DETECTION OF LEPTOSPIRAL ANTIBODIES IN ANIMAL SERA BY MEANS OF FRACTIONATED ANTIGENIC EXTRACTS

N. C. FATHALLA AND J. D. COGHLAN

Leptospira Reference Laboratory, Central Public Health Laboratory, Colindale Avenue, London NW9 5HT

ISOLATION of leptospires from blood culture is likely to be successful only during the first week of the illness. The laboratory diagnosis of human and animal leptospirosis otherwise has to be based on serological tests. Microscopic and macroscopic agglutination, complement fixation (CF), haemagglutination and direct and indirect immunofluorescence are all tests that can be applied to the detection of leptospiral antibodies (Turner, 1968). The interpretation of the results of these tests at different times can indicate whether a patient's illness is or has been due to acute leptospirosis. The titres of antibody and the classes of immunoglobulin directed to the various leptospiral strains used in the tests may indicate the serogroup if not the serotype (serovar) of the infecting strain.

Difficulties occur because each serotype is characterised by its own particular combination of antigenic factors. Some of these antigens are common to all serotypes—pathogens and saprophytes (i.e., they are genus specific), some are common to only a few serotypes which consequently make up a serogroup (group-specific antigens) and others are unique to the serotypes in which they occur (serotype-specific antigens).

During the early stages of infection or immunisation, antibodies to all these antigenic factors are produced and, because of the shared antigens, a considerable amount of cross reaction occurs between the various strains used as antigens in the serological tests. This is particularly marked in the microscopic agglutination test, in which many different leptospiral suspensions representing all the known serogroups are routinely used so that leptospirosis can be diagnosed whatever the serotype of the infecting strain. As the infection clears and the antibody levels begin to decline, the homologous antibodies predominate, usually enabling the serogroup of the infecting strain to be determined. If all the known serotypes within the serogroup are included in the serological tests, the serotype of the strain may also be revealed, although this is not always possible. In any case, it may be many weeks or even months before the cross reactions are eliminated and the nature of the infecting strain becomes clear from the residual antibodies.

Work on the antigenic constitution of leptospires has in the past been directed mainly towards a clarification of the taxonomy of the genus and to immunisation procedures rather than to improving diagnosis. Various processes have been used in attempts to extract and purify leptospiral structures and the antigens associated with them, e.g., outer-envelope antigens have been studied by Anderson and Johnson (1968), Auran, Johnson and Ritzl (1972) and Palit, Hamilton and Gulasekharan (1974); cell-wall antigens by Yanagawa and Faine (1966), Shinagawa and Yanagawa (1972) and Imamura, Matsui and Ashizawa (1974), and axial-filament antigen by Chang and Faine (1970, 1973, 1974). Extracts obtained by treatment with sodium dodecyl sulphate (SDS) were examined by immunodiffusion and complement fixation (Yana-

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gawa and Takashima, 1972), and by electronmicroscopy (Kida *et al.*, 1976). The treatment with SDS dissolves most of the cell, and the resulting extract contains soluble antigens derived from all parts of it.

With the aim of improving the serological diagnosis of leptospirosis by early identification of the infecting strain we fractionated SDS extracts of different leptospires by ultracentrifugation and examined, by immunological tests, the interaction between each fraction and homologous and heterologous antisera. In developing this technique we used animal sera in order to preserve our collection of positive human sera. This is the report of our investigation with animal sera. We are now investigating the application of the test to human sera.

MATERIALS AND METHODS

Strains of Leptospira. All three species of *Leptospira* were represented in the investigation. *Leptospira interrogans*, which incorporates the pathogenic serotypes (serovars), was represented by the following serotypes: *icterohaemorrhagiae* (strain RGA) and *copenhageni* (strain Wijnberg), both of serogroup Icterohaemorrhagiae; *canicola* (strain Canicola) and *schuffneri* (strain Vleermuis 90C) of serogroup Canicola; *bangkinang* (strain Bangkinang 1) of serogroup Autumnalis; *hebdomadis* (strain Hebdomadis) and *hardjo* (strain Hardjoprajitno) of serogroup Hebdomadis; *ballum* (strain Mus 127), *castellonis* (strain Castellón 3) and *arborea* (strain Arborea) of serogroup Ballum. *L. biflexa*, which includes mainly non-pathogenic serotypes, was represented by serotype *patoc* (strain Patoc 1) of serogroup Semarang. *L. illini* (species *incertae sedis*) was represented by serotype *illini* (strain 3055).

Culture medium. The strains were grown in Ellinghausen and McCullough's (1965) liquid medium supplemented with 2% pooled and inactivated rabbit serum. To obtain enough growth of each strain for each extraction, 2 ml of the culture was inoculated into 70 ml of the medium and incubated at 28°C for 5–7 days until the maximum density, about 10^8 cells/ml, was attained.

Extraction of cultures. The cells were harvested by centrifugation at 9000 *g* for 30 min, washed once with 0.01M phosphate-buffered saline pH 7.2 (PBS) and resuspended in 0.5 ml of PBS. To each suspension was added 0.5 ml of 2% sodium dodecyl sulphate (BDH, Poole, Dorset) and the mixtures were allowed to stand overnight at 37°C before being centrifuged at 12 000 *g* for 30 min. The supernates constituted the SDS "extracts" and the sediments the "cell residue".

Sucrose-density-gradient ultracentrifugation of the SDS extract was by the method of Kida *et al.* (1976) with modifications. SDS extract 0.6 ml was applied to a preformed sucrose gradient (8 ml of 10–40% solutions in PBS) underlayered with 0.4 ml of 60% sucrose solution and overlaid with 1.0 ml of PBS. After centrifuging at 170 000 *g* for 90 min., 10 fractions of 1 ml were collected, starting from the top of the tube. Because it was difficult to separate completely the supernate of the SDS extract from the sediment, fraction 10 sometimes contained some insoluble material which was shown by microscopy to be cell residue.

Serological tests. The microscopic agglutination test (MAT) was by the method of Turner (1968). The antigens were 5–7-day cultures of leptospires killed by the addition of neutralised formaldehyde solution in a final concentration of 0.02% (v/v). A battery of such antigens was arranged in pools of one or more strains, each pool representing one of the 16 recognised serogroups within the species *L. interrogans*. Three additional strains were included to represent two serogroups of *L. biflexa* and the single serotype within the species *L. illini*. Equal volumes of serum dilution and antigen suspension were added to the wells of a plastic agglutination plate from a standardised pasteur pipette. The test was kept at room temperature overnight and read by transferring drops of the antigen-antibody mixture to a slide for examination for agglutinated leptospires by dark-field microscopy at $\times 100$ magnification.

Complement-fixation tests (CFT) were by the method of Bradstreet and Taylor (1962) with long fixation (overnight at 4°C). A compound genus-specific antigen, as prepared for distribu-

tion to diagnostic laboratories by the Leptospira Reference Laboratory, was used. This consists of cultures of six different serotypes of *Leptospira*: *canicola*, *sumatrana*, *jules*, *georgia*, *wolfii*, and *patoc*, killed by addition of thiomersal to give a final concentration of 1 in 10 000 and harvested by centrifugation. The compound antigen was treated by acidification and boiling for 30 min. to abolish any anticomplementary effect (L.H. Turner and A.F. Mohun, 1970, unpublished notes). Doses of complement of 2 haemolytic units (2 HC50) were used and the titre of the serum was judged from the dilution resulting in 50% fixation.

Simultaneously with the normal MAT and CFT, the same tests were performed with PBS diluent containing 0.02M dithiothreitol (DTT) which breaks down the IgM molecule. The difference between the titres of the tests with and without DTT indicated the nature of the immunoglobulin present, i.e., the reaction with DTT was due to IgG whereas without DTT the reactions were likely to be due to the combined effects of IgM and IgG.

Immunodiffusion tests were by double diffusion in petri dishes containing 1% agar in distilled water (Weir, 1973).

Rabbit antisera. Rabbits were immunised by injections into the marginal ear vein of 2 ml of 7-day-old cultures of leptospires in Ellinghausen and McCullough's (1965) medium at weekly intervals for 6 weeks. The rabbits were bled before each inoculation so that the antibody response could be monitored. If the titres had reached a high enough level, the rabbits were bled out 1 week after the sixth inoculation and the serum was separated.

Dog sera. Sera from four dogs were obtained from Dr T.W.A. Little, Central Veterinary Laboratories, Ministry of Agriculture, Fisheries and Food, New Haw, Weybridge, Surrey. The dogs had all been diagnosed as suffering from leptospirosis. Two specimens were taken from dog no. 1 on days 1 and 14 after onset. Single specimens were obtained from the other three dogs during the first week of illness (dogs nos. 2 and 4) and 1 month after onset (dog no. 3).

Cow serum was sent to us by Dr W.A. Ellis, Veterinary Research Laboratories, Stormont, Belfast. It was taken from a cow on the day that it suffered an abortion thought to be due to leptospiral infection.

RESULTS

Preliminary tests with homologous rabbit antisera

Specimens of serum taken from a rabbit at intervals during the process of immunisation against strain Hond Utrecht IV of serotype *canicola* were monitored by MAT and CFT against strain Hond Utrecht IV before and after treatment with DTT (table I). By the 7th day after the first injection of leptospires only IgM appeared to be responsible for the serological activity because the DTT-treated serum was negative in both tests. By the 14th day IgM and IgG titres were recorded and the IgG titres increased to reach peaks of more than 10 000 and 2560 in the MAT(DTT) and CFT(DTT) respectively by the 35th day. One week after the sixth injection, i.e., the 42nd day, the antibody titres as revealed by MAT and CFT on DTT-treated and untreated sera were apparently due wholly to IgG.

Fractions of the SDS extract of strain Hond Utrecht IV were prepared and tested for their serological activity against the same series of immune rabbit serum. Table II shows that although fractions 1, 2, 7, 8, 9 and 10 were strongly reactive in the CFT from the 7th day, fractions 3, 4, 6 and 7 failed to react until day 21, i.e., after the third injection, by which time IgG was present as shown in table I. The activity against fraction 5 was delayed until the 35th day, 1 week after the fifth injection.

TABLE I

Microscopic agglutination and complement-fixation tests showing the activity of IgM and IgG in the serum of a rabbit during immunisation against the homologous strain Hond Utrecht IV (Canicola serogroup)

Time of collection of serum (days after start of immunisation)	Antibody titre in the test indicated			
	MAT	MAT (DTT)	CFT	CFT (DTT)
7	1000	< 10	320	< 10
14	30000	100	2560	160
21	30000	1000	5120	320
28	10000	10000	5120	1280
35	10000	10000	10240	2560
42	1000	1000	640	640

MAT=microscopic agglutination test; CFT=complement-fixation test; (DTT)=serum treated with di-thiothreitol.

Antigenic specificity of extracts

Having shown that the 10 fractions reacted in the CFT with the homologous antiserum, we attempted to test the specificity of similar fractions from other strains.

The 10 fractions obtained from an extract of strain Mus 127 of serotype *ballum* were tested by the CFT against rabbit antiserum to each of the three serotypes within the Ballum serogroup, and against antisera to strains within two different serogroups, *viz.* Icterohaemorrhagiae and Hebdomadis of *L. interrogans* and against antiserum to strain Patoc 1 of *L. biflexa*. The results are shown in table III. All fractions 1–10 fixed complement with antisera to the three serotypes within the Ballum serogroup. Fraction 1 reacted with antisera to all strains of pathogenic serogroups but not with Patoc 1 antiserum. It therefore appears to be species specific for *L. interrogans*. Fraction 2 reacted with antisera to all strains, *i.e.*, it is genus specific. Fractions 3–9 reacted only with antisera to the three strains of the Ballum serogroup and are therefore serogroup specific. Fractions 8 and 9 gave the highest titre, 20 480, with serotype *ballum* and may therefore contain a serotype-specific antigen. Fraction 10 appeared to be species specific for *L. interrogans* strains.

The test was repeated with strain Castellón 3 (table IV) and Arborea (table V) antigen fractions and the previous results were confirmed, *i.e.*, fraction 1 was found to be species specific, fraction 2 genus specific, fractions 3–9 of strain Castellón 3 and fractions 4–10 of strain Arborea were group specific and fraction 9 indicated clearly the serotype specificity in every case. Fraction 10 was variable in its specificity. That obtained from strains Mus 127 and Castellón 3 was species specific whereas fraction 10 of strain Arborea was serogroup specific.

Fractions of strains of the two species *L. biflexa* and *L. illini* reacted differently. From strain Patoc 1 (table VI) only fractions 1, 2, 8, 9 and 10 reacted in the CFT. Fractions 1 and 2 gave much higher titres with Patoc 1

TABLE II
Complement-fixation titres of rabbit homologous sera against extracts of strain *Hond Utrecht IV* (*Canicola serogroup*)

Time of collection of serum (days after start of immunisation)	Complement-fixation titre against									
	fraction no.									
	1	2	3	4	5	6	7	8	9	10
7	160	320	<10	<10	<10	<10	160	1280	2560	640
14	5120	2560	<10	<10	<10	<10	80	10240	20480	80
21	10240	10240	320	320	<10	1280	5120	20480	20480	160
28	1280	2560	160	320	<10	640	320	20480	20480	320
35	1280	5120	640	1280	160	2560	1280	20480	20480	640
42	640	1280	320	160	2560	320	10240	20480	20480	320

SDS = unfractionated sodium dodecyl sulphate extract.

TABLE III
Specificity of fractions of SDS extract of serotype *ballum* strain *Mus 127* (*serogroup Ballum*) in the complement-fixation test with rabbit homologous and heterologous antisera

Rabbit antiserum raised against			Complement-fixation titre against											
serogroup	serotype	strain	fraction no.										SDS	
			1	2	3	4	5	6	7	8	9	10		
Ballum	<i>ballum</i> <i>castellonis</i> <i>arborea</i>	Mus 127	10240	20480	2560	5120	5120	5120	1280	1280	20480	20480	80	1280
		Castellón 3	320	320	320	5120	5120	5120	2560	320	640	5120	5120	1280
		Arborea	40	640	1280	5120	5120	5120	2560	640	1280	640	1280	<10
Icterohaemorrhagiae	<i>copenhageni</i>	Wijnberg	640	160	<10	<10	<10	<10	<10	<10	<10	<10	320	<10
Hebdomadis	<i>hebdomadis</i>	Hebdomadis	10240	640	<10	<10	<10	<10	<10	<10	<10	<10	5120	<10
Biflexa	<i>patoc</i>	Patoc 1	<10	20480	<10	<10	<10	<10	<10	<10	<10	<10	<10	160

SDS = unfractionated sodium dodecyl sulphate extract.

TABLE IV
Antigenic specificity of fractions of SDS extract of serotype castellonis strain Castellón 3 (serogroup Ballum)

Rabbit antiserum raised against		Complement-fixation titre against									
		fraction no.									
		1	2	3	4	5	6	7	8	9	10
Ballum	<i>ballum</i>	20480	2560	2560	5120	320	2560	1280	1280	320	320
	<i>castellonis</i>	20480	2560	1280	5120	2560	589	10240	10240	10240	320
	<i>arborea</i>	20480	2560	2560	1280	1280	2560	320	320	320	640
	<i>copenhagani</i>	1280	640	<10	<10	<10	<10	<10	<10	<10	<10
	<i>hebdomadis</i>	1280	5120	<10	<10	<10	<10	<10	<10	<10	640
Biflexa	<i>patoc</i>	<10	20480	<10	<10	<10	<10	<10	<10	<10	160

SDS = unfractionated sodium dodecyl sulphate extract.

TABLE V
Antigenic specificity of fractions of SDS extract of serotype arborea strain Arborea (serogroup Ballum)

Rabbit antiserum raised against		Complement-fixation titre against									
		fraction no.									
		1	2	3	4	5	6	7	8	9	10
Ballum	<i>ballum</i>	40	1280	640	320	320	640	320	320	5120	640
	<i>castellonis</i>	40	320	1280	320	320	320	320	320	2560	640
	<i>arborea</i>	2560	640	320	320	320	320	320	640	10240	2560
	<i>copenhagani</i>	40	320	320	<10	<10	<10	<10	<10	<10	<10
	<i>hebdomadis</i>	1280	320	160	<10	<10	<10	<10	<10	<10	<10
Biflexa	<i>patoc</i>	<10	320	<10	<10	<10	<10	<10	<10	<10	40

SDS = unfractionated sodium dodecyl sulphate extract.

antiserum than with the other antisera and in this respect they appear to be species specific for *L. biflexa* although their genus specificity was also revealed by the positive reactions with all antisera. Fractions 3, 4, 5, 6 and 7 of strain Patoc 1 failed to react in the CFT but fractions 8 and 9 appeared as before to be serotype specific.

Fraction 1 of strain 3055 of serotype *illini* failed to react with any antiserum even the homologous one (table VII). Fraction 2 was genus specific and fractions 8 and 9 serotype specific as before. Unlike the other species, fraction 10 of *illini* reacted strongly with all antisera and so appeared to be genus specific. Only one of two existing strains of this species was available so that confirmatory tests were not possible.

Identification of infecting strain by use of pooled antigen fractions

Adjacent fractions of the SDS extracts of the various leptospiral strains were pooled and an attempt was made to use the pools as a means of identifying the serotypes of the leptospires causing infection in animals by immunodiffusion and CF tests. With the aim of providing a genus-specific antigen, pool I was made up of fractions 1, 2 and 3; for serogroup specificity, pool II comprised fractions 4, 5 and 6, and pool III was made up of fractions 7, 8 and 9 to provide an antigen that could be serotype specific. Fraction 10, which probably was impure, was included in the tests as pool IV. Tests were done on specimens of serum from four naturally infected dogs and an infected cow.

The routine agglutination test had failed to identify the serotype or even the serogroup of the leptospires causing the infection in dog no. 1 in the early stages, because there were many cross reactions among 16 antigen pools representing the various serogroups used in the MAT (table VIII). A later specimen, however, showed it to be an *Icterohaemorrhagiae* infection. By use of the four pools of antigen fractions, differences were revealed in the reactions in the immunodiffusion and CF tests in the first specimen that indicated the identity of the infecting strains (table IX). Thus the first serum of dog no. 1 was shown to have antibodies specific for the serotype-specific fraction of serotype *copenhageni* (*Icterohaemorrhagiae* serogroup) because only pool III of *copenhageni* developed a precipitation line and reacted to a high titre in the CFT against the dog serum taken in the acute stage of the illness.

The routine MAT on the serum specimens of dogs nos. 2 and 3 (table X) indicated recent *Canicola* and *Icterohaemorrhagiae* serogroup infections respectively. These were confirmed by the use of pooled antigenic fractions of serotypes *canicola* and *schueffneri* of the *Canicola* serogroup and of serotypes *icterohaemorrhagiae* and *copenhageni* of the *Icterohaemorrhagiae* serogroup which showed by immunodiffusion and CF tests that the infections were probably due to strains of serotypes *canicola* and *copenhageni* respectively.

Although conclusions could not be drawn from MAT results with serum from dog no. 4 (table X), which showed many cross reactions, an immunodiffusion line specific for serotype *schueffneri* resulted with pool-III fractions of that serotype and was confirmed by a titre of 21 500 in the CFT.

TABLE VI
Antigenic specificity of fractions of SDS extract of serotype patoc strain Patoc 1 (Biflexa complex)

Rabbit antiserum raised against			Complement-fixation titre against										SDS	
serogroup	serotype	strain	fraction no.											
			1	2	3	4	5	6	7	8	9	10		
Biflexa	<i>patoc</i>	Patoc 1	10240	20480	<10	<10	<10	<10	<10	<10	<10	640	160	80
Illini	<i>illini</i>	3055	320	320	<10	<10	<10	<10	<10	<10	<10	<10	640	2560
Icterohaemorrhagiae	<i>copenhageni</i>	Wijnberg	640	640	<10	<10	<10	<10	<10	<10	<10	<10	<10	160
Hebdomadis	<i>hebdomadis</i>	Hebdomadis	160	160	<10	<10	<10	<10	<10	<10	<10	<10	640	160
Ballum	<i>ballum</i>	Mus 127	160	160	<10	<10	<10	<10	<10	<10	<10	<10	160	640

SDS = unfractionated sodium dodecyl sulphate extract.

TABLE VII
Antigenic specificity of fractions of SDS extract of serotype illini strain 3055

Rabbit antiserum raised against			Complement-fixation titre against										SDS	
serogroup	serotype		strain	fraction no.										
	1	2		3	4	5	6	7	8	9	10			
Illini	<i>illini</i>	3055	<10	20480	320	320	320	320	1280	2560	20480	640		
Biflexa	<i>patoc</i>	Patoc 1	<10	10240	<10	<10	<10	<10	<10	<10	20480	640		
Icterohaemorrhagiae	<i>copenhageni</i>	Wijnberg	<10	1280	<10	<10	<10	<10	<10	<10	20480	160		
Hebdomadis	<i>hebdomadis</i>	Hebdomadis	<10	320	<10	<10	<10	<10	<10	<10	20480	640		
Ballum	<i>ballum</i>	Mus 127	<10	10240	<10	<10	<10	<10	<10	<10	20480	160		

SDS = unfractionated sodium dodecyl sulphate extract.

TABLE VIII

Routine microscopic agglutination and complement-fixation tests on sera from dog no. 1*

Antigen	Microscopic-agglutination titre of		Complement-fixation titre of	
	Serum no. 1	Serum no. 2	Serum no. 1	Serum no. 2
Suspension of leptospire of serogroup:				
Icterohaemorrhagiae	1000	1000	...	...
Javanica	300	100	...	...
Canicola	1000	300	...	...
Pyrogenes	1000	300	...	...
Ballum	300	1000	...	...
Autumnalis	1000	300	...	...
Australis	1000	100	...	...
Grippotyphosa	1000	100	...	...
Hebdomadis	300	100	...	...
Sejroe	300	100	...	...
Mini	300	100	...	...
Bataviae	1000	300	...	...
Tarassovi	300	100	...	...
Pomona	300	100	...	...
Cynopteri	300	100	...	...
Biflexa	300	300	...	...
Routine diagnostic antigen†	...	...	2560	2560

* Serum specimens 1 and 2 were collected on days 1 and 14 of illness, respectively.

† See *Materials and methods* for composition.

TABLE IX

Immunodiffusion and complement-fixation tests on the serum of dog no. 1, collected on the day of onset of illness, against pooled antigenic fractions of the serotypes that reacted strongly in the routine microscopic agglutination test

Serotypes fractionated	Number of precipitation lines in immunodiffusion test with antigenic fraction pool no.*				Complement-fixation titre with antigenic fraction pool no.*			
	I	II	III	IV	I	II	III	IV
<i>icterohaemorrhagiae</i>	2	2	0	2	20480	2560	160	10
<i>copenhageni</i>	2	2	2	1	20480	2560	5120	320
<i>canicola</i>	2	0	0	0	20480	320	80	10
<i>ballum</i>	2	0	0	0	5120	320	80	10
<i>autumnalis</i>	2	0	0	0	640	80	40	10

Bold type indicates that similar results were obtained from the second serum specimen, collected on the 14th day of illness, confirming that infection was due to a strain of serotype *copenhageni* of serogroup Icterohaemorrhagiae.

* Specificities: pool I, genus; pool II, group; pool III, type; pool IV, mixed.

The results of the routine MAT on the cow serum made it obvious that the animal had suffered from a recent infection by a strain of the Hebdomadis (Sejroe) serogroup (table XI). The titre was 1000. There was little cross reaction among the various serogroups, except with the three Hebdomadis groups (Hebdomadis, Sejroe and Mini), and because of this and the relatively

TABLE X
Comparison of routine agglutination and complement-fixation tests with immunodiffusion and complement fixation by pooled antigenic fractions, against sera from dogs with clinical leptospirosis

Dog no.	Result of routine agglutination test against leptospirae of serogroup																	Routine complement-fixation titre	Antigen derived from serotype	Number of precipitation lines in immunodiffusion test with antigenic fraction pool no.*			Complement-fixation titre with antigenic fraction pool no.*																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																												
	Dilution of serum	ICT												Javanica	Canicola	Pyrogenes	Ballum			Autumnalis	Australis	GT	Hebdomadis	Sejroe	Mini	Bataviae	Tarassovi	Pomona	Cynopteri	Biflexa	I	II	III																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																		
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ICT and *ict* = Icterohaemorrhagiae and *icterohaemorrhagiae* respectively; GT = Grippityphosa; + = 100% agglutination; ± = 50% agglutination; - = no reaction. Results in bold type are those that indicate the serotype of the infecting strain.

* Specificities: pool I, genus; pool II, group; pool III, type.

TABLE XI
Comparison of routine agglutination and complement-fixation tests with immunodiffusion and complement fixation by pooled antigenic fractions,
against serum from a cow with clinical leptospirosis

Results of routine microscopic agglutination test of leptospire of serogroup																	Routine complement-fixation titre	Serotypes fractionated	Number of precipitation lines in immunodiffusion test with antigenic fraction pool no. *			Complement-fixation titre with antigenic fraction pool no. *					
Dilution of serum																			I	II	III	I	II	III			
	ICT	Javanica	Canicola	Pyrogenes	Ballum	Autumnalis	Australis	GT	Hebdomadis	Sejroe	Mini	Bataviae	Tarassovi	Pomona	Cynopteri	Biflexa		<i>copenhageni</i>									
30	+	-	+	-	-	-	-	-	+	+	+	+	+	-	-	+		2	0	0	10240	160	-				
100	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-		2	0	0	5120	80	-				
300	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-		2	2	0	10240	2560	640				
1000	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-		2	2	0	10240	2560	640				
3000	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-		2	2	2	10240	2560	5120				

Results in bold type are those indicating that the infection was probably due to serotype *hardjo* of serogroup Sejroe, as shown by the microscopic agglutination test. Other footnotes as in table X.

low titre, it is not considered likely that the infection was still in the acute phase despite the abortion that took place on the day on which the specimen was taken.

The immunodiffusion and CF tests with antigen-fraction pools confirmed the impression obtained from the results of the MAT and identified the causative organism as belonging, or being closely related, to serotype *hardjo* of Hebdomadis (Sejroe) serogroup. This was confirmed by the isolation of a strain identified as *hardjo* from the aborted fetus.

DISCUSSION

By means of immunodiffusion and CF tests we have shown that the 10 fractions obtained from each of the leptospiral strains examined contained three different antigenic components that are identifiable as relatively genus specific, group specific and serotype specific. By pooling fractions 1, 2 and 3, fractions 4, 5 and 6 and fractions 7, 8 and 9 these genus-specific, serogroup-specific and serotype-specific antigens may be obtained for diagnostic purposes. Trials of single and pooled fractions for the purpose of identifying the serotype of the infecting strains in the early stages of infection have been promising. Fraction 1 of the strains representing the three serotypes of the Ballum serogroup of *L. interrogans* reacted in the CFT with antisera to all strains of pathogenic leptospira examined whatever their serogroup. They did not however react with the antiserum to the saprophytic strain Patoc 1 of *L. biflexa*. Fraction 1 of strain Patoc 1 on the other hand reacted with all antisera of all strains whether of *L. interrogans*, *L. biflexa* or *L. illini*. This inconsistency may reflect the fact that although the main constituent of fraction 1 of strain Patoc 1 is present in all other serotypes, it may not be present in sufficient amounts in fraction 1 of the pathogenic strains to bring about a positive reaction with the homologous antibodies in Patoc 1 antiserum. Serotypes of the various serogroups within *L. biflexa* will have to be tested to confirm the species specificity of fraction 1.

The fact that fraction 1 of strain 3055 (serotype *illini*) had no reactivity with antisera against other pathogenic or saprophytic leptospiral strains confirms the validity of the conclusion of the Subcommittee on the Taxonomy of *Leptospira* (1978) that the strain does not belong either to *L. interrogans* or *L. biflexa* but represents a *species incertae sedis* named *Leptospira illini*.

Fraction 2 of strain 3055 was reactive in the CF test with all antisera against pathogenic and saprophytic strains. It appears therefore that whereas fraction 1 represents a species-specific antigenic component, fraction 2 is specific for the genus *Leptospira* as a whole.

Although fractions 3–9 from the three serotypes of serogroup Ballum reacted only with the Ballum serogroup antisera, fractions 8 and 9 showed considerably higher titres with the homologous serotype antiserum and therefore probably contain the serotype-specific main factor, while the low-titre reactions of antisera to other serotypes within the Ballum serogroup to the same fractions reveal the additional presence of serogroup-specific factors.

These results may explain the observations of Cacciapuoti (1976) who reported the regular development during the course of leptospiral infections of high-titre agglutinating and complement-fixing antibodies which are variably associated with one or more serotype antigens apparently not related to the infecting strain.

SUMMARY

Sodium dodecyl sulphate extracts of the reference strains Mus 127, Castellón 3 and Arborea of the Ballum agglutinogenic serogroup of *Leptospira interrogans* (the species of pathogenic leptospira), of strain Patoc 1 of the saprophytic species of *L. biflexa*, and of strain 3055 of *illini* serotype, the sole representative of *L. illini*, were each fractionated by ultracentrifugation in a sucrose density gradient into 10 fractions. The fractions were tested by complement fixation and immunodiffusion against the sera of animals during the process of immunisation and during the course of naturally occurring infections.

The fractions could be divided into three main pools of serological activity: pool I (fractions 1, 2 and 3), pool II (fractions 4, 5 and 6), pool III (fractions 7, 8 and 9). Pool I was species/genus specific; fraction 1 tended to be species specific while fraction 2 reacted with antisera to all strains whether pathogenic or saprophytic. Pool II was serogroup specific and reacted only with antisera to members of the same serogroup. Pool III was serotype (serovar) specific and revealed the identity of the infecting strain at an early stage of infection.

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