Preparation, use and maintenance of antigens and antisera

MICROSCOPIC AGGLUTINATION TEST (MAT) FOR THE DIAGNOSIS OF LEPTOSPIROSIS AND SEROTYPING OF LEPTOSPIRES

H. Korver

Department of Biomedical Research,
Royal Tropical Institute, Meibergdreef 39,
11005 AZ Amsterdam,
The Netherlands

Get PDF file

The Microscopic agglutination test (MAT) is the basis of serological diagnosis and classification. In 1926 Schuffner and Mochtar described the phenomenon of agglutination and lysis with human and animal sera and made their dilution series with drops from one and the same pipette. The reaction was read after 16-20 hours.
Since then the method was improved by others (Borg-Petersen and Fagroeus, 1949; Wolff, 1954; Carbrey, 1960 and Cole et al., 1973). They tried to standardize factors like incubation time, incubation temperature, reading of the end point titer and density and age of the culture. To discuss these variables it is necessary to define the microscopic agglutination test which is still used today for different purposes.

This definition could be as follows:

Serial dilution of serum kept in contact with an equal volume of a well grown suspension of leptospires at a certain temperature for a certain period of time and read microscopically by estimating 50% agglutination as the end point titer of he reaction mixture.

The various variables are underlined in this definition and shall be discussed. This paper also explains the limitations of the MAT and of the interpretation which may be made of the results of such tests.

Serial dilution

In the twenties of this century investigators in the field of leptospirosis used a method which at that time could not be standardized due to tool-to-tool variance of equipment. Over the years progress was made when more sophisticated standard volume pipettes became commercially available.

Well grown suspension of leptospires

The titers of agglutination tests may be effected by the quality of the antigen suspensions and the strains used. Borg-Petersen and Fagroeus (1949) tried to standardize the density of the leptospira antigen. They compared their antigens with one of the tubes of the McFarland scale. Culture densities can also be estimated by spectrophotometry, darkfield microscopy and counting of leptospires. Within certain limits these methods can be used but we have to keep in mind that the thickness of length of the leptospires are sometimes different among leptospira strains. It is a well known fact that when the antigen density decreases the serum titer increases sometimes considerably. The agglutinability of certain leptospira strains, especially those which are already maintained and subcultured in reference collections for decades, may also affect the MAT results. Local strains and fresh isolates for a certain serovar tend to react better than reference strains for the same serovar.
This may be an advantage especially when a serum sample is taken very early in the disease and antibody levels are very low. In general, in many laboratories 5-7 days old subcultures are being used. The influence of the age of a culture is of lesser importance although by increasing the age of a culture a slight decrease in serum titer can be found. Many laboratories like to use live leptospires and stead of formalin killed ones. The advantage of live leptospires is the ability to react higher in he MAT than killed antigens. The disadvantage of live antigen is the fact that many strains should be subcultured every week to have optimal conditions for the performance of the test. An advantageous circumstance of the use of killed leptospires is that the risk of infection is avoided and formalized cultures can be kept in sealed tubes for several weeks but their sensitivity should be controlled every two weeks. Carbrey (1960) concluded in his study about the relative importance of variable factors in the MAT that a fourfold dilution in antigen density could cause a twofold increase in serum titer. In the same study increasing the age of an antigen culture was thought to decrease its sensitivity.

Temperature and time

Some investigators claim that agglutination occurs within several minutes at room temperature (Ryu, 1970) but most laboratories use an incubation period of as lest two hours for their test. Incubation at temperatures between 20C (room temperature) and 37C show no significant changes in titer values. In general, at lower temperatures longer time is needed for incubation.

End point titer

The degree of agglutination ranges from 100% when no free leptospires can be discovered to nil. The end-point of the MAT with live leptospires is the final dilution of serum at which 50% of the leptospires are agglutinated i.e. 50% of leptospires remain free as compared with a control suspension. The estimation of 50% agglutination in relation to 50% free leptospires is not always very clear. The transition from clumps of leptospires to free non-agglutinating leptospires is sometimes not abrupt but diminishes too gradually over a few dilution which makes it difficult to determine the end-point titer. Furthermore, there is not only a person to person but also a lab to lab variability due to the application of different criteria. For the reading of MAT titers it is highly recommended to use a darkfield microscope with a high contrast between the leptospires and the dark background.

Serodiagnosis

General remarks

In leptospirosis antibodies can be demonstrated in the serum by MAT. The MAT is the keystone for a correct diagnosis (Wolff, 1954).
One can start to detect antibodies to leptospira 7-10 days after the onset of the disease. When receiving a blood sample during the first week of the disease, it is important to examine a second sample after another 1-2 weeks to see if there is seroconversion or a rise in titer. Seroconversion or a rise in titer may be indicative for current leptospirosis.
In the agglutination tests for serodiagnosis usually representative group antigens are used. The number and choice of stains should be adapted to the aim of the investigations. Local modifications are necessary for diagnosis as well as for epidemiological purposes. When a serum reacts with strains of different serological types it is generally assumed that the infection was caused by a leptospira of the same serologic type as the strain which gives the highest titer. This conclusion is valid in epidemiological studies provided the test is performed with strains of all serological types which cause leptospirosis in the study population and provided the serum sample is taken sufficiently late after the disease. Cross reacting antibodies will decrease more rapidly in titer than values with the homologous agent.
Borg-Petersen (1949) remarked that in the first few weeks of the disease it is not uncommon that heterologous reactions are stronger than the homologous reactions. This phenomenon is called "paradoxical reaction" and was thoroughly studied by Kmety (1957). For example, during the acute phase of Icterohaemorrhagiae infections paradoxical reactions may occur with heterologous serovars such as sejroe, bratislava or andamana.
One should never depend entirely upon the serologic reactions of the patients serum in determining the infecting serotype.
A negative MAT reaction even on serial samples does not rule out the possibility of infection, since the patient may be infected with a serotype not included in the battery of antigens.
Furthermore early antibiotic treatment may suppress the development of leptospiral antibodies. Paradoxical reactions with certain strains act as a signal for an acute leptospirosis. Serodiagnosis of leptospirosis in not particularly difficult by MAT, but reliability depends on the availability of a large number of representative strains of Leptospira and on specialized experience.
Apart from the variables inherent in the serological tests the antibody response of the host to leptospiral infections is also variable.
The results must be considered for each individual case and evaluated in relation to all other, especially clinically and epidemiological data.

Serotyping

General

Antigen-antibody reactions are used for the identification of strains. The antigenic structure of leptospires is complex. Serogroups are comprised of serovars which cross agglutinate to high titers with each others antisera (Dikken and Kmety, 1978). Serogroups cannot be defined accurately and have no official status.
Serogroups serve the practical purpose of combining strains with antigenic similarity. Grouping is a necessity in view of the large number of over 200 leptospiral reference strains which for practical reasons cannot be used or assessed individually. There is no sharp definition of a serogroup and in the past serovars have shifted between serogroups due to blurred delineation between groups.
The definition of a serovar which was first formulated by Wolff and Broom (1954) was not only for systematic purposes but also for practical application and describing host-parasite relationships.
The same authors summarized and extended the work of designing a scheme of serogroups and serotypes on which the current classification is still based.
The conventional method of serotyping involves two procedures.
First the unknown strain is used as antigen suspension in titration with a range of rabbit antisera representing all recognized serogroups to determine the group status of a strain and to investigate the relationship of the unknown strain with other reference strains within the same serogroup.

Arrangements into serogroups has limitations. Strains might be allocated to either of two serogroups. Some of the serogroups have become so extended that they should be rearranged. Two examples are the division of the original Hebdomadis group into Hebdomadis, Mini and Sejroe serogroups and the division of the original Autumnalis group into Autumnalis, Djasiman and Louisiana serogroups.

The second procedure involves comparing cross agglutination-adsorption reactions of the unknown strain and its antiserum with relevant reference strains and their antisera.

Cross agglutination

The basic taxon is the serovar which is represented by a reference strain. Serotypes which belong to the same serogroup cross react at high titers.

Commonly a strain representing a new serovar reacts with a certain antiserum but sometimes strains react at high titers with more than one antiserum.

Further tests with antisera against other serovars belonging to the serogroups under study should resolve the problem.

Cross absorption

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 titer regularly remains in at least one of the two antisera in repeated tests.

The amount of antigen to be used for absorption is a very important factor (Wolff and Broom, 1954; Kmety et al., 1970).

It is important that the quantitative relationship between antigen and immune serum to be absorbed should be well balanced to avoid possible non specific absorption by excessive amounts of antigen (Babudieri, 1971). Therefore in our laboratory variable amounts of antigen are mixed with the antiserum. This antiserum is standardized to a MAT titer of 1:5,120. The absorbed antiserum whose titer with the absorbing strain is approaching zero (and should not exceed 1% of the pre-absorption titer) is used for he agglutination test with the homologous strain. Tests are repeated several times, if necessary.

Typing methods always depend on the definition of the basic taxon (Dikken and Kmety, 1978). This means that in typing leptospires at present, the 10% limit criterion is decisive.

This rule leaves a 0-10% margin of difference for strains belonging to the same serovar (Faine, 1982). The cross agglutination absorption test is laborious and time-consuming which restricts the identification of strains mainly to specialized laboratories.

Reference laboratories have searched many years for a quick typing of leptospira strains. This can be done by factor analysis (Kmety 1967; Dikken and Kmety, 1978) and identification of isolates with monoclonal antibodies (Terpstra et al., 1985; Terpstra et al., 1987).

Factor analysis

The official definition does not exactly characterize each serovar, but rather states the degree of serological difference necessary for the description of a strain to represent a new serovar. Kmety's factor analysis (1967) represents a more detailed study of the antigenic structure of each serovar which is characterized bs its own particular combination or mosaic of main and minor antigenic factors. Factor sera are prepared by absorbing with one or more different antigen suspensions until they react only with one serovar, a subgroup or serogroup.

Factor analysis is a highly refined method to study degrees of antigenic similarities between strains (Kmety, 1967). Reaction comparison allows a rapid provisional determination of the serovar status of the strain (Dikken and Kmety, 1978).

The preparation of factor sera is laborious and reproducibility may be weak due to batch-to-batch variation. Still, factorsera are useful for a quick presumptive typing.

Monoclonal antibodies (MCAs)

Characterization with monoclonal antibodies is related to conventional typing and based on the recognition of characteristic antigen patterns of serovars by panels of MCAs.

The specificity of MCAs is limited among other things by the antigenic structure of the immunizing strain and the immunological repertoire of the mouse (Terpstra, 1991).

A strain may be characterized by a specific combination of traits (mosaic of epitopes).

One of the advantages of working with MCAs is that a large number of strains can be typed in a short time.

More than half of the more than two hundred presently recognized serovars can now be identified to serovar level.

Mislabeled strains can be traced accurately and more easily than with the use of ordinary rabbit antisera. Differences in agglutination profiles obtained with a panel of MCAs may be indicative for new serovars and even differences between strains belonging to the sam serovar may be observed (Terpstra et all, 1985).

Peripheral laboratories can type quickly and easily when equipped with panels of MCAs, adjusted to the locally circulating strains.

Current developments

The composition of a panel of serovars recommended for use in the MAT depends upon the purpose of examination. Generally it is advisable for the average diagnostic laboratory to keep the test simple. The success and reliability of the laboratory investigations depend largely on antigens of good quality.

Most important factors in the MAT are the antigen density and the definition of the end-point. Formalized antigens usually remain stable for a period of several weeks (Turner, 1868; Sulzer and Jones, 1974). They should be checked with positive and negative sera before use and watched closely for spontaneous agglutination. Leptospires can be grown in large quantities in shaking incubators in appropriate media.

References
1. Babudieri B. Proposed standardization of the agglutination-adsorption test for leptospira. Bull Wld Hlth Org, 1971;44:795-810.
2. Borg-Petersen C, Fagroeus A. The influence of the antigen density and other factors on the serum titer in the agglutination-lysis-test for Leptospirosis. Acta Path, 1949; XXVI:4.
3. Carbrey EA. The relative importance of variable factors in the agglutination-lysis test. 64th Annual Proceedings United States Livestock Sanitary Association, 1960.
4. Cole JR, Sulzer CR, Pursell AR. Improved micro technique for the Leptospiral Agglutination Test. Applied Microbiol 1973; 25,6:976-980.
5. Dikken H, Kmety E. Serological typing methods of leptospires. In: Bergan T, Norris JR (eds) Methods in Microbiology, Academic Press, 1978; 11.
6. Faine S. Tuidelines for the Control of Leptospirosis. WHO Offset Publ 1982; NO. 67.
7. Kmety E. Betrachtungen zum Problem der paradoxen Reaktion und deren Bedeuting in der Serodiagnostik einiger Leptospirosen. Zbl Bakt Par Infekt Hyg 1957; I, 170:597-608.
8. Kmety E. Faktorenanalyse von Leptospiren der Icterohaemorrhagiae und einiger verwaandter Serogruppen. Bratislava Slovak Academy of Sciences, Thesis, 1967.
9. Kmety E, Galton MM, Sulzer CR. Futher standardization of the agglutinin absorption test in the serolog of leptospirosis. Bull WHO, 1970; 42:733-738.
10. Ryu E. Rapid microscopic agglutination test for leptospira without non-specific reaction. Bull Off Int Epiz 1970; 73(1-2):49-58.
11. Schuffner W, Mochtar A. Experiments on the differential characters of Leptospira strains with introductory remarks on the process of agglutination and analysis. Kon Akademie v Wetenshappen, Amsterdam, Proceedings 1926; XXX,1.
12. Sulzer CR, Jones WL. Leptospirosis, methods in laboratory diagnosis. DHEW Publication No (CDC) 74-8275, 1974.
13. Terpstra WJ. Typing of leptospira from the perspective of a reference laboratory. Acta Leidensia, 1991; 60:79-87.
14. Terpstra WJ, Korver H, Leewmen J Vvan, Klatser PR, Kolk AHJ. The classification of Sejroe group serovars of Leptospira interrogans with monoclonal antibodies. Zbl Bakt Hyg. 1985; A259:498-506.
15. Terpstra WJ, Korver H, Schoone GJ, Leewen J van, Jonge S de, Kolk AHJ. Comparative classification of Leptospira serovars of the Pomona group by monoclonal antibodies and restriction endonuclease analysis. Zbl Bakt Hyg 1987; A266:412-421.
16. Turner LH. Leptospirosis II. Trans R Soc Trop Med Hg 1968; 62,6:880-899.
17. Wolf JW. The Laboratory diagnosis of leptospirosis. Thomas Publishers, USa, 1954
18. Wolf JW, Broom BR. The Genus Leptospira Noguchi, 1917, Problems of classification and a suggested system based on antigenic analysis. Documenta Med Georg Trop 1954; 6:78-95.

Microscopic Agglutination Test For The Detection Of Antibodies To Leptospires Practical view

Principle: Antibodies in the test serum react with antigens on the surface of the bacteria and agglutinate them.

Materials: Microtitre plates; Incubator 30C; Loop; Microscope slides; Microscope (equipped with objective 29x, dark field condenser and 10x eyepieces); Microtitre pipettes.

Reagents:
Leptospira cultures; PBS, pH7.2

Procedure:

1. Fill all 96 wells of a microtitre plate with 50 uL PBS.
2. Add another 40 uL PBS to the wells of column 2.
3. Add 10 uL of antiserum to the wells of column 2 (serum dilution is now 1:10).
4. Dilute by pipetting 50 uL from one well to the next. Discard the final 50 uL.

5. Add 50 uL Leptospira culture to all wells (dilution in wells from column 2 is now 1:20) and cover the microtitre plate.
6. Mix thoroughly on a micro shaker for a few seconds.
7. Incubate for 2-4 hours at 30C or overnight at room temperature.

Reading the results:

The serum-antigen mixtures are examined under a dark field microscope for agglutination. This can be done by transferring one drop of mixture to a microscope slide.
The endpoint (titer) is taken as that dilution which gives 50% agglutination, leaving 50% of the cells free. Compare with a control suspension of leptospires diluted 1:2 in PBS without serum (column 1).
N.B. Agglutination is not always visible under the microscope.

back to top