LEPTOSPIROSIS IN THE PIG

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SUMMARY

A study was made of leptospiral infection in pigs. The epidemiology of this disease and whether or not cell mediated immunity (CMI) is part of the immune response of pigs to *pomona* infection were investigated.

A number of serological techniques were used in this study. In a trial to measure the precision of the serological method, it was found that 93% of the sera tested varied by only $\pm$1 doubling dilution. An instrument was developed to aid the removal of samples from serology test-plates for darkfield examination and it was shown that the titres determined using this instrument were no different from those using the traditional capillary tube method. It was also found that the MA titres of sera from conventionally collected blood samples were the same as those estimated on sera collected from heart clots.

The isolation and identification of leptospires was used to define the epidemiology of leptospirosis in pigs. An efficient cultural method was developed, utilising a stomacher to homogenise kidneys. P80 medium was found to be superior to Stuart's and Fletcher's medium, with respect to the isolation of leptospires from pig kidneys.

A cultural and serological survey of leptospirosis was conducted in young and adult pigs from an abattoir. Serovar *pomona* was isolated from 38/84 (45%) of the kidneys from the young pigs, and from 1/65 (2%) of the adult kidneys. However 87% of the young pigs and 86% of the adult pigs were serologically positive at 1/12 to *pomona*. In this survey serovar *tarassovi* was isolated for the first time in New Zealand. In contrast to *pomona*, the prevalence of *tarassovi* culture-positive animals was 1/84 (1%) in the young group and 3/65 (5%) in the adult pigs. Twenty-one percent of the

young pigs and 25% of the adult pigs had MA titres to *tarassovi* of 1/12 or more. Although many of the sera reacted with serovars other than *pomona* and *tarassovi*, no other serovars were isolated. It was concluded that pigs in New Zealand are reservoir hosts for *pomona* and *tarassovi*, but that they do not become life-long carriers of these serovars. The MA titres to these serovars are maintained long after infection has been eliminated. It was also considered that *pomona* antibody cross-reacts with many other serovars. In this investigation it was found that the genus-specific microscopic agglutination test (MAT) using serovar *biflexa* (CDC) as antigen was of no value for detecting either infected pigs, or pigs which had titres to parasitic leptospires. It was also found that in many of the kidneys from which *pomona* was isolated either no gross leptospiral-like lesions were apparent, or only minor lesions were observed. It was concluded that infected kidneys that had no gross lesions constituted a potential public health risk.

Using abattoir samples, a number of tests were evaluated with respect to the ability to predict renal infection with serovar *pomona*. It was concluded that in a population with a moderate to high prevalence of infection, at a MA titre of 1/384 both the sensitivity and specificity will be approximately 85%. The usefulness of darkfield examination of urine and urine culture was limited because of low sensitivities of these parameters. In the sample collected from juvenile animals, 67% of the culture-positive animals had urine homologous MA titres of 1/4 or more, and 95% of the culture-negative animals were test-negative at this level. However, as older recovered animals were not included in this survey, it was considered that the specificity of this parameter was over-estimated.

The pattern of leptospiral infection in a pig herd was studied. This was a part of a collaborative study of leptospirosis infection in pigs, cattle and wildlife in this area. It was concluded that serovar *pomona* infection was endemic in this pig herd, and that the focus of infection was in those pigs aged between 6 and 12 months. The sera of the *pomona* infected pigs cross-reacted with many other serovars, and some marked paradoxical heterologous titres to *copenhageni* were observed. There was no evidence of current infection in the older sows nor in the cattle or wildlife in this area. It was concluded that pig to pig transmission of *pomona* infection occurred, and this was facilitated by the system of management, and the design of the buildings used to house the young stock.

Serological surveys were conducted in two other pig herds where serovar *tarassovi* had previously been isolated. There was serological evidence of extensive infection with both *pomona* and *tarassovi* in both herds. The younger animals were found to be the main reservoir of infection for both these serovars. There was no evidence of any reciprocal cross-protection between *pomona* and *tarassovi*.

A further serological survey of leptospirosis in pigs was conducted, using sera from 234 adult pigs selected from all major districts in New Zealand. It was concluded that infection with serovars *pomona* and *tarassovi* occurred commonly and it was estimated that 53% of the animals sampled had been infected with *pomona* and 33% with *tarassovi*. The prevalence of both *pomona* and *tarassovi* infection is higher in the North Island than in the South Island. *Pomona* infected herds are the most common, followed by herds infected by both *pomona* and *tarassovi*, with *tarassovi* infection alone being the least common. There was no evidence that infection with serovars other than *pomona* or *tarassovi* commonly occurs in pigs in New Zealand.

An abortion storm in pigs was investigated and shown to be due to *pomona* infection. In total 22% of the mated sows aborted. Leptospires were isolated from many tissues from the aborted piglets, but the most convenient tissue to use was vitreous humor. In these abortions a marked acute placentitis was observed. Antibiotic therapy did not prevent abortions.

In vitro lymphocyte transformation was used to investigate whether or not cell mediated immunity (CMI) was present in pigs that had been naturally infected with *pomona*. Lymphocyte microcultures from 18 pigs from a herd known to be endemically infected with *pomona* were prepared. Cells were stimulated with phytohaemagglutinin (PHA), a sonicated *pomona* extract and a sodium deoxycholate-derived *pomona* antigen. The time-responses of antigen stimulated cells over 144 hours were also studied. The PHA responses of the older pigs which had been infected with *pomona* were the same as those of the younger non-infected pigs. The activities of the non-stimulated cultures of these groups were also the same. The cells of all the animals were also transformed *in vitro* by the antigen extracts with dose-responses occurring in most cases. However the maximum responses of the pigs which had been infected with *pomona* were significantly greater than those which had not been infected. It was concluded that transformation in response to the *pomona* antigens had occurred in the cell cultures from animals which had been infected, suggesting that CMI is part of the immune response of the pig to *pomona* infection. It was also considered that the antigen extracts contained a low concentration of non-specific mitogens.

In a further study of these *in vitro* lymphocyte activities, the responses of B cell-depleted cultures to the *pomona* antigen extracts were investigated. The removal of B cells from porcine blood lymphocytes was achieved by nylon wool fractionation and confirmed by enumerating the cells exhibiting surface immunoglobulin. Although the

non-fractionated cells responded to both antigen extracts the eluted cells did not. Supplementation of cultures of eluted cells with adherent cells did not increase the level of transformation achieved. It was therefore concluded that the antigen-reactive cells in this *in vitro* system were probably B cells. Thus, the postulate that CMI is part of the immune response of the pig to infection with serovar *pomona* was not confirmed. However these findings were in accord with the observation that the essence of the resistance to infection with the parasitic leptospires lay with the ability of the animal to secrete antibody soon after infection.

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INTRODUCTION

The chapters that follow a review of the literature, describe firstly the serological and culture techniques which were used to study leptospiral infection in pigs, and secondly an attempt to define the epidemiology of leptospirosis in pigs in New Zealand. After this an investigation of cell mediated immunity in pigs which had been infected with serovar *pomona* is described. During the study a number of experiments were conducted with the aim of defining the optimal conditions of cell culture for these transformation experiments, and a number of these have been reported in the appendices.

CHAPTER I

REVIEW : PORCINE LEPTOSPIROSIS

INTRODUCTION

Leptospires are bacteria with a characteristic helical morphology (Hovind-Hougen, 1976), and with the other spirochaetes are incorporated in the Family *Spirochaetacea*. Although this study is concerned solely with parasitic organisms it should be noted that most of the leptospires are saprophytes (Smibert, 1974; Willcox, 1976). Two leptospiral complexes, *biflexa* and *interrogans*, incorporating the saprophytes and parasites respectively, have been proposed, but there are no completely reliable methods to distinguish leptospires on this basis, and therefore all leptospires are currently considered to be one species, *Leptospira interrogans* (Turner, 1976). Stable antigenic leptospiral variants have been isolated, and those which cross agglutinate at high titre with one another's antisera are regarded as belonging to the same serogroup. Furthermore, two strains are considered to be different serovars or serotypes, if after absorption with the heterologous strain at least 10% of the homologous titre remains present in each of the two antisera (W.H.O., 1967).

Although leptospirosis in pigs was not recorded until approximately 20 years after the discovery of parasitic leptospires (Klarenbeek and Winsser, 1937), infection in pigs has since been frequently reported in many countries (Alexander, *et al.*, 1964). The serovars most commonly isolated from pigs throughout the world are *pomona*, *tarassovi*, *canicola* and *copenhageni*, but there are also reports of at least another 16 serovars being occasionally cultivated from pig tissues (see Table 1).

GENERAL CONCEPTS OF LEPTOSPIROSIS

It has been observed that particular leptospiral serovars are frequently isolated from certain animal species; for example, in many countries serovar *copenhageni* has been isolated from *Rattus norvegicus*, serovar *ballum* from *Mus musculus*, serovar *pomona* from pigs, and serovar *hardjo* from cattle (U.S. Dept of Health, Education and Welfare, 1966, 1975). Evidence indicates that these animals become chronically infected with the particular serovar and excrete organisms in their urine over a long period (Willcox, 1976). Where such a situation prevails it has been proposed that the animal should be termed a reservoir or maintenance host of that particular serovar (Roth *et al.*, 1963).

This relationship between an animal and a serovar is considered to be the end result of a process of biological adaptation in which a saprophytic leptospire adopted a particular parasitic niche. It is suggested that during this process each serovar evolved its unique antigenic structure (van der Hoeden, 1958, Willcox, 1976). In other words, serovars, as they are known today, are the result of host-parasite adaptation.

It may be concluded from the high frequency with which particular serovars are isolated from their reservoir hosts as compared with their frequency of isolation from other animals, that serovars spread primarily among the members of their reservoir host population. They may spread to other populations, but are not maintained within them. Roth *et al.*, (1963) have proposed that these animal species which are only occasionally infected be termed accidental or incidental hosts for the particular serovars in question. As no leptospires have been reported as being maintained in human populations, man must be considered an incidental host of all pathogenic serovars.

In order to understand the dynamics of leptospiral infection both within and between these animal populations, the host-parasite or intrinsic factors, and various environmental or extrinsic factors must be examined. The intrinsic factors will be considered first.

The course of leptospiral infections in all animals and with all serovars is essentially the same. In the chronic phase of leptospirosis, leptospires are excreted in the urine, and susceptible animals become infected either directly from this source, or indirectly via the environment (Turner, 1967; Hanson, 1976). Animals have been successfully infected experimentally by inoculating leptospires on to abraded skin (Michna, 1965; Hathaway, 1978), the nasal mucosa (Ferguson and Powers, 1956; Chaudary *et al.*, 1966 a, b; Golota *et al.*, 1964) and oral mucosa (Chaudary *et al.*, 1966 a), and there is ample chance for this to occur naturally. Although considered to be of less significance, venereal transmission has been conclusively demonstrated in cattle (Sleight and Williams, 1961) and there is circumstantial evidence that this mode of transmission occurs in mice (Kemenes and Széky, 1966). Transmission *via* blood sucking ectoparasites has been demonstrated under laboratory conditions (Burgdorfer, 1956; Schlossberger and Langbein, 1952) but this is probably of no importance in field infections (Turner, 1967). One requirement of the host-parasite relationship between a serovar and a reservoir host, is that the reservoir host be highly susceptible to infection by the serovar, thus ensuring that the infection is maintained.

Two to 16 days after infection a leptospiraemia occurs, and this period of the disease is usually termed the acute phase. During this period many different clinical manifestations have been reported, depending on the animal species and the serovar involved (Hanson, 1976). A leptospiraemia followed by kidney colonisation must occur if the cycle of infection within a reservoir host

population is to continue, and to avoid an end host situation it is essential that the animal does not die as a result of infection. In the case of some wildlife species even a mild debility could result in death due to secondary factors, such as predation.

The clearance of leptospires from the blood coincides with the rise in specific humoral antibody and this signifies the start of the leptospiruric or chronic phase of the disease (Faine, 1962). The number of viable leptospires shed in the urine and the duration of leptospiruria vary considerably not only between serovars in one species, but also between animals of the same species (Hanson, 1976). It may last a matter of days or it can continue for months or even years, and the number of organisms excreted and the period of shedding are important features in the epidemiology of leptospirosis (Roth *et al.*, 1963).

Various facets of the host-parasite relationship have been examined experimentally; for example Chernuka *et al.*, (1974) found that there were considerable differences in the ability of a number of serovars not only to infect various feral animals, but also in the ability to colonise the renal tubules and produce leptospiruria. This was taken as confirmation that particular serovars were adapted to certain animal hosts. Likewise the *in vitro* susceptibility of various serovars to complement-mediated lysis appears related to the ability of a serovar to parasitise an animal (Johnson and Muschel, 1966). It was found that saprophytic leptospires were rapidly lysed by normal serum with complement, whereas parasitic leptospires were resistant to this nonspecific immune mechanism. It was concluded by these authors that these results were in accord with other findings among gram-negative enteric bacteria, which suggested an association between serum susceptibility and virulence (Muschel, 1960). It can be hypothesised that parasitic leptospires have evolved a mechanism to resist these nonspecific leptospiricidal

factors. There is also evidence that the presence of specific antibody in urine affects the number of viable leptospires shed (Morse *et al.*, 1958).

The continued maintenance of a serovar in an animal population depends on extrinsic or environmental factors as well as the intrinsic host-parasite interaction. In feral animals Roth *et al.*, (1963) suggest that population density has an affect on the spread of infection. In domestic animals, the general livestock management and the design of the buildings used to house animals are important factors, because they may influence the direct or indirect contact that occurs between infected and susceptible animals (Buddle and Hodges, 1977).

An environment contaminated with leptospires is considered to be a major source of infection (Turner, 1967; Willcox, 1976), and therefore the survival of the organism outside the host will markedly affect the spread of infection both between reservoir hosts and from reservoir hosts to accidental hosts. Leptospires do not form spores and are more susceptible to noxious, physical and chemical influences than many other bacteria (van der Hoeden, 1958). They will not survive dessication, and they lose viability at extremes of pH and temperature (Okazaki and Ringen, 1957; Gorden-Smith and Turner, 1961; Ryu and Liu, 1966). However, these last two groups of workers reported that susceptibility of leptospires to these physical conditions varies to some degree with each serovar, and for this reason environmental conditions could be expected to influence the prevalence of serovars in particular geographical areas.

In summary, therefore, the result of this host-parasite-environment interaction is the maintenance of a serovar by particular animal populations. Where there is an abundance of animal species and conditions are favourable for transmission there would be expected to be the opportunity for leptospires to become adapted to a number

of animals (Cernuha and Kokovin, 1967). This situation occurs in many tropical areas, and it is indeed here that many of the 116 recognised serovars (Turner 1976) have been isolated (U.S. Dept of Health, Education and Welfare, 1966, 1975).

In the field, the interaction between animal populations and various leptospiral serovars is likely to be very complex (Roth *et al.*, 1963). An animal species may be the reservoir host for one or more serovars and the incidental host for yet other leptospiral serovars. Furthermore, more than one reservoir host for a particular serovar may occur in the same geographical area, and the prevalence of infection in each is unlikely to be the same. Thus, major and minor reservoir hosts may be identified in one area.

In view of the fact that the course of infection in all animals, and with all serovars is essentially similar, Roth *et al.* (1963) consider that evidence of present or past infection, as determined by cultural and serological methods respectively, provides a means of determining which animals are important reservoir hosts for leptospires. These authors considered that a high prevalence of renal infection, as determined by kidney culture, not exceeded by the serological evidence of infection with that serovar (the serological rate) suggests persistent infection with little or no mortality, features of a reservoir host. In contrast a high serological rate, many times greater than the kidney culture rate, suggests mild transitory infection as would occur in an incidental host.

SEROVAR *POMONA* INFECTION

Serovar *pomona* was originally isolated from a dairy farmer in Queensland, Australia, in the late 1930's (Clayton *et al.*, 1937) and the first isolation from pigs is attributed to Mochtar (1940) who was working in Batavia (now Jakata). Shortly after this, Gsell (1952) incriminated *pomona* as the aetiological agent of "Swineherd's Disease" and described infection both in man and pigs.

Of all the leptospiral infections of pigs, serovar *pomona* infection has been the most frequently reported (see Table 1), and the best documented. The course of this infection in pigs indicates that there is a high degree of host-parasite adaptation and pigs are recognised all over the world as important reservoir hosts of *pomona*.

(a) Infection and Pathogensis

Pigs are very easy to infect experimentally with *pomona*. Successful infections have resulted from the inoculation of organisms into the eye, nose, mouth and vagina (Ferguson and Powers, 1956; Golota *et al.*, 1964; Chandhary *et al.*, 1966 a, b) with as few as 100 leptospires in some cases being sufficient to initiate infection (Chaudhary *et al.*, 1966 a, b).

Leptospiraemia occurs between 2 and 10 days after infection and during this acute phase leptospires can be isolated not only from the blood but also from the liver, spleen, kidney, brain and spinal cord (Morse *et al.*, 1958; Sleight and Lundberg, 1961; Ferguson and Powers, 1956; Chaudhary *et al.*, 1966 a, b).

In contrast to sheep and cattle, in which a haemolytic anaemia is often seen (Hanson, 1976), in the leptospiraemic phase of *pomona* infection in pigs no outstanding haematological changes occur (Ferguson and Powers, 1956;

Morse *et al.*, 1958). However, during these experimental studies it was observed that some pigs exhibited a slight neutrophilia and band cells entered the circulation.

During the acute phase of the infection, the only consistent clinical manifestation reported is a mild fever lasting 1 to 2 days. Some animals may also appear depressed and/or anorexic (Ferguson and Powers, 1956; Morse *et al.*, 1958; Fennestad and Borg-Peterson, 1966). In most cases the symptoms reported were so benign that they would not normally have been noticed.

Leptospires first appear in the urine 10 to 20 days after infection (Ryley and Simmonds, 1954 b; Fennestad and Borg-Peterson, 1966; Hodges, 1973; Hodges *et al.*, 1976 and maximum shedding of organisms is observed for 1 to 3 weeks after this (Morse *et al.*, 1958; Hodges, 1973). During this early period very large numbers of viable leptospires may be excreted (Morse *et al.*, 1958). Following this period of marked leptospiruria, small numbers of leptospires are shed intermittently for 10 to 50 days (Ryley and Simmonds, 1954 a, b; Mitchell *et al.*, 1966; Hodges, 1973), but exceptional cases of intermittent shedding for as long as 16 months have been reported (Mitchell *et al.*, 1966). This chronic renal infection does not appear to affect the general health of pigs, and the weight gain of young pigs is not adversely affected (Morse *et al.*, 1958).

(b) Urine Changes

The urine of pigs infected with serovar *pomona* has not been reported as being outstandingly different to that of uninfected pigs (Morse *et al.*, 1958). During the acute phase some pigs show a positive occult blood test (Sleight *et al.*, 1960) and Miller *et al.* (1977) found that infected pigs consistently had more dilute urine than non-infected controls. It was concluded that this

dilution effect was not the result of renal damage, but was secondary to a greater water consumption by the infected animals. A reason for this change in water intake was not suggested by these workers.

The changes in homologous urinary microscopic agglutination (MA) titres have been studied in detail by Morse *et al.*, (1958). Although it appears that an irregular number of the infected animals was sampled at various times, it would seem from this report that in most cases *pomona* agglutinating antibody, at titres of 1/5 to 1/10, was first detected 2 to 3 months after infection, and that the highest titres (1/1000) occurred after 3 to 4 months. Urine MA titres were still demonstrable, although low, after 1 year. The urine titres were not correlated to the serum MA titres and on this evidence it was concluded that the urinary antibody was not a result of leakage of antibody from the circulation, but was excreted by "reticuloendothelial" cells within the kidney.

(c) Serum Antibody Response

The changes in serum MA titres in experimentally infected pigs have been followed by Ryley and Simmonds (1954 b), Ferguson and Powers (1956), Morse *et al.*, (1958), Fennestad and Borg-Peterson (1966), and Hodges (1973). In addition Mitchell *et al.*, (1966) monitored the MA titres of 8 sows for 14 months after natural *pomona* infection. From these reports it would seem that low concentrations of serum agglutinins (titres of 1/20 to 1/200) first appear about 1 week after infection, and rise to maximum concentrations (titres of 1/1000 to 1/80 000) over 3 to 4 weeks. However, MA titres as high as $1/10^7$ and $1/10^8$ have been recorded in this period (Morse *et al.*, 1958; Sleight *et al.*, 1960). Titres then gradually subside to between 1/100 and 1/1000 and may remain at this level for at least one year.

Complement-fixing antibodies also appear one to two weeks after infection but have disappeared by 6 to 7 weeks (Hodges, 1973). It was therefore suggested by this author that the complement-fixation test might be useful in detecting new infections.

(d) Pathology

In pigs killed during the acute phase of *pomona* infections, only minor lesions have been found. Petechial and ecchymotic haemorrhages may be seen in the lungs, and the livers may be slightly pale and friable (Burnstein and Baker, 1954). The only consistently recorded histological lesions are slight renal tubular damage and infiltration by a small number of lymphocytes in the kidney (Burnstein and Baker, 1954; Sleight *et al.*, 1960; Chaudhary *et al.*, 1966 a). However, a meningoencephalitis involving principally the cerebrum and lymphocytic infiltration of the adrenals have also been recorded by Sleight *et al.* (1960), and focal liver necrosis was found by other workers (Burnstein and Baker, 1954).

In the chronic phase of *pomona* infection, lesions have been found only in the kidneys and renal lymph nodes. The gross and microscopic changes that occur have been described in detail by Langham *et al.* (1958) and to a lesser extent by Burnstein and Baker (1954). In essence small greyish white spots 1 to 2 mm in diameter develop on the surface of the kidney approximately 4 weeks after infection, and these gradually increase in number and size to involve areas of both the cortex and medulla. It has been shown histologically that the white foci consist predominantly of lymphocytes, plasma cells and macrophages, and these cells are found infiltrating between the renal tubules and surrounding blood vessels. In addition, segments of the nephron become atrophic and necrotic and in many instances are completely destroyed. Glomeruli are reported as exhibiting only minor changes, but it should be noted that

no ultrastructural studies of glomeruli have been conducted. The lesions described have been considered to be those of a mild progressive interstitial nephritis. The reported enlargement of the renal lymph nodes has been shown histologically to be due to oedema.

(e) Effect on Fertility

Serovar *pomona* is considered to be a common cause of abortions in pigs (Dunne, 1970). Bryan *et al.* (1953) appears to have been the first to have actually isolated *pomona* from aborted piglets and it has since been associated with abortions in pigs by many other workers, including Bohl *et al.*(1954), Ryley and Simmonds (1954 a), Powers *et al.* (1956), and Mitchell *et al.* (1966). In New Zealand serovar *pomona* infection has been frequently reported as being the likely cause of abortions in pigs (Anon., 1957, 1958, 1960 b, 1961, 1974). However, in some of these reports the leptospires isolated from the aborted tissues were not fully identified.

The case histories of 7 pig herds in which it was considered that *pomona* infection was responsible for abortions have been reviewed by Powers *et al.* (1956). The abortions usually occurred in the last three weeks of gestation, typically whole litters of dead piglets were expelled, sometimes a few of the foetuses were mummified, and occasionally one or two piglets were alive at birth but died within 48 hours. Where there were abortions there was usually a history of pigs being recently brought into the herd, and "abortion storms" with from 20% to 100% of sows aborting over a short period were not uncommon.

Experimental infection of pregnant sows has not consistently led to reproductive failure. Pigs infected early in their pregnancies farrow normal litters (Ferguson and Powers, 1956), and sows in the chronic phase of leptospirosis appear to be able to breed quite normally

(Mitchell *et al.*, 1966). Ferguson and Powers (1956) also infused serovar *pomona* into the uteri of 6 gilts around the time that they were mated. This resulted in renal infection, as indicated by leptospiruria, in 4 of the 6 animals, but did not appear to have any affect on their reproductive performance.

However, abortions similar to those observed in field cases have been reported following experimental infection of sows with *pomona* in late pregnancy. In these cases dead piglets were generally expelled 1 to 4 weeks after the termination of the leptospiraemic phase (Ryley and Simmonds, 1954 a,b; Fennestad and Borg-Peterson, 1966). In some experiments, expulsion of foetuses and membranes was not observed, but the sows suddenly came into oestrus shortly before their expected farrowing time (Ferguson and Powers, 1956). Breeding behaviour similar to this has also been observed in *pomona* epidemics in breeding herds (Powers *et al.*, 1956) and foetal death with resorption has been suggested as an explanation for this phenomenon.

The only consistent lesions seen in aborted foetuses are focal areas of necrosis 1 to 4 mm diameter in the liver. In cases where foetal death has occurred before abortion, the piglets are autolysed and excess clear or blood-tinged fluid may be seen in the pleural and peritoneal cavities (Ryley and Simmonds, 1954 b; Fennestad and Borg-Peterson, 1966). Jubb and Kennedy (1970) also report that a focal myocarditis with coagulative necrosis and a mononuclear cell infiltrate may occur. In addition, there may be mononuclear cell infiltrates beneath the epicardium and endocardium, and many minor foci of interstitial nephritis.

The chorio-allantoic portion of the foetal membranes is usually thickened and oedematous, or maybe necrotic and brown (Ryley and Simmonds, 1954 b).

Following a *pomona* abortion there is no impairment of fertility even though the sows may still be shedding leptospires in their urine (Mitchell *et al.* 1966) and field observations suggest that once a sow has aborted it will not abort again (Powers *et al.*, 1956).

The results of an experimental infection (Tammemagi *et al.*, 1961) and field observations (Burki and Wisemann, 1963) suggest that *pomona* infection does not affect the fertility of boars, but it would appear that they may act as an important source of infection for other pigs (Powers *et al.*, 1956).

(f) The epidemiology of *pomona* infection in pigs

Although the pig is recognised as a major maintenance host for *pomona*, other important reservoir hosts have also been demonstrated. In some areas *pomona* appears to be maintained within cattle populations (Cernuha and Kokovin, 1967), while in others various feral animal populations are epidemiologically important. For example in North America the skunk is a major reservoir host of *pomona* (Roth *et al.*, 1963; Mitchell *et al.*, 1966), and in Denmark the field mouse, *Apodemus agrarius*, acts as an important source of infection (Borg-Peterson and Fennestad 1956).

The transmission of *pomona* between various reservoir hosts does not appear to have been investigated in detail. Burnstein and Baker (1954) studied the relationship between cattle and pigs, and they reported that *pomona* infected calves shed small numbers of leptospires and were unable to transmit infection to susceptible swine with which they were housed. In contrast, infected swine easily passed *pomona* to susceptible calves indicating that pigs are more significant sources of infection than cattle.

There have been few detailed investigations of the epidemiology of *pomona* infection within pig herds. It has been observed that infection in piglets in the first 2 to 3 months of their life is rare, even when their mothers are excreting leptospires. This protection is due to colostral-derived antibody (Fish *et al.*, 1963; Chaudary *et al.*, 1966 b) and when this antibody wanes piglets can then become infected from the sow (Fish *et al.*, 1963). The duration of this protection is dependent upon the amount of colostral antibody which is transferred to the piglet at birth, and the amount of antibody in colostrum is directly related to the serum antibody concentration of the sow (Chaudary *et al.*, 1966 b).

The pattern of infection of *pomona* in young pigs on an intensively managed farm was examined by Buddle and Hodges (1977). They observed that infection was limited to a particular pig house where the effluent drainage was such that newly introduced susceptible stock were exposed to the urine of older animals. By protecting the introduced animals from this effluent, infection could be avoided. They concluded that infected animals had been introduced into this house at some earlier date and that the system of effluent drainage was responsible for the continuance of infection. As in the other studies, no infection was found in piglets from birth to weaning.

Venereal transmission in pigs has not yet been conclusively demonstrated. In one experiment a boar which was shedding *pomona* failed to infect the susceptible gilts with which he was mated (Tammemagi *et al.*, 1961). The four boars that were infected in this project began excreting leptospires later than usual, (25, 28 and 31 days), and only passed small numbers of organisms, many of which were non-motile, over a period of 2 to 3 weeks. It is not clear whether this was due to a *pomona* strain of low virulence or whether the course of infection in boars is different from that in other classes of pigs.

As gilts have been successfully infected by infusing *pomona* into the vagina (Ferguson and Powers, 1956) it would seem that venereal transmission can indeed occur.

SEROVAR *TARASSOVI* INFECTION

Known at first as *Leptospira mitis* (Johnson, 1942), then as *Leptospira hyos* and now as *Leptospira interrogans* serovar *tarassovi* (Turner, 1967), this leptospiral strain has been isolated from animals throughout the world (U.S. Dept of Health, Education and Welfare, 1966, 1975). Like *pomona*, it has been frequently isolated from pigs (see Table 1), and appears very well adapted to this species. It has, however, been isolated from many wild-life species including *Rattus rattus* and *Rattus norvegicus*.

Sows have been successfully infected experimentally with *tarassovi* (Ryley and Simmonds, 1954; Tammemagi and Simmonds, 1956, 1958) and it was found that the course of *tarassovi* infection in the pig, like *pomona*, is essentially asymptomatic. Some animals had a slight fever during the leptospiraemic phase, but this was not a constant feature. Leptospiruria began 2 to 3 weeks after infection and intermittent shedding of leptospires for periods from 10 to 70 days were recorded. Agglutinins at titres of approximately 1/100 usually appeared one to two weeks after infection, rose to 1/300 to 1/1000 over the next month and then declined to 1/100 to 1/300, and remained at this level for a long period.

Although the clinical symptoms associated with serovar *tarassovi* infection are benign, there is considerable evidence from field studies that infection of pregnant sows can result in abortions, stillbirths and high neonatal mortality (Baryshev and Drozhzhin, 1963; Kemenes and Szeky, 1970; Tammemagi and Simmonds, 1958; Anon., 1976). This has not yet been confirmed by the experimental infection of pregnant sows and gilts

(Ryley and Simmonds, 1954; Tammemagi and Simmonds, 1956, 1958). There appears to be nothing published on the spread of infection from other *tarassovi* hosts to pigs, or from pigs to other animals. Similarly the epidemiology of *tarassovi* infection within pig herds has not been studied in any detail.

SEROVAR *CANICOLA* INFECTION

Among the domestic animals the dog is traditionally considered to be the reservoir host of serovar *canicola* (van der Hoeden, 1958). There are, however, a number of reports of *canicola* being maintained within pig populations for long periods independently of dogs (Seiler *et al.*, 1956; Coghlan *et al.*, 1957; Michna, 1962; Shenberg *et al.*, 1977).

Canicola appears to be very well adapted to the pig. During the acute phase of infection there may be a febrile response, and some animals may have a white or blood tinged mucoid vaginal discharge (Michna, 1962). However, in most instances, as with *pomona* and *tarassovi*, the course of *canicola* infection in the pig is largely asymptomatic (Michna, 1965; Coghlan *et al.*, 1957). This is in contrast to the severe illness, "Stuttgart's Disease," that usually occurs in dogs after *canicola* infection (van der Hoeden, 1958).

It has been demonstrated experimentally that the pig is very susceptible to infection with serovar *canicola*, and that they excrete organisms for up to 90 days after infection (Coghlan *et al.*, 1957; Michna, 1962, 1965). As is seen with *pomona*, homologous agglutinating titres may rise as high as 1/3000 or 1/30 000 in the early stages of infection (Michna, 1962, 1965; Kemenes *et al.*, 1962).

SEROVAR *COPENHAGENI* INFECTION

Serovar *copenhageni*, the aetiological agent of Weil's Disease in man (Alston and Broom, 1958), has been isolated quite frequently from pigs (Table 1) and *copenhageni* titres have been found in numerous serological surveys (Alexander *et al.*, 1964). It should be noted, however, that in the early stages of *pomona* infection, paradoxical heterologous titres to *copenhageni* may occur (Morse and Allen, 1956) and therefore, as *pomona* infection in pigs is very common in many countries, this serological data may not reflect the true prevalence of *copenhageni* infection.

Although there is scant data concerning the course of *copenhageni* infection in pigs, it does appear to be different from that described for *pomona, tarassovi* and *canicola*. A severe illness has been reported in the acute phase with infected pigs often dying (Field and Sellers, 1951; Nisbet, 1951; Gilka, 1957). There is insufficient information to deduce the degree of adaptation of *copenhageni* to pigs. It is possible that the pig, like man, is essentially an accidental host. The frequent reports of *copenhageni* infection in pigs may simply reflect the close physical association between pigs and *Rattus norvegicus*, the major maintenance host for this serovar (van der Hoeden, 1958).

INFECTION WITH OTHER SEROVARS

Serovar *grippotyphosa* has been implicated in abortion storms in pigs in North America and it has been demonstrated that infection late in pregnancy will induce an abortion similar to that seen with *pomona* (Hanson, 1971). This serovar is associated primarily with various feral rodent populations and occasionally with cattle (U.S. Dept of Health, Education and Welfare, 1966, 1975), and there are no reports of pig populations maintaining *grippotyphosa* for long periods.

Woods *et al.* (1962) attempted to infect pigs experimentally with serovar *ballum*. Many wildlife species were known to be infected with this serovar, and it was considered by Woods *et al.* that pigs were likely to be exposed to infection. However, in this study there was no evidence that infection was established, and it was concluded that the strain of *ballum* used had lost its virulence. In an extensive survey in Czechoslovakia, Kmety *et al.* (1956) isolated serovar *ballum* from the kidneys of a healthy pig, indicating that natural infection may occur.

Fennestad and Borg-Peterson (1966) have also tried to infect pregnant sows with serovars *sejroe* and *saxkoebing*. Previous serological surveys had indicated that pigs in Denmark became infected with these serovars. Both of the sows infected with serovar *sejroe* had a fever and were depressed 2 to 4 days after infection, and their homologous MA titres rose to 1/3000. However, leptospiruria was not observed in either animal. One sow farrowed 4 stillborn, 2 weak and 6 normal piglets, whereas the other farrowed 1 stillborn, 1 weak and 5 normal piglets. Although leptospires were not cultured from any piglets, they were seen histologically in the tissues of stillborn piglets from both litters. The sows inoculated with *saxkoebing* did not show any clinical signs indicative of a septicaemia, did not exhibit leptospiruria, and developed homologous titres of only 1/300 and 1/1000. These animals farrowed normal healthy litters.

LEPTOSPIROSIS IN NEW ZEALAND

Kirschner and Grey isolated serovar *copenhageni* from rats in 1951, and at about the same time *pomona* was isolated from cattle (Anon., 1951). Up to the present time four other serovars, *hardjo* (Lake, 1973), *ballum* (Anon., 1967), *tarassovi* (Ryan and Marshall, 1976) and *balcanica* (Marshall *et al.*, 1976) have been reported as occurring in this country. A summary of the serovars

which have been isolated from man, domestic and feral animals is presented in Table 2.

The occupational group most affected by leptospirosis in New Zealand is the dairy farmer and the name "Dairy Farm Fever" has become synonymous with leptospirosis in rural areas (Christmas *et al.*, 1974 a). On a national scale the major losses sustained in domestic livestock production due to leptospirosis have been abortions in cows and deaths of calves from "red water fever" (Salisbury, 1954; Jamieson *et al.*, 1970). It is therefore understandable why the major thrust of leptospirosis research in New Zealand has been centred on cattle, with wildlife and porcine leptospirosis being virtually ignored. Only recently have systematic studies of leptospirosis in wildlife been conducted (Brockie, 1977 a, b), and although the pig was recognised very early as an important carrier of *pomona* (Ensor and McClure, 1953; Webster and Reynolds, 1955; Salisbury, 1954) few investigations have been undertaken.

As early as 1951 it was recognised that pigs in New Zealand were infected with *pomona*. Kirschner *et al.*, (1952) reported that there were positive titres to *pomona* in the sera of pigs which had been sent to an abattoir for slaughter, and that two pigs in contact with one of the first recorded outbreaks of "red water fever" in calves also had positive *pomona* titres.

Pomona titres as well as *tarassovi* titres were found in another survey of pigs conducted at a later date also by Kirschner (Kirschner, 1954).

The only regular reports of leptospirosis in pigs up to the present time come from the Ministry of Agriculture and Fisheries Animal Health Laboratories (Anon., 1957, 1958, 1960 b, 1961, 1974, 1976). Serovar *pomona* or sometimes merely "leptospiral" infections in these reports were associated with abortions and neonatal losses.

Serovar *tarassovi* was isolated for the first time in the early part of this study (Ryan and Marshall, 1976) and there has now been one report of an abortion storm in pigs in which this serovar was implicated (Anon., 1976).

Only one extensive serological survey of leptospirosis in pigs has been conducted. Blood was collected from "chopper" (adult) pigs sent for slaughter at 6 abattoirs in the North and South Islands and the serum from these samples tested for the presence of *pomona* and *tarassovi* agglutinins (Russell and Hansen, 1958). The conclusion from the investigation was that both these serovars were present throughout New Zealand and that of the animals sampled 5% were infected with *pomona* and 4% with *tarassovi*. Russell and Hansen's survey will be considered in greater detail in Chapter VI.

CONCLUSIONS

There is little known about two fundamental aspects of the epidemiology of leptospirosis in pigs in New Zealand. Firstly only limited surveys of this disease in pigs have been conducted, and thus one cannot state with certainty all the serovars which infect pigs in this country. Secondly the overall pattern of leptospiral infection in pig herds has not been thoroughly investigated. It was decided that a major objective of this study should be to elucidate these points. It was envisaged that this would be accomplished using the serum microscopic agglutination test (Galton *et al.*, 1965) and by culturing and identifying the serovars involved (Turner, 1970). Therefore important prerequisites were the development of reliable and efficient serological and cultural techniques. In addition as studies in pig herds would depend on being able to predict the prevalence of infection by analysing blood and/or urine samples, an evaluation of these diagnostic tests was also indicated. All the laboratory techniques were evaluated in order that the data collected during the epidemiological studies could be properly appraised.

In the literature on leptospirosis there is little reference to cell mediated immunity (CMI). There have been attempts to use skin tests to diagnose infection, and in some cases responses similar to delayed hypersensitivity have been observed (see Chapter X). However CMI in naturally infected animals has not been studied using more reliable *in vitro* methods such as lymphocyte transformation (W.H.O., 1973). As CMI is an important means whereby animals overcome and resist many bacterial infections (Bladen, 1974; Campbell, 1976) it was decided that this immune mechanism should be investigated. There always remained the possibility that cellular immunity was an important factor controlling the pattern of leptospiral infection in pig populations.

TABLE 1

LEPTOSPIRAL SEROVARS ISOLATED FROM PIGS

(a) Serovar *pomona*

Country	Reference
Argentina	Myers *et al.*, 1973.
Australia	Ryley and Simmonds, 1954 a.
Brazil	Santa Rosa *et al.*, 1973.
Bulgaria	Mitov *et al.*, 1961.
Canada	Boulanger *et al.*, 1959.
Ceylon	Nityananda and Harvey, 1971.
Chile	Fuensalida and Contreras, 1959.
China	Hou Tsung - Ch'ang *et al.*, 1957.
Czechoslovakia	Kmety *et al.*, 1956.
East Germany	Horsch *et al.*, 1966.
France	Kolochine-Erber and Mailloux, 1960.
Hungary	Fuzi *et al.*, 1957.
Italy	Austoni, 1966.
Indonesia	Collier, 1948.
Malaysia	WHO/FAO, 1966.
Peru	Herrer *et al.*, 1960.
Portugal	Fraga de Azevedo and Monteiro da Costa, 1955.
Phillipines	Topacio *et al.* 1971.
New Zealand	de Jong and Fowler, 1968.
Rumania	Combiescu and Sturdza, 1957.
Switzerland	Gsell, 1952.
Thailand	WHO/FAO, 1966.
U.S.S.R.	Malakhov *et al.*, 1973.
U.S.A.	Burnstein and Baker, 1954.
Venezuela	Bella *et al.*, 1966.
Yugoslavia	Zaharija and Peric, 1966.

TABLE 1 (cont.)

(b) Serovar *tarassovi*

Country	Reference
Argentina	Myers *et al.*, 1973.
Australia	Ryley and Simmonds, 1954a.
Brazil	Santa Rosa *et al.*, 1962.
Czechoslovakia	Kmety *et al.*, 1956.
Hungary	Fuzi *et al.*, 1957.
Italy	Farina, 1962.
Netherlands	Minkenhof *et al.*, 1968.
New Zealand	Ryan and Marshall, 1976.
Peru	Herrer *et al.*, 1960.
Rumania	Nicolescu *et al.*, 1970.
U.S.S.R.	Malakhov *et al.*, 1973.

(c) Serovar *canicola*

Country	Reference
Argentina	Myers *et al.*, 1973.
Brazil	Guida, 1948.
Britain	Coghlan *et al.*, 1957.
Chile	Fuensalida and Contreras, 1959.
Czechoslovakia	Kmety *et al.*, 1956.
Hawaii	U.S. Dept of Health, Education and Welfare, 1966.
Israel	Shenberg *et al.*, 1977.
Ireland	McErlean, 1964.
Peru	Herrer *et. al.*, 1960.
Rumania	Sturdza, 1966.
South Africa	Rensberg, 1973.
Taiwan	Tsai and Kundin, 1971.
Thailand	WHO/FAO, 1966.
U.S.A.	Ward *et al.*, 1956.
U.S.S.R.	Matveeva *et al.*, 1977.
Venezuela	Bella *et al.*, 1966.

TABLE 1 (cont.)

(d) Serovar *copenhageni*

Country	Reference
Belgium	Van Riel *et al.*, 1957.
Brazil	Santa Rosa *et al.*, 1962.
Britain	Nisbet, 1951.
Czechoslovakia	Halása, 1958.
Netherlands	Klarenbeck and Winsser, 1937.
Phillipines	Carlos *et al.*, 1970.
Poland	Wachnik, 1958.
Rumania	Combiescu and Sturdza, 1957.
U.S.S.R.	Malakhov *et al.*, 1973.

(e) Miscellaneous Serovars

	Serovar	Country	Reference
(1)	*australis*	Taiwan	Tsai and Kundin, 1971.
(2)	*bataviae*	China	Anon., 1960a.
(3)	*ballum*	Czechoslovakia	Kmety *et al.*, 1956.
(4)	*balcanica*	U.S.S.R.	Matveeva *et al.*, 1977.
(5)	*autumnalis*	Phillipines	U.S. Dept of Health, Education and Welfare, 1966.
(6)	*guidae*	Brazil	Guida, 1948.
	"	Rumania	Nicolescu *et al.*, 1970.
(7)	*grippotyphosa*	U.S.A.	Hanson *et al.*, 1971.
(8)	*javanica*	Taiwan	Tsai and Kundin, 1971.
(9)	*moldaviae*	U.S.S.R.	Matveeva *et al.*, 1956.
(10)	*monjakov*	U.S.S.R.	Matveeva *et al.*, 1956.
	"	Ceylon	Nityananda and Harvey, 1971.
(11)	*sejroe*	Czechoslovakia	Kmety *et al.*, 1956.
	"	Hungary	Fuzi *et al.*, 1957.
	"	Rumania	Combiescu, 1958.
(12)	*vietnam*	Vietnam	Chernuka *et al.*, 1969.

TABLE 2

ISOLATIONS OF LEPTOSPIRAL SEROVARS IN NEW ZEALAND

Serovar	Animal	Reference
pomona	man	Christmas *et al.*, 1976b.
hardjo	"	" " " "
ballum	"	Anon., 1967.
pomona	pigs	de Jong and Fowler, 1968.
tarassovi	"	Ryan and Marshall, 1976.
hardjo	cattle	Lake, 1973.
pomona	"	Anon., 1951.
ballum	"	Ris *et al.*, 1973.
copenhageni	cattle	Dodd and Brakenridge, 1966.
copenhageni	rats	Brockie, 1977.
ballum	"	" "
ballum	mice	" "
ballum	hedgehogs	Brockie and Till, 1977.
pomona	sheep	Hartley, 1952.
balcanica	opossum	Marshall *et al.*, 1976.
*hardjo**	"	Brockie, 1975.
*hardjo**	"	De Lisle *et al.*, 1975.
pomona	cat	Harkness *et al.*, 1970.
pomona	dog	Te Punga and Bishop, 1953.

*Not serotyped by agglutination/absorption.

CHAPTER II

SEROLOGICAL METHODS : A DESCRIPTION AND AN EVALUATION

INTRODUCTION

In this study serological procedures were used to diagnose leptospiral infection in animals, and to identify the leptospires which were cultured from both animal tissues and the environment. The serological techniques used are described in this chapter.

The microscopic agglutination test (MAT) was originally developed by Schuffner and Mochtar in 1926, and with some modifications this is still the most commonly used serological test used in leptospirosis research and diagnosis. The test is carried out with suspensions of living cultures of leptospires, or with cultures killed by the addition of formalin and the degree of agglutination of the organisms in sequentially diluted serum is estimated using dark-ground microscopy. The accepted end-point of an agglutination reaction is the final dilution of serum in which 50% of the leptospires are agglutinated (Turner, 1968). It has been demonstrated that the MAT detects antibody of both the IgM and IgG classes (Tong *et al.*, 1971; Morris and Hussaini , 1974), and that the agglutinating antibodies are produced in response to antigens located in several structures of the leptospire, including the outer envelope (Auran *et al.*, 1972) and cell wall (Faine *et al.*, 1974).

In the early stages of some leptospiral infections, microscopic agglutinating (MA) titres are encountered for one or more heterologous serovars which are as high or higher than the homologous titre (van der Hoeden, 1958). These paradoxical heterologous titres subside after the acute phase of infection. In addition, stable characteristic cross-reactions or co-agglutination titres have been demonstrated between certain serovars of

different serogroups. The co-agglutination pattern of various serovars has been investigated, and lists giving the degree of cross-reactivity that occurs have been published (Alston and Broom, 1958).

Many other serological tests have been developed for the purpose of diagnosing leptospiral infection in man and animals, and these have been reviewed by Turner (1968). As these other procedures were not used in this study they will not be considered here.

It was recognised, even by the earliest investigators of leptospirosis in man and animals, that infection with different antigenic variants occurred, and standardised serological procedures have been developed to differentiate between these leptospiral strains (Turner, 1968; Kmety *et al.*, 1970). Cross-agglutination tests are used to distinguish between those strains belonging to different serogroups, whereas agglutination-absorption tests are used to identify different serovars or serotypes. As has been discussed in Chapter I, the isolation and identification of leptospires is an essential element of an investigation of the epidemiology of leptospirosis.

MATERIALS AND METHODS

The Microscopic Agglutination Test

The leptospiral microscopic agglutination test (MAT), as modified to use standard microtitre equipment (Galton *et al.*, 1965; Cole *et al.*, 1973), was used for all the serological investigations in this study. Serum was initially diluted with physiological saline 1:3 in a microtitre plate. This plate, which was known as the Serum Reference Plate (SRP), was either used on the same day, or was sealed with cellophane and stored at -20C until required. On the day that the agglutination tests were to be performed, a series of microtitre plates, the

Test Plates (TP), were prepared by pipetting 25 µl of physiological saline into each well, using a semi-automatic dispensing machine (Minipipetter*). The SRP was placed on the tray of a Minidilutor* and 25 µl of the diluted test serum picked up with the dilutor heads. The SRP was removed from the Minidilutor tray, a test plate replaced, and a sequential doubling dilution across or down the plate performed. After the dilution had been completed, 25 µl of antigen (see below) was dispensed into each well, using a modified cornwall syringe ** to which a Minipipetter dispensing head had been attached (Fig. 2.1). Test plates were incubated at 37C for 1½ hours, before examining the contents of the wells under the darkfield microscope.

This procedure resulted in the lowest serum dilution (after antigen had been dispensed) being 1/12. Doubling dilutions either to 1/1536 could be produced across the test plate, or to 1/24576 by moving down the plate. The final serum dilutions in the wells were: 1/12 (well 1), 1/24 (well 2), 1/48 (well 3), 1/96 (well 4), 1/192 (well 5), 1/384 (well 6), 1/768 (well 7), 1/1536 (well 8), 1/3072 (well 9), 1/6144 (well 10), 1/12288 (well 11), 1/24576 (well 12).

The MA titre of a serum was taken as that dilution in which 50% of leptospires had been agglutinated (Turner, 1958). In the early experiments a sample from each serum dilution was removed from the wells in the test plate by means of a capillary tube. As this was a very time consuming process, an instrument was developed (Fig. 2.2) so that samples from 8 wells could be picked up simultaneously and deposited on a microscope slide. This instrument, which was used in most of the serological surveys, was made of stainless steel and consisted of 8 rods which were inserted in a block, such that a sample of the contents from the 8 wells could be picked up on the end of each rod.

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* Cook Engineering Co., Alexandria, Virginia, U.S.A.

** Becton and Dickinson & Co., Rutherford, New Jersey, U.S.A.

All the antigens used in this study were grown in liquid P80 medium (Ellinghausen and McCullough, 1965b; Johnson and Harris, 1967) and only used when the density was 1 to 5 x 10^8 cells/ml. The density of the antigen was assessed by dark-ground microscopy.

Samples of high titre rabbit antisera produced against the serovars *pomona* (Pomona), *tarassovi* (mitis-Johnson), *ballum* (M127), *hardjo* (Hardjoprajitno), *copenhageni* (M20), *australis* (Ballico), *autumnalis* (AkiA), *bataviae* (Swart), *canicola* (Hond Utrecht), *pyrogenes* (salinem), and *grippotyphosa* (MosV) were also incorporated in the SRP as antigen controls.

Fig. 2.1 The cornwall syringe as modified for dispensing antigen into microtitre plates.

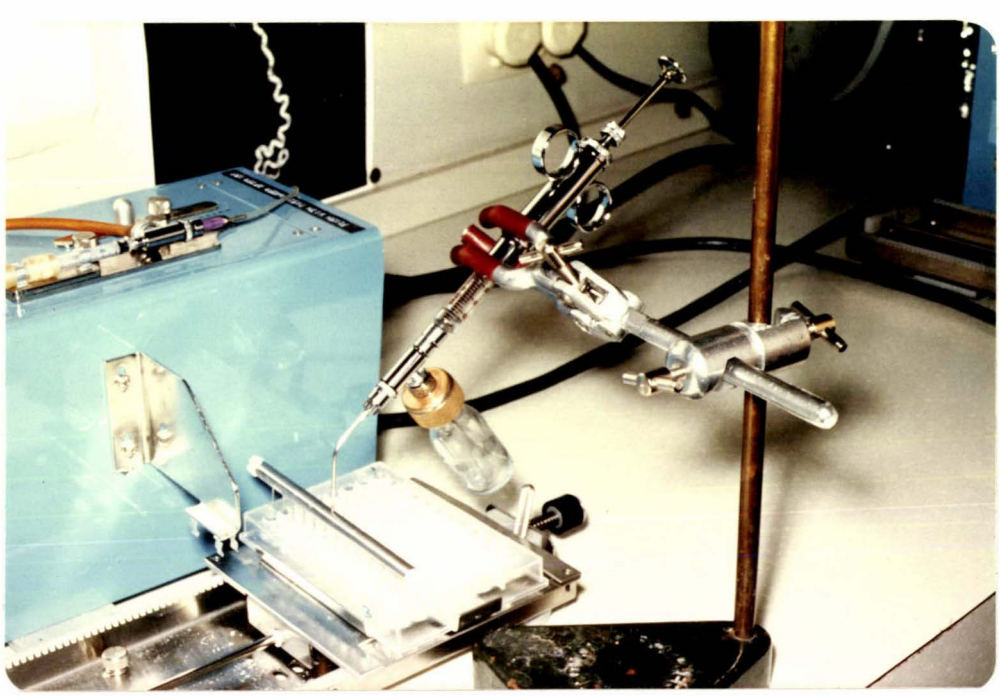
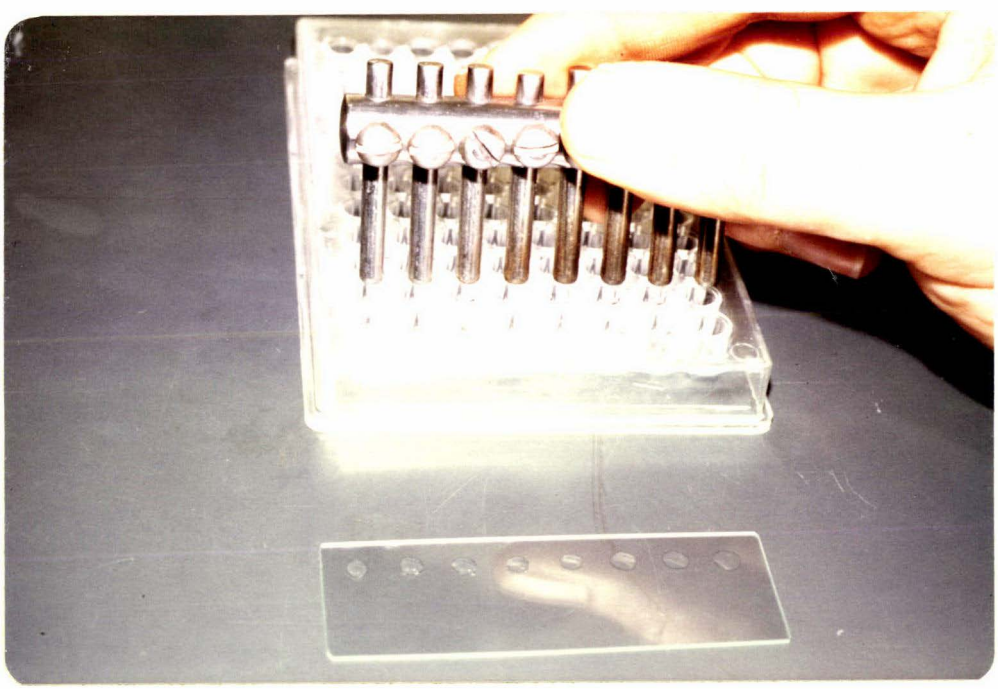


Fig. 2.2 The instrument developed to withdraw samples from microtitre plates for dark field examination.



In all statistical analyses, and to calculate the geometric mean titres of groups of animals, a system of coded titres was used, instead of the $\log_{10}$ transformed data (Paul and White, 1973). A titre of 1/12 was coded as 1, 1/24 as 2, 1/48 as 3, 1/96 as 4 etc. This code is directly proportional to the log transformations of the reciprocals of titres, and therefore can be used in all analyses. The actual geometric mean titres were calculated from the coded geometric mean titres with the formula which relates these parameters, $1/t = \text{antilog}\ (0.30x + 0.78)$, where 1/t is the titre, and x the coded titre.

Identification of Leptospires isolated

The medium used to isolate leptospires from animal tissues and the environment was a semi-solid medium containing $0.15\%_{\Delta}^{\text{agar}}$ (Chapter III), and it was therefore necessary to passage the isolates into a liquid medium before attempting their identification. The isolates were sub-cultured frequently until a high growth rate was achieved, such that a density of between 1 and 5 x 10^8 organisms per ml occurred after 5 to 10 days incubation at 30C. The strain was then serogrouped by determining the cross-agglutination pattern using the high titre rabbit antisera noted above. Test plates containing antisera were prepared as has been described, and 25 µl of a culture of the unknown strain was dispensed into the wells. The MA titre against each of the sera was estimated by dark-ground microscopy.

During the study a number of isolates were selected and sent to the Leptospirosis Reference Laboratory, Centre for Disease Control, Atlanta, Ga., U.S.A., where they were serotyped by agglutination-absorption (Kmety *et al.*, 1970).

Precision of the Serological Method

The precision of the serological technique used in this study was measured. A series of 72 pig sera were selected from the -20C storage bank, and the *pomona* MA titre was determined twice using the method described above.

A comparison of the methods used to remove samples from the test plates for darkfield examination.

A trial was conducted in order to ensure that the method used to remove a sample from a test plate well did not have any affect on the estimate of the MA titre. Eighteen sera were processed as described above, and the *pomona* titre estimated using capillary tubes to remove a sample from each well. These results were compared with those obtained when a sample was removed with the instrument shown in Fig. 2.2.

A comparison of the heart clot serum MA titres and the conventional serum MA titres.

In a number of surveys where samples were collected at an abattoir, it was not practicable to collect blood at the time of slaughter. Instead, the MA titre was estimated using the serum which exuded from clots of blood which had been collected from the heart. To confirm that the MA titre of the heart clot serum, and serum from conventionally collected blood were the same, paired samples were collected from 19 pigs and the titres against serovar *pomona* estimated.

The removal of fat from serum

The presence of numerous fat globules in the sera of some pigs made it impossible to accurately estimate the degree of agglutination which had occurred. In these cases it was necessary to defat the serum samples before testing.

An experiment was, therefore, conducted to assess the efficacy of various defatting procedures, and the effect the procedures had on the MA titres.

Ten fatty sera were selected from the storage bank, and aliquots of each were defatted by ultracentrifugation (12 000 xg for 30 minutes), membrane filtration* (pore size 0.45 µm), and treatment with chloroform. The chloroform treatment consisted of mixing each serum with an equal volume of chloroform, shaking the mixture vigorously, and then allowing it to settle overnight. The defatted serum rose to the top of the mixture, and could be easily removed.

In addition, the *pomona* MA titres of 12 sera before and after the defatting procedures were determined.

RESULTS

Precision of the Serological Method

The MA titres of the sera tested varied from 1/24 to 1/3072. In 35 instances (49%), the titre of each serum was found to be the same on both occasions. In 32 cases (44%) a difference in one dilution had occurred, and in 5 (7%) there was a difference of 2 dilutions (Table 2.1).

A statistical analysis of the coded titres indicated that there was no significant difference between the mean coded titres ($t=1.69$, 71 *d.f.*). The 95% confidence limits of the difference between the coded mean titres was -0.40 to +0.06.

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* Millipore Corporation, Bedford, Massachusetts, U.S.A.

Variation in titre due to the method used to remove a sample from the test plates for DGE.

The MA titres of the sera varied from 1/12 to 1/1536.

The difference in paired samples is summarised in Table 2.2. The variation in each serum was similar to that observed above, and there was no significant difference between the coded mean titres (*t=1.29, 17 d.f.*).

Comparison of heart clot and conventional serum MA titres.

The serum samples obtained from heart clots were haemolysed but this did not affect the reading of the test.

The titres ranged from 1/48 to 1/3072. The variation between samples was within the normal range (Table 2.3), and there was no significant difference between the coded means of the heart clot samples and those collected in the conventional manner (*t=0.44, 18 d.f.*).

The removal of fat from serum

All the methods resulted in the successful removal of fat from the sera.

Although 10 of the 12 (83%) of the sera treated either with chloroform or by centrifugation varied only $\pm$ 1 dilution from the pre-treatment titre, there was a tendency for a reduction of 1 dilution (Table 2.4). The difference in geometric mean after the chloroform treatment was significant (*t=2.97, 11 d.f., P=0.01*). Similarly after centrifugation there was a significant difference (*t=2.17, 11 d.f., P=0.05*). The 95% confidence limit after chloroform treatment was -0.78 to -0.56, and after centrifugation -0.73 to -0.43.

There was no significant difference in the geometric mean titres after filtration ($t=1.30$, 11 $d.f.$), and the variation in titre between samples was within the normal range (Table 2.4).

DISCUSSION

Large numbers of sera could be handled very efficiently with the serological system used throughout this study. In a normal working day as many as 150 sera could be titrated against an antigen by one person. It was a convenient system, and the use of microtitre plates both for the storage of the diluted serum and for testing, enabled easy identification of individual sera.

At the outset it was decided that in the interests of efficiency all dilutions of serum should be made in the microtitre wells, and only the semi-automatic machines (Minipipetter and Minidilutor) should be used to dispense saline and dilute serum. An initial tube dilution of serum would have been necessary in order to achieve the more usual doubling dilution sequence of 1/25, 1/50, 1/100 etc. The dilution series chosen is the closest to the more commonly used one that could be prepared with only 25 µl aliquots, and with the limited capacity of the microtitre wells.

The coded system of titres used in this study was introduced because of the ease with which geometric mean titres could be calculated, and statistical tests accomplished. It should be noted that this code is standardised against the dilution series used throughout this study. Other workers in this laboratory also used a similar system of coded titres (Hathaway, 1978; Hellstrom, 1978), but the serum dilution series they adopted was different. The coded titres in those studies cannot, therefore, be compared directly with the coded titres in this study.

In the trial conducted to measure the precision of the serological technique 93% of the paired sera varied by only $\pm$ 1 doubling dilution. This level of precision was considered to be acceptable. The size of the 95% confidence limit (0.46) would suggest that a difference of up to 0.5 could occur by chance alone between the mean coded titres of two identical populations.

The results of the other trials showed that the titres determined using the new instrument for taking samples from the test plate wells were no different from those using capillary tubes, and that the MA titres of sera from conventionally collected blood samples are the same as those estimated on sera collected from heart blood clots.

Although all the treatments successfully removed the fat from the serum, the chloroform treatment was the most convenient method. The membrane filters blocked very quickly, allowing only a small volume of serum to be collected. After centrifugation the layer of fat, which had formed on the top of the sample, often broke up, and when this happened the serum had to be processed again. Chloroform treatment was therefore the method of choice, but it should be noted that this treatment resulted in a reduction of approximately 1 dilution in the MA titre.

SUMMARY AND CONCLUSIONS

1. The serological techniques used in this study of leptospirosis are described.

2. In a trial to measure the precision of the serological method, it was found that 93% of the sera tested varied by $\pm$ 1 doubling dilution.

3. The titres determined using the new instrument for removing samples from the test-plates were no different from those using the traditional capillary tube method.

4. The MA titres of sera from conventionally collected blood samples were the same as those estimated on sera collected from heart clots.

5. Chloroform treatment was the most convenient method to use for defatting sera. This treatment resulted in a reduction of the MA titre by one doubling dilution.

Table 2.1 - The precision of the serological method. The difference between the coded MA titres of each of 72 sera which were tested twice using the method described in Chapter II.

Difference between coded titres	-2	-1	0	+1	+2
Number of Sera	2	23	35	9	3

Table 2.2 - The differences between the coded MA titres of each of 18 sera after removing a sample for darkfield examination from the test plates with capillary tubes and the new instrument (Fig. 2.2).

Difference between coded titres	-2	-1	0	+1	+2
Number of Sera	1	4	11	2	0

Table 2.3 - The use of heart clot serum to determine the serum MA titre. A comparison in 19 animals of the coded MA titres of the serum collected from heart blood clots and of the serum taken off a conventional blood sample.

Difference between coded titres	-2	-1	0	+1	+2
Number of Animals	1	4	8	4	2

Table 2.4 - The change in coded MA titres observed in sera after centrifugation, filtration or treatment with chloroform.

Change in coded titre	-2	-1	0	+1	+2
Centrifugation	2	4	5	1	0
Filtration	1	3	5	3	0
Chloroform	2	4	6	0	0

CHAPTER III

CULTURAL METHODS : A DESCRIPTION AND AN EVALUATION

INTRODUCTION

The problem of cross-reactions between leptospiral serovars within the same serogroup places severe limitations on the epidemiological usefulness of serological surveys. Cultural isolation and identification of the serovars occurring within the populations under study are, therefore, necessary. Cultural methods should also be as efficient as possible, and their sensitivity defined.

This chapter describes the cultural methods used in this study of leptospirosis, and the effect that various factors had on the *in vitro* growth of leptospires.

Traditionally the parasitic leptospires have been cultivated in serum-enriched media such as those of Fletcher (1928) and Stuart (1946). Analyses of the key components in rabbit serum that are necessary for the stimulation of the growth of the parasitic leptospires *in vitro*, led to the development of a medium containing an oleic-albumin complex, vitamin B_{12}, thiamine, and ammonium chloride which did not require a serum supplement (Ellinghausen and McCullough, 1965a). It was later found that bovine albumin and polysorbate 80 (Tween 80) together could replace the oleic-albumin complex (Ellinghausen and McCullough, 1965b), and a further modification of this medium, "EMJH" (Johnson and Harris, 1967) has superseded the serum containing media in many laboratories. Ellinghausen (1976) has suggested that this class of medium should be called polysorbate 80 medium (P80 medium), and this term has been used throughout this thesis.

There have been a number of attempts to produce a completely synthetic medium (Vogel and Hutner, 1961; Stalheim and Wilson, 1964; Baseman *et al.*, 1966;

Shenberg, 1967) but none have been shown to support the growth of all leptospires consistently (Ellinghausen, 1976).

Leptospires grow slowly in culture and are very sensitive to changes in medium composition. It has been found that growth is stimulated and maintained, especially in primary cultures, when agar is incorporated in the medium (Ellinghausen, 1976). It is not known why agar enhances the growth of leptospires, but it has been suggested that it may absorb toxic metabolites or inhibitory substances in the inoculum (Cerva, 1967). The contamination of cultures by other bacteria often presents a problem. For this reason, there have been attempts to produce an additive which would selectively inhibit bacterial contaminants. For example, 5-fluorouracil (5-FU) (Johnson and Rogers, 1964), and a combination of sulphathiazole, neomycin and actidione (Cousineau and McKiel, 1961) have both been suggested as useful additives to leptospiral culture media.

MATERIALS AND METHODS

GENERAL DESCRIPTION

Culture from organs

The organ, or portion of an organ to be cultured, was removed aseptically from the animal carcase and homogenised (20% wt./vol.) in sterile Stuart's basal medium (SBM) (Stuart, 1946). One ml of the homogenate was removed, and 2 or 3 serial 10-fold dilutions made also in SBM. From each of these dilutions 0.25 to 0.30 ml was then inoculated into 5 ml of a semi-solid (0.15% agar) leptospiral cultural medium. The cultures were incubated at 30C, and examined for leptospires at various times by dark-ground microscopy over a period of 3 months.

The larger tissue samples were homogenised using a stomacher*. The sample was placed into a heavy grade plastic bag, which had been sterilized by γ-irradiation. Stuart's basal medium was poured in (20% wt./vol.), and the tissue was then homogenised with the stomacher. Pig kidney samples (20 to 25 g), whole piglet kidneys, and portions of piglet livers (10 to 12 g) were prepared in this manner.

The smaller tissue samples were placed inside a sterile 5 or 10 ml disposable syringe, and mascerated by forcing it out through the nozzle into a volume of Stuart's basal medium. The suspension was then shaken vigorously. This method was used with hamster kidneys and livers (2 to 3 g), and with piglet brain samples (3 to 5 g).

Culture from body fluids

(i) Blood culture

Pigs were bled from either an ear vein or the anterior vena cava into oxalated vacuum tubes** and one or two drops of this sample were used to inoculate semi-solid medium.

(ii) Pericardial, pleural and peritoneal fluid culture

The pericardial, pleural and peritoneal fluids from aborted piglets were routinely cultured for leptospires. Samples of these fluids were collected from the foetus with a Pasteur pipette and 1 or 2 drops were inoculated into semi-solid medium.

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* Colworth 400, A.J. Seward, V.A.C. House, London, England.

** Vacutainer, Becton and Dickinson, Rutherford, New Jersey, U.S.A.

(iii) Vitreous humor culture

The vitreous humor of aborted piglets was examined by dark-ground microscopy as well as being cultured for leptospires. The eyelids of the piglet were removed, the eye swabbed with alcohol and then flamed with a bunsen burner for 5 to 10 seconds. A sterile 16 or 18 gauge needle was pushed through the cornea and into the posterior chamber. A sample of vitreous humor was sucked out, diluted in 10 ml of SBM, and 3 or 4 drops of the diluted material were used to inoculate semi-solid medium.

(iv) Urine culture

A mid-stream sample of urine was collected from pigs in field studies. In abattoir surveys 10 ml of urine was collected at evisceration directly from the bladder with a sterile disposable syringe.

One drop of undiluted urine was transferred to 10 ml of sterile SBM, an approximate 1:500 dilution. One or 2 drops of the undiluted and diluted urine were inoculated into semi-solid medium. Five millilitres of the undiluted urine was then centrifuged at 400 g for 3 minutes in order to sediment gross particulate matter. The supernatant was then taken up in a sterile disposable syringe, and the syringe attached to a swinnex filter* which had been loaded with a membrane filter* (pore size 0.45 µm). One to 2 ml of the urine was initially expressed and discarded, and then 3 or 4 drops of the subsequent filtrate inoculated directly into semi-solid medium.

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* Millipore Corporation, Bedford, Massachusetts, U.S.A.

When hamsters were available, they were used to aid the isolation of leptospires from urine samples. One half of a millilitre of undiluted pig urine was injected intraperitoneally (IP) into a hamster, and kidney and liver samples from any of these hamsters which died were cultured for leptospires. All surviving hamsters were killed at 21 days and a kidney from each was also cultured.

Culture from contaminated sources

Occasionally it was necessary to attempt the isolation of leptospires from highly contaminated materials, such as soil and effluent. Dry materials were first suspended in Stuart's basal medium, and the mixture shaken vigorously. All samples were centrifuged at 400 g for 4 to 5 minutes to sediment the particulate matter; 0.5 ml of the supernatants was then either inoculated I/P into hamsters, or filtered using the "floating membrane" technique (Fowler, 1970), and then cultured in semi-solid medium. The kidney and liver from any hamsters dying were cultured as described above. All surviving hamsters were killed after 21 days and a kidney from each of these animals was also cultured.

PRELIMINARY SURVEYS

After the first two cultural surveys of leptospirosis in pigs had been completed, the results were analysed in order that the minimum culture requirements could be defined. The surveys are briefly described here, and results are presented in the following section.

In the first survey 73 kidneys from porker and bacon weight pigs were sampled, and cultured in Fletcher's, Stuart's and P80 media using kidney homogenate dilutions of 1/10, 1/100 and 1/1000. Cultures were examined each week for 3 months, and the density of leptospires noted. The results were analysed to determine whether the dilution

of the kidney homogenate had an effect on either the number of isolates obtained, or the contamination rate of the cultures. In addition, the numbers of isolates obtained with each medium were compared.

In the second survey, kidney and urine samples from 56 pigs were cultured. The 1/10 dilution of the kidney homogenate was inoculated into P80 medium and into P80 medium containing 200 µg 5-FU/ml**. The urine samples were cultured as described above, using semi-solid P80 medium, and P80 medium containing both 200 µg 5-FU/ml and 400 µg 5-FU/ml.

EXPERIMENTS WITH A SEROVAR SELECTIVE MEDIUM

The preliminary results of the first surveys indicated that serovar *pomona* infection had a high prevalence in the pig population. The question arose as to whether other serovars were also infecting these animals, but were not being isolated because their growth was being overwhelmed by *pomona*. In order to investigate whether a medium could be produced that would eliminate *pomona*, but still allow other serovars to grow, the growth inhibiting effect of high titre rabbit *pomona* anti-serum (MA titre 1/24576) was tested against serovars *pomona*, *ballum*, *hardjo* and *copenhageni*.

Culture tubes* containing 5 ml of liquid P80 medium were prepared, and 1/2 ml aliquots of serially diluted rabbit *pomona* antiserum were added. The rabbit antiserum had been membrane filtered, and the final dilutions in the medium were 1/10, 1/50, 1/500 and 1/5000. Dense liquid

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* Kimax, Owens, Illinois, U.S.A.

** Sigma, St. Louis, Missouri, U.S.A.

cultures of serovars *pomona*, *ballum*, *hardjo* and *copenhageni* were prepared. 1/4 ml of the culture containing serovar *pomona* was inoculated into medium containing the rabbit antiserum. Likewise, cultures of each of the other serovars were inoculated into the medium containing the rabbit *pomona* antiserum. Control cultures of normal P80 medium were also established. The number of organisms in all the prepared cultures (incubated at 30C) was estimated at weekly intervals over 2 months.

RESULTS

The effect of dilution of kidney homogenate on the number of isolates obtained.

Isolates were obtained in P80 medium from 30 of the kidneys sampled. In 29 cases leptospires were cultured in the medium inoculated with the 1/10 dilution of homogenate. In 26 cases they were cultured in the medium inoculated with the 1/100 dilution, and in 18 cases in the medium inoculated with the 1/1000 dilution of homogenate.

When the numbers of isolates which were obtained from each of the homogenate dilutions were compared, they were found to be significantly different ($\chi^2=14.07$, 2 d.f. $P<0.005$).

In Stuart's and Fletcher's media a similar pattern was observed.

The effect of dilution of kidney homogenate on the contamination rate of cultures.

A total of 657 cultures was prepared, and of these 23 (3.5%) were observed to be contaminated when they were examined after 1 week of incubation. Nine (4.1%) of the cultures which were inoculated from the 1/10 dilution of kidney homogenate were contaminated, and 7 (3.2%) from both the 1/100 and 1/1000 dilutions of homogenate.

There was no systematic pattern of contamination, with the 23 contaminated cultures coming from 16 different kidneys.

When the number of contaminated cultures in each of these 3 classes (i.e. the classes inoculated from the 1/10, 1/100, and 1/1000 dilutions of kidney homogenate) was compared, they were found to be not significantly different ($\chi^2=0.36$, *2 d.f.*).

The effect of medium on the number of isolates obtained.

Of the 32 leptospiral strains isolated from the sample of pig kidneys used in this experiment, 31 were shown to be serovar *pomona* and 1 serovar *tarassovi*.

Thirty (94%) grew in the P80 medium, 25 (78%) in the Stuart's and 20 (63%) in Fletcher's. The 2 strains which were not obtained in the P80 medium grew in Stuart's medium.

When the numbers of isolates which were obtained with each medium were compared they were found to be significantly different ($\chi^2=9.14$, *2 d.f.*, $P=0.01$). However, the number of strains isolated with the P80 medium was not significantly different to that isolated with the Stuart's medium ($\chi^2=2.07$, *1 d.f.*).

The effect of 5-fluorouracil on the number of isolates obtained.

In this survey 56 kidneys were cultured, and a total of 15 strains of serovar *pomona* isolated. All 15 grew in the P80 medium without 5FU, and 12 were isolated in the medium containing 5FU.

Six of the cultures without 5FU were contaminated, and only 1 in the medium with 5FU.

There was no significant difference between the number of strains isolated in each medium (χ^2=*0.20, 1.d.f.*). The contamination rates were too low for a statistical analysis to be conducted.

The growth pattern of leptospires in primary culture.

In some cultures leptospires were observed after only 4 days of incubation. The longest period before organisms were observed in the cultures which had been inoculated from the 1/10 dilution of the kidney homogenate was 27 days. In contrast some of the cultures inoculated from the 1/100 and 1/1000 dilutions of the homogenates took 48 days. Leptospires were seen in most of the cultures of the 1/10 and 1/100 homogenate dilutions within 21 days.

Leptospires did not appear for merely brief periods in culture. After 3 months, organisms could still be found in all positive cultures, regardless of when they were first seen.

The culture of leptospires from urine.

Serovar *pomona* was isolated from 6 of the 56 specimens of urine which were collected in this survey. The number of strains of serovar *pomona* which were isolated from the undiluted and diluted urine, in media containing no 5-FU, 200 µg 5-FU/ml and 400 µg 5-FU/ml is summarised in Table 3.1. Five of the leptospiral strains were isolated from undiluted urine, and 6 from diluted; while 4 strains grew in the media containing no 5-FU, and 6 with 5-FU. There was no significant difference between the number of isolates obtained in each of the 6 classes (χ^2=*2.90, 5 d.f.*); nor was there any significant difference between the number of isolates obtained in media with no 5-FU and the number obtained with media supplemented with 5-FU (χ^2=*1.43, 1 d.f.*).

All of the cultures which had been inoculated with samples from these 6 specimens of urine were free from contaminating bacteria.

The effect of the dilution of urine, and of the addition of 5-FU to media on the contamination of cultures is summarised in Table 3.2. Of the 336 cultures of urine put up in this survey, 6 (2%) were found to be contaminated when they were examined for the first time after 3 weeks incubation. Five of these contaminated cultures were in media which had been inoculated with undiluted urine, and only 1 in media which had been inoculated with the diluted urine. Four were in media which contained no 5-FU, and 2 in media with 200 µg 5-FU/ml.

Leptospires were not isolated from any of the urine samples which had been filtered, and none of the cultures which had been inoculated with the filtered urine samples were contaminated.

The effect of *pomona* antiserum on the growth of serovars *hardjo, ballum, copenhageni* and *pomona*.

The growth of serovars *hardjo, ballum* and *copenhageni* followed the same pattern as that seen in the media without any *pomona* antiserum. A marked increase in the number of organisms over the first 1 to 2 weeks of incubation occurred, and then the number gradually decreased.

The growth of serovar *pomona* in media containing the highest concentration of the anti-*pomona* rabbit serum (1/10, 1/50 and 1/500 dilutions) was initially suppressed. Instead of a marked increase in the density of organisms, followed by a gradual decline, the number of leptospires steadily decreased over the first 1 month of incubation. However, after this period the number of organisms gradually increased, such that after approximately 1½ months of incubation the density of organisms in the

cultures containing *pomona*, *hardjo*, *ballum* and *copenhageni* was about the same.

The growth of serovar *pomona* in the media containing the lowest concentration of antiserum (1/5000) was the same as that in media without antiserum.

DISCUSSION

It has been suggested that kidney tissue contains lipids which may inhibit the growth *in vitro* of leptospires, and that a high dilution of the kidney homogenate may be necessary to overcome this problem (Turner, 1970). In these cultural surveys, there was no evidence of such an effect. In fact, the significantly increased number of isolates obtained with the more concentrated kidney homogenate, suggests that a greater number of isolates might be obtained from a more concentrated homogenate. However, there was a distinct disadvantage using undiluted homogenate to inoculate the culture media. The high density of the tissue suspension in the medium made it extremely difficult to see the leptospires by dark-ground microscopy. For this reason, the method adopted for the remainder of the study was to inoculate culture medium from the 1/10 and 1/100 dilutions of the kidney homogenate.

An important feature of this technique of kidney culture was that the isolation of leptospires was not limited by the contamination of cultures. It has been suggested that such contamination can be a problem, and that substantial dilution of the kidney homogenate may be necessary to avoid this hazard. However, the results obtained in this survey indicate that the contamination rate of cultures was unaffected by dilution of the homogenate. This finding, together with the sporadic occurrence of the contamination suggests that airborne organisms, or other occasional breakdowns in the aseptic technique were responsible for these cases.

Although the number of isolates obtained with P80 medium was not significantly ($P<0.05$) greater than the number obtained with Stuart's medium, the results are in accord with the observation of others (Turner, 1970), that P80 medium is superior to the serum containing media. For this reason, it was decided to use P80 as the primary isolation medium for the remainder of this study. Fletcher's and Stuart's medium were used, however, to grow organisms for inoculating into rabbits to produce antiserum; also, if a primary isolate could not be readily adapted to liquid P80 medium, it was inoculated into both Fletcher's and Stuart's in an attempt to adapt it to one of the serum containing media instead.

The results obtained in the second survey indicate that 200 µg 5-FU/ml may in fact suppress the growth of leptospires *in vitro*. In addition, it would appear that this concentration effectively inhibits the growth of contaminating bacteria. Thus, although the addition of 5-FU may aid the primary isolation of leptospires by reducing contamination, any advantage could be offset by the suppression of leptospiral growth. As the isolation of leptospires was such an important part of this investigation, it was decided that both P80 medium, and P80 medium with 200 µg 5-FU/ml would be used in future cultural surveys. This is in line with the recommendation of Turner (1970).

Turner (1970) also recommends that primary cultures should be examined for leptospires by darkfield microscopy at least weekly. However, the steady growth of leptospires observed in the semi-solid medium indicates that this is not necessary. Therefore, in subsequent studies, primary cultures were routinely examined after 3 weeks, 7 weeks and 10 or 11 weeks incubation.

With leptospires being isolated from only 6 urines it is not possible to draw any definite conclusions regarding this aspect of urine culture. However, it was surprising to find such a variable success rate within each category. As contamination was not a limiting factor, a possible explanation for this is that the leptospires, which were few in number in these samples, were clumped, and that isolation was dependent on the chance event that the inoculum contained a sufficient number of organisms to become established in primary culture.

The number of urine cultures which were contaminated was too low for a statistical analysis. However, the results do suggest that the contamination rate may be reduced by diluting the urine, and by using medium containing 5-FU.

In this survey, membrane filtration of urine yielded no leptospiral isolates and it would seem that the reason for this failure was low numbers of organisms in the urine. This conclusion is supported by the report of Nervig and Ellinghausen (1978) who found that the minimum number of leptospires required before filtration (pore size 0.45 μm) would permit recovery was 10^3 to 10^4 organisms/ml. It should be noted here, however, that although no isolates were obtained in this survey, membrane filtration of dense leptospiral cultures in liquid media was a useful method of eliminating any contaminating bacteria.

In summary, therefore, the results of this urine culture evaluation study suggested that urine should be inoculated into a number of cultures, and that contamination might be reduced by diluting urine and using media containing 5-FU. It was, therefore, decided to adopt the same technique used in this survey for the subsequent field investigations. However, as membrane filtration yielded no isolates this technique was not used when attempting to isolate leptospires from urine specimens.

It was hoped that the high concentration of hyperimmune homologous antiserum *in vitro* would completely eliminate *pomona* organisms but still allow the other serovars to grow. If this had occurred a primary isolation medium containing anti-*pomona* serum could have been prepared, and used in parallel with normal primary isolation media to investigate whether dual infection with *pomona* and other serovars occurred in pigs. However, as the growth of *pomona* was only suppressed for a short period in medium containing antiserum, it is to be expected that *pomona* would survive in primary cultures, and after inoculation into secondary cultures uninhibited growth of *pomona* would occur. It would be unlikely that a cultural method such as this would allow the selective isolation of other serovars in cases of dual infection. For this reason it was decided not to pursue this line of investigation any further.

SUMMARY AND CONCLUSIONS

1. The cultural techniques used in this study of leptospirosis are described.

2. Most leptospiral isolates were obtained from cultures which had been inoculated with the most concentrated kidney homogenate used in this experiment.

3. The rate of contamination of cultures was very low.

4. Most leptospiral isolates were obtained in P80 medium, and it was decided to use this medium for the remainder of the study.

5. It was considered adequate to routinely examine cultures after 3 weeks, 7 weeks, and 10 or 11 weeks incubation.

Table 3.1 - The recovery of leptospires (serovar *pomona*) from undiluted and diluted urine in media containing no 5-FU, 200 µg 5-FU/ml and 400 µg 5-FU/ml.

Specimen No.	Undiluted Urine			Diluted Urine		
	0 5FU	200 5FU	400 5FU	0 5FU	200 5FU	400 5FU
345	+	+	+	-	-	+
352	+	-	-	-	-	-
354	-	-	-	-	+	+
359	-	+	+	-	+	+
372	-	+	+	+	+	+
373	+	+	+	-	+	+
Total	3	4	4	1	4	5

Table 3.2 - The number of contaminated cultures in media containing no 5-FU, 200 µg 5-FU/ml, 400 µg 5-FU/ml which had been inoculated with undiluted and diluted urine.

	Undiluted Urine			Diluted Urine		
	0 5FU	200 5FU	400 5FU	0 5FU	200 5FU	400 5FU
Number	3	2	0	1	0	0

CHAPTER IV

AN ABATTOIR SURVEY OF LEPTOSPIROSIS IN THE PIG

INTRODUCTION

Serological surveys for leptospirosis in pigs have previously been conducted in New Zealand by Kirschner (1952, 1954) and Russell and Hansen (1958), but only two antigens, *pomona* and *tarassovi*, were used in these studies. Many of the samples tested reacted with these antigens, and it was concluded that pigs commonly became infected with both these serovars. The only leptospiral serovar which has been isolated from pigs in this country is *pomona* (de Jong and Fowler, 1958), but no extensive cultural surveys have been undertaken.

This chapter describes a serological and cultural survey of leptospirosis in pigs at an abattoir. The objective of the study was to determine whether or not pigs became infected with any of the other serovars which are known to infect domestic and feral animals in New Zealand or in other countries, and to establish whether or not pigs are reservoir or incidental host for any of these serovars. The survey technique used in this study was that which has been successfully used in wildlife studies by Roth *et. al.* (1963).

During this study two other aspects of leptospiral infection in pigs were also investigated. Firstly, the MA titres to serovar *biflexa* in the infected and non-infected pigs were compared with titres obtained when using specific antigens with a view to determining whether or not this serological test could be used as a "Genus" specific screening test (Turner, 1968). Serological tests using antigens prepared from saphrophytes such as *biflexa* (CDC) have been found to be useful in man for detecting infection with the parasitic leptospires (Cox *et al.*, 1958; Mailloux, 1967; Sulzer and Jones,

1973). Secondly, in order to confirm that the criteria used at meat inspection for the detection of leptospiral infected kidneys were valid, the gross renal lesions in culture-positive and culture-negative kidneys were compared.

MATERIALS AND METHODS

Tissues for this study were collected from both young and adult pigs at an abattoir. The young pigs were between 5 and 10 months old. The adult animals were mainly cull sows. Included in this survey were pigs from many different farms located in the southern half of the North Island.

BLOOD SAMPLES

Collection

(i) Young Pigs: Blood samples were collected from the young pigs immediately after each animal had been stunned. Generally, 30 to 35 animals from a number of different farms were sampled during each visit to the abattoir. A metal tag was placed in the ear of approximately half of the animals sampled, so that these carcases could be subsequently identified at the time of removal of the kidneys. More bloods than kidneys were taken, as a greater number of serum samples could be processed in the laboratory than could kidney samples.

(ii) Adult Pigs: Only a limited number of adult pigs were slaughtered at this abattoir, and it was not practicable to wait in the stunning area to collect blood samples from these. Instead, the serum MA titres were estimated from the serum derived from heart blood clots (see Chapter II). After the heart had been removed, it was opened and any clots of blood in the ventricles or auricles removed.

Serology

The serum MA titres were determined by the method described in Chapter II, employing the following serovars: *pomona* (Pomona), *tarassovi* (mitis-Johnson), *ballum* (M127), *hardjo* (Hardjoprajitno), *copenhageni* (M20), *australis* (Ballico), *autumnalis* (Akiyama A), *bataviae* (Swart), *canicola* (Hond Utrecht), *pyrogenes* (Salinem), *grippotyphosa* (Moskva V), and *biflexa* (CDC).

As has been described in Chapter II, the titre of a serum was taken as that dilution where 50% of the leptospires were agglutinated. In the presentation of results the terms positive and negative were used in relation to each serovar, positive being the description applied to a serum showing 50% or greater agglutination at a particular dilution and negative the description applied to a serum showing less than 50% agglutination at a particular dilution.

Geometric mean titres (gmt) for both groups of animals were determined by decoding the arithmetic mean of the coded titres.

The coded titres to *biflexa* of the serum from culture-positive and culture-negative animals were compared using the "t" test. Similarly the coded *biflexa* titres of the sera which were test-positive at 1/12 to *pomona* were also compared with the coded *biflexa* titres of the sera which were test-negative at 1/12 to *pomona*.

KIDNEY SAMPLES

Collection

(i) Young animals: A kidney, with the capsule still intact, was removed from each tagged carcase, and placed in a plastic bag. The ear tag was removed and placed in the bag with each kidney for later identification.

(ii) Adult Animals: A kidney from each of the adult animals was removed and placed in a plastic bag with the bottle containing the clots of blood which had been collected from the heart. Matched numbers were assigned to each of these samples before the tissues were processed.

Culture

The kidneys were cultured as described in Chapter II, and any leptospiral isolates obtained were identified by cross-agglutination using hyperimmune rabbit sera. These rabbit antisera had been prepared against known laboratory strains (Chapter II). A selection of the isolated leptospires was also sent to the Leptospirosis Reference Laboratory, Centre for Disease Control Atlanta, Ga., U.S.A. for confirmatory identification.

Pathology

Before a sample from each kidney was cultured, the capsule was stripped and the cortex scrutinised for lesions suggestive of a leptospiral infection. The lesions considered most likely to be due to leptospirosis are grayish white foci ranging in size from the limit of visibility up to 1 cm in diameter (Langham *et al.*, 1958; Sleight *et al.*, 1960). In addition to recording gross lesions, sections of each kidney were taken for histopathological examination.

RESULTS

SEROLOGY

Most of the sera collected from both the young and adult pigs reacted to more than one serovar. At a dilution of 1/12 less than 12% of the sera were positive to *ballum*, *hardjo*, and *canicola*, between 22% and 37% were positive to *tarassovi*, *copenhageni*, *australis*, *bataviae*, *pyrogenes* and *grippotyphosa*, 68% positive to *autumnalis* and 87%

positive to *pomona*. In regard to all these serovars, many of the titres did not exceed 1/12 or 1/24, with the result that the proportion positive at 1/48 was substantially below that at 1/12. In particular, most of the titres to *grippotyphosa*, *bataviae*, *australis*, *ballum* and *copenhageni* were 1/24 or less (Table 4.1).

The range of the MA titres to each of the serovars varied considerably, especially with the sera from the young pigs. In this group serovar *pomona* titres ranged from 1/12 to 1/98 304, and the *autumnalis* titres from 1/12 to 1/24 576. In the adults the highest *pomona* and *autumnalis* titres were 1/1536 and 1/192 respectively. Although an occasional young pig had a high titre against one of the other serovars, titres to these were low in the majority of both young and adult animals. The geometric mean titres (gmt) shown in Table 4.2 reflect this finding. It can be seen that with the exception of the *pomona* and *autumnalis* gmt's, the range of geometric mean titres was from 1/12 to 1/35. The *pomona* and *autumnalis* gmt's were 1/533 and 1/108, and 1/51 and 1/18 in the young and adult samples respectively. None of the adult pigs were positive at 1/12 to serovar *canicola*.

There was a tendency for a particular serum sample to react with many serovars, or with none at all. This was particularly evident in the sera which had been obtained from the young pigs. However, 5 of these animals, which were negative at 1/12 to *pomona*, had titres of either 1/48 or 1/96 against other serovars. Two of these were *tarassovi* titres, two *copenhageni* titres and one a *pyrogenes* titre. Of all the adult sera which were negative at 1/12, only one reacted with another serovar, and this animal had a *tarassovi* titre of 1/48.

The regression analyses on data obtained from the young pigs indicates that there is a significant association ($P<0.001$) between the *pomona* titres and those to *australis*,

autumnalis, *bataviae* and *grippotyphosa*. In the adult pigs there are significant associations between titres to *pomona* and those to *australis* ($P<0.001$), *autumnalis* ($p<0.001$), *pyrogenes* ($P<0.001$),and *grippotyphosa* ($P<0.001$). In the young pigs there was no significant association between *pyrogenes* titres and *pomona* titres ($P=0.50$), and in the adult group there was no significant association between *bataviae* titres and *pomona* titres ($P=0.10$). The regression line calculated from these analyses (Figs 4.1 and 4.2) indicates that in all the cases in which there is a significant regression, the association is such that low titres against these serovars are related to very high *pomona* titres. The variation in the titres which can be attributed to the regression on the *pomona* titres (r^2) is between 5% and 30% in all but one case (Tables 4.3 and 4.4.)

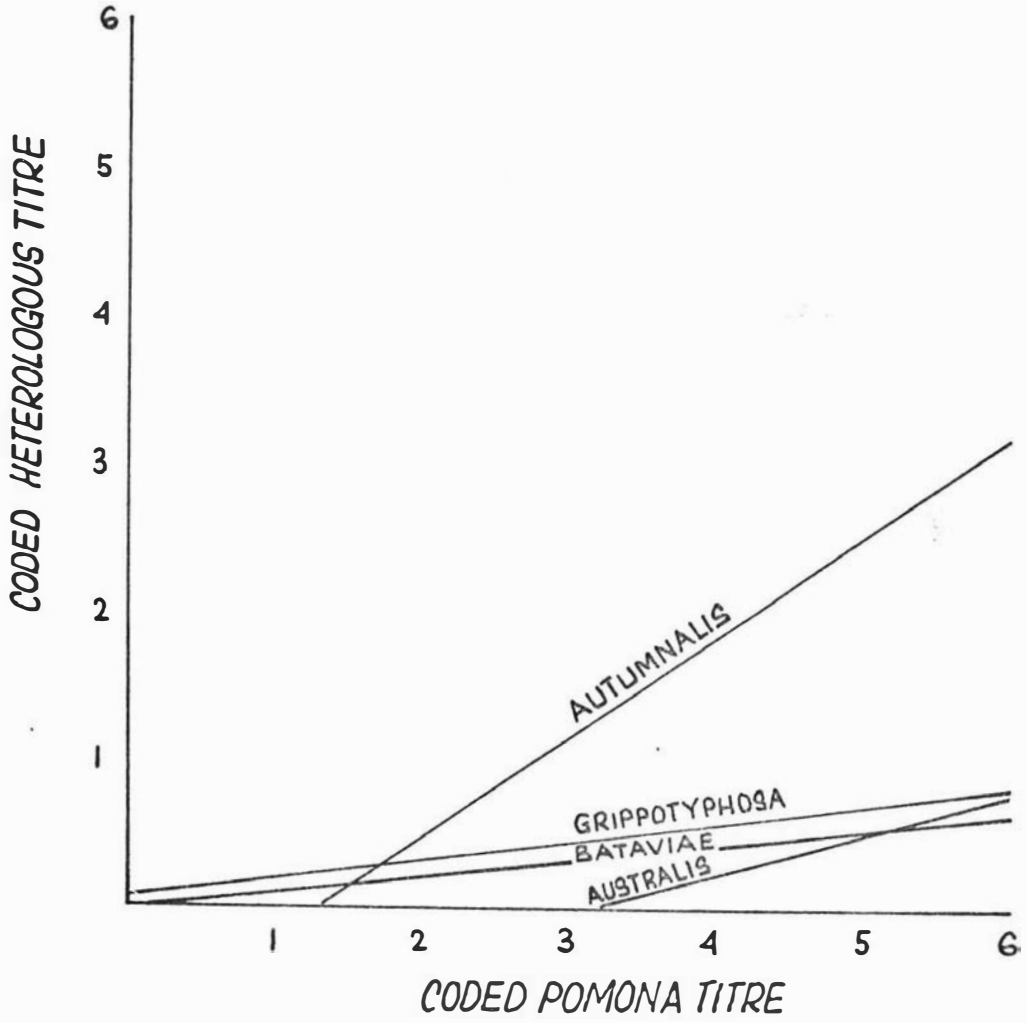
KIDNEY CULTURE

Kidneys were collected from 84 young pigs, and 65 adults, a total of 149. Serovar *pomona* was isolated from 38 (45%) of the kidneys from the young pigs and from one (2%) of the adult samples. In contrast, serovar *tarassovi* was cultured from only 1 (1%) of the juvenile group and from 3 (5%) of the adult kidneys. The combined culture positive rates for all pigs were therefore 39/149 (26%) for serovar *pomona* and 4/149 (3%) for serovar *tarassovi*.

The serovar *biflexa* titres of the culture-negative and culture-positive animals.

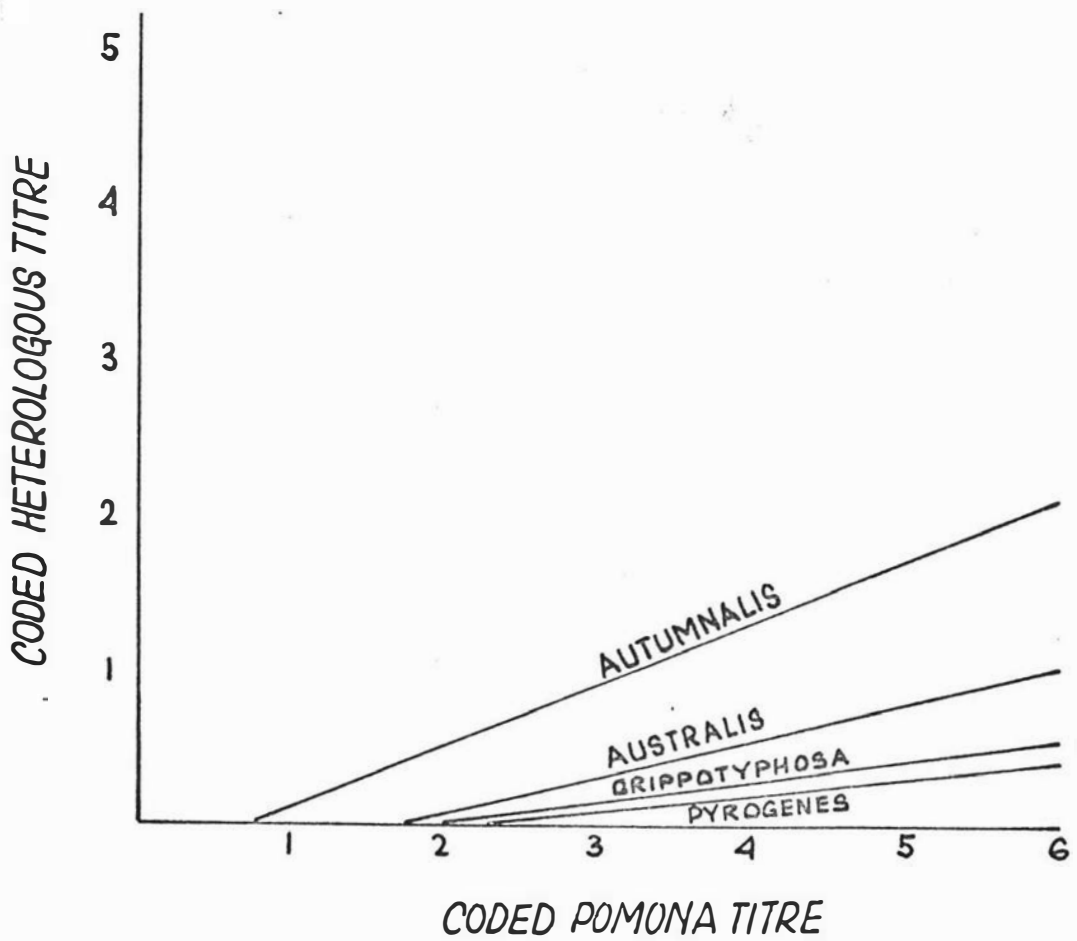
The coded mean titre of the young pigs which were culture-positive was 1.66, and that of the culture-negative ones was 2.09. There was no significant difference between these coded mean titres ($t=1.62$, 82 *d.f.*).

Fig. 4.1 The association between the coded *pomona* MA titres and the coded heterologous MA titres in young pigs.



SEROLOGY CODE
1=1/12, 2=1/24, 3=1/48, 4=1/96, 5=1/192,
6=1/384, 7=1/768, 8=1/1536, 9=1/3072,
10=1/6144, 11=1/12288, 12=1/24576.

Fig. 4.2 The association between the coded *pomona* MA titres and the coded heterologous MA titres in adult pigs.



SEROLOGY CODE
1=1/12, 2=1/24, 3=1/48, 4=1/96, 5=1/192,
6=1/384, 7=1/768, 8=1/1536, 9=1/3072,
10=1/6144, 11=1/12288, 12=1/24576.

The serovar *biflexa* titres of the animals which were either positive or negative at 1/12 to *pomona*.

The coded mean *biflexa* titre of the young pigs which were positive at 1/12 to *pomona* was 2.08 and that of the negative ones 2.11. These were not significantly different ($t = 0.07$, 139 *d.f.*).

RENAL PATHOLOGY

Gross lesions considered to be due to leptospirosis were recorded in 12 of the young and adult kidneys (Table 4.5). No gross lesions were detected in 20 out of 28 (71%) from which leptospires were isolated. Very minor lesions which might have remained undetected by routine meat inspection were found in a further 5 of the 28 (18%).

Histopathological examination of the kidneys with grossly visible white focal lesions showed that the lesions consisted of interstitial infiltrations of mononuclear cells, including lymphocytes, macrophages and plasma cells. In addition there was some evidence of necrosis and atrophy of renal tubules. Similar microscopic lesions were found in some sections from the infected kidneys showing no gross lesions.

DISCUSSION

Concurrent serological and cultural surveys for leptospirosis have been shown in a study of feral animals by Roth *et al.* (1963) to be an effective means of distinguishing between animals which are reservoir hosts for various serovars, and those which become infected from other reservoir hosts. According to these workers, if an important reservoir of infection occurs in an animal population, the prevalence of renal infection will be approximately the same as the proportion of animals which are serologically positive for that serovar. Conversely a high prevalence of serologically positive animals

which is many times greater than the prevalence of renal infection is considered to indicate a mild transitory infection. However, the results of this abattoir study suggest that this interpretation cannot be applied to the domestic pig.

In this survey, serovar *pomona* was isolated from 26% of the animals sampled, whereas 86% of the pigs were serologically positive at 1/12 to *pomona*. Similarly *tarassovi* was isolated from 3% of the animals sampled and 22% were serologically positive. Strictly applying the criteria set down by Roth *et al.*, it could be concluded from these results that the domestic pig is not a reservoir host for serovar *pomona* or serovar *tarassovi*. However taking into consideration the frequency with which these organisms have been isolated from pigs in both this study and other surveys (Table 1.1), and the results of both field investigations (Burnstein and Baker, 1954; Boulanger *et al.*, 1959; Buddle and Hodges, 1977) and experimental infections (Tammemagi and Simmonds, 1956, 1958; Morse *et al.*, 1958; Hodges, 1973) it is unlikely that this is the case. In most countries pigs are regarded as a major reservoir host of both *pomona* and *tarassovi*.

It would seem that this method of defining a reservoir host cannot be satisfactorily applied to pigs with *pomona* infection because in this population the infection is much more prevalent in a particular age group. Pigs do not become life-long carriers of *pomona*, but the homologous MA titres appear to persist long after infection has been eliminated. As a result of these two factors the prevalence of serologically positive animals is very much greater than the prevalence of infection. Furthermore, the pig population sampled in this survey might be regarded as an aggregation of a number of sub-populations since a number of farms were included in the survey. Criteria such as those of Roth *et al.*, which are applicable to more homogenous populations of wild

animals might therefore not be strictly applicable to the current survey. Another consideration is that in wildlife species, the duration of renal infection in a reservoir host may well approach the natural life span of the animal, and in this situation Roth *et al's.* criteria are more likely to be valid.

The serological and cultural results for *tarassovi* are more difficult to interpret. If *tarassovi* had not been isolated from these pigs, it may well have been concluded that the *tarassovi* titres were cross-reactions to *pomona*. Unlike the *pomona* titres which dominated the agglutination pattern of virtually every serum, none of the *tarassovi* titres were particularly high. However, in the absence of any other reservoir host for *tarassovi* having been demonstrated in New Zealand, it may be assumed that the pig is the reservoir host for this serovar. In contrast to *pomona*, *tarassovi* infection in pigs appears to be sporadic.

Roth's method of identifying reservoir hosts is dependent on the efficiency with which leptospires may be isolated from renal tissue, and it can be argued that a disparity between *pomona* and *tarassovi* culture-positive animals, and the proportion of animals which are serologically positive is due to this factor. However, as has been discussed in Chapter III, there would appear to be few factors which might have limited the isolation of leptospires as profoundly as these figures suggest.

At present in New Zealand, in addition to serovars *pomona* and *tarassovi*, only *ballum* (Brockie, 1977), *hardjo* (Lake, 1973), *balcanica* (Marshall *et. al.*, 1976) and *copenhageni* (Kirschner and Gray, 1951) have been isolated from domestic or feral animals. Extensive serological surveys in feral animals (Hathaway, 1978), cattle (Hellstrom, 1978) sheep (Marshall, R.B., unpublished), dogs (Baluyat, C., unpublished), and cats (Shophet, R., unpublished) have been undertaken, and there is no indication that any of

these animal species are reservoir hosts for other serovars. It would, therefore, appear to be highly unlikely that the titres to *autumnalis, australis, bataviæ, canicola, pryogenes* and *grippotyphosa* observed in this survey of pigs were due to infection with the homologous serovar.

There was little evidence that the pigs sampled at this abattoir had been infected with *ballum, hardjo* or *balcanica*. Virtually all of the titres to *ballum* and *hardjo* were 1/12 or 1/24, and these were always associated with high *pomona* titres. If infection with either of these organisms was a common occurrence in pigs, a greater range of titres would have been observed. In addition, some titres to *ballum* and *hardjo* in the serum samples which were negative to *pomona* would also have been expected. It is therefore reasonable to conclude that the *ballum* and *hardjo* titres were due to cross-reactions. As *balcanica* cross-reacts with *hardjo* to a very high titre in other animals (Hathaway, 1978), it may be assumed that a *balcanica* antigen would have reacted as did the *hardjo* (Hardjoprajitno) antigen, and therefore, for the same reasons it would appear that these pigs had not been infected with *balcanica*.

By way of a contrast, some high titres to *copenhageni* were recorded, and in 2 cases pigs without *pomona* titres had *copenhageni* titres of 1/48 and 1/96. In addition, the *copenhageni* titres did not follow a consistent pattern with respect to the *pomona* titres. *Rattus norvegicus* is the reservoir host for this serovar in New Zealand (Brockie, 1977a) and there would be ample opportunity for pigs to become infected. All of this might suggest that the *copenhageni* titres were due to infection with this serovar. On the other hand, it is well documented that certain strains of serovar *pomona* induce the secretion of agglutinins that react with *copenhageni* at high titre (Morse and Allen, 1956; Alston and Broom, 1958). In a later investigation of a *pomona* infected pig herd definite paradoxical heterologous titres to

copenhageni were observed in those animals which had been recently infected with serovar *pomona* (see Chapter VI). It is therefore not possible to interpret with certainty the significance of the *copenhageni* titres found in this survey.

The highly significant regressions (Tables 4.3 and 4.4), and the nature of the association (Figs 4.1 and 4.2) between the *pomona* titres and those to *australis*, *autumnalis*, *bataviae*, *grippotyphosa* and *pyrogenes* also indicates a cross-reaction phenomenon. In some cases the amount of the variation attributed to the regression on the *pomona* titres was not high. Nevertheless this is to be expected as the degree of cross-reaction depends on the relative amounts of various classes of antibody present in the sera (Hellstrom, 1978), and this changes markedly during the course of the infection (Tong *et al.*, 1971; Hathaway, 1978; Hellstrom, 1978). This phenomenon may also explain why differences between the young and adult pigs occurred.

The serological results arising from the use of the *biflexa* antigen were disappointing. It was not possible to differentiate serologically between culture-positive and culture-negative animals. The *biflexa* titres of those which were serologically positive to *pomona* were the same as those which were negative. Thus, it would seem that the MAT using *biflexa* as the antigen has little value as a screening test to detect pigs which are infected or indeed those which have positive homologous titres. Using a number of saprophytes as antigens, Ris (1975) also found in cattle that the MAT was of no value as a genus-specific screening test.

The finding that no gross lesions were visible in over 70% of the pig kidneys from which leptospires were isolated has considerable public health implications. As a result of these findings a further survey was conducted, in which pig kidneys on sale in shops were cultured. It was found that either *pomona* or *tarassovi* could be cultured from 25% of these kidneys (Lord, B.L., unpublished). In the household kitchen kidneys usually undergo a considerable amount of handling and cutting. Housewives may therefore be exposed to a previously unsuspected and serious risk of contracting leptospirosis.

SUMMARY AND CONCLUSIONS

1. A cultural and serological survey of leptospirosis was conducted in young and adult pigs from an abattoir. The sera collected were subjected to the microscopic agglutination test (MAT) using 11 parasitic serovars and 1 saprophyte, serovar *biflexa* (CDC), as antigens. Kidneys from both groups were cultured for leptospires.

2. Serovar *pomona* was isolated from 38/84 (45%) of the kidneys from the young pigs and from 1/65 (2%) of the adult kidneys. However, 87% of the young pigs and 86% of the adult pigs were serologically test-positive at 1/12 to *pomona*.

3. Serovar *tarassovi* was isolated for the first time in New Zealand. In contrast to *pomona*, the prevalence of culture-positive animals was 1/84 (1%) in the young group, and 3/65 (5%) in the adult pigs. 21% of the young pigs, and 25% of the adult pigs had MA titres to *tarassovi* of 1/12 or more.

4. Although many of the sera reacted with serovars other than *pomona* and *tarassovi*, none of these were isolated.

5. It was concluded that pigs in New Zealand are reservoir hosts for *pomona* and *tarassovi*, but that they do not become life-long carriers of these serovars. The MA titres to these serovars appear to be maintained long after infection has been eliminated.

6. It was concluded that *pomona* antibody cross-reacts markedly with many other serovars. There was strong evidence that the pigs sampled had been infected only with serovars *pomona* or *tarassovi*, although it is possible that some had been infected with serovar *copenhageni*.

7. It was considered that the method described by Roth *et al.* (1963) to define the role of an animal species in the epidemiology of leptospirosis could not be satisfactorily applied to the domestic pig population.

8. The MAT using serovar *biflexa* (CDC) as antigen appeared to be of no value in pigs for detecting either infected animals, or animals which had positive titres to the parasitic leptospires.

9. In many of the kidneys from which *pomona* was isolated, either no gross leptospiral-like lesions were apparent, or only minor lesions were observed. It was concluded that infected kidneys showing no gross lesions constituted a potential public health risk.

Table 4.1 - The prevalence of MA titres in the young and adult pigs, and in the total (combined) sample.

Serovar	Young Pigs 1/12	Young Pigs 1/48	Adult Pigs 1/12	Adult Pigs 1/48	Combined 1/12	Combined 1/48
pomona	87%*	72%	86%	58%	86%	67%
tarassovi	21%	7%	25%	5%	22%	6%
ballum	14%	1%	8%	0%	12%	1%
hardjo	4%	1%	9%	0%	5%	1%
copenhageni	29%	7%	18%	0%	26%	5%
australis	30%	10%	26%	2%	29%	7%
autumnalis	74%	44%	55%	8%	68%	32%
bataviae	27%	7%	26%	2%	27%	5%
canicola	5%	0%	0%	0%	3%	0%
pyrogenes	49%	21%	12%	0%	37%	14%
grippotyphosa	42%	10%	6%	2%	30%	8%

* prevalence =

$$\frac{\text{Number of sera test-positive at 1/12 or 1/48.}}{\text{Total number of sera in that group}} \quad \frac{100}{1}$$

Table 4.2 - The geometric mean titres, and the maximum titres with each of the antigens which were used in the microscopic agglutination tests.

Antigen	Young Pigs gmt*	Young Pigs max. titre**	Adult Pigs gmt	Adult Pigs max. titre
pomona	1/533	1/98304	1/51	1/1536
tarassovi	1/28	1/384	1/19	1/48
ballum	1/16	1/48	1/12	1/12
hardjo	1/32	1/96	1/14	1/24
copenhageni	1/21	1/1536	1/14	1/24
australis	1/35	1/12288	1/14	1/48
autumnalis	1/108	1/24576	1/18	1/192
bataviae	1/23	1/192	1/15	1/48
canicola	1/15	1/24	<1/12	<1/12
pyrogenes	1/35	1/1536	1/12	1/12
grippotyphosa	1/22	1/192	1/20	1/48

*gmt = geometric mean titre of the animals test-postive at 1/12.

**max. titre = maximum titre attained with that antigen.

Table 4.3 - Young Pigs. The regression of heterologous coded MA titres on coded *pomona* titres of 1/12 or more.

Serovar	*b*	*c*	*r*	r^2	*t*	*P*(122 d.f.)
tarassovi	-0.013	0.533	-0.041	0.002	-0.470	>0.500
ballum	0.008	0.180	0.047	0.002	0.502	>0.500
hardjo	0.038	-0.071	0.137	0.019	1.522	0.100
copenhageni	0.001	0.626	-0.002	0.000	0.035	>0.500
australis	0.270	-0.871	0.550	0.302	7.266	<0.001
autumnalis	0.661	-0.825	0.756	0.571	12.759	<0.001
bataviae	0.095	0.024	0.310	0.096	3.613	<0.001
canicola	-0.002	0.099	-0.030	0.001	-0.345	>0.500
pyrogenes	0.036	1.20	0.071	0.005	0.776	0.500
grippotyphosa	0.123	0.083	0.372	0.138	4.431	<0.001

b = regression coefficient; *c* = regression equation constant; *r* = correlation coefficient; *P* = probability

Table 4.4 - Adult Pigs. The regression of heterologous coded MA titres on coded *pomona* titres of 1/12 or more.

Serovar	*b*	*c*	*r*	r^2	*t*	*P*(63d.f.)
tarassovi	-0.053	0.503	-0.116	0.013	-0.856	0.400
ballum	-0.002	0.660	-0.014	0.002	-0.032	>0.500
hardjo	-0.029	0.213	-0.113	0.013	-0.846	0.400
copenhageni	0.030	0.158	0.088	0.008	0.654	0.500
australis	0.224	-0.352	0.530	0.281	4.597	<0.001
autumnalis	0.398	-0.301	0.508	0.258	4.332	<0.001
bataviae	-0.101	0.669	-0.228	0.052	-1.720	0.100
canicola	-	-	-	-	-	-
pyrogenes	0.083	-0.148	0.402	0.162	3.237	0.005
grippotyphosa	0.129	-0.275	0.387	0.150	3.078	0.005

b = regression coefficient; *c* = regression equation constant; *r* = correlation coefficient; *P* = probability

Table 4.5 - The occurrence and nature of kidney lesions considered to be indicative of leptospirosis.

Lesions	Culture +	Culture -
(1) No leptospiral-like lesions	20 (71%)	98 (96%)
(2) 1 to 3 white foci 1 to 3 mm diameter	5 (18%)	4 (4%)
(3) Moderate Nos of white foci 1 to 3 mm diameter	1 (4%)	0
(4) Large Nos of white foci 1 to 3 mm diameter or larger	2 (8%)	0
Total recorded	28	102

CHAPTER V

DIAGNOSTIC PARAMETERS IN PORCINE *POMONA* INFECTION

INTRODUCTION

In this study of leptospirosis in pigs, it was planned to investigate the epidemiology of *pomona* infection in a pig herd, where pigs could not be killed and their kidneys cultured for leptospires. Instead, one or more diagnostic tests were going to have to be used to define the pattern of infection. In order that the results from these tests could be interpreted wisely, an evaluation study was conducted.

Two important attributes of a test are it's sensitivity (Se) and specificity (Sp), where sensitivity is the percentage of diseased animals which are test-positive, and specificity is the percentage of disease-free animals which are test-negative. (Thorner and Remein, 1961; Schwabe *et al.*, 1977; Martin, 1977). In other words the sensitivity is the ability of a test to give a true positive result, whereas specificity is the ability to give a true negative result.

When the magnitude of the sensitivity and the specificity are known, the real prevalence (RP) of a disease in a group of animals can be estimated from the apparent prevalence (AP); the apparent prevalence being the percentage of the group which is test-positive. The formula which is used to calculate the real prevalence is as follows:

$$RP = \frac{AP + Sp - 1}{Se + Sp - 1}$$

The derivation of this formula is shown in Appendix I. It should be noted that in situations where the sum of the sensitivity and the specificity equals unity, this expression cannot be used. This does not occur commonly

(Schwabe *et al.*, 1977), but when it does the denominator of the above equation is zero, and division by zero has no mathematical meaning.

The isolation of *pomona* from the kidney was chosen in this study as the most appropriate base line against which to judge other tests as indicators of infection. The tests under study were the homologous MA titre of serum, the homologous MA titre of urine, the direct demonstration of leptospires in urine by darkfield microscopy, and the cultural isolation of *pomona* from urine.

MATERIALS AND METHODS

The association between serum MA titres and kidney culture was investigated in Survey A, and in a second study, Survey B, the urinary parameters were evaluated.

SURVEY A

BLOOD SAMPLES

Collection

Blood samples were collected from both juvenile and adult animals as described in Chapter IV. In the adults sera was taken off clots retrieved from the heart (Chapter II).

Serology

Sera were subjected to the microscopic agglutination test using serovar *pomona* (Pomona) as the antigen (Chapter II).

KIDNEY SAMPLES

Collection

One kidney, with the renal capsule still intact, was collected from each carcase. Care was taken to ensure that the blood sample and kidney from each carcase were matched.

Kidney Culture

The kidneys were cultured for leptospires as described in Chapter III. The leptospiral isolates which were obtained were identified by cross-agglutination. A selection of strains was also sent to the Leptospirosis Reference Laboratory, Centre for Disease Control, Atlanta, Ga, U.S.A. for confirmatory identification.

ANALYSIS OF THE SEROLOGICAL RESULTS

The sensitivity of the MA test at each titre was calculated using the MA titres of those animals from which serovar *pomona* was isolated. The specificity of the MA test at each titre was calculated using the MA titres of those pigs which were culture-negative.

SURVEY B

Tissues for this survey were also collected at an abattoir. It was found prior to this survey that the only animals which still had a useful amount of urine in their bladders at evisceration were the entire males and castrates. As adult boars were only rarely killed at this abattoir, all samples came from juvenile boars and castrates.

URINE SAMPLES

Collection

The bladder was removed at evisceration and 10 ml of urine was collected through the bladder wall with a sterile disposable syringe.

Darkfield examination

A drop of urine was examined by darkfield microscopy. Samples revealing organisms showing typical leptospiral morphology and typical motility were classed as positive.

Culture

Undiluted and diluted (1:500) urine were cultured in semi-solid P80 medium and semi-solid P80 medium containing 200 and 400 µg 5-FU/ml as described in Chapter III.

The urinary MA titre

The method used to determine the *pomona* MA titre of the samples of urine was the same as that used for serum (Chapter II), with the exception that no initial dilution was made in the serum reference plate. Thus, the sequence of doubling dilutions in the test-plates was 1/4, 1/8, 1/16, etc.

KIDNEY SAMPLES

Collection

After a urine sample had been collected, one kidney was removed from the carcase. Care was taken to ensure that each urine sample could be matched with each kidney.

Kidney culture

The kidneys were cultured for leptospires as described in Chapter III, and any isolates obtained were identified by cross-agglutination (Chapter II).

ANALYSIS OF THE RESULTS FROM SURVEY B

The sensitivity of each of these diagnostic tests was calculated by determining the proportion of the infected animals which were positive (i.e. positive darkfield examination, or positive urine culture, or a urinary MAT of 1/4 or greater). Specificity was calculated by determining the proportion of culture-negative animals which were negative (i.e., negative darkfield examination, or negative urine culture or with a urinary MA titre of less than 1/4).

RESULTS

SURVEY A

BLOOD SAMPLES

Eighty two blood samples were collected from juvenile animals and 65 from adult animals. The range of *pomona* MA titres was from zero (less than 1/12) to greater than 1/24 576. The frequency distribution of titres peaked at a titre of zero and at a titre of 1/48 (Fig. 5.1).

KIDNEY SAMPLES

Serovar *pomona* was isolated from 37 out of the 147 kidneys (25%) which were collected in this survey. Of these 36 came from the 82 juvenile samples and 1 came from the 65 adult samples.

ANALYSIS OF THE SEROLOGICAL RESULTS

The frequency distributions of the *pomona* titres in the culture-positive and culture-negative groups were quite different (Fig. 5.2). In the culture-positive groups most of the titres were evenly distributed between 1/192 and 1/24 576. In contrast, most of the culture-negative group had titres between zero and 1/384, and the distribution was bimodal with peaks at zero and 1/48.

The sensitivities and specificities at each titre are given in Table 5.1. All data have been expressed as percentages. The range in sensitivity is 95% at 1/12 to 27% at 1/24 576. Five of the 37 animals which were culture-positive had "titres" of greater than 1/24 576. Thus, the sensitivity at this level is 5/37 (14%). In contrast, the specificity ranges from 16% at 1/12 to 98% at 1/24 576. As illustrated in Fig. 5.3, the lines representing sensitivity and specificity intersect at a titre of 1/384. At this point both the sensitivity and specificity are 85%; that is to say 85% of the animals from which *pomona* was isolated had titres of 1/384 or greater, while 85% of those which were culture-negative had titres less than 1/384.

SURVEY B

URINE SAMPLES

Matched urines were collected from 57 pigs. Leptospires were observed in 2 samples, and serovar *pomona* was cultured from 6, including the 2 which were observed to be darkfield-positive. Twelve of the urine specimens had MA titres of 1/4 or greater.

KIDNEY SAMPLES

Serovar *pomona* was isolated from 15 out of the 57 (26%) kidneys collected.

ANALYSIS OF RESULTS FROM SURVEY B

All the darkfield and culture-positive urines were from animals which were also kidney culture-positive. Thus, the sensitivity of urine darkfield examination was 2/15 (13%) and the specificity 42/42 (100%), while for urine culture the sensitivity was 6/15 (40%) and the specificity also 42/42 (100%). Two of those animals which were kidney culture-negative had urine titres of 1/4 or greater. Therefore, a urinary *pomona* MA titre of 1/4 or more had a sensitivity of 10/15 (67%) and a specificity of 40/42 (95%).

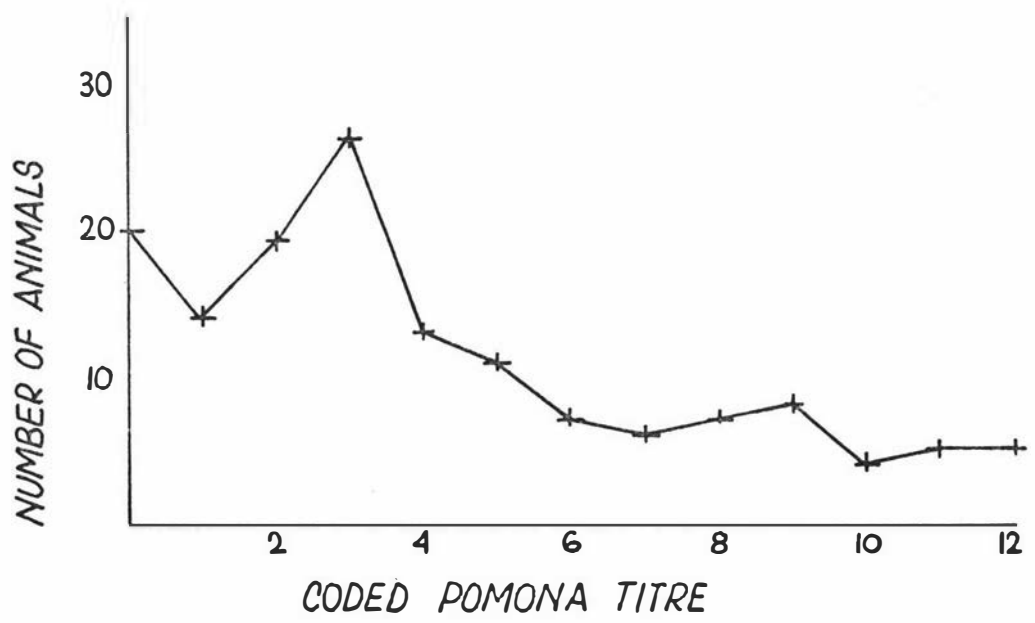
The analysis of the urine evaluation study is summarised in Table 5.2 and illustrated in Fig. 5.4.

DISCUSSION

There are two important facets of these evaluation studies which should be considered when discussing the validity of the results obtained in these surveys. The first is whether or not the group of animals which was sampled was accurately divided into infected and non-infected categories. The second is whether or not these samples of culture-positive and culture-negative animals were truly representative of those occurring in the populations in which it was planned to use these diagnostic tests.

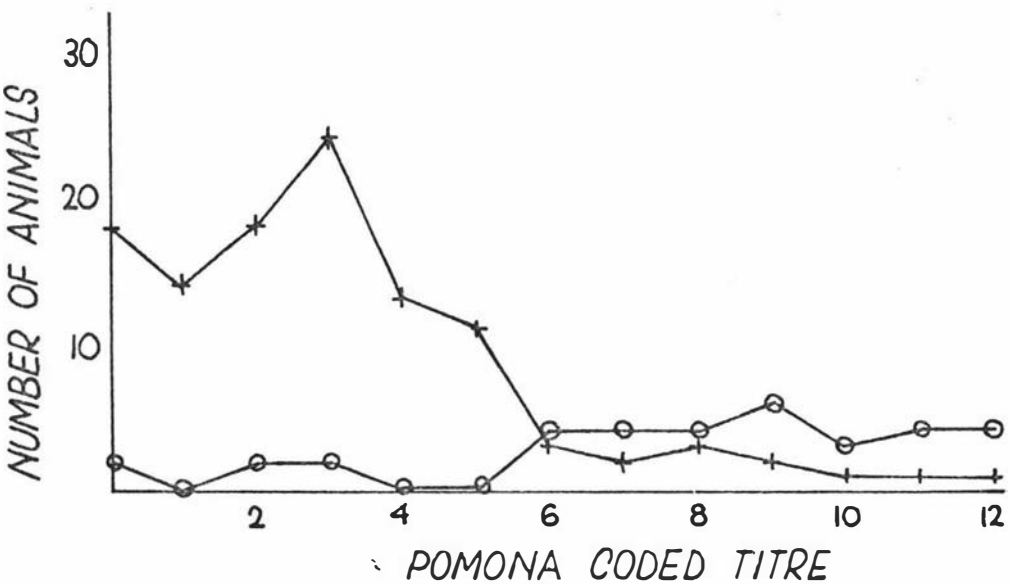
The first point calls into question the efficiency of the kidney culture technique. Possible limiting factors are the poor survival of the leptospires after the death of the pig, an inadequate culture medium, an insufficient number of leptospires in the kidney homogenate inocula, and the contamination of cultures by other bacteria.

Fig. 5.1 The frequency distribution of the *pomona* MA titres of the pigs sampled at an abattoir.



SEROLOGY CODE
1=1/12, 2=1/24, 3=1/48, 4=1/96, 5=1/192,
6=1/384, 7=1/768, 8=1/1536, 9=1/3072,
10=1/6144, 11=1/12288, 12=1/24576.

Fig. 5.2 The frequency distribution of the *pomona* MA titres in those pigs which were kidney culture - positive and kidney culture - negative.

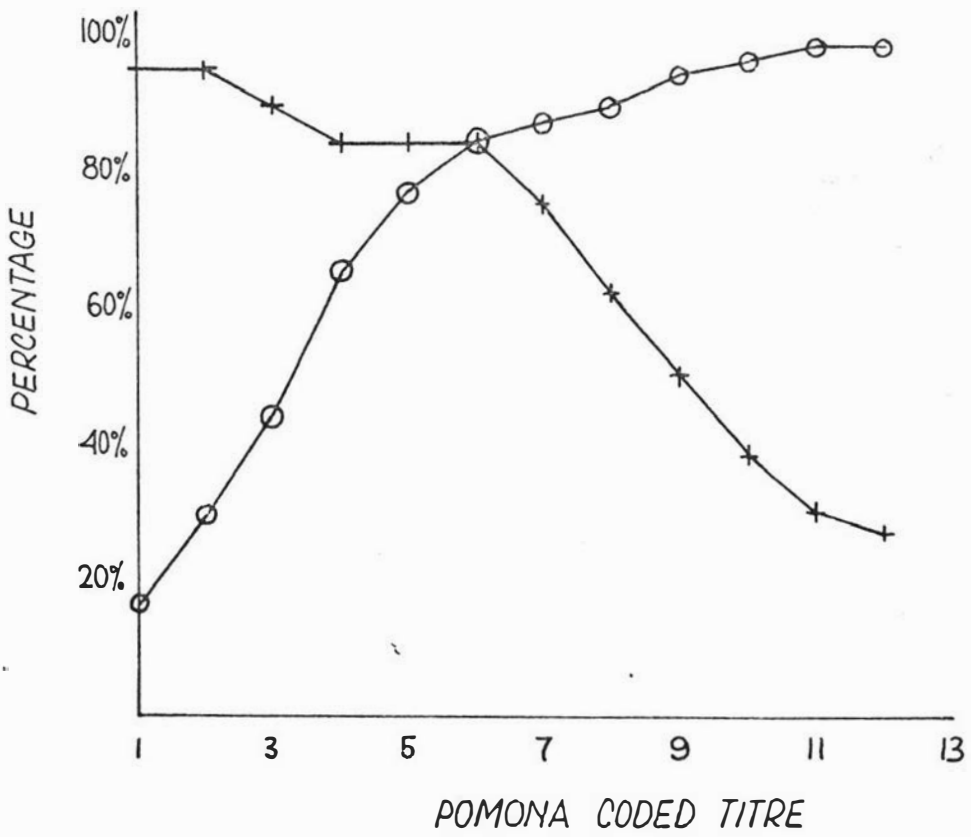


SEROLOGY CODE
1=1/12, 2=1/24, 3=1/48, 4=1/96, 5=1/192,
6=1/384, 7=1/768, 8=1/1536, 9=1/3072,
10=1/6144, 11=1/12288, 12=1/24576.

\+ = culture-negative

o = culture-positive

Fig. 5.3 The association between sensitivity, specificity and the homologous serum MA titres in *pomona* infection in pigs.

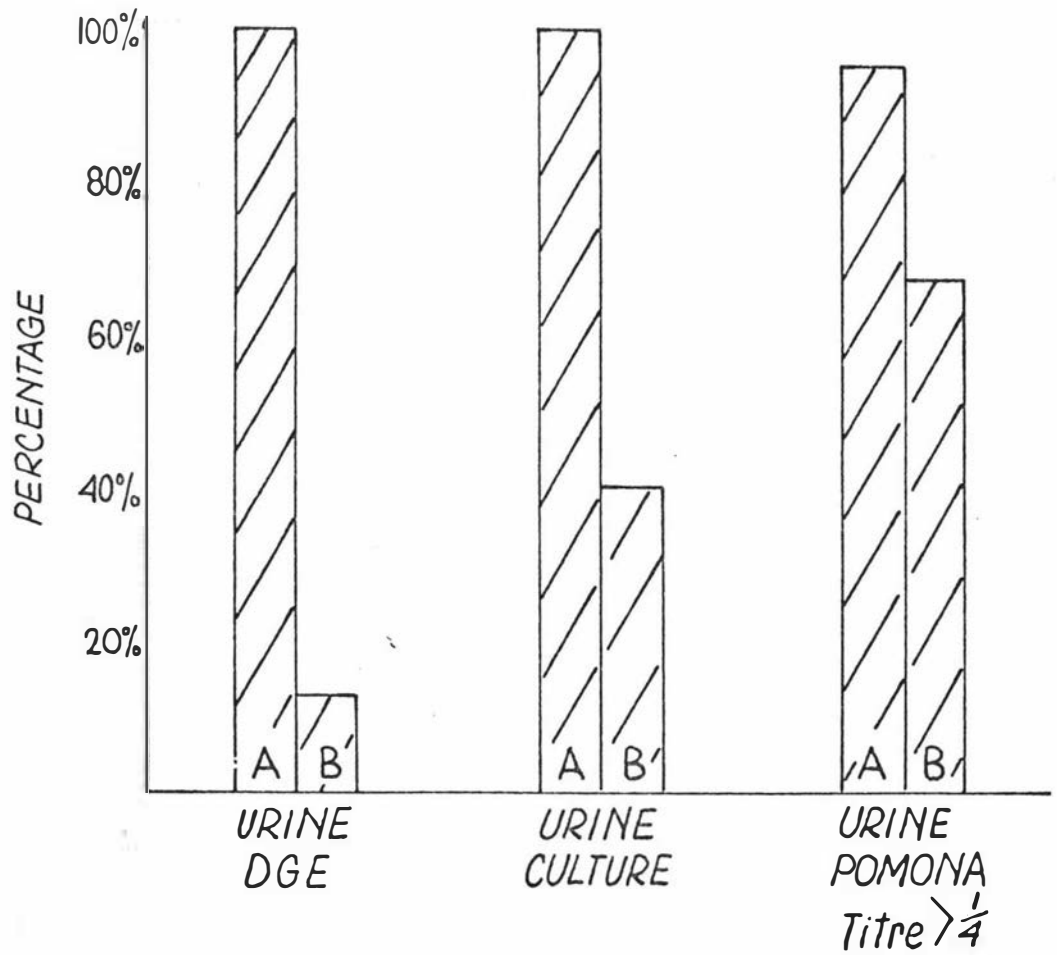


SEROLOGY CODE
1=1/12, 2=1/24, 3=1/48, 4=1/96, 5=1/192,
6=1/384, 7=1/768, 8=1/1536, 9=1/3072,
10=1/6144, 11=1/12288, 12=1/24576.

○ = specificity

\+ = sensitivity

Fig 5.4 The sensitivities and specificities of urine dark-field examination, urine culture and the urinary homologous MA titre in *pomona* infection in pigs.



B = sensitivity

A = specificity

However, on the basis of work reported in Chapter III, the only factor likely to have limited the isolation of *pomona* was a low number of organisms in inocula.

Although it should be recognised that not all of the infected pigs were detected, there is some experimental evidence that suggests all those pigs likely to shed a significant number of leptospires were. Firstly, it has been reported that a consistent pattern of leptospiruria occurs after pigs have been infected with *pomona*. Leptospires first appear in the urine approximately 2 weeks after infection and many organisms are shed for 1 to 3 weeks following this (Ryley and Simmonds, 1954, a, b; Morse *et al.*, 1958; Hodges, 1973; Hodges *et al.*, 1976). The number of organisms in the urine then decreases markedly and in most cases intermittent shedding occurs for only 10 to 50 days. A sudden resurgence of pronounced leptospiruria has not been observed. Secondly, after experimental infection (Morse *et al.*, 1958; Fish *et al.*, 1963) and natural infection (Mitchell *et al.*, 1966) it has been found that serovar *pomona* can be cultured from the kidneys of pigs which had long ceased shedding leptospires. These observations suggest that all those pigs likely to be important shedders of *pomona* will be detected by kidney culture, and, therefore, the results of this survey have valid epidemiological implications.

It appears that the culture-positive animals were an adequate sample to use to estimate the sensitivity of these diagnostic tests. Although a statistically defined random sample of abattoir pigs was not taken, animals of different ages and from many farms, were sampled. It is therefore likely that pigs at different stages of *pomona* infection were included, and the serological results suggest that this was indeed the case. The range of MA titres was similar to that which has been observed after experimental infection (Morse *et al.*, 1958; Ferguson and Powers, 1956) and

natural infection (Mitchell *et al.*, 1966), and the even frequency distribution (Fig. 5.2) suggests that pigs at different stages of infection were indeed adequately represented.

Although it appears that the sensitivities of these diagnostic tests can be used with confidence, the same cannot be said about the specificities which were calculated at each titre. This is because pigs free of *pomona* infection may or may not have MA titres depending on their age and their previous exposure to infection. Firstly, many neonatal piglets have MA titres as a result of colostral antibody (Fish *et al.*, 1963; Chaudhary *et al.*, 1966 b). Secondly, those pigs which have lost their colostral antibody, but have not been infected with serovar *pomona*, will be serologically negative, assuming of course that they do not have coagglutination titres. Thirdly, those that have been infected but have eliminated infection, are likely to still have MA titres (Ryley, 1956, Morse *et al.*, 1958, Mitchell *et al.*, 1966). In this serological survey, animals in categories two and three were sampled, and the bimodal frequency distribution of the culture-negative group (Fig. 5.2) is the result of the different MA titres of animals in these sub-groups.

Because of this possible variation in the homologous MA titre of *pomona*-free pigs, the frequency distribution will vary according to both the age of pigs in the groups, and the proportion of animals that have been infected with *pomona*. For the same reasons, the specificities at each MA titre may vary from group to group. However a useful and valid approximation can still be derived from the data collected in this survey.

The results both in this survey and in another survey of leptospirosis in a herd where all the pigs had been infected with *pomona* (Chapter VI), suggested that the titres of pigs which have recovered from a *pomona* infection are 1/12 or more. This is also in accord with the observations of other workers (Ryley, 1956; Morse *et al.*, 1958; Mitchell *et al.*, 1966). If it is assumed that the animals which were serologically positive at 1/12 are representative of those that have been infected with *pomona*, it would appear that the specificity at 1/384 will be 82% (75/92) in a population where all the pigs have been infected. This estimate of the specificity was confirmed in a later study of an endemically infected herd (Chapter VI). Five out of 25 of the pigs which were 2 years and older were still positive at 1/384. As the duration of renal infection with serovar *pomona* is approximately 4 months in most pigs (Morse *et al.*, 1958) it is most unlikely that these animals would still be infected. Therefore, the estimate of specificity from this herd is 80% (20/25), about the same as that found in the abattoir survey. As the titres of piglets due to colostral antibody exceed 1/100 only over the first few weeks of birth, it would appear that in populations where most of the animals have been infected the specificity at 1/384 is likely to be between 80 and 90%. As has been suggested 85% would seem to be a reasonable compromise figure. It should be noted, however, that this is only an approximation and any data calculated using it should be viewed with this in mind.

In the second survey in which the urinary parameters alone were evaluated, samples from juvenile animals only were available. However, as all non-infected pigs, regardless of whether they have never been infected, or have been infected and recovered, will clearly not shed leptospires in their urine, this sampling bias will not affect the validity of the specificities of darkfield examination of urine or urine culture. On the other

hand, it has been reported that pigs which have recovered from infection with *pomona* may still have urinary homologous MA titres of 1/10 or more (Morse *et al.*, 1958). Therefore, as older recovered animals were not included there must be some doubt concerning the validity of the specificity calculated. It may well be that the specificity of 95% is an overestimate.

The low sensitivities of urine darkfield examination, and urine culture limit the usefulness of these tests for estimating the prevalence of infection. As a result of the low sensitivities, any small change in the apparent prevalence will make a marked difference in the estimate of real prevalence. Leptospires are shed only intermittently in urine, and therefore it can be expected that the apparent prevalence of infection in small groups of pigs would be subject to considerable fluctuation.

In conclusion it should be noted that these diagnostic tests are based on probabilities, and therefore only in large samples will an accurate estimate of the real prevalence of infection be achieved. In small samples some error is to be expected. However, the benefit of a study such as this is that it allows a better appreciation of the likely size and source of such errors, and therefore enables a more reasoned conclusion to be made.

SUMMARY AND CONCLUSIONS

1. Using matched specimens from abattoir pigs, a study was made of the relationship of the serum *pomona* MA titre, urine darkfield examination, urine culture and the urine *pomona* MA titres with the cultural isolation of leptospires from the kidney.

2. The sample of culture-positive animals was considered to be an adequate sample to calculate the sensitivities of the microscopic agglutination test, and the urinary parameters.

3. It was concluded that the specificity of the microscopic agglutination test at low titres might vary according to the age structure of a sample, and the amount of infection in the population. However, in a population with a moderate to high prevalence of infection, at a titre of 1/384 both the sensitivity and specificity will be approximately 85%.

4. The usefulness of darkfield examination of urine and urine culture was limited because of low sensitivities of these parameters.

5. In the sample collected from juvenile animals, 67% of the culture-positive animals had urine MA titres of 1/4 or more, and 95% of the culture-negative animals were test-negative at this level. However, as older recovered animals were not included in this survey it was considered that the specificity of this parameter was overestimated.

Table 5.1 - The proportion of culture-positive animals which were test-positive at each titre (the sensitivity), and the proportion of culture-negative animals which were test-negative at each titre (the specificity).

Pomona titre	1/12	1/24	1/48	1/96	1/192	1/384	1/768	1/1536	1/3072	1/6144	1/12288	1/24576
Sensitivity	95%	95%	89%	84%	84%	84%	75%	62%	50%	38%	30%	27%
Specificity	16%	29%	44%	65%	77%	85%	87%	89%	95%	97%	98%	98%

Table 5.2 - The proportion of culture-positive animals with urine also test-positive (positive darkfield examination, positive urine culture or urinary MA titre test-positive at 1/4), (the sensitivity); and the proportion of culture-negative animals with urine test-negative, (the specificity).

Urine parameter	Darkfield examination	Culture	Urine MAT 1/4
Sensitivity	13%	40%	67%
Specificity	100%	100%	95%

CHAPTER VI

THE EPIDEMIOLOGY OF LEPTOSPIROSIS IN A PIG HERD

INTRODUCTION

There have been few systematic studies of the epidemiology of leptospirosis infection in pig herds. Some workers consider that herds become infected as a result of contact with other infected animal species. For example, the dog has been held responsible for *canicola* outbreaks (Shenberg *et al.*, 1977), the rat for the occasional *copenhageni* infections (Field and Sellers, 1951), and the skunk for *pomona* epidemics (Mitchell *et al.*, 1966). No domestic or wild animal has yet been shown to be responsible for introducing leptospirosis into pig herds in New Zealand, although the hedgehog has been suggested as a wild animal reservoir of *pomona* infection (Webster, 1957).

Little is also known about the pattern of leptospiral infection within pig herds. Buddle and Hodges (1977) have reported a case of a farm where all the pigs in a particular building eventually became infected with serovar *pomona*. These authors considered that this was due to the drainage of urine from the older infected animals into the pens where the younger susceptible pigs were first introduced. It has also been reported that infected boars act as important disseminators of leptospirosis in pig herds (Burki and Wiesmann, 1963). Piglets rarely become infected, even when their mothers are excreting leptospires, owing to the protection afforded by colostral antibody (Fish *et al.*, 1963; Mitchell *et al.*, 1966; Chaudhary *et al.*, 1966 b).

This chapter describes an investigation into the pattern of leptospiral infection in a pig herd. Animals from this farm were sampled at an abattoir to confirm that leptospiral infection was present in the herd. A serological survey of the whole herd was then conducted

and later the older animals were studied more intensively, in order to determine whether or not they were an important reservoir of infection.

This study was part of a collaborative survey of leptospirosis in domestic animals and wildlife on two farms operated by the university. Concurrent studies of other species on these properties were conducted so that a complete picture of the dynamics of leptospiral infection in animals could be developed.

MATERIALS AND METHODS

The Massey University pig farm which was used in this study is an intensively managed unit and during this investigation the size of herd was between 600 and 650 pigs. The herd was composed of two groups; the grower population, which consisted of the young animals (birth to 7 or 8 months), and the breeding herd, which was made up of the unmated gilts, sows and boars. The breeding herd was managed in such a way as to provide a continuous supply of piglets for the grower side of the operation, while the grower animals were either sold as replacement breeding stock, or were slaughtered for pork or bacon.

The management of both the grower and breeding herds followed a regular pattern. All the sows were farrowed in separate pens inside the farrowing unit. The piglets were weaned after 6 weeks, and the sows were returned to the breeding herd area. The piglets from several litters were usually mixed together, and then moved into the weaning unit. After 4 to 6 weeks in the weaning unit, the piglets were moved to a temporary holding area for 2 weeks (grower unit I) before entering a large fattening house, grower unit II. The pigs which were to be slaughtered for pork were selected from those in grower unit II after about 6 to 8 weeks, whereas those gilts destined to be sold as breeding replacements were moved

into grower unit IV. The balance of the animals in grower unit II, those to be slaughtered for bacon, were either kept in this unit, or shifted to grower unit III.

The plan of the buildings used to house the grower herd, and the plan of the effluent drains in these units is illustrated in Fig. 6.1. Of particular note is that effluent in the weaning unit, grower unit II and grower unit IV drained from pen to pen. The ages of the grower pigs at the various stages in the production system are summarised in Table 6.1.

Over the period of this investigation there were from 40 to 50 sows, 5 to 10 unmated gilts and 4 to 5 boars in the breeding herd. The replacement animals for this herd were only rarely selected from the grower population. Virtually all replacement gilts, and all the boars were brought in from another farm, the animals arriving in batches of 4 to 6 every 3 to 4 months. On arrival, the gilts were put out onto pasture for 3 to 4 weeks before being brought in to be mated and introduced into the sow herd. After mating the sows and gilts were placed in individual pens for 3 weeks, and then moved into a large holding area with 4 to 6 animals in each pen. They were held in this area for a further 3 to 4 weeks before being put out onto pasture for the remainder of their gestation period. Two to three days before their anticipated farrowing date the sows were brought into the grower herd area and placed in pens in the farrowing unit.

The plan of the pens used to hold the breeding population is also illustrated in Fig. 6.1. In these units effluent could only drain from pen to pen in the area used for holding groups of sows and gilts after mating.

There was a rapid turnover of animals in both the breeding and grower herds on this farm. Approximately 20% of the sow herd was replaced annually, and young boars

were introduced each 3 to 4 months, with the oldest boar being culled. Similarly there was a rapid movement of animals through the grower units, with pigs being either sent for slaughter or sold to other farmers.

On the farm the gilts in the breeding herd were vaccinated with a killed *pomona - tarassovi* vaccine* approximately 1 to 2 months after their first mating.

Pigs from the Massey farm were included in the abattoir survey reported in Chapter IV. Results of kidney culture and serology on the slaughtered pigs from the Massey herd have accordingly been presented again in this chapter, along with other epidemiological information obtained by on-farm studies.

ABATTOIR SAMPLES

The collection and processing of abattoir samples has been reported in Chapter IV.

FARM STUDIES

Two consecutive investigations were carried out on this farm. The first was a serological survey of the whole herd (Survey A), whereas the second (Survey B) was an intensive study of the breeding herd using both serological and cultural techniques. However, during Survey A, the urine from a small number of animals, and samples of soil and effluent were also cultured for leptospires.

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*"Leptovax-Bivalent." ICI-Tasman, Private Bag, Upper Hutt, New Zealand.

SURVEY A (WHOLE HERD)

SEROLOGICAL STUDIES

Because of the high replacement rate of pigs in both the breeding and grower herds, the bleeding of the same animals on more than one occasion presented considerable difficulties. It was therefore decided to sample at random both the breeding and grower populations every 3 to 4 months. The whole herd was surveyed in November 1975, March 1976 and June 1976. In addition because many of the older sows were culled in the latter part of 1976 and many gilts introduced into the breeding herd, the sow herd was surveyed again in December 1976.

Collection of Samples

Ten animals in each of the buildings used to house the grower population were bled from the anterior vena cava. Five pens in each unit were selected at random, and 2 pigs in each pen were chosen by counting animals against a table of random numbers. At the time of the third sampling (June 1976) the weaning unit had been closed, and all the animals normally held there had been moved to grower unit I. Thus, the weaning unit was only surveyed twice, and the June 1976 survey of grower unit II took in an age group from 6 to 14 weeks, instead of the usual 12 to 14 weeks. Furthermore, as many of the pens in grower units III and IV were often not in use, 5 pens from this combined area were selected and 2 pigs in each pen sampled. To summarise, the subgroups of the grower population which were sampled were:-

(a) Farrowing unit
(b) Weaning unit
(c) Grower unit I
(d) Grower unit II
(e) Grower units III and IV.

All the sows in the breeding herd were individually identified, and a list of these animals, including the gilts which had been recently mated, was prepared immediately before each survey time. Ten animals were randomly selected from this list and sampled. In addition, five of the sows which had been selected for the first survey (November 1975) were also bled during the subsequent surveys.

As there were always less than 10 boars and 10 unmated gilts, all animals in these classes were sampled.

Serology

The MA titres of all animals were determined using the method described in Chapter II.

URINE CULTURES

Diluted and undiluted urine was inoculated into semi-solid P80 medium, with and without 5 - fluorouracil as described in Chapter III.

ENVIRONMENTAL SAMPLING

During the course of this investigation, samples of soil and surface water from the paddocks where the breeding herd was kept, and effluent samples were cultured for leptospires. These highly contaminated samples were cultured either by diffusing them through membrane filters* as described by (Fowler 1970), or by injecting the sample intraperitioneally into hamsters, and then subsequently culturing the hamster kidney and liver as described in Chapter II.

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* Millipore Corporation, Bedford, Massachusetts, U.S.A.

SURVEY B (BREEDING HERD)

COLLECTION OF SAMPLES

In February 1977, blood and urine samples were collected from 16 randomly selected sows, all the boars (5) and all the unmated gilts (4). The gilts were sampled within 12 hours of their arrival on the farm.

All the animals were resampled in April 1977, and all the sows and gilts, but not the boars in June 1977. The boars could not be re-sampled for reasons beyond the author's control.

Four more gilts arrived from another herd in May 1977, and blood and urine samples were also collected from these animals within 12 hours of their arrival. In addition, the gilts were bled again in August 1977 during the course of another experiment and these MAT results have also been included here.

SEROLOGY

The MA titres to *pomona*, *tarassovi*, *ballum*, *hardjo* and *copenhageni* were determined using the method described in Chapter II.

URINE EXAMINATION

All urine samples were examined for leptospires by darkfield microscopy, cultured for leptospires and the *pomona* MA titre determined as described previously (Chapters II and III). In addition, in April and June hamsters were available for experimental use, and ½ ml of undiluted urine from each pig sampled was injected into a hamster intraperitoneally. Kidney and liver samples from any hamster which died were cultured for leptospires, and after 21 days all the remaining hamsters were killed and their kidneys cultured (Chapter III).

OTHER LABORATORY EXAMINATIONS

During this second study, one of the gilts aborted. Vitreous humor, kidney and liver samples from the aborted piglets were cultured for leptospires as described in Chapter III.

RESULTS

ABATTOIR SAMPLES

SEROLOGY

The *pomona* MA titres of the juvenile pigs ranged from 1/384 to 1/24 576. None of these animals had titres of 1/12 or more to *tarassovi*. However reactions to *hardjo*, *copenhageni*, *australis*, *bataviae*, *pyrogenes*, and *grippotyphosa* were present (Table 6.1). As discussed in Chapter IV, all of these reactions were associated with high *pomona* titres. One pig had a *copenhageni* titre as high as 1/1536.

All 4 of the animals from the breeding herd had MA titres to *pomona* ranging from 1/48 to 1/384 (Table 6.1). Other serovars were agglutinated at low titre only.

CULTURE

Serovar *pomona* was isolated from 8 out of 9 (89%) juvenile pigs. No leptospires were isolated from the 4 adult animals sampled.

FARM STUDIES

SURVEY A

SEROLOGY

(a) Farrowing unit

Between 20 and 80% of the sera from the piglets in the farrowing unit were positive to *pomona* at a titre of 1/12 or greater (Table 6.12). Both piglets from each litter samples were either positive or negative to *pomona*. The range of the titres, corrected for the chloroform treatment which was necessary (see Chapter II), was from 1/24 to 1/384. (Table 6.3 and Table 6.13). In contrast to that observed with most of the sera from the abattoir, the sera from the farrowing unit piglets did not react with any of the other serovars.

The average geometric mean titre to *pomona* was 1/18 (Table 6.14, Fig. 6.2). Many animals were serologically negative, and therefore the average geometric mean titre of the positive animals was higher, being 1/110 (Table 6.15). Three piglets were encountered with a titre of 1/384 or greater, and the estimated real prevalence of *pomona* in this population, as estimated by the method described in Chapter V, was 2% with a standard error also of 2% (Table 6.16, Fig. 6.3).

(b) Weaning unit

The agglutination pattern of the sera collected from animals from the weaning unit was similar to that observed in the farrowing unit samples. Forty to fifty percent of the sera were positive at 1/12 to *pomona* (Table 6.4 and 6.12) and the range of these titres was from 1/12 to 1/96 (Table 6.4 and Table 6.13). In addition reactions to the other serovars were observed, all at titres of 1/12 or 1/24.

Overall, most of the *pomona* titres were less than those encountered in pigs from the farrowing unit, with an average geometric mean titre of only 1/13 (Table 6.14) Fig. 6.2). The average geometric mean titre of the positive animals was only 1/34, substantially below that observed in the farrowing unit (Table 6.15). All the *pomona* titres were below 1/384, and therefore both the apparent prevalence and estimated real prevalence of *pomona* infection was zero (Table 6.16, Fig. 6.3).

(c) Grower Unit I

The general pattern was for the prevalence and range of the *pomona* titres in these animals to be less than those in the weaning unit. Between 20% and 50% of sera were positive at 1/12, but all titres were 1/48 or less (Tables 6.12 and 6.13.) The sera from 2 animals also agglutinated other serovars at titres of 1/12 or 1/24 (Table 6.5).

The geometric mean titres to *pomona* reflect these trends (Table 6.14 and 6.15). The average geometric mean titre for all animals was 1/8, and for the positive animals 1/15. As no animals were serologically positive at 1/384, the estimated real prevalence of *pomona* infection was zero (Table 6.16, Fig. 6.3).

(d) Grower Unit III

Although many pigs in this unit were either negative at 1/12 to *pomona*, or had *pomona* titres of 1/12 or 1/24, a number had high *pomona* titres (Table 6.6). The range of *pomona* titres was 1/12 to 1/3012. In most instances it was found that both the animals from the same pen had either negative or low *pomona* titres or had high titres. Those with high *pomona* titres were the oldest members of the grower unit II population. It was also found that the high *pomona* titre sera agglutinated many other serovars, and that the agglutination pattern was similar to that observed in the abattoir surveys (Chapter III).

Because as few as 30% of the samples from animals in grower Unit II were serologically positive (Table 6.12) the average geometric mean titre to *pomona* of all animals, (1/39), is much less than the average geometric mean titre of positive animals, (1/219). A moderate number of animals had titres of 1/384 or more, leading to an estimated prevalence of *pomona* infection over the study period of 19% $\pm$ 10% (Table 6.16, Fig. 6.3).

(e) Grower units III and IV.

The agglutination pattern of the sera from these groups was similar to that observed in the juvenile group in the abattoir survey (Chapter III). A few animals had negative or low *pomona* titres, but most had very high titres (Table 6.7). As was seen in the other groups, all of the sera with high *pomona* titres also agglutinated many other serovars.

The average geometric mean titre to *pomona* for all animals was 1/331 (Table 6.14, Fig. 6.2) and the average geometric mean titre for the positive ones was 1/603. Many pigs had titres of 1/384 or greater, and therefore the estimated real prevalence of *pomona* infection was high (74% $\pm$ 13%) (Table 6.16, Fig. 6.3).

(f) Sows

The ages of the sows in the breeding herd were between 9 months and 5 years, and the age distribution of the animals sampled varied considerably (Table 6.17). In particular towards the end of 1976 many older sows were culled and this altered the age structure considerably.

All of the sows sampled were positive to *pomona* at 1/12 (Tables 6.8 and 6.9) and the range of titres to *pomona* was from 1/24 to 1/768 (Tables 6.13). Although the *pomona* titres of animals of the same age varied

considerably, it was evident that there was a trend for the titre to decrease as their age increased (Fig. 6.4). The regression of the coded *pomona* titres on age was significant ($t = 3.02$, 36 *d.f.*, $P = 0.005$), and 20% of the variation in MA titre could be attributed to the effect of age. The regression equation ($y = 5.81 - 0.49x$) suggested that the *pomona* titre of the sows decreased by approximately half a doubling dilution each year.

The sow sera also agglutinated many other serovars at titres between 1/12 and 1/768. The higher titres occurred with serovar *autumnalis*, most of the other titres being only 1/12 or 1/24 (Table 6.8).

Over the investigation period the average geometric mean titre to *pomona* was 1/135 (Table 6.14, Fig. 6.2). Many of the sows, especially the recently introduced young sows were positive at 1/384 to *pomona*.

The estimated real prevalence of *pomona* infection was 18% $\pm$ 12% (Table 6.16, Fig. 6.3).

Of the 5 sows selected for repeated sampling 3 were still in the herd after 12 months. The *pomona* MA titres of the older animals were stable over this period, whereas that of the youngest of the three animals decreased from 1/384 to 1/96 (Table 6.18).

(g) Boars

At each sampling time there were only 4 boars on the farm, and from 2 to 4 of these were positive at 1/12 to *pomona* on each occasion. (Tables 6.10 and 6.12). In the November 1975 sampling, the range of *pomona* titres was from 1/24 to 1/1536, whereas on the other two occasions *pomona* titres of only 1/12 to 1/48 were encountered. Some of the boar sera also reacted with the other serovars, showing a similar agglutination pattern to that observed in other pigs known to be infected with serovar *pomona* (Table 6.2).

The average geometric mean titre to *pomona* of all animals was 1/37, while that of the boars which were positive at 1/12 was 1/72 (a coded titre of 3.6 $\pm$ 0.9). The average and standard error of the estimated real prevalence of *pomona* infection was 17% $\pm$ 17% (Table 6.16, Fig. 6.3).

(h) Gilts

All of the unmated gilts had positive *pomona* titres (Table 6.11), and the range was from 1/24 to 1/24 576, with many being in the high range (Tables 6.11 and 6.13). Like all of the sera collected from the juvenile pigs at the abattoir (see above), the gilt sera also agglutinated many other serovars (Table 6.11). In most cases *copenhageni* titres were low (1/12 to 1/48), even when the *pomona* titres were very high. However, in the case of the gilts that were bled in November 1975, and in March 1976, some moderate to high *copenhageni* titres (1/192 to 1/1536) were observed. When these animals were retested 4 months later their sera was negative at 1/12 to *copenhageni*.

The average geometric mean titre to *pomona* of the unmated gilts over the study period was 1/1230 (Tables 6.14, 6.15 and Fig. 6.2), while the average estimated prevalence of *pomona* infection was 86% $\pm$ 14% (Tables 6.16 and Fig. 6.3).

URINE CULTURE

Serovar *pomona* was isolated from 2 of the 4 unmated gilts which had both *copenhageni* and *pomona* titres of 1/1536 or greater.

ENVIRONMENTAL SAMPLING

Serovar *pomona* was isolated from effluent flowing from grower unit II and from the area where the boars were penned (Fig. 6.1). In addition, *pomona* was also isolated on one occasion from surface water in the paddock where the unmated gilts were held.

Three other spirochaetes were isolated using the membrane diffusion technique of Fowler (1970). However these organisms were not agglutinated by any of the antisera used for the preliminary identification of leptospires in these studies. Furthermore, it was not possible to infect hamsters with these isolates, and all three grew very much more rapidly *in vitro* than parasitic leptospires. It was therefore concluded that these were saprophytes.

SURVEY B

SEROLOGY

(i) Sows

The age of animals in this sample varied from 9 months to 4 years, with half of the sows being under 2 years old (Table 6.19). All were positive at 1/12 to *pomona*, and the range of titres was from 1/24 to 1/3072. (Table 6.19). As was observed in previous surveys of the sow herd, the younger animals had the higher titres.

Over the 4 month study period the geometric mean titre to *pomona* of the sows remained between 1/331 and 1/288. However, the geometric mean titre of the animals less than 1 year old decreased from 1/933 to 1/660 and then to 1/537 over this period. Many of the younger animals had titres in excess of 1/384, and the average of the estimated real prevalence of *pomona* infection was 55% $\pm$ 1%.

The sera from a few animals also agglutinated the other serovars used in this investigation. Two had persistent titres of 1/24 and 1/48 to *tarassovi*, but the titres to the other serovars were only 1/12.

(ii) Boars

The 3 older boars (43/58, 43/68, 135/7), all had high *pomona* titres in February. Boar 43/68 was culled before the April survey, but the two other boars still had high *pomona* titres at that time (Table 6.21). The *pomona* titre of the replacement boar, number 135/59, was negative at 1/12 on arrival, and 3 months later there was still no serological evidence of infection.

The *pomona* titre of boar 43/69 was negative in February, but this animal had a titre of 1/12 in April.

The boars did not have titres in excess of 1/12 to serovars *ballum*, *copenhageni*, *tarassovi* or *hardjo*.

(iii) Gilts

All but one of the gilts which arrived on the farm in February had *pomona* titres in excess of 1/1536. Over 7 months the titres of these animals steadily decreased to 1/384 (Table 6.20). One gilt, number 110/6, was negative at 1/12 when it arrived on the farm. However, it became infected with *pomona* in July or August and aborted 6 piglets in early September. The *pomona* titre of this animal was 1/384 in August, and in excess of 1/1536 at the time of the abortion.

All the gilts which arrived later in this investigation (May 1977) had *pomona* titres greater than 1/1536 on arrival. These animals were not sampled after this time.

With the exception of the *copenhageni* reactions, only titres of 1/12 against the other serovars were encountered. However, two of the gilts which arrived in May 1977 (86/9 and 16/59) had *copenhageni* titres of 1/96.

URINE EXAMINATION

(i) Sows

Leptospires were not observed in any of the urine samples. Many of the cultures of the undiluted and diluted urine in P80 medium without 5-FU were overgrown with contaminating bacteria. However, this occurred with only a small number of cultures in the 5-FU containing medium, and the greatest number of isolates were obtained from this medium. Serovar *pomona* was isolated from one animal (number 30/67) consistently over the 5 month period. In the April survey serovar *pomona* was also isolated from the urine of sow 13/8 (Table 6.22). The hamsters injected with urine from 30/67 and 13/8 remained normal, and no leptospires were isolated from their kidneys at 21 days.

Pomona agglutinins were not encountered consistently in the urine of any animal. At various times 5 sows had urinary titres of 1/4 or greater. Four of these animals were about 1 year old, while the fifth was 4 years old. The urine from which *pomona* was isolated had MA titres of less than 1/4 (Table 6.22).

(ii) Boars

It was not possible to collect a midstream urine sample from the boars. They usually voided small amounts of urine, allowing it to dribble from the prepuce. As a result, most of the urine cultures were heavily contaminated with other bacteria. However, serovar *pomona* was isolated from the kidney of a hamster which had been injected with the urine collected from a boar

(number 43/69) during the April sampling. No leptospires were observed in any of the urine samples by dark-ground microscopy.

(iii) Gilts

Large numbers of leptospires were observed in the urine of two of the gilts (numbers 86/9 and 86/59) which arrived in May. No leptospires were seen in any other urine specimens.

Serovar *pomona* was isolated from the urine of one of the February replacements (number 5/57), and from the urines of the May replacements (86/9 and 86/59) in which leptospires had also been observed (Table 6.20).

The urines from all the May replacements had *pomona* titres in excess of 1/4. However, as was observed with the sow samples, the urine from the other group of gilts did not always have *pomona* MA titres. Two, 5/57 and 130/8, had titres of 1/4 or greater on one occasion (Table 6.20).

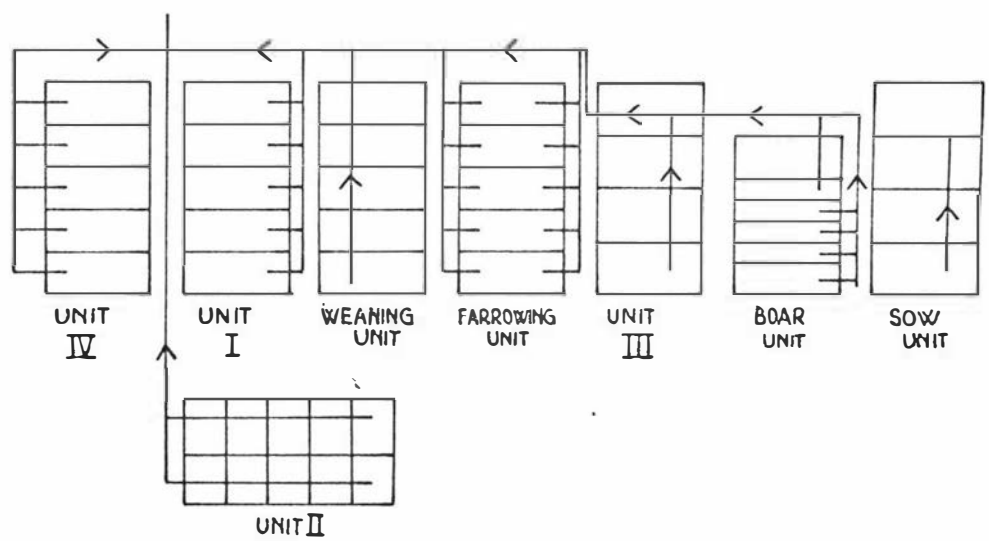
OTHER LABORATORY EXAMINATIONS

Serovar *pomona* was isolated in pure culture from the vitreous humor of all the piglets aborted by gilt 110/6.

DISCUSSION

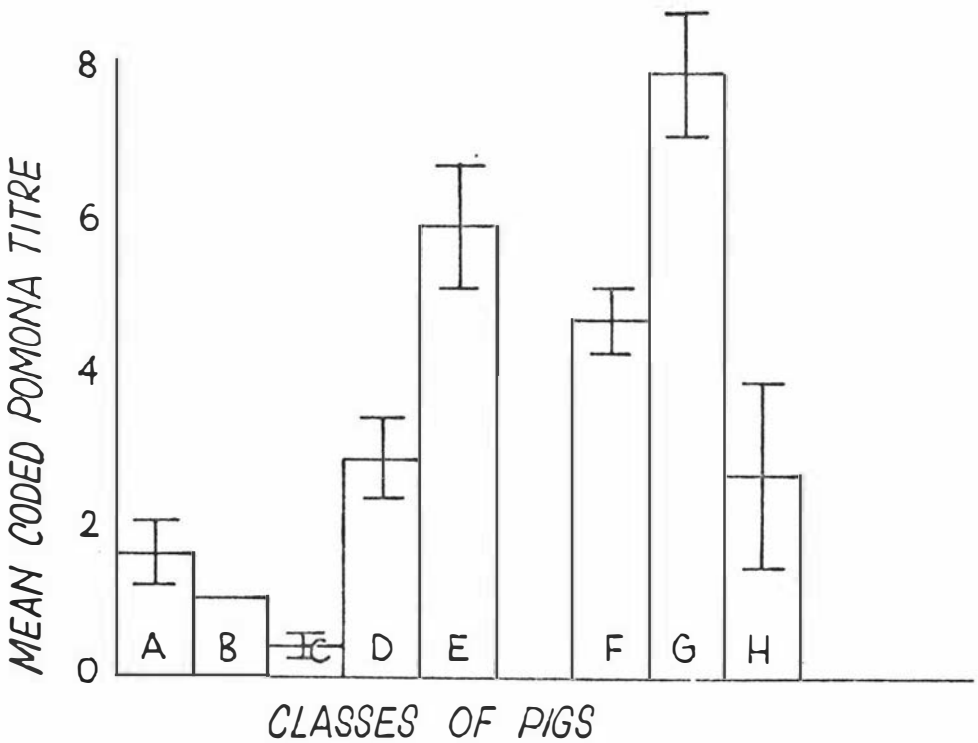
With the exception of one juvenile pig which had a high *copenhageni* titre, the abattoir results were similar to those occurring with *pomona* infected pigs from other farms (Chapter IV). The agglutination patterns were also similar, and the prevalence of renal infection with *pomona* with respect to age was the same as that observed in these animals.

Fig. 6.1 A schematic plan of the Massey University pig farm showing the pattern of open drainage.



→ = flow of effluent

Fig. 6.2 The average and standard error of the mean coded titres of all pigs in each group sampled in the epidemiological studies.

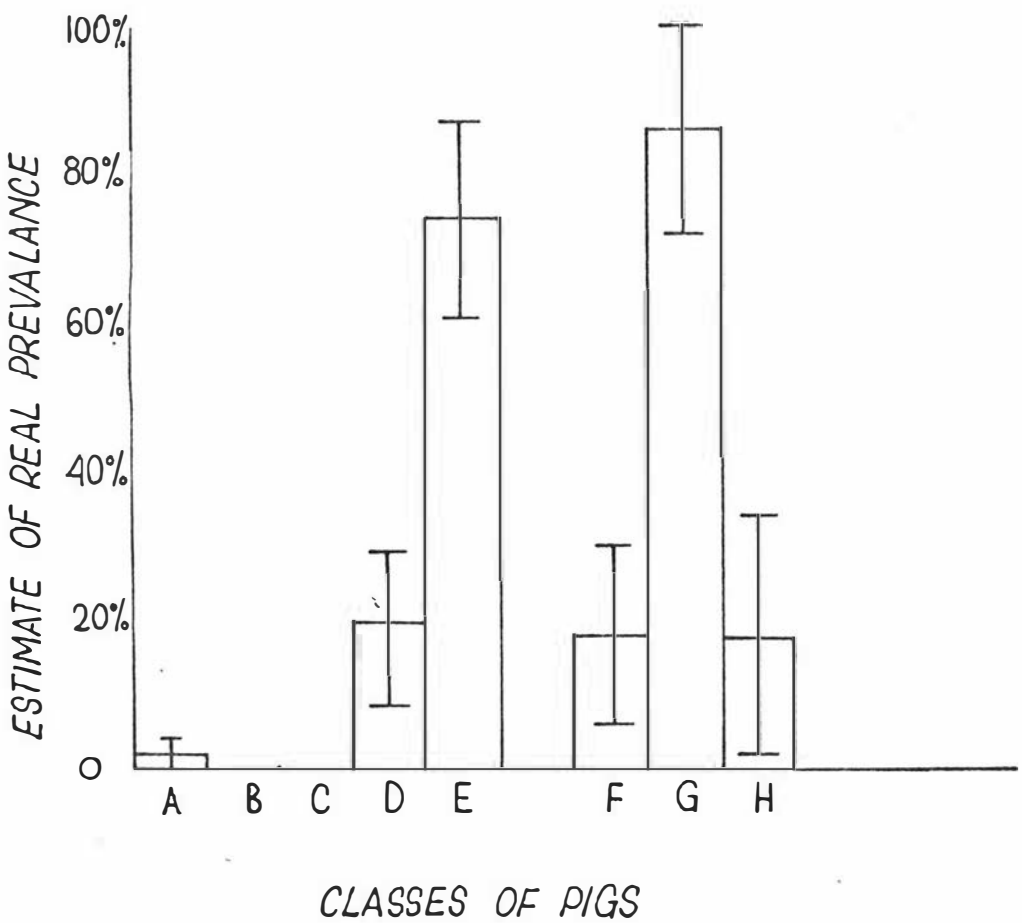


SEROLOGY CODE
1=1/12, 2=1/24, 3=1/48, 4=1/96, 5=1/192,
6=1/384, 7=1/768, 8=1/1536, 9=1/3072,
10=1/6144, 11=1/12288, 12=1/24576.

A = farrowing unit piglets)
B = weaning unit piglets)
C = grower unit I pigs) grower herd
D = grower unit II pigs)
E = grower unit III + IV pigs)

F = sows)
G = gilts) breeding herd
H = boars)

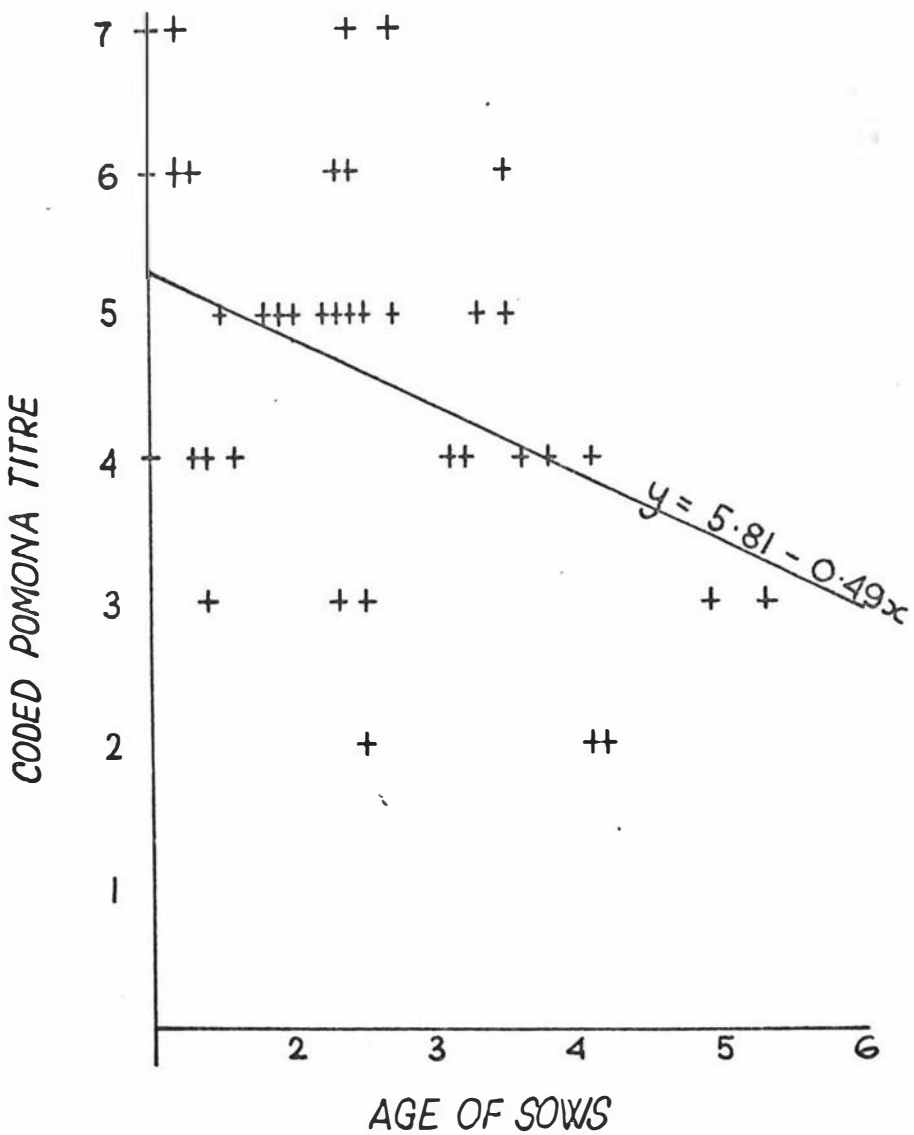
Fig. 6.3 The average and standard error of the estimated real prevalences of *pomona* infection in each group sampled.



A = farrowing unit piglets)
B = weaning unit piglets)
C = grower unit I pigs) grower herd
D = grower unit II pigs)
E = grower unit III + IV pigs)

F = sows)
G = gilts) breeding herd
H = boars)

Fig. 6.4 The association between the coded *pomona* MA titres and age in the sow herd.



SEROLOGY CODE
1=1/12, 2=1/24, 3=1/48, 4=1/96, 5=1/192,
6=1/384, 7=1/768, 8=1/1536, 9=1/3072,
10=1/6144, 11=1/12288, 12=1/24576.

The results of the serological survey of the whole herd indicated that there was a focus of *pomona* infection in the animals aged between about 6 and 12 months. Although some of the neonates had moderately high *pomona* titres, the results point to these being due to colostral antibody, rather than to active infection.

The steady decline in MA titres to *pomona* observed in the older sows suggests that by the time the sows were 12 to 18 months old they had eliminated their *pomona* infection. The negative kidney culture results of the small number of adults which were sampled at the abattoir confirms this conclusion. Furthermore, there was no evidence that pigs older than about 18 months were shedding leptospires in their urine.

In most cases the gilts were already infected when they were first mated. This is in contrast to the situation described by Burki and Wiesmann (1963) who reported that sows became infected from boars at mating.

Serovar *ballum* was isolated from the rats, mice and hedgehogs which were commonly found about the piggery, and serovar *balcanica* was isolated from opossums in this area (Hathaway, 1978). In the cattle on the adjacent farm endemic infection with serovar *hardjo* was found (Hellstrom, 1978). No wildlife species had *pomona* titres, and only a few older cows had low *pomona* titres. It is clear from the results presented in this chapter that *pomona* infection is endemic in the pig farm under study. The only evidence indicating transmission to other species in the same area is the observation that low titres to *pomona* occurred in the cattle. These low titres probably indicate a past *pomona* infection in the cattle which did not persist in the herd.

The endemic state of *pomona* infection on the pig farm depends upon the direct or indirect contact of young susceptible pigs with older infected animals. The effluent channels running from pen to pen in grower units II and III, and the mixing of stock of different ages would ensure that infection was maintained in this herd. This is a similar situation to that observed by Buddle and Hodges (1977).

Of interest is the fact that effluent in the weaner house also runs from pen to pen but animals in this group were not infected. It would seem very unlikely that infection had not been introduced into this area sometime previously. It is presumed therefore that residual colostral antibody of the weaners ensured that they did not become infected.

As the titres against *tarassovi*, *ballum*, *hardjo* and *canicola* were either negative or very low it was concluded that the pigs in this herd had not become infected with these serovars. It was also concluded that the MA titres against *autumnalis*, *bataviae*, *pyrogenes*, *australis* and *grippotyphosa* were cross-reactions of *pomona* antibody. It is reasonable to expect that if infection with these serovars had occurred, the homologous titres would have not been so well correlated with the *pomona* titres.

Initially it was considered that the high *copenhageni* titres, which were demonstrated in the sera of one abattoir animal and in the sera from the unmated gilts, indicated infection with this serovar. In the abattoir surveys, and in other animals sampled in this study of leptospirosis in pigs, high *pomona* titres were never associated with high *copenhageni* titres, and therefore dual infection seemed likely. However, as these *copenhageni* titres had disappeared after 4 months, and the sera did not coagglutinate serovar *canicola* as it characteristically does in cases of *copenhageni* infection

(Alston and Broom, 1958), it was concluded that the titres observed were paradoxical heterologous reactions. *Pomona* strains with a similar propensity to induce high *copenhageni* titres early in infection have been reported elsewhere (Morse and Allen, 1956).

The results of the effluent and soil studies were disappointing. With so many infected animals present it was expected that leptospires would have been isolated from many samples. However, it is significant that *pomona* was isolated from surface water in the paddock where the unmated gilts were kept. These animals were shedding large numbers of leptospires at that time. In a paddock on the dairy farm adjacent to this area *pomona* was also isolated from surface water (Hellstrom, 1978). The pigs would therefore seem to be an important potential source of *pomona* infection for the cattle.

Over the 18 month period of this investigation, the reproductive performance of the sows and boars was also recorded. Any sows returning to oestrous after mating were bled and the MA titres to *pomona*, *ballum*, *copenhageni*, *hardjo* and *tarassovi* were determined. Any aborted piglets were necropsied and tissues cultured for leptospires. However, the only effect which could be conclusively attributed to leptospirosis was the abortion which occurred in gilt 110/6. The MA titres of the sows which returned to oestrous were similar to those which farrowed normally. Only 3 other abortions were reported and in these there was no evidence of leptospirosis. The fertility of boars could not be correlated to the presence or absence of positive *pomona* titres, or to leptospiruria. Overall it would seem as if leptospirosis infection was not having a major effect on fertility in this herd.

Late returns to oestrous were a problem in this herd. This type of infertility, without evidence of an abortion

has also been observed in other *pomona* infected herds. (Powers *et al.*, 1956), and has also been reported following experimental infection with this serovar (Ferguson and Powers, 1956). It has been suggested that embryonic death due to infection with *pomona* and resultant embryonic resorption is the cause of this syndrome. It has also been suggested that in leptospirosis a chronic infection of the uterus may occur and lead to reproductive losses such as this (Hanson, 1977). In order to examine this postulate further work not reported here in detail was undertaken. Uteri and kidneys from a number of gilts were collected at an abattoir and samples of each organ were cultured for leptospires. Sections of each uterus were also taken and examined for lesions. However, no leptospires were isolated from the uterine tissues, and no abnormalities of the uteri were seen even though leptospires were isolated from 35% (7/20) of the kidney samples. It would, therefore, appear that although leptospires may localise in the endometrium during the acute phase (Dozsa and Sahu, 1970) organisms are eliminated rapidly and no lasting effects on fertility will occur. As virtually all the sows were infected well before their first mating, it would seem unlikely that leptospirosis was the cause of these late returns in this herd.

Because infection occurs in the gilts at an early age, the vaccination programme employed on the farm against leptospirosis was of little use. In addition, if the case of gilt 110/6 is indicative of the level of resistance resulting from vaccination, it would also seem that where animals have not been infected, vaccination does not induce sufficient protection. Hodges *et al.* (1976) also found that the same vaccine did not protect against *pomona* infection in pigs.

In an earlier survey (Chapter V), it was found that a screening test based on the homologous urinary MA titre might be a useful indicator of renal infection. However, the variable urinary titres observed in this field study indicate that this would be a very unreliable diagnostic test.

SUMMARY AND CONCLUSIONS

1. The pattern of leptospiral infection in a pig herd was studied. This was a part of a collaborative study of leptospirosis infection in pigs, cattle and wildlife in the same area.

2. It was concluded that serovar *pomona* was endemic in the pig herd and that the focus of infection was in those pigs aged between 6 and 12 months.

3. The sera of the *pomona* infected pigs cross-reacted with many other serovars. Some striking paradoxical heterologous titres to *copenhageni* were observed.

4. The *pomona* MA titres rose to high levels shortly after infection (1/1536 to 1/24576), but decreased to between 1/24 and 1/768 within 6 to 7 months, and then decreased by approximately half a doubling dilution per year.

5. Most of the gilts introduced from another farm had been recently infected with *pomona*. Likewise many of the gilts on this farm offered for sale as replacement stock were infected with *pomona*.

6. There was no evidence of current infection in the older sows.

7. Pig to pig transmission of *pomona* infection was facilitated by the system of management, and the design of the buildings used to house the young stock.

Table 6.1 The age of pigs in the various units on the farm being studied.

HOUSE	AGE
Farrowing Unit	Birth - 6 weeks
Weaner Unit	6 - 12 weeks
Grower Unit I	12 - 14 weeks
Grower Unit II	14 weeks +
Grower Unit III	20 weeks +
Grower Unit IV	20 weeks +

Table 6.2 The coded MA titres of the grower and breeding pigs sampled at the abattoir.

		pm	ta	bl	ha	cp	au	at	bt	ca	py	gr
	1	10			1		2	5	2		3	2
	2	7					2	4	2		3	1
	3	7						3	1		3	1
	4	6						4	1			
Grower	5	12			4		5	8			5	4
Herd	6	9			2		1	4	1		2	1
	7	9				8	5	8	4		5	3
	8	6						2				
	9	8				4	4	7	5		1	
	10	3		1	1		1	1	1			1
Breeding	11	5				1						
Herd	12	6				1	1	1				
	13	3										

pm=*pomona*, ta=*tarassovi*, bl=*ballum*, ha=*hardjo*, au=*australis*, at=*autumnalis*, bt=*bataviae*, ca=*canicola*, py=*pyrogenes*, gr=*grippotyphosa*.

1=1/12, 2=1/24, 3=1/48, 4=1/96, 5=1/192, 6=1/384, 7=1/768, 8=1/1536, 9=1/3072, 10=1/6144, 11=1/12288, 12=1/24576.

Table 6.3 The coded MA titres of the piglets in the farrowing unit.

Date	Animal	pm	ta	bl	ha	cp	au	at	bt	ca	py	gr
Nov.	A1	3										
1975	A2	6										
	A3	6										
	A4	4										
	A5	2										
	A6	2										
	A7											
	A8											
	A9											
	A10											
March	B1	2										
1976	B2	3										
	B3											
	B4											
	B5	5										
	B6	3										
	B7											
	B8											
	B9											
	B10											
June	C1											
1976	C2											
	C3	6										
	C4	5										
	C5											
	C6											
	C7											
	C8											
	C9											
	C10											

pm=*pomona*, ta=*tarassovi*, bl=*ballum*, ha=*hardjo*, au=*australis*, at=*autumnalis*, bt=*bataviae*, ca=*canicola*, py=*pyrogenes*, gr=*grippotyphosa*.

1=1/12, 2=1/24, 3=1/48, 4=1/96, 5=1/192, 6=1/384, 7=1/768, 8=1/1536, 9=1/3072, 10=1/6144, 11=1/12288, 12=1/24576

Table 6.4 The coded MA titres of the piglets in the weaning unit.

		pm	ta	bl	ha	cp	au	at	bt	ca	py	gr
Nov.	A1											
1975	A2				1							
	A3	4			2			1	1			
	A4	1										
	A5											
	A6	4						2	1		2	
	A7											
	A8											
	A9	1										
	A10	2										
Mar.	B1											
1976	B2											
	B3											
	B4											
	B5	1					1					
	B6											
	B7	4					1					
	B8	3										
	B9	2					1					
	B10											

pm=*pomona*, ta=*tarassovi*, bl=*ballum*, ha=*hardjo*, au=*australis*, at=*autumnalis*, bt=*bataviae*, ca=*canicola*, py=*pyrogenes*, gr=*grippotyphosa*.

1=1/12, 2=1/24, 3=1/48, 4=1/96, 5=1/192, 6=1/384, 7=1/768, 8=1/1536, 9=1/3072, 10=1/6144, 11=1/12288, 12=1/24576.

Table 6.5 The coded MA titres of the piglets in grower Unit I.

Date		pm	ta	bl	ha	cp	au	at	bt	ca	py	gr
Nov.	A1											
1975	A2	1										
	A3	1										
	A4											
	A5											
	A6											
	A7											
	A8	3										
	A9	1										
	A10	1										
Mar.	B1	2										
1976	B2											
	B3											
	B4											
	B5											
	B6											
	B7											
	B8	1										
	B9											
	B10											
June	C1											
1976	C2											
	C3								1			
	C4	1					2	2			2	
	C5											
	C6											
	C7											
	C8	1										
	C9	1										
	C10											

pm=*pomona*, ta=*tarassovi*, bl=*ballum*, ha=*hardjo*, au=*australis*, at=*autumnalis*, bt=*bataviae*, ca=*canicola*, py=*pyrogenes*, gr=*grippotyphosa*.

1=1/12, 2=1/24, 3=1/48, 4=1/96, 5=1/192, 6=1/384, 7=1/768, 8=1/1536, 9=1,3072, 10=1/6144, 11=1/12288, 12=1/24576.

Table 6.6 The coded MA titres of the pigs in grower Unit II.

Date	Animal	pm	ta	bl	ha	cp	au	at	bt	ca	py	gr
Nov.	A1	3										
1975	A2											
	A3	1										
	A4	9					4	6	2		4	3
	A5	9					3	6	1		3	
	A6	7						3	1		1	
	A7	7							2		1	1
	A8											
	A9											
	A10											
Mar.	B1	2										
1976	B2	7					1	5				
	B3	7					1	5				
	B4	7					1	5	1			
	B5											2
	B6											
	B7	1										
	B8											
	B9	2										
	B10	1										
June	C1											
1976	C2							1				
	C3	5										
	C4	5				1		1				
	C5	7					1	4	2	1	2	
	C6											
	C7											
	C8											
	C9											
	C10											

pm=*pomona*, ta=*tarassovi*, bl=*ballum*, ha=*hardjo*, au=*australis*, at=*autumnalis*, bt=*bataviae*, ca=*canicola*, py=*pyrogenes*, gr=*grippotyphosa*.

1=1/12, 2=1/24, 3=1/48, 4=1/96, 5=1/192, 6=1/384, 7=1/768 8=1/1536, 9=1/3072, 10=1/6144, 11=1/12288, 12=1/24576.

Table 6.7 The coded MA titres of the pigs in grower units III and IV.

		pm	ta	bl	ha	cp	au	at	bt	ca	py	gr
Nov.	A1	9			5		3	7	4			4
1975	A2	9			2		3	5	2		4	
	A3	3										
	A4	7						2				
	A5	7						2				
	A6	3										
	A7	2						1				
	A8	8			2			4	3		2	2
	A9	9			2			6	4		2	2
	A10	9			4			4	2		4	3
Mar.	B1											
1976	B2											
	B3	8				1	4	8	1			
	B4											
	B5	2										
	B6											
	B7	7						2	1		1	
	B8											
	B9	8	2			2		5	2		3	1
	B10	9	1			1		2	1		1	
June	C1	7						3				
1976	C2	6				1		2				
	C3	7						4				
	C4	9					1	5	1		2	
	C5	1										
	C6	4						3				
	C7	9					1	4	1		1	
	C8	8						5	1			
	C9	6						4				
	C10	9						2				

pm=*pomona*, ta=*tarassovi*, bl=*ballum*, ha=*hardjo*, au=*australis*, at=*autumnalis*, bt=*bataviae*, ca=*canicola*, py=*pyrogenes*, gr=*grippotyphosa*.

1=1/12, 2=1/24, 3=1/48, 4=1/96, 5=1/192, 6=1/384, 7=1/768, 8=1/1536, 9=1/3072, 10=1/6144, 11=1/12288, 12=1/24576.

Table 6.8 The coded MA titres of the sows.

		pm	ta	bl	ha	cp	au	at	bt	ca	py	gr
Nov.	A1	5		1			2	2			1	
1975	A2	5					2	4				
	A3	7					4	4	3		4	3
	A4	5			1		2	2			1	1
	A5	3										
	A6	6	1		1		2	5	1			
	A7	2						1			1	
	A8	5						1				
	A9	6						5			1	
	A10	4						3	1		1	1
Mar.	B1	7	1			3	2	7	2	2	2	2
1976	B2	5	1		1			4	2			
	B3	2				1	1	5			1	
	B4	3				1		3	1			
	B5	3						1			3	
	B6	4	2				1	5			1	1
	B7	4	2			1	2	3	3	2	1	
	B8	4	1				3	4	1			
	B9	4	1				1	3				3
	B10	3					1	5				
June	C1	4				1		3				
1976	C2	2					1	3				
	C3	3						1			2	
	C4	3						1				
	C5	6					1	3			2	
	C6	5						3			2	
	C7	4						3				
	C8	5	1					2				1
	C9	3				2	2	1				1
	C10	5						4				

pm=*pomona*, ta=*tarassovi*, bl=*ballum*, ha=*hardjo*, au=*australis*, at=*autumnalis*, bt=*bataviae*, ca=*canicola*, py=*pyrogenes*, gr=*grippotyphosa*.

1=1/12, 2=1/24, 3=1/48, 4=1/96, 5=1/192, 6=1/384, 7=1/768, 8=1/1536, 9=1/3072, 10=1/6144, 11=1/12288, 12=1/24576

Table 6.9 The coded MA titres of sows sampled in December 1976.

	pm	ta	bl	ha	cp	au	at	bt	ca	py	gr
D1	5	1		1		-	-	-	-	-	-
D2	6					-	-	-	-	-	-
D3	6					-	-	-	-	-	-
D4	7					-	-	-	-	-	-
D5	4					-	-	-	-	-	-
D6	4	1				-	-	-	-	-	-
D7	7	1				-	-	-	-	-	-
D8	4					-	-	-	-	-	-
D9	5					-	-	-	-	-	-
D10	6					-	-	-	-	-	-

Table 6.10 The coded MA titres of the boars

Date	Animal	pm	ta	bl	ha	cp	au	at	bt	ca	py	gr
Nov.	A1	6			1		3	4			3	
1975	A2	5					1	3			1	1
	A3	2		1	2							
	A4	8			3	3	2	2			2	
Mar.	B1	3	1			1		4	1			1
1976	B2		3			3		1				
	B3	1					1	5	1		1	1
	B4	1						3				
June	C1	2						2	1		1	
1976	C2		1					2				
	C3	3						3			2	
	C4											

pm=*pomona*, ta=*tarassovi*, bl=*ballum*, ha=*hardjo*, au=*australis*, at=*autumnalis*, bt=*bataviae*, ca=*canicola*, py=*pyrogenes*, gr=*grippotyphosa*.

1=1/12, 2=1/24, 3=1/48, 4=1/96, 5=1/192, 6=1/384, 7=1/768, 8=1/1536, 9=1/3072, 10=1/6144, 11=1/12288, 12=1/24576.

Table 6.11 The coded MA titres of the gilts.

Date		pm	ta	bl	ha	cp	au	at	bt	ca	py	gr
Nov.	A1	10	1	2	3	8	3	6	4		3	1
1975	A2	8			4	8	1	4	2		4	
	A3	9		1		8	5	7	2		4	1
	A4	11		1		8	7	8	8		7	4
	A5	12			5	4	5	8	3		2	4
	A6	9		1	5	1	1	6	2		3	2
	A7	4			3	1		1			1	
Mar.	B1	5			1		2	6	2			2
1976	B2	2	1		1		3	1	1		1	1
	B3	9						4	6			
	B4	8	2	1	1		4	5	2	1	1	2
	B5	6	2		1	2	2	6	1	1	4	1
	B6	9	2				3	2	2		1	
	B7	10	1		1	4	5	8	1	1	1	3
	B8	5				4		5				
	B9	4	1			4	3	7	1		1	2
June	C1	6		1			3	4				
1976	C2	10	4	1			3	6	2	1	4	1
	C3	8		1			2	6	1		4	1
	C4	7					1	3				1

pm=*pomona*, ta=*tarassovi*, bl=*ballum*, ha=*hardjo*, au=*australis*, at=*autumnalis*, bt=*bataviae*, ca=*canicola*, py=*pyrogenes*, gr=*grippotyphosa*.

1=1/12, 2=1/24, 3=1/48, 4=1/96, 5=1/192, 6=1/384, 7=1/768, 8=1/1536, 9=1/3072, 10=1/6144, 11=1/12288, 12=1/24576.

Table 6.12 The prevalence in each group of animals serologically positive at 1/12 to *pomona*.

Population	Prevalence test-positive at 1/12			
	November 1975	March 1976	June 1976	December 1976
Farrowing Unit	80%	60%	20%	-
Weaning Unit	50%	40%	-	-
Grower Unit I	50%	20%	30%	-
Grower Unit II	60%	70%	30%	-
Grower Unit III + IV	100%	60%	100%	-
Sows	100%	100%	100%	100%
Gilts	100%	100%	100%	-
Boars	100%	75%	50%	-

Table 6.13 The range of titres to *pomona* in each group.

Population	Minimum Titre	Maximum Titre
Farrowing Unit	1/24	1/384
Weaning Unit	1/12	1/96
Grower Unit I	1/12	1/48
Grower Unit II	1/12	1/3072
Grower Unit III + IV	1/12	1/3072
Sows	1/48	1/768
Gilts	1/48	1/24576
Boars	1/12	1/1536

Table 6.14 The average and standard error of the mean coded titres of all pigs in each group, and the average geometric mean titres (gmt's).

Group	Average (mean coded titres)	Average gmt
Farrowing Unit	1.6 ± 0.4	1/18
Weaning Unit	1.1 ± 0.1	1/13
Grower Unit I	0.4 ± 0.1	1/8
Grower Unit II	2.7 ± 0.5	1/39
Grower Unit III + IV	5.8 ± 0.8	1/331
Sows	4.5 ± 0.4	1/135
Boars	2.6 ± 1.3	1/37
Gilts	7.7 ± 0.8	1/1230

Table 6.15 The average of the mean coded titres and the average of the geometric mean titres (gmt's) of those pigs which were positive at 1/12, in each group.

Group	Average (mean coded titres)	Average gmt
Farrowing Unit	4.2 ± 0.7	1/110
Weaning Unit	2.5 ± 0.1	1/34
Grower Unit I	1.3 ± 0.2	1/15
Grower Unit II	5.2 ± 0.7	1/219
Grower Unit III + IV	6.7 ± 0.1	1/603
Sows	4.5 ± 0.4	1/135
Boars	3.6 ± 0.9	1/72
Gilts	7.7 ± 0.8	1/1230

Table 6.16 Apparent (AP)* and estimated real prevalence (RP)** of *pomona* infection in each population at each sampling period, and the average and standard error (SE) over the study period.

Population	Nov. 1975 AP	Nov. 1975 RP	Mar. 1976 AP	Mar. 1976 RP	June 1976 AP	June 1976 RP	Dec. 1976 AP	Dec. 1976 RP	Average RP + SE
Farrowing Unit	20%	7%	0	0	0	0	-	-	2% ± 2%
Weaner Unit	0	0	0	0	0	0	-	-	0
Grower Unit I	0	0	0	0	0	0	-	-	0
Grower Unit II	40%	36%	30%	21%	10%	0	-	-	19% ± 10%
Grower Unit III & IV	70%	79%	50%	50%	80%	93%	-	-	74% ± 13%
Sows	30%	21%	10%	0	10%	0	50%	50%	18% ± 12%
Gilts	86%	100%	56%	58%	100%	100%	-	-	86% ± 14%
Boars	50%	50%	0	0	0	0	-	-	17% ± 17%
Total	34%	27%	19%	6%	27%	17%	-	-	17% ± 6%

* Apparent prevalence = percentage of pigs in each group with titre 1/384 or greater.

** Estimated real prevalence = $\frac{\text{AP + specificity - 1}}{\text{specificity + sensitivity - 1}}$, where

specificity = sensitivity = 85%. (See Chapter VI).

Table 6.17 Sow Herd. Age distribution of the sows in each sample.

Age (Years)	November 1975	March 1976	June 1976	December 1976
0 - 1.0	1	-	-	2
1.1 - 2.0	2	3	3	3
2.1 - 3.0	2	2	4	3
3.1 - 4.0	3	3	-	2
4.1 - 5.0	2	1	2	-

Table 6.18 *Pomona* MA titres of sows that were repeat sampled from November 1975 to December 1976.

Number & Age	November 1975	March 1976	June 1976	December 1976
3 - 6 (2.7 yrs)	1/192	1/96	1/96	1/192
124 - 59 (2.4 yrs)	1/768	1/192	(Died, not necropsied)	
52 - 7 (3.5 yrs)	1/384	1/192	1/384	(Culled)
160 - 56 (4.1 yrs)	1/24	1/24	1/48	1/48
78 - 58 (1.0 yr)	1/384	1/192	1/96	1/96

Table 6.19 The age of the sows in February 1977 and the coded *pomona* MA titre at each sampling.

Sow Number	Age (Years)	Feb.	April	June
27 - 6	0.8	7	7	6
13 - 8	0.9	5	5	4
95 - 8	1.0*	6	7	7
30 - 67	1.0*	8	7	7
96 - 58	1.1	9	8	-**
15 - 59	1.1	7	6	8
95 - 59	1.4	9	7	8
55 - 56	1.7	7	7	6
13 - 9	2.0	6	6	8
53 - 6	2.0*	5	4	4
78 - 58	2.4	4	5	4
124 - 6	3.0	7	7	7
118 - 9	3.1	3	3	3
61 - 5	3.3	2	3	4
3 - 6	3.9	5	5	5
23 - 9	3.9	3	3	4

* Age estimated from date of arrival on farm.

** Animal culled from herd before June sampling.

Table 6.20 Gilts: coded *pomona* MA titres, results of urine culture, and the *pomona* MA titres of urine.

Gilt No.	*Pomona* MA titre					Urine Culture			Urine MA titre ≥ 1/4		
	Feb.	April	June	Aug.	Sept.	Feb.	April	June	Feb.	April	June
99/6	8	8	7	6	N.S.	-	-	-	-	-	-
130/8	8	8	8	6	N.S.	-	-	-	+	-	-
110/6	0	0	3	6	8	-	-	-	-	-	-
5/57	8	8	7	6	N.S.	+	-	+	-	+	-
86/9*	N.S.	N.S.	8	N.S.	N.S.	N.S.	N.S.	+	N.S.	N.S.	+
86/59*	N.S.	N.S.	8	N.S.	N.S.	N.S.	N.S.	+	N.S.	N.S.	+
16/59*	N.S.	N.S.	8	N.S.	N.S.	N.S.	N.S.	-	N.S.	N.S.	+
23/6*	N.S.	N.S.	8	N.S.	N.S.	N.S.	N.S.	-	N.S.	N.S.	+

* Arrived at farm May 1977

N.S. No sample; (+) = serovar *pomona* isolated from urine, or urine MA titre 1/4 or greater; (-) = urine culture negative, or urine MA titre less than 1/4.

Table 6.21 The boars: the coded MA titres in February and April, 1977.

Boar Number	February	April
135 - 7	8	9
43 - 58	7	7
43 - 68	8	N.S.*
135 - 59	0	0
43 - 69	0	1

* N.S. = No Sample

Table 6.22 Sow Herd: Results of urine culture, and the *pomona* MA titres of urine.

Sow No.	February Culture Titre >½	April Culture Titre >½	June Culture Titre >½
27/6		+	+
95/59			
55/56			
118/9			
30/67	+	+	+
78/58			
61/5			
96/58		+	N.S.* N.S.
15/59			+
95/8		+	
13/9			
124/6			
3/6			
13/8		+	
53/6			
23/9		+	

* No sample

+ = Serovar *pomona* isolated or urinary titre greater than ½.

CHAPTER VII

SEROVAR *TARASSOVI* INFECTION IN PIGS

INTRODUCTION

The survey of leptospiral infection in pigs conducted in the first part of this study (Chapter IV) indicated that *pomona* occurred very commonly, while the occurrence of *tarassovi* infection appeared to be sporadic. It was therefore decided to conduct a more detailed serological investigation in the herds where *tarassovi* infection had been found, in order to elucidate the pattern of infection with this serovar.

MATERIALS AND METHODS

COLLECTION OF SAMPLES

Blood samples were collected from pigs in two herds (A and B) in which serovar *tarassovi* had been isolated. In Herd A, a random sample of 16 weaner pigs (aged between 8 and 12 weeks), 25 grower pigs (aged between 5 and 9 months), and 10 sows were bled on the farm. In Herd B, blood samples were collected at an abattoir from 21 grower pigs and 14 sows.

SEROLOGY

The serum MA titres against *pomona* (Pomona) and *tarassovi* (mitis-Johnson) were determined as described in Chapter II.

RESULTS

HERD A

This herd proved to be infected not only with *tarassovi*, but also with *pomona*, and the pattern of the *pomona* titres in each age group were similar to those observed in another closely studied pig herd (Chapter VI). The majority of the weaner pigs had low, or negative *pomona* titres, the grower population a wide range of titres, from zero to greater than 1/1536, and the sows only a narrow range of titres between 1/12 to 1/192 (Fig. 7.1).

Overall the pattern of serovar *tarassovi* titres was the same as that seen with *pomona*. The weaner animals had titres less than 1/12, the growers from 1/12 to 1/1536, and the sows from 1/12 to 1/384. With only one exception all sows had both *pomona* and *tarassovi* titres.

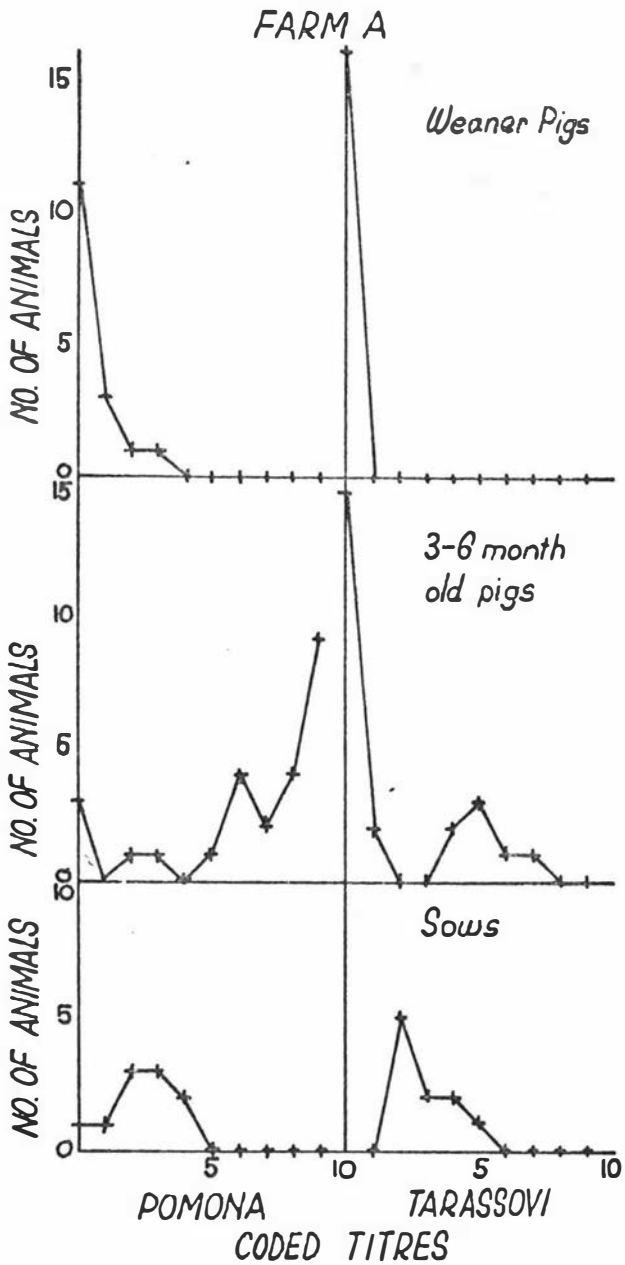
HERD B

Herd B also proved to be infected with both *pomona* and *tarassovi*. However, the *pomona* titres of the juvenile and adult animals in this herd were very similar to one another (Fig. 7.2). As in Herd A, with only one exception all the sows had both *pomona* and *tarassovi* titres.

THE RELATIONSHIP BETWEEN THE *POMONA* AND *TARASSOVI* TITRES

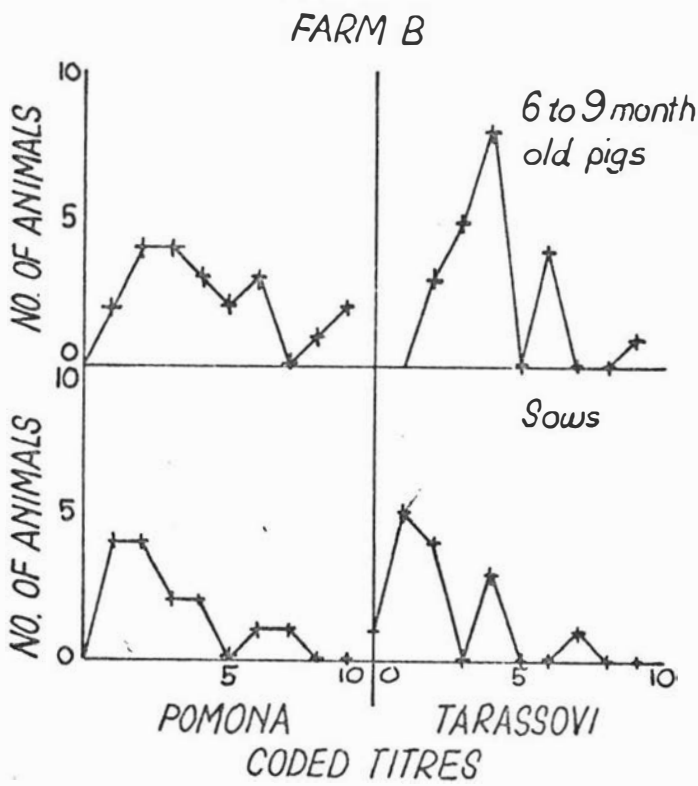
The scatter diagram of the *pomona* and *tarassovi* titres of the sera from both Herd A and B suggests that there is no relationship between these parameters. The correlation coefficient (r) calculated from these data was 0.040, and this is non-significant at the 5% level (*60 d.f.*).

Fig. 7.1 The frequency distribution of *pomona* and *tarassovi* MA titres in a known *tarassovi* infected farm.



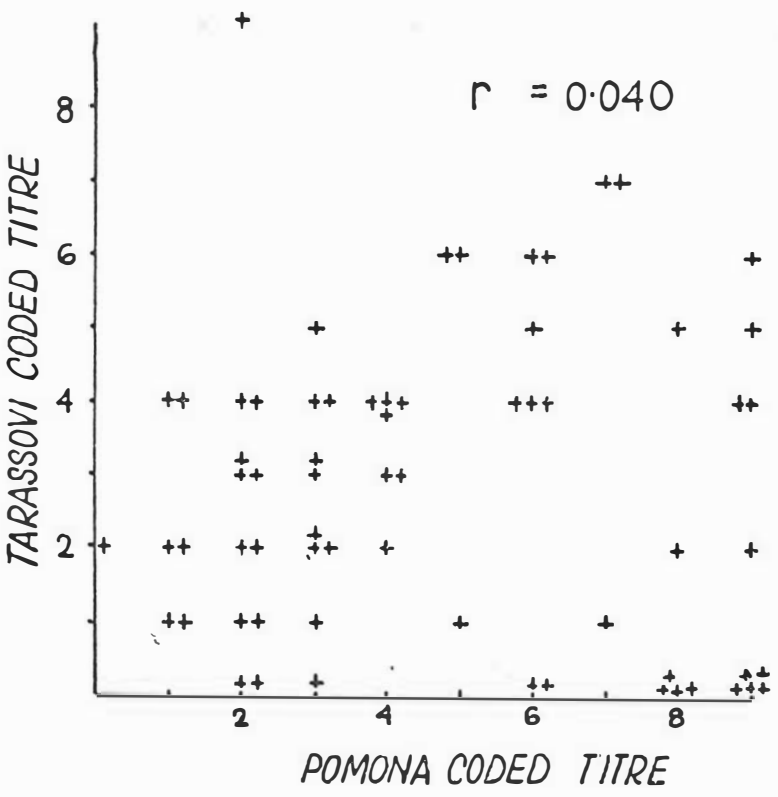
SEROLOGY CODE
1=1/12, 2=1/24, 3=1/48, 4=1/96, 5=1/192, 6=1/384, 7=1/768, 8=1/1536, 9=1/3072, 10=1/6144, 11=1/12288, 12=1/24576.

Fig. 7.2 The frequency distribution of *pomona* and *tarassovi* MA titres in a known *tarassovi* infected farm.



SEROLOGY CODE
1=1/12, 2=1/24, 3=1/48, 4=1/96, 5=1/192,
6=1/384, 7=1/768, 8=1/1536, 9=1/3072,
10=1/6144, 11=1/12288, 12=1/24576.

Fig. 7.3 The lack of association between the coded *pomona* and *tarassovi* MA titres in pigs from two *tarassovi* infected herds.



SEROLOGY CODE
1=1/12, 2=1/24, 3=1/48, 4=1/96, 5=1/192,
6=1/384, 7=1/768, 8=1/1536, 9=1/3072,
10=1/6144, 11=1/12288, 12=1/24576.

DISCUSSION

The results of the serological surveys conducted in these herds suggest that the pattern of *tarassovi* infection is remarkably similar to that of *pomona*. It appears that the major focus of infection is in the grower population, and that all the pigs in these herds eventually became infected with *tarassovi*. As the pattern of *tarassovi* is similar to that of *pomona* it might be assumed that the epidemiology of *tarassovi* is also similar. However, *tarassovi* infection has been reported in many other species, including rats, mice and cattle (U.S. Department of Health, Education and Welfare, 1966, 1975) and it is possible that the epidemiology of this serovar could be very different. More extensive investigations of other species in areas where *tarassovi* infection in pigs occurs should be conducted.

In these herds, there was no indication of any reciprocal cross-protection, and many animals appear to have been currently infected with both serovars. Although little is known about the interaction between *pomona* and *tarassovi* infection in pigs, studies in laboratory animals also suggest that there is no reciprocal cross-protection after infection with either serovar (Alston and Broom, 1958; Plesko, 1974). Thus it seems likely that animals which have been infected with *pomona* will still be susceptible to *tarassovi*, and that *tarassovi* infection can spread in herds such as the one described in Chapter IV, where *pomona* infection is endemic.

The converse of this situation has been reported, an epidemic of *pomona* occurring in a herd where the animals had been infected with *tarassovi* 2 years previously (Kemenes and Suveges, 1976).

In an experiment not detailed in this thesis, 2 pregnant sows from a herd where endemic *pomona* was known to occur were experimentally inoculated with a recent isolate of *tarassovi*. However, the results were inconclusive owing, it would seem, to prior exposure of these animals to *tarassovi*. Further study of *tarassovi* infection in pigs, especially with regard to possible effects on fertility, needs to be undertaken.

SUMMARY AND CONCLUSIONS

1. Serological surveys in two herds where serovar *tarassovi* had been previously isolated were conducted.

2. There was serological evidence of extensive infection with *tarassovi* in both herds. As with *pomona*, the younger animals appeared to be the main reservoir of infection.

3. There was no evidence of any reciprocal cross-protection between *pomona* and *tarassovi*.

CHAPTER VIII

A NATIONAL SEROLOGICAL SURVEY OF LEPTOSPIROSIS IN PIGS

INTRODUCTION

This chapter describes and discusses the results of a serological survey of leptospirosis infection in pigs from throughout New Zealand. It was conducted in order to ascertain whether or not serovar *pomona* and serovar *tarassovi* infection were present in pigs from different parts of the country, and also to see if infection with any other serovars commonly occurred in pigs.

Only three other serological surveys for leptospirosis in pigs have been conducted in New Zealand. In 1952 and 1954, Kirschner reported that he had found serological evidence of *pomona* and *tarassovi* (*hyos*) infection in pigs in the South Island. In 1958 Russell and Hansen published the results of a national survey of leptospirosis in pigs in which sera from adult animals were titrated against *pomona* and *tarassovi* antigens.

MATERIALS AND METHODS

COLLECTION OF SAMPLES

The sera for this survey were selected from the New Zealand Ministry of Agriculture and Fisheries pig serum bank, which is held at the Wallaceville Animal Health Reference Laboratory, Upper Hutt. The 5000 sera in this bank were from adult pigs, mainly sows, and were collected between 1975 and 1977. The animals bled were not a statistically defined random sample of adult pigs in New Zealand, but all important pig-raising areas were adequately represented.

Because of the manner in which the sera had been stored, it was not practicable to take a random sample of the sera in the collection. Instead storage bags were haphazardly chosen and one serum taken from each submission pack until 39 samples from each of the Ministry of Agriculture and Fisheries veterinary districts (Anon., 1978) had been obtained.

SEROLOGY

The MA titres against serovars *pomona* (Pomona), *tarassovi* (mitis-Johnson), *ballum* (M127), *hardjo* (Hardjoprajitno), *copenhageni* (M20), *australis* (Ballico), *autumnalis* (AkiA), *bataviae* (Swart), *canicola* (Hond Utrecht), *pyrogenes* (Salinem) and *grippotyphosa* (Mos V) were determined using the serological method described in Chapter II.

RESULTS

SEROVAR *POMONA* TITRES

The MA titres to *pomona* ranged from 1/12 to greater than 1/1536 (Table 8, Fig. 8.1). As in all the other serological surveys of leptospirosis in pigs, the *pomona* titres were the most prevalent, with 65% (153/234) of sera having titres of 1/12 or more, and 53% (124/234), having titres of 1/24 or more. Thirty four out of the 234 (15%) had titres of 1/384 or more, and thus the estimated real prevalence of *pomona* infection in this group was zero (Chapter V).

Sixty six percent (101/153) of the sera from the North Island districts were positive at 1/24, whereas 28% (23/78) from the South Island were positive at this level. These prevalence figures are significantly different ($\chi^2 = 24.55$, *1 d.f.*, $p < 0.005$).

There were marked differences between veterinary districts in the number of sera that reacted with the *pomona* antigen, and there was a trend for positive reactions to decrease from North to South. Eighty-seven percent of the samples from the Auckland district were positive, 57% from the Hamilton, Palmerston North and Hastings regions, 31% from Christchurch and 28% from Dunedin (Table 8.2).

When the coded *pomona* titres of 1/12 or more were compared with the corresponding coded titres to the other serovars, significant regression coefficients were obtained with the data to *copenhageni* ($P = 0.01$), *australis* ($P < 0.001$) *bataviae* ($P < 0.001$), *pyrogenes* ($P < 0.001$), *grippotyphosa* ($P < 0.001$), and *autumnalis* ($P < 0.001$). These regression coefficients were all positive (Table 8.1), and the nature of the association such that high *pomona* titres were related to low titres to these serovars (Fig. 8.2).

SEROVAR *TARASSOVI* TITRES

The distribution of the MA titres to *tarassovi* was similar to that seen to *pomona* (Fig. 8.3). The *tarassovi* titres ranged from 1/12 to greater than 1/1536, with 35% of all sera being positive at 1/12, and 21% positive at 1/24 (Table 8.5).

Twenty eight percent of the samples from the North Island, but only 8% of the South Island sera were positive at 1/24. There was no significant difference between the numbers positive in each of the veterinary districts in the North Island ($\chi^2 = 1.27$, 3 *d.f.*), and although the South Island figures were too low to allow a statistical analysis, the proportion which were positive in the Christchurch and Dunedin districts were of the same order (10% and 5% respectively).

As with the *pomona* titres, a regression analysis between the *tarassovi* titres of 1/12 or more, and the titres to the

other serovars was conducted. However, there was a significant regression ($P < 0.001$) only between the *tarassovi* titres and those to *bataviae* (Table 8.4). In this case the association was also one of low titres to *bataviae* being associated with high titres to *tarassovi* (Fig. 8.4).

The correlation coefficient between the coded *tarassovi* titres and the coded *pomona* titres was only 0.053.

In 140 (60%) of the samples the *pomona* and/or *tarassovi* titres were 1/24 or more. Of these 92 (66%) had only *pomona* titres of 1/24 or more and 15 (11%) had only *tarassovi* titres of this level. The balance 33 (24%) had both *pomona* and *tarassovi* titres of 1/24 or more.

SEROVAR *HARDJO* TITRES

10% of the samples were positive at 1/12 to the *hardjo* antigen, but 86% of these were only 1/12, and all were less than 1/96 (Table 8.6).

SEROVAR *COPENHAGENI* TITRES

Titres of 1/12 or more were encountered in 19% of sera, but 80% of these were only 1/12, and few were greater than 1/24 (Table 8.7). Two animals had titres of 1/96 and 1/384, but both also had *pomona* titres of greater than 1/1536.

SEROVAR *BALLUM* TITRES

Thirty eight animals had *ballum* titres of 1/12 or more (Table 8.8), but 92% of these were only 1/12 and most were associated with high *pomona* titres.

SEROVAR *AUSTRALIS* TITRES

Although there were some moderately high titres to serovar *australis*, all of these were associated with very high *pomona* titres. However, most of the *australis* titres were only 1/12 or 1/24 (Table 8.9).

SEROVAR *AUTUMNALIS* TITRES

Although 68% of the sera were positive at 1/12 or greater to *autumnalis* (Table 8.10), and many of these titres were quite high, there was a high correlation between the *autumnalis* titres and the *pomona* titres ($r = 0.626$, Table 8.3). No *autumnalis* titres occurred in sera where there were no *pomona* titres.

SEROVAR *BATAVIAE* TITRES

Positive *bataviae* titres were found in 9% of the samples (Table 8.11), but all were less than 1/96, and were associated with moderate or very high *pomona* or *tarassovi* titres.

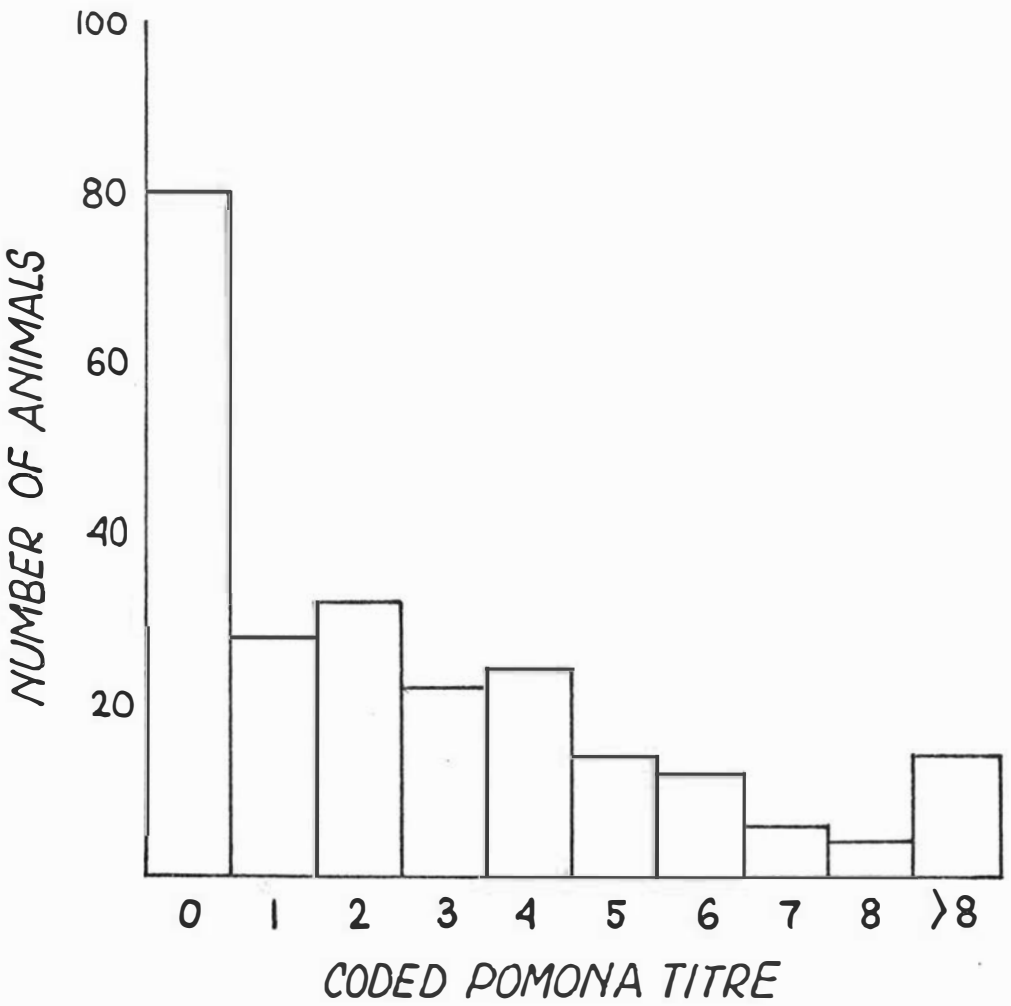
SEROVAR *PYROGENES* TITRES

In common with many of the other serovars, the *pyrogenes* titres were associated with high *pomona* titres, and the regression of *pomona* coded titres on the coded *pyrogenes* titres was highly significant ($P < 0.001$).

SEROVAR *CANICOLA* TITRES

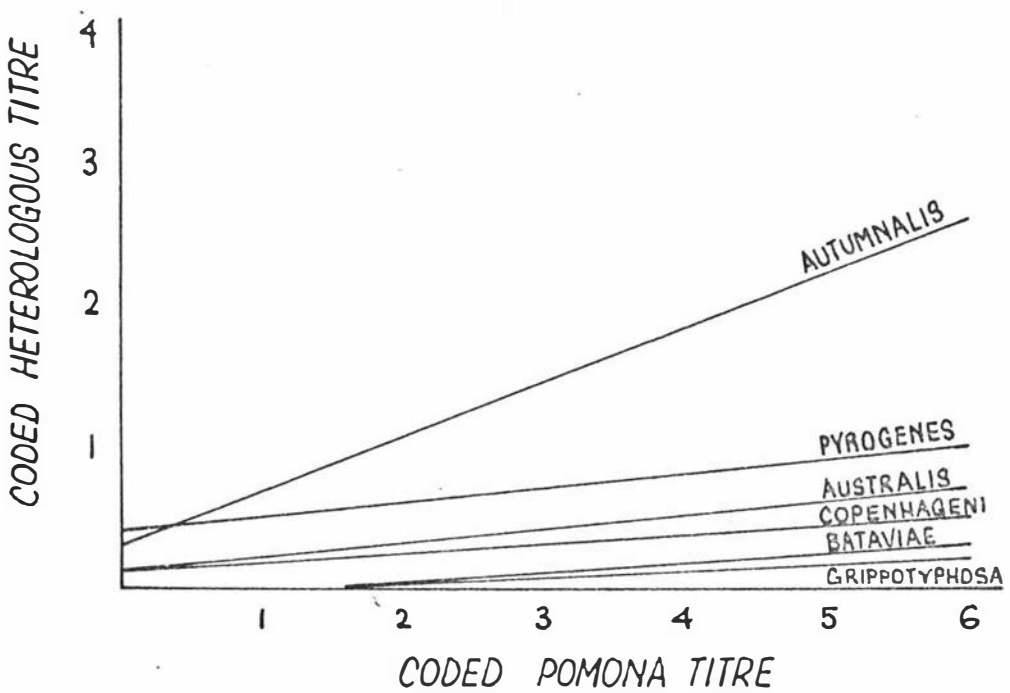
None of the samples were positive to *canicola* at 1/12.

Fig. 8.1 The frequency distribution of *pomona* MA titres in the sample of sera from pigs from throughout New Zealand.



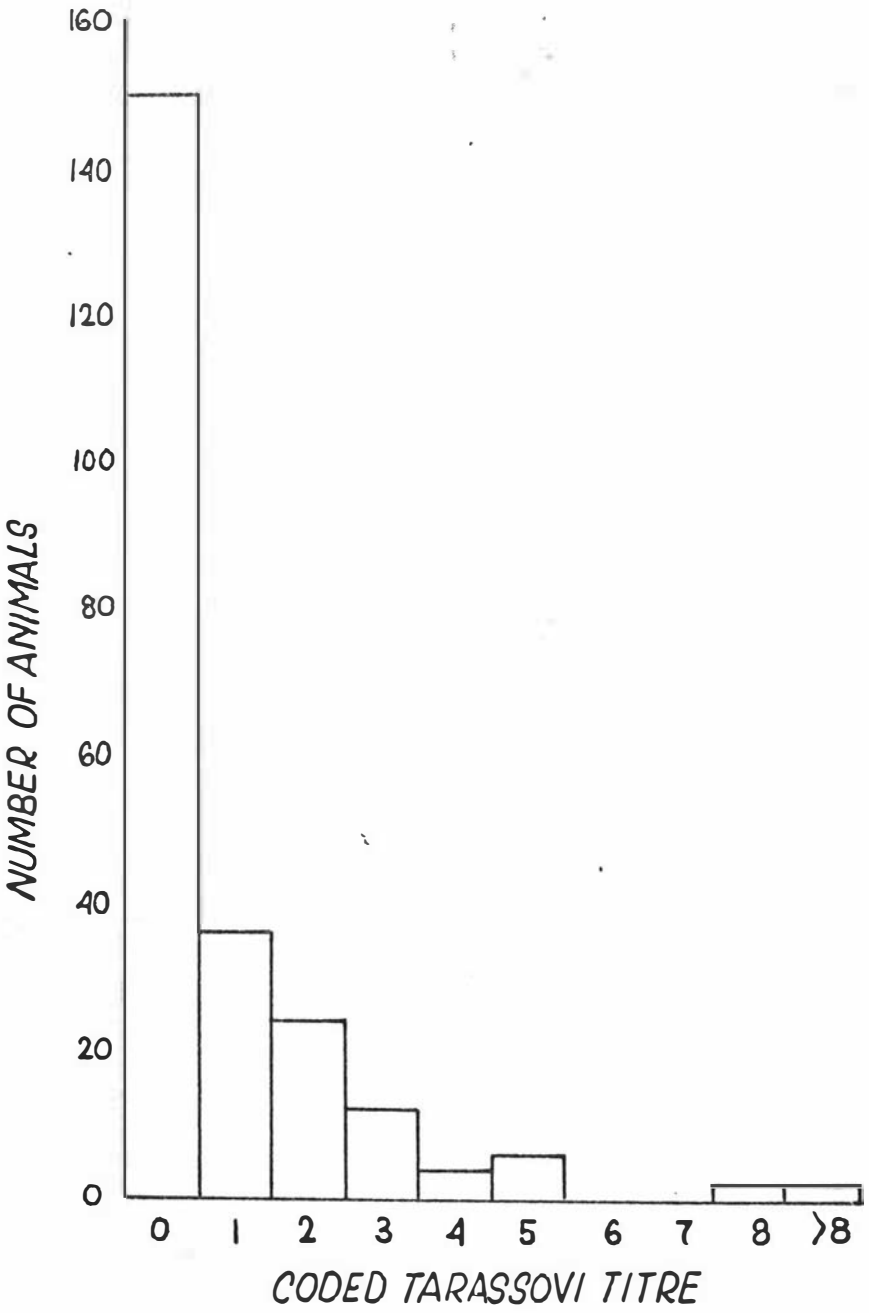
SEROLOGY CODE
1=1/12, 2=1/24, 3=1/48, 4=1/96, 5=1/192,
6=1/384, 7=1/768, 8=1/1536, 9=1/3072,
10=1/6144, 11=1/12288, 12=1/24576.

Fig 8.2 The association between the coded *pomona* MA titres and the coded heterologous titres from the total New Zealand sample.



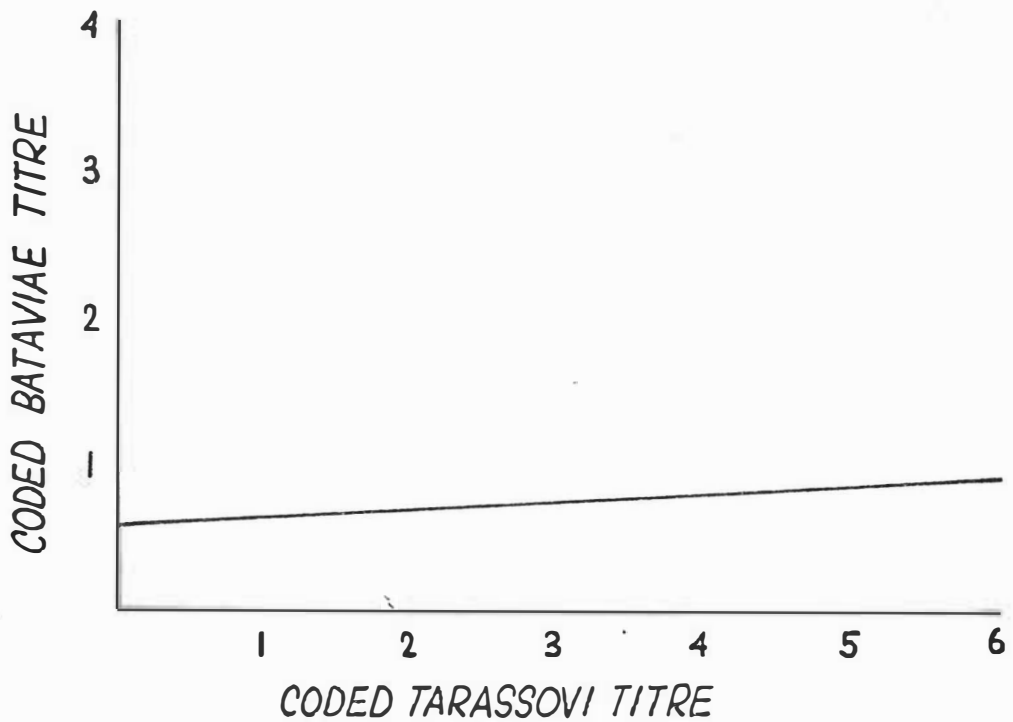
SEROLOGY CODE
1=1/12, 2=1/24, 3=1/48, 4=1/96, 5=1/192,
6=1/384, 7=1/768, 8=1/1536, 9=1/3072,
10=1/6144, 11=1/12288, 12=1/24576.

Table 8.3 The frequency distribution of *tarassovi* MA titres in the sample of sera from pigs from throughout New Zealand.



SEROLOGY CODE
1=1/12, 2=1/24, 3=1/48, 4=1/96, 5=1/192,
6=1/384, 7=1/768, 8=1/1536, 9=1/3072,
10=1/6144, 11=1/12288, 12=1/24576.

Fig. 8.4 The association between the coded *tarassovi* MA titres and the coded *bataviae* MA titres in sera from the New Zealand sample.



SEROLOGY CODE
1=1/12, 2=1/24, 3=1/48, 4=1/96, 5=1/192,
6=1/384, 7=1/768, 8=1/1536, 9=1/3072,
10=1/6144, 11=1/12288, 12=1/24576.

SEROVAR *GRIPPOTYPHOSA* TITRES

Nine per cent of the samples had positive titres to *grippotyphosa*, but 85% of these were only 1/12 and the maximum titres were 1/48 (Table 8.13). All the *grippotyphosa* titres were associated with moderate or high *pomona* titres.

DISCUSSION

It is a fundamental principle of statistics that if sample results are to be related back to a population, then each sample should be randomly selected from the population. Although no conscious bias entered into the decision as to which sows were bled, or which sera were taken from the serum bank, they were clearly not from a true random sample of all the sows in New Zealand. However, the total sow population in New Zealand is only approximately 50 000 (Anon., 1977), and therefore, sera tested in this survey constituted about 0.5% of the total population. Although care must be exercised when drawing conclusions from these results about leptospiral infection in the New Zealand sow population, the possible inadequacy of the sample is compensated for by the relatively high proportion of the total population examined.

A further problem with this investigation is the interpretation of MA titres. Earlier work completed in this study (Chapters IV and VI) suggests that homologous titres of 1/24 or more to *pomona* are indicative of present or past infection with this serovar. The serological surveys in herds where *tarassovi* had been isolated (Chapter VII) indicate that although homologous titres may not rise as high as *pomona* titres during the early phase of infection, most of the recovered animals had *tarassovi* titres of 1/24 or more. Hence, it would seem that *tarassovi* titres of 1/24 or greater may also be

taken as evidence of present or past infection with this serovar. However, the nearest comparable serum dilution used by Russell and Hansen (1958) was 1/10, and therefore, the number of samples which were positive at 1/12 had to be used when comparing the results obtained in this current survey with those of the earlier study.

It is also important to note that presence of MA titres does not necessarily mean that the animal was in a herd infected with either *pomona* or *tarassovi*. It is a common practice to purchase replacement breeding stock, and because most pigs are infected at a young age (Chapters VI and VII), a positive test may merely indicate that the animal was originally derived from a herd where leptospirosis infection occurred.

This serological survey shows that serovar *pomona* infection occurs in pigs throughout New Zealand, and that the prevalence of infection in the North Island is greater than that in the South Island. The decreased prevalence in southern areas may be an effect of environmental temperature. Low temperatures may inhibit the environmental survival of leptospires and therefore reduce transmission. This effect of temperature on survival of these organisms should be examined further. Having regard to the sampling procedure, it is not possible to be completely sure that the prevalence of infection in the Auckland veterinary district is so much greater than that of the other districts, but this should also be investigated further. Clearly, however, pigs are an important potential reservoir of *pomona* infection for man and other animals throughout New Zealand.

The prevalence of *pomona* titres of 1/12 or more (65%) was significantly greater in this survey than the 43% at 1/10 reported by Russell and Hansen (1958) ($\chi^2 = 38.01$, $P < 0.005$). They also found marked differences between the North and South Island reaction-rates. Forty six

percent (471/1018) of the sera from the North Island had titres of 1/10 or more, whereas only 16% (13/80) of the South Island sera had titres of this level. This difference is significant ($\chi^2 = 25.91$, 1 d.f. $P < 0.005$) and is more evidence that the difference noted in this present survey is real, and not a sampling artifact.

Although it was not an objective of this survey to estimate the prevalence of *pomona* infection in this sample, it is of interest that Russell and Hansen found that 5% of their sample had *pomona* titres of 1/200 or more, which they considered was indicative of current infection. If the estimated values of specificity and sensitivity at a titre of 1/192 (Chapter V) are applied to their data, the estimated real prevalence of infection in their sample is zero, the same as the estimate in this survey. It would therefore, seem that the pattern of *pomona* infection with respect to the age of pigs has remained essentially unchanged over 20 years.

Serovar *tarassovi* was isolated for the first time in New Zealand during the early part of this study (Ryan and Marshall, 1976), and the results of this serological survey indicate that some pigs throughout the country are infected with this serovar. The range of titres from 1/12 to 1/1536 to *tarassovi*, and the poor correlation to the *pomona* titres is good evidence that many of these sows had been infected with *tarassovi*.

As with *pomona* infection, the results suggest that infection with *tarassovi* is more common in the North Island than the South Island. In 1958 Russell and Hansen also found a higher prevalence of *tarassovi* infection in the North Island (40%) than in the South Island (19%).

Although the prevalence of *pomona* titres had increased quite markedly since 1958, there is no significant difference between the number of sera with titres to *tarassovi* of 1/10 or more at that time, and that found in this survey ($\chi^2 = 0.97$, *1 d.f.*). It would seem therefore that the sporadic occurrence of *tarassovi* infected herds has not changed over this period.

As has been discussed there is good evidence that pigs positive at 1/24 to *pomona* or *tarassovi* have been infected with either of these serovars. It would therefore appear that approximately 40% of the animals originated from herds where there was *pomona* infection only, 15% where there was infection with both *pomona* and *tarassovi*, 6% where there was *tarassovi* only, and the balance (39%) where no infection with either occurred.

It would appear that infection with serovars other than *tarassovi* and *pomona* is rare in New Zealand. No positive titres to *canicola* were found, and those against *hardjo*, *bataviae*, *ballum* and *grippotyphosa* were usually only 1/12 and only rarely greater than 1/48. In virtually all cases the titres to these serovars were associated with high *pomona* or *tarassovi* titres. Some of the titres to the other serovars were higher and more prevalent, but in all these cases there was a significant association between them and the *pomona* titres. This group included the reactions to *copenhageni*, *australis*, *pyrogenes*, and *autumnalis*. In addition the regression of the *tarassovi* titres on the *bataviae* titres was also highly significant. Cross reactions between *tarassovi* and *bataviae* have been reported in other studies (Alston and Broom, 1958). It would therefore appear that at the present time serovars *pomona* and *tarassovi* are almost exclusively responsible for leptospiral infection in pigs in New Zealand.

SUMMARY AND CONCLUSIONS

1. The sera from 234 adult pigs selected from all major districts in New Zealand were titrated against 11 parasitic leptospiral serovars.

2. It was concluded that infection with serovar *pomona* and *tarassovi* occurred in pigs throughout New Zealand, and it was estimated that 53% of the animals sampled had been infected with *pomona*, and 33% with *tarassovi*.

3. The prevalence of both *pomona* and *tarassovi* infection is higher in the North Island than in the South Island.

4. *Pomona* infected herds are the most common, followed by herds infected by both *pomona* and *tarassovi*, with *tarassovi* infection alone being the least common.

5. There was no evidence that infection with serovars other than *pomona* or *tarassovi* commonly occurs in pigs in New Zealand.

Table 8.1 The distribution of coded *pomona* titres.

Coded Titre	0	1	2	3	4	5	6	7	8	9
Number	81	29	32	22	23	13	11	6	3	14
Frequency	35%	12%	14%	9%	10%	6%	5%	3%	1%	6%

Table 8.2 The number and prevalence of *pomona* titres equal to or greater than 1/12, 1/24 and 1/384.

Titre	Total	AK*	HM*	HS*	PN*	CH*	DN*
1/12	153	36	28	20	30	19	20
	65%	92%	72%	51%	77%	49%	54%
1/24	124	34	24	17	26	12	11
	53%	87%	62%	44%	67%	31%	28%
1/384	34	2	15	4	8	3	2
	15%	5%	38%	10%	21%	8%	5%

*AK = Auckland, HM = Hamilton, HS = Hastings, PN = Palmerston North, CH = Christchurch, DN = Dunedin

Table 8.3 The correlation and regression of coded *pomona* titres on the coded heterologous titres.

Serovar	*b*	*c*	*r*	r^2	*t*	Significance
hardjo	-0.009	0.189	-0.039	0.002	-0.493	N/S
copenhageni	0.067	0.088	0.211	0.045	2.649	P=0.01
tarassovi	0.043	0.784	0.073	0.005	0.888	N/S
ballum	-0.011	0.276	-0.055	0.003	-0.682	N/S
australis	0.097	0.044	0.340	0.113	4.425	P<0.001
bataviae	0.070	-0.093	0.319	0.102	4.129	P<0.001
canicola	-	-	-	-	-	-
pyrogenes	0.101	0.401	0.285	0.081	3.615	P<0.001
grippotyphosa	0.050	-0.039	0.277	0.077	3.535	P<0.001
autumnalis	0.386	0.323	0.626	0.392	9.879	P<0.001

b = regression coefficient, *c* = regression equation constant, *r* = correlation coefficient

Table 8.4 The correlation and regression of coded *tarassovi* titres on the coded heterologous titres.

Serovar	*b*	*c*	*r*	r^2	*t*	Significance
pomona	0.080	2.782	0.053	0.003	0.478	N/S
hardjo	0.025	0.110	0.081	0.007	0.733	N/S
copenhageni	-0.085	0.587	-0.160	0.026	-1.463	N/S
ballum	-0.005	0.311	-0.001	0.000	-0.014	N/S
australis	-0.013	0.518	-0.029	0.001	-0.264	N/S
autumnalis	0.115	1.465	0.136	0.019	1.249	N/S
canicola	-	-	-	-	-	-
bataviae	0.208	-0.140	0.468	0.219	4.800	P<0.001
pyrogenes	0.040	-0.660	0.079	0.006	0.726	N/S
grippotyphosa	-0.009	-0.198	-0.030	0.001	-0.284	N/S

b = regression coefficient, *c* = regression equation constant, *r* = correlation coefficient

Table 8.5 The number and prevalence of *tarassovi* titres equal to or greater than 1/12 and 1/24.

Titre	AK	HM	HS	PN	CH	DN	Total
1/12	22	12	15	15	8	9	81
	56%	31%	38%	38%	28%	23%	35%
1/24	13	12	10	9	4	2	50
	33%	31%	26%	23%	10%	5%	21%

Table 8.6 The number and prevalence of *hardjo* titres equal to or greater than 1/12 and 1/24.

Titre	AK	HM	HS	PN	CH	DN	Total
1/12	5	8	3	3	2	0	21
	13%	21%	8%	8%	5%	0	9%
1/24	2	0	1	0	0	0	3
	5%	0	3%	0	0	0	1%

Table 8.7 The number and prevalence of *copenhageni* titres equal to or greater than 1/12 and 1/24.

Titre	AK	HM	HS	PN	CH	DN	Total
1/12	12	9	7	9	6	1	44
	31%	23%	18%	23%	15%	3%	19%
1/24	1	3	3	2	0	0	9
	3%	8%	8%	5%	0	0	4%

Table 8.8 The number and prevalence of *ballum* titres equal to or greater than 1/12 and 1/24.

Titre	AK	HM	HS	PN	CH	DN	Total
1/12	10	5	5	9	5	4	38
	26%	13%	13%	23%	13%	10%	16%
1/24	0	1	0	1	0	1	3
	0	3%	0	3%	0	3%	1%

Table 8.9 The number and prevalence of *australis* titres equal to or greater than 1/12 or 1/24.

Titre	AK	HM	HS	PN	CH	DN	Total
1/12	11	10	14	14	7	7	63
	28%	26%	36%	18%	18%	18%	27%
1/24	1	4	3	3	0	0	11
	3%	10%	8%	8%	0	0	5%

Table 8.10 The number and prevalence of *autumnalis* titres equal to or greater than 1/12 and 1/24.

Titre	AK	HM	HS	PN	CH	DN	Total
1/12	28	27	28	34	21	22	160
	72%	69%	72%	87%	54%	56%	68%
1/24	8	15	18	24	6	8	81
	21%	38%	46%	62%	15%	21%	38%

Table 8.11 The number and prevalence of *bataviae* titres equal to or greater than 1/12 and 1/24.

Titre	AK	HM	HS	PN	CH	DN	Total
1/12	3	10	1	2	2	2	20
	8%	26%	3%	5%	5%	5%	9%
1/24	2	1	1	1	11	0	6
	5%	3%	3%	3%	3%	0	3%

Table 8.12 The number and prevalence of *pyrogenes* titres equal to or greater than 1/12 and 1/24.

Titre	AK	HM	HS	PN	CH	DN	Total
1/12	22	23	17	24	16	14	116
	56%	59%	44%	62%	41%	36%	50%
1/24	4	2	2	4	3	4	19
	10%	5%	5%	10%	8%	10%	8%

Table 8.13 The number and prevalence of *grippotyphosa* titres equal to or greater than 1/12 and 1/24.

Titre	AK	HM	HS	PN	CH	DN	Total
1/12	0	7	2	7	4	0	20
	0	18%	5%	18%	10%	0	9%
1/24	0	2	0	1	0	0	3
	0	5%	0	3%	0	0	1%

CHAPTER IX

AN ABORTION STORM DUE TO INFECTION WITH SEROVAR *POMONA*

INTRODUCTION

If sows become infected with serovar *pomona* in late pregnancy, they often abort (Ryley and Simmonds, 1954, a, b; Fennestad and Borg-Peterson, 1966) and if infection is introduced into a susceptable breeding herd, many sows may abort over a short period (Powers *et al.*, 1956).

In 1977 a new piggery was established in the Manawatu, and shortly after the first gilts were placed in this unit an abortion storm occurred. This was subsequently shown to be due to infection with serovar *pomona* and the opportunity was taken to investigate this problem in detail.

MATERIALS AND METHODS

SEROLOGY

Blood samples were collected from all the gilts which aborted, within one or two days of the abortion. In addition a number of the gilts from each group sent to the piggery (Fig. 9.1) were bled. The MA titres to *pomona* (Pomona) *tarassovi* (mitis-Johnson), *hardjo* (Hardjoprajitno), *ballum* (M127), and *copenhageni* (M20) were determined using the method described in Chapter II.

CULTURE

In the early phases of this investigation samples of kidney, liver, brain, pericardial fluid and vitreous humor from 2 or 3 piglets from each aborted litter were cultured for leptospires as described in Chapter III.

In addition, samples of placentae were homogenised in Stuart's basal medium (Stuart, 1946) and the homogenate was inoculated into P80 medium (Chapter III).

After a diagnosis of *pomona* infection had been established samples of kidney and vitreous humor alone from the aborted piglets were cultured for leptospires.

PATHOLOGY

A wide range of tissues from the aborted piglets were taken for histopathological examination. Sections from paraffin embedded blocks were stained with haematoxylin and eosin (H and E), and with a modification of the Warthin-Starry method for spirochaetes (Young, 1969).

RESULTS

GENERAL FINDINGS

Groups of from 8 to 13 gilts, both mated and unmated, were brought onto this farm over a 4 month period. Most of the animals (74/96 = 77%) came from 2 pig units in Tauranga, the balance (22/96 = 23%) coming from 1 pig farm in Christchurch (Fig. 9.1).

The first abortion occurred on August 6 1977 and the last on October 13 1977, with 16 out of the 73 (22%) mated gilts aborting (Fig. 9.1). Only animals from the Tauranga farms aborted. The gilts from Christchurch farrowed normal healthy litters.

The first abortions in each of the consignments of pigs sent to this Manawatu farm usually occurred from 3 to 5 weeks after their arrival. However, in one group, one animal aborted after 6 weeks and a further 3 after approximately 8 weeks.

After 6 of the gilts in the first consignment had aborted oxytetracycline was mixed with the meal at a rate of 10 kg/tonne and this mixture was fed for two weeks. After an interval of one week, the medicated meal was fed for a further week (Fig. 9.1). In addition all gilts in the fourth to seventh consignments from Tauranga were injected intramuscularly with 1-2g of streptomycin, and vaccinated with a formalized *pomona-tarassovi* bacterin before being dispatched to the new piggery.

SEROLOGY

The *pomona* titres of the gilts which aborted were between 1/96 and 1/24 576, with most being between 1/384 and 1/1536. The titres to the other serovars were negative or very low.

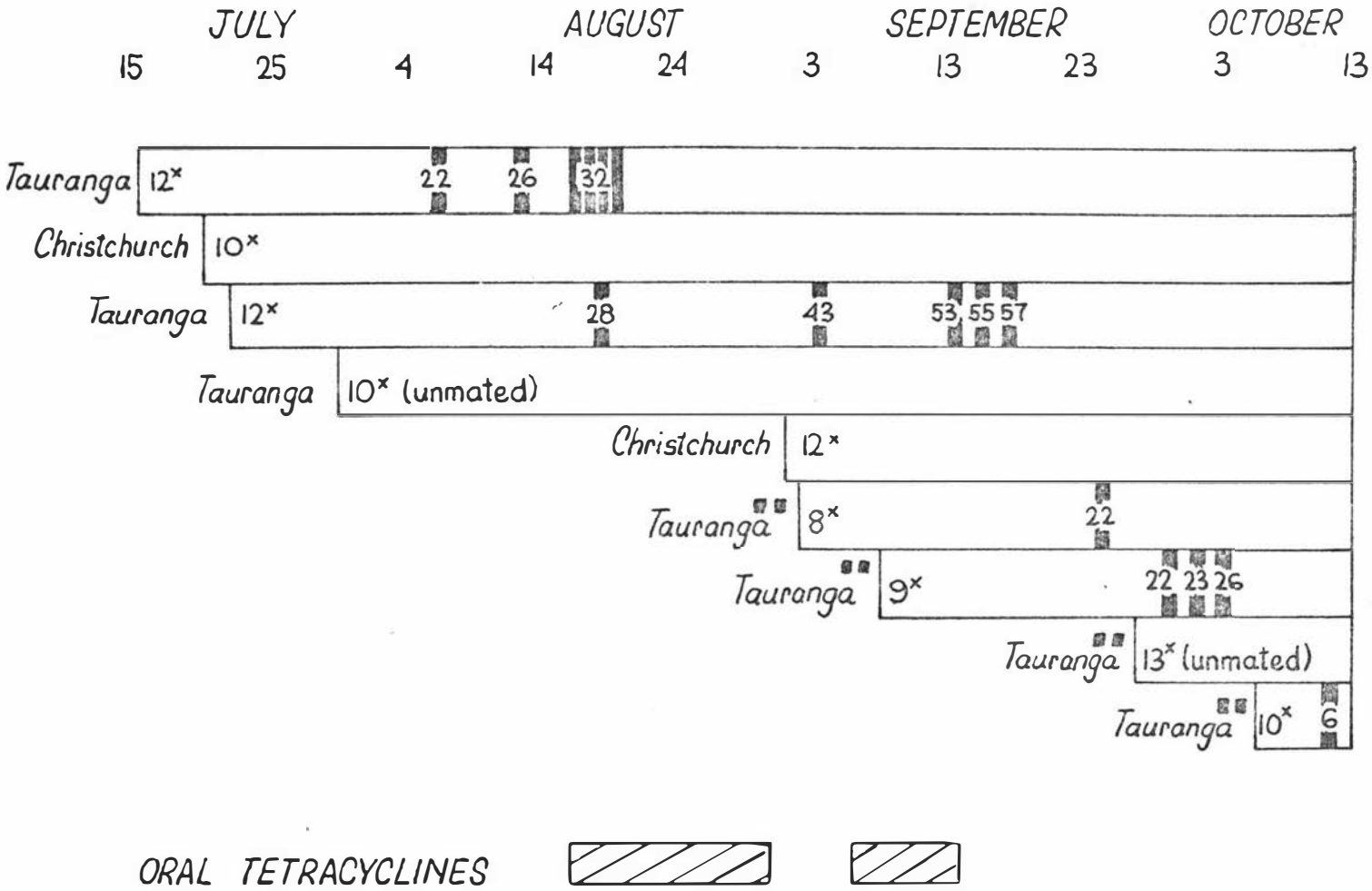
The last consignment of gilts from Tauranga was the only group sampled at the time of arrival. Many of these animals had *pomona* titres of greater than 1/6144, and were shedding leptospires in the urine.

The other consignments were sampled within a month of their arrival. All but three of the pigs (7%) had *pomona* titres of 1/12 or more. However, most of those which had come from Christchurch were low (a geometric mean titre of 1/135) whereas many of the pigs from Tauranga had very high titres (a geometric mean titre of 1/1230).

CULTURE

The cultural results from the first three abortions are reported here. The complete results will be published later (B L Stevenson, pers. com.). However, the results obtained in this preliminary investigation are similar to those subsequently obtained with the later samples.

Fig. 9.1 A summary of events that occurred on a pig farm during a *pomona* abortion storm.



x = No. of pigs in each consignment ■■= Pigs also injected I/M with streptomycin and vaccinated.
22 (etc) = 22 (etc) days between introduction to farm and abortion.

Serovar *pomona* was isolated from the liver, kidney, brain and pericardial fluid of all the aborted piglets examined. In addition leptospires were observed by darkfield microscopy in the vitreous humor of approximately threequarters of the aborted piglets, and *pomona* was isolated from all of the vitreous humor samples.

Pomona was also isolated from the placenta of two of these abortion cases.

Serovar *pomona* was isolated from the urine of the gilts from both Tauranga and Christchurch.

PATHOLOGY

No gross lesions were observed in any of the aborted piglets which were examined. However, on histological examination focal areas of necrosis could be seen in liver sections. There was also evidence of a mild interstitial nephritis, but no lesions were observed in any sections of brain, spleen, lung, heart or skeletal muscle.

Leptospires could be seen in the silver stained sections of liver and kidney, but not in those of brain, spleen, lung, heart or skeletal muscle.

Areas of the placentae were grossly oedematous. Histologically, oedema and an acute placentitis were seen (Fig. 9.2). In some sections focal accumulations of leptospires could be seen in the silver-stained sections (Fig. 9.3).

DISCUSSION

This report dramatically illustrates the possible consequences of mixing pregnant gilts from different farms. In the abattoir surveys, and in the field investigations (Chapter VI and VII) it was found that the main focus of *pomona* infection in pigs was in the animals aged between 6 and 12 months. This abortion storm appears to have resulted from the mixing of infected gilts with susceptible ones.

It would seem that most of the gilts which aborted were infected about the time when they were transported from Tauranga, although in one group secondary spread appears to have occurred after arrival. The gilts from Christchurch farrowed normally even though many were infected as shown by the positive urine cultures. This phenomenon has also been reported elsewhere (Ferguson and Powers, 1956; Mitchell *et al.*, 1966) and illustrates that abortions occur only when sow and gilts are infected in late gestation.

It is not possible to be sure just how effective the antibiotic therapy was in preventing further abortions. However the results indicate that in many cases both oxytetracycline and streptomycin at the dose rates used did not prevent abortion. More work is needed to define the most effective use of antibiotic therapy in circumstances such as those encountered on this farm.

The presence of leptospires in the vitreous humor of the aborted piglets, in sufficient numbers to be seen by direct darkfield microscopy, and the ease of isolation uncontaminated with other bacteria was a noteworthy finding. This would appear to be a most useful technique to use when investigating abortions in pigs.

Fig. 9.2 Section of pig placenta showing the inflammatory cell infiltrate and staining of leptospires. (Modified Warthin-Starry x 180).

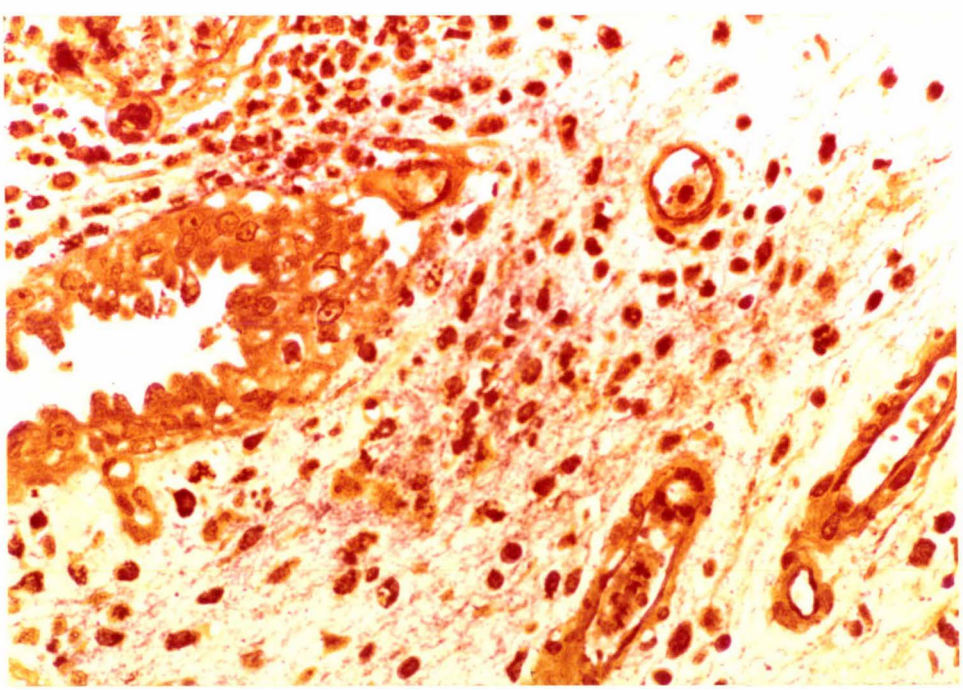
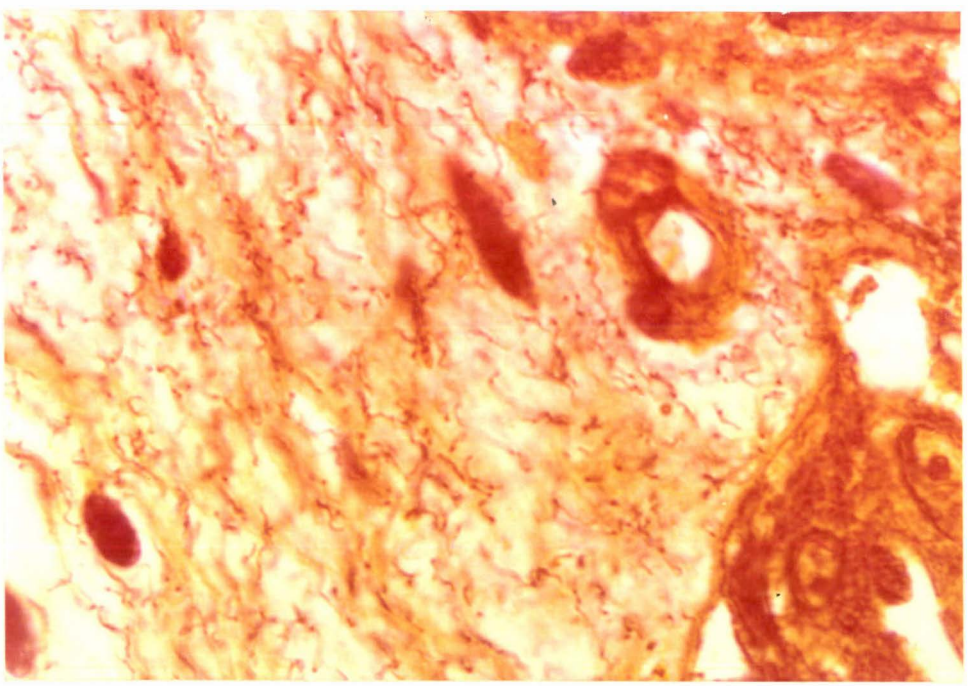


Fig. 9.3 Section of pig placenta showing individual leptospires. (Modified Warthin-Starry x 650).



The acute placentitis observed in this investigation has not been previously described. The lesions were discrete and could have been missed if a number of blocks of the placenta had not been sectioned. Leptospires were even more difficult to find, and the focus of leptospires illustrated was certainly not a common feature.

SUMMARY AND CONCLUSIONS

1. An abortion storm in a pig herd was shown to be due to *pomona* infection.

2. The *pomona* titres of the pigs which aborted were between 1/96 and 1/24 576, with most being between 1/384 and 1/1536.

3. Although leptospires could be isolated from many of the tissues from the aborted piglets, the most convenient tissue to use was vitreous humor.

4. Abortions were not prevented by the feeding of oxytetracyclines (10 kg/tonne meal), nor by injecting the gilts with 1-2g of streptomycin.

5. It was concluded that the abortion storm resulted from the mixing of *pomona* infected gilts with non-infected susceptible pregnant gilts.

6. In the abortions investigated in this herd, serovar *pomona* infection was associated with an acute placentitis.

CHAPTER X

LYMPHOCYTE TRANSFORMATION STUDIES

INTRODUCTION

The role of antibody in immunity to leptospirosis is established and has been reviewed by Alexander (1976). There is evidence that cell mediated immunity (CMI) may also be part of an animal's defence to leptospiral infection. The mononuclear cell infiltrates seen in the kidneys of pigs both in this study (Chapter IV) and also reported by others (Burnstein and Baker, 1954; Langham *et al.*, 1958) appear histologically similar to those associated with delayed hypersensitivity reactions. Furthermore, although the intradermal inoculation of infected animals with homologous antigen may induce either an immediate reaction (Torten *et al.*, 1967; Ben-Efraim and Torten, 1969) or an Arthus' type response (Kuppuswamy *et al.*, 1960; Blendon *et al.*, 1961; Obiger and Schonberg, 1972, 1974), characteristic delayed hypersensitivity reactions have been described (Torten *et al.*, 1967; Ben-Efraim and Torten, 1969). As little is known about the role of CMI in leptospirosis, a study of this aspect of the immune reponse was undertaken.

The involvement of CMI in man and animals is most frequently assessed by intradermal skin testing with an antigen extract. However, because of the occurrence of the reactions due to antibody, equivocal results are often obtained by this technique (W.H.O., 1973). Consequently, an *in vitro* correlate of CMI was used in this study, namely, lymphocyte transformation (Ling and Kay, 1975).

Preliminary studies in which lymphocytes from *pomona* infected pigs were cultured with *pomona* extracts showed that in a number of cases the lymphocytes underwent transformation. These preliminary experiments were conducted using tissue-culture tubes,* a method which limited the number of cultures which could be processed efficiently. Subsequent investigations of lymphocyte transformation were carried out in a microculture system.

The investigations are described as "Experiment I" and "Experiment II." In Experiment I a number of pigs of various ages was selected randomly from a herd which was endemically infected with *pomona*, and the *in vitro* responses of their lymphocytes to two *pomona* extracts were tested. In Experiment II, the time-responses of lymphocytes from two young pigs which had been naturally infected with *pomona* were investigated. In both experiments, as a control of cultured cell viability and as an estimate of latent T cell capacity, the lymphocytes were also cultured in the presence of a known T cell mitogen, phytohaemagglutinin (PHA). (Anderson *et al.*, 1972; Greaves and Bauminger, 1972).

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* "Kimax," Owens, Illinois, U.S.A.

MATERIALS AND METHODS

ANIMALS

EXPERIMENT I

Eighteen pigs from a *pomona* infected herd (Chapter VI) were chosen at random. The animals were classed as follows:

Animal Code	Age	Group
A - C	2 - 4 years)	
D - F	12 - 18 months)	I
G - I	5 - 6 months)	
J - R	6 - 12 weeks	II

The results of an epidemiological investigation on this farm indicated that the Group I animals would have been exposed to *pomona* whereas those in Group II would not have been (Chapter VI).

EXPERIMENT II

Cells from two gilts (coded S and T) from this herd were used to investigate the time-responses of the antigen treated cultures. As a result of a previous study (Chapter VI) it was known that both animals had been infected with serovar *pomona*.

SEROLOGY

The serum MA titres to serovars *pomona* (Pomona), *ballum* (M127), *tarassovi* (mitis-Johnson), *copenhageni* (M20) and *hardjo* (Hardjoprajitno) were determined for each animal as described in Chapter II.

COLLECTION OF LYMPHOCYTES AND VIABILITY TESTING

Pigs were bled from the anterior vena cava. Equal amounts of heparinised blood and saline were mixed and layered onto ficoll-diatrizoate* gradients (Boyum, 1968), and centrifuged at 400X g for 30 minutes. The mononuclear cells were harvested, washed once with isotonic saline, and twice with Eagle's Minimal Essential Medium** before being diluted in the culture medium to a density of 1×10^6 cells per ml.

0.2 ml of the concentrated cell suspension was added to 1.8 ml of 0.2% trypan blue*** in isotonic saline. Viable mononuclear cells, those excluding dye, were counted in a haemocytometer.

CULTURE MEDIA AND INCUBATION

Preliminary experiments (Appendix II) to determine the ability of various media to support the growth of porcine lymphocytes indicated that TC199** was the medium of choice. Consequently this medium supplemented with heat-inactivated (56 ^{o}C/30 minutes) foetal bovine serum+ (FBS) to a final concentration of 5% (Appendix VI) was used in the experiments described here. One ml of an antibiotic solution (containing 1% streptomycin, 1% kanamycin and 10^4 IU penicillin/ml) and one ml of a solution containing 30 mg glutamine were added to each 100 ml of medium.

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* "Lymphoprep," Nyegaard, Oslo, Norway

** Wellcome, Beckenham, England

*** BDH, Laboratory Chemicals, Poole, England

\+ Labserve, P O Box 12502, Auckland, New Zealand

0.2 ml volumes of the cell suspension containing 1×10^6 cells/ml were dispensed into the wells of micro-culture* plates, the plates were covered with loose fitting lids,** and the assembled units incubated at 37 °C in a humidified atmosphere of 5% CO_2/95% air.

Preliminary experiments had shown that maximum isotope uptake occurred after 96 hours (Appendix VI). Consequently this culture time was generally used. However, when studying the time-responses of the cultured cells (Experiment II) incubation periods of 24, 48, 96, 120 and 144 hours were used.

PROCESSING OF CULTURES

Lymphocyte transformation was assessed by measuring the incorporation of tritiated thymidine*** (^{3}H-t-dR, 1mCi/ml, specific activity 2Ci/mmol). The 0.2 ml cultures were pulsed with 0.2 µCi of the isotope 16 hours prior to harvesting (Appendix III), and harvested with a multiple cell harvester+. A technical point considered in the harvesting of cells, namely, the necessity for washing the harvested cells with trichloroacetic acid, is described in Appendix V.

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* Unit 3040, Microtest II, Falcon, Division of Becton, Dickinson and Co., Oxnard, Ca., U.S.A.

** Unit 3041, Falcon, Division of Becton, Dickinson and Co., Oxnard, Ca., U.S.A.

*** The Radiochemical Centre, Amersham, England

\+ Mini-Mash, Dynatech Singapore (Private) Ltd, 21B Goldhill Plaza, Singapore 11.

The fibreglass filters were dried and placed in scintillation vials. Ten millilitres of scintillation fluid was added to each vial. The scintillation fluid was prepared by dissolving 8 g of 2,5-diphenyloxazole*, 200 mg of 1,4-bis-(5-phenyloxazole) benzene* and 600 ml of Triton X-100 in 1400 ml of toluene.

The vials were counted in a liquid scintillation spectrometre** and the counts per minute (cpm) over a 10 minute period measured.

STIMULATION OF LYMPHOCYTE CULTURES

Two extracts of serovar *pomona* were used to stimulate lymphocyte cultures:

Sonicated *pomona* extract (SA)

Five hundred millilitre volumes of P80 medium+ were inoculated with a dense culture of *pomona* (Pomona) and incubated at 30C for 8 days. Before harvesting the cultures were checked for bacterial contamination by inoculating onto blood agar and into peptone broth blood culture tubes.++ The leptospires were harvested (22 500 g x 45 minutes) and washed three times in sterile isotonic saline. The concentrated cells, cooled to between 4C and 8C, were sonicated+++ intermittently at maximum power until no intact leptospires could be seen by darkfield microscopy.

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* Sigma, St Louis, Missouri, U.S.A.

** Beckman, P O Box 3100, Fullerton, California, U.S.A.

\+ EMJH, Difco, Detroit, Michigan, U.S.A.

++ Becton-Dickinson & Co., Rutherford, New Jersey, U.S.A.

+++ Soniprobe, Dave Instruments Ltd, Acton, London W3, U.K.

The sonicated material was sterilised by filtration* (pore size 0.22 µm), and 3 serial 10 fold dilutions made in phosphate buffered saline (PBS). The SA-*pomona* extracts were held at -20C until required.

Sodium deoxycholate-derived *pomona* extract (SD)

This preparation was as described by Manev and Siromashkova (1970). Five hundred millilitre volumes of *pomona* (Pomona) were grown, harvested and checked for bacterial contamination as described above. The washed cells were resuspended in 150 ml of 0.01 N Sorenson's phosphate buffer, pH7.2. Sodium deoxycholate** (1.5 g) was added and the cells held at 42C for 5 hours. The mixture was dialysed against water for 4 days and then centrifuged at 1500 x g for 20 minutes. The supernatant was collected and lyophilised. Sixty milligrams of the lyophilised antigen extract was dissolved in 6 ml of PBS and the solution sterilised by filtration* (pore size 0.22 µm). Three serial 10 fold dilutions in PBS were made, and all SD-*pomona* extracts were held at -20C until required.

EXPERIMENTAL DESIGN AND THE CONTROLS USED IN THE TRANSFORMATION EXPERIMENTS

Twenty µl amounts of the antigen preparations were dispensed into 3 cell cultures. In all experiments, 3 cultures were treated with PHA (1 µg/ml) to assess the latent T cell responsiveness. This concentration of PHA was found in preliminary experiments to induce maximum cell transformation. "Non-stimulated" controls were also included, these being 3 lymphocyte cultures to which 20 µl of PBS were added.

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* Millipore Corporation, Bedford, Massachusetts, U.S.A.

** Sigma, St Louis, Missouri, U.S.A.

Cell cultures free of isotope, cultures without cells but containing medium and the stimulating agent, and cultures containing medium alone were also set up.

ANALYSIS OF THE TRANSFORMATION EXPERIMENTS

The logarithm (base 10) of the cpm was determined, and the geometric mean (Mg) of each triplicate was calculated by averaging the log transformed data. In most cases the difference between the geometric means (the gmd) of the stimulated and non-stimulated cultures was used to compare the effects of the stimulating agents. Additionally, the stimulation index (SI) was determined, by calculating the antilog of the geometric mean difference. Where appropriate the standard deviations (SD) or standard errors (SE) of these parameters were also calculated.

RESULTS

SEROLOGY

EXPERIMENT I

Sera reacted consistently only with the *pomona* antigen. Some samples agglutinated *ballum* and *copenhageni* at dilutions of 1/12 or 1/24, but in all these cases the *pomona* titres were 1/384 or greater.

All the mature sows (A, B and C), and the gilts (D, E and F) had *pomona* titres ranging from 1/196 to 1/1536. The pigs from grower unit III (G, H and I) had titres of 1/24, 1/192 and 1/12 respectively. Three of the piglets from the weaning unit (J to R) had *pomona* titres of 1/12 or 1/24, but all the rest had titres of less than 1/12 (Table 10.1).

EXPERIMENT II

The *pomona* titres of the gilts used in the time-response studies were 1/192 and 1/24.

ACTIVITIES OF THE PHA-STIMULATED AND NON-STIMULATED CONTROL CULTURES

EXPERIMENT I

All the lymphocyte cultures were transformed by PHA (1 µg/ml) (Table 10.2, Fig. 10.2). The responses of the cells of the older Group I animals were not significantly different to those of the younger Group II animals ($t = 0.29$, 16 *d.f.*). The average geometric mean difference between the PHA-stimulated and non-stimulated cultures was 2.43, equivalent to a stimulation index of 263.

The activities of all the non-stimulated cultures were similar (Table 10.2), and there was no significant difference between the Group I and Group II animals ($t = 0.43$, 16 *d.f.*).

EXPERIMENT II

The cells from the two gilts were transformed *in vitro* by PHA (1 µg/ml) and the time-responses were similar to those observed in the preliminary experiments (Table 10.8 and 10.9, Figs 10.5 and 10.6).

RESPONSE TO LEPTOSPIRAL ANTIGENS

(i) Sonicated antigen

Lymphocyte cultures treated with the sonicated *pomona* antigen varied in their degree of transformation, a marked dose-response effect occurring in many cases (Table 10.3, Appendix VI). The peak responses to this antigen

occurred with the undiluted material in 5 animals, the dilution of 1/10 in 8 animals, the 1/100 dilution in 4 animals and the 1/1000 dilution in one animal.

The peak responses of some of the antigen-treated cultures were not significantly different from the activity of the non-stimulated controls (Table 10.4). This was seen in 5 out of the 9 Group II animals. The peak responses of the older Group I animals were all significantly greater ($P < 0.05$) than those of the corresponding non-stimulated controls.

The maximum responses, in terms of the geometric mean difference, of the cells from the Group I animals were significantly greater than those of the Group II animals (Fig. 10.2) ($t = 6.44$, 16 d.f., $P < 0.001$). The average geometric mean difference of the older animals was 0.67, equivalent to a stimulation index of 4.7 whereas that of the younger animals was 0.15, a stimulation index of 1.4.

(ii) Sodium deoxycholate extracted antigen

Sodium deoxycholate-derived antigen stimulated the lymphocyte cultures in that the peak responses attained were generally significantly greater than those of the non-stimulated controls ($P < 0.05$) (Table 10.5, Appendix VI). Exceptions were animal G (Group I) and animals K and L (Group II) (Table 10.6), where there was no significant differences between the log counts of the stimulated and non-stimulated cultures. Since peak responses occurred with the undiluted extract this was used for the last 6 animals tested (M to R), and in all subsequent experiments.

On comparing the responses of the Group I and Group II animals, it was apparent that the older animals responded significantly more than did the younger animals (Fig. 10.3) ($t = 3.43$, 15 d.f., $P < 0.005$). The average geometric mean

difference of the older animals was 0.68, equivalent to a stimulation index of 4.8. The corresponding figures for the Group II animals were 0.34 and 2.2 respectively.

(iii) The correlation between the responses to the sonicated and sodium deoxycholate extracts

The peak responses observed with the sonicated and sodium deoxycholate-derived extracts were highly correlated ($r=0.85$), and the correlation coefficient was statistically significant ($P < 0.01$) (Table 10.7).

The graph of these responses (Fig. 10.4) indicates that although the degree of stimulation of the cells from the older animals (Group I) was similar with both extracts, the responses of the cells from the younger (Group II) animals to the sodium deoxycholate antigen were greater than those to the sonicated preparation.

(iv) Time-responses of lymphocyte cultures to the sonicated antigen, and the sodium deoxycholate derived antigen (Experiment II)

After 48 hours incubation the peak responses occurred with the 1/10 dilution of the sonicated antigen, and with the undiluted sodium deoxycholate derived antigen. The time-response curves of the cultures stimulated with these preparations followed those of the non-stimulated cultures (Tables 10.8 and 10.9, Figs 10.5 and 10.6). However, with both animals the rate of increase over the first 48 hours of incubation was greater than than of the non-stimulated controls, and after this time the decline in the antigen-stimulated cultures was not as marked. As a result of these changes in the stimulated and non-stimulated cultures, the maximum geometric mean differences occurred at 96 or 120 hours, and not at 48 hours, the time when the maximum retention of isotope occurred

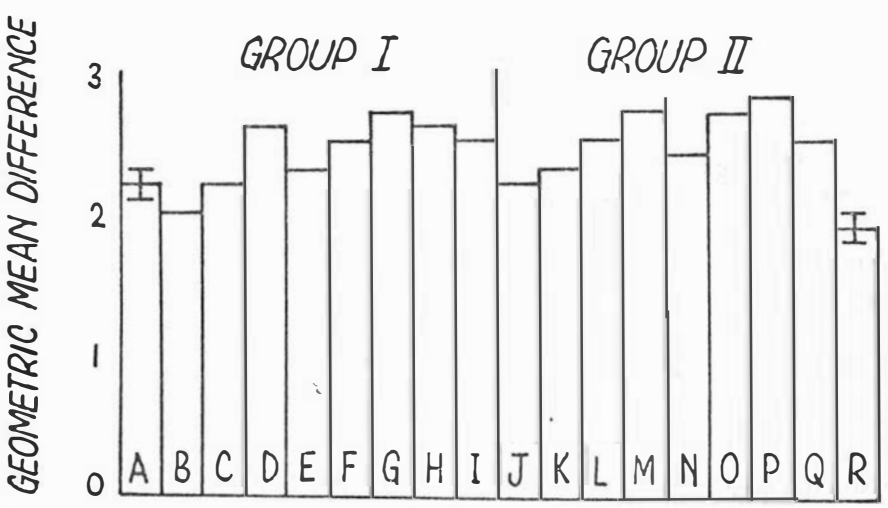
(Table 10.10, Figs 10.7 and 10.8). At 96 hours the geometric mean differences with each antigen dilution were of the same order as those observed in Experiment I.

DISCUSSION

The significant finding of these experiments was that lymphocytes from the older (Group I) pigs were transformed by *pomona* antigens to a significantly greater degree than were lymphocytes from the younger (Group II) pigs. The cells from all the animals responded to PHA, and the activities of the non-stimulated cultures and the PHA responses of both groups were the same. From this it may be assumed that the culture system was capable of supporting the proliferation of mitogen-sensitive cells and that the latent T cell activities of the two groups were the same. The serological results indicated that while the older pigs had experienced *pomona* infection the younger pigs had not. The association between increased lymphocytes transformation and elevated *pomona* titres with age suggested a cellular involvement in the response of the pig to *pomona* infection.

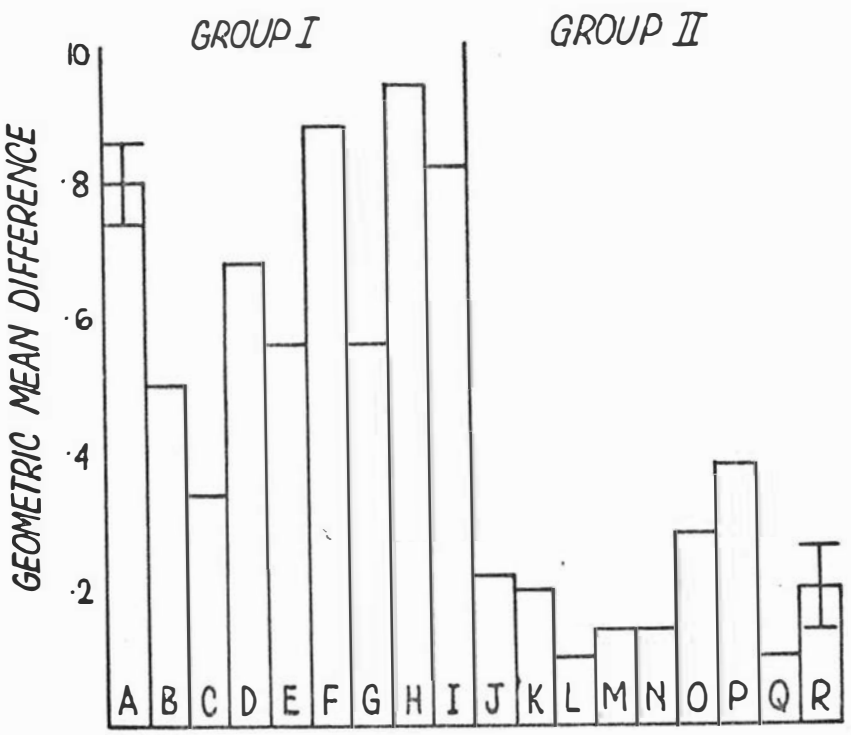
The time-responses of the antigen-stimulated and non-stimulated cultures showed that the results obtained were not due to the added antigen extracts simply influencing the continued viability of the cultured cells. The greater response over the first 48 hours incubation confirmed that the antigens had a stimulatory effect on the cell cultures. The slight responses given by some Group II animals to the *pomona* extracts could be due to the presence of non-specific mitogens in these materials. Such non-specific factors have been reported in a number of bacterial species (Peavy *et al.*, 1973; Coutinoho *et al.*, 1975).

Fig. 10.1 The PHA responses of the Group I (presumed infected) and Group II (presumed non-infected) pigs.



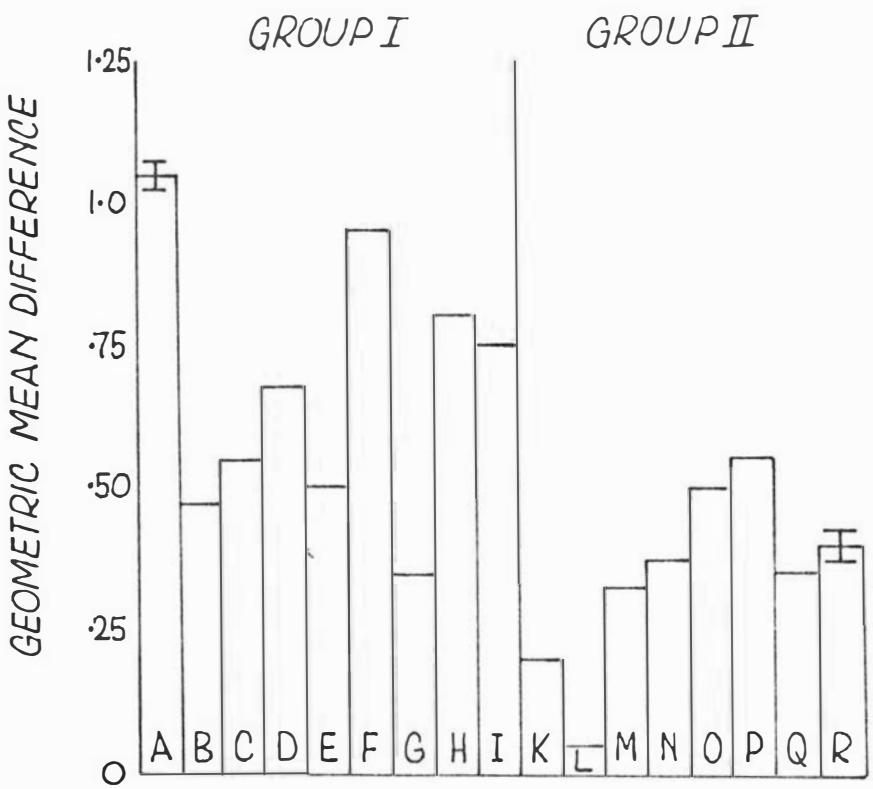
Average standard error of geometric mean differences shown (A and R).

Fig. 10.2 The maximum responses of the Group I (presumed infected) and Group II (presumed non-infected) animals to the sonicated antigen extract.



Average standard error of the geometric mean differences shown (A and R).

Fig. 10.3 The maximum responses of the Group I (presumed infected) and Group II (presumed non-infected) animals to the sodium deoxycholate antigen extract.



Average standard error of geometric mean differences shown (A and R).

Fig. 10.4 The association between the maximum responses to the sonicated antigen, and the maximum responses to the sodium deoxycholate-derived extract.

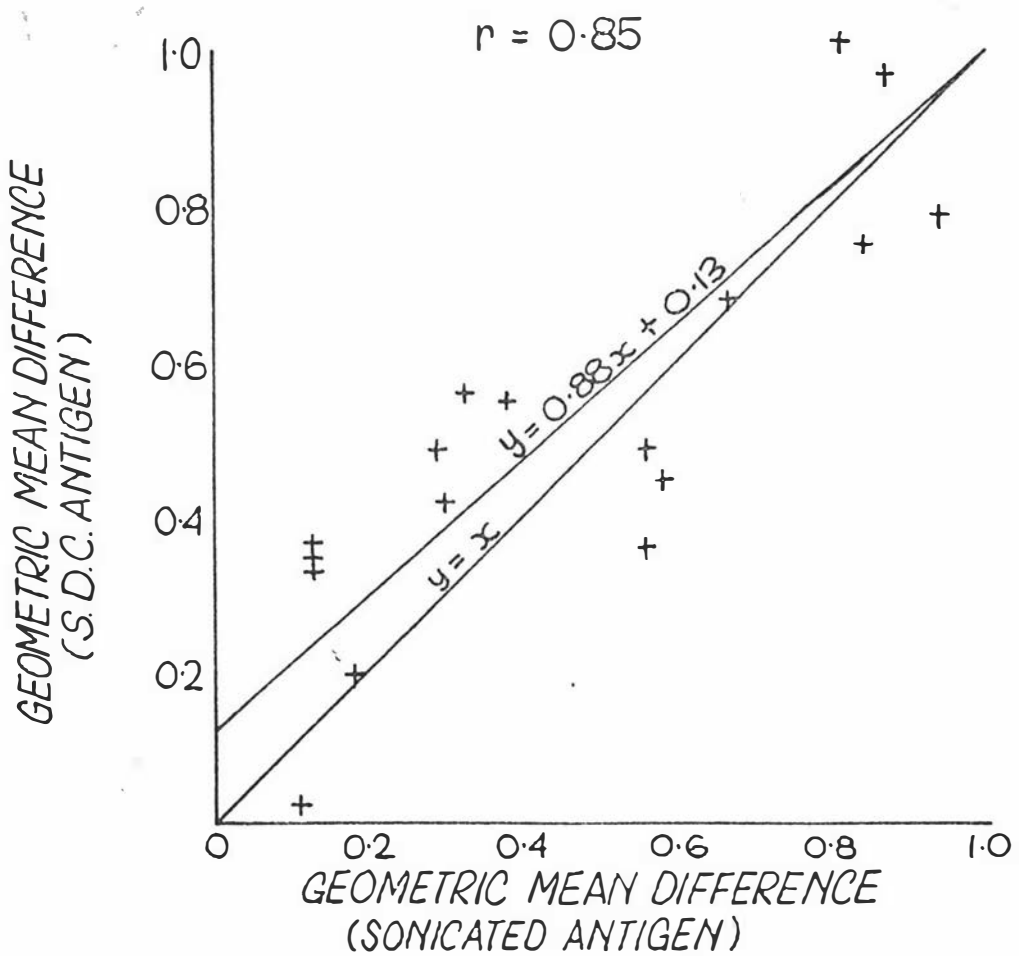
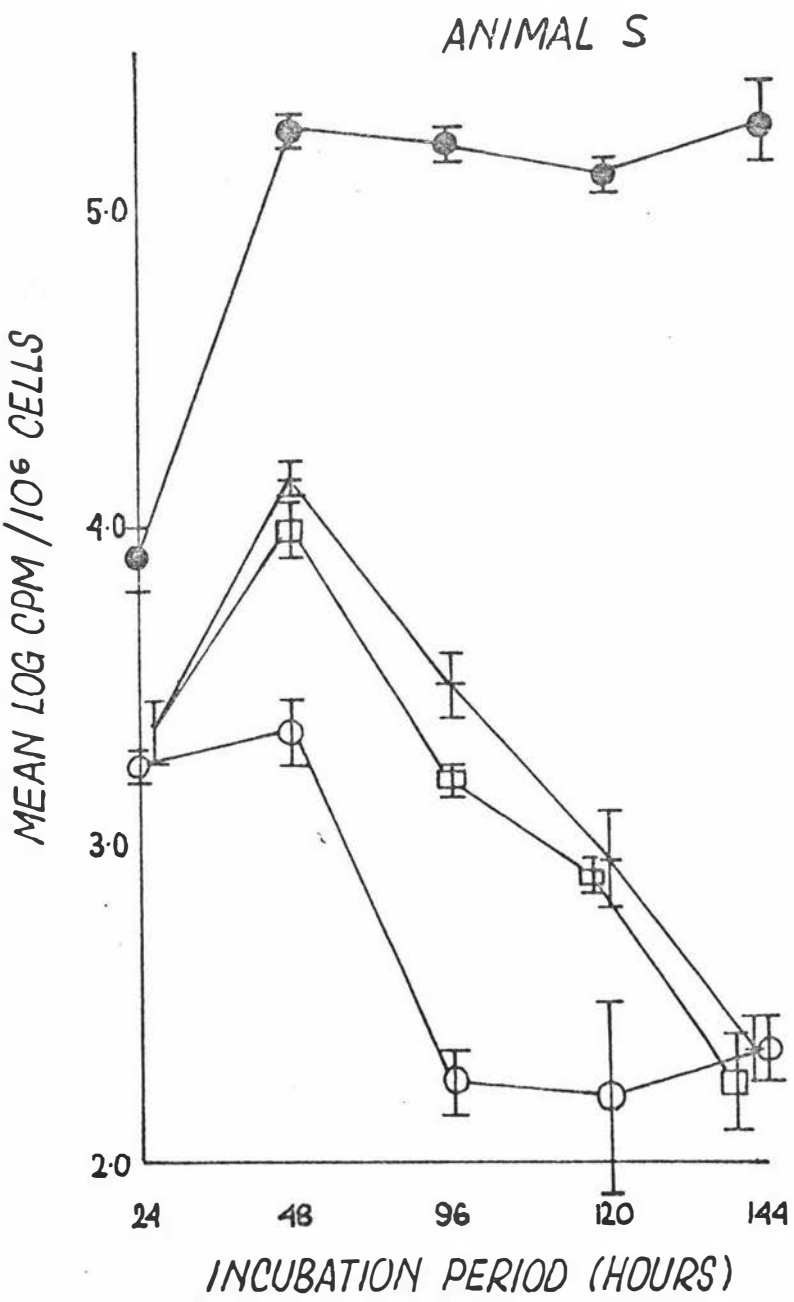
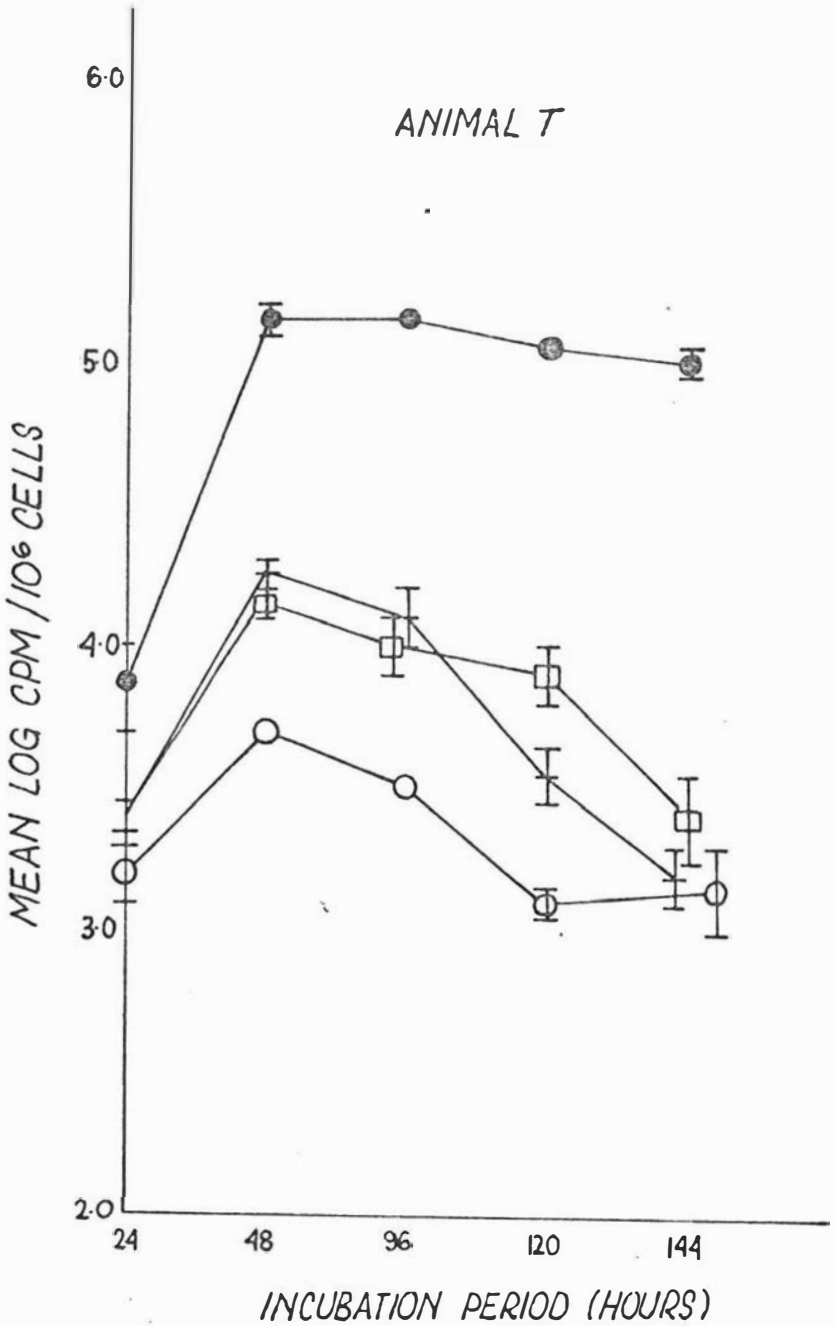


Fig. 10.5 The optimal antigen *in vitro* lymphocyte responses, and PHA responses over 144 hours.



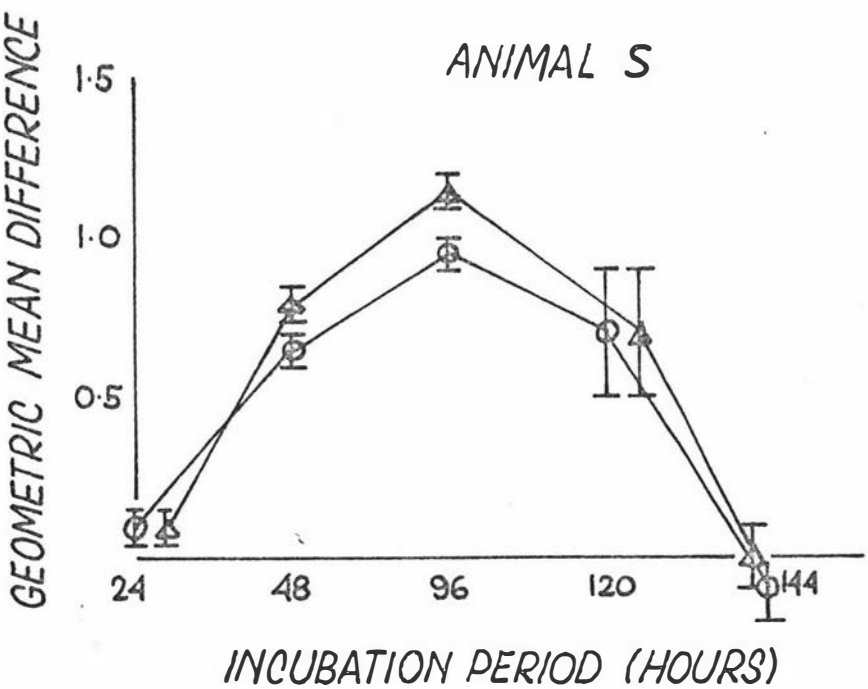
● = PHA
o = non-stimulated
\+ = sodium deoxycholate-derived *pomona* antigen
□ = sonicated *pomona* antigen

Fig. 10.6 The optimal antigen *in vitro* lymphocyte responses, and PHA responses over 144 hours.



● = PHA
o = non-stimulated
\+ = sodium deoxycholate-derived *pomona* antigen
□ = sonicated *pomona* antigen

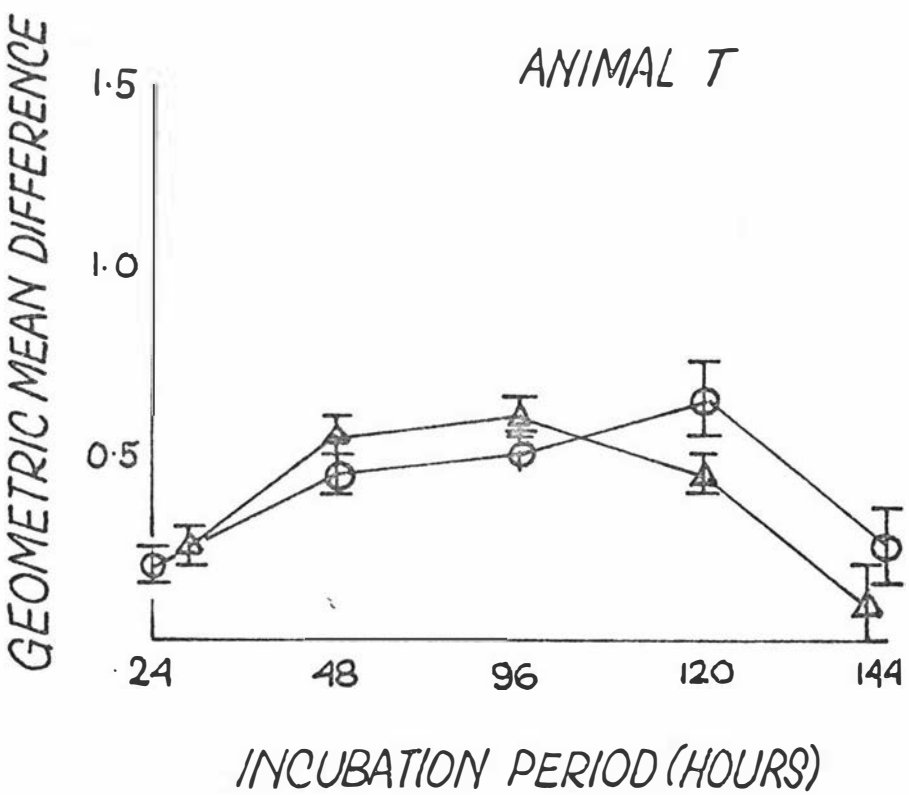
Fig. 10.7 The differences between geometric means of the antigen treated and non-stimulated cultures over 144 hours.



Δ = sodium deoxycholate-derived *pomona* antigen

o = sonicated *pomona* antigen

Fig. 10.8 The differences between geometric means of the antigen treated and non-stimulated cultures over 144 hours.



Δ = sodium deoxycholate-derived *pomona* antigen

o = sonicated *pomona* antigen

The serological results of these randomly selected animals confirmed that the pattern of leptospirosis infection in this herd had not changed from that which had been delineated in a previous study (Chapter VI). Of the serovars used in the MAT's, there was evidence of *pomona* infection only, the low titres against *ballum* and *copenhageni* being typical cross-reactions (Chapter VI). The high *pomona* titres of the old and young sows indicated that they had all been infected with *pomona*. The 9 young piglets were selected from different pens in the weaning unit, and the lack of any high titres confirmed that *pomona* infection was absent from this group. The low titres in 3 of these piglets were probably due to colostral antibody (Chapter VI). The MA titres of 2 of the older pigs (G and I) were atypically low. These animals were from Grower Unit III (See Chapter VI), where previously it had been found that most pigs had *pomona* titres of 1/384 or greater. Taking into consideration the age of pigs G and I, it is unlikely that the titres recorded were due to residual colostral antibody. It is also unlikely that they had been infected and recovered since the results of experimental *pomona* infection in the pig suggest that the homologous MA titres would not have decreased to this low level in such a short time (Morse *et al.*, 1958; Mitchell *et al.*, 1966; Hodges, 1973). The likely reason for these titres is that these pigs had become infected immediately prior to sampling.

The high correlation between the maximum responses to the antigen extracts suggest that the same mitogenic factors were operating in both preparations. Such a high correlation ($r = 0.85$) in an experiment where much variation might be expected was surprising.

Although these *in vitro* responses were evidence of a role for CMI *in vivo*, it was decided to confirm this postulate by establishing the nature of the responding cells. The results of this investigation are reported in the following chapter.

SUMMARY AND CONCLUSIONS

1. Lymphocyte microcultures from 18 pigs from a herd known to be endemically infected with *pomona* were prepared. Cells were stimulated with PHA, a sonicated *pomona* extract and a sodium deoxycholate-derived *pomona* extract. The time-responses of antigen stimulated cells over 144 hours were also investigated.

2. The PHA responses of the older pigs which had been infected with *pomona* were the same as those of the younger non-infected pigs. The activities of the non-stimulated cultures of these groups were also the same.

3. The cells of all the animals were transformed *in vitro* by the antigen extracts, with a dose-response occurring in most cases. The maximum responses of the pigs which had been infected were significantly greater than those which had not been infected.

4. It was concluded that transformation in response to the *pomona* antigens had occurred in the cell cultures from animals which had been infected, suggesting that CMI is part of the immune response of the pig to *pomona* infection.

5. It was also considered that the antigen extracts contained non-specific mitogens.

Table 10.1 Experiment I. The *pomona* titres of the pigs sampled.

Group	Animal Code	*pomona* titre
	A	1/48
	B	1/1536
	C	1/96
	D	1/96
I	E	1/1536
	F	1/768
	G	1/24
	H	1/192
	I	1/12
	J	1/12
	K	1/12
	L	0
	M	1/24
II	N	0
	O	0
	P	0
	Q	0
	R	0

Table 10.2 Experiment I. The PHA (1 µg/ml) and non-stimulated culture activity.

Group	Animal Code	PHA		Non-stimulated		gmd	SE	SI
		Mg	Sg	Mg	Sg			
I	A	5.18	0.12	2.94	0.04	2.24	0.07	174
	B	5.35	0.01	3.37	0.01	1.98	0.01	95
	C	5.26	0.06	3.07	0.06	2.19	0.05	155
	D	5.35	0.04	2.74	0.04	2.61	0.03	407
	E	5.30	0.06	3.01	0.14	2.29	0.09	195
	F	5.37	0.21	2.88	0.14	2.49	0.15	309
	G	5.43	0.02	2.74	0.07	2.69	0.04	490
	H	5.30	0.01	2.68	0.05	2.62	0.03	417
	I	5.45	0.04	2.91	0.11	2.54	0.07	347
II	J	5.39	0.16	3.21	0.14	2.18	0.12	151
	K	5.53	0.02	3.19	0.15	2.34	0.09	219
	L	5.57	0.01	3.04	0.09	2.53	0.05	339
	M	5.34	0.18	2.67	0.04	2.67	0.11	468
	N	5.35	0.06	2.91	0.10	2.44	0.07	275
	O	5.40	0.12	2.74	0.04	2.66	0.07	457
	P	5.41	0.01	2.58	0.10	2.83	0.01	676
	Q	5.29	0.21	2.82	0.04	2.47	0.12	295
	R	4.64	0.10	2.78	0.04	1.86	0.06	72

Mg = geometric mean
Sg = standard deviation of geometric mean
gmd = geometric mean difference
SE = standard error of the gmd
SI = stimulation index

Table 10.3 Experiment I. The responses to the sonicated *pomona* extract.

Group	Animal Code	SA_0 Mg	SA_0 Sg	SA_1 Mg	SA_1 Sg	SA_2 Mg	SA_2 Sg	SA_3 Mg	SA_3 Sg
I	A	2.65	0.30	3.56	0.03	3.75	0.07	3.48	0.09
	B	3.01	0.28	3.47	0.07	3.86	0.02	3.78	0.02
	C	2.67	0.06	3.23	0.21	3.40	0.06	3.34	0.05
	D	3.41	0.01	3.33	0.10	3.06	0.08	2.80	0.05
	E	3.31	0.07	3.48	0.09	3.57	0.13	3.21	0.08
	F	3.56	0.11	3.75	0.10	3.69	0.10	3.21	0.13
	G	3.30	0.04	3.10	0.01	2.84	0.03	2.77	0.07
	H	3.62	0.10	3.51	0.04	3.17	0.07	2.89	0.03
	I	3.73	0.02	3.72	0.07	3.27	0.01	3.10	0.02
II	J	3.17	0.13	3.43	0.04	3.37	0.06	3.28	0.08
	K	3.30	0.01	3.43	0.04	3.24	0.04	3.38	0.17
	L	3.15	0.06	3.06	0.01	3.01	0.06	2.98	0.05
	M	2.76	0.03	2.80	0.10	2.71	0.07	2.78	0.03
	N	2.93	0.02	3.04	0.07	2.94	0.06	2.98	0.03
	O	2.87	0.04	3.03	0.03	2.90	0.04	2.87	0.01
	P	2.98	0.01	2.96	0.02	2.86	0.06	2.82	0.08
	Q	2.68	0.08	2.93	0.06	2.92	0.12	2.92	0.12
	R	2.79	0.04	2.98	0.05	2.88	0.04	2.82	0.05

Mg = geometric mean
Sg = standard deviation of geometric mean
SA_0 = sonicated antigen, undiluted
SA_1 = " " 1/10 dilution
SA_2 = " " 1/100 dilution
SA_3 = " " 1/1000 dilution

Table 10.4 Experiment I. Summary of the analysis of the responses to the sonicated antigen.

Animal Code		gmd	SE	SI	**t(4.d.f.)	Animal Code		gmd	SE	SI	t(4d.f.)
A	*0	-0.29	0.17	0.51		J	0	-0.04	0.11	.91	
	1	0.62	0.03	4.17			+ 1	0.22	0.08	1.66	2.62
	+ 2	0.81	0.05	6.46	16.12		2	0.16	0.09	1.45	N/S
	3	0.54	0.06	3.47	p<0.001		3	0.07	0.09	1.17	
B	0	-0.36	0.16	0.44		K	0	0.11	0.09	1.29	
	1	0.10	0.04	1.26			1	0.15	0.09	1.41	
	+ 2	0.49	0.01	3.09	9.89		2	0.05	0.09	1.12	p=0.05
	3	0.41	0.01	2.57	p<0.001		+ 3	0.19	0.13	1.55	2.80
C	0	-0.40	0.05	0.40		L	+ 0	0.11	0.06	1.29	1.75
	1	0.16	0.13	1.45			1	0.02	0.05	1.05	N/S
	+ 2	0.33	0.05	2.14	4.88		2	-0.03	0.06	0.93	
	3	0.27	0.05	1.86	p=0.010		3	-0.10	0.06	0.79	
D	+ 0	0.67	0.02	4.68	27.60	M	0	0.09	0.03	1.23	
	1	0.59	0.06	3.89	p<0.001		+ 1	0.13	0.06	1.35	2.01
	2	0.32	0.05	2.09			2	0.04	0.05	1.10	N/S
	3	0.06	0.04	1.15			3	0.11	0.03	1.29	
E	0	0.30	0.09	2.00		N	0	0.02	0.06	1.05	
	1	0.47	0.10	2.95			+ 1	0.13	0.07	1.35	1.71
	+ 2	0.56	0.11	3.63	5.31		2	0.03	0.07	1.07	N/S
	3	0.20	0.09	1.58	p=0.01		3	0.07	0.06	1.17	
F	0	0.68	0.10	4.79		O	0	0.13	0.03	1.35	
	+ 1	0.57	0.10	7.41	8.41		+ 1	0.29	0.03	1.95	9.85
	2	0.81	0.10	6.46	p=0.01		2	0.16	0.03	1.45	p<0.001
	3	0.33	0.11	2.14			3	0.13	0.02	1.35	
G	+ 0	0.56	0.05	3.63	9.74	P	0	0.36	0.01	2.29	
	1	0.36	0.04	2.29	p<0.001		+ 1	0.38	0.01	2.40	29.93
	2	0.10	0.04	1.26			2	0.28	0.04	1.91	p<0.001
	3	0.03	0.06	1.07			3	0.24	0.05	1.74	
H	+ 0	0.94	0.06	8.71	15.08	Q	0	-0.14	0.05	0.72	
	1	0.83	0.04	6.76	p<0.001		+ 1	0.11	0.04	1.29	2.60
	2	0.49	0.05	3.09			2	0.10	0.07	1.26	N/S
	3	0.21	0.03	1.62			3	0.10	0.07	1.26	
I	+ 0	0.82	0.06	6.61	13.29	R	0	0.01	0.03	1.02	
	1	0.81	0.08	6.46	p<0.001		+ 1	0.20	0.04	1.58	5.12
	2	0.36	0.06	2.29			2	0.10	0.03	1.26	p=0.01
	3	0.19	0.06	1.55			3	0.04	0.04	1.10	

* 0 = undiluted antigen
1 = 1/10 diluted antigen
2 = 1/100 diluted antigen
3 = 1/1000 diluted antigen

** Value of "t" and level of significance for the maximum geometric mean difference

gmd = geometric mean difference

SE = standard error of the gmd

SI = stimulation index

\+ = maximum gmd

Table 10.5 Experiment I. The responses to the sodium deoxycholate extract.

Group	Animal Code	DA_0 Mg	DA_0 Sg	DA_1 Mg	DA_1 Sg	DA_2 Mg	DA_2 Sg	DA_3 Mg	DA_3 Sg
I	A	3.99	0.02	3.04	0.22	3.00	0.05	3.00	0.05
	B	3.85	0.07	3.53	0.03	3.46	0.12	3.38	0.08
	C	3.63	3.05	3.03	0.14	3.16	0.12	3.04	0.14
	D	3.42	0.07	2.76	0.06	2.84	0.02	2.78	0.05
	E	3.50	0.04	3.06	0.09	3.13	0.05	3.07	0.05
	F	3.84	0.04	2.84	0.07	2.92	0.03	2.93	0.06
	G	3.10	0.04	2.61	0.04	2.78	0.08	2.82	0.06
	H	3.47	0.03	2.75	0.03	2.94	0.13	2.80	0.07
	I	3.66	0.06	2.82	0.07	2.92	0.14	2.86	0.06
II	J	–	–	–	–	–	–	–	–
	K	3.39	0.08	3.12	0.08	3.20	0.06	3.23	0.06
	L	3.08	0.04	2.84	0.07	2.91	0.10	2.89	0.08
	M	3.00	0.04	-	-	-	-	-	-
	N	3.28	0.12	-	-	-	-	-	-
	O	3.23	0.06	-	-	-	-	-	-
	P	3.13	0.05	-	-	-	-	-	-
	Q	3.17	0.19	-	-	-	-	-	-
	R	3.20	0.03	-	-	-	-	-	-

Mg = geometric mean

Sg = standard deviation of geometric mean

DA_0 = sodium deoxycholate antigen, undiluted

DA_1 = " " " 1/10 dilution

DA_2 = " " " 1/100 dilution

DA_3 = " " " 1/1000 dilution

Table 10.6 Experiment I. Summary of the analysis of the responses to the sodium deoxycholate antigen.

Animal Code	gmd	SE	SI	**t(4d.f.)
A + *0	1.05	0.03	11.22	3950
1	0.01	0.13	1.26	P<0.001
2	0.06	0.04	1.15	
3	0.06	0.04	1.15	
B + 0	0.48	0.04	3.02	7.70
1	0.16	0.02	1.45	p=0.005
2	0.09	0.07	1.23	
3	0.01	0.05	1.02	
C + 0	0.56	0.05	3.63	8.55
1	-0.04	0.09	0.91	p=0.005
2	0.09	0.08	1.23	
3	-0.03	0.09	0.93	
D + 0	0.68	0.05	4.79	13.61
1	0.02	0.04	1.05	p<0.001
2	0.10	0.03	1.26	
3	0.04	0.04	1.10	
E + 0	0.49	0.08	3.09	6.05
1	0.05	0.10	1.12	p=0.005
2	0.12	0.09	1.32	
3	0.06	0.09	1.15	
F + 0	0.96	0.08	9.12	11.21
1	-0.04	0.09	0.91	p<0.001
2	0.04	0.08	1.10	
3	0.05	0.09	1.12	
G + 0	0.36	0.05	2.29	2.64
1	-0.13	0.05	0.74	N/S
2	0.04	0.06	1.10	
3	0.08	0.05	1.20	
H + 0	0.79	0.03	6.17	22.49
1	0.07	0.03	1.17	p<0.001
2	0.26	0.08	1.82	
3	0.12	0.05	1.32	
I + 0	0.75	0.07	5.62	11.02
1	-0.09	0.08	0.81	p<0.001
2	0.01	0.10	1.02	
3	-0.05	0.07	0.89	

Animal Code	gmd	SE	SI	t(4d.f.)
K + 0	0.20	0.10	1.58	2.08
1	-0.07	0.10	0.85	N/S
2	0.01	0.09	1.02	
3	0.04	0.09	1.10	
L + 0	0.04	0.06	1.10	0.69
1	-0.20	0.07	0.63	N/S
2	-0.13	0.08	0.74	
3	-0.15	0.07	0.71	
M + 0	0.33	0.03	2.14	11.24 p<0.001
N + 0	0.37	0.09	2.34	4.07 p=0.025
O + 0	0.49	0.04	3.09	11.14 p<0.001
P + 0	0.55	0.03	3.55	20.12 p<0.001
Q + 0	0.35	0.11	2.24	3.19 p=0.05
R + 0	0.42	0.03	2.63	14.13 p<0.001

* 0 = undiluted antigen
1 = 1/10 diluted antigen
2 = 1/100 diluted antigen
3 = 1/1000 diluted antigen

** Value of "t" and level of significance for the maximum geometric mean difference

gmd = geometric mean difference

SE = standard error of the gmd

SI = stimulation index

\+ = maximum gmd

Table 10.7 Experiment I. The maximum geometric mean differences occurring with the antigen extracts.

SA max. gmd	DA max. gmd
0.81	1.05
0.49	0.45
0.33	0.56
0.67	0.68
0.56	0.49
0.87	0.96
0.56	0.36
0.94	0.79
0.82	0.75
0.22	ND*
0.19	0.20
0.11	0.04
0.13	0.33
0.13	0.37
0.29	0.49
0.38	0.55
0.11	0.35
0.30	0.42

SA = sonicated antigen
DA = sodium deoxycholate antigen
max. gmd = maximum geometric mean difference
*ND = not done

Table 10.8 Experiment II. The time-responses of cultures treated with the antigen extracts. Animal T .

Treatment		Incubation Time (hours)				
		24	48	96	120	144
Non-stimulated	Mg	3.20	3.71	3.50	3.10	3.13
	Sg	0.09	0.01	0.02	0.05	0.13
PHA	Mg	3.87	5.17	5.16	5.04	4.97
	Sg	0.15	0.03	0.01	0.02	0.03
SA_1	Mg	3.38	4.15	4.01	3.73	3.39
	Sg	0.03	0.03	0.07	0.11	0.16
DA_0	Mg	3.43	4.27	4.11	3.54	3.20
	Sg	0.05	0.06	0.12	0.07	0.10

Mg = geometric mean
Sg = standard deviation of the geometric mean
SA_1 = sonicated antigen, 1/10 dilution
DA_0 = sodium deoxycholate antigen, undiluted

Table 10.9 Experiment II. The time-responses of cultures treated with the antigen extracts. Animal S.

Treatment		Incubation Time (hours) 24	48	96	120	144
Non-stimulated	Mg	3.24	3.35	2.23	2.21	2.33
	Sg	0.04	0.07	0.07	0.33	0.07
PHA	Mg	3.38	5.23	5.21	5.12	5.23
	Sg	0.08	0.04	0.03	0.05	0.08
SA_1	Mg	3.34	3.99	3.18	2.90	2.23
	Sg	0.11	0.07	0.03	0.03	0.13
DA_0	Mg	3.36	4.13	3.48	2.93	2.35
	Sg	0.07	0.02	0.07	0.16	0.10

Mg = geometric mean

Sg = standard deviation of the geometric mean

SA_1 = sonicated antigen, 1/10 dilution

DA_0 = sodium deoxycholate antigen, undiluted

Table 10.10 Experiment II. The time responses to the antigen preparations which induced the highest geometric mean differences.

Animal	Antigen		Incubation Time (hours) 24	48	96	120	144
S	SA_1	gmd	0.10	0.64	0.95	0.69	-0.10
		SE	0.07	0.06	0.04	0.19	0.09
		SI	1.26	4.37	8.91	4.90	0.79
	DA_0	gmd	0.12	0.78	1.25	0.72	0.02
		SE	0.05	0.04	0.06	0.21	0.07
		SI	1.37	6.03	17.78	5.25	1.05
T	SA_1	gmd	0.18	0.44	0.51	0.63	0.26
		SE	0.05	0.02	0.04	0.07	0.12
		SI	1.51	2.75	3.24	4.27	1.82
	DA_0	gmd	0.23	0.56	0.61	0.44	0.07
		SE	0.06	0.04	0.07	0.05	0.09
		SI	1.70	3.63	4.07	2.75	1.17

SA_1 = sonicated antigen, 1/10 dilution
DA_0 = sodium deoxycholate antigen, undiluted
gmd = geometric mean difference
SE = standard error of the gmd
SI = stimulation index

CHAPTER XI

LYMPHOCYTE TRANSFORMATION : EXPERIMENTS WITH B CELL DEPLETED LYMPHOCYTE CULTURES

INTRODUCTION

Although *in vitro* lymphocyte transformation in response to antigens is generally considered to be a result of T lymphocyte blastogensis and proliferation (W.H.O., 1973; Ling and Kay, 1975), it has also been shown that on appropriate stimulation B cells *in vitro* can also proliferate (Bloom, 1971; Elfenbein *et al.*, 1972; Luzzati *et al.*, 1973).

Since the *in vitro* transformation responses to the *pomona* antigens described in Chapter X could have been a secondary response of either T or B cells, it was considered that the lymphocyte subset activated in these experiments should be defined. The responses of B cell-depleted cultures were therefore compared with those of normal non-fractionated cell cultures. The selective removal of B cells was achieved by nylon wool filtration. The reduction in B cells so achieved was confirmed by demonstrating a decrease in the number of cells with membrane associated immunoglobulin, as shown by immunofluorescence.

Nylon wool columns also retain macrophages (Luquetti and Janossy, 1976) and since it has been demonstrated that adherent cells, presumably macrophages, are essential for a wide range of *in vitro* cell activities (Waldron *et al.*, 1973; Rosenstriech and Rosenthal, 1974; Lipsky *et al.*, 1976), the eluted cell populations were replenished with adherent cells.

MATERIALS AND METHODS

THE DEPLETION OF PERIPHERAL BLOOD LYMPHOCYTES OF B CELLS ON NYLON WOOL COLUMNS.

Mononuclear cells were separated from heparinised pig blood as described previously (Chapter X). The cells were washed twice in Eagle's minimal essential medium, and resuspended in 2 ml of TC199 containing 5% heat inactivated (56 ^{o}C/30 minutes) foetal bovine serum (FBS) and antibiotics. This medium was used in all column separation experiments, and in subsequent microcultures.

The 2 ml cell suspension was loaded onto a nylon wool column which had been prepared as described by Julius *et al.* (1973). The cells were washed into the nylon wool with 1 ml of medium and the column sealed and left for 45 minutes at 37 ^{o}C.

The column was then washed with warm (37 ^{o}C) medium, and the first 25 ml collected. The eluted cells were pelletted for 10 minutes at 300xg, resuspended in 5 ml of medium, and the number of viable cells estimated by trypan blue exclusion.

The eluted cells were either reacted with fluorescein congugated anti-pig-γ-globulin to detect cells displaying surface immunoglobulin, or were cultured as described in Chapter X.

SUPPLEMENTATION OF COLUMN-DERIVED CELL CULTURES WITH ADHERENT CELLS

Column-derived cells, presumably lacking adherent cells, were reconstituted with adherent cells in the following manner. Microcultures of non-fractionated lymphocytes, prepared as described in Chapter X, were incubated at 37 ^{o}C for one hour to allow adherent cells to bind to the

plastic (Ling and Kay, 1975). The non-adherent cells were then flushed out with medium and 0.2 ml of the suspension of eluted cells added to the wells.

Controls, consisting of cultures containing adherent cells and medium, were included in each experiment to confirm that non-adherent lymphocytes had been removed.

STIMULATION OF B CELL-DEPLETED CULTURES

Mononuclear cells from 2 young sows (coded U and V) which had been infected with *pomona*, and which had MA titres of 1/384 and 1/1536, were harvested as described above. Cultures of the following cell compositions were established:-

(i) Non-fractionated cells.
(ii) Column-eluted cells alone.
(iii) Column-eluted cells with adherent cells.
(iv) Adherent cells alone.

Triplicates of each culture were stimulated with optimal levels of PHA (1 µg/ml), sonicated *pomona* antigen (1/10 dilution), and sodium deoxycholate antigen (undiluted). Cultures were harvested after 96 hours incubation.

PREPARATION OF ANTI-PIG-GAMMA-GLOBULIN (ANTI-PGG)

The sera from 4 pigs were pooled and precipitated 3 times with 35% ammonium sulphate (Herbert, 1974). The final precipitate was dissolved in PBS, dialysed against physiological saline, and concentrated to 30 mg/ml by dialysis against polyethylene glycol (M.W. = 20 000).

A 1:10 dilution of the concentrated PGG in PBS was emulsified in Freund's complete adjuvant*. Two rabbits were given 3 fortnightly injections of 0.5 ml of this material. Ten days after the final injection the rabbit antisera were collected and pooled.

PREPARATION OF FLUORESCEIN ISOTHIOCYANATE (FITC) LABELLED ANTI-PGG (FL-ANTI-PGG).

The pooled anti-PGG was precipitated with ammonium sulphate and the resulting gamma-globulin fractionated and labelled with fluorescein isothiocyanate** by the method of Thé and Feltkramp (1970, a,b). Following absorption with pig liver powder***, the conjugated material was stored at -20 ^{o}C.

DETECTION OF SURFACE IMMUNOGLOBULIN

Mononuclear cells were separated from peripheral blood, as described previously, washed three times in physiological saline and resuspended in saline at a concentration of 15 x 10^{6} cells/ml. Doubling dilutions from 1/2 to 1/16 of the Fl-anti-PGG were prepared, and 0.1 ml of each dilution mixed with 0.1 ml of the cell suspension. The cells were labelled for 30 minutes, washed 3 times in saline and viewed by blue darkground fluorescence microscopy. In preliminary experiments viable cells were labelled at 4 ^{o}C and washed with saline also at 4 ^{o}C. In the later experiments, the washed cells were first held at 56 ^{o}C until all were non-viable as judged by the trypan blue exclusion test (Chapter X). Cells were then

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* Difco, Detroit, Michigan, U.S.A.

** Sigma, St Louis, Missouri, U.S.A.

*** Wellcome, Beckenham, England

labelled at 37 ^{o}C, and washed with saline at ambient temperature.

Sheep lymphocytes, similarly harvested and processed, acted as controls for labelling specificty.

RESULTS

THE IDENTIFICATION OF B LYMPHOCYTES BY IMMUNOFLUORESCENCE

When viable ficoll-separated cells were used, spots and caps of fluorescence were seen on the cell surfaces. When heat-killed cells were used, a distinct membrane fluorescence was observed on approximately 10% of cells. The heat-killed cells also showed a faint diffuse cytoplasmic fluorescence, which was quite unlike the fluorescence associated with the cell membrane.

The control sheep mononuclear cells exhibited only faint cytoplasmic fluorescence; no cells showed the distinctive membrane fluorescence which was seen on the pig cells.

Similar results were observed with all the dilutions of the Fl-anti-PGG.

THE RETENTION OF PORCINE B LYMPHOCYTES ON NYLON WOOL COLUMNS

Peripheral blood was collected from 3 pigs, and the proportions of the ficoll-separated cells which were labelled by the Fl-anti-PGG in each case were 40/409 (9.8%), 83/711 (11.7%) and 36/385 (9.4%), an average percentage of 10.3%. After the cells had been eluted through nylon wool columns, it was found that the proportion of labelled cells in each case was 2/121 (1.6%), 6/325 (1.9%) and 4/169 (2.4%), an average of 2.0%.

THE *IN VITRO* RESPONSES TO PHA AND TO THE *POMONA* EXTRACTS

With the exception of the cultures containing adherent cells alone, the PHA responses of the various cell populations were similar (Table 11.1, Figs 11.1 and 11.2). The activities of the cultures of adherent cells alone were not significantly different to those of the non-stimulated cultures ($t_u = 1.03$, $t_v = 0.70$, 4 *d.f.*).

The starting cell population (non-fractionated) was transformed *in vitro* by the antigen extracts. The geometric mean differences with the sonicated preparation were 0.38 and 0.39 for animals U and V respectively (Table 11.2, Figs. 11.3 and 11.4). Less marked responses to the sodium deoxycholate extract were recorded, the geometric mean differences in each case being 0.08 and 0.24 respectively (Table 11.2, Figs 11.5 and 11.6) for animals U and V.

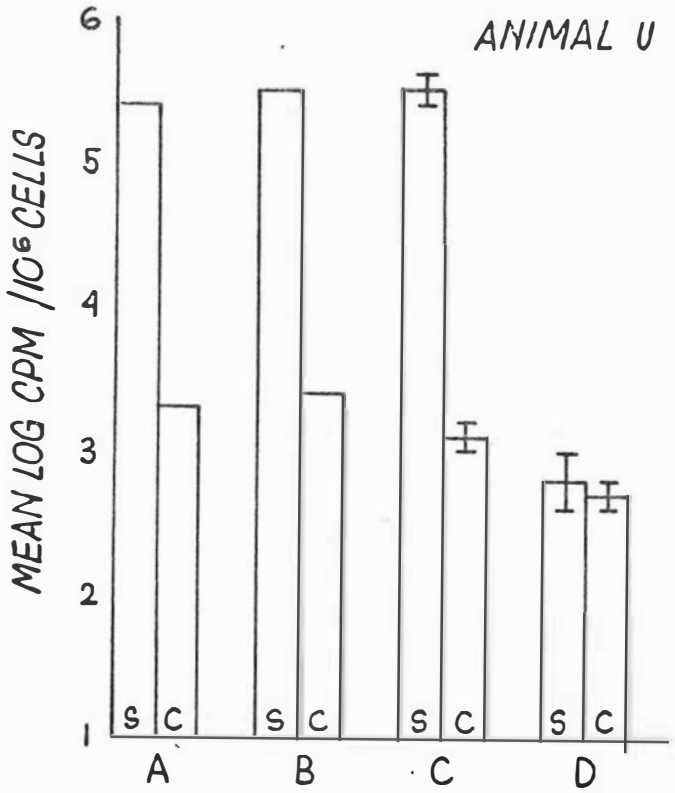
There was no evidence of transformation in response to the antigens in those cultures containing either column-eluted cells alone or column-eluted cells supplemented with adherent cells (Table 11.2, Figs 11.3 to 11.6). As is seen from these figures, in some cases the background cellular proliferation usually observed in non-stimulated cultures was suppressed.

There was no evidence of cellular proliferation in response to the antigen extracts in cultures containing adherent cells alone (Table 11.2, Figs 11.3 to 11.6).

DISCUSSION

While the non-fractionated lymphocytes from pigs which had been infected with serovar *pomona* were transformed *in vitro* by the *pomona* antigen extracts, nylon wool fractionation of these cells gave a population of cells unresponsive to the antigens used. Nylon wool columns retain both adherent cells (macrophages) and

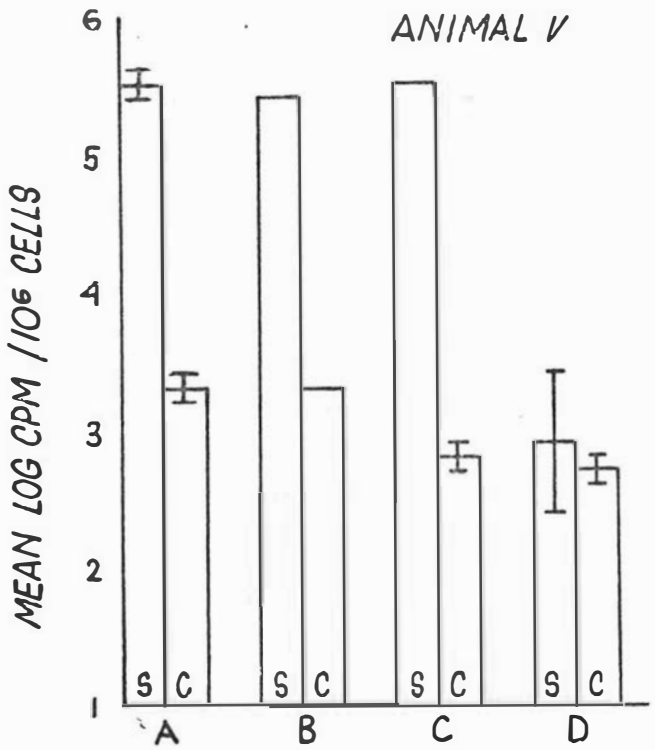
Fig. 11.1 The *in vitro* responses to PHA of B cell-depleted cultures.



A = non-fractionated cells
B = eluted + adherent cells
C = eluted cells alone
D = adherent cells alone
S = PHA
C = non-stimulated

(standard error of mean log cpm shown only where it is 0.05 or greater).

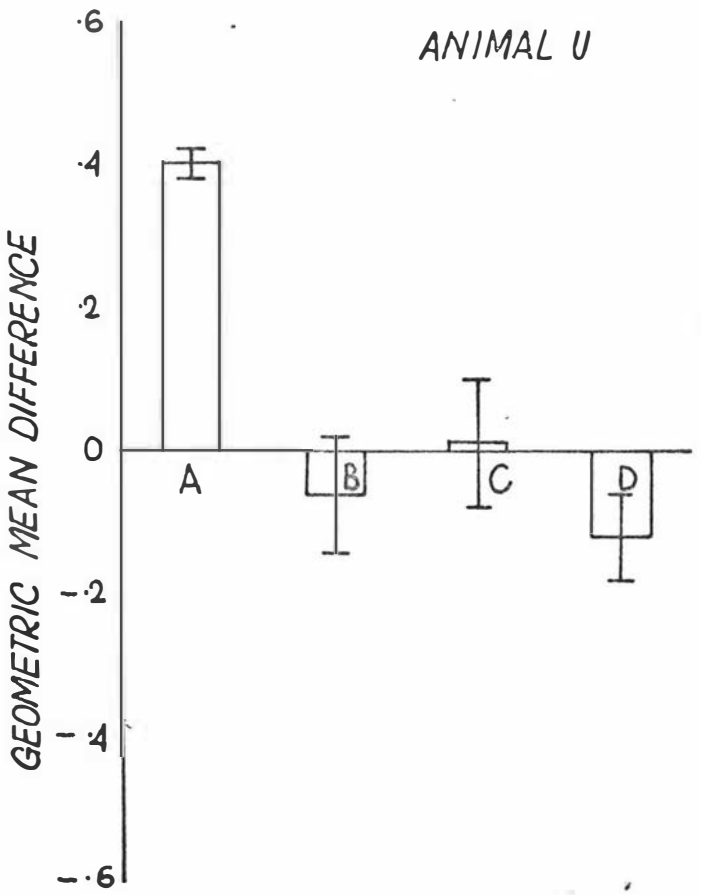
Fig. 11.2 The *in vitro* responses to PHA of B cell-depleted cultures.



A = non-fractionated cells
B = eluted cells + adherent cells
C = eluted cells alone
D = adherent cells alone
S = PHA
C = non-stimulated

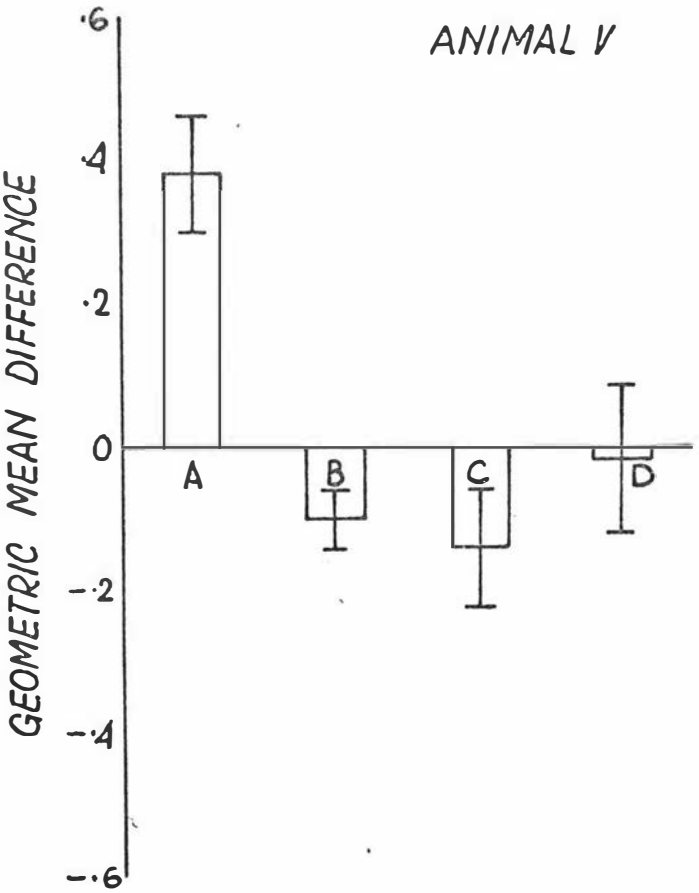
(standard error of mean log cpm shown only where it is 0.05 or greater)

Fig. 11.3 The *in vitro* responses to the sonicated *pomona* extracts of B cell-depleted cultures.



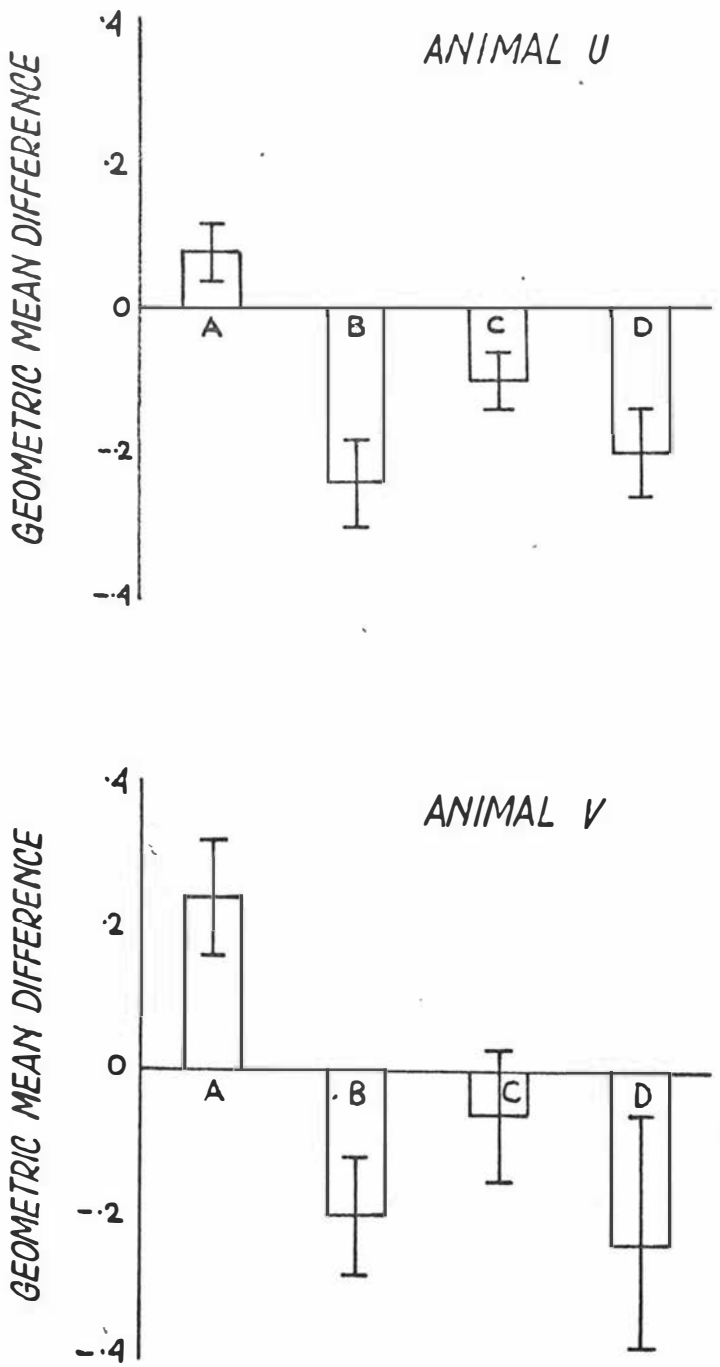
A = non-fractionated cells
B = eluted + adherent cells
C = eluted cells alone
D = adherent cells alone

Fig. 11.4 The *in vitro* responses to the sonicated *pomona* extracts of B cell-depleted cultures.



A = non-fractionated cells
B = eluted + adherent cells
C = eluted cells alone
D = adherent cells alone

Figs 11.5 and 11.6 The *in vitro* responses to the sodium deoxycholate-derived antigen of B cell depleted cultures.



A = non-fractionated cells C = eluted cells alone

B = eluted + adherent cells D = adherent cells alone

B lymphocytes (Julius *et al.*, 1973; Luquetti and Janossy, 1976).

When attempting to replace macrophages in the eluted cell cultures, after the lymphocytes had been flushed out, large numbers of adherent cells could be seen attached to the bottom of the microcultures. Thus, it would appear that the lack of responsiveness to the antigens by the eluted cells could not be explained by a deficiency of macrophages. All cultures of eluted cells responded equally well to PHA, indicating that they contained immuno-competent T lymphocytes.

There are two possible explanations for these results. Either the fractionation method used removed an essential T-helper cell population, or alternately T cells are not involved in the antigen-driven cellular proliferation. A requirement for a T-helper subset in antigen induced T cell mitogensis has not been demonstrated (Blanden, 1974; Campbell, 1976). What was clear was that the decrease in cellular activity was associated with the removal of B lymphocytes, 80% of which were removed by this fractionation technique, as judged by immunofluorescence. This suggests that B cells and not T cells were the antigen-reactive sub-population in this *in vitro* system.

It was expected that the PHA responses of the cultures containing eluted cells alone would be less than those containing either non-fractionated cells or those of eluted cells supplemented with adherent cells. A requirement for macrophages in PHA-induced transformation has been demonstrated by other workers (Lipsky *et al.*, 1976). It would seem that pig T lymphocytes are less demanding in their requirements for macrophages in PHA-induced transformation than are the cells from other species.

An extension to this work is indicated by these results. The proliferative response to the *pomona* antigens of the cell population retained on the nylon wool columns should be investigated to confirm whether or not the antigen-reactive cells are B cells. Additionally the cells seen in the lesions of *pomona* infected pig's kidneys should be further identified.

SUMMARY AND CONCLUSIONS

1. The responses of B cell-depleted cultures to the *pomona* antigen extracts were investigated. The removal of B cells from porcine blood lymphocytes was achieved by nylon wool fractionation and confirmed by immunofluorescence.

2. Although the non-fractionated cells responded to both the sonicated and sodium deoxycholate-derived antigens, the eluted cells did not. Supplementation of cultures of eluted cells with adherent cells did not increase the level of transformation achieved.

3. It was concluded that the antigen-reactive cells in this *in vitro* system were probably B cells. Thus, the postulate that CMI is part of the immune response of the pig to infection with serovar *pomona* was not confirmed.

Table 11.1 The activities of the cell cultures treated with PHA, the 1/10 dilution of the sonicated antigen and the undiluted sodium deoxycholate antigen.

Animals	Cells cultured		Stimulated with Non-stim.	PHA	SA_1*	DA_0**
U	Non-fractionated	Mg	3.25	5.42	3.64	3.33
		Sg	0.03	0.04	0.03	0.06
	Eluted + Mϕ	Mg	3.43	5.46	3.07	2.96
		Sg	0.04	0.04	0.12	0.02
	Eluted alone	Mg	3.06	5.48	3.37	3.21
		Sg	0.05	0.05	0.15	0.08
	Mϕ alone	Mg	2.70	2.80	2.58	2.51
		Sg	0.07	0.15	0.08	0.05
V	Non-fractionated	Mg	3.25	5.45	3.63	3.49
		Sg	0.11	0.07	0.06	0.04
	Eluted + Mϕ	Mg	3.30	5.44	2.70	2.78
		Sg	0.02	0.02	0.06	0.13
	Eluted alone	Mg	2.84	5.45	3.20	3.09
		Sg	0.10	0.01	0.05	0.14
	Mϕ alone	Mg	2.72	2.91	2.73	2.49
		Sg	0.09	0.48	0.17	0.23

Mϕ = adherent cells
Mg = geometric mean
Sg = standard deviation of the geometric mean
* SA_1 = sonicated antigen, 1/10 dilution
** DA_0 = sodium deoxycholate derived antigen, undiluted

Table 11.2 Summary of the analysis of the responses of the cells from animals U and V to the antigen preparations.

Animal	Antigen	Cells cultured	gmd	SE	SI
U	SA_1	Non-fractionated	0.39	0.02	2.45
		Eluted + Mϕ	-0.06	0.07	0.87
		Eluted alone	0.01	0.08	1.02
		Mϕ alone	-0.12	0.06	0.76
	DA_0	Non-fractionated	0.08	0.04	1.20
		Eluted + Mϕ	-0.22	0.05	0.60
		Eluted alone	-0.10	0.03	0.79
		Mϕ alone	-0.19	0.05	0.65
V	SA_1	Non-fractionated	0.38	0.07	2.40
		Eluted + Mϕ	-0.10	0.03	0.79
		Eluted alone	-0.14	0.07	0.72
		Mϕ alone	0.01	1.02	0.11
	DA_0	Non-fractionated	0.24	0.07	1.74
		Eluted + Mϕ	-0.10	0.03	0.79
		Eluted alone	-0.14	0.07	0.72
		Mϕ alone	-0.23	0.59	0.14

Mϕ = adherent cells
SA_1 = sonicated antigen, 1/10 dilution
DA_0 = sodium deoxycholate antigen, undiluted
gmd = geometric mean difference
SE = standard error of the gmd
SI = stimulation index

CHAPTER XII

GENERAL DISCUSSION

In many countries pigs, along with various rodents, are regarded as reservoir hosts for many leptospiral serovars. It was therefore surprising to find at the outset of this study that so little work on leptospirosis in pigs had been conducted in New Zealand. This is especially so, in view of the situation that pigs are generally regarded as important hosts of *pomona* and that infection with this serovar was of importance in this country in both the public health (Josland *et al.*, 1957; Christmas *et al.*, 1974 a, b) and animal production spheres (Salisbury, 1954; Jamieson *et al.*, 1970). The likely reason for this lack of research is that the pig industry was regarded as being of only minor economic importance. It appears to have been overlooked that diseases associated with pigs may have wide ranging effects.

Although a great deal is known about *pomona* infection in pigs, this cannot be said about infection with the other serovars such as *tarassovi*. In the course of this study an attempt was made to remedy this situation with regard to *tarassovi*, but the experimental animals failed to become infected owing, it would seem, to prior exposure to this serovar. This highlighted an important requirement in studies of leptospirosis, the need to have a ready supply of animals known not to have been exposed to any leptospiral serovars. In view of the ubiquitous nature of leptospiral infection in both wildlife and domestic animals, the practice of selecting animals for experimental infection studies on the basis of a negative MA titre is unsatisfactory.

Two of the techniques which were developed and used extensively in this study deserve comment. They are the "stomacher" method of homogenising pig kidneys (Chapter III), and the serological method (Chapter II). Much of the success of the culture surveys may be attributed to the former, while the latter enabled a rapid and efficient testing of sera for anti-leptospiral agglutinins.

The studies conducted in this laboratory in pigs, in wildlife (Hathaway, 1978), and in cattle (Hellstrom, 1978) confirmed the general concepts of leptospirosis which were enunciated in Chapter I. Various species were found to be infected only with certain serovars, even though there was ample opportunity for infection with other serovars to occur. This has particular importance when considering either control or eradication of leptospiral infection in domestic animals. For this reason these epidemiological findings should be confirmed by experimental methods. Studies of the factors that might affect the infectivity of leptospires in the various domestic species are particularly relevant to such investigations.

An important result in this current study was the elucidation of the extent and type of leptospiral infection in the pig population in New Zealand; i.e. that *pomona* and *tarassovi* infection occurs very commonly. The results demonstrated that for an accurate assessment to be made it is necessary to conduct both cultural and serological surveys. The diverse cross-reactions observed in pigs which had been infected with *pomona* might have led one to believe that pigs were infected with many serovars. It seems that the pig's reputation for being a reservoir host of many serovars may have arisen from such serological data, and may therefore be unwarranted. The results also emphasise the need to test sera with an appropriate range of antigens, to

titrate the serum out to the end-points, and to appraise the total agglutination pattern of each serum sample.

The studies of the relationship between kidney infection with *pomona* and both the homologous MA titres and the shedding of leptospires (Chapter V) point to problems when attempting to diagnose current infection. The sensitivity and specificity of the microscopic agglutination test indicate that errors in the diagnosis will occur frequently. The strict interpretation placed on the MA titres to various serovars by various government agencies (Anon., 1977) appears to be unfounded. Treatment with streptomycin would be a more effective means of ensuring that pigs are free of leptospirosis (Stalheim, 1967).

After *tarassovi* infection, the homologous MA titres do not appear to rise as high as those seen after *pomona* infection, and the serological criteria which might apply to the latter are not applicable to *tarassovi*.

In the pig herd which was studied in detail (Chapter VI), *pomona* infection only caused minor losses in production. If it is assumed that the events on this farm are typical of those occurring on other endemically infected properties, *pomona* infection does not appear to affect the general health of young pigs, and as gilts are usually infected well before breeding, losses due to abortions are minimal. Although a balance has been achieved within such herds, the situation is precarious and changes in husbandry procedures could easily and rapidly alter this situation.

The pathogenicity of *tarassovi* infection in pigs, and the pattern of infection in pig herds is very similar to *pomona*. For these reasons, it appears that what has been said about *pomona* is equally applicable to *tarassovi* infection. However, the course of *tarassovi* infection

in pigs is not well documented and this should be the subject of further study. In addition, the interaction between *pomona* and *tarassovi* infection in pigs should be more closely examined.

Other than a cause of abortions, there is no conclusive evidence that leptospirosis has any other effect on fertility. In this study on many occasions pigs which were infected with *pomona* bred normally. However, in view of the contention that leptospirosis results in chronic infertility (Hanson, 1977), there would be merit in comparing the fertility of animals in infected and non-infected herds.

There are other important consequences of this infection in pigs. As cattle (Hellstrom, 1978), sheep (Marshall, R.B. unpublished), and wildlife (Brockie, 1977; Hathaway, 1978) do not appear to be reservoir hosts of *pomona*, *pomona* infection in man and other domestic animals is likely to have come directly or indirectly from pigs. Likewise *tarassovi* infection in man and animals can almost certainly be attributed to the endemic infection in the pig population.

There are many reasons why leptospirosis in pigs should be eradicated or at least controlled, and on a herd basis this would not be a difficult task to accomplish. As there are no known wildlife reservoirs of either *pomona* or *tarassovi* in New Zealand, these infections could be eliminated either *via* antibiotic therapy (Dobson, 1971), or by simply ensuring that the transmission from the older infected pigs to the younger susceptible ones is stopped. The only likely problem is that infection could be reintroduced by replacement animals. A quarantine programme involving treatment with antibiotics and blood testing of new introductions to the herd could be employed to ensure against this event. However, an efficient vaccination programme would be a better alternative.

In contrast to the high levels of immunity occurring in pigs after infection with *pomona*, vaccines and vaccination programmes currently recommended for this serovar do not appear to provide sufficient protection. In this study and others (Hodges *et al.*, 1976) it was noted that vaccinated pigs were still susceptible to infection. These observations were among the reasons for investigating the role of cell mediated immunity (CMI) in the pig in the course of *pomona* infection. It seemed a reasonable proposition that CMI was protective and only developed sufficiently after natural infection. As there is good evidence that both T cells (Pearmain *et al.*, 1963; Mills, 1966; Oppenheim, 1968; Ling and Kay, 1974) and B cells (Bloom 1971; Elfenbein *et al.*, 1972; Luzzati *et al.*, 1973) may be transformed by antigens *in vitro*, and both may produce lymphokines *in vitro* (Papageorgiou *et al.*, 1972; Astor *et al.*, 1973; Rocklin *et al.*, 1974; Mackler *et al.*, 1974; Florentin *et al.*, 1975), these responses cannot be regarded as definitive evidence of CMI *in vivo*. Likewise, although skin testing is an effective means of investigating CMI in some diseases, in many cases it is of little value (W.H.O., 1973). When immediate and Arthus' type responses also occur, as in leptospirosis (see Chapter XII), the appraisal of delayed hypersensitivity reactions becomes especially difficult. With this in mind it was decided to investigate CMI in this study by demonstrating *in vitro* transformation in animals that had been infected with *pomona*, and having done so then to attempt to define the cell type responsible using B cell-depleted lymphocyte cultures. As B cell responses to most antigens are dependent on the concomitant activity of helper T cells (Claman and Chaperon, 1969; Gershon, 1974), the response to antigen of T cell-depleted cultures would not have been a satisfactory method of investigating this matter.

Although a criticism of the lymphocyte transformation experiments might be that in some instances only a small number of animals was tested, there is strong evidence that the antigen reactive cells *in vitro* were B cells and not T cells. This observation is in accord with the conclusions of others (Johnson and Muschel, 1966; Johnson and Harris, 1967; Adler and Faine, 1976), that the essence of the resistance to infection with parasitic leptospires is the ability of the animal to produce specific antibody within a short time of infection. It would also appear that the problems with *pomona* vaccines referred to above are not related to their failure to induce CMI *in vivo*. However, it may well be that the *in vitro* responses which were demonstrated in this study might be a useful parameter against which to judge the efficacy of vaccines.

There is a disagreement as to whether anti-leptospiral immunoglobulin found in urine is the result of leakage from the circulation (Faine, 1963; Lane and Faine, 1963), or is secreted by the mononuclear cells commonly seen in the kidneys of animals in the chronic phase of leptospirosis (Morse *et al.*, 1958; Morrison and Wright, 1976). In the absence of evidence of T cell transformation *in vitro*, it would seem unlikely that these renal infiltrates are the result of delayed hypersensitivity. Rather, it would appear that the lymphocytes are in the main, B cells, whose function is the production of antibody locally. This could be investigated by studying the surface membrane characteristics of the lymphocytes in these lesions, as described by Miller *et al.* (1975).

It would seem appropriate that this thesis should be concluded with a recommendation for continued work on leptospirosis in pigs in New Zealand, with a view to eliminating this important focus of *pomona* and *tarassovi* infection. As discussed in Chapter VIII there

is evidence that widespread infection in pigs has been present in this country for at least 20 years, and during this period disease in man, and losses in livestock production due to infection with these serovars have been considerable. It would be unfortunate if this situation was allowed to continue.

APPENDIX I

THE ASSOCIATION BETWEEN REAL PREVALENCE, APPARENT PREVALENCE, SENSITIVITY AND SPECIFICITY

The relationship between infection and a diagnostic test can be expressed in the following figure:

		DISEASE		
		+	-	
TEST	+	x	P-x	P
	-	A-x	y	A+y-x
		A	T-A	T

By definition sensitivity (Se) $= \frac{x}{A}$ (1)

and specificity (Sp) $= \frac{y}{T-A}$ (2)

Furthermore $y + P - x = T-A$ (3)

thus $y = T-A-P+x$

Substituting in equation (2) for y

$$T-A-P+x = Sp\ (T-A) \quad (4)$$

Likewise substituting in equation (4) for x

$$T-A-P+SeA = Sp(T-A) \quad (5)$$

$$SeA + SpA - A = SpT - T + P$$

$$A = \frac{SpT - T + P}{Se + Sp - 1}$$

$$\frac{A}{T} = \frac{Sp - 1 + P/T}{Se + Sp - 1}$$

But $\frac{A}{T}$ = Real Prevalence, RP

and $\frac{P}{T}$ = Apparent Prevalence, AP

Therefore $RP = \frac{AP + Sp - 1}{Se + Sp - 1}$

APPENDIX II

THE EFFECT OF CULTURE MEDIA ON LYMPHOCYTE TRANSFORMATION IN VITRO

As a media effect on human lymphocyte transformation has been described (Luquetti and Janossy, 1976), it was decided to investigate whether or not porcine lymphocytes were similarly affected by changes in culture medium.

MATERIALS AND METHODS

Three culture media, Eagle's Minimal Essential Medium*, TC199* and a 1:1 mixture of McCoy's and Lieberwitz's media** (M+L) were compared to determine their ability to support the growth of PHA-stimulated (10 µg/ml) porcine blood lymphocytes. Macrocultures were prepared in which $1x10^6$ cells were cultured in screw-topped tubes. All media were supplemented with foetal bovine serum (10%) and antibiotics. Cultures were incubated for 3 days and pulsed with 1 µCi of tritiated thymidine for 16 hours prior to cell harvesting.

RESULTS AND DISCUSSION

There were marked media effects on both the activity of the stimulated and non-stimulated cultures (Table A2.1, Fig. A2.1). The cpm of the stimulated cells in MEM were considerably lower than those either in TC199 or the M+L mixture. However, there was no significant difference between the geometric means of the stimulated cells cultured in TC199 and M+L ($t = 0.45$, 4 $d.f.$). Conversely, the non-stimulated cultures in M+L had a

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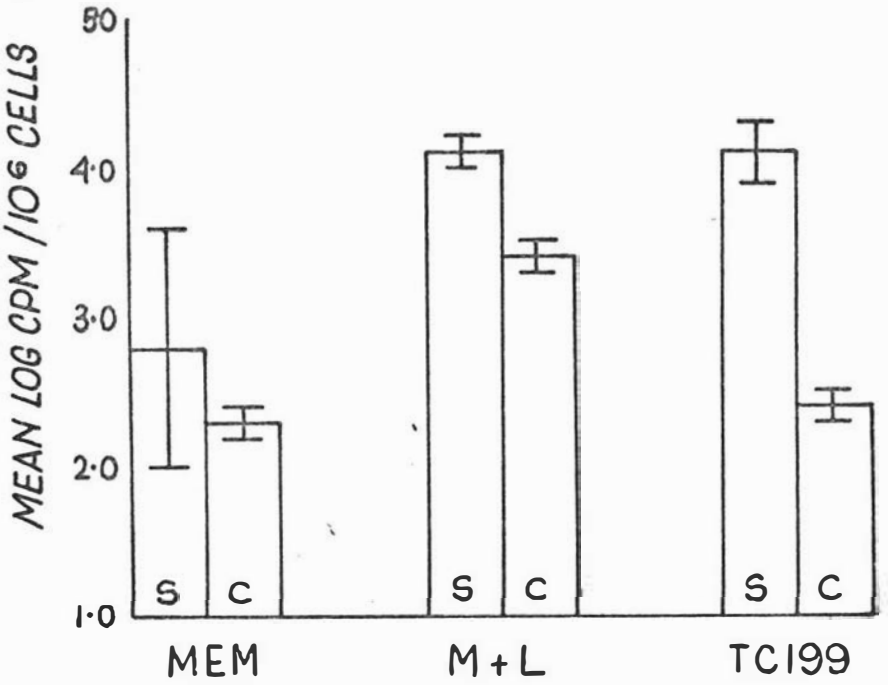
* Wellcome, Beckenham, England

** Flow Laboratories, Irvine, Scotland

significantly higher activity than those in TC199 ($t = 18.90$, 4 d.f. $P < 0.001$), indicating some degree of cell transformation in this medium.

TC199 medium appeared to be the medium of choice and was used in all subsequent transformation experiments.

Fig. A2.1 The effect of culture medium on *in vitro* lymphocyte transformation.



MEM = minimal essential medium
M+L = McCoy and Lieberwitz medium
S = PHA treated
C = non-stimulated

Table A2.1 The effect of medium on the activity of cultures.

Medium*	MEM		M+L		TC199	
PHA**	+	-	+	-	+	-
Mg	2.80	2.28	4.11	3.43	4.05	2.27
Sg	0.82	0.13	0.10	0.08	0.19	0.07

*MEM = Minimal Essential Media
*M+L = McCoy and Lieberwitz, 1:1 mixture
** + = stimulated (10 µg PHA/ml)
\- = non-stimulated control
Mg = geometric mean
Sg = standard deviation of geometric mean

APPENDIX III

THE EFFECT OF PULSE TIME ON THE INCORPORATION OF TRITIATED THYMIDINE

In order to establish the condition under which maximum incorporation of tritiated thymidine would occur, the effect of pulse time of the activity of PHA stimulated cultures was investigated.

MATERIAL AND METHODS

Triplicate porcine lymphocyte cultures containing 10 µg PHA/ml were set up and incubated for 3 days. The cultures were harvested after pulsing with tritiated thymidine for either 30 minutes, 5 hours, 10 hours, 16 hours or 24 hours.

RESULTS AND DISCUSSION

The amount of isotope incorporated by the PHA stimulated cells was at a maximum after a pulse period of 16 hours. The non-stimulated cells retained more isotope as the pulse time increased, with an approximate 50% increase over the 24 hour period (Table A3.1, Fig. A3.1). The geometric mean differences were 0.73 (5 hours), 0.74 (10 hours), 0.94 (17 hours) and 0.89 (24 hours).

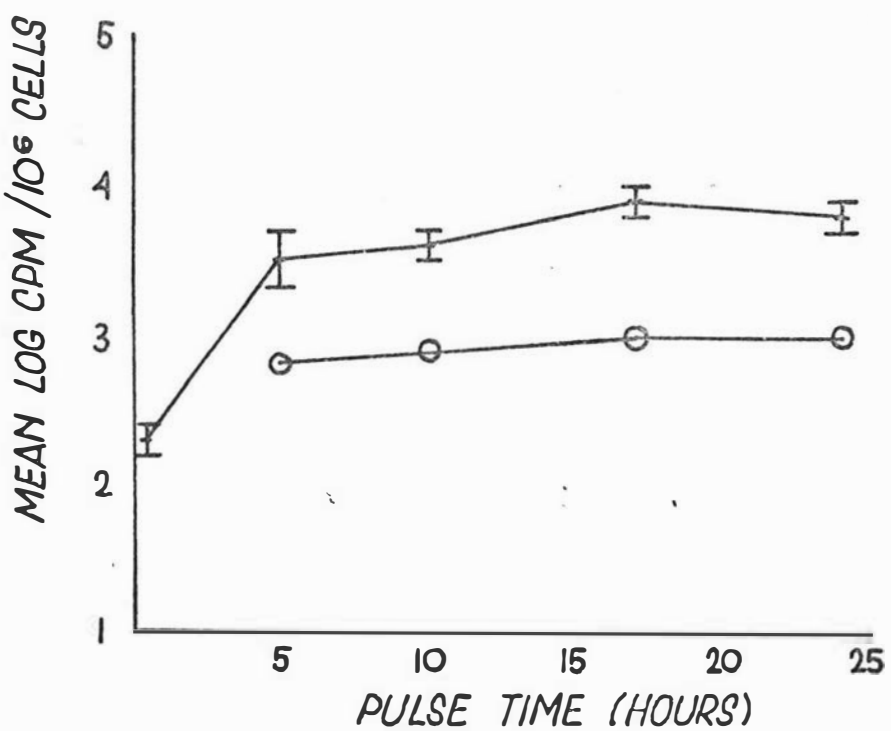
A 16 hour pulse period was used in subsequent transformation experiments.

Table A3.1 The effect of pulse time on the incorporation of tritiated thymidine by stimulated (+PHA) and non-stimulated (-PHA) cell cultures.

PHA	Pulse Period	30 min	5 hrs	10 hrs	16 hrs	24 hrs
+	Mg	2.27	3.49	3.62	3.89	3.84
	Sg	0.11	0.13	0.03	0.02	0.11
-	log cpm*	ND**	2.76	2.88	2.95	2.95

Mg = geometric mean
Sg = standard deviation of geometric mean
* = only 1 non-stimulated culture harvested at each time
** = not done

Fig. A3.1 The effect of pulse time on the uptake of tritiated thymidine by transformed lymphocytes.



\+ = PHA treated

o = non-stimulated

APPENDIX IV

THE EFFECT OF WASH VOLUME ON THE RETENTION OF NON-INCORPORATED ISOTOPE BY FIBREGLASS FILTERS

As part of an investigation to establish the conditions under which the maximum difference between stimulated and non-stimulated cultures would occur, the retention of isotope on fibreglass harvesting filters was examined.

MATERIAL AND METHODS

1 ml volumes of Eagle's Minimal Essential Medium* containing 1 μCi of tritiated thymidine were prepared. Each solution was poured onto a fibreglass filter which was then flushed with 1 to 32 ml of water, 3 samples being processed for each of the wash volumes used. The filters were dried and counted as described in Chapter X.

RESULTS AND DISCUSSION

Isotope was readily flushed from the fibreglass filters, and with wash volumes greater than 10 ml there was only a small decrease in the residual activity (Table A4.1), Fig. A4.1). The reciprocal of the mean cpm was highly correlated to the wash volume ($r = 0.98$), indicating that the amount of isotope left was inversely proportional to the wash volume.

For the remaining experiments a 12 ml wash volume was used to remove non-incorporated isotope from the filter discs.

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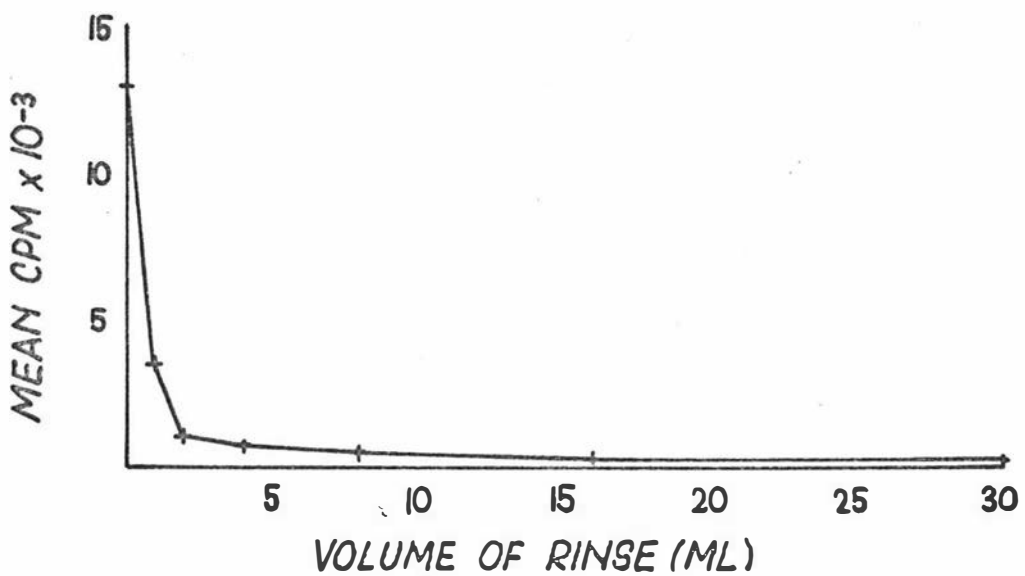
* Wellcome, Beckenham, England

Table A4.1 The retention of isotope on fibreglass filters.

Wash Volume (ml)	0	1	2	4	8	16	32
M (cpm)	13033	3526	1029	696	410	313	163
S	895	285	133	130	75	19	12

M = mean
S = standard deviation
cpm = counts per minute

Fig. A4.1 The effect of wash volume on the retention of tritiated thymidine by fibreglass filters.



APPENDIX V

THE EFFECT OF A TRICHLOROACETIC ACID (TCA) WASH ON THE ACTIVITY OF STIMULATED AND NON-STIMULATED LYMPHOCYTE CULTURES

Trichloroacetic acid (TCA) washes were an essential step in the original methods of measuring the amount of isotope retained by cells. The labelled DNA was precipitated along with a protein carrier, and this material was dissolved in scintillation fluid (Hughes and Caspary, 1970). With the introduction of filtration rather than precipitation to harvest the DNA, the acid wash appears to have been carried over from the original method and accepted as being necessary, in spite of the observation by the developers of multiple-cell harvesters that saline washes alone gave the same results as sequential washes of saline, TCA and methanol (Hartzman *et al.*, 1972). There is evidence, however, that if whole blood cultures are used, a TCA wash may be necessary in order to avoid colour quenching problems during scintillation counting (Sanderson, 1974; Liquetti and Janossy, 1976).

In this present study it was found that in order to wash the isotope-labelled filtrate with TCA, it would have been necessary to modify the multiple-cell harvester available. It was decided, therefore, to investigate whether a TCA wash was an essential requirement in cell harvesting. An experiment using the macroculture system is described.

MATERIALS AND METHODS

Thirty two 1 ml pig lymphocyte cultures were established in culture tubes*, sixteen of these were stimulated with 10 µg PHA, while 0.1 ml of phosphate buffered saline (PBS) was dispensed into the remainder which served as controls. During harvesting the filtered cells from half of the PHA stimulated cultures, and from half of the controls were flushed successively with 4 ml of saline, 4 ml of 1% of TCA and 4 ml of 5% TCA. The remaining cultures were washed with 12 ml of distilled water only. The activities of the stimulated and control cultures were then compared.

RESULTS AND DISCUSSION

The geometric means of the stimulated and non-stimulated cultures are summarised in Table A5.1. There was no significant difference between the activities of the stimulated cultures ($t = 1.04$, *12 d.f.*), nor between the activities of the non-stimulated cultures ($t = 0.67$, *12 d.f.*).

As there were no significant differences between the counts of the filtrates washed with TCA, and those washed with water only, in all subsequent experiments the filtered cells were washed with water only. With regard to colour quenching, the filtrates were always flushed with an excess of water and the quench ratios were always inspected to ensure that this was not a factor causing variation in the cpm's recorded.

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* "Kimax," Owens, Illinois, U.S.A.

Table A5.1 The effect of a TCA wash on the radioactivity of a filtered cell pellet.

PHA		Wash + TCA	Wash Water Only
+	Mg	4.65	4.73
	Sg	0.17	0.11
-	Mg	2.82	2.76
	Sg	0.13	0.17

Mg = geometric mean
Sg = standard deviation of geometric mean

APPENDIX VI

THE EFFECT OF FOETAL BOVINE SERUM ON LYMPHOCYTE TRANSFORMATION

In preliminary experiments which were conducted to establish the optimal conditions of culture for lymphocyte transformation, the non-stimulated controls were occasionally transformed. For this reason the effect of supplementing the culture medium with foetal bovine serum (FBS) was studied.

MATERIALS AND METHODS

Lymphocytes from 2 pigs were prepared and cultured in TC199 medium containing 0%, 5% and 10% FBS. Half of the cultures were stimulated with PHA (1 µg/ml), the other half being non-stimulated controls. The cells were harvested after 24, 48, 96 and 120 hours incubation.

RESULTS AND DISCUSSION

The PHA-stimulated cells cultured in TC199 with FBS incorporated more isotope than did those in medium without FBS. However over the 120 hour incubation period marked time-responses were observed (Table A6.1, Figs A6.1 and A6.2).

The activities of the non-stimulated cultures in medium without FBS declined more or less steadily over the 120 hour period. The counts of the non-stimulated cultures containing FBS increased slightly over the first 48 hours (a difference in geometric means of 0.47 in both animals, equivalent to a stimulation index of 3.0), but then

decreased more rapidly than the cultures without FBS. By 96 hours in all but one case the geometric means of the non-stimulated cultures were similar.

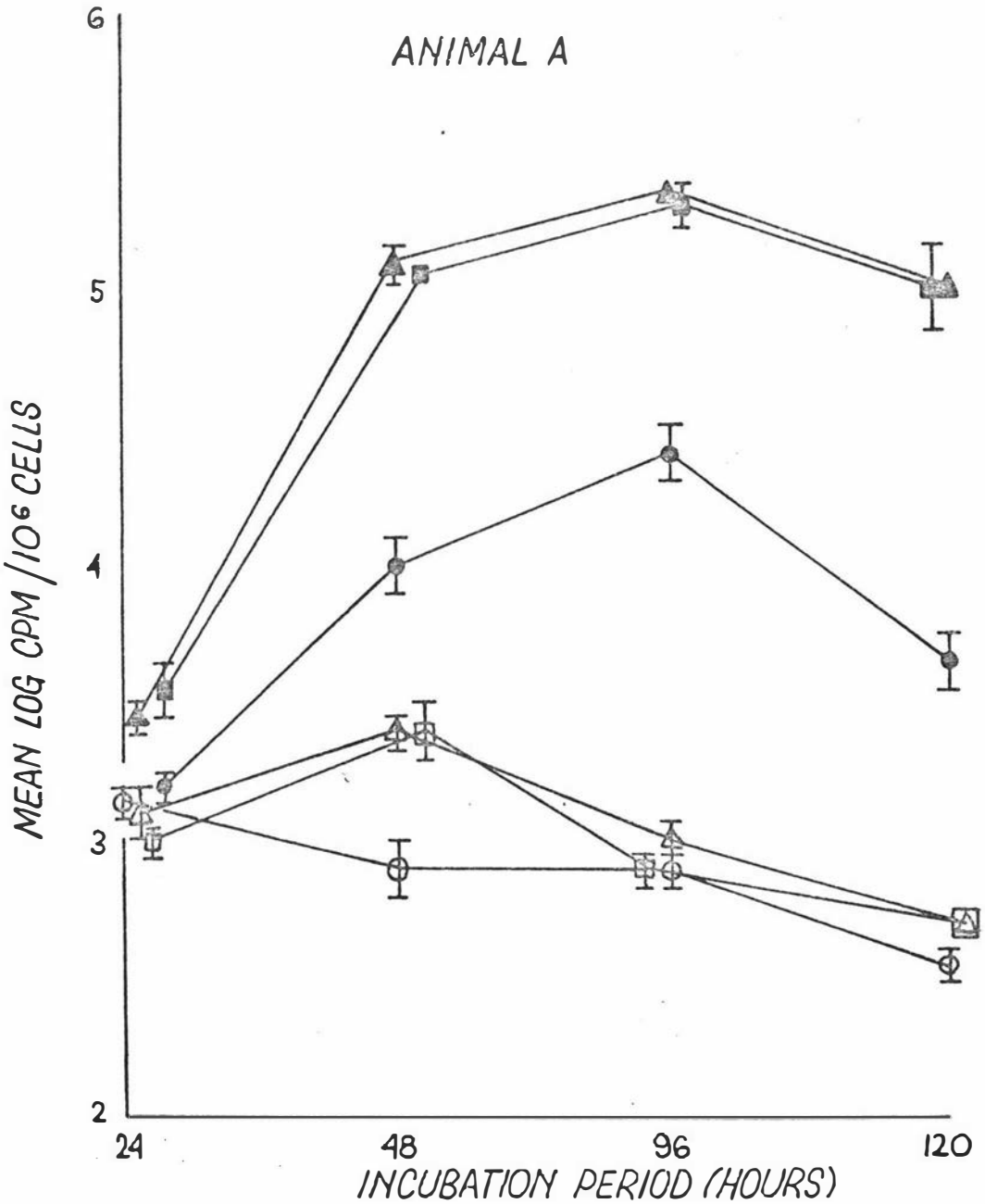
For the remainder of the transformation experiments the culture medium was supplemented with FBS to a final concentration of 5%, and the microcultures were harvested after 96 hours incubation.

Table A6.1 The effect of supplementing culture medium with foetal bovine serum (FBS).

PHA 1 µg/ml	FBS Vol:Vol%		Incubation Period (hours)							
			24		48		96		120	
			A	B	A	B	A	B	A	B
+	10%	Mg	3.55	3.87	5.04	5.08	5.29	5.32	5.00	4.70
		Sg	0.07	0.11	0.00	0.08	0.06	0.08	0.13	0.70
+	5%	Mg	3.44	3.90	5.07	5.30	5.35	5.34	5.02	4.88
		Sg	0.04	0.03	0.05	0.05	0.02	0.05	0.02	0.08
+	0%	Mg	3.18	3.63	3.97	3.75	4.38	4.52	3.64	3.75
		Sg	0.04	0.05	0.09	0.06	0.08	0.05	0.07	0.12
-	10%	Mg	3.00	3.23	3.34	3.38	2.91	3.36	2.69	2.79
		Sg	0.03	0.12	0.07	0.02	0.06	0.07	0.01	0.08
-	5%	Mg	3.12	3.29	3.36	3.45	3.00	3.04	2.69	2.96
		Sg	0.08	0.04	0.04	0.04	0.06	0.03	0.02	0.08
-	0%	Mg	3.13	3.38	2.88	2.95	2.92	3.01	2.55	2.61
		Sg	0.07	0.02	0.09	0.02	0.06	0.07	0.03	0.05

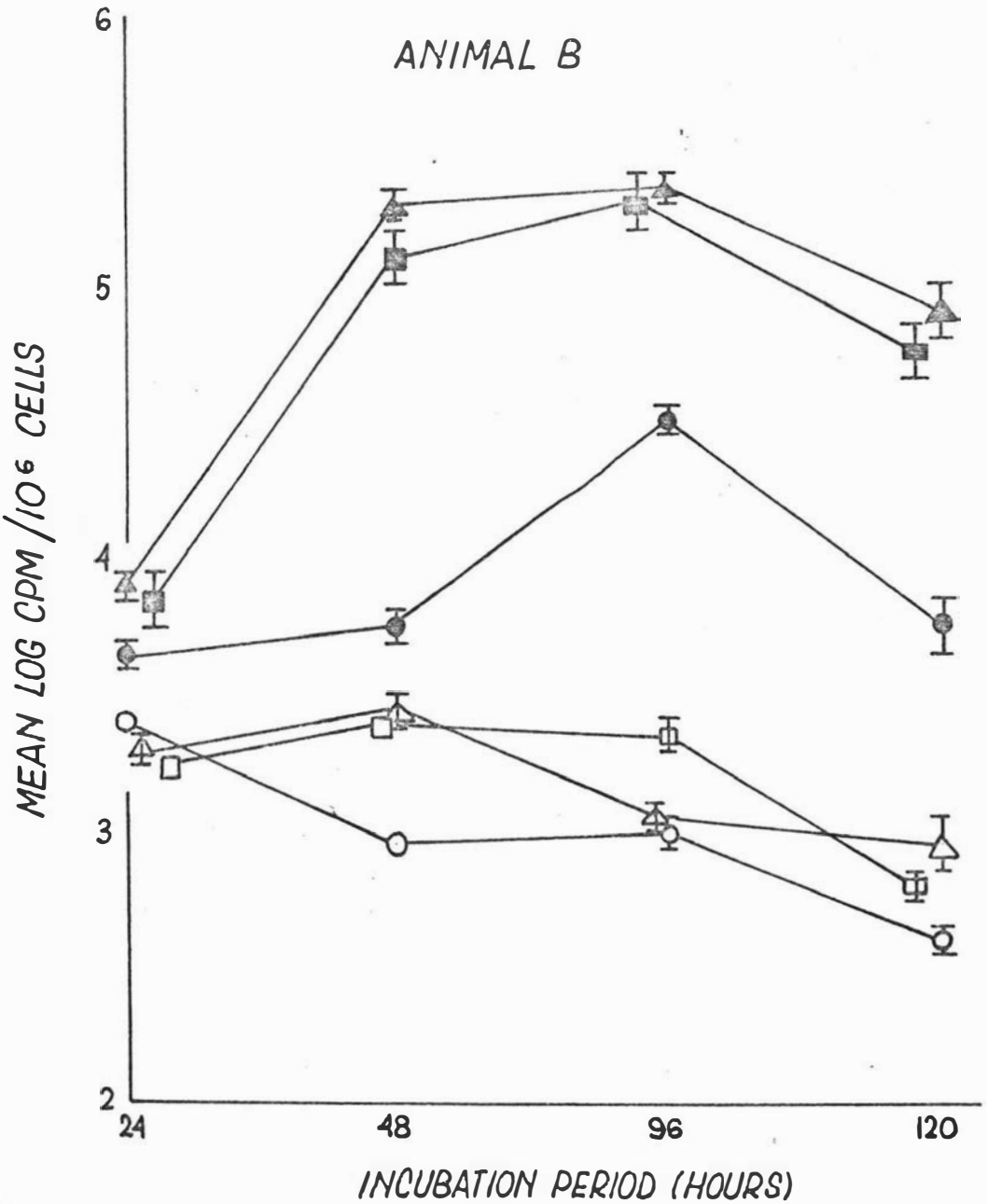
Mg = geometric mean
Sg = standard deviation of the geometric mean
\+ = PHA stimulated cultures
\- = Non-stimulated controls
A = Animal A
B = Animal B

Fig. A6.1 The effect of the concentration of foetal bovine serum (FBS) in culture medium on *in vitro* lymphocyte transformation.



● = 0% FBS, PHA (1 µg/ml) o = 0% FBS non-stimulated
▲ = 5% " " " △ = 5% " "
■ = 10% " " " □ = 10% " "

Fig. A6.2 The effect of the concentration of foetal bovine serum (FBS) in culture medium on *in vitro* lymphocyte transformation.



● = 0% FBS, PHA (1 µg/ml) o = 0% FBS, non-stimulated
▲ = 5% " " " △ = 5% " "
■ = 10% " " " □ = 10% " "

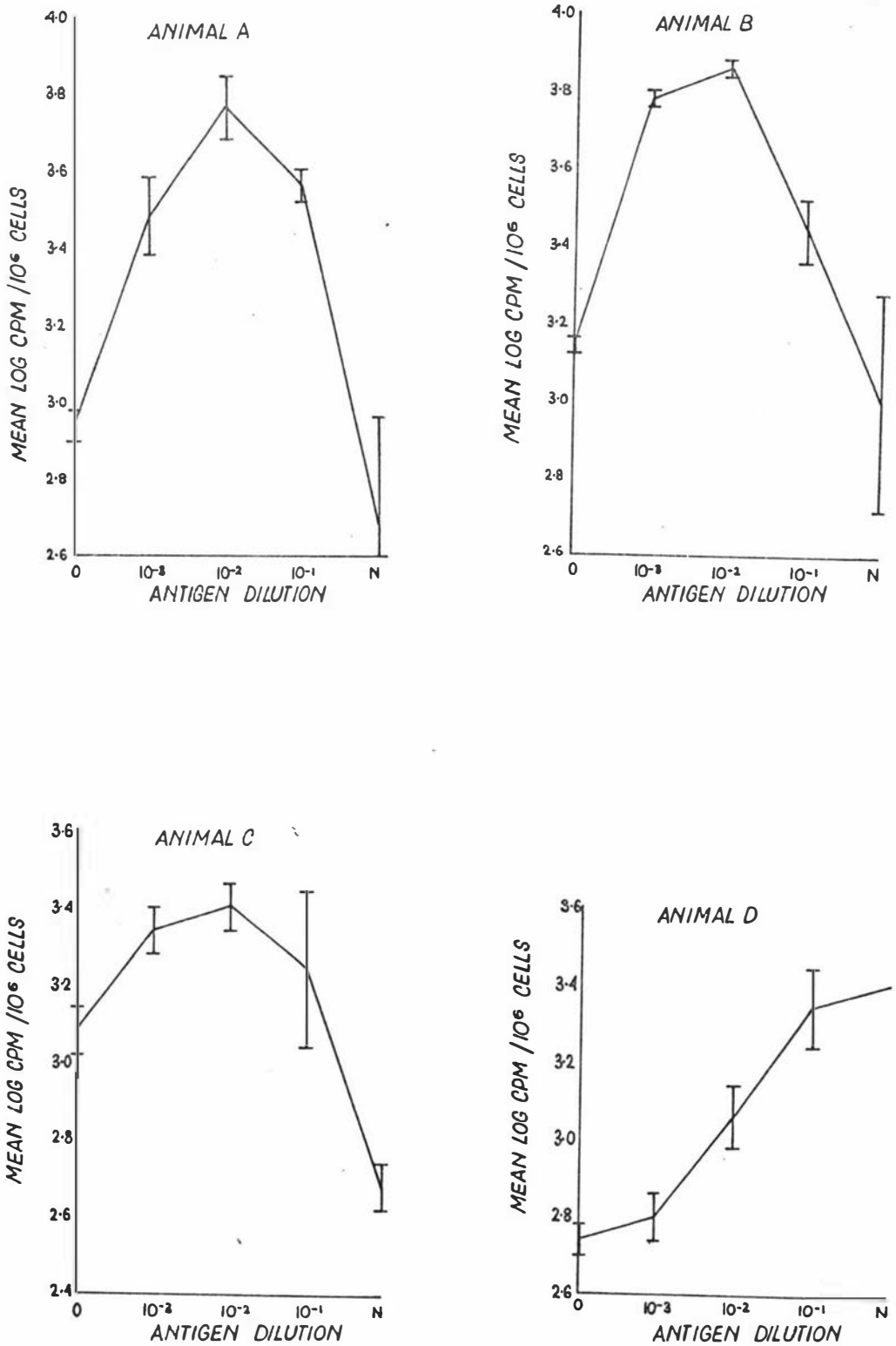
APPENDIX VII

THE *IN VITRO* DOSE-RESPONSES OF LYMPHOCYTES TO THE SONICATED AND SODIUM DEOXYCHOLATE-DERIVED *POMONA* ANTIGEN EXTRACTS

Group I pigs (presumed infected) coded A to I.

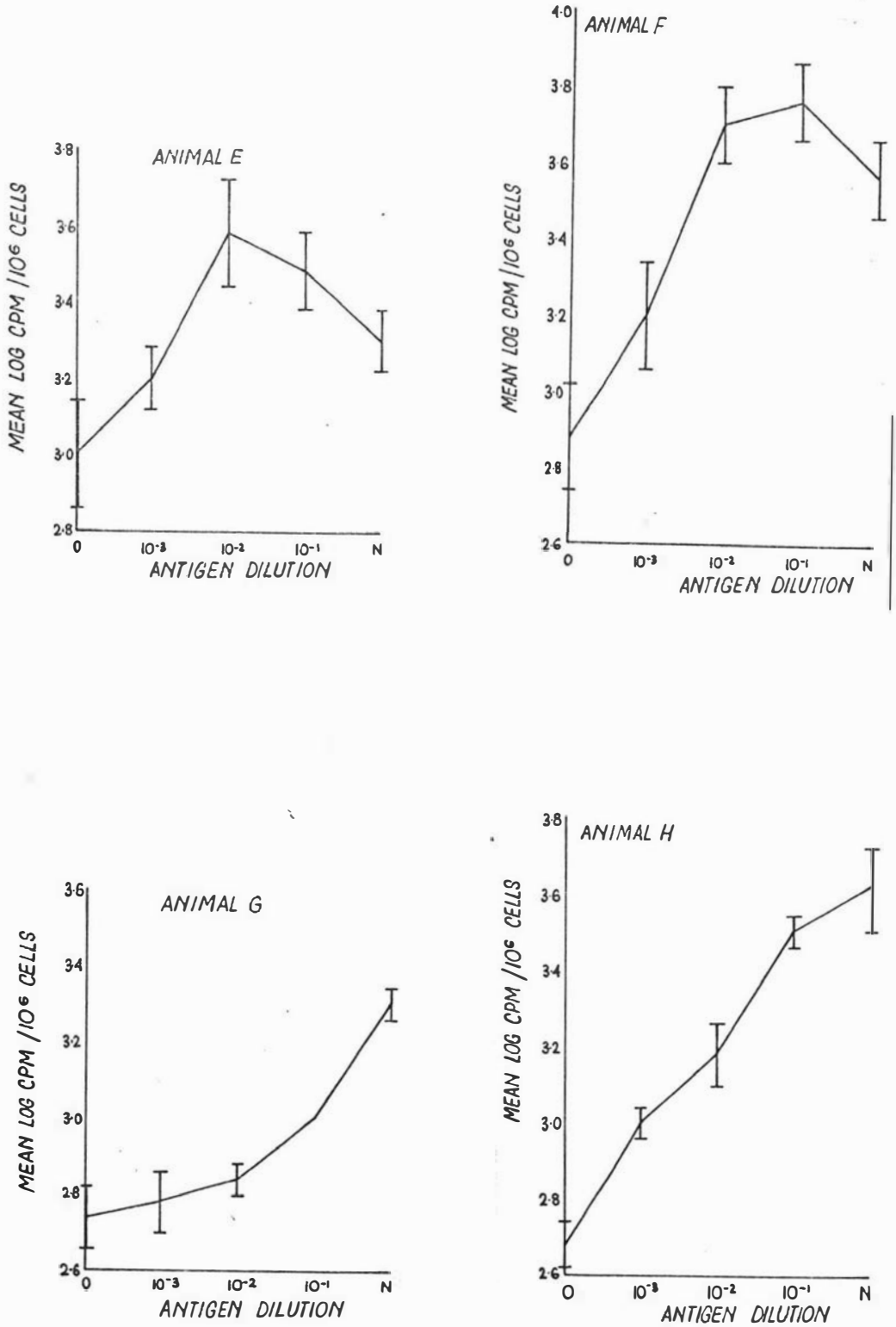
Group II pigs (presumed non-infected) coded J to R (standard deviation plotted only if it was greater than 0.02.)

Fig. A7.1 The *in vitro* lymphocyte responses to the sonicated *pomona* extract.



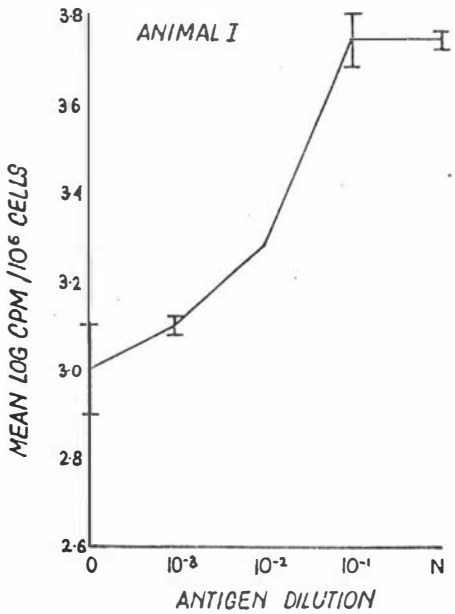
0	=	non-stimulated
10^{-3}	=	1/1000 dilution of antigen
10^{-2}	=	1/100 dilution of antigen
10^{-1}	=	1/10 dilution of antigen
N	=	undiluted antigen

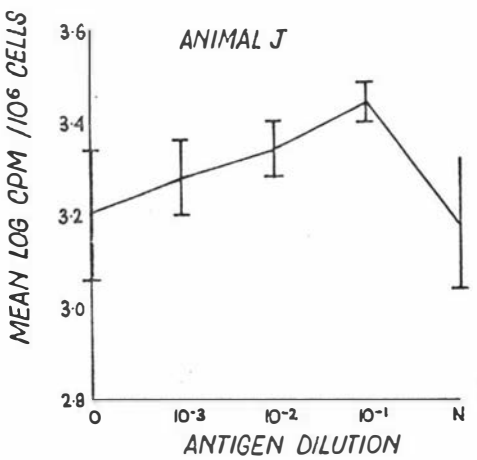
Fig. A7.1 (cont.) The *in vitro* lymphocyte responses to the sonicated *pomona* extract.

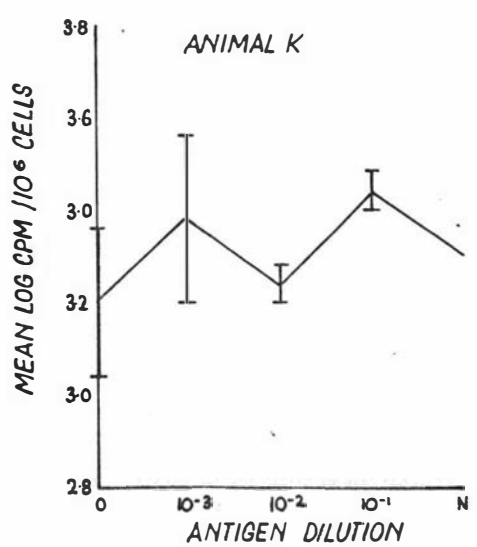


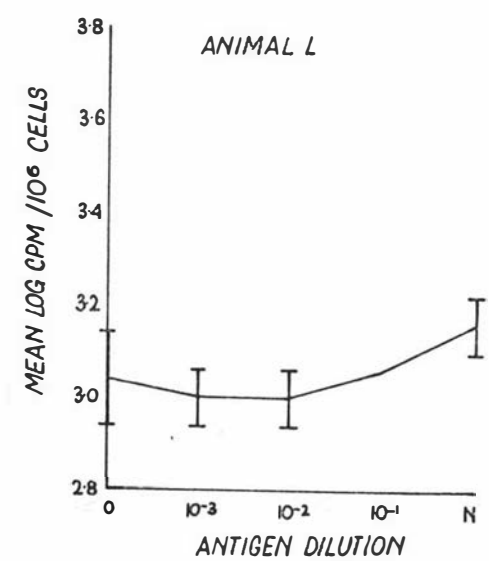
o = non-stimulated
10^{-3} = 1/1000 dilution of antigen
10^{-2} = 1/100 dilution of antigen
10^{-1} = 1/10 dilution of antigen
N = undiluted antigen

Fig. A 7.1 (cont.) The *in vitro* lymphocyte responses to the sonicated *pomona* extract.



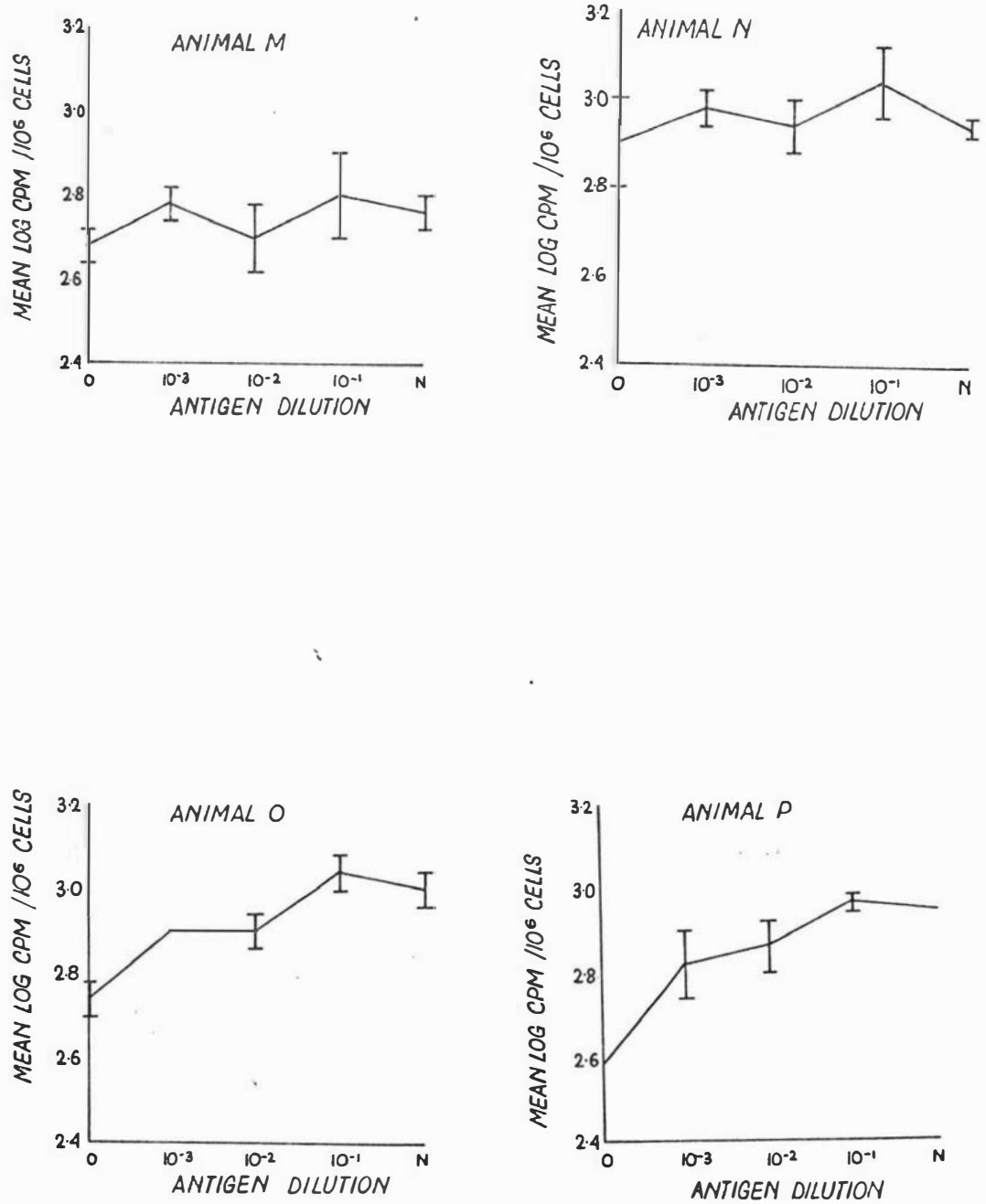






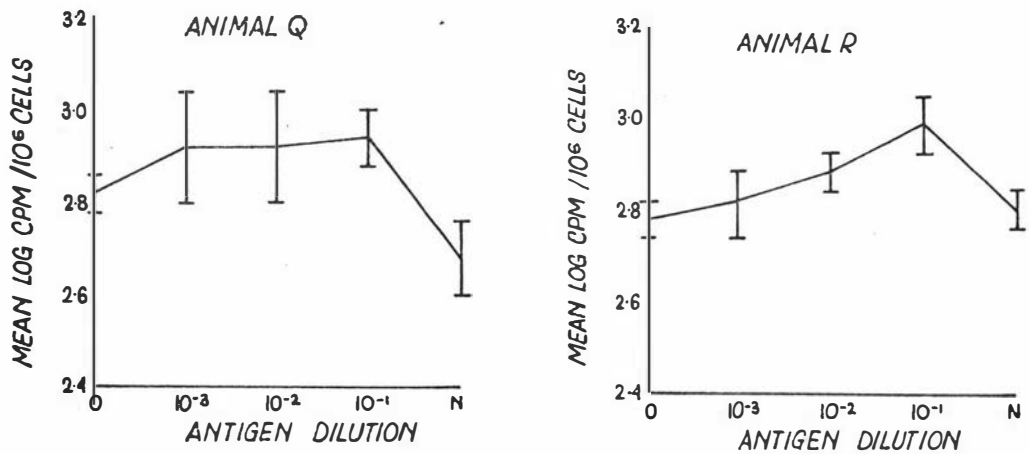
o = non-stimulated
10^{-3} = 1/1000 dilution of antigen
10^{-2} = 1/100 dilution of antigen
10^{-1} = 1/10 dilution of antigen
N = undiluted antigen

Fig. A7.1 (cont.) The *in vitro* lymphocyte responses to the sonicated *pomona* extract.



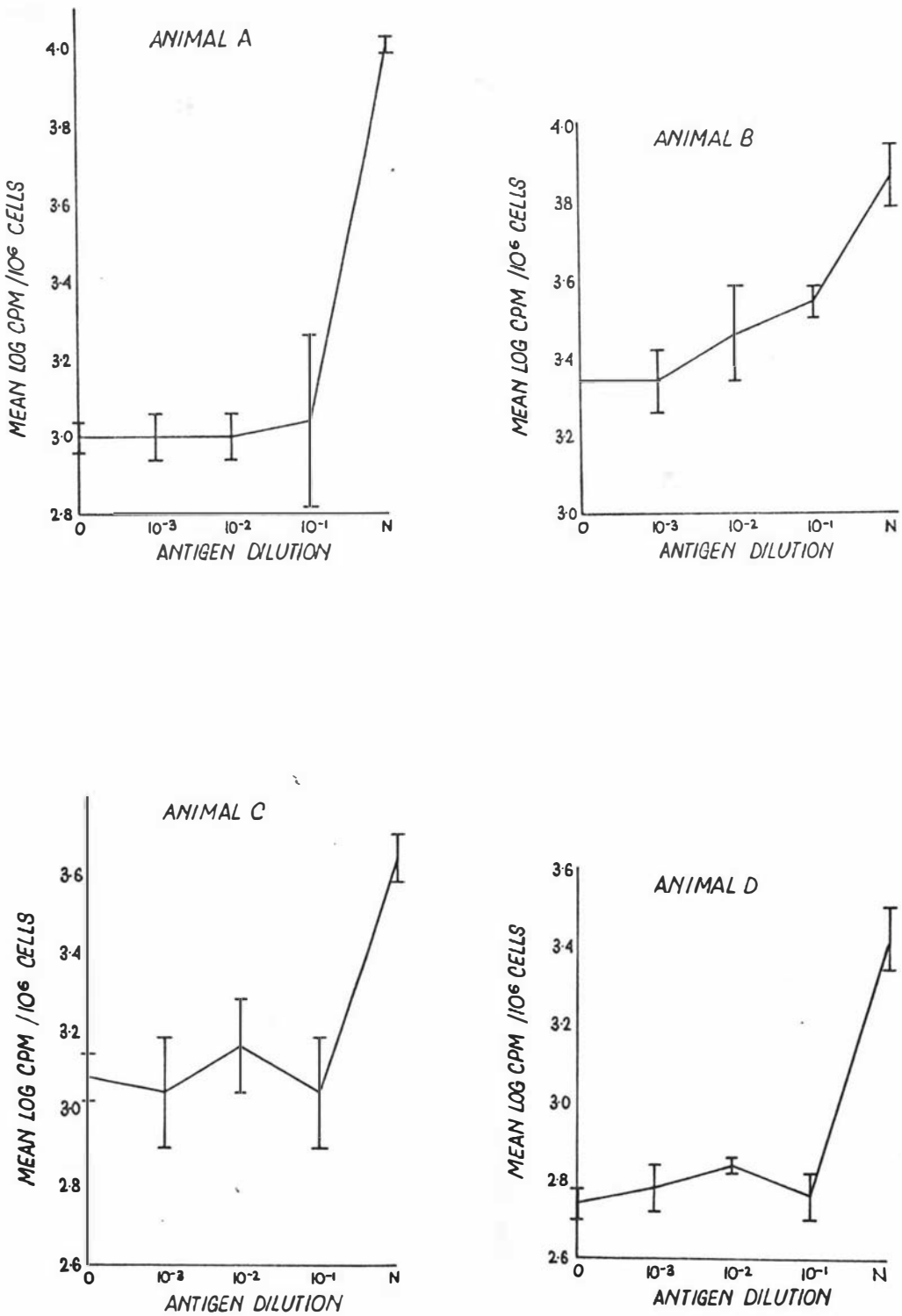
O = non-stimulated
10^{-3} = 1/1000 dilution of antigen
10^{-2} = 1/100 dilution of antigen
10^{-1} = 1/10 dilution of antigen
N = undiluted antigen

Fig. A 7.1 (cont.) The *in vitro* lymphocyte responses to the sonicated *pomona* extract.



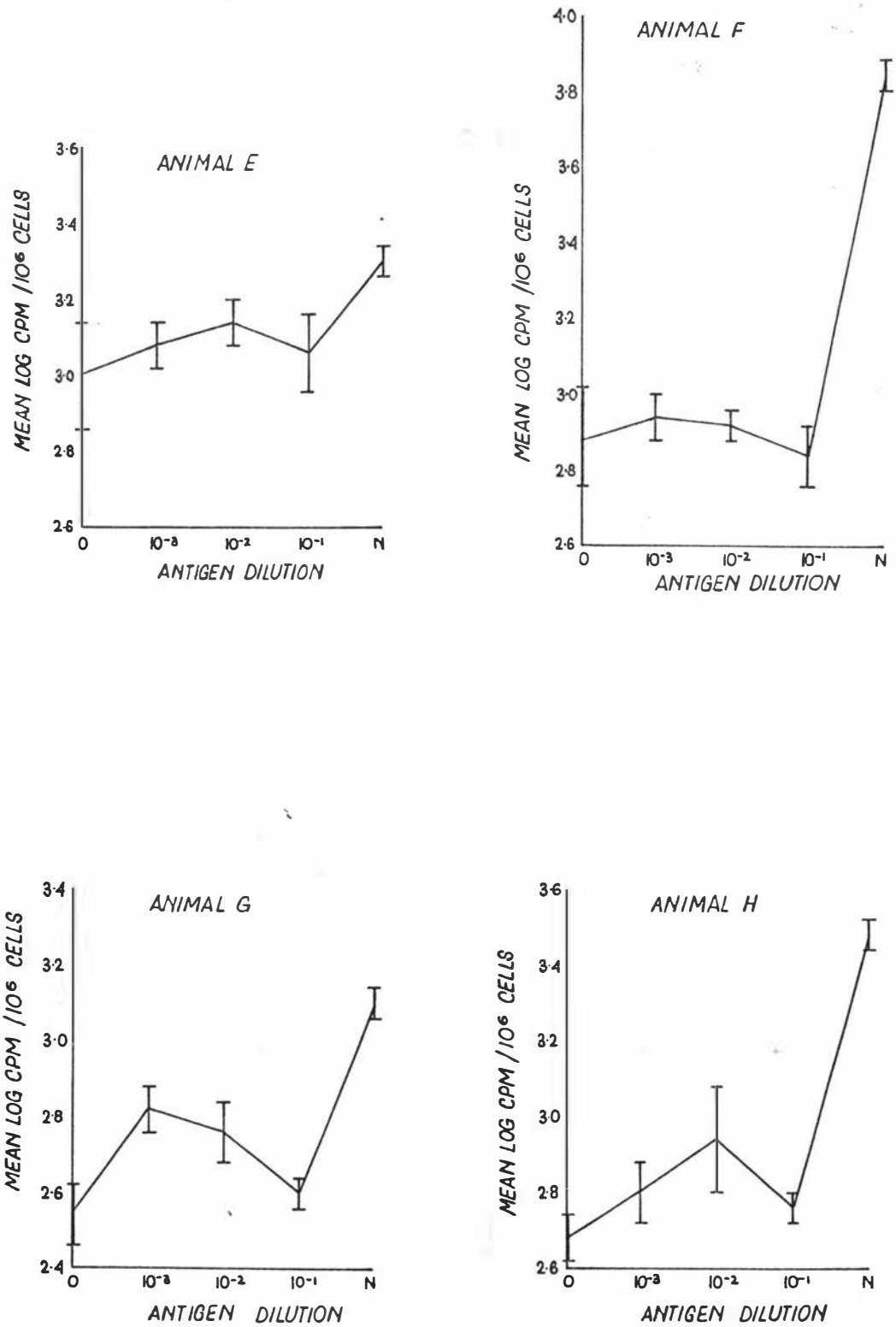
o = non-stimulated
10^{-3} = 1/1000 dilution of antigen
10^{-2} = 1/100 dilution of antigen
10^{-1} = 1/10 dilution of antigen
N = undiluted antigen

Fig. A 7.2 The *in vitro* lymphocyte responses to the sodium deoxycholate-derived *pomona* extract.



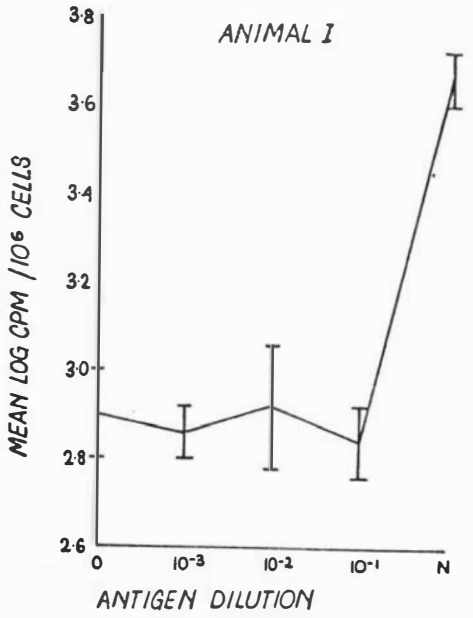
o = non-stimulated
10^{-3} = 1/1000 dilution of antigen
10^{-2} = 1/100 dilution of antigen
10^{-1} = 1/10 dilution of antigen
N = undiluted antigen

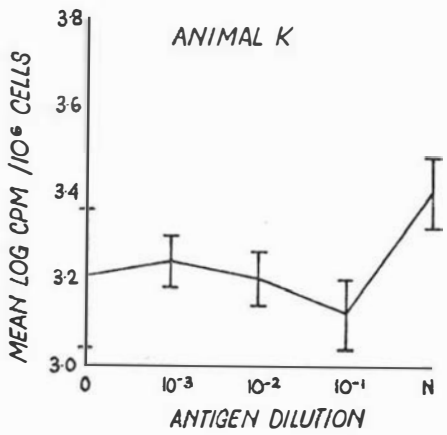
Fig. A 7.2 (cont.) The *in vitro* lymphocyte responses to the sodium deoxycholate-derived *pomona* extract.

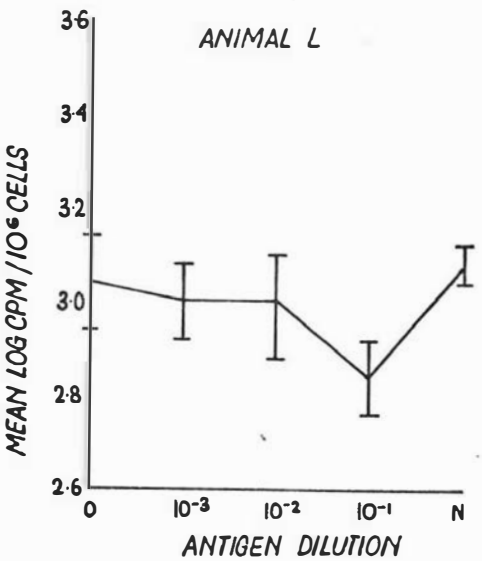


o = non-stimulated
10^{-3} = 1/1000 dilution of antigen
10^{-2} = 1/100 dilution of antigen
10^{-1} = 1/10 dilution of antigen
N = undiluted antigen

Fig. A7.2 (cont.) The *in vitro* lymphocyte responses to the sodium deoxycholate-derived *pomona* extract.







o = non-stimulated
10^{-3} = 1/1000 dilution of antigen
10^{-2} = 1/100 dilution of antigen
10^{-1} = 1/10 dilution of antigen
N = undiluted antigen

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