COMPARISON OF SEROLOGICAL TESTS FOR THE DETECTION OF LEPTOSPIRA ANTIBODIES IN IMMUNISED ANIMALS

By

R RAVIKUMARAN NAIR

THESIS

Submitted in partial fulfilment of the requirement for the degree

Master of Veterinary Science

Faculty of Veterinary and Animal Sciences
Kerala Agricultural University

Department of Microbiology

COLLEGE OF VETERINARY AND ANIMAL SCIENCES
MANNUTHY - TRICHUR

1980

DECLARATION

I hereby declare that this thesis entitled "COMPARISON OF SEROLOGICAL TESTS FOR THE DETECTION OF LEPTOSPIRA ANTIBODIES IN IMMUNISED ANIMALS" is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship, or other similar title, of any other University or Society.

Mannuthy, R.Ravikumaran Nair

CERTIFICATE

Certified that this thesis, entitled "COMPARISON OF SEROLOGICAL TESTS FOR THE DETECTION OF LEPTOSPIRA ANTIBODIES IN IMMUNISED ANIMALS" is a record of research work done independently by Sri.R.Ravikumaran Nair under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to him.

24.9.8

Dr.P.K.Abdulla,B.V.Sc.,M.V.Sc.
(Toronto)
(Chairman, Advisory Board)
Professor,
Department of Microbiology.

Mannuthy,

ACKNOWLEDGEMENT

I wish to record my most sincere gratitude to Dr.P.K.Abdulla, B.V.Sc., M.V.Sc. (Toronto), Professor and Head, Department of Microbiology, under whose guidance this work was carried out.

I express my sincere thanks to the members of the Advisory Committee, Dr.S.Sulochana, Associate Professor, Department of Microbiology, Dr.V.Jayaprakasan, Assistant Professor, Department of Microbiology and Dr.K.M.Ramachandran, Associate Professor, Department of Pathology for their valuable suggestions and continued interest rendered throughout this study.

I am gratefully indebted to Dr.K.T.Punnoose, Associate Professor, Dr.P.C.James, Dr.R.Madhusoodanan-Pillai, Assistant Professors, Dr.M.C.George and Dr. G.Krishnan Nair, Junior Assistant Professors and all the members of the Department of Microbiology.

Grateful acknowledgement is made to Dr.P.G.Nair, Dean, Faculty of Veterinary and Animal Sciences for the facilities provided for the study.

I am grateful to Dr.T.G.Rajagopalan, Professor of Animal Management for his personal interest and many folded help during this work.

My thanks are also due to Dr.A.D.Joy, Officer In-Charge Small Animal Breeding Station for the timely supply of Laboratory animals, and to Dr.Y.Ganeswara Rao and Dr.G.Sukumara Pillai, M.V.Sc Scholars, Department of Parasitology for their esteemed help and assistance.

I am highly thankful to Dr.K.Rajagopal, Amul Dairy, Anand, Gujarat, for providing me the necessary reprints of technical publications for the present study.

Grateful acknowledgement is made to Dr.T.N.Vaishnav, Director of Animal Husbandry, Gujarat State and Government of Gujarat for deputing me for the study.

My thanks are due to Shri.Mohanakumar.K.C for getting the manuscript neatly typed.

R.RAVIKUMARAN NAIR

CONTENTS

....

INTRODUCTION

INTRODUCTION

Leptospirosis is a disease primarily of animals and secondarily of man caused by a group of spirochaetal organisms of the genus Leptospira. It was first recognised as a human disease, and since then been found to occur in dogs, cattle, sheep, swine and many species of wild life. The occurrence of this disease in cattle was first recognised in Russia in 1935. (Michin and Azinow 1935). The economic losses incurred to livestock industry due to this disease are from abortion, decreased milk production and death of calves and rarely adults.

Because of the lack of specific signs for the disease, one method of diagnosing leptospirosis is by detection of antibodies in the serum of infected or convalescent animals. A number of procedures are employed for the detection of leptospiral antibodies in the serum. The usual serological tests are Microscopic agglutination, plate and capillary agglutination, complement fixation, florescent antibody technique and passive haemagglutination.

The Microscopic agglutination test has been widely preferred and used for serodignosis of leptospirosis in most of the laboratories. Although this test is specific to the serotype used, the procedure of the test involves maintenance of large number of live leptospiral serotypes

which is time consuming and offers risk of infection to operators. The plate and capillary tests described by Stoenner (1954) are comparatively easier to perform than the Microscopic agglutination test since formalinised pooled leptospiral antigens are used for the tests and continuous maintenance of live cultures is not required. However the stability and sensitivity of these antigens have been questioned by several workers (Singh 1970; Sandhu and White 1972). The complement fixation test involves more complex procedures thus confining its application only in specialised laboratories. Fluorescent antibody staining technique can also be employed for diagnosis of leptospirosis. But it has been restricted to the generic level because the preparation of serotype conjugates is not perfected sufficiently for definitive diagnostic use. (White and Ristic 1959).

The passive haemagglutination test described by Chang and Mc comb (1954) has been reported to be sensitive and specific to determine the antibody level in the hyperimmune sera prepared in rabbits (Palit and Sharma 1971). However very few reports are available on the comparative evaluation of passive haemagglutination and the standard microscopic agglutination test. A detailed correlative study in this field has not been carried out so far. Therefore the

the following work was undertaken to compare the sensitivity stability and potency of passive haemagglutination test to the established microscopic agglutination test utilising rabbit hyperimmune sera as the source of antibody

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Incidence

Weil (1886) was the first to recognise leptospiral jaundice on the basis of clinical findings. The term "Weil's disease" was first used by Glodschmidt (1887) to designate the form of infectious jaundice described by Weil. Since then Weil's disease was reported from many parts of the world (Jaeger 1892: Chowdry 19031: Cockayne 1932: and Boggs 1915).

Leptospirosis as a disease of cattle was first recognised in Russia by Michin and Azinow (1935). Jungherr (1944) reported the death of three cows due to leptospirosis and he also expressed that cattle, dogs and rodents should be included in the concept of contagious cycle of leptospirosis in U.S.A. Stuard (1949) showed that fourty per cent of the Glasgow house dogs hav been infected with L.canicola. There are also reports of leptospirosis among domestic and wild animals from various parts of the world (Keast et al. 1963: Michna 1967: Watson et al.1976; Salt and Little (1977) Corrigall 1978 and Davindon; Higgins et al. 1980).

Inada et al. (1916) were the first to demonstrate these organisms in the liver tissue of a guineapig which had been infected with blood from a patient suffering from Weil's disease.

Based on serological survey, various workers have shown that leptospirosis is prevalent among sheep, goat, cattle, horses and dogs in different parts of India. Ayyar (1932) reported an outbreak of leptospiral jaundice among Madras hounds. The animals were suffering from anorexia, discharge from nostrils, conjunctivitis, passing faeces with blood and sudden death. The outbreak was caused by L.icterohaemorrhagiae and this forms the first record of leptospirosis in India. During an investigation Adinarayanan et.al (1960) found that leptospirosis due to serotypes L.sejroe, L.medanensis and L.saxkoebing was responsible for a disease condition among cattle population in Uttar Pradesh. But they could not isolate the organisms as the animals were treated with arsenicals and antibiotics. However, Das and Ghose (1961) observed an unusual disease among cattle population in West Bengal, locally known as yellow disease. The clinical symptoms and other findings were suggestive of leptospirosis.

Pargaonker (1957) detected leptospiral infection in 10.6% of the rats from the city of Hyderabad. He could demonstrate the organisms in kidney by direct smear examination stained with Fontana's method or sections stained with Lavaditi's techniques.

The presence of leptospiral infection among sheep and goats in Andhra Pradesh was reported by Pargaonker (1964

He found that out of fifty serum samples from sheep and goats with history of abortions examined, seventeen had antibodies to serotypes of L.pomona, L.hyos and L.hebdomadis groups with serum titres ranging from 300 to 10,000.

Palit and Sharma (1971) collected a total of 283 seru samples from different species of animals from different par of India and screened for antibodies to L.pomona, L.hebdomad L.autumnalis, L.icterohaemorrhagiae, L.canicola and L.poi. They could observe a significantly high titre for L.pomona with buffaloe sera (1:1000 to 1:3000) by the microscopic agglutination test.

Tripathy (1977) observed the presence of leptospiral agglutinins in cattle, sheep and goats. About 44.37%, 17.56 and 50% respectively of 151 cattle, 74 sheep and 138 goat serum samples showed the presence of leptospira agglutinins to antigen pool consisting of L.autumnalis, L.pomona and L.wolffi. Rapid microscopic slide agglutination test was used in this trial. He also found that agglutinins to L.autumnalis were more common in all the animals when testec with individual antigen.

Serological evidence for leptospirosis in horses was made by Rajasekhar et. al.(1977). These workers could obse antibodies to L.icterohaemorrhagiae and L.wolffi in horse sera by MA test.

Adinarayanan and James (1980) reported the isolation of leptospires from farmstock and wildlife in Kerala. A total of 28 strains were recovered comprising 14 isolate from pigs, eight from bandicoots and one each from an aborted bovine foetus, a sheep, a goat, a rat and mangoose, besides the solitary isolate from the sewer effluents in the isolation ward of the local piggery. Among the strains that could be typed were the members of the sero groups, L.autumnalis, L.hebdomadis, L.javanica and L.tarassovi. These are the first isolations from the concerned species in the Indian subcontinent.

Serology

There are various procedures employed for the serodiagnosis of leptospirosis. Meyer et al.(1939) demonstrated agglutination test using porceline plates. He used both live and formalin killed antigen.

Newman (1950) evaluated the laboratory diagnostic procedures such as complement fixation and agglutination tests while studying canine leptospirosis in Michigan area.

Ward (1954) described certain fundamental facts to be taken into account before the serological diagnosis of leptospirosis. He emphasised that the determination of specific serotype cannot be made, only on the basis of serology.

The application of serology for the diagnosis of leptospirosis was described by Stoenner (1955). He compared the serological findings in herds of cattle recently infected with L.pomona with findings in herds with residual serological evidence of past infection. Howrath (1956) evaluated the macroscopic tube agglutination test for the diagnosis of leptospirosis. The evaluation of the test was made by routine testing of 7313 sera samples during a five year period. He described this test as highly specific for the detection of leptospiral infection.

A more simplified method employing only a glass plate and a standard dropper that was supplied with commercial antigen was described by Freeman (1957). Galton et al.(1958 demonstrated a rapid macroscopic slide screening test for the serodiagnosis of leptospirosis. A drop of each of the pooled antigens were mixed with 0.01 ml of undiluted serum and the results were recorded on the basis of macroscopic clumping of antigen.

Boulanger (1958) observed that the results of the plate and tube agglutination tests using formalized antigen were similar to those of agglutination test, but the clinical history of each individual animal and of the herd are necessary for the evaluation of the results. Muraschi (195 described a simple macroscopic tube agglutination test, usi

an antigen composed of polystrene latex particles and formol killed leptospires. Employing a rapid slide agglutination test Watson et al.(1976) have shown that 6.8% of the sera tested from 60 dogs had a significant titre 1:100 or greater.

Microscopic agglutination (MA) test for the serodignosi of leptospirosis was first developed by Schuffner and Mochtar (1927). Stoenner (1955) made a detailed evaluation on the effects of density of antigen, method of preparation of serum dilutions, strain of leptospira, incubation period and age of the culture on the results of agglutination test. He found that antigen of low cell content yielded higher titre than tests with antigen containing a heavy concentration of leptospires. The use of antigen diluted between 1:4 to 1:8 resulted in end points about five fold greater than titres obtai with undiluted antigen. The geometric mean titre of sera examined in ten fold serial dilutions was about four times greater than that of sera examined in two fold serial dilution. In his study six strains of L.pomona showed uniform sensitivity but variations were observed among strains of L.canicola and L.icterohaemorrhagiae. The age of the culture had little effect on the sensitivity of the test. When tests were read after two hours of incubation at 37°C, end points of only three of the 20 sera were one dilution lower, but reactions were not as complete as those observed in tests incubated for four hours at 37°C. Stoenner and Davis (1967)

modified the leptospiral plate antigen. They found that plate tests performed with these antigens were as sensitive as the MA test. Morse and Allen (1956) observed cross agglutination reaction between L.pomona and L.icterohaemorrhagiae in naturally infected cattle. Expert group of WHO (1967) considered MA test as the standard test for the serological diagnosis of leptospirosis in man and animals.

The observations made by Higgins and Cayouette (1978) showed that serological analysis is necessary to differentiate leptospirosis from similar diseases and the serotype involved. These workers tested serum samples from different species of animals, using MA test employing six different sero type namely L.pomona, L.icterohaemorrhagiae, L.hardjo, L.sejroe, L.grippotyphosa and L.canicola.

The effects of vaccination with two vaccines of L.pomona were studied by Ris (1978). He found that the calves vaccinated twice with one commercial L.pomona vaccine had serum titres upto 1:300 and 1:20 by MA and CFT respectively. A second commercial vaccine did not elicit any detectable antibodies, but all the vaccinated as well as unvaccinated calves showed a rapid rise in antibody levels, detectable with all the three tests, after challenge with a high dose of L.pomona.

Turner (1967) reported that the word "lysis" used in the agglutination test is not applicable because the organisms do not get lysed in this test, as believed earlier. Hence the test is called as the microscopic agglutination with live antigen (MAL) and microscopic agglutination test with killed antigen (MAK).

Fluorescent antibody technique was used by many workers for the diagnosis and identification of leptospiral serotypes (Dacres,1961; Coffin and Maestrone,1961 and Stalheim, 1971).

Although Ezell et al. (1952) have shown the association of a strain specific soluble antigen for Leptospira it was Chang and Mccomb (1954) who first isolated an Erythrocyte sensitizing substance (ESS) that can be used for passive haemagglutination (PHA) test for detecting leptospiral antibodies. They successfully isolated the leptospiral ESS from five strain of leptospirae (L.icterohaemorrhagiae, L.canicola, L.pomona, L.hebdomadis and L.autumnalis). The principles and techniques of performing PHA test for the detection of leptospirosis was also laid down by them. They found that the leptospiral ESS was genus specific and not serotype specific as against the observation of Ezell et al. (1952) with the soluble antigen

Cox (1955) described a haemolytic reaction which involved sheep red blood cells sensitized with leptospiral extracts, leptospiral antiserum and complement. Later on Chang et al. (1957) showed that erythrocyte lysis (HL) test can be performed with ESS as antigen for sensitization of the red cells and was more sensitive than the PHA test.

The use of extracts from non pathogenic L.biflexa as antigen in the HL test was described by Cox in 1957. He found that it could be used for HL test for the diagnosis of leptospirosis. The method of standardization and stabilization of these extracts were also described by him. In 1957 Cox et al. evaluated the use of HL test employing L.biflexa extract as antigen in the serodiagnosis of human leptospirosis and suggested that the MA test could be advantagiously replaced with HL procedure.

In 1958, Cox et al. studied the antibody response to different types of antigens of L.biflexa such as whole cell suspension HL antigen, HL antigen adsorbed on to rabbit RBC and leptospiral extract prepared with 50% and 70% ethanol and 50% ether. It was found that the whole cell suspension stimulated both MA and HL antibodies; the HL antigen homologous antibody and cell extract MA antibodies. They also found that the HL antibody system is not operative in leptospiral agglutination, precipitation and complement fixation

reactions. Characterization of the HL antigen revealed that it contains polysaccharide which is probably complexed, in some low molecular weight nitrogenous material. Sharp (1958) observed that an ESS prepared from L.australis reacted with antibodies to 26 pathogenic serotypes. He also showed that sera from 104 patients with proven leptospiral infection had rising antibody titre to the above antigen. Eighty six of 90 patients considered to be infected with leptospirae on the basis of rising agglutination lysis titres exhibited rising titres for ESS antibody in the sensitized erythrocyte lysis (SEL) test. No ESS antibody was detected by the SEL test in paired sera and 182 of 198 patients with non leptospiral pyrexia. In another experiment 99 of 104 proven leptospiral infections, ESS antibody appeared along with or before the HL antibody, but in 9 of 24 patients the ESS antibody disappeared before the HL antibody. High titres of ESS antibody were obtained with SEL tests on sera stored at -20°C upto five years. He also showed that the SEL test on paired sera was useful in the diagnosis of leptospirosis, particularly when the epidemic necessiates the examination of large number of patients.

Palit and Gulasekharam (1973) used a genus specific substance extracted from a water leptospira for sensitization of sheep RBC. They found that such sensitized cells could be preserved by freeze drying without any effect on its serological activity. It was also suggested that these

freeze dried sensitive reagent could be used in the screening test for diagnosis of leptospirosis.

The use of PHA test for the diagnosis of human leptospirosis was evaluated by Sulzer et al.(1975). A soluble alcohol extract of an Andamana strain adsorbed to human 'O' negative erythrocytes and preserved by pyruvic aldehyde fixation was used as the antigen. They showed that the over all sensitivity and specificity of PHA test was 92 and 95 per cent respectively in comparison with 69% and 83% with the presumptive slide agglutination test.

Morris et al.(1977) studied the antibodies involved in PHA test. They found that PHA titres were parallel to the MA titres during the primary response. In the anamnestic response PHA titres rapidly declined and were not detectable by the end of the 24th week of study, although antibodies were detectable by MA test. The PHA titres were usually lower than MA titres but there was little correlation between the two tests. Disulphide bond reduction and gel filtration studies in both experimental as well as field cases indicated that I_gM was the major immunoglobulin class detected by the PHA test while the MA test detected both I_gM and I_gG antibodies.

A comparative study of common serological tests used for the diagnosis of leptospirosis was reported by Abdulla (1964). He observed that the plate and capillary agglutination test using formalized antigen gave results closely comparable to the microscopic agglutination test. The stability, sensitivity and the specificity of the antigens used for the tests were found to be satisfactory.

In a comparative study of macroscopic slide agglutination test with microscopic agglutination test, Solorzano (1967) observed considerable differences between MA test and rapid macroscopic slide agglutination test. There were higher number of positive reactors to L.wolffi live antigen in the MA test than with L.sejroe formalinized antigen. He also found a lower number of reactors to L.canicola and L.icterohaemorrhagiae with the MA test than with the macroscopic agglutination test. These results were considered to be due to the differences in the sensitivity of formalinized antigens of L.sejroe from that of L.canicola and L.icterohaemorrhagiae.

Singh (1970) evaluated the agglutination lysis, plate agglutination and complement fixation tests for their efficacy in screening sheep experimently infected with L.pomona. He found that agglutination lysis and complement fixation tests were superior to plate test and

among these two tests, the lysis test appeared to be preferable because of its sensitivity and simplicity.

Negi et al. (1971) studied the antibody response of cattle to L.pomona. The response was measured by HA, MA and hamster protection tests. They found that calves vaccinated with L.pomona bacterin produced both I_gG and I_gM classes of antibodies that persisted for one year. Although the vaccinated calves did not develop serum microscopic agglutination titres using a starting dilution of 1:100, they had protective antibodies as determined by the hamster protection test. However haemagglutination test was able to detect L.pomona antibodies in serum samples that were negative by routine MA test.

Palit and Sharma (1971) made a comparative study of MA, PHA and CF tests using rabbit and buffaloe calf hyperimmune sera for the detection of leptospiral antibodies. The antigens used for MA, PHA and CF tests were live leptospires, Chang and Mccombs antigen and washed merthiolated leptospires respectively. Three different serotypes of leptospires were employed in this study (L.icterohaemorrhagiae, L.pomona and L.autumnalis). The MA test in rabbit and buffaloe calf immune sera showed high homologous titres and low or negligible heterologous reaction. The PHA and CF test in rabbit serum showed high cross reaction, but the same with buffaloe serum was only negligible.

The use of whey and serum for MA test for the detection of leptospira antibodies was studied by Hussaini(1976). Whey and serum from 123 animals from three herds showed that whey agglutination test is a dependable one, as there was a close relation between the whey and serum agglutination test. He also found that a titre of 1:4 giving 50% agglutination, using whey can be considered as diagnostic titre as it compared well the 1:100 titre for serum.

Dawe et al. (1976) evaluated the MA, PHA test and serum neutralization tests for the detection of leptospiral antibodies. Of the tests done, only the MA and neutralization tests could give any difference in the antibody responses between principal and control groups. The antibody titres determined by neutralization test were higher than those determined by the homologous MA. The results of their study suggested that the PHA test using heterologous antigen is not a valid method for detecting antileptospiral antibodies in swine.

MATERIALS AND METHODS

MATERIALS AND METHODS

(1) Glassware

Glassware used for preparation of medium were chemically cleaned by soaking overnight in sulphuric dichromate cleaning mixture, prepared by adding 35 ml of saturated potassium dichromate to 1000 ml of sulphuric acid. The following day glassware were removed from the above solution and washed well in running tap water and then rinsing in distilled water. They were filled to the neck with 0.1M phosphate buffer (PH 7.2), autoclaved at 120°C for 20 minutes, cooled, the buffer solution discarded; again autoclaved at 120°C for 20 minutes, and then stored at room temperature until used.

(2) Rabbit sera for media preparation.

About 30-40 mls of blood was removed aseptically from healthy rabbits free from leptospiral antibodies, by cardiac puncture and dispensed in 10 ml quantities in one ounce screw capped vials. The clot was allowed to contract at room temperature. When serum was seperated, the vials were inverted several times so that some of the cells were resuspended. The serum cell suspension was seperated and haemolysis was then induced by rapid freezing at -20°C and thawing at 37°C. These haemolysed serum samples were inactivated at 56°C for

30 minutes and were sterilized by filtration. The sterility was tested by plating on blood agar and incubating at 37°C for 24-48 hours. The above sterile, haemolysed serum was stored at -20°C in 10 ml quantities in screw capped vials for further use.

2 (b) Bovine sera

Blood collected from five to six months old bull calves free from leptospiral antibodies were processed in the same way as described for rabbit sera.

(3) Medium

Throughout this work, Korthof's medium was used for the propagation of the antigen. One litre of the medium was prepared as follows:-

Reagents

Peptone (Bactoneopeptone)	..	0.8 gram
Nacl	..	1.4 grams
Na_2 Co_3	..	0.02 gram
Kcl	..	0.04 gram
$Cacl_2$	..	0.04 gram
KH_2 Po_4	..	0.24 gram
Na_2 HPo_4 $2H_2o$	..	0.88 gram
Double distilled water	..	to 1000 ml

After dissolving all the ingredients, the medium was heated in a waterbath for 20 minutes to precipitate the Phosphates and then cooled. The precipitate was removed by filtration through Whatman filter paper No.12. The resulting medium was bottled in 90 ml quantities in screw capped bottles. The medium thus dispensed was autoclaved at 120°C for 15 minutes and stored at room temperature until used. Before use the medium was supplemented with haemolysed rabbit serum/haemolysed bovine serum at 10% level and distributed in three to five ml quantities in one ounce screw capped vials and stored at 4°C.

(4) Antigen

Leptospira serotypes used in these experiments were obtained from Institute of Tropical Medicine, Amsterdam. The serotypes used were Leptospira autumnalis and L.pyrogenes. These two serotypes were maintained in this laboratory in Korthof's/Fletchers medium.

Propagation of antigen for Microscopic agglutination test

Inoculation of the medium was done with a sterile Pasteur pipette using an inoculum of about one tenth volume of the medium. The inoculated tubes were incubated at 30°C in a BOD incubator to ensure maximum growth of the organisms. These cultures were used as antigen

for microscopic agglutination test after 7-10 days of incubation, when it was found to contain 1.8×10^7 organisms per ml. The purity, motility and growth were checked periodically under dark field illumination.

(5) Hyperimmune sera

The method of Palit and Sharma (1971) was followed for raising hyperimmune sera in rabbits. Hyperimmune sera against L.autumnalis and L.pyrogenes were raised seperately in adult rabbits. Before immunization, rabbits were screened for the presence of leptospiral antibodies. Three healthy adult rabbits free from leptospiral antibodies were used for each serotype. The rabbits were given a total of six intravenous injections at five day intervals. The initial dose of 1 ml was raised uniformly at each injection in such a way that the final dose was 6 ml (Table I). At intervals the rabbits were examined culturally by inoculating two drops of blood in Korthof's medium.

At weekly intervals, starting from the date of inoculation, blood samples (about 5 ml each) were collected from the ear vein of each animal till 49th day of inoculation. The sera seperated from these samples were inactivated at 56°C for 30 minutes and stored at -20°C for antibody titration after adding merthiolate to a final

concentration of 1:10,000.

A week following the last injection about 15 ml of blood was collected from each animal by cardiac puncture and allowed to stand overnight at room temperature. The serum seperated was inactivated at 56°C for 30 minutes and stored at -20°C after adding merthiolate to a final concentration of 1:10,000. These serum samples formed the hyperimmune sera for different tests.

(6) Preparation of Antigen-erythrocyte sensitizing substance (ESS) for passive haemagglutination test.

Leptospira autumnalis and L.pyrogenes were grown in litre volumes of Korthof's medium supplemented with 10% rabbit serum/bovine serum. The inoculated media were incubated for seven to ten days at 30°C. The growth and purity were tested before they were removed for concentration. The growth was found satisfactory between the seventh and tenth day of inoculation. For concentration, the culture was dispensed in sterile test tubes in 10 ml quantities and centrifuged at 250g (14000 rpm) for 45 minutes. Immediately after centrifugation the sediments of leptospires were removed and pooled. One litre of leptospiral culture yielded about 500 mgm of wet cell which was resuspended in five ml of phosphate buffered saline PH 7.2 (PBS).

Preparation of Leptospiral ESS

In 5 ml of the leptospiral suspension in PBS, 0.25 gm of pure sodium taurocholate was dissolved and incubated first at 37°C for 2 hours and then at 4°C for 16-20 hours. Following the incubation, 45 ml absolute alcohol was added and the alcohol bile salt-leptospira mixture was mixed thoroughly and kept for flocculation at 4°C for two hours. The precipitate was collected by centrifugation at 1500 rpm for five minutes and resuspended in five ml of the PBS, PH 7.2. The resulting faintly turbid solution constituted the leptospiral ESS.

Sheep red blood cells

Sheep red blood cells were collected in Alsever's solution (Cruickshank 1968) from healthy adult sheep from the slaughter house Kuriachira, Trichur. The RBC were then washed thrice in PBS and preserved at 4°C until used.

(7) Titration of ESS

The optimum dilution of antigen required for maximum sensitization of the red cells for the PHA test was determined by the conventional checker board titration described by Palit and Gulasekharam (1973). A serial two fold dilutions of the ESS preparation were made in PBS, starting with an initial dilution of 1:2. To one ml of the diluted ESS, 0.1 ml of the 10% sheep RBC was added. The ESS erythrocytes mixture was kept at 37°C in water bath

for one hour. The erythrocytes were kept dispersed in the diluted ESS by gently shaking the mixture in every 15 minutes. The sensitized erythrocytes thus prepared were washed twice in PBS by centrifugation at 1500 rpm for five minutes, resuspended in one ml of the same diluent. Positive and negative sera were diluted serially in a perspex haemagglutination plate using PBS and to each dilution sensitized erythrocytes were added at the rate of 0.1 ml to 0.4 ml of the serum dilutions. Then the plate was kept at 30°C for 16-20 hours. Agglutination was indicated by clearly visible aggregates or agglutinates (Table II)

The optimum dilution of the antigen required for sensitization was the highest dilution of the antigen which when coated on to the cells showed haemagglutination with the highest dilution of the homologous antiserum. In the final test the sheep RBC was sensitized with the predetermined optimum dilution of the antigen and then reacted with the test serum samples for determination of antibody titres. The highest dilution of serum showing haemagglutination was considered as the titre of the serum.

(8) Sensitization of sheep red cells for PHA test.

Ten per cent suspension of RBC in PBS was used for sensitization. To one ml of the diluted antigen, 0.1 ml of the red cell suspension was added and the mixture was allowed to react at 37°C for one hour with intermittent shaking. These cells were washed twice in PBS and resuspended in the same buffer to get a final concentration of one per cent cells. This constituted the sensitized sheep RBC for PHA tests.

Test procedure for microscopic agglutination test.

Each serum sample was diluted with PBS in two fold dilutions starting with an initial dilution of 1:50 in perspex haemagglutination plates. With a pipette from each diluted serum sample, a drop was transferred to another perspex plate. To the transferred drop of diluted serum, one drop of antigen was added thus doubling the dilution of the serum. In all the test procedures, positive and negative controls were kept. After mixing by gentle shaking the plates were incubated at 30°C for 3-4 hours and then a loopful of the mixture was examined under dark field microscope for evidence of agglutination. The end point was taken as the highest final dilution of the serum, in which about 50% or more of the leptospires were agglutinated.

Procedure for passive haemagglutination.

Serial two fold dilutions of the antiserum were made in PBS, starting with an initial dilution of 1:5 in 'U' bottom perspex plates. To each well containing four columes diluted serum, one volume of one per cent sensitized sheep RBC was added. Appropriate controls were also set. The antigen antibody mixture was incubated at 37°C for one hour, then a room temperature for overnight before the results were recorded. The highest dilution of serum showing haemagglutination was considered as the end titre of the serum.

Stability and potency of antigen.

The ESS antigens were stored at -20°C for three months and again tested against the same serum samples mentioned above in order to evaluate the stability and potency of the antigens.

RESULTS

RESULTS

Throughout this work Korthof's medium supplemented with 10% rabbit serum was used for the propagation of antigen. After inoculation, the cultures were incubated at 30°C. Seven to ten days old cultures were found to contain 1.8×10^7 organisms per ml which was used for microscopic agglutination test. (Figures 1 &2). The organisms were also grown in Korthof's medium supplemented with bovine serum but the growth obtained was not satisfactory to the required level, so that, medium enriched with rabbit serum was used for the preparation of antigens for different tests.

Leptospiraemia could not be detected in any of the inoculated rabbits, when blood was cultured at various intervals of the experimental period. The optimum dilution of the antigen required for maximum sensitization of the red cells for PHA test as determined by checker board titration was found to be 1:2 for both the serotypes employed in this study.

The results of microscopic agglutination and passive haemagglutination tests conducted on the sera collected from six rabbits at various intervals are summarised in tables III and IV. None of the serum

samples collected before the experiment revealed leptospiral antibodies against the two serotypes tested. On the seventh day of inoculation, the samples collected from all the three animals inoculated with L.autumnalis had a uniform titre of 1:400 whereas animals inoculated with L.pyrogenes showed only a low titre of 1:100 by MA test. However both the groups had the same antibody titre of 1:5 by passive haemagglutination test. By the fourteenth day there was a marked increase in the antibody titre both by microscopic and passive haemagglutination tests. Sera from all the three rabbits inoculat with L.autumnalis had a titre of 1:800 and 1:10 by MA and PHA tests respectively. On the other hand one of the three rabbits immunized with L.pyrogenes had a higher MA titre of 1:400 compared to the other two animals where the titre obtained was only 1:200. However all the three animals showed a PHA titre of 1:10. The rise of antibody titre in the serum samples collected subsequently were uniform. The maximum titre of 1:1280 for L.autumnalis was attained on day 21 and it remained the same until the 35th day except in one animal where a decrease in the titre was noticed (1:6400). In comparison, maximum PHA titre (1:160) was reached only on

DISCUSSION

DISCUSSION

Throughout this work Korthof's medium supplemented with 10% rabbit serum was used for the propagation of the antigen. Haemolysed bovine serum was also tried as an enrichment in the medium which was found to be unsuitable for obtaining maximum growth in shorter period when compared to rabbit serum enrichment. The added advantages of rabbit serum to support better growth of leptospira could be due to high content of Vitamin B12 and comparatively low agglutinin content (Turner 1970). Addition of small amounts of haemoglobin solution to the medium has been reported to improve substantially the growth of leptospirae. (Wolff 1954; Czekalowski; 1954 and Faine 1959). During the present study also haemolysed rabbit serum was constantly used to have better growth in the medium. This was achieved by induced haemolysis of RBCs by frequent freezing and thawing of serum containing blood. By about seven days of incubation both the serotypes of leptospirae had produced good growth, (1.8×10^7 organisms per ml) which was used for MA test.

Leptospira autumnalis and L.pyrogenes were selected for preparation of antisera during this study.

L.autumnalis has been reported to be an important serotype prevalent in different parts of India (Tripathy 1977). He has observed the presence of leptospira agglutinins in cattle, sheep and goats. About 44.37%, 17.56% and 50% respectively of 151 cattle, 74 sheep and 138 goat serum samples showed the presence of leptospira agglutinins to antigen prepared against L.autumnalis, L.pomona and L.wolffi. He also observed that agglutinins to L.autumnalis were more common in all the animals, when tested with individual antigen. Adinarayanan and James (1980) reported the isolation of the serotypes L.autumnalis, L.hebdomadis, L.javanica and L.tarassovi from farm-stock and wild life in Kerala. In both these reports L.autumnalis was found to be the predominant serotype occurring in our country. Although reports on the occurrence of L.pyrogenes in our country are very few, this serotype was also included in this work for a comparative evaluation of serological reactions.

The optimum dilution of the antigen required for maximum sensitization of the sheep blood cells for PHA test as determined by checker board titration described by Palit and Gulasekharam (1973) was found to be 1:2

for the two serotypes employed in this study.

The microscopic agglutination test has been widely used for serodiagnosis in most of the laboratories, though it is having certain disadvantages like maintanance of large number of serotypes and the risk of infection to the operators while handling cultures particularly of pathogenic leptospires. The PHA test has been reported to be sensitive and specific to determine the antibody level in the sera of infected animals. This test overcomes the disadvantages of MA test. It is easy to perform and in addition ensures the use of nonviable antigenic fraction of a cell. Moreover once prepared, it can be stored for longer periods without loosing the antigenicity.

For PHA test the antigen was extracted by the technique described by Chang and McComb (1954) and Palit and Gulasekharam (1973). It has been established that oral sensitivity and specificity of PHA test was 92 and 95% respectively in comparison with 69 and 83% with slide agglutination test (Sulzer et al. 1975). Morris et al. (1977) have observed that the PHA test had always a low titre when compared to MA titres. In the present study also PHA titres were far below when compared to MA titres. A 1:400 reaction by MA titre corresponded to 1:5 of PHA

in the case of L.autumnalis although 1:100 by MA test had a corresponding titre of 1:5 by PHA in the case of L.pyrogenes. At the same time a gradual rise of antibody was noticed by PHA test till 35th day by both the serotypes, the titres being 1:160 in the case of L.autumnalis and 1:80 in the case of L.pyrogenes. At the same time a peak titre was noticed by both the serotypes on 21st day by MA test which remained stationary till 35th day and showed a tendency to decline thereafter. The serum collected from two rabbits showed the maximum antibody level even on 42nd day by PHA test (1:160) whereas the MA titres showed a decline from 1:12800 to 1:6400 in the case of L.autumnalis. However L.pyrogenes infected animals had a uniform fall in the antibody level from 35th day, the titres falling to 1:1600 by MA test and 1:40 by PHA test on 42nd day. The results obtained tend to show that PHA titres after reaching the maximum level remained detectable for longer period when compared to MA titres. Turner (1968) in his studies on the level of antibodies in the leptospira infected animals have observed that PHA antibody titres could be detected much earlier to MA titres. In the present study the serum collected from the inoculated animals was examined

on 7th day for agglutinins. The PHA titres during this period were found to be 1:5 in both the group of rabbits. The earlier appearence of agglutinins could not be ascertained since the first bleeding was conducted on 7th day of inoculation. On the whole the results obtained by the two tests were closely comparable. Similar observations have also been made by Morris et al. (1977) in their studies on antibodies involved in PHA titres.

The stability and potency of the PHA antigens were found to be unaltered even after keeping at -20°C for a period of three months. On retesting the stored antigens gave similar results indicating that the potency and stability are retained without any deterioration. The sera samples which were collected periodically were also preserved at -20°C for a period of three months. There was no drop in antibody titre even when the samples were tested repeatedly at varying periods.

Palit and Gulasekharam (1973) have reported the use of sheep red cells sensitized with extracts from Leptospira which was freeze dried and used subsequently for performing haemagglutination test to detect

leptospiral antibodies. The process of freeze drying has not altered the sensitivity of the reagent. The result of the present work indicate that the PHA antigens and serum can be conveniently stored at -20°C keeping its sensitivity for a period of at least three months.

SUMMARY

SUMMARY

A comparative study was carried out on the sensitivity of MA and PHA tests using hyperimmune sera prepared in rabbits. For MA test antigen was prepared by growing Leptospira serotypes, L.autumnalis and L.pyrogenes in Korthof's medium enriched with 10% haemolysed rabbit serum for a period of seven to ten days. Seven to ten day old cultures concentrated by centrifugation at 14,000 rpm for 45 minutes was used for antigen extraction for PHA test. The ethanol extracted antigen (ESS) coated on to sheep RBC was used for PHA test.

Hyperimmune sera for the above two tests were prepared by immunizing rabbits by multiple intravenous injections. Antibody titres were determined in all serum samples collected at weekly intervals from seven days following the first inoculation upto 49th day. Both the tests employed were found to be sensitive to determine the antibody level in the hyperimmune sera. Although titres obtained by PHA test were far below the MA test, the results obtained by both the tests were closely comparable. In general an MA titre of 1:12800 attained by L.autumnalis on 35th day of inoculation corresponded to 1:160 by PHA test. Similarly a titre of 1:3200 by MA test had a

corresponding reaction of 1:80 by PHA in the case of L.pyrogenes. It was also observed that the PHA titres after reaching the maximum level remained detectable for longer period when compared to MA titres.

The erythrocyte sensitizing substance prepared from both the serotypes and the sera samples collected periodically from immunized rabbits were preserved at -20°C at varying length of time, upto three months. There was no deterioration in the stability or potency of ESS or sera on storage.

REFERENCES

REFERENCES

Abdulla, P.K (1964). A comparison of serological tests commonly used for diagnosis of leptospirosis. Kerala Vet. 3:1-7.

Adinarayanan,N., Jain, N.C. and Chandiramani,N.K.(1960). Studies on leptospirosis among bovines in India (A priliminary report on the occurrence in cattle based on serological evidence). Indian Vet.J., 37: 251-254.

Adinarayanan,N. and James, P.C.(1980). Studies on leptospirosis in farmstock and wild life in Kerala. Isolation of leptospira from divergent classes of "Animal Hosts" with an outline of cultural procedures. Indian Vet.J.57: 496-500.

Alston, J.M. and Broom,J.C.(1958). Leptospirosis in man and animals. E&S Livingstone Ltd, Edinburg and London.

Ayyar, V.K.(1932). A note on an outbreak of leptospiral jaundice among the Madras hounds. Indian J.Vet. 2: 160-169.

Boggs, T.R.(1915), Infectious jaundice. A system of Medicine edited by W.Osler and T.Mccare, 2nd edition; 1:1014 cited by Alston, J.M. and Broom, M.C. (1958).

Boulanger,P. (1958) Agglutination test on leptospirosis in cattle. Canada.J.Publi.Hlth.49:142-147.

Chang, R.S.M., and McComb,D.E. (1954). Erythrocyte sensitizing substances from five strains of leptospirae. Am.J.Trop.Med.Hyg., 3: 481-489.

Chang,R.S., Smith,D.J.W, McComb,D.E. Sharp,C.F. and Tong, J.I. (1957). The use of erythrocytes sensitizing substance in the diagnosis of leptospirosis II. The sensitized erythrocyte lysis test. Am.J.Trop.Med and Hyg.,6: 101-107.

Cockayne, E.A.(1932). Catarrhal jaundice, sporadic and epidemic,and its relation to acute yellow atrophy of the liver. Quart.J.Med.6 (1): cited by Alston, J.M. and Broom, J.C.(1958).

Chowdry, A.K. (1903). Jaundice at Portblair, Andaman Islands.Ind.Med.Gaz. 38:409.

Coffin, D.L. and Maestrone,G. (1962). Detection of Leptospires by fluorescent antibody. Am.J.Vet.Res., 23: 159-164.

Corrigall,W. (1978). Naturally occurring leptospirosis in red deer. Vet.Rec.,103 (4): 75-76.

Cox, C.D. (1955), Haemolysis of sheep erythrocytes sensitized with leptospiral extracts. Proc.Soc.Exp. Biol. and Med., 90: 610-615.

Cox,C.D. (1957). Standardization and stabilization of an extract from Leptospira biflexa and its use in the haemolytic test for leptospirosis. J. Infect.Dis., 101: 203-209.

Cox, C.D, Alexander, A.D. and Murphy,L.C. (1957) Evaluation of the haemolytic test in the serodiagnosis of human leptospirosis. J.Infect.Dis., 101: 210-218.

Cox,C.D, Stover,R.C. and Treicks, R.W. (1958), Serologic studies on haemolytic antigen from Leptospira. Proc.Soc.Exp.Biol. and Med.,98: 265-269.

Czekalowski, J.W, McLeod, J.W and Rodican,J. (1954) observations on the nutrition of leptospirae based on the development of Dinger's Phenomenon. J.Gen.Microbiol., 10: 199-206.

Dacres, W.G. (1961). Fluorescin-labelled antibody technique for identification of leptospiral serotypes. Am.J.Vet.Res.,22: 570-572.

Das, M.S. and Ghose,G.K. (1961). Some observations on an unusual disease of cattle popularly called yellow disease. Indian Vet.J.,38(9):448.

Davidson, J.N. and Hish,D.C.(1980). Leptospirosis in lambs. J.Am.Vet.Med.Ass.,176(2): 124-125.

Dawe,D.L., Shotts,E.B., Brown,J. and Sulzer,C.R.(1976). Proc.of 4th Int.Nat.Pig.Vet.Soc.Cong.America., D.D.8.

Ezell,S.B.,Hoag, W.G., Warner,A.R., Yager,R.H. and Gochenour,H.S.(1952) soluble specific leptospiral complement fixing antigens, Proc.Soc. Exper.Biol. and Med., 80: 220-223.

Faine,S.(1959). Iron,as a growth requirement for pathogenic Leptospira. J.Gen.Microbiol., 20: 246-251.

Freeman,A. (1957) Simplified leptospirosis diagnosis. Vet.Med., 52: 589-591.

Galton, M.M., Powers,D.K., Hall, A.D. and Cornell, R.G. (1958). A rapid Macroscopic slide screening test for the serodiagnosis of leptospirosis. Am.J.Vet.Res.19: 505-512.

Goldschmidt,F. (1887). Ein.Beitrag Zur neuen. Infection. Skrankhait Weils. Dtsch.Arch.Klin.Med.40 238, cited by Alston, J.M. and Broom, J.C.(1958).

Higgins,R. and Cayoutte,P. (1978). Serological diagnosis of Leptospirosis in the province of Quebec.Can. Vet.J.19(1): 13-16.

Higgins,R. and Cayoutte.F., Hoquet,F. and Lasalle,F.D. (1980). Serological studies on leptospirosis in domestic animals in Quebec.Can.J.Comp.Med., 44: 229-231.

Howrath,J.A. (1956). A macroscopic tube-agglutination test for leptospirosis. Am.J.Vet.Res., 10: 189-192.

Hussaini,S.N. (1976). A comparative study on the use of Whey and serum for microscopic agglutination test for the detection of Leptospira antibodies. Br.Vet.J., 132(6): 621-626.

Inada, R., Ido,Y., Hoki,R.,Kaneko,R. and Ito,H.(1916). The etiology, mode of infection and specific therapy of Weil's disease spirochaetosis itero haemorrhagica). J.Exp.Med., 23:377.

Jaeger,H.(1892). Die actiologie des infectiosen fieberhaftin Icterus (Weilsche Krankheit). Z.Hyg. Infektr., 12: 525. cited by Alston,J.M. and Broom,J.C.(1958).

Jungherr,E. (1944). Bovine leptospirosis. J.Am.Vet.Med. Ass., 105: 276-281.

Keast, J.C., Forbes, B.R.V., and Wannan,J.S. (1963). Bovine leptospirosis in New South Wales including the results of a 10 year survey. Aust. Vet.J., 40 (1): 19-26.

Meyer, K.F., Anderson, B.T. and Eddie,B. (1939). Canine Leptospirosis in the United States. J.Am.Vet. Med.Ass., 95: 710-729.

Michin, N.A., ad Azinow,S.A. (1935).spirocheatal jaundice of cattle in North Caucasus (Translated title Vander Hoeden.J.). Epizootology of leptospirosis. Advances in Veterinary Science. 4: 277-339. (1958).

Michna,S.W., (1967). Animal Leptospirosis in the British Isles. A serological survey, Vet.Rec.,80 (13): 394-400.

Morris,J.A.,Gill,J.E., and Hussaini,S.N. (1977). An examination of antibodies active in the indirect haemagglutination test for bovine leptospirosis. Br.Vet.J.,133(1): 17-24.

Morse, E.V., and Allen.V. (1956). Serological cross-agglutination reaction between Leptospira pomona and L.icterohaemorrhagiae. Am.J.Vet. Res., 17 (64): 563-568.

Muraschi, T.F. (1958). Latex-leptospiral agglutination test. Proc.Soc.Exp.Biol.Ny., pp.235-238. Cited from Vet.bull. Appendix (1959).

Negi, S.K., Meyers, W.L. and Segre,D. (1971). Antibody response of cattle to Leptospira pomona. Response as measured by haemagglutination, microscope agglutination and Hamster protection tests. Am.J.Vet.Res., 32 (1): 1915-1920.

Newman,J.P. (1950). Studies of canine leptospirosis. I. Evaluation of laboratory diagnostic procedures. II. Serological determination of incidence of latent infection in the Lansing, Michigan area. (1950). Am.J.Vet.Res., 10: 405-410.

Palit,A. and Gulasekharam,J. (1973). Genus-specific leptospiral antigen and its possible use in laboratory diagnosis. J.Cli.Path., 26: 7-16.

Palit, A and Sharma,G.L. (1971a). Comparison of Microscopic agglutination, indirect haemogglutination and complement fixation tests in rabbits and buffaloe calf hyperimmune sera for detection of leptospiral antibodies. Br.Vet.J., 127: 154-161.

Palit,A. and Sharma,G.L. (1971b). Serological evidence of the incidence of leptospirosis among livestock in India. Indian Vet.J., 48 (8): 773-779.

Pargaonker, V.N. (1957). Rats as carriers of Leptospira. Indian Vet.J., 34 (3): 205-208.

Pargaonker,V.N. (1964). Leptospirosis in sheep and goats in Andhra Pradesh, Indian Vet.J.,41: 441.

Rajasekhar, M., Kesavamurthy,B.S, and Nanjiah,R.D. (1977). Leptospiral antibodies in the serum of horses in Karnataka State. Indian Vet. J.,54 (1): 74.

Ris,D.R. (1977). The Serology of calves vaccinated and challenged with Leptospira interrogans serotype pomona. Agglutination and complement fixation reactions. N.Z.Vet.J., 25 (1):10-1

Salt,G.F.H., and Little, T.W.A.(1977). Leptospires isolated from wild mammals caught in the West of England. Res.Vet.Sci., 22 (1): 126-127.

Sandhu, T.S. and White, F.H. (1972). Evaluation of a Macroscopic plate test and an indirect immunofluorescence test to detect leptospira antibodies in bovine serum. Can.J.Comp.Med., 36 (1): 34-37.

Schuffner, W., and Mochtar,A., Versuche Zur Aufteilung Von Leptospirenstammen mit einleitenden Bemerkungen Uber den Verlauf Von Agglutination and lysis. Zbl.Bakt.101: 405.cited by Alston, J.M. and Broom, M.C. (1958).

Sharp,C.F. (1958). Laboratory diagnosis of leptospirosi with sensitized erythrocyte lysis test. J. Path.Bact., 76: 349-356.

Singh,S.P. (1970). A comparative study of serological diagnostic tests of leptospirosis. Indian Vet.J.47 (10): 817-819.

Solorazano,R.F.(1967). A comparison of the rapid Macroscopic slide agglutination test with the microscopic agglutination test for leptospirosis. Cornell Vet.,57: 239-249.

Stalheim, O.H.V. (1971). Leptospirosis diagnosis of immunofluorescence. Improved procedure for antigen preparation.Am.J.Vet.Res., 32 (1): 2107-2109.

Stoenner,H.G. (1954). Application of the capillary tube test and a newly developed plate test to the serodiagnosis of bovine leptospirosis. Am.J.Vet.Res., 15: 434-439.

Stoenner,H.G. (1955). Application of serology for the diagnosis of leptospirosis. Proc.Am.Vet. Med. Ass., 172-176. (Biol.Abstr. 31:32075).

Stoenner,H.G. and Davis,E. (1967). Further observations on leptospiral plate antigens. Am.J.Vet.Res., 28 (122): 259-266.

Stuart,R.D. (1946). Canine leptospirosis in Glasgow. Vet.Rec., 58 (12):131-132.

Sulzer,C.R., Glosser,J.W., Rogers, F., Jones, W.L. and Frix,M.(1975). Evaluation of an indirect haemagglutination test for diagnosis of human leptospirosis. J.Clin.Microbiol., 2: 218-221.

Tripathy, S.B. (1977). Serological prevalence of lepto spirosis in cattle,sheep and goats. Indian Vet.J.54 (7): 684-685.

Turner,L.H. (1967). Leptospirosis. I. Trans.Roy.Soc. Trop.Med.Hyg., 61: 842-855.

Turner,L.H.(1968). Leptospirosis II. Trans.Roy.Soc. Trop.Med.Hyg., 62: 880-899.

Turner,L.H. (1970). Maintenance, isolation and demonstration of leptospirae. Trans.Roy.Soc.Trop. Med. and Hyg., 64: 623-646.

Ward,M.K. (1954). Some problems in the laboratory diagnosis and control of leptospirosis. Vet. Med., 49: 147-152.

Watson, A.D.J., Wannan,J.S.,Porges,W.L. and Testoni,F., (1976). Leptospiral agglutinins in dogs in Sydney. Aust.Vet.J., 52(9): 425-426.

Weil,A. (1886). Ubereine eigentumliche, mit Milztumor Icterus and Nephritis einhergehende, akute infekton-skrankheit. Dtch.Arch.Klin.Med.39: 209-232. Cited by Alston,J.M. and Broom.J.C. (1958).

White,F.H. and Ristic.M. (1959), Detection of Leptospira pomona in guineapig and bovine urine with fluorescent labelled antibody. J.Infect.Dis., 105: 118-123.

W.H.O. (1967). Current problems in Leptospirosis. Research Report of W.H.O. Expert Group: World Health Organization Techniques. Rep.Ser.380.

Wolff.J.W. (1954). The laboratory diagnosis of leptospirosis. Charles.C.Thomas Publishers. Spring field, Illinois.

TABLES

Table I

Schedule of inoculation for raising hyperimmune sera in rabbits

Days	Dose	Route
1st day	1 ml	I/V
5th day	2 ml	"
10th day	3 ml	"
15th day	4 ml	"
20th day	5 ml	"
25th day	6 ml	"

Table II

Determination of optimum dilution of antigen for sensitization of sheep erythrocytes

Antigen dilutions	Antiserum dilution								
	5	10	20	40	80	160	320	640	1280
1:2	+	+	+	+	+	-	-	-	-
1:4	+	+	+	+	+	-	-	-	-
1:8	+	+	+	+	-	-	-	-	-
1:16	+	-	-	-	-	-	-	-	-
1:32	-	-	-	-	-	-	-	-	-

Table III

MA and PHA titres of serum samples from rabbits immunised against *L.autumnalis*

Days after inoculation	*L.autumnalis*					
	Rabbit I		Rabbit II		Rabbit III	
	MA	PHA	MA	PHA	MA	PHA
0 day	Negative	-	-	-	-	-
7th day	1:400	1:5	1:400	1:5	1:400	1:5
14th day	1:800	1:10	1:800	1:10	1:800	1:10
21st day	1:12800	1:40	1:12800	1:40	1:12800	1:40
28th day	1:12800	1:40	1:12800	1:40	1:12800	1:40
35th day	1:12800	1:160	1:12800	1:160	1:6400	1:160
42nd day	1:6400	1:160	1:6400	1:160	1:6400	1:80
49th day	1:3200	1:80	1:3200	1:80	1:3200	1:40

Table IV

MA and PHA titres of serum samples from rabbits immunised against *L.pyrogenes*

Days after inoculation	*L.pyrogenes*					
	Rabbit I		Rabbit II		Rabbit III	
	MA	PHA	MA	PHA	MA	PHA
0 day	Negative	-	-	-	-	-
7th day	1:100	1:5	1:100	1:5	1:100	1:5
14th day	1:200	1:10	1:200	1:10	1:400	1:10
21st day	1:3200	1:40	1:3200	1:40	1:3200	1:40
28th day	1:3200	1:40	1:3200	1:40	1:3200	1:40
35th day	1:3200	1:80	1:3200	1:80	1:3200	1:80
42nd day	1:1600	1:40	1:1600	1:40	1:1600	1:40
49th day	1:1600	1:20	1:1600	1:40	1:800	1:20

FIGURE-3 MICROSCOPIC AGGLUTINATION AND PASSIVE HAEMAGGLUTINATION TITRES OF LEPTOSPIRA AUTUMNALIS IN IMMUNISED RABBITS.

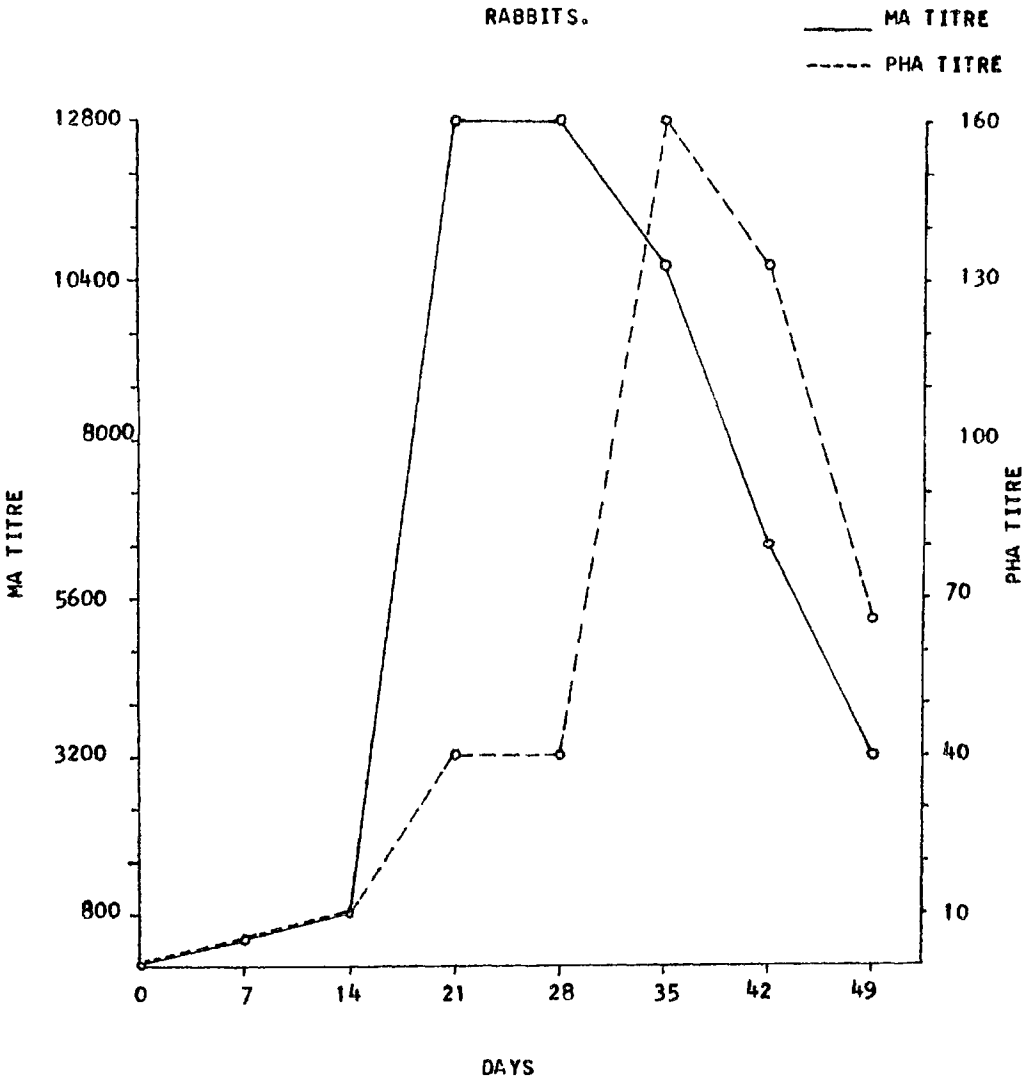
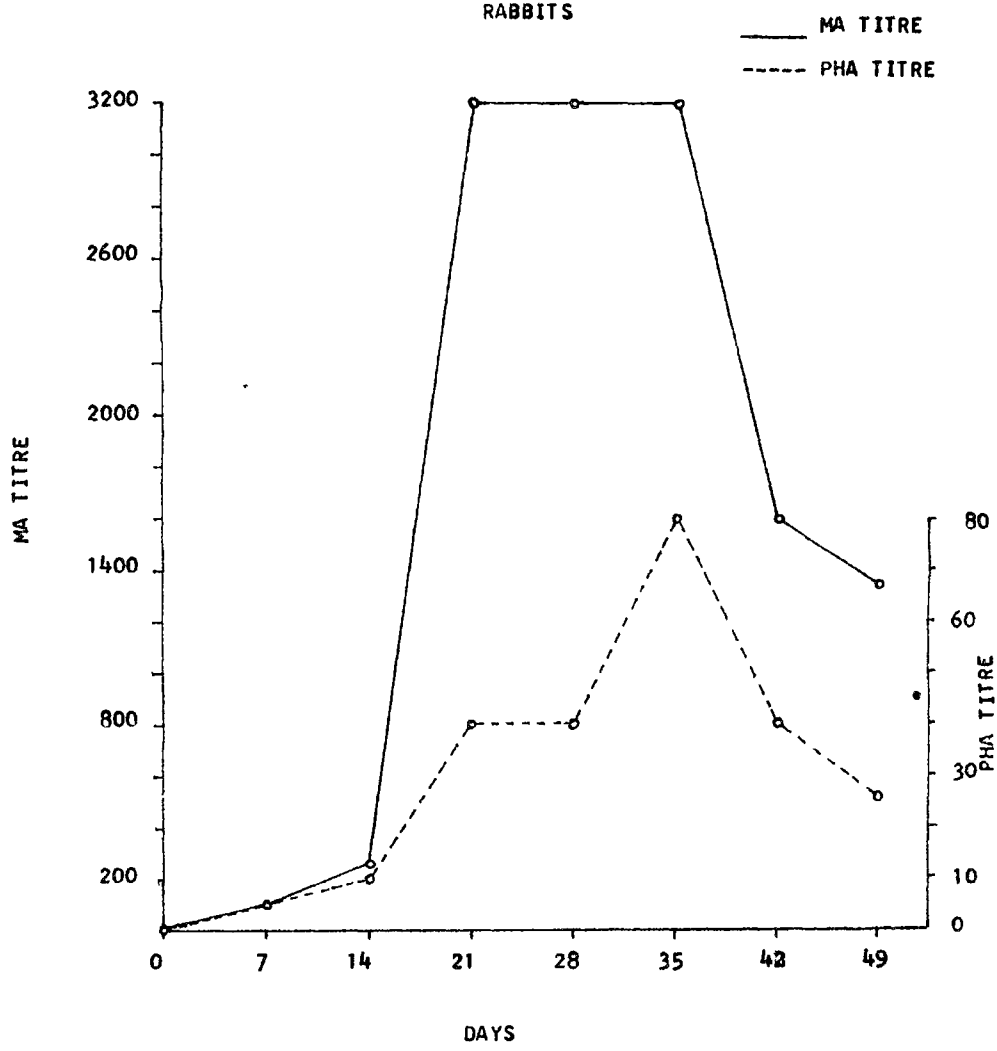
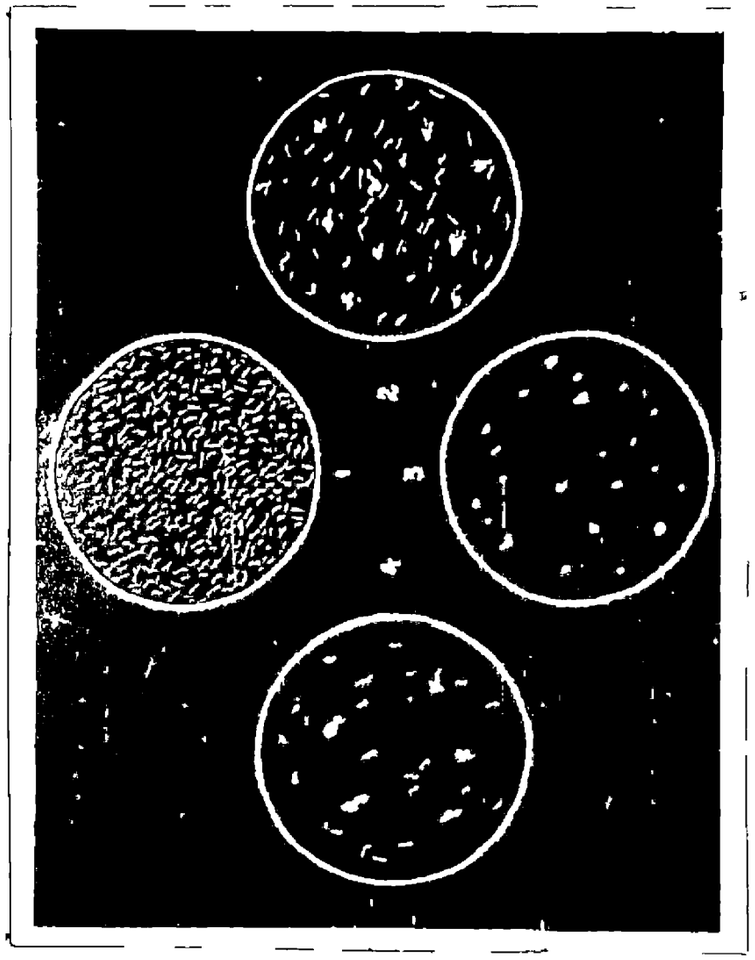
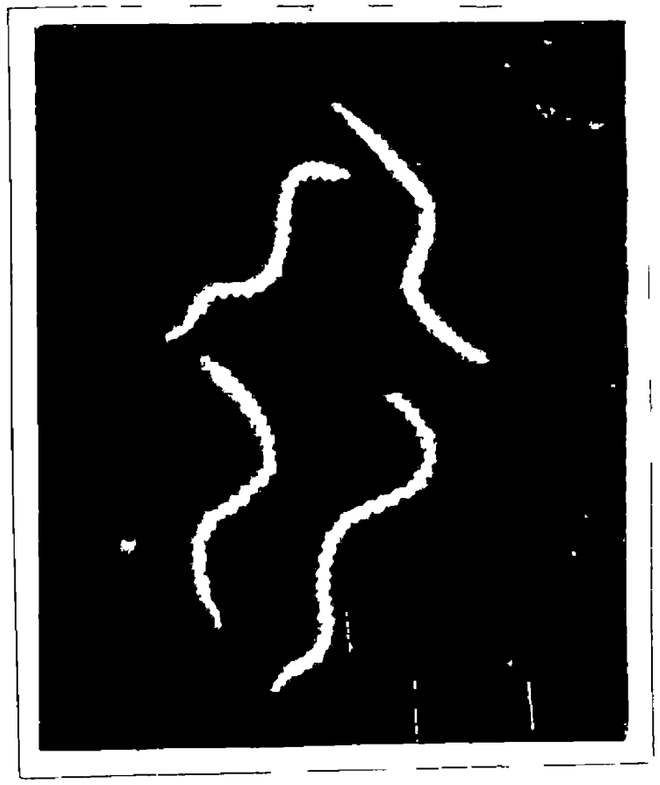


FIGURE-4 MICROSCOPIC AGGLUTINATION AND PASSIVE HAEMAGGLUTINATION TITRES OF LEPTOSPIRA PYROGENES IN IMMUNISED RABBITS



PLATES





COMPARISON OF SEROLOGICAL TESTS FOR THE DETECTION OF LEPTOSPIRA ANTIBODIES IN IMMUNISED ANIMALS

By

R RAVIKUMARAN NAIR

ABSTRACT OF A THESIS

Submitted in partial fulfilment of the requirement for the degree

Master of Veterinary Science

Faculty of Veterinary and Animal Sciences
Kerala Agricultural University

Department of Microbiology

COLLEGE OF VETERINARY AND ANIMAL SCIENCES
MANNUTHY - TRICHUR

1980

ABSTRACT

Leptospirosis is a widespread disease of man and animals and is of considerable economic importance besides being a public health problem. The leptospira infection in man and animals may be confirmed either by isolation of the organisms or by detection of specifi antibodies in the serum and tissues of infected animals. Isolation of Leptospira is time consuming and beyond the scope of many diagnostic laboratories.

In the present study the sensitivity of passive haemagglutination test was compared with the establishe microscopic agglutination test utilizing rabbit hyper-immune serum as the source of antibody.

Leptospira serotypes were grown in Korthof's medi enriched with 10% haemolysed rabbit serum. By 7-10 day satisfactory concentration of the organisms was obtaine and was used for MA test. Passive haemagglutination test was carried out employing ethanol extracted antige from concentrated leptospiral cultures. The PHA test was carried out after determining the optimum dilution of antigen required to sensitize sheep erythrocytes.

Hyperimmune sera to both serotypes were raised in rabbits by a series of intravenous inoculations.

Serum samples for antibody titration was collected at weekly intervals from seven days following the first injection till the 49th day.

Antibody titration by MA and PHA tests have sho that all the three animals inoculated with L.autumna had a uniform titre of 1:400 on the seventh day where the other three animals inoculated with L.pyrogenes showed a low titre of 1:100 by MA test. The PHA titr of both the groups remained the same ie 1:5. The max mum titre of 1:28000 for L.autumnalis was attained on the 21st day and remained unchanged until 35th day. maximum PHA titre was attained only on 35th day (1:16 The rabbits inoculated with L.pyrogenes showed a maxi titre of 1:3200 by MA and 1:80 by PHA. The results o ned tend to show that PHA titres after reaching the maximum level remained detectable for longer period w compared to MA titres.

Erythrocyte sensitizing substance from both the serotypes and the sera samples collected periodically from immunized rabbits were preserved at -20°C at var ing length of time upto three months. There was no deterioration in the stability or potency of ESS or s on storage.