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Essentially protein-free O-carbohydrate or O-polysaccharide antigens are separated from a species, or serogroup of a species, of bacteria of the genus Legionella, and coupled to an activated chromatographic column. The column so prepared is used to purify raw antibodies to the same species or serogroup of a species, of Legionella. The resulting antigen-specific antibodies are used in immunochemical assays for detecting Legionnaires disease, Pontiac fever and other Legionella-caused diseases in humans and for detecting Legionella bacteria in environmental samples. A rapid, highly specific and sensitive immunoassay for Legionella pneumophila serogroup 1 conducted in an immunochromatographic test device is described in detail.

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Essentially protein–free O–carbohydrate or O–polysaccharide antigens are separated from a species, or serogroup of a species, of bacteria of the genus *Legionella*, and coupled to an activated chromatographic column. The column so prepared is used to purify raw antibodies to the same species or serogroup of a species, of *Legionella*. The resulting antigen–specific antibodies are used in immunochemical assays for detecting Legionnaires disease, Pontiac fever and other *Legionella*–caused diseases in humans and for detecting *Legionella* bacteria in environmental samples. A rapid, highly specific and sensitive immunoassay for *Legionella pneumophila* serogroup 1 conducted in an immunochromatographic test device is described in detail.

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METHOD FOR DETECTION OF LEGIONELLA BACTERIA EMPLOYING PURIFIED ANTIGEN-SPECIFIC ANTIBODIES

This invention relates to essentially protein-free carbohydrate, including polysaccharide antigens, separated from bacteria of the genus Legionella, and especially from serogroups and/or strains of Legionella pneumophila, including the O-polysaccharide antigen of L. pneumophila serogroup 1, and to the use of these antigens in the affinity purification of polyvalent antibodies to corresponding Legionella organisms. More particularly, the invention encompasses coupling the carbohydrate or polysaccharide antigen separated from a Legionella bacterium to an activated chromatographic column and using that column for affinity purification of the polyclonal antibodies to the same species, or the same serogroup of a species, of Legionella. The invention further encompasses the use of the affinity-purified polyvalent antibodies produced in immunochemical assays for the detection of Legionella-caused diseases such as Legionnaires disease and Pontiac fever in human patients and for the detection of environmental sources of Legionella infectious agents.

BACKGROUND

Legionnaires' disease, a pneumonia-like human infection caused by gram-negative bacteria of the genus Legionella, is virtually impossible to differentiate on a reliable clinical basis (involving assessment of patient symptoms without laboratory tests) from pneumonia and other similar lung infections. Because the disease may produce lung abscesses, infections in other bodily organs or bacteremia, and its mortality rate is significantly increased by delay in commencing appropriate therapy, there is a need for rapid and reliable diagnostic tests which has to date been only partially met. Stout, J.E. and Yu, V.L., "Legionellosis", 337, N. Eng. J. Med. 682-687 (1997). Efforts to develop such tests have been hampered by the fact that there are a number of Legionella species, at least some of which are known to have a number of distinct serogroups.

Legionnaires' disease was first recognized in the summer of 1976 and a number of techniques for detecting Legionella ("L.") pneumophila, which is now known to account for some 90% of cases (Stout and Yu, supra), have been developed in the interim. In general, these tests are time-consuming and incapable of identifying more than one serogroup of the 15 serogroups known so far to fall within the L. pneumophila species. It should be noted, however, that, as reported by Stout and Yu, supra, more than 80% of reported cases of Legionnaires' disease are attributable to L. pneumophila serogroup 1 -- a fact which makes the development of a rapid, reliable immunoassay for that entity of particular importance and has led researchers to focus on this as a priority.

The early efforts to establish the identity of the causative agent of Legionnaires' disease depended largely upon culturing the bacteria for 5 to 6 days and examining the culture microscopically. Efforts to speed up the identification process led to numerous immunochemical tests of varying sensitivity and specificity including, inter alia, the presently commercially available EQUATE™ radioimmunoassay ("RIA"), and the Binax enzyme immunoassay ("EIA"), both of which are sold in kit form by applicants' assignee, Binax, Inc. As indicated by Hackman, B.A. et al. in "Comparison of Binax Legionella Urinary Antigen EIA Kit with Binax RIA Urinary Antigen Kit for Detection of Legionella pneumophila Serogroup I Antigen", 34 J. Clin. Microbiol. 1579-1580 (1996), both of these assays were found to be specific for L. pneumophila Serogroup I antigen. The reported sensitivity of the EIA was 77%; that of the RIA was higher. The article indicates that each can be performed within "less than 3 h[ours] from beginning to end"; in Binax's own tests each requires at least 2 1/2 hours to perform. More information about this EIA assay appears in Kazandjian, D. et al., "Rapid Diagnosis of Legionella pneumophila Serogroup I Infection with Binax Enzyme Immunoassay Urinary Antigen Test", 35 J. Clin. Immunobiol. 954-956 (1997).

BRIEF DESCRIPTION OF THE INVENTION

Perhaps the most significant advantage of the immunochromatographic test

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("ICT") described herein is its ability to give a test result within a 15-minute time span for the presence or absence of L. pneumophila Serogroup I (or its antigen), which result is of high specificity and sensitivity. The speed with which this test can be reliably conducted to yield a result of high specificity and sensitivity is believed to be due to the strongly reactive nature of the affinity purified antibodies prepared in accordance with this invention. The superior reactive properties of these antibodies, in turn, is believed to be attributable to the use for affinity purification of the novel, essentially protein-free O-polysaccharide antigen of L. pneumophila serogroup 1 which is also a part of this invention.

Another ICT test has been made possible by the separation, according to this invention, of an antigen of L. pneumophila serogroup 5 which is of non-proteinaceous, carbohydrate nature and is common to multiple serogroups of L. pneumophila. The presence of such a common antigen has been suggested in the scientific literature; see e.g., Nolte, F.S. et al., "Electrophoretic and Serological Characterization of the Lipopolysaccharides of Legionella pneumophila", 52 Infection and Immunity 676-681 (1986); Otten, S. et al., "Serospecific Antigens of Legionella pneumophila", 167 J. Bacteriol. 893-904 (1986); Barthe, C. et al., "Common Epitope on the Lipopolysaccharide of Legionella pneumophila Recognized by a Monoclonal Antibody", 26 J. Clin. Microbiol. 1016-1023 (1988); Knirel, Y.A. et al., "The Structure of the O-specific Chain of Legionella pneumophila Serogroup I", 227 Eur. J. Biochem. 239-245 (1994). Heretofore there has been no clear report of any separation of such an antigen for use in developing a useful broad spectrum immunochemical assay.

This invention comprises the extraction from any Legionella bacterium, and especially from a bacterium of any of the serogroups of L. pneumophila of an essentially protein-free carbohydrate antigen, the preparation of a conjugate of this antigen with a spacer protein, the coupling of the conjugate to an affinity column, the use of that column for the purification of polyvalent antibodies to the corresponding Legionella bacterium, such as a bacterium of a serogroup of L. pneumophila and the use of the antibodies thus purified in an ICT

immunoassay for detecting Legionella bacteria or their antigens, including antigens of L. pneumophila serogroups or their corresponding bacteria, as more specifically described hereinafter.

In particular, the invention includes the separation of an essentially protein-free O-polysaccharide antigen specific to L. pneumophila serogroup 1, its use in the affinity purification of the polyvalent antibody specific to the same microorganism and the use of that affinity purified antibody in an ICT immunoassay of high specificity and high sensitivity that is performable within 15 minutes.

In another embodiment, the invention includes the separation from L pneumophila serogroup 5 of an essentially protein-free carbohydrate antigen, and its use in the affinity purification of the polyclonal antibody specific to the L. pneumophila serogroup 5 antigen (i.e., an antibody that was obtained from a rabbit immunized with the said antigen); this antibody showed an ability to cross-react with antigens of the L. pneumophila serotypes 1, 2 and 4 in addition to the antigen of serotype 5.

In accordance with a further embodiment, the invention provides a method for obtaining an essentially protein free O-polysaccharide antigen from Legionella pneumophila serogroup 1 bacteria, which comprises the steps of:

(a) harvesting bacterial cells from a culture of a L. pneumophila serogroup 1 in the form of a wet cell pellet;

(b) suspending the wet cell pellet in 0.1M NaOH for about 45 minutes with stirring;

(c) adjusting the pH of the suspension to an acid pH and centrifuging the suspension;

(d) separating the supernatant resulting from the centrifugation of step (d) and adjusting the pH of the supernatant to approximate neutrality;

(e) adding a broad spectrum protease enzyme preparation to the supernatant and digesting residual proteins in the supernatant for at least about 15 hours;

(f) adjusting the pH of the digestion mixture of step (e) to an alkaline pH,

applying the mixture to a size exclusion column equilibrated with a weakly alkaline solution, pooling material eluted in the first peak and adjusting the pH of the pooled material to approximate neutrality, to produce an essentially protein free polysaccharide antigen.

In accordance with another embodiment, the invention provides an essentially protein free, O-polysaccharide antigen obtained from L. pneumophila serogroup 1 bacteria by the method of the preceding paragraph.

In accordance with a further embodiment, the invention provides a method for the purification of polyclonal antibodies to L. pneumophila serogroup 1 comprising:

(a) obtaining an essentially protein free O-polysaccharide antigen from L. pneumophila serogroup 1 by the method described above;

(b) conjugating said antigen through a spacer molecule to an activated chromatographic affinity column;

(c) subjecting polyclonal antibodies to L. pneumophila serogroup 1 to affinity chromatography on the column from step (b); and

(d) eluting antibodies bound to said column to produce purified antibodies.

In accordance with another embodiment, the invention provides purified antibodies to L. pneumophila serogroup 1 and its O-polysaccharide antigen, purified by the method of the preceding paragraph.

In accordance with a further embodiment, the invention provides an immunochromatographic ("ICT") test device for the detection, in a sample of fluid, of the O-polysaccharide antigen of L. pneumophila serogroup 1, which device comprises a housing having a view window and containing a strip of bibulous material, wherein the strip comprises:

(1) a first zone having a sample introduction point at or near its free end, wherein there is movably deposited at a location close to, but slightly removed from, said sample introduction point, a portion of polyvalent antibodies raised in an animal against either (i) L. pneumophila

serogroup 1 bacteria or (ii) the O-polysaccharide antigen of L. pneumophila serogroup 1, which antibodies have been tagged with a tag that exhibits a colour change upon formation of a tagged antibody-antigen-untagged antibody "sandwich", and

(2) a second zone located under the view window of the device and following said first zone, wherein a capture line has been formed on said strip, near its end opposite from the sample introduction point, by immovably striping a portion of antibodies that are nontagged, but otherwise identical to those present in said first zone, across the width of said strip, and wherein the antibodies in both zones of said strip have been treated by (i) passing them over a chromatographic affinity column to which is coupled, through a spacer molecule, an essentially protein free O-polysaccharide antigen from L. pneumophila serogroup 1 bacteria obtained by the method described above; and (ii) then eluting from said column, with an eluting agent, those antibodies that bound to said antigen.

In accordance with a further embodiment, the invention provides an immunochromatographic (ICT) assay for detecting the presence of L. pneumophila serogroup 1 O-polysaccharide antigen in a fluid comprising:

(a) introducing an aliquot of a sample of the fluid into the first zone of the device of the preceding paragraph at the sample introduction point;

(b) allowing the sample to flow along the strip and pick up the movably deposited tagged antibodies present in said first zone;

(c) allowing the sample and tagged antibodies to continue to flow, together, along the strip and mingle thoroughly, thereby forming tagged antibody-antigen conjugates if the O-polysaccharide antigen of L. pneumophila serogroup 1 is present in the sample;

(d) allowing the flowing mixture of tagged antibodies, sample and any already formed tagged antibody-antigen conjugates to flow into the second zone of said test device and contact the immovable capture line located therein; and

(e) after 15 minutes from the time at which the sample was introduced to said strip, observing whether a color change, caused by massing of the tag along the capture line of the test device, has occurred, thereby denoting that tagged antibody-antigen-untagged antibody "sandwiches" have formed along that line and hence, that the O-polysaccharide antigen of L. pneumophila serogroup 1 is present in the sample.

DESCRIPTION OF THE DRA WINGS

Figure 1 and its related Figures lA, lB and LC hereof show the structure of a typical ICT device which is suitably adapted to perform the L. pneumophila serogroup 1 specific assay, as described in Examples VII, VIII and IX hereof.

Figure 2 hereof shows the results of Western blot analyses of phosphate-buffered saline extracts of L. pneumophila serogroups 1, 2, 4 and 5 with L. pneumophila serogroup *5,* essentially protein-free carbohydrate antigen-affinity-purified polyclonal antibody specific to serogroup *5* antigen, which analysis is described in Example X hereof.

DETAILED DESCRIPTION OF THE INVENTION

Previous experience in the art, including experience with the Binax RIA assay for L. pneumophila Serogroup 1 antigen sold under the trademark EQUATE and the Binax EIA assay for the same antigen, has shown that antigens of the

Legionella species, including antigens of various L. pneumophila serogroups, are more conveniently detectable in specimens from the urine of patients infected with a Legionella microorganism than in specimens of blood, sputum or other fluids. This is in part because the Legionella antigens usually appear in urine within 1-3 days after infection of a human patient whereas their appearance at a detectable level in blood may occur later, and also in part because patients infected with Legionnaires' disease often do not produce much sputum. The ICT test which forms a part of this invention can be configured to run on blood, sputum or some other fluid such as cerebrospinal fluid, or on aqueous samples of environmental origin. It is noted that urine is generally the preferred sample fluid for diagnosis of human patients because it can be obtained non-invasively and easily, even in the doctor's office, and it is not as readily contaminated with other microorganisms, e.g. oral microflora present in sputum, which are innocuous but may affect the results obtained.

Broadly speaking, the ICT test for L. pneumophila serogroup 1 antigen that is specifically a part of the present invention may be designed to be run in any known disposable ICT device disclosed in the art. Preferably, the test is conducted using an ICT device of the type disclosed in U.S. Patent No. 6,168,956 of Howard Chandler, assigned to Smith-Kline Diagnostics, Inc. but exclusively licensed to Binax, Inc. in a wide area of applications that includes the present diagnostic field --i.e., diseases of the respiratory system. The device is suitably impregnated with the affinity purified polyvalent antibodies herein disclosed which are specific to the antigen of L. pneumophila serogroup 1 antigen. Positive results of the assay are shown by the appearance of a color upon reaction of suitably labelled antibodies with the antigen. Suitable labels may be any of those known in the art to produce visible color when antibodies conjugated thereto react with antigen, including finely divided metallics and various other labelling materials. Colloidal gold is the preferred label.

The invention contemplates that essentially protein free carbohydrate or

antigens may similarly be separated from each of the Legionella bacteria, including from bacteria of other Legionella species and from bacteria of other serogroups of L. pneumophila, that such antigens may similarly be utilized in the affinity purification of polyvalent antibodies to the corresponding Legionella species or serogroup bacteria and its antigen, and that these affinity-purified antibodies may be utilized in ICT and other immunoassays as specifically disclosed herein for affinity-purified antibodies to L. pneumophila serogroup 1.

Preliminary to the preparation of the device is obtaining the antibodies and effecting their affinity purification. The antibodies that may be used in this invention are conventional polyvalent (also called "polyclonal") antibodies obtained by the well known process of immunizing a rabbit, goat or other animal to a Legionella antigen, e.g., L. pneumophila antigen of known serogroup and bleeding the animal after the passage of an appropriate time period to obtain serum containing the desired antibodies. See, e.g., Cherry, U.B. and McKinney, R.M., pp. 91-104 in Jones, G.L. and Herbert, G.A. (Eds.), "Legionnaires'' the disease, the bacterium and the methodology, (Center for Disease Control, Atlanta, 1979). In this invention, relative to the ICT immunoassay for L. pneumophila serogroup 1 disclosed herein, the rabbit or other animal is immunized to L. pneumophila serogroup 1 antigen and the polyvalent antibodies recovered from its blood are antibodies to L. pneumophila serogroup 1.

The polyvalent antibodies to be used in this invention are further subjected to affinity purification on a specially prepared chromatographic column which employs the essentially protein-free carbohydrate (including polysaccharide) antigen of the same bacterium against which the antibodies are reactive as the purifying agent for them.

Preliminary to the affinity purification of the antibodies, it is therefore necessary according to this invention to prepare an essentially protein free purified carbohydrate antigen from a culture of known Legionella bacteria of the desired species or serogroup of a species.

The following examples I and II explain how the purified protein free polysaccharide or carbohydrate antigen is obtained.

Example I

Bacteria of L. pneumophila Serogroup 1 (strain Philadelphia-1) were obtained from Centers for Disease Control and Prevention (Atlanta, GA) and cultured on charcoal-yeast extract agar plates obtained from Northeast Laboratory (Waterville, ME) for a period of 72 hours at 37°C. Cells were harvested with phosphate-buffered saline ("PBS") at pH 7.2 containing 0.2% of NaN_3 and collected by centrifugation at 8000 rpm for 25 minutes. The resulting cell pellet was stored at -20°C. until used.

Wet cells from this pellet were suspended in 20 ml. 0.1 M NaOH per gram of wet cells and stirred at room temperature for 45 minutes. The pH of the solution was then adjusted with concentrated acetic acid to 3.0 and the solution was subjected to centrifugation at 8000 rpm for 20 minutes. The supernatant from this step was then neutralized with aqueous NaOH and dialyzed against distilled water.

The resulting dialyzate was concentrated 10 times on a rotary vacuum evaporator and then sonicated for 5 minutes in an ultrasonic bath.

Proteinase K, in a concentration of 0.2 mg. per ml. of the concentrated product, was added to digest the remaining proteins and the mixture was incubated at 40°C. overnight. The next step was the addition of further Proteinase K, in a concentration of 0.1 mg. per ml., to the mixture, followed by further overnight incubation at 40°C. This second incubation was followed by concentration of the product on a rotary evaporator to a small volume, adjustment of its pH to 10-11 with 0.2% triethylamine and application of the thus-treated mixture to a column of Sephacryl™ S-200 from Pharmacia, equilibrated with 0.02% triethylamine. Material eluted in the first peak was pooled, adjusted with 0.1 NHCl to approximately neutral pH and dialyzed against distilled water for 18 hours, followed by lyophilization.

The yield of O-polysaccharide antigen from 16. 5 grams of wet cells of L. pneumophila serogroup 1 strain Philadelphia-1 was 62 mg.

It should be noted that in place of Proteinase K, any broad spectrum protease enzyme preparation may be used. What is important in this procedure is the elimination of protein from the antigen to the maximum possible extent within reasonable limits of time feasibility.

Example II

Example I was repeated using L. pneumophila serogroup 5 strain Dallas IE as the bacteria in the culture step. 11. 5 wet grams yielded 21 mg. of a carbohydrate antigen. At a later time, the procedure was again repeated, this time using L. pneumophila serogroup 5 (strain U8W) as the bacteria in the culture step.

It is within the scope of this invention to repeat the culturing and extraction steps as herein described on any other Legionella bacterium and especially on bacteria of any other of the serogroups of L. pneumophila, i.e., on any of serogroups 2,3, 4 and 6-15 inclusive, to obtain a carbohydrate antigen essentially free of proteinaceous material.

In order to couple the essentially protein free O-polysaccharide product of Example I or the carbohydrate of Example II to a chromatographic column for use in affinity purification of polyvalent antibodies to the corresponding bacterium from which the carbohydrate antigen was extracted, it is necessary to complex it with a protein spacer molecule suitably prepared to ensure that it will stably bond to both the carbohydrate antigen and the column and will itself remain inert during the affinity purification step. A modified bovine serum albumin ("BSA") was chosen as the preferred spacer molecule and was prepared as in Example III :

Example III -- Preparation of Protein Spacer Molecule

An 0.5M aqueous solution of hydrazine dihydrochloride from Aldrich Chemical Co., Inc. (Milwaukee, WI) was prepared and its pH was adjusted to 5. 2

with dry NaOH. The solution was then mixed with dry BSA from Sigma Chemical Co. to a final concentration of 25 mg. per ml. of BSA. After the BSA was completely dissolved, N-(dimethylaminopropyl)-N^1-ethylcarbodiimide hydrochloride obtained from Fluka Chemical Co. (St. Louis, MO) was added to a final concentration of 2.5 mg. per ml. The mixture was stirred overnight at ambient temperature and then dialyzed for a period of about five days against distilled water at 4°C, with daily changes of the water.

In lieu of this modified BSA, it is contemplated that other appropriate spacer molecules may be used.

The conjugation of the O-polysaccharide antigen from serogroup 1 of L. pneumophila to the spacer molecule was conducted as follows:

Example IV -- Conjugation of O-polysaccharide Antigen to Spacer Molecules

L. pneumophila serogroup 1 essentially protein-free O-polysaccharide antigen was dissolved in distilled water in a concentration of 4-5 mg/ml and the pH was adjusted to 5.0 with 1 M HCl. The modified BSA solution prepared as in Example III was adjusted to pH 5.0 with 1 M HCl and slowly added, in a weight ratio of 4-to-1 of the O-polysaccharide antigen solution, to the latter. After 5 minutes of stirring, about 100-200 mcl of distilled water containing the N-(dimethylamino-propyl-N^1-ethylcarbodiimide hydrochloride referred to in Example III was added in a weight ratio of 1:2 to the O-polysaccharide antigen/BSA solution. The resulting mixture was stirred at ambient temperature overnight.

To separate the conjugate of the O-polysaccharide antigen with modified BSA from any unreacted materials present, the reaction mixture was chromatographed on a Sepharose™ CL-4B column equilibrated with a buffer of 0.1 M NaH_2PO_4:0.5 M NaCl. All of the chromatography fractions were subjected to testing for antigen activity using the commercial Binax EIA test referred to above. All fractions that showed antigen activity in the tested were pooled in and used for affinity column preparation as shown in Example V.

Example V -- Coupling of Conjugate to Activated Chromatographic Column

An immunoadsorbent gel was prepared by conventionally activating Sepharose CL-4B with cyanogen bromide. The ligand of O-polysaccharide antigen with modified BSA, prepared as in Example IV, was coupled to it using procedures known in the art, e.g., as described in Hermanson, G.T., et al. in Immobilized Affinity Ligand Techniques, 53-56 (Academic Press, Inc. 1992). The gel was then packed in a column and washed successively with 5-10 volumes per volume of gel with PBS of pH 7.2, triple strength PBS of pH 7.2 and 0.2 M glycine-HCl of pH 2.5. The resulting activated column was used, as described below, for affinity purification of polyvalent antibodies to L. pneumophila serogroup 1 antigen. The column after elution should be stored in PBS or anther neutral buffer until used.

Instead of cyanogen-bromide activated Sepharose, spherelose and various commercially available activated columns could be used in this step.

Example VI -- Affinity Purification of Antibodies

The activated column described in Example V was used for the affinity chromatography of rabbit-anti-L. pneumophila serogroup 1 polyvalent antibodies to L. pneumophila serotype 1 antigen according to the method described by Harlow E. and Lane, D. in Antibodies: A Laboratory Manual at 313-315 (Cold Spring Harbor Laboratory, 1988). Elution of the antibodies from the column was effected with 0.2M glycine-HCL buffer of pH 2.5. Alternative eluants such as 3M NaSCN or 0.1M Et_3N could be substituted.

These affinity-purified antibodies were utilized in an ICT test specific to L. pneumophila serogroup 1 as described in the following example.

Example VII -- ICT Device and Its Preparation

A. Preparation of Test Device:

A test device comprising a hinged cardboard housing equipped with a window to allow the viewing of both the test results and control results was

prepared as shown in Figure 1. The device has a recess into which is placed a preformed plastic swab well for receiving the sample-wetted swab on the right-hand (labeled 1 in the drawing). An overlabel shown in Figure 1A is then placed over the entire right-hand side of the device. The overlabel has been equipped with two holes -- a lower one (marked B on Figure 1A) into which the saturated swab is to be inserted and an upper one (marked B on Figure 1A) toward which the swab will be pushed after insertion thereof into the hole B. The position of the overlabel with its holes A and B, and the swab well cooperate to hold the swab in a proper position during the assay and to promote the expulsion of sorbed liquid from the swab.

A preassembled test strip (marked C on Figure 1) described below, is inserted into the recess (labeled 2 on Figure 1) and held in place by an adhesive applied to the bottom thereof. An overlabel shown in Figure 1B is placed atop the left-hand side. It has been equipped with a single hole (marked D in Figure 1B) which mates to the right-hand side hole A when the device is closed for performance of the assay.

The assembled device is stored in a sealed pouch with desiccant until it is used. Prior to sealing the pouch and storing, a lightly adhesive tape is placed on the outer edge of the right-hand half of the device.

B. Construction and Preparation of the Preassembled Test Strip

Figure 1C shows the construction of the preassembled strip. It is comprised of a conjugate pad of sorbent material in which a conjugate of gold particles and the affinity-purified rabbit anti-Legionella pneumophila serogroup 1 antibodies described above have been impregnated. In contact with this pad is a nitrocellulose pad onto which a capture line for the sample which reacts with the conjugate has been established by embedding a stripe of affinity-purified rabbit anti-L. pneumophila serogroup 1 antibodies, prepared as described above. The nitrocellulose pad also has a downstream control line established by striping the pad with goat anti-rabbit immunoglobulin (IgG). Following the nitrocellulose pad,

the strip is ended by an absorbent pad which serves as a reservoir for liquid. All of these pads are backed by an adhesive strip when the device is ready to ship.

The conjugate pad is normally made from non-woven polyester or extruded cellulose acetate. To prepare this pad for use in the assay, gold particles of 50 nm. diameter are conjugated to affinity-purified rabbit anti-Legionella pneumophila serotype 1 antibodies prepared as described above. The conjugation is effected using a known method such as that described by DeMay in Polak, J.M. and Van Norden, S. (Eds.), Immunochemistry: Modern Methods and Application, (Wright, Bristol, England, 1986). The gold conjugate particles are mixed with a drying agent consisting of aqueous 5mM sodium tetraborate of pH 8.0 containing 1.0% BSA, 0.1% Triton™ X-100, 2.0% Tween™ 20, 6.0% sucrose and 0.02% sodium azide. The pad is heated sufficiently to remove all of the liquid present and stored in a low-humidity environment pending assembly of the test strip. These pads and their treatment are especially chosen so that the pads will hold the dry conjugate and will release it only when later wetted by sample.

The nitrocellulose pad is first treated by embedding a stripe of affinity purified rabbit anti-L. pneumophila serotype 1 antibodies in a first portion thereof, using a carrier solution of phosphate buffered saline. These antibodies act as the capture line. In a second portion of the pad downstream of the first one in the assembled test device, the control line is established by striping goat anti-rabbit IgG in the same carrier solution on the surface of the pad. The nitrocellulose pad is then subjected to desiccation at 18-25° C to promote permanent absorption of the protein stripes thereto.

The absorbent pad used is of a commercially available cellulosic material sold under the name Ahlstrom 939™. This pad requires no special treatment.

C. Kit Preparation

As sold in commerce, the test device containing the finished test strip is assembled. In practice, a number of devices are packaged with a commensurate number of swabs fashioned from fibrous Dacron™ and a bottle of "Reagent A" equipped with a top adapted to deliver Reagent A dropwise. "Reagent A" is a

solution of 2.0% Tween 20, 0.05% sodium azide and 0.5% sodium dodecyl sulfate in a 0.05 M sodium citrate-sodium phosphate buffer of pH 6.5. Positive and negative controls are also included in each kit.

The use of the finished test devices to identify L. pneumophila serogroup 1 O-polysaccharide antigen is illustrated in the following example VIII:

Example VIII — Conducting the ICT Test for L. pneumophila Serogroup 1 O-Polysaccharide Antigen

In practice, the swab furnished with each device is dipped into the liquid sample, completely immersing the swab head. The use of the swab to act as a filter for undissolved solids, semisolids and colloids present in liquid biological samples such as urine, blood, lymph, etc. and also in liquid environmental samples is the subject of U.S. Patent No. 6,548,309 of Norman Moore and Vincent Sy filed March 19, 1998. The swab is inserted into the hole at the bottom of the device (hole B of Figure 1A) and gently pushed upward so that the swab tip is visible in the top hole (hole A of Figure 1A). The Reagent A vial is held vertically above hole B and two drops of Reagent A are slowly added. The adhesive liner is then immediately peeled from the right edge of the device and the device is closed and securely sealed, thus pressing the swab in the swab well against the gold conjugate pad. After 15 minutes, the result can be read in the window of the device. A negative sample -- i.e., one containing no L. pneumophila serogroup 1 O-polysaccharide antigen -- will exhibit only the control line in the top half of the window. A positive sample containing the target antigen will show two lines, the lower one of which is the patient (or sample) line; even a faint sample line indicates the presence of the target antigen in the sample. If no line appears in the window after 15 minutes, or only a sample line appears in the lower part of the window, the test is invalid and must be repeated.

Using the procedure described above, the devices prepared as described in Example VII were tested in the ICT procedure just described against 300 patient

urine samples, 100 of which had been previously diagnosed as having L. pneumophila serogroup 1 infection.

These ICT tests according to this invention were conducted under circumstances such that the previous diagnoses were unknown to personnel performing the ICT tests. Overall, 98% of the ICT tests agreed with the previous positive diagnoses. Also overall, 98% of the urine samples previously diagnosed as negative for L. pneumophila serogroup 1 O-polysaccharide antigen gave results in agreement therewith when tested by the ICT procedure described herein, using the ICT device described in Example VII.

Example IX -- Use of the ICT to Test Environmental Samples

Applicability of this same test to environmental samples suspected of containing L. pneumophila serogroup 1 was also investigated as follows:

Water was seeded with L. pneumophila serogroup 1 bacteria obtained from a commercial source. The mixture was concentrated by filtering through a 0.22 μm filter. A swab dipped in the sample was applied to the device, the device was closed and the assay was allowed to proceed. A positive result was observed within less than 15 minutes.

Example X - Western Blot Immunoassay For Detection of Cross-Reactive Carbohydrate Antigens of L. Pneumophila Serogroups 1, 2, 4 and 5

In order to perform the Western Blot immunoassay using a kit purchased from Bio-Rad Laboratories, L. pneumophila serogroup 5 cells were cultured as in Example II. A suspension of these cells was solubilized with 1 % sodium dodecylsulfate in the presence of 10 mM mercaptoethanol at 100°C for 5 minutes. The solubilized cells were treated with protease K and then subjected to electrophoretic separation of protein according to standard procedures provided by Bio-Rad.

The carbohydrate antigen from L. pneumophila serogroup 5 was conjugated to the spacer molecule described in Example III hereof in the manner described in

Example IV and applied to an activated Sepharose column as described in Example V. This column was then used for the affinity purification of polyvalent rabbit antibodies specific to the carbohydrate antigen of L. pneumophila serogroup 5 (which were conventionally obtained from serum of a rabbit previously injected with the protein-containing of L. pneumophila serogroup 5) using the procedure of Example VI.

The Western immunoblot analysis was performed using a reagent kit from Bio-Rad and according to directions from this manufacturer. Briefly, the PBS extract of cells of L. pneumophila antigens 1, 2, 4 and 5 was subjected to the SDS-PAGE in 12% polyacrylamide gel blocked with 1% BSA with PBS transferred onto a nitrocellulose membrane. After this step, the membrane was incubated with affinity purified antibodies specific to carbohydrate of L. pneumophila serogroup 5. The membrane, washed as recommended by the manufacturer, and incubated with horseradish peroxidase conjugated to goat-anti-rabbit antibodies provided by Bio-Rad. After washing, the membrane was developed with a substrate system of 0.022 M 4-chloro-1 naphthol and 0.0012 M N, N-dimethyl-p-phenylene-diamine monohydrochloride in 0.1 M sodium citrate buffer of pH 6.9 containing 2.9 mM of hydrogen peroxide. Figure 2 hereof shows the Western blot assay results compared with that of the prestained SDS-PAGE standard (in Lane 5) for the affinity purified antibodies of serogroup 5 of L. pneumophila against PBS extracts containing antigens of L. pneumophila as follows:

Lanes 1 and 7--L. pneumophila serogroup 2 (strain Togus-1)

Lanes 2 and 8--L. pneumophila serogroup 4 (strain Los Angeles-1)

Lanes 3 and 6--L. pneumophila serogroup 1 (strain Philadelphia-1)

Lanes 4 and 9--L. pneumophila serogroup 5 (strain U8W).

It is pointed out that the affinity purified antibodies for Lanes 1-4 were affinity purified on a column to which carbohydrate antigen from L. pneumophila serogroup 5 (strain U8W) was attached while those for Lanes 6-9 were affinity purified in the same manner on a column having attached carbohydrate antigen of L. pneumophila serogroup 5 (strain Dallas IE).

Figure 2 clearly demonstrates that affinity purified antibodies as herein disclosed of L. pneumophila serogroup 5 react with antigens of L. pneumophila serogroups 1, 2, and 4 in addition to those of serogroup 5.

An ICT assay as described above in which affinity purified antibodies from L. pneumophila serogroup 5 are substituted for affinity purified antibodies from L. pneumophila serogroup 1 is contemplated.

Those skilled in the art of immunochemistry generally, and especially those skilled in immunoassays, will recognize that other materials and ingredients and at times, other procedural steps, can readily be substituted for those specifically recommended herein. A vast array of literature, both patent and non-patent, discusses the design and use of reliable, one-time-use, disposable immunoassay test devices that could be substituted for the preferred ICT device described and recommended herein. It is not intended that the present invention should be limited with respect to substitutable assay devices, materials, ingredients or process steps except insofar as the following claims may so limit it.

We claim:

1. A method for obtaining an essentially protein free O-polysaccharide antigen from Legionella pneumophila serogroup 1 bacteria, which comprises the steps of:

(a) harvesting bacterial cells from a culture of a L. pneumophila serogroup 1 in the form of a wet cell pellet;

(b) suspending the wet cell pellet in 0.1M NaOH for about 45 minutes with stirring;

(c) adjusting the pH of the suspension to an acid pH and centrifuging the suspension;

(d) separating the supernatant resulting from the centrifugation of step (d) and adjusting the pH of the supernatant to approximate neutrality;

(e) adding a broad spectrum protease enzyme preparation to the supernatant and digesting residual proteins in the supernatant for at least about 15 hours;

(f) adjusting the pH of the digestion mixture of step (e) to an alkaline pH, applying the mixture to a size exclusion column equilibrated with a weakly alkaline solution, pooling material eluted in the first peak and adjusting the pH of the pooled material to approximate neutrality, to produce an essentially protein free polysaccharide antigen.

2. The method of claim 1 wherein the wet cell pellet is suspended in 20ml 0.1M NaOH per gram of wet cells.

3. The method of claim 1 or 2 wherein, in step (c), the pH is adjusted to about 3.0.

4. The method of any one of claims 1 to 3 wherein, in step (f), the pH is adjusted to between about 10 and about 11.

5. The method of any one of claims 1 to 4 wherein the pooled, pH-adjusted material of step (f) is lyophilized.

6. An essentially protein free, O-polysaccharide antigen obtained from L. pneumophila serogroup 1 bacteria by the method of any one of claims 1 to 5.

7. A method for the purification of polyclonal antibodies to L. pneumophila serogroup 1 comprising:

(a) obtaining an essentially protein free O-polysaccharide antigen from L. pneumophila serogroup 1 by the method of any one of claims 1 to 5;

(b) conjugating said antigen through a spacer molecule to an activated chromatographic affinity column;

(c) subjecting polyclonal antibodies to L. pneumophila serogroup 1 to affinity chromatography on the column from step (b); and

(d) eluting antibodies bound to said column to produce purified antibodies.

8. Purified antibodies to L. pneumophila serogroup 1 and its O-polysaccharide antigen, produced by the method of claim 7.

9. An immunochromatographic ("ICT") test device for the detection, in a sample of fluid, of the O-polysaccharide antigen of L. pneumophila serogroup 1, which device comprises a housing having a view window and containing a strip of bibulous material, wherein the strip comprises:

(1) a first zone having a sample introduction point at or near its free end, wherein there is movably deposited at a location close to, but slightly removed from, said sample introduction point, a portion of polyvalent antibodies raised in an animal against either (i) L. pneumophila serogroup 1 bacteria or (ii) the O-polysaccharide antigen of L. pneumophila serogroup 1, which antibodies have been tagged with a

tag that exhibits a colour change upon formation of a tagged antibody-antigen-untagged antibody "sandwich", and

(2) a second zone located under the view window of the device and following said first zone, wherein a capture line has been formed on said strip, near its end opposite from the sample introduction point, by immovably striping a portion of antibodies that are nontagged, but otherwise identical to those present in said first zone, across the width of said strip, and wherein the antibodies in both zones of said strip have been treated by (i) passing them over a chromatographic affinity column to which is coupled, through a spacer molecule, an essentially protein free O-polysaccharide antigen from L. pneumophila serogroup 1 bacteria obtained by the method of any one of claims 1 to 5; and (ii) then eluting from said column, with an eluting agent, those antibodies that bound to said antigen.

10. The device of claim 9 wherein the tag is finely divided gold.

11. An immunochromatographic (ICT) assay for detecting the presence of L. pneumophila serogroup 1 O-polysaccharide antigen in a fluid comprising:

(a) introducing an aliquot of a sample of the fluid into the first zone of the device of claim 9 or 10 at the sample introduction point;

(b) allowing the sample to flow along the strip and pick up the movably deposited tagged antibodies present in said first zone;

(c) allowing the sample and tagged antibodies to continue to flow, together, along the strip and mingle thoroughly, thereby forming tagged antibody-antigen conjugates if the O-polysaccharide antigen of L. pneumophila serogroup 1 is present in the sample;

(d) allowing the flowing mixture of tagged antibodies, sample and any already formed tagged antibody-antigen conjugates to flow into the second zone of said test device and contact the immovable capture line located therein; and

(e) after 15 minutes from the time at which the sample was introduced to said strip, observing whether a color change, caused by massing of the tag along the capture line of the test device, has occurred, thereby denoting that tagged antibody-antigen-untagged antibody "sandwiches" have formed along that line and hence, that the O-polysaccharide antigen of L. pneumophila serogroup 1 is present in the sample.

12. The process of claim 11 wherein the fluid is a bodily fluid.

13. The process of claim 12 wherein the bodily fluid is human urine.

14. The process of claim 12 or 13 wherein the sample is obtained from a human exhibiting clinical signs of acute respiratory disease.

15. The process of claim 11 wherein the fluid is water.

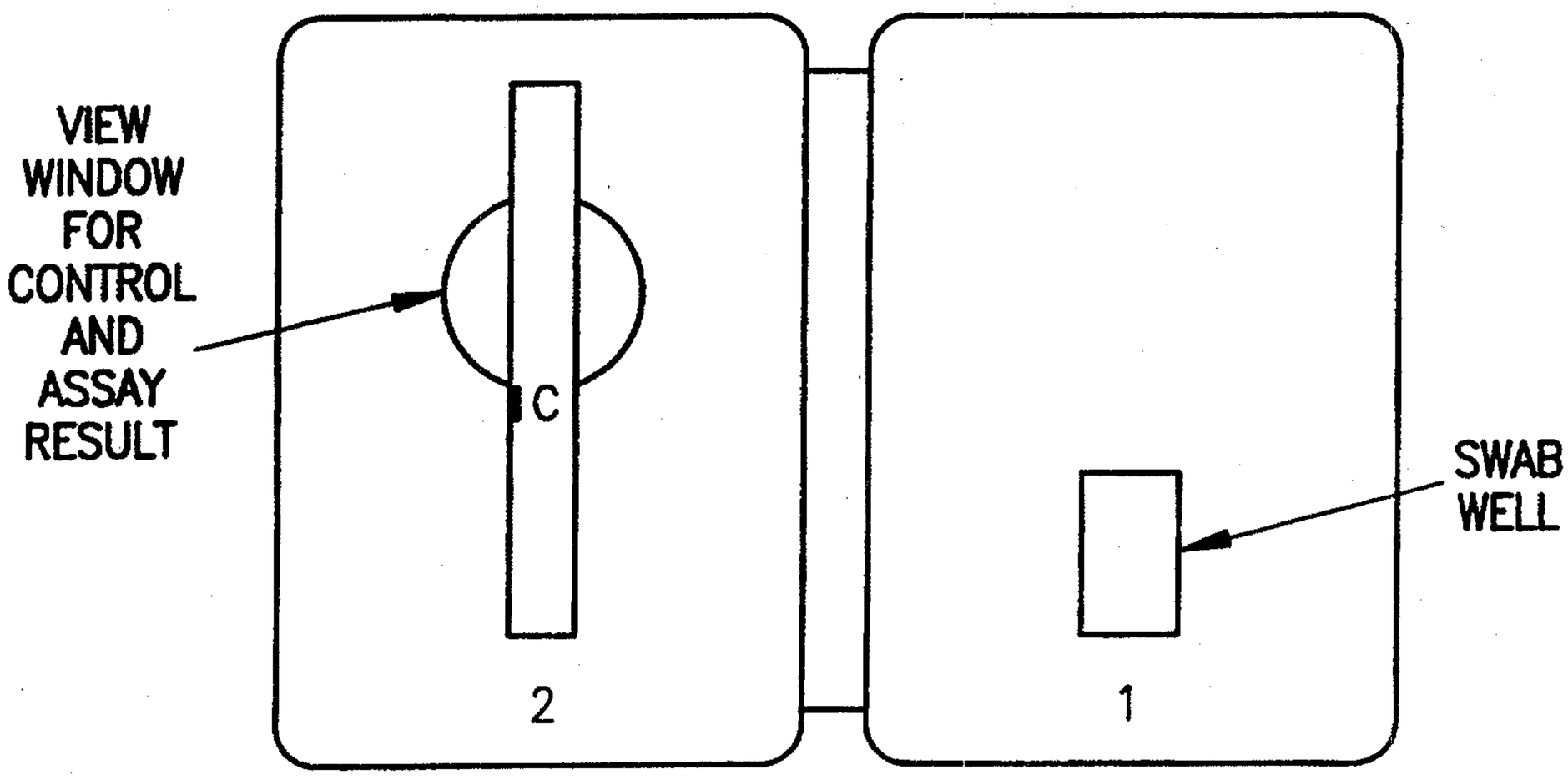


FIG. 1

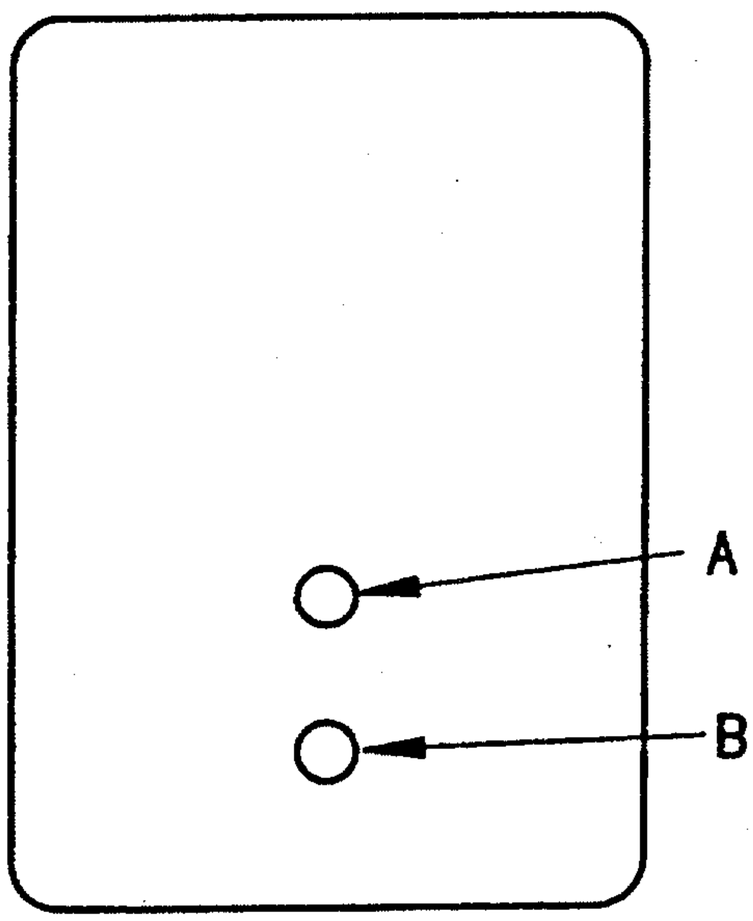


FIG. 1A

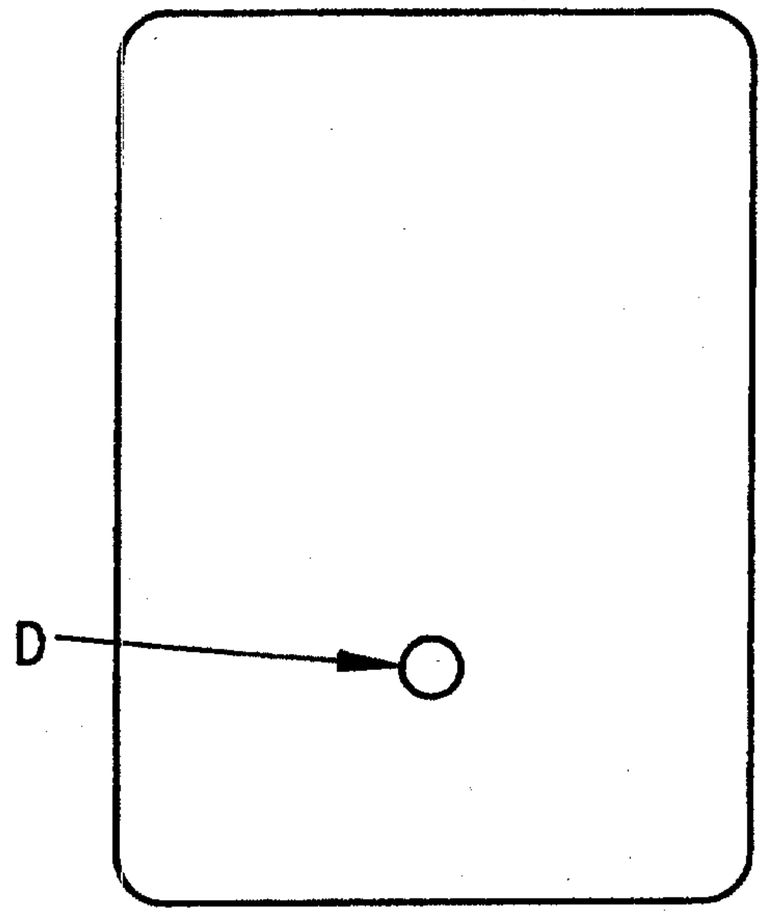


FIG. 1B

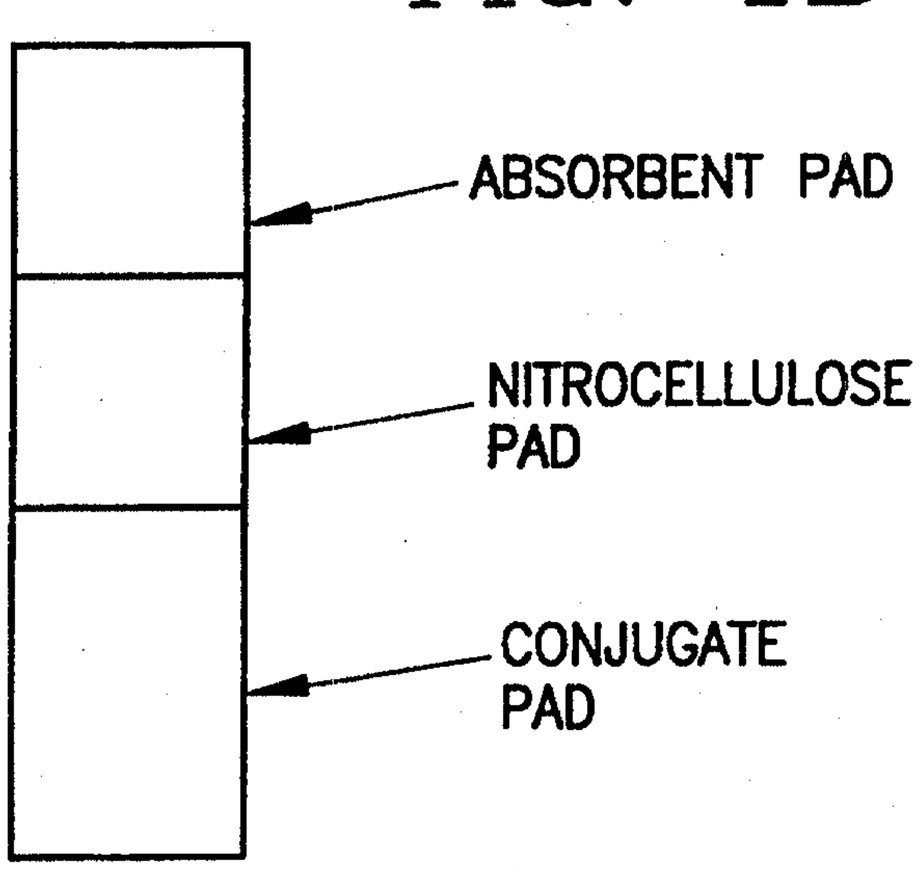
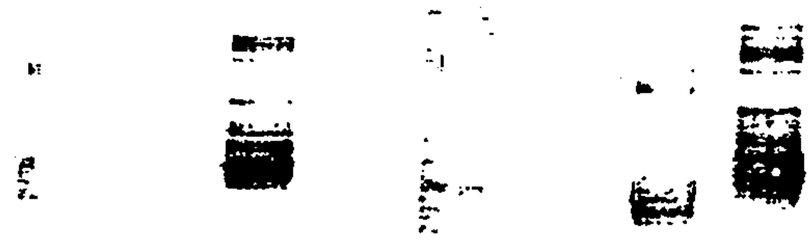


FIG. 1C



1 2 3 4 5 6 7 8 9

FIG. 2