Nov. 28, 1961 M. L. LITTMAN ET AL 3,010,880

DEVICE FOR DETERMINING BACTERICAL SENSITIVITIES

Filed May 8, 1959 2 Sheets-Sheet 1

Fig 1.

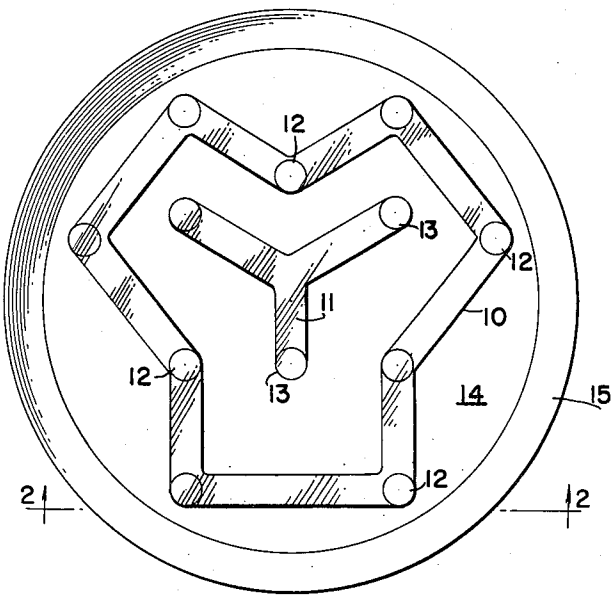
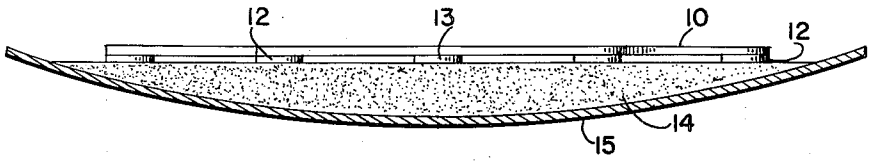


Fig 2.



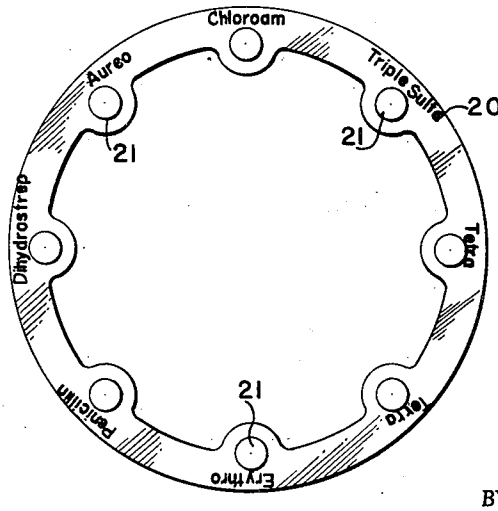


Fig 3.

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Fig 4.

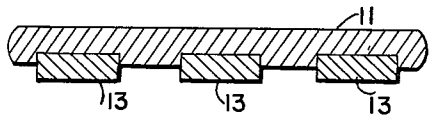


Fig 5.

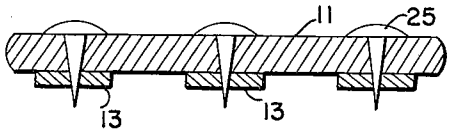


Fig 6.

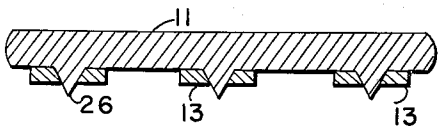


Fig 8.

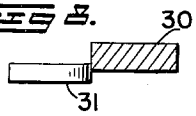
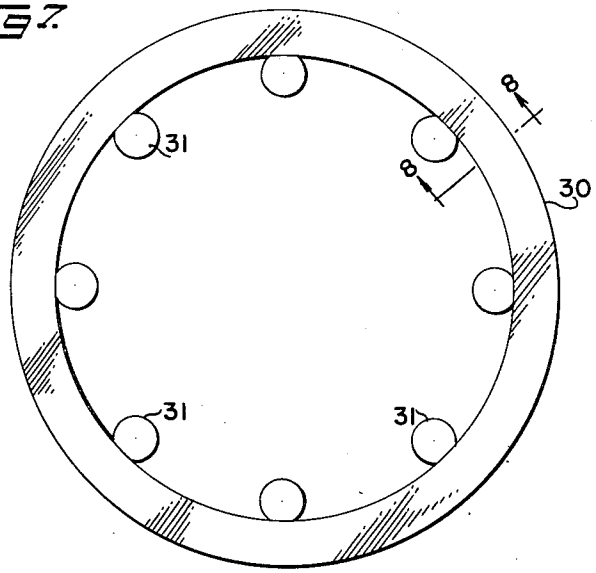


Fig 7.



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DEVICE FOR DETERMINING BACTERIAL SENSITIVITIES

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9 Claims. (Cl. 195—103.5)

This invention relates to an improved multiple disk testing device for determining the in vitro sensitivities of microorganisms to therapeutic agents. In another aspect, this invention concerns a device for ascertaining microorganism types through their sensitivity to inactivating agents, e.g. bacteriophages.

In the past few decades, sulfa drugs, penicillin, tetracycline and dozens of other antibacterial agents have become available to the doctor for the treatment of infections due to staphylococci, pneumococci, streptococci, gram-negative rods, and other microorganisms. The antibacterial agents vary in potency, toxicity, specific effect and other attributes. Moreover, an agent which has been effective against a group of microorganisms in the past, may lose that effectiveness in time because of the emergence of resistant strains of that microorganism. For instance, the evolution of resistant strains of Staphylococcus has become a serious public health problem, especially in the operating rooms and maternity wards of hospitals.

The infiltrated paper disk technique has evolved as one answer to the problem of determining the in vitro sensitivity of an organism to the various antibacterial agents. In this procedure, a plate of solid culture medium, e.g. Mueller-Hinton blood agar or any other suitable growth medium is inoculated with a specimen obtained from the infected human or animal, one or more paper disks impregnated with antibacterial agents are placed on the agar and the agar plate is incubated. If the organism in the specimen is sensitive to the antibacterial agent, it will fail to grow in the area about this agent and that zone will remain clear. If the organism is resistant to the antibacterial agent, it will continue to grow adjacent to the disk as well as elsewhere on the plate and no clear zone will develop.

An improvement over the single infiltrated paper disk is a multiple absorbent paper disk, containing several different antibacterial agents at various locations on a single paper sheet. The several agents may be located at the ends of paper spokes emanating from a central paper hub, as described in U.S. Patent No. 2,787,581, and Reissue No. 24,557, or they may be spaced on an absorbent paper ring, paper polygon, paper tree, etc. The multiple absorbent paper disk is valued because it saves labor, in that the several antibacterial agents are pre-positioned with relation to each other.

There are several objectionable features to the multiple absorbent paper disk made from a single sheet of absorbent paper. A serious disadvantage is the difficulty of obtaining the desired concentrations simultaneously of each of the several antibacterial agents on their respective disks. United States and foreign regulatory agencies now require that the strength of antibacterial agents in paper disks be controlled within fairly narrow limits. If, for example, 10% of impregnations of single paper disks result in batches of disks having antibacterial concentrations outside the acceptable limits, then a batch of single disks will have a 90% probability of being acceptable. On the other hand, multiple paper disks containing 8 spaced antibiotic areas will have only $(0.09)^8$ or approximately a 43% chance of being acceptable, since the entire disk must be rejected if only a single component is outside the prescribed concentration.

Another very serious objection to the multiple absorbent paper disk is that the paper connecting the several impregnated disk areas lies on the culture medium even in an area immediately adjacent the impregnated areas. Since a primary function of the multiple absorbent paper disk is in evaluating the effectiveness of antibacterial agents against the growth of aerobic bacteria, it is apparent that the paper inter-connecting the several impregnated areas can prevent any useful result being obtained where the paper connector is attached to the impregnated areas. This is particularly disadvantageous in the detection of small zones. When the connecting paper lies on the culture medium there is less chance that regular or symmetrical growth will be obtained.

A further disadvantage of the multiple absorbent paper disk is that the water-soluble antibacterial agents in the impregnated areas have a marked tendency to diffuse into the interconnecting paper, particularly in the presence of water. This diffusion obscures the growth zone boundaries, making large and irregular zones, and even causes overlapping of the zones of inhibition of the various antibacterial agents which can prevent adequate analysis. There are other disadvantages associated with the multiple absorbent paper disk, for instance, the inter-connecting paper is opaque which prevents the doctor or technician from observing what is happening beneath the paper.

We have invented a multiple impregnated area device which overcomes various difficulties associated with the use of the multiple absorbent paper disk. Instead of being cut from a single sheet of absorbent paper, our device has a hydrophobic, i.e. essentially non-absorbent to water, holder to which are affixed a plurality of separate water absorbent pieces or disks, each containing its unique antibacterial agent or mixture of agents in approved concentrations. In the highly preferred form of the invention the water absorbent pieces are impregnated with the different water-soluble antibacterial agents prior to being attached to the holder. Thus the separate pre-manufactured batches of impregnated paper pieces can be evaluated and if found to have an unacceptable amount of antibacterial agent can be discarded without necessitating the refusal of the entire holder containing various other antibacterial agents in the proper amounts. Our device could be made by placing the unimpregnated water absorbent paper pieces on the hydrophobic holder and then adding the several antibacterial agents to the separate pieces but, of course, this manner of manufacture is materially less desirable.

In addition to our device being useful in determining the efficacy of antibacterial agents, it can be used in ascertaining microorganism types. In this embodiment the separate water absorbent pieces are impreganted with different specific bacteriophages. The prepared holder can be placed on a nutrient medium seeded with a specific bacteria type and upon incubation a clear area resulting around a given water absorbent piece shows the identity of the bacteria seed.

It is important that the impregnated water absorbent pieces on our holder project from the holder in essentially the entire area immediately surrounding the pieces. The holder will thus be held away from the nutrient medium to provide contact with air and more representative growth or inhibition in the medium around the pieces will result. The hydrophobic holder is preferably of sufficient thickness and rigidity to prevent it from sagging, so that substantially its entire area will be held away or separate from the nutrient medium. Less rigid holders can be used, however, and the portions interconnecting the impregnated pieces may contact the nutrient medium although the projection of the pieces from the holder will still keep the holder spaced away from the nutrient medium in the areas immediately surrounding the impregnated pieces.

The holder in our device is an essentially hydrophobic material which prevents the antibacterial agents from diffusing away from the impregnated water absorbent pieces and into the holder. Instead the composition of the holder insures or compels the diffusion of the agents into the nutrient medium where they are intended to go. The holder should be of a material which does not cause significant inhibition or stimulation of any aerobic microorganism being tested. The holder may be symmetrical or asymmetrical in shape and, of course, is designed to carry a plurality of the impregnated paper pieces. The holder may be opaque or transparent but the latter is preferred. The holder material may be colored and may be imprinted in part without substantial impairment of visibility. Suitable holder materials are exemplified by aluminum, glass, polystyrene, cellulose acetate, cellulose acetate-butyrate, ethyl cellulose, polyethylene and the like.

The impregnated pieces are water absorbent and usually will be of paper in circular form. Other shapes and materials can be used less advantageously, in fact it has been recommended that standardized paper disks of ¼" diameter be employed, see "The Assay and Control of Antibiotic Sensitivity Discs," by A. Kirshbaum, J. Kramer and B. Arret, Department of Health, Education and Welfare, Food and Drug Administration, Washington, D.C., November 1958. Thus while square, hexagonal or other shaped pieces may be used we prefer round pieces to insure round zones. The pieces can be connected to the holder by any suitable means. For instance, the single, standardized pieces or paper disks may be attached to the interconnecting, hydrophobic holder with adhesives, two-sided pressure-sensitive tape, or mechanical fasteners, or they may be wedged into receptacles in the interconnecting material, impaled on spikes, etc. We have found both solvent and bodied cements suitable for bonding the disks to the matrix or holder.

Our invention may be further understood by reference to the drawings in which:

FIGURE 1 is a plan view of two embodiments of our device located on a nutrient medium in a dish;

FIGURE 2 is a side view of the device of FIGURE 1 taken along lines **2—2**;

FIGURE 3 is a plan view of a form of the invention using a ring holder;

FIGURES 4 to 6 illustrate in partial, sectional side view the three-pronged holder of FIGURE 1 with different means for securing the impregnated pieces on the holder;

FIGURE 7 represents still another embodiment of the invention; and

FIGURE 8 is a cross-sectional view taken on lines **8—8** of FIGURE 7.

FIGURES 1 and 2 show a polygonal holder **10** for nine paper disks **12** and a hub-and-spoke holder **11** for three paper disks **13**, which can be used simultaneously to determine the sensitivities of an organism to 12 different antibacterial agents. The holders are on an agar nutrient medium **14** in dish **15**, and as shown the holders are separated from the agar by essentially the thickness of the single attached paper disks. It may be advisable to leave at least one disk blank to guarantee the organism a chance to grow in the event that the organism is sensitive to all the agents. Any number and combination of disks can be placed on the hydrophobic holders.

In FIGURE 3 is illustrated a circular holder **20** containing eight paper disks **21** each impregnated with a different antibacterial agent as specified in the figure. The paper disks are glued on what might be considered the underside of the holder ring **20** although in actual use the dish containing the nutrient medium on which the disks **21** rest may be in inverted position as has been suggested to avoid undesirable effects of humidity.

In FIGURES 4 to 6, three different embodiments of the three-pronged holder of FIGURE 1 are shown. In FIGURE 4 the paper disks are pressed into recesses on the holder and these disks project from the underside of the holder. In FIGURE 5 the holder **11** is pierced through by thumbtacks **25** and the paper disks are impaled onto the spike of the thumbtacks at the underside of holder **11**. The embodiment of FIGURE 6 is somewhat similar to that of FIGURE 5 except that spikes or sharp projections **26** are an integral part of the holder **11**.

In FIGURES 7 and 8 we have illustrated a circular holder ring **30** inside of which are attached eight paper disks **31** each impregnated with a different antibacterial agent. As shown particularly in FIGURE 8 the disks **31** project from the bottom of the holder ring **30**.

Our invention will be illustrated further by the following specific examples which are not to be considered limiting:

Example 1

Single absorbent paper disks containing, respectively, 2 units of penicillin, 50 mcg. nitrofurantoin, and 50 mcg. triple sulfa (sulfadiazine, sulfamerazine and sulfamethazine) are bonded at spaced positions on one side of a flat straight strip or piece of molded, crystal-clear, rigid polystyrene, using a cement made by dissolving polystyrene in toluene. Toluene evaporates from the paper disks leaving them firmly bonded to the polystyrene. A plate of Mueller-Hinton blood agar is inoculated with *Staphylococcus aureus,* American Type Culture Collection No. 6358, and the polystyrene multiple disk device is placed on the agar so that all the disks are in contact with the agar. After 24 hours of incubation, a creamy tan growth of *Staphylococcus aureus* develops over the entire surface of the agar except in a circle of approximately ½" around the nitrofurantoin, indicating that the organism is inhibited by nitrofurantoin but not by penicillin or triple sulfa. The test is repeated with the same paper disks, culture medium and organism, but without the hydrophobic polystyrene holder device, and identical results are obtained.

Example 2

A ring was cut from flexible, blue-tinted cellulose acetate film, and eight paper disks impregnated with antibacterial agents were attached with solvent cement at equal spacings on one side of the ring. The paper disks contained one of each of the following antibacterial agents: 2 units penicillin, 5 mcg. chloroamphenicol, 50 mcg. triple sulfa, 5 mcg. terramycin, 2 mcg. erythromycin, 5 mcg. tertacyline, 2 mcg. dihydrostreptomycin, 5 mcg. aureomycin. The same organism as in Example 1 was streaked over Muller-Hinton blood agar, and the cellulose acetate multiple disk device was placed on the agar so that all disks were in contact with the agar. After 24 hours' incubation, a creamy tan growth of *Staphylococcus aureus* developed over the surface of the agar except near all 8 antibiotics, indicating that all 8 were inhibitory to the organism. The test was repeated with the same paper disks, culture medium and organism but without the non-absorbent cellulose acetate holder device, and identical results were obtained.

Example 3

A nine-disk non-absorbent holder along the style of FIGURE 1 is cut from translucent polyethylene sheet 0.020" thick, and nine paper disks containing different antibiotics are appended with two-sided pressure sensitive tape at spaced apart locations on one side of the holder. Upon inoculation of blood agar with *Proteus vulgaris,* placing the disks and holder on the agar and incubating, a pattern of growth was obtained which is similar to that afforded when individual paper disks are placed carefully in analogous positions on another plate of seeded agar without the nonabsorbent holder.

Example 4

One quarter inch diameter circular paper disks containing different water-soluble heat-stable, antibacterial

agents are sealed at spaced apart positions to the coated side of a strip of cellophane coated on one side with a heat-sealable, moisture resistant coating. The compositing is effected by pressing the cellophane and disks for one second in a jaw sealer or press heated to 250° F. The multiple disk device is tested and found satisfactory.

Example 5

A three-pronged holder of clear, rigid acrylic plastic is made in the style shown in FIGURE 1, with three recesses 0.010" deep on one side. Paper disks 0.026" thick are impregnated with different antibiotic solutions and pressed into the cavities while wet (see FIGURE 4), after which the multiple disk was dried under vacuum. When compared with individual disks for antibacterial sensitivity, the multiple disk shows no significant difference.

Example 6

The test of Example 5 is repeated, except that dry, standardized disks are employed. Test results are the same.

Example 7

A sheet of "Pliofilm" chlorinated rubber is pierced from one side with three spaced apart thumbtacks. Paper disks containing standardized amounts of different antibacterial agents are impaled on the points of the tacks (see FIGURE 5), and the resultant multiple disk is pressed upon a plate of inoculated agar until the disks make contact. After incubation, a sensitivity pattern is obtained which is similar to that when the three disks are placed on the agar separately.

Example 8

A transparent polystyrene holder (see FIGURE 6) comprising spaced apart spikes projecting from one side of a flat surface is utilized in the same manner as the holder of Example 7 with similar results.

Example 9

To a holder as described in Example 2 are affixed eight paper disks each of which is impregnated with a different type specific bacteriophage, only one of which is effective against *Staphylococcus aureus*. The holder disks

are placed on an agar nutrient medium seeded with a test strain of *Staphylococcus aureus*. After incubation a clear zone around only the disk containing the bacteriophage for *Staphylococcus aureus* identified the test strain.

We claim:

1. A device for determining microorganism sensitivities, which comprises a hydrophobic holder and a plurality of spaced apart, water absorbent members on said holder, said absorbent members having a substantially flat surface projecting from said holder to support said device on a nutrient medium with the holder portions immediately surrounding said members being out of contact with said medium, said absorbent members having thereon materials exhibiting different sensitivities with respect to microorganisms.

2. The device of claim 1 in which the materials exhibiting sensitivity are water-soluble antibacterial substances.

3. A device for determining bacterial sensitivities which comprises an essentially flat hydrophobic holder, a plurality of separated paper disks mounted on one surface of said holder and projecting from said surface to support said device on a nutrient medium, said paper disks having thereon water-soluble antibacterial agents exhibiting different sensitivities and said paper disks being positioned on said surface of said hydrophobic holder so as to lie flat on said nutrient medium.

4. The device of claim 3 in which the holder is in the form of a ring.

5. The device of claim 3 in which the holder is transparent

6. The device of claim 3 in which the holder is rigid.

7. The device of claim 3 in which the paper disks are in recesses in the holder.

8. The device of claim 3 in which the paper disks are impaled on sharp projections from said holder.

9. The device of claim 3 in which the paper disks are held on the holder by adhesive.

References Cited in the file of this patent

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