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WORLD HEALTH ORGANIZATION
TECHNICAL REPORT SERIES

No. 113

**DIAGNOSIS AND TYPING IN
LEPTOSPIROSIS**

Report of a Study Group

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WORLD HEALTH ORGANIZATION

PALAIS DES NATIONS

GENEVA

DECEMBER 1956

STUDY GROUP ON LEPTOSPIROSIS

Amsterdam, 2-4 November 1955

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The report of this study group was originally issued in mimeographed form as document WHO/Zoon/41, 8 February 1956.

PRINTED IN SWITZERLAND

DIAGNOSIS AND TYPING IN LEPTOSPIROSIS

Report of a Study Group

In December 1952, a symposium on leptospirosis was held in Washington, D.C., under the auspices of the US Army Medical Service Graduate School. The proceedings of this meeting, which was attended by leading leptospirosis workers of the USA and Europe, have since been published.¹ Immediately following the symposium, WHO convened an informal meeting of some of the experts to explore the knowledge to date of epidemiological aspects of the disease, and to determine where international action was needed to increase the effectiveness of public-health control measures. It was evident that one of the great difficulties in the field of leptospirosis was the lack of agreement among laboratory workers on diagnostic methods and on the classification of leptospire. The lack of simplified survey techniques and uniform laboratory methods which would permit better comparison of results obtained in different countries has been one of the greatest drawbacks in this field. Recently, WHO undertook work to clarify for international purposes the laboratory aspects of leptospirosis, and a meeting of a study-group on leptospirosis was convened by WHO to discuss the results obtained to date and to plan for further work in this connexion. The Study-Group met at the Institute for Tropical Hygiene and Geographical Pathology, Amsterdam, from 2 to 4 November 1955. Professor J. W. Wolff was elected Chairman of the meeting and Dr J. C. Broom Rapporteur.

1. CLASSIFICATION

It was agreed that the method at present in use in many leading leptospirosis laboratories which divides the genus *Leptospira* into serotypes on the basis of agglutigen characters, as determined by agglutination and cross-absorption reactions with immune rabbit sera, is the best available (see Annex 2, page 11).

It was further agreed that no alteration should be recommended in the criteria (accepted by the *Leptospira* Subcommittee of the International

¹ United States of America, Army Medical Service Graduate School (1953) *Symposium on the leptospiroses*, Washington, D.C. (Medical Science Publication No. 1)

Committee on Bacteriological Nomenclature at the Sixth International Congress of Microbiology, 1953)¹ for differentiating one serotype from another. Thus two strains are considered to belong to different serotypes if, after cross-absorption with adequate amounts of heterologous antigen, 10% or more of the homologous titre regularly remains in each of the two antisera.

The Study-Group appreciated that limits lower than 10% have been advocated, and in certain instances can be used with validity for finer differentiation of serotypes. The use of these lower limits would provide a method of bringing out the minor antigenic differences which may be present among strains which belong to the same serotype on the 10% criterion. If constant, these differences might be of epidemiological significance. In addition, the Study-Group fully appreciated the fact that biochemical methods of antigenic fractionation of leptospire, or other methods, may in the future provide a new and more satisfactory basis of differentiation.

2. REFERENCE STANDARD SERA AND REFERENCE LABORATORIES

During the past year a collaborative effort, assisted by WHO, was undertaken by eight laboratories² to assay the homologous agglutinin content of antisera to six serotypes of leptospire. This work was undertaken as a pilot study to work out procedures whereby reference standard sera might be prepared in the future for international use. The results of the assays were studied and the Study-Group came to the following conclusions:

(1) The preparation of such sera would serve a very valuable function in international work on leptospirosis.

¹ *Int. Bull. bact. Nom. Taxon.*, 1954, 4, 115

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(2) The specific antiserum should be prepared, if possible, for all the well-established serotypes and subtypes (biotypes). These sera would be prepared in dried form and would conform as far as possible to the procedures used for the preparation of international standard sera. This would involve uniform preparations, ampouling, freeze-drying, etc., and subsequent check-testing by the various collaborating laboratories.

(3) The reference standard sera would be placed at the disposal of leptospirosis reference laboratories, established in collaboration with WHO and FAO to serve different regions of the world. It was suggested that reference laboratories be established in Amsterdam, London, Rome, Tokyo, and Washington, D.C., and that additional laboratories be established in the future, when desirable, to serve various geographical areas.¹

(4) The reference standard sera would be used principally to check periodically the purity of the basic leptospiral strains supplied from the type collection held at Amsterdam which are accepted as representing the original type species.

(5) The reference laboratories would supply cultures of the strains upon request to national laboratories or other recognized laboratories working in the field of leptospirosis in their region. Whenever possible, the reference laboratories would assist these other leptospirosis laboratories in identifying and classifying new strains which have given difficulty, and would render other services, when feasible, such as supplying locally prepared specific antisera, and accepting trainees for limited periods to learn laboratory procedures in leptospirosis.

3. DIAGNOSTIC METHODS

3.1 Culture

The most definite method of diagnosing leptospirosis is the isolation of the organism, and blood culture provides one of the best means of accomplishing this purpose. (The preparation of suitable media, such as Fletcher's, Vervoort's, and Korthof's, has been described in a monograph by Wolff.²) Blood specimens should be taken as early as possible in the course of the illness, preferably during the first few days. A few drops of blood should be added to 5-6 ml of culture medium, and the culture can then be sent to a suitable laboratory for examination. It is preferable to incubate these cultures at 37°C and to examine them at intervals of 4 to 7

¹ The leptospirosis reference laboratories designated by WHO and FAO up to 30 November 1956 are listed in Annex 1 (page 10). — ED.

² Wolff, J. W. (1954) *The laboratory diagnosis of leptospirosis*, Springfield, Ill., p. 19

days for at least one month ; where no incubator is available, lower temperatures (about 25°C) can be used. For blood cultures it might be convenient to use serum bottles with rubber diaphragms. Fluid or whole blood can be used for the inoculum, or the blood-clot can be ground and a few drops added to the culture bottles. Sometimes it is advisable to defibrinate the blood, especially when complement-fixation or agglutination tests are to be performed on the serum.

The administration of antibiotics to a patient does not necessarily affect the efficacy of blood cultures. However, it is strongly advisable to take the first blood culture before the administration of antibiotics. Several blood specimens should be taken during the febrile period.

Cerebrospinal fluid or aseptically obtained urine can be cultured in the same manner as whole blood, using an inoculum of 0.5 ml to 5 ml of culture medium.

3.2 Animal inoculations

Where material which is not bacteriologically sterile is involved, animal inoculations are advisable, and the material should be injected as soon as possible after the specimen has been obtained. Young guinea-pigs, weighing about 200 g, are most commonly used, but many serotypes do not produce fatal infection in these animals. It is therefore desirable to examine by darkfield microscope a drop of peritoneal fluid withdrawn between the second and the seventh day following inoculation of the specimens. If positive, blood can be taken by heart-puncture for culture and future identification of the strain. Some laboratories routinely culture the blood of inoculated guinea-pigs on the seventh day following inoculation of the specimen.

Blood samples should be taken from the guinea-pig one month after inoculation and examined for the possible presence of leptospiral antibodies.

The use of 1- to 2-day-old chicks has been very successful for the isolation of the organism. These birds are inoculated intraperitoneally with urine and other possibly contaminated materials. The chick's heart-blood is examined under the darkfield microscope 6 days after inoculation.

Nine- to eleven-day-old embryonated eggs can be used for isolation of leptospire from uncontaminated materials. Allantoic fluid is examined when the embryo appears to be sluggish on candling.

Young hamsters (3-6 weeks old) and meriones (about 6 weeks old) have been found particularly useful for the isolation of certain serotypes where the use of guinea-pigs has not been wholly satisfactory, for instance with *L. canicola* and *L. grippityphosa*.

It is inadvisable to use white mice or rats for attempted isolation of leptospire because these animals are frequently found to be natural carriers.

Workers inexperienced with *Leptospira* should not attempt to make a diagnosis of leptospirosis by direct darkfield examination only, because artefacts similar to *Leptospira* are commonly observed.

A rough guide to the expectations of recovery of leptospire by various methods using blood and other specimens withdrawn at different times following the onset of the disease is given in the following table.

MOST SUITABLE TECHNIQUES FOR THE DIAGNOSIS OF LEPTOSPIROSIS

Method	Stage of disease	Specimens examined				
		Blood	Cerebro-spinal fluid	Urine	Liver tissue	Kidney tissue
Darkfield examination	First 8 days Later	+	+	+	+	+ +
Animal inoculation	First 8 days Later	+	+	+	+	+ +
Direct culture	First 8 days Later	+	+		+	+ +
Serological test	First 8 days Later	(+) +	(+) +			

(+) = result likely to be negative but valuable for comparison with test at a later date.

3.3 Serological tests

3.3.1 *The agglutination-lysis test*

The agglutination-lysis test using living cultures is considered to be the best single test for the demonstration of leptospiral antibodies. The essentials of the technique are described in Wolff's monograph,¹ but laboratories frequently differ with respect to the criteria for an end-point reading. The Study-Group considers that the end-point is the highest dilution of serum in which a distinct difference is apparent in comparison with the control, as well as with the next higher dilution of serum. These differences consist, either singly or in combination, of the degree of agglutination and the number of free organisms.

¹ Wolff, J. W. (1954) *The laboratory diagnosis of leptospirosis*, Springfield, Ill., p. 39

3.3.2 *Straight agglutination tests (microscopic)*

The use of living cultures may present difficulties and, in these circumstances, killed antigens may be substituted. Formalin-killed suspensions of leptospire are most commonly used. Difficulties are often encountered with killed antigens because they are not stable for any fixed period. Killed antigens, when used, should always be controlled for evidence of auto-agglutination or loss of sensitivity. (This applies equally to live antigens.)

In screening-procedures, reduction of work is possible by combining three serotypes as a single antigen. Another method is to combine up to five serum specimens tested against single serotype antigens. The detection of positive reactions in such combinations would then have to be followed by testing single serotype antigens or single sera, respectively.

In routine tests it is almost impossible to include all known antigenically different strains, so that the number of strains must be limited according to local laboratory facilities, at the same time covering the widest possible diagnostic field. The choice and number of strains included in investigations may therefore vary and depend on: (a) the scope and special character of the investigation, and (b) the distribution of the different leptospiral infections known or suspected to occur in the area.

For more definite typing purposes, the agglutination-lysis test is preferable to that with killed antigens because the cross-reactions obtained with killed antigens may differ from those of the agglutination-lysis test.

Various modifications of macroscopic tests with killed antigens have been described, such as plate and capillary-tube tests, etc., but the Study-Group considers that more work is required before a definite recommendation can be made.

3.3.3 *Interpretation of agglutination tests*

For diagnostic purposes in individual cases of illness, the most significant feature for a positive diagnosis is the finding of a rising titre in two serum specimens taken several days apart, provided the first is taken early in the course of the disease. On single specimens no definite statement can be made, but a relatively high titre found in conjunction with clinical signs is presumptive evidence of leptospirosis. Some workers have reported low levels or absence of antibodies when antibiotics have been administered very early in the disease.

When making serological surveys it should be borne in mind that the length of time for which antibodies persist, and the height of the titres, vary in different individuals, and are also affected by the serotype of the original infection.

Normally, screening-tests with both living and killed antigens, using the microscope, can be carried out at a single dilution of 1/100 of serum, and a positive reaction at this dilution can be considered as evidence of past or present infection with the leptospiral organism.

3.3.4 *Complement-fixation tests*

Several complement-fixing antigens have been developed for the detection of leptospiral antibodies. These differ widely in their methods of preparation and in the broadness of their antigenic spectra. Some antigens seem to be essentially either serotype- or group-specific, while others are intermediate in their cross-reactions.

The antigen-antibody systems concerned differ from those of the agglutination-lysis and agglutination phenomena. From the practical standpoint this is of significance, as complement-fixing antibodies are sometimes detectable earlier than agglutinating antibodies; moreover, they fall to undetectable levels much sooner than agglutinating antibodies. This restricts the utilization of complement-fixation techniques to the serodiagnosis of acute or recent leptospirosis and precludes their use in serological surveys directed towards estimation of prior infection with leptospores.

At the present stage of development, complexities of preparation and standardization of antigens, and the exacting techniques required in the performance of leptospiral complement-fixation examinations, limit their utilization to research laboratories familiar with the particular complement-fixing antigens they employ. In these laboratories, complement-fixation tests have proved extremely valuable in investigations of acute febrile disease in man and animals. These complement-fixation techniques, however, are not at present recommended for use by routine diagnostic laboratories.

3.3.5 *Other fluids*

Agglutinins can also be detected in the cerebrospinal fluid, urine, milk, and the aqueous humour of the eye. In special circumstances examination of these fluids may provide useful information.

Annex 1

WHO/FAO LEPTOSPIROSIS REFERENCE LABORATORIES

Following the recommendations made by the Study Group on Leptospirosis (see page 5), WHO/FAO Leptospirosis Reference Laboratories have been designated,¹ in agreement with the governments concerned, at the following places :

Institute for Tropical Hygiene and Geographical Pathology
(Royal Tropical Institute)
Mauritskade 57 A
Amsterdam, Netherlands

The Wellcome Laboratories of Tropical Medicine
183-193 Euston Road
London N.W.1, England

Division of Veterinary Medicine
Walter Reed Army Institute of Research
Washington, D.C., USA

Department of Viral and Rickettsial Diseases
National Institute of Health
Tokyo, Japan

The functions of the reference laboratories are : (a) to supply cultures of *Leptospira* strains and specific antisera upon request to national laboratories or other recognized laboratories working in the field of leptospirosis in their region ; (b) to assist these other leptospirosis laboratories, wherever possible, in identifying and classifying new strains which have given difficulty, and to render other services such as supplying locally prepared specific antisera ; (c) if feasible, to accept trainees for limited periods to learn laboratory procedures in leptospirosis.

It is anticipated that early in 1957 standard reference sera in dried form will have been prepared for the major serotypes of *Leptospira* and will be available, along with cultures of the homologous serotype, for supply to laboratories, as indicated in the preceding paragraph.

¹ Negotiations are under way to designate additional reference laboratories. The work of all these laboratories is being co-ordinated by the Veterinary Public Health Section, WHO, Geneva, from which further information can be obtained.

