METHOD FOR THE DIAGNOSIS OF INFECTIONS WITH DETECTION OF LIPOPOLYSACCHARIDE ANTIGENS

Method for diagnosing infections by agents with distinct lipopolysaccharide antigens in which clinical samples are applied to a support which absorbs the lipopolysaccharide antigen and then the support, carrying the antigen, is treated with a solution of antibody (especially monoclonal antibody) which has affinity for the antigen and a means for rendering it detectable, especially a radio-label. The absorbing support's preferred material is nitrocellulose, and its preferred form is a membrane. Accuracy and efficiency are improved by treating samples, before application to the absorbing support, with a proteolytic enzyme which is later deactivated. Treatment of the support or membrane with a solution of high-protein content, after it has absorbed the antigen, gives further improvement. The method is easily used by the immune blot technique and can be used as a genus-specific method for diagnosing Chlamydia (C.trachomatis and C.psittaci).
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Method for the diagnosis of infections with detection of lipopolysaccharide antigens.

This invention relates to a diagnostic method useful in biological screening procedures, more particularly in methods for detecting the presence of infectious agents, and especially for the diagnosis of bacterial infections such as those of chlamydial infections, for example those of *Chlamydia trachomatis*.

A known technique for the diagnosis of infectious agents which cause disease is to employ a procedure by which some antigenic component of the infectious agent (usually an infectious organism) is immunologically recognised. This has the advantage that it does not require the use of culture procedures (which can be slow) or the use of viable organisms in the laboratory, so that the agents or organisms do not have to be maintained in a viable state until they reach a laboratory, and enables results to be obtained more speedily.

The most recent procedures are described as Enzyme Linked Immuno-Sorbent Assays ("ELISA" tests), and kits for carrying them out are available commercially. In these tests, the antigen derived from the infectious agent is first captured from a fluid sample by means of a plastic carrier which has an affinity for the antigen and then the presence of the captured antigen on the carrier is detected by treatment with an antibody which reacts with it. The detecting antibody is usually "tagged" with an ancillary agent which can subsequently be used to reveal its presence and position on the carrier; in ELISA tests the antibody is conjugated with an enzyme which, when contacted with an appropriate substrate or reagent, generates a colour which indicates the presence of the enzyme and, therefore, also of the antigen to which the antibody binds.

Conventionally, the plastic carrier does not itself have any affinity for the desired antigen, and has to be made receptive to it by first being coated with a layer of material which incorporates an antibody to which the desired antigen
attaches. Consequently, the system is relatively complicated because of the involvement of so many components.

ELISA tests are usually carried out in "microtitre wells" in plastic plates or strips, which make them very suitable for the bulk testing of large numbers of samples. Like all new rapid diagnostic tests, it is important that they are both sensitive (so that they do not miss the truly positive samples) and also specific (so that they do not produce false positive reactions) if they are to provide an efficient and satisfactory alternative to older conventional diagnostic methods.

We have now found that these problems can be overcome for the diagnosis of infectious agents, for example bacteria, with a distinct lipopolysaccharide antigen, notably those of the genus Chlamydia, by utilising the ability of a contacting absorptive medium (especially nitrocellulose) which acts as an efficient medium for capturing lipopolysaccharide antigens. Indeed, such a medium has proved to be more efficient for this purpose than a plastic base coated with a "capture" antibody, as is used in a conventional ELISA test.

Nitrocellulose membranes are used regularly as absorbents for nucleic acids. They are also used for protein separation procedures, in which the proteins are first separated from each other in a polyacrylamide gel and then transferred from the polyacrylamide gel to a nitrocellulose membrane, placed in contact with the gel, by application of an electrical potential across the two - i.e. by electrophoretic action.

Hitherto, however, it has not been realised that a nitrocellulose membrane is sensitive enough to detect the low concentration of antigen present in clinical material. It is particularly surprising that it can absorb a lipopolysaccharide antigenic component derived from an infectious organism as opposed to a proteinaceous antigenic component.

Thus according to the present invention we provide an improved diagnostic method for the diagnosis of infection by an infectious agent with a distinct lipopolysaccharide antigen, in which the sample under examination is first intimately applied
to a contacting absorptive medium which has an affinity for a lipopolysaccharide, which then absorbs the lipopolysaccharide antigen of the infectious agent, and the resulting support carrying the absorbed antigen then is treated with a solution containing an antibody which (a) has an affinity for the said antigen and also (b) is associated with a means whereby the presence of the antibody (and consequently of any antigen to which it is attached) can be rendered detectable.

The term "contacting absorptive medium" is used here as meaning that the medium exerts its affinity for the antigen, without being restricted to any specific mode or mechanism by which it does so.

The invention is especially applicable to infectious agents with a distinct epitope on a lipopolysaccharide antigen, and organisms for which it is especially useful include those of the genus *Chlamydia*.

The method of the present invention is useful for the biological screening of large numbers of samples with ease and speed. Taking the case of the chlamydial organisms as an example, it also has the advantage that, as it relies upon the detection of the heat-stable lipopolysaccharide antigen, which is genus-specific. Thus, for example, it can be used to diagnose chlamydial infections other than those of *Chlamydia trachomatis* alone, which affects humans. Especially it can also be used to diagnose infections of *Chlamydia psittaci*, which affects animals and birds, and so can be valuable for diagnostic work in the veterinary field. This is in contrast to the methods dependent upon the protein antigens, which are less heat-stable and are not genus-specific but specific only to an individual species, for example *Chlamydia trachomatis*.

Another advantage of the method is that samples for test can be transported and stored under much less stringent conditions than those which usually need to be used when the viability of an organism has to be maintained, and mild cooling (for example a few degrees below 0 degrees C.) may be used instead of deep-freezing.
The sample for examination should be in the form of a dispersion and/or solution of the sample material in an aqueous medium and may be prepared by extracting the initial clinical sample material with water or an aqueous buffer solution.

This aqueous mixture containing the sample material is then preferably heated to assist in extracting the desired soluble lipopolysaccharide antigen, to kill any viable infectious agents or organisms present and destroy infectivity, and also to assist in insolubilising or deactivating other components of the sample which could interfere with the accuracy of the diagnosis. The time and temperature of heating may vary, but conveniently at least 10 minutes at about the boiling point (100 degrees C.) may be used.

In our experience, we have found that false positive reactions can occur when there is protein A in the clinical specimen. This problem can arise, in the case of Chlamydia, when other organisms are present in addition to Chlamydia, notably in the case of eye swabs when there is Staphylococcus aureus infection of the conjunctiva. Since Chlamydia trachomatis sometimes infects the eyes of babies and young adults, it is important to have a Chlamydia diagnostic test can be used satisfactorily on conjunctival swabs.

We have now found that an enzyme treatment of the specimen can be used to destroy proteinaceous materials (for example protein A) present, and even to destroy it completely. The antigen detected in the diagnostic method of the present invention is a lipopolysaccharide, which resists treatment with proteolytic enzymes, and we have found that treatment of the clinical specimens with such an enzyme (protease) before it is applied to the contacting absorptive medium improves both the sensitivity and the specificity of the test. Moreover, this treatment not only improves the ability of the test to detect the lipopolysaccharide antigen, but also enables specimens to contact or pass through the contacting absorptive medium more easily, so that it is less likely that the specimen will clog the contacting absorptive medium and centrifugation of the
specimen as part of its preparation can be made no longer necessary.

This modification has very significantly improved our test.

Thus according to a further feature of our invention we also provide an improvement in the said diagnostic method which comprises treatment of the clinical sample, before it is applied to the contacting absorptive medium, with a proteolytic enzyme.

The proteolytic enzyme used may be any of those known in the art, and may be used under conventional conditions, for example temperature, media and times, which are sufficient to enable it to act to attack and break down the proteinaceous materials present.

We also prefer that, after the enzyme treatment, the specimen should be treated in order to deactivate the enzyme as this might otherwise adhere to the contacting absorptive medium and destroy a labelled (e.g. radio-labelled) monoclonal antibody used at the later stage of the test procedure. This deactivating treatment may be a heating treatment, especially at 100 degrees C or higher; conveniently, this may be achieved by boiling or, still more conveniently, by steam treatment in a pressure vessel.

The intimate contacting of the sample under examination with the contacting absorptive medium may be achieved by using the contacting absorptive medium in a form of high surface area. Most preferably this is in the form of a thin film, conveniently termed a membrane, but other forms of high surface area may be used.

The liquid sample may be contacted with the contacting absorptive medium in any way which allows adequate intimacy of contact and sufficient time for the lipopolysaccharide antigen to be taken up by the contacting absorptive medium. This can be done by allowing the liquid sample to stand in contact with the contacting absorptive medium but the preferred method is for the liquid sample to be impelled through a layer, and
especially a film (membrane), of the contacting absorptive medium. This can be done by application of a pressure differential between the sides of the layer or film, so that the liquid is either blown or sucked through it.

The contacting absorptive medium may be any material known in the art to have an affinity for materials or products of a lipopolysaccharide nature. The preferred contacting absorptive medium is nitrocellulose, as this has exceptional efficiency and convenience in use.

The nitrocellulose (which may alternatively be described as cellulose nitrate) should preferably be in a pure form, as additives can interfere.

The contacting absorptive medium, especially when in the form of a film or membrane (for example of nitrocellulose) should be sufficiently permeable to allow the liquid of the sample to pass through it at a convenient speed. This can be achieved very conveniently by using a film (for example of pure nitrocellulose) having pore sizes of the order of about half a micron, but materials having larger or smaller pore sizes may be used if desired.

The contacting absorptive medium carrying the absorbed antigen is preferably washed thoroughly (conveniently with water) before being treated with a solution of an antibody in the next stage.

The antibody then used to treat antigen-bearing contacting absorptive medium may be any which has an affinity for the lipopolysaccharide antigen, but is preferably a monoclonal antibody for increased specificity and sensitivity.

The means, associated with the said antibody, whereby the presence of the antibody (and consequently of any antigen to which it is attached) can be rendered detectable may be any means which results in the antibody being capable of being detected, directly or indirectly. The preferred means, however, is for the antibody to be radio-labelled (i.e. rendered radioactive) so that its presence can be detected either directly by the radiation emitted or indirectly by the
effect of the emitted radiation on a photographic emulsion or other photosensitive material. The radio-labelling can be carried out with any radioisotope which can be incorporated in the antibody without adversely affecting its antibody properties (i.e. its affinity for the antigen), and preferably has a half-life which allows for convenient and safe handling in the laboratory. An example of this is labelling by treating the antibody with radioactive iodine (Isotope $^{125}$I). The technique for radio-labelling with radioactive iodine can be carried out for example by the technique described by Hunter and Greenwood (Nature, 1962, Volume 194, page 495). This radio-labelling provides a highly sensitive label and requires only very small amounts of the antibody. These labelled (and especially radio-labelled) antibodies are also provided as new products according to the present invention.

The contacting absorptive medium (for example a film of nitrocellulose), after it has absorbed the lipopolysaccharide antigen from the sample, should then be then treated with a material which blocks all the remaining attachment sites and prevents it trapping any further antigens or materials which can obscure the absorbed lipopolysaccharide antigen. The most convenient reagent for this purpose is a solution of high protein content, which may be buffered appropriately if desired.

The invention is especially well suited for use in an immune blot technique (or dot immunobinding technique) in which impermeable plates or sheets of glass or plastic, provided with a series of holes, are used to carry out tests on a number of samples simultaneously. For this, two such plates are clamped together with the series of holes in each substantially in register with each other, and with a sheet of the contacting absorptive medium (for example nitrocellulose film) clamped between them. The samples to be assessed are then put into the wells formed by the holes in one plate and the liquid is drawn through the film of contacting absorptive medium by applying suction below the second plate. The lipopolysaccharide antigen
is trapped in the film layer and then, when the plates are removed from it, the film can be treated with a solution of the antibody bearing its labelling material. The trapped antigen is thus labelled in its position on the film, and the film can be developed or used to provide a visible or measurable record of the range of samples.

When the labelling is by way of a radio-labelled antibody, it is very convenient to prepare the "record" by placing the contacting absorptive medium (for example nitrocellulose film), bearing its labelled "spots" of trapped antigen with the attendant radio-labelled antibody, against a sheet of radiation-sensitive material — for example X-Ray film — and then developing the exposed material in the customary fashion. This has the advantage of producing a record of many samples in a form which lends itself very well to being kept as a permanent record and examined quantitatively as well as qualitatively. Also, if the amount of radioactivity present is small and the initial exposure to the radiation-sensitive film is too dark or too faint to be studied adequately, the procedure can be repeated again using fresh film and a different exposure, and this may be repeated as often as is desired so long as the radioactivity is still present.

It is particularly useful, however, as it is very sensitive and can enable an observer to distinguish very readily those samples which show a strong reaction.

For practical purposes there is also the advantage that, in addition to increased sensitivity, the immune blot test is simpler to perform than the ELISA test as fewer washing steps are involved. Once each specimen has been placed in its individual well, the whole film of contacting absorptive medium, for example nitrocellulose membrane, (which can, for example, test about 90 specimens) is processed and the washing and subsequent treatment of individual wells, which is necessary in ELISA tests, is avoided.

The invention is not restricted to this specific form of multi-sample test, but may if desired be applied to tests using
fewer samples — even individual samples in individual wells — if desired.

The invention is illustrated but not limited by the following Examples, in which the parts and percentages are by weight unless stated otherwise.

EXAMPLE 1:

Preparation of clinical sample for test:
20 A swab taken of a mucous surface to be assessed is treated with an aqueous phosphate buffer solution containing sucrose, as transit medium, and this is then heated for 15 to 30 minutes at 100 degrees C. in a boiling water bath. This helps to solubilise the material for test, and also destroys any infectivity. The liquid is then centrifuged lightly, to remove any insoluble materials which could cause clogging of the membrane in the subsequent stages.

Test procedure:
25 The resulting liquid is then put into a "well" of a plate as used for dot immunobinding techniques. Such plates are well known and are available commercially under the name "Bio-dot" (Bio-Rad Laboratories Ltd., Watford WD1.8A, United Kingdom), and comprise a sheet of plastic or glass in which a multiplicity of holes have been made (commonly about 90 holes).

Two such plates are clamped together with a thin sheet of nitrocellulose membrane (pure nitrocellulose, available as BA85 grade, and with pore size of approximately 0.45 micron) between them, so that the holes in the two plates correspond and form a series of cavities ("wells") each with the nitrocellulose membrane across it.

The quantity of liquid per well is usually about 400 microlitres, and liquid derived by the above procedure from a variety of samples may be put into the different wells. Control samples of known content (usually at least one which is inert and one which contains a known amount of the antigen
sought) are put into some of the wells to act as standard. Then the liquid in the various wells is drawn through the nitrocellulose membrane by suction from below the plates.

The nitrocellulose membrane is then separated from the plates and soaked for about 1 hour in a phosphate-buffered saline solution containing 5% of powdered skimmed milk. This high protein solution serves to block all the remaining attachment sites on the membrane. The membrane is then washed very thoroughly with water to remove all surplus protein.

The washed membrane is then treated with a very dilute aqueous solution of a monoclonal antibody which has an affinity for the lipopolysaccharide antigen and also has been radio-labelled with radioactive iodine (\(125^I\)) by the method of Hunter and Greenwood (Nature, 1962, Volume 194, page 495), by soaking in the solution for about 2 hours at ambient temperature.

The membrane is then removed from the antibody solution and washed very thoroughly, especially to remove all radioactive material which is not attached to the antigen on the nitrocellulose membrane, and dried.

The dried membrane is then put into an intensifying screen and used to expose a sheet of X-ray photographic film for 12 to 24 hours, after which time the X-ray film is developed in the customary manner and examined for the degree of darkening in positions corresponding to the various wells and samples.

Wherever radio-labelled antibody has reacted with antigen (i.e. a positive clinical specimen) a black dot develops on the film, and the density of the dot is dependent upon the amount of antigen trapped and on the time of exposure of the X-ray film to the treated nitrocellulose film. The rest of the X-ray sheet remains unchanged, so positive reactions can be detected easily and quickly by eye.

If the effect on the X-ray film is too faint, the nitrocellulose film can be used to expose another sheet of X-ray film for a longer time so as to obtain a darker dot to assist evaluation.
**EXAMPLE 2:**

**Preparation of clinical sample for test:**

Clinical specimens (for example swabs taken of a mucous surface to be assessed, for example from urethra, cervix or conjunctiva) are placed in conventional Chlamydia transport medium (sucrose-phosphate buffer with appropriate antibiotics) and transported to the laboratory. The specimens are numbered, and aliquots are removed for culture if comparative studies are being carried out.

Protease (proteinase K, Sigma P-0390, obtained from Sigma Chemicals) is then added to the remainder of the specimen (final concentration 250 μg ml⁻¹) which is vortexed (conveniently on a "Whirsimixer," for about 10 seconds) and then held at 56 degrees C. for 30 minutes to allow the enzyme to act. The enzyme is then deactivated by steam treatment in a pressure cooker for 15 minutes.

The prepared specimen is then ready for loading into the Bio-dot plate for the test procedure.

**Test procedure:**

A 96 well Biodot microfiltration apparatus (Bio-Rad Laboratories Ltd., Watford WD1.8A, United Kingdom) is then assembled with nitrocellulose membrane moistened with phosphate buffered saline (PBS) and specimens are added to the wells (0.4 ml of specimen, 1 specimen per well), then drawn through the membrane by suction. The membrane is removed and placed in blocking solution (PBS which contains 5% W/V skimmed milk powder) for 30 minutes at 37 degrees C., washed in PBS containing 0.05% V/V of "Tween 20" (PBS-T) ("Tween 20" is a commercially available surfactant) then incubated with 125I-labelled genus monoclonal antibody diluted in PBS-T (2-3 x 10⁶ cpm) for 2.5 hours at 37 degrees C. or at room temperature overnight. The membrane is then thoroughly washed in PBS-T, dried and autoradiographed with pre-flashed Kodak X-Omat AR film and "Super-rapid" screens (exposure time 24 hours at -70
degrees C.). A two-fold titration of a cell culture stock of *Chlamydia trachomatis* is included as positive control on each membrane. The dilutions are adjusted to yield a range of dots of varying intensity with the end-point at the fifth dilution. Clinical specimens which produce a reaction equal to or greater than the end-point are considered positive.

The monoclonal antibody used was the genus monoclonal antibody J12 used in the commercially available Boots Celltech antigen detection kit ("IDEIA"), which was made and characterised by Dr. Margaret Thornley in Cambridge.

Assessment of the results showed that this procedure was very much more sensitive than the commercial ELISA ("IDEIA"), and also more sensitive than the corresponding test procedure in which the enzyme (protease) treatment was omitted. Sensitivity, specificity, and positive and negative predictive values were all good.

This procedure, using the enzyme treatment, was also appreciably more sensitive than culture methods for diagnosis of ocular *Chlamydia trachomatis* infection. The reason for this is not clear, but is probably because the procedure overcomes the effect of the topical antibiotics (for example chloramphenicol) which are frequently used to treat patients at the time that swabs are taken.
CLAIMS:

1. Diagnostic method for the diagnosis of infection by an infectious agent with a distinct lipopolysaccharide antigen, in which the sample under examination is first intimately applied to a contacting absorptive medium which has an affinity for a lipopolysaccharide, which then absorbs the lipopolysaccharide antigen of the infectious agent, and the resulting support carrying the absorbed antigen then is treated with a solution containing an antibody which (a) has an affinity for the said antigen and also (b) is associated with a means whereby the presence of the antibody (and consequently of any antigen to which it is attached) can be rendered detectable.

2. Diagnostic method as claimed in Claim 1 wherein the infectious agent is one with a distinct epitope on a lipopolysaccharide antigen.

3. Diagnostic method as claimed in Claim 1 or Claim 2 wherein the infectious agent is an organism of the genus Chlamydia.

4. Diagnostic method as claimed in Claim 3 wherein the organism is *Chlamydia trachomatis*.

5. Diagnostic method as claimed in Claim 3 wherein the organism is *Chlamydia psittaci*.

6. Diagnostic method as claimed in any one of Claims 1 to 5 wherein the sample for examination is in the form of a dispersion and/or solution in an aqueous medium.

7. Diagnostic method as claimed in any one of Claims 1 to 6 wherein the clinical sample, before it is applied to the contacting absorptive medium, is treated with a proteolytic enzyme, especially a protease.

8. Diagnostic method as claimed in Claim 7 wherein, after the treatment with the proteolytic enzyme and before it is applied to the contacting absorptive medium, the specimen is treated in order to deactivate the enzyme.
9. Diagnostic method as claimed in Claim 8 wherein the deactivating treatment is a heating treatment at 100 degrees C or higher.

10. Diagnostic method as claimed in any one of Claims 1 to 9 wherein the intimate contacting of the sample under examination with the contacting absorptive medium is achieved by using the contacting absorptive medium in a form of high surface area.

11. Diagnostic method as claimed in Claim 10 wherein the contacting absorptive medium is in the form of a membrane.

12. Diagnostic method as claimed in any one of Claims 1 to 11 wherein the liquid sample is impelled through a layer, and especially a membrane, of the contacting absorptive medium.

13. Diagnostic method as claimed in any one of Claims 1 to 12 wherein the contacting absorptive medium is nitrocellulose.

14. Diagnostic method as claimed in any one of Claims 1 to 13 wherein the antibody used to treat antigen-bearing contacting absorptive medium is a monoclonal antibody.

15. Diagnostic method as claimed in any one of Claims 1 to 14 wherein the means associated with the said antibody, whereby the presence of the antibody (and consequently of any antigen to which it is attached) can be rendered detectable, is radio-labelling.

16. Diagnostic method as claimed in Claim 17 wherein the 25 antibody is labelled with radioactive iodine (Isotope ¹²⁵I).

17. Diagnostic method as claimed in any one of Claims 1 to 16 wherein the contacting absorptive medium, after it has absorbed the lipopolysaccharide antigen from the sample, is then treated with a material which blocks substantially all the remaining attachment sites and prevents it trapping any further antigens or materials which can obscure the absorbed lipopolysaccharide antigen.

18. Diagnostic method as claimed in Claim 17 wherein the material which blocks substantially all the remaining attachment sites is a solution of high protein content.
19. Diagnostic method as claimed in any one of Claims 1 to 18 wherein the method is used for an immune blot technique (or dot immunobinding technique).

20. Diagnostic method substantially as described.

21. An antibody which (a) has an affinity for a distinct lipopolysaccharide antigen of an infectious agent and also (b) is associated with a means whereby the presence of the antibody (and consequently of any antigen to which it is attached) can be rendered detectable.

22. An antibody as claimed in Claim 21 which is a monoclonal antibody.

23. An antibody as claimed in Claim 21 or Claim 22 which is radio-labelled.
**INTERNATIONAL SEARCH REPORT**

**International Application No** PCT/GB88/00561

### I. CLASSIFICATION OF SUBJECT MATTER

According to International Patent Classification (IPC) or to both National Classification and IPC

**IPC**: G 01 N 33/569, 33/571, 33/543

### II. FIELDS SEARCHED

**Classification System**

**Classification Symbols**

| IPC 4 | G 01 N |

### III. DOCUMENTS CONSIDERED TO BE RELEVANT

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### IV. CERTIFICATION

Date of the Actual Completion of the International Search 28th September 1988

Date of Mailing of this International Search Report 25 NOV 1988

International Searching Authority EUROPEAN PATENT OFFICE

Signature of Authorized Officer P.C.G. VAN DER PUTTEM

Form PCT/ISA/210 (second sheet) (January 1985)
### III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

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<td>Analytical Biochemistry 137, p. 129-133, published 1984 (W. BRADBURY et al) &quot;Detection of Lipopolysaccharides in Polyacrylamide Gels by Transfer to Nitrocellulose Followed by Immun autoradiography with Antibody and 125I-Protein A: &quot;LPS Blotting&quot;. Whole document</td>
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