FURTHER OBSERVATIONS ON TESTING MILK FOR PENICILLIN

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(Received for publication March 31, 1960)

Positive results can be obtained most rapidly by using penassay seed agar or equivalent in not over 6 ml. amounts per plate, preincubating for one hour, using 0.5 disc and incubating at 37°C after "spotting." Lower incubation temperatures prolong the time required for zone formation. Heat-shocking of the spore suspension also generally hastens growth. Refrigeration of poured plates before use is not necessary, but with penassay seed agar has little delaying effect until after 5 days.

Some workers have felt that the standard disc assay method (9), which calls for incubation of the disc-planted plates at 35°C. for 4-6 hours, is too time-consuming. Arret and Kirshbaum (1) have described a "simplified and rapid method" which they claim will detect concentrations of penicillin as low as 0.05 I. U./ml. in 2-3 hours at 37°C. Johns (4) has criticized their original method as being less simple, less reliable and less sensitive than the standard procedure. Arret and Kirshbaum (2) have since recommended that poured plates be refrigerated, instead of being held at 15°C., for 3 to 5 days before being used. This has removed the writer's chief objection to the method. There still remain, however, other objections such as their requirement of a 37°C. incubator, the thicker layer of agar (10 ml vs. 6 ml. in the official method), and the failure to indicate that the plating medium, spore suspension and penicillinase discs are all available commercially. They also failed to recommend heating milks to avoid false positive zones due to naturally occurring inhibitory substances (8).

Milk plant laboratories are now doing the bulk of the testing for antibiotics in milk. They would like the results as soon as possible. However, when milk supplies continue to arrive until late in the day, it is not convenient to complete all tests the same day. For the late arrivals at least two alternatives suggest themselves. Poured plates may be "spotted" with the milk-soaked discs and either (a) incubated overnight, or (b) refrigerated overnight, then placed in the incubator the first thing next morning. The former method suffers from a slight disadvantage in that the zones of inhibition are sometimes partially obscured by a secondary growth of colonies of the test organism, and the zones are slightly smaller than

when first visible. It has, however, been employed successfully by H. P. Hood & Sons of Boston since July 1959, using whey agar and incubation at 32°C. for 14 to 24 hours (10). Results are available first thing in the morning and positive findings are reported to fieldmen when they telephone in for other laboratory results.

With the latter method the sensitivity is increased, as the antibiotic has a greater opportunity to diffuse into the agar layer before the test organisms begin to grow.

The present paper reports recent studies on modifications of the standard method (9) aimed at getting results in less than 4 hours. These will be discussed individually. With few exceptions, tests were run on milk shown to be free from antibiotics, to which penicillin G was added to give a concentration of 0.05 I. U./ml. Incubation was ordinarily at 35°C., using 6 ml of Bacto penassay seed agar per plate and 0.5 No. 740-E, S & S discs. Bacto subtilis spore suspension B453 was used in all tests.

Plating Medium

The standard method (9) gives a choice of whey agar, Bacto B34, or penassay seed agar, Bacto B263, or penicillin assay seed agar, BBB, for poured plates. Growth on whey agar has been very much slower than on penassay seed agar. However, with 0.5 discs on the latter we have been able to detect 0.025 I. U. penicillin/ml. quite readily.

Temperature of Incubation

Most milk plant laboratories have incubator space at 35° and/or 32°C., but few also have space at 37°C. Comparative tests, some of which are shown in Table 1, indicated that with penassay seed agar zones were usually detectable with 0.5 disc in 2 to 3 hr. at 37°C., in 3 to 3½ hr. at 35°C., and in 3½ to 4 hr. at 32°C. Similar differences were observed with whey agar; thus, there is no serious objection to the use of temperatures lower than 37°C.

1 Contribution No. 36 from the Dairy Technology Research Institute.
3 days refrigeration compared with 5 hours when found a definite slowing down with refrigerated plates when compared with freshly poured ones. In later, more extensive, studies (Table 2) this effect was successfully employed ("Spotting" agar there was a definite disadvantage. For most freshly poured (Table 1).

Overnight incubation at room temperature has been countered.

Refrigeration of Poured Plates Before Use

Arret and Kirshbaum now (2) recommend refrigerating poured plates not less than 3 or more than 5 days before using. In our earlier studies (5) we found a definite slowing down with refrigerated plates when compared with freshly poured ones. In later, more extensive, studies (Table 2) this effect was scarcely noticeable with penassay seed agar until after 5 days refrigeration at 4°C. Whey agar plates, on the other hand, have required 7 hours after 2 and 3 days refrigeration compared with 5 hours when freshly poured (Table 1).

There was no apparent advantage in using only refrigerated plates as prescribed by Arret and Kirshbaum (2) for penassay seed agar, while with whey agar there was a definite disadvantage. For most laboratories it would be convenient to pour a batch of penassay seed agar plates at one time, use some the same day and refrigerate the remainder for subsequent use within 5 days.

Preliminary Incubation of Poured Plates Before "Spotting"

When plates were incubated at 35°C, for 1 hour before the milk-soaked discs were "spotted" thereon, zone formation was detectable from 30 to 90 minutes earlier. In this laboratory this procedure has not resulted in a loss of sensitivity, as reported by Arret and Kirshbaum (1).

Heat Shocking of Spores

The standard procedure (9) specifies melting the plating medium, cooling to 50-55°C, then introducing the spore suspension and pouring the plates. If the spore suspension is heat-shocked by adding it to the melted medium at 70°C and maintaining the medium at this temperature for 15 minutes, zones are frequently detectable 30 to 60 minutes earlier than with the official procedure. This effect has been observed even in plates refrigerated for 8 days before use. However, considerable variability has been noted. In one test heat-shocking saved over 90 minutes, while in another 30 minutes longer were required. There are evidently some unrecognized variables here which have not been controlled; nevertheless, this procedure does seem to be of value in hastening growth of the test organism.

Sensitivity

The increased sensitivity obtained by using (a) thinner layers of seeded agar and (b) 0.5" paper discs has been reported by various workers (3, 5, 6, 7). The standard procedure (9) calls for the use of 6 ml. per plate. Recent comparisons between plates poured with 6 and with 10 ml. of seeded medium showed a sharp drop in sensitivity with 10 ml., especially when 0.25" discs, as recommended by Arret and Kirshbaum (1), are used. In fact, in several tests we were unable to detect 0.05 I. U./ml. penicillin with the small-

Table 1 - Rates of Zone Development on Pen assay Seed Agar and on Whey Agar. Milk contained 0.05 I. U. penicillin/ml; 0.5" discs; plates incubated at 32°C, 35°C, and 37°C.

<table>
<thead>
<tr>
<th>Days refrigerated at 4°C</th>
<th>32°C</th>
<th>35°C</th>
<th>37°C</th>
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<tbody>
<tr>
<td>Penassay Seed Agar</td>
<td>0</td>
<td>3.5</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>3.5</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3.7</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>3.9</td>
<td>3.1</td>
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<tr>
<td>Whey Agar</td>
<td>0</td>
<td>5.0</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>5.8</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>&gt;7</td>
<td>6.8</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>&gt;7</td>
<td>7</td>
</tr>
</tbody>
</table>

Table 2 - Effect of Previous Refrigeration of Poured Plates on Rate of Zone Formation. Penassay seed agar; 0.5" discs; milks containing 0.05 I. U. penicillin/ml; incubation at 35°C.

<table>
<thead>
<tr>
<th>Days refrigeration</th>
<th>No. of tests</th>
<th>Hours incubation to detect zone of inhibition</th>
<th>Average</th>
<th>Range</th>
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<tbody>
<tr>
<td>0</td>
<td>6</td>
<td>3.33</td>
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<tr>
<td>1</td>
<td>6</td>
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<td>3.0-4.0</td>
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</tr>
<tr>
<td>2</td>
<td>6</td>
<td>3.7</td>
<td>3.0-5.0</td>
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</tr>
<tr>
<td>3</td>
<td>5</td>
<td>3.5</td>
<td>3.0-4.5</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>3.6</td>
<td>2.5-5.5</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>3.63</td>
<td>3.0-4.0</td>
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</tr>
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<td>6</td>
<td>4</td>
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</tr>
<tr>
<td>7</td>
<td>3</td>
<td>4.5</td>
<td>4.0-5.0</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>2</td>
<td>5.25</td>
<td>5.0-5.5</td>
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</tr>
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</table>
Further Observations on Testing Milk for Penicillin

In our recent studies the difference in sensitivity between the two sizes of discs has been less than we found in tests with aureomycin (5), or that reported by others (3, 7) with penicillin. However, with the 0.5" disc we have invariably detected a concentration of penicillin of 0.025 I. U./ml; with the 0.25" disc this concentration sometimes gave a negative or doubtful reaction, even when incubation was continued for several hours longer. Up to 2 hours longer incubation was frequently required to detect zone formation with the smaller disc.

While it is recognized that more of the smaller discs can be accommodated on the surface of a poured plate, this must be balanced against the greater ease of handling the 0.5" disc, and the larger quantity (some six times as great) and greater uniformity of the amount of milk absorbed.

Acknowledgement

I am indebted to D. J. Swan for help in running the tests in the present series of studies.

References


Proposed Model Act

For the Registration of Sanitarians

Editor's Note: One of the projects of the Sanitarian's Joint Council has been the development of a model act for the registration of Sanitarians. At its meeting on June 18, 1960, the Council approved this Act printed below. Representatives of APHA, NAS and IAMFS constitute the Joint Council.

An Act Relating To The Preservation And Protection Of The Public Health And Providing For The Registration Of Sanitarians And Sanitarians-In-Training; Providing For The Establishment Of A Board Of Registration And Prescribing Its Powers, Duties And Functions; Dealing With Qualifications, Appointment, Removal, Compensation, And Expenses Of Members Thereof; Providing For Qualifications, Examinations, And Registration Of Sanitarians And Sanitarians-In-Training; And For Issuance, Renewal, And Reinstatement Of Certificates Of Registration; And Fixing Fees Therefor; Authorizing Revocation Of Certificates; Providing For Expenditures Of Funds Collected Under Provisions Of This Act; Fixing Purposes For Which Such Funds May Be Used; And Providing A Penalty.

Be It Enacted By The Legislature Of The State Of ____________________________:

Section 1. State Board Of Registration: There is hereby created a Board of Registration to register qualified sanitarians whose duties in public health and environmental sanitation require a knowledge of physical, biological, and sanitary sciences and whose professional pursuits and duties are necessary to the promotion of life, health, and prosperity of the State's citizens.

Section 2. Definitions: The words and phrases defined below shall, when used in this Act, have the following meaning unless the context clearly indicates otherwise:
(a) "Board"—means the Board of Registration for Sanitarians, hereby created.
(b) "Sanitarian"—is a person who by education and experience in the physical, biological, and sanitary sciences is qualified to carry out educational, investigational and technical duties in the field of sanitation.
(c) "Registered Sanitarian"—is a sanitarian registered in accordance with the provisions of this Act.
(d) "Sanitarian-in-Training"—is a person registered as a sanitarian-in-training under the provisions of this Act.
(e) "Certificate of Registration"—is a document issued as evidence of registration and qualification to practice