THE ROUTINE APPLICATION OF A MICROTECHNIQUE FOR THE DEMONSTRATION OF LEPTOSPIRAL ANTIBODIES

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THE detection of leptospiral antibodies by the conventional microscopic agglutination (MA) test is laborious and time consuming. It involves transferring samples of the completed agglutination test on to slides for microscopic examination and this exposes the operator to the live leptospirae used as antigens.

The advantages of the microtechnique described by Galton *et al.* (1965) over the MA test are as follows. (1) Small volumes of sera and antigens (total volume 50 μ l) are used. (2) Dilution techniques with microdiluters are rapid. (3) The test can be read directly on the plate. (4) There is reduced exposure to live antigens.

Galton *et al.* (1965) compared the microtechnique with the MA test and the results obtained on 635 sera showed 87% agreement. Later, Cole, Sulzer and Pursell (1973) examined 298 sera and found only 77% agreement. The microtechnique has been used in this laboratory over a period of 3 years and the experience gained suggests that it is a useful method that may be of interest to other workers.

MATERIALS AND METHODS

Serum samples

The microtechnique was used to examine sera received from outbreaks of abortion and from herds in which high titres of leptospiral antibodies had previously been demonstrated.

Equipment

The following items were used: polystyrene disposable Microtitre Plates with 96 wells, and lids (Dynatech, Billingshurst, Sussex, England); a Terumo Microsyringe 100 μ l with 2- μ l calibrations (Shandon-Southern, Camberley, Surrey, England); Titertek Pipette Droppers 25 μ l and Microdiluters 25 μ l, and the Titertek Microtitration System Instruction Manual (Flow Laboratories, Irvine, Scotland).

A jet of clean compressed air was used to blow out any dust or plastic particles from the wells of the plate and from the inner surface of the lid before use. The plates were used approximately six times, or until they were so badly scratched that reading of the test became difficult. After use, the plates were sterilised by overnight immersion in a 1 in 20 dilution of a hypochlorite solution (Chlorox; Imperial Chemical Industries), drained, rinsed several times in tap water, and then de-ionised water, and dried in the inverted position in a drying cabinet. The Titertek Microdiluters were favoured over other types because they produced only minimal scratching of the wells.

Antigen suspension

The antigens were live, 7-day-old cultures grown in Korthof's medium at 30°C. These cultures contained *c.* 2×10^8 leptospirae per ml. Checks on the numbers of leptospirae were made with a nephelometer, and for each serovar (serotype) used a graph was prepared to relate nephelometer units to viable count. Where necessary the cultures were diluted to contain *c.* 2×10^8 organisms per ml. The cultures were grown in 10-ml volumes of medium

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from inocula that consisted of 1-ml volumes of 7-day-old cultures. Polysorbate medium was tried as a growth medium but proved unsatisfactory because of a tendency for members of the *Hebdomadis* group to produce clumps of leptospirae ("brood nests").

Performance of the test

All sera examined were first screened at a dilution of 1 in 100. Any serum positive at this titre was then titrated in twofold steps from 1 in 100 to 1 in 3200, or to a higher dilution if necessary. The sera were screened routinely against four serovars that had been detected by previous serological examinations in the Carmarthen district. The four serovars used were *wolfii* or *hardjo* (*Hebdomadis* serogroup), *icterohaemorrhagiae*, *pomona* and *canicola*. Four wells were used for each serum, thereby allowing 24 sera to be examined against four antigens on each plate, the arrangement being as shown in fig. 1.

The sera were screened as follows. A 1 in 50 dilution in phosphate buffered saline (PBS), pH 7.2, was made by drawing up 98 μ l of PBS and 2 μ l of serum into the microsyringe. This was then discharged into the first of the four appropriate wells, e.g., A1 (see fig. 1) and mixed by filling and discharging the syringe three or four times. Care had to be taken to avoid bubble formation, as this prevented accurate measurements being made. By means of the microsyringe, 25 μ l of the 1 in 50 serum dilution was transferred to each of three remaining wells e.g., B1, C1 and D1 (fig. 1). A drop of antigen suspension was added to each appropriate well as shown in fig. 1, e.g., *wolfii* was added to wells A1 to A12 and E1 to E12 inclusive; the addition of antigen suspension was made with a 25- μ l pipette dropper, to give a final concentration of 1 in 100 and a final volume of 50 μ l. Each plate was then sealed with a lid, gently tapped to mix the contents of the wells and incubated at 37°C for 2.5 h. Hyperimmune control sera prepared against each antigen used were titrated with each batch of tests. The tests were then read as described later.

Any serum producing agglutination of 75% or more leptospiral cells in the screening test was then titrated from 1 in 100 to 1 in 3200 in two-fold steps. Any serum positive at the 1 in 3200 dilution was later titrated in two-fold steps to find the last dilution that gave more than 50% agglutination.

Six wells were used for each serum titrated, thereby allowing 16 sera to be examined against a single antigen on each plate, the arrangement being as shown in fig. 2. The titrations

		Plate _____												Date _____	
Ref numbers		1	2	3	4	5	6	7	8	9	10	11	12		
L. wolfii	A													A	L. wolfii
L. ictero	B													B	L. ictero
L. pomona	C													C	L. pomona
L. canicola	D													D	L. canicola
L. wolfii	E													E	L. wolfii
L. ictero	F													F	L. ictero
L. pomona	G													G	L. pomona
L. canicola	H													H	L. canicola
Ref numbers		1	2	3	4	5	6	7	8	9	10	11	12		

FIG. 1.—Arrangement of Microtitre Plate for screening examinations.

												Plate _____	
												Date _____	
						100	200	400	800	1600	3200		
Ref. numbers	1	2	3	4	5	6	7	8	9	10	11	12	Ref. numbers
A													A
B													B
C													C
D													D
E													E
F													F
G													G
H													H
	1	2	3	4	5	6	7	8	9	10	11	12	
													Antigen _____

FIG. 2.—Arrangement of Microtitre Plate for titration examination.

were so arranged that separate plates were used for each antigen. To each of the first two wells, e.g., A1 and A2 (fig. 2), were added 25 μ l of a 1 in 50 dilution of the appropriate serum, diluted and dispensed as before with the microsyringe. To each well, except the first of the six, e.g., wells A2–A6 inclusive, were added 25 μ l of PBS with a 25- μ l dropping pipette. A 25- μ l microdiluter was used, according to the technique described in the Microtitration Instruction Manual, to dilute the serum from the second to the sixth well, e.g., A2 to A6, to give a range of dilutions in twofold steps from 1 in 50 to 1 in 1600. When a full plate of tests had to be diluted, eight diluters could be handled together, so that eight serial dilutions could be completed as quickly as one. Antigen was added and the procedure was then as described above.

Reading the test

After incubation the plates were placed on a black glass plate attached to the stage of a dissecting microscope with $\times 15$ widefield eyepieces and a 32-mm objective. The microscope lamp was adjusted so that the light struck the plate from above, at an angle of approximately 45° (Galton *et al.*, 1965). The degrees of agglutination were recorded as 1, 2, 3 and 4, figures that represented the agglutination of 25%, 50%, 75% and 100% of leptospirae, respectively. The scoring of the degrees of agglutination was originally made by examining a large number of positive sera by the microtechnique, and making additional readings by transferring samples on to microscope slides and observing them under darkground illumination. After sufficient experience had been gained, examination under darkground illumination was discontinued, apart from occasional checks to confirm that the accuracy of the microtechnique readings was being maintained.

Under the dissecting microscope, the agglutinates appeared as white, slightly translucent globular particles, and with the light favourably adjusted, the free, motile leptospirae were observed as tiny refractile particles in the background. The knowledge that free leptospirae should be visible prevented the recording of false negative reactions in instances in which antigen had been omitted in error.

Occasionally low dilutions of certain sera, usually those with high titres, gave 100% agglutination reactions consisting mainly of large numbers of small agglutinates; at higher dilutions the agglutinates were of the usual large-globular type.

DISCUSSION

The microtechnique was found to be a suitable means of detecting leptospiral antibodies in large numbers (>8000) of serum samples. It gave results that allowed accurate comparisons to be made between individual animals, and between different geographical areas. The microtechnique-dilution and direct-reading procedures enabled the tests to be completed in approximately a quarter of the time required for the MA test. The use of automatic multidispensers and multidiluters would reduce the time even more. The results were read directly on the plates, thus avoiding the transfer of samples to microscope slides for observation of agglutination, and so reducing the exposure of the operator to live antigens. This is an even more important feature when the microtechnique is used for determining the serovar of a freshly isolated leptospiral strain. The simplicity of the microscopical apparatus allowed the microtechnique to be performed with existing equipment; the black glass plate was obtained from a local glazier.

When the disposable polystyrene plates were used once only, the cost per sample screened was approximately 0.5 new penny; when the plates were sterilised, washed and used several times the cost per sample was greatly reduced and the efficiency of the test was unaffected.

SUMMARY

The microtechnique of Galton *et al.* (1965) for the routine screening of blood samples for leptospiral antibodies was used to examine more than 8000 serum samples from animals that had aborted and from herds in which leptospiral antibodies had previously been demonstrated. The method gave consistently accurate results. In respect of ease and speed of performance, it was superior to the microscopic agglutination test and it required only minute volumes of serum and antigen suspension. Its most important advantage was that the agglutination reaction could be read directly on the plate, and the removal of samples for microscopical examination was therefore unnecessary.

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