SOME SEROLOGICAL STUDIES ON LEPTOSPIRES WITH PARTICULAR REFERENCE TO IMPROVEMENT OF DIAGNOSTIC TECHNIQUES.

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SUMMARY:

High-titred antisera to three serotypes of *Leptospira* and ESS were prepared in rabbits and subjected to HA, MA and immunodiffusion tests. The antisera were absorbed with ESS and subjected to HA and MA tests to observe the degree of anti-ESS reactivity. The MA test with monovalent antisera gave high titres (4096 to 8192) with homologous antigens. Very low titres were recorded with polyvalent and anti-ESS antisera and when heterologous antigen were used with monovalent antisera. The MA test showed broad reactivity against each antiserum (32 to 128). Absorption of the antisera with ESS considerably reduced the titres in both HA and MA tests compared to pre-absorption titres. FITC conjugates prepared from the antisera were applied to homologous serotypes to evaluate staining capacities. Generally, the specific fluorescence obtained was unsatisfactory. In only one instance (FITC/ anti-*patoc* with *patoc* organisms) was there bright staining. The other homologous staining regimes produced only faint fluorescence and with heterologous combinations staining was very faint or non existent. When the antisera were subjected to immunodiffusion against ESS there were precipitin line against all antisera employed except anti-*patoc*.

INTRODUCTION:

The disease of animals and man now known to be caused by infection with *leptospira* species was first recognised as a clinical entity in human medicine. Leptospirosis was suggested in 1887 by Goldschmidt from four cases of febrile jaundice with relapse described by Adolf Weil, (Joshua and Broom 1949). Knowledge acquired recently in several parts of the world indicated that a variety of Leptospira possess the potential for infecting a diversity of domestic and wild animal species as well as humans.

Leptospiral infections may be diagnosed in the laboratory by either isolating the organisms or by detection of specific antibodies. The isolation of the strain is the most desirable method but has some draw-backs depending on the stage of illness, type of material collected, the availability of suitable medium, and laboratory facilities for handling animals (Ellinghausen and McCullough 1965) The isolation of *leptospires* from infected animals and man is time consuming and most diagnostic laboratories cannot cope with.

The detection of specific leptospiral antibodies is more convenient when compared to the isolation procedures. The microscopic agglutination test using live *leptospires* as antigens is the standard reference test for the leptospiral diagnosis (World Health Organisation 1967). This test also has some disadvantages e.g. a large number of cultures of serotypes must be kept, it is time consuming and also it involves the handling of live organisms (World Health Organisation 1967).

Work carried out by Chang and McComb (1954) and Cox et al (1957) showed that a particular leptospiral extract behaved like a genus specific antigen in detecting antibodies against homologous serotypes of *leptospires*. An ethanol precipitated fraction extracted from *leptospira* sp. (serotype *biflexa* strain patoc) was found to be a mixture of genus specific and type specific antigens Palit et al (1973). The purpose of this work was to assess the genus - specific leptospiral antigen for use in laboratory diagnosis. It was also aimed to develop a genus-specific fluorescent antibody technique using *L. patoc* enthanol extract antigen in rabbits.

MATERIALS AND METHODS

Experimental animals:

Mature rabbits which weighed 3 to 4kg (New Zealand White) were housed individual wire cages, fed on rabbit pellets (17% protein) and water was provided *ad libitum*. They had no previous history of leptospirosis. Healthy Merino sheep obtained from the Animal Health Station, Oonoonba-Australia were used for blood collection

Leptospira strains:

Cultures of *L. hardjo* and *L. pomona* were obtained from the Animal Health Station, Oonoonba. A culture of *Leptospira biflexa patoc 1 (Semaranga* serogroup) was obtained from Commonwealth Serum Laboratories, Parkville, Victoria. The organisms were maintained in Ellinghausen's medium as modified by Johnson and Harris (1967).

Biological solutions:

Isotonic saline was prepared by dissolving 8.50 g of NaCl in 1 litre of distilled water, where a phosphate buffered saline (PBS) was prepared as per Goldman (1968). Alsever's solution was prepared by dissolving 20.5 g dextrose, 8.0 g sodium citrate, 0.55 g citric acid, 4.2 g sodium chloride in 1 litre of distilled water. Fluorescein isothiocyanate (Isomer I, crystalline) was obtained from the Baltimore Biological Laboratory Cockeysville, Maryland, U.S.A.

Preparation of Erythrocyte - Sensitizing Substance (ESS)

Erythrocyte-sensitizing substance was prepared by the method of Chang and McComb (1954) with some minor modifications. *L. biflexapatoc* organisms were grown in 3 to 5 litres volumes in Ellingahausen medium. The in oculated medium was left at room temperature for 8 days after which the concentration of organisms was about 4×10^7 *leptospires*®ml. The organisms were harvested from the culture by centrifugation at 4600 g for 30 minutes (20°C to 25°C). They were then suspended in PBS, washed twice and the sedimented *leptospires* were resuspended in sufficient PBS to give an optical density of about 0.25 (wave lenght 650 mu). The *leptospires* were thus concentrated about 10-fold to 40-fold.

The washed leptospira suspension (100 ml) was mixed with 100 ml of absolute ethanol at 0°C and shaken for about 4 hours. The mixture was centrifuged at 4600 g for about 20 minutes and the sediment discarded. To the supernatant flued 800 ml of absolute ethanol was added. The precipitate formed after 24 hours at 4°C was collected by centrifugation (600 g, 20 minutes), and redissolved in 100 ml PBS. This antigen suspension was divided into 20 ml aliquots and stored at — 20°C.

Preparation of immune sera:

Antisera against the three leptospira serotypes *(patoc, pomona* and *hardjo)* were prepared in rabbits by twice weekly intravenous injection (1 ml) of 7 to 10 day-old, dense living cultures of the organisms for 6 weeks. Blood samples were collected before the first inoculation and 1 week after the last. Serum was prepared and kept frozen until used.

A polyvalent antiserum against *L. patoc, L. pomona* and *L. hardjo* was prepared in one rabbit. This was done by injecting 2 ml of a heat killed mixture of the above mentioned *leptospira* serotypes emulsified in Freund's complete adjuvant, intramuscularly at four sites. Ten days later the injection was repeated with the same dose. Blood samples were collected before the first injection and two weeks after the last.

Anti-ESS serum was prepared by injecting a rabbit (4 intramuscular sites) with 3 ml of ESS emulsified in 1 ml of Freund's complete adjuvant. Ten days later the injection was repeated using the same dose. The rabbit was bled by cardiac puncture 2 weeks after the last injection.

Absorption of antisera with ESS antigen:

Equal volumes of each antiserum *(L. patoc, L. pomona* and *L. hardjo)* were mixed with equal volumes of ESS and left to react at 37°C for about 2 hours, centrifuged at 4600 g for 20 minutes and the supernatant collected. This procedure was repeated twice.

Sensitization of fresh sheep erythrocytes:

Erythrocytes preserved in Alsever's solution were washed three times with PBS. The washed erythrocytes were packed by centrifuging the cell suspension (600 g, 15 minutes). A 10% suspension of cells was prepared in PBS. To 1 ml of ESS of various dilutions (1:2, 1:4 etc.) was added 0.1 ml of a 10% erythrocyte suspension. The mixture was allowed to react (37°C, 1 hour) and shaken at 15 minute intervals. After 1 hour, the cells were washed twice in PBS and resuspended in 1 ml of th same diluent to make a 1% sensitized erythrocyte suspension.

Indirect haemagglutionation test:

The indirect haemagglutination (HA) test was carried out as described by Palit and Gulasekharam (1973) with minor modifications. A 1% solution of ESS — sensitized erythrocytes was prepared as previously described. The haemagglutination test was performed in U-bottom perspex haemagglutination trays. All sera used were heat inactivated for 30 minutes at 56°C. Serial two-fold dilutions were made of the test sera (rabbit). To 0.4 ml of diluted serum, 0.1 ml of 1% sensitized erythrocytes was added, the plates shaken gently and incubated (37', 1 hour). The results were recorded after overnight incubation at room temperature. The end point was taken as the highest dilution of serum showing at least 50% haemagglutination. Negative controls were run for antigen and antibody.

Microscopic agglutination (MA) test:

Two-fold serial dilutions (0.2 ml) of serum samples were made in PBS using multiple depression plastic plates. An equal volume of live, active 7 day-old culture of *leptospires* was added to each serum dilution and incubated (37°C, 1 to 2 hours). A negative control (0.2 ml PBS + 0.2 ml antigen) was included. Agglutination results were read using dark-ground microscopy. The highest dilution of the serum-antigen mixture in which 50% of the *leptospires* were agglutinated was taken as the end point (World Health Organization 1967).

Fluorescent Antibody (FA) Techniques:

The fluorescent antibody technique as described by Hodges and Ekdahl (1973) was used with some modifications. Fluorescein isothiocyanate (FITC) was dissolved in 0.1 m Na_2HPO_4 (1.25 mg of FITC/ml) and antiserum globulins slowly added to the FITC solution to produce an FITC: protein ratio of (1:20). After addition of FITC solution the pH was immediately raised to 9.5 with 0.04M NaOH and conjugation was continued at room temperature for one hour.

One drop of an actively growing culture of *leptospires* (4 to 7 day old) was spread on a glass slide, dried in an incubator at 37°C, then acetone fixed for 10 minutes at room temperature. The dried smears were covered with optimally diluted conjugate and incubated (37°C, 1 hour) in a humid chamber for 1 hour. The unbound conjugate was removed by rinsing in PBS and the film air dried. The preparation was mounted in glycerol-saline (9 parts glycerol to 1 part physiological saline) and examined on the same day.

Controls for the specificity of fluorescence consisted of (i) treating the smear with unlabelled leptospiral antiserum prior to staining with fluorescent antibody, (ii) using conjugate from normal rabbit globulin and (iii) applying conjugates to smears of staphylococci.

Double diffusion in agar:

A modification of the method of Ouchterlony (1958) was used for double diffusion tests. Wells (2 mm diameter) were punched in normal immunoelectrophoresis plates using a gel punch. The holes were punched in a circle (1 cm or 2 cm in diameter) with a central well for antigen. The antigen and antisera were placed in wells, and allowed to react for 24 to 36 hours, thereafter they were washed and stained.

RESULTS:

Microscopic agglutination:

Monovalent, polyvalent and anti-ESS sera were prepared from rabbits using the 3 serotypes (*patoc, pomona, hardjo*) and ESS, after determination of optimal antigen dilution by the chequer — board titration technique the rabbit sera were tested in the HA assay before and after absorption with ESS. Results for the MA tests of unabsorbed rabbits antisera are presented in Table 1. In the microscopic agglutination test with homologous antigen, each of the monovalent antisera had titres of 4096 or 8192. However, titres for these antisera with heterologous antigen were significantly lower (8 to 32). The polyvalent and anti-ESS sera had very low titres (0 to 32).

Following absorption of the antisera with ESS the titres were found to be considerably reduced (0 to 32) compared to pre-absorption titres. (Table I)

Passive haemagglutination:

Results for the chequer-board titration to determine the optimal dilution of antigen for use in the HA assay are shown in Table 2. The optimal dilution for the ESS preparation was 1/16 and this antigen dilution was associated

with a titre of 128 for the homologous antiserum. Subsequently a 1:16 dilution of the ESS preparation was used to sensitize erythrocytes for the haemagglutination test.

Results for the HA test using the various antisera before and after absorption with ESS are presented in Table 3. These results clearly demonstrated that the ESS test gave broad reactivity and absorption of the antisera with ESS removed a considerable proportion of the HA antibody titre.

Evalution of FITC conjugates:

Each of the 5 rabbit antisera were conjugated to fluorescein isothiocynate as described in the Materials and Method section. Degrees of fluorescent brightness were recorded as 4 bright, 3 faint, 2 very faint and 0 no fluorescence. The optimal dilution of each conjugate was the greatest dilution producing bright fluorescence against the homologous serotype. Optimal dilutions of the conjugates were then tested against homologous and heterologous organisms.

Results of the immunofluorescence assays are summarised in Table 4. The optimal dilutions of the conjugates were in the range 1:4 to 1:8. Optimal dilutions of *patoc, pomana* and *hardjo* stained brightly or faintly with homologous sertypes but did not stain, or stained very faintly, with heterologous serotypes. Anti-ESS conjugated with FITC produced very faint fluorescence with *patoc 1* organisms but no fluorescence with *hardjo* and *pomona* serotypes. The polyvalent conjugate showed very faint fluorescence with the 3 serotypes. Each of the three controls gave no fluorescence.

Immunodiffusion assay:

The results for the double diffusion test are given in Table 5. The ESS antigen showed precipitin lines with polyvalent, anti-ESS, *pomona* and *hardjo* antisera but no precipitin lines were observed with *patoc 1* or with the controls (normal rabbit antisera and PBS).

DISCUSSION:

Recent interest in leptospirosis has focussed attention on the need for a safe genus-specific serologic test that can be readily performed by the ordinary diagnostic laboratory.

The microscopic agglutination test is the reference test for test for serological diagnosis of leptospirosis (World Health Organisation 1965, 1967). In this study the test was used to detect leptospiral antibodies in rabbit hyperimmune sera. The test showed high titres for homologous reactions but there were only moderate heterologous reactions recorded. This confirmed the high degree of sensitivity and serotype specificity of the microscopic agglutionation test. Polyvalent and anti-ESS sera gave low titres with the test suggesting that infection with live organisms is far more efficacious in eliciting high antibody titres for this particular assay. It is also possible that heat-labile antigens are necessary for the production of high titres of antibody in the MA test. If this is the case then the method of preparation of the polyvalent antiserum would have caused low titres.

When the antisera were absorbed with ESS the subsequent MA titres were greatly reduced. It is apparent from this result that ESS is an important antigen for the MA test. It has been reported (Auran *et al. 1972)* that ESS is derived from the envelope of the organisms and it is therefore likely that this portion of the *leptospire* is bound by antibody molecules during agglutination reactions. These data support the results of Palit *et al.* (1974) who showed that washed, live *patoc 1* organisms removed from antisera almost all of the specific antibody responsible for both HA and MA reactions.

In the indirect haemagglutination test the rabbit hyperimmune sera showed moderately low but uniform titres. This finding demonstrated the high degree of broad reactivity of the HA test carried out using *patoc 1* ethanol extracted antigen. Thus the antigen exhibited genus specificity in this serological assay. The ethanol extract contains erythrocyte sensitizing carbohydrates (Chang and MacComb 1954, Cox 1975). It has been reported that 1gM antibody is responsible for antibody titres in the HA test (Palit and Gulasekharm 1973). Therefore the sharp decline in HA antibody titres following absorption of the antisera with ESS suggests that much of the 1gM antibody activity is directed against ESS or closely related antigens.

In most previous studies of direct FA techniques for leptospirosis diagnosis, much attention has been focussed on homologous serotypes and little has been done to develop genus-specific FA techniques. Cross reactivity has been noted by Chernukha and Korn (1965) who found cross-reactions between fluorescein-labelled antibodies to *grippotyphosa* and other pathogenic serotypes within the group. Recently, Hodges and Ekdahl (1973) found faint staining with heterologous serotypes. In this study it was found that the conjugate from the monovalent antisera were highly serotype-specific although there was minor cross staining exhibited especially with *hardjo* and *pomona* antisera conjugates. These results are contrary to those of Chernukha and Korn (1965) Hodges and Ekdahl (1973) who found cross-reactivity. It was apparent from the present experients that none of the monovalent antisera conjugates would be suitable as screening reagents.

The anti-ESS conjugate stained very faintly with *patoc* only. It had been hoped that the anti-ESS conjugate would show broad reactivity against all serotypes. Failure of the anti-ESS conjugate to react satisfactorily with the 3 serotypes suggested that it was unlikely to be of value in immunofluorescent screening assays, although ESS is clearly of considerable importance in serological assays such as the HA assay. The ESS-absorbed antisera conjugates showed no fluorescence. This result indicates that the ESS had removed specific antibody which had produced moderate staining prior to absorption. Thus, although antibody to ESS or related antigens plays some role in immunofluorescene of leptospiral ortanisms, the results which have been obtained in this study do not suggest that the FA technique with this antigen could be developed into a genus-specific test suitable for diagnostic purposes.

In double immunodiffusion tests using ESS as antigen, a single precipitin line developed with each of the antisera except anti-*patoc*. This was indeed a surprising result as the ESS had been extracted from *patoc* organisms. Since the anti-*patoc* antiserum gave a high titre (128) in the HA assay this would suggest that non-infectious *patoc* arganisms induced agglutinating but not precipitating antibody against ESS. It is therefore possible that infection of animals with non-pathogenic *leptospires* such as *patoc* induces IgM antibody (agglutinating but not IgG antibody (precipititating). This may be due to rapid elimination of non-pathogenic strains from the animals. If such is the case then this observation may provide the basis for a diagnostic test to differentiate animals which have been in contact with pathogenic or non-pathogenic *leptospires*.

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Table I:

Antibody titres for rabbit serum samples tested by the Microscopic Agglutination (MA) test.

ANTISERA	LIVE ANTIGEN — MA TITRE					
	PATOC		POMONA		HARDJO	
	Pre-absorption	Post-absorption	Pre-absorption	Post-absorption	Pre-absorption	Post-absorption
Polyvalent	32	4	8	0	4	2
Anti-ESS	32	2	32	2	0	2
Pomona	32	0	8192	8	16	0
Patoc	4096	2	8	0	32	0
hardjo	8	2	16	0	4096	16

ESS = Erythrocyte Sensitizing Substance.

Table 2:

Results of chequer-board titration for determination of optimal dilution of ESS antigen for sensitization of sheep erythrocytes.

Antigen dilution	Antiserum (Homologous) Dilution						
	1:8	1:16	1:32	1:64	1:128	1:256	1:512
1:8	+	+	+	+	+	—	—
1:16	+	+	+	+	+	—	—
1:32	—	—	—	—	—	—	—
1:64	—	—	—	—	—	—	—
1:128	—	—	—	—	—	—	—
1:256	—	—	—	—	—	—	—

Table 3:

Results of (Haema agglutination) test with heterologous and homologous rabbit hyperimmune sera.

ANTISERA	HA TITRES	
	Pre absorption	Post absorption
Polyvalent	128	2
Anti-ESS	64	0
pomona	128	32
patoc	128	0
hardjo	32	0

ESS = Erythrocyte sensitizing substance.

Table 4:

Results of Flourescence antibody technique applied to homologous and heterologous smears.

	Fluorscein Conjugated Antisera								
	patoc	pomona	hardjo	Anti-ESS	Polyvalent	Normal rabbit serum	ESS absorbed patoc	ESS absorbed pomona	ESS absorbed hardjo
Organisms									
Patoc	4	3	3	2	2	0	0	0	0
Pomona	0	3	3	0	2	0	0	0	0
hardjo	2	2	3	0	2	0	0	0	0
staphylococci	0	0	0	0	0	0	0	0	0

Key: 4 Bright; 3 Faint; 2 Very Faint; 0 No Fluorescence.
ESS = Erythrocvte sensitizing substances.

Table 5:

Results of double diffusion tests in agar gel using the ESS antigen with rabbit antisera.

Antigen	Antisera	Precipitine line
ESS	Polyvalent	present
ESS	Anti-ESS	present
ESS	*pomona*	present
ESS	*hardjo*	present
ESS	*patoc*	absent
ESS	PBS Control	absent
ESS	NRS* Control	absent

*NRS = Normal rabbit serum.
ESS = erythrocyte sensitizing substance.