A STUDY OF *LEPTOSPIRA INTERROGANS* INFECTION IN DEER AND GOATS IN NEW ZEALAND

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STEPHEN HARRY FLINT

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ABSTRACT

In order to determine the prevalence of leptospirosis in deer and goats both serological tests and the culturing of bacteria from urine samples were used. The serological tests enabled an assesment to be made as to the nature and extent of antibody levels. To ensure confidence in the serological results, it was necessary to validate the standard microscopic agglutionation test (MAT). Repeat tests demonstrated reproducible results that were within one two-fold serial dilution. Enzyme linked immunosorbent assays (ELISA) were investigated as alternatives for both the detection of antibodies and *Leptospira* antigens. These assays demonstrated greater sensitivity than the MAT for the detection of antibodies, but were less sensitive than standard methods for the detection of antigen.

Using the MAT, antibodies to *australis, ballum, bratislava, copenhageni, hardjo, pomona,* and *tarassovi* were detected in serological surveys of deer and goats. In deer, the most frequently recorded antibody titres were to *ballum, bratislava* and *copenhageni*. As 87% of the antibody titres <80, there appears to be a low level of active infection. In some areas there was a high prevalence of antibody titres to *hardjo*. In goats, 70% were found to have antibody titres ≥10 to one or more serovars with antibodies to *ballum* and *bratislava* the most frequently recorded. As 90% of the antibody titres were <80, there appears to be a low level of active infection in goats.

Antibodies to *bratislava*, a serovar that has not been isolated in New Zealand, were widespread in both deer and goats. The possibility

that these resulted from mixed infections was considered but not resolved. Unsucessful attempts were made to purify mixed cultures using specific antisera. The possibility of serological cross reactions of antibodies to other serovars with *bratislava* was supported by the increased serological response of deer and goats to vaccination with *hardjo* and *pomona* antigens. Western blot studies identified several common antigens between *bratislava* and *pomona*.

A study of a deer farm showed a high prevalence of antibody titres to *hardjo* corresponding to a similar prevalence of antibody titres to *balcanica* believed to be due to the antigenic similarity of these two serovars. *Balcanica* was isolated from urine samples from these deer and is believed to be the first isolation of this serovar from deer.

Studies of six goat farms showed low antibody levels and no *Leptospira* were isolated from urine samples.

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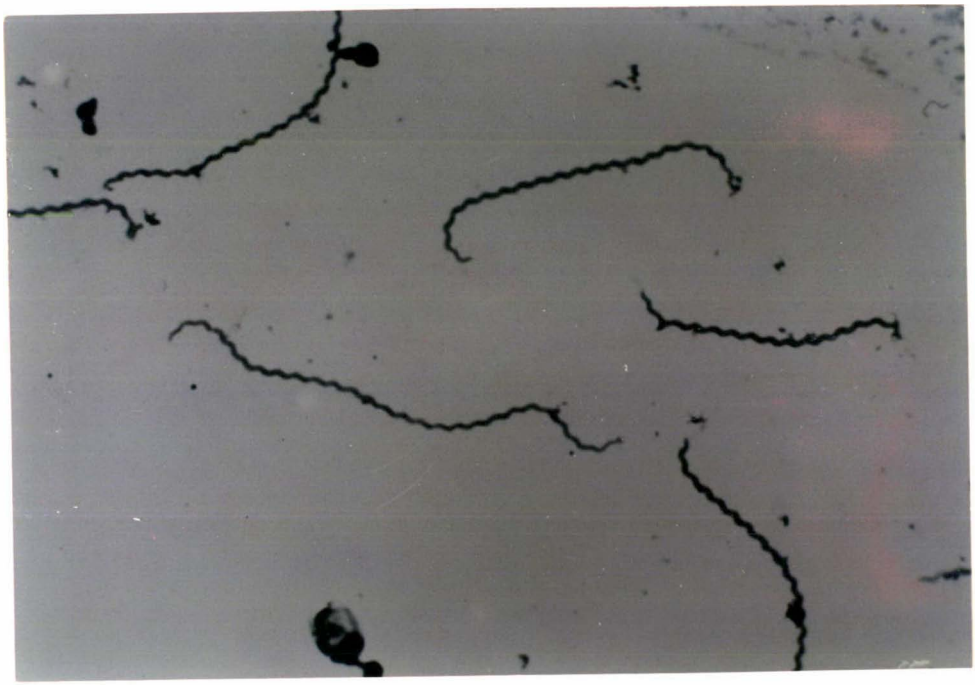
FRONTPIECE : *Leptospira interrogans*



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INTRODUCTION

Leptospirosis is recognised internationally as a serious human disease resulting in death in the most severe cases. This zoonosis is maintained in a wide range of animal populations in which it is often exists as a chronic, inapparent infection. Acute infection may occur in some animal populations producing classical signs of jaundice, fever, conjunctivitis, anorexia, central nervous system disorders, and liver and renal failure. Abortions and neonatal death occur in some cases.

Leptospira interrogans, spirochaete bacteria, are responsible for this disease. More than 180 serological varieties, termed serovars, have been identified, each of which have specific maintainance host animal populations. These maintainance hosts may carry the infection for an extended period, shedding the bacteria in their urine providing a source of infection for their own and other species. The infection in the maintainance host is often inapparent whereas accicental hosts may develop an acute disease.

In New Zealand, six serovars of the *interrogans* species have been isolated from domestic and feral animals. *Hardjo, pomona* and *tarassovi* are maintained in domestic animals. *Balcanica, ballum* and *copenhageni* are maintained in wildlife.

The disease in New Zealand is of concern due to the economic loss from death and ill health of farmed animals and the economic and social implications of the debilitating human illness. New Zealand had the highest incidence of human leptospirosis in the world in 1971 with 800 cases. Research into the disease in cattle, the primary source of the human infection in New Zealand, enabled

control of the disease. This is beleived to have been responsible for the reduction in the annual number of human cases reported to approximately 100.

There are several possible sources of these remaining 100 cases. Deer and goats are capable of carrying the infection and have therefore been identified as potential sources of human infections.

There is an increasing interest in the diseases of deer and goats with the recent increase in the farming of these animals. Stock losses cause concern as these are often particularly valuable animals. The number of reports of clinical disease attributed to leptospirosis in deer is increasing.

As there have been no detailed studies on leptospirosis in deer and goats in New Zealand, this study was aimed at determining the prevalence of leptospirosis using serological investigations and cultural isolation of *Leptospira* from urine samples.

In the course of this investigation, it was necessary to validate some experimental techniques, in particular the microscopic agglutination test to detect antibodies to *Leptospira*. Enzyme linked immunosorbent assays were investigated as alternative methods for the more rapid identification of specific antibody and *Leptospira*.

Investigation into the antigenic relationships between certain serovars was prompted by the serological results obtained during this study. This investigation involved attempting to purify cultures containing more than one serovar using specific antisera, monitoring the serological response to vaccination, the separation of proteins and the identification of protein antigens.

CHAPTER 1

LITERATURE REVIEW

1.1 Introduction and Classification

Leptospirosis is a disease affecting both animals and humans caused by bacteria of the genus *Leptospira* . This is in the order *Spirochaetales* and is described in section 1 of Bergey's Manual of Systematic Bacteriology (Johnson and Faine 1984). *Leptospira* are flexible, motile, helicoidal rods that are obligatory aerobes. Three species, *biflexa, illini,* and *interrogans* are included in this genus. The *interrogans* species is primarily responsible for leptospirosis. Differentiation within this species is based on cross-agglutination absorption tests which have been used to identify about 180 serological varieties termed serovars. These are more conveniently divided into serogroups containing closely related serovars.

Members of the *biflexa* species are only rarely associated with infection. *L. interogans* can be differentiated further from *L. biflexa* by its DNA composition, more fastidious growth requirements and greater sensitivity to the growth inhibitory action of 8-azaguanine and divalent cations. *L. interrogans* also has unique serological characteristics (Johnson and Faine 1984).

L. illini is phenotypically similar to *L. biflexa* but quite different in its DNA base composition, serological characteristics and some morphological features (Johnson and Faine 1984).

Leptospirosis was first described clinically as 'Weils' disease in the 1880's as a tribute to Professor Adolf Weil who recognised this 'infectious jaundice' as a distinctive entity (Alston and Broom 1958). The causative organism was not isolated until 1915 (Inada Ido 1915). Since that time, leptospirosis has been diagnosed in most countries of the world.

1.2 Leptospirosis- The Disease in Animals

Initially, leptospirosis was considered to be a sporadic infection of rats and dogs caused by a relatively small number of serovars (Alston and Broom 1958). Investigations have shown leptospirosis to be common, but generally an inapparent infection of many different species of wildlife and domestic stock and is associated with a large number of different serovars. Some animal species serve as inapparent reservoirs and can act as a source of infection for other animals. The chronic, inapparent infection of animals is usually localised in the kidneys and may occur in animals that have passed through an acute stage. In the chronic carrier state, leptospires are excreted in the urine for a varying length of time. This urinary shedding of leptospires may be intermittent or continuous depending on the serovar and animal species (Faine 1982).

Acute leptospirosis is initially manifest as a febrile illness, accompanied by fever, with signs of depression, malaise, anorexia and conjunctivitis. Later signs, which are characteristic of leptospirosis, include bleeding, jaundice, central nervous system disorders and liver and renal failure. Abortion, stillbirth and mastitis in lactating animals can also occur (Faine 1982).

Leptospirosis shows a natural nidality where each serovar has specific maintenance host populations which act as reservoirs of infection (Blackmore and Hathaway 1980). Other animals, including humans, are accidental hosts, becoming infected through direct contact with urine or tissues from infected animals or by indirect contact via an environment contaminated with infected urine (Wilcox 1976).

1.3 Leptospirosis - The Disease in Humans

Leptospirosis in humans has a number of clinical signs and symptoms (Feign and Anderson 1975). Commonly it manifests as a sudden febrile illness with headache, prostration, severe myalgia and conjunctival suffusion. Haemorrhage and jaundice may appear in very severe cases. There may also be a rash on the palate, meningism, renal failure and mental depression. If not treated in the first two to three days it may become more severe and ultimately lead to death due to renal failure in the most severe of cases.

Leptospires enter the body through cuts and abrasions, the conjunctiva and mucous membranes from where they are spread, via the bloodstream, where they multiply until they become opsonized and phagocytosed. Growth may continue until high concentrations of leptospires are found in all tissues of the body (Turner 1967). Antibiotic treatment is effective in the first few days of infection (Faine 1982).

1.4 Leptospirosis in New Zealand

Six serovars of the *Leptospira interrogans* species have been isolated from domestic and feral animals in New Zealand. Under New Zealand conditions, *hardjo, pomona,* and *tarassovi* are harboured in domestic livestock populations. *Hardjo* is maintained in cattle (Hellstrom 1978) whereas *pomona* and *tarassovi* are maintained in pigs (Ryan 1978). *Balcanica, ballum* and *copenhageni* are maintained in wildlife reservoirs. The opossum *(Trichosaurus vulpecula)* is considered to be the maintenance host for *balcanica* (Hathaway 1978). Hedgehogs *(Erinaceus europeus)*, mice *(Mus musculus)*, and black rats *(Rattus rattus)* are the maintenance hosts for *ballum* (Hathaway 1978). *Copenhageni* is maintained in brown rats *(Rattus norvegicus)* (Hathaway 1978). One other serovar, *australis* has been isolated in New Zealand but as yet no animal reservoir has been identified in this country. This serovar, was isolated from a Northland farmer by Thompson (1980).

1.4.1 Cattle

Pomona was the first serovar to be associated with leptospirosis in cattle in New Zealand with an isolation reported in 1950 (Filmer 1951). The clinical importance of the disease was recognised with reports of death in young calves and haemoglobinuria in cattle (Salisbury 1954; Jamieson *et al* 1970). Abortions (Te Punga and Bishop 1953) and agalactia (Lake 1975) have also been reported. Adult cows may also be asymptomatic carriers (Salisbury 1954). A national survey by Hellstrom (1978) found 85/480 (18⅔) with antibody titres ≥24 to *pomona.* Cattle infected with *pomona* shed the organism in their urine for one to four months (Hellstrom 1978). The source of *pomona* infection in cattle is believed to be primarily due to either direct or indirect contact with pigs (Mackintosh 1981).

Serological evidence of *hardjo* infection in cattle was first reported in the late 1960's (Jamieson 1970). The first confirmed

isolation was made in 1971 (Lake 1973). Serological surveys have been conducted by Lake (1973), Brockie (1976) and Hellstrom (1978). The most thorough study was the work of Hellstrom (1978) which showed antibody titres ≥17 in 128/158 (81%) of cattle herds and 290/480 (60%) individual cattle. The use of *hardjo* as an antigen in serological tests from the begining of the 1970's has confirmed that this is the most commonly occurring serovar in cattle in New Zealand (Hellstrom 1978).

Hellstrom (1978) studied the epidemiology of *hardjo* infection in town supply cattle and showed a continuous cycling of the infection in dry stock, including the yearling heifers. A propagating epidemic occurred in the yearlings in late winter and spring which resulted in leptospiruria persisting for up to 14 months enabling the infection to be maintained.

Serological studies of 35 factory supply herds in Taranaki (Anon 1980) indicated that infection was cycling in the yearlings of half the herds, while in the remainder it cycled in the milking herd. These are probably the most common endemic cycles of *hardjo* infection in New Zealand cattle, as 90% of dairy cattle in New Zealand are on factory supply farms (Mackintosh 1981).

In yearling cattle *hardjo* infection is generally asymptomatic (Marshall *et al* 1979b). There are some reports of pyrexia, agalactia, flaccid udders and yellow milk secretions in milking cows (Lake 1975). Abortions due to *hardjo* infection have been reported internationally (Little *et al* 1980), but have not been confirmed in New Zealand (Hellstrom 1978). This is believed to be due to a difference in strains (Mackintosh 1981).

It is possible that some of the antibody titres to *hardjo* in cattle may be due to *balcanica* infection as it is not possible to distinguish between the antibody titres produced by these two serovars (Helstrom 1978). *Balcanica* was isolated from cattle in 1980

(Mackintosh *et al* 1980), but the low level of leptospiruria observed made the possibility of long term transmission between cows seem unlikely. It was concluded, therefore, that cattle were unlikely to act as maintenance hosts for this serovar (Mackintosh 1981).

The potential ability of cattle to act as carriers of *ballum* is unknown. Ris *et al* (1973) isolated *ballum* from two, three month old healthy calves. Hellstrom (1978) found that 17/480 (4%) of New Zealand cattle had antibody titres ≥17 to *ballum*.

Copenhageni infection appears to be sporadic. *Copenhageni* has been isolated from cattle in New Zealand (Dodd and Brackenridge 1960) where it was associated with severe clinical signs of weakness, laboured breathing and a pendulous abdomen. The serological survey of cattle by Hellstrom (1978) showed 11/480 (2%) with antibody titres ≥17 to *copenhageni* and all positive sera originated from the North Island. Asymptomatic infections in cattle have been reported by Ris *et al* (1973) who suggested that calves may acquire infection through contact with infected rat urine.

There is no evidence to suggest that *tarassovi* is important clinically, or that cattle act as maintenance hosts for this serovar (Ryan and Marshall 1976).

The most important serovar infecting cattle in New Zealand, *hardjo*, can be controlled through vaccination (Marshall *et al* 1979b).

1.4.2 Sheep

Hardjo was first isolated from New Zealand sheep by Bahaman *et al* (1980a). *Hardjo* was isolated from the kidneys of 3/180 of the sheep studied in a survey of the North Island.

A serological survey of sheep in the lower North Island showed a prevalence of 182/928 (20%) with antibody titres ≥24 to *hardjo* (Bahaman 1981)

Clinical disease associated with *hardjo* infection has not been reported and experimental infections (Hathaway and Marshall 1979) failed to produce clinical signs and leptospiruria. The duration of leptospiruria from natural infections and the ability of sheep to act as maintenance hosts for *hardjo* are not known (Mackintosh 1981).

Sheep are susecptible to infection with *pomona* and this may cause death in lambs, although it is usually asymptomatic in adult sheep (Salisbury 1954). Experimental infections produce leptospiruria for up to three months, with natural infections causing leptospiruria for up to 9 months (Webster and Reynolds 1955). A survey of sera from the lower North Island showed that 71/928 (8%) of sheep had antibody titres ≥24 to *pomona* (Bahaman 1981).

The serological prevalence of *tarassovi* in sheep is also low. A survey in the lower North Island revealed a prevalence of 66/928(7%) with antibody titres ≥24 (Bahaman 1981). There are no reports of clinical disease and no evidence that sheep may act as maintenance hosts for *tarassovi*.

There are no reports of a natural *balcanica* infection in sheep, although some of the antibody titres to *hardjo* may be due to *balcanica* infection because of the serological cross reactions due to the close serological relationship between these serovars (Hellstrom 1978). Experimental infection of sheep with *balcanica* has produced leptospiruria (Derfee and Presidente 1979).

Bahaman (1981) recorded antibody titres ≥24 to *ballum*, *copenhageni* and *australis* in 55/928(6%), 40/928(4%) and 23/928 (2%) respectively of the sheep surveyed.

Vaccination of sheep with a vaccine containing *hardjo* antigen has been shown to reduce urinary shedding of leptospires due to *hardjo* infection (Marshall 1979a).

1.4.3 Pigs

The association of leptospirosis with pigs was first recognised by Kirschner *et al* (1952) where the disease in cattle and humans on dairy farms could be related to contact with pigs.

Most of the information on leptospirosis in pigs resulted from surveys by Ryan (1978). A sample of 234 adult sows from throughout New Zealand showed 150 (64%) with antibody titres ≥12 to *pomona* (Ryan 1978). Earlier serological prevalence data from Russell and Hanson (1958) showed 484/1125 (44%) with antibody titres ≥10 to *pomona*.

A survey of pigs from abattoirs in the lower North Island showed that 72/84 (86%) of 5 to 10 month old pigs and 57/65 (88%) of pigs older than 10 months had antibody titres ≥12 to *pomona* (Ryan 1978). *Pomona* was isolated from 38/84 (45%) of the kidneys from the 5 to 10 month old group but only 1/65 (2%) of those from the group older than 10 months.

Pomona is endemic in 6 to 12 month old pigs (Ryan 1978) and although leptospiruria has been recorded as persisting for 12 to 24 months (Mitchell *et al* 1966) it is not lifelong .

Pomona infection in young pigs is usually asymptomatic (Ryan 1978), but in pregnant pigs abortions and stillbirths have been recorded (Powers *et al* 1956). In New Zealand it is believed that pigs are the maintenance hosts for *pomona* and therefore act as the primary source of infection for other animals (Ryan 1978).

Tarassovi was first reported in pigs by Kirschner (1954). He surveyed 100 adult pigs and found that 6% had antibody titres ≥150 to *tarassovi*. Russell and Hanson (1958) showed that 431/1125 (38%) of pigs they surveyed had antibody titres ≥10 to *tarassovi*. Similar results were reported by Ryan (1978) in his national survey of 234 pigs, in which 82 (35%) had antibody titres ≥12 to *tarassovi*.

The first isolation of *tarassovi* from pigs in New Zealand was reported by Ryan and Marshall (1976) from a kidney culture. Subsequent investigations revealed both *tarassovi* and *pomona* to be endemic in the pig herd from which the isolation originated and both infections cycled in young pigs. *Tarassovi* is not recognised as a major problem on pig farms in New Zealand which is different from the situation on European pig farms (Ryan 1978).

The extensive survey of pigs by Ryan (1978) revealed no serological or cultural evidence of *hardjo*. However, in 2/234 (1%), antibody titres ≥24 were found to *ballum* and 9/234 (4%) of the population had antibody titres ≥24 to *copenhageni*. Ryan concluded that these serovars were minor causes of leptospirosis in New Zealand pigs.

Vaccination against *pomona* has been shown to be an effective means of reducing urinary shedding of leptospires resulting from *pomona* infection in pigs (Hodges *et al* 1985).

1.4.4 Goats

Surveys of goats in many countries have revealed agglutinins to several different serovars (reviewed by Schollum and Blackmore 1981). Comparatively few investigations have been carried out in New Zealand. Daniel (1967) examined sera from 150 feral goats in New Zealand at 1/10 and 1/200 dilutions and found one with an antibody titre ≥200 to *pomona*. Three reacted to *tarassovi* at a 1/10 dilution. All others were negative for antibodies to *andaman, australis A,*

australis B, canicola, grippotyphosa, icterohaemorrhagiae, pomona and *tarassovi*.

Hellstrom and O'Hara (1979) recorded antibody titres ≥200 to *copenhageni, hardjo*,and *pomona* in 2/300, 23/400 and 1/150 respectively in the domestic goats surveyed. Presumably the number of animals exhibiting antibody titres would have been higher if lower antibody titres had been recorded.

Schollum and Blackmore (1981) reported antibody titres ≥24 to *balcanica, ballum* and *hardjo* in 13/116, 5/116 and 12/116 respectively, in a study of New Zealand feral goats. Tests for antibodies to *australis, copenhageni, pomona,* and *tarassovi* were all negative. Although results for both *balcanica* and *hardjo* were given, they were indistinguishable due to their antigenic similarity (Hathaway *et al* 1978). *Balcanica* and *hardjo* were recovered from the kidneys of 3/101 and 1/101 of these animals, respectively.

1.4.5 Deer

There are few studies on the prevalence of leptospirosis in New Zealand deer (Mackintosh 1984). Daniel (1966) tested 109 sera from feral deer for antibodies to *andaman, australis A, australis B, canicola, grippotyphosa, icterohaemorrhagiae, pomona* and *tarassovi* at 1/10 and 1/200 dilutions. One animal showed an antibody titre ≥200 to *pomona*. Antibody titres ≥10 were recorded for *andaman, icterohaemorrhagiae, pomona* and *tarassovi* in 4/109, 3/109, 4/109 and 1/109 deer, respectively. In later studies, Daniel (1967) found one antibody titre ≥200 to *andaman* and one antibody titre to *tarassovi* in sera from 279 feral deer, using the same serovars and dilutions of sera. Hathaway *et al* (1981) also failed to find evidence of leptospiral infection in 31 feral deer using a miniumum serum dilution of 1/24, testing for antibodies to 12 different serovars (*australis, autumnalis, ballum, bataviae, biflexa, canicola,*

copenhageni, grippotyphosa, hardjo, pomona, pyrogenes, and *tarassovi*).

In 1980, the death of a 4 year old red hind was diagnosed as being due to a *pomona* infection. Five out of 12 deer associated with this animal had antibody titres to *ballum, hardjo, pomona* and *tarassovi* (Anon 1980c).

Fairley (1984) reported antibody titres ≥200 to *pomona* in 9/12 farmed deer while investigating deaths in deer that had been attributed to *pomona* infection. One deer had an antibody titre of 100 to *hardjo*. All 12 sera were screened for antibody titres ≥100 to *ballum, copenhageni, hardjo, pomona,* and *tarassovi*.

Ingles (1984) reported Icterohaemorrhagiae serogroup antibody titres of ≥50 in 6/24 (25%) and to *pomona* in 8/24 (33%) of farmed deer. A survey of deer in the same area by the Ministry of Agriculture and Fisheries, detected Icterohaemorrhagiae serogroup antibodies of unspecified titre in 47/108 deer on 7/9 farms (Ingles 1984).

Flint *et al* (1986) found 6/27 farmed deer with antibody titres of ≥32 to *hardjo* in a herd where some animals were shedding *hardjo* and *copenhageni* in their urine. No antibody titres ≥32 were detected to *ballum, copenhageni, pomona,* or *tarassovi*.

Antibody titres ≥24 to *balcanica, hardjo* and *pomona* were found in 2/16, 2/16 and 10/16 domestic deer, respectively, by Fairley *et al* (1986) while investigating a haemolytic disease associated with a *pomona* infection. The antibodies to *balcanica* and *hardjo* cannot be distinguished due to extensive cross reactions between these two serovars (Hathaway *et al* 1978). None of these deer had antibody titres ≥24 to *ballum, copenhageni,* and *tarassovi*.

1.4.6 Other Domestic Animals

There have been few studies of leptospirosis in dogs in New Zealand. The first isolation was of *pomona* from a dog on a dairy farm which was experiencing an abortion storm in the cattle herd (Te Punga and Bishop 1953). Two other dogs had antibodies to *pomona* although none of them showed signs of disease. Salisbury (1954) found that 7/63 (11%) samples of dog sera submitted to an Animal Health Laboratory for diagnostic testing had antibodies of unspecified titre to *pomona*. Seven dogs, from three dairy farms that had concurrent epidemics of *pomona* in their cows or calves, had antibody titres ≥192 to *pomona* (Mackintosh 1981).

The first report of antibody titres to *hardjo* in dogs occurred in two dogs on a dairy farm (Anon 1972). Mackintosh (1981) studied 64 cattle dogs from 37 dairy farms and showed 28 (44%) had antibody titres ≥24 to at least one serovar. Antibodies to *hardjo* were present in 18 (28%) of these dogs. Six (9%) had antibodies to *ballum* and four (6%) had antibodies to *pomona*.

In a similar survey of 47 city dogs (Mackintosh 1981) only two (4%), one (2%) and one (2%) had antibody titres ≥24 to *australis*, *hardjo* and *pomona*, respectively.

These results reflect the greater exposure of rural dogs to rodent, cattle and pig populations which maintain *ballum*, *hardjo* and *pomona* respectively.

An outbreak of clinical leptospirosis due to *copenhageni* was investigated in a pack of 38 hounds (Mackintosh 1981). Twenty six (68%) were infected, based on the development of antibody titres ≥24 to *copenhageni*. Four showed clinical signs and two died. The infection was presumed to have originated from contact with rats. Several sub-clinical infections with *tarassovi* were also detected in 9/38 (24%) of hounds with low (≤24) antibody titres to *tarassovi*.

Tarassovi was isolated from four hounds. This infection may have been due to contact with pig urine during hunting.

Mackintosh (1981) experimentally infected five dogs with *tarassovi* which caused sub-clinical infection and leptospiruria in all animals for up to ten months. It is unlikely, however, that dogs act as maintenance hosts for *tarassovi* in New Zealand due to the low intensity of leptospiruria and the lack of evidence of dog to dog transmission in the study of the hound pack (Macktinosh 1981).

From these studies it appears that dogs in New Zealand do not maintain leptospiral infection and therefore, do not represent an important source of infection for other animals.

Similarly, domestic cats in New Zealand are not regarded as important in the epidemiology of leptospirosis, although there is some evidence of leptospirosis occurring in this species. For example, *pomona* was isolated from a cat on a dairy farm by Harkness *et al* (1970).

A thorough serological study was performed by Shophet (1979) where sera from 225 feral and domestic cats were screened against 11 serovar antigens. Twenty (9%) of the sera gave antibody titres ≥24 to leptospira. The most frequently recorded antibody titres were to *ballum, copenhageni, hardjo* and *pomona* in 4, 5, 5, and 4 cats respectively. The antibody titres to *ballum* and *copenhageni* were presumed to have originated from predator prey transmission between cats and rodents. *Hardjo* and *pomona* are thought to have originated from contact with other animals or a contaminated environment. The low serological prevalence in this study suggests that *ballum* infection is uncommon.

There is only one report of leptospirosis in horses in New Zealand (Anon 1977a). This describes abortions due to *pomona* which appear to be rare.

1.4.7 Wildlife

Leptospirosis is widespread in New Zealand's wildlife. This has been demonstrated primarily by the studies of Brockie (1975; 1977), Brockie and Till (1977) and Hathaway (1978).

The studies of Hathaway (1978) revealed that opossums(*Trichosurus vulpecula*) acted as maintenance hosts for the *balcanica* serovar. He cultured 57 Sejroe serogroup isolates from 154 animals (37%), eight of which were confirmed to be *balcanica*. Infection was found to be restricted to sexually mature animals which suggests direct contact transmission rather than indirect environmental transmission.

These results complement those of de Lisle *et al* (1975) where antibody titres ≥200 to *hardjo* were found in 17/26 (65%) of opossum sera and isolates believed to be *hardjo*, were made from five of these animals. Distinguishing between *balcanica* and *hardjo* is not possible by routine typing procedures and it has been suggested that these isolates were in fact *balcanica* (Hathaway 1978).

Brockie (1975) produced similar results in a survey of 146 opossums, of which 38 (26%) revealed antibody titres of 100 to *hardjo* and two antibody titres of 100 were found to *ballum* and *autumnalis*. Isolates were obtained from 2/146 kidney cultures and these were provisionally typed as *hardjo*.

Earlier work by Salisbury (1954) was limited to *pomona* studies and no antibodies were found in the 56 sera studied. No further details were given.

Preliminary studies of the brown rat by Kirschner and Gray (1951) found that 8/53 (15%) had serological evidence of *copenhageni* exposure and this serovar was isolated from 2/53 kidneys. Forty seven black rats were also studied and no serological or cultural

evidence of leptospirosis was found. Sera were tested for antibodies to *australis, canicola, copenhageni* and *bataviae*. The minimum serum dilution used was not given. Unfortunately, there were no tests for antibodies to *ballum*.

Blakelock and Allen (1956) in a survey of rats in Wellington city found no evidence of *Leptospira* antibodies at a minimum serum dilution of 1/10 using *australis, canicola, hyos,* and *pomona* antigens. Antibodies to *ballum* and *copenhageni*, which are now known to be the serovars endemic in rats were not tested for in this survey.

Shortridge (1960) reported 'positive or suspicious' antibody titres to *copenhageni* in 8/75 (11%) of the sera tested from rats, presumably brown rats. No further details were given.

Ballum serogroup infections were found to be endemic in black rats, as well as, house mice and hedgehogs (Hathaway 1978). The differention of age specific prevalences in these groups of animals implies that direct contact is important for transmission.

Hathaway (1978) investigated rats in the lower North Island. Five of 21 (24%) black rats had antibody titres ≥24 to *ballum*. Previously, Brockie (1977) found 1/16 (9%) of black rats seropositive but a minimum serum dilution of 1/100 was used. Hathaway (1978) found 5/155 (3%) of brown rats with antibody titres ≥24 to *ballum*. This was the only serovar isolated from the culture of kidneys. Isolations were made from 63/232 (27%) of brown rats and 17/52 (6%) of black rats. Although the serological prevalence was low, the cultural prevalence was high. In the studies of Brockie (1977), *copenhageni* isolations were made from 5/79 (6%) of brown rats. *Copenhageni* in rats appears to be confined to certain geographic locations. Brockie's study was centred in the Waikato region. Brockie also isolated *ballum* from 8/79 (10%) of brown rats and 4/16 (25%) of black rats. Hathaway (1978) concluded that the brown rat is a maintenance host for *copenhageni* and black rats are the normal

maintainance hosts for *ballum* although *ballum* is maintained in brown rat populations in synanthropic foci. The prevalence of *ballum* infection is population density dependent.

The most recent work in rats is that of Carter and Cordes (1980), where 112 urban and 48 rural (132 brown and 28 black) rats were examined for leptospirosis. Urine isolations were made from 51 (32%) of these rats. *Ballum* was isolated from 15 (9%) (12 brown, 3 black) and *copenhageni* was isolated from 36 (23%) (33 brown and 3 black).

The house mouse is considered a maintenance host for *ballum*. Brockie (1977) isolated *ballum* from 9/73 (12%) mice in rural areas of the North Island. Serological prevalence was low with only two of these nine animals with antibody titres ≥100 to *ballum*.

Hathaway (1978) found no serological evidence of leptospirosis in 12 house mice using a minimum serum dilution of 1/24. He tested for antibodies to *australis, autumnalis, ballum, bataviae, biflexa, canicola, copenhageni, grippotyphosa, hardjo, pomona,* and *pyrogenes.*

Hathaway (1978) found no serological or cultural evidence of leptospirosis in his study of 20 mustelids. These included ferrets (*Putorious putorious*) (9), stoats (*Mustela erminea*) (8) and weasels (*Mustela nivalis*) (3). The same sera dilutions and antigens were used for the serological tests as for the mouse study.

Feral cats were also studied by Hathaway (1978). No leptospiral isolates were made but there was serological evidence of exposure to *pomona*. The serological tests were performed as described previously.

Hedgehogs are known to be infected with *ballum*. Brockie and Till (1977) isolated the Ballum serogroup from 5/78 (7%) of hedgehogs from dairy farms in the lower North Island. Hathaway (1978) found 6/12 (50%) of hedgehogs with antibody titres ≥24 to *ballum* in

animals from rural farmed land. *Ballum* was isolated from 1/5 hedgehogs, all of which had antibody titres ≥24 to *ballum*.

Nine lagomorphs studied by Hathaway (1978) showed no serological evidence of leptospirosis using a minimum serum dilution of 1/24 and the 12 antigens used previously. All attempts to culture *Leptospira* were also negative.

Wild ungulates were studied by Daniel (1967) and only 1/393 (0.25%) goats had an antibody titre ≥200 to *pomona* and three goats and one deer had antibody titres ≥10 to *tarassovi*. No other evidence of leptospirosis was found. These sera were all tested at 1/10 and 1/200 dilutions for antibodies to *andaman, australis A, australis B, canicola, grippotyphosa, icterohaemorrhagiae, pomona,* and *tarassovi*.

Similar results were reported by Hathaway (1978) with no serological evidence of leptospirosis in sera from 38 wild ungulates. He concluded that leptospirosis in wild ungulates occurs at only very low levels.

1.4.8 Humans

Kirschner and Gray (1951) reported the first serologically confirmed case of leptospirosis in New Zealand. This occurred in a dairy farmer who had a high antibody titre to a representative of the Icterohaemorrhagiae serogroup. His farm was heavily infested with brown rats. This coincided with the establishment of a Leptospirosis Reference Laboratory at the University of Otago (McDonald *et al* 1985).

A year later, Kirschner *et al* (1952) reported the first isolation of *pomona* from 12 patients all of whom were associated with dairy farms. Six of these patients had been associated with an outbreak of 'redwater' in calves (Bruere 1952).

Leptospirosis was made a notifiable disease in 1952. From November 1951 to November 1952, sera from 315 patients were tested (McDonald *et al* 1985). Sixty eight (22%) were diagnosed as having leptospirosis. Most were dairy farm workers or meat workers (Faine and Kirchner 1953). Of these 68, one (2%), nine (13%) and 59 (87%) had antibody titres to *canicola*, *icterohaemorrhagiae* and *pomona*, respectively. The *canicola* seroconversion was reported by West and Whitehead (1953) and is the only report of *canicola* antibodies in humans in New Zealand. The sample produced an antibody titre of 9600. No source of infection was identified.

Faine and Kirchner (1953) showed leptospirosis to be widespread in New Zealand with a prevalence of 0.0035%. Kirschner (1954) reported an almost two fold increase in the incidence during the second year of the reference laboratory's operation. Some sera from patients with clinical symptoms of leptospirosis and from high risk occupational catagories, did not react against *canicola*, *icterohaemorrhagiae*, or *pomona* antigens used in the serological test. This lead to the inclusion of the *mitis (tarassovi)* antigen. Three patients were found to be serologically positive to this antigen only.

In September 1954, leptospirosis diagnostic services were relocated to the National Health Institute (NHI) and in the following two years, 774 human sera were tested (Josland *et al* 1957). Antibody titres ≥300 to *icterohaemorrhagiae*,*pomona* and *tarassovi* were found in 8, 39, and 6 respectively. The possibility of four more serovars was suggested with the demonstration of antibodies reacting with *australis*, *autumnalis*, *medanensis*, and *sentoti* antigens (Josland *et al* 1957). *Ballum* was first isolated from two dairy farm workers during a survey of clinical cases (Anon 1967). The first human isolation of *hardjo* in New Zealand was made in 1971 (Christmas *et al* 1974). This serovar, formerly of the Hebdomadis serogroup, is now classified as belonging to the Sejroe serogroup (Faine 1982).The most recent new serovar isolated from humans in New Zealand is

australis. This was isolated in 1977 by Thompson (1980) from a dry stock farmer suffering from clinical leptospirosis.

In the 1970's over 99% of the cases reported to the Department of Health were diagnosed as *hardjo* or *pomoma* (Brockie 1976). These were mainly dairy farmers or their families (Robinson 1975). Other occupational groups frequently associated with leptospirosis were pig farmers, stock truck drivers, abattoir workers and veterinarians (Kirschner and Maguine 1957, Robinson 1975, Schollum and Blackmore 1982). Serological surveys have confirmed that rural people are at greater risk (2-4%) than urban dwellers (Thompson 1979). Meat inspectors had an overall serological prevalence of 10.2% with *pomona* and *tarassovi* accounting for 85% of these titres (Blackmore *et al* 1979). A study by Blackmore and Schollum (1980), showed that 6.3% of meat workers had antibody titres ≥24 to *Leptospira* with *pomona* being the most common. They also established a relationship between abattoir workers who had contact with pig carcasses and the presence of antibodies to *pomona* and *tarassovi*.

The number of cases reported to the New Zealand Department of Health peaked at 800 in 1971. Between 1971 and 1980 an average or 400 cases were reported annually. The average number of annual cases reported fell below 200 between 1982 and 1985. This reduction coincided with the introduction of *hardjo/pomona* vaccines to control the disease in cattle (Marshall 1979b, Flint and Liardet, 1980). Ninety nine cases were reported in 1986 (New Zealand Department of Health 1987). There is a lack of information concerning the source of these remaining cases. Possibilities include domestic animals, such as deer and goats. However, in these later two species the disease is not well understood.

CHAPTER 2

VALIDATION OF THE MICROSCOPIC AGGLUTINATION TEST FOR THE DETECTION OF ANTIBODIES TO *LEPTOSPIRA*

2.1 Introduction

In 1917, Martin, Pettit and Vaudremer observed that sera of patients, who had suffered from leptospirosis, agglutinated leptospira cells. This agglutination reaction, which is maximal when a pure culture of *Leptospira* is mixed with homologous antiserum, is the basis of *Leptospira* serology. The Microscopic Agglutination Test (MAT), developed by Schuffner and Mochter (1926), is the origin of current methods used to detect antibodies to these organisms. There are numerous variations to this serological test. One widely accepted variation using microtitre plates, was first reported by Galton *et al* (1965) and later extensively modified by Cole *et al* (1973).

Although the MAT is the standard reference procedure for the serological diagnosis of leptospirosis, antibody titres obtained within laboratories and more frequently, between laboratories, often reveal unacceptable discrepancies (i.e. greater than one two fold dilution difference). For example, Bettleheim and Pearce (1986) reported on results submitted from 17 different laboratories within New Zealand from tests performed on identical batches of sera and for which the normal distribution of titres ranged from <25 to 51,200. These disparities between results may be explained by variations in the test methods employed by these different laboratories.

A standard procedure for the MAT has been documented in the WHO Technical Report Series (1967) and by Stallman (1984). These procedures are, however, sufficiently lacking in precise detail as to produce differing results. In addition, many of the methods used in different laboratories are well outside the general guidelines.

No reports have been found of studies assessing the suitability, particularly the reproducibility, of the MAT in measuring leptospiral antibodies in deer and goat sera. This study was aimed at establishing the critical variables and determining the reproducibility of the MAT using deer and goat sera.

2.2 Materials and Methods

2.2.1 Culture Medium

The medium used for the cultivation of all the *Leptospira interrogans* antigens used in this study is described in Appendix I.

2.2.2 Antigens

The antigens used were the following serovars of *L. interrogans* (Table 2.1):

Table 2.1 Antigens used in the Microscopic Agglutination Test

SEROVAR	STRAIN
australis	Ballico
ballum	Mus 127
bratislava	Jez
copenhageni	M20
hardjo	Hardjoprajitno, (avirulent)
hardjo	NZ12, (virulent)
pomona	Pomona
tarassovi	Peripelican

Antigen cultures were prepared by inoculating culture medium with approximately 10% of a viable culture and incubating at 29°C. Before use, cells were counted microscopically using a bacterial counting chamber[1] and if necessary, diluted using phosphate buffered saline (PBS) (Appendix II).

2.2.3 Sera

Deer sera were collected in 1984 from red deer hinds on two properties in the Nelson district.

Domestic goat sera were obtained from the Wallaceville Animal Research Centre[2] and were collected from animals throughout New Zealand.

The feral goat samples came from the Waikato region and had been previously tested in 1981 (Schollum and Blackmore, 1981) and stored at -20°C.

1 Petroff Hausser and Son, Philadelphia, USA.
2 Ministry of Agriculture and Fisheries, Wallaceville Animal Research Centre, Upper Hutt, New Zealand.

2.2.4 Equipment Validation

The precision and accuracy of a Dynatech automatic dispenser[3] and modified Cornwall syringe dispenser[4] were determined by comparing the weight of 96 well microtitre plates before and after the addition of a measured volume of PBS. The dispenser volumes were set at 25ul. Precision was determined by comparing the variation from the mean delivery over ten repeat tests. Accuracy was determined by comparing the variation of the mean delivery from the expected result. The accuracy of individual dispenser nozzles was determined by the removal of the diluent from each row in turn and reweighing.

The precision and accuracy of a micropipette[5] and diluters[6] for the transfer of 25ul was tested using the Diluter Delivery Tester.[7]

2.2.5. The Microscopic Agglutination Test

A serum reference plate (SRP) was prepared by dispensing 60ul of PBS, using the automatic dispenser, into each of the 96 wells of a Dynatech Microtitre plate.[8] Sera to be tested were added in 40ul volumes using the micropippette into ten individual wells in each row of the SRP to produce an initial dilution of 1/2.5. Forty microlitres of reference serum was added to the eleventh well of each row as a positive control. To the twelfth well, 40ul of PBS was added as a negative control. The SRP was covered, sealed with adhesive tape and stored at -20°C for later use.

3 Cooke Laboratory Products, 900 Slaters Lane, Alexandria, Virginia 22314, USA.
4 Becton, Dickinson and Co., Rutherford, New Jersey 07070, USA.
5 Gilson, France.
6 Flow Laboratories
7 Cooke Laboratoty Products, 900 Slaters Lane, Alexandria, Virginia 22314, USA.
8 Cooke Laboratory Products, 900 Slaters Lane, Alexandria, Virginia 22314, USA.

The diluter was then used to transfer 25ul of 10 sera and the reference serum from one row of the SRP 1/2.5 dilutions to the first row of the TP, producing serum dilutions of 1/5. The diluter was used to mix each sample and produce sequential doubling dilutions down the plate. The dilutions if each serum sample ranged from 1/5 in the first well to 1/640 in the last. Live antigen, diluted to 2 x 10^8 cells/ml, was added to each well in 25ul volumes using the modified Cornwall syringe dispenser.

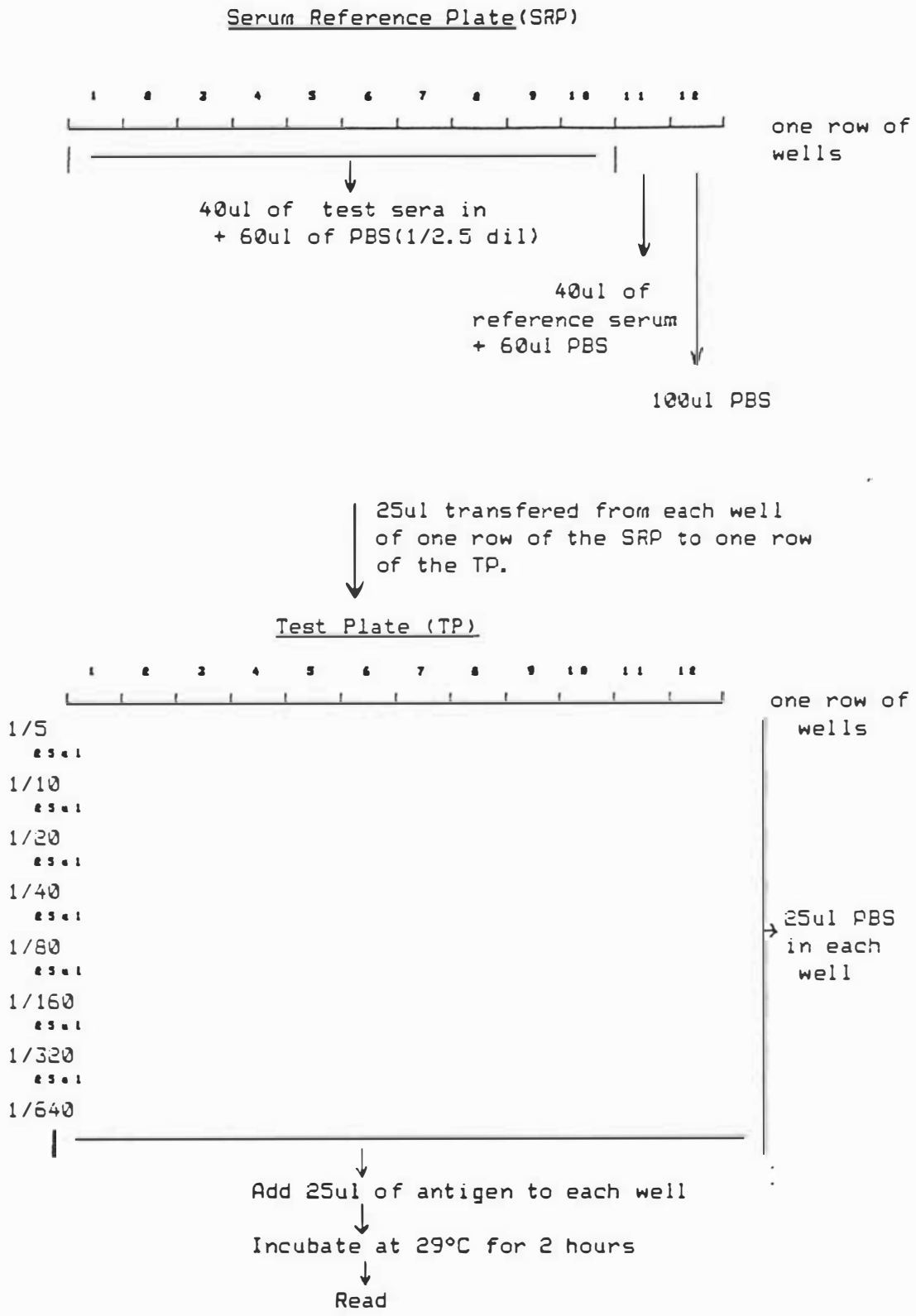
This produced a final dilution series which ranged from 1/10 to 1/1200 and an antigen concentration of 1 x 10^8 cells/ml. Each plate was then covered and incubated at 29°C for two hours. After incubation an aliquot was removed from each well and placed onto a glass slide for dark field examination. The titre of an agglutination reaction was taken as the greatest dilution of serum in which 50% of the leptospires were agglutinated.

The MAT procedure is outlined in Figure 2.1

Where inactivated antigen was used, 0.1% Formalin[9] was added to a live culture one hour before use.

9 ICI New Zealand

Figure 2.1 The Microscopic Agglutination Test Procedure



2.3 Results

The automatic dispenser, calibrated to deliver 25ul over all 96 wells, produced a mean delivery of 1.9ml, with a standard deviation (S) of 0.073, for each plate over 10 replicates. This was 21% less than expected so the accuracy was 79%. Precision, calculated from the variation from the individual eight nozzles was approximately 79%.

The Modified Cornwall Syringe 12 channel dispenser, also calibrated to deliver 25ul into each well of a microtitre plate or 2.4ml over all 96 wells, produced a mean delivery of 2.46ml (S = 0.027) for each plate over ten replicates. This is 0.06ml greater than the correct volume and so its accuracy is 97.5%. Precision was within 96.8%.

The 25ul diluters and micropippetter were tested for accurate delivery using the Diluter Delivery Tester. All were found to meet the manufacturer's criteria.

Microscopic agglutination tests on ten deer sera were used to compare both dispensers for diluent dispensing and determine whether variations in accuracy affected the results. Eight out of ten titres to *hardjo* were within one two-fold dilution (Table 2.2).

A series of 12 replicate tests for antibodies to *hardjo* in ten deer sera, produced results which were within one two-fold dilution (Table 2.3).

Two batches of 55 deer sera were tested for antibodies to *hardjo* (Tables 2.4 and 2.6). Samples were selected from these for triplicate tests (Tables 2.5 and 2.7). Antibody titres were within one two fold dilution.

Table 2.2 Agglutination Tests Comparing Antibody Titres to *Hardjo* using Two Dispensers

SERUM TEST NUMBER	ANTIBODY TITRES	
	AUTOMATIC DISPENSER	CORNWALL SYRINGE
1	20	20
2	160	160
3	640	320
4	640	320
5	640	80
6	160	640
7	640	320
8	320	320
9	320	320
10	1280	640

Table 2.3 Reproducibility of the MAT. Antibody Titres to *Hardjo*.

SERUM TEST NUMBER	REPLICATE TEST NUMBER											
	1	2	3	4	5	6	7	8	9	10	11	12
1	80	80	80	80	40	40	80	40	20	20	20	20
2	10	10	10	40	20	20	20	20	10	40	10	20
3	<10	<10	<10	10	<10	<10	<10	<10	<10	10	10	10
4	10	20	20	20	10	20	20	20	20	10	20	20
5	<10	<10	<10	10	10	<10	<10	<10	<10	<10	<10	<10
6	40	10	40	10	20	40	20	40	10	40	40	40
7	10	10	10	10	10	20	20	20	10	10	10	10
8	320	80	80	80	80	160	160	160	80	80	80	160
9	<10	<10	10	10	10	<10	<10	<10	<10	<10	<10	<10
10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10

Table 2.4 MAT Results from Batch 'A' of 55 Deer Sera

ANTIBODY TITRES TO *Hardjo*	NUMBER
0	17
10	16
20	10
40	6
80	4
160	2

Table 2.5 Results from Triplicate Tests on Ten Deer Sera from Batch 'A'

SERUM TEST NUMBER	ANTIBODY TITRES TO *Hardjo* IN THREE TESTS		
	1	2	3
1	10	20	10
2	20	20	10
3	20	20	20
4	<10	<10	<10
5	<10	<10	<10
6	20	20	20
7	20	20	20
8	10	<10	10
9	40	40	40
10	20	20	20

Table 2.6 MAT Results from a Second Batch 'B' of 55 Deer Sera

ANTIBODY TITRES TO *Hardjo*	NUMBER
0	27
10	13
20	6
40	2
80	5
160	1
320	1

Table 2.7 Results from Triplicate Tests on Ten Deer Sera from Batch'B'

SERUM TEST NUMBER	ANTIBODY TITRES TO *Hardjo* IN THREE TESTS		
	1	2	3
1	<10	<10	<10
2	<10	<10	10
3	40	80	80
4	160	80	80
5	<10	10	10
6	<10	<10	<10
7	20	40	20
8	40	40	20
9	<10	<10	<10
10	160	160	80

Sixty four goat sera tested using the MAT produced antibody titres >10 to *australis* in two sera (2), *ballum* (37), *bratislava* (20), *copenhageni* (4), *hardjo* (3), *pomona* (11) and *tarassovi* (6).

Twenty sera retested for antibodies to *ballum* produced 19 results within one two-fold dilution of the previous result (Table 2.8).

Table 2.8 Reproducibility of the MAT using Goat Sera

SERUM TEST NUMBER	ANTIBODY TITRES TO *Ballum* IN DUPLICATE TESTS	
	1	2
1	<10	<10
2	<10	<10
3	40	20
4	<10	<10
5	40	20
6	20	20
7	320	320
8	20	20
9	<10	<10
10	10	20
11	<10	<10
12	20	20
13	80	80
14	20	20
15	80	40
16	<10	<10
17	<10	<10
18	40	20
19	40	10
20	20	20

In the following tests (Tables 2.9 to 2.15), deer sera were used to determine the effect of varying test conditions on the antibody titres to *hardjo*.

Ten-fold differences in the final antigen density resulted in lower antibody titres than the standard 1 x 10^8 cells /ml (Table 2.9).

Table 2.9 Antibody Titres to *Hardjo* with Ten Fold Variations in the Antigen Concentration

SERUM TEST NUMBER	ANTIBODY TITRES TO *Hardjo* Antigen concentration (cells/ml)		
	1×10^7	1×10^8	1×10^9
1	<10	10	<10
2	<10	40	20
3	<10	320	80
4	<10	320	80
5	<10	20	<10
6	<10	320	160
7	10	160	40
8	10	320	80
9	<10	40	40
10	10	320	320

Trials varying the antigen concentration over a smaller range, confirmed results obtained using 10-fold differences. That is, lower antibody titres were obtained using antigen densities greater or less than 1×10^8 cells/ml (Table 2.10).

Cultures inactivated by adding 0.1% Formalin produced lower antibody titres than live cultures when used as antigen in the MAT (Table 2.11).

Table 2.10 Antibody Titres to *Hardjo* Obtained Using Different Antigen Concentrations

SERUM TEST NUMBER	ANTIBODY TITRES TO *Hardjo* Antigen Concentration					
	1 x 10^7	4 x 10^7	1 x 10^8	2 x 10^8	4 x 10^8	1 x 10^9
1	20	40	20	20	20	<10
2	10	<10	80	40	40	20
3	10	<10	320	320	320	80
4	10	80	320	320	160	80
5	10	80	80	40	20	10
6	<10	80	320	320	160	80
7	<10	160	320	320	160	80
8	10	80	80	40	40	20
9	10	40	40	40	40	20
10	10	320	320	320	160	80

Table 2.11 Comparison of Live and Inactivated Antigen

SERUM TEST NUMBER	ANTIBODY TITRES TO *Hardjo*	
	Live	Inactivated
1	20	10
2	160	80
3	640	<10
4	640	80
5	640	40
6	160	160
7	640	320
8	320	320
9	320	320
10	1280	640

In a test comparing the use of a recent isolate and laboratory adapted culture as antigen in the MAT, all antibody titres were within one two-fold dilution (Table 2.12).

Table 2.12 A Comparison of Antibody Titres Obtained Using Different Antigen Strains

SERUM TEST NUMBER	ANTIBODY TITRES TO *Hardjo*	
	Recent Isolate	Laboratory Culture
1	20	10
2	80	80
3	320	320
4	640	640
5	160	80
6	640	640
7	320	640
8	320	320
9	320	320
10	1280	640

Four day old cultures produced higher antibody titres than 10 and 21 day old cultures when used as antigen in the MAT (Table 2.13)

Table 2.13 Comparison of Antibody Titres Obtained Using Cultures of Different Ages

SERUM TEST NUMBER	ANTIBODY TITRES TO *Hardjo* Age of Culture (Days)		
	4	10	21
1	10	<10	<10
2	80	40	40
3	320	320	80
4	640	80	80
5	80	10	10
6	640	160	160
7	640	40	160
8	320	80	80
9	320	40	160
10	640	640	320

The incubation time required for maximum antibody titres varied inversely with the incubation temperature. At 29°C, maximum antibody titres were recorded after three hours incubation (Table 2.14).

Table 2.14 Antibody Titres Obtained at Different Incubation Times at 29°C

SERUM TEST NUMBER	ANTIBODY TITRES TO *Hardjo* Incubation Time (Hours)			
	1	2	3	4
1	10	10	10	20
2	40	40	80	80
3	<10	320	640	320
4	40	320	320	320
5	20	20	20	40
6	<10	320	640	640
7	40	160	320	320
8	320	320	320	320
9	40	40	160	160
10	160	320	1280	1280

Maximum antibody titres were also produced incubating at 16°C for 18 hours or 37°C for two hours.

Tests read again after storage at 4°C overnight produced identical results.

Tests comparing PBS and culture medium as diluents resulted in 9/10 antibody titres within one two-fold dilution (Table 2.15).

Table 2.15 A Comparison of Antibody Titres Obtained Using PBS or Culture Medium as Diluents

SERUM TEST NUMBER	ANTIBODY TITRES TO *Hardjo* Diluents	
	PBS	Culture Medium
1	20	20
2	160	160
3	320	160
4	320	320
5	80	320
6	640	640
7	320	640
8	320	640
9	320	320
10	640	640

The precision of the end point estimation was confirmed by checks using other observers.

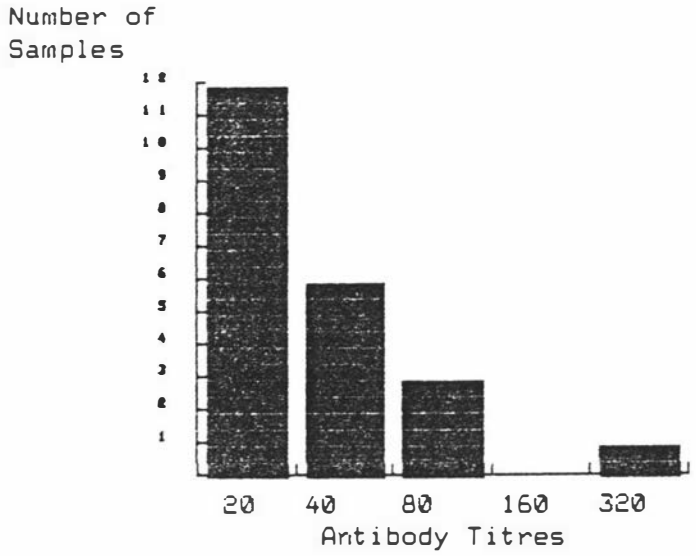
In 50% of the deer sera tests, a prozone effect was observed, with no agglutination at some of the lower serum dilutions and agglutination at higher dilutions. This was eliminated in 5/6 sera by heating the sera to 56°C for 30 minutes before testing, or by prolonging the incubation of the test for 30 hours at 16°C.

Current results on deer and goat sera show some differences in the results of previous tests in which different conditions were used (Figure 2.2).

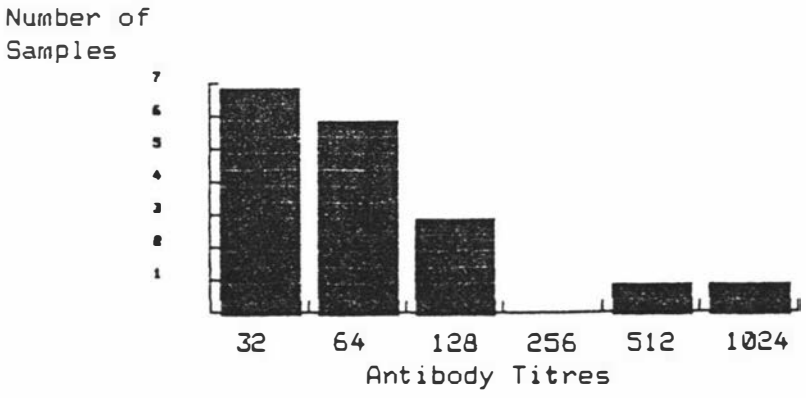
Figure 2.2 A Comparison of Results from Two Laboratories using the MAT

Identical Deer sera were Titrated for Antibodies to *hardjo* at the Massey Laboratory (A) and the Upper Hutt Laboratory (B)

(A) Massey Laboratory (22/46 Antibody titres ≥20)



(B) Upper Hutt Laboratory (18/46 Antibody titres ≥32)



2.4 Discussion

This investigation of the MAT has revealed those factors which are most likely to affect results and has demonstrated satisfactory reproducibility, that is within one two-fold dilution, using a standardized method for the determination of antibody titres in deer and goat sera.

Tests on the precision and accuracy of the equipment available for use in the MAT were necessary to ensure that variations would not affect results in subsequent experiments. The precision of the two dispensers, based on weight comparisons, was similar at 91% and 98%. Although the difference in accuracy was greater at 79% and 97.5% this had no effect on the MAT results in comparative trials (Table 2.2).

The Diluter Delivery Tester is essentially a blotter and is less reliable, although more convenient, than weight measurements, for determining the precision and accuracy of the diluters. Replicate MAT results using these diluters were within one two-fold dilution, so any undetected variations in the accuracy had a negligible effect on the test results.

The MAT method used was based on the following World Health Organisation (WHO) guidelines (WHO Expert Group 1967).

1. Prepare serial two-fold dilutions of test sera.

2. Add equal volumes of a four to 14 day old culture of 2×10^8 cells/ml to give a final concentration of 1×10^8 cells/ml.

3. Incubate at 22 to 37°C for two hours.

4. Examine microscopically for 50% agglutination.

Within these guidelines, the following, more clearly defined procedure was used initially in this study.

1. Prepare two-fold serial dilutions of test sera from 1/10 to 1/1280 in PBS.

2. Add equal volumes of antigen consisting of a four day old culture of 2 x 10^8 cells/ml.

3. Incubate at 29°C for two hours.

4. Examine microscopically for 50% agglutination.

However, as a result of the trials varying the test conditions, the incubation parameters were altered to 37°C for two hours.

Twelve repeat tests on ten deer sera demonstrated satisfactory reproducibility with all results within one two-fold variation (Table 2.2). Possible bias in the reading of the results was eliminated by the randomization and coding of the sera by an independent operator.

Two further lots of ten deer sera produced results within one two-fold dilution in triplicate tests (Tables 2.5 and 2.7).

Reproducibility was demonstrated in repeat tests on twenty goat sera with 19/20 results within the acceptable limits (Table 2.8).

Variations in test conditions established the critical variables in the method. Antigen concentration variations produced the greatest effect on the antibody titres recorded (Tables 2.9 and 2.10). This emphasises the need for accurate cell counts to be performed on each antigen before use in the MAT.

Formalin inactivated cultures produced lower antibody titres than live cultures in the MAT (Table 2.11). Tests using inactivated cultures were also more difficult to read.

There was no difference in results when laboratory adapted and freshly isolated *hardjo* cultures were compared as antigens (Table 2.12).

Four day old cultures produced maximum and reproducible antibody titres (Table 2.13). Ten and 21 day old cultures produced lower antibody titres and results were more difficult to read.

Maximum antibody titres were obtained after two hours incubation at 37°C. Longer incubation was required at lower temperatures. At 29°C, three hours were required to obtain maximum antibody titres (Table 2.14). Although maximum antibody titres were obtlined after 18 hours incubation at 16°C, the end-point was difficult to interpret.

There was no detectable difference in results using PBS or culture medium as diluent (Table 2.15).

It may not always be possible to use the ideal conditions for the MAT. If, for example, a culture cannot be grown to a sufficient cell density in four days, an older culture or a lower cell concentration may have to be used. In such circumstances, trials should compare the alternatives to determine which produces the most reproducible results.

No difficulty was experienced in determining the end-point of 50% agglutination using standard test conditions. This was reflected in the reproducibility trials and confirmed by checking with other observers. Difficulties were experienced when older and more concentrated antigens were used. Referring to a 1/2 dilution of the PBS control wells, where the number of free cells approximates that

of a 50% agglutination, assisted the identification of the end point.

Prozones are chracterised by the absence of agglutination at the lower serum dilutions and agglutination at higher dilutions.

Prozones were observed in approximately 50% of the deer sera with antibody titres to *hardjo*, but not in tests on goat sera.

The only recent report of prozones was that of Malkin (1984) in tests on sheep and goat sera. No reports of prozones using deer sera have been found.

Prozone effects can cause a misinterpretation of results. The cause of prozones is attributed to the presence of blocking antibodies or excess antibody preventing the formation of a lattice (Tizzard, 1984). The prozone can be eliminated by treating the sera at 56°C for 30 minutes before testing, as reported by Malkin, (1984) and confirmed in his study.

Extending the MAT incubation to 18 hours also reduced the prozone.

The differences in results obtained in tests on deer and goat sera, compared with earlier tests, can be explained by differences in the test methods.

The deer sera had been previously tested using different equipment and more importantly, there were differences in the test methods. The procedure used in the earlier tests used a minimum serum dilution of 1/32 rather than 1/10 used in this study. Antigen concentration was visually assessed by holding the culture up to an indirect light source, instead of using a counting chamber. The antigen cultures were four to seven days old as opposed to a standard four day old culture.

Previous tests on goat sera followed the method of Cole *et al* (1973). Their method differed from the one used in this study in that a minimum dilution of 1/24 was used, the antigen concentration was visually determined and again cultures were between four and seven days old.

From the results of this present study, it is recommended that test conditions be carefully controlled to help prevent variations in results. The use of standard sera and repeat tests for reproducibility would ensure reliability. The agglutination test, as standardized in this study, provides reproducible results with both deer and goat sera.

2.5 Conclusions

1. A standardized technique was found to enable reproducible MAT results with deer and goat sera.

2. Variations in antigen density have the greatest effect on antibody titres measured with the MAT. An antigen strength of 2 x 10^8 cells/ml is recommended.

3. Four day old cultures should be used as antiges for maximum, reproducible MAT results.

4. A test incubation temperature of 37°C for two hours is recommended as this produces maximum, reproducible, easily read MAT antibody titres in the shortest time.

5. End-point determination is assisted by referring to a 1/2 dilution of the antigen.

6. Treating sera at 56°C for 30 minutes before testing or prolonging the test incubation to 18 hours will eliminate prozone effects observed with deer sera in the MAT.

CHAPTER 3

LEPTOSPIROSIS IN GOATS

3.1 Introduction

There is little information about the prevalence of leptospirosis in goats in New Zealand (Daniel 1967, Hellstrom and O'Hara 1979, Schollum and Blackmore 1981). The reports that are published provide evidence that leptospirosis does occur, but no national surveys or detailed studies have been performed to determine the extent of either the infection or the disease. This information is vital in order to determine the role that goats play in the epidemiology of leptospirosis. The importance of goats as a source of infection for humans and other animals may become greater with the increase in the farming of goats in New Zealand.

The aim of this part of the study was to determine the prevalence of leptospirosis in goats. A national survey of randomly selected sera for leptospiral antibodies was followed by studies of six farms.

Three parts of the study were designed to look for serological evidence of infection and three were designed to search for serological and cultural evidence.

3.2 Materials and Methods

3.2.1 Sample Selection

The sera for the national survey were selected from 4000 samples sent to the Ministry of Agriculture and Fisheries (MAF) Wallaceville Animal Research Centre as part of the scheme to accredit goat flocks free from caprine arthritis encephalitis (CAE) (MacDairmid 1984). These samples had been randomly collected from animals nation wide and stored at -20°C. Using random number tables (RAND Corp. 1955), 64 samples were selected for leptospirosis testing.

The sample size was set arbitrarily as no suitable data of previously reported prevalence was available. A sample of this size was considered manageable for a detailed serological study. The sample size for such a study would normally be based on the size of the entire population, the estimated prevalence and the percentage certainty required (Faine 1982).

Age, sex and breed of the animals from which the sera were collected for the national survey were not specified.

The properties selected for the farm studies were chosen on the basis of the cooperation of the farmers and to some extent, for their accessibility to Massey University.

Sera from 110 mixed saanan breed goats, of unspecified age and sex, were obtained from property number one, located near Wanganui.

Sera from 72 feral goats, all approximately two years of age and of unspecified sex, were obtained from the second property, situated in Paraparaumu.

Sixty two sera were obtained from property number three, located in the Waikato. These were randomly selected from a mixed age group of angora cross does. *Pomona* infection was suspected on this property after abortions had occurred and serological evidence obtained by the MAF.

Sera and urine samples were collected from 40, approximately two year old, angora cross does on property number 4 in Featherston.

Another 40 angora cross does from four age groups ranging from 12 to >48 months were sampled for their sera and urine on property number 5 in the Pohangina Valley.

Mixed Saanan milking goats on property 6, near Palmerston North, provided sera and urine samples from 40 does in 8 different age groups ranging from one to nine years. Leptospirosis was suspected on this property five years earlier but this was never confirmed.

3.2.2 Serology

The MAT, as described in Chapter 2, was used in this study.

For the national survey, sera were screened for antibodies to 20 of the serogroups listed in Table 3.1. A 1/10 dilution of each serum was prepared for screening. Sera were tested, using doubling dilutions from 1/10, for antibodies to six of the serovars that have been isolated in New Zealand. *Bratislava* was included since antibodies were detected in the screening test.

The percentage of samples with antibody titres ≥10 to each serovar and the geometric mean titres (GMT) ≥10 for each serovar were calculated.

Table 3.1 Antigens used in the Serogroup Screening Test

SEROGROUP	REPRESENTATIVE SEROVAR	STRAIN
Australis	*australis*	Ballico
Australis	*bratislava*	Jez-bratislava
Autumnalis	*autumnalis*	Aki Yami A
Ballum	*ballum*	Mus 127
Bataviae	*bataviae*	Swart
Butembo	*butembo*	Butembo
Canicola	*canicola*	Hond Utrecht IV
Celledoni	*celledoni*	Celledoni
Cynopteri	*cynopteri*	3522c
Djasiman	*sentot*	Sentot
Grippotyphosa	*grippotyphosa*	Moskova V
Hebdomadis	*hebdomadis*	Hebdomadis
Icterohaemorrhagiae	*copenhageni*	M20
Javanica	*javanica*	Veldrat-Bataviae 46
Louisiana	*louisiana*	LSU 1945
Mini	*mini*	Sari
Panama	*panama*	CZ 214K
Pomona	*pomona*	Pomona
Pyrogenes	*pyrogenes*	Salinem
Sejroe	*hardjo*	Hardjoprajitno
Shermani	*shermani*	1342K
Tarassovi	*tarassovi*	Peripelican

3.2.3 Culture Preparation

Urine samples were collected in sterile specimen containers after an intravenous injection of 'Frudix'[1] diuretic (1ml/10kg body weight). A 0.1ml aliquot of urine was transfered to 10ml of a transport media (Appendix III). This preparation was then used to inoculate two types of culture media: semi solid EMJH (Appendix I), and Ellis medium (Appendix IV). Two 5ml aliquots of each medium was inoculated with 0.02ml and 0.1ml volumes of the inoculated transport medium. When inoculated, the culture media were incubated at 29°c for three months. Microscopic examinations were made at approximately two weekly intervals to observe growth.

3.3 Results

3.3.1 Serological Survey

The national survey screening test detected antibodies to Serogroups Ballum, Bratislava, Icterohaemorrhagiae, Pomona, Tarassovi and Sejroe.

In the national survey, seventy percent of all sera responded with antibody titres ≥10 to at least one of the serovars tested. All results were within 95% confidence limits using tables relating prevalence and percentage certainty to sample size in Faine (1982).

Antibody titres to *bratislava* were the most prevalent in the national survey. Thirty one percent of the samples had titres ≥10 (Table 3.2).

The next most frequently recorded antibody titres were to *ballum* (25%).

The highest antibody titres recorded were to *hardjo* with a GMT of 126.

1 Pfizers, Auckland, New Zealand.

Table 3.2 Serological Survey of the National Herd
(64 Samples)

SEROVAR	ANTIBODY TITRES								PERCENTAGE	GMT
	10	20	40	80	160	320	640	1280		
australis									0	
ballum	7	14	4	1		1			25	22
bratislava	12	4	2	2					31	16
copenhageni	5	1	1						11	13
hardjo			1	1			1		5	126
pomona	5		1	1					11	16
tarassovi		3							5	20

The most frequently recorded combination of antibody titres detected was to *ballum* and *bratislava* (Table 3.3) All other samples with multiple antibody titres included *ballum* and *bratislava*.

Table 3.3 Serological Survey of the National Herd
Antibody Titres to Multiple Serovars

Serovars	Number of Sera
ballum, bratislava	9
ballum, bratislava, pomona	5
ballum, pomona	3
ballum, tarassovi	2
bratislava, pomona	1
bratislava, copenhageni	1
ballum, bratislava, copenhageni	1
ballum, bratislava, tarassovi	1
ballum, bratislava, copenhageni, hardjo, tarassovi	1

3.3.2 Farm Studies

The serology results from the first farm study differed from those of the national herd in that there were more antibody titres to *ballum, hardjo* and *tarassovi* (Table 3.4). Antibody titres to *ballum* were the most frequently recorded (67/110, 61%). The highest antibody titres were to *bratislava* (1280), *hardjo* (640), and *pomona* (1280). The highest GMTs were to *hardjo* (47) and *pomona* (42).

A predominance of antibody titres to *ballum* and *bratislava* was seen in the results from the second farm study (Table 3.5). Antibody titres to *ballum* (19/75, 25%) and *bratislava* (28/75, 37%) were the most frequently recorded. The highest antibody titres were recorded to *hardjo* (640) with a GMT of 72.

Table 3.4 Farm Study 1 Serology Results
(110 Samples)

SEROVAR	ANTIBODY TITRES								PERCENTAGE	GMT
	10	20	40	80	160	320	640	1280		
australis	3	1							4	12
ballum	23	25	10	5	4				61	22
bratislava	12	3		4			1	1	19	23
copenhageni	8	3	1	2					13	17
hardjo	2	1	2	3		1	2		10	47
pomona	6	1	3	2	1	1	1	2	15	42
tarassovi	9	3	1	1	1				14	17

Table 3.5 Farm Study 2 Serology Results
(75 Samples)

SEROVAR	ANTIBODY TITRES								PERCENTAGE	GMT
	10	20	40	80	160	320	640	1280		
australis									0	
ballum	11	6	1	1					25	25
bratislava	11	10	6	1					37	37
copenhageni	6	4	1						15	15
hardjo	1	1	1	2		1	1		9	9
pomona	9	2							15	15
tarasssovi		1							1	1

Property number 3 also had a high prevalence of antibody titres to *ballum* (33/62, 53%) but lower to *bratislava* (10/62, 16%) (Table 3.6). Antibody titres to *hardjo* were the next most frequently recorded (16/62, 25%). A comparatively large number of sera from animals on this property had antibodies to *tarassovi* (14/62, 20%). The highest titres recorded were to *hardjo* (640) and *tarassovi* (320).

Antibody titres to *ballum* (8/40, 23%), *bratislava* (12/40, 30%), and *copenhageni* (11/40, 28%), all occured with a similar frequency to each other in the fourth farm study (Table 3.7). There were no antibody titres to *hardjo* in this study.

The fifth farm study demonstrated a high percentage of antibody titres to *copenhageni* (32/40, 80%), although the GMT was low (15) (Table 3.8).There was also a high prevalence of antibody titres to *ballum* (19/40, 48%). The highest antibody titres recorded were to *bratislava* (320) and *tarassovi* (320).

A study of age prevalence distribution of antibody titres was possible on property number 5 and showed that the 36 month old group had the greatest number of animals with antibodies to one or more serovars (29/40, 72%) (Table 3.9). The 12 month old group had the least number of animals with antibody titres to any of the serovars tested (11/40, 27%).

All antibody titres from farm study number 6 were low (≤80) (Table 3.10). The most frequently recorded antibodies were to *copenhageni* (31/40, 78%). The highest antibody titre was also recorded for this serovar (80). There was also a large number of low (≤40) antibody titres to *bratislava* (20/40, 50%).

No leptospires were cultured from any of the urine samples.

Table 3.6 Farm Study 3 Serology Results
(62 Samples)

SEROVAR	ANTIBODY TITRES								PERCENTAGE	GMT
	10	20	40	80	160	320	640	1280		
australis	2	2							6	14
ballum	12	10	5	6					53	22
bratislava	6	1		3					16	20
copenhageni	2	2	1						8	17
hardjo	3	2	5	5			1		25	42
pomona	1	3	1						8	20
tarassovi	5	6	2			1			20	21

Table 3.7 Farm Study 4 Serology Results
(40 Samples)

SEROVAR	ANTIBODY TITRES								PERCENTAGE	GMT
	10	20	40	80	160	320	640	1280		
australis									0	0
ballum	6	2			1				23	16
bratislava	6	1	2	2	1				30	24
copenhageni	6	2	3						28	17
hardjo									0	0
pomona				1					3	80
tarassovi	2								6	10

Table 3.8 Farm Study 5 Serology Results
(40 Animals)

SEROVAR	ANTIBODY TITRES								PERCENTAGE	GMT
	10	20	40	80	160	320	640	1280		
australis	6	1							18	11
ballum	12	3	3	1					48	15
bratislava	6	1		1		1			23	20
copenhageni	18	9	4	1					80	15
hardjo			1						3	40
pomona	4								12	10
tarassovi	4	1		1		1			21	24

Table 3.9 Farm Study 5 Age Distribution of Antibody Titres

SEROVAR	AGE GROUP (MONTHS)			
	12	24	36	) 48
australis	1	2	2	1
ballum		4	8	6
bratislava		1	6	2
copenhageni	9	11	8	6
hardjo		1		
pomona			2	2
tarassovi	1	1	3	1
TOTAL	11	20	29	18

Table 3.10 Farm Study 6 Serology Results
(40 Samples)

SEROVAR	ANTIBODY TITRES								PERCENTAGE	GMT
	10	20	40	80	160	320	640	1280		
australis									0	0
ballum	7	2							23	12
bratislava	12	6	2						50	14
copenhageni	22	4	4	1					78	14
hardjo									0	0
pomona	6	1							18	11
tarassovi	5								13	10

3.4 Discussion

The sera chosen for the national survey provided a representative sample of the national herd. One area of bias resulted from the voluntary nature of the CAE accreditation scheme. Farmers most concerned with animal health were most likely to participate.

A high level of exposure to *Leptospira* was indicated by the 70% prevalence of antibody titres ≥10 to one or more serovars. Ninety percent of the antibody titres were <80.

The most frequently recorded antibody titres were to serovars *ballum* and *bratislava*. The response to *ballum* was most likely due to contact with infected rodents or through contamination of food and water supplies with their urine. The cause of the antibody response to *bratislava* was unknown as this serovar has never been isolated in New Zealand.

Antibodies to *bratislava* have been reported in human sera in New Zealand but it has been suggested that this is due to cross-reactions between *bratislava* and *pomona* (McDonald *et al* 1985).

It is possible that the antibodies observed in this survey were a result of cross-reactions with another serovar. An examination of sera with antibodies to multiple serovars (Table 3.3) showed that antibodies to *ballum* and *bratislava* occurred together more often than any other combination. This may represent an antigenic cross-reaction or indicate that both serovars originated from the same source.

Ballum and *bratislava* are both associated with hedgehogs in other countries (Broom and Coghlan 1960). *Bratislava* has also been reported in dogs, pigs and horses (Ellis 1985). Recently this serovar was isolated from aborted domestic pigs in Northern Ireland (Ellis *et al* 1985).

The relevance of antibody titres to *bratislava* will be discussed further in Chapter 6.

It is often difficult to draw accurate comparisons between the results of different workers as test conditions vary. The most recent work in New Zealand goats was that of Schollum and Blackmore (1981) in which sera from captured feral stock were tested for antibodies to *australis, balcanica, ballum, copenhageni, hardjo, pomona,* and *tarassovi.*

Schollum and Blackmore's work differed from this study in that they did not test for *bratislava* and the minimum serum dilution was 1/24, rather than 1/10.

Balcanica was not included as an antigen in the present study as this serovar is so closely related to *hardjo* that the antibody response is usually indistinguishable (Hathaway 1978).

Schollum and Blackmore (1981) found 15/166 (13%) of goat sera with detectable antibodies. Antibodies to serovar *hardjo* were detected in 12/116 (10%) goat sera and antibodies to *balcanica* were detected in 13/116 (11%) of goat sera demonstrating the close serological relationship between these serovars. Antibody titres to *balcanica* were all within one two fold dilution of the antibody titres to *hardjo* for individual animals.

The low number of antibodies to *hardjo* (5%) detected in this current survey of domestic goats probably originated from contact with cattle while the antibody titres in the feral stock studied by Schollum and Blackmore (1981) are more likely to be due to *balcanica* originating from contact with opossums.

Hellstrom and O'Hara (1979) recorded 23/400 (6%) domestic goats with antibody titres ≥200 to *hardjo*.

In the study of Schollum and Blackmore (1981), 5/116 (4%) of the animals possessed antibodies to *ballum*, compared to the 25% recorded in this survey. No antibodies to any of the other serovars were detected.

Some explanation is required to account for the other antibodies detected in the national survey. The antibody titres to *copenhageni* presumably indicates exposure to infected rodents. Contact with pigs was the most likely source of exposure to *pomona* and *tarassovi*.

Hellstrom and O'Hara (1979) only recorded antibodies to *copenhageni* 3/870 domestic goats, however, they used 1/200 as their minimum serum dilution.

Antibody titres to *ballum* and *bratislava* were the most frequently recorded in the sera from the first farm study. The serological results differed from those of the national herd in that there was a higher prevalence of antibodies to *ballum, hardjo* and *tarassovi* and a lower prevalence of antibody titres to *bratislava*. This may be explained by the unusual management of stock on this property. There were frequent stock movements on this property, the farmer choosing to graze stock belonging to other farmers rather than purchasing goats herself. This type of management would increase the likelyhood of stock being exposed to a wider range of serovars than would be the case on properties with more stable populations. There were no other species farmed on this property so contact with other domestic stock did not provide an immediate source of exposure to leptospires.

Antibody titres to *ballum* and *bratislava* were the most frequently recorded in sera from the first farm. The high prevalence of antibody titres to *ballum* (61%) implies a high level of contact with infected rodents. The range of antibody titres to *bratislava* was

greater than was found in the national survey, with two high titres of 640 and 1280 perhaps indicating active infection.

The high antibody titres (>160) to *hardjo* and *pomona* may also indicate active infection.

The few low antibody titres to *australis* are probably the result of cross-reactions with other serovars.

Unfortunately no urine samples were obtained from these goats.

Although the animals on property number 2 were of feral stock, they were maintained in a domestic environment which may explain the results more closely resemble those of the national survey of domestic stock than those of recently captured feral goats studied by Schollum and Blackmore (1981).

As antibody titres to *ballum* and *bratislava* were the most frequently recorded, contact with infected rodents may have been important.

The high antibody titres to *hardjo* (>160) may indicate recent exposure to either *hardjo* through contact with cattle or *balcanica* from contact with opossums.

The suspected history of *pomona* infection on farm number 3, was not supported by the serology results.

There was a large difference between the number of animals with antibody titres to *ballum* (53%) compared with titres to *bratislava* (16%) on property 3 which indicates that there may be less antigenic similarity between these two serovars than was first suspected from the national survey.

The frequency of antibody titres to *hardjo* on farm number 3 was five times that for the national herd indicating a higher than average exposure to this serovar or a related serovar such as *balcanica*.

There was also a higher than average frequency of antibody titres to *tarassovi* recorded which is most likely to be due to contact with pigs.

Antibody titres to *ballum, bratislava,* and *copenhageni* were the most frequently recorded on property number 4. These results imply that contact with rodents is the most likely source of exposure to *Leptospira* on this property. This was supported by frequent sightings of rats. This property is primarily a sheep farm with no other domestic animals to act as possible reservoirs of infection. Although only one age group (2 years) was sampled, these results are probably representative of the whole group.

The lack of any leptospiral isolates from urine samples indicates that there was no active infection in this group. It is possible that younger age groups may be harbouring active infection.

Farm study number 5 was the most complete with a number of different age groups being studied. Antibody titres to *copenhageni* and *ballum* were the most frequently recorded implying contact with rodents as the a possible source of exposure to these serovars. Antibody titres in general were low (≤80) which may indicate the absence of active infection. The two antibody titres of 320 to *bratislava* and *tarassovi* may indicate a current, infection.

The age distribution of antibody titres (Table 3.9) shows only 11/40 12 month old goats with antibody titres ≥10 indicating that this group is either not as susceptible or not as exposed to infection as the older groups. The high frequency of antibody titres in the 36 and >48 month old groups implies that antibodies persist for several years or that constant re-exposure to these serovars maintains low antibody levels.

Although other animals on this property (cattle, horses and sheep) were not available for sampling, the antibody titres of the goats did not suggest that these animals were acting as reservoirs of *Leptospira* infection.

Leptospirosis had been suspected five years before on property 5, when there were several cases of haemoglobinurea in the goats and a single case of human leptospirosis. However, there was no serological evidence to suggest that this infection was still active in the herd. All antibody levels were low and most of them were to *copenhageni*, indicating that rodents were the most likely source of infection.

Sheep and cattle grazed on property number 5 were not sampled but there was no serological evidence in the goats to suggest that these other animals might be important as sourcesof infection.

With only low antibody titres being recorded in all the animals sampled, it was perhaps not surprising that no *Leptospira* were isolated from the urine.

Internationally, leptospirosis in goats is rarely reported although there is ample evidence to show that goats can become infected experimentally (Morse and Langham 1958; Tripathy *et al* 1985a, 1985b) and by natural exposure (van der Hoeden 1953). Serovars from the Australis, Grippotyphosa, Hebdomadis and Pomona serogroups have been isolated in many countries (Torten 1979).

Few serological surveys have been reported. In one the prevalence of leptospiral agglutinins was estimated at only 5 to 10 per cent (Schollum and Blackmore 1981)

The serological prevalence determined from the present survey of the national herd in New Zealand was 70%. Comparisons of serological prevalence rates must be interpreted with caution however because of

differences in the test techniques. The high frequency of antibody titres to *ballum* and *copenhageni* in all these studies implies that rodents are the primary reservoir of *Leptospira* for goats in New Zealand.

The numerous antibody titres to *bratislava* need further investigation as they have not been previously reported in goats nor has the serovar been isolated in New Zealand.

Although leptospiruria has been detected in goats (Morse and Langham 1958), no *Leptospira* were cultured from any of the urine samples in studies reported here. It is possible that *ballum, bratislava* and *copenhageni* are not shed in goat urine and that spread of infection only occurs from the maintenance population. Another possibility is that infection may be transmitted between goats venereally. This has been suggested as a possible means of transmission for *bratislava* in pigs after isolations from aborted piglets and the genital tracts of adult pigs (Ellis *et al* 1985).

The sensitivity of the culture techniques used in this study must also be questioned. The concentration of viable *Leptospira* in goat urine may have been below detectable levels. This aspect will be discussed further in Chapter 6.

The results from this study support the general conclusions made by other workers (Hellstrom and O'Hara 1979), that leptospirosis is not important in goats in New Zealand as no active infections were demonstrated. There was, however, serological evidence of widespread exposure to *Leptospira*.

3.5 Conclusions

1. Seventy per cent of domestic goats in New Zealand had antibodies to one or more *Leptospira* serovars.

2. Antibodies to *ballum, bratislava* and *copenhageni* predominated.

3. Antibodies to *australis, hardjo, pomona* and *tarassovi* were present.

4. More antibodies to *Leptospira* were detected in animals older than 24 months

5. No *Leptospira* were isolated from goat urine.

CHAPTER 4

LEPTOSPIROSIS IN DEER

1.1 Introduction

Although there are few documented studies of leptospirosis in New Zealand deer (Chapter 1), lately there has been an increase in the number of cases reported which probably reflects the recent growth in domestic deer farming. There are 3000 registered farms containing 400,000 deer (N.Z. Dept of Statistics).

This study of leptospirosis in deer was prompted by two factors. The first was the economic loss due to abortions and neonatal deaths attributed to leptospirosis. Secondly, deer are a possible reservoir of infection for humans and may account for some of the approximately 100 human cases of leptospirosis recorded in New Zealand annually. In this country, *hardjo, pomona* and *copenhageni* have all been isolated from deer urine.

Leptospirosis in deer due to at least nine serovars has been reported in the following countries; Australia (*hardjo, pomona, grippotyphosa*), the UK (*icterohaemorrhagiae, bataviae, ballum*), the USA (*canicola, grippotyphosa, pomona, pyrogenes*), USSR (*grippotyphosa, pomona*), France (unspecified), Yugoslavia (*ballum, pomona*), West Germany (*ballum, grippotyphosa, icterohaemorrhagiae*), and China (*romania*) (Mackintosh 1984).

This study was designed to determine the prevelance of leptospirosis in domestic deer in New Zealand. It involved a serological survey of animals from both the North and South Islands and a study of a deer farm situated at Gladstone, in the Wairarapa district. In the deer farm study both serological and cultural techniques were used.

4.2 Materials and Methods

4.2.1 Sample Selection

Samples of sera were obtained from two North Island and three South Island sources. The sample size and content varied, depending on sample availability from each source.

Sera from 50 red deer were obtained from Invermay Agriculture Research Centre[1] in the South Island. Forty of these were from hinds and nine from stags. These deer were in three age categories; yearlings, 18 months, and aged.

A deer slaughter plant in Mosburn, provided sera from seventy eight red deer stags from seven different South Island properties (A-G). These were grouped into four age categories; 2 years, 3 years, 4 years and above.

A Nelson deer farm provided sera from 6-12 month old red deer hinds.

1 Invermey Agriculture Research Centre, Private Bag, Mosgiel.

The Ruakura Animal Health Laboratory supplied 120 sera from fallow and red deer of unspecified age and sex, from the Waikato region.

Twenty four sera, from two year old red deer hinds from Taranaki, were obtained from the deer slaughter plant in Hastings.

The deer farm in Gladstone was selected for a detailed study because of its proximity to Massey University and the cooperative attitude of the farmer. Two visits, three months apart, were made to this property to take blood and urine samples. Thirty red deer hinds were sampled on the first visit. These were in three age categories; 6 months, 18 months, and older. Samples from 42 red deer, 32 hinds and 10 stags, were taken on the second visit. These animals, now three months older, were grouped into five age categories; 9, 21, 33, 45, and 59 months. All the stags were nine months of age and represented a group not sampled on the first occasion.

4.2.2 Serological Techniques

The MAT described in Chapter 2 was used in this study.

The deer sera from Ruakura and Mosburn were screened for antibodies to the 20 serogroups listed in Table 3.1 (Chapter 3). A 1/10 dilution of each serum was used for screening.

Sera from all sources were titrated using doubling dilutions starting from 1/10, and tested for the presence of antibodies to six of the serovars known to occur in New Zealand (*australis, ballum, copenhageni, hardjo, pomona, tarassovi*). The sera were also titrated for antibodies to *bratislava* as antibodies to this serovar were detected in the screening tests. Sera obtained during the farm study were tested using the same seven serovars and serovar *balcanica*.

The percentage of samples with detectable antibodies to each serovar and the geometric mean titres (GMT) for those sera with antibody titres ≥10 were calculated from the results of these tests.

4.2.3 Culture Preparation

Urine samples were collected in sterile specimen containers after an intravenous injection of 'Frudix'[2] diuretic (1ml/10kg body weight). A 0.1ml aliquot of urine was transfered to 10ml of a transport media (Appendix III). This preparation was then used to inoculate two types of culture media: semi solid EMJH (Appendix I), and Ellis medium (Appendix IV). Two 5ml aliquots of each medium were inoculated respectively with 0.02ml and 0.1ml volumes of the inoculated transport medium. The inoculated culture media were incubated at 29°C for three months. Microscopic examinations were made at approximately two weekly intervals to observe growth.

4.2.4 Culture Typing

Leptospiral isolates were identified by two methods; serum agglutination and bacterial restriction endonuclease analysis (BRENDA) (Marshall *et al* 1981).

Cultures isolated from urine samples were grown in liquid EMJH medium by transfering 0.5ml of culture to 5ml of liquid EMJH and incubating at 29°c for 7 to 14 days until growth was observed by microscopic examination. This was repeated until cultures containing ≥2 x 10^8 cells/ml with no signs of clumping after five days of incubation were obtained.

The cells were counted microscopically using a bacterial counting chamber.[3] The cell suspension was adjusted to 2 x 10^8 cells/ml by diluting in PBS (Appendix II). This cell suspension was used as

2 Pfizers, Auckland, New Zealand.
3 Petroff Hauser and Son, Philadelphia, USA

antigen and reacted against 12 standard reference antisera of known antibody titre. Antisera prepared against *australis, autumnalis, balcanica, ballum, bataviae, bratislava, canicola, copenhageni, grippotyphosa, hardjo, pomona,* and *tarassovi* were obtained from the WHO/FAO Leptospirosis Collaborating Laboratory, Public Health Laboratory, Brisbane. The isolates were identified to their serogroup level based on their agglutination with the test antisera.

Definitive identification was achieved using BRENDA. The isolates were repeatedly cultured to achieve pure cultures with a final volume of 50ml. These cultures were centrifuged at 10,000g for 30 minutes and resuspended in PBS (Appendix II). This was repeated twice to wash the cells free of media components. The cells were centrifuged again and the pellet resuspended in 1ml of 10mM Tris HCl ethylenedamine tetracetic acid sodium salt (EDTA) buffer (TEB)(Appendix XV).

Each preparation was incubated for 15 minutes at 37°C with 100ul of Grade 1 lysozyme[4](3mg/ml). Following this, 100ul of a 10% aqueous solution of sodium lauryl sulphate (SDS)[5]and 100ul of an aqueous solution of pronase[6](10mg/ml) was added and incubated further at 50°c for 18 hours. The protease solution was heated to 56°c for 30 minutes before use to destroy any endonucleases.

Sodium perchlorate was added to a final concentration of 1M. Incubation was continued at 50°C for 60 minutes. The mixture was then made up to 5ml with STE (Appendix XVI). DNA was extracted from this mixture with a solution of phenol, chloroform and isoamyl alcohol (25:24:1) (Appendix XVII) that had been saturated with STE.

DNA extraction was performed by adding 5ml of the phenol, chloriform isoamylalcohol mixture to 5ml of the sample and mixing gently by tipping backwards and forwards for approximately 1-2 minutes. The

4 Sigma Chemical Co. St. Louis, Missouri 63178, USA.
5 BDH, Poole, England.
6 Sigma Chemical Co. St. Louis, Missouri 63178, USA.

layers were separated by centrifugation at 3000g for 15 minutes. The top aqueous layer was removed and the extraction repeated until no further precipitating protein was evident. The sample was then pipetted into dialysis tubing. The DNA extract was dialysed against a tris EDTA dialysis buffer (TE) (Appendix XVIV) at 4°C for two days. The DNA sample was then stored at 4°C until required.

The purity of the DNA was determined by reading the optical density (OD) of the dialysed extracts in a spectrophotometer against a blank containing dialysis buffer. Optical density (OD) readings were taken in 10nm steps between 230nm and 280nm. Phenol contamination was indicated by a peak reading at 270nm. In this case more dialysis was necessary. Protein contamination was indicated by a 260/270 ratio of <1.8. If this occurred, the whole extraction procedure was repeated from the protease step.

The nucleic acid concentration was determined by the relationship; 1.0 OD reading = 50ug/ml at 260nm. The DNA content was estimated at 70% to 80% of the nucleic acid. This was confirmed by reading the OD of a solution containing 2ug of nucleic acid from the test sample, 200ul of 10X concentrated STE (Appendix XVII) and 800ul of ethidium bromide (diluted to 12.5 ug/ml) made up to 2ml with distilled water. The OD at 260nm of this solution was compared with a standard curve prepared with 0 to 1 ug/ml of calf thymus DNA. A volume of sample containing 2ug of DNA was digested with 20ul of 5x enzyme buffer (Appendix XIX) and 4 units of ECoR1 restriction endonuclease were made up to 100ul with water. This mixture was incubated at 37°C for 30 minutes followed by incubation at 65°c for 30 minutes. A 1/20 volume of 5M NaCl and two volumes of absolute ethanol were added, the solution mixed and stored at -20°c for 30 minutes. This was centrifuged in a microfuge[7] at 15,000g for 5 minutes. The supernatant was removed and the deposit rinsed with 70% ethanol and dried. The sample was resuspended in 45ul of sample buffer (Appendix XX) and incubated for 18 hours at room temperature.

7 Eppendorph Geratebau, West Germany.

An electrophoresis gel was prepared from 0.7g of ultra pure agarose[8] boiled in 100ul of electrophoresis buffer (Appendix XXI) and poured onto a 200 x 155 mm glass plate bounded by cellophane adhesive tape. Well formers were placed in the gel during pouring. When the agarose base set, the well formers and tape were removed and the plate was placed on a flat bed electrophoresis apparatus[9]). The wells were washed with electrophoresis buffer (Appendix XXI), the samples were added and the wells topped up with electrophoresis buffer.

Electrophoresis was allowed to continue until the methylene blue tracking dye, which had been added to the sample buffer, had migrated 125mm.

The gel was removed from the glass slide and placed on an ultra violet light box and photographed using Tri X film.[10]

The restriction patterns of the isolates were compared with those of standard reference strains.

4.4 Results

4.4.1 Serological Survey

The prevalence of leptospirosis in New Zealand domestic deer was estimated using the serology results originating from five different sources.

The screening tests on sera from Ruakura and Mosburn showed that antibody titres were present to all the serovars that have been isolated in New Zealand. In addition, antibodies to *bratislava*, a serovar that has not been isolated in New Zealand, were detected.

8 LKB, Bromma, Sweden
9 LKB, Bromma, Sweden
10 Kodak New Zealand Ltd

The highest antibody titres in the Invermay Agricultural Research Centre herd were to *hardjo* with a GMT of 380 (Table 4.1). These titres occurred in 62% of the samples. Antibody titres to *copenhageni* and *bratislava* were the most prevalent within this group, being detected in 96% and 86% of the samples respectively. Antibody titres to *ballum* and *tarassovi* occurred more frequently in the older groups while antibody titres to *hardjo* were found to be more prevalent in the yearling and 18 month old groups (Table 4.2).

Sera collected at the Mosburn deer slaughter plant had the highest antibody titres to *hardjo* with a GMT of 224 although the proportion of animals with titres (18/78, 23%) was smaller than those from the Invermay herd. Antibody titres to *copenhageni, ballum, tarassovi* and *bratislava* were the most prevalent in the Mosburn deer with 76/78 (97%), 69/78 (88%), 65/78 (83%) and 58/78 (74%) respectively having antibody titres ≥10 (Table 4.3).

The age distribution of antibody titres is difficult to interpret accurately due to variations in the size of the groups (Table 4.4). Antibody titres to *hardjo* occurred most frequently in the 3 year old group (13/22, 59%). Antibody titres to *pomona* occurred most frequently in the 2 year old group.

The distribution of antibody titres between the seven properties showed that most of the antibody titres to *hardjo* were confined to one property, 'C', from which all animals sampled were in the 3 year old group (Table 4.5).

Antibody titres to all other serovars were similar on all properties.

Similar results were seen in the deer sera from the Nelson farm. The highest antibody titres recorded on this farm were to *hardjo* although the GMT was somewhat lower at 23 (Table 4.6). However, the proportion with antibody titres to *hardjo* was higher at 68/88 (77%).

Table 4.1 Serology Results for the Invermay Deer
(50 samples)

SEROVAR	ANTIBODY TITRES											%	GMT
	10	20	40	80	160	320	640	1280	2560	5120	10240		
australis	4	1										10	11
ballum	13	5	2	1								42	15
bratislava	15	8	11	9								86	14
copenhageni	10	17	12	8		1						96	27
hardjo	4	1	1	2	2	5	13	1		1	1	62	380
pomona	9	1										20	11
tarassovi	8	3										22	12

Table 4.2 Age Distribution of Antibody Titres ≥10 for the Invermay Deer Herd

	Yearlings	18 Months	Aged
australis	0	5 (14%)	0
ballum	2 (22%)	15 (41%)	4 (80%)
bratislava	9 (100%)	31 (86%)	3 (60%)
copenhageni	9 (100%)	34 (94%)	5 (100%)
hardjo	6 (66%)	23 (63%)	2 (40%)
pomona	2 (22%)	8 (22%)	0
tarassovi	1 (11%)	8 (22%)	2 (40%)
TOTAL SAMPLED	9	35	5

Table 4.3 Serology Results for the Mosburn Deer

(78 samples)

SEROVAR	ANTIBODY TITRES								PERCENTAGE	GMT
	10	20	40	80	160	320	640	1280		
australis	32	2							44	11
ballum	12	39	14	4					88	22
bratislava	27	22	5	4					74	17
copenhageni	7	35	21	13					97	69
hardjo	1	2	1	3		1	7	3	23	224
pomona	7	7	2						21	16
tarassovi	33	18	10	4					74	17

Table 4.4 Age Distribution of Antibody Titres ≥10 for the Mosburn Deer

SEROVAR	AGE GROUP			
	2 yrs	3 yrs	4 yrs	)4yrs
australis	20 (44%)	6 (27%)	3 (75%)	2 (29%)
ballum	44 (98%)	18 (82%)	4 (100%)	4 (57%)
bratislava	38 (84%)	14 (63%)	4 (100%)	2 (29%)
copenhageni	43 (95%)	20 (90%)	4 (100%)	7 (100%)
hardjo	3 (7%)	13 (59%)	1 (25%)	1 (14%)
pomona	13 (29%)	2 (9%)	0	1 (14%)
tarassovi	36 (80%)	17 (77%)	4 (100%)	7 (100%)
TOTAL SAMPLED	45	22	4	7

Table 4.5 The Property Distribution of Antibody Titres from the Mosburn Deer

SEROVAR	PROPERTY						
	A	B	C	D	E	F	G
australis	0	3	15	4	15	4	32
ballum	0	5	12	4	15	4	30
bratislava	0	2	12	4	14	4	22
copenhageni	1	7	14	4	15	4	30
hardjo	0	1	13	1	0	0	3
pomona	0	1	1	0	6	1	6
tarassovi	0	8	12	3	15	4	17
TOTAL SAMPLED	1	7	15	4	15	4	32

Table 4.6 Serology Results for the Nelson Deer

(88 samples)

SEROVAR	ANTIBODY TITRES								PERCENTAGE	GMT
	10	20	40	80	160	320	640	1280		
australis	2	1							3	13
ballum	34	1	1						41	11
bratislava	27	17	4	3					58	16
copenhageni	8			1					10	13
hardjo	24	22	12	7	2	1			77	23
pomona	3								3	10
tarassovi	11	1							14	11

Antibody titres to *ballum* and *bratislava* were the most frequently recorded in the Ruakura sera at 30/120 (23%) and 24/120 (20%) respectively. Although there were only a few serum samples with antibody titres to *hardjo* (12/120, 10%) these were the highest antibody titres recorded in the Ruakura group (Table 4.7).

Sera from the Taranaki hinds all had low antibody titres (≤ 80). The most frequently recorded were to *ballum, copenhageni* and *bratislava* at 16/24 (67%), 7/24 (29%) and 6/24 (25%) respectively (Table 4.8).

The combined results from the five serological studies showed that the order of prevalence for antibody titres ≥10 was *bratislava* (53%), *ballum* (52%), *copenhageni* (49%), *hardjo* (36%), *tarassovi* (23%), *australis* (15%) and *pomona* (13%).

4.4.2 Farm Study

The results from the first sampling of the Gladstone deer farm showed that the highest antibody titres were to *balcanica*, with a GMT of 227 followed by *hardjo* with a GMT of 225 (Table 4.9). Antibody titres to *balcanica* were detected in 21/30 (70%) and to *hardjo* in 20/30 (67%) of the sera. The most prevalent antibody titres were to *ballum* (29/30, 97%). Antibody titres to *tarassovi* and *bratislava* were also recorded at high frequencies at 20/30 (67%) and 16/30 (53%) respectively. There were no antibody titres to *pomona* in this group.

An examination of the age distribution of antibody titres revealed that most occurred in the 18 month old group (Table 4.10).

Samples taken three months later had a lower GMT to both *balcanica* (147) and *hardjo* (147) and antibody titres were recorded in a smaller proportion of animals (19/42, 45%) (Table 4.11). The most frequent antibody titres were to *ballum, tarassovi, australis,*

copenhageni, and *bratislava* at 38/42 (90%), 36/42 (86%), 36/42 (86%), 34/42 (81%) and 79% (33%) respectively.

Antibody titres to *balcanica* and *hardjo* were absent in the nine month old group but present in 100% of the 21 month old group (Table 4.12).

Table 4.7 Serology Results for the Ruakura Deer

(120 samples)

SEROVAR	ANTIBODY TITRES							PERCENTAGE	GMT
	10	20	40	80	160	320	640	1280		
australis	4								3	10
ballum	11	5	8	4					23	23
bratislava	12	3	6	2	1				20	23
copenhageni	9	4	4						14	16
hardjo	7	4						1	10	18
pomona	7	3	4	2		2			13	28
tarassovi	3								3	10

Table 4.8 Serology Results from the Taranaki Deer

(24 samples)

SEROVAR	ANTIBODY TITRES								PERCENTAGE	GMT
	10	20	40	80	160	320	640	1280		
australis	1	1	1						13	20
ballum	5	7	4						67	19
bratislava	4		1	1					25	18
copenhageni	5	2							29	12
hardjo	1			1					8	28
pomona	1	1							8	14
tarassovi	1								4	10

Table 4.9 Serology Results from the First Sampling of Deer from the Gladstone Property

(30 samples)

SEROVAR	ANTIBODY TITRES								PERCENTAGE	GMT
	10	20	40	80	160	320	640	1280		
australis	6								20	10
balcanica	5	3	3	4	3	1	1	1	70	227
ballum	9	4	7	9					97	29
bratislava	7	6	1	2					53	18
copenhageni	9	3							40	12
hardjo	4	1	3	6	2	1	1	2	67	225
pomona									0	0
tarassovi	7	6	1						67	12

Table 4.10 Age Distribution of Antibody Titres ≥10 from the First Sampling of Deer from the Gladstone Property

SEROVAR	AGE GROUP (months)					
	6		18		71-95	
australis	0		8	(88%)	5	(63%)
balcanica	5	(38%)	9	(100%)	7	(87%)
ballum	12	(92%)	9	(100%)	8	(100%)
bratislava	3	(23%)	8	(89%)	5	(63%)
copenhageni	2	(15%)	4	(44%)	5	(63%)
hardjo	4	(31%)	9	(100%)	7	(87%)
pomona	0		0		0	
tarassovi	7	(54%)	7	(78%)	6	(75%)
TOTAL SAMPLED	13		9		8	

Table 4.11 Serology Results from the Second Sampling of Deer from the Gladstone Property

(42 samples)

SEROVAR	ANTIBODY TITRES								PERCENTAGE	GMT
	10	20	40	80	160	320	640	1280		
australis	13	11	10	2					86	20
balcanica	1	2	4	3			8	1	45	147
ballum	1	8	19	7	2	1			90	43
bratislava	11	12	10						79	20
copenhageni	11	16	5	2					81	19
hardjo	1	2	3	4		1	7	1	45	147
pomona	4	4	1						21	16
tarassovi	8	11	13	4					86	26

Table 4.12 Age Distribution of Antibody Titres ≥10 from the Second Sampling of Deer from the Gladstone Property

SEROVAR	AGE GROUP (MONTHS) (f=female, m=male)					
	9(f)	9(m)	21(f)	33(f)	45(f)	59(f)
australis	7 (70%)	10 (100%)	7 (100%)	5 (100%)	5 (100%)	4 (100%)
balcanica	0	0	7 (100%)	5 (100%)	5 (100%)	4 (100%)
ballum	8 (80%)	9 (90%)	7 (100%)	5 (100%)	5 (100%)	4 (100%)
bratislava	8 (80%)	8 (80%)	7 (100%)	5 (100%)	5 (100%)	4 (100%)
copenhagen	7 (70%)	7 (70%)	7 (100%)	5 (100%)	5 (100%)	2 (50%)
hardjo	0	0	7 (100%)	5 (100%)	5 (100%)	4 (100%)
pomona	1 (10%)	3 (30%)	2 (29%)	3 (60%)	0	0
tarassovi	8 (80%)	10 (100%)	7 (100%)	5 (100%)	5 (100%)	2 (50%)
TOTAL SAMPLED	10	10	7	5	5	4

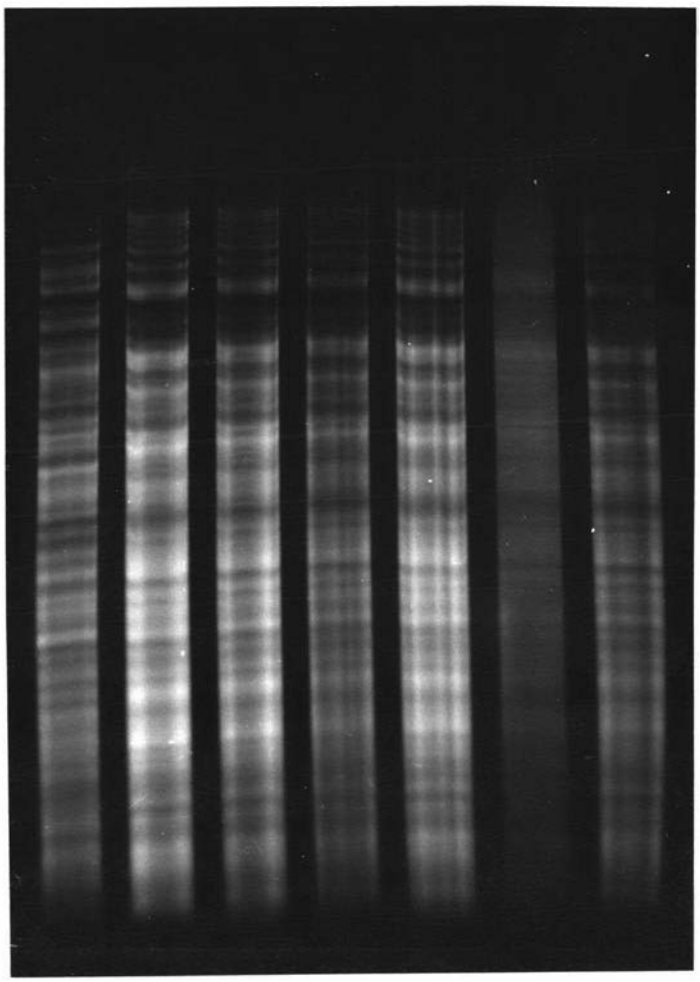
All the antibody titres to *balcanica* were within one doubling dilution of the antibody titre to *hardjo*.

Leptospira were isolated from urine samples of 5/9 of the 18 month old deer and 1/8 of the 71-95 month old deer from the first sampling. From the second sampling, 3/7 and 2/5 *Leptospira* isolations were made from urine samples from the 21 and 33 month old groups respectively. All isolates agglutinated with antisera to *balcanica* and *hardjo*. The BRENDA patterns confirmed that all the isolates were *balcanica* (Figure 4.1).

Figure 4.1 BRENDA of Isolates from Deer of the Gladstone Property

From left to right:

LANE	CULTURE
1	*Balcanica* reference strain - 1627 Burgas
2	Deer Isolate from an 18 month old animal
3	Deer Isolate from an 18 month old animal
4	Deer Isolate from an 18 month old animal
5	Deer Isolate from an 18 month old animal
6	Deer Isolate from an 18 month old animal
7	Deer Isolate from a 6 year old animal



4.4 Discussion

Tests on deer sera from five sources in New Zealand revealed that antibody titres to *ballum, bratislava* and *copenhageni* were the most prevalent and occurred with about equal frequency to one another. This result was similar to that obtained from the study of goats (Chapter 3). Rodents are the most likely source of infection for *ballum* and *copenhageni* as these represent the maintainance hosts for these serovars in New Zealand (Hathaway, 1978). Antibody titres to *bratislava* may have originated from contact with the same source as their prevalence is similar to that of *ballum* and *copenhageni*. Serovar *bratislava* has never been isolated in New Zealand and so an alternative explanation for these titres is required. It is possible that the antibody titres to *bratislava* are the result of cross reactions with similar antigens of other serovars. This will be discussed in more detail in Chapter 6.

The low level antibody titres to *australis, pomona* and *tarassovi* also may result from cross reactions with other serovars.

The high prevalence and magnitude of antibody titres to *hardjo* may represent exposure to either *balcanica* or *hardjo*. It is normally impossible to distinguish between the antibody response to these two serovars (Hathaway, 1978). The isolation of *hardjo* from deer has been reported (Fairley *et al* 1984). Cattle were also grazed on the Gladstone property in paddocks separate from the deer but on higher ground providing the possibility of the spread of *hardjo* through the natural watershed. Although these cattle were not studied for evidence of leptospirosis, cattle are the recognised maintainance hosts for this serovar and *hardjo* is endemic in cattle in many New Zealand herds (Hellstrom 1978). There was, however, no evidence of infection due to *hardjo* in the Gladstone deer.

The isolation of *balcanica* from deer urine in this study is the first confirmation of this infection in deer. The BRENDA patterns of

these isolates are the same as those obtained from New Zealand opossums but differ from the type strain *balcanica*, 1627 burgas, isolated in the Bulkan states in Central Europe by Janev as reported by Mateev and Manev (1974). Opossums are the most likely source of infection being the recognised maintainance hosts for this serovar. Contact with opossums on the Gladstone property may well have occurred although it is possible that the older stock became infected before capture and passed the infection on to the other stock. If the infection has spread in this way then transmission between deer must have occurred for many years as all deer less than 71 months of age were born and raised on the property. All the deer on the Glasdstone property share the same races and yard facilities which probably facilitates the spread of infection.

All the *balcanica* isolates in this study were obtained from animals aged 18 months or older. It is likely that deer younger than 18 months are susceptable to infection with *Leptospira*, as antibodies to other serovars were present.

The age specific prevalence of antibody titres to *balcanica* and *hardjo* in sera obtained from animals on the property studied indicated that the younger animals were not infected. Only 38% and 31% of sera with antibody titres to *balcanica* and *hardjo* respectively occurred in the six month old group. No antibody titres to these serovars were recorded in animals from the same group on the second visit. It is possible that the antibodies detected in the six month old group represented residual maternal antibodies although studies in other species, for example, cattle, have demonstrated that these antibodies normally wane to undetectable levels before six months (Hellstrom 1978).

The serological results from the Mosburn deer showed a similar pattern for the age specific prevalence of antibodies to *hardjo* with only 7% of deer in the 1-2 year age group having these antibodies.

This increased to 59% in the 2-3 year age group. Similarly, there were few antibody titres to *hardjo* in the hinds from Taranaki, which were described as being 'approximately two years old'. The results from the Invermay herd differed in that there was a high percentage (66%) of yearling hinds with antibody titres to *hardjo*.

Similarly, 77% of the deer from Nelson at six months of age had produced antibody titres to *hardjo* up to 320.

It is difficult to interpret the results from Ruakura as the ages of the animals sampled were not specified. There were, however, few antibody titres to *hardjo* (14%).

The age specific prevalence of *balcanica* infection in deer observed on the Gladstone property, is different from that observed for *balcanica* infection in cattle (Mackintosh 1981). It appeares that yearling cattle are either more susceptable, are grazed in areas more likely to contain a source of infection, or that the older animals are immune due to antibodies to *hardjo* resulting from past exposure.

There are several possible explanations for the absence of infection in deer less than 18 months old, some of which are unique to the Gladstone property. All deer on this property are kept in separate age groups, preventing any direct contact between groups. They are rarely moved and when moved, the paddocks are left without stock to recover for two to three weeks. This would prevent contact with urine recently shed from infected animals.

The different age groups of deer on the Gladstone property are grazed separately but sometimes on adjacent paddocks. There is, under these conditions, insufficient contact to enable spread between stock. Mackintosh (1981) showed that *hardjo* infection from properties with infected cattle failed to spread to cattle on a

neighbouring, even though there was natural drainage of water from the property with infected cattle.

It is possible that all the deer on the Gladstone property were exposed to *balcanica* before the birth of the youngest group. If *balcanica* infection produces only a low intensity of urinary shedding in deer, the younger stock may not be infected by contact with the older infected animals.

At the time of this study, the spread of infection to the younger animals may have been affected by climate as the district experienced a drier than normal winter in 1987. Only forty nine percent ot the normal rainfall was recorded from May to September (Heerdegen 1987). Late winter to early spring has been shown to be the most favourable time for the spread of *leptospira* in cattle (Hellstrom 1978). Climate influences the survival of *leptospira* outside the host animal and therefore has an influence on its spread (Faine 1982). The amount of ground water is also important in affecting spread (Hellstrom 1978).

One possible explanation for the absence of infection due to *balcanica* in the the younger animals is that spread of this serovar may require intimate contact between animals, such as that associated with mating. According to Yerex and Spiers (1987), the first mating in deer occurs when they are at least 15 months old. Behaviour associated with mating has been suggested as the cause of spread of *balcanica* infection in the opossum where infection only occurs in sexually mature animals (Hathaway 1978). If intimate contact between deer is required for the spread of infection due to *balcanica*, as is the case for opossums, this may be the normal method of spread for this serovar.

If the age specific prevalence of *balcanica* infection in the deer on the Gladstone property is a feature of *balcanica* infection in deer, it appears likely that at least some of the antibody titres to

hardjo observed in the serological studies of deer from other sources were due to exposure to *balcanica.* This is most likely in the Mosburn deer where there is a similar age distribution of antibody titres to *hardjo* as the deer from the Gladstone property.

4.5 Conclusion

1 There is serological evidence in deer for widespread exposure to *ballum, bratislava,* and *copenhageni.*

2 Evidence of exposure to *balcanica* or *hardjo* was seen on all properties studied.

3 *Balcanica* was isolated from deer for the first time.

4 *Balcanica* infection was found to occur somewhere from nine to 18 months of age in deer on the Gladstone property and its spread is possibly associated with mating.

CHAPTER 5

THE USE OF ENZYME LINKED IMMUNOSORBENT ASSAYS FOR THE DETECTION OF ANTIBODIES TO LEPTOSPIRA AND LEPTOSPIRA ANTIGEN.

5.1 Introduction.

The diagnosis of leptospirosis at present largely relies on both serological techniques to detect circulating antibodies and the isolation of *Leptospira*. This chapter describes the use of enzyme linked immunosorbent assay (ELISA) techniques as diagnostic methods.

The standard serological test, the MAT, was described in detail in Chapter 2. This has many disadvantages and as a result it is a specialised test not readily performed by routine diagnostic laboratories. Technical problems with this test include the use of live cultures as antigens in many laboratories which means a large battery of cultures needs to be maintained and also presents a risk of infection to laboratory workers. The need to use a darkfield microscope to determine the end point for the MAT titre makes the reading of these tests time consuming. To obtain reproducible results, an experienced operator is required as the end point determination of this test is subjective.

Isolation of *Leptospira* from infected fluids and tissues is required for the definitive diagnosis of leptospirosis. This is time consuming as inoculated culture media must be incubated for several months and examined microscopically at frequent intervals in order to detect growth. The sensitivity of culture techniques relies on the quality of the media and is fraught with difficulties which mainly arrise from the introduction of microbial contamination. Isolates must also be cloned and cultured several times to obtain a pure culture and must achieve a cell density of at least 10^8 cells/ml before they can be serologically typed. This means it may take up to six months to isolate and type *Leptospira*. To reduce the time taken to make this diagnosis, it was decided to explore other methods based on the ELISA.

Enzyme labelled antibodies were first used in a manner analogous to immunoflouorescence for the microscopic localization and

identification of antigens in biological tissues (Nakane and Pierce 1966). Later, Miles and Hales (1968) described how enzymes could be used instead of isotopes in an immunometric assay. The first detailed descriptions of enzyme immunoassay were published independently by Van-Weemen and Schuurs (1971) and by Engvall and Perlman (1971).

The Enzyme linked immunosorbent assay (ELISA) is a versitile tool that is used for the identification of a variety of antigens and antibodies in both the medical and veterinary fields (Voller *et al* 1976).

Antigen detection by ELISA generally takes the form of one of two basic methods, the competitive system or the double antibody sandwich.

The competitive system involves coating a solid phase with specific antibody. A sample containing an unknown amount of antigen is then mixed with a known amount of enzyme labelled antigen. This mixture is then allowed to react with the antibody coated solid phase. After washing to remove the unreacted material, a substrate is added and the inhibition of uptake of the enzyme labelled antigen is measured by the inhibition of substrate degradation. This is proportional to the amount of antigen in the sample. The disadvantage of this method is that each different antigen must be conjugated to an enzyme.

The double antibody sandwich method requires only labelled antibody. The solid phase is coated with specific antibody. The sample containing antigen is added and attaches to the coated solid phase. After washing, enzyme labelled specific antibody to the antigen is added and attaches to any antigen-antibody complexes on the solid phase. After washing, to remove unreacted material, a substrate is added. The proportion of substrate degraded is proportional to the amount of antigen in the test sample.

An indirect ELISA is used to measure antibody levels to infectious agent such as *Leptospira*. The solid phase is coated with antigen and incubated with the serum sample to be assayed for antibody. Antibody becomes attached to to the antigen on the solid phase. After washing, an enzyme labelled anti-species globulin is added which attaches to any antibody-antigen complex on the solid phase. Substrate is added and the rate of degradation of this is proportional to the amount of antibody in the test solution.

Although the test principles are simple, there is much work required to determine the optimum conditions for a successful diagnostic test. There are numerous possible permutations of variables such as the type of solid phase, solid phase coating conditions, washing regimes, dilutions of reagents and substrate selection.

A variety of materials have been used for the solid phase (Voller *et al* 1976). Disposable polystyrene, polypropylene or polyvinyl microtitre plates are the most convenient to use as large numbers of samples may be handled conveniently. Other materials include cellulose, cross linked dextrans, polyacrylamide, glass and silicone rubber which have been used in the form of beads, tubes, disks or microtitre plates.

The coating of the solid phase is normally achieved through passive adsorption of antigen or antibody, but covalent coupling using cyanogen bromide or fixation with acetone or cross linking with glutaraldehyde may also be used. The aim is to obtain a reproducible coating of the solid phase with the antigen or antibody, yet preventing non specific binding of subsequent reagents. Non specific binding is reduced by using blocking solutions, usually of protein, which bind to remaining areas on the solid phase and do not react with test reagents. Washing solutions and diluents usually contain a wetting agent, such as Tween 20, which reduces non specific uptake.

The method of preparation of the antigen for attachment to the solid phase is important. The antigen must be pure to prevent competition with contaminants for attachment. To remove all but the firmly fixed material, the solid phase must be thoroughly washed.

Test samples are diluted in a buffer containing wetting agents and additional protein to prevent non specific binding in subsequent steps. The optimum dilution of test samples and the incubation times and temperature must be determined through a series of tests

Washing steps are essential to remove unreacted material. Several washes are normally required and standing time in the washing solution is an important part of the process.

Conjugates are anti-globulins attached to enzymes. Both components need to be highly reactive. A variety of enzymes may be used. Alkaline phosphatase (AP) and horse raddish peroxidase (HRP) are the most common. The enzyme and antibody are coupled by one of several methods. The glutaraldehyde method (Avrameus 1969) and the periodate method (Wilson and Nakane 1978) are the most often used for this purpose.

A series of tests are required to determine the appropriate dilution of conjugate and the incubation conditions needed to produce optimum results. A washing step is essential after the conjugate reaction is complete.

The appropriate enzyme substrate is added which changes colour in the presence of the enzyme. Substrates need to be safe, inexpensive and stable. The substrate of choice for alkaline phosphatase is p-nitrophenyl phosphate. For peroxidase conjugates, hydrogen peroxide (H_2O_2) is used in conjunction with orthophenylenediamine (OPD), 5 amino salicylate or diaminobenzidine. For high sensitivity assays, OPD is considered best (Voller 1978).Substrate degradation is determined by a reaction rate analyzer or by the amount of colour

change in a given time. This is achieved by stopping the reaction after a set time with either a strong acid or alkali. The assay results may be expressed as being either positive or negative following a visual appraisal. Alternatively, absorbance values from a spectrophotometer, which are proportional to the antigen or antibody in the sample being assayed, may be used.

ELISA results may also be expressed as titres by serally diluting the sample to a predetermined end point.Reference positive and negative samples must always be included to achieve accurate and reproducible results.

The earliest reports of antigen quantitation by ELISA were those of Holmgren and Svennerholm (1973) who sucessfully measured the toxins of *Vibrio cholera*.

The ELISA is most widely applied to the measurement of specific antibody with one of the first applications being to measure antibody to *Salmonella* antigens (Carlson *et al* 1972).

For leptospirosis, the ELISA has several advantages over the MAT. It uses killed antigen, is less tedious to perform, can be read objectively and can be readily used for the testing of many samples. Different immunoglobulin classes can also be differentiated easily by using the appropriate conjugate.

The first recorded use of an ELISA for leptospirosis diagnosis was for the detection of IgM and IgG antibodies to *Leptospira* in human sera (Adler *et al* 1980). This involved coating the wells of microtitre plates with a sonicated suspension of Leptospiral cells and using a HRP conjugate and an H_2O_2/OPD substrate. Both IgM and IgG antibodies to *Leptospira* were detected in patients infected with *copenhageni, hardjo,* or *pomona.* Adler *et al* (1981) used a similar test to detect antibodies to *hardjo* in sheep finding a 95%

correlation between the MAT and ELISA for the detection of antibodies.

Cursons and Pyke (1981) suggested a diffusion in gel ELISA as an alternative to the MAT because of its simplicity, sensitivity and versitility. They used this test in a survey of human sera (Cursons *et al* 1982).

An ELISA similar to that used by Adler *et al* (1980) was used to detect the bovine IgM and IgG response to *hardjo* after infection or immunization (Adler *et al* 1982b).

An ELISA similar to that used by Adler *et al* (1980) was used by Waltman and Dawe (1983) to detect Leptospiral antibodies in swine sera. They reported 100% sensitivity, 86% specificity and a predictive accuracy of 91.3% with results directly correlating with the MAT.

Thierman and Garrett (1983) used a phenol extract as the antigen in an ELISA to detect antibodies to *hardjo* and *pomona* in cattle sera. They showed that this test was more sensitive than the MAT.

Adler and Faine (1983) demonstrated the presence of both genus and species specific non agglutinating antigens which could be detected only by ELISA.

An outer envelope preparation was used by Hartman *et al* (1984c) in an ELISA for the detection of canine antibodies to *canicola* with which the stage of the disease could be monitored. A similar test was used by Hartman *et al* (1984a) to monitor the response of dogs following vaccination against *Leptospira*.

Hartman *et al* (1984b) found the outer envelope antigen to be serotype specific in the ELISA.

Fairbrother (1984) used a variety of antigens in the ELISA to demonstrate the serological relationship between different antigens. Alkali extracts, sonicated antigen and ethanol precipitates were used.

A short publication by Setyawan (1984) describes the use of an ELISA using deoxycholate extract type specific leptospiral antigens to detect antibodies in rabbit and cattle sera. The *hardjo* antigen gave 80% sensitivity and 96.5% specificity in differentiating rabbit antibodies to *hardjo*.

Terpstra *et al* (1985) used a polysaccharide extract in the ELISA for the detection of human antibodies to *Leptospira* and concluded that this ELISA was useful for the rapid diagnosis of human leptospirosis.

Sonicated antigen as prepared as by Adler *et al* (1980), was used by Cousins *et al* (1985) to measure the antibody response of cattle to *Leptospira*. The levels of antibody detected by ELISA and the MAT neither correlated with each other nor with the periods of urinary shedding.

An ELISA using a sheath extract as the antigen was used by Cousins and Robertson (1986) to detect IgM and IgG antibody to *Leptospira* in sheep with good correlation between IgG antibodies measured by the ELISA and the MAT ($r = 0.9018$, $P > 0.001$).

There are no reports on the use of ELISA for the detection of antibodies to *Leptospira* in deer and only one brief account of its use for the detection of goat antibodies to *Leptospira* (Setyawan 1984).

The following work was based on methods used by Adler *et al* (1980) as these have been the most widely used to detect antibodies to *Leptospira*. The poor correlation between this type of ELISA and the MAT and urinary shedding, reported by Cousins *et al* 1985, was of little concern as a more sensitive test may also give a poor correlation yet be of greater use in the detection of *Leptospira* antibodies for epidemiological studies.

5.2 Materials and Methods

5.2.1 Solid Phase

Polyvinylchloride flat bottomed 96 well microtitre plates. Dynatech[1] and Nunc[2]brands were used.

5.2.2 Antigen and Antisera Preparation for Plate Coating

Cultures of *Leptospira* were grown in EMJH media (Appendix I) for four to five days at 29^{o}C. The cells were counted under a dark field microscope using a Petroff Hausser bacterial counting chamber[3]and cultures centrifuged at 10,000g for 10 minutes. The supernate was removed and the cells suspended in 0.06M carbonate buffer pH9.6 (Appendix XIII) to give a concentration of 5 x 10^{7} cells/ml. This was sonicated at 20 hz for two periods of 15 seconds immediately before use to coat the solid phase.

For the detection of antigen by ELISA, rabbit antisera to *Leptospira* were diluted to 1/400 in 0.06M carbonate buffer for plate coating.

1 Cooke Laboratory Products, 900 Slaters Lane, Alexandra, Verginia 22314, USA
2 Nunc, Denmark
3 C.A. Hausser and Son, Philadelphia, USA

5.2.3 Test Antisera Collection

Blood was taken from deer and goats and left to clot. Sera were extracted by centrifugation at 3000g for 10 minutes and stored at -2^0C until required for testing. All sera used for ELISA had been previously tested for antibodies to *Leptospira* using the MAT.

5.2.4 Antisera Production for Conjugate Preparation

Where commercial conjugates were not available, rabbit antisera were used for conjugate preparation.

Rabbit antiserum to *hardjo* was prepared following a series of inoculations of three month old New Zealand white rabbits. The inocula consisted of 1×10^9 cells/ml from seven day old cultures of the Bovis strain of *hardjo* prepared in EMJH medium (Appendix I). The cells were counted and 0.1ml of Formalin[4](40% formaldehyde) added to inactivate the cells. This was then centrifuged at 10,000g for 30 minutes, the supernate removed and the cells resuspended in PBS (Appendix II). The centrifugation and resuspension was repeated twice to wash the cells free of culture medium. The cell suspension was diluted with PBS (Appendix II) to give a final concentration of 1×10^9 cells/ml. One millilitre of this cell suspension was added to 1ml of Freunds complete adjuvant[5]and a water in oil emulsion created by forcing the mixture repeatedly through a double ended hyperdermic needle between two syringes. Once a stable emulsion was formed, as demonstrated by the formation of a discrete droplet that did not dissociate on the surface of water, it was injected intramuscularly into rabbits in four, 0.5ml volumes at four separate sites.

4 ICI New Zealand Ltd.
5 Difco Laboratories, Detroit, Michigan, USA.

A second inoculation was given 21 days later, intramuscularly at four different sites using 0.5ml volumes. This inoculum consisted of an emulsion prepared as for the first vaccination but using Freunds incomplete adjuvant.[6]

Fourteen days later, live *hardjo* cells from a seven day old culture of the Bovis strain grown in EMJH medium (Appendix I), were counted, then centrifuged at 10,000g for 30 minutes, the supernate removed, and the cells resuspended in PBS (Appendix II). The centrifugation and resuspension steps were repeated twice to remove any media components, the suspension adjusted to give 1×10^9 cells/ml, and 1ml injected intraveniously into the rabbit. This inoculation was repeated weekly, and 1ml blood samples were also taken at weekly intervals from an ear vein. Sera were extracted from the blood samples by centrifugation at 3,000g for 10 minutes, and tested for antibodies to *hardjo* using the MAT. When antibody titres were ≥300,000, 50ml of blood was taken by cardiac puncture and the sera extracted as described and stored at -20°C until required for conjugate preparation.

Anti-deer serum was prepared from New Zealand white rabbits inoculated with deer IgG which was separated from complete serum by the method of Corthier *et al* (1984).

The separation of IgG involved adding 5ml of saturated ammonium sulphate solution to 5ml of deer serum which was free from antibodies to *Leptospira* determined by using the MAT. This was incubated at 4°C for 60 minutes, centrifuged at 3,000g for 10 minutes, and resuspended in 0.025M Tris- HCl buffer pH 8.8 containing 0.035M NaCl (Appendix V). This was dialysed against this buffer at 4°C for 18 hours, then applied to a 1.6cm x 10cm DEAE-Trisacryl-M column,[7] that had been equilibrated with the Tris- HCl

6 Difco Laboratories, Detroit, Michigan, USA
7 LKB, Bromma, Sweden

buffer with a flow rate of 50ml/hr. The release of IgG was indicated by an ultra violet monitor.[8] Samples containing IgG were pooled.

The column was prepared for further work by desorbing other proteins by eluting with 0.025M Tris-HCl, pH 8.8 containing 1M NaCl (Appendix VI), followed by an equilibration with three volumes of Tris-HCl buffer containing 0.035M NaCl (Appendix V).

The IgG fraction pool was assayed for protein content according to the method of Bradford (1976). A 0.1ml sample was added to 5ml of Bradford Reagent (Appendix VII). After mixing and standing for two minutes, the absorbance was read at 595nm. A standard curve was prepared by testing solutions of bovine serum albumin containing a series of two fold increases in the concentration of protein from 10 to 100ug/ml using the Bradford method. The protein content of the IgG sample was estimated by comparing the absorbance result with the standard curve.

The protein content was adjusted to 10ug/ml by diluting with PBS (Appendix II) or concentrating using an ultrafilter[9] with a 10,000mw limit membrane (Amicon YM10)[10] and the sample was checked for purity by using immunoelectrophoresis (Graber and Williams 1953). A 1% agar (Oxoid Ionagar)[11] solution was made in 0.5% sodium barbitone buffer pH8.6 (Appendix VIII) and 10ml was poured while still hot onto a $10cm^2$ glass plate that had been thoroughly cleaned with detergent and alcohol. The glass plate was left at $4^{o}C$ for 18 hours then 0.5cm troughs were cut with a scalpel blade and 0.5cm diameter wells were cut with a punch at a distance of 1cm from the troughs (Figure 5.1). Initially, the agar was removed from the wells only and sufficient sample added to fill the wells. The samples were electrophoresed

8 LKB, Bromma, Sweden
9 Amicon USA
10 Amicon, USA
11 Oxoid, Basingstoke, Hampshire, England

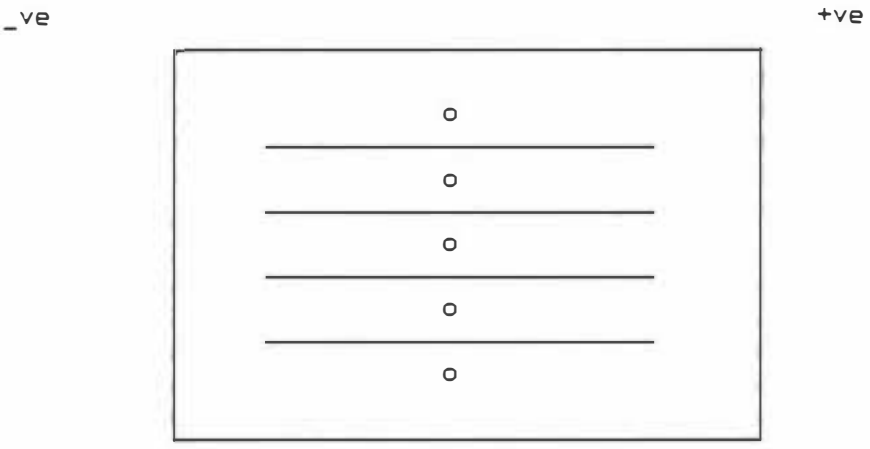
through the gel by applying 10v/cm (100v, 15mA) at 10°c for three to four hours using the LKB 2117 Multiphor.[12] A litre of 0.5% sodium barbitone buffer pH8.6 covering each electrode was connected to the gel with 10cm^2 wicks of filter paper.

Complete deer serum and the IgG fraction were electrophoresed on the same plate for comparison.

After electrophoresis, the agar was removed from the troughs and these were filled with 100ul of whole anti deer serum. The plate was then put in a humid chamber at room temperature for 18 hours. The lines of precipitation were recorded visually before the plate was dried by covering the gel with moist filter paper followed by another glass plate and a 1kg weight. After 18 hours at 37°c, the filter paper was removed from the dried gel by wetting and pealing from the dried gel. The gel was covered in Coomassie Blue stain (Appendix VIX) and left for 30 minutes at room temperature. The stain was removed and the gel soaked in 10% acetic acid to remove the stain from the gel to leave the precipitin lines visible. Four changes of acetic acid were needed with 30 minutes between changes to destain the gel. The gel was photographed with Ectachrome film.[13]

12 LKB Bromma, Sweden
13 Kodak, New Zealand

Figure 5.1 Immunoelectrophoresis Plate



Once pure deer IgG was obtained, 1ml containing 10ug of protein was added to 1ml of Freunds complete adjuvant and a water in oil emulsion created by forcing the mixture between two syringes connected by a double ended hypodermic needle as desctribed earlier. This was used to inoculate a rabbit intramuscularly at four different sites using 0.5ml at each site.

Twenty eight days later, 1ml of deer IgG containing 10ug of protein was combined with Freunds incomplete adjuvant and a water in oil emulsion prepared as before. This was injected intramuscularly at four different sites in 0.5ml volumes.

After a further 28 days, a series of intravenous inoculations of deer IgG containing 10ug/ml of protein were given on three successive days, with doses of 0.5ml, 1ml, and 2ml respectively. Thereafter, 2ml inoculations were given intravenously every seven days until a maximum antibody response was obtained. The antibody response was measured using the Ouchterlony double diffusion technique (Ouchterlony 1949) at seven day intervals.

A 1ml blood sample was taken from an ear vein and the sera extracted to determine the antibody titre to deer IgG using the Ouchterlony double diffusion technique. A glass plate $10cm^2$ was coated with 10ml of 1% agar (Oxoid Ionagar in sodium barbitone buffer pH8.6 (Appendix VIII). Wells, 0.5cm in diameter were cut using a template to give a pattern as in Figure 5.2 with a distance of 2mm between each well.

The agar was removed from each well using a pasteur pipette attached to a vacuum. The central well was filled with deer IgG and the outer wells were filled with ten fold dilutions of antisera from 1/10 to 1/100,000 in PBS (Appendix II). The plate was incubated in a humid atmosphere at room temperature for 24 hours, then dried, washed, stained and photographed as for the immunoelectrophoresis plates.

5.2.5 Conjugate Preparation

The method used was based on that of Wilson and Nakane (1978).

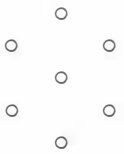
Horse radish peroxidase (HRP,RZ 3)[14] was prepared for conjugation by dissolving 4mg in 1ml of distilled water, adding 0.2ml of freshly made 0.1M sodium periodate, and stirring at room temperature for 20 minutes. This was then dialysed against 1mM sodium acetate buffer, pH4.4 (Appendix X) at 4°C for 18 hours.

Immunoglobulin (8mg), which had been purified using DEAE Trysacryl-M and assayed for protein content as described in 5.2.4, was dissolved in 1.0ml of 0.01M carbonate buffer pH9.5 (Appendix X).

To the HRP solution, 20ul of 0.2M carbonate buffer (Appendix XII) was added and this was immediately followed by the immunoglobulin solution. This was stirred for 2 hours at room temperature. A fresh sodium borohydride solution containing 4mg/ml was made and 0.1ml of this added to the HRP/immunoglobulin mixture and allowed to stand at 4°C for 2 hours.

14 Sigma Chemical Co., St. Louis, Missouri 63178, USA

Figure 5.2 Gel Diffusion Plate



An equal ammount of saturated ammonium sulphate was added, and the solution centrifuged at 10,000g for 10 minutes. The supernate was discarded and the precipitate was resuspended in a 50% saturated ammonium sulphate solution. This was centrifuged again, the precipitate resuspended as before, centrifuged for a third time and the precipitate resuspended in PBS (Appendix II). This was dialysed for 18 hours against PBS (Appendix II) at 4°C. Bovine serum albumin was added to a final concentration of 1% and the conjugate filtered through a 0.22um nitrocellulose membrane.[15] An equal quantity of glycerol was added for storage at -20°C.

5.2.6 Tests for Conjugate Activity

To test the activity of the antiserum to deer conjugate, a microtitre plate was coated with 0.2ml of normal deer serum at a 1/400 dilution prepared in 0.06M phosphate buffer pH8.6 (Appendix XIII) by incubating at 4°C for 18 hours. The coating solution was then removed by inverting the plates. The wells of the plates were washed three times by filling with washing solution (Appendix XIV), standing for five minutes, then refilling with washing solution using a Nunc Immunowash.[16] Two fold serial dilutions of conjugate from 1/50 to 1/3200 were made in a PBS/Tween 20/BSA diluent (Appendix XIV). Fifty microlitre volumes of each dilution were added to each of three wells and the plates were incubated at 37°C for 60 minutes.

The plates were then washed six times as described above.

The substrate was prepared by dissolving 0.005g of OPD in 0.25ul of methanol, adding 12.25 ml of water and 25ul of 3% H_2O_2. This was

15 Millipore Corporation, Bedford, Massachusetts 01730, USA
16 Nunc, Denmark

added in 50ul volumes to each well and incubated at room temperature in the dark for 20 minutes.

The reaction was stopped by adding 0.25ul of 8N H_2SO_4 sulphuric acid to each well.

The optical density was read at 486nm against blank wells with no coating antiserum or antigen using a plate reader (SLT 210).[17]

A HRP Protein A conjugate[18] was tested as above for use as a possible alternative to the antiserum to deer conjugate.

The antiserum to *hardjo* conjugate was tested using a similar technique but the plates were coated with a suspension *hardjo* cells described in 5.2.2. instead of serum.

5.2.7 Chequerboard Titrations

Chequerboard titrations were used to determine the optimum concentration of antisera and conjugate to use.

Microtitre plates were coated with antigen as previously described in 5.2.6.

Antiserum to the antigen was diluted in serial two fold steps from 1/100 to 1/3200 in PBS/Tween 20/BSA (Appendix XIV).

Serum from the same species with no antibodies to the antigen was diluted as above.

The dilutions of sera with and without antibodies to the antigen, as measured by the MAT, were added in 100ul volumes to the wells of the microtitre plates, as in Figure 5.3.

17 Salzburger Electronik Industrie, SLT Instruments, Grundig, West Germany

The plates were incubated at 37°C for 60 minutes, then washed three times as described in 5.2.6.

Serial two-fold dilutions of conjugate from 1/100 to 1/3200, were prepared in PBS/Tween 20/BSA (Appendix XIV). These were added to the plates as in Figure 5.3, and the plates incubated at 37°C for 60 minutes.

The plates were washed as before and 50ul volumes of a freshly prepared substrate prepared, as described in 5.2.6, added to each well. The plates were incubated at room temperature in the dark for 20 minutes and the reaction stopped by adding 25ul of 8N H_2SO_4 to each well.

The optical density was read at 486nm using a spectropohotometer (SLT 210 reader), and the optimum dilutions of serum and conjugate determined by the combination that produced the greatest difference between positive and negative sera.

18 Miles Laboratories Inc. Elkhart, Indiana 46514, USA

Figure 5.3 Chequerboard Titration

	Serum Dilutions											
	Negative						Positive					
Conjugate Dilutions	1/3200	1/1600	1/800	1/400	1/200	1/100	1/3200	1/1600	1/800	1/400	1/200	1/100
1/3200												
1/1600												
1/800												
1/400												
1/200												
1/100												

5.2.8 An Indirect ELISA Procedure for the Detection of Goat Antibodies to *Leptospira*

This procedure was based on that of Adler *et al* (1980). Antigen, prepared as described in 5.2.2, was added in 200ul volumes to all wells of a microtitre plate. This was incubated at 4°C for 18 hours.

The antigen was then removed by inverting the plate, and the plate was washed three times by filling the wells with PBS/Tween 20/BSA (Appendix XIV) and emptying after five minutes, using the Nunc Immunowash.

Test sera were added in 100ul volumes. For most tests, the optimum dilution of serum in PBS/Tween 20/BSA (Appendix XIV), which had previously been determined using a chequerboard titration, was added to each of three wells for each sample. Alternatively, sera were titrated in serial two fold dilutions from 1/50 to 1/3200 in PBS/Tween 20/BSA (Appendix XIV) and 100ul of each dilution added to each of three wells. The plates were incubated at 37°C for 60 minutes.

The plate was washed six times as described previously, then 50ul of the appropriate dilution of rabbit antiserum to goat peroxidase conjugate, (Evai Bois[19] and Miles[20] which had been determined by a chequerboard titration, was added to each well and the plate was incubated at 37°C for 60 minutes.

The plate was washed six times as before.

The substrate was prepared by dissolving 0.005g of OPD in 0.25ml of methanol, adding 12.25ml of water and 25ul fo 3% H_2O_2. This was added

19 Evai Bois, Petworth, West Sussex, England
20 Miles Laboratories Inc. Elkhart, Indiana 46514, USA

in 50ul volumes to each well and incubated at room temperature in the dark for 20 minutes. The reaction was stopped by adding 25ul of 8N H_2SO_4.

The optical density was read using the SLT 210 plate reader at 486nm against blank wells containing no antiserum.

The procedure is summarised in Figure 5.4

5.2.9 The Double Antibody Sandwich ELISA Procedure to Detect *Leptospira*

A microtitre plate was coated with hyperimmune bovine antiserum to *Leptospira* prepared as described in 5.2.2. Quantities of 200ul were added to each well and the plate was incubated at 4°C for 18 hours.

The plate was emptied and washed three times, as described previously.

Leptospira cultures, incubated for seven days in EMJH (Appendix I), were counted using a bacterial counting chamber,[21] and serial ten fold dilutions made in PBS (Appendix II), from 10^8 to 10^1 cells/ml. Each dilution was added to each of three wells in 0.1ml quantities. The plate was incubated at 37_oC for 60 minutes, then emptied and washed six times.

Antiserum to *hardjo* was added to each well in 100ul volumes at a dilution determined previously using a chequerboard titration, and the plate was incubated at 37°C for 60 minutes.

The plate was then emptied and washed six times before the appropriate dilution of conjugate, which had been determined by a

21 C.A. Hauser and Son, Philadelphia, USA

chequerboard titration, was added in 50ul volumes to each well. The plate was then incubated for a further 60 minutes at 37°C.

The plate was washed six times, and the substrate, prepared as in 5.2.8, was added in 50ul volumes and the plates were incubated in the dark at room temperature for 20 minutes. The reaction was stopped by adding 25ul of 8N H_2SO_4 to each well. The optical density was read on the SLT210 reader at 486nm.

This procedure is summarised in Figure 5.5

5.2.10 A Single Antibody ELISA Procedure to Detect *Leptospira*

A single antibody ELISA procedure was also used which followed the procedure of the double antibody sandwich to the stage just before the addition of the antiserum to *hardjo.* At this point 50ul volumes of rabbit antiserum to *hardjo* peroxidase conjugate, diluted to the optimum concentration as determined by a chequerboard titration, was added. The plate was incubated at 37°C for 60 minutes. After washing six times, the substrate was added and the reaction allowed to procede as described previously.

This procedure is summarised in Figure 5.6

Figure 5.4 The ELISA for Detecting Antibodies to *Leptospira*.

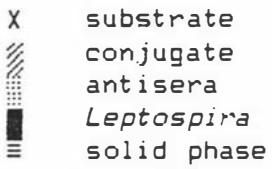


Figure 5.5 The Double Antibody Sandwich Procedure

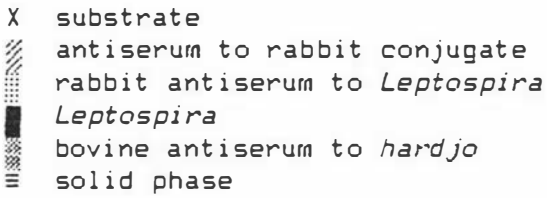
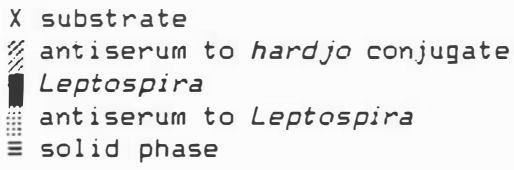


Figure 5.6 The Single Antibody ELISA Procedure



5.2.11 Tests Performed

The reproducibility of the ELISA for the detection of goat antibodies to *hardjo* was determined by testing one serum with antibodies to *hardjo* measured by the MAT and another without detectable MAT antibodies to any of the six serovars of *Leptospira* used in this test. These were tested in four diferent plates, with tests performed in triplicate on each plate. Two different brands of plate, Dynatech and Nunc, were compared for use in this test.

Ninety four goat sera were assayed by ELISA, using a single serum dilution, for goat antibodies to *hardjo*.

Sixty four sera from a national survey of New Zealand goats (Chapter 3) were tested for antibodies to *hardjo* using the single serum dilution ELISA and these results were compared with the MAT.

The ELISA using titrated sera was performed on sixteen sera and these results were compared with those of the single serum dilution ELISA.

Antibodis to *pomona* were detected using the single serum dilution ELISA. Twenty three sera were tested.

The double antibody sandwich ELISA was used to test for *hardjo*. Different strengths of coating serum were tested to try to increase the sensitivity.

The single antibody ELISA was compared with the double antibody sandwich method for the detection of *hardjo* antigen.

5.2.12 Statistical Analysis

To compare the MAT and ELISA for measuring the antibody response to *hardjo*, contingency tables were prepared (Piazza 1979) (Table 5.1).

Table 5.1 Contingency Table for the Analysis of Serological Data

		MAT +	MAT −	
ELISA	+	a	b	R_1
	−	c	d	R_2
		C_1	C_2	N

$R_1 = a+b$

$R_2 = c+d$

$C_1 = a+c$

$C_2 = b+d$

$N = a+b+c+d$

The comparative sensitivity of the ELISA compared to the MAT is a/C_1.

The comparative specificity of these is d/C_2.

The comparative sensitivity of the MAT compared to the ELISA is a/R_1.

The comparative specificity of these is d/R_2

The Chi squared (X^2) test for independence was performed on this data, to test the nul hypothesis that there is no statistical association between these two tests.

The X^2 test, based on the contingency table above is :

$$X^2 = \frac{[(ad - bc)^2 - 0.5N]^2}{R_1 R_2 C_1 C_2}$$

The correlation coefficient (r) was calculated to determine the extent of the association.

The formula, based on the information in the contingency table is:

$$r = \frac{(ad - bc)}{(R_1 R_2 C_1 C_2)^{1/2}}$$

5.3 Results

5.3.1 Conjugate Preparation

Rabbit antiserum to *hardjo* produced an antibody titre of 327,680 measured by the MAT.

The IgG fraction of deer serum was eluted from the DEAE Trysacryl-M-column in a discrete peak measured by the UV monitor (Figure 5.7).

Immunoelectrophoresis of the pooled fractions from the DEAE TRYSACRYL M column shows the sample is pure Figure 5.8

The standard curve for the estimation of protein by the Bradford method is shown in figure 5.9.

Figure 5.7 Elution Peak from Deer Sera Adsorbed onto DEAE TRYSACRYL M

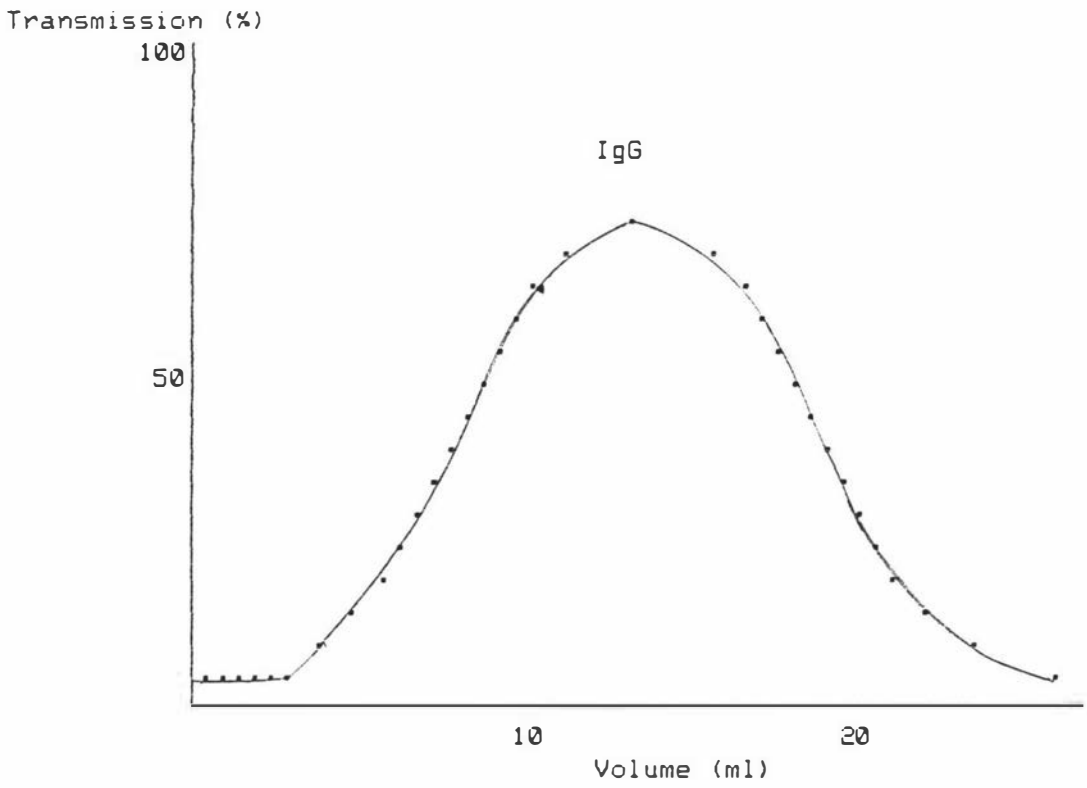


Figure 5.8 Immunoelectrophoresis Plate of Fractionated Serum

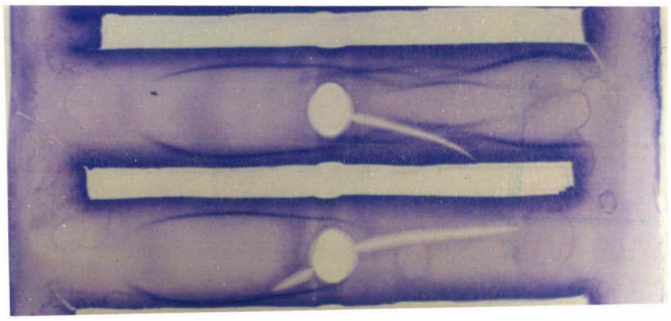
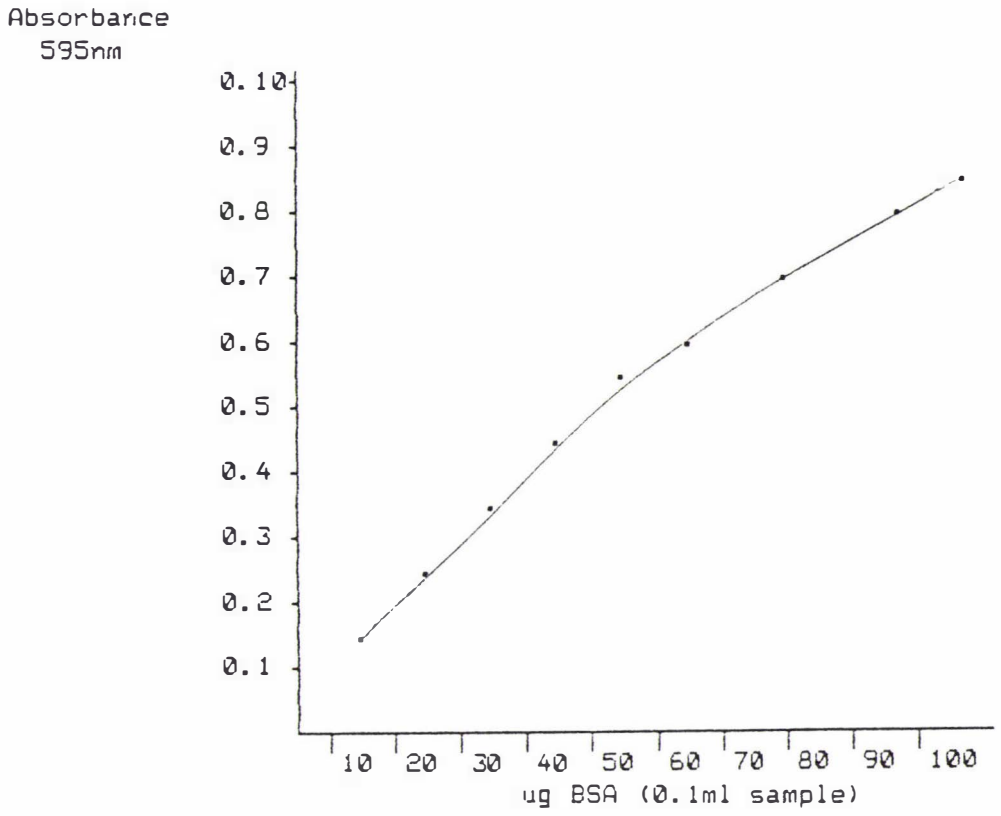


Figure 5.9 Standard Curve for Protein Estimation



The pooled fractions from the column contained 4mg/ml protein and were concentrated to 10mg/ml by ultrafiltration for use.

The rabbit antiserum to deer IgG, produced a gel difusion titre of 1000. A series of inoculations over 120 days were required to reach this level.

Three attempts at the preparation of a peroxidase labelled rabbit antisera to deer IgG conjugate failed to produce a product that reacted with the substrate in an indirect ELISA to detect deer serum antibodies to *hardjo*.

HRP conjugated protein A (Miles) failed to react with deer serum so this could not be used as an alternative to the antiserum to deer conjugate.

Rabbit antiserum to *hardjo* was conjugated to HRP, reacting at a dilution of 1/50 with an optical density reading of >1.0 on plates coated with *hardjo*.

5.3.2 Chequerboard Titrations

Chequerboard titrations of Evai Bois pig antiserum to goat IgG peroxidase conjugate failed to distinguish between the presence or absence of goat IgG in the test described in 5.2.7. All reactions were weak with optical density readings of <0.1.

The Miles rabbit antiserum to goat IgG peroxidase conjugate clearly distinguished between the presence and absence of goat IgG with an optimum test dilution of 1/200 (Table 5.2, Figure 5.10)

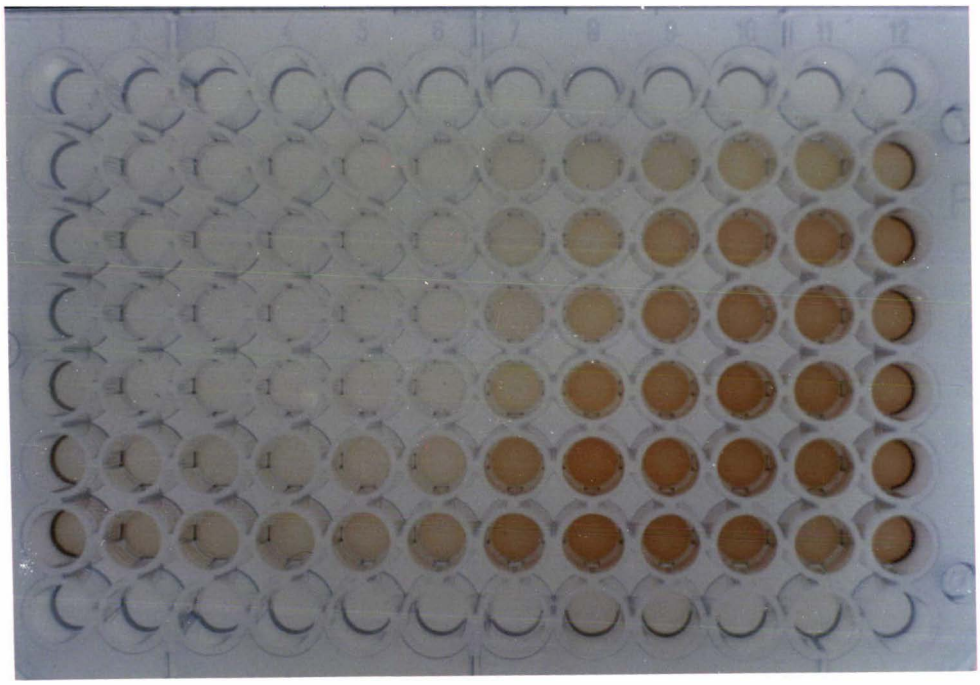
Table 5.2 A Chequerboard Titration to Determine the Optimum Concentration of Conjugate and Test Sera for use in the ELISA to Detect Antibodies to *Hardjo*

	Sera Dilutions					
Conjugate Dilution	1600	800	400	200	100	50
1600	0.015	0.058	0.112	0.129	0.132	0.009
800	0.041	0.083	0.121	0.177	0.192	0.013
400	0.037	0.107	0.130	0.178	0.193	0.029
200	0.056	0.135	0.190	**0.207**	0.158	0.039
100	0.097	0.100	0.105	0.153	0.142	0.030

Results are expressed as the differences between positive and negative sera.

The greatest difference in bold type indicates the optimum dilutions of sera and conjugate to use.

Figure 5.10 A Chequerboard Titration to Determine the Optimum Concentration of Conjugate and Test Sera for use in the ELISA to Detect Antibodies to *Hardjo*



5.3.3 Tests for Goat Antibodies to *Leptospira*

The reproducibility of this test was demonstrated by the results in Table 5.3

Table 5.3 Reproducibility of the ELISA for the Detection of Antibodies to *Hardjo*

Plate Number	Sera	Repeat Tests
1	a	0.250, 0.280, 0.241
	b	1.147, 1.133, 1.135
2	a	0.196, 0.205, 0.258
	b	1.249, 1.199, 1.198
3	a	0.147, 0.226, 0.211
	b	1.275, 1.298, 1.271
4	a	0.257, 0.288, 0.265
	b	1.093, 1.110, 1.070

Tests of 95 sera to compare the ELISA and MAT produced the results summarised in Tables 5.4 and 5.5.

Table 5.4 Comparison of the ELISA and MAT

Number of Samples	MAT Titre to Hardjo	Mean ELISA OD for Antibodies to Hardjo
17	<10 (no antibodies to other serovars)	0.407
54	<10 (with antibodies to other serovars)	0.472
2	20	0.837
5	40	0.913
9	80	0.996
3	160	1.061
4	640	1.458
1	1280	1.121

Table 5.5 Contingency Table of Results for the ELISA and the MAT

ELISA \ MAT	+ ⟩10	− ⟨10	
+	24	18	42
−	0	53	53
	24	71	95

The Chi squared result is 0.40 which makes P > 0.05 implying that the ELISA and MAT are statistically associated.

The correlation coefficient of 0.65 shows a positive but incomplete correlation between these tests.

The comparative sensitivity of the ELISA to the MAT is 100% and the comparative specificity is 75%.

The comparative sensitivity of the MAT to the ELISA is 57% and the comparative specificity is 100%.

The results show the ELISA to be more sensitive than the MAT.

The titration of sera in the test for goat antibodies to *hardjo* produced the results in Table 5.6.

Table 5.6 A Comparison of the ELISA (using the titration method) and the MAT for Detecting Antibodies to *Hardjo*

MAT Titre	ELISA Titre (OD>0.2)
<10	50
20	400
40	1600
80	800
160	3200
320	3200
640	3200

Using an OD of 0.5 as an indication of the presence of goat antiserum IgG to *hardjo*, based on the results of earlier tests, the sera from the national survey showed 9/64 (16%) with antibodies to *hardjo*. This compares with 3/64 (5%) with antibodies to *hardjo* measured by the MAT (Chapter 3).

The results of the single sera dilution ELISA for detecting antibodies to *pomona* in Tables 5.7 and 5.8 showed similarities with the test for goat antibodies to *hardjo*. The test destinguished between the presence or absence of antibodies but the ELISA readings do not correspond to the MAT for the same sera. Cross reactions with antibodies to other serovars only produced a small increase in the ELISA reading.

Table 5.7 A Comparison of the ELISA and the MAT for the Detection of Antibodies to *Pomona*

Number of samples	MAT Titre	Mean ELISA OD
5	<10 (no other antibodies	0.317
9	<10 (other antibodies	0.409
4	40	1.117
3	80	1.293
1	160	1.054

Table 5.8 Contingency Table for the Comparison of the ELISA and MAT for the Detection of Antibodies to *Pomona*

		MAT + ›10	MAT − ‹10	
ELISA	+ ›0.500	9	3	12
	− ‹0.500	0	11	11
		9	14	23

As for the test for *hardjo* antibodies there is a statistical association between these tests (X^2 = 0.46, P > 0.05) and there is a correlation coeficient of 0.77.

The comparative sensitivity of the ELISA to the MAT is 100% and the comparative specificity is 79%.

The comparative sensitivity of the MAT to the ELISA is 75% with a comparative specificity of 100%.

The results show that the ELISA is more sensitive than the MAT.

5.3.4 Detection of *Hardjo*.

The double antibody sandwich test for *hardjo* indicated that 10^6 cells/ml was the lowest concentration detected (Table 5.9).

Table 5.9 ELISA Results for the Detection of *Hardjo*

Hardjo (cells/ml)	Mean ELISA OD
10^8	0.715
10^7	0.426
10^6	0.263
10^5	0.196
10^4	0.204
10^3	0.186

There was some variation in the sensitivity of this test using different antisera to coat the plate. The serum with the highest antibody titre to *hardjo* measured by the MAT produced the most sensitive assay but the sensitivity of the test was not directly related to the level of agglutinating antibodies in the coating sera (Table 5.10).

Table 5.10 ELISA Results for the Detection of *Hardjo* using Different Coating Sera.

Hardjo (cells/ml)	Mean ELISA OD Coating Sera			
	A	B	C	D
108	1.134	0.465	0.273	0.211
107	0.867	0.334	0.208	0.153
106	0.323	0.167	0.142	0.141
105	0.144	0.116	0.103	0.138
104	0.112	0.144	0.095	0.123
103	0.102	0.135	0.116	0.129
0	0.088	0.086	0.106	0.091

The MAT titres of the coating sera were:

A = 163,840
B = 20,480
C = 5,120
D = 320

Rabbit antiserum with a titre of 327,680 was used at two different dilutions to coat the ELISA plates. Comparative tests using these plates showed that this serum was just as effective at 1/400 as 1/100 for the detection of *hardjo* antigen (Table 5.11).

Table 5.11 A Comparison of Different Dilutions of Plate Coating Sera and Conjugate for use in the ELISA to Detect *Hardjo*

Dilution of coating sera	*Hardjo* (cells/ml)	Mean ELISA OD at a Conjugate Dilution of; 1/200	1/400
1/100	10^8	0.715	0.400
	10^7	0.420	0.213
	10^6	0.263	0.156
	10^5	0.196	0.168
	10^4	0.204	0.127
	10^3	0.186	0.169
1/400	10^8	0.525	0.293
	10^7	0.337	0.217
	10^6	0.235	0.137
	10^5	0.197	0.109
	10^4	0.190	0.112
	10^3	0.186	0.186

The sensitivity of the ELISA for detecting *hardjo* antigen was not increased using cells disrupted by sonication (Table 5.12).

Table 5.12 Comparing the ELISA test to Detect *Hardjo* as Whole Cells and Cells Disrupted through Sonic Treatment.

Hardjo (cells/ml)	Mean ELISA OD	
	Whole Cells	Sonic Treated Cells
10^8	0.673	1.456
10^7	0.235	0.643
10^6	0.122	0.192
10^5	0.113	0.089
10^4	0.094	0.086
10^3	0.099	0.071

The single antibody ELISA procedure was less sensitive than the double antibody sandwich test for detecting *hardjo* (Table 5.13)

Table 5.13 The Single Antibody ELISA for the Detection of *Hardjo*.

Hardjo (cells/ml)	Mean ELISA OD
10^8	0.332
10^7	0.065
10^6	0.002

5.4 Discussion

The ELISA has been used to measure antibodies to *Leptospira* in sera from humans (Adler *et al* 1980), sheep (Adler *et al* 1981), cattle (Adler *et al* 1982b), pigs (Waltman and Dawe 1983), dogs (Hartman *et al* 1984b) and rabbits (Setyawan 1984). There are no reports of these tests being used to measure the antibody response in deer and only a brief report of their use in testing for goat antibodies (Setyawan 1984).

It was decided to use antiserum made against deer and goat IgG to test for antibodies to *Leptospira*. IgG antibodies are known to persist for longer than the IgM class in many species particularly cattle (Cousins *et al* 1985), and are therefore of greater interest in epidemiological studies.

There is no commercially available enzyme conjugate for deer ELISA studies. This may be due to a low demand for such a product or to some difficulty in its preparation.

The periodate method of conjugation (Wilson and Nakane 1978) used in this study, failed to produce a functional antiserum to deer IgG

peroxidase conjugate, although this is understood to be the most reliable method available (Wordinger *et al* 1983). The same method was used to produce a functional antiserum to *hardjo* peroxidase conjugate. The procedure for the production of IgG followed established techniques. The purification of deer IgG using DEAE Trisacryl M ion exchange chromatography has been reported for rat, rabbit, sheep, pig, cattle, horse, and dog sera (Corthier *et al* 1984). Purification of sera from goats and cats has been shown to be less successful, while mouse and hare sera are not able to be purified using this method.

The succesful separation of deer IgG was demonstrated by immunoelectrophoresis producing a precipitin line representing IgG.

The response of rabbits to the immunization programme with purified IgG was slow and antibodies never reached high levels in the gel diffusion assay. The low level of antibody may explain the poor results in the preparation of the conjugate.

It is possible that another species of animal may have produced a better response.

There is an absence of published details on methods of raising antisera with much reliance being put on trial and error (Ritchie 1986)

The attempt to use Protein A peroxidase to react with deer IgG was aimed at avoiding the need for an antiserum to deer conjugate. Staphlococal Protein A interacts with the Fc fragment of IgG molecules from several species including humans, dogs, swine, and rabbits (Biancifiori and Cardaras 1983). Protein A peroxidase conjugate has been used to detect Leptospiral antibodies in swine and dogs with results reported to be equal to or better than those obtained using the MAT (Biancifori and Cardaras 1983).

For the detection of goat antibodies to *hardjo* and *pomona* one commercial conjugate (Miles ICN) produced acceptable results but another (Evai Bois) conjugate was unsuitable.

Several other problems were encountered in setting up this test. There was a difference in the reproducibility of the results from tests performed in Dynatech and Nunc plates. To prevent the non specific binding of reagents onto the plate, a reliable washing regime needed to be established and a protein block (BSA) was essential in all diluents.

The test for goat antibodies to *Leptospira* showed a high sensitivity and was sufficiently serovar specific to preclude confusing cross reactions. This specificity was demonstrated by the similar results obtained for antisera with MAT titres >10 with and without detectable antibodies to other serovars. Another highly specific ELISA was reported by Hartman *et al* (1984b) using an outer envelope preparation for coating plates.

There are also some reports of IgG ELISA cross reactions. Adler *et al* 1980 reported a one way cross reaction between *pomona* sera and *hardjo* antigen. Cross reactions between *hardjo* and *tarassovi* using the IgG ELISA were reported by Cousins *et al* (1985). In studies of human sera for antibodies to *Leprospira*, Terpstra *et al* (1985), observed cross reactions from patients with *grippotyphosa, hardjo* and *icterohaemorrhagiae* infections. There are no reports of investigations studying cross reactions between a large number of Leptospiral antigens in ELISA tests.

In our study, antibodies to *ballum* are the most prevalent in goats (Chapter 3) so these would be the most likely to interfere with the tests for other antigens if there were a sufficient antigenic similarity.

Both genus and species specific non agglutinating antigens, that could be detected by ELISA, were reported by Adler and Faine (1983). Although it has been suggested by Adler *et al* (1981) that this ELISA and MAT may detect totally different antigens, there is a significant association between these tests ($P > 0.05$).

There is a positive correlation between these tests for the presence of antibodies to both *hardjo* (65%) and *pomona* (77%). A 95% correlation between the MAT and the IgG ELISA was reported by Adler *et al* (1981) for antibodies to *hardjo* in sheep. Cousins and Robinson (1986) reported a 90% correlation between the same tests for antibodies to *hardjo* in sheep. A 91% correlation was reported by Adler *et al* (1982) between these tests for bovine antibodies. Waltman and Dawe (1983) reported a direct correlation between the two tests. Their data, when calculated, shows an 88% correlation. While the correlations for the presence or absence of antibodies are high, studies of the correlations of the levels of antibody measured by the two tests are low. Adler *et al* (1981) showed an 11% correlation between the antibody levels measured by each test. Similarly in our data (Tables 5.4 and 5.7), an increasing MAT does not necessarily correspond to an increasing ELISA result.

The sensitivity of the ELISA was greater than the MAT for both *hardjo* and *pomona* antibodies. This was demonstrated in the comparison of 98 goat sera for antibodies to *hardjo* and in the results from the 64 sera of the national survey, where antibodies to *hardjo* were also tested by each method. The greater sensitivity of the ELISA was also demonstrated by comparing the tests of the 23 sera for antibodies to *pomona*.

The sensitivity of the ELISA for detecting bovine IgG to *Leptospira* was shown to be similar to the MAT with more sensitive results being

obtained using antisera to complete globulin (Thierman and Garrett 1983).

As expected with tests of greater sensitivity, there is some loss of comparative specificity. Whether this is due to the detection of false positives or because the ELISA is a more accurate test indicating the presence of infection can only be determined through detailed investigations in conjunction with in depth studies of the disease.

The double antibody sandwich ELISA was able to detect a lower concentration of antigen than the single antibody test.

A competitive ELISA was not attempted as the antiserum to *hardjo* conjugate produced weak reactions with the substrate and therefore would not be very effective in a competitive system.

The double antibody ELISA reliably detected a concentration of 10^6 cells/ml. This limit varied with different coating sera but the reactions for concentrations below 10^6 cells/ml. were similar. With different dilutions of coating sera the limit of antigen detection varied from 10^6 to 10^4 but again all results for concentrations less than 10^6 were treated with caution as all the readings were similar.

In the comparison of whole cell and sonic treated cells, the ELISA detected a limit of 10^6 cells/ml which was more clearly defined for the sonic treated cells than the whole cells. It may be argued that the detection limit for whole cells was 10^5.

A rapid and sensitive method of detecting *Leptospira* in samples of animal tissues and fluids would greatly assist all *Leptospira* studies. This ELISA, however, will not reliably detect cell concentrations below the level detected by standard techniques. Dark

field microscopic examinations will detect 10^4 cells/ml although this technique is laborious and made more difficult when contaminants are present (Faine 1982).

Cultures of infected tissues and fluids will detect as little as 20 cells/ml although this may take several months (Ellinghausen 1973)

Other techniques such as the immunofluorescent techniques have been used but serotype specific tests of this sort are laborious if multiple serovars are to be considered (Faine 1982).
Immunofluorescence has been reported to be more sensitive than dark field microscopy but there are problems with non specific fluorescence (Coffin and Maestrone 1962).

Both isolation and fluorecent methods depend on viable leptospires surviving in specimens that often arrive in the laboratory several hours or days after their collection (Faine 1982).

A chemiluminescent immunoassay, described by Waitkins and Hookey (1986), may provide an accurate and rapid method of detecting leptospires in biological fluids. Further investigations into this method are recommended by the authors.

Leptospira have been identified in tissue using an immunoperoxidase staining procedure involving a double antibody sandwich technique (Ellis *et al* 1983). They also used this technique for the detection of *Leptospira* in smears made from different infected tissues and fluids with a good correlation between these and culture results. More work is needed to determine the limit of detection using this method and its use on a wider range of biological materials.

An avidin - biotinylated HRP staining procedure was used by Jennings *et al* (1983) for the microscopic visualization of spirochetes. This

may assist visualization of spirochetes in difficult specimens, especially if there is much contamination. However, it is not likely to bring about a great increase in ones ability to detect *Leptospira* above other microscopic techniques such as dark field examination or silver staining (Faine 1982).

Inakov *et al* (1977) used a complement fixation test to detect about 10^6 *Leptospira* /ml in urine.

Radioimmune assay was reported by Adler *et al* (1982a) to have a detection limit of between 10^4 and 10^5 organisms/ml, but it loses sensitivity in biological fluids.

Biological fluids such as urine and serum have inhibitory components which increase the difficulty of obtaining adequate immunoassay sensitivity.

Bahaman, Marshall and Moriarty (1980b) found radioimmunoassay to be a simple, rapid technique for the detection of *Leptospira*, but a minimum of only 3.6 $x10^7$ cells/ml could be detected.

Chappel *et al* (1985) tried to improve the sensitivity further by investigating the technique of enzymatic radioimmunoassay, which is like enzymeimmune assay but employs a radioactive substance, combining both the features of enzyme immunoassay and radioimmunoassay, potentially increasing the sensitivity. This was used to detect *pomona* in pig urine but did not offer any increased sensitivity over the other diagnostic methods such as culture and dark field microscopy. Unfortunately the sensitivity in terms of the numbers of cells detected is not given.

It is possible to detect 1.1 x 10^3 *Leptospira*/ml using DNA hybridization techniques (Miller *et al* 1987). Terpstra *et al* (1987)

combined the specificity of DNA hybridization with the visualization of *Leptospira* morphology using biotin labelled DNA probes. Unfortunately, the authors did not state the sensitivity of the technique.

It is possible that an enzyme amplification system such as that used by Self (1985) for increasing the sensitivity of tests using alkaline phosphatase, may increase the sensitivity of an ELISA for detecting *Leptospira*.

For the ELISA to be useful for the routine diagnosis of leptospirosis, it must be able to detect fewer cells than was achieved in the present study.

5.5 Conclusions

1 The ELISA may be used as an alternative to the MAT for the detection of goat antibodies to *Leptospira*.

2 The ELISA is more sensitive than the MAT for detecting goat antibodies to *hardjo* and *pomona*.

3 There is some difficulty in producing an antiserum to deer IgG peroxidase conjugate.

4 Deer IgG may be purified using DEAE Trysacryl M.

5 Staphloccal Protein A did not react with deer IgG.

6 The ELISA will detect 10^6 *Leptospira* cells/ml in PBS.

CHAPTER 6

STUDIES OF THE ANTIGENIC SIMILARITIES BETWEEN *BRATISLAVA* AND OTHER SEROVARS

6.1 Introduction

The serological studies of deer and goats reported in this thesis have demonstrated a widespread distribution of sera reacting with *bratislava*.

Antibodies to *bratislava* have been reported as occurring in human sera (McDonald *et al* 1985) and pig sera (Bolt 1987) in New Zealand. Internationally, *bratislava* has been isolated from hedgehogs (Broom and Coghlan 1960), dogs, pigs and horses (Ellis 1985). It has been associated with abortions in pigs (Ellis *et al* 1985).

It has been suggested by McDonald *et al* (1985), however, that the antibody titres to *bratislava* seen in human sera may be due to cross reactions with antibodies directed against other serovars, particularly *pomona*.

The results in Chapters 3 and 4 show that antibodies to *ballum* and *bratislava* often occur with a similar frequency, a finding which suggests the possibility that cross reactions may occur not only with *pomona* but with *ballum* as well.

The possibility that *bratislava* infections may occur at a low level in the population must not be overlooked. The failure to isolate the organism may be due to one or more factors, namely, a low level of infection, difficulties in growing the organism in conventional medium, or difficulties associated with isolating this serovar from a multiple infection containing other serovars which may overgrow *bratislava*.

The work described in this chapter investigates a method of isolating *bratislava* from a mixed culture. Antiserum to *pomona* was used to inhibit the growth of the homologous serovar.

Deer and goats were monitored to observe any serological response to *bratislava* which may be produced as a result of vaccinating with other antigens.

The proteins of eight different *Leptospira* were compared using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-

PAGE) to determine the degree of similarity between *bratislava* and other serovars.

Western blots of the SDS-PAGE separated proteins were reacted with different antisera against three serovars to identify common protein antigens.

6.2 Materials and Methods

6.2.1 Growth Inhibition using Specific Antiserum

This method was based on that of Tripathy *et al* (1971).

A 1200ml batch of semi-solid EMJH medium (Appendix I) was prepared and split into three 400ml batches labelled A, B and C. Batch A was dispensed in 4ml quantities. This was the control batch with no antiserum added. Rabbit antiserum to *pomona* with an antibody titre $> 8 \times 10^9$ was heated to 56°C for 30 minutes to destroy complement. This was filtered through a sterile 0.22um nitrocellulose filter[1] and added to a final dilution of 1/100 to batch B which had been held at 50°C. This was mixed and dispensed in 4ml volumes. The antiserum was also added to batch C to a final dilution of 1/1000, mixed and dispensed in 4ml volumes.

Individual five day old cultures of *bratislava* and *pomona* were prepared in EMJH liquid medium (Appendix I). The cells were counted using a bacterial counting chamber[2] and were serially diluted in sterile PBS (Appendix II) ten fold from 10^7 cells/ml to 10^3 cells/ml

Two bottles of medium from each batch (A, B, C,) were inoculated with 0.4ml of culture from each dilution of *bratislava* culture. Similarly, duplicate cultures were made from each dilution of *pomona*. In addition, cultures were prepared in each batch of medium

1 Millipore Corporation, Bedford, Massachusetts 01730, USA
2 C.A. Hausser and Son, Philadelphia, USA

from inocula consisting of 0.4ml of mixtures from combinations of *bratislava* and *pomona*.

All cultures were incubated at 29°C for three months and examined weekly for growth by dark field microscopy. Positive cultures were repeatedly cultured in liquid EMJH medium (Appendix I) in preparation for serological and BRENDA typing as described in Chapter 4.

6.2.2 The Serological Response of Deer and Goats Following Vaccination

The serological response of deer and goats to *australis, ballum, bratislava, copenhageni, hardjo, pomona,* and *tarassovi* was monitored following vaccination. The animals selected for this work were on properties where the farmer or veterinarian perceived a need for vaccination against leptospirosis and agreed to cooperate by supplying blood samples for testing.

Serum samples were taken from forty red deer hinds of six to twelve months of age immediately before the vaccination of 20 of these with 2ml of a commercial vaccine[3] containing *hardjo, pomona* and *copenhageni* antigens. The sera were tested for antibodies to the seven serovars listed above using the MAT (Chapter 2). Twenty eight days later, sera were again taken from all animals and tested for antibodies to *Leptospira*. A second vaccination of 2ml was given to the 20 animals vaccinated previously. Fourteen days later, blood samples were taken from all animals and tested for antibodies to *Leptospira*. The geometric mean titres (GMT) were calculated for each serovar in both groups at each sampling.

Sera were taken from a mixed age flock of 48 angora cross does and tested for antibodies to the seven serovars as for the deer. All were vaccinated with 2ml of a commercial vaccine[4] containing *hardjo*

3 'Lepto 3', Coopers Animal Health N Z Ltd. Upper Hutt.
4 'Leptavoid', Coopers Animal Health NZ Ltd. Upper Hutt.

and *pomona* antigens. Sera were taken from all animals 34 days later and tested for antibodies to *Leptospira*. At this time they were vaccinated again with 2ml of the same vaccine. Forty five days later, sera were taken to test for antibodies. The GMT for each serovar at each sampling was calculated for each serovar.

6.2.3 The Separation of Leptospiral Proteins using Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Leptospira cultures, which had been cloned by culturing from limit dilutions, were grown in 100ml of EMJH liquid medium (Appendix I) to a concentration of between 10^8 and 10^9 cells/ml. These were centrifuged at 10000g for 15 minutes, the supernatant discarded and the precipitate resuspended in PBS (Appendix II). This was repeated three times to wash the cultures free of culture medium. The precipitate from the final centrifugation was resuspended to a volume of 10 ml. The cells were then disrupted by sonication at 20 Hz for two minutes.

The protein content of each preparation was estimated by the method of Bradford (1976) described in Chapter 5 but using the modification of Ionas (1983). This modification simulates SDS treatment by using 0.2M NaOH to solubilise the proteins. Protein estimations of the SDS treated samples were not possible as SDS interferes with the test (Ionas 1983).

The SDS-PAGE discontinuous buffer system developed by Laemmli (1970) was used to separate the proteins. An acrylamide solution was polymerised between two glass plates and then placed vertically between upper (negative electrode - the cathode) and lower (positive electrode - the anode) buffer reservoirs.

Each glass plate measured 13cm x 17cm x 0.55cm. One plate had a 2cm deep, 10cm long notch cut to permit gel contact with the upper buffer reservoir.

The plates were thoroughly cleaned by washing in detergent and hot water, drying and wiping with methanol.

Threads of petroleum jelly were piped from a syringe along the sides and bottom of both plates. Three spacers, 0.15cm thick were placed on the sides and bottom of one plate and the other plate placed on top and pressed down firmly to give a good seal. The plates were clamped into a vertical perspex stand using bulldog clips. The notch in the glass plate was placed at the top facing away from the stand.

A running gel of 10% acrylamide was prepared as described in Appendix XXII. This was poured between the glass plates to a depth of 11cm, taking care to avoid air bubbles. This was immediately overlayed with distilled water to produce a sufficiently anaerobic environment to enable the acrylamide solution to polymerise. The gels were left for 30 minutes at room teamperature for the polymerisation to occur.

The stacking gel was prepared as described in Appendix XXIII. The water was poured off the top of the running gel and the exposed surface washed twice with aliquots of freshly prepared stacking gel acrylamide. The stacking gel solution was poured on top of the running gel to completely fill the space to the top of the glass plates. A perspex comb, with eight 7.5mm x 17mm long teeth spaced 3mm apart, was inserted between the glass plates into the stacking gel so that the bottom of the teeth were 8mm above the running gel. Care was taken to avoid air bubbles on the teeth of the comb. Polymerization was left to occur at room temperature for ten minutes. The comb was then removed and the wells washed with Tris - glycine buffer (Appendix XXIV).

The gel sandwiched between two glass plates was removed from the perspex stand and the bottom spacer was carefully removed.

Thick threads of petroleum jelly were applied to the electrophoresis apparatus, immediately below the upper reservoir and midway between the two reservoirs. This ensured a liquid tight seal between the reservoirs, permitting current flow only through the gel. The gel sandwich was then pushed onto the electrophoresis apparatus and held with bulldog clips with the plate notch facing into the upper buffer reservoir.

The reservoirs were filled with Tris - glycine buffer (Appendix XXIV). Bubbles were eliminated from the lower gel surface using a syringe full of the buffer.

The samples were prepared for electrophoresis by mixing 100ul of lysed cell suspension, 25ul of SDS sample buffer (Appendix XXV), 12.5ul of bromophenol blue tracking dye (Appendix XXVI) and boiling for three minutes to solubilize the proteins. The samples were cooled and centrifuged at 3000g for three minutes to remove any insoluble material. These sample prepararions were stored at -20°C if not required for immediate use.

A volume of each sample needed to give the required protein concentration, as determined by the protein assay, was added to each well using a micropipette[5].

A current of 15mA was applied to the gel until the tracking dye reached the interface of the stacking and running gels. The current was then reduced to 10mA until the tracking dye reached the bottom of the running gel. The electricity was then disconnected.

The gel was removed from the electrophoresis apparatus, the two perspex spacers on either side removed and the plates levered apart using a spatula. The lower left corner of the gel was removed to ensure correct orientation of the gel in later stages. The stacking gel was removed with a spatula and discarded. The running gel was

5 Gilson, France

allowed to fall into a plastic container of isopropanol stain (Appendix XXVI). This was left rocking for three hours at room temperature.

The stain was siphoned off and the gel was destained by rinsing and soaking in 10% aqueous acetic acid. The acetic acid solution was decanted off and replaced every two to three hours until the background of the gel became clear. The gel was washed with distilled water until no acetic acid smell remained. Selected gels were photographed using Tri X film.[6]

A series of samples of the *pomona* serovar containing protein concentrations ranging from 20ug to 160ug in 20ug steps were separated by SDS-PAGE to determine the optimum concentration to use in further work.

The proteins of seven serovars (*australis, ballum, bratislava, copenhageni, hardjo, pomona,* and *tarassovi*) plus an additional *hardjo* strain Bovis, were separated by SDS-PAGE.

6.2.3 Western Blots

This method was based on that of Towbin *et al* (1979), Burnette (1981), and Tamura *et al* (1985).

Leptospira proteins were separated using SDS-PAGE as described above with the exception that the gels were not stained. Once electrophoresis was complete one glass plate was removed and the gel overlayed with a sheet of nitrocellulose[7] that had been cut to the size of the gel slab and marked in pencil with lanes for each sample. Each lane was numbered for future reference. The nitrocellulose was soaked for 10 minutes in transfer buffer (Appendix XXVIII) before laying on the gel. A clean glass rod was

6 Kodak
7 Bio Rad, Richmond, California 94804, USA

rolled over the nitrocellulose sheet to remove any air trapped between the sheet and the gel. Filter paper, which had been moistened in transfer buffer (Appendix XXVIII), was laid on top of the nitrocellulose sheet and a layer of synthetic sponge placed on top of this. The glass plate was removed from the other side of the gel and replaced with another sheet of moistened filter paper and synthetic sponge. This sandwich was placed between two porous plastic sheets from the 'Trans Blot' cell[8] and was inserted into the Trans Blot cell with the nitrocellulose sheet closest to the anode. The 'Trans Blot' cell was then filled with transfer buffer (Appendix XXVIII) and the current set at 300 mA for 18 hours.

The nitrocellulose sheet was then removed and dried at 37°C for 60 minutes, then cut with scissors along the lines separating the bands of electrophoresed protein. The strips were placed in separate containers and washed in PBS containing 0.05% Tween 20. Each strip was then soaked in 5% bovine serum albumin (BSA) for 60 minutes at room temperature to block any sites on the nitrocellulose likely to cause non specific binding of antisera or conjugates. The BSA was removed and the strips washed three times in PBS/Tween 20. Appropriate antisera to *Leptospira*, diluted in PBS/Tween 20 containing 1% BSA were added and allowed to react at 29°c on a rocker for 60 minutes. The strips were then washed five times in PBS/Tween 20. A peroxidase conjugate[9], diluted to 1/200 in PBS/Tween 20 was added and the nitrocellulose strips incubated on the rocker at 29°C for a further 60 minutes. The strips were then washed three times in PBS/Tween 20 and the substrate (Appendix XXVII) was added. This was allowed to react at 29°C on the rocker for 15 minutes. If the reaction was not sufficient after this time, an additional 10ul of H_2O_2 was added to the substrate covering each strip and the incubation was continued for a further 15 minutes at 29°C. The reaction was stopped by rinsing the nitrocellulose strips in tap water. The strips were dried then photographed with Tri-X[10] film.

8 Bio Rad, Richmond, California 94804, USA
9 Miles Laboratories Inc., Elkhart, Indiana 46514, USA
10 Kodak, New Zealand

Table 6.1 Western Blots

Lane Number	Antigen	Antisera to *Leptospira*	Antisera to Species Peroxidase Conjugate
1	*bratislava*	Rabbit antisera to *pomona*	Goat antisera to Rabbit
2	*pomona*	Rabbit antisera to *pomona*	Goat antisera to Rabbit
3	*bratislava*	Goat antisera to *pomona*	Pig antisera to goat
4	*pomona*	Goat antisera to *pomona*	Pig antisera to goat
5	*bratislava*	Goat antisera to *bratislava*	Pig antisera to goat
6	*pomona*	Goat antisera to *bratislava*	Pig antisera to goat
7	*bratislava*	Goat antisera to *hardjo*	Pig antisera to goat
8	*pomona*	Goat antisera to *hardjo*	Pig antisera to goat

Protein bands of *bratislava* and *pomona* were reacted with antisera in Western blots as in Table 6.1.

6.3 Results

6.3.1 Growth Inhibition using Specific Antiserum

The presence of growth detected by darkfield microscopic observation at the end of the three month period is detailed in Table 6.2.

Antisera to *pomona* inhibited the growth of this serovar in tubes 1, 2 and 3 where *pomona* only was used as the inoculum. Some growth was apparent in most tubes early in the experiment but the effect of the antisera appeared after two weeks incubation.

The *pomona* antisera did not affect the growth of *bratislava* in those tubes inoculated with *bratislava* only.

An examination of the serovars isolated from this experiment showed that the inhibition of *pomona* growth in a mixed culture was incomplete (Table 6.3). *Pomona* was isolated more frequently than *bratislava* overall. The antiserum to *pomona* was more effective in limiting the growth of this serovar at a dilution of 1/100 than 1/1000.

Where *pomona* did overgrow the *bratislava* in mixed culture, *bratislava* could not be detected in the isolates prepared for serotyping.

The serological typing of these isolates was confirmed by the BRENDA result (Figure 6.1)

Table 6.2 Microscopic Examination of the Growth Inhibition Trial

Inocula Log^{10} cells/ml		Dilution of Antiserum to *Pomona*		
bratislava	*pomona*	No Serum	1/100	1/1000
0	7	+	−	+
0	6	+	−	+
0	5	+	−	+
0	4	−	−	−
0	3	−	−	−
7	0	+	+	+
6	0	+	+	+
5	0	−	+	−
4	0	−	−	−
3	0	−	−	−
7	7	+	+	+
6	7	+	+	+
5	7	+	+	+
4	7	+	−	−
3	7	+	−	−
7	6	+	+	+
6	6	+	+	+
5	6	+	+	+
4	6	+	−	+
3	6	+	−	+
7	5	+	+	+
6	5	+	+	+
5	5	+	+	+
4	5	+	−	+
3	5	+	−	+
7	4	+	+	+
6	4	+	+	+
5	4	−	+	−
4	4	−	−	−
3	4	−	−	−
7	3	+	+	+
6	3	+	+	+
5	3	−	−	−
4	3	−	−	−
3	3	−	−	−

(+) = growth (−) = no growth

Table 6.3 Serotyping of Isolates from the Growth Inhibition Trial

Table 6.3 Serotyping of Isolates from the Growth Inhibition Trial

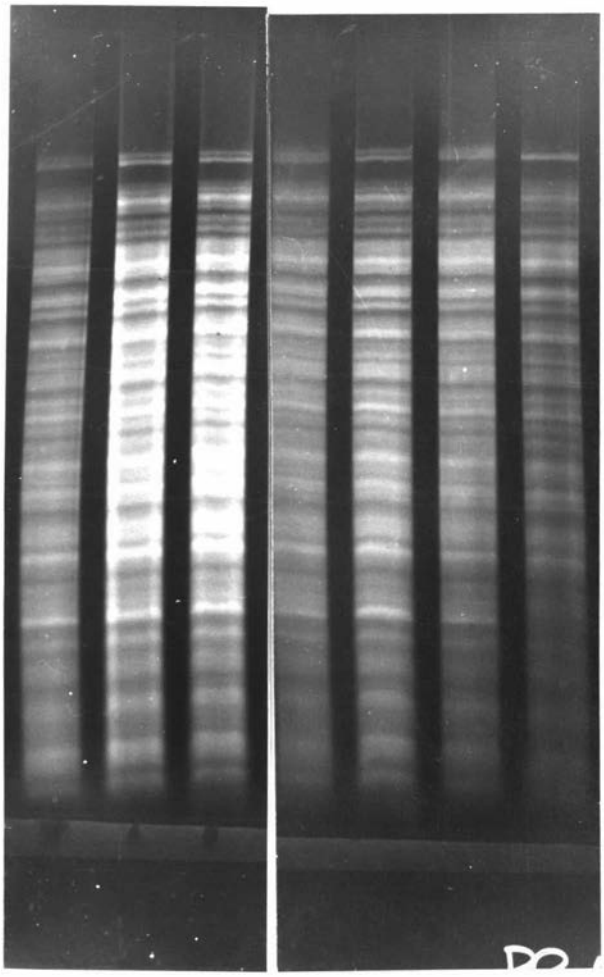
Inocula Log^{10} cells/ml		Antiserum Dilution		
bratislava	*pomona*	No Serum	1/100	1/1000
0	5	P	-	P
6	0	B	B	-
7	7	P	B	P
6	7	P	P	P
5	7	P	P	P
7	6	P	B	P
6	6	P	P	P
5	6	P	-	P
7	5	-	B	B
6	5	P	B	B
5	5	P	-	P
4	5	P	-	P
3	5	P	-	P
7	4	-	B	-
6	4	-	B	-
5	4	-	B	-
7	3	-	B	-
6	3	-	B	-

P = *pomona* isolate B = *bratislava* isolate
(-) = no isolate

Figure 6.1 **BRENDA Patterns of the Isolates from the Growth Inhibition Trial**

From left to right:

LANE	SEROVAR
1	*Pomona* reference strain VDH
2	*Pomona* isolate
3	*Pomona* isolate
4	*Pomona* isolate
5	*Pomona* isolate
6	*Pomona* isolate
7	*Pomona* reference strain VDH



6.3.2 The Serological Response of Deer and Goats to Vaccination

The vaccination of deer and goats produced antibody titres to those serovars contained in the vaccines. In addition, there was a corresponding, although lower, rise in the antibody titres to *bratislava*. The antibody titres to the other serovars tested remained substantially the same as they were before vaccination (Tables 6.4 and 6.5).

Table 6.4 The Serological Response of Deer to Vaccination.

SEROVAR	GEOMETRIC MEAN TITRE					
	Vaccinated Group			Non Vaccinated Group		
	DAY			DAY		
	0	28	43	0	28	43
australis	5	5	5	5	5	5
ballum	6	5	5	5	5	5
bratislava	8	9	31	8	9	8
copenhageni	5	15	121	5	5	5
hardjo	7	12	25	7	7	7
pomona	6	12	112	6	6	6
tarassovi	5	5	5	5	5	5

Table 6.5 The Serological Response of Goats to Vaccination

SEROVAR	GEOMETRIC MEAN TITRE		
	DAY		
	0	34	79
australis	5	5	6
ballum	11	12	10
bratislava	6	35	36
copenhageni	5	8	11
hardjo	8	130	59
pomona	6	190	1313
tarassovi	9	8	9

6.3.3 The Separation of Leptospiral Proteins by SDS-PAGE

The results of SDS-PAGE of a range of protein concentrations showed the clearest separation was obtained using 100ug of protein (Figure 6.2). This was used in all subsequent work.

The protein bands produced by all strains of *Leptospira* differed from one another (Figure 6.3). Although common bands were present, none of the strains tested was found to have any greater degree of similarity than any other.

6.3.4 Western Blot Analysis of the Protein Antigens

Three protein bands from SDS-PAGE which were common to both *bratislava* and *pomona*, reacted with goat antibodies to *bratislava*, *hardjo* and *pomona*. More protein bands from *bratislava* and *pomona* reacted with goat antisera to *bratislava* or *pomona* than with goat antisera to *hardjo*. The rabbit antiserum to *pomona* also reacted with several antigens from both *bratislava* and *pomona* (Figure 6.4).

Figure 6.2 The Titration of *pomona* Protein in SDS-PAGE

From left to right:

LANE	PROTEIN (ug)
1	20
2	40
3	60
4	80
5	100
6	120
7	140
8	160

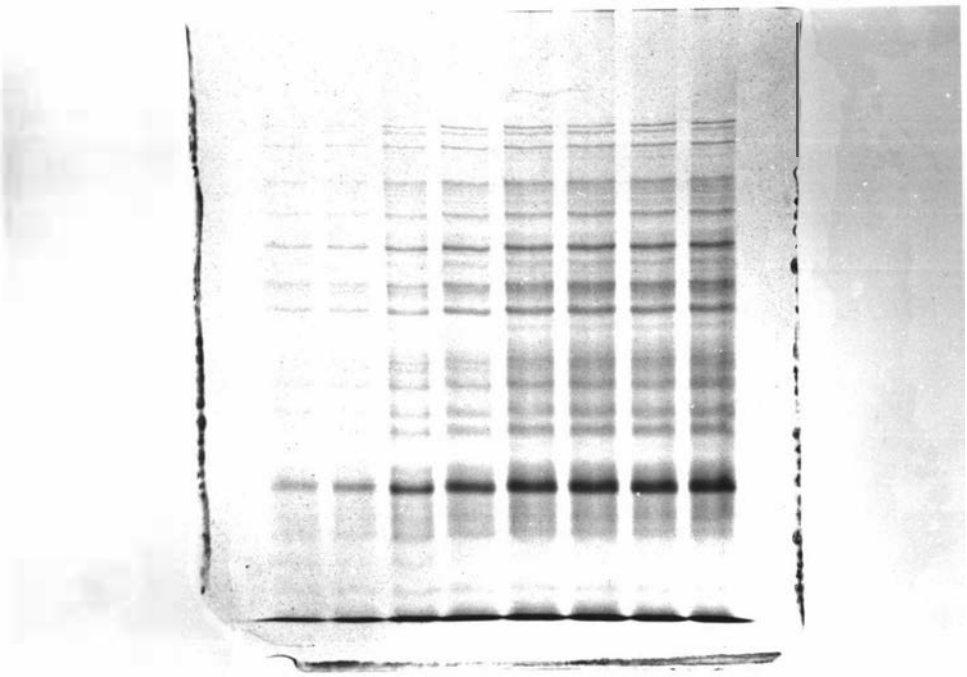


Figure 6.3 SDS-PAGE of Eight Different Strains of *Leptospira*

From left to right:

LANE	SEROVAR
1	*Australis*
2	*Ballum*
3	*Bratislava*
4	*Copenhageni*
5	*Hardjo* (Prajitno)
6	*Hardjo* (Bovis)
7	*Pomona*
8	*Tarassovi*

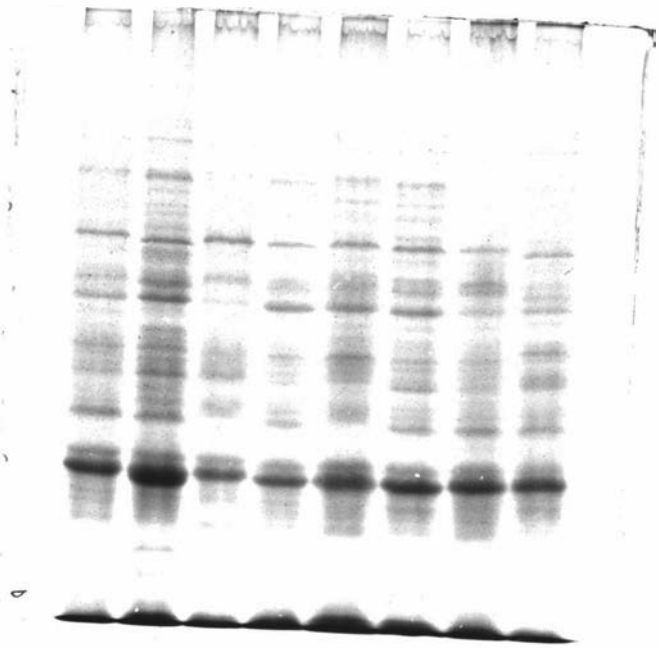
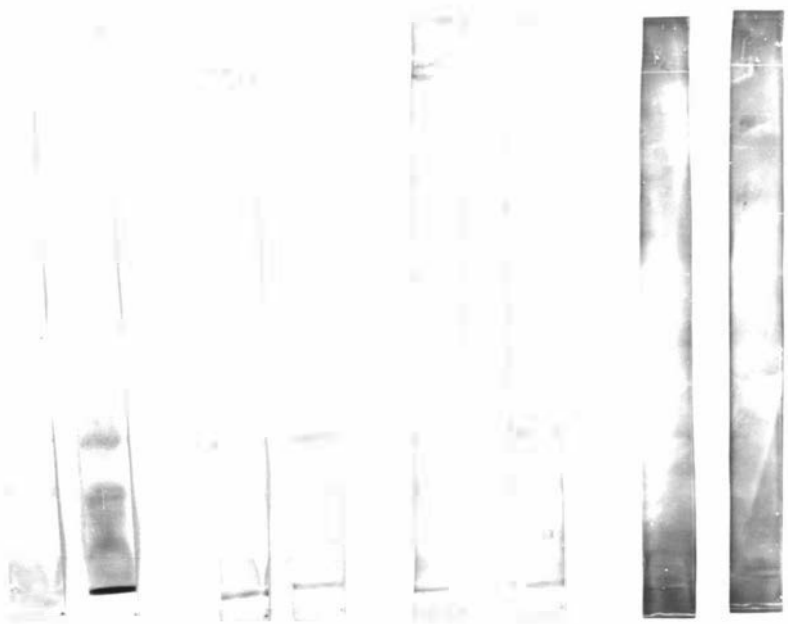


Figure 6.4 Western Blots of *bratislava* and *pomona*.

From left to right:

LANE	ANTIGEN	ANTISERA
1	*Bratislava*	Rabbit antiserum to *pomona*
2	*Pomona*	Rabbit antiserum to *pomona*
3	*Bratislava*	Goat antiserum to *pomona*
4	*Pomona*	Goat antiserum to *pomona*
5	*Bratislava*	Goat antiserum to *bratislava*
6	*Pomona*	Goat antiserum to *bratislava*
7	*Bratislava*	Goat antiserum to *hardjo*
8	*Pomona*	Goat antiserum to *hardjo*



6.4 Discussion

Growth inhibition of *pomona* with specific antisera was only partially successful in limiting the growth of *pomona*. The antisera worked satisfactorily in cultures inoculated with *pomona* only but failed to limit *pomona* growth in many of the mixed cultures.

The inhibition of *pomona* growth has been demonstrated using antisera with much lower MAT titres, than used in this study. Tripathy *et al* (1971) were able to demonstrate growth inhibition using antisera from vaccinated cattle which had antibody titres of <100.

In these studies incomplete inhibition of *pomona* growth in mixed culture may have been due to the effect of the other serovar, in this case *bratislava*. Rabbit sera has been used for many years as an essential ingredient of many leptospiral media such as those of Fletcher (1928), Korthof (1932) and Stuart (1949). It is possible that the small amount of antiserum to *pomona* used in the growth inhibition test may have been used as a nutrient by *bratislava* in the early stages of growth, allowing *pomona* to grow and take over the culture in the later stages. This assumes that antibody to *pomona* has little effect on the growth of *bratislava*. To avoid this problem, cultures could be prepared from limit dilutions of the growth inhibition cultures before the antiserum loses its effect.

The results of this test confirm that *pomona* will overgrow *bratislava* in mixed cultures. To isolate *bratislava* in such situations it would be necessary to in some way limit the growth of *pomona*.

These results show that this batch of EMJH media was only capable of supporting growth from samples containing 10^6 cells/ml of *bratislava* or 10^5 cells /ml of *pomona*. Samples containing less than this would not have been detected using this culture medium. If *bratislava* was

is shed in urine samples at concentrations less than 10^6 cells/ml it would not have been detected in cultures.

The results from the antibody response in deer and goats, following vaccination, indicated that there was some cross reaction between *hardjo* and/or *pomona* and *bratislava*. The level of antibodies detected in the deer and goats to the antigens contained in the vaccine, was similar to that observed in other species (Marshall *et al* 1979a and 1979b) including deer (Wilson and Schollum 1984).

In this trial, antibody titres to most serovars which were not contained in the vaccine did not change. However, an increase in the antibody titres to *bratislava* was observed and the time of this increase corresponded with the increase in the antibody titres to serovars contained in the vaccines. This suggests that the antibodies generated by vaccination were responsible for the reactions to *bratislava*. This provides some evidence of antigenic similarity which may help explain the presence of antibody titres to *bratislava* observed in the serological surveys.

It is unfortunate that there was a lack of control over the management of the vaccination programmes. There was no 'control' group of non vaccinated goats and variation existed in the timing of the samplings of the goats compared with the deer. However, some useful information was gained from this limited experiment.

The protein patterns observed in the SDS-PAGE separation of all the *Leptospira* strains were heterogenious and of no help in distinguishing antigenic similarity.

Western blot studies were more useful in distinguishing common antigens. Their value in determining the antigenic relationships amongst *Leptospira* has been reported previously (Chapman *et al* 1986). Three protein bands reacted with all sera and probably represent common genus specifc antigens. Three major protein bands

of genus specific antigen were also recognised by Chapman *et al* (1986) in a more thorough study using several fractionation techniques.

A strong similarity between *bratislava* and *pomona* was demonstrated by the reaction of goat antisera to *bratislava* with similar protein bands from both *bratislava* and *pomona*. The reaction of goat antisera to *pomona* with similar protein bands from both *bratislava* and *pomona* supports the hypothesis that there is antigenic similarity. Goat antisera to *hardjo* reacted to fewer proteins from *bratislava* and *pomona* indicating a greater difference. It would have been interesting to perform Western blots using antisera with antibodies to *ballum* only as *ballum* and *bratislava* antibodies were frequently detected together. Unfortunately there were no samples of goat sera containing antibodies to *ballum* only.

Although this test has established a similarity between *bratislava* and *pomona*, there are several areas that need clarification. It is possible that the sera thought to contain antibodies to only one serovar may have antibodies to others not detected by the MAT which may react in the Western blot. It is interesting that goat antisera to *hardjo* reacted with only three bands and there was a likelihood that this sera contained undetected antibodies to other serovars. Also a similar result to that obtained using goat antibodies to *pomona* was obtained with rabbit antisera to *pomona* which, it could be argued, would be unlikely to contain contaminating leptospiral antibodies.

These blots were repeated with different batches of sera and on each occa sion produced the same results. The purity of the *Leptospira* preparations is difficult to ensure and although cultures cloned from limit dilutions were used, it is still possible that a pure culture had not been achieved.

While this work does not conclusively explain the presence of antibodies to *bratislava*, it does provide strong evidence supporting the hypothesis that *bratislava* and *pomona* share common antigens.

6.5 Conclusions

1 A growth inhibition test using specific antisera is not a reliable method of isolating *bratislava* from a mixed culture.

2 The EMJH medium and culture methods used in this study would detect a lower limit of 10^6 cells/ml of *bratislava* and 10^5 cells/ml of *pomona*.

3 Immunization of deer and goats with two commercial vaccines containing *hardjo* and *pomona* antigens produced a rise in antibody titre to *bratislava* as well as to *hardjo* and *pomona*.

4 SDS-PAGE is unsuitable for detecting similarities between strains of *Leptospira*.

5 Western blots showed similar antigens in *bratislava* and *pomona*.

CHAPTER 7
GENERAL DISCUSSION

This study was designed to determine the prevalence of leptospirosis in deer and goats in New Zealand. It is involved serological surveys and detailed farm studies using serological techniques and culture isolation from urine samples. In pursuing this objective it was necessary to validate experimental techniques, in particular to ensure the reproducibility of the Microscopic Agglutination Test (MAT). Experiments were designed to determine the value of Enzyme Linked Immunosorbent Assay (ELISA) as an alternative method for detecting antibodies to *Leptospira*. The ELISA was also applied to the detection of *Leptospira* organisms. Experiments were carried out to look at the antigenic relationships between *bratislava* and other serovars in an attempt to explain the widespread presence of antibodies to *bratislava*.

Validation of the Microscopic Agglutination Test

The standard serological technique recommended by WHO for studies of *Leptospira* infections is the MAT. Although this test is widely used, there are discrepencies in the results from repeat tests and in the results from different laboratories (Bettleheim and Pearce (1986). It is therefore necessary to standardise the method used in a particular study in order to ensure that the results are reproducible.

Firstly, the precision and accuracy of the equipment used in this study was established. The precision for two diluent dispensers was high at >90%. The accuracy varied for the two types of dispenser used in the laboratory at 79% and 97.5% respectively. Using the same equipment and a standardised method, reproducible results, within one two fold dilution, were obtained in repeat tests.

Although guidelines for carrying out the MAT have been published (WHO Expert group 1967), there is still room for variation in the detailed methods of the test. These variations in the test conditions were identified as critical variables and control of these enabled a more stringent procedure to be developed to ensure reproducibility and maximum antibody titres.

Differences in the antigen concentration produced the greatest variation in the test results emphasising the need to standardize at 2×10^8 cells/ml.

Live cultures produced higher titres and were easier to read than when formalin inactivated antigens were used.

Four day old cultures produced maximum antibody titres which were reproducible. Cultures older than ten days resulted in lower antibody titres and tests that were more difficult to read.

Maximum antibody titres were obtained after two hours incubation at 37°C with longer incubation times being required to obtain the same result at lower temperatures.

Factors that did not affect the results were the type of diluent used (PBS or culture medium) and the type of culture (recent field isolates or laboratory adapted strains).

The interpretation of the MAT end point is subjective but reproducible results are obtained by the same operator or when different operators are trained to interpret the 50% end point in the same way.

Prozones were observed and may be a cause of concern in the interpretation of the results. Prozones were identified by observing the results from several dilutions of serum and were eliminated by

heating the serum to 56°C for 30 minutes or by extending the incubation time of the test to 18 hours.

The following standard method was found to produce maximum antibody titres, which were within one two fold dilution, when applied to repeated tests:

1. Prepare two-fold serial dilutions of test sera from 1/10 to 1/1280 in PBS.

2. Add equal volumes of antigen consisting of a four day old culture of 2×10^8 cells/ml.

3. Incubate at 37°C for two hours.

4. Examine microscopically for 50% agglutination.

In addition, the use of a control serum of known antibody titre and the inclusion of a sample of antigen diluted 1/2 to approximate the cell density that exists at the 50% agglutination end point, are employed to assist with the interpretation of the end point.

Leptospirosis in Goats

Leptospirosis has rarely been reported in goats although there is evidence to show that goats can and do become infected (Morse and Langham 1958).

A survey of sera from a sample of goats, representative of the national herd, showed a 70% prevalence of antibodies ≥10 to one or more of the following serovars; *australis, ballum, bratislava, copenhageni, hardjo, pomona,* and *tarassovi*. Ninety percent of these antibody titres were <80 suggesting a low level of active infection.

The most frequently recorded antibody titres were to *ballum* and *bratislava*. The antibody titres to *ballum* most likely originated from food and water contaminated by urine from infected rodents as these animals act as maintainance hosts for this serovar. As antibody titres to *bratislava* occurred with a similar prevalence and were frequently associated with antibody titres to *ballum* in individual animals, both serovars may have originated from a similar source. Alternatively, both serovars may be closely related antigenically so that antibodies to *ballum* cross react with *bratislava*. *Bratislava* has not yet been isolated in New Zealand.

There was only a 5% prevalence of goats with antibodies to *hardjo* in this serological study. These antibodies probably resulted from infection acquired as the result of contact with infected cattle, the recognised maintainance host for this serovar.

Antibody titres to *copenhageni* presumably resulted from contact with infected urine from brown rats (*Rattus norvegicus*), the maintainance hosts. Antibodies to *pomona* and *tarassovi* are most likely to have originated from contact with infected urine from pigs, the normal maintainance hosts for these serovars.

Comparing the results of this study with those of other workers is difficult because of the different test conditions employed. The most recent work in goats was that of Schollum and Blackmore (1981) where 13% of feral goats were found with antibody titres to ≥24 to *Leptospira*.

Serological results from six farm studies also showed a high prevalence of antibody titres to *ballum* as observed in the national survey. The prevalence of antibody titres to all the other six serovars varied and the association between antibody titres to *ballum* and *bratislava* observed in the national survey was only seen in samples from two properties (2 and 4).

The variation in the prevalence of antibody titres to different serovars presumably reflects the differences in the exposure of the goats to the various maintainance host populations. For example, the high prevalence of antibody titres to *copenhageni* on properties 5 and 6 (80% and 70% respectively) presumably indicates a greater exposure to rodents infected with this serovar. Similarly, the higher prevalence of antibody titres to *hardjo* on properties 1 and 3 (10% and 25% respectively), compared with the results of the national survey, presumably reflects contact with infected cattle in which this serovar is endemic. The management of the stock on proprety number 1, where there is a high turnover of goats coming from a variety of locations, would increase the chance of some goats introducing the infection to this farm from contact with cattle on other properties. Property number 3 is located in the Waikato, a major dairy farming district where contact with cattle is more likely than in some other parts of the country.

The age distribution studies in goats from one property showed that age may be a factor influencing susceptability or exposure to infection. Fewer animals less than 12 months of age had antibody titres ≥10 to *Leptospira* than did the older stock. Unfortunately, age distribution studies could not be conducted on the other properties. It is possible that younger animals are less susceptable or not as likely to be exposed to infection as the older groups. However, as all goats on this property were grazed together, it was thought that all age groups would have been exposed to the same sources of infection.

The serovars for which there is most serological evidence of exposure in goats in this country are *ballum, bratislava* and *copenhageni*. However, *Leptospira* were not isolated from any of the urine samples. One explanation for this may be that these serovars are incapable of producing a urinary shedding infection in goats. Another possible explanation is that goats may shed these organisms

at such low concentrations as to be undetedtable by the culture techniques used in this study.

Although leptospirosis has been reported in goats and there is widespread serological evidence of exposure, this disease is not a problem in goats in New Zealand.

Leptospirosis in Deer

Deer sera from five locations in New Zealand were tested for antibodies to *australis, ballum, bratislava, copenhageni, hardjo, pomona,* and *tarassovi*. Antibodies to *ballum, bratislava,* and *copenhageni* were the most prevalent. Contact with infected rodents is again the most likely source of exposure to *ballum* and *copenhageni*. The prevalence of antibody titres to *bratislava* was similar to that for *ballum* and *copenhageni* which suggests the possibility that antibodies to *ballum* and *copenhageni* may cross react with *bratislava*. Alternatively, *bratislava* may also exist in rodents but has not yet been isolated in New Zealand.

Antibody titres to *australis, pomona* and *tarassovi* were all ≤80 and these may result from cross reactions with antibodies to other serovars.

Antibody titres to *hardjo* were widespread with prevalences of up to 77% in the serological studies. Some of these titres may have been due to *balcanica* as exposure to *balcanica* produces antibody titres that are normally indistinguishable from those to *hardjo* (Hathaway 1978). This was demonstrated in the detailed farm study on the Gladstone property where *balcanica* was isolated from urine samples and the MAT antibody titres to *balcanica* and *hardjo* were all within one serial dilution of each other. This is the first isolation of *balcanica* from deer. The origin of *balcanica* infection in these deer is likely to have been infected opossums as these have been shown to be its maintainane host. Contact with infected opossums may have

occurred either on the farm or the original stock may have become infected before capture. If the original captured stock were the source of infection for this herd, then deer must be capable of maintaining *balcanica* infection for at least 71 months when the last captured stock were introduced.

Infection with *balcanica* appeared to be restricted to animals of at least 18 months of age. No isolates were made from animals less than 18 months old. This was reflected in the absence of antibody titres to *balcanica* and *hardjo* in the nine month old group. Presumably these younger stock were susceptable to infection with *Leptospira* as antibodies to other serovars were present.

The absence of infection in the younger stock may have been due to several factors. Firstly, stock management involved keeping the different age groups in separate paddocks which prevented direct contact between the groups. The older stock may have been exposed to infection before the birth of the younger stock and this type of management would have prevented the infection spreading further. Secondly, if *balcanica* produces low intensity urinary shedding of *Leptospira* in deer, there may been insufficient numbers of organisms for the spread of infection between deer. Thirdly, an extremely dry late winter and early spring may have prevented the spread of infection during this particular year as ground water is known to be an important factor in the spread of *Leptospira* between other species such as cattle (Hellstrom 1978). Finally, intimate contact between animals, as occurs during mating, could be required for the spread of *balcanica* infection in deer. This degree of intimacy of contact is thought to be necessary for the transmission of *balcanica* infection in opossums (Hathaway 1978).

It appears that the age specific prevalence of *balcanica* infection in deer is widespread as antibody titres to *hardjo* in samples from other areas showed a similar age distribution to those of the Gladstone property.

Enzyme Linked Immunosorbent Assay (ELISA) for the Detection of Antibodies to *Leptospira*.

ELISA systems have been used to detect antibodies to many infective agents including *Leptospira*. Such tests have numerous advantages over the MAT used for measuring antibodies to *Leptospira*. In particular they are faster to perform and provide a more objective result than the MAT.

Experiments were designed to develop tests using peroxidase conjugated antiserum to goats that would detect antibodies to *Leptospira*. Once the test conditions were established using known positive and negative sera, as determined by the MAT, a test of high sensitivity and good serovar specificity could be performed. Although the correlation between the ELISA and the MAT for the presence and absence of antibodies to *hardjo* and *pomona* was good, the correlation for the magnitude of the antibody levels measured by the two tests was poor. This may be explained by the fact that the two tests may use totally different antigens (Adler *et al* 1981). The difference may also be due to the different class of antibodies measured by the two tests. The MAT is believed to measure both IgM and IgG antibodies (Hellstrom 1978). The ELISA used in this study measured only the IgG antibodies. This was chosen as IgG antibodies are known to persist for longer than the IgM class in many species, particularly cattle (Cousins *et al* 1985), and are therefore of greater interest in epidemiological studies.

An ELISA for the detection of deer antibodies to *Leptospira* was more difficult to develop as there is no commercially available antiserum to deer conjugate. Deer IgG was successfully purified using ion exchange chromatography. Rabbits were chosen for the immunization programme to produce antiserum to deer IgG. Unfortunately their response to deer IgG using a standard immunization programme was

poor. Other species may be more suitable for raising antiserum to deer.

Once a level of antibodies had been obtained in the rabbits, their sera was used to conjugate to horse radish peroxidase enzyme according to the standard method of Wilson and Nakane (1978). A functional conjugate was not produced with this antiserum even though the conjugation method was validated by using another test.

It appears that there is some difficulty in producing an antiserum to deer conjugate, and a range of different animal species will have to be tried for the immunization programme.

Protein A peroxidase conjugate was used as an alternative to the antiserum to deer conjugate. Protein A interacts with the Fc fragment of IgG molecules from several species (Biancifiori and Cardaras 1983). This conjugate, however, did not react in an ELISA for deer IgG.

A rapid and sensitive method for the detection of *Leptospira* in samples of animal tissues and fluids would greatly assist all *Leptospira* studies. Two ELISA systems were compared for the detection of *Leptospira;* single and double antibody sandwich tests. The double antibody sandwich ELISA reliably detected a minimum concentration of 10^6 *hardjo* cells/ml. The single antibody ELISA using an antiserum to *hardjo* conjugate produced weaker reactions and would only detect 10^8 cells/ml. These ELISAs, however, will not detect cell concentrations below the level detected by standard techniques such as dark field microscopy (10^4 cells/ml) and culture (20 cells/ml). In addition, although cells were diluted in PBS for this study, biological fluids such as urine, may have inhibitory components which would increase the difficulty in obtaining adequate immunoassay sensitivity.

Other workers are developing a variety of techniques for the detection of *Leptospira*. The most promising of these is DNA hybridization which is reported to detect concentrations of 1.1 $x10^3$ cells/ml (Miller *et al* 1987).

Growth Inhibition Tests Using Specific Antisera

A growth inhibition test using specific antisera to *Leptospira* has been shown to prevent the growth of the *Leptospira* serovar to which the serum antibodies are directed (Tripathy *et al* 1971). This method was used in an attempt to purify a mixed culture of *pomona* and *bratislava*. These serovars were chosen as agglutinating antibodies to both which are often detected together (McDonald *et al* 1985). The explanation for this association may be that *pomona* antibodies cross reacting *bratislava* or that dual infections occur. Specific antisera may be able to limit the growth of one serovar in a mixed culture, allowing the other to grow. In a mixed culture there is the possibility of one serovar becoming dominant due to its better growth in culture medium. If this situation were to occur it would be difficult to isolate and type the slower growing serovar.

Rabbit antiserum to *pomona* incorporated into culture medium, limited the growth of *pomona* when inoculated with this serovar only. In mixed cultures containing *bratislava* and *pomona* the antiserum was less successful in inhibiting the growth of *pomona*. One explanation for this is that the other serovar, *bratislava*, may utilise the rabbit antisera as a nutrient early in the incubation of the culture, allowing *pomona* to survive and overgrow the *bratislava*.

It appears that serum inhibition is unsuitable for preventing growth of one serovar in a mixed culture.

The Response of Deer and Goats to Vaccination

Deer and goats were vaccinated with commercially available vaccines designed to protect against leptospirosis due to *hardjo* or *pomona*. The antibody responses to these and five other serovars were monitored. The antibody responses to *hardjo* and *pomona* were similar to those reported in other species (Marshall *et al* 1979a and 1979b) including deer (Wilson and Schollum 1984). There was no change in the antibody levels to most other serovars except *bratislava* where there was an increase in the antibody titres following vaccination. This finding suggests that antibodies which are the result of vaccination cross react with *bratislava* and this provides some evidence of antigenic similarity between at least one of the serovars contained in the vaccine and *bratislava*. This implies that antibodies made against *hardjo* and/or *pomona* in deer and goats may have reacted with *bratislava*.

Sodium Dodecyl Sulphate Polyacrylamide Electrophoresis (SDS-PAGE) Separation of Proteins from Different Serovars

SDS-PAGE of proteins from different serovars attempted to demonstrate any antigenic similarities.

The protein patterns were heterogeneous and of no help in distinguishing antigenic relationships.

Western Blot Studies of *Leptospira*

Leptospiral proteins were separated by SDS-PAGE, transfered to nitrocellulose and reacted with antisera and and an antiserum enzyme conjugate to highlight specific bands. The value of this technique for the detection of antigenic similarities between *Leptospira* has been reported by Chapman *et al* (1986).

In this present study, three protein bands were found to react with all sera tested and presumably represent common genus specific antigens as reported by Chapman *et al* (1986).

A closer similarity between *bratislava* and *pomona* was demonstrated by the reaction of a greater number of protein bands from both these serovars reacting with antiserum to *bratislava*. Similarly, antiserum to *pomona* reacted with several bands ofboth the *bratislava* and *pomona* protein electrophoretic profiles. Antiserum to *hardjo* reacted with only three bands of the *bratislava* and *pomona* profiles.

This provides further evidence to support the hypothesis that the antibody titres to *bratislava* observed in this study were the result of cross reactions to *pomona*.

Conclusion

This study has demonstrated the widespread presence of deer and goat antibodies to *Leptospira* serovars known to be present in New Zealand. It represents the first nationwide study of the serological prevalence of leptospirosis in these domestic animals. Antibodies to *bratislava*, a serovar not yet isolated in New Zealand, were also widespread in these animals and evidence was presented suggesting that these may result from cross reacting antibodies to other serovars. *Balcanica* was isolated from deer urine and this represents the first report of this infection in deer. *Leptospira* were not isolated from the urine of any of the goats under study.

APPENDIX 1

EMJH MEDIUM

Ref; Johnson and Seitzer (1977)

The following stock solutions were required

Stock Solutions			Amount (g/100ml)
Ammonium chloride	(NH_4Cl)	[B.D.H.]	25
Zinc sulphate	($ZnSO_4$ $7H_2O$)	[M & B]	0.4
Magnesium chloride	($MgCl_2$ $6H_2O$)	[Analar]	1.5
Calcium chloride	($CaCl_2$ $2H_2O$)	[Analar]	1.5
Ferrous sulphate	($FeSO_4$ 7H2O)	[Analar]	0.5
Copper sulphate	($CuSO_4$ $5H_2O$)	[Analar]	0.3
Sodium pyruvate		[B.D.H.]	10
Glycerol		[B.D.H.]	10
Tween 80		[Sigma]	10
Thiamine	(Vitamin B_1)	[Sigma]	0.5
Cyanocobalamin	(Vitamin B_{12})	[Sigma]	0.02

Basal Medium

The following were dissolved in 996ml of distilled water;

Disodium hydrogen orthophosphate	(Na_2HPO_4)	[Analar]	1.0g
Potassium dihydrogen phosphate	(KH_2PO_4)	[Analar]	0.3g
Sodiun chloride	(NaCl)	[Analar]	1.0g

The following stock solutions, prepared as above, were added;

Ammonium chloride	1.0ml
Thiamine	1.0ml
Sodium pyruvate	1.0ml
Glycerol	1.0ml

The pH was adjusted to 7.4 with the addition of sodium hydroxide (NaOH) or hydrochloric acid (HCl).

The base medium was then sterilized by autoclaving at 121°C for 20 minutes.

Albumin Supplement

Twenty grams of Fraction V bovine albumin was added to 100ml of distilled water. The following stock solutions (prepared as above) were added;

Calcium chloride	2.0ml
Magnesium chloride	2.0ml
Zinc sulphate	2.0ml
Copper sulphate	0.2ml
Ferrous sulphate	20.0ml
Cyanocobalamin	2.0ml
Tween 80	25.0ml

The pH was adjusted to 7.4 by the addition of sodium hydroxide or hydrochloric acid. Distilled watwe was then added to bring the volume to 200ml and the solution was sterilized by filtration.

Complete Medium

The complete medium was prepared by adding 30ml of the albumin suplement to 270ml of basal medium. Before dispensing into the required volumes, 0.33ml of a 2mg/ml solution of neomycin was added.

A stock solution of 5-fluorouracil was added to some batches of media to reduce contamination. A stock solution of 5-flurouracil was prepared by adding 1.0g of 5-fluorouracil to 50ml of distilled water. This was placed in a 60°C water bath to dissolve and the pH was adjusted to 7.6 by the addition of 1N HCL. The solution was made up to 100ml with distilled water and was sterilized by filtration through a 0.22u membrane filter. This was stored at 4°C until required for addition to the culture medium. Before dispensing the complete medium, 1ml of the 5-fluorouracil stock solution was added to 100ml of medium.

Semi solid medium was prepared by adding 0.75g of agar (Difco bacteriological grade) to 450ml of basal medium. This was autoclaved at 121°C for 20 minutes and cooled to approximately 40°C before the aseptic addition of 50ml of albumin supplement, neomycin and 5 fluorouracil. This was then dispensed into the required volumes.

The complete medium was incubated at 37°C for at least three days to ensure sterility.

APPENDIX II

PHOSPHATE BUFFERED SALINE (PBS)

The following were dissolved in two litres of distilled water;

Sodium chloride (NaCl)	15.3g
Disodium hydrogen orthophosphate	1.448g
Potassium dihydrogen phosphate	0.42g

The pH was checked to be 7.2-7.4.

This was then dispensed and autoclaved at 121°C for 15 minutes.

APPENDIX III

TRANSPORT MEDIUM

This was prepared by dissolving 1.0g of Fraction V bovine albumin to 100ml of distilled water. When dissolved, 1ml of a 5 fluorouracil solution, prepared as in Appendix II, was added. This solution was then sterilized by filtration through a 0.22u membrane filter before dispensing in 4ml volumes.

APPENDIX IV

ELLIS MEDIUM

Ref; Ellis, W.A.; Montgomery, J.A.; Cassells, J.A. (1985)

The following stock solutions were prepared by dissolving each in 100ml of distilled water:

Zinc sulphate	0.4g
Calcium chloride	1.0g
Magnesium chloride	1.0g
Glycerol	10.0ml
Vitamin B12	0.02g
Thiamine chloride	0.5g
Manganese sulphate [B.D.H.]	0.3g

These were stored at 4°C until required.

The following additional stock solutions were prepared in distilled water immediately before use for the preparation of the supplement:

Ferrous sulphate	0.5g/100ml
Tween 80	20.0ml/180ml
Tween 40	20.0ml/180ml
5-Flurouracil	1.0g/100ml
Nalidixic acid	0.1g/100ml

The supplement was prepared by adding the following to 50ml of sterile distilled water:

Fraction V bovine albumin	10.0g
Lactalbumin hydrolysate [Difco]	1.0g
Superoxide dismutase	0.1g
Sodium pyruvate	0.04g

This was stirred at slow apeed to prevent foaming and when fully dissolved, the following stock solutions were added in the following order:

Thiamine chloride	1.0ml
Calcium chloride	1.0ml
Magnesium chloride	1.0ml
Zinc sulphate	1.0ml
Manganese sulphate	1.0ml
Ferrous sulphate	10.0ml
Vitamine B12	1.0ml
Tween 80	9.0ml
Tween 40	3.5ml

This was stirred for one hour to ensure that all the ingredients were dissolved. The pH was adjusted to 7.4 using 10% sodium hydroxide and the final volume was made up to 96ml using sterile distilled water.

To this, 4ml of fresh rabbit serum which had been heated to 56°C for 30 minutes, was added.

Naladixic acid and 5-fluorouracil were added in 20ml volumes to the supplement before sterilizing by filtering through a 0.22u membrane filter.

The complete medium was prepared by dissolving the following in 998ml of distilled water:

Disodium hydrogen orthophosphate	1.0g
Potassium dihydrogen phosphate	0.3g
Sodium chloride	1.0g
Ammonium chloride stock solution	1.0ml
Glycerol stock solution	1.0ml

The pH was adjusted tp 7.4 using 10% sodium hydroxide solution.

Note that where components that were also used in the preparation of EMJH medium were required, the same types and sources were used as detailed in Appendix I.

To 860ml of the basal medium, 1.5g of bacteriological grade agar was dissolved and sterilized by by autoclaving at 121°C for 20 minutes. This was then cooled to approximately 50°c before aseptically adding 140ml of supplement, mixing and dispensing into 5ml volumes.

APPENDIX V

0.025M TRIS HCL BUFFER + 0.035M NaCL

Ref: Corthier, *et al* (1984)

Six grams of Trizma base were added to one litre of distilled water. The pH was adjusted to 8.8 with 0.05M HCL and the volume was made up to 2 litres.

To this, 3.63g of sodium chloride were added and dissolved.

APPENDIX VI

0.025M TRIS HCL BUFFER + IM NaCL

Ref: Corthier *et al* (1984)

The Tris buffer was prepared as in Appendix V.

To this, 108.9g of sodium chloride were added.

APPENDIX VII

BRADFORD REAGENT

Ref: Bradford (1976)

In 50ml of 95% ethanol, 100mg of Coomassie Brilliant Blue G250 was dissolved. To this , 100ml of 85% phosphoric acid was added ant the solution diluted to 1000ml. This was filtered through a Whatman number 1 filter paper and stored in a dark glass bottle until required.

APPENDIX VIII

SODIUM BARBITONE BUFFER

Ref: Hellstrom (1978)

This buffer was prepared by dissolving 20.62g of sodium barbitone in 1500ml of distilled water, adjusting the pH to 8.6 with 0.5N HCl and making the volume up to 2000ml with distilled water.

APPENDIX XIX

COOMASSIE STAIN FOR AGAROSE GELS

Ref: Wallenborg and Anderson (1978)

This was made by dissolving 1g of Coomassie brilliant blue R250 stain in a solution of 90ml of ethanol, 20ml of acetic acid and 90ml of water. This was stored at room temperature and used several times to stain agarose gels.

APPENDIX X

0.001M SODIUM ACETATE BUFFER

Ref: Munro (1970)

This was prepared by combining 283ml of 0.001M acetic acid with 100ml of 0.001M NaOH and diluting to 1000ml. The ph was checked to be 4.4.

APPENDIX XI

0.01M CARBONATE BUFFER

Ref: Munro (1970)

Two solutions were prepared as follows:

Solution A contained 0.84g of sodium bicarbonate dissolved in 1000ml of distilled water.

Solution B contained 1.06g of sodium carbonate dissolved in 1000ml of distilled water.

The two solutions were combined in the following proportions:

Solution A 45.3ml
Solution B 18.2ml

The volume was made up to 1000ml with distilled water and the pH checked to be 9.6 at 25°C.

APPENDIX XII

0.2M CARBONATE BUFFER

Ref: Munro (1970)

Two solutions A and B were made as follows:

Solution A contained 16.8g of sodium bicarbonate dissolved in 1000ml of distilled water.

Solution B contained 21.2g of sodium carbonate dissolved in 1000ml of distilled water.

The complete buffer was prepared by combining 45.3ml of solution A and 18.2ml of solution B and making the volume up to 1000ml with distilled water.

The pH was checked to be 9.6 at 25°C.

APPENDIX XIII

0.06M CARBONATE BUFFER

Ref: Munro (1970)

Two solutions A and B were prepared as follows:

Solution A; 5.04g of sodium bicarbonate were dissolved in 1000ml of disstilled water.

Solution B; 6.36g of sodium carbonate were dissolved in 1000ml of distilled water.

The complete buffer was prepared by combining 62.8ml of solution A with 12.4ml of solution B and making up the volume to 1000ml with distilled water.

The ph was checked to be 9.6 at 0°C.

APPENDIX XIV

PBS, TWEEN 20 ELISA WASHING SOLUTION AND DILUENT

PBS was prepared as in Appendix II.

To 1000ml of PBS, 0.5ml of Tween 20 was added. This was the washing solution used between each step in the ELISA tests.

For diluting sera and conjugates, 1g of Fraction V bovine albumin was added to 100ml of the PBS/Tween 20 washing solution.

APPENDIX XV

Tris-HCl EDTA Buffer (TEB)

Ref: Marshall *et al* (1981)

TEB was prepared by combining 100mM Tris-HCl (pH 7.5) and 100mM ethylene diamine tetracetic acid sodium salt (EDTA).

APPENDIX XVI

Sodium Chloride Tris-HCl EDTA Buffer (STE)

Ref: Marshall *et al* (1981)

This buffer was prepared as a 10X stock solution. This consisted of the following:

5M NaCl	20ml
1M Tris HCl pH 7.5	50ml
0.2M EDTA	5ml

These were combined and made up to a volume of 1000ml with distilled water.

APPENDIX XVII

PHENOL:CHLOROFORM:IAA EXTRACTION MIXTURE

Ref: Marshall et al (1981)

Phenol was melted in hot tap water then combined with chloroform and isoamyl alcohol in the following proportions:

Phenol	25ml
Chloroform	24ml
Isoamyl alcohol	1ml

The phenol was of high purity and the mixture was made fresh each day.

APPENDIX XVIII

Tris EDTA Buffer (TE BUFFER)

Ref: Marshall et al (1981)

This consisted of 10ml of 1M tris-HCl (pH7.5) and 5ml of 0.2M EDTA which was combined and diluted to 1000ml with distilled water.

APPENDIX XIX

ENZYME BUFFER

Ref: Boehringer, Mannheim; Manufactures Specifications.

This consisted of a solution containing the following:

100mM Tris-HCl (pH 7.5)
50mM sodium chloride
10mM magnesium chloride

APPENDIX XX

SAMPLE BUFFER

Ref: Marshall *et al* (1981)

This consisted of a combination of the following:

1M Tris-HCl (pH 7.5)	100ul
0.2M EDTA	50ul
10% Sodium dodecyl sulphate (SDS)	50ul
Glycerol	2ml

This was made up to a volume of 10ml with distilled water.

APPENDIX XXI

ELECTROPHORESIS BUFFER

Ref: Marshall *et al* (1981)

This was a solution consisting of:

40mM Tris acetate pH 7.8
5mM Sodium acetate
1mM EDTA

To each 1500ml of this buffer, 300ul of an ethidium bromide solution containing 2.5mg/ml was added.

APPENDIX XXII

POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE) RUNNING GEL

Ref: Ionas (1983)

The following stock solutions were prepared:

A. Lower Tris Buffer

This consisted of the following:

Trizma base	18.17g
10% SDS in distilled water	4ml
12N HCl to pH 8.8	
Distilled water to 100ml	

The Trizma base was added to 70ml of distilled water and the pH was adjusted to 8.8 with 12N HCl. SDS was added and the solution made up to 100ml with distilled water. The pH was checked and adjusted if necessary.

B. Running Gel Acrylamide

This contained the following:

Acrylamide	30.0g
Methylene bis acrylamide	0.5g
Distilled water to 100ml	

The acrylamide solution was prepared by dissolving the acrylamide in 70ml of distilled water. The methylene bis acrylamide was then added and the solution was made up to 100ml with distilled water.

C. Ammonium Persulphate

This contained the following:

Ammonium persulphate	0.1g
Distilled water	1.0ml

The ammonium persulphate was dissolved in the distilled water immediately before use.

The running gel was a 10% acrylamide gel prepared by combining the following solutions in the order given with thorough mixing after each addition.

Lower Tris buffer	5.0ml
Running gel acrylamide	6.7ml
Distilled water	8.3ml
Ammonium persulphate	0.3ml
N,N,N',N'- tetramethylethylenediamine (TEMED)	0.01ml

APPENDIX XXIII

PAGE STACKING GEL

Ref: Ionas (1983)

The following stock solutions were prepared:

A. Upper Tris Buffer

Trizma base	6.06g
10% SDS in distilled water	4ml
12N HCl to pH6.8	
Distilled water to 100ml	

The Trizma base was added to 70ml of distilled water and the pH was adjusted to 6.8 with 12N HCl. SDS was added and the solution made up to 100ml with distilled water. The pH was checked and adjusted if necessary.

B. Stacking Gel Acrylamide

Acrylamide	30.0g
Methylene bis acrylamide	1.6g
Distilled water to 100ml	

The acrylamide solution was prepared by dissolving the acrylamide in 70ml of distilled water. The methylene bis acrylamide was then added and the solution was made up to 100ml with distilled water.

C. Ammonium Persulphate

Ammonium persulphate	0.1g
Distilled water	1.0ml

The ammonium persulphate was dissolved in the distilled water immediately before use.

The stacking gel was prepared by mixing the following in the order given, mixing thoroughly after each addition.

Upper Tris buffer	2.5ml
Stacking gel acrylamide	1.5ml
Distilled water	6.0ml
Ammonium persulphate	0.03ml
TEMED	0.01ml

APPENDIX XXIV

TRIS GLYCINE RESERVOIR BUFFER

Ref: Ionas (1983)

The following were dissolved in 2000ml of distilled water:

Trizma base	6.07g
Glycine	28.8g
SDS	2.0g

The pH was checked to be 8.3 and the buffer stored at 4°c until required.

APPENDIX XXV

SDS SAMPLE BUFFER

Ref; Ionas (1983)

The following were combined:

2-Mercaptoethanol	10.0ml
SDS	6.0g
Upper Tris buffer	25.0ml

Distilled water was added to make the volume up to 100ml and the buffer was stored at 4°C until required.

APPENDIX XXVI

BROMOPHENOL BLUE TRACKING DYE

Ref: Ionas (1983)

This consisted of the following:

Bromophenol blue	0.005g
Glycerol	40ml
Distilled water to 50ml	

These were combined and stored at room temperature until required.

APPENDIX XXVII

ISOPROPANOL COOMASSIE STAIN FOR PAGE

Ref: Ionas (1983)

The following were mixed and stored at room temperature until required:

Isopropanol	250ml
Glacial acetic acid	100ml
Coomassie brilliant blue R250	0.4g
Distilled water	1000ml

APPENDIX XXVIII

TRANSFER BUFFER FOR WESTERN BLOTS

Ref: Gershoni and Palade (1983)

A 25mM phosphate buffer was used and prepared by mixing the following in 1000ml of 20% methanol:

Potassium dihydrogen phosphate	0.13g
Disodium hydrogen orthophosphate	0.047g

The pH was checked to be 6.5

APPENDIX XXIX

WESTERN BLOT SUBSTRATE

Ref: Hawkes *et al* (1982)

A substrate concentrate was made by dissolving 3mg of 4-chloro-1-naphthol in 1ml of methanol.

A substrate buffer was prepared by combining the following:

100mM Tris HCl (pH 7.4)	8ml
Distilled water	8ml
Sodium chloride	0.18g

The complete substrate was prepared by combining 10ml of substrate buffer with 2ml of substrate concentrate and 10ul of 30% hydrogen peroxide.

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