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SELECTIVE MEDIA FOR THE ISOLATION OF *PASTEURELLA PESTIS*

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ABSTRACT

KNISELY, RALPH F. (U.S. Army Biological Laboratories, Fort Detrick, Frederick, Md.), LOIS M. SWANEY, AND HAROLD FRIEDLANDER. Selective media for the isolation of *Pasteurella pestis*. J. Bacteriol. **88**:491–496. 1964.—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 hr 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.

A selective medium for the isolation of *Pasteurella pestis* from material heavily contaminated with other organisms was described by Morris (1958). 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.

The present study was primarily concerned with the development of an azide medium that permits excellent recovery and growth of *P. pestis* in 48 hr from material heavily contaminated with other organisms. Test media evaluated were: (i) plain azide, (ii) enriched azide, (iii) Bile Salts, (iv) Morris medium, and (v) 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.

MATERIALS AND METHODS

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 (1961).

Preparation of cells. Test inocula of each strain were prepared by suspending the growth (24 hr at 26 C) from two BAB slants in 10 ml of 0.06 M phosphate buffer (pH 7.3). Dilutions containing approximately 10^3 organisms per ml were prepared for plating on selective media. Triplicate plates were inoculated with 0.1 ml of suspension and incubated at 26 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), Abbott Laboratories, North Chicago, Ill.; novobiocin (Albamycin), The Upjohn Co., Kalamazoo, Mich.; ethyl violet, National Aniline Division, Allied Chemical Corp., New York, N.Y.; cycloheximide, The Upjohn Co.; nystatin, E. R. Squibb & Sons, New York, N.Y.; hemin, Eastman Organic Chemicals, Rochester, N.Y.; sodium azide, Eastman Kodak Co., Rochester, N.Y.

Antibiotic solutions were freshly prepared. Sterile solutions of other compounds were prepared and stored at 4 C. Hemin was freshly prepared by dissolving in 0.05 N NaOH and was autoclaved at a pressure of 5 psi for 30 min. Calcium ion was added as $CaCl_2 \cdot 6H_2O$. Nystatin was suspended in 70% alcohol or dissolved in propylene glycol.

Selective media. The composition of selective

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media is presented in Table 1. Ingredients were added after autoclaving the base agar and cooling to 45 C. Tryptose Agar was employed in the Morris medium, and BAB was used with the other media. The Morris medium was adjusted to pH 7.6 with 2 N NaOH. The pH of the remaining media, after addition of supplements, was approximately 6.9 (unadjusted). Test media were generally used 24 hr after preparation, but satisfactory results could be obtained after storage for 1 week at 4 C.

Preparation of Fildes' digest. Fresh sheep blood was obtained aseptically and defibrinated with glass beads. The method of Fildes (1920) was followed with the use of Difco pepsin, and the digestion was allowed to proceed with occasional shaking for approximately 5 hr in a water bath at 55 C.

Survey for selective ingredients. Strains (19 virulent and 15 avirulent) of *P. pestis* were tested for sensitivity to various antibiotics and dyes. Sensitivity discs (Difco) were employed for antibiotic studies. Antibiotics that failed to inhibit *P. pestis* were tested for their effect on soil organisms. A variety of concentrations and combinations were evaluated at various pH levels.

Soil studies with P. pestis. Soil was considered as representing a specimen heavily contaminated with other bacteria. Comparative studies were conducted with 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. Samples (1 g) of soil were inoculated with the test strain and suspended in a total volume of 10 ml of phosphate buffer (pH 7.3). Suspensions or dilutions were vigorously shaken, and 0.1 ml was plated on triplicate plates. For plating on BAB control plates, 0.1 ml of a 10^{-3} dilution of the suspension was employed.

Identification of P. pestis colonies. Colonies on plates were carefully examined with a stereoscopic microscope (12×), by use of oblique illumination. Representative colonies possessing typical morphology of *P. pestis* were subcultured on Tryptose Agar for more detailed study.

RESULTS

Survey for selective ingredients. Extensive testing showed that a selective medium for *P. pestis* could be prepared by utilizing BAB in combination with sodium azide, erythromycin, cycloheximide, nystatin, 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.

Recovery of P. pestis on plain azide, Bile Salts, and Morris media. Recovery efficiencies after 72 hr of incubation on plain azide, Bile Salts, Morris, and BAB media are compared in Table 2. BAB served as the control medium. Maximal recovery of all strains was generally obtained on the Morris and BAB media in 48 hr, whereas a period of 72 hr 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.

Recovery of P. pestis on enriched azide and Morris media. Recovery with enriched azide media

TABLE 1. *Composition of selective media*

Medium	Base medium	Concn (μg/ml)					
		Erythromycin	Ethyl violet	Cycloheximide	Nystatin	Sodium azide	Hemin
Plain azide	BAB	40	1	100	100	7	—
Bile Salts*	BAB	40	1	100	100	—	—
Enriched azide†	BAB	40	1	100	100	5	40
Morris medium‡	Tryptose Agar	5	—	100	—	—	—

* Bile salts (1,500 μg/ml) added.

† Additions to enriched azide medium consisted of 0.1% glucose and 0.1 M Ca^{++} as $CaCl_2 \cdot 6H_2O$.

‡ Supplements to the Morris medium consisted of 10 μg/ml of novobiocin; 5 μg/ml of Potassium Tellurite; and 5% (v/v) peptic digest of sheep blood (medium adjusted to pH 7.6; remaining media are unadjusted at pH 6.9).

in 48 hr was markedly superior to that of the Morris medium (Table 3). In addition, the recovery rates were improved over those obtained with plain azide medium (Table 2). Maximal recovery on enriched azide was obtained in 48 hr. The colonies were large and possessed characteristic morphology.

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

Recovery of P. pestis from soil samples. The recovery of avirulent *P. pestis* (A1122) was higher on plain azide than on the Morris medium with the use of 700 and 1,400 cells per gram of soil (Table 4). Similar results were obtained with enriched azide and the virulent Alexander strain (Table 5). Figure 2 illustrates growth of soil organisms from soil suspensions A and B on plain and enriched azide and the Morris medium. To recover *P. pestis* from soil A and B by plating on BAB media, it was necessary to use an inoculum of 2.5×10^5 and 1×10^5 *P. pestis* cells per gram of soil, respectively.

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*

TABLE 2. *Recovery of Pasteurella pestis after 72 hr of incubation (26 C) on three selective media*

Test strains*	Per cent recovery†			
	Plain azide medium‡	Bile Salts medium‡	Morris medium‡	Control (BAB)
Avirulent (14)...	80	96	29	100
Virulent (14)....	85	85	61	100

* Avirulent: A-12, Tjiwidej, Soemedang, EV-76, A1122, TRU, A-4, Java, 556a, M23B, A-16, 53-H-1, M33, and AD5-S.

Virulent: Alexander, Charleston, Kuma, Washington, Yokohama, M41-1, Powell, MPG, Shasta, M23, Saka, 556, H5, and H15.

† Plate counts obtained by averaging three plates for each strain.

‡ Range of recovery was as follows: plain azide medium, not less than 58% recovery except for one avirulent strain (4%) and one virulent strain (25%); Bile Salts medium, not less than 50% recovery for all strains; Morris medium, less than 50% recovery for 11 avirulent strains and 3 virulent strains.

TABLE 3. *Recovery of Pasteurella pestis after 48 hr of incubation (26 C) on enriched azide and Morris media*

Test strains*	Per cent recovery†		
	Enriched azide medium‡	Morris medium‡	Control (BAB)
Avirulent (14)........	95	27	100
Virulent (12).........	105	39	100

* Avirulent: A-12, Tjiwidej, Soemedang, EV-76, A1122, TRU, A-4, Java, 556a, M23B, A-16, M-33, A1224, and AD5-S.

Virulent: Alexander, Charleston, Kuma, Washington, Yokohama, Powell, M41-1, MPG, Shasta, M23, Saka, and 556.

† Plate counts obtained by averaging three plates for each strain.

‡ Recovery on enriched azide medium was not less than 74% for any strain. Recovery of the avirulent strains on Morris medium ranged from 0 to 57% and recovery of the virulent strains from 0 to 80%.

ATCC-11303, *Escherichia coli* B, *Pasteurella mastidis* 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% and of *Shigella sonnei* 49% of the recovery on the BAB control.

Paracolobactrum coliforme, *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% on plain azide, 94% on enriched azide, and 45% on the Morris medium. *Pseudomonas aeruginosa* recovery was 100% on plain azide, 90% on enriched azide, and 22% 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.

Statistical analysis. Analyses of variance were performed, and comparisons were made at the 5% 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 2 and

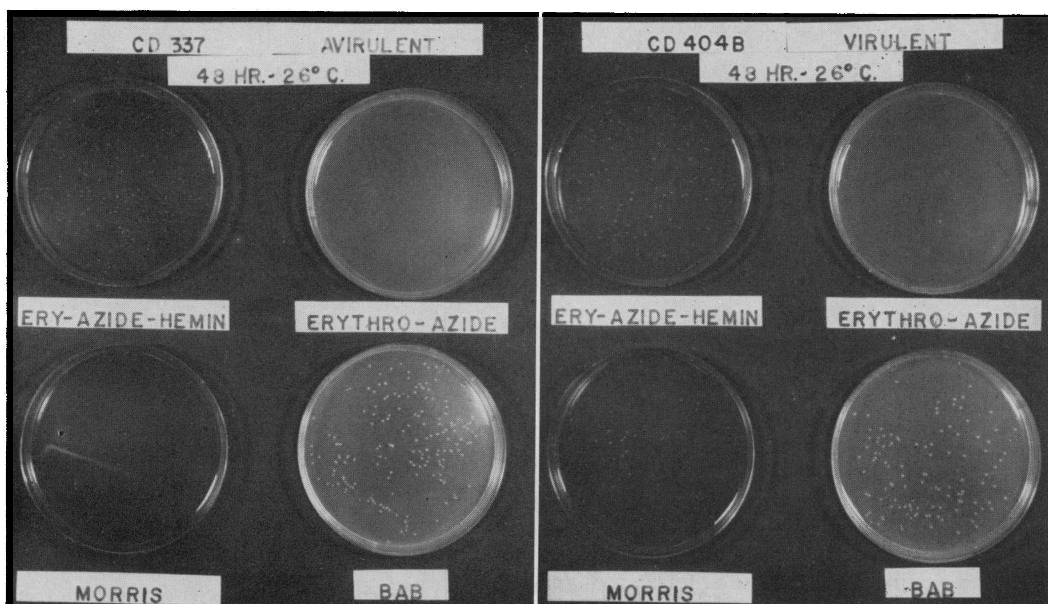

FIG. 1. *Recovery of Pasteurella pestis strain A1122 (CD337) and Alexander strain (CD404B) on enriched azide (top, left), plain azide (top, right), and Morris medium after 48 hr of incubation. The BAB plate serves as a control.*

TABLE 4. *Average recovery of avirulent Pasteurella pestis (A1122) and soil organisms from soil A on plain azide and Morris media**

Initial inoculum of *P. pestis* cells per gram of soil	Dilution of soil suspension plated	Avg recovery of *P. pestis*		Avg recovery of soil organisms	
		Plain azide	Morris	Plain azide	Morris
700	Undiluted†	7.9	2.3	202	321
	Undiluted	2.4	0.8	103	117
	1:2	2.9	0.9	49	50
1,400	Undiluted	6.8	2.9	185	192
	1:2	3.6	1.5	90	87
	1:5	1.9	0.7	45	35

* Counts obtained from three samples (triplicate plates averaged).

† A 0.2-ml amount of undiluted suspension was plated; 0.1-ml quantities were plated from all other suspensions and dilutions.

3). The recovery of *P. pestis* from soil A (Table 4) on plain azide was significantly higher than on the Morris medium for both levels of inocula. The analysis indicated that there were no significant differences between these two selective media in the inhibition of the soil organisms from soil A.

The analysis of the data in Table 5 showed that there was a significantly higher recovery of *P. pestis* on the enriched azide medium compared with the Morris medium. There was no significant difference in inhibition of soil organisms by either of the selective media.

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

TABLE 5. *Recovery of virulent Pasteurella pestis Alexander strain and soil organisms from soil B on enriched azide and Morris media**

Initial inoculum of *P. pestis* cells per gram of soil	Dilution of soil suspension plated	Avg recovery of *P. pestis*		Avg recovery of soil organisms	
		Enriched azide	Morris	Enriched azide	Morris
595	Undiluted	4.7	0.9	47	23
	1:2	2.8	0.4	26	16
1,190	Undiluted	8.0	1.7	22	15
	1:2	3.8	0.9	10	8

* Counts obtained from three samples (triplicate plates averaged).

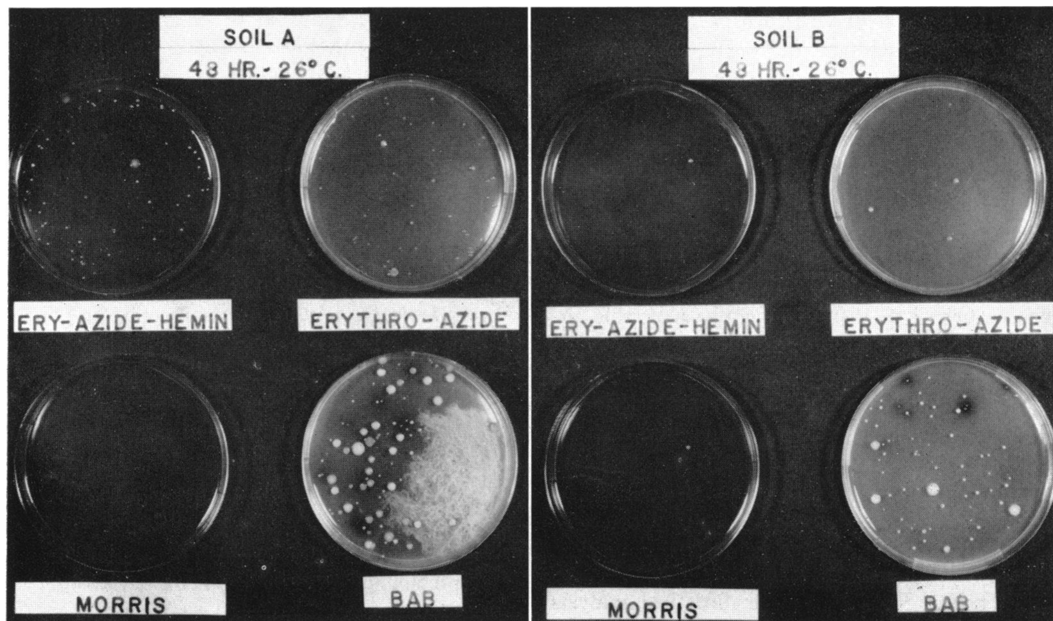

FIG. 2. *Relative inhibition of soil organisms on enriched azide (top, left), plain azide (top, right), and Morris medium. The selective media were inoculated with 0.1 ml of a 10^{-1} dilution of soil suspension. The BAB was inoculated with 0.1 ml of a 10^{-3} dilution of soil suspension. Note the spreading-type colony on the BAB plate of soil A.*

this medium prepared in our laboratory varied from 27% for the avirulent strains to 60% for the virulent strains when compared with BAB controls. The recovery of individual strains of *P. pestis* frequently varied each time the medium was prepared.

Markenson and Ben-Efraim (1963) described an oxgall medium for identification of *P. pestis*. This medium requires 2% defibrinated rabbit blood. Preliminary results with this medium indicate that it is less inhibitory for contaminating organisms than is azide media. Recovery of 12 virulent strains of *P. pestis* averaged 56% 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, (Drennan and Teague, 1917; Meyer and Batchelder, 1926; Quan, Von Fintel, and McManus, 1958). A medium for the cultivation of *Pasteurella* and *Brucella* species with brilliant green as the inhibitory agent was described by Levin, Trupin, and Cabelli (1962). 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 (1930). 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 (1934).

The plain azide medium requires 72 hr 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 hr 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 (1949) reported that hematin was the factor in pepsonized 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 (1959) and Higuchi and Smith (1961). 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.

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