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METHOD OF DETECTING OR CLASSIFYING MICROORGANISMS
USING AGAR REAGENT SHEETS
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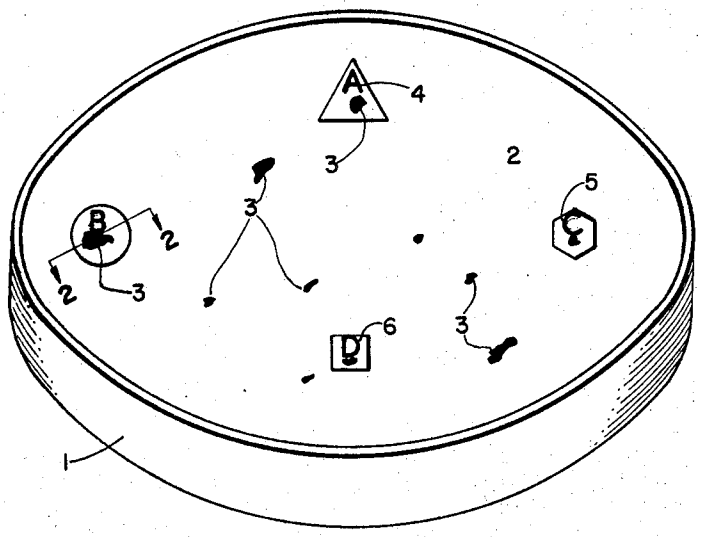


FIG. 1

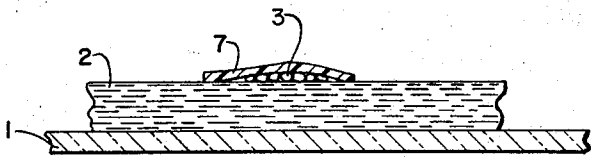


FIG. 2

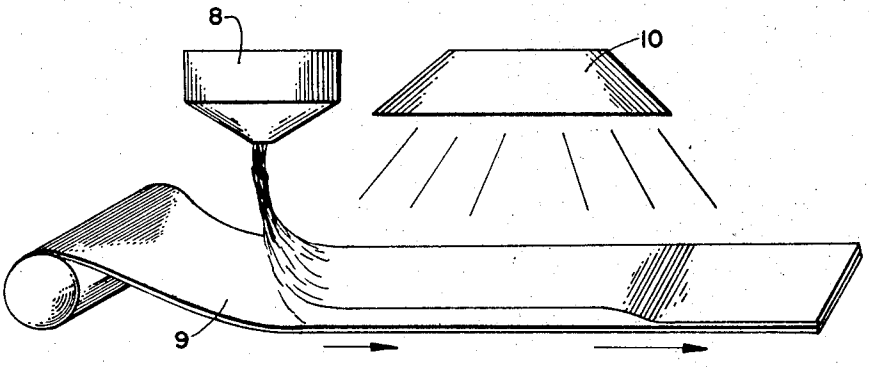


FIG. 3

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METHOD OF DETECTING OR CLASSIFYING MICROORGANISMS USING AGAR REAGENT SHEETS

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Continuation-in-part of application Ser. No. 542,978, Mar. 4, 1966, which is a continuation-in-part of Ser. No. 286,532, June 10, 1963. This application Oct. 11, 1967, Ser. No. 687,405

5 Claims. (Cl. 195—103.5)

ABSTRACT OF THE DISCLOSURE

A dried transparent agar sheet having a test reagent responsive to some product elaborated by the microorganism to be detected incorporated therein. The test sheet is used as such, or attached to a sheet of transparent film, or by placing on conventional agar plates having colonies of microorganisms growing thereon.

This application is a continuation-in-part of my previous application Ser. No. 542,978, filed Mar. 4, 1966, and now abandoned, which in turn is a continuation-in-part of my now abandoned earlier application Ser. No. 286,532, filed June 10, 1963.

This invention relates to microbiological assaying and particularly to a novel means and method of performing microbiological assays in a simple and expeditious manner.

It is well known in the prior art that dried paper discs impregnated with various chemotherapeutic agents or biological or chemical agents have been used for biological assays. In the prior art small discs, approximately 1 centimeter in diameter, are cut from a sheet of filter or blotting paper, and submerged in a solution or suspension having in it a known concentration of the antibiotic or chemical agent. The discs are removed, drained, and dried and can be stored for extended periods of time. Bacterial infected material, either taken from a patient or in pure culture, is evenly spread on the surface of a suitable solid culture medium incorporating agar as a gelling agent, and contained in a petri dish using the usual laboratory methods. The antibiotic-impregnated discs are placed on the surface of the medium and the petri dish is incubated at the optimum temperature for growth of the microorganisms; 37° C. is optimum for most microorganisms pathogenic for men. During the period of incubation the antibiotic, or chemical, or biological agent impregnated into the disc diffuses out into the medium. The concentration of agent is greater near the perimeter of the disc than it is some distance away from the edge of the disc. If the bacteria on the medium are resistant to the antibiotic, or other chemotherapeutic agent, they will grow right up to the edge of the disc impregnated with that inhibitor. If, on the other hand, the bacteria are susceptible to the agent there will be a clear area free of microbial colonies surrounding the edge of the disc.

The occurrence of strains of antibiotic-resistant bacteria from originally antibiotic-resistant strains has made it necessary to assay for antibacterial activity more than one antibiotic and, as a result, more than one disc is used when testing a culture.

The impregnated disc method of the prior art has certain disadvantages which will be apparent; particularly, the disc is made of filter paper, or blotting paper, which are opaque materials. As a result, chemical reactions, due to the diffusion of enzymes or gaseous or other matter from bacterial colonies, may obscure color changes or color discharges or other physical phenomena, such as the formation of gas bubbles, which take place directly under

the disc and are therefore not visible, so that the only effect which can be observed is that which is beyond the diameter of the disc.

Also, when chemotherapeutic or chemical agents are soaked up into paper discs prepared as described in the prior art, it is difficult to be sure that the same amount of chemotherapeutic or chemical agent is being absorbed in every paper disc at every time discs are prepared.

It is the primary objective of the present invention to overcome these difficulties by using, in place of the opaque paper or filter paper or absorbent paper, a transparent medium of which the testing agent, such as chemical or biological or chemotherapeutic agent, is an integral part.

Another object of the preent invention is to overcome the problem of making sure that an exactly correct and uniform amount of testing agent, such as chemotherapeutic or chemical or biological agent is in each disc.

Another object of the present invention is to provide a sheet material for the disc which will be completely compatible with the medium used for growing the bacterial culture, and thus avoid any possibility of the disc material itself exerting an inhibitory or stimulating effect on the microorganisms since the disc material is a substance foreign to the nutrient agar.

My invention lies essentially in the use of a medium consisting of a dried transparent sheet of agar in which testing agents, such as chemotherapeutic or chemical or biological agents, form a constituent part. The testing agents employed would be similar to those used to test microorganisms such as a bacterial or fungal culture, virus, rickettsiae, or a cell or tissue culture in a more conventional nutrient medium, i.e., are responsive to some product elaborated by the microorganism to be detected. In one specific aspect of the present invention, oxalated human plasma is incorporated in agar and dried down to sheet form in a vacuum dessicator at room temperature, i.e., about 24° C. to avoid damage to the plasma. Discs cut from the agar sheet, thus incorporating the oxalated human plasma, can be used in testing *Staphylococcus aureus* for bound coagulase activity. A platinum bacteriologic transfer wire loop full of an aqueous suspension of the *Staphylococcus aureus* is gently rubbed, by the wire loop, onto the dry agar disc. The fibrin formed by the action of bacterial bound coagulase on the plasma fibrinogen, pulls the *Staphylococcus aureus* cells together in a clumping reaction of the organisms; this is a characteristic test for virulence of this organism. The clumping is immediately apparent to the naked eye. The principal advantage of this procedure over the standard procedure, in which a petri dish or a tube of nutrient medium is used to first grow a colony (or large numbers) of the strain of *Staphylococcus aureus*, the organisms then being transferred to a glass slide and mixed with a drop of the plasma to give the clumping effect, is that no glass slide need be used, and the plasma is in a stable form within the dried agar discs, and only a very small amount of plasma is used per disc. This avoids the waste of substantial quantities of liquid plasma which cannot be kept for later tests in the standard method. Also, the disc is always immediately ready for use.

The present invention is more particularly designed for reactions which take place on an agar medium in a petri dish and give rise to a visible physical phenomenon, such as color change that is either a color discharge, or a change in one of the chemical reagents contained in the disc, from one color to another, or a reaction in which it is advisable to view the reaction which takes place beneath the disc containing the chemotherapeutic or chemical or biological agent, such as the disappearance of a bacterial colony, or failure of growth, or the development of opacity due to a precipitation reaction,

visible to the naked eye, or the entrapment of bubbles of gas evalved by action of the bacteria upon the chemical agents incorporated into the disc.

To illustrate this aspect of my invention reference is made to the accompanying drawings wherein:

FIGURE 1 is a perspective view of a petri dish holding a bacterial culture;

FIGURE 2 is an enlarged cross-sectional view of the petri dish with the culture and material in place;

FIGURE 3 is a diagrammatic illustration of a machine for making the material of the invention.

Referring to FIGURES 1 and 2, a standard petri dish 1 containing a standard culture medium 2, such as nutrient agar, has streaked on it a bacterial specimen which gives rise, after proper incubation, to visible colonies of bacteria 3. The pieces of dried transparent agar testing sheet 4, 5, 6 and 7 are preferably made of the same materials contained in the culture medium used in the petri dish, but are in dried transparent sheet form. Incorporated into the dried transparent agar sheet is the particular chemotherapeutic or chemical or biological agent which it is desired to use to test the bacteria or other microorganism being tested, either for sensitivity, or for the production of enzyme or other by-products of growth of microorganism.

In making the dried agar sheet according to the present invention, the test agent or reagent is preferably incorporated into the dried agar sheet by first incorporating a predetermined fixed amount of the reagent into a quantity of standard agar in aqueous solution and permitting the agar to gel and dry. The latter can be accomplished by pouring the agar medium into a flat bottomed shallow dish and allowing the agar medium to stand until it dries to a thin transparent film. Preferably, the drying is expedited by putting the dish in an oven at 42° C. The dried agar sheet is removed from the flat bottomed shallow dish and cut to form pieces of the sizes required. Each piece will contain a predetermined amount of test reagent as a constituent of the sheet based on the presence of a fixed amount of reagent per unit area of the sheet.

The transparent agar sheet of the present invention is semipermeable to air and moisture. This allows the bacterial culture beneath the agar sheet disc to grow in semi-aerobic condition and if the bacteria requires, allows air to enter to the microorganisms. By varying the concentration of agar in the solution from which the sheet is made, or the amount of solution used to make the sheet, the thickness of the agar sheet can be varied and the degree of permeability to gases controlled.

A continuous method of making the dried reagent sheet is diagrammatically illustrated in FIGURE 3. Container 8 holds the liquid agar containing a predetermined amount of reagent which is dropped onto a moving sheet of plastic material 9, such as cellophane, polyethylene, cellulose acetate, or vinyl copolymer. The moving sheet passes the sheet of agar under a drying apparatus 10, which may be a heat lamp, or battery of heat lamps, or a drying oven. As it issues from the drying apparatus, the agar has been dried to a thin sheet containing the reagent in a predetermined amount as a constituent of the sheet itself. The reagent sheet is then stripped from the plastic sheet and cut into convenient sizes for use.

The agar film of this invention, after being dried on transparent cellophane or plastic film, can be die cut together with the film so as to have the cellophane or plastic film as a reinforcement and an added film for the entrapment of gases. If used in this fashion, the agar is in contact with the microorganisms, and the film serves to make it more easy to handle and to make the discs distinctively.

It is apparent that the reagent sheets of the present invention can be cut into many different shapes, and different sheets also may contain many different chemical, or chemotherapeutic test agents. The sheets may be cut into pieces of varying shapes to identify the particular

chemical or biological, or chemotherapeutic agent contained therein. For example, as shown in FIGURE 1, the shape of the pieces can be triangular, as in piece 4, hexagonal as in piece 5, square as in piece 6, or a circular disc as in piece 7. In addition, specific code letters can be placed on the pieces. Also as shown in FIGURE 1, piece 4 has impressed on it the letter A, piece 5 has impressed on it the letter C, piece 6 has impressed on it the letter D, and piece 7 has impressed on it the letter B.

As a general example of the preparation of an agar sheet according to my invention, purified agar is dissolved in hot water to form a solution of the desired concentration. For example, 1.5 gm. per 100 cc. has been found to be a satisfactory concentration for most purposes. It is desirable to use a higher concentration of agar, e.g., approximately 4 to 5 gm. per 100 ml. of water when preparing a dried agar sheet for use in gas entrapment tests. Thermostable chemicals, biological agents, antibiotics, or chemotherapeutic agents, as well as dyes, or pH indicators, in the desired amounts, are added to the agar solution which is then poured into a flat bottomed shallow dish and allowed to cool and harden. Varying either the depth or concentration of the agar in the solution as poured into the dishes will control the thickness of the resultant dried agar sheet.

Preferably, the agar solution is poured into a plastic petri dish. Plastic petri dishes do not hold or retain heat as do glass petri dishes and the agar dries more uniformly throughout. The material in contact with the plastic dish is not subjected to excessive heat and the resultant dried agar is more readily peeled from the bottom of the dish.

It may be necessary or desirable to add relatively thermolabile materials to the agar, i.e., materials that can tolerate 50° C. but not higher temperatures. Such materials are added to the agar solution after it has been permitted to cool to 48° C. just before the solution is poured into the flat bottomed dishes.

It may be necessary or desirable to add to the agar chemicals, biological agents, antibiotics, or chemotherapeutic testing agents that are labile at 48° C. Such materials are poured atop the cooled agar immediately after it has been poured into the flat bottomed dishes but before the agar has had time to dry. The applied materials diffuse into the agar.

Reagent materials that may be incorporated into the agar include the following:

Sugars with dye pH indicators.
Desoxyribonucleic acid with toluidine blue.
Coagulase reacting plasma.
Antibody.
Sodium desoxycholate with dye markers to indicate colony dissolution for differentiation of *Diplococcus pneumoniae.*
Sodium nitrate, for nitrite formation tests.
Oxidase reactant materials for the Neisseria oxidase test.
Antibiotics.
Bacteriophages.
Chemotherapeutic agents.
Dyes for Brucella speciation, and
Other biochemicals, chemicals, or reagents for which tube, or plate tests, are usually used in microbiological testing.

If sterility of materials is required, the agar in aqueous solution is autoclaved at 120° C. for fifteen minutes under 15 lbs. pressure before being poured into sterile flat bottomed dishes and exposed to drying in sterile air. Such autoclaving of agar, plus added components, is employed if the added components are stable at the autoclave temperature of 120° C. Otherwise, only the agar is autoclaved and the thermolabile components to be added are separately sterilized such as by filtration (Seitz, diatomaceous earth, porcelain, fritted glass, or membrane filters),

before being added to the agar as discussed above under sterile conditions.

Another method of sterilization can be used regardless of whether or not the chemicals, biological materials, antibiotics, or chemotherapeutic testing agents are thermolabile. The agar is dissolved by boiling in distilled water at 100° C., poured into sheets, and the chemicals, biochemicals, antibiotics, or chemotherapeutic testing agents are poured atop the cooled and gelled, but not dried, agar and permitted to diffuse into the agar. After the agar has hardened, the resultant agar-reagent sheets are sterilized by exposure to ethylene oxide in a gas sterilizer, then dried by being held in vacuo in a dessicator.

As discussed above, the agar that had been poured into petri dishes to harden and dry is most expeditiously dried by moderate warming under vacuum or in a current of dry air. When drying agar containing heat labile materials such as plasma, serum complement and penicillin, vacuum dessication at room temperature or even lower temperatures should be utilized.

The following are examples of agar-reagent sheets that have been made and proved efficacious on testing. In each example the ingredients listed are mixed uniformly with water in proportions indicated as percent by weight and the composition dried to a thin brittle sheet.

Example 1

	Percent
Agar	1.5
Lactose	1
Phenol red adjusted to pH 7.6 with dilute sodium hydroxide	0.025

Discs cut from the above sheet, when placed atop fresh heavy inocula for 18–24 hour old colonies on nutrient agar of *Escherichia coli*, have indicated microbial lactose fermentation to acid by the phenol red atop and about the colonies changing to the acid yellow color within three hours after the lactose has been broken down by the specific enzymes of these bacteria. Gas formation by the bacteria carrying out this fermentation was detected by the visible bubbles of gas seen trapped under the test agar disc, when the agar concentration of the prepared disc was 4.5% rather than 1.5%.

Example 2

	Percent
Agar	1.5
Mannitol	1
Phenol red adjusted to pH 7.6 with dilute sodium hydroxide	0.025

The mannitol agar sheet, placed atop a culture of *Staphylococcus aureus* grown on nutrient agar plates, has indicated bacterial mannitol fermentation, by phenol red atop and diffused about the colonies, changing to the acid yellow color within four hours after the mannitol was fermented by the specific enzymes of these bacteria.

Example 3

	Percent
Agar	1.5
Sodium desoxycholate	1
Gentian violet	0.01

The sodium desoxycholate agar sheet, placed atop colonies of twenty-four hour cultures of *Diplococcus pneumoniae* and *Streptococcus viridans* grown on 5% sheet blood agar at 37° C., differentiated between the two microorganisms by dissolving the colonies of *Diplococcus pneumoniae*. The gentian violet stained the streptococcal colonies violet and, therefore, even more clearly indicated the difference in reaction between these colonies and those of the pneumococci.

Example 4

When a disc of agar test sheet prepared as in Example 3 was placed atop freshly streaked cultures of *Diplococcus pneumoniae* and *Streptococcus viridans* covering the surface of a blood agar plate, it differentiated between the two organisms in that the bile salt diffused from the agar disc and, in the case of *Diplococcus pneumoniae*, inhibited growth of the organisms in a wide area about the limited area of the agar disc.

As exemplified below, dried agar sheets can also be prepared with food or food supplements incorporated into the agar. Such food or food supplements can be meat infusion, blood digest, vitamins, or blood. The food or food supplements that have been incorporated into the discs themselves are available to the microorganisms when the discs are placed atop the microorganisms, and then permit the observation of differential growth of those microorganisms that require such food or food supplement to manifest growth. Thus, for example, when a nutritionally deficient basal growth medium in petri dishes is streaked with a microorganism whose growth requirements are not satisfied by the constituents of this basal medium, then my dried agar discs, each containing different supplemental growth factors can be used on top of such inoculated basal medium to distinguish those microorganisms that will manifest growth if particular food supplements are made available to them.

Example 5

A basal medium consisting of Brain-Heart infusion agar in a petri dish is inoculated on its surface with *Haemophilus influenzae* which will grow only when haemin and coenzyme A are both available. By making up the dried agar sheets with haemin alone and coenzyme A alone, and another sheet with both haemin and coenzyme A, one can speciate the Haemophilus genus to distinguish *Haemophilus influenzae* from other Haemophilus species. Speciation is accomplished in a simple and inexpensive manner using only one petri dish in place of several that would otherwise be required.

A particular example of the aspect of the present invention, which involves the use of the bacterial test on the dried agar sheet without requiring growth of microorganisms, is in its use in the bound coagulase test. This test is one of the most important of the procedures used to determine whether an isolated strain of Staphylococcus is potentially virulent. The test determines the presence of a factor, bound coagulase, that is closely associated with the bacterial cell surface. Possession of the bound coagulase enables the organisms to transform human plasma fibrinogen to fibrin. The fibrin, as it is formed, pulls the bacterial cells together and causes them to appear clumped.

Example 6

Plasma is incorporated into a disc of agar as described below. The droplet of emulsified bacterial culture that is rubbed over the surface of the disc causes the bacterial to clump into large clumps, visible to the naked eye, when those bacteria contain bound coagulase. The reaction is instantaneous.

To prepare the bound coagulase test reactant discs, 0.6 mg. of dried commercial agar is added to 13.5 ml. of water. The agar is dissolved by placing the agar-water mixture in a flask in a boiling water bath. The agar solution is cooled to 46° C. and 1.5 ml. of freshly drawn oxalated human plasma, or other fibrinogen-containing plasma, is added to the mixture and thoroughly mixed by stirring. The contents of the flask are poured into a 75 mm. diameter petri dish, preferably made of plastic, and permitted to cool and gel. The dish is placed in a vacuum jar at room temperature, and the agar-plasma gel is converted into a dry sheet by evaporation of the water under vacuum. The dried agar-plasma sheet is cut into discs approximately 1 cm. in diameter for use in the test.

Among the important advantages of my invention are the following: It permits the packaging of single, reactive, light-weight units of a microbiological test medium. It also permits the setting up of microbiological, medical, or public health, or industrial field laboratory units where at

least a portion of the testing materials are ready for immediate use. Since the agar sheet media can be prepared in advance and are stable on storage, the invention saves the time required to prepare conventional microbiological test materials. This would be of great value in the event of an emergency situation. The present invention also minimizes the amount of materials, particularly test reagents, required for a given test.

Discs cut from dried agar sheets have the advantage of being relatively transparent and of embodying as a constituent part thereof reacting materials that may be water soluble. The discs, when placed atop plates of nutrient agar on which microorganisms, fungi, bacterial, protozoa, etc., have been either freshly inoculated, or previously grown to colony size, by virtue of the reactants present, can react either with the bacteria, or their extra-cellular products, such as enzymes, or different gases, so as to give reactions of identity for the microorganisms. It has been found that when the dried agar discs, as described above, are placed on freshly inoculated plates of agar the reaction occurs even more rapidly than when the dried agar discs are placed on fully grown colonies of bacteria. The discs permit, by virtue of their transparency, the observation of single colony effects by seeing the results of testing on single colonies below the discs. Moreover, tests done with the minute amounts of reactant materials in the agar discs placed atop microbial or fungal colonies have the added advantage of being rapid tests, requiring only a small fraction of the time otherwise required for standard tests, where the microbes or fungi have to act on a relatively large amount of test material before a reaction is visible.

The dried agar sheets or discs of the present invention can be used independently of living biological systems, i.e., in addition to being used on nutrient agar in petri dishes, the sheets or discs can be used to test for any material that will give visible reaction with the reagent present. For example, the dried agar sheets of the present invention can also be used in electrophoresis and in chromatography as well as the other uses described herein.

It is important to note that the agar sheets of the present invention are thin dry brittle sheets incapable of supporting bacterial growth per se. These dry storage-stable sheets should not be confused with conventional agar plates which are dried only to the extent of permitting excess surface moisture to evaporate primarily to prevent undue colony spreading. The prior art cautions against the further drying of conventional agar plates.

I claim:

1. A method of detecting or classifying microorganisms which comprises placing a piece of dry transparent agar sheet having a test reagent visually responsive to a product elaborated by the said microorganisms incorporated therein over a colony of microroganisms growing on a nutrient agar medium, and observing the visual response.

2. A method according to claim 1 wherein the visual response is a color change.

3. A method according to claim 1 wherein the visual response is gas formation and entrapment of gas bubbles.

4. A method of testing for possible virulence of staphylococcus organisms which comprises emulsifying a culture of the organism to be tested with water, spreading the emulsion over a film of dried agar having a fibrinogen-containing plasma incorporated therein, and observing clumping of the organisms.

5. A method of detecting gas formation in bacteria which comprises covering said bacteria which are growing on a nutrient agar medium with a transparent dried agar sheet containing a reagent which will form gas under the influence of the specific enzymes of said bacteria, and observing the gas formation through the transparent dried agar sheet.

References Cited

UNITED STATES PATENTS

2,904,474	9/1959	Forg	195—103.5
3,206,317	9/1965	Golber	195—103.5 X

ALVIN E. TANENHOLTZ, *Primary Examiner.*

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