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TECHNICAL MANUSCRIPT 126

A SELECTIVE MEDIUM FOR THE ISOLATION OF PASTEURELLA PESTIS

FEBRUARY 1964

UNITED STATES ARMY BIOLOGICAL LABORATORIES
FORT DETRICK
A SELECTIVE MEDIUM FOR THE ISOLATION OF *Pasteurella pestis*
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ABSTRACT

Several selective media are described that were successfully used to isolate virulent and avirulent strains of Pasteurella pestis from material heavily contaminated with other organisms. These media are comparatively easy to prepare, consist of readily available ingredients, and usually require no adjustment of the pH. One of the selective media described permits excellent recovery and the growth of large, easily distinguishable colonies of P. pestis in 48 hours at 26°C, and also allows the detection of fewer numbers of P. pestis organisms in soil than a previously recommended selective medium. The inhibition of other organisms frequently present in clinical specimens is described.
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I. INTRODUCTION

A selective medium for the isolation of Pasteurella pestis from material heavily contaminated with other organisms has been described by Morris. Initial studies in this laboratory with the Morris medium showed marked variability with respect to recovery of virulent and avirulent strains as well as with individual strains in each group.

Our study is primarily concerned with the development of an azide medium that permits excellent recovery and growth of P. pestis in 48 hours from material heavily contaminated with other organisms. Test media evaluated in this study were (a) plain azide, (b) enriched azide, (c) Morris medium, and (d) blood agar base (BAB) as a control medium. The efficiency of a selective medium was evaluated on the basis of maximal recovery and growth of P. pestis organisms from known concentrations of test inocula. In addition, the effectiveness of the medium was also determined by the extent of inhibition of soil and other common contaminating organisms. The ease of preparation and the availability of ingredients were also important considerations.

II. MATERIALS AND METHODS

A. BACTERIAL STRAINS

Virulent and avirulent strains of P. pestis were employed in quantitative recovery experiments. The virulent strains were assayed for the proportion of avirulent cell types by the oxalate plate method of Higuchi and Smith.

B. PREPARATION OF CELLS

Test inocula of each strain were prepared by suspending the growth (24 hours at 26°C) from two blood agar base slants in 10 milliliters of 0.05M phosphate buffer of pH 7.3. Dilutions containing approximately $10^5$ organisms per milliliter were prepared for plating on selective media. Triplicate plates were inoculated with 0.1 milliliter of suspension and incubated at 26°C.

C. ANTIBIOTICS, CHEMICALS, AND CULTURE MEDIA

All culture media, potassium tellurite, and bile salts No. 3 were obtained from Difco. The following antibiotics and chemicals were obtained from the sources indicated: erythromycin (erythromycin lactobionate),
Abbot: novobiocin (Albamycin), Upjohn; ethyl violet, National Aniline Division of Allied Chemical and Dye Corporation; actidione, Upjohn; mycostatin, E.R. Squibb; hemin, Eastman Organic Chemicals; sodium azide, Eastman Kodak Company.

Antibiotic solutions were freshly prepared. Sterile solutions of other compounds were prepared and stored at 4°C. Hemin was dissolved in 0.05N NaOH and autoclaved. Calcium ion was added as CaCl₂.6H₂O. Mycostatin was suspended in 70 per cent alcohol or dissolved in propylene glycol.

D. SELECTIVE MEDIA

The composition of selective media is presented in Table I. Ingredients were added after autoclaving the base agar and cooling to 45°C. Tryptose agar was employed in the Morris medium; BAB was used with the other media. The Morris medium was adjusted to pH 7.6 with 2N NaOH. The pH of the remaining media after addition of supplements was approximately pH 6.9 (unadjusted). Test media were generally used 24 hours after preparation, but satisfactory results could be obtained after storage for one week at 4°C.

<table>
<thead>
<tr>
<th>TABLE I. COMPOSITION OF SELECTIVE MEDIA</th>
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<tr>
<td>Concentration, µg/ml</td>
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<tr>
<td>Base Medium</td>
</tr>
<tr>
<td>Plain Azide</td>
</tr>
<tr>
<td>Bile Salts&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Enriched Azide&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Morris Medium&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

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<sup>a</sup> 1500 micrograms of bile salts added per milliliter.
<sup>b</sup> Additions to enriched azide medium consisted of 0.1 per cent glucose and 0.1 M Ca++ as CaCl₂.6H₂O.
<sup>c</sup> Supplements to the Morris medium consisted of 10 micrograms of novobiocin per milliliter; 5 micrograms of potassium tellurite per milliliter and 5 per cent (v/v) peptic digest of sheep blood (medium adjusted to pH 7.6; remaining media are unadjusted at pH 6.9).
E. PREPARATION OF FILDES' DIGEST

Fresh sheep blood was obtained aseptically and defibrinated with glass beads. The method of Fildes\(^3\) was followed using Difco (N.F.) pepsin, and the digestion was allowed to proceed with occasional shaking for approximately five hours in a water bath at \(55^\circ\text{C}\).

F. SURVEY FOR SELECTIVE INGREDIENTS

Nineteen virulent and 15 avirulent strains of \(P.\) \textit{pestis} were tested for sensitivity to various antibiotics and dyes. Sensitivity discs (Difco) were employed for antibiotic studies. Antibiotics that failed to inhibit \(P.\) \textit{pestis} were tested for their effect on soil organisms. A variety of concentrations and combinations were evaluated at various pH levels.

G. SOIL STUDIES WITH \(P.\) \textit{PESTIS}

Soil was considered as representing a specimen heavily contaminated with other bacteria. Comparative studies were conducted utilizing plain and enriched azide media, bile salts medium, and Morris medium. A local soil sample (Soil A) and a garden sample (Soil B) were employed in these experiments. Each soil was passed through screens of decreasing pore size to remove large clumps and other debris. The moisture content was not adjusted for this experiment. One-gram samples of soil were inoculated with the test strain and suspended in a total volume of 10 milliliters of phosphate buffer (pH 7.3). Suspensions or dilutions were vigorously shaken and 0.1 milliliter was plated on triplicate plates. For plating on BAB control plates; 0.1 milliliter of a \(10^{-3}\) dilution of the suspension was employed.

H. IDENTIFICATION OF \(P.\) \textit{PESTIS} COLONIES

Colons on plates were carefully examined with a stereoscopic microscope (12X), using oblique illumination. Representative colonies possessing typical morphology of \(P.\) \textit{pestis} were subcultured on tryptose agar for more detailed study.
III. RESULTS

A. SURVEY FOR SELECTIVE INGREDIENTS

Extensive testing showed that a selective medium for *P. pestis* could be prepared by utilizing blood agar base in combination with sodium azide, erythromycin, actidione, mycostatin and ethyl violet. The addition of hemin improved the recovery of *P. pestis* on this medium. Optimal results were obtained by lowering the sodium azide concentration and adding glucose and calcium ion.

B. RECOVERY OF *P. PESTIS* ON PLAIN AZIDE, BILE SALTS AND MORRIS MEDIA

Recovery efficiencies after 72 hours of incubation on plain azide, bile salts, Morris and BAB media are compared in Table II. Blood agar base served as the control medium. Maximum recovery of all strains was generally obtained on the Morris and BAB media in 48 hours; a period of 72 hours was required with the other media. Atypical colonies of *P. pestis* were produced on the bile salts medium. A higher recovery was obtained with bile salts and plain azide media than with the Morris medium.

C. RECOVERY OF *P. PESTIS* ON ENRICHED AZIDE AND MORRIS MEDIA

Recovery with enriched azide media in 48 hours was markedly superior to that with the Morris medium (Table III). In addition, the recovery rates were improved over those obtained with plain azide media (Table II). Maximum recovery on enriched azide was obtained in 48 hours. The colonies were large and possessed characteristic morphology.

Figure 1 shows the relative size and numbers of a virulent (Alexander) and avirulent (A1122) strain of *P. pestis* on enriched azide and Morris media.

D. RECOVERY OF *P. PESTIS* FROM SOIL SAMPLES

The recovery of avirulent *P. pestis* (A1122) was higher on plain azide than on the Morris medium utilizing 700 and 1400 cells per gram of soil (Table IV). Similar results were obtained with enriched azide and the virulent Alexander strain (Table V). Figure 2 shows growth of soil organisms from soil suspensions A and B on plain and enriched azide and the Morris medium. In order to recover *P. pestis* from Soil A and B by plating on BAB media, it was necessary to use an inoculum of $2.5 \times 10^9$ and $1 \times 10^9$ *P. pestis* cells per gram of soil, respectively.
TABLE II. RECOVERY OF *P. PESTIS* AFTER 72 HOURS OF INCUBATION (26°C) ON THREE SELECTIVE MEDIA

<table>
<thead>
<tr>
<th>Test Strains</th>
<th>Plain Azide Medium</th>
<th>Bile Salts Medium</th>
<th>Morris Medium</th>
<th>Control (BAB)</th>
</tr>
</thead>
<tbody>
<tr>
<td>14 avirulent</td>
<td>80</td>
<td>96</td>
<td>29</td>
<td>100</td>
</tr>
<tr>
<td>14 virulent</td>
<td>85</td>
<td>85</td>
<td>61</td>
<td>100</td>
</tr>
</tbody>
</table>

a. Plate counts obtained by averaging three plates for each strain.
c. Range of recovery was as follows: Plain azide medium - not less than 58 per cent recovery except for one avirulent strain (4 per cent) and one virulent strain (25 per cent); Bile salt medium - not less than 50 per cent recovery for all strains; Morris medium - less than 50 per cent recovery for 11 avirulent strains and 3 virulent strains.

---

TABLE III. RECOVERY OF *P. PESTIS* AFTER 48 HOURS OF INCUBATION (26°C) ON ENRICHED AZIDE AND MORRIS MEDIA

<table>
<thead>
<tr>
<th>Test Strains</th>
<th>Per Cent Recovery</th>
<th>Control (BAB)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Enriched Azide Medium</td>
<td>Morris Medium</td>
</tr>
<tr>
<td>14 avirulent</td>
<td>0</td>
<td>27</td>
</tr>
<tr>
<td>12 virulent</td>
<td>105</td>
<td>39</td>
</tr>
</tbody>
</table>

a. Plate counts obtained by averaging three plates for each strain.
c. Recovery on enriched azide medium was not less than 74 per cent for any strain. Recovery of the avirulent strains on Morris medium ranged from 0 to 57 per cent and recovery of the virulent strains from 0 to 80 per cent.
Figure 1. Recovery of *P. pestis* Strain Al122 (CD337) and Alexander Strain (CD404B) on Enriched Azide (Top, Left) and Plain Azide (Top, Right) and Morris Medium after 48 Hours of Incubation. The BAE plate serves as a control.
Figure 2. Relative inhibition of Soil Organisms on Enriched Azide (Top, Left), Plain Azide (Top, Right), and Morris Medium. The selective media were inoculated with 0.1 milliliter of a $10^{-4}$ dilution of soil suspension. The BAB was inoculated with 0.1 milliliter of a $10^{-5}$ dilution of soil suspension. Note the spreading-type colony on the BAB plate of soil A.
TABLE IV. AVERAGE RECOVERY OF AVIRULENT \textit{P. Pestis} (A1:22) AND SOIL ORGANISMS FROM SOIL A ON PLAIN AZIDE AND MORRIS MEDIA

<table>
<thead>
<tr>
<th>Initial Inoculum of \textit{P. Pestis} Cells Per Gram of Soil</th>
<th>Dilution of Soil Suspension Plated</th>
<th>Average Recovery of \textit{P. Pestis}</th>
<th>Average Recovery of Soil Organisms</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Undiluted (b)</td>
<td>Plain Azide</td>
<td>Morris Azide</td>
</tr>
<tr>
<td>700</td>
<td>Undiluted</td>
<td>7.9</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>1:2</td>
<td>2.4</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>1:5</td>
<td>2.9</td>
<td>0.9</td>
</tr>
<tr>
<td>1400</td>
<td>Undiluted</td>
<td>6.8</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>1:2</td>
<td>3.6</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>1:5</td>
<td>1.9</td>
<td>0.7</td>
</tr>
</tbody>
</table>

\(a\). Counts obtained from three samples (triplicate plates averaged).

\(b\). 0.2 milliliter of undiluted suspension plated; 0.1 milliliter quantities plated from all other suspensions and dilutions.

TABLE V. RECOVERY OF VIRULENT \textit{P. Pestis} Alexander Strain AND SOIL ORGANISMS FROM SOIL B ON ENRICHED AZIDE AND MORRIS MEDIA

<table>
<thead>
<tr>
<th>Initial Inoculum of \textit{P. Pestis} Cells Per Gram of Soil</th>
<th>Dilution of Soil Suspension Plated</th>
<th>Average Recovery of \textit{P. Pestis}</th>
<th>Average Recovery of Soil Organisms</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Undiluted</td>
<td>Enriched Azide</td>
<td>Morris Azide</td>
</tr>
<tr>
<td>595</td>
<td>Undiluted</td>
<td>4.7</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>1:2</td>
<td>2.8</td>
<td>0.4</td>
</tr>
<tr>
<td>1190</td>
<td>Undiluted</td>
<td>8.0</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>1:2</td>
<td>3.8</td>
<td>0.9</td>
</tr>
</tbody>
</table>

\(a\). Counts obtained from three samples (triplicate plates averaged).
E. INHIBITION OF OTHER CONTAMINANTS

The following organisms were inhibited on plain azide media and the Morris medium: *Aerobacter aerogenes*, *Alcaligenes faecalis* ATCC-8750, *Escherichia coli* ATCC-11303, *Escherichia coli* B, *Pasteurella mastoidis* ATCC-10899, *Pasteurella multocida* ATCC-9656, *Shigella sonnei* 16, *Streptococcus faecalis*, and *Vibrio comma* VM-7708. The enriched azide media inhibited all these strains except *Aerobacter aerogenes*, *E. coli* B, and *Shigella sonnei*. Recovery of *E. coli* on this medium was 56 per cent and of *Shigella sonnei* 49 per cent of the recovery on the BAB control.

*Paracolobacterium coliorme*, *Proteus vulgaris*, and *Salmonella typhosa* A-60 were inhibited on the Morris medium but not on the plain or enriched azide media. Recovery of six strains of *Pasteurella pseudotuberculosis* (ATCC 6902, 6903, 6904, 6905, 913, and 11960) averaged 74 per cent on plain azide, 94 per cent on enriched azide, and 43 per cent on the Morris medium. *Pseudomonas aeruginosa* recovery was 100 per cent on plain azide, 90 per cent on enriched azide, and 22 per cent on the Morris medium. All percentages are in comparison with recovery on BAB plates. Strains not specifically identified were stock strains that had been used for student instruction.

F. STATISTICAL ANALYSIS

Analyses of variance were performed and comparisons made at the five per cent probability level. The statistical analysis of the data showed that the recovery of virulent and avirulent strains of *P. pestis* was significantly higher on plain and enriched azide and bile salts media than on the Morris medium (Tables II and III). The recovery of *P. pestis* from Soil A (Table IV) on plain azide was significantly higher than on the Morris medium for both levels of inoculum. The analysis indicated that there was no significant difference between these two selective media in the inhibition of the soil organisms from Soil A.

The analysis of the data in Table V showed that there was a significantly higher recovery of *P. pestis* on the enriched azide medium compared with that on the Morris medium. There was no significant difference in
IV. DISCUSSION

The Morris medium was difficult to prepare, required adjustment of the pH, and did not allow for efficient recovery of *P. pestis*. Recovery on this medium prepared in our laboratory varied from 27 per cent for the avirulent strains to 60 per cent for the virulent strains when compared with blood agar base controls. The recovery of individual strains of *P. pestis* frequently varied each time the medium was prepared.

Markenson and Ben-Efraim describe an oxgall medium for identification of *P. pestis*. This medium requires two per cent defibrinated rabbit blood. Preliminary results with this medium indicate that it is less inhibitory for contaminating organisms than azide media. Recovery of 12 virulent strains of *P. pestis* averaged 56 per cent compared with recovery on BAB. This medium also has the disadvantage of being opaque.

Previous attempts by other investigators to isolate *P. pestis* on selective media from various specimens were not entirely satisfactory. A medium for the cultivation of *Pasteurella* and *Brucella* species utilizing brilliant green as the inhibitory agent was described by Levin, Trupin, and Cabelli. This medium requires the use of a peptic digest of bovine hemoglobin.

The bile salts medium permitted good recovery of *P. pestis*, but colony morphology was atypical. Similar variations in colony morphology on a bile salts medium were described by LaRose. Recognition of *P. pestis* colonies among soil colonies on this medium was more difficult than with other selective media. The bile salts medium is therefore recommended only in special situations where the contaminants are particularly sensitive to bile salts. The use of bile solution as an enrichment fluid for the isolation of *P. pestis* was reported by Kirschner.

The plain azide medium requires 72 hours for recovery of *P. pestis*, but has the advantage of being more inhibitory than the enriched azide medium for certain types of contaminating organisms. The enriched azide medium has an advantage of allowing excellent recovery of *P. pestis* in 48 hours and growth of large easily distinguishable colonies.

Hemin served as a satisfactory enrichment in the azide medium. It eliminated the necessity of obtaining sheep blood and the time required to prepare the digest. Herbert reported that hematin was the factor in pepsinized blood responsible for increased growth of *P. pestis* in agar media.

The correlation of virulence of *P. pestis* with calcium ion was reported by Higuchi, Kupferberg, and Smith and Higuchi and Smith. Glucose and calcium ion were added in an attempt to improve the azide medium. Although
the addition of calcium and glucose does not significantly increase the recovery of *P. pestis*, it does aid in obtaining large, uniform colonies possessing a characteristic morphology. The advantages of the proposed azide media are easy preparation, utilizing readily available ingredients, and the possibility of direct isolation of *P. pestis* from heavily contaminated material. The growth of many organisms frequently encountered in clinical specimens is inhibited on these media.
LITERATURE CITED


