THE SEROLOGICAL AND CULTURAL PREVALENCE IN SHEEP OF LEPTOSPIRAL INFECTION IN THE NORTH ISLAND OF NEW ZEALAND

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ABSTRACT

A survey of sheep from the North Island of New Zealand was conducted for leptospiral infection. The results of the serological examination showed 20.5 percent of the sheep had titres (≥ 1:48) to Hebdomadis serogroup, 3.8 percent to serovar *pomona*, 2.6 percent to serovar *tarassovi*, 2.3 percent to serovar *copenhageni* and 2.7 percent to serovar *ballum*. No titres of 1:48 or greater were detected to serovar *australis*. It was shown that a minimum dilution of 1:24 resulted in many non-specific or cross-reaction. A minimum dilution of 1:48 was more accurate for detecting the serological prevalence of agglutinins to leptospires in ovine sera. In the cultural survey, serovar *hardjo* was isolated from three animals in one group of sheep. It was considered that the Hebdomadis titres were more likely to represent previous infection with *hardjo* than with *balcanica*.

Based on the serological and cultural examinations from the general survey and a study farm, a pattern of infection was recorded. The serological prevalence and the geometric mean titre (GMT) of different age groups of sheep from different farms and the lack of success on obtaining further isolates of *hardjo* indicated that sheep are not the maintenance host for this serovar in New Zealand. Although infection of sheep by serovar *hardjo* is not uncommon, it is a sporadic occurrence and endemic infection is unlikely to occur.

Preliminary investigations on the use of radioimmunoassay in detecting leptospires or leptospiral antigens in urine are presented.

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TABLE OF CONTENTS

TABLE OF CONTENTS CONTINUED

LIST OF TABLES

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES CONTINUED

LIST OF FIGURES CONTINUED

CHAPTER ONE

OVINE LEPTOSPIROSIS: A REVIEW OF THE LITERATURE

The earliest mention of leptospiral infection was in the paper by Larrey (1812), but it was only in 1915 that the organisms causing leptospirosis were discovered. In 1915, the Japanese workers (Inada *et al*, 1916) managed to isolate and identify *Leptospira interrogans* serovar *icterohaemorrhagiae*. Since then, numerous works on leptspirosis have been recorded. Now leptospirosis is recognised as a disease of world wide significance. The new understanding exists through better knowledge of the infection in animals and man, together with improved laboratory techniques for diagnosis.

Leptospiral infection in sheep has not been studied extensively. One reason is that it has often been considered to be of little importance. The earliest record of leptospiral infection in sheep was in 1932, an experimental infection of sheep by Petzetakis *et al*, (1932). These workers managed to infect sheep with *Spirochaeta icterohaemorrhagiae*, now known as *Leptospira interrogans* serovar *icterohaemorrhagiae*, by intratesticular inoculation. Since then, several natural infections of sheep with leptospirosis have been reported. Some of the early reports came from Austria (Wirth, 1937), Germany (Schlossberger, 1950), Israel (Bernkopf and Olitzki, 1948), Palestine (Fruend, 1947) and U.S.S.R. (Awrorow, 1941). One report (Nefedev, 1949) mentioned an outbreak of leptospirosis (the serovars were not mentioned) among cattle and sheep in some regions of the U.S.S.R.. Vaccines were introduced in this outbreak to control the infection with an apparently high degree of success.

During the past fifty years, reports of serological surveys for the presence of leptospiral antibodies have been reported from almost every part of the world. One of the earliest serological surveys of leptospiral infection in sheep was by

van der Hoeden (1953). He found serological evidence of infection of sheep in Israel with *Leptospira grippotyphosa* All the affected animals, except for one ram, were healthy. The ram was off its feed and had a titre of 1:30,000 to serovar *grippotyphosa*. The kidneys from one healthy animal with high titre, showed greyish spots and histologically the kidneys showed interstitial nephritis. Cultures set up were all found to be negative.

Mochmann (1955) found serological evidence of infection in sheep in Germany to *Leptospira interrogans* serovar *canicola*, whilst van Riel and van Riel (1956) in Central Africa showed significant agglutination titres of serum samples from sheep to serovar *bataviae* and members of the Icterohaemorrhagiae and Cynopteri serogroups. In Argentina, 113 out of the 889 (12.7 percent) sheep sera examined by Cacchione *et al*, (1963) were seropositive to either *ballum*, *hebdomadis*, *autumnalis*, *icterohaemorrhagiae*, *tarassovi* or *pomona*. Michna (1967) in Scotland observed the presence of agglutinating antibodies in the sera of Scottish sheep to *ballum*, *icterohaemorrhagiae*, *sejroe* and *canicola*, with titres ranging from 1:30 to 1:1,000.

In the United States, Beamer *et al*, (1953) described an outbreak involving 108 young ewes with deaths and abortions. Postmortem examination of five of the ewes revealed icterus, swollen kidneys and marked hematuria. Cultures of various organs for pathogenic microorganisms were negative. Dark-field examination of the stomach contents of one foetus revealed motile organisms morphologically resembling leptospires. The organisms were suggestive of leptospires but could not be identified with certainty. Some of the serum samples were positive to leptospires (the serovar was not mentioned). Although leptospires were not demonstrated by cultural methods, the authors believed that the symptoms, lesions, microscopic agglutination reactions and the demonstration of leptospiral forms from an aborted foetus warranted a diagnosis of leptospirosis.

Following the report of Beamer *et al*, (1953) on the outbreak of ovine leptospirosis, a survey in the State of Illinois was conducted by Chappell *et al*, (1961). The survey showed low prevalence (5.3 percent) of leptospiral infection in the sheep examined. Sera that were examined in this survey reacted to *tarassovi*, *grippotyphosa*, *icterohaemorrhagiae*, *pomona*, *canicola* and *wolffi*. A more recent survey by Smith and Armstrong (1975) showed a much higher prevalence of leptospiral infection in sheep in the United States. Sixteen percent of the sheep demonstrated agglutinating antibodies for *Leptospira interrogans* serovar *pomona*.

Since these initial reports of leptospiral infections in sheep, serovar *pomona* has been recognised as a major cause of ovine leptospirosis in the United States. In 1973, another outbreak in two flocks of lambs with fatal hemolytic anemia due to *Leptospira interrogans* serovar *pomona* was reported by Smith and Armstrong (1975). In this case, the ewes in both flocks appeared to be clinically normal, but the lambs had hemoglobinemia and hemoglobinuria. When postmortem examinations were carried out, all the organs other than the kidneys were normal. These were enlarged, pale brown and friable. Numerous dark brown streaks and scattered pin-point white spots were observed throughout the renal cortices. The microscopic findings were consistent with focal to diffuse subacute interstitial nephritis.

Two major episodes of acute haemolytic disease associated with high mortality in lambs occurred in 1978 (Davidson and Hirsh, 1980). Clinical signs were severe depression, dyspnoea and tachycardia. The gross postmortem findings were hemoglobinuria and icterus. Serovar *pomona* was proposed as the causative organisms because of the high titres to this serovar in the lambs and ewes in the flocks. It appears that leptospirosis in lambs in the United States is quite often accompanied by fatal hemolytic anemia.

A similar observation was made by Bokori *et al*, (1960) in Hungary, where fever, hemoglobinuria, anemia and icterus occurred among three to four-month old dying lambs. *Leptospira interrogans* serovar *pomona* was isolated from recovered animals. Leptospiruria lasted for about two months and the infection is believed to be contracted from cattle.

A more recent report (Andreani *et al*, 1974) in Italy, recorded a naturally occurring outbreak of infection in 1000 sheep associated with abortions. Affected animals had high serological titres to *Leptospira interrogans* serovar *hardjo*. Bacteriological tests were negative and leptospires were not isolated from guinea pigs infected intra-peritoneally with urine of seventeen ewes showing high serological titres. Positive serological responses were also detected among cattle, buffaloes and dogs on the same farm. Microscopic agglutination tests performed on 207 serum samples showed a positive reaction in 102 (49.2 percent) of the sheep, with titres ranging from 1:500 to 1:200,000 to serovar *hardjo*. It was concluded that the outbreak was due to *hardjo* infection.

Clinical leptospirosis in sheep has seldom been described in Australia. However, there were occassional reports of ovine infection with *Leptospira interrogans* serovar *pomona* (Sullivan, 1974). Serological surveys in New South Wales (Hoare and Claxton, 1972) and in Victoria (Durfee and Presidente, 1979) revealed a high prevalence of agglutinating antibodies to serovar *hardjo* in sheep. McCaughan *et al*, (1980) reported the isolation of leptospires belonging to the Hebdomadis serogroup from two flocks of sheep in Victoria in 1978. Serum samples from both flocks had high prevalence of agglutinating antibodies to serovar *hardjo*. Unfortunately, the isolates could not be serotyped. It was suspected that the source of infection for the sheep was the cattle on the farms.

A survey in Victoria by Gordon (1980) recorded a high serological prevalence of serogroup Hebdomadis infection in sheep. There was a forty percent overall reaction from the 1000 sera tested and the findings indicate serovar *hardjo* might be endemic in sheep in Australia. Sporadic and sudden deaths occurred in a flock of 5000 wethers in 1979. A live sheep with microscopic agglutination titres of 1:512 to serovar *hardjo* was autopsied and the histopathological findings were consistent with leptospirosis. The kidneys were found to be enlarged and pale and there was a chronic interstitial nephritis. Random serum samples from the flock showed high microscopic agglutination titres to serovar *hardjo*. Leptospires were detected by darkfield examination in the urine of three sheep (11.5 percent) and subsequently, they were identified as *Leptospira interrogans* serovar *hardjo* by the cross-agglutination absorption tests.

Numerous experimental investigations (Alexander *et al*, 1971), (Hathaway and Marshall, 1979), Lindqvist *et al*, 1958), (Langham *et al*, 1958), (Morse *et al*, 1957), (Smith *et al*, 1970) of leptospiral infections in sheep have been carried out and these studies have shown that sheep are susceptible to experimental infection. It is believed by some workers (Chappell *et al*, 1961), (Sullivan, 1974), (Zaharija and Todorovic, 1964) that sheep are resistant to leptospiral infection. The choice of sheep as experimental animals may be due to economic factor as well as the fact that sheep are both easy to maintain and handle.

Clinical manifestations of severe illness were not evident in sheep experimentally infected with *Leptospira interrogans* serovar *pomona* by Morse *et al*, (1957). The most prominent indication was fever of 40^{o} to 42^{o}C. Leptospiremia was readily demonstrated within five to eight days following infection. Leptospiruria was consistent after the third week of infection and lasted for about 62 days. Darkfield microscopy consistently failed to reveal the presence of leptospires in urine samples which were later proved positive by animal inoculation. The number of leptospires present in the urine must have been very few.

Similar results of sheep experimentally infected with serovar *pomona* were obtained by Lindqvist *et al*, (1958), Langham *et al*, (1958) and Dozsa and Sahu (1970). These workers also noted that their experimentally infected sheep produced mild symptoms and without very extensive tissue changes. The most significant lesions were macroscopic grayish-white circumscribed foci and streaks measuring from one to four millimetres in diameter. Langham *et al*, (1958) observed white foci in the kidneys of almost all their experimentally infected animals. Microscopically these areas were characterised by infiltrations of lymphocytes, some plasma cells and a few macrophages.

Linqvist *et al*, (1958) experimentally produced *pomona* infections in pregnant ewes. The animals apparently did not show the severe symptoms and mortality observed in natural outbreaks. Abortion is a common sequel to *pomona* infection in both cattle and pigs. None of the experimentally infected ewes aborted. Apparently, foetal invasion by leptospires is not common in leptospiral infection of pregnant ewes. The conditions under which experimentation is conducted may be an important factor accounting for the differences in the experimental and natural infections. In natural conditions, sheep may be subjected to a number of predisposing stress factors.

Smith *et al*, (1970) produced ovine foetal leptospirosis by infecting pregnant ewes with organisms belonging to serovar *pomona* through the middle uterine artery. A hypothesis was presented on the pathogenesis of foetal infection as indicated by the distribution of leptospires in the placental and foetal materials. Foetal death they believed was due to direct invasion of leptospires. Autolytic changes preceeded the death of the foetus and abortion was delayed until the autolytic effects extended to the placenta. The autolytic changes would interfere with the demonstration of leptospires by the usual laboratory methods and as placentomes are the least affected by autolysis , they

suggested that close attentions should be given to their examination.

An experimental infection of sheep with *Leptospira interrogans* serovar *balcanica* was set up by Durfee and Presidente (1979). All the sheep developed microscopic agglutinating titres to serovar *hardjo* and transient leptospiruria was seen between days fourteen and 25 post-inoculation. However, none of the ewes showed any evidence of clinical disease.

OVINE LEPTOSPIROSIS IN NEW ZEALAND.

Leptospirosis as a clinical disease was first recorded in New Zealand by workers at the Wallaceville Laboratory (ANON 1951). It was shown that the disease of calves and sheep commonly referred to as "Redwater", was in fact leptospiral infection due to *Leptospira interrogans* serovar *pomona*. "Redwater" was a condition well-known to farmers in New Zealand for many years it may therefore be assumed that leptospirosis is not a new disease but one which has been in the country for many years.

Since this initial report, leptospirosis has been reported on a number of occasions. Kirschner and Gray (1951) reported the presence of *Leptospira icterohaemorrhagiae* and *Leptospira canicola* in rats and dogs in the Dunedin area. At about the same time, several outbreaks of ovine leptospirosis were reported, (Hartley, 1952), (Salisbury, 1954), (Webster and Reynolds, 1955). All the outbreaks were due to *Leptospira interrogans* serovar *pomona*. Icterus, haemoglobinuria, and high mortality were the main clinical features reported. However, inapparent infections were also recorded.

The report by Hartley (1952) was of an outbreak of leptospirosis in cattle and sheep in 1950. This was a severe outbreak with death of twelve lambs and two ewes. The affected animals showed marked generalised icterus.

Cultures were set up but failed to grow any pathogenic organisms from the liver and kidneys. Urine was examined by darkfield microscopy, but did not reveal any leptospires. On the other hand, Levaditi-stained sections of liver and kidneys showed variable numbers of typical leptospires. In another outbreak investigated by Hartley (1952), *Leptospira interrogans* servar *pomona* was isolated. Numerous viable leptospires were present in the urine of a live lamb when examined by darkfield microscopy. A high microscopic agglutinating titre against serovar *pomona* was obtained from the serum of a recovered sheep.

Salisbury (1954) reported the outbreak occurring in 1953 of ovine leptospirosis due to serovar *pomona* in a flock of seventy animals. There were eight deaths (11.4 percent) among the lambs. Urine samples were collected from 145 ewes and lambs and thirteen (nine percent) were shown to be positive.

A severe outbreak of leptospirosis towards the end of 1951 in a large stud flock of 400 ewes and their lambs was investigated by Webster and Reynolds (1955). There was a mortality of eighteen percent, with morbidity approaching 100 percent in the lambs. Symptoms were mild in the older sheep, most of which gave no clinical evidence of infection, though a few had slight transient haemoglobinuria. In another outbreak towards the end of 1953, thirty unweaned lambs out of 162 (eighteen percent) died with the characteristic symptoms of leptospirosis. Again, ewes showed no clinical signs of infection, though some had leptospiruria. From these two outbreaks, Webster and Reynolds (1955) were able to isolate organisms belonging to serovar *pomona*. In two separate sheep experiments using these field isolates, no clinical symptoms except transient fever were observed in either the inoculated ewes or the lambs. Leptospiruria was observed from most of the animals and persisted for about three months. They observed that the majority of the naturally infected animals ceased excreting leptospires after two or three months, but there were some with

persistent leptospiruria. In one particular animal, leptospires were present consistently in the urine for nine months. Another interesting feature of the trials is that vaccinated animals resisted challenge three weeks after vaccination.

Since these initial reports, ovine leptospirosis has rarely been reported. This could be due to overt outbreaks or that there have been no investigations in this species. No other serovars other than serovar *pomona* have been associated with sheep in New Zealand. A recent report by Ris (1975) showed evidence of infection in sheep by members of the Hebdomadis serogroup. Of 344 sheep sera collected since 1968 from ten flocks in the southern half of the North Island, 65 percent had positive titres (1:100 or more) to *hardjo* using the microscopic agglutination test. More than half of these reactors had titres over 1:1000. In eight of these flocks, the prevalence of positive titres was 75 to 100 percent while in the remaining two flocks, it was 30 and 42 percent. In another flock of 93 ewes, *hardjo* titres of 1:300 or higher, were detected in 95 percent of the flock. Several had titres of 1:10,000 or higher and 44 percent had 1:1,000 or higher. One year earlier only ten percent of these ewes had titres of 1:300 or higher. No abnormalities that could have been associated with leptospirosis were seen in either the ewes or the lambs. It appears that the infection was widespread in these samples of sheep. In spite of the high serological prevalence in these sheep to members of the Hebdomadis serogroup, symptoms were either absent or not observed. This indicates that the pathogenicity for sheep by this serovar is low. Attempts to isolate the organisms were unsuccessful.

Titres to Hebdomadis serogroup are commonly found in cattle, sheep and goats, horses and possums in New Zealand (Hathaway and Marshall, 1979). However, the only definite maintenance hosts for the two serovars within this serogroup which have been identified in New Zealand are cattle for *Leptospira*

interrogans serovar *hardjo* and possums for *Leptospira interrogans* serovar *balcanica*.

In a small survey made by Blackmore *et al*, (1976) at an artificial breeding centre in the Manawatu, serological examination of sheep sera provided evidence of agglutinating antibodies of four (14.3 percent) of the sheep. However, no mention was made of which serovar the sheep were reacting to. Twenty eight sheep kidney homogenates were examined culturally and by direct darkfield microscopy. No evidence of infection was seen directly, although five of the cultures were found to contain organisms. None of the cultures were able to be serotyped due to poor growth. There was a high prevalence of both *hardjo* and *pomona* infections in the cattle population in the centre and it was claimed that the low prevalence of leptospiral infection in the sheep indicated that the sheep were not a significant source of infection for the cattle.

Hodges (1974) experimentally infected lambs with *Leptospira interrogans* serovar *pomona* in order to assess the importance of sheep in the epidemiology of leptospirosis in farm animals. A wide variation in clinical signs was noted in the infected lambs. During the early stages of infection a febrile response and severe hemoglobinuria were noted in otherwise clinically normal lambs. Haemolytic anaemia was observed in four of the six lambs inoculated with serovar *pomona*. Agglutinating antibodies were demonstrated after four days of infection, but leptospires were not seen in any urine sample. The failure to detect leptospiruria in sheep by darkfield microscopy is in agreement with the findings of Beamer *et al*, (1953), Morse *et al*, (1957) and Lindqvist *et al*, (1958). A similar clinical picture was shown by Millar *et al*, (1979) in their sheep experimentally infected with serovar *pomona*.

In an attempt to gain information on whether sheep could act as a maintenace host for either serovar *hardjo* or *balcanica*, a small experiment was undertaken by Hathaway and Marshall (1979). All the animals infected showed a serological response and titres to either the homologous or heterologous serovar were similar over the three-week period of the experiment. However, no clinical symptoms of infection were shown by the infected animals. Renal infection and leptospiruria were readily established with serovar *hardjo* but not with *balcanica*. The possibility of serovar *hardjo* being responsible for the Hebdomadis serogroup titres which are found in sheep in New Zealand was suggested. The preliminary results showed that sheep are unlikely to be a maintenance host for serovar *balcanica*.

Similar results were obtained by Mackintosh *et al*, (1981) in their experimental infection of sheep with *Leptospira interrogans* serovar *balcanica*. Even though all the animals had seroconverted by the fourteenth day after inoculation, no clinical signs except for mild fever were seen. Only one animal was leptospiruric. The control animals which had been running together with the infected animals, apparently were not infected by close contact. Durfee and Presidente (1970) in Australia were also able to infect sheep with serovar *balcanica*, but renal infection was demonstrated in their animals.

A vaccination trial in sheep by Marshall *et al*, (1979) showed a high degree of success, with 78 percent of the animals vaccinated resisting heavy challenge with leptospires. The vaccinated animals showed serological response after challenge, but this declined more rapidly than in the sheep in the unvaccinated group. The trial demonstrated that vaccination of sheep effectively reduced the number of animals likely to become infected with serovar *hardjo*.

CHAPTER TWO

THE SEROLOGICAL PREVALENCE OF NATURALLY OCCURRING INFECTIONS OF LEPTOSPIROSIS IN SHEEP

INTRODUCTION

Leptospirosis is a disease affecting all species of domestic animals, including sheep and it has been recognised as an important worldwide zoonosis. Leptospirosis in sheep has not been studied extensively and it has often been regarded of little importance. Overseas reports (Brewer *et al*, 1960), (Cafferena *et al*, 1971), (Chulovskii, 1971), (Michna, 1967), (Shigidi, 1974) have shown the prevalence to range from zero to 51 percent in sheep examined for leptospiral agglutinating antibodies (Table 2-1).

Workers in Austria (Sebek *et al*, 1976), Chile (Zamora *et al*, 1975), India (Palit *et al*, 1971) and the U.S.S.R. (Dragomir *et al*, 1975) showed there was a low prevalence of sheep with agglutinating antibodies to leptospiral infection, whilst sheep from Australia (Gordon, 1980), Turkey (Brewer *et al*, 1960) and Yugoslavia (Zaharija, 1979) had indications of a comparatively high prevalence of leptospiral infection.

Serological surveys of leptospiral infection in sheep from the various countries reported the presence of agglutinating antibodies to a number of serovars (Table 2-2). Perhaps, the three most commonly occurring serovars in sheep are serovar *icterohaemorrhagiae* (Chappell *et al, 1961*), (Michna, 1967), (van Riel and van Riel, 1956); serovar *pomona* (Davidson and Hirsh, 1980), (Salisbury, 1954), (Smith and Armstrong, 1975) and serovar *hardjo* (Andreani *et al*, 1974), (Gordon, 1980), Hoare and Claxton, 1972).

TABLE 2-1: WORLD SURVEYS OF THE PREVALENCE OF LEPTOSPIRAL INFECTION IN SHEEP

Country	No. of Sera Examined	Minimum Titres Used	Percentage of Positives	Reference
Afghanistan	88	1:800	2.3	Sebek *et al* (1978)
Africa (Central)	72	1: 30	4.2	van Riel and van Riel (1956)
Australia	1000	?	40	Gordon (1980)
	505	1:100	19.6	Hoare & Claxton (1972)
Argentina	889		12.7	Cacchione *et al* (1963)
Austria (Tyrol)	189	1:800	0.5	Sebek *et al* (1978)
Banat	107	1:100	12.2	Zaharija and Todorovic (1964)
Bulgaria	1900	?	1.7	Popov & Rashev (1962)
Chile	182	1:100	7.1	Zamora *et al* (1975)
Germany	15	?	6.7	Schlossberger (1950)
	231	?	61	Fritzsche & Rohl (1963)
	815	?	0.1	Graubmann *et al* (1970)
India	162	1:100	5.5	Arora (1976)
	51	1:100	54.9	Ball & Sheikh (1958)
	90	1:100	0	Palit & Sharma (1971)
	102	1: 30	11.7	Pargaonker & Ramakrishna (1963)
	155	1: 10	3.2	Rajasekhar & Nanjiah (1971)
Israel	34	1: 10	47	van Hoeden (1953)
Italy	207	1:500	49.2	Andreani *et al* (1974)
	1151	1:500	7.2	Ciuchini *et al* (1980)
Mongolia	128	1:800	1.6	Sebek (1974)
Morocco	616	?	28	Mailloux (1969)
New Zealand	174	?	9.2	Salisbury (1954)
	344	1:100	65	Ris (1975)
Scotland	167	1: 10	10.2	Michna (1967)

Cont'd..

TABLE 2-1: CONTINUED

Country	No. of Sera Examined	Minimum Titres Used	Percentage of Positives	Reference
Spain	?	?	16	Vizcaino *et al* (1978)
Sudan	200	1: 16	2	Shigidi (1974)
Turkey	49	1:100	47.8	Brewer *et al* (1960)
U.S.A.	561	1: 50	5.3	Chappell *et al* (1961)
Uruguay	289	?	10.4	Caffarena *et al* (1971)
U.S.S.R.	2300	1:100	8.51	Chulovskii (1971)
	906	?	0.8	Dragomir *et al* (1975)
	?	?	6	Nuikin & Tsvetkova (1972)
Yugoslavia (Croatia)	254	1:100	33.2	Zaharija (1979)

TABLE 2-2: THE VARIOUS LEPTOSPIRAL SEROVARS REPORTED TO HAVE INFECTED SHEEP.

Country	Presence of Agglutinating Antibodies to Serovars	Reference
Afghanistan	*ballum, javanica*	Sebek *et al*, (1978)
Africa (Central)	*icterohaemorrhagiae, bataviae, butembo*	van Riel & van Riel (1956)
Argentina	*autumnalis, ballum, hebdomadis, icterohaemorrhagiae, pomona, tarassovi*	Cacchione *et al*, (1963)
Australia	*hardjo*	Hoare & Claxton (1972)
	medanensis, kremastos, mini, icterohaemorrhagiae, esposito, autumnalis	Spradbrow (1964)
Austria	*icterohaemorrhagiae, sorex*	Sebek *et al*, (1976)
Chile	*ballum, copenhageni, javanica, icterohaemorrhagiae, pomona*	Zamora *et al*, (1975)
Germany	*icterohaemorrhagiae*	Schlossberger (1951)
India	*autumnalis, pomona*	Ball & Sheikh (1958)
Iran	*grippotyphosa, tarassovi, pomona*	Maghami (1970)
Israel	*grippotyphosa*	Hoeden (1953)
Italy	*hardjo, mini, sejroe, poi, saxkoebing*	Ciuchini *et al*, (1980)
Mongolia	*tarassovi, hebdomadis*	Sebek (1974)
Morocco	*icterohaemorrhagiae, canicola, ballum, pyogenes*	Mailloux (1970)
New Zealand	*hardjo*	Ris (1975)
	pomona	Salisbury (1954)
Scotland	*ballum, canicola, sejroe, icterohaemorrhagiae*	Michna (1967)
Turkey	*ballum, autumnalis, sejroe, grippotyphosa*	Brewer *et al*, (1960)
Yugoslavia	*australis, hebdomadis, poi, tarassovi, icterohaemorrhagiae, salinem, sejroe, pomona*	Zaharija (1979)
U.S.S.R.	*grippotyphosa*	Akhmedov *et al*, (1979)

Agglutinating antibodies to serovar *pomona* in sheep have been reported on many occassions overseas and in New Zealand (Hartley, 1952), (Zaharija, 1979), (Zamora *et al*, 1975), (Webster and Reynolds, 1955). Only lately, reports of a high prevalence of agglutinating antibodies in sheep to serovar *hardjo* have been reported (Gordon,1980), (Andreani *et al*, 1974), (Ris, 1975).

In New Zealand, serological surveys (Table 2-3) of leptospiral infection have been carried out in most of the domestic animals and in wildlife. The surveys in cattle (Hellstrom, 1978), pigs (Ryan, 1978) and possums (Hathaway, 1978) recorded a high prevalence of leptospiral infection in these species. Sixty percent of the cattle population were shown to have experienced *hardjo* infection, with eighty percent of the cattle herds in New Zealand having had leptospiral infection at one time or another. Serovars *pomona* and *tarassovi* are the two main serovars affecting pigs in New Zealand. It has been shown that nearly half of the pig population have experienced either *pomona* or *tarassovi* infection (Ryan, 1978). Amongst wildlife species, the possum (*Trichosurus vulpecula*) is the most important as regards leptospiral infection. Hathaway (1978) showed that 85 percent of the possum population had agglutinating antibodies to serovar *balcanica*.

There is also evidence of leptospiral infection in feral deer (Daniel, 1966), feral goats (Schollum and Blackmore, 1981), and feral pigs (Mackintosh per. comm.) but these species appear to be less important as carriers of leptospires. Because a detailed survey to establish the prevalence of leptospiral infection in sheep in New Zealand had not been carried out, it was decided to carry out an extensive investigation in this species to determine the prevalence of agglutinating antibodies for the various leptospiral serovars.

TABLE 2-3: SEROLOGICAL PREVALENCE OF LEPTOSPIRAL INFECTION IN DOMESTIC ANIMALS AND WILDLIFE IN NEW ZEALAND (Percentage).

ANIMAL HOST	NO. OF SERA EXAMINED	*hardjo*	*balcanica*	*pomona*	*tarassovi*	*ballum*	*copenhageni*	*australis*	OVERALL PREVALENCE	REFERENCE
Cattle										
National	480	60		18	9	4	2		66	Hellstrom (1978)
Manawatu	480	73		5	2	1	1			Hellstrom (1978)
Pigs	65			86	25					Ryan (1978)
Dogs	65	27.7		6.2	1.5	9.2	3.1		45	Mackintosh *et al* (1980)
Cats	225	2.2	0.4	1.8	0	1.8	2.2	0	8.8	Shophet (1979)
Feral goats	116	10.3	11.2			4.3			13.3	Schollum & Blackmore (1981)
Possums	600		55						85	Hathaway (1978)
Norway rats	79					12.7	32			Brockie (1977)
Ship rats	16					25			20	Brockie (1977)
Mice	67					14.9				Brockie (1977)
Hedgehogs	78			3.8	1.3	35.9	9.0		89.7	Brockie & Till (1977)
Deer	109			0.9					0.9	Daniel (1966)

AIMS

The main aim of the survey was to determine the serological prevalence of leptospiral infection in sheep of the North Island of New Zealand. The survey had a number of objectives, namely: -

1. To determine the overall serological prevalence of leptospiral infection in the ovine population.

2. To ascertain which serovars affect sheep and their relative importance.

3. To find out which age group of sheep is affected most and to deduce when sheep picked up infection.

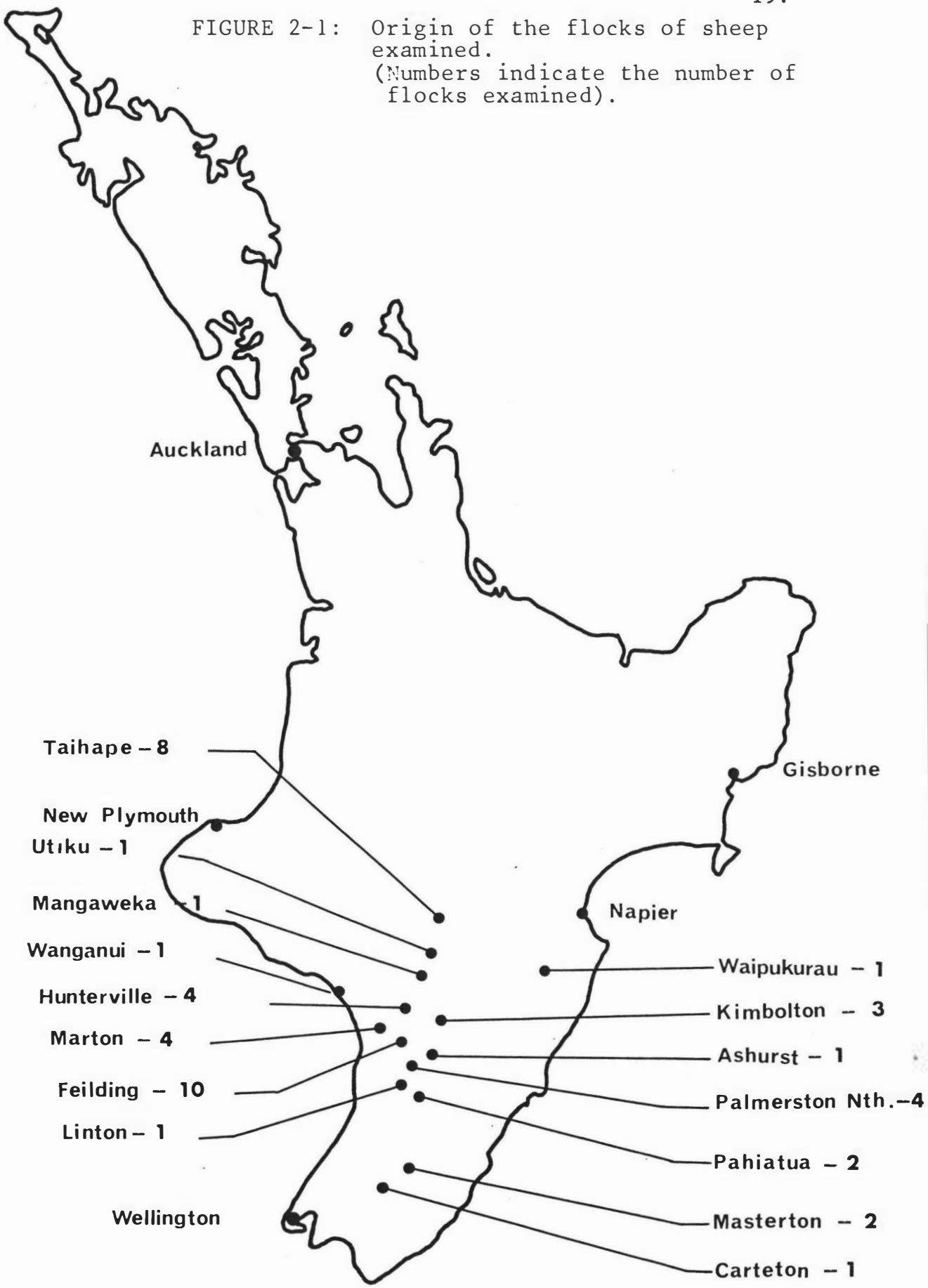
4. To examine the distribution of the agglutinating antibodies in individual flocks of sheep.

MATERIALS AND METHODS

From 28th March, 1979 to 18th August, 1979, blood samples were collected from sheep going to two Freezing Works in the Manawatu. Both these works process sheep from the southern half of the North Island. The first twenty animals were selected from a line of at least 100 animals submitted for slaughter at the Freezing Works. All the animals apparently were healthy when killed. As the animals were being bled, blood samples were immediately collected into clean universal bottles. Altogether 928 blood samples from 42 flocks of sheep were collected. The flocks were from various parts of the North Island (Figure 2-1), some originating as far North as Taihape, Waipukurau to the East and Carterton to the South. The flocks were categorised into three age groups namely;

a) lambs, animals less than one year old with no permanent teeth,

FIGURE 2-1: Origin of the flocks of sheep examined.
(Numbers indicate the number of flocks examined).



b) hoggets, animals about one to two years old, having two to four permanent incisors and
c) ewes, those animals more than two years old and having not less than six permanent incisors.

The name and address of the farmer from whom the sheep originated was obtained, so that a subsequent questionnaire could be sent to obtain details of the farm including other stock contact.

The blood samples were held overnight at 4°C and the sera separated from the blood clot by centrifugation at 2,000 rpm for five minutes. These serum samples were then placed in bijoux bottles and held at -20°C until tested by the leptospiral microscopic agglutination test (MAT). This is a modification of the method described by Cole *et al*, (1973). The lowest serum dilution tested was 1:24. Serial doubling dilutions of sera after the addition of the antigens ranged from 1:24 to 1:3072. The antigens grown in liquid JS medium* were four to eight-day old live cultures of serovars *hardjo, balcanica, pomona, tarassovi, ballum* and *copenhageni* with estimated density of 10^8 organisms per ml. These serovars were considered because they are known to be endemic in New Zealand. Serovar *australis* was later included in the battery after Thompson (1980) described a case in man, the first isolation of this serovar in this country.

The whole procedure was prepared in plastic microtitre plates with round bottom wells (Microtitre)** using a semi-automated minidiluter (Dynatech)**. Each well contained 25 ul of diluted serum and 25 ul of antigen, resulting in a dilution of 1:24. The plates were then incubated at 37°C for ninety minutes before examining for agglutination.

* Appendix Four
** Appendix One

Tests were read by transferring a drop from each well onto a glass microscope slide, using a multiple dipper designed by Ryan (1978). The drops were examined by dry dark-field microscopy at a magnification of 120X. A positive reaction was regarded as one in which fifty percent or more of the leptospires were agglutinated. The titre endpoint was taken as the last well in which fifty percent or more agglutination was observed.

RESULTS

Thirty eight (351/928) percent of the sheep examined had titres to one or more leptospiral serovars ranging from 1:24 to 1:3072. The proportion of the sheep demonstrating agglutinating antibodies to the seven serovars are summarised in Table 2-4 and Figure 2-2.

Out of these 351 positive sera, 182 (19.6 percent) sera gave titres to serovar *hardjo*, 193 (20.8 percent) sera to serovar *balcanica*, 71 (7.7 percent) sera to serovar *pomona*, 66 (7.1 percent) sera to serovar *tarassovi*. 55 (5.9 percent) sera to serovar *ballum*, 40 (4.3 percent) sera to serovar *copenhageni*, and 23 (2.5 percent) sera to serovar *australis*. These figures were obtained when titres at dilution of 1:24 and above were considered as significant titres.

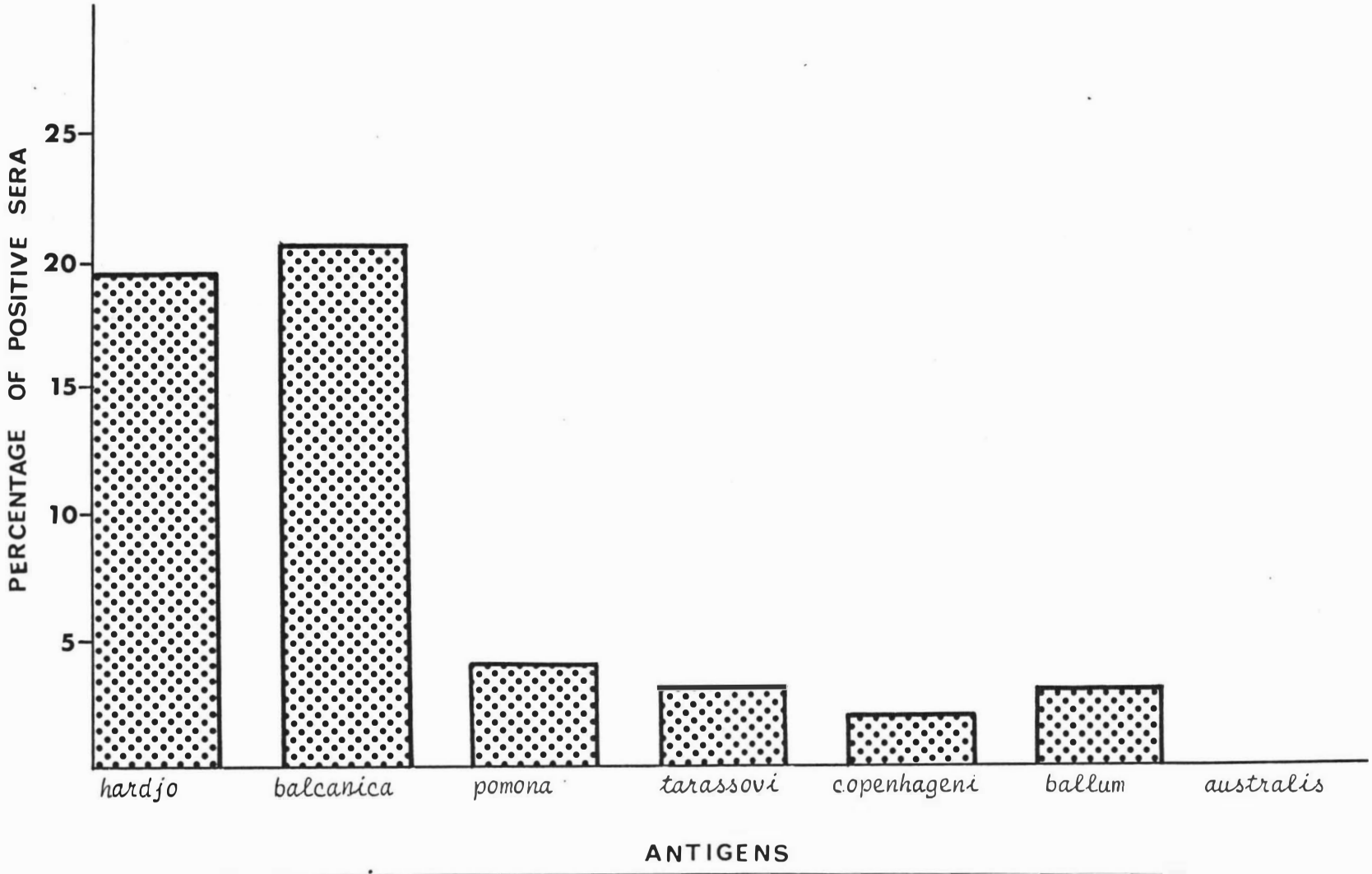
A large proportion of the positive sera reacted to members of the Hebdomadis serogroup. The titres to serovar *hardjo* formed a major portion of the positives and it is clear that serovar *hardjo* is the most important serovar affecting sheep in New Zealand. The prevalence of MAT titres ($\geq$ 1:24) to serovar *hardjo* among the flocks varied from zero to 94.1 percent (Table 2-5). Of the various flocks of sheep examined, only two of the flocks came from farms without any cattle on their properties. It is suspected that the sheep contracted their *hardjo* infections from cattle, but

TABLE 2-4: DISTRIBUTION OF TITRES TO LEPTOSPIRAL ANTIGENS IN A SEROLOGICAL SURVEY OF SHEEP FROM THE NORTH ISLAND OF NEW ZEALAND

ANTIGENS	NEGATIVES	NUMBER OF SERA POSITIVE (RECIPROCAL OF TITRES)								POSITIVE	PERCENTAGE POSITIVE AT 1:24	PERCENTAGE POSITIVE AT 1:48	GMT	RANGE
		24	48	96	192	384	768	1536	3072					
hardjo	747	9	26	50	54	25	10	5	2	181	19.5	18.5	1:264	1:24-1:3072
balcanica	736	2	8	31	56	59	22	9	5	192	20.7	20.5	1:432	1:24-1:3072
pomona	856	37	25	5	2	2	-	-	1	72	7.8	3.8	1: 94	1:24-1:3072
tarassovi	862	42	17	3	2	2	-	-	-	66	7.1	2.6	1: 49	1:24-1: 384
copenhageni	888	19	15	2	1	-	2	1	-	40	4.3	2.3	1:111	1:24-1:1536
ballum	872	31	15	4	4	-	1	1	-	55	5.9	2.7	1: 89	1:24-1:1536
australis	905	23	-	-	-	-	-	-	-	23	2.5	0	1: 24	1:24

(Total sera examined = 928)

FIGURE 2-2: The proportions of the sheep sera demonstrating titres (≥1:48) to the seven leptospiral antigens.



this may not be the only mode of infection as these two flocks of sheep still demonstrated *hardjo* titres in their sera in the absence of cattle contact.

Positive titres to serovars *pomona* and *tarassovi* were next in numerical importance. These two serovars have pigs as their maintenance host and as such, we can expect titres to these two serovars to be nearly the same in prevalence (7.7 percent for serovar *pomona* and 7.1 percent in case of serovar *tarassovi*). There was a general distribution of agglutinating antibodies to serovar *pomona* in the sheep surveyed regardless of the presence or not of pigs on the farms. The prevalence of infection for serovar *pomona* is from zero to 22.7 percent among the flocks of sheep, whilst it is from zero to 39.1 percent to serovar *tarassovi*.

The other three serovars make up the remaining small portion of positive sera. Two flocks of sheep had high number of titres to serovar *copenhageni*. Nearly half of the titres ($\geq$ 1:24) to serovar *copenhageni* were single reactions. Cross-reactions, where they occurred, were mainly with serovar *hardjo* and these cross-reactions were found to be significant ($p = 0.001$).

All the *australis* titres obtained in this survey were at dilution of 1:24. This suggests that they were either cross-reactions or non-specific titres. If natural infection had occurred, a distribution of titres of different levels, similar to those associated with *hardjo* and the other serovars would have been expected.

The sixteen flocks of lambs from the various parts of the southern half of the North Island showed a prevalence of leptospiral infection ranging from zero to 52.4 percent (Table 2-5). Three of these flocks were negative to all the seven serovars tested. The highest prevalence of leptospiral infections among the flocks of lambs was to serovar *tarassovi*. In general, the prevalence of infection was low among the flocks of lambs.

TABLE 2-5: PREVALENCE OF AGGLUTINATING ANTIBODIES (1:24 OR MORE) TO LEPTOSPIRAL INFECTION IN INDIVIDUAL FLOCKS

GROUP ID	NO. ANIMALS IN GROUP OF SHEEP	NO OF SERA EXAMINED.	PERCENTAGE OF GROUP EXAMINED	NO OF SERA POSITIVE*	PERCENTAGE OF ANIMALS POSITIVE
AGE GROUP - LAMBS					
1	115	20	17.4	-	0
2	80	20	25.0	-	0
3	230	21	9.1	-	0
4	442	20	4.5	1	5
6	501	20	4.0	3	15
12	90	21	23.3	6	28.6
13	146	21	14.4	2	9.5
14	186	21	11.3	11	52.4
15	204	21	10.3	5	23.8
16	61	16	26.2	1	6.3
17	70	20	28.6	5	25
18	500	20	4.0	2	10
28	147	22	15.0	10	45.5
38	92	20	21.7	5	25
44	60	19	31.7	7	36.8
45	67	20	29.9	3	15
AGE GROUP - HOGGETS					
5	105	20	19.0	7	35
19	56	22	39.3	10	45.5
20	80	22	27.5	8	36.4
23	140	23	16.4	5	21.7
26	30	22	73.3	15	68.2
27	185	22	11.9	18	81.8
29	130	20	15.4	5	25
32	21	20	95.2	12	60
33	33	21	63.6	5	23.8
35	45	22	48.9	-	0
36	55	22	40	13	59.1

Cont'd..

TABLE 2-5: CONTINUED

39	91	23	25.3	7	30.4
40	30	19	63.3	3	15.8
41	95	20	21.1	14	70
AGE GROUP - EWES					
7	36	23	63.9	16	69.6
8	47	20	42.6	9	45
9	60	21	35	6	28.6
10	39	22	56.4	11	50
11	1144	20	1.7	14	70
21	17	17	100	16	94.1
22	45	24	53.3	14	58.3
24	44	22	50	17	77.3
25	30	22	73.3	7	31.8
30	20	18	90	10	55.6
31	32	23	71.9	9	39.1
34	20	20	100	8	40
37	16	16	100	4	25
42	80	20	25	13	65
43	29	20	69	14	70

* Positive titres to any of the seven antigens.

In the hogget age group, only one flock was found to be negative to all the seven serovars. All the other thirteen flocks had agglutinating antibodies to one or more serovars. The highest prevalence of infection was 81.8 percent, with titres to the Hebdomadis serogroup making up nearly all of the positives.

All the flocks in the ewe age group were positive to leptospiral infection. The prevalence of infection in the flocks of ewes ranged from 25 to 94.1 percent.

Nearly two-thirds (sixty percent) of the flocks tested had serological evidence of leptospiral infection in over fifty percent of their members. The majority of the positive sera reacted to serovars *hardjo* and *balcanica* antigens.

Multiple reactions were obtained from 82 sera and they were mainly of titres of less than 1:48 (Table 2-6). Some of the low titres were considered to be cross-reactions. A certain degree of cross-reactivity can be expected and in most instances, the serovar giving the highest reaction was accepted as the one causing the infection in the animal, in order to allow a diagnosis to be made. However, a few had titres to two or more serovars of approximately the same titre, in these instances, the serovar involved could not be determined except by cross-absorption tests and these were not carried out.

DISCUSSION

In this survey, the lowest serum dilution used was 1:24 and any agglutination in serum dilutions of 1:24 or more was accepted as evidence that the sheep was either currently or had previously been infected with leptospirosis. Broom and McIntyre (1948) stated that specific agglutination of leptospires by serum does not occur in the absence of infection (present or past) and that agglutination, even if present in low dilution is proof of such infection. This is

TABLE 2-6: DISTRIBUTION OF TITRES (≥ 1:24) TO ONE OR MORE SEROVARS IN A RANDOM SAMPLE OF NORTH ISLAND OVINE SERA.

		Prevalence
1. Titres to one serovar		
hardjo (balcanica)	132	14.2 percent
pomona	40	4.3
tarassovi	42	5.4
ballum	26	2.8
copenhageni	18	1.9
australis	11	1.2
Total	269	29 percent
2. Titres to two titres		
hardjo x *pomona*	16	1.7
hardjo x *tarassovi*	12	1.3
hardjo x *ballum*	12	1.3
hardjo x *copenhageni*	12	1.3
hardjo x *australis*	4	0.4
pomona x *tarassovi*	3	0.3
pomona x *copenhageni*	2	0.2
pomona x *australis*	1	0.1
tarassovi x *ballum*	2	0.2
tarassovi x *copenhageni*	2	0.2
ballum x *copenhageni*	2	0.2
ballum x *australis*	1	0.1
Total	69	7.4 percent
3. Titres to three serovars		
hardjo x *pomona* x *ballum*	1	0.1
hardjo x *pomona* x *tarassovi*	1	0.1
hardjo x *tarassovi* x *ballum*	1	0.1
hardjo x *tarassovi* x *copenhageni*	1	0.1
hardjo x *ballum* x *copenhageni*	2	0.2
hardjo x *ballum* x *australis*	1	0.1
pomona x *tarassovi* x *ballum*	2	0.2
pomona x *ballum* x *copenhageni*	1	0.1
Total	10	1.1 percent

Cont'd..

TABLE 2-6: CONTINUED

		Prevalence
4. Titres to four serovars		
hardjo x pomona x ballum x australis	1	0.1 percent
hardjo x pomona x ballum x copenhageni	1	0.1
hardjo x australis x ballum x copenhageni	1	0.1
Total	3	0.3 percent
5. Titres to two or more serovars		
	82	8.8 percent

supported by Bernkopf (1946) in Israel who maintained that in MAT titres to leptospiral serovars does not exceed 1:20. Blackmore *et al*, (1976) considered all sera with a positive reaction however slight, to the antigens in the test as significant. They mentioned that the adoption of a higher titre as a cut-off point could give a false impression of the true prevalence of the disease in flocks. Titres considered as significant and quoted by other workers (Chappell *et al*, 1961), (Michna, 1967), (Sebek *et al*, 1976), (Zaharija, 1979) have varied from 1:10 to 1:800 and often appeared to have been selected arbitrarily without any additional support.

In the majority of the serological surveys of sheep in various countries, the serological prevalence of agglutinating antibodies to leptospiral infection was shown to be in the region of zero to fifteen percent (Chappell *et al*, 1961), (Caffarena *et al*, 1971), (Michna, 1967), (Zamora *et al*, 1975). In New Zealand, Ris (1975) showed a high

prevalence of leptospiral infection in sheep with the prevalence ranging from thirty to 100 percent in flocks of sheep examined. Several of the sera that Ris (1975) examined had titres as high as 1:10,000. This present survey found that 37.8 percent of the sheep examined showed evidence of leptospiral infection. This is much higher than most reported overseas. One of the reasons is probably due to the choice of titres of 1:24 as positive evidence of previous leptospiral infection, whereas most overseas workers have chosen much higher titres as indicative of infection. Sebek (1974), Sebek *et al*, (1976), (1978) had only titres of 1:800 and above to be of significance.

Chappell *et al*, (1961) and Morse *et al*, (1957) suggest that the methods of husbandry used in sheep farming in New Zealand are responsible for the high prevalence of leptospiral infection in sheep. They pointed out that in New Zealand because of the lush wet pastures, more sheep are grazed per hectare and that cattle, the maintenance host for serovar *hardjo* in New Zealand, are often pastured with the sheep. This facilitates easy interspecies transmission of leptospiral organisms.

A review of the available literature relating to serological surveys for leptospiral infection clearly indicates that ovine leptospirosis is a subject often overlooked in many parts of the world. The findings in this present survey, denote a reasonably high proportion of sheep in New Zealand to have experienced infection. The significance of the findings have not been considered in relation to the epidemiology of leptospiral infection in man and other animals and it warrants further research.

In all, antibodies to twenty leptospiral serovars have been detected in sheep sera overseas. In this survey, the serovar of greatest significance belonged to the Hebdomadis serogroup and with the isolation of serovar *hardjo* organisms (Bahaman *et al*, 1980), serovar *hardjo* was proven to be the

causal organisms. Serovars *pomona, tarassovi, ballum, copenhageni* and *australis* follow in decreasing order of importance. Cattle are considered the maintenance host for serovar *hardjo* in New Zealand (Blackmore, 1979), (Hathaway and Marshall, 1979), (Hellstrom, 1979) and it has been suggested that sheep contracted leptospiral infection from cattle (Beamer *et al*, 1953), (Hodges, 1974), (McCaughan *et al*, 1980). However, Morter and Morse (1956) working in the United States were not able to show that this inter-species infection occurred. In their transmission experiment, calves were unable to transmit the infection to sheep although other animals (calves, pigs, goats) became infected. No clear cut answers as to the source of infection for sheep, came from this present survey.

All the farms from where the sheep originated had cattle on their properties, except for two. It was not possible therefore to make a correlation between the prevalence of leptospiral infection in the sheep and the prescence of cattle. The sheep in the two farms without cattle on their properties still showed agglutinating antibodies to serovar *hardjo*. A cohort study of sheep from farms with cattle and farms without cattle on their properties would give a better indication to this situation.

Surveys (Gordon, 1980), (Durfee and Presidente, 1979), (Hoare and Claxton, 1972), (Ris, 1975) have shown that sheep are frequently infected with leptospires from the Hebdomadis serogroup and the possibility that serovar *hardjo* infection may be endemic in sheep has been suggested (Gordon, 1980), (Marshall *et al*, 1979a). Experimentally, Hathaway and Marshall (1979) have shown that inapparent serovar *hardjo* infection is quite readily established in sheep. When only the ewes are considered, 100 percent of the flocks examined in this survey were found to be positive, with a prevalence ranging from fourteen to 68 percent. In the flocks examined by Ris (1975) the prevalence was much higher and many of the sheep in his survey had very high titres. This present

survey supports the suggestion by Marshall *et al*, (1979a) that serovar *hardjo* might be endemic in sheep. The high prevalence of serological reactions to serovar *hardjo* coupled with the large percentage of farms with reactors indicated that there is a widespread exposure of the sheep population in the North Island to serovar *hardjo*.

From the results obtained, many of the *balcanica* titres appeared higher than the *hardjo* titres by one or two dilution. It is not possible to differentiate serologically between serovars *hardjo* and *balcanica* infection (Hellstrom, 1978). We believed the titres to the Hebdomadis serogroup were caused by organisms belonging to serovar *hardjo*. Hathaway and Marshall (1979) have experimentally infected sheep with serovars *hardjo* and *balcanica* and showed that *hardjo* titres were lower than the *balcanica* titres. This is similarly shown by Mackintosh *et al*, (1981). The *hardjo* antigen used in the serological tests was a laboratory adapted strain of *hardjoprajitno* which gives comparatively lower agglutinating titres. In the field, serovar *hardjo* organisms are suspected to be slightly different from this laboratory strain of serovar *hardjoprajitno* and may therefore, give a higher titre especially if isolates of serovar *hardjo* were used as the antigen. In a survey of cattle in Taranaki, Bahaman and Marshall (unpublished data) using laboratory adapted strain *hardjoprajitno* and field isolates of serovar *hardjo* (08/1 cattle isolates) in parallel, found the field isolates gave titres one to two dilutions higher in comparison to the laboratory strain of *hardjoprajitno*. These factors and also the isolation of organisms belonging to serovar *hardjo* from this survey confirm that the Hebdomadis serogroup titres occurring in sheep were actually due to serovar *hardjo*.

There was a general distribution of agglutinating antibodies to serovar *pomona* among the flocks of sheep surveyed regardless to the presence of pigs or not on the farms. The prevalence of the agglutinating antibodies to serovar *pomona* varies from zero to 27.3 percent among the flocks. The highest

prevalence of infection to serovar *pomona* was 27.3 percent and incidentally this particular farm did have a problem with feral pigs. Whether the high prevalence of *pomona* infection is associated with the presence of the feral pigs is as yet unknown. All the sheep farms with pigs except for one, had only a small number of pigs. The one farm which had over 2,000 heads of pigs of varying age had a ten percent prevalence of *pomona* infection. The practice of spraying pig effluent on to pastures was carried out on this particular farm and yet the flock of sheep from this farm had indications of a low prevalence of infection with serovar *pomona*.

The prevalence (7.1 percent) of leptospiral infection in the sheep due to serovar *tarassovi* is almost the same percentage as that due to serovar *pomona*. Both serovars have pigs as their maintenance hosts (Blackmore, 1979). Serovar *pomona* infection in sheep in New Zealand has been reported sporadically (Christiansen, per. comm.) and is perhaps on the decline. This is perhaps directly due to lesser number of farms having pigs. This indicates that serovar *tarassovi* might be more important than serovar *pomona* in the future. This is supported by the fact that there was a higher number of reactors to serovar *tarassovi* than expected, as disclosed in this survey. However, serovar *tarassovi* has not yet been isolated from sheep in New Zealand.

In Chile, serovar *copenhageni* has been shown to be the major organism affecting sheep (Zamora *et al*, 1975). In this present survey, we found two flocks had high titres to serovar *copenhageni*. This is interesting as serovar *copenhageni* has not yet been shown to be established in the southern half of the North Island. Nearly half of the agglutinating antibodies to serovar *copenhageni* were single reactors and the majority of the cross-reactions were with members of the Hebdomadis serogroup. (Table 2-7)(Table 2-8). The concurrent occurrence of titres to serovars *copenhageni* and *hardjo* is found to be significant ($p < 0.001$).

TABLE 2-7: DISTRIBUTION OF SINGLE AND DUAL TITRES TO THE SEVEN SEROVARS AT DILUTION OF 1:24

	A	B	C	D	E	F	TOTAL	G
hardjo (A)	132	16*	12*	12*	12*	4*	188	29.8
pomona (B)	16	40	3	2	-	1	62	35.5
tarassovi (C)	12	3	42	2	2	-	61	31.1
copenhageni (D)	18	2	2	18	2	-	38	52.6
ballum (E)	12	-	2	2	26	1	43	39.5
australis (F)	4	1	-	-	1	11	17	35.3
H	70.2	64.5	68.9	47.4	60.5	64.7		

TABLE 2-8: DISTRIBUTION OF SINGLE AND DUAL TITRES TO THE SEVEN SEROVARS AT DILUTION OF 1:48

	A	B	C	D	E	F	TOTAL	G
hardjo (A)	166	3**	4**	4**	4**	-	181	8.3
pomona (B)	3	35	-	1	-	-	39	10.3
tarassovi (C)	4	-	20	1	1	-	26	23.1
copenhageni (D)	4	1	1	11	2	-	19	42.1
ballum (E)	4	-	1	2	16	-	22	30.4
australis (F)	-	-	-	-	-	-	-	0
H	91.7	89.7	76.9	57.9	69.6	0		

G - Percentage of sera with dual titres
H - Percentage of sera with single titre
* - ρ = significant
** - ρ = insignificant

The low prevalence of the other three serovars showed that they were sporadic infections. Titres to these serovars at dilution of 1:24 were probably non-specific titres or cross-reactions. Evidence was presented (Tables 2-7 and 2-8) that ovine sera at dilution of 1:24 were not specific and those at 1:48 were shown to be significant. Again there is always the possibility that the low titres might be from animals that have been infected for some time and the low titres were residual titres.

All the *australis* titres obtained in this survey were at dilution of 1:24 or less suggesting that the titres were either cross-reactions due to the other serovars or just non-specific titres. Otherwise, there would bound to be a few titres greater than 1:24. Since there were no *australis* titres higher than 1:24, we can take that the *australis* titres were non-specific titres.

If we consider titres at dilution of 1:24 as non-specific titres as in the case of serovar *australis*, then we have to consider the titres of 1:24 to the other antigens as non-specific too. Taking serovar *ballum* for example, on omitting titres of 1:24, the prevalence to this serovar will be 2.7 percent instead of 5.9 percent. The prevalence of serovar *ballum* infection in cattle in New Zealand is four percent (Hellstrom, 1978) and considering the chances of cattle contracting *ballum* infection the same or higher than sheep, we would expect the prevalence to serovar *ballum* in sheep to be the same or lower than that of cattle. This is true consistent with these present findings if one omits titres to serovar *ballum* at dilution of 1:24, otherwise, the prevalence of serovar *ballum* in sheep is much higher than that of cattle.

The distribution of titres in the different age groups (lambs, hoggets and ewes) and the geometric mean titre (GMT), (Tables 2-9 and 2-10), indicates that infections were picked

TABLE 2-9: THE PREVALENCE OF LEPTOSPIRAL INFECTION IN THE THREE AGE GROUPS OF SHEEP (Percentage of the animals sero-positive)

	Lambs	Hoggets	Ewes	Overall
hardjo	3.1	23.6	32.1	19.6
balcanica	2.9	23.2	36.4	20.8
pomona	4.0	9.1	9.7	7.7
tarassovi	6.2	3.7	11.4	7.1
copenhageni	3.7	4.0	5.2	4.3
ballum	3.4	10.1	4.5	5.9
australis	1.6	4.4	1.3	2.5
Overall				37.8

TABLE 2-10: THE GEOMETRIC MEAN TITRE (GMT) OF LEPTOSPIRAL INFECTION IN THE THREE AGE GROUPS OF SHEEP

	Lambs	Hoggets	Ewes
hardjo	312*	200*	115*
balcanica	566	312	230
pomona	53	37	43
tarassovi	32	48	34
copenhageni	57	57	24
ballum	80	35	42
australis	24	24	24

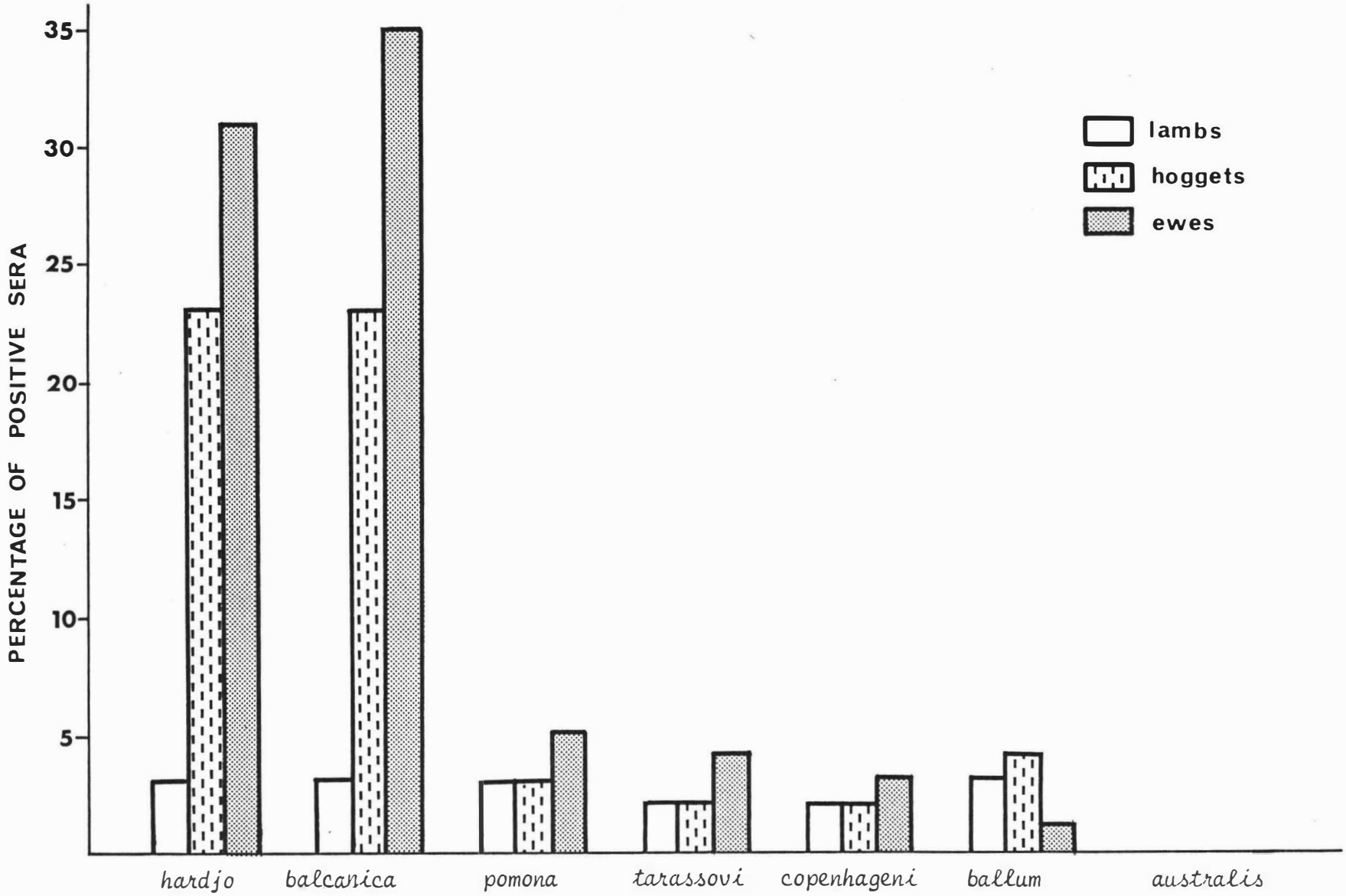
* Reciprocal of titres

up mainly in the hogget age group, that is, in those animals around eight months to two years of age. A large proportion of the lambs in general were found to be negative. A similar observation was made by Zaharija (1979) and in his survey, all the lambs examined were negative to leptospiral infection. It is hypothesised that when maternal antibodies to leptospires were present in the colostrum, the lambs were able to resist leptospiral infection but when these maternal antibodies declined and the lambs were weaned, the lambs were then susceptible to infection. Thus, we expect low number of infected lambs. This is supported by the fact that there is a high GMT in lambs and it decreases progressively through the hogget and ewe age groups respectively. High titre occurred in the young animals soon after they became infected (Hellstrom and Blackmore, 1980). Conversely, there is a rise in the serological prevalence from the lamb age group to the hogget and ewe age groups. This is very obvious in the case of serovar *hardjo* infection, from 3.1 percent serological prevalence in the lambs to 23.6 percent in the hoggets and on to 32.1 percent in the ewes (Figure 2-3).

However, there are several reports (Hartley, 1952), (Davidson and Hirsh, 1980) of outbreaks of leptospiral infection in lambs but in nearly all the cases, the serovar affecting the animals was serovar *pomona*. This serovar is not endemic in sheep and as such, ewes are not naturally immunized and hence the lambs do not get the benefit of protection through the colostrum. On the other hand, serovar *hardjo* is commonly found in cattle and sheep in New Zealand and therefore the lambs are protected through maternal antibodies in the colostrum. Unweaned lambs are therefore protected to some extent from infection by this passive immunization.

The prevalence of leptospiral antibodies in sheep as indicated by these serological studies suggests that the infection is quite significant but of comparatively lesser importance than that of cattle and swine. This difference among classes of livestock is difficult to explain. One

FIGURE 2-3: Distribution of titres to the antigens tested according to the different age groups of sheep (n = 928). (Titres ≥ 1:48)



possibility is that sheep might be more resistant than the other species of farm animals. However, evidence available seems to be contradictory. It appears something other than natural resistance is involved as to why sheep seems to be affected less often than cattle and pigs.

SUMMARY

A serological survey employing the microscopic agglutination test for leptospiral infection was conducted on 928 sheep sera from 42 randomly chosen flocks of the southern half of the North Island of New Zealand. The following information was gained from this survey:

1. An overall reactor rate of 37.8 percent was demonstrated among the sera. The prevalence of leptospiral infection among the flocks ranged from zero to 94.1 percent and indicated that sheep is quite commonly affected.

2. At dilution of 1:24, reactions were obtained with all the seven serovars employed as antigens but at 1:48, no titres were obtained with serovar *australis*.

3. The most common reaction obtained was to serovar *hardjo*. Reactions to the other serovars were observed in sera of less than 18.2 percent of the sheep tested. Sixty-two percent of the sheep did not react to any antigens The high prevalence of serological reactions to serovar *hardjo* coupled with the large percentage of the flocks with at least some reactors indicate that there is widespread exposure of the sheep population to serovar *hardjo*. The significance of the reactions to the remaining serovars is unclear. Except for serovar *pomona*, the other serovars have not yet been isolated from sheep in New Zealand and do not appear to be associated with clinical disease.

4. Evidence was presented that ovine sera at dilution of 1:24 were not specific and those at 1:48 were shown to be significant.

5. Low prevalence of infection in the lambs but with a high geometric mean titre suggest that infection is picked up mainly by hoggets. The percentage of infected animals is considered to depend on their susceptibility (age), their behaviour and husbandry and to a great extent, on the intensity of natural foci in the region.

6. Practically all the flocks had evidence of infection and therefore, it is unfounded to consider that sheep as a species are relatively resistant to leptospiral infection.

CHAPTER THREE

THE CULTURAL PREVALENCE IN SHEEP ON NATURALLY-OCCURRING LEPTOSPIRAL INFECTION

Six serovars have been successfully isolated from sheep from various countries (Table 3-1). Andreani *et al*, (1974) in Italy were unable to demonstrate any leptospiral organisms from sheep with apparently high titres to serovar *hardjo*, in an outbreak of leptospirosis. Similarly, Beamer *et al*, (1953) and Davidson and Hirsh (1980) were not able to isolate or demonstrate any leptospires from sheep in the outbreaks occurring in the United States. In Israel, van der Hoeden (1953) showed greyish spots in the kidneys of sheep with high leptospiral titres. Interstitial nephritis and pericholangitis were evident histologically but no leptospires were seen. Cultures set up remained sterile.

Hodges (1974) was not able to observe any leptospires in urine samples in his experimentally infected sheep with serovar *pomona*. This failure to detect leptospiruria in sheep by darkfield microscopy was shared by Beamer *et al*, (1953), Morse *et al*, (1957) and Lindqvist *et al*, (1958) but contrasts with the experiences of Hartley (1952) and Webster and Reynolds (1955). Dozsa and Sahu (1970) demonstrated only small numbers of leptospires in the kidneys of experimentally infected sheep, using the Levaditi stain or Warthin-Starry stain. There have been many instances that leptospires could be demonstrated from tissues of known infected sheep (Smith *et al*, 1970).

On the other hand, Hakioglu (1956) in Turkey was able to identify leptospires in sections of kidneys from sheep with jaundice and haemoglobinuria. The isolation of leptospires from the testis and epididymis of rams by Smith *et al*, (1960) suggested that venereal transmission of leptospiral organisms may occur in sheep.

TABLE 3-1: LEPTOSPIRAL SEROVARS THAT HAVE BEEN ISOLATED FROM SHEEP OVERSEAS

SEROGROUP	SEROVAR	COUNTRY	YEAR ISOLATED	REFERENCE
Ballum	*ballum*	Argentina	1963	Cacchione *et al*, (1963)
Javanica	?	Phillippines	1966	ANON (1966)
Grippotyphosa	*grippotyphosa*	Iran	1957	Rafyi & Maghami (1957)
	"	Kenya	1958	Burdin *et al*, (1958)
Pomona	*pomona*	Australia	1953	Seddon (1953)
	"	Bulgaria	1959	Mitov & Yankov (1959)
	"	Hungary	1960	Bokori *et al*, (1960)
Tarassovi	*tarassovi*	U.S.S.R.	1965	Semenova & Solosenko (1965)
Hebdomadis	*hardjo*	Australia	1980	Gordon (1980)

A recent report (Gordon, 1980) recorded the detection of leptospires by darkfield microscopy in the urine of sheep in Australia. Isolation of leptospires were made from the kidneys of sheep, but cultures of liver were negative. The isolates were later identified as serovar *hardjo*.

In New Zealand, outbreaks of leptospirosis in sheep have been reported on a number of occassions (Hartley, 1952), (Salisbury, 1954), (Webster and Reynolds, 1955) and in all these outbreaks, serover *pomona* was incriminated as the causal organisms. Salisbury (1954) and Webster and Reynolds (1955) managed to isolate the leptospiral organisms belonging to serovar *pomona* from those infected sheep. No other serovar has been isolated from sheep in New Zealand. Attempts to isolate leptospiral organisms from sheep have been made by Blackmore *et al*, (1976) and Ris (1975), but none were successful.

Up to the present time seven leptospiral serovars have been isolated in New Zealand (Table 3-2). It is only recently that serovar *australis* has been isolated and this was from a human patient (Thompson, 1980). In spite of efforts to find a probably source of infection, there has not been any serological or cultural reports of its presence in New Zealand in either man or animals. Out of the seven serovars reported to have been isolated in New Zealand, only six are known to be endemic in either domestic animals or wildlife. It is interesting to note that only serovars *balcanica*, *copenhageni* and *ballum* have as their maintenance hosts species of wildlife (Table 3-3).

Three serovars are endemic in domestic animals in New Zealand. These are serovars *hardjo*, *pomona* and *tarassovi*. Serovar *hardjo* is maintained by cattle whilst serovars *pomona* and *tarassovi* are maintained by pigs (Blackmore, 1979), (Hellstrom, 1980). Sheep have been incriminated as secondary hosts for serovars *pomona* and *hardjo* (Hellstrom, 1980). As

TABLE 3-2: LEPTOSPIRAL SEROVARS THAT HAVE BEEN ISOLATED IN NEW ZEALAND

SEROGROUPS	SEROVARS	ANIMAL HOSTS	REFERENCE
Pomona	*pomona*	cattle	a. ANON (1951)
			b. Salisbury (1954)
		dogs	a. Te Punga & Bishop (1953)
			b. Mackintosh *et al*, (1980)
		sheep	a. ANON (1951)
			b. Webster & Reynolds (1955)
			c. Salisbury (1954)
		pigs	a. deJong & Fowler (1968)
		cats	a. Harkness *et al*, (1970)
		man	a. Kirschner *et al*, (1952)
			b. Till (1968)
Ballum	*ballum*	cattle	a. Ris *et al*, (1973)
Ictero-haemorrhagiae	*copenhageni*	cattle	a. deJong & Fowler (1968)
			b. Ris *et al*, (1973)
	ictero-haemorrhagiae(?)	cattle	a. Dodd & Brakenridge (1960)
		man	a. Kirschner (1966)
		Norway rats (*Rattus norvegicus*)	a. Kirschner & Gray (1951)
Biflexa	*miranda*	stagnant water	a. Ris & Fowler (1968)
Tarassovi	*tarassovi*	pigs	a. Ryan & Marshall (1976)
		man	a. Till (1977)
		dogs	a. Mackintosh *et al*, (1980)

Cont'd..

TABLE 3-2: CONTINUED

SEROGROUPS	SEROVARS	ANIMAL HOSTS	REFERENCE
Australis	*australis*	man	a. Thompson (1980)
Hebdomadis	*hardjo*	man	a. Christmas *et al*, (1974)
		cattle	a. Lake (1973)
		sheep	a. Bahaman *et al*, (1980)
		goats	a. Schollum & Blackmore (1981)
	?	possums (*Trichosurus vulpecula*)	a. Brockie (1975)
	balcanica	possums (*Trichosurus vulpecula*)	a. Marshall *et al*, (1976)
		goats	a. Schollum & Blackmore (1981)
		cattle	a. Mackintosh *et al* (1980)

TABLE 3-3: MAINTENANCE AND SECONDARY HOSTS FOR LEPTOSPIRES IN NEW ZEALAND

SEROVARS	MAINTENANCE HOSTS	SECONDARY HOSTS
hardjo	cattle	sheep, goats, horses, man.
balcanica	possums (*Trichosurus vulpecula*)	cattle, goats
pomona	pigs	cattle, sheep, cats, dogs, horses, man
tarassovi	pigs	cattle, dogs, man
ballum	mice (*Mus musculus*)	cattle, cats, brown rats, man
	black rats (*Rattus rattus*)	
	hedgehogs (*Eranaceus europeus*)	
copenhageni	brown rats (*Rattus norvegicus*)	cattle, pigs, dogs, man

mentioned earlier, serovar *pomona* is the only one so far that has been isolated from sheep. Prior to this study, serovar *hardjo* had not been isolated from sheep, but there was serological evidence to suggest that *hardjo* infection in sheep was quite common in some areas of New Zealand (Hathaway and Marshall, 1979), (Marshall *et al*, 1979), (Ris, 1975).

AIMS

The main aim of this survey is to determine the cultural prevalence of sheep to naturally occurring leptospiral infection. The survey has several objectives, namely:

1. To find out the overall cultural prevalence of sheep to leptospiral infection.

2. To find out which age groups of sheep are shedding leptospiral organisms in their urine.

3. To determine the distribution of leptospiral organisms in the various flocks of sheep surveyed.

4. To correlate the cultural and the serological prevalence of sheep to leptospiral infection.

5. To find out when the animals acquired their leptospiral infection.

6. To determine the possibility of sheep as a maintenance host for leptospiral organisms.

METHODS AND MATERIALS

Collection of samples

Blood samples were collected from the first twenty sheep in each line of at least 100 animals at the Freezing Works, the identity of the "slaughtering chain" and the "line" or flock of animals were noted. The numbers were labelled on tags

placed on the first two animals in the line and this facilitated the identification of the carcases for the recovery of the kidneys. The carcases from which the blood samples were taken were then traced at the end of the slaughtering chain and 180 kidneys from twelve flocks were obtained from the sheep sent to the two Freezing Works. These twelve flocks, as mentioned in Chapter Two, were randomly selected and originated from various places in the southern half of the North Island.

Cultural procedure

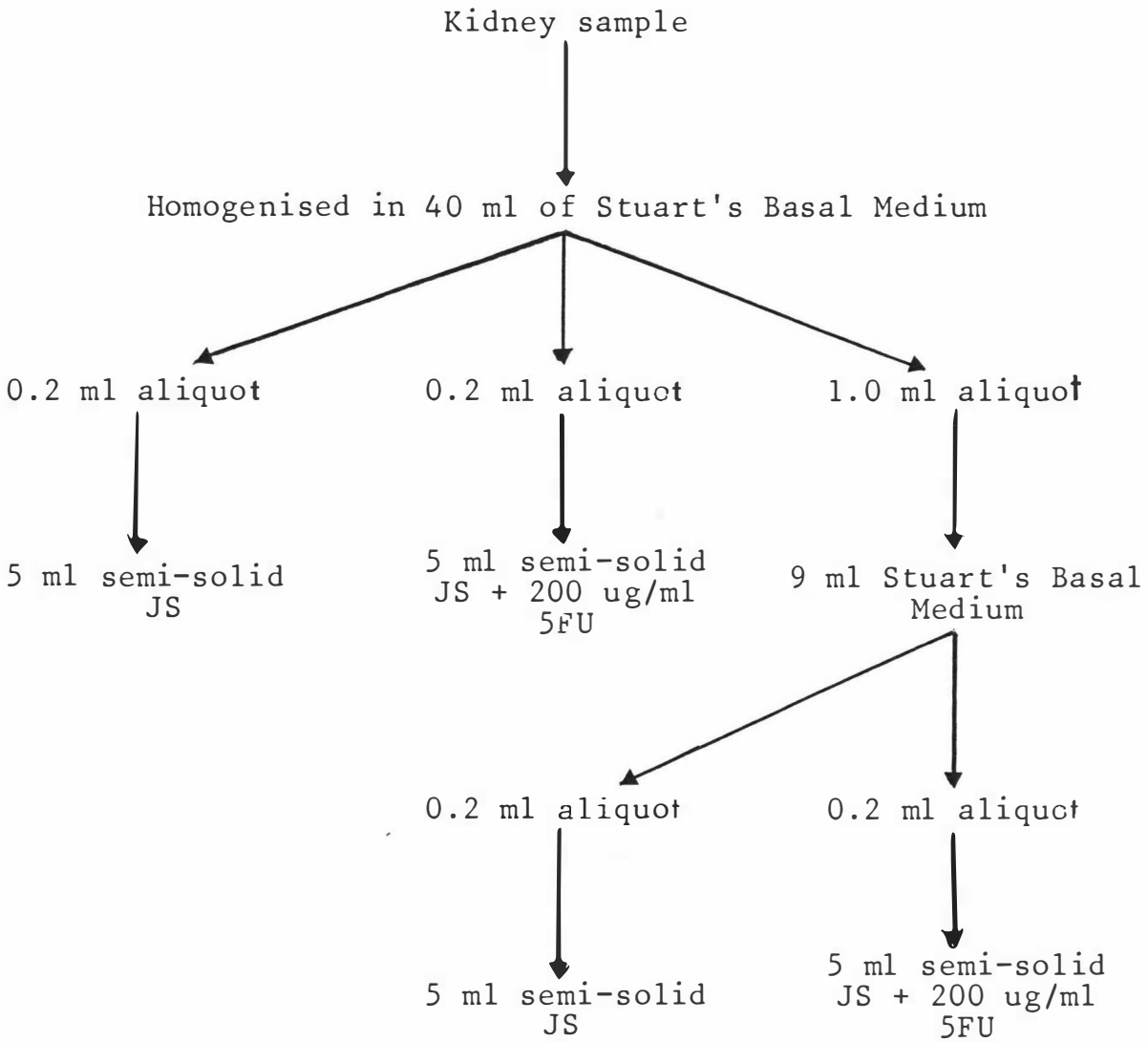
Within four hours of the animals being slaughtered, the kidney samples were processed. An approximately three cubic centimetre of each kidney were cut up aseptically, rinsed with alcohol in a funnel and then placed in a gamma-sterilised polythene bag containing 40 ml of sterile Stuart's Basal Medium (BBM)**.

A portion of kidney was taken from the cortex as the usual site of leptospires is the proximal convoluted tubules of the kidney. It was then homogenised with the aid of a stomacher (Colworth 400)*, until it was completely mascerated. A 0.2 ml aliquout of the homogenate was cultured in 5 ml of semi-solid JS (Difco) Medium**. Another 0.2 ml aliquot was cultured in the same medium but with the addition of 200 ug/ml of 5-flurouracil (5FU)**. A ten-fold dilution of the homogenate was made in 10 ml bijoux bottles containing sterile Stuart's Basal Medium. Further 0.2 ml aliquots of the mixture were sub-cultured into JS media with and without 5FU (Figure 3-1). This made a total of four cultures for every kidney sampled.

The bottles of kidney cultures were then incubated at 30°C for twelve weeks. The effect of temperature upon leptospiral growth in vitro is poorly understood. Replication of leptospires in most hosts takes place at 37°C or higher

* Appendix I
** Appendix II

FIGURE 3-1: THE PROCEDURE INVOLVED IN THE CULTURING OF KIDNEY SAMPLES



and yet for efficient laboratory propagation, they prefer incubation at 30°C. The cultures were examined by darkfield microscopy weekly from the second to the fourth week and then fortnightly until the twelfth week. If no leptospiral organisms were observed by the twelfth week, then that culture was considered to be negative and discarded. Any isolates obtained were subcultured into a liquid JS medium and passaged weekly into fresh bottles of liquid JS until pure and dense (generally in the order of 1×10^8 leptospiral organisms per ml) cultures were obtained.

Isolates obtained in this study were identified as to their serogroup using the microscopic agglutination test (MAT) and a battery of twelve hyperimmune sera: *hardjo*, *ballum*, *canicola*, *tarassovi*, *copenhageni*, *australis*, *pomona*, *bataviae*, *autumnalis*, *pyrogenes*, *grippotyphosa* and *balcanica* (Table 3-4). These antisera were obtained from the Center for Disease Control, Atlanta, Georgia, U.S.A.

The isolates were inoculated into laboratory rabbits to obtain rabbit anti-isolate hyperimmune sera using the method of Tan (1970), (Appendix V). The isolates together with their rabbit derived hyperimmune sera were sent to the WHO Leptospira Reference Laboratory, Center for Disease Control for serovar identification by cross agglutinin-absorption tests. The isolates were also typed by restriction endonuclease analysis, a method developed by Marshall *et al*, (1981).

RESULTS

In this present survey, out of the 180 kidneys cultured, only three had leptospiral organisms. This gives a cultural prevalence of less than two percent of the sheep. Altogether twelve flocks of sheep were examined, four of the samples were made up of lambs, another four were hoggets and the others were ewes. All three isolates were from one

TABLE 3-4: SEROTYPING OF THE ISOLATES USING RABBIT HYPERIMMUNE SERA

HYPERIMMUNE SERA	ISOLATES		
	S5-7	S5-9	S5-10
hardjo	3072*	1536	3072
ballum	48	-	-
canicola	24	-	-
tarassovi	-	-	-
copenhageni	-	-	-
australis	-	-	-
pomona	-	-	-
bataviae	-	-	-
autumnalis	-	-	-
pyrogenes	24	48	-
grippotyphosa	96	48	-
balcanica	3072	1536	1536

* Reciprocal of titre

of the hogget flocks. This gives a culture-positive prevalence of five percent for animals of this age group (Table 3-5).

Three of the fifteen (twenty percent) kidneys from this particular flock of sheep had leptospiral organisms. The three isolates were initially serotyped using hyperimmune sera which were supplied by Center for Disease Control, United States (Table 3-4). They indicated that all three isolates belonged to the Hebdomadis serogroup. During serotyping using the hyperimmune sera, titres were also obtained to a few of the other sera, but these were low and were therefore considered as cross-reactions.

TABLE 3-5: PERCENTAGE OF THE KIDNEYS EXAMINED CULTURALLY POSITIVE TO LEPTOSPIRAL INFECTION.

SOURCE	NO. OF KIDNEYS EXAMINED	POSITIVES	PERCENTAGE POSITIVE
Lambs (4 flocks)	60 kidneys	0	0
Hoggets (4 flocks)	60 kidneys	3	5
Ewes (4 flocks)	60 kidneys	0	0
Total (12 flocks)	180 kidneys	3	1.7

Rabbit hyperimmune sera were prepared against each isolate (Appendix V) and these hyperimmune sera together with cultures of the strains isolated, were sent to the WHO Leptospira Reference Laboratory, Center for Disease Control, U.S.A. for serovar identification using the cross agglutinin-absorption tests. The tests were performed by Ms K. Sulzer and the isolates were shown to be *Leptospira*

interrogans serovar *hardjo*. These are believed to be the first isolations of serovar *hardjo* from naturally infected sheep in New Zealand.

As a matter of interest, the isolates were also serotyped by the restriction endonuclease analysis, a method developed by Marshall *et al*, (1981). Essentially, the method consists of extraction of DNA from a homogenous population of leptospires, digestion of the DNA with a restriction endonuclease and electrophoresis of the digested DNA in an agarose gel. By this method, it was shown that all three isolates were *Leptospira interrogans* serovar *hardjo* (Figures 3-2 and 3-3).

Of the three isolates, only one of them (5-7) originated from an animal with a titre to the *hardjo* antigen. This animal had a titre of 1:1536 to serovar *hardjo* and 1:3072 to serovar *balcanica*. This is indicative of current or recent leptospiral infection. The serum cross-reacted with serovar *pomona* antigen (1:24) and serovar *ballum* antigen (1:48) (Table 3-6). The other two isolates originated from animals with no titres to serovar *hardjo*. The two isolates either came from animals infected much earlier but having persistent renal infection or from animals with very recent infection in which titres had not yet been established. It is more likely that the two animals were recently infected and that their titres were not established yet. As discussed in Chater Two, infection generally occurred within the hoggets and it is expected that if there are any isolates, they would be from the hoggets as in these cases.

The isolates were avirulent to hamsters as they did not kill weanling hamsters within three weeks. The sera obtained from these hamsters gave titres of 1:192 to 1:768 to serovar *hardjo* (Table 3-7). All kidneys from the hamsters were culturally positive for leptospiral organisms.

FIGURE 3-2: Restriction endonuclease analysis of one (S5-7) of the isolates and the seven serovars that have been isolated in New Zealand. From left to right: *lambda*, *ballum*, sheep *hardjo* isolate (S5-7) *hardjoprajitno*, *balcanica*, *copenhageni*, *australis*, *tarassovi* and *pomona*.

TABLE 3-6: THE SEROLOGICAL AND CULTURAL PREVALENCE OF SHEEP IN FLOCK NUMBER FIVE

ANIMAL ID	SEROLOGY							CULTURES
	hardjo	*balcanica*	*pomona*	*tarassovi*	*ballum*	*copenhageni*	*australis*	
S5-1	-	-	-	-	-	-	-	-
S5-2	-	-	-	-	-	-	-	-
S5-3	-	-	-	-	-	-	-	-
S5-4	-	-	-	-	-	-	-	-
S5-5	-	-	-	-	-	-	-	-
S5-6	-	-	-	-	-	-	-	-
S5-7	1536*	3072	24	-	48	-	24	+
S5-8	-	-	-	48	-	-	-	-
S5-9	-	-	-	-	-	-	-	+
S5-10	-	192	24	-	-	-	-	+
S5-11	48	48	-	-	-	-	-	-
S5-12	-	-	-	-	-	-	-	-
S5-13	-	192	-	-	-	-	-	-
S5-14	-	-	-	-	-	-	-	-
S5-15	-	-	-	-	-	-	-	-
S5-16	-	-	-	-	-	-	-	ND
S5-17	-	-	-	-	-	-	-	ND
S5-18	-	-	-	-	-	-	-	ND
S5-19	-	-	-	-	-	-	-	ND
S5-20	-	-	-	-	-	-	-	ND

* Reciprocal of titre

ND Not done

FIGURE 3-3: Restriction endonuclease analysis of the three sheep *hardjo* isolates and five different serovars of the Hebdomadis serogroup. From left to right: *lambda*, *haemolytica*, *medanensis*, *szwajizak*, *hebdomadis*, *kremastos*, sheep *hardjo* isolates *S5-7*, *S5-9* and *S5-10*.

TABLE 3-7: THE SEROLOGICAL AND CULTURAL STUDIES OF HAMSTERS BEING INFECTED WITH THE OVINE HARDJO ISOLATES

SEROLOGY

HAMSTER ANTI-ISOLATE SERA		ANTIGENS						
S5-7	a.	3844*	384	96	-	-	-	-
	b.	192	192	-	-	-	-	-
S5-9	a.	768	768	-	-	-	-	-
	b.	192	192	-	-	-	-	-
S5-10	a.	384	384	-	-	-	-	-
	b.	384	48	-	-	-	-	-

* Reciprocal of titre

CULTURES

(Hamster kidneys in JS media).

		WEEK OF INCUBATION				
		1st	2nd	3rd	4th	5th
S5-7	a.	-	+	+	+	+
	b.	-	+	+	+	+
S5-9	a.	-	-	-	+	+
	b.	-	-	+	+	+
S5-10	a.	-	+	+	+	+
	b.	-	-	+	+	+

\+ Positive for leptospiral organisms

The number of culture-positives is too small to deduce a correlation between serological and cultural prevalence of sheep to leptospiral infection. In the flock (S5) from where the isolates originated, the serological prevalence of sheep to *hardjo* infection is ten percent whilst the cultural prevalance of the same animals to serovar *hardjo* is twenty percent.

DISCUSSION

Leptospires are slow-growing organisms having a generation time of approximately 24 hours at 30^{o}C (Johnson and Rogers, 1964). The combination of this slow growth and the requirement for a rich medium at a neutral pH, predisposes the cultivation of leptospires to problems of contamination. This is particularly true when attempting to isolate leptospires from natural sources. Various methods have been developed to detect leptospiral infection but cultural propagation is still the most reliable method available. There are however, factors which may affect the success rate of the culture procedure. Some of these are listed below:

a) fastidious requirements of the leptospiral organisms,

b) the small numbers of organisms in the tissue or urine samples,

c) the toxic effects of tissue or urine components on the leptospires, and

d) cultural contamination from other organisms in the samples.

In this survey, 3.8 (27/720) percent of the cultures were contaminated, but none of the kidneys were discounted from the survey. If more than two of the four cultures of any kidney sample are contaminated then that kidney is discounted from the survey.

The results from this cultural examination showed that serological tests, such as the MAT, are by themselves not sufficient to confirm a leptospiral infection. As in these cases, two of the animals were having *hardjo* organisms in their kidneys had no detectable titres to serovar *hardjo*. This also indicates the need when carrying out serological surveys to use a sufficiently low serum dilution as positive to detect any level of antibody which might indicate infection. Otherwise, infection resulting in low titres with or without renal infection can be missed and thereby give a false impression of the disease in the flocks.

Some of the bacterial contaminants and artifacts appeared very much like leptospires and can be especially confusing if they are motile or being moved by brownian movement. Leptospires are slender (0.1 x 6.0 um) organisms and are characterised by very active "spinning" flexuous motility and usually have a hooked end. (Johnson and Sieter, 1977). Initially, it is quite difficult to differentiate between the contaminants and the leptospires but after gaining experience, the leptospiral organisms can quite readily be identified by the characters described above in darkfield microscopy.

Blackmore *et al*, (1976) cultured and examined by darkfield microscopy 28 sheep kidney homogenates. No evidence of infection was seen directly although five of their cultures were found to contain organisms. Unfortunately, none of these cultures were serotyped because of their poor growth.

Previous studies failed to isolate serovar *hardjo* from sheep and this may be due in part to difficulty of isolation with the then available culture media and methodology, rather than actual absence of infection in the sheep population. It is noted that serovar *hardjo* is difficult to grow in culture, especially during the initial adaptation from their natural environment to culture. On several occassions in this survey, the isolates dwindled from a dense culture to a few organisms for no apparent reason.

The isolation of leptospires from kidneys therefore demands a medium in which growth from small inocula can be effectively initiated. As a result of extensive nutritional studies on the leptospires, media have been formulated that meet the above requirements. In our laboratory, JS medium (Appendix IV) has been found to be very suitable for the isolation of leptospires from naturally infected animals. Good results were obtained by adding the pyrimidine analoque 5-fluorouracil (5FU) to the media. This serves to control the overgrowth of many bacterial contaminants, thus enhancing the establishment of the leptospires in the culture medium.

As hypothesised in Chapter Two, infection is picked up by the animals generally after the age of six months, that is, after the animals are weaned and deprived of passive protection through ingestion of colostrum. Carter and Cordes (1980) reported a similar finding in which infected female rats were unable to infect their young and one reason given was because of colostral antibodies conferring passive protection of long duration. In this cultural survey, as expected, the three isolates were isolated from hoggets. The pathogenesis of leptospiral infection appears to be that infected animals first become leptospiraemic, then developed titres to the organisms, the organisms become established in the kidneys and consequently, the infected animals become leptospiruria.

It has been shown by Webster and Reynolds (1955) and Morse (1957) that leptospiruria in sheep due to serovar *pomona* lasts approximately two months. Serovar *hardjo* is less virulent to sheep than serovar *pomona*, it is therefore postulated that serovar *hardjo* is better adapted to sheep than serovar *pomona* and consequently, the leptospiruria would be much longer. The ability to develop the renal carrier state is considered to represent a high degree of adaptation of host and parasite to one another. The length and intensity of leptospiruria in sheep has to be established and until then, the role of sheep in the epidemiology of

leptospirosis cannot be defined.

Kidney infection and leptospiruria in experimentally infected sheep due to serovar *hardjo* have been shown by Hathaway and Marshall (1979) to be readily established. However, Sullivan (1952) in Australia showed only a transient leptospiruria in his experimentally infected sheep with serovar *hardjo*. It has been noted that serovar *hardjo* has a surprisingly restricted host range and infection is virtually limited to cattle with occassional accidental transmission to man. Currently, the only evidence of *hardjo* infection of feral animals is a sporadic incidence in feral goats (Schollum and Blackmore, 1981).

Serovar *hardjo* has been frequently reported in cattle from many parts of the world (Milner *et al*, 1980), (Mitchell *et al*, 1960), (Gordon 1979), (Hussain *et al*, 1978) and recently, the isolation of serovar *hardjo* organisms from sheep was reported in Australia (Gordon, 1980). In New Zealand, the isolation of serovar *hardjo* has only been reported from cattle (Lake, 1973), man (Christmas *et al* 1974) and feral goats (Schollum and Blackmore, 1981). Although serological evidence of infection has been recorded in sheep (Ris, 1975), it is only in this present survey that serovar *hardjo* organisms have been conclusively demonstrated from sheep in New Zealand.

This cultural survey confirmed the presence of serovar *hardjo* infection in sheep in New Zealand and has drawn attention to the possibility of sheep as a maintenance host for serovar *hardjo* and to the possible role of sheep in the epidemiology of the infection.

The isolation of serovar *hardjo* from sheep and the demonstration of a high prevalence of sheep with leptospiral titres suggest that leptospiral infection may be more common in sheep in New Zealand than has been suspected. It indicates that sheep may constitute a potential source of

infection for other animals and man. The relative importance of sheep as a maintenance host or short term carrier of leptospires has yet to be investigated.

The small number of isolates obtained indicates that there was a poor correlation between infection and MAT titres. The ratio between culture-positive and seropositive animals is approximately 1:10. The cultural prevalence of sheep to *hardjo*infection was 1.7 percent whilst the serological prevalence was 19.6 percent. In this present survey, it is suspected that the two sheep with renal infection but which had no serological titres had just become infected and that a measurable antibody level had not yet become established.

The overall low cultural prevalence of less than two percent of the sheep in this survey to leptospiral infection may be taken as an indication that sheep are not a maintenance host for serovar *hardjo* in New Zealand. A maintenance host is defined as an animal which is capable of acting as a natural source of infection for its own species (Blackmore and Hathaway, 1980). To be a maintenance host, the animals have to demonstrate the following characters:

a) High susceptibility to infection, that is, low infective dose,

b) Low pathogenicity of the serovar for the host,

c) Long term kidney infection and subsequently, long period of leptospiruria, and

d) Natural transmission within the host species.

From this survey, therefore it is not possible to determine whether or not sheep are a maintenance host for serovar *hardjo*. The only feature which seems to be disclosed from this survey is that serovar *hardjo* appears to be of low pathogenicity for sheep. The three isolates are all derived from apparently healthy animals.

Morse *et al*, (1957) housed infected sheep in the same pens as healthy sheep, this did not result in transmission of infection to the in-contact animals. It appears that sheep generally shed fewer leptospires in their urine and often sporadically. This therefore would lessen the risk of transmission from sheep to sheep (intra-species transmission). It has been shown in one investigation by Blackmore *et al*, (1976) that sheep did not contribute to bovine outbreaks. Even if sheep to sheep transmission does occur they are unlikely to be important reservoirs because of the intermittent nature and short duration of leptospiruria (small numbers of organisms shed and small volume of urine). Cattle must still be considered the major source of *hardjo* infection because of the volume of their urine and the extent and duration of leptospiruria.

If sheep are not a maintenance host for serovar *hardjo*, then the infection in sheep is bound to be from cattle which have been proven to be a maintenance host for serovar *hardjo*. Furthermore, sheep and cattle often graze the same pastures and the possibility of inter-species transmission is easily understood.

Because of the low cultural prevalence of leptospires in sheep it can be interpreted that they are unlikely to be a maintenance host for serovar *hardjo* in New Zealand. However, the one flock from which shedding animals were found had a twenty percent cultural prevalence of sheep to leptospiral infection. This shows that under certain circumstances it is possible for a high proportion of sheep within a flock to be shedders. Therefore, given the right conditions, sheep could help to maintain *hardjo* infection.

CONCLUSION

1. This is believed to be the first report of naturally-occurring *hardjo* infection in sheep in New Zealand. It confirmed the belief (Chapter Two) that serovar *hardjo* is the major serovar affecting sheep.

2. The cultural prevalence of sheep to leptospiral infection is less than two (3/180) percent. This indicates that sheep are not a significant source of infection and thereby, not a natural maintenance host for serovar *hardjo* or any other serovars.

2. All the flocks of sheep, with one exception, were culturally negative to leptospiral infection. The one flock with positive cultures to leptospiral infection demonstrated that it is possible to get a high number of sheep within a flock shedding leptospiral organisms.

4. It was shown that hoggets were the important age group of animals shedding leptospires and it is suspected that infection is mainly picked up by animals in this age group.

5. There was a poor correlation between the cultural (two percent) and the serological (19.7 percent) prevalence of sheep to leptospiral infection on comparing the two surveys.

CHAPTER FOUR

AN EPIDEMIOLOGICAL STUDY OF LEPTOSPIROSIS DUE TO LEPTOSPIRA INTERROGANS SEROVAR HARDJO IN A PARTICULAR FLOCK OF SHEEP

Since the outbreaks of leptospirosis in sheep recorded by Hartley (1952) and Webster and Reynolds (1955) in the 1950's, there were no reports of major clinical leptospirosis in sheep in New Zealand. From 1974 to 1979, only 53 cases of leptospirosis were recorded by the Animal Health Division's six diagnostic laboratories (Christiansen, per. comm.). Twenty two of these cases were diagnosed on clinically normal sheep whilst the rest were associated with various clinical signs. Most of the cases were diagnosed by the mere presence of serum titres to leptospires. Three serovars were serologically identified and they were serovars *pomona*, *hardjo* and *copenhageni*.

Serovar *pomona* has been recognised as the major serovar affecting sheep in New Zealand. Ris (1975) showed that the majority of sheep he examined had agglutinating antibodies to serovar *hardjo*. In Chapter Two, it was similarly shown that the majority of the seropositive sheep had positive titres to serovar *hardjo*. It could have been that serovar *hardjo* has been affecting sheep for some time in New Zealand, but because of the occult or inapparent infection by serovar *hardjo*, it was probably not diagnosed or reported.

In the preceeding chapters, it has been shown serologically and by cultural isolation that serovar *hardjo* is the serovar responsible for most of the leptospiral infection in sheep. Nearly twenty percent of the sheep examined had agglutinating antibodies to serovar *hardjo*. Agglutinating antibodies to the other six serovars made up only a small portion of the positive titres.

During investigations into the serological and cultural prevalence of sheep to leptospiral infection, three isolates of serovar *hardjo* were obtained from a flock of hoggets making the cultural prevalence of sheep in this flock to serovar *hardjo* infection, twenty percent. This flock originated from a farm in Waituna West in the Manawatu. This was the only farm having animals with positive renal infection to leptospires.

The isolation of serovar *hardjo* organisms from this flock of hoggets prompted the present investigation and it was decided to examine the farm and its sheep flock in greater details.

AIMS

The main aim of this present investigation was to examine the epidemiology of leptospiral infection in a farm where a few of the sheep have been proven to have renal infection due to serovar *hardjo* . Some of the objectives of the investigation were:

1. To examine the distribution of agglutinating antibodies among the three age groups of sheep on the farm and whether they conformed to the serological pattern observed in Chapter Two.

2. To study the dynamics of the infection in this population of sheep.

3. To assess the infection, whether it was a sporadic or an endemic one.

4. To compare the serological and cultural findings.

5. To find out whether there were any animals shedding leptospiral organisms in their urine (leptospiruric).

MATERIALS AND METHODS

The first sampling of sheep from this particular farm (Farm No S5) was done on 5 April, 1979 at the Freezing Works in Feilding. Twenty blood samples were collected in universal bottles from a line of 105 hoggets sent for slaughter at the Freezing Works. Fifteen kidneys were obtained from the carcases of the animals whose blood were sampled. Arrangement was made to collect an identified serum sample and a kidney from the same animals. The blood and kidney samples were immediately processed on arrival at the laboratory.

Twenty two blood samples were collected from another line of thirty hoggets at the Freezing Works in the second sampling on 23 July, 1979. This second line was made up of culled animals originating from the same farm.

On 25 November, 1980 blood were randomly sampled from live sheep on the farm. Urine samples were obtained from the animals by encouraging them to urinate by holding the mouth and nostrils firmly closed with the hands. This is the method advocated by Webster and Reynolds (1955).

The study farm is situated in Waituna West in the Manawatu. The farm is located in a predominantly hilly area. The drainage on the farm varied from well-drained areas to poorly-drained areas in certain parts. Varied sources of water supply were available on the farm, from troughs and dams to streams and marshes.

There were approximately 11,000 heads of sheep on the farm in June, 1979. There was no other stock except for 880 head of beef cattle. These were mature steers which usually run together with the sheep.

Altogether 249 blood samples and 95 urine samples were obtained from the sheep on the farm.

61	blood samples	and	16	urine samples	from	lambs,
48	"	"	25	"	"	hoggets,
140	"	"	54	"	"	ewes .

All sera were stored frozen until tested by the microscopic agglutination test (MAT). Appropriate aliquots (two drops) of the urine samples were inoculated into semi-solid JS media. Two concentrations of 5-fluorouracil (200 mg/ml and 400 mg/ml) were incorporated in the JS media to combat bacterial contamination. Preliminary study showed that media without 5FU were unsuitable for culturing urine for leptospiral organisms due to contamination rates that resulted from their use.

The urine samples were also examined by direct darkfield microscopy for the presence of leptospiral organisms.

RESULTS

Serum samples from the three age groups of sheep on the farm were tested for the presence of agglutinating antibodies to leptospires. Positive titres ($\geq$ 1:48) were obtained from 142 (57.0 percent) animals. Of these 142 animals, 109 (43.8 percent) had positive titres to serovar *hardjo*. Only a small number of animals had agglutinating antibodies to the other serovars (Table 4-1, Figure 4-1).

There were great differences in prevalence among the animals in the three age groups to leptospiral infection. Except for a few reactors to serovars *ballum* and *australis*, all the lambs were negative to leptospiral infection. This is in contrast to the prevalence in the hoggets and ewes (Table 4-2). Significant increase in prevalence among the animals to Hebdomadis serogroup infection from the hogget age group to the ewe age group was shown (Figure 4-2).

TABLE 4-1: DISTRIBUTION OF TITRES TO LEPTOSPIRAL ANTIGENS IN A SEROLOGICAL SURVEY OF SHEEP FROM A STUDY FARM

ANTIGENS	NEGATIVES	24	48	96	192	384	768	1536	3072	POSITIVES	PERCENTAGE POSITIVE AT 1:24	PERCENTAGE POSITIVE AT 1:48	GMT	RANGE
hardjo	107	33	52	41	13	2	1	-	-	142	57.0	43.8	1.79	1:24-1:768
balcanica	97	14	46	50	31	7	3	1	1	152	61.0	54.4	1.130	1:24-1:1536
pomona	232	12	3	2	-	-	-	-	-	17	6.8	2.0	1.37	1:24-1:96
tarassovi	238	8	3	-	-	-	-	-	-	11	4.4	1.2	1.31	1:24-1:28
copenhageni	245	1	2	-	1	-	-	-	-	4	1.6	1.2	1.78	1:24-1:192
ballum	239	7	1	1	-	1	-	-	-	10	4.0	1.2	1.70	1:34-1:38
australis	240	9	-	-	-	-	-	-	-	9	3.6	0	1.24	1:24

(Total sera examined = 249)

FIGURE 4-1: The proportion of sheep in the study farm demonstrating titres (≥1:48) to the seven leptospiral antigens.

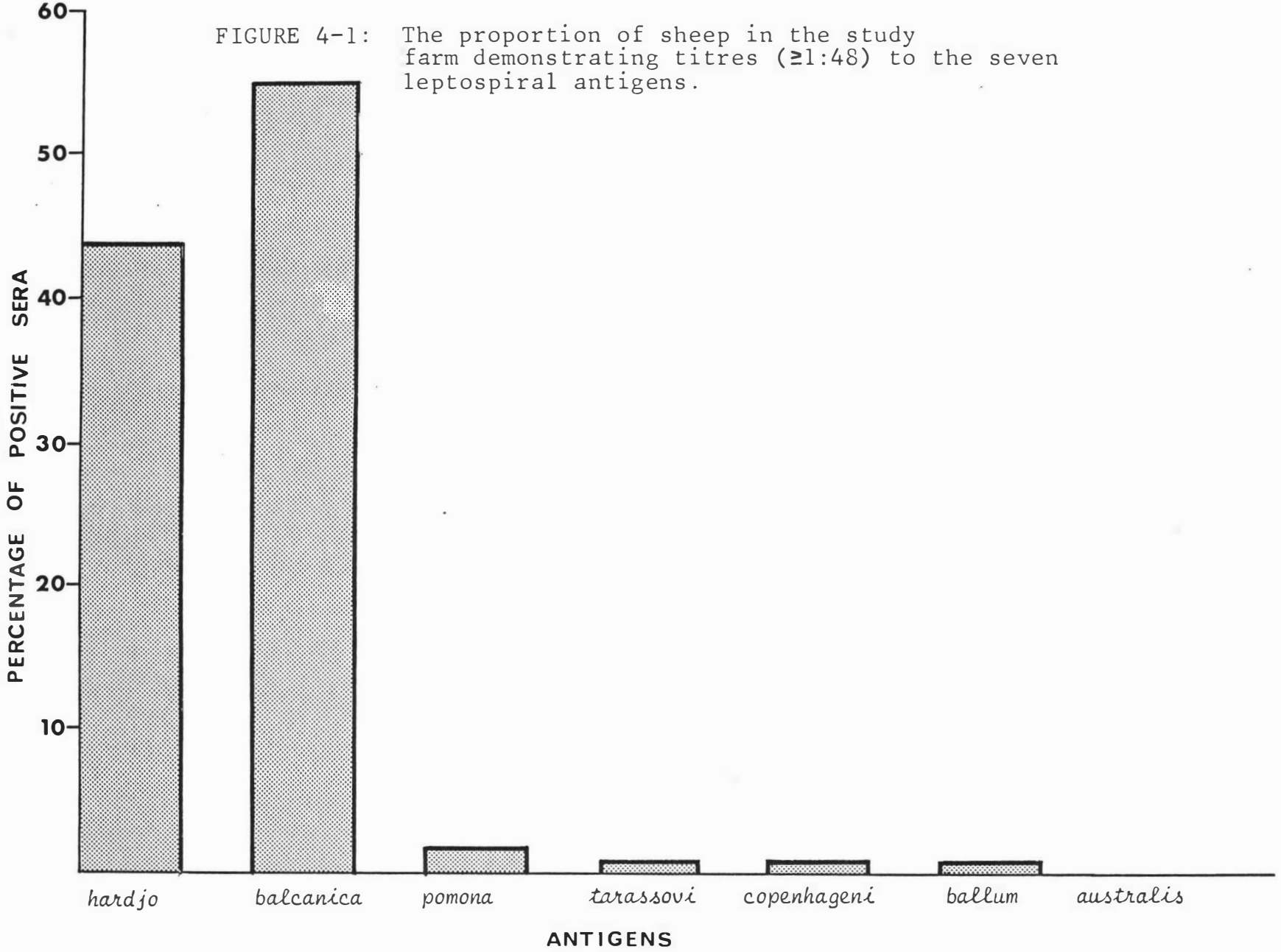
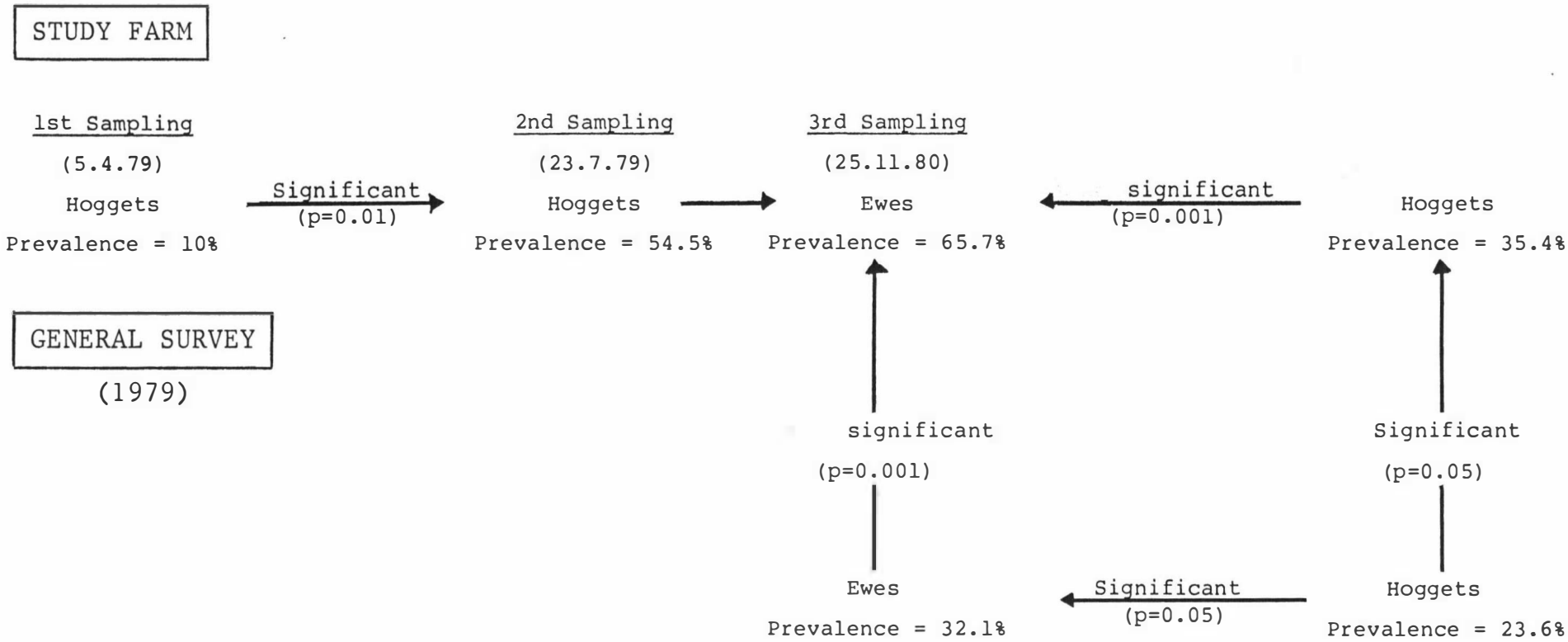


TABLE 4-2: THE PREVALENCE OF LEPTOSPIRAL INFECTION AND THE GEOMETRIC MEAN TITRES IN THE THREE AGE GROUPS OF SHEEP FROM A STUDY FARM (TITRES ≥1:48)

		hardjo	*balcanica*	*pomona*	*tarassovi*	*copenhageni*	*ballum*	*australis*
LAMBS	Prevalence	-	-	-	-	-	3.3	-
	GMT	-	-	-	-	-	1:72	-
HOGGETS	Prevalence	35.4	43.8	4.2	-	-	2.1	-
	GMT	1:164	1:293	1:72	-	-	1:384	-
EWES	Prevalence	65.7	83.6	2.1	2.1	2.1	-	-
	GMT	1:83	1:104	1:64	1:48	1:96	-	-

FIGURE 4-2: THE DYNAMICS OF THE *HARDJO* INFECTION IN THE STUDY FARM.



The overall serological prevalence of sheep from Farm No. S5 to serovar *hardjo* was as follows: -

5 April, 1979	Hoggets	10	(2/20) percent seropositive
23 July, 1979	Hoggets	54.5	(12/22) percent seropositive
25 November, 1980	Lambs	0	(0/61) percent seropositive
	Hoggets	35.4	(17/48) percent seropositive
	Ewes	65.7	(92/140) percent seropositive

None of the urine samples were positive for leptospiral organisms when examined by direct darkfield microscopy or culture propagation. This indicated that either the animals were not leptospiruric or that the number of leptospiral organisms in the urine was too low to be detected by either procedure.

DISCUSSION

There were only sporadic records of sheep with leptospiral infection in New Zealand (Christiansen, per comm.) and none where a leptospira of the Hebdomadis serogroup was isolated. Serological surveys (Ris, 1975), (Chapter Two) have revealed a relatively high number of sheep in New Zealand had agglutinating antibodies to serovar *hardjo*.

Results from the serological examination revealed a high prevalence of agglutinating antibodies to the Hebdomadis serogroup in the sheep sampled, with 109 out of the 249 sera (44 percent) positive to serovar *hardjo* antigen and 138 out of the 249 sera (55 percent) positive to serovar *balcanica* antigen (Table 4-1). The high prevalence of sheep to leptospiral infection paralleled the leptospiral infection in cattle in New Zealand. This present investigation supported the findings in Chapter Two that serovar *hardjo* infection occurred frequently in sheep in New Zealand. The high prevalence (44 percent) of sheep in this survey farm

and in the general survey to serovar *hardjo* and also the high percentage (64.4 percent) of the flocks with at least some reactors, indicated that there was widespread infection of sheep in New Zealand to serovar *hardjo*.

This present investigation showed that nearly all the lambs sampled from the survey farm were negative to leptospiral infection. As explained in Chapter Two, lambs probably had passive immunity which was derived by ingestion of colostrum and they became infected on being weaned. It was therefore expected that most of the animals infected would be from the hogget and the ewe age groups. Nearly thirty-five percent of the hoggets and sixty-six percent of the ewes from this survey farm had agglutinating antibodies to serovar *hardjo* (Table 4-2). The distribution of titres to leptospiral infection from this survey farm, on the whole, is similar to that of the general survey (Figure 4-3).

A high GMT is indicative of recent infection as titres of agglutinating antibodies to leptospiral infection fall significantly over a period of time. Comparing the distribution of the prevalence and GMT of the animals in the three age groups in the survey farm with the general survey, it was shown that animals in the survey farm were infected at hogget age. This is similarly shown in the general survey except for a few lambs which could be just nearing hogget age (Figure 4-4). It could be generalised that leptospiral infection in sheep is picked up mainly by animals in the hogget age group.

The serological results obtained from this survey farm indicated that most of the animals had agglutinating antibodies to serovar *hardjo*. If considering the ewes only, all the flocks from the general survey had reactors to serovar *hardjo* (Table 4-3). Ris (1975) in his survey also showed that all the flocks he examined had seropositive animals and often with high titres. Basing on these findings, it appears serovar *hardjo* infection in sheep in

FIGURE 4-3: The percentage of sheep positive to leptospiral infection (titres 1:48 or more).

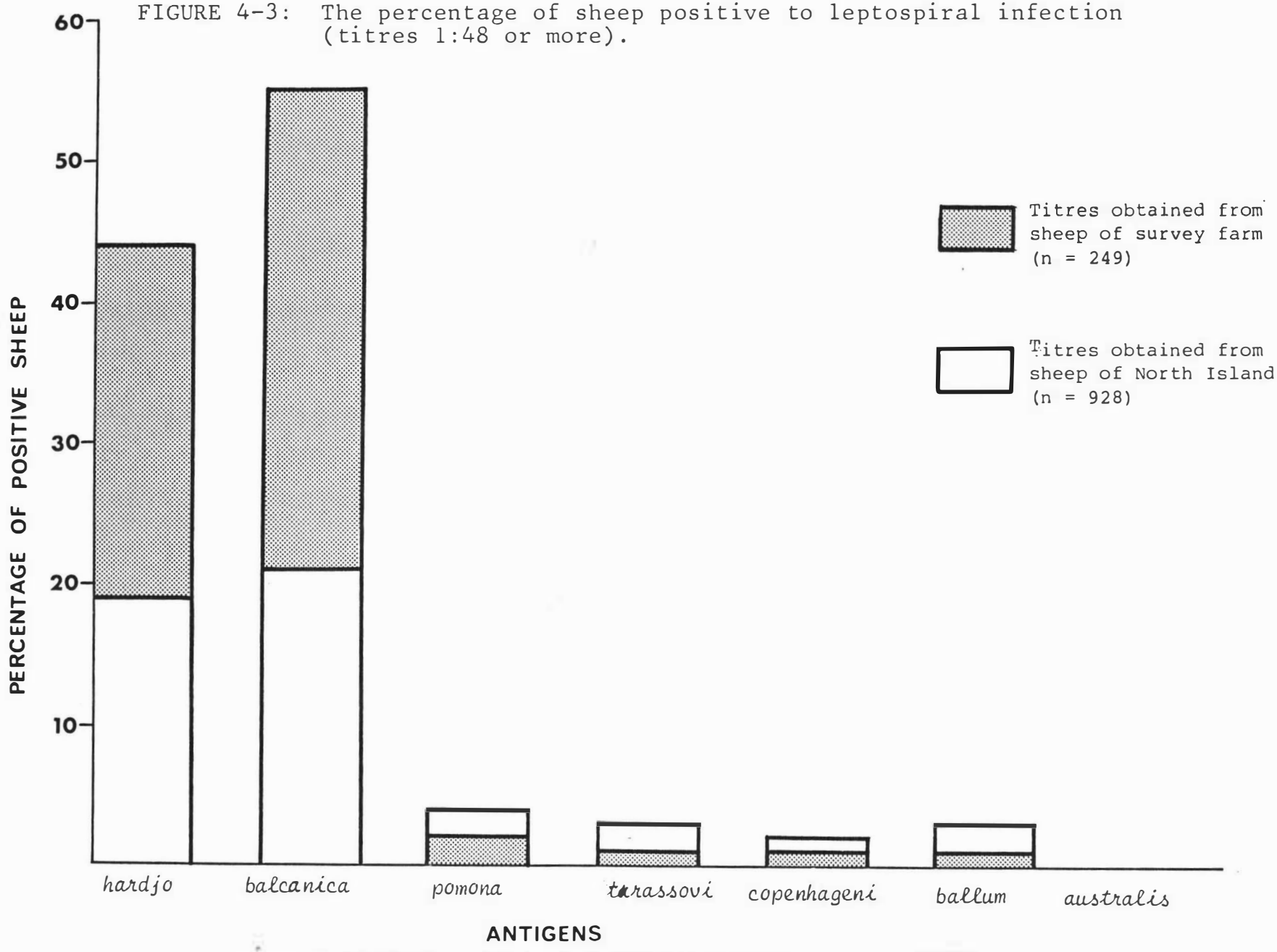
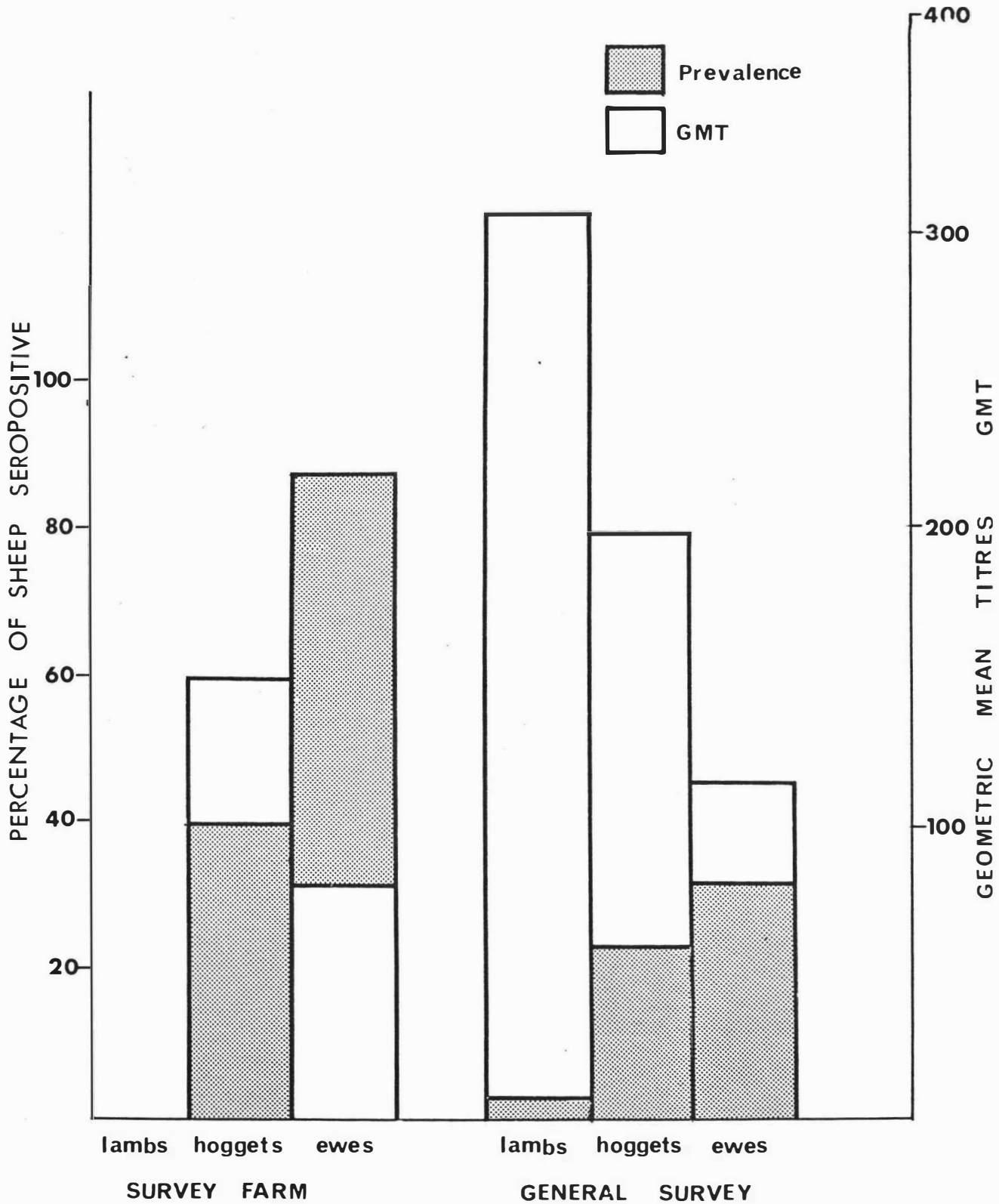


TABLE 4-3: THE PROPORTION OF FARMS HAVING SHEEP WITH TITRES (≥1:48) TO THE DIFFERENT SEROVARS

Serovar	No. of Farms with Seropositive Sheep (n = 45)	Percentage of Farms with Seropositive Sheep	No. of Groups of Sheep of Different Ages Seropositive			Range in Sero Prevalence of Infected Groups
			Lambs (n=16)	Hoggets (n=14)	Ewes (n=15)	
hardjo	29	64.4	3 (18.8)	11 (78.6)	15 (100)	4.8 - 77.3
pomona	23	51.1	5 (31.3)	8 (57.1)	10 (66.7)	4.3 - 22.2
tarassovi	13	28.9	2 (12.5)	6 (42.9)	5 (33.4)	4.5 - 21.7
copenhageni	10	22.2	2 (12.5)	3 (21.4)	5 (33.4)	4.2 - 23.8
ballum	19	42.2	7 (43.8)	8 (57.1)	4 (26.7)	4.3 - 12.5

FIGURE 4-4: The prevalence of leptospiral agglutinating antibodies to *hardjo* among the three age groups of sheep in the study farm and general survey.



New Zealand is widespread. The significance of the infection to the sheep industry is at present not known.

The distribution of titres obtained from the 249 sheep on the study farm was shown in Table 4-1, whilst Table 4-2 showed the serological prevalence in the different age groups. It was shown in Figure 4-2 that significantly higher prevalence rates occurred in the hoggets ($p = < 0.05$) and the ewes ($p = < 0.001$) on the study farm as compared with those in the general survey.

Despite the high prevalence of animals from the survey farm with titres to serovar *hardjo*, there was no bacteriological evidence in all the urine samples examined. Because of the possibility of intermittent urinary shedding of leptospires in sheep, it should be appreciated that a negative result as in this case, does not eliminate the possibility that the animals were infected. Altogether 95 urine samples were obtained from the three age groups of sheep and were cultured immediately on arrival at the laboratory. There were a few contaminated samples. The low number of contaminated samples was probably because 5-fluorouracil (5FU) was incorporated in all the cultures. Two concentrations of 5FU were applied. Even then, some bacterial contaminants managed to survive these high concentrations of 5FU.

Two of the cultures from the ewe age group had bacterial contaminants which were similar in appearance to leptospires. The contaminating organisms were slender, nearly the same length as the average leptospires and were motile. For confirmation, 0.5 ml of each culture was individually inoculated into weanling hamsters. None of the hamsters showed any symptoms of leptospiral infection and they were euthanised after three weeks post-inoculation. The kidneys were cultured and were negative for leptospiral organisms. Sera from the hamsters were negative to leptospiral infection when tested by the MAT.

No symptoms of leptospiral infection were noted among all the animals in the study farm. The cultures of kidney samples from this survey farm (Chapter Three) revealed that a high (three out of fifteen) proportion of the animals had renal infection with leptospires. As such, it was expected that some of the animals would be leptospiruric. Results from the urine cultures indicated that none of the animals were shedding leptospiral organisms in their urine. It was suspected that if the animals were leptospiruric, they must be shedding low numbers of organisms and therefore were not being detected by direct darkfield microscopy or culture propagation. Length and intensity of leptospiruria in sheep is one aspect which has received insufficient investigation. Alexander *et al*,(1971) had briefly experimentally infected sheep with serovar *hardjo*. They showed that leptospires could not be isolated from the blood or urine of these animals. All the animals, however, had evidence of infection as demonstrated by serological tests. This is the converse to the experimental investigation by Hathaway and Marshall (1979). Hathaway and Marshall (1979) showed sheep were easily infected with serovar *hardjo* and *balcanica* and leptospiruria was observed (three weeks post-inoculation) in most of the animals infected with serovar *hardjo*.

Variability in duration and persistence of leptospiruria was a feature of *hardjo* infection and there was a tendency for an intermittent excretion pattern. Experimentally infected sheep experienced only a transient leptospiruria indicating that ovine infection is unlikely to be important in the spread of the disease (Sullivan, 1976).

There is a unique feature related to leptospiral infection in sheep. Although there can be a high serological prevalence of sheep with leptospiral infection, the cultural prevalence is often negative or low (Alexander *et al*, 1971), (Ris, 1975), (Andreani *et al*, 1970). This suggests that

either;

a) sheep recover early from the renal infection,

b) they are intermittent shedders of leptospiral organisms or,

c) there are very low numbers of the leptospires in the urine.

The low prevalence of agglutinating antibodies to the other serovars among the animals on the study farm and those from the general survey indicated that infection in sheep by the other serovars is insignificant. It is believed that some of the titres were probably non-specific titres or cross-reaction titres.

The presence of agglutinating antibodies to the other serovars could be associated with:

a) subclinical or inapparent specific infection past or present,

b) cross-reaction due to the other serovars, that is, antigens shared by two or more serovars,

c) non-specific agglutinins which resulted in false positive reactions.

Hellstrom (1978) has clearly established that the maintenance host for serovar *hardjo* in New Zealand is cattle. No other animal host has been implicated as a carrier able to transmit these organisms to other animals or man. It is not known what is the source of infection for the sheep in this study farm. Cattle present on the survey farm if they were infected with serovar *hardjo* are the most likely source of infection for the sheep. It has already been shown that cattle were able to excrete leptospires for more than twelve months and in much bigger numbers of leptospires (Hellstrom, 1978), (Mackintosh, 1981). It was noticed that the cattle on the survey farm ran freely with the sheep population and hence transmission of leptospiral infection

from cattle to the sheep was likely. Cattle have been suspected as the source of leptospiral infection for sheep overseas (Bokori *et al*, 1960), (Davidson and Hirsh, 1980).

It is hypothesised that when some of the sheep became infected on contact with the cattle, the sheep in turn propagated the infection to the other sheep. Hence the infection now became self propagated within the sheep population (Figure 4-5).

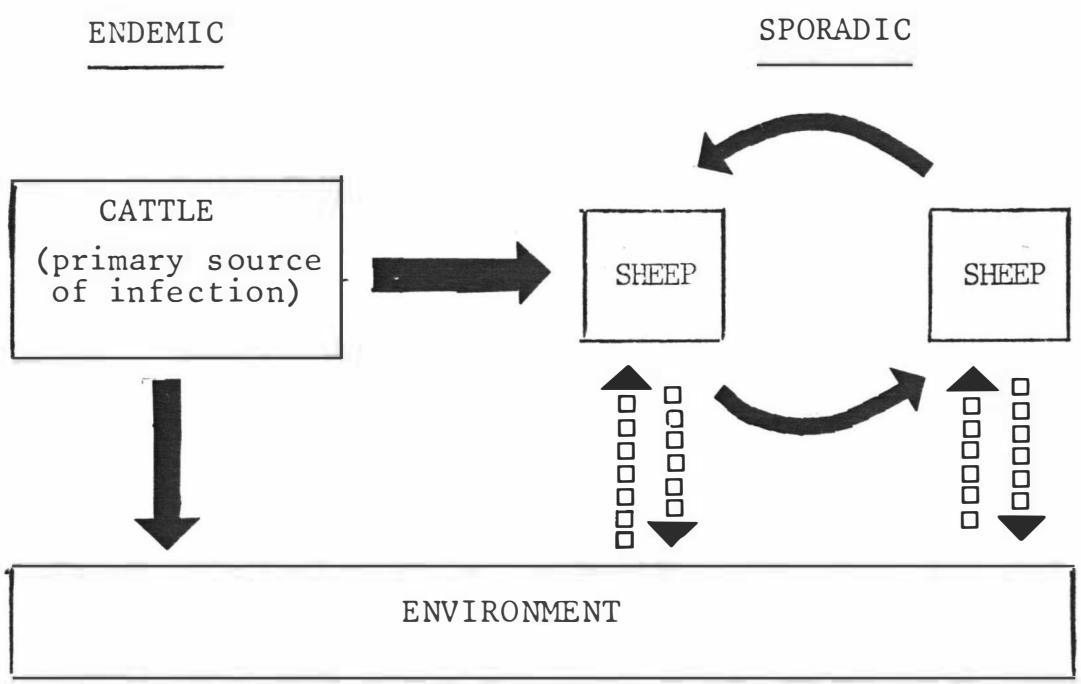


FIGURE 4-5: A HYPOTHETICAL DYNAMIC OF LEPTOSPIRAL INFECTION IN A STUDY FARM

The sheep to sheep transmission was probably responsible for the widespread occurrence of agglutinating antibodies in this sheep population. It appeared however that the infection was a self limiting epidemic.

Roth *et al*, (1963) stated that a serological rate many times greater than the kidney culture rate, suggested mild transitory infection of the type seen in an incidental host. The results obtained from this present investigation seems to fit into the category. The serological prevalence of leptospiral infection in sheep on the study farm as well as those in the general survey were shown to be high. In contrast, the leptospiral cultural prevalence in the animals, in both surveys were very low. Hellstrom (1980) has indicated that sheep in New Zealand are secondary host for serovars *hardjo* and *pomona*. It is difficult to describe the infection in this survey farm. Whether the infection was a sporadic, endemic or even epidemic one was difficult to assess. All evidence seems to relate the leptospiral infection in the survey farm to a sporadic one.

Figure 4-2 showed that there was a significant increase in the prevalence of animals having experienced leptospiral infection during subsequent samplings. There was a very significant increase in prevalence of animals from the hoggets to the ewes in the survey farm. There was also a very significant increase in prevalence of agglutinating antibodies in ewes in the survey farm to that of the ewes in the general survey. This showed that the infection was an epidemic, that is, unusually frequent occurrence of infection.

The prevalence of sheep from both surveys to *hardjo* having serological evidence of infection and their GMT is shown in Figure 4-4. The patterns from the survey farm and the general survey were almost the same. In the survey farm, the prevalence of animals with *hardjo* infection were much

higher compared to that of the general survey but the GMT of the survey farm were lower than that of the general survey. One would expect that with higher prevalence, the GMT would be higher too. This was not so, and this was because most of the animals in the survey farm had low titres. The low titres may indicate that the animals have had the infection for some time. It could be postulated that it was the end of the epidemic and that most of the animals had declining titres.

SUMMARY

In a serological and cultural survey of 249 sheep from a study farm, 142 (57.0 percent) of the animals sampled were positive for agglutinating antibodies by the microscopic agglutination test. Of these 142 animals, 109 (44 percent) had agglutinating antibodies to the serovar *hardjo*. A small number of titres were obtained to the other serovars. An attempt was made to isolate leptospiral organisms from some of the animals by urine cultures. However, none of the 95 urine samples taken from those animals were positive. It appears that either the animals were not leptospiruric or the number of leptospires in the urine were too small to be detected by culture propagation.

1. There was a significant difference in prevalence among the three age groups of sheep. Nearly all the lambs were negative and it was the converse with the ewes.

2. It was shown that animals with agglutinating antibodies to serovar *hardjo* were widespread on the farm. This supported the belief that serovar *hardjo* is the major serovar affecting sheep in New Zealand. No definite conclusion was reached as to the status of the infection. The infection seems to be a sporadic epidemic infection.

3. The bacteriological and serological prevalence of sheep from a farm that has been shown to have animals with positive renal infection were compared. The serological study showed a high prevalence of the animals to *hardjo* infection whilst no evidence of infection was obtained from urine cultures.

4. The high number of animals with titres to serovar *hardjo* suggested that sheep are highly susceptible to *hardjo* infection. However, it is doubtful whether sheep could maintain the infection.

5. In this cross-sectional study, attempts were made to define the epidemiology of leptospiral infection in sheep. The serological and cultural studies showed that sheep were not suitable as a maintenance host for leptospires. The dynamics of the infection in this survey farm was presented.

CHAPTER FIVE

DIAGNOSIS OF LEPTOSPIRAL INFECTION

INTRODUCTION

Bacteriological diagnosis is to a great extent based on serology and the same applies to the diagnosis of leptospiral infection. It relies a great deal on serological tests, like the microscopic agglutination test (MAT) for example. The MAT is the most commonly employed laboratory diagnostic technique used in the studies of leptospiral infection. It is a relatively sensitive test but rather laborious and time consuming. On the whole, diagnostic techniques based on the detection of antibodies have two disadvantages, namely:

a) delay before detectable levels of antibody are produced, and

b) problems of interpretation due to the persistence of antibodies from previous infection.

Diagnostic procedures used in leptospiral studies are similar to those used in other bacterial infections and are based upon:

a) culture and/or animal inoculation for isolation of the etiologic agent,

b) visualization of the organisms in tissues or body fluids or

c) serological tests to demonstrate antibody production.

A variety of techniques are available. Each technique has its advantage and disadvantages and no single test is sufficiently sensitive to be used on its own. Briefly, some of the techniques commonly used in leptospiral studies are:

1. Darkfield microscopy

This is direct visualization of the leptospires. The number of leptospires found in body fluids are usually very small and if small numbers are present the chance of seeing an organism may be remote. This probability however, may be enhanced by the use of appropriate concentration techniques. Blood and urine are perhaps most commonly viewed by direct microscopy.

2. Histopathological and direct staining methods

The staining of tissues to demonstrate leptospires depends upon techniques which utilize silver impregnation or silver deposition on tissue sections. Demonstration of leptospires in tissue is extremely important where cultural or serological procedures have not been effective or are not possible.

3. Fluorescent antibody technique

Its most common use has been in the demonstration of leptospires in urine using the method developed by White and Ristic (1959). This method is particularly useful since it permits formalin treatment of the urine or tissue prior to examination.

4. Direct culture

A variety of media is available for the culture of leptospires. Recovery of leptospires is often plagued by contaminants which may overgrow the leptospires. The use of 5-fluorouracil (5FU) or neomycin has greatly enhanced recovery rates of leptospires when added to isolation media.

5. Animal inoculation

The use of animals for the isolation of leptospires should be restricted to recovery attempts from tissues or fluids which are grossly contaminated with bacteria. The use of animals for isolation of leptospiral organisms is rather time consuming.

6. Macroscopic agglutination test

Macroscopic agglutination tests have been developed primarily for the screening of large numbers of diagnostic sera. Essentially these are formalin fixed and washed leptospiral cells which have been resuspended to a predetermined density in a suitable buffered carrier. After mixing of the serum and the antigen, the mixture is shaken and then viewed for agglutination.

7. Microscopic agglutination test

It is the standard serological test for the detection of leptospiral antibodies in sera. The test is performed by diluting the serum to be examined usually in two-fold dilutions followed by addition of a standard amount of actively growing leptospiral culture. This mixture is allowed to incubate and then examined for agglutination.

The only conclusive evidence of infection is, however, the isolation of the leptospiral organisms from the samples, whether they be organs, tissues, blood or urine. As infected animals are known to excrete leptospires in their urine for considerable periods during the course of the infection (Hellstrom, 1978), (Ryan, 1978), (Webster and Reynolds, 1955), urine is probably the most accessible source for their isolation or detection.

Various methods have been used to detect leptospiruria and cultural propagation is considered the most reliable method yet available. This, as mentioned in Chapter Three, is fraught with problems created by the requirements of the organisms. Cultures often take several weeks to accomplish and success to a large extent depends on the relative freedom of the urine samples from gross bacterial contamination.

Darkfield microscopy is the alternative method to detect leptospires in urine. A limitation of this procedure is that on most occassions it is necessary to examine many microscopic fields before detecting a single leptospira. This is true when there are less than 10^4 leptospiral organisms per ml of urine. Centrifugation of the urine may assist in detection of the small number of leptospires.

One of the greatest handicaps in the control of leptospiral infection in domestic animals is the lack of a practical diagnostic test which will recognise those animals that are responsible for spreading the infection. This refers to the animals having renal infection and shedding leptospiral organisms in their urine. This also holds true for sheep. Many workers (Beamer *et al*, 1953), (Hodges, 1974), (Morse *et al*, 1957) have failed to detect leptospires in naturally as well as experimentally infected sheep with leptospiral infection. The main reason for this is probably due to the fact that sheep shed low numbers of leptospires and therefore detection by darkfield microscopy and even cultural propagation is difficult.

The diagnosis of leptospiral infection with early laboratory confirmation is highly desirable. The search for a quick, specific and sensitive method of diagnosis of leptospiral infection is therefore essential. Radioimmunoassay seems a promising method. It is not a suitable method to be used by an ordinary diagnostic laboratory, but it could be very useful for screening animals in surveys or similar projects.

Solid-phase radioimmunoassay has been successfully developed to detect antigens of *Brucella abortus* (Wilson *et al*, 1977) and gonococcal antigens(Thornley *et al*, 1979). It has been shown to be a sensitive method of detecting bacterial antigens.

Radioimmunoassays employing immunosorbents (antigens or antibodies coupled to an isolate polymer) were introduced by Catt *et al*, (1966) and by Wide and Porath (1966). The classical radioimmunological system originally described by Yallow and Berson (1960) is based upon the ability of an antibody to bind its antigen labelled with a radioactive isotope and the competitive inhibition of the reaction by the unlabelled antigen. The antibody-bound labelled antigen is separated from the unbound and the radioactivity in one or both of these two fractions is measured. By the use of antibodies coupled to an insoluble polymer this separation procedure was simplified and can be made for instance by simple centrifugation.

The form of the solid phase can be varied with subsequent change of the technical procedure for the separation of the antibody-bound and free antigen. In the early techniques reported the insoluble immunosorbents consisted of small particles which were added to each tube in the form of a suspension. Radioimmunoassay systems using antibodies coupled to thin discs or absorbed to plastic tubes have also been developed (Catt and Tregear, 1967), (Catt *et al*, 1967).

This present investigation follows the method of radioimmunoassay by Thornley *et al*, (1979), Wide (1969) and Wilson *et al*, (1977). Briefly, the assay system in this investigation is illustrated as in Figure 5-1. This is the "double antibody sandwich technique" using polymer-coupled antibody to which antigens in the urine or simulated samples are bound and after subsequent washing, the labelled antibodies are added.

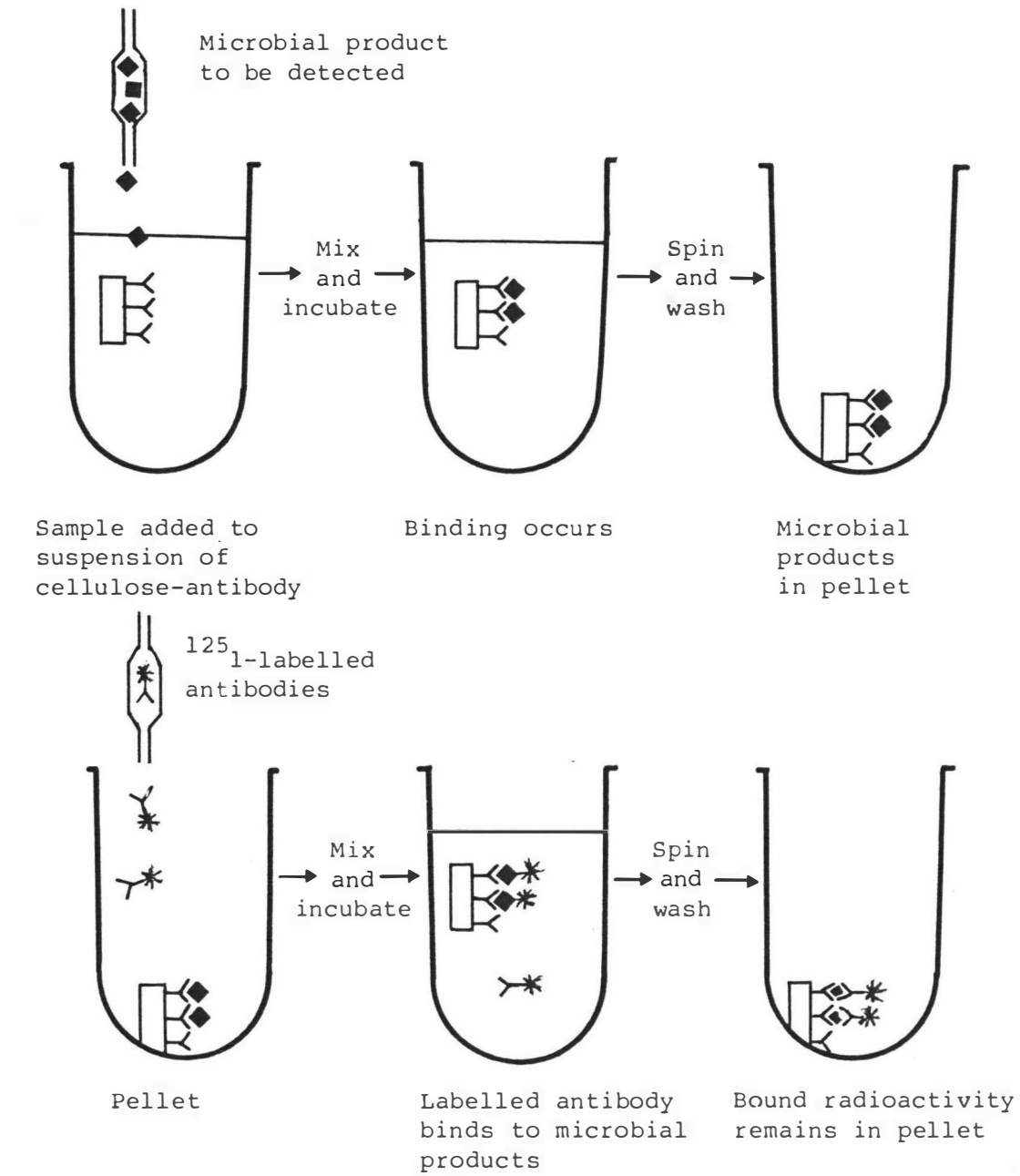


FIGURE 5-1: Principle of the solid phase radioimmunoassay.
(From Thornley *et al*, 1979).

The amount of labelled antibodies bound to the polymer-antibody complex is related to the amount of specific antigen present in the urine.

The investigation described here is designed to determine whether it is possible to detect leptospiral organisms or their antigenic components in urine by radioimmunoassay.

AIMS

The aim of this investigation is to determine whether it is possible to detect leptospiral organisms or their antigenic components in urine or simulated samples by the solid-phase radioimmunoassay. The specific objectives are as follows: -

a) To determine whether the procedure is able to detect whole (unlysed) and/or sonicated leptospiral organisms in urine and hence detect the carrier animals.

b) To find out the sensitivity of the procedure, that is, to find out the lowest number of organisms that it can specifically detect.

c) To determine the minimal time to run the procedure so as to obtain results quickly which is essential in diagnosis and control programmes.

d) To evaluate the usefulness of the procedure for diagnostic work and surveys.

METHODS AND MATERIALS

The following materials were prepared for the assay: -

a) Antigen for the assay

This consisted of a culture of *Leptospira interrogans* serovar *hardjo*. It was a laboratory strain which was obtained from the Center for Disease Control, Georgia, U.S.A. and which is being maintained for diagnostic work in the Leptospirosis Laboratory, Department of Veterinary Pathology and Public Health, Massey University. It was grown in liquid JS media.

b) Antigen for the production of rabbit hyperimmune serum

This rabbit hyperimmune serum was obtained from laboratory rabbits inoculated with serovar *hardjo* isolate (sheep 535) by the method of Tan (1970) (Appendix V). The hyperimmune serum had microscopic agglutinating titre of 1:24,000 to serovar *hardjo*.

c) Immunoglobulin fractions

Immunoglobulin fractions extracted from the rabbit hyperimmune serum were obtained by the method of Ion Exchange Chromatography by means of a system of gradient elution (Appendix VI). The first fraction eluted is IgG and it is pooled and concentrated using polyethylene glycol. The concentration of the pooled IgG was determined by spectrophotometry (UNICAM SP500)* and adjusted to a protein concentration of 10 mg per ml and then stored at $-20^{o}C$ until needed. The IgG fraction was checked for the absence of other serum proteins by immunoelectrophoresis against goat anti-whole-rabbit serum (Appendix VI).

d) Cellulose-*hardjo* antibody complexes for use as a solid phase reagent

Initially, the method of Wide (1969) was followed.

* Appendix I

The microcrystalline cellulose (Whatman)** was activated with cyanogen bromide (CNBr)** and then linked with the *hardjo* antibody. The mixture was then dried with the aid of acetone and stored as dry powder at -20°C (Appendix VII). Later, CNBr-activated sepharose (Pharmacia)** was utilised as the solid phase reagent.

e) Radio-labelled gammaglobulin (IgG)

The gammaglobulin was labelled with radioactive sodium iodide (^{125}I) (Amersham)** by a modified method of MacConhey and Dixon (1966) (Appendix VIII).

Assay Procedure

The procedure followed is that of Wilson (1977) and Thornley *et al*, (1979) and is illustrated as in Figure one. A pipette was used to dispense one millilitre portions from a vigorously stirred suspension of the cellulose antibody complex (the stock solution was diluted 1 in 400 with the assay buffer) into small plastic tubes (Consolidated Plastics)*. Assay buffer is that of Thornley *et al*, (1979) (Appendix III). Each tube received the equivalent of 0.125 mg (dry weight) of the complex. Normal rabbit serum (20 ul) prepared in the laboratory was added and the tubes were incubated at room termperature and at the same time rotated end-over-end using a blood cell suspension mixer (Matburn)* for at least 30 minutes. The sample (100 ul) or a simulated solution of antigen in PBS to be assayed was then added and incubation with rotation was continued overnight (17 hours).

* Appendix I

** Appendix II

The tubes were centrifuged for three minutes at 4,000 rpm in a centrifuge (Sorvall)* and the supernates were discarded by means of a suction device so that approximately 0.25 ml of the mixture remained in each tube. The pellets were washed three times with the assay buffer (1.5 ml each wash). After removing the third wash supernate, labelled antibody (100 ul) was added together with more assay buffer (400 ul) and normal rabbit serum (20 ul). The tubes were incubated with rotation for a further three hours.

The cellulose-antibody complex was centrifuged and washed three times with assay buffer as before and then any bound radioactivity was counted in the automatic gamma-counter (Nuclear Enterprises)*. Tubes containing cellulose-antibody complex but no antigen were included as reference control to which the counts in other tubes could be related.

The results are expressed as radioactivity bound (percentage) given by:

$$\frac{S}{B} \times 100,$$

where S is the radioactivity retained by the tube containing the sample and B is the radioactivity retained by the tube containing no antigen.

RESULTS

Assay No 1

Simulated samples of leptospiral antigen to be assayed were made up of five day old serovar *hardjo* organisms. The organisms were placed in urine, water and JS medium to give antigen concentration varying from 10^1 to 10^9 leptospiral cells per ml. The results of the assay did not show any relationship.

* Appendix I

FIGURE 5-2: Assay No 1
Radioimmunoassay of leptospiral cells (suspended in urine (△), distilled water (○) and JS medium (□)).

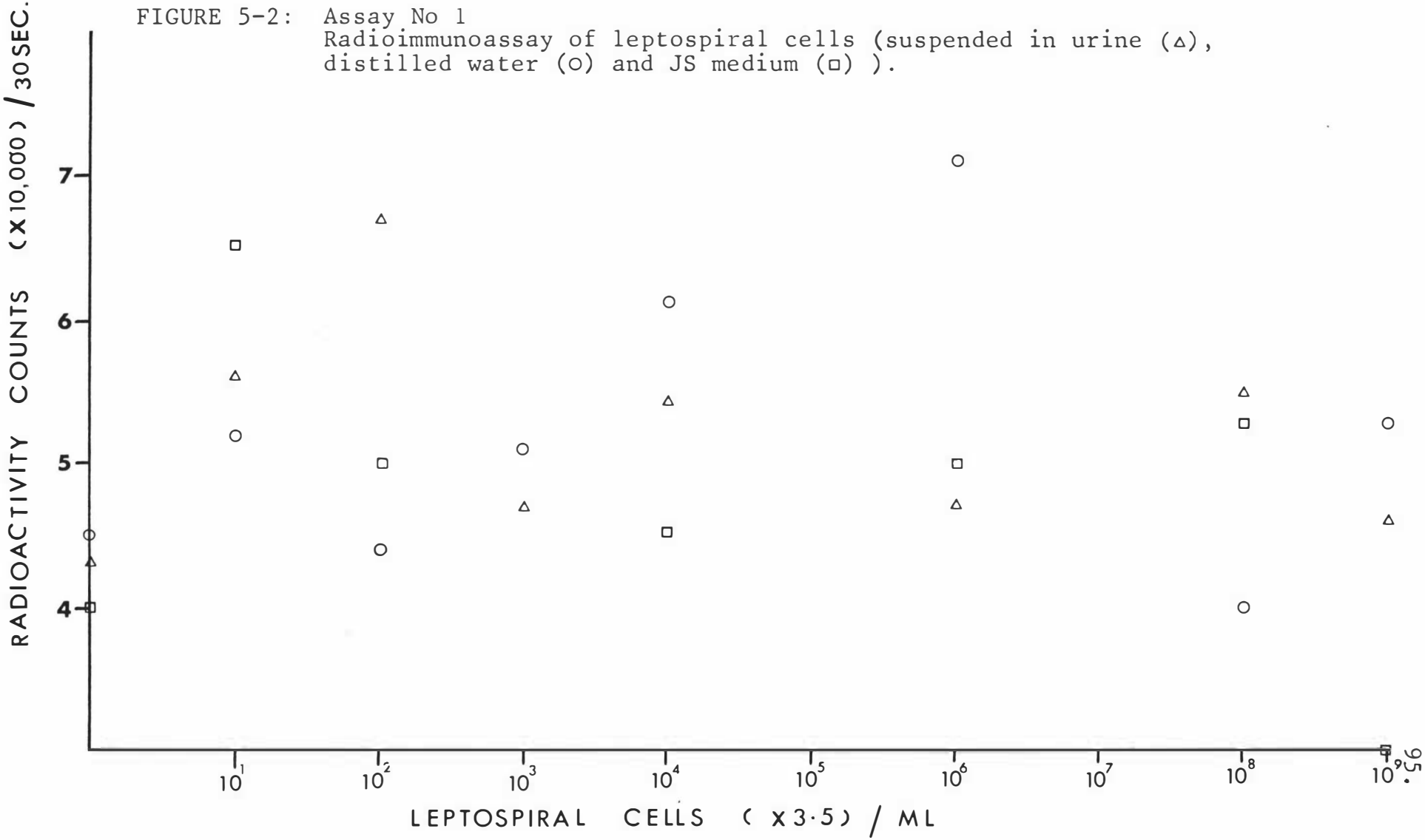


FIGURE 5-3: Assay No 2
Radioimmunoassay of leptospiral cells in urine.

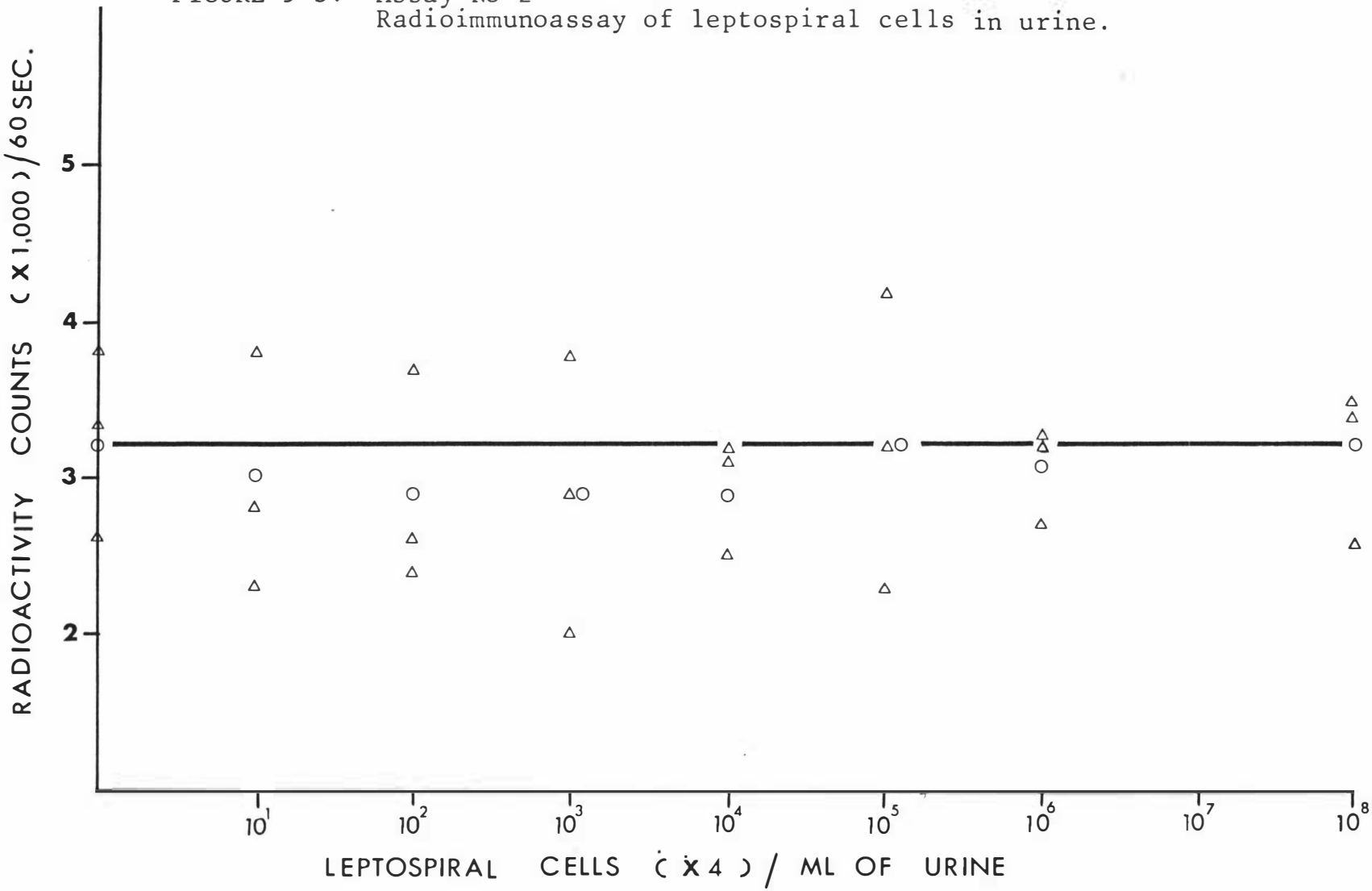


FIGURE 5-4: Assay No 3
Radioimmunoassay of leptospiral cells suspended in assay buffer.

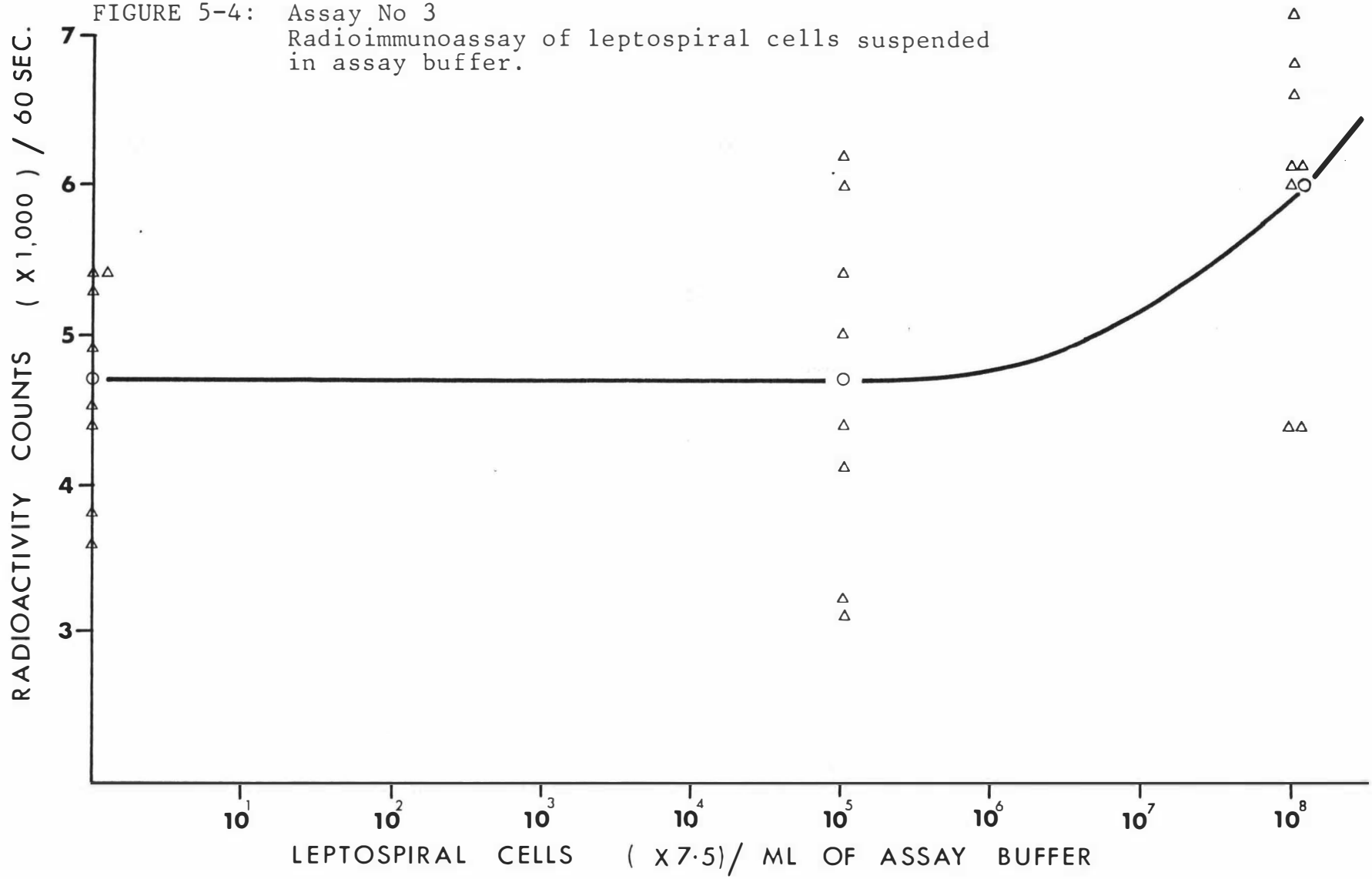
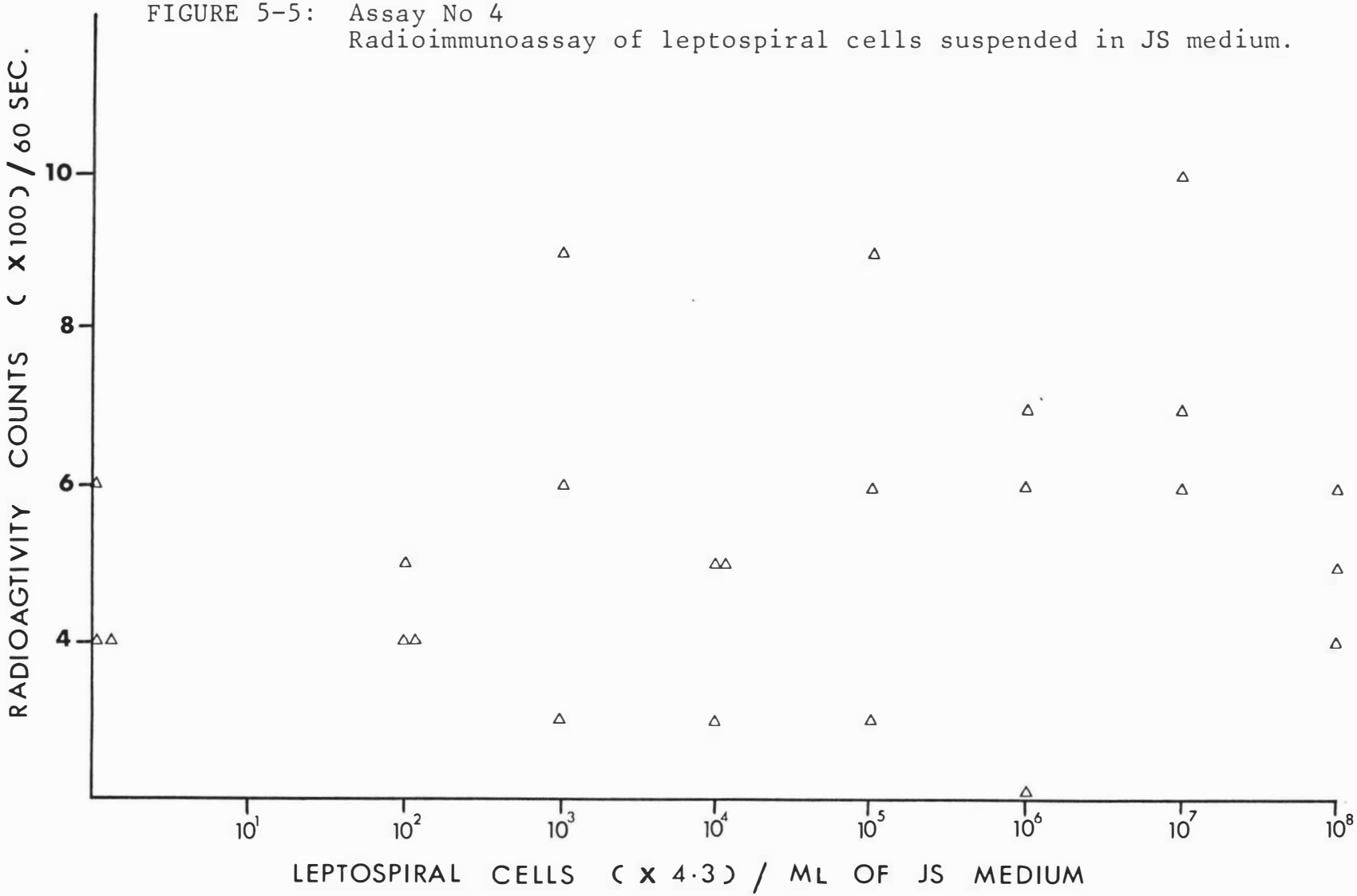


FIGURE 5-5: Assay No 4
Radioimmunoassay of leptospiral cells suspended in JS medium.



Assay No 2

The assay was repeated with the antigens suspended in urine only. Samples were assayed in triplicates, ranging from 10^1 to 10^8 leptospiral cells per ml. No significance finding was obtained from this assay.

Assay No 3

The antigens were suspended in assay buffer instead of urine. This is to find out whether urine had any inhibitory effects on the assay. The assay was run in eight samples for each concentration of antigen. This would show whether there was any specificity in the assay. Results showed that the assay is sensitive when the antigen concentration is 10^6 or more leptospiral cells per ml. On the whole, the procedure was not specific.

Assay No 4

No significance results were obtained when the antigens were suspended in JS medium.

Assay No 5

Antigens in the form of sonicated leptospiral cells did not give any significance results but higher specificity was obtained from the assay.

Assay No 6

Sonicated and lyophilised leptospiral cells were used as antigens. No significance results were observed.

Assay No 7

Procedure for Assay No 6 was repeated but the amount of normal rabbit serum was increased from 20 ul to 100 ul. With this increase, it was expected that the background readings would be lowered. No significant results were obtained.

FIGURE 5-6: Assay No 5
Radioimmunoassay of sonicated leptospiral cells suspended in urine.

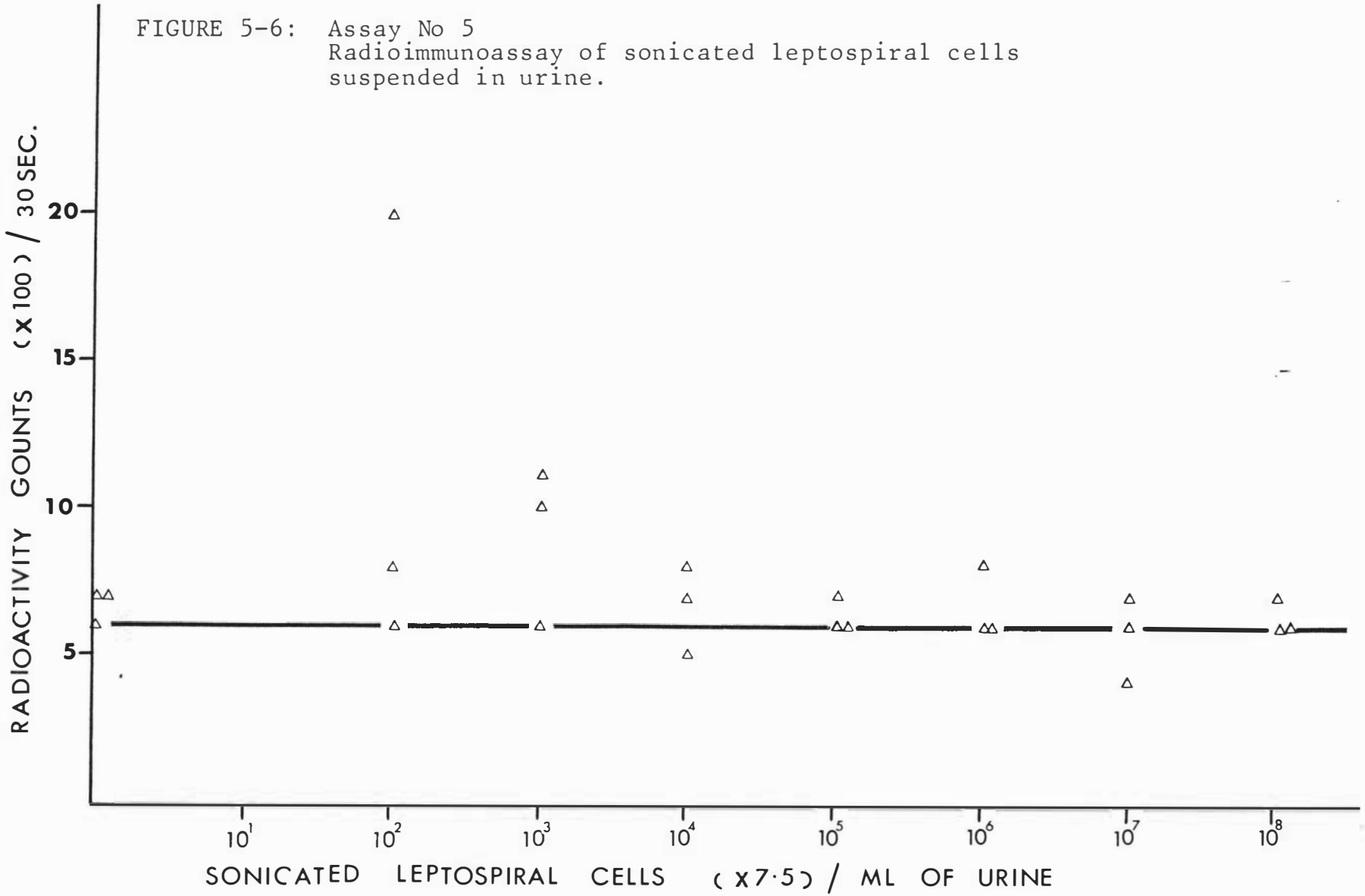


FIGURE 5-7: Assay No. 6.
Radioimmunoassay of sonicated and lyophilised leptospiral cells suspended in buffered saline.

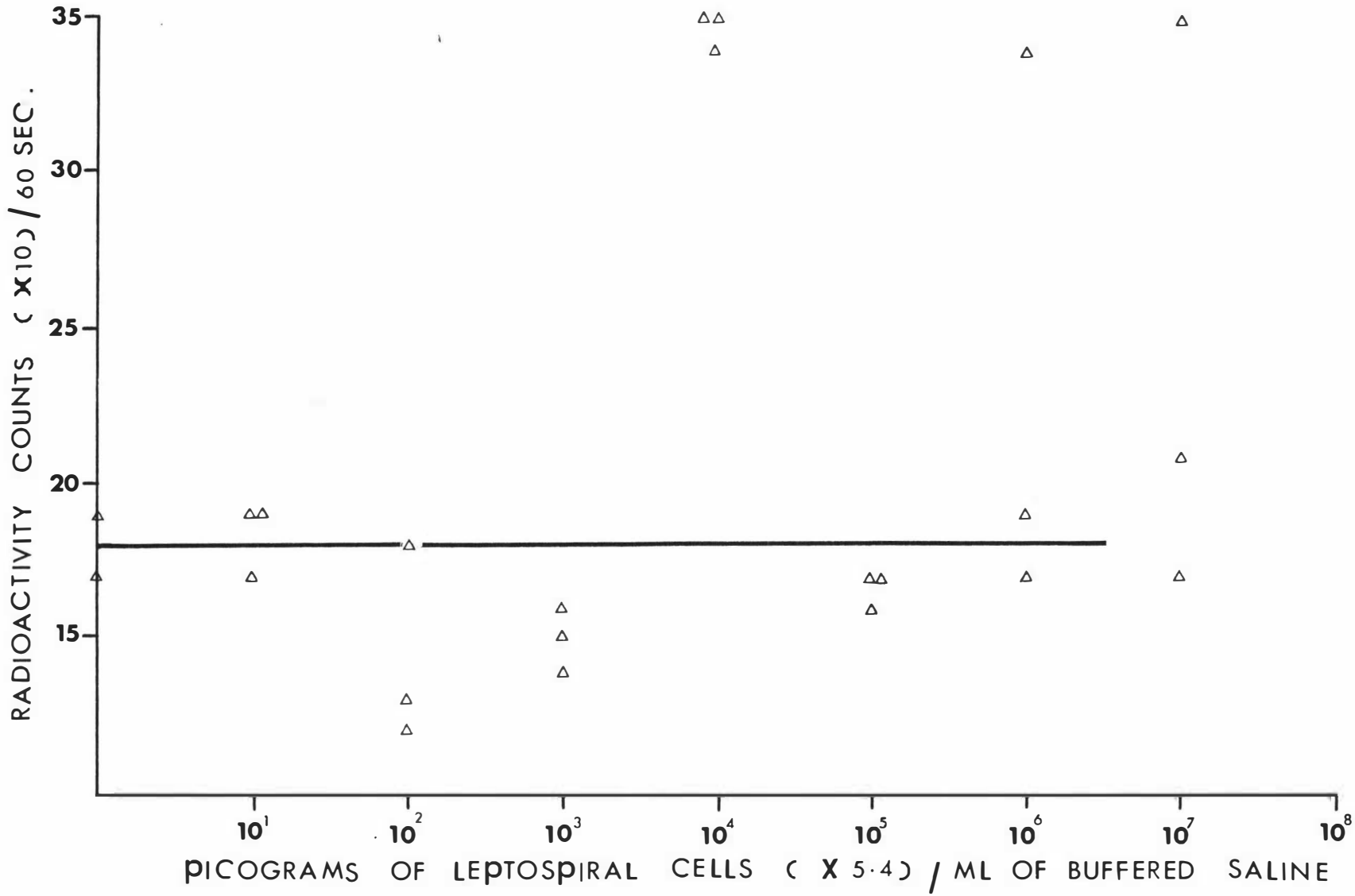


FIGURE 5-8: Assay No 7
Radioimmunoassay of sonicated and lyophilised leptospiral cells suspended in buffered saline (100 ul of normal rabbit serum instead of 20 ul).

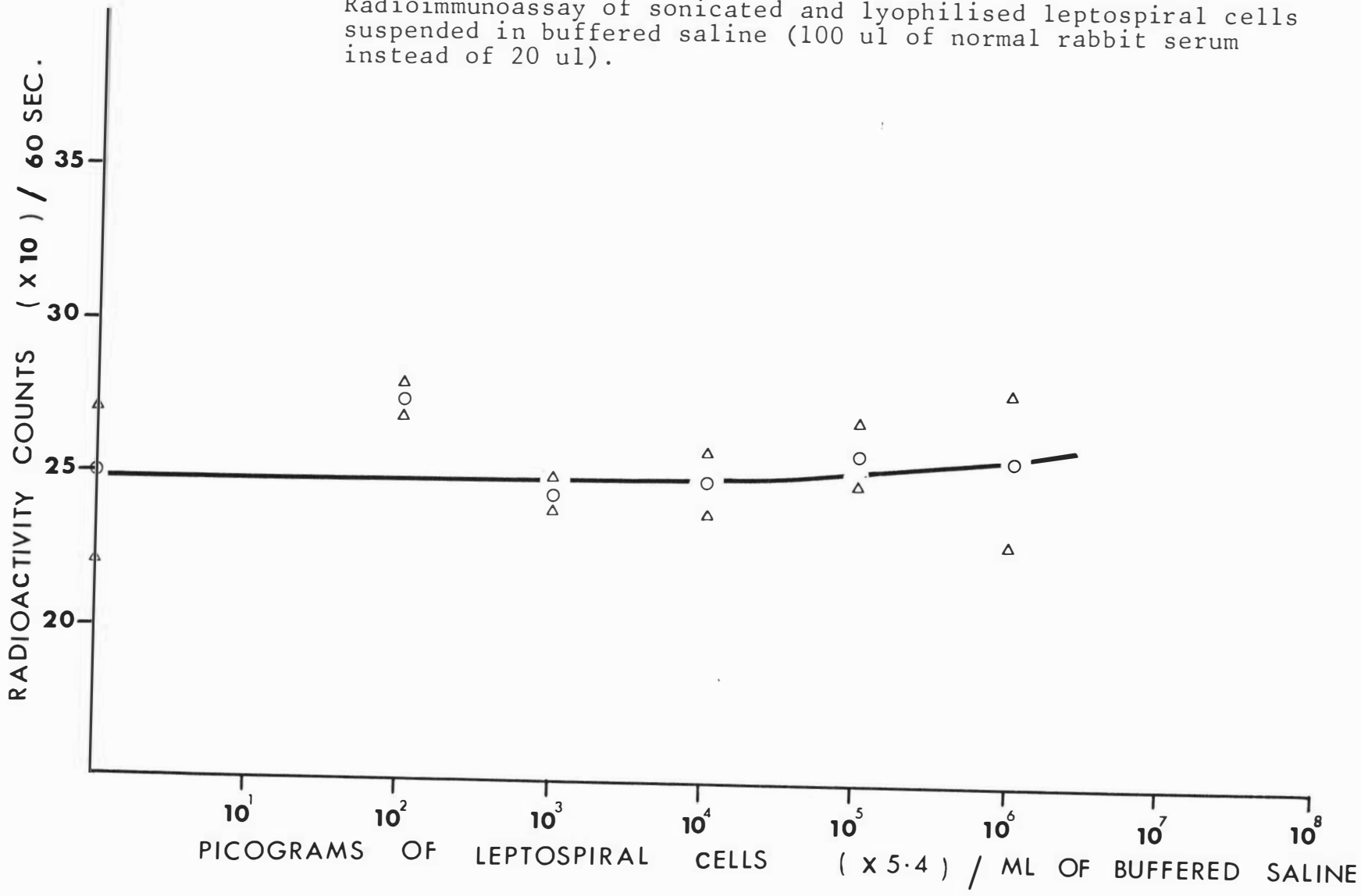
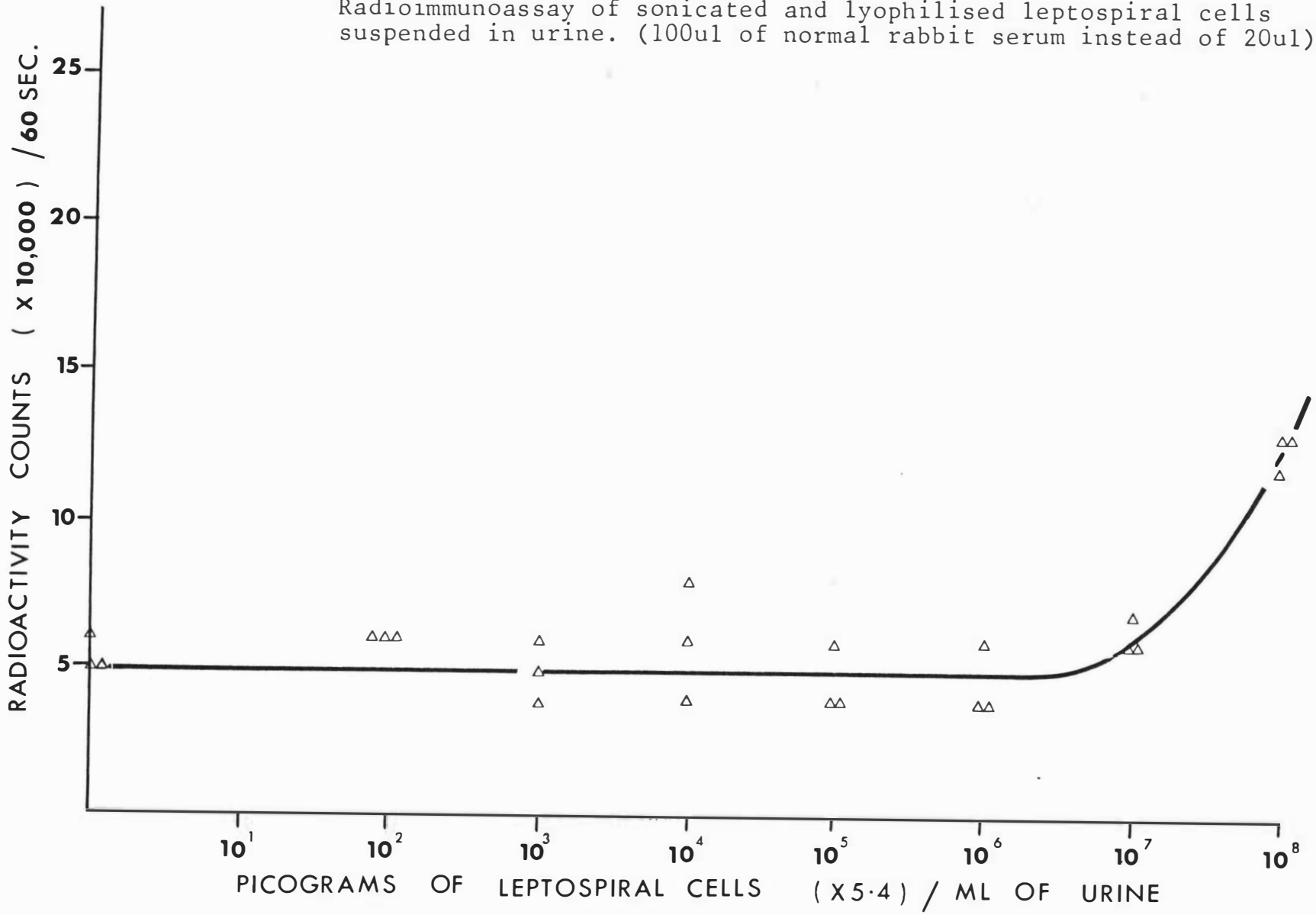


FIGURE 5-9: Assay No 8
Radioimmunoassay of sonicated and lyophilised leptospiral cells suspended in urine. (100ul of normal rabbit serum instead of 20ul).



Assay No 8

The same procedure was applied as for assay 6 and 7 however the antigen concentration was increased. The results showed that the assay is sensitive at antigen concentration of 10^7 picograms of leptospiral protein or more. Apparently at antigen concentration of 10^6 picograms of leptospiral protein or less, the assay was insensitive and the radio-activity counts were the same as the background readings. Results showed higher specificy of the assay.

Assay No 9

The assay was repeated as Assay No 8. The antigens were in five copies so as to determine the specificity of the assay. Results showed high specificity of the procedure.

Assay No 10

In this assay, the time of incubation was altered. Instead of overnight incubation, thirty minutes were given for the antigens to react with the cellulose-antibody complex. The time of the second incubation was also lowered. Thirty minutes were allowed for the labelled antibody (^{125}I-Ab) to react with the cellulose-antibody-antigen complexes. The results showed that the short periods of incubation did not affect the assay. High specificity was recorded but the assay is still only sensitive when the antigen concentration is above 10^7 picograms of leptospiral protein per millilitre.

Asay No 11

Similar results were obtained when aliquots of the complete complexes were assayed.

Assay No 12

The labelled antibody were diluted four times. Similar results were obtained. The assays was just as specific as in previous assays. The sensitivity still lies in antigen concentration of 10^7 picograms or more of leptospiral protein per millilitre.

FIGURE 5-10: Assay No. 9
Radioimmunoassay of sonicated and lyophilised leptospiral cells suspended in urine.

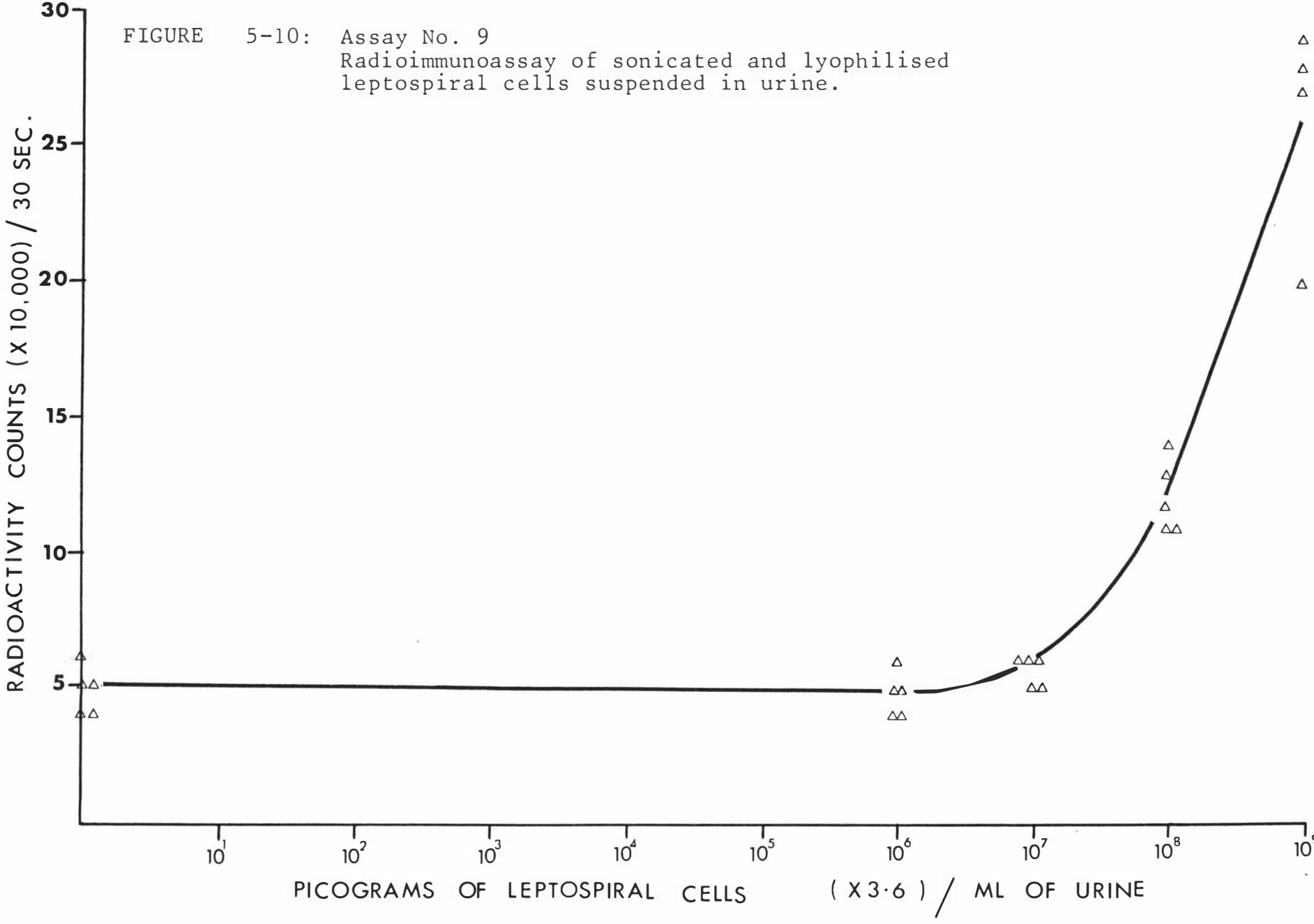


FIGURE 5-11: Assay No 10
Radioimmunoassay of sonicated and lyophilised leptospiral cells suspended in urine. (Time of incubation reduced from 20 hours to one hour).

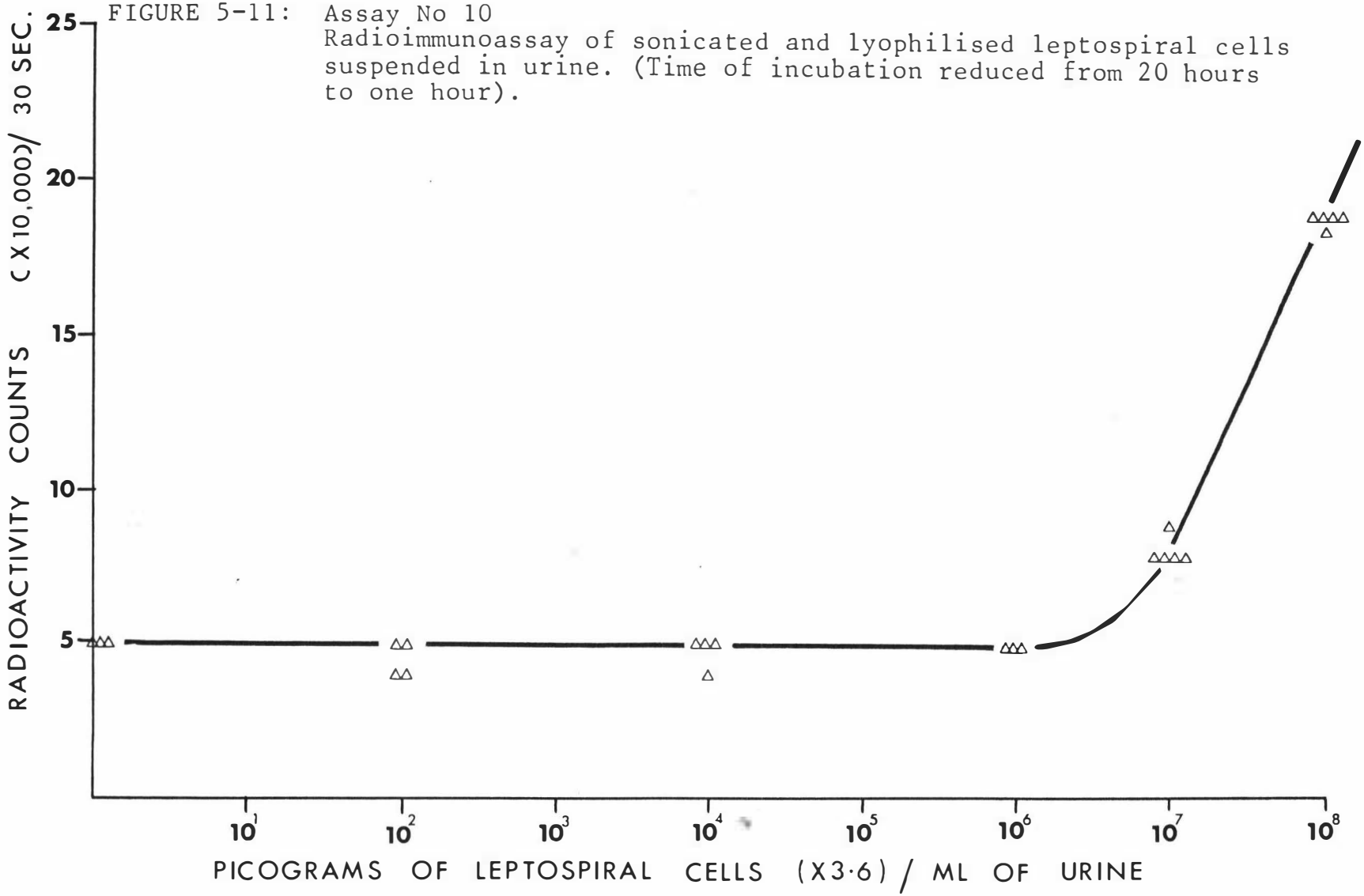


FIGURE 5-12: Assay No 11
Radioimmunoassay of sonicated and lyophilised leptospiral cells suspended in buffered saline (△ full assay counts, ○ counts of 300 ul aliquots).

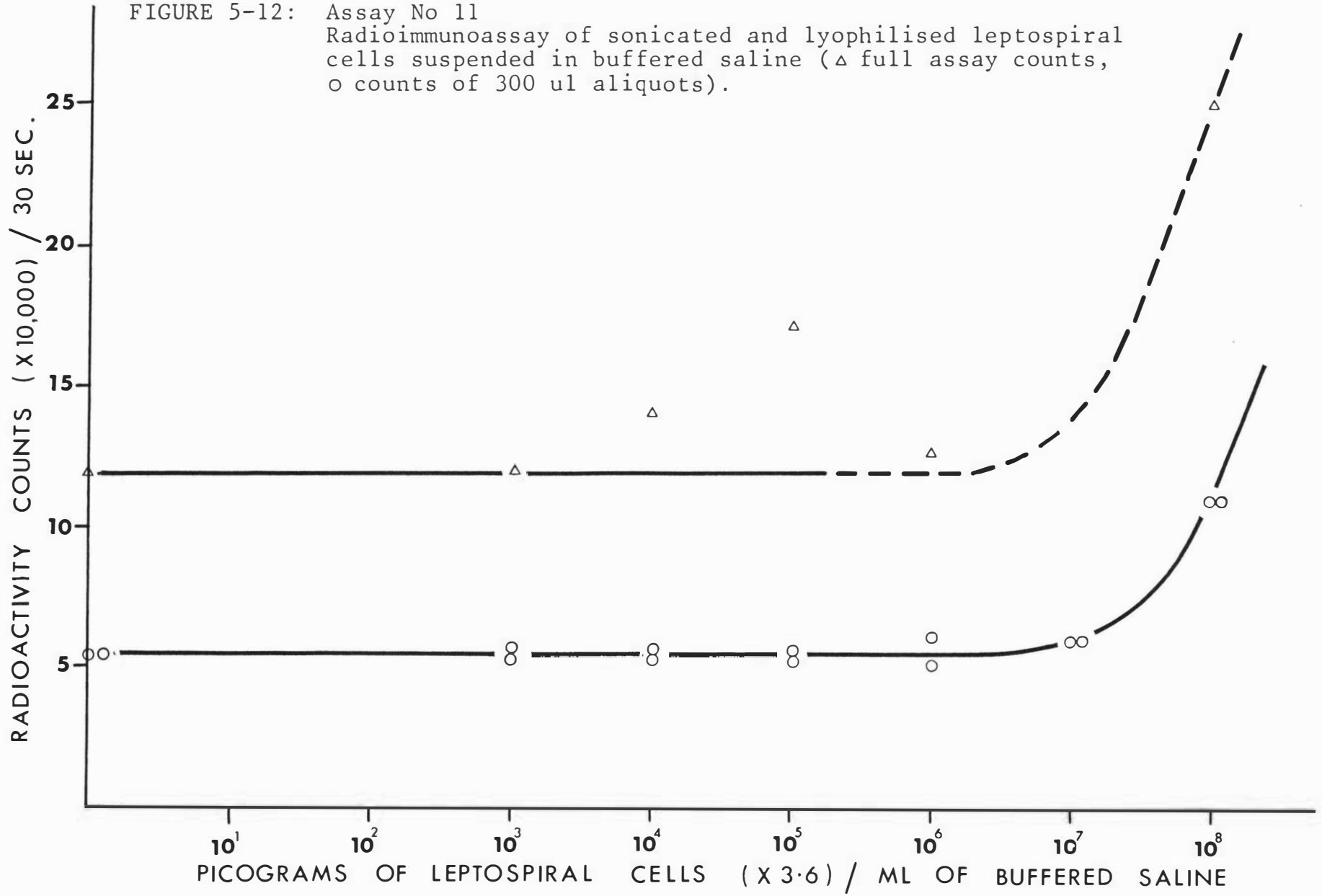
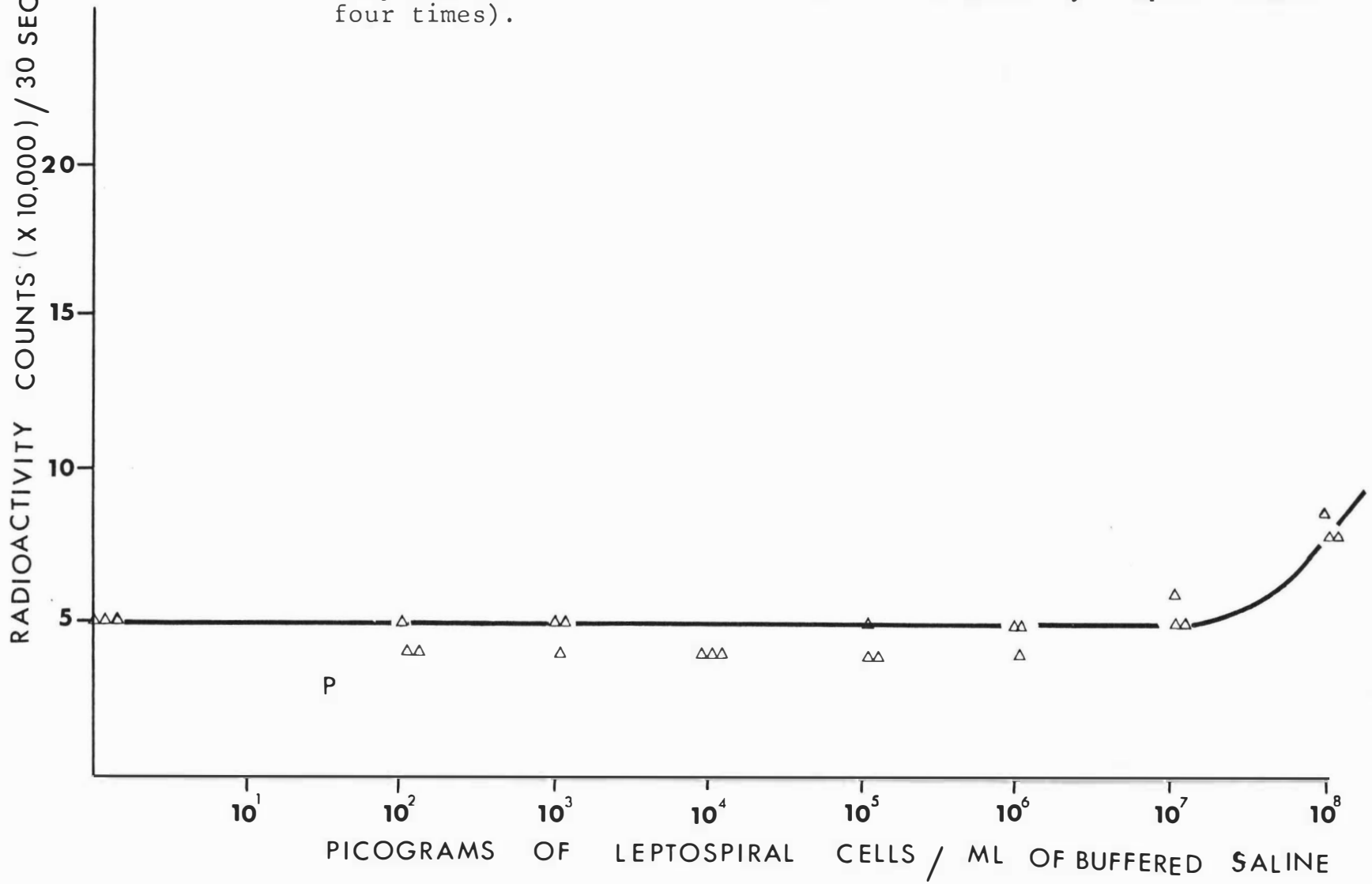


FIGURE: 5-13: Assay No 12
Radioimmunossay of sonicated and lyophilised leptospiral cells suspended in buffered saline. (Labelled antibody complex diluted four times).



DISCUSSION

Leptospirosis in domestic animals is of great economic concern largely because of the abortions which frequently follow infection of pregnant animals. In many instances abortions occur without any detectable signs of illness. Diagnosis in such cases depends upon the ability to demonstrate leptospires in the foetal and placental materials. Otherwise, the infection must be diagnosed presumptively from clinical history and the results of serological tests.

The general appearance of normal health in infected non-pregnant animals may make it difficult to detect infection. Infected animals may only have a brief febrile response, and the possibility of not observing this under natural conditions is very obvious.

The incidence of infection in any geographic area may depend on several factors and one of these, is the proportion of the animal population excreting leptospires at any one time. The spread or transmission of leptospires among animals is almost exclusively through the medium of infected urine, either directly to in-contact animals or indirectly to other animals via the environment.

The failure to control leptospiral infection overseas, is partly due to presence of a large number of serovars and their animal hosts. New Zealand is fortunate to have only three of the six endemic serovars affecting its livestock. This makes control of infection a lot easier. Vaccination is one of the important means of controlling leptospiral infection. The presently available vaccines were effective in establishing immunity to clinical disease but apparently did not provide complete protection against the development of the renal carriers (Johnson and Sieter, 1977). Introduction of a carrier may therefore render current measures useless. Inability to detect these carriers is probably a factor for the failure to control leptospiral infection.

Numerous reports have been published describing the leptospiral carrier state in animals. Because infected animals are known to excrete leptospires in their urine for considerable periods (Hellstrom, 1978), (Ryan, 1978). (Webster and Reynolds, 1955) during the course of the disease, the urine is probably the most accessible animal material for the isolation of leptospires and detection of infection. The usual method for isolating leptospires from urine or any other sample is however too long and laborious to undertake in large surveys. As a result, most studies concerning the prevalence of leptospiral infection have been serological surveys with relatively few attempts to determine the extent of leptospiruria or to compare actual shedding with the presence of leptospiral agglutinating antibodies.

Urine therefore becomes the body fluid of choice for the isolation of leptospires and detection of infection and carrier animals. Shedding of organisms may be intermittent and the concentration of leptospires may be low. This is true in the case of sheep. This makes it difficult to pick out the animals shedding the organisms. Often direct darkfield microscopy and culture fails to detect these carrier animals.

The confirmatory diagnosis of leptospiral infection has proved difficult and largely depends on attempts to isolate the pathogen or to demonstrate leptospires in tissues by silver impregnation, fluorecent antibody techniques or by direct darkfield microscopy. The MAT is the usual procedure for detecting leptospiral agglutinating antibodies, measuring antibody titre and identifying unknown leptospiral isolates. As mentioned earlier, the procedure is tedious, time consuming and requires large quantities of antigen. There is therefore a need to find a much more convenient method of diagnosis.

Methods based on the immunological identification of the microbial antigen have potential advantages in speed and specificity. Radioimmunoassay is a method of great sensitivity that has been applied successfully in the screening of blood donors in Australia for Hepatitis B antigen (Prince *et al*, 1973), (Alter *et al*, 1973). It may be used to detect antigen in a great variety of specimens like serum, saliva, faeces and urine (Irwin *et al*, 1975).

Thornley *et al*, (1979) and Wilson *et al*, (1977) have shown that radioimmunoassay is a very useful technique to detect small number of organisms. It could possibly be just as sensitive and specific a technique for the detection of leptospiral organisms in urine samples. It is forseen that such a technique would be very useful in surveys where large number of urine samples have to be screen for leptospiral organisms. In this situation, the urine samples could be pooled and concentrated by centrifugation and finally assayed by radioimmunoassay. Speed in processing the samples following their collection is not particularly important as radioimmunoassay should be equally sensitive and specific at picking dead or lysed organisms.

Conventional radioimmunoassay procedures which employ labelled antigen require prior knowledge of the chemical nature of the substance to be detected and are most satisfactory for homogeneous substances of a single molecular species. By means of labelled antibodies, these difficulties are largely avoided. Detailed knowledge of the likely antigen is not essential provided that antiserum containing specific antibodies can be produced. Another advantage is that the radiochemical manipulations are carried out on easily purified and characterised immunoglobulin.

The concept of solid phase radioimmunoassay depends on the use of an insoluble polymer to which specific antibody may be attached. By this means the radioimmunoassay procedure has been considerably simplified, as the separation of bound and free tracer antigen can be effected by washing the solid phase at the completion of the immune reaction. The washed solid phase antibody-labelled antigen complex may then be counted to determine the bound tracer.

Results from this present assay were consistent in showing that the assay is not a sensitive procedure. The assay was applied to simulated samples of leptospiral protein varying from 10^1 to 10^9 picograms per ml. It was shown that the assay is only able to detect leptospiral protein of not less than 10^6 picograms per ml. This corresponds to approximately 9×10^6 leptospiral organisms per ml. Comparing this with direct darkfield microscopy, this is not a sensitive procedure. Darkfield microscopy is able to detect as low as 10^4 leptospiral organisms per ml of sample (Mackintosh, per. comm.).

On the whole, the assay is found to be quite specific. Results obtained although not sensitive, showed consistent radioactivity counts at the various concentration of leptospiral protein. The effectiveness of the assay is dependent on the quality of the reagents that are used. Provided that the antibody fraction has adequate specificity and their radiolabelling does not reduce the binding properties, it should be possible to use the assay for measurements in the picogram range.

The rabbit hyperimmune serum when tested with serovar *hardjo* antigen had a microscopic agglutinating titre of 1:24,000, but the IgG fraction when tested had only an agglutinating titre of 1:1536 to serovar *hardjo*. A gammaglobulin fraction with a much higher specific activity is essential to increase the sensitivity of the assay. This is one component of the assay described in this chapter which was suspected of being the cause of the low sensitivity.

It should be possible to increase the sensitivity in several ways:

1) labelled antibody of higher specific activity would give some improvement,
2) affinity purification of the labelled antibody could lower the background level,
3) antibody of greater affinity could result in increased radioactivity surviving the multiple washes and being retained in the final pellets.

It is not known how much of the leptospiral material is being measured. The antigen is made up of sonicated and lyophilised leptospiral cells. It is possible that only a fraction of the material was available as antigen and if that is true then the sensitivity of the assay would probably be much higher with a pure antigen. From the various trials of the assay, assay curves (Figure 5-1 to 5-12) obtained were almost identical. The trials showed that the shape of the assay curves is determined by the level of antigen in the samples. The assay curves were identical even when the following changes were made in the procedure:

a) instead of 20 ul, 100 ul of normal rabbit serum were used in the assay,
b) aliquots of 300 ul of the final complex were measured for radioactivity instead of centrifuging and meaasuring the pellets for radioactivity,
c) the ^{125}I-antibody complex was diluted four times.

Even with all these changes, the results of the assay were apparently little affected. Good assay curves were obtained, but the sensitivity of the assay was still low, that is, it could detect only 10^6 picograms of leptospiral protein or higher amount.

It was shown that the time required for the incubation of the complexes in the procedure can be cut down to a time of thirty minutes with a second incubation of thirty minutes instead of the overnight incubation followed uy a three hour period. There was no change in the results with the shortened incubation periods. This is significant as results of the assay could be obtained earlier and this would have advantages in control and surveillance programmes.

With improved sensitivity, radioimmunoassay would have great advantage over culture methods. Radioimmunoassay would not be affected even when the leptospires were dead or lysed. This normally happened when there was delay of submission of the samples. It would also be unaffected by contaminated samples.

Three areas of the assay which need to be investigated further are as follows: -

a) the use of pure and more specific antigen instead of sonicated and lyophilised leptospiral cells,

b) ways of lowering the background readings, present background readings were quite high,

c) IgG fraction of higher specificity should be used.

SUMMARY

A rapid and reproducible method of detecting leptospiral antigens by solid phase radioimmunoassay is described:

1) It is shown that the procedure is relatively simple to perform. However, at this stage, it is not sufficiently sensitive for the precise measurement of leptospiral antigens below 10^6 picograms per ml. The procedure would be useful as a diagnostoc procedure if its sensitivity and specificity could be further increased.

2) The assay for leptospiral antigens appeared to be relatively insensitive compared with other bacterial antigens (Thornley *et al* 1979), (Wilson *et al*, 1977) and one possibility is that the antigen being assayed was derived from whole leptospiral cells and probably only a small fraction is being measured in the assay.

3) A further improvement of the procedure was presented. The time of assay was cut down from twenty hours (overnight and three hours) to just one hour (thirty minutes and thirty minutes).

CHAPTER SIX

GENERAL DISCUSSION

The random survey of sera from sheep of the North Island in this study represents the first reported attempt to assess in sheep the prevalence of the six leptospiral serovars known to be endemic in New Zealand.

A serological prevalence of titres to serovar *hardjo* of nineteen percent in sheep of all ages and evidence of infection on twenty-seven out of forty-two different farms indicated that infection by serovar *hardjo* is not uncommon. It was therefore shown that serovar *hardjo* is the most prevalent serovar affecting sheep in New Zealand. The other serovars were insignificant and occurred sporadically when sheep happened to come in contact with the maintenance hosts.

In comparison with overseas surveys, the prevalence of positive titres in sheep from this survey was relatively high. The significance of this high prevalence of sheep to leptospiral infection is not yet known. However, it was established in this present investigation that the *hardjo* infection was not endemic in the sheep population.

In the course of this investigation, it was shown that sheep were unlikely the maintenance host for serovar *hardjo* in New Zealand. One condition in the maintenance of a particular serovar in a particular host population is that a significant proportion of the infected animals remain carriers sufficiently long to infect each new generation. In this cross sectional study, it was shown that sheep could not maintain the infection for a long duration and therefore they were unlikely to be a maintenance host. Thus, the role of sheep in the continuing survival of leptospires in the New Zealand environment, appears to be unimportant.

The *hardjo* titres observed in the sheep were probably attributable to short term infections accompanied by transient leptospiruric phase. It is not known how long sheep are able to excrete leptospiral organisms in their urine. This has to be resolved by further investigation. Evidence obtained in this study, however, indicated that the leptospiruria was of short duration. In this survey, high proportion of the sheep population had contact with cattle and it appears that infection in sheep resulted from close contact with cattle. It is assumed that cattle were the primary source of infection for the sheep. It is therefore suggested that although sheep are obviously naturally susceptible to sporadic infection with *hardjo*, they are unlikely to be a significant source of reinfection for cattle in a herd in which the infection is being controlled by vaccination or other control methods.

In relation to other stock, sheep probably play an insignificant role in the transmission of leptospiral infection. It is suspected that infection in sheep is a dead end.

In relation to public health, leptospiral infection in sheep probably pose little problem. This conclusion is based on the low cultural prevalence of sheep in the general survey to leptospiral infection and the fact that all the sheep examined in the study farm were not leptospiruric. Thus, people dealing with sheep particularly those working in the freezing works and shearing sheds have little chance of picking leptospiral infection from sheep.

It is difficult to assess the effect of leptospiral infection on the economy of the sheep industry. Firstly, it is not known what are the effects of *hardjo* infection on sheep under natural conditions. There were no reports of abortions in sheep due to *hardjo* infection except for one reported by Andreani *et al*, (1974). The abortions mentioned by Andreani *et al*, (1974) were suspected to be due to serovar *hardjo*. Infections due to serovar *hardjo* were often inapparent and probably had little effect on the animals.

Leptospiral infection in sheep will probably have little effect on the control or eradication programmes of leptospiral infection in other stock. The control of *hardjo* infection in cattle will help to eliminate the *hardjo* infection in sheep assuming the source of infection in sheep came from cattle.

The need to diagnose leptospiral infections as soon as possible after the infection has occurred, is essential. In the later part of this thesis, some work on the use of radioimmunoassay to detect leptospires or their antigens in urine samples, is included. There are good indications that the procedure, with further improvement, might be useful in surveys and control programmes in detecting leptospiruria in animals.

APPENDIX I

LIST OF EQUIPMENT AND APPARATUS

	EQUIPMENT AND APPARATUS	MAKER OR SUPPLIER
1.	Blood cell suspension mixer (Matburn).	Matburn Ltd., Emibold St., London, England.
2.	Colworth 400	A.J. Seward and Co Ltd., 6 Stamford St., London SE1 9UE, England.
3.	ISCO Model 170 Golden Retriever Model	Instrumentation Specialties Co., P.O. Box 5347, Lincoln, Nembraska 68505, U.S.A.
4.	Gilson "pipetman"	Gilson France S.A. 72, Nue Gambetta 95400 Villiers-le Bal, France.
5.	Plastic microtitre plates (Microtitre)	Dynatech Singapore Ltd., 21B Goldhill Plaza, Singapore 11.
6.	Petroff-Hauser	C.A. Hauser and Son, Philadelphia, U.S.A.
7.	Nuclear Enterprises NE 1600 (Gamma counter)	B.R. Homersham Ltd., Electrical and Mechanical Engineers, Auckland, N.Z.
8.	Semi-automated minidiluter (Dynatech)	Cook Laboratory Products, 900 Slaters Lane, Alexandria, Virginia 22314, U.S.A.
9.	Soniprobe Type 1130A/1	Dawe Instruments Ltd., Western Avenue, Ascorn, London W3, England.

APPENDIX I CONTINUED

EQUIPMENT AND APPARATUS	MAKER OR SUPPLIER
10. Sorvall GLC-1 (General Laboratory Centrifuge)	Dupont Instruments Sorvall, Peck's Lane, Newtown, Connecticut 06470, U.S.A.
11. Super mixer	Lab-Line Instruments Inc., Melrose Park, Illinois, U.S.A.
12. Test tubes (polystyrene) (for RIA)	Consolidated Plastics, Upper Hutt, N.Z.
13. Turner Fluorometer Model No 111	G.K. Turner Associates Ltd, Palo Alto, California, USA.
14. UNICAM SP500 Series 2	Pye Unicam Ltd., York Street, Cambridge, England.

APPENDIX II
LIST OF MATERIALS

	CHEMICALS	SOURCE
1.	Agar (Difco)	Difco, Detroit, Michigan, U.S.A.
2.	B.D.H.	British Drug House Chemicals Ltd., Poole, England.
3.	Chloramine-T (B.D.H.)	British Drug Houses Ltd., BDH Laboratory Chemicals, Poole, England.
4.	CNBr-activated Sepharose	Pharmacia Fine Chemicals, Uppsala, Sweden.
5.	Cyanogen bromide (CNBr)	ICN Pharmaceuticals Inc., Life Sciences Group, Plainview, New York, U.S.A.
6.	5-Flurouracil (5FU)	Sigma Chemical Co., P.O. Box 14508, St. Louis, Missouri 63178, U.S.A.
7.	Goat anti-rabbit whole serum	Miles Yeda, Miles Laboratories Ltd., P.O. Box 37, Stokes Poges, Slough, England SL2 4KY
8.	Iodine 125 (^{125}I)	New England Nuclear 549 Albany St., Massachusetts 02118, U.S.A.
9.	JS Medium (Difco)	Difco Laboratories, Detroit, Michigan, U.S.A.
10.	M. & B.	May and Baker Ltd., Dagenham, England.

APPENDIX II CONTINUED

CHEMICALS	SOURCE
11. Pentex-Miles	Miles Laboratories Inc., Bedford, Mass. 01739, U.S.A.
12. Sodium metabisulphite (B.D.H.)	British Drug Houses Ltd., BDH Laboratory Chemicals, Poole, England.
13. Stuart's Basal Medium Bacto. Leptospira Medium, (B.B.M.)	Difco Laboratories, Detroit, Michigan, U.S.A.
14. Whatman DE52 (Diethylaminoethyl cellulose)	Whatman Ltd., Springfield Mill, Maidstone, Kent, England.
15. Sigma	Sigma Chemical Co., P.O. Box 14508, St. Louis, Missouri 63178, U.S.A.

APPENDIX III

PREPARATION OF BUFFER (FORMULA)

1. Phosphate Buffer:

Stock solutions were prepared as follows: -

Solution A: Dissolve 312.02g $NaH_2PO_4.2H_2O$ (M & B) in 2000 ml of distilled water.

Solution B; Dissolve 716.40g $Na_2HPO_4.12H_2O$ (M & B) in 2000 ml of distilled water.

1.0M Phosphate buffer can be prepared by combining x ml of solution A and y ml of solution B.

x	y	pH
45.0	55.0	6.9
39.0	61.0	7.0
33.0	67.0	7.1
28.0	72.0	7.2
23.0	77.0	7.3
19.0	81.0	7.4
16.0	84.0	7.5
13.0	87.0	7.6
10.5	90.5	7.7
8.5	91.5	7.8
7.0	93.0	7.9
5.3	94.7	8.0

0.6M, 0.1M and 0.01M phosphate buffers were prepared by diluting 1.0M buffer with distilled water.

2. Phosphate Buffered Saline (P.B.S.)

0.1M NaCl
0.005M Potassium phosphate
pH 7.2

APPENDIX III CONTINUED

3. PBS/BSA

0.1M NaCl
0.005M Potassium phosphate
1% Bovine serum albumin
pH 7.2

4. Assay Buffer (RIA)

500 ml of 0.9% (w/v) NaCl aqueous solution
500 ml of 0.1M Sodium phosphate pH 7.5
5 ml of 10% (w/v) Sodium azide aqueous solution
2 gm of bovine serum albumin
11 gm of Tween 20

APPENDIX IV

PREPARATION OF JS MEDIUM ACCORDING TO THE METHOD OF JOHNSON AND SEITER (1977)

Analytical grade reagents and double-distilled deionised water were used in the preparation of this medium. Glassware was thoroughly washed in an automatic laboratory washing machine and rinsed with distilled water before being autoclaved at 121°C for 20 minutes.

STOCK SOLUTIONS: Fresh stock solutions of chemicals were prepared for each batch of medium as follows: -

		grams per 100 ml deionised water
NH_4Cl	(B.D.H.)*	25.0
$ZnSO_4.7H_2O$	(M & B) *	0.4
$MgCl_2.6H_2O$	(B.D.H.)	1.5
$CaCl_2.2H_2O$	(B.D.H.)	1.5
$FeSO_4.7H_2O$	(B.D.H.)	0.5
$CuSO_4.5H_2O$	(B.D.H.)	0.3
Sodium pyruvate	(B.D.H.)	10.0
Glycerol	(B.D.H.)	10.0
Tween 80	(Sigma) *	10.0
Thiamine.HCl	(Sigma)	0.5
Cyanocobalamin	(Sigma)	0.02

ALBUMIN SUPPLEMENT: The albumin supplement was prepared by dissolving 20g bovine albumin fraction V powder (Pentex-Miles)* in 100 ml deionised distilled water. While this was stirred the following stock solutions were slowly added: -

$MgCl_2$	2.0 ml
$CaCl_2$	2.0 ml
$ZnSO_4$	2.0 ml
$CuSO_4$	0.2 ml
$FeSO_4$	20.0 ml
Cyanocobalamin	2.0 ml
Tween 80	25.0 ml

* Appendix II

APPENDIX IV CONTINUED

When the powder was completely dissolved the pH was adjusted to 7.4 and the solution brought to a final volume of 200 ml by the addition of deionised distilled water. The solution was then sterilised by filtration using a 0.22um filter (Millipore) and stored in 30 ml batches in sterile glass bottles. The sterility of the solution was checked by the addition of 1 ml albumin supplement to 10 ml nutrient broth and incubation at 37°C for 24 hours, after which time it was examined for bacterial contamination.

BASAL MEDIUM: To 996 ml of deionised distilled water the following were added:

Na_2HPO_4 (anhydrous)	B.D.H.	1.0 g
KH_2PO_4 (anhydrous)	B.D.H.	0.3 g
NaCl	B.D.H.	1.0 g

plus the following stock solutions:

NH_4Cl	1 ml
thiamine	1 ml
sodium pyruvate	1 ml
glycerol	1 ml

The pH of the resulting solution was adjusted to 7.4, and the solution was decanted into 270 ml screw capped bottles which were autoclaved at 121°C for 20 minutes and stored until used.

LIQUID MEDIUM: Liquid medium was prepared by adding 30 ml of albumin supplement to 270 ml of basal medium.

SEMISOLID MEDIUM: Semisolid medium was prepared by adding 0.5 g of agar (Difco, Bacto-Agar) to 270 ml of basal medium. This was autoclaved at 121°C for 20 minutes, then cooled to 56°C before the addition of 30 ml of albumin supplement.

APPENDIX IV CONTINUED

Medium was dispensed in 5 ml aliquots into screw-capped McCartney bottles.

All batches of medium were checked for bacterial contamination by incubation at 37°C for three days and 27°C for three days and then examined.

All new batches of basal medium and albumin supplement were tested to see that they supported the growth of a recent isolate of *hardjo*.

SELECTIVE MEDIUM: Selective media were prepared by the addition of 200 or 400 ug 5FU/ml to liquid or semisolid medium. A stock solution of 5FU (Sigma) was prepared by the addition of 1.0 g of 5FU to 50 ml of distilled water. This was placed in a 56°C water bath to dissolve the 5FU, and the pH was then adjusted to 7.4 - 7.6 by the addition of 1M HCl. The solution was then made up to 100 ml by the addition of deionised distilled water and sterilised by filtration through a 0.22 um filter (Millipore). Twenty ml aliquots of 5FU solution were held at 4°C until required, when they were dissolved by placing in a 56°C water bath prior to their addition to prepared medium. To prepare medium with 200 ug 5FU/ml 6.0 ml of 5FU stock solution were added to 300 ml of medium; and 12.0 ml 5FU were added to 300 ml medium to produce a final concentration of 400 ug/ml.

APPENDIX V

PRODUCTION OF RABBIT HYPERIMMUNE SERUM

The rabbit hyperimmune serum was produced in adult laboratory rabbits according to the method of Tan (1970).

Day 1 - A seven day culture grown in liquid JS was heat-fixed for thirty minutes at 56°C. The culture should contain not less than 10^8 leptospires per ml and four ml of the culture were inoculated into the rabbit through the ear vein.

Day 6 - The above procedure was repeated.

Day 12 - The rabbits were inoculated with a seven-day old, live culture of not less than 10^8 leptospires per ml through the ear vein.

Day 18 - The rabbits were anaesthetised with ether and then bled by cardiac puncture. The blood was let to clot and the serum separated by centrifugation. The serum was then stored in appropriate containers at -20°C.

APPENDIX VI

PRODUCTION OF THE IMMUNOGLOBULIN FRACTIONS

The antibody activity in antisera is predominantly contained in the gammaglobulin (IgG) fraction and for this reason, the IgG fraction was selected to be used as the antibody component of the radioimmunoassay. The method of extracting the fractions is by Whatmans Advanced Ion Exchange Celluloses (diethylaminoethyl-52).*

a) Preparation of the DE-52 column

Whatmans pre-swollen diethylaminoethyl cellulose (DE-52) is chosen for the column. Eighty grams of DE-52 cellulose and 500 ml of 0.02M phosphate buffer of pH 8 were mixed. This mixture has a pH of 10 and it is lowered to pH 8 by adding 0.2M NaH_2PO_4 solution. It is stirred slowly for fifteen minutes. The cellulose is slowly poured into the column and then degassed with the aid of a vacuum device. The column is run overnight with 0.02M phosphate buffer solution.**

b) Elution of the Fractions

The column is firstly standardised by running 0.02M phosphate buffer solution (pH 8) through the column. The sample (2 ml) of rabbit hyperimmune serum to serovar *hardjo* was centrifuged to clear any debris and dialysed in 0.02M phosphate buffer solution overnight before being run through the column. The first fraction eluted with 0.02M phosphate buffer solution is IgG. The other fractions were obtained by elution with 0.03M, 0.05M and 0.1M phosphate buffer solutions.** The fractions were pooled and concentrated with the aid of polyethylene glycol. On completion, the column was washed with IM NaCl and finally with 0.02M phosphate buffer solution.

* Appendix II

** Appendix III

APPENDIX VI CONTINUED

c) Concentrating the Fractions

Individual fractions were pooled into dialysing tubes and placed in a tray. They were then covered with polyethylene glycol for about two hours. The amount of each fraction required was approximately two to three ml.

d) Examination of the Fractions for Purity

Immunoelectrophoresis on agar gels was employed to examine the immunoglobulin fractions for any contaminating or excess protein. Each fraction was placed separately in wells in the gel, whilst goat anti-rabbit serum (Miles Yeda)** was placed in the troughs. The goat anti-rabbit serum acted as the antibody to the fractions. On electrophoresis, the fractions and the antibody diffused into the gel forming precipitin lines where the two met. The IgG fraction produced only one distinct line of precipitation which indicated that the fraction was pure IgG and therefore considered suitable for use as the antibody component of the radioimmunossay.

e) Protein Concentration of the Fractions

The protein concentration of the gammaglobulin fraction was measured using a spectrophotometer (UNICAM).*** The gammaglobulin fraction was found to contain 10 mg of protein per ml.

** Appendix II

*** Appendix I

APPENDIX VII

PREPARATION OF CELLULOSE - HARDJO ANTIBODY COMPLEXES FOR USE AS A SOLID PHASE REAGENT

The procedure followed the method of Wide (1969).

a) Cyanogen bromide (CNBr)* activation of the insoluble polysaccharides.

The cyanogen bromide activation is carried out in a fume cupboard. Five grams of cyanogen bromide is transferred to a flask and distilled water is added until a final concentration of 2.5 percent (w/v) is achieved. The cyanogen bromide solution (40 ml for each gram of polymer) and the cellulose are mixed and the pH of this mixture is raised by adding 1M NaOH solution until pH 10.5 is achieved. The mixture is then filtered with a glass filter having a pore size of one micron. About 500 ml of ice cold distilled water is added to wash the cellulose suspension. The cellulose particles are dried on the glass filter by washing with acetone. A concentration of approximately fifty percent is first used and the concentration is gradually increased until pure acetone is finally used. The acetone is allowed to evaporate at room temperature, thus leaving the cellulose dry. The dry powder is weighed and then stored at $-20^{o}C$.

b) Coupling of the *hardjo* antibody to the activated polymer.

For every 100 mg of activated cellulose, 0.1 ml of the *hardjo* antibody and 0.4 ml of 0.1 M $NaHCO_3$ solution were added. The mixture is mixed by rotating slowly for one to three days at $+4^{o}C$ or for 24 hours at room temperature. This immunosorbent material is first washed by adding two ml of 0.5M $NaHCO_3$, mixed by rotation and poured into a centrifuge tube. This washing procedure is repeated

* Appendix II

APPENDIX VII CONTINUED

twice and then it is made up to ten ml with 0.5M $NaHCO_3$ solution and mixed by rotation for twenty minutes. It is then centrifuged, the supernatant discarded and ten ml of 0.5M $NaHCO_3$ solution added. The washing procedure is repeated as before. The particles are now washed with ten ml of an 0.1M acetate buffer of pH 4 for one hour. During a second wash with this buffer and before the solution is rotated, it is homogenised for ten seconds in an ultrasonic disintegrator (Soniprobe).* Finally, it is rotated for 18 to 20 hours. The particles are now washed twice in ten ml of the assay buffer. A final centrifugation and decantation of the supernatant is followed by the addition of ten ml of assay buffer. This concentrated stock solution is ready for use. It may be kept at $4^{o}C$ if it is going to be used immediately or stored at $-20^{o}C$.

* Appendix I

APPENDIX VIII

RADIO-LABELLING OF THE GAMMAGLOBULIN (ANTI-HARDJO IgG) WITH SODIUM IODIDE (^{125}I)

A quantity (2 mg) of gammaglobulin in 0.2 ml of 0.05M phosphate buffer solution (pH 7) was labelled with one mCi of ^{125}I (IMS-30)* by the method of McConahey and Dixon (1966). The reaction took place in the microvial supplied by Amersham and in a fumehood. This method involved the oxidation of the mixture of IgG and ^{125}I with Chloramine-T (BDH)*. After the addition of Chloramine-T, an equal amount of Sodium metabisulphite (BDH)* was added to neutralise any remaining oxidising agent and stop the reaction. Non-protein bound iodide was removed by dialyis in the cold against phosphate buffered saline. The final product was diluted to ten ml with PBS/BSA in plastic tubes and kept at $4^{o}C$ until being used.

Procedure: -

Rabbit gammaglobulin	2 mg in buffer
^{125}I (Amersham)	1 mCi
Chloramine-T (pH 7.5)	50 ug in 0.1 ml buffer

Wait 2 minutes

Sodium metabisulphite (pH 7.5) 50 ug in 0.1 ml buffer

Dialyse three times against PBS (0.1M NaCl and 0.005M Potassium phosphate, pH 7.2)

Dilute to 10 ml with PBS/BSA (1%)

Chloramine-T and Sodium metabisulphite solutions.
50ug of above reagent / 0.1 ml of buffer
500 ug / 1 ml
5 mg /10 ml
10 mg /20 ml of buffer (make fresh)

* Appendix II

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