

Dec. 19, 1967

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OF ACETYLMETHYLCARBINOL
Filed April 4, 1967

3,359,180



BARRIER ZONE 4.

REAGENT ZONE 3.
(SOLUTION A)

BARRIER 2.

MEDIUM ZONE 1.
(SOLUTION B)

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DIAGNOSTIC PREPARATION FOR THE DETECTION OF ACETYLMETHYLCARBINOL

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Filed Apr. 4, 1967, Ser. No. 628,385

11 Claims. (Cl. 195-103.5)

ABSTRACT OF THE DISCLOSURE

A method for differentiating and identifying acetylmethylcarbinol (AMC) producing Enterobacteriaceae from non-AMC producing Enterobacteriaceae which utilizes a bibulous strip containing a medium zone capable of supporting the growth of Enterobacteriaceae, separated by a hydrophobic barrier from a reaction zone containing an acid addition salt of L-arginine and an alkali metal salt of α -naphthol sulfonic acid. The strip is inserted into a tube containing an unknown organism isolated in pure culture with the medium zone of the strip immersed in the organism suspension. After incubation, an alkali metal hydroxide solution is added to the test tube. A positive test is indicated by the production of a red color in the reaction zone of the bibulous test strip.

BACKGROUND OF THE INVENTION

This application is a continuation in part of applicants' copending U.S. patent application Ser. No. 427,272, filed on Jan. 22, 1965, which describes a diagnostic preparation for the rapid detection of acetylmethylcarbinol (AMC) produced by certain micro-organisms. The diagnostic preparation described in Serial No. 427,272 utilizes a bibulous strip with a medium zone consisting of Brain Heart Infusion Broth, trypticase and D-glucose separated by a hydrophobic barrier from a reaction zone consisting of L-arginine and α -naphthol. The diagnostic test strip prepared according to this formulation provided satisfactory results when used to detect the presence of certain AMC producing Enterobacteriaceae. However, it was subsequently found that the test strips prepared with these ingredients exhibited a relatively short shelf life. The α -naphthol in the reagent zone was somewhat unstable as it had a tendency to sublime, thereby spreading to all of the remaining portions of the strip. Also, it was found that the α -naphthol was soluble in the organic solvent in which the hydrophobic barrier material is dissolved, thus it was possible for the α -naphthol to spread to the remaining portions of the test strip. It was also discovered that the α -naphthol was toxic to the organisms which were growing in the medium zone of the strip; therefore, when the α -naphthol spread to the medium zone of the bibulous strip, it tended to destroy the usefulness of this test procedure.

The production and the subsequent detection of acetylmethylcarbinol (AMC) from glycolysis products of certain organisms of the Enterobacteriaceae forms the basis of the Voges-Proskauer or V-P test. This test is a well-known laboratory diagnostic method for the differentiation and identification of the various organisms which belong to this group. For example, two organisms of this coliform group, i.e., *Aerobacter* and *Klebsiella*, produce acetylmethylcarbinol and on this basis may be distinguished from *Escherichia coli* and *E. freundii*, which do not. However, to carry out this classical test or its more modern adaptations requires the use of incubation periods of a duration too long for rapid identification of the organisms.

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Thus, one of the preferred methods for detecting acetylmethylcarbinol is the Barritt modification (J. Path. 42: 441-454, 1936). In this method the unknown organism isolated in pure culture from a specimen such as stool, blood or urine is grown in a glucose-peptone-phosphate buffer broth for 48 hours. To 1 ml. of the medium is then added 0.2 ml. 40% potassium hydroxide and 0.6 ml. 5% naphthol in ethanol. The development of a pink to red color in 2-5 min., becoming crimson in 30 minutes to 4 hours indicates the presence of acetylmethylcarbinol (AMC).

Another modification of the V-P test is the Benjaminson method (M. A. Benjaminson, B. C. deGunsman, and A. J. Weil, J. Bact. 87,234-235, 1964). In this test, isolates grown on triple sugar iron (TSI) agar slants are suspended in 0.5 ml. of 0.5% aqueous creatine solution and then naphthol and 40% KOH are added. A pink to red color which develops in 5 minutes is positive for AMC.

It is obvious that there are many disadvantages to these methods. In the Barritt modification, time of incubation is dependent on the volume of reagents and is critical. In addition, pure cultures must be grown in specialized media following primary isolation of the culture. In the method of Benjaminson et al., organisms can be taken directly from a TSI agar slant only if acid has been produced on the slant. The use of organisms from a TSI slant is considered advantageous since this media is usually incorporated into the scheme for separation of the Enterobacteriaceae. However, if acid is not produced on the slant, the testing of these non-lactose fermentors becomes impractical. Hence, the test is limited in practice to the *Aerobacter-Klebsiella* group to the exclusion of other AMC producers.

In all bacterial infections the rapid and accurate identification of the infectious agent is of paramount importance so that proper therapy can be promptly initiated. The above-described tests are obviously impractical when rapid diagnosis is essential. In addition, the necessity for the preparation of the various specific reagents required for the tests further hampers the ability to achieve rapid identification. Also, the α -naphthol used in the tests outlined above is not a stable reagent; therefore, it is necessary that this reagent be prepared frequently and in small quantities.

SUMMARY OF THE INVENTION

This invention relates to a novel method for the differentiation and identification of acetylmethylcarbinol producing Enterobacteriaceae utilizing a bibulous strip containing a medium zone and a reagent zone. The strip is inserted into a tube containing unknown organisms isolated in pure culture; the tube containing the strip is then incubated, after which an alkali metal hydroxide solution is added to the tube with a resultant color change in the reaction zone of the strip indicating the presence of AMC producing Enterobacteriaceae.

DESCRIPTION OF THE DRAWING

The drawing shows a diagrammatic representation of the bibulous test strip with the medium zone and the reagent zone separated thereon by a hydrophobic barrier zone with a second hydrophobic barrier zone contiguous to the reagent zone on the opposite side of the reagent zone from the first barrier zone.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The description of this invention may be more easily understood by reference to FIGURE I, which is a diagrammatic representation of the invention. The figures in parenthesis appearing herein relate to FIGURE I.

This invention relates to an improved diagnostic method for differentiating and identifying acetylmethylcarbinol (AMC) producing Enterobacteriaceae from non-AMC producing Enterobacteriaceae which utilizes a bibulous strip with a medium zone containing a medium capable of supporting the growth of Enterobacteriaceae separated by a hydrophobic barrier zone from a reagent zone which contains (a) a compound containing the guanidino group and (b) an alkali metal salt of α -naphthol sulfonic acid.

The concentrations expressed herein are all parts by weight of the total volume of the aqueous solutions applied to the bibulous strip material commonly expressed as a weight/volume solution.

The bibulous test strip of the present invention is prepared by impregnating one zone of the strip, hereinafter called reagent zone 3, with an aqueous reagent system which contains (a) a compound containing the guanidino group, such as l-arginine or its mono, di, or tri acid addition salts, agmatine, creatine, etc., wherein the acid addition salts of l-arginine are those formed with strong mineral acids such as hydrochloric or nitric, and (b) an aqueous solution of an alkali metal salt of α -naphthol sulfonic acid such as:

1-naphthol-8-sulfonic acid sodium salt,
1-naphthol-5-sulfonic acid sodium salt,
1-naphthol-2-sulfonic acid potassium salt,
1-naphthol-3,6-disulfonic acid disodium salt, and
1-naphthol-4-sulfonic acid sodium salt.

The alkali metal salts of naphthol sulfonic acid are soluble in water but insoluble in the organic vehicles in which the hydrophobic barrier material is dissolved. When applied to the test strip, these salts are stable and do not sublime from one zone of the test strip to another.

The reagent system contains an aqueous solution of from about 2% to about 3% w./v. of a compound containing the guanidino group with from about 3% to about 4% w./v. of an alkali metal salt of α -naphthol sulfonic acid added thereto. The reagent system, which preferably is made up of the sodium salt of 1-naphthol-8-sulfonic acid and l-arginine monohydrochloride, is applied to the reagent zone 3 of the strip in excess amounts. The ingredients of the reagent zone function as color developers when they come into contact with AMC. Therefore, the amount of these two ingredients which is necessary for the reaction zone of the test strip is a function of the amount of AMC produced by the micro-organisms. Consequently, the amount of these two ingredients in the reagent zone is not critical, so long as they are present in excess amounts. The preferred concentration of the ingredients in the reagent zone of the strip is 0.3 mg. of 1-naphthol-8-sulfonic acid sodium salt per strip and 0.2 mg. of l-arginine per strip. The ingredients in the reagent system may be applied in the relative proportions of about 1 to about 5 parts of an alkali metal salt of α -naphthol sulfonic acid to about 1 part by weight of a compound containing the guanidino group. The preferred ratio in which these ingredients are applied is about 1.2 parts of an alkali metal salt of α -naphthol sulfonic acid, preferably 1-naphthol-8-sulfonic acid sodium salt to about 1 part by weight of a compound containing the guanidino group, preferably l-arginine monohydrochloride. These ingredients may be applied to the bibulous strip with a syringe microbiuret No. S 1/4T (Model No. SB2), Micro-Meter Instrument Co., Cleveland, Ohio.

Impregnated in another zone of the strip, hereinafter called the medium zone 1, is an aqueous nutrient medium containing ingredients capable of supporting the growth of Enterobacteriaceae, which ingredients may be a peptone-dextrose-potassium phosphate mixture, with D-glucose and sodium pyruvate optionally included therein as desirable adjuncts which promote the rapid production of AMC. In the practice of this invention, the peptone-dextrose, potassium phosphate mixture is preferably ob-

tained in a prepared form known as Bacto MR-VP Medium in which the peptone-dextrose-potassium phosphate is present in a ratio of 7 parts peptone-5 parts dextrose-5 parts potassium phosphate.

The concentration of the peptone-dextrose-phosphate mixture in the nutrient medium with which the medium zone 1 is impregnated is not critical and may vary from about 5% to about 25% by weight of the total weight of the nutrient medium. Where the optional ingredients D-glucose and sodium pyruvate are in the nutrient medium with which the medium zone 1 is impregnated, they may be included in a concentration varying from 5% to 25% by weight of the total weight of the nutrient medium; however, it is preferred that the D-glucose be maintained at a concentration of about 20% to 25% by weight of the nutrient medium to give optimum results.

The reagent zone 3 and the medium zone 1 are separated by a barrier zone 2 containing hydrophobic barrier material. Another barrier zone 4 containing hydrophobic barrier material is contiguous to reagent zone 3 and on the opposite side of reagent zone 3 from zone 2.

The hydrophobic barrier zone 2 must be capable of isolating the ingredients of the medium zone 1 from the ingredients of the reagent zone 3 during the incubation period hereinafter described. Barrier zone 4 is preferably present in order to prevent excessive migration of the reagent and diffusing of the color formed in a positive test. The ingredients in barrier zones 2 and 4 must be microbiologically inert with respect to the Enterobacteriaceae. Any substance which will form a waterproof barrier of this type may be used. Suitable materials include waxes, lacquers, and a colorless acrylic resin composition known as "Krylon 150 Crystal Clear." The "Krylon" material is particularly preferred. It is supplied in a toluene vehicle and may be diluted for ease of application with additional toluene or other hydrocarbon thinners, such as ethyl, methyl or propyl alcohol U.S.P. It has been found that barrier solution made from about 75% to 100% v./v. Krylon and 0 to 25% v./v. diluent is suitable. A particularly preferred combination is prepared from 85% v./v. Krylon with 15% v./v. ethyl alcohol U.S.P.

Bibulous materials which can be employed as the strip carriers are those materials which by means of capillary action are able to draw a liquid upward and materials such as filter paper, felt, porous ceramic strips, woven or matted glass fiber and the like are suitable.

The bibulous material is normally cut into narrow strips to facilitate packaging in small bottles or other containers from which they may be dispensed as needed.

In use, the end of the bibulous material impregnated with the nutrient medium, the medium zone 1, is inserted into a suspension of a pure culture of unknown bacteria contained in a test tube, and while in this position in the tube, both the tube and strip are incubated together at 37° C. for about 4 hours. The suspension of bacteria need not have been grown in special media or incubated for long duration for this test although cells grown in a medium containing relatively high concentrations of glucose give a better reaction. After incubation, a few drops of an aqueous alkali metal hydroxide are added to the tube taking care not to wet the reagent zone. The concentration of the alkali metal hydroxide solution is preferably 40% although greater amounts of more dilute solutions can be used.

The tube contents are then gently mixed after which the hydroxide treated material is allowed to come in contact with the reagent zone. A positive test for the presence of AMC is indicated by a magenta cherry red color which is quite prominent in the reagent zone. This cherry red color appears in about 5 to 30 minutes.

The advantages and convenience of the above-described invention are quite outstanding in view of the fact that the test for AMC can be carried out under conditions employing a very much shorter incubation time than pre-

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viously considered necessary. In addition, this test has also been found to be more sensitive for detecting the AMC production of certain bacteria than conventional techniques.

The following examples are included in order to further illustrate the invention:

Example 1

(A) *Preparation of reagent system (Solution A).*—2.6 grams of l-arginine is dissolved in about 100 ml. of water. To this is added with stirring of 3.3 grams 1-naphthol-8-sulfonic acid, sodium salt.

(B) *Preparation of nutrient medium (Solution B).*—17 grams of dehydrated Bacto MR-VP Medium, 15 grams of sodium pyruvate, 37.5 grams of D-glucose are dissolved together in 150 ml. of distilled water with the aid of gentle heating until the solution is complete.

(C) *Application to bibulous material.*—In preparing the test strip the initial step is the application of two barrier zones 2 and 4 which act to prevent the migration of aqueous solutions which are subsequently applied. These barrier zones are applied to a suitable filter paper such as Eaton-Dykman #623 by employing a solution of a lacquer which produces a water-impervious barrier. After allowing the lacquer solution to dry, the reagent zone 3, consisting of Solution A, of Example 1, and the medium zone 1, consisting of Solution B, of Example 1, as described above are then applied to the paper. The zones thus formed are then allowed to dry. The filter paper is then cut into strips.

Example 2

USE OF THE REAGENT STRIPS

1 to 2 loopfuls of the unknown bacterium isolated in pure culture, which was previously grown on an agar slant, is suspended in 0.3 ml. of isotonic saline. A test strip prepared according to Example 1 is placed in the tube with the medium zone 1 immersed in the suspension of the organism. The test strip and the organism suspension are incubated at 37° C. for about 4 hours. After the incubation period, several drops of 40% potassium hydroxide are added to the tube, taking care to avoid wetting the reagent zone 3. The tube is gently agitated and the suspension is then allowed to come in contact with the reagent zone 3. A positive test for the presence of acetylmethylcarbinol is indicated by the formation of a pink to red color in the reagent zone.

Test strips, prepared as described in Example 1 and used in the manner described in Example 2, were compared with conventional testing methods used to detect the presence of acetylmethylcarbinol. The results obtained are shown in Table 1:

TABLE 1.—CONVENTIONAL VOGES-PROSKAUER AND PAPER STRIP TEST REACTIONS AMONG REPRESENTATIVE GENERA AND SPECIES IN THE ENTEROBACTERIACEAE

Organism	# of strains	Paper Strip	Conventional V-P Test
<i>Escherichia coli</i>	23		
<i>Aerobacter aerogenes</i>	3		
<i>Klebsiella pneumoniae</i>	8		
<i>Serratia marcescens</i>	1		
<i>Klebsiella-Aerobacter-Serratia</i>	39		

It is understood that the foregoing detailed description is given merely by way of illustration and that many variations may be made therein without departing from the spirit of our invention.

Having described our invention, what we desire to secure by Letters Patent is:

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1. A diagnostic preparation for the rapid and positive detection of acetylmethylcarbinol which comprises a strip of bibulous material impregnated with:

(a) a medium zone capable of supporting the growth of Enterobacteriaceae;

(b) a reagent zone comprising:

(i) a member selected from the group consisting of:

l-arginine,

the mono-, di, or tri acid addition salts of l-arginine,

agmatine,

and creatine; and

(ii) an alkali metal salt of α -naphthol-sulfonic acid, and

(c) a hydrophobic barrier zone separating said medium zone and said reagent zone.

2. A diagnostic preparation according to claim 1 which has a second hydrophobic barrier zone contiguous to said reagent zone, and on the opposite side of said reagent zone from said (c) barrier zone.

3. A diagnostic preparation according to claim 1 wherein said reagent zone comprises l-arginine monohydrochloride and an alkali metal salt of α -naphthol sulfonic acid.

4. A diagnostic preparation according to claim 3 wherein said reagent zone comprises 1.2 parts by weight of an alkali metal salt of α -naphthol sulfonic acid to 1 part of l-arginine monohydrochloride.

5. A diagnostic preparation according to claim 3 wherein said alkali metal salt of naphthol sulfonic acid is 1-naphthol-8-sulfonic acid sodium salt.

6. A diagnostic preparation according to claim 1 wherein said medium zone comprises peptone, dextrose and potassium phosphate.

7. A diagnostic preparation according to claim 6 wherein the ingredients of said medium zone are present in a ratio of 7 parts peptone-5 parts dextrose-5 parts potassium phosphate.

8. A diagnostic preparation according to claim 6 wherein the optional ingredients D-glucose and sodium pyruvate are also included in the medium zone.

9. A diagnostic preparation according to claim 8 wherein the optional ingredients in the medium zone are present in an amount of from about 5 to about 25 parts by weight of sodium pyruvate and from about 20 to about 25 parts by weight of D-glucose.

10. A diagnostic preparation according to claim 2 wherein the hydrophobic barrier zones comprise an acrylic resin coating composition.

11. A process for the detection of acetylmethylcarbinol produced by micro-organisms which comprises allowing the medium zone of a test strip as defined in claim 1 to come in contact with a suspension of a pure culture of an unknown bacterium for about 4 hours at 37° C., adding an aqueous solution of an alkali metal hydroxide to said bacterial suspension and allowing the alkali hydroxide treated bacterial suspension to come in contact with the reagent zone of said test strip, and observing the reagent zone for color change.

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