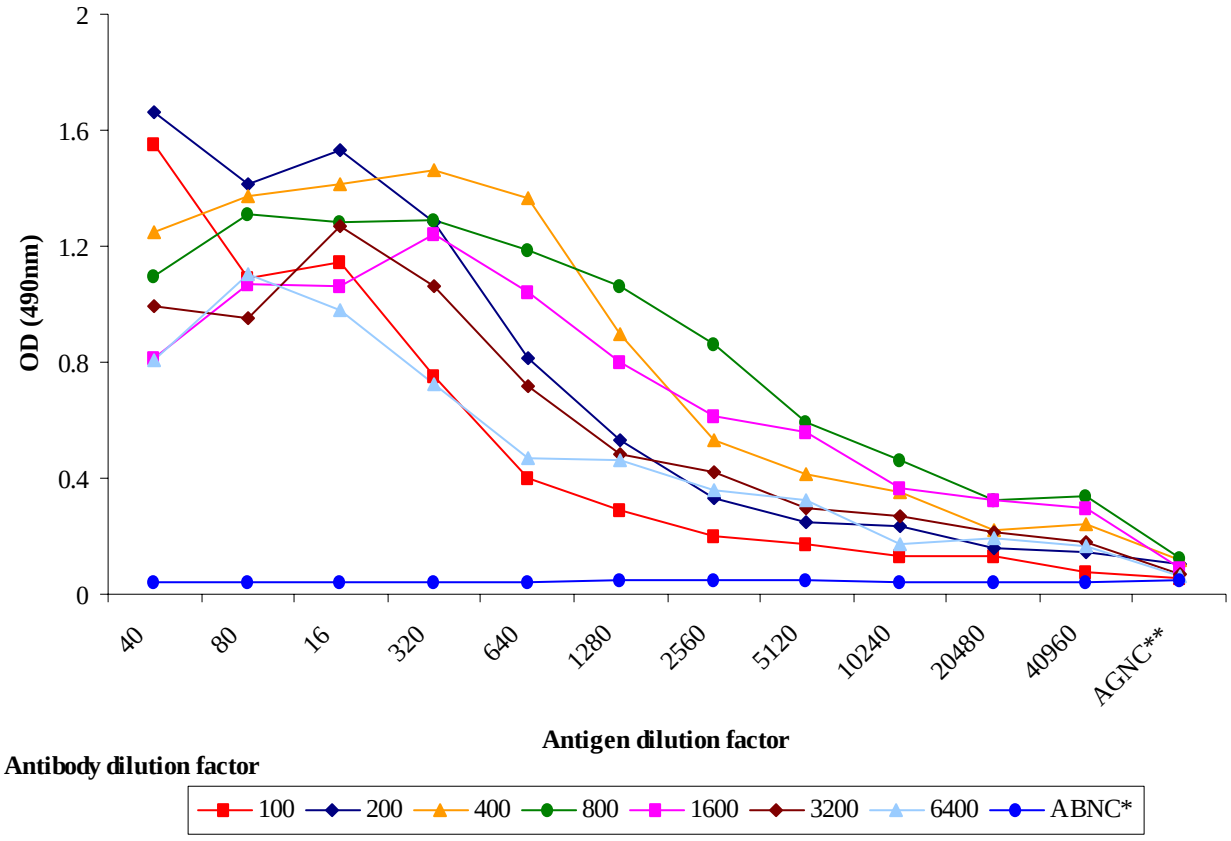
*ABNC = Antibody Negative Control **AGNC = Antigen Negative Control

Figure 18b. Optical density values of serial dilutions of antigen preparation two and anti-sera (ELISA methodology one). Antigen preparation concentration is represented by a serial two-fold dilution of a stock solution of 0.70µg.mL. Antibody concentrations are unknown but each line represents a different dilution of anti-sera (ELISA methodology 2i.).



*ABNC = Antibody Negative Control **AGNC = Antigen Negative Control

Figure 18c. Optical density values of serial dilutions of antigen preparation two (0.70µgmL) and anti-sera (ELISA methodology one), using an antigen concentration of 1/40 instead of 1/100 (ELISA methodology 2i.).



*ABNC = Antibody Negative Control **AGNC = Antigen Negative Control

CHAPTER FOUR

DISCUSSION

This study was undertaken to develop a sensitive and specific serological test for use in epidemiological studies and/or monitoring of *Salmonella* Brandenburg exposure in New Zealand sheep. Because bacteriological sampling does not always provide an accurate indication of infection (Wray, CA and WA, 2000), and due to the relative insensitivity of conventional serological tests (Kim *et al*, 1991), the ELISA was thought to be the most sensitive and specific serological test available. Although the ELISA has been shown to be more sensitive and specific than other methods (Cooper *et al*, 1989; Nicholas and Cullen, 1991), problems relating to the specificity of the ELISA still exist.

Antigen preparation

The antigens used in an ELISA are the crucial element for determining the specificity of the assay (Crowther, 1995). The successful isolation of a specific *Salmonella* Brandenburg antigen is critical for the development of a specific ELISA. As previously mentioned *Salmonella* Brandenburg and *Salmonella* Typhimurium both belong to serogroup B, and are known to share two common somatic O antigens. Because O antigens make up the polysaccharide portion of LPS, there were reservations in preparing the commonly used LPS-based antigen. This was due to the possibility of cross-reactions between *Salmonella* Brandenburg and *Salmonella* Typhimurium antibodies. Antigen preparations were therefore based on a method described by Zamora *et al* (1999). The protocol contained a simple and fast purification step (filtration and concentration) which excluded cross-reacting LPS from the antigen preparation. The antigen preparation is thought to be mainly composed of fimbria and flagella.

Removal of LPS from antigen preparation one and two was achieved by allowing the supernatant to pass through a syringe filter, which removes pyrogens from the protein solution. The agglutination tests for both antigen preparations one and two turned out negative indicating the absence of LPS. Both positive and negative controls were used to check the

reliability of the agglutination test and both results showed that the test was reliable.

Antigen Preparation One

Antigen preparation one was a mixture of fimbrial, flagella and outer membrane protein fractions and numerous clear protein bands were seen ranging in a molecular weight between 28.8KDa to 103KDa (Figure 6).

Specificity of Antigen preparation one

In order to check the specificity of antigen preparation one the preparation was probed with various *Salmonella* anti-sera using the Western Blot technique. The procedure was first optimised using a positive control. Positive colour reactions were seen with both 1/50 and 1/500 dilutions of the anti-pdg serum (Figure 7), indicating that all reagents were active, and that the system itself was robust and responsive.

Antigen preparation one was electro-transferred onto a nitrocellulose membrane and probed with anti-*Salmonella* Brandenburg serum obtained from a previous *Salmonella* vaccine trial. A positive colour reaction was seen for about 10-15 protein bands ranging from 43.2 – 129KDa. In order to check the specificity of the antigen preparation, nitrocellulose membranes containing the antigen proteins were probed with anti-serum containing only *Salmonella* Typhimurium and *Salmonella* Hindmarsh antibodies. Colour reactions were seen only with the anti-serum containing all three *Salmonella* serovars. The absence of a colour reaction in the anti-serum containing only *Salmonella* Typhimurium and Hindmarsh indicated that the antigen/antibody complexes forming with anti-serum containing all three serovars must be due to *Salmonella* Brandenburg antibodies. From these results it was concluded that the antigen protein components were specific for *Salmonella* Brandenburg antibodies.

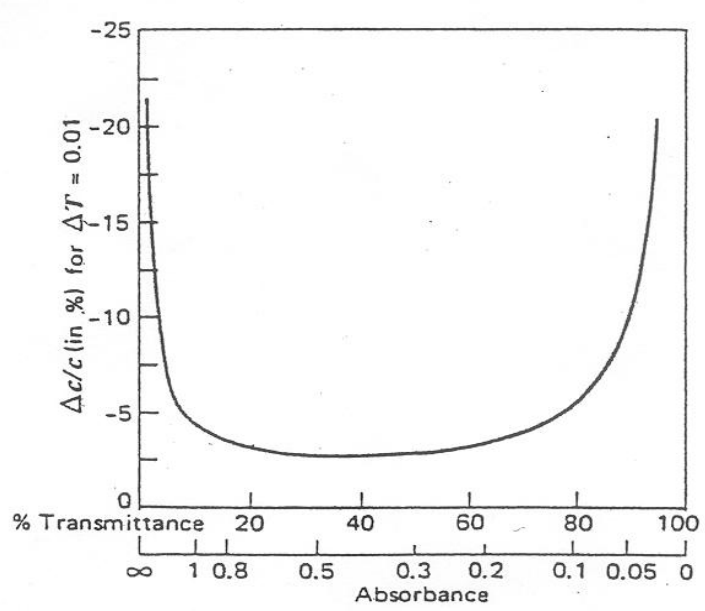
Indirect ELISA of Antigen Preparation One

Antigen preparation one was then used as a capturing antigen for the development of a specific *Salmonella* Brandenburg indirect ELISA. A checkerboard titration was performed in order to optimise the procedure and to produce a standard curve for the system. A 2-fold dilution range of antigen was administered across all the rows and a 2-fold dilution range of

anti-serum was counter-administered down all the columns (Figure 5).

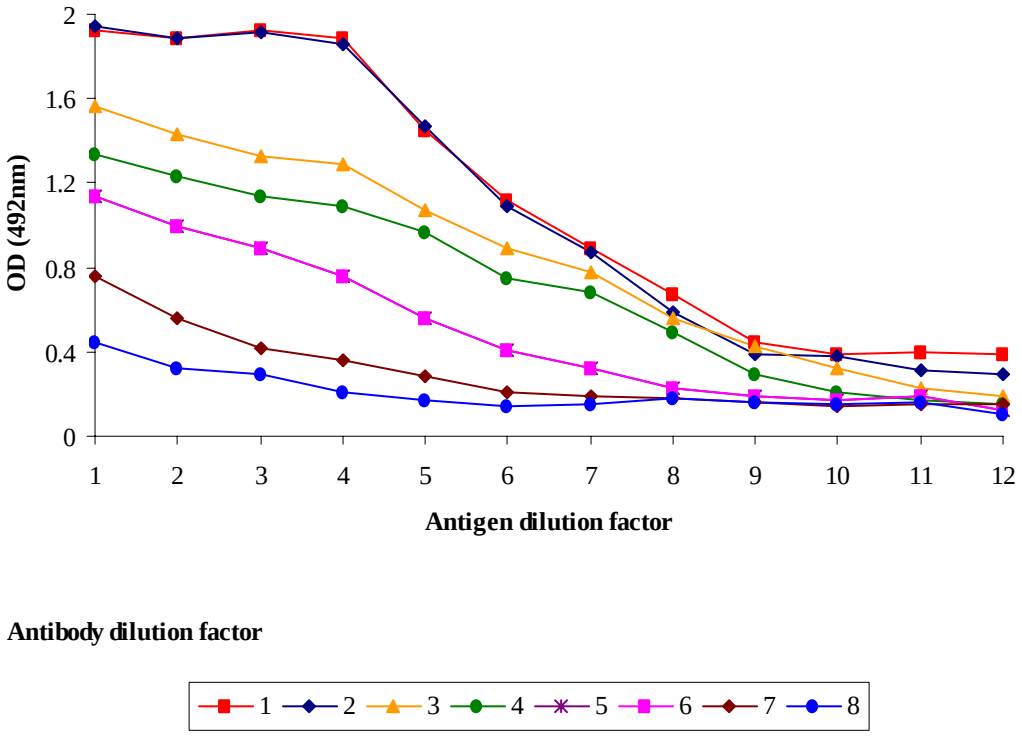
In a checkerboard titration, the optical density readings should range between 0.1 and 1.5, to accurately quantify specific binding between the antigen and antibodies (Figure 19).

Figure 19. The relative concentration error as a function of transmittance for a 1% uncertainty in percentage transmittance (The Beers Lambert Law).



An almost constant minimum error occurs between 0.2 – 0.7 nm. In order to prevent large errors in spectrophotometry readings the optical density readings should fall within 0.1 to 1.5 (Binnie, JE. 1991). Consequently antigen preparations and anti-sera were diluted or concentrated, so that the minimum and maximum amount of functional antibody, which after forming an immuno-complex, would give an optical density readings of between 0.1 and 1.5 Abs at a wavelength of 490nm. The ideal standard curve, generated in order to ensure correct optimisation of the indirect ELISA, should produce a sigmoidal curve as seen in Figure 20.

Figure 20. The ideal indirect ELISA standard curve.



The standard curve will show a plateau region (maximal colour development) representing complete antibody saturation of the bound antigen. This will be followed by gradual decrease of the optical density as the antibodies are titrated and no longer saturate the antigen. Finally the last antibody dilutions will produce low optical density readings that will be equivalent to the plate background. The optimal concentration of antigen that could be used as a single dilution to detect and possibly quantify antibodies will show good binding across the whole range of anti-serum dilutions.

Response to high background optical densities

Figure 8a shows the results of the initial checkerboard titration, where each line represents a different dilution range of antigen (1/100 – 1/102400) plotted against a dilution range of antibody (1/100 - 1/6400). The maximal colour development gave an optical density of about 0.7 with no linear response to changes in antigen concentration. The antigen negative control (AGNC) showed a reasonably high background noise, indicating a certain amount

of non-specific interaction. Non-specific interactions are known as non-specific binding effects and reduce the sensitivity of the immunoassay. After the antigen has adhered to the well surface the plates are washed to remove any unbound antigen. This can leave free sites on the solid phase that the antigen is not bound to. Other proteins, for example antibodies, are then able to adsorb and bind to the plate surface i.e. interact non-specifically. Addition of a blocking reagent after the antigen has been passively adsorbed to the plate has been shown to reduce any potential non-specific binding (Crowther,1995). Therefore a modification was made to the ELISA methodology, where a blocking step was added to the original indirect ELISA protocol. Comparisons between the addition of a blocking step after the adsorption of the antigen (Figure 8b.) and the original protocol (Figure 8a) showed that the non-specific binding effects were reduced with the addition of a blocking step. This can be seen be clearly seen in the antigen negative control (AGNC), where there was no antigen added to the wells. With the addition of a blocking step the non-specific binding of other proteins was reduced to a minimum, as indicated by the low absorbency readings of the antigen negative control. The blocking step was considered to be an important additional step for the indirect ELISA and was added to all further protocols.

Response to repeated low optical densities

While the addition of a blocking step reduced the effect of non-specific binding, the indirect ELISA consequently produced repeated low optical density readings. The production of a weak signal could be due to various human or experimental errors.

Human errors would include such examples as:

- Improper storage of reagents resulting in degradation.
- Omission of reagents or the addition of reagents in the wrong order.
- Incorrect use of reagents.
- Cross contamination of the wells reduced signals

Experimental errors would include such examples as:

- Insufficient amounts of antigen could be present.
- Antigen did not bind well to the plate.
- The conditions to obtain optimal binding of antigen were incorrect.
- Non optimised conditions for blocking and washing techniques.

- The primary antibody could not bind to antigen
- A poor interaction between the antibody and denatured antigen.
- Insufficient incubation times with secondary antibody or substrate.
- Lot and vender variations in micro-titration plates resulting in poor signal quality.

Human errors were eliminated by repeating the experiments twice and checking that the reagents were stored correctly and were for current use. The following procedures were implemented in an attempt to investigate possible experimental sources of error.

Direct ELISA

Due to the vast possibility of parameters that could be causing the problem, a direct ELISA was carried out. A direct ELISA was used to check:

- that the secondary antibody was forming an immuno-complex with the primary antibody.
- that the substrate-enzyme complex reaction was producing a colour reaction.

The direct ELISA showed a number of high optical density responses indicating that there were no problems with the recognition of primary antibody, concentration or incubation times of secondary antibody or the concentration of the chromaphore (Figure 9). It was therefore concluded that the low optical density readings obtained in previous experiments must be due to the first steps of the protocol used for the indirect ELISA.

In the indirect ELISA the optical density readings are dependent on the amount of antigen that has been attached to the wells. This is dependent on the adsorption characteristics of the plastic, the concentration of the antigen, and the ability of the primary antibody to recognise the antigen. In order to eliminate these as being responsible for the problems with the assays, washing and blocking conditions were first investigated to order to determine optimal conditions for binding the antigen to the plate, and the primary antibody to the antigen.

Washing conditions

The washing step in an indirect ELISA is very important. Its primary objective is to remove any bound or unbound (free) reagents from the wells. Generally the washing buffer used in

the washing procedure contains PBS (Phosphate Buffered Saline 0.1M, pH 7.4) and 0.05% detergent (Tween-20) and is administered to all wells for three minutes. The process is usually repeated three times. Most antigen-plate, antigen-antibody interactions will stand up to these washing conditions. The possibility that the antigen had been removed in the washing procedure was considered first.

Washing buffers do not always contain detergents. When detergents are added to washing buffers they can cause excessive froth. Air bubbles can prevent the washing buffer from contacting the well surface and prevent the washing buffer from removing bound or unbound antigen. Strong detergents also have the ability to denature the antigen, making it unrecognisable to various primary antibodies. The amount of washing seems to vary between different protocols. If the antigen is poorly bound to the well surface, excessive amounts of washing may cause the antigen to leach from the well. All of these factors could cause a low optical density response. Three experiments were undertaken to discover whether the washing procedure was removing antigens, and was therefore the reason for low optical density readings.

The first modification undertaken compared the use of two different detergents. No differences could be seen between the use of Tween-20 (Figure 10a) and Thesit, a milder detergent (Figure 10b). A weak response was seen for both plates.

The second modification to the protocol compared the use of a washing buffer containing detergent (Figure 11a) and a washing buffer containing no detergent (Figure 11b). Again a weak colour reaction was seen for both plates.

The third modification to the protocol used three washes (Figure 12a) compared to one (Figure12b). Both plates produced repeated low optical density readings.

All three modifications in the washing procedure made no difference to the repeated low optical density readings, and therefore it was concluded that the washing procedure had no significant effect on the low optical density readings.

Blocking conditions

Because the various changes in the washing conditions did not increase the optical density readings, the blocking conditions to obtain optimal binding of the antigen to the plate or the primary antibody to the antigen were next investigated. As already discussed blocking measures have to be taken in order to prevent non-specific binding of proteins. Blocking agents work by competing with other proteins for available sites. Due to the competitive nature of the blocking buffer, it is also possible that a concentrated blocking buffer could bind to both the antigen and the plastic, rendering it impossible for the primary antibody to bind. Therefore another modification was made to the protocol. Two different concentrations of gelatine blocking buffer were compared (0.5% and 0.25%). As shown in Figures 13a and 13b, only a weak signal was observed for both plates.

The use of different blocking buffers has been reported for various immuno-detection methods. Skim milk was therefore tried as a blocking reagent (Figure 14b) instead of gelatine (Figure 14a). Again, little difference was seen between the two different protocols, indicating that the problem was not due to blocking.

Capturing antigen: Antigen preparation one

As already mentioned, the value of the optical density readings is dependent on the amount of antigen that has been attached to the wells. This in turn is dependent on the adsorption characteristics of the plastic, and the concentration of the antigen. Because the possibilities of poor blocking and washing conditions had been eliminated, it seemed most likely that the problem was due to the antigen. All further investigations involved modifications of antigen preparation. Firstly antigen was applied at a higher concentration, as the low response could simply be due to the fact that not enough antigens were adhering to the plate. Therefore antigen preparation one was applied at a concentration of 1/40 instead of 1/100. The consequence of increasing the antigen concentration proved to be of little benefit (Figures15a and 15b). Both plates still showed consistent low optical densities.

A number of possible problems can exist with the capturing antigen, for example:

1. The primary antibody may not be binding efficiently to the antigen.

The binding of the antibody to the antigen is dependent on a variety of close interacting forces, such as;

- Electrostatic forces, where oppositely charged groups of proteins interact.
- Hydrogen bonding, where formation of hydrogen bridges between atoms form.
- Van der waals forces, where interaction between electron clouds form.
- Hydrophobic forces, where there is an association of non-polar and hydrophobic groups so that contact with water is minimized.

Antibodies that recognise antigen epitopes perfectly will tend to have a high affinity for the antigen, while antibodies that recognise epitopes imperfectly will tend to have a low affinity. The low optical density response may be due to poor antibody recognition of the antigen. Another possibility is that the antigen could have been adhering to the plate in a bad orientation. Hydrophobic residues are usually oriented to the plastic surface and therefore it is possible that the antigen epitope may be on the same side that binds to the well surface, and may therefore be only partially exposed. This would also result in a low optical density response. The same situation would occur if the antigen was denatured in any way.

2. The antigen could not be adhering to the plates.

Most proteins adsorb to the plastic surfaces as a result of hydrophobic interactions between non-polar proteins and the plastic matrix. It is possible that the antigen may not be binding to the plate and hence causing a number of low optical density responses.

The hydrophobicity of the plastic-protein interaction can be manipulated to increase binding and ensure firmer interaction between the antigen and the plastic. Partial denaturation of the antigen would expose hydrophobic regions and is another possibility to ensure firmer binding. However, as previously discussed, partial denaturation may also decrease the affinity between the antigen and antibody.

The rate and extent of coating the antigen onto the plates can depend on (Crowther, 1995):

- The diffusion coefficient of the attached molecule
- The ratio of the surface area being coated to the volume of the coating solution

- The concentration of the substance being adsorbed
- The temperature: the rate of "collision" of antigen and antibody is important to achieve interaction.
- The time of adsorption

Generally a concentration range of 1-10µg/mL of protein in a volume of 50µL is a good guide to the level of protein needed to saturate the sites of a plate well (Crowther, 1995). However this will depend on the purity of the antigen preparation. If the coating solution contains only a small amount of specific antigen, then the amount of specific antigen is reduced compared to its proportion in the mixture. Other proteins will take up the sites, and because the plates have a saturation level, the use of a crude antigen may lead to a poor assay result. Crude antigen preparations can be unsuitable for direct adsorption onto a plate, especially if other proteins of a higher protein concentration can compete for sites on the plastic. It must also be taken into consideration the total number of molecules that can bind to the surface, and the amount that can bind and remain biologically active. This is dependent on the nature of the antigen and the surface.

In order to try and to determine if the crude nature of the antigen preparation was the problem, an experiment was carried out using ovalbumin antigen as a capturing antigen. Ovalbumin antigen had previously been used as a positive control in the Western Blot. Pure ovalbumin diluted to the concentration of antigen preparation one was crudely mixed with BSA (Bovine Serum Albumin) to try and reproduce the conditions of the antigen preparation. Despite the fact that a crude antigen was used, the results clearly showed a high optical density response (Figure 16a and 16b). It also showed that the antigen was able to bind efficiently to the plate with the current ELISA conditions.

3. The antigen may need to be more concentrated.

Antigen preparation one is of a relatively crude and non-characterised nature. Therefore the specific antigen required for a reasonable response may be at a low concentration compared to other antigens and may only attach a low concentration of antibody. Due to the risk that the crude antigen preparation was simply not concentrated enough, a second antigen preparation was prepared. The same protocol was followed as in antigen preparation one,

except for the following:

- A larger amount of nutrient broth was prepared and inoculated. This was simply to ensure further concentrated antigen preparation could be obtained.
- The suspension was spun down in an ultracentrifuge as the suspension was too concentrated and could not be administered through syringe filters.

The above deviations from the antigen preparation protocol are believed to have minimal impact on the final outcome.

Antigen Preparation Two

Antigen preparation two had a concentration of 0.70 µg/µL. This antigen preparation was three times more concentrated than antigen preparation one.

Specificity of antigen preparation two

A Western Blot was undertaken to investigate the specificity of antigen preparation two. Specific *Salmonella* anti-sera from experimentally infected rabbits were now available and were used for the Western Blot. Antigen preparation two was first exposed to anti-*Salmonella* Brandenburg, anti-*Salmonella* Typhimuium and anti-*Salmonella* Hindmarsh serum. A positive colour reaction was seen for both *Salmonella* Brandenburg and Typhimurium serum. This indicated that the second antigen preparation was not specific for *Salmonella* Brandenburg, as both *Salmonella* Brandenburg and Typhimurium antibodies were recognising the antigen. Antigen preparation two was again administered through the syringe filter and the Western Blot repeated. Positive colour reactions were again seen for both *Salmonella* Brandenburg and Typhimurium serum.

Further investigation of specificity of antigen preparation one and two

The specificity of the rabbit serum was further investigated by incubating a membrane containing proteins from antigen preparation one and two in the three different anti-sera. The results showed that proteins from both antigen preparation one and two formed an immuno-complex with anti-*Salmonella* Brandenburg and Typhimurium serum. These results indicated that both antigen preparations one and two were cross-reacting with *Salmonella* Brandenburg and Typhimurium antibodies.

Both antigen preparations were further investigated by performing the western Blot with serum from a previous *Salmonella* Brandenburg vaccine trial, which originally indicated that antigen preparation one was specific for *Salmonella* Brandenburg. The results both indicated that antigen preparation one and two were specific for *Salmonella* Brandenburg antibodies. Positive colour reactions were only seen with the serum containing all three *Salmonella* serotypes, where no colour reaction was seen with serum containing *Salmonella* Typhimurium and Hindmarsh antibodies. This evidence supports the original conclusions that antigen preparation one was specific for *Salmonella* Brandenburg and also suggests that antigen preparation two is specific.

The conflicting specificity information seemed to be resulting from the use of different sera, not through the use of another antigen preparation. At the time of the preparation of the first antigen, serum from a previous vaccine trial was the only serum available for use. Due to confidentiality issues, limited details were available from the clinical trial that took place. Therefore more confidence is obtained through the use of the experimentally infected rabbit serum than the serum used from the vaccine trial.

Indirect ELISA of Antigen Preparation Two

Despite the specificity problems of the antigen preparations, the second antigen preparation was still investigated as a capturing antigen for the indirect ELISA. Antigen preparation two was adhered to the plates as described in the original protocol (Figures 18a, 18b and 18c). The results clearly show that with the more concentrated antigen preparation (antigen preparation 2) a higher optical density response can be produced. A sigmoidal curve with a plateau region showing antigen saturation and a low background level indicating a minimum amount of non-specific binding was obtained. Therefore it was concluded that the antigen preparation needed to be more concentrated in order to produce a higher optical density response.

Limitations of study

In order to develop a useful and reliable serological test for use in epidemiological studies and/or monitoring of *Salmonella* Brandenburg exposure in New Zealand sheep, it is important that the test is sensitive and specific. The main limitation in this study was the

preparation of a specific *Salmonella* Brandenburg antigen for the indirect ELISA. Because *Salmonella* Brandenburg and *Salmonella* Typhimurium both belong to serogroup B and share common O somatic antigens, the use of commonly used LPS as capturing antigen was thought to be inappropriate. Therefore an antigen preparation was prepared that was mainly composed of flagella and fimbria proteins (Zamora *et al*, 1999). Unfortunately the antigen preparation was of a relatively crude and non-characterised nature and could only produce a reasonable optical density response at a high concentration. Because the antigen preparation had to be highly concentrated, the final volume of the antigen preparation was often minimal and difficult to handle. Despite these difficulties the final antigen preparation was able to produce a reasonable optical density response.

Future Direction

This study has produced an indirect ELISA for the detection of *Salmonella* antibodies. However, the specificity of the ELISA for *Salmonella* Brandenburg antibodies remains in doubt. The various western blot procedures showed inconsistent results, where the inconsistency seemed to be resulting from the use of different sera. Future direction would involve further investigation of the specificity of the antigen preparation, through the use of different sera or through the development of a more pure and specific antigen. Various western blots showed a number of protein bands that were specific for *Salmonella* Brandenburg antibodies. These various proteins could be purified and concentrated to prepare a more pure antigen preparation. Both antigen preparations one and two were of a relatively crude nature and needed to be adhered to the ELISA plates at a high concentration in order to obtain a reasonable optical density response. If the antigen preparation was of a more purified nature, it may produce an improved optical density response due to improved specificity. A reasonable optical density response and improved specificity are both factors that are critical for the successful development of a sensitive and specific serological test for use in epidemiological studies and/or monitoring of *Salmonella* Brandenburg exposure in New Zealand sheep.

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CHAPTER FIVE

CASE CONTROL STUDY

Introduction

Salmonella Brandenburg has been isolated sporadically from cattle, pigs, dogs, and birds within New Zealand for a number of years (Bailey, 1997). In 1996 a Canterbury sheep farm experienced an outbreak of abortions and a number of deaths amongst pregnant ewes (Bailey, 1997). The causative organism was identified as *Salmonella* Brandenburg, an uncommon isolate amongst New Zealand sheep. Since then further outbreaks of *Salmonella* Brandenburg associated disease have occurred in sheep flocks throughout the southern regions of the South Island. Animal Health Laboratory records show that in 1997, 17 farms in mid Canterbury and one in Southland experienced epidemics of abortions and deaths in sheep due to *Salmonella* Brandenburg. In 1998, over 100 farms were affected, and for the first time cases were reported in Otago. In 1999, 2000 and 2001 a total number of 297, 401 and 216 infected farms were seen throughout Canterbury, Otago and Southland (Clark *et al*, 2000). *Salmonella* Brandenburg is now recognised as a common sheep isolate in the South Island. *Salmonella* Brandenburg has also caused major financial loss (Roe, 1999) and has been recognised as a zoonosis (Clark *et al*, 1999), creating greater public awareness of the organism and its ability to cause disease.

Salmonella Brandenburg infection is believed to occur mainly in the autumn/spring period and primarily in pregnant ewes. Abortion usually occurs after three months of gestation and it is more likely to occur in twin and multiple bearing ewes. In an affected flock clinical disease has been shown to take a course of about 16-20 days, with cases peaking about 8-10 days after identification of the first case (Smart, 1999). In a new area, abortion rate and death rate of clinically infected ewes can be reasonably high, with 5-20% of affected flocks aborting, and 10-100% of aborting ewes dying (Clark *et al*, 2000). It is believed that infection of sheep may occur primarily through ingestion of *Salmonella* organisms. Ewes have been shown to excrete *Salmonella* Brandenburg for up to 6 months (Clark, 2000), and *Salmonella* organisms can survive in the environment for up to 3 months (Tannock and Smith, 1971). Furthermore, *Salmonella* is able to survive in dust (Robinson, 1967).

Therefore infection may occur through the licking and smelling of aborted foetuses, ingestion of contaminated pasture or water, or ingestion of sheep yard dust.

Little is known about the specific epidemiology of infection of this disease. There is a definite need to understand the epidemiology of this disease in order to faciliate development of effective control and prevention practices.

The *Salmonella* Brandenburg survey described in this paper involved gathering information from affected (case) and unaffected (control) farms, which allowed comparisons between the two groups of farms. This research project was designed to focus on the identification of environmental, management and animal risk factors associated with *Salmonella* Brandenburg in sheep.

Materials and Methods

Farm selection

Farms were eligible for inclusion in the case control study if they were sheep clients of selected veterinary clinics agreeing to participate. Four veterinary clinics in the southern region of the South Island, all with large numbers of sheep farming clients, and that had previously dealt with *Salmonella* Brandenburg disease, were contacted and asked to participate in the case control study (Appendix V). Three veterinary clinics agreed to partake.

A list of sheep farming clients was obtained from each clinic. Those farms that were known to have experienced *Salmonella* Brandenburg disease (ewe abortion and ewe mortality) in 2000 were identified and defined as case farms. Putative control farms were defined as farms believed not to have been affected by *Salmonella* Brandenburg in the year 2000. All case farms were selected for inclusion in the study. Within each practice region, three times as many putative control farms as case farms were randomly selected with the expectation that some control farms would be re-classified as case farms during the information gathering process. Numbers of case and control farms from each veterinary clinic are shown in Table 4.

Table 4. Numbers of case and control farms from each veterinary clinic.

Veterinary Clinic	Case farms (n)	Control farms (n)	Total
A	24	72	96
B	79	237	316
C	45	135	180
Total	148	444	**592**

Questionnaire

A questionnaire (Appendix VI) was designed to gather information on farm characteristics, flock sizes, numbers of animals affected by disease, management practices and other potential risk factors for disease (at farm and flock levels). Questions regarding *Salmonella* Brandenburg disease in other animal species and humans were also incorporated into the questionnaire.

Data Collection

A total of 592 questionnaires were mailed to farmers throughout Southland and South Otago at the end of the 2000 lambing season, each with an attached letter of explanation and a stamped addressed envelope for return postage of the completed questionnaire. A total of 148 surveys were sent to case farms and 444 surveys to putative control farms. Three reminder letters (Appendix VII) were sent to non-responders, at approximately three-week intervals after the initial questionnaire in an attempt to increase the response rate. The third reminder contained another copy of the questionnaire. Two to four weeks after the third reminder all remaining non-responders were contacted by telephone to ask them to complete the questionnaire and post it to the EpiCentre.

Data Management

Data were entered into a custom designed Microsoft 97 Access® database for subsequent manipulation and analysis. The data were first checked for data quality. Entry errors, implausible data, and data completeness were all assessed. Five percent of the total number of pages for all 405 surveys returned were randomly checked for error in data entry by comparing entered values with the corresponding values on the paper copy of the

questionnaire. This resulted in 223 pages containing a total of 6080 cells being checked. Errors were recorded and corrected. All cells were also checked for implausible data by sorting the data in each variable in both ascending and descending order and checking the extreme values at each end. If an implausible value was found, it was checked against the hard copy of the survey form and corrected if necessary. Finally the database was checked for data completeness. Missing data were assessed for each question by quantifying the percentage of respondents that did not reply to a question or part of a question.

Data Analysis

A descriptive analysis was completed to provide summary statistics for all variables in the data set, giving an overview of the disease situation and the study population for the year 2000. Univariate and multivariate analyses were conducted to assess associations between measures of *Salmonella* Brandenburg disease and various risk factors. Analyses were conducted at two different levels:

1. At the farm level: Farms were defined as cases or controls based on presence or absence of *Salmonella* Brandenburg associated disease during the 2000-year. Analyses were conducted at the farm level to identify possible risk factors associated with the likelihood of a farm being a case farm.
2. Within affected (case) farms: For each affected farm estimates were obtained of the number of sheep in affected flocks, numbers with clinical disease due to *Salmonella* Brandenburg, and numbers dying due to *Salmonella* Brandenburg disease. Analyses were performed within case farms only to identify possible risk factors associated with severity of disease as determined by morbidity and mortality estimates.

Univariate analysis

Unadjusted odds ratios and 95% confidence intervals were produced by entering every independent variable one at a time into a logistic regression model with the appropriate outcome variable. For farm level analyses the outcome variable was a binary variable that coded for case or control (0=control farm, 1=case farm). For analyses within affected farms, a binomial outcome variable was produced measuring the proportion of mated ewes on the farm that experienced disease (recovered plus dead). Any variable with a wald statistic p-value of less than 0.25 in the univariate screening models, was retained for inclusion in the multivariate model.

Multivariate analysis

All selected variables from univariate analyses at the farm level and within affected farms were entered into initial multivariate models. A backward stepwise selection process was employed for model development. At each step the variable with the highest wald statistic p value was omitted and the model re-calculated. This was continued until the final main effects model only included variables that were significant using a threshold p-value for inclusion of 0.1. Biologically plausible two-way interactions were then added one at a time to the model and retained only if they were associated with a p-value less than 0.1. D-scale corrections were applied to logistic regression outputs to account for clustering in the data (McDermott and Schukken, 1994). Separate models were generated for the farm level analyses and for the within affected farm level analyses. Standardised residuals from each model were generated and examined for unusual patterns and extreme values. Data points associated with large standardised residuals were checked against the original paper questionnaire for accuracy and validity. Model fit for final models was assessed using the Hosmer-Lemeshow goodness of fit statistic and the ratio of the deviance to the degrees of freedom for the model. Analyses were performed in SPSS version 10.1® for Windows and SAS Proc GENMOD (SAS version 8.1® for Windows).

Results

Data collection

Of the 592 surveys sent out, 83% of the case farms and 73% of the control farms replied, with an overall response rate of 75%. A total of 405 respondents were eligible for inclusion in the case control study, while 40 farms were discarded. Discarded respondents included farmers that did not wish to partake in the study, farmers that were no longer sheep farmers or farmers that had limited sheep stock numbers (lifestyle blocks). Fifty-four of the putative control farms that were believed to be unaffected with *Salmonella* Brandenburg in 2000 season, were reported by respondents to have been affected. These farms were re-defined as case farms and the resulting final distribution of respondent farms between cases and controls are presented in Table 5.

Data management

Data Quality

Of the 223 pages containing 1582 data cells that were randomly checked for data entry error, 30 cells (2%) contained data entry error or digit transposition error.

Missing data

A range of 4 to 12 risk factors within the Hogget, Two-tooth and the Mixed Age ewe data were excluded from analysis because they contained more than 30% missing data (Appendix VIII).

Data Analysis

Table 5 shows the final number of case and control farms, and ewe populations in the dataset used for all analyses.

Table 5. Summary of sheep numbers on respondent farms

Summary	Farm (n)	Hogget (n)	TT and MA (n)
Case	176	108,324	449,232
Control	229	126,990	504,186
Total	405	235,314	953,418

Of the 176 affected farms, 8 farms (4.5%) reported *Salmonella* Brandenburg disease in hoggets, with an average risk of 5.3% for abortions and 1.0% for ewe deaths. In contrast, 78 (45.1%) and 172 (97.1%) of affected farms reported outbreaks in two-tooth (TT) and mixed age (MA) ewes respectively. The risk of ewe death was 2.5% for TT ewes and 2.4% for MA ewes (Table 6). Table 7 shows the presence and absence of clinically diseased sheep in different age classes.

Table 6. Number of sheep in different age classes affected and dying on farms reporting *Salmonella* Brandenburg disease during 2000.

Summary	Farms (n)	Ewes in affected mob (n)	Diseased N (%)	Death n (%)
Hogget	8	3,115	162 (5.2)	31 (1.0)
Two Tooth	78	55,890	3130 (5.6)	1397 (2.5)
Mixed Age	172	272,425	11174 (4.3)	6538 (2.4)

Table 7. Presence (+) or absence (-) of clinically diseased sheep in different age classes on 405 farms in the South Island of New Zealand.

Mixed Age	Two Tooth	Hogget	Farm (n)
+	+	+	5
+	+	-	69
+	-	+	2
+	-	-	96
-	+	+	1
-	+	-	3
-	-	+	0
-	-	-	229
			Total 405

Univariate analysis

Twelve to nineteen risk factors were found to be significantly associated ($P<0.25$) with the occurrence or severity of disease (Appendix IX).

Multivariate analysis

Table 8. Logistic regression analysis of factors associated with occurrence of *Salmonella* Brandenburg disease in mixed age ewes.

Variable	Category		P Value	OR (95% CI)
Main effects:				
Ewe total (unit = 100 ewes)	*Continous*		<0.001	1.04 (1.02-1.06)
Strip grazing	*No*		-	1
	Yes and no BF		0.002	6.07 (1.97-18.67)
	Yes and BF		<0.001	9.79 (4.07-23.51)
Crop	*Not fed*		-	1
	Fed		0.008	0.41 (0.22-0.79)
OR for interaction terms (reporting the effect of terrain in each level of crop):				
Crop*Terrain	*No Crop*	*Flat Terrain*		1
		Hill Terrain		0.347 (0.21-0.73)
	Fed Crop	*Flat Terrain*		1
		Hill Terrain		0.895 (0.28-2.42)

BF=Backfencing

Table 9. Logistic regression analysis of factors associated with occurrence of *Salmonella* Brandenburg disease in t wo-tooth ewes.

TT Risk Factor	Category	P Value	OR (95% CI)
Strip grazing	*No*	-	1
	Yes - no BF	0.385	1.59 (0.56-4.54)
	Yes - BF	0.001	3.37 (1.64-6.96)

Table 10. Logistic Regression analysis of factors associated with reported severity of *Salmonella* Brandenburg disease in mixed age ewes on affected farms

Variable	Category		P Value	OR (95% CI)
Main effects:				
Time of shearing	>*July*		0.025	0.63 (0.42-0.94)
	<*July*		-	1
Time of ram removal from ewes	>*June*		0.081	1.43 (0.96-2.14)
	<*June*		-	1
OR for interaction terms (reporting the effect of vaccination in each level of time of shearing) :				
Shear*Vaccinate	*Shear > July*	*No Vaccination*		1
		Vaccinated		0.78 (0.34-1.83)
	Shear < July	*No Vaccination*		1
		Vaccinated		0.36 (0.19-0.70)

BF=Backfencing

Table 11. Logistic Regression analysis of factors associated with reported severity of *Salmonella* Brandenburg disease in Two-tooth ewes on affected farms

Variable	Category		P Value	OR (95% CI)
Main effects				
Vaccinated against Salmonella	*Yes*		0.003	0.47 (0.28-0.77)
	No		-	1
Time of crutching	*>July*		0.041	0.61 (0.37-0.98)
	<July		-	1
Strip grazing	*No*		-	1
	Yes - no BF		0.063	5.14 (0.92-28.9)
	Yes - BF		0.093	3.89 (0.80-18.97)
Hay	*Fed*		0.019	4.42 (3.01-6.47)
	not fed		-	1
OR for interactions (reporting the effect of total # of yardings in each level of hay):				
Total Yardings*Hay	*Fed hay*	*Total # of yardings*		0.64 (0.22-1.82)
	No hay	*Total # of yardings*		0.99 (0.68-1.48)

Discussion

This research has identified several key risk factors that appear to be associated with the occurrence and or severity of *Salmonella* Brandenburg disease. Statistical analyses were performed separately for Mixed age, Two-tooth and Hogget ewes that were lambing in the 2000 season, because of possible differences in disease epidemiology and management methods for different age classes. Logistic regression analyses focussed on MA and TT data only, as very few farms had *Salmonella* Brandenburg infection in their Hoggets (Table 6), no farm had hogget infection alone (Table 7), and information relating to Hoggets had more missing data (Appendix VIII).

At the farm level, strip grazing with or without backfencing (BF) and increased stocking numbers appeared to increase the risk of disease in sheep. Both of these factors are likely to be associated with intensively managed farms. Intensive farming methods often result in

high stress levels in stock and consequently may increase shedding of *Salmonella* organisms (Barham *et al*, 2002). Therefore the practice of strip grazing with or without back fencing and/or running a sheep mob at higher stocking density may expose sheep to increased levels of stress and exposure to potential pathogens. Stress has been recognised as a pre-disposing factor for *Salmonella* shedding and infection of animals (Clegg *et al*, 1983; Littlewood, 1984), where it has been related to a number of different factors. Examples of potentially stressful management procedures that may increase *Salmonella* shedding and infection include: sudden changes in nutrition (MacDonald and Brown, 1974; Sayed *et al*, 1998), holding sheep in yards, transportation (Neilson *et al*, 1985; Barham *et al*, 2002; Groenstoel *et al*, 1974), high stocking rates (Bruere and West, 1993), and severe weather conditions (Hunter and Izsak, 1990; MacDonald and Brown, 1974). Feeding crop and having hilly terrain appeared to decrease the risk of disease within the mixed age ewes. While feeding crop is considered to be another form of controlled winter feeding, crop fields are often not back-fenced and the sheep are not subjected to frequent movement. This may reduce the amount of stress associated with this feeding management practice relative to strip grazing. The protective effect of hilly terrain observed could also be representative of less intensive farm management, since hilly terrain is often not managed as intensively as flat. In the 1970s and 80s an effect of terrain was reported in *Salmonella* Montevideo abortion storms in sheep in Scotland. Less intensive farming in the West seemed to present fewer opportunities for disease introduction and dissemination than the more intense farming in the East (Reilly *et al*, 1985). The interaction of crop feeding and terrain is consistent with different farm management practices. Hilly terrain appeared more protective in farms that fed no crop than in farms that fed crop. It is likely that the combination of hilly terrain and no crop feeding are representative of farms that are managed in a less intensive manner than flat terrain farms that feed crop. While farming on hilly terrain appears to be associated with less intense farming, any increase in intensity of farming methods, such as feeding crop, appears to increase the risk of disease. While the interaction term is marginally significant (P=0.081), both variables appear to contribute more to the risk of disease as independent main effects based on the lower P-values associated with the main effect terms compared to the interaction term.

Additional analyses were performed to investigate risk factors influencing the severity of disease within affected farms. Major findings included an increase of severity of disease

with controlled winter grazing, the feeding of hay and the removal of rams after June. As previously mentioned, stressful conditions may result from controlled winter-feeding and predispose the ewes to severe disease. Supplementary feed, such as hay, and the removal of rams after June may also be related to stress. Supplementary feed is often offered when pasture feed levels are scarce or of poor quality and sheep are therefore under nutritional stress. Feeding of contaminated hay, may be another possible reason for increased disease severity (Robinson, 1970). Feeding of hay may also lead to the clustering of animals, allowing for easier spread of infection. The removal of rams requires yarding and handling and there may be some importance in the temporal relationship between the timing of this event and either exposure to the causative organism or susceptibility to development of clinical disease in already exposed animals. Yarding of animals, which can also be generally stressful, has previously been found to be associated with *Salmonella* infection (Neilson *et al*, 1985). Both these factors may therefore be indirectly related to the severity of disease.

Vaccination against *Salmonella* with killed vaccines Salvexin® or Salvexin B+® (Schering Plough) for both Mixed age and Two-tooth ewes appeared to be protective. A field survey conducted in 2000 showed that vaccination of Two-tooths and Mixed age ewes with both Salvexin® and Salvexin B+ ® reduced the incidence of disease and deaths when *Salmonella* Brandenburg disease occurred (Marchant *et al*, 2002). Our results also indicate that vaccination does appear to reduce the severity of disease within an affected farm. Yarding, pre-lamb shearing and winter crutching of ewes would be generally viewed as stressful events for sheep; however, pre-lamb shearing and winter crutching appeared to reduce the severity of disease for both mixed age and two-tooth ewes. This result appears to be in contrast to the other findings, as stress generally appears to be increasing the risk of occurrence or severity of disease. While pre-lamb shearing/crutching is a stressful event, ewes are often given better shelter and are fed well post-shearing and these influences may explain the reduction in the severity of disease. This finding is consistent with a previous report indicating that supplementary food given to sheep infected with *Salmonella* Dublin appeared to reduce disease (Baker *et al*, 1971).

The effect of vaccination on disease severity in affected mixed age ewe flocks was influenced by timing of shearing as evidenced by the interaction between these two variables. Caution should be used in interpreting the interaction term since the P-value was

higher than those reported for the main effects of vaccination and shearing. Vaccination did not appear to be effective in those flocks that shore ewes after July while it was effective at reducing disease severity in flocks shorn before July. Shearing ewes closer to lambing is associated with a protective main effect and the reduction in disease severity due to this effect may be responsible for the inability to detect a protective effect of vaccination in these animals. Vaccination and crutching after July was associated with reduced disease severity in affected Two-tooth ewe flocks though shearing was not found to be influential.

As previously indicated strip grazing was associated with a tendency for increased disease severity and this is considered likely to be mediated by either stress-associated mechanisms or increased exposure. Feeding of hay increased the severity of disease in affected flocks. This effect may be mediated by hay quality and contamination or by factors such as feeding on contaminated ground or in high stocking density areas and once again stress and exposure are likely to be involved. In those flocks that fed hay and were yarded more often, disease severity tended to be reduced compared to flocks that yarded less often. Increased number of yardings for a flock may be an indirect indicator of the general level of farm management and animal observation. The main effect of number of yardings is non-significant and any effect attributable to yardings appears to be expressed mainly in those flocks that fed hay.

Since the initial outbreak of *Salmonella* Brandenburg disease in New Zealand sheep in 1996, various reports have identified possible factors associated with *Salmonella* Brandenburg disease. In the past *Salmonella* transmission has been shown to occur through foodstuffs (Al-Hindawi and Taha, 1979), pasture, water (Hunter and Izsak, 1990; Robinson, 1970), and dust (Robinson, 1967). Black backed gulls (scavengers), sourced from affected and non-affected farms have been shown to carry *Salmonella* Brandenburg organisms in their intestinal contents and may have acted as potential reservoirs of infection (Clark *et al*, 1999). Other studies have shown increased environmental contamination with organisms isolated from rivers (Keller, M. *per comm.*) and sheep yard dust (Clark, 2000). Environmental stress factors such as severe winters have also been implicated in the association of *Salmonella* outbreaks (Hunter and Izsak, 1990). All of these additional factors were not considered in the case control study due to limitations in availability or collection of data.

The use of a case control approach, as described in this paper, facilitates rapid and cost effective collection of data in the preliminary investigation and understanding of disease epidemiology. Retrospective case control studies may be subject to recall bias, and findings of association do not necessarily indicate causality. Caution is therefore prudent when drawing conclusions from this study. The high response rate for this survey clearly shows a high level of farmer concern about the disease and is considered to support the findings of this study as being representative of the sheep farming population of the southern regions of the South Island of New Zealand.

This study has identified a number of risk factors associated with the occurrence or severity of disease that appear to be related either directly or indirectly to stress or exposure mechanisms. Many of the main effects found are associated with intensive farming conditions, such as controlled winter feeding, high stocking numbers and farming on flat terrain. Other related stressful events include yarding of sheep for various management practices such as shearing or removal of rams. Unfortunately many of the risk factors found are common farm management methods. Measures to reduce animal stress and minimise exposure to potentially contaminated environments may help to reduce the risk or severity of disease. The strongest associations seen in the final models were those factors relating to controlled winter grazing. Strip grazing gave the highest odds for disease and therefore may have a causal association with the disease. Because strip grazing is a common farming practice, it would be unreasonable to discourage the practice. Instead a more detailed understanding of the various management methods is needed to gain a better understanding of the relationship between management practices and disease risk. In this way modifications in management practices may be identified that can reduce disease risk while maintaining farm productivity.

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OVERALL CONCLUSION

Little is known about the specific epidemiology of *Salmonella* Brandenburg in New Zealand sheep. As a result, control of this disease has been largely based on subjective evidence and general epidemiological principals. This study focused on two different aims, where both focused on a common goal: to provide information to help produce control and prevention methods for *Salmonella* Brandenburg disease within New Zealand sheep.
The aims were as follows:

- To develop a serological test for use in epidemiological studies and in monitoring future control efforts targeting *Salmonella* Brandenburg in New Zealand sheep.
- To identify factors associated with the occurrence and severity of *Salmonella* Brandenburg outbreaks in New Zealand sheep.

The development of an ELISA test for detecting antibodies to *Salmonella* Brandenburg organisms in sheep plasma/serum was thought to be a potential alternative method, compared to bacteriological culture, for the diagnosis of *Salmonella* infection. It is a quick and potentially sensitive and specific test that could be utilised successfully as a serological test for use in epidemiological studies and in monitoring control programs.

Because various diagnostic serological techniques have resulted in a high level of antibody cross-reactivity, an antigen preparation mainly composed of flagella and fimbria proteins was prepared in order to try and reduce possible specificity problem. The antigen preparation was of a relatively crude and non-characterised nature and could only produce a reasonable optical density response at a high concentration. Unfortunately while the ELISA was responsive, various specificity tests showed inconsistent results. A reasonable optical density response and specificity are both factors that are critical for the development of a sensitive and specific serological test. Therefore, further investigation of the specificity of the antigen preparation, through the use of different sera or through the development of a more pure and specific antigen is needed in order to produce a successful serological test for use in epidemiological studies and/or monitoring of *Salmonella* Brandenburg exposure in New Zealand sheep.

A case control study was undertaken in order to identify factors associated with *Salmonella* Brandenburg disease in New Zealand sheep. Details of disease prevalence and farm management methods were collected and associations between possible risk factors and *Salmonella* Brandenburg were evaluated using odds ratios. This study identified a number of risk factors associated with the occurrence or severity of disease that appear to be related either directly or indirectly to stress or exposure mechanisms. Many of the main effects were found to be associated with intensive farming conditions. Measure to reduce animal stress and minimisation of exposure to potentially contaminated environments may help reduce the risk or severity of disease. Unfortunately, many of the risk factors found were also common farm management methods, such as controlled winter grazing. It would be unreasonable to discourage these practices and therefore further research into various management methods is needed in order to obtain a better understanding of various management practices and disease risk.

This study produced a range of information and contributed meaningfully to the understanding of the epidemiology of *Salmonella* Brandenburg infection in New Zealand sheep. Further research is required to maintain progress towards identification and implementation of effective prevention and control mechanisms for this disease.

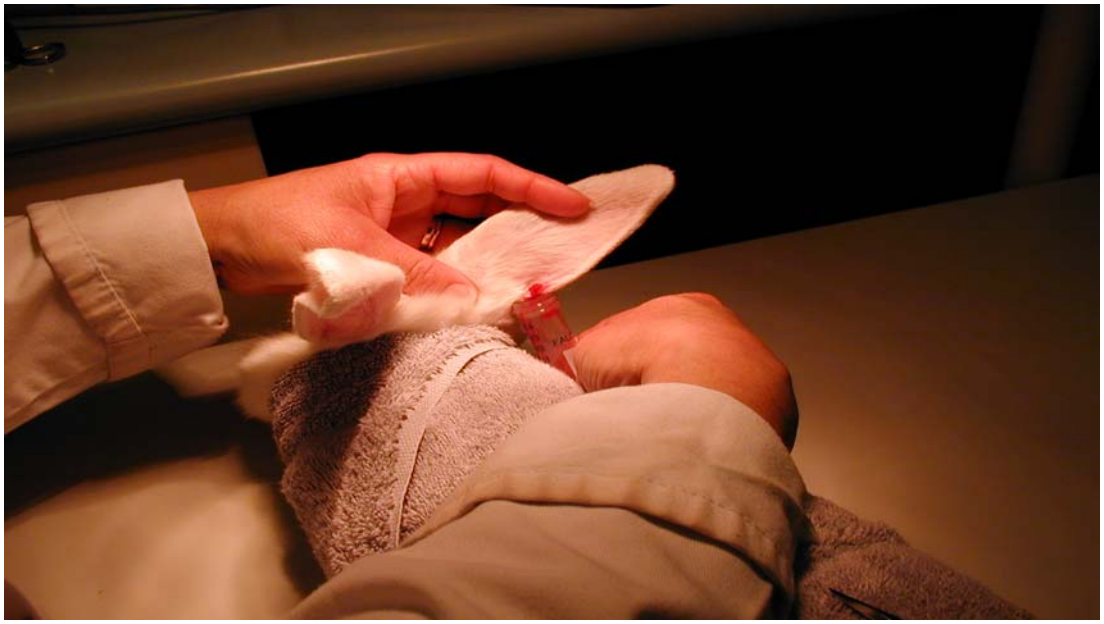
APPENDICES

Appendix I

Housing of experimentally infected rabbits at SAPU (Small Animal Unit)

Appendix II

Bleeding of experimentally infected rabbits from the marginal ear vein.



Appendix III

Equipment and Materials used in the development of a serological test for *Salmonella* Brandenburg.

Protein Concentration

Equipment

Varian Spectrophotometer (Cary-10)

Materials

Bradford reagent

100mg Coomassie Blue G-250 dissolved in 50mL of 95% ethanol, mixed with 100mL 85% phosphoric acid, diluted to 1L with milli-Q water.

Protein Concentration standards

0.05, 0.10, 0.20, and 0.40 mg/mL BSA (Bovine Serum Albumin) standards

Electrophoresis

Equipment

Mini-PROTEAN® Electrophoresis Cell

Materials

Resolving gel

3.5mL distilled water

2.5mL 1.5M Tris-HCl

100µL 10% SDS

4mL Acrylamide/Bis (30%)

100µL Ammonium persulphate (10%)

5µL Temed

Stacking gel

6.1mL distilled water

2.5mL 0.5M Tris-HCl, pH 6.8

100µL 10% SDS

1.3mL Acrylamide/Bis (30%)

100µL Ammonium persulphate (10%)

10µL Temed Need ingredients

Electrode buffer

80mL of 5X electrode buffer with 450mL of distilled water

Staining solution

0.1% (w/v) Coomassie Brilliant Blue R250 (40% methanol / 10% acetic acid in water)

Western blot

Equipment

Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad)

Materials

Transfer buffer

10mM of CAPS (Sigma®) transfer buffer was prepared by adding 2.213g of CAPS to 1 L of distilled water. The pH was adjusted to pH 11.

TBS pH7.4

20mM of Tris HCl and 500mM of NaCl was added to 1.5L of deionised water. The pH was adjusted to pH 7.5 using HCl before adding distilled water to a volume of 2L.

Washing buffer

500µL of Tween 20 was added to 1L of TBS and stored at 4ºC.

Blocking Buffer

5% of skim milk was added to the washing buffer.

Primary Antibody

Serum from *Salmonella* affected or unaffected sheep/rabbits.

Secondary antibody

Anti-Sheep IgG (whole molecule) Peroxidase conjugate developed in donkey (Sigma®)

Anti-Rabbit IgG (whole molecule) Peroxidase conjugate developed in goat (Sigma®)

Antibody dilutient

Blocking buffer

Substrate

4-Chloro-1-Napthphol 30mg Tablets (Sigma®)

ELISA Development

Equipment

ELISA plate spectrophotometer (Anthos htll)

Materials

Plates

96 Well Microtitre Plate (Greiner®)

Coating buffer

Carbonate-BiCarbonate Buffer Capsules (Sigma®)

Dissolved one capsule in 100mL of de-ionised water for 0.05M buffer, pH 9.6.

Washing buffer

Tween 20 (Sigma®)

Phosphate Buffered Saline, pH 7.4 (Sigma®)

One sachet was dissolved in 1L of water and 200µL of Tween 20 was added.

Blocking buffer

Gelatin (Type A: From Porcine Skin) (Sigma®)

Gelatin was added to sufficient volume of coating buffer to achieve the desired concentration eg 0.04g of gelatin added to 80mL of coating buffer to produce 0.05% solution. Gentle heat was used to facilitate dissolving of gelatin in the buffer.

Antibody dilution

Antibodies were diluted in blocking buffer.

Primary antibody

Sera from *Salmonella* Brandenburg affected/unaffected sheep/rabbits were used.

Secondary antibody

Anti-Sheep IgG (whole molecule) Peroxidase conjugate developed in donkey (Sigma®)

Anti-Rabbit IgG (whole molecule) Peroxidase conjugate developed in goat (Sigma®)

Substrate buffer

Phosphate-Citrate Buffer Tablets (Sigma ®)

One tablet was dissolved in 100mL of de-ionised water.

Substrate

o-Phenylenediamine 20mg Dihydrochloride Tablets (Sigma®)

The substrate was prepared immediately before use and in wrapped tinfoil to avoid degradation caused by UV rays. 1 OPD tablet was added to 50mL of substrate buffer and was followed by the addition of 20µL of 30% H_2O_2.

Stopping solution

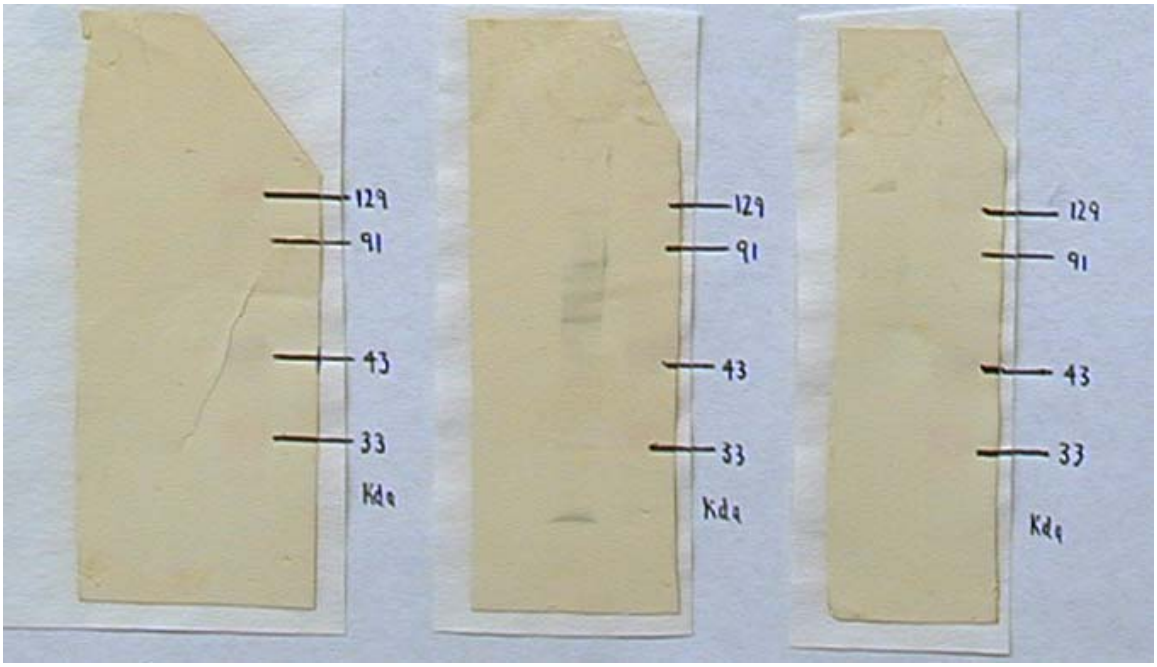
2.5M H_2SO_4 was prepared in distilled water.

Appendix IV

Western Blots of Antigen Preparations in Order to Determine Specificity

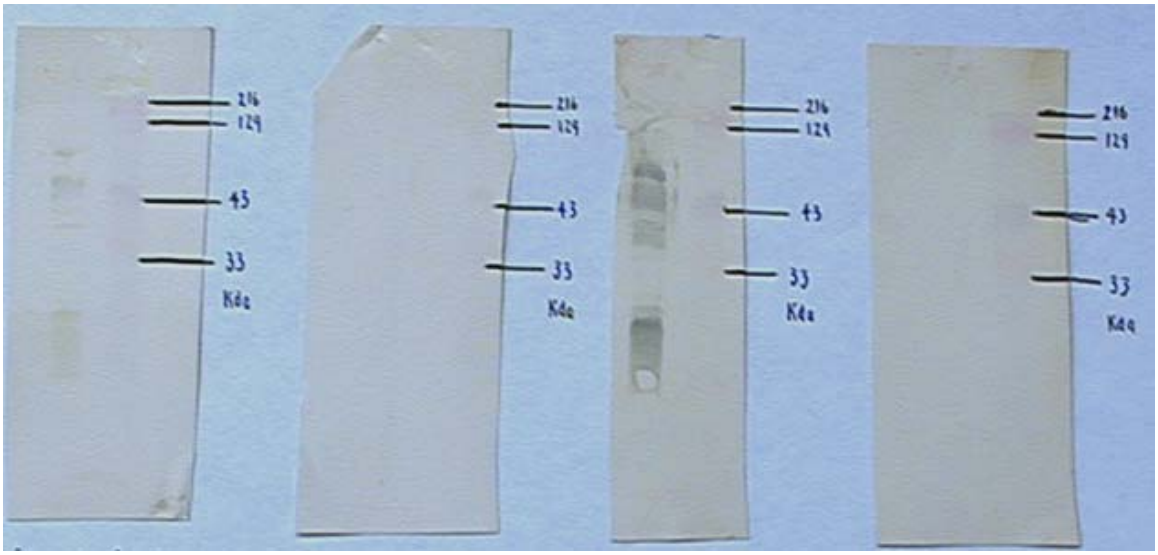
A. Western Blot of Antigen Preparation one (AP1) incubated in a 1/50 dilution of serum obtained from a previous vaccine trial.

Left membrane: incubated in serum containing no *Salmonella* Brandenburg antibodies (negative control).
Middle membrane: incubated in serum containing *Salmonella* Brandenburg, *Salmonella* Typhimurium and *Salmonella* Hindmarsh antibodies.
Right membrane: incubated in anti-serum containing *Salmonella* Typhmiurium and *Salmonella* Hindmarsh antibodies.



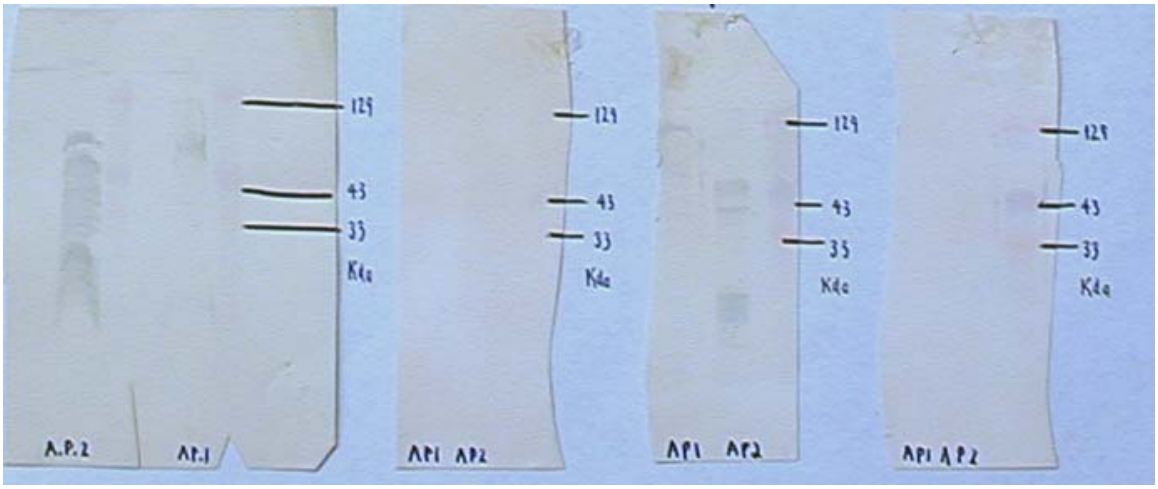
B. Western Blot of Antigen Preparation Two (AP2) incubated in 1/50 dilution of serum obtained from rabbits experimentally challenged with specific *Salmonella* serotypes.

First membrane: incubated in anti-serum containing *Salmonella* Brandenburg antibodies.
Second membrane: incubated in anti-serum containing *Salmonella* Hindmarsh antibodies
Third membrane: incubated in anti-serum containing *Salmonella* Typhmurium antibodies.
Fourth membrane: incubated in serum containing no *Salmonella* antibodies.



C. Western Blot of Antigen Preparation One (AP1) and Two (AP2) incubated in 1/50 dilution of serum obtained from rabbits experimentally challenged with specific *Salmonella* serotypes.

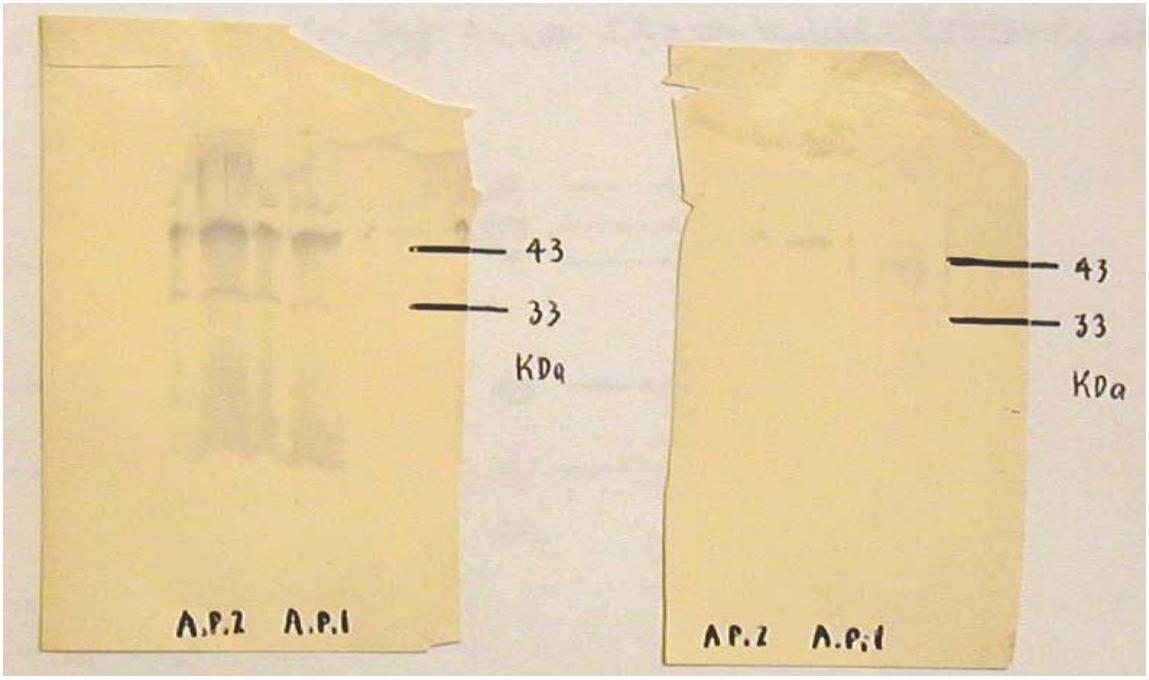
First membrane: incubated in anti-serum containing *Salmonella* Brandenburg antibodies.
Second membrane: incubated in anti-serum containing *Salmonella* Hindmarsh antibodies
Third membrane: incubated in anti-serum containing *Salmonella* Typhmurium antibodies.
Fourth membrane: incubated in serum containing no *Salmonella* antibodies.



D. Western Blot of Antigen Preparation one (AP1) and two (AP2) incubated in 1/50 dilution of serum obtained from a previous vaccine trial.

Left membrane: incubated in anti-serum containing *Salmonella* Brandenburg, *Salmonella* Typhimurium, *Salmonella* Hindmarsh antibodies.

Right menbrane: incubated in anti-serum containing *Salmonella* Typhmurium and *Salmonella* Hindmarsh.



Appendix V

Veterinary clinics that participated in the *Salmonella* Brandenburg case control survey.

Central Southland Veterinary Services
Clinic Moores Road
WINTON
03 236 8115

Gore Vets
22 Traford St
GORE
03 208 9020

Clutha Vets Animal Health Centre
BALCLUTHA
03 418 1280

Appendix VI

Questionnaire used in the Salmonella Brandenburg case control survey.

Salmonella brandenburg in New Zealand sheep
Questionnaire by the EpiCentre, Massey University

This questionnaire is one of a series of research projects with the following goal:

to assist in the development and implementation of practical and cost-effective strategies for prevention and control of *S.brandenburg* disease on New Zealand sheep farms.

Why do we need to research this issue?

- On farm losses: S.brandenburg is continuing to cause devastating losses in the sheep industry.
- Market access: NZ lamb shipments have been rejected by European countries.
- Human health: Cases of disease due to S.brandenburg have increased dramatically.
- We need to know more about this disease. Effective control depends on a better understanding of what happens on affected and unaffected farms.

How will another questionnaire help?

- Most previous questionnaires have concentrated on farms where the disease has occurred. This is only half the story. We need detailed and accurate information from **farms which have experienced the disease and** from **farms which have not experienced the disease**. This approach will help explain why some farms experience the disease and others do not and also why the severity of disease on affected farms is so variable.
- The results of this questionnaire will be used to help develop control and prevention strategies.

What else is being done?

- This project is part of a large scale initiative investigating multiple aspects of S.brandenburg disease and incorporating efforts from farmers, industry bodies, government agencies, practising veterinarians and scientists.

Completing the questionnaire

Your assistance will help the industry to achieve the goal outlined above. Please take the time to complete this questionnaire and mail it back in the enclosed, prepaid envelope.

It is especially important that you complete the questionnaire even if you did not experience any disease due to S.brandenburg this year.

Confidentiality of information supplied

We give an undertaking that all information will be treated by us as strictly confidential. No information will be used in any way that could reasonably be expected to identify any individual farm or flock or farmer.

Key contacts:

Nigel Perkins BVSc, MS, FACVSc (project leader) and Joanne Kerslake BSc
EpiCentre, Massey University
Phone: 06 350 5270 Fax: 06 350 5716
email: N.R.Perkins@massey.ac.nz

Salmonella brandenburg questionnaire

1. Date form completed

2. Property or station name

3. Name of farm owner

4. Name of farm manager (person responsible for day to day decisions) – if different to farm owner in question 2 above:

5. Location address

Please do not give a PO Box number or Rural Delivery number.

road name where farm is located

nearest town or locality

6. Contact phone, fax and email

phone

mobile phone

fax

email

7. What is the land area of the farm in hectares?

8. Please indicate if the farm consists of more than one block of land geographically separated by land owned by other persons.

Yes (more than one block) ☐ No (single land unit) ☐

9. What is the approximate land area of the farm used for sheep farming? (hectares)

☐

10. Estimate the number of sheep wintered on the farm in 2000.

	Rams (2-tooth and over)	Ewe hoggets		Ewes (2 tooth and over)		Ram and wether hoggets	Wethers (2-tooth and over)
		Put to ram in 2000	Dry- not put to ram in 2000	Put to ram in 2000	Dry- not put to ram in 2000		
Romney							
Coopworth							
Perendale							
Corriedale							
Halfbred							
Merino							
Borderdale							
Drysdale							
Border leicester							
Cheviot							
South suffolk							
Suffolk							
Texel							
Poll Dorset							
Finn							
Other							

11. Did you purchase ewe replacements during the 2000 season?

Ewe age class	**Yes**	**No**	**From where (please tick)**	
			another property	**saleyard**
Hoggets				
2-Tooths				
Mixed age ewes				

12. Estimate the numbers of farmed animals other than sheep which wintered on the farm during 2000.

Cattle breed	number of young < 12 mnths old	Heifers >12 mnths old	Cows	Steers >12 mnths old	Bulls
Dairy breeds					
Beef breeds					

Deer	number of young < 12 mnths old	Hinds	Stags

Pigs	breeding sows	

Farmed Birds	broilers	
	laying hens	
	geese	
	ducks	
	turkeys	
	other	

Working farm dogs	Total number	

Horses	Total number	

Other (please list)	Total number

13. When did mating start and finish during the 2000 season?

Ewe age class	Date rams went in	Date rams came out	Not mated this year
Hoggets	/ / 2000	/ / 2000	
2-Tooths	/ / 2000	/ / 2000	
Mixed age ewes	/ / 2000	/ / 2000	
	Record the date		Tick the box

14. Please record hogget and ewe shearing and crutching dates for 2000.

Hogget	Dates for hoggets
Crutching	/ / 2000
	/ / 2000
Shearing	/ / 2000

Ewes	Dates for ewes
Crutching	/ / 2000
	/ / 2000
Shearing	/ / 2000

15. Types of terrain where pregnant ewes graze during the period from start of mating to lambing (you may tick more than one box)

Type of grazing land	Tick the box
mainly flat to rolling downlands	
mainly moderate to steep hill country	
mixture of both flat and hill country	

16. Sources of water for pregnant ewes during the period from start of mating to lambing (you may tick more than one box).

Water source	Tick the box
running water (stream, creek, river)	
surface water (rain water run off) in a dam	
dam water reticulated into troughs	
underground water (artesian, spring)	
town water	
no water provided	
other	

17. Do you share reticulated water with adjacent farms?

Yes	
No	

18. What is the dominant pasture type used for feeding pregnant ewes through the winter up to lambing?

19. Please indicate whether you feed supplementary feed to ewes during pregnancy. (You may tick more than one box).

Feed type	Ewe age class			Indicate type or brand	Months when fed	Fed on ground?
	Hogget	2-Tooth	Mixed age ewes			
none fed						
Hay						Y / N
Haylage						Y / N
Silage						Y / N
Grain						Y / N
Crop						Y / N
Sheep nuts						Y / N
Other						Y / N
	Tick the box					Circle

20. Do you strip graze pregnant ewes between mating and lambing?

Ewe age class	Yes	No
Hogget		
2-Tooth		
Mixed age ewes		

21. If you answered yes to question 20, please provide details about your strip grazing practices.

Ewe age class	Date strip grazing began	Do you back fence?		Ave no. of days per shift			Date set stocking resumed before lambing
		Yes	No	June	July	August	
Hogget	/ / 2000						/ / 2000
2-Tooth	/ / 2000						/ / 2000
Mixed age ewes	/ / 2000						/ / 2000
				approx number of days per shift			

22. Did you vaccinate breeding ewes against Salmonella in 2000? (Tick the box)

Ewe age class	Yes	No
Hoggets		
2-Tooths		
Mixed age ewes		

23. If you answered Yes to question 22, please provide details of vaccinations used and whether you gave one or two injections. If you vaccinated all animals in one age class (ie all 2-Tooth ewes), record All in the space for number of animals. If you vaccinated only a portion of the ewes in one age class, please record the number of animals vaccinated.

Ewe age class	Details	Product used		
		Salvexin only	Salvexin+B only	Salvexin and Salvexin+B
Hoggets	number of injections (1 or 2)			2
	number of animals			
2-Tooths	number of injections (1 or 2)			2
	number of animals			
Mixed age ewes	number of injections (1 or 2)			2
	number of animals			

24. If you vaccinated ewes against Salmonella, please indicate when the last vaccine was administered.

Ewe age class	During mating	Before lambing		During lambing (September)
		Before August 1	During August	
Hoggets				
2-Tooths				
Mixed age ewes				

Please tick the box

25. Record the number of times ewes were yarded and the main reasons for yarding, during the months of June, July and August of 2000.

Month	Ewe age class	Number of yardings (0, 1, >1)	Main reason(s) shearing, crutching, drench, vacc, dip/jet, drafting
June	Hoggets		
	2-Tooths		
	Mixed age ewes		
July	Hoggets		
	2-Tooths		
	Mixed age ewes		
August	Hoggets		
	2-Tooths		
	Mixed age ewes		

26. Did you have any abortions in pregnant ewes in the 2000 lambing season?

Yes	
No	

if no, go to question 31

27. If you answered YES above, did any of the aborting ewes die from the same disease, around the time of abortion?

Yes	
No	

28. Was *Salmonella brandenburg* diagnosed as a cause of the abortions/deaths?

Yes	
No	

29. If S.brandenburg was diagnosed as a cause of disease this year, how was the diagnosis made?

Method of diagnosis of S.brandenburg	Tick the box
Veterinarian + lab samples	
Veterinarian without lab samples	
Farmer diagnosis based on the following signs	tick below
rotting aborted lambs	
sick & dying ewes	
Other (please specify)	

30. Please estimate the number of ewes affected by disease due to S.brandenburg during the 2000 season.

	Hogget	2-Tooth	Mixed Age
Total number of ewes in affected mobs			
Total number of ewes aborted & survived			
Total number of ewes aborted and died			
Total number of ewes which died before aborting			
When did brandenburg abortions start?	tick the appropriate box below		
1st half of July or earlier			
2nd half of July			
1st half of August			
2nd half of August			
1st half of Sept			

31. Did you have S.brandenburg disease in your ewe flock during the last 3 lambing seasons?

Did S.brandenburg disease occur ?		Ewe age class		
		Hogget	2-Tooth	Mixed age ewes
in 1999	Yes			
	No			
	Don't know			
in 1998	Yes			
	No			
	Don't know			
in 1997	Yes			
	No			
	Don't know			

tick the box

32. If you experienced S.brandenburg disease in your ewe flock last year (1999 lambing season), what did you do with ewes which aborted and then recovered?

	Hoggets	2-Tooth	Mixed age ewes
Kept all surviving ewes to breed this year			
Culled some but not all			
Culled all ewes which aborted or were ill			

tick the box

33. Do you suspect or know that S.brandenburg associated disease occurred in any other animal types on the farm during the 2000 season?

Animal	Disease present ?	S.brandenburg confirmed by lab?	List major clinical signs of disease
Calves up to 12 months			
Other cattle			
Dogs			
Deer			
Pigs			
Poultry			
Horses			
Cats			
Other			

tick the box

34. How many people live and work on the farm?
Include family members and children if they live on the farm. Include all permanent and casual labourers who work on the farm on a regular basis but exclude short term and contract labour such as fencers, shearers etc.

35. Of the people covered by question 34, how many work or interact regularly with sheep?

36. Of the people covered by question 34, have any experienced gastrointestinal disease (nausea, vomiting, or diarrhoea) during 2000?

Yes	
No	

if No, go to question 40.

37. If the answer to 36. was YES, indicate when the disease occurred.

Person	Month disease occurred
1	
2	
3	
4	

38. Please provide information regarding the severity of the disease in affected people.

Person	Age (years)	Duration of illness (days)	Days off work or school	Received medical treatment	Required hospitalisation	S.brandenburg diagnosed by doctor
1						
2						
3						
4						
				Tick the box where appropriate		

39. Please indicate the level of contact with sheep for each person who has experienced gastrointestinal disease in 2000. Tick the box where appropriate.

Person	None	Occasional	Frequent
1			
2			
3			
4			

40. We are very interested in receiving your comments and observations on your experiences with Salmonella brandenburg. Issues of particular interest to this project include your ideas and observations about those factors which you feel may be involved in the spread of the disease <u>and</u> those which you think may help prevent the spread or help control disease once it has occurred on a property.

Thank you for your co-operation in this project.
Your assistance in this project is helping the industry combat this disease.

Appendix VII

Reminder lettters sent to farmers regarding the return of the case control survey.

15th January 2001

EpiCentre,
I.V.A.B.S,
Massey University,
Private Bag 11222,
Palmerston North.

To Whom It May Concern:

A friendly reminder! Our records show that you have not yet responded to a *Salmonella* Brandenburg Questionnaire that was sent out to you in December 2000. If you have already sent the questionnaire back prior to receiving this letter, then thank you very much for your time and effort, and please disregard this letter.

Your response and input is very important to obtain a decent representation of *Salmonella* Brandenburg infected and non-infected farms. The return of your survey forms will help us achieve a greater understanding of the disease, which will assist us in the development of cost-effective and practical control and prevention strategies, aimed at reducing or eliminating the impact of this disease.

Again I thank you very much for your participation in this survey. It is much appreciated. Looking forward to your response.

Joanne Kerslake BSc

15th February 2001

EpiCentre,
I.V.A.B.S,
Massey University,
Private Bag 11222,
Palmerston North.

To Whom It May Concern:

A friendly reminder! Our records show that you have not yet responded to a *Salmonella* Brandenburg Questionnaire that was sent out to you in December 2000. If you have already sent the questionnaire back prior to receiving this letter, then thank you very much for your time and effort, and please disregard this letter.

Your response and input is very important to obtain a decent representation of *Salmonella* Brandenburg infected and non-infected farms. The return of your survey forms will help us achieve a greater understanding of the disease, which will assist us in the development of cost-effective and practical control and prevention strategies, aimed at reducing or eliminating the impact of this disease.

Again I thank you very much for your participation in this survey. It is much appreciated. Looking forward to your response.

Joanne Kerslake BSc

15th March 2001

To Whom It May Concern

Our records show that you have not yet responded to a *Salmonella Brandenburg* Questionnaire that was sent out to you in December 2000. We need your response to ensure successful completion of this project.

Another copy of the questionnaire has been enclosed for your convenience. Please complete the questionnaire and return in the postage paid envelope.

Again I thank you very much for your participation in this survey.

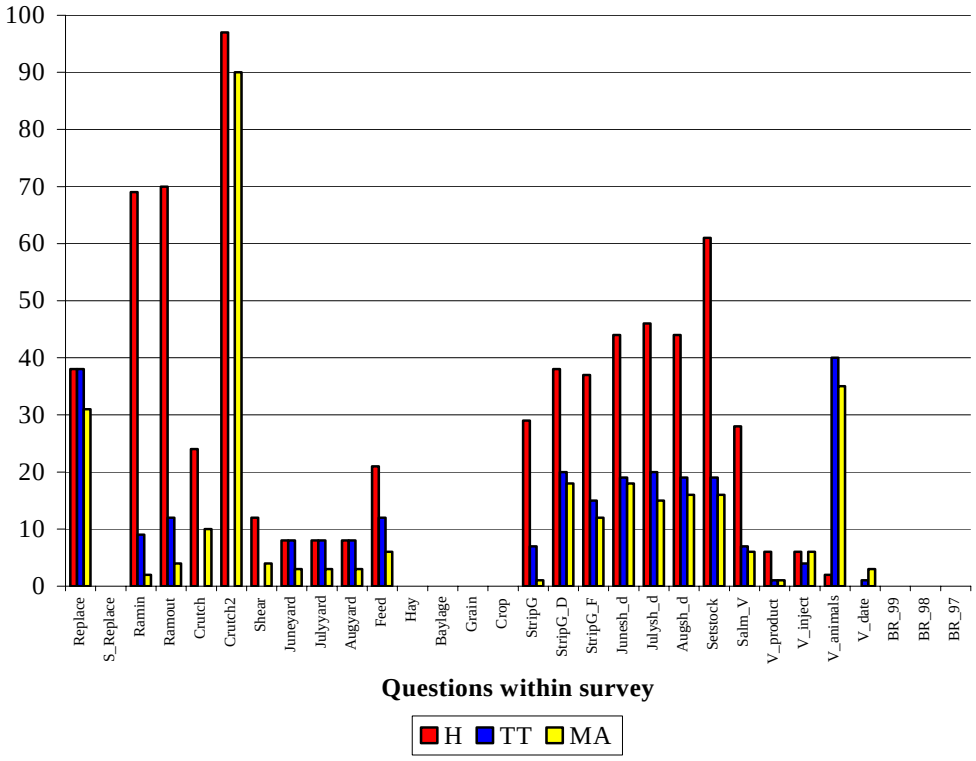
If you have already replied prior to receiving this letter, then thank you very much for your time and effort, and please disregard this letter.

Joanne Kerslake BSc
EpiCentre
The Wool Building
Massey University
Private Bag 11222
Palmerston North

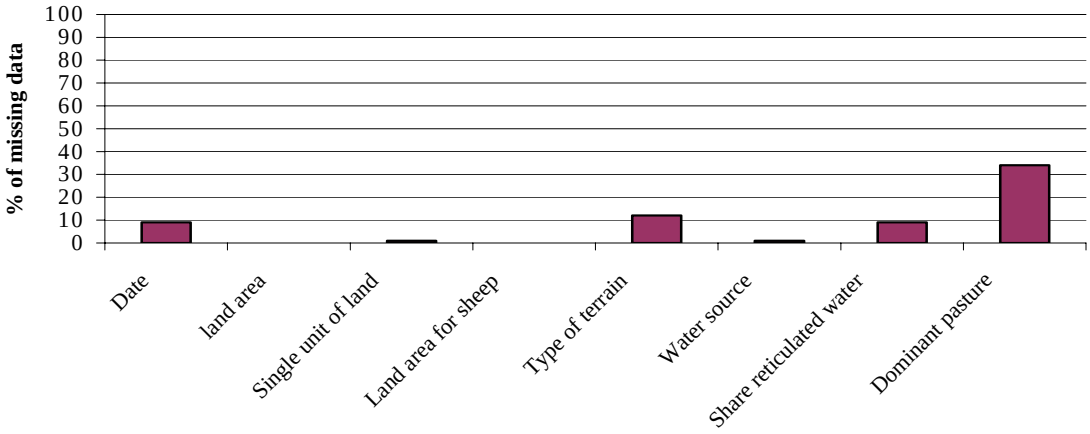
Appendix VIII

The amount of missing data in the case control survey

Percentage of missing data for Hogget, Two-tooth and Mixed age ewe related questions.



Percentage of missing data for farm related questions.



Appendix IX

Univariate analysis of case control data

Unadjusted odd ratios (ORs) and 95% confidence intervals (CI) for mixed age ewe factors associated with likelihood of a farm being a case farm than a control farm. Comparisons are made between categories with a reference category for each variable.

Variable	Category	Case (n)	Control (n)	P value	OR (95% CI)
Strip grazed	Yes	120	170	<0.001	7.14 (3.15-16.19)
	No	7	50		1
SB infection in 1999	Yes	44	15	<0.001	3.98 (2.10-7.54)
	No	101	137		1
Stocking rate	>13 sheep/Ha	73	61	0.003	1.90 (1.25-2.89)
	<13 sheep/Ha	104	165		1
Stocking rate	Continous			0.008	1.07 (1.02-1.12)
Date setstocked	September	89	96	0.011	0.52 (0.32-0.86)
	August	64	36		1
Type of terrain	Hill	57	100	0.013	0.60 (0.39-0.90)
	Flat	120	125		1
Fed Silage	Yes	115	120	0.017	1.69 (1.09-2.45)
	No	62	106		1
Total no. of ewes	Continous			0.029	1.00 (0.99-1.00)
August yarding	Yes	152	170	0.051	1.71 (1.00-2.94)
	No	24	46		1
Fed Crop	Yes	44	74	0.085	0.68 (0.44-1.05)
	No	133	152		1
Backfenced while strip grazing	Yes	149	116	0.101	1.83 (0.89-3.79)
	No	14	20		1
No. of August shifts/day	>1day	32	37	0.136	0.66 (0.38-1.14)
	1day	123	94		1
June yarding	Yes	95	102	0.184	0.76 (0.88-1.96)
	No	81	114		1
Date ram removed	>July	37	55	0.236	0.79 (0.47-1.21)
	<July	139	155		1
SB infection in 1998	Yes	14	10	0.236	1.67 (0.77-2.77)
	No	120	143		1
Vaccinated with S+B	Yes	31	32	0.278	1.35 (0.78-2.32)
	No	132	189		1
Vaccinated for Salmonella	Yes	42	46	0.324	1.27 (0.79-2.05)
	No	122	170		1
Fed Hay	Yes	160	118	0.392	1.19 (0.80-1.77)
	No	77	108		1

Variable	Category	Case (n)	Control (n)	P value	OR (95% CI)
Total no. of yardings	Continous			0.448	1.07 (0.90-1.27)
No. of June shifts/day	>1day	64	60	0.45	0.83 (0.52-1.33)
	1day	92	72		1
No. of vaccine shots admin.	2	27	27	0.454	1.41 (0.57-3.52)
	1	12	17		1
Date ram put out	>mid April	81	109	0.459	0.86 (0.58-1.28)
	<mid April	95	110		1
Vaccinated with S+SB	Yes	4	8	0.495	0.65 (0.19-2.21)
	No	159	208		1
No. of July shifts/day	>1day	52	49	0.502	0.85 (0.52-1.38)
	1day	104	83		1
Fed Grain	Yes	39	56	0.52	0.86 (0.54-1.39)
	No	138	170		1
Crutching date	>July	56	75	0.54	0.87(0.57-1.35)
	<July	106	124		1
Date strip grazing began	>June	34	32	0.555	0.85 (0.49-1.47)
	<June	118	99		1
SB infection in 1997	Yes	4	0	0.569	565.66 (<0.01->1.00)
	No	128	147		1
Supplemetary Fed	Yes	166	201	0.744	0.83 (0.26-2.61)
	No	6	6		1
July yarding	Yes	99	118	0.748	1.07 (0.72-1.59)
	No	77	98		1
Replacement of stock	Yes	37	32	0.786	0.93 (0.53-1.60)
	No	116	93		1
Vaccinated with Salvexin	Yes	6	7	0.816	1.14 (0.38-3.46)
	No	157	209		1
Date last vaccine shot administered	Aug	3	3	0.834	1.20 (0.23-6.29)
	<Aug	36	43		1
Shearing Date	>July	53	69	0.885	1.04 (0.64-1.68)
	<July	63	85		1
Water Source	High Risk	126	102	0.931	1.02 (0.62-1.68)
	Low Risk	35	46		1

Unadjusted odd ratios (ORs) and 95% confidence intervals (CI) for mixed age (MA) ewe factors associated with severity of disease on a case farm. Comparisons are made between categories with a reference category for each variable.

Variable	Category	Farms (n)	P value	OR (95% CI)
SB infection in 1999	Yes	43	0.000	0.49 (0.34-0.71)
	No	98	0.000	1
Vaccinated for Salmonella	Yes	41	0.008	0.61 (0.43-0.88)
	No	118	0.008	1
Vaccinated with S+B	Yes	31	0.008	0.58 (0.38-0.86)
	No	127	0.008	1
Date setstocked	September	87	0.015	0.69 (0.52-0.93)
	August	62	0.015	1
2 Vaccine shot administered	Yes	27	0.021	0.61 (0.40-0.93)
	No	131	0.021	1
Date ram removed	>July	36	0.026	1.43 (1.04-1.96)
	<July	135	0.026	1
Shearing Date	>July	51	0.037	0.68 (0.47-0.98)
	<July	62	0.037	1
Fed Crop	Yes	43	0.038	0.68 (0.48-0.98)
	No	129	0.038	1
Fed Grain	Yes	39	0.056	1.36 (0.99-1.85)
	No	133	0.056	1
SB infection in 1998	Yes	14	0.073	0.56 (0.31-1.05)
	No	117	0.073	1
June yarding	Yes	98	0.113	0.80 (0.60-1.05)
	No	80	0.113	1
Date strip grazing began	>June	33	0.126	1.29 (0.56-1.07)
	<June	114	0.126	1
1 Vaccine shot administered	Yes	12	0.268	0.68 (0.34-1.34)
	No	146	0.268	1
July yarding	Yes	97	0.396	1.13 (0.84-1.51)
	No	74	0.396	1
Total no. of yardings	Continous		0.465	1.05 (0.92-1.19)
Replacement of stock	Yes	35	0.498	1.12 (0.81-1.56)
	No	115	0.498	1
Backfenced while strip grazing	Yes	145	0.507	1.23 (0.44-1.50)
	No	13	0.507	1
No. of August shifts/day	>1day	50	0.526	1.01 (0.79-1.57)
	1day	102	0.526	1

Variable	Category	Farms (n)	P value	OR (95% CI)
Strip grazed	Yes	165	0.539	1.30 (0.56-3.05)
	No	7	0.539	1
Stocking rate	>13 sheep/Ha	70	0.539	0.91 (0.68-1.22)
	<13 sheep/Ha	102	0.539	1
SB infection in 1997	Yes	4	0.545	0.68 (0.20-2.36)
	No	125	0.545	1
Date ram put out	>mid April	79	0.566	0.92 (0.69-1.22)
	<mid April	92	0.566	1
Crutching date	>July	55	0.599	1.08 (0.81-1.45)
	<July	103	0.599	1
Fed Silage	Yes	113	0.687	0.94 (0.70-1.26)
	No	59	0.687	1
Fed Hay	Yes	95	0.694	0.95(0.71-1.25)
	No	77	0.694	1
No. of July shifts/day	>1day	30	0.716	1.06 (0.78-1.43)
	1day	121	0.716	1
No. of June shifts/day	>1day	61	0.764	1.05 (0.78-1.43)
	1day	91	0.764	1
Total no. of ewes	Continous		0.804	1.00 (1.00-1.00)
Supplemetary Fed	Yes	161	0.845	0.93 (0.42-2.01)
	No	6	0.845	1
Stocking rate	Continous		0.917	1.00 (0.96-1.04)
Vaccinated with S	Yes	5	0.959	0.98 (0.40-2.36)
	No	153	0.959	1
August yarding	Yes	147	0.978	0.99 (0.66-1.50)
	No	24	0.978	1

Unadjusted odd ratios (ORs) and 95% confidence intervals (CI) for two-tooth (TT) ewe factors associated with likelihood of a farm being a case farm than a control farm. Comparisons are made between categories with a reference category for each variable.

Variable	Category	case (n)	control (n)	P value	OR (95% CI)
Total no. of ewes	Continous			<.001	1.00 (1.00-1.00)
Strip grazed	Yes	59	153	<.001	8.50 (3.28-22.03)
	No	5	41	<.001	1
Date setstocked	September	79	86	<.001	0.35 (0.20-0.61)
	August	63	24	<.001	1
Fed Grain	Yes	28	35	0.984	0.99 (0.58-1.71)
	No	144	179	0.984	1
Crutching date	>July	46	57	0.882	0.9 (0.61-1.53)
	<July	112	134	0.882	1
Water Source	High Risk	122	157	0.875	0.96 (0.58-1.60)
	Low Risk	34	42	0.875	1
Date ram removed	>July	37	43	0.777	0.93 (0.55-1.54)
	<July	124	139	0.777	1
Shearing Date	>July	51	68	0.767	0.94 (0.60-1.45)
	<July	113	141	0.767	1
Vaccinated with S+SB	Yes	8	8	0.655	1.26 (0.46-3.42)
	No	164	206	0.655	1
July yarding	Yes	95	104	0.637	1.11 (0.73-1.68)
	No	71	86	0.637	1
Date ram put out	>mid April	94	112	0.629	0.90 (0.59-1.38)
	<mid April	69	74	0.629	1
SB infection in 1997	Yes	3	0	0.626	518.01 (<0.01->1.00)
	No	116	122	0.626	1
Supplemetary Fed	Yes	155	171	0.568	1.36 (0.47-3.91)
	No	6	9	0.568	1
Fed Crop	Yes	51	70	0.52	0.87 (0.56-1.34)
	No	121	144	0.52	1
No. of vaccine shots admimistered	2	52	51	0.464	0.46 (0.15-1.43)
	1	11	5	0.464	1
Replacement of stock	Yes	19	11	0.436	1.37 (0.62-3.02)
	No	116	92	0.436	1
Date strip grazing began	>June	33	32	0.308	0.75 (0.42-1.31)
	<June	108	78	0.308	1
Vaccinated with S	Yes	9	6	0.227	1.91 (0.67-5.48)
	No	163	208	0.227	1
Fed Hay	Yes	87	95	0.226	1.28 (0.86-1.92)
	No	85	119	0.226	1

Variable	Category	case (n)	control (n)	P value	OR (95% CI)
No. of August shifts/day	>1day	31	33	0.2	0.69 (0.39-1.22)
	1day	109	80	0.2	1
No. of June shifts/day	>1day	54	51	0.19	0.71(0.43-1.18)
	1day	89	60	0.19	1
SB infection in 1998	Yes	15	9	0.182	1.80 (0.76-4.29)
	No	109	118	0.182	1
Total no. of yardings	Continous			0.091	1.17 (0.98-1.39)
August yarding	Yes	142	149	0.084	1.62 (0.93-2.83)
	No	24	41	0.084	1
Vaccinated with S+B	Yes	49	44	0.071	1.54 (0.96-2.46)
	No	123	170	0.071	1
Backfenced while strip grazing	Yes	138	101	0.045	2.32 (1.02-5.28)
	No	10	17	0.045	1
Vaccinated for Salmonella	Yes	66	58	0.041	1.58 (1.02-2.45)
	No	98	136	0.041	1
Fed Silage	Yes	99	99	0.028	1.58 (1.05-2.36)
	No	73	115	0.028	1
June yarding	Yes	97	88	0.023	1.63 (1.07-2.48)
	No	69	102	0.023	1
Type of terrain	Hill	56	97	0.011	0.58 (0.38-0.88)
	Flat	116	117	0.011	1
Stocking rate	Continous			0.01	1.07 (1.02-1.12)
SB infection in 1999	Yes	28	11	0.004	1
	No	98	117	0.004	1
Stocking rate	>13 sheep/Ha	72	59	0.003	1.89 (1.24-2.89)
	<13 sheep/Ha	100	155	0.003	1

Unadjusted odd ratios (ORs) and 95% confidence intervals (CI) for two-tooth (TT) ewe factors associated with severity of disease on a case farm. Comparisons are made between categories with a reference category for each variable.

Variable	Category	Farm (n)	P Value	OR (95% C.I.)
Vaccinated for Salmonella	Yes	33	0.001	0.44 (0.27 - 0.70)
	No	44		1
Vaccinated with S+B	Yes	4	0.005	0.48 (0.28 - 0.80)
	No	74		1
Total no. of yardings	Continous		0.009	1.34 (1.07 - 1.67)
Backfenced while strip grazing	Yes	67	0.014	0.46 (0.25 - 0.85)
	No	5		1
2 Vaccine shot administered	Yes	24	0.019	0.52 (0.30 - 0.90)
	No	51		1
SB infection in 1998	Yes	10	0.055	0.46 (0.21 - 1.02)
	No	52		1
SB infection in 1999	Yes	17	0.071	0.58 (0.33 - 1.05)
	No	46		1
1 Vaccine shot administered	Yes	7	0.110	0.43 (0.15 - 1.21)
	No	68		1
Replacement of stock	Yes	10	0.112	1.60 (0.90 - 2.86)
	No	52		1
Fed Hay	Yes	46	0.121	1.46 (0.91 - 2.35)
	No	32		1
Crutching date	>July	25	0.123	0.67 (0.40 - 1.11)
	<July	47		1
Fed Grain	Yes	16	0.151	0.65 (0.36 - 1.17)
	No	62		1
Strip grazed	Yes	76	0.165	0.40 (0.11 - 1.46)
	No	1		1
SB infection in 1997	Yes	2	0.181	0.10 (<0.01 - 2.96)
	No	58		1
June yarding	Yes	44	0.229	0.76 (0.48 - 1.19)
	No	34		1
Stocking rate	>13 sheep/Ha	27	0.263	1.30 (0.82 - 2.05)
	<13 sheep/Ha	51		1
Date setstocked	September	40	0.273	0.77 (0.48 - 1.23)
	August	30		1
Date ram removed	>July	59	0.312	1.38 (0.39 - 1.35)
	<July	16		1
Fed Silage	Yes	46	0.345	0.80 (0.51 - 1.27)
	No	32		1
Stocking rate	Continous		0.465	0.97 (0.90 - 1.05)

Variable	Category	Farm (n)	P Value	OR (95% C.I.)
Supplemetary Fed	Yes	72	0.491	1.52 (0.46 - 5.05)
	No	4		1
August yarding	Yes	68	0.537	0.80 (0.39 - 1.64)
	No	10		1
No. of August shifts/day	>1day	18	0.558	1.16 (0.70 - 1.92)
	1day	51		1
Date strip grazing began	>June	19	0.565	1.16 (0.70 - 1.92)
	<June	52		1
Total no. of ewes	Continous		0.651	1.00 (0.99 - 1.00)
No. of June shifts/day	>1day	28	0.671	1.11 (0.69 - 1.79)
	1day	40		1
Shearing Date	>July	19	0.690	1.12 (0.65 - 1.94)
	<July	56		1
Vaccinated with S	Yes	26	0.761	0.82 (0.24 - 2.88)
	No	52		1
Date ram put out	>mid April	42	0.880	1.04 (0.64 - 1.68)
	<mid April	34		1
July yarding	Yes	43	0.896	0.97 (0.61 - 1.54)
	No	35		1
Fed Crop	Yes	25	0.963	1.01 (062 - 1.66)
	No	53		1