Vibrios from Fish Pen Slime Which Mimic

*Escherichia coli* on Violet Red Bile Agar

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Organisms from fish pen slime which mimicked coliforms and *Escherichia coli* on Violet Red Bile Agar were identified as members of the genus *Vibrio* on the basis of metabolic and morphological characteristics.

Violet Red Bile (VRB) Agar is known as a selective medium for the direct enumeration of coliforms in milk, poultry, and dairy products (5, 7, 12, 15, 16, 18). Discrepancies do exist, however, as to the true value of VRB Agar for the direct detection and isolation of coliforms and *E. coli*. The American Public Health Association (1) recommends counting as coliforms those dark red colonies having a diameter of 0.5 mm or greater, usually with a zone of precipitate. However, such a criterion might well be questioned. Hartman (8) observed that small red colonies could be produced by *Proteus*, *Alcaligenes*, intermediates, and *Achromobacter*. These small red colonies are supposedly “atypical,” yet Hartman found many such colonies confirming as coliforms. Hartman also found “typical large” colonies that could not be confirmed (9). Jones, working with dairy products, also found colony size on VRB not to be a valid criterion for differentiating coliforms from non-coliforms (11). Barber and Fram (3), Koburger (13), and even the American Public Health Association (1) indicate the uncertainty which may develop when attempting to determine coliform densities in various foods with VRB Agar.

In this study, organisms having a population density of 10⁷ per square inch of surface slime on storage pens of fishing trawlers, which had been selectively enumerated on VRB Agar as coliforms, were subjected to more detailed studies for identification.

**MATERIALS AND METHODS**

**Source of cultures.** Forty red colonies on VRB Agar derived from fish pen slime were purified and transferred to Nutrient Agar (NA) slants from which further studies were performed.

**Biochemical studies.** Gelatin liquefaction was determined in tubes of 12% nutrient gelatin incubated at 35°C for 6 days. The method of Hugh and Leifson (10) was used for detecting aerobic and anaerobic production of acid from 1% glucose, arabinose, mannose, sucrose, lactose, salicin, trehalose, esculin, maltose, and mannitol. The production of gas from glucose, lactose, and trehalose was determined by using tubes of Nutrient Broth containing 1% of each sugar and Durham fermentation vials. Starch hydrolysis was determined by inoculating plates of NA, containing 0.2% soluble starch, followed by 48 hr of incubation at 35°C and then flooding with Lugol's iodine. The chola red test was performed.

| No. of cultures | Indole production | Methyl red | Acetoin production | Citrate utilization | Gas in BGB Broth | Growth on EMB Agar (24 hr) |
|-----------------|------------------|------------|--------------------|-------------------|-----------------|--------------------------|
| 2               | +                | -          | -                  | -                 | -               | -                        |
| 16              | -                | +          | -                  | -                 | -               | -                        |
| 4               | -                | +          | +                  | 1+ 3              | 1+ 3           | -                        |
| 1               | +                | +          | +                  | +                 | +               | +                        |
| 3               | -                | -          | +                  | 1+ 1              | -               | -                        |
| 2               | +                | +          | +                  | +                 | +               | +                        |
| 5               | -                | -          | +                  | -                 | -               | -                        |
| 2               | +                | +          | +                  | -                 | -               | -                        |
| 3               | -                | -          | +                  | +                 | -               | -                        |
| 1               | -                | +          | +                  | +                 | -               | -                        |

* Abbreviations: IMViC, indole, methyl red, Voges-Proskauer, citrate; BGB, Brilliant Green Lactose Bile Broth; EMB, Levine Eosine Methylene Blue Agar.

* Numerical values indicate number of cultures. Symbols: +, all cultures positive; -, all cultures negative.
filter-paper discs, treated with a saturated solution of the agent, which were placed onto seeded surfaces of NA plates.

**Morphology.** Gram stains were performed on NA cultures incubated for 24 hr at 35°C. Broth cultures were observed under phase-contrast microscopy to determine morphology and motility. Flagella were observed by using the following procedure. Cells from 18-hr NA slants were suspended in distilled water to yield a cloudy suspension; several drops were placed onto a tilted slide and the excess fluid was allowed to run off. After air-drying, the slides were immersed in reagent I for 15 min. This step serves the functions of both fixative and mordant. Reagent I consisted of: distilled water, 100.0 ml; tannic acid, 5.0 g; FeSO₄ (saturated solution), 1.5 ml; formaldehyde, 2.0 ml; and 1% NaOH, 1.5 ml.

The slides were covered next with reagent II until a brown color appeared and were then washed with distilled water and air-dried. Reagent II contained: AgNO₃, 5.0 g; H₂O, 100.0 ml; and NH₄OH (several drops added to 10 ml of AgNO₃ solution; when precipitate clears add to 90 ml of remaining solution).

**Pathogenicity.** Cultures were grown in Nutrient Broth for 18 hr at 35°C, harvested, washed three times in 0.1% phosphate-buffered saline (pH 7.0), and then adjusted to an absorbance of 0.50 at 650 nm (5.0 × 10⁶ cells/ml) with a Bausch & Lomb Spectronic-20 colorimeter. The live-cell suspension (0.5 ml) was injected intraperitoneally into 3-week-old mice. A second group of mice received the cultures through oral feeding. Controls were inoculated with sterile buffered saline intraperitoneally, and the second group was fed sterile Nutrient Broth.

**Hemolytic properties.** Cultures were grown on agar containing Blood Agar Base and 5% sterile

### TABLE 2. Characteristics of 16 cultures which mimic E. coli on Violet Red Bile Agar

| Characteristic                        | Cultures |
|--------------------------------------|----------|
|                                      | 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 |
| Gram stain                           | - - - - - - - - - - - - - - |
| Number of polar flagella             | 1 1 1 1 1 1 4 4 1 1 1 1 1 1 1 |
| Indole                               | - - - - - - - - - - - - - - |
| Methyl red test                      | + + + + + + + + + + + + + + + |
| Acetyl-methyl-carbinol               | - - - - - - - - - - - - - - |
| Citrate                              | - - - - - - - - - - - - - - |
| Starch hydrolysis                    | + + + + + + + + + + + + + + |
| Nitrate reduction                    | + + + + + + + + + + + + + + |
| H₂S                                  | - - - - - - - - - - - - - - |
| Urease                               | - - - - - - - - - - - - - - |
| Growth at pH 9                       | + + + + + + + + + + + + + + |
| Gelatin liquefaction                 | + + + + + + + + + + + + + + |
| Cytochrome oxidase                   | + + + + + + + + + + + + + + |
| 4% NaCl tolerance                    | + + + + + + + + + + + + + + |
| 6% NaCl tolerance                    | - - - - - - - - - - - - - - |
| Pigment                              | - - - - - - - - - - - - - - |
| Sensitive to 0/129                    | - - - - - - - - - - - - - - |
| Hemolytic (alpha)                    | + + + + + + + + + + + + + + |
| Growth at 0°C                        | - - - - - - - - - - - - - - |
| Growth at 20°C                       | + + + + + + + + + + + + + + |
| Growth at 40°C                       | - - - - - - - - - - - - - - |
| Eosin Methylene Blue Agar.           | - - - - - - - - - - - - - - |
| Brilliant Green Broth                | - - - - - - - - - - - - - - |
| Anaerobic growth                     | + + + + + + + + + + + + + + |
| Litmus milk                          | P P P P P P A A P P P P P P |
| Acid production from                 |                      |
| Glucose                              | + + + + + + + + + + + + + + |
| Arabinose                            | + + + + + + + + + + + + + + |
| Mannose                              | + + + + + + + + + + + + + + |
| Sucrose                              | + + + + + + + + + + + + + + |
| Lactose                              | + + + + + + + + + + + + + + |
| Salicin                              | - - - - - - - - - - - - - - |
| Trehalose                            | + + + + + + + + + + + + + + |
| Esculin                               | - - - - - - - - - - - - - - |
| Maltose                              | + + + + + + + + + + + + + + |
| Mannitol                             | + + + + + + + + + + + + + + |
| Cholera red test                     | - - - - - - - - - - - - - - |

*a* P, peptonized; –, negative reaction; +, positive reaction; A, acidic reaction.
defibrinated horse blood. Plates were examined after 4 days at 35 C.

Salt tolerance. Cultures were inoculated into Nutrient Broth containing 0, 0.5, 1, 2, 4, 6, and 8% NaCl and incubated at 2, 20, 25, and 35 C. After 5 days of incubation, the optical density at 660 nm was determined with a Bausch & Lomb Spectronic-20 colorimeter.

RESULTS AND DISCUSSION

None of the 40 cultures produced gas in Brilliant Green Lactose Bile Broth and all were unable to produce other than faint growth on Levine Eosine Methylene Blue Agar after incubation for 4 days at 35 C. These results indicated the necessity of applying such confirmatory media after isolation of coliforms from VRB Agar, and the erroneous conclusions are incurred if only the IMViC tests are applied after isolation. From the results (Table 1), none of the isolates was a coliform. The 16 cultures (Table 1) with IMViC reactions of -- + -- representing the predominant forms were subjected to further biochemical and morphological characterization (Tables 2 and 3; Fig. 1–3).

Biochemical results (Table 2) showed that the 16 isolates were able to reduce nitrate to nitrite and ferment glucose, arabinose, mannose, sucrose, lactose, trehalose, maltose, and mannitol without gas in the Hugh and Leifson test. Gelatin was liquefied, and cytochrome oxidase was present. Citrate was not used as a sole source of carbon, nor were H$_2$S, urease, or indole produced. Litmus milk was peptonized, and starch was hydrolyzed. The ability of these organisms to grow at a pH of 9.0 is thought to be of considerable taxonomic significance.

The results of salt tolerance studies are shown in Table 3. Maximum optical densities in Nutrient Broth were obtained in the presence of 2% NaCl at 25 C. When incubation temperatures were lowered to 2 C or raised to 35 C, the maximal optical densities were obtained with 1% NaCl. The highest salt concentration tolerated was 4%, with growth occurring in the absence of NaCl.

Morphological studies indicated that the isolates were gram-negative rods, polarly flagellated and pleomorphic. Vibrio forms, spheroplasts, swollen cells, and elongated filaments were observed when the concentration of NaCl exceeded 1% (Fig. 2 and 3).

It is apparent from these results that our isolates can justifiably be classified as vibrios. The isolates in question resemble V. parahaemolyticus, V. proteus, V. ichthyodermis, and V. anguillarum. V. parahaemolyticus was ruled out since the organisms under study failed to produce indole, grow in 7% NaCl, grow above 40 C, or be pathogenic to mice. A positive methyl red test and insensitivity to 0/129 provided further evidence that the isolated vibrios were not V. parahaemolyticus. Because of cytochrome oxidase production,
a positive methyl red test, and the inability to produce indole or H₂S, the organisms could not be classified as *V. proteus*. Strong similarities exist between these pen slime organisms and *V. ichthyodermis*. They differ however, in the following reactions: methyl red test, indole production, and the fermentation of lactose and arabinose, which distinguished these isolates from *V. anguillarum*. One culture, no. 6 (Table 2), was fed and injected intraperitoneally to mice and
failed to yield any infection. All cultures, however, were able to produce large zones of alpha hemolysis on blood-agar plates (Fig. 1).

Davis and Park (6) suggest placing those organisms that meet the following criteria in the genus *Vibrio*: formation of spheroplasts, pleomorphic forms, the presence of a single polar flagellum, growth at pH 9.0, sensitivity to the vibriostatic
agent 0/129, fermentative in the Hugh and Leifson test, no fluorescence under ultraviolet light, and no gas from glucose. Susceptibility to 0/129 and the reaction in the cholera red test were listed as variable. The preceding characteristics were noted by Sakazaki, Iwanami, and Fukumi (19) in their study on *V. parahaemolyticus*. The IAMS Subcommittee on Taxonomy of Vibrios in 1966 (17) suggested as a provisional description for the genus *Vibrio*: "Gram-negative asporogenous rods, with single rigid, curved or straight rods; single polar flagellum; oxidase positive; produce acidity, without gas, from glucose.

Similarities exist between the isolated vibrio species, but at this time the only conclusion that may be drawn is that the organisms are members of the genus *Vibrio* and are present in the slime associated with the storage pens of commercial trawlers. From the results above, it is obvious that erroneous conclusions are possible when using VRB Agar as a direct enumeration medium for detecting coliforms and *E. coli* on fishing vessels when confirmed only with the IMVIC tests.

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**LITERATURE CITED**

1. American Public Health Association. 1967. Standard methods for the examination of dairy products, 12th ed. Amer. Pub. Health Ass., Inc.
2. American Public Health Association. 1965. Standard methods for the examination of water and wastewater, 12th Ed. Amer. Pub. Health Ass., Inc., New York.
3. Barber, F. W. and H. Fram. 1955. The problem of false coliform counts on fruit ice cream. J. Milk Food Technol. 18:88-90.
4. Beam, W. W. 1959. Effect of excess nitrate on tests for indole and the cholera red reactions. J. Bacteriol. 77: 328-330.
5. Canale-Parola, E., and Z. J. Ordal. 1957. A survey of the bacteriological quality of frozen poultry pies. Food Technol. 11:578-582.
6. Davis, G. H. F., and R. W. A. Park. 1962. A taxonomic study of certain bacteria currently classified as *Vibrio* species. J. Gen. Microbiol. 27:101-119.
7. Fanelli, M. J., and J. C. Ayres. 1959. Methods of detection and effect of freezing on the microflora of chicken pies. Food Technol. 12:294-300.
8. Hartman, P. A. 1958. The selectivity of autoclave-sterilized violet red bile agar. Food Res. 23:532-535.
9. Hartman, P. A. 1960. Further studies on the selectivity of violet red bile agar. J. Milk Food Technol. 23:45-48.
10. Hugh, R., and E. Leifson. 1953. The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various Gram negative bacteria. J. Bacteriol. 66:24-26.
11. Jones, G. A., D. L. Gibson, and K. J. Cheng. 1966. Characterization of bacteria which produce colonies atypical of the coliform group on violet red bile agar. J. Milk Food Technol. 29:316-318.
12. Kachikian, R., C. R. Fellers, and W. Litsky. 1959. A bacterial survey of frozen breaded shrimp. J. Milk Food Technol. 22:310-312.
13. Koburger, J. A. 1964. Isolation of *Mima polymorpha* from dairy products. J. Dairy Sci. 47:666.
14. Kovacs, N. 1956. Identification of *Pseudomonas pyocyanea* by the oxidase reaction. Nature (London) 178:703.
15. Litsky, W., I. S. Fagerson, and C. R. Fellers. 1957. A bacteriological survey of commercially frozen beef, poultry, and tuna pies. J. Milk Food Technol. 20:216-219.
16. Machala, W. E. 1961. A bacteriological investigation of frozen foods in the Oklahoma area. J. Milk Food Technol. 24:323-327.
17. Minutes of IAMS Subcommittee on Taxonomy of Vibrios. 1966. Int. J. Syst. Bacteriol. 16:135-142.
18. Ross, A. D., and F. S. Thatcher. 1957. Bacteriological content of market precooked frozen foods in relation to public health. Food Technol. 12:369-371.
19. Sakazaki, R., S. Iwanami, and H. Fukumi. 1963. Studies on the enteropathogenic, facultatively halophilic bacteria, *Vibrio parahaemolyticus*. Jap. J. Med. Sci. Biol. 16:161-188.
20. Society of American Bacteriologists. 1957. Manual of microbiological methods. McGraw-Hill Book Co., Inc., New York.