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- [54] BACTERIAL CONTROLS AND PREPARATION THEREOF
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[57] ABSTRACT

Bacteria are preserved by centrifuging a broth culture, mixing the bacterial sediment with gelatin, diethylaminoethyl dextran and monosodium glutamate, and drying at ambient temperature on a non-adhering support surface. The product is useful as a control for test procedures and reagents.

14 Claims, No Drawings

BACTERIAL CONTROLS AND PREPARATION THEREOF

BACKGROUND OF THE INVENTION

The detection and identification of bacteria in specimens of body fluids from patients by diagnostic or clinical microbiology laboratories requires complex media and reagents. Frequently, in order to perform the required tests, the laboratories prepare the media and reagents for their own use. This can cause uncertainties as to the results of the tests since many of the media and reagents are relatively unstable.

In order to ensure that the tests as run in the laboratories are performing accurately and consistently, the laboratories must check their procedures and reagents with cultures of the specific bacteria being detected. Since these cultures have predictable biochemical properties, the laboratories are able to evaluate their procedures and reagents. In order to do this, the laboratories must acquire a set of cultures and perpetuate them. Since the cultures are generally rather unstable and must be subcultured frequently, an element of uncertainty is injected into the situation because each time an organism is subcultured, mutations may occur. Thus, the clinical bacteriologist requires a stable, readily available economic source of viable standard-reacting microorganisms.

The usual method of preserving bacteria by freeze-drying (lyophilization) is not suitable for routine quality control cultures because of the inconvenience and expense involved. For example, a vial of lyophilized bacteria must be used immediately after reconstitution, but since the amount of inoculum obtained is small and adequate for a limited number of tests, the expense involved is prohibitive. The preparation of lyophilized cultures is difficult and expensive, therefore they are usually used to propagate a larger, fresh culture rather than being used directly in a bacteriological procedure.

Hence, there is also a need for a stable, inexpensive bacteriological control in unit dosage form that permits direct use in a bacteriological test.

SUMMARY OF THE INVENTION

This invention relates to stable bacterial control discs suitable for direct use in bacteriological tests as well as methods for preparing and using the discs.

DETAILED DESCRIPTION OF THE INVENTION

It has been discovered that suitable bacterial controls can be formed in stable form as discs by vacuum desiccation of bacterial cultures suspended in a nutrient medium containing a nutrient gelatin and materials which stabilize the viability of the bacteria. Controls for other microorganisms such as fungi can also be formed in a similar manner. This invention, however, is directed to bacterial controls.

The type of bacteria which are particularly suitable for use in this invention are those non-spore forming bacteria which remain viable upon desiccation and are stable under conditions of accelerated stability tests. While many bacteria are suitable under these criteria, those which are preferred are the common gram negative and gram positive bacteria generally tested for by the clinical bacteriologist. Examples of some suitable organisms are *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterobacter cloacae*, *Salmonella typhimurium*, *Proteus vulgaris*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Streptococcus pyogenes* and the like.

The suspending medium which has been found to be most efficacious and is thus preferred contains about 90 percent to about 97 percent by weight of a nutrient gelatin, about 0.005 percent to about 0.015 percent by weight of diethylaminoethyl dextran and about 2 percent to about 10 percent by weight of monosodium glutamate. The latter two materials stabilize the viability of the bacteria. However, the invention comprehends the use of other materials which stabilize the viability of the bacteria. Thus, the diethylaminoethyl dextran and monosodium glutamate can be replaced by an equal total amount of either glucose, sucrose, lactose, mannitol or polyvinylpyrrolidone. Furthermore, if monosodium

glutamate is used, equal amounts of dextran sulfate or dextran can be used to replace the diethylaminoethyl dextran. Also, if the concentration of the nutrient gelatin is increased to up to about 99.1 to about 99.5 percent, then all the previously mentioned stabilizers can be replaced by cysteine, thiourea, glutathione and monothioglycerol.

The nutrient gelatins which are suitable for use in the compositions of this invention are generally commercially available materials, e.g., Bacto Nutrient Gelatin (Difco Co.) and Nutrient Gelatin (Baltimore Biological Laboratory). Any other commercially available nutrient gelatin is suitable provided it is amenable to desiccation and forms discs with the required physical properties. While the amount of nutrient gelatin can vary between about 90 to about 97 percent by weight of the suspending medium, it is preferred to use compositions containing about 96 to 97 percent. More would result in poor bacterial stability and less would result in discs with undesirable physical properties.

The amount of diethylaminoethyl dextran, dextran sulfate or dextran used in combination with monosodium glutamate should be sufficient to provide stability to the viability of the bacteria, usually about 0.005 to 0.015 percent by weight is sufficient with about 0.01 percent preferred. It is preferred to use diethylaminoethyl dextran. The remainder of the composition when a dextran is used is monosodium glutamate which should be present in a concentration of from about 2 to 10 percent, with about 3 percent preferred.

It has been found that nutrient gelatin, diethylaminoethyl dextran and monosodium glutamate in the amounts indicated are most preferred since the stability of the resulting discs is excellent.

The bacterial control discs are prepared under aseptic conditions, to avoid contamination. The discs are prepared by desiccating, under vacuum, droplets of the suspending medium containing the bacteria. The droplets, when thus treated become transformed into firm discs. The discs are approximately circular in shape and weight from about 2.8 to about 4.0 mg. This shape and size is determined by the size of the droplets and the surface upon which they are dried. Preferably discs weighing about 3.2 mg. are formed. The term "disc" is used to mean any dried droplet, formed according to this invention.

It is preferable to dry the droplets on a sterile non-adhering surface, e.g., a waxed Petri dish, for easy removal of the resulting discs.

Conveniently, the droplets on the sterile non-adhering surface are dried under a vacuum of about 500-600 mm. Hg. at room temperature. This can be accomplished in a desiccator jar containing CaSO_4 and usually takes about 72 hours. Other conventional vacuum drying means and conditions can be used with equivalent results.

The discs which are formed can be stored in small sterile screw-capped vials containing a desiccant, e.g., silica gel, CaCl_2 , CaSO_4 . Other sterile containers can be used, however, the vials are convenient for labelling, shipping, storage and use in the laboratory.

The organism is prepared for desiccation by growing the organism in a suitable broth, e.g., Tryptic Soy Broth medium, for about 15-24 hours at about 30°-40°C., preferably 37°C. The culture is then centrifuged and the bacterial sediment which results is then suspended in the gelatin suspending medium and dispensed in drops to a non-adhering surface and vacuum desiccated.

The discs which result from the aforementioned process contain about 10^5 to 10^8 viable organisms per disc and the viability of the organism is protected without allowing its biochemical characteristics to be altered. This stability is evidenced by an accelerated stability test wherein the viability of the organism was retained after the discs were subjected to temperatures of 100°C. for from 5 hours to 7 hours. This effect is significant since the same bacteria would ordinarily be killed by such temperatures. Thus, the bacteria are excellent for control purposes and this invention permits laboratories to

TABLE I.—REACTIONS OF CONTROL ORGANISMS IN PRIMARY IDENTIFICATION TESTS

	Gram stain	Oxi- dase	O/F (glu- cose)*	Gas from glucose	Methyl red	Voges-Proskauer	H ₂ S (TSI)	KCN	Urease (Chris- tensen's)	Citrate (Simmons')	Lysine decarbox- ylase	Ornithine decarbox- ylase	Arginine dihydro- lase	Malonate	Coag- ulation	Cata- lase	6.5% NaCl	DNAse	Nitrate reduc- tion
<i>Staphylococcus aureus</i>	+	—	F	—	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	+	+	+	+	+
<i>Staphylococcus epidermidis</i>	+	—	F	+	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	+	+	+	+	+
<i>Enterobacter cloacae</i>	—	—	F	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Salmonella typhimurium</i>	—	—	F	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Proteus vulgaris</i>	—	—	F	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Escherichia coli</i>	—	—	F	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Pseudomonas aeruginosa</i>	—	+	O	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

1 48 hours, weak at 24 hours.
2 Tip of slant positive.
3 Questionable weak positive at 72 hours.
4 Slow positive; negative at 24 hours; positive at 72 hrs.
*O = Oxidative; F = Fermentative. NA = Not Applicable. NT = Not Tested.

TABLE II.—CARBOHYDRATE FERMENTATION REACTIONS OF CONTROL ORGANISMS

	Adon- itol	Abab- inose	Cello- biose	Dulci- tol	Glyce- rol	Inositol	Lactose	Inulin	Maltose	Manni- tol	Raffi- nose	Rham- nose	Salicin	Sorbitol	Sucrose	Treha- lose	Xylose
<i>Staphylococcus aureus</i>	—	—	—	—	(+) ¹	—	+	—	+	+	—	—	—	—	+	+	—
<i>Staphylococcus epidermidis</i>	—	—	—	—	(+) ²	—	+	—	+	+	—	—	—	—	+	+	—
<i>Enterobacter cloacae</i>	—	+	+	+	(+) ³	—	+	—	+	+	—	—	—	—	+	+	—
<i>Salmonella typhimurium</i>	—	+	+	+	(+) ⁴	—	+	—	+	+	—	—	—	—	+	+	—
<i>Proteus vulgaris</i>	—	+	+	+	(+)	—	+	—	+	+	—	—	—	—	+	+	—
<i>Escherichia coli</i>	—	+	+	+	(+)	—	+	—	+	+	—	—	—	—	+	+	—
<i>Pseudomonas aeruginosa</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

1 Positive after 3-6 days.
2 Slight positive at 72 hours; positive seven days.
3 Negative 72 hours; positive eight days.
4 Weak positive 48 hours; positive six days.
5 Negative 72 hours; positive seven days.
Note: (+) = delayed positive.

have better quality control for their tests and more accurate tests with high confidence in the results.

In order to utilize the bacteria in the discs as a control for a diagnostic test, the proper organism must be selected. It is preferable to select one which is negative for the test as well as one which is positive. The disc containing the organism is then either added directly to the test media or is placed in a suitable liquid growth medium, e.g., Tryptic Soy Broth and incubated for about 2 to 24 hours. In the latter procedure, one or more loopfuls of the suspension of bacteria in the Broth can then be used in the test procedure to determine its sufficiency and accuracy. In the former direct procedure, the discs are used directly in the test procedure to determine its sufficiency and accuracy.

Table I lists some of the primary identification tests for bacteria and indicates the reaction of the control organisms.

These data can be used, for example, to correlate the results of the oxidase test for *Pseudomonas aeruginosa* and to determine if it is properly run in the laboratory. Thus, if the control test is positive using the bacteria control disc containing *Pseudomonas aeruginosa* as the organism and negative with a disc containing *E. coli*, for example, then the test procedures in the laboratory are correct.

Table II lists some of the carbohydrate fermentation reactions of the control organisms.

These data can be used, for example, to correlate the results of the raffinose fermentation test and to determine if it is correctly run in the laboratory. Thus, if the control disc containing *Enterobacter cloacae* is positive and a control disc containing *P. vulgaris* is negative, then the test procedures in the laboratory are correct.

The following table contains a tabulation of the recommended organisms for positive and negative control of bacteriological tests.

TABLE III

Table of Recommended Organisms for Positive and Negative Control of Bacteriological Tests

List of Organisms		Recommended Organism	
1. <i>Escherichia coli</i>		5. <i>Staphylococcus epidermidis</i>	
2. <i>Enterobacter cloacae</i>		6. <i>Salmonella typhimurium</i>	
3. <i>Proteus vulgaris</i>		7. <i>Pseudomonas aeruginosa</i>	
4. <i>Staphylococcus aureus</i>		8. <i>Streptococcus pyogenes</i>	
Test		Positive	Negative
1. Oxidase		7	1 or 2
2. Indole		1	2 or 3
3. Methyl Red		1	2
4. Voges-Proskauer		2	1
5. Citrate utilization		2 or 6	1
6. Hydrogen sulfide (TSI)		6	1 or 2
7. Urease		3	1
8. Lysine decarboxylase		6	2 or 3
9. Ornithine decarboxylase		2	3
10. Arginine dihydrolase		2	3
11. Catalase		any	none
12. Coagulase		4	5
13. KCN		2	1
14. DNAase		4	5
15. Glucose fermentation		1	7*
16. Lactose fermentation		1	3
17. Mannitol fermentation		4	5
18. β hemolysis on blood agar		8	5
19. Bacitracin disc		8	—
20. Sodium hippurate		4	8

*Organism No. 7 is negative for fermentation of glucose but will degrade this carbohydrate oxidatively.

All the tests listed in the foregoing tables are known tests and the manifestations of the positive and negative reactions which indicate the presence or absence of an organism either by growth, staining or change of color in the media are known to the art.

The following examples illustrate the invention. Temperatures are in degrees C.

EXAMPLE 1

Bacteriological control discs are prepared as follows:

The identified organism is grown in 10 ml. Tryptic Soy Broth medium for 16 to 18 hours at 37°. The Tryptic Soy Broth is composed of 17 grams of pancreatic digest of casein, 3 grams of soy bean peptone, 5 grams of sodium chloride, 2.5 grams of dipotassium phosphate, 2.5 grams of glucose and 1,000 ml. of distilled water. The final pH is about 7.3.

The resulting culture is centrifuged and the supernatant liquid is decanted and discarded. The remaining bacterial sediment is suspended in 4 ml. of a sterile mixture consisting of 96.99 percent nutrient gelatin; 0.01 percent diethylaminoethyl dextran and 3 percent monosodium glutamate, by weight.

The suspended organisms are aseptically dispensed with a Pasteur pipette in drops of about 0.02–0.05 ml. onto a sterile waxed Petri dish. The dishes containing the droplets of organisms are placed in a desiccator jar containing CaSO_4 and the jar is evacuated to 500–600 mm. Hg. and allowed to stand at room temperature, about 20°–25° for 72 hours. The droplets become transformed into firm discs when dried. The discs are aseptically dislodged from the waxed surface and placed in small sterile screw-capped vials containing a silica gel desiccant. About 30 discs are put into each vial. The vials are suitable for use as a kit and can be transported or stored.

EXAMPLE 2

This example illustrates the use of the discs.

a. Indirect Method

A disc is aseptically removed from a vial with sterile forceps and placed in a sterile screw-capped tube containing 2 ml. of sterile Tryptic Soy Broth and incubated at 37° for 1–2 hours. A suspension of viable organisms is formed.

One or two loopfuls of the suspension are used to inoculate the appropriate biochemical test media, depending on the nature of the specific organism being identified and test being controlled. The results are then observed and the quality of the test determined.

b. Direct Method

A disc is added directly to the test medium as, for example, in the following coagulase test:

Two discs, one with *Staphylococcus aureus* and one with *Staphylococcus epidermidis* are placed in separate test tubes containing rabbit plasma.

In three hours, the plasma clotted in the tube containing *S. aureus*, while *S. epidermidis* gave a negative test.

By means of this test the bacteriologist can determine if his coagulase test procedure is correct and performed properly. Analogously, the procedures for the other tests described herein which are directed to detecting these and other organisms can be evaluated.

EXAMPLE 3

The stability of the organisms prepared in the discs is determined by an accelerated stability test calculated to detect the viability of the organism and conducted as follows:

Single discs are placed in each of 8 sterile 13 × 100 mm. covered (not airtight) test tubes. One of the tubes serves as a growth control and is not subject to heat. The remaining tubes are placed in a constant temperature oil bath. When the internal temperature of an empty control tube placed in the bath at the same time reaches 100°, the timing starts.

At hourly intervals, up to 7 hours, one tube containing a disc is removed. Immediately after removal, 3 ml. Tryptic Soy Broth is added to the tube. After the addition of the Broth to

each tube, they all reincubated overnight at 37° and observed the following day for turbidity, i.e., growth.

Broth cultures containing the corresponding organism as used in the discs and subjected to the same temperature conditions are killed in less than one hour. Typical results from the accelerated stability test are as follows:

Bacterial disc	Hours at 100°							
	0	1	2	3	4	5	6	7
<i>P. aeruginosa</i>	+	+	+	+	+	+	+	+
<i>E. cloacae</i>	+	+	+	+	+	+	+	+
<i>J. typhimurium</i>	+	+	+	+	+	+	+	+

NOTE: +=growth.

We claim:

1. A composition suitable for forming by vacuum desiccation at ambient temperature into a stable disc for preserving bacteria comprising a nutrient gelatin and materials which stabilize the viability of the bacteria wherein said materials are diethylaminoethyl dextran and monosodium glutamate.
2. The composition of claim 1 containing on a weight basis from about 90 to about 97 percent nutrient gelatin, from about 0.005 to about 0.015 percent diethylaminoethyl dextran and the remainder monosodium glutamate.
3. The composition of claim 1 containing on a weight basis 96.99 percent nutrient gelatin, 0.01 percent diethylaminoethyl dextran and 3 percent monosodium glutamate.
4. A stable dry disc prepared by vacuum desiccation at ambient temperature containing bacteria and a suspending medium for said bacteria comprising nutrient gelatin and materials which stabilize the viability of said bacteria wherein said materials are diethylaminoethyl dextran and monosodium glutamate.
5. The disc of claim 4 containing between about 10⁵ and 10⁸ viable bacteria per disc.
6. The disc of claim 5 wherein said suspending medium contains on a dry weight basis about 96.99 percent nutrient gelatin, about 0.01 percent diethylaminoethyl dextran and about 3.0 percent monosodium glutamate.
7. A method of producing a stable disc for preserving bacteria which comprises adding bacteria to be preserved to the composition of claim 1 and vacuum desiccating the mixture at ambient temperature on a sterile, non-adhering, supporting member.

8. A method of producing a stable disc for preserving bacteria which comprises adding bacteria to be preserved to the composition of claim 2 and vacuum desiccating the mixture at ambient temperature on a sterile, non-adhering, supporting member.

9. A method of producing a stable disc for preserving bacteria which comprises adding bacteria to be preserved to the composition of claim 3 and vacuum desiccating the mixture at ambient temperature on a sterile, non-adhering, supporting member.

10. A method of preserving identified bacteria which comprises forming a composition containing said identified bacteria and a suspending medium for said bacteria comprising nutrient gelatin and materials to stabilize the viability of said bacteria wherein said materials are diethylaminoethyl dextran and monosodium glutamate and then vacuum desiccating at ambient temperature discrete droplets of the composition on a sterile non-adhering supporting surface.

11. The method of claim 10 wherein the composition before desiccation comprises identified viable bacteria, about 90 to about 97 percent by weight nutrient gelatin, about 0.005 to about 0.015 percent by weight diethylaminoethyl dextran and the remainder monosodium glutamate.

12. A method of evaluating the accuracy of biochemical bacterial differentiation and identification procedures and reagents which comprises using identified bacteria in the form of a stable gelatin disc as a process control, by observing the activity thereof with said procedures and reagents wherein said disc is prepared by vacuum desiccation at ambient temperature from a composition comprising, prior to desiccation, said bacteria, nutrient gelatin, diethylaminoethyl dextran and monosodium glutamate.

13. A diagnostic reagent kit comprising a closed aseptic container having therein stable preserved identified viable bacteria in discs comprising said bacteria, nutrient gelatin, diethylaminoethyl dextran and monosodium glutamate, said discs weighing about 2.8 to about 4.0 mg. and wherein said composition prior to desiccation at ambient temperature thereof comprises said bacteria, about 90 to about 97 percent by weight nutrient gelatin, about 0.005 to about 0.015 percent by weight diethylaminoethyl dextran and about 2 to 10 percent by weight of monosodium glutamate.

14. The disc of claim 4 wherein the bacteria is identified bacteria.

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