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 noncoliforms (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 $10^5$ 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, salicine, 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.

**TABLE 1. IMViC reactions and growth in confirmatory media of 40 coliform-like colonies on Violet Red Bile Agar plates**

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

by the method of Beam (4). The indole, methyl red, Voges-Proskauer, and citrate (IMViC) tests were performed by the standard methods of the American Public Health Association (2). The production of H₂S was determined from growth in tubes of Peptone Iron Agar. Nitrate reduction was assessed by the method in the Manual of Microbiological Methods (20). The method of Kovacs was used to determine the presence of cytochrome oxidase (14). Sensitivity to the vibriostatic agent 0/129 was tested by using...
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

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**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     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 Bile Broth           | -     -     -     -     -     -     -     -     -     -     -     -     -     -     -     - |
| Anaerobic growth                     | +     +     +     +     +     +     +     +     +     +     +     +     +     +     +     + |
| Litmus milk                          | P     P     P     P     P     P     A     P     P     P     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 H₂S, 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. para-haemolyticus, V. proteus, V. ichthyodermis, and V. anguillarum. V. para-haemolyticus 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. para-haemolyticus. Because of cytochrome oxidase production,

![Fig. 1. Typical vibrio culture from storage pen slime on blood-agar plate. Note zone of alpha hemolysis at center of plate distant from area of greatest growth density.](http://aem.asm.org/)
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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