

A Medium for Presumptive Identification of *Vibrio anguillarum*

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A medium (VAM) for differentiation of *Vibrio anguillarum* is described. The presence of bile salts, the high pH, and the high NaCl concentration select mainly for *Vibrio* species. The high salinity and the ampicillin select for a fraction of *Vibrio* species, and sorbitol fermentation differentiates among those vibrios still able to grow. One hundred ninety-seven of 227 strains of *V. anguillarum* were identified with this medium. Only 3 of 66 strains of *Vibrio* that were not *V. anguillarum* or *V. anguillarum*-like were recognized with this medium, and any of 7 non-*Vibrio* strains related to fish diseases or *Escherichia coli* grew on the medium. It is our contention that the medium described here constitutes an efficient instrument for presumptive detection of *V. anguillarum* in pathological and environmental samples.

Vibrio anguillarum is a gram-negative, facultatively anaerobic rod, which is the main causative agent of vibriosis in fish. This disease produces high mortality in marine aquaculture and consequent economic losses.

The characterization of *V. anguillarum* by biochemical tests usually takes longer than the time available for identification when outbreaks of vibriosis occur in fish populations and must be quickly brought under control to avoid mortalities (4, 6). The serological techniques using polyclonal antibodies can give false-positive results because of cross-reaction among the members of the genus *Vibrio*, leading to erroneous classification (3, 7). Any procedure that is faster and more specific is desirable to improve the identification of *V. anguillarum* in environmental samples. The development of highly specific molecular techniques (2, 11, 13) or selective media may be an alternative to the present methods. Available *Vibrio*-selective media allow isolation of the *Vibrio* genus (1, 5, 8, 12, 15). However, marine samples present a great diversity of *Vibrio* species and the selective media available do not provide sufficient differentiation when the studies are focused on a particular species if it is present in small numbers or is at a metabolic disadvantage (5). On the other hand, some selective and differential agar media have been developed for certain *Vibrio* spp., providing successful isolation of those species: *Vibrio parahaemolyticus*, *Vibrio alginolyticus* (9), and *Vibrio cholerae* (14). A differential plating medium would be very useful for the isolation and differentiation of *V. anguillarum*.

We have developed a medium (VAM) for presumptive identification of *V. anguillarum* strains. It can be used either for pathological or for environmental studies, replacing any *Vibrio*-selective medium when the isolation of *V. anguillarum* is sought, or for the preliminary identification of isolates obtained in any rich medium: marine agar (MA) and Trypticase soy agar (TSA) complemented with 1.5% NaCl (TSA2), etc. After assay of the different compositions and growth conditions, the selected medium has the following composition: sorbitol, 15 g; yeast extract, 4 g; bile salts, 5 g; NaCl, 35 g; ampicillin, 10 mg; cresol red, 40 mg; bromothymol blue, 40 mg; agar, 15 g; and distilled water, 1,000 ml. All the ingredients with the exception of ampicillin were dissolved by agitation and boiling. After the medium was cooled to 50°C, the pH was adjusted to 8.6 (± 0.2) with 5 M NaOH, before the addition of

ampicillin. This medium does not need to be autoclaved. The VAM was poured into petri dishes. These must be stored lid down at 15°C. The viability of VAM expires in around 3 weeks as the ampicillin loses its activity. The uninoculated VAM is violet-blue. The identification of *V. anguillarum* on VAM is based on the color change of the colonies because of the fermentation of sorbitol. *V. anguillarum* colonies are bright yellow, with a yellow diffusion halo.

Thirty collection strains were used to evaluate the VAM. Ten of them were *V. anguillarum* strains (Table 1). Moreover, 271 recent environmental *Vibrio* isolates from different sources were tested. They were biochemically classified. Moreover, *V. anguillarum* isolates were serotyped. The inocula were prepared from culture grown overnight on TSA2 or MA. After suspension in marine phosphate-buffered saline (PBS) (PBS complemented with 1.5% NaCl) and standardized to a McFarland solution standard of 2, the strains tested were plated on VAM. Inoculated plates were incubated at 23°C ($\pm 2^\circ\text{C}$) and examined after 24, 48, and 72 h of incubation.

Detection of *V. anguillarum* in mixed cultures was examined by mixing equal proportions of previously calibrated pure suspensions of *V. alginolyticus* and *V. anguillarum*. Three media were inoculated with 100 μl of the mixed suspension: TSA2, thiosulfate-citrate-bile-sucrose (TCBS), and VAM. Plates were incubated at 23°C ($\pm 2^\circ\text{C}$) and examined at 24, 48, and 72 h.

To determine the efficiency of recovery of CFU, counts of *V. anguillarum* on VAM were also compared with counts on MA and on TCBS. To this end, a set of 10-fold dilutions of a standardized suspension of *V. anguillarum* was prepared in marine PBS. Duplicate plates of each medium were inoculated with 100 μl of each dilution. The CFU were counted after 48 h of incubation at 23°C ($\pm 2^\circ\text{C}$).

VAM was also evaluated for an experimental vibriosis in red sea bream (*Sparus aurata*). Kidney and intestine samples were taken from infected and control fish. Water samples from the tanks were also taken. TSA1 (TSA complemented with 0.5% NaCl), TCBS, and VAM plates were incubated at 23°C ($\pm 2^\circ\text{C}$) and were examined at 24, 48, and 72 h after inoculation. The identity of *V. anguillarum* differentiated on VAM plates was confirmed biochemically and with a specific 16S rDNA probe by colony hybridization (11) (data not shown).

A high percentage of *V. anguillarum* strains (86.78% [197 of 227]) produced bright yellow, round, flat colonies with a yellow halo after 48 h of incubation. Some *V. anguillarum* strains needed 72 h. Most other isolates did not grow on VAM. Among those that grew, most produced round, flat, blue

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TABLE 1. Collection strains used in this study

Species	Strain(s) ^a
<i>Aeromonas hydrophila</i>	CECT 398
<i>Aeromonas salmonicida</i>	ATCC 27013
<i>Aeromonas sobria</i>	CECT 837
<i>Escherichia coli</i>	ATCC 11303
<i>Plesiomonas shigelloides</i>	CECT 597
<i>Pseudomonas aeruginosa</i>	ATCC 15442
<i>Vibrio alginolyticus</i>	ATCC 17749
<i>Vibrio anguillarum</i>	ATCC 43305, ATCC 43306, ATCC 43307, ATCC 43308, ATCC 43309, ATCC 43310, ATCC 43311, ATCC 43312, ATCC 43313, ATCC 43314
<i>Vibrio campbellii</i>	ATCC 25920
<i>Vibrio costicola</i>	CCM 2811
<i>Vibrio damsela</i>	ATCC 33539
<i>Vibrio fluvialis</i>	ATCC 33812
<i>Vibrio harveyi</i>	ATCC 14126
<i>Vibrio ordalii</i>	ATCC 33509
<i>Vibrio parahaemolyticus</i>	ATCC 17802
<i>Vibrio pelagius</i>	ATCC 25916
<i>Vibrio proteolyticus</i>	ATCC 15338
<i>Vibrio splendidus</i>	ATCC 33125
<i>Vibrio vulnificus</i>	ATCC 3147
<i>Yersinia ruckeri</i>	CECT 955
<i>Pasteurella piscicida</i>	CNEVA 10831*

^a ATCC, American Type Culture Collection; CECT, Colección Española de Cultivos Tipo; CCM, Czechoslovak Collection of Microorganisms; CNEVA, Centre National d'Etudes Vétérinaires et Alimentaires. *, this strain was kindly provided by F. Baudin-Laurencin.

colonies. Eleven of 66 environmental and collection *Vibrio* strains other than *V. anguillarum* grew on VAM, producing yellow colonies. These isolates were characterized as follows: eight as *V. anguillarum*-like, one as *Vibrio fluvialis*, one as *Vibrio harveyi*, and one as a nondetermined *Vibrio* sp. None of eight non-*Vibrio* strains grew on VAM.

In the experiment with mixed cultures, the differentiation of *V. anguillarum* was much clearer and more consistent on VAM than on TCBS. *V. anguillarum* was easily differentiated from *V. alginolyticus*, which produced round blue colonies without the usual swarming that this species presents in other media.

The colony counts of *V. anguillarum* on the three media presented certain differences. The viable total colony counts per milliliter for MA, VAM, and TCBS were 2.1×10^8 , 4.8×10^7 , and 1.53×10^7 , respectively. The counts for each medium are the averages from 28 experiments performed with 28 different strains. MA yielded higher counts than TCBS or VAM, thus showing an effect of the selective medium on stressed bacteria. VAM allowed slightly greater recovery rates than TCBS.

VAM plates inoculated with either kidney or intestine samples from infected fish or the water of their tank presented yellow colonies which were confirmed as *V. anguillarum*. A single colonial morphology, corresponding to that of *V. anguillarum*, developed on VAM plates inoculated with kidney samples from infected fish. Several different colonies were produced on VAM from intestine and water samples, but *V. anguillarum* colonies were easily distinguished, since they were the only ones to become yellow. This differentiation was not possible with TCBS. No *V. anguillarum* was detected on VAM

plates inoculated with kidney, intestine, or water samples from noninfected fish. However many colonies with the characteristic aspect of *Vibrio* species were detected in plates of TCBS inoculated with intestine or water samples.

The use of sorbitol as the main carbon source, together with ampicillin in a basal medium with inhibitors for bacteria other than *Vibrio* species allows a clear differentiation of *Vibrio* species which ferment sorbitol and present resistance to ampicillin. Most *Vibrio* strains with those characteristics belong to *V. anguillarum*. Taxonomical studies (6) reported 77% of strains of *V. anguillarum* able to ferment sorbitol and to present resistance to ampicillin. These capabilities are not frequent among bacteria belonging to the families *Vibrionaceae* and *Aeromonadaceae*. However, low percentages of isolates of a few species of those families have been reported (6) to ferment sorbitol and to present resistance to ampicillin: i.e., *V. fluvialis* (20%), *V. harveyi* (4%), *Vibrio metschnikovii* (10%), *Aeromonas hydrophila*. (37%), *Aeromonas caviae* (5%), and *Aeromonas sobria* (2%). Only some strains of the three *Vibrio* species mentioned above can grow on VAM, because of the high concentrations of NaCl, the high alkalinity, and the presence of bile salts in the medium. It is important to emphasize that in environmental studies some *Vibrio* species other than *V. anguillarum*, especially *V. fluvialis*, *V. harveyi*, and *V. metschnikovii*, can grow on VAM, presenting yellow round colonies. That accounts for the presumptive nature of the VAM. In such cases, the presence of *V. anguillarum* will be confirmed by a few biochemical tests: arginine dihydrolase, lysine decarboxylase, *o*-nitrophenyl- β -D-galactoside, Voges-Proskauer, and oxidase. If the composition of VAM is used to make an enrichment broth for *V. anguillarum*, less restrictive conditions, such as lower salinity and pH (2% and 7 to 8, respectively) may be used, according to the physiological studies for this species (10).

In summary, VAM allows the partial selection and further presumptive differentiation of *V. anguillarum* among other vibrios much more easily than the methods available so far. In addition, *V. anguillarum* grows slightly better on VAM than on TCBS. Therefore, VAM allows a better differentiation when used as a primary isolation medium for this species with samples containing a large number of other vibrios. It provides simple, differential, and reliable detection of *V. anguillarum* and is of great value in direct plating of water or other kinds of samples from the marine environment and in preliminary identification of bacterial isolates.

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REFERENCES

1. Akiyama, S., K. Takizawa, H. Ichinoe, S. Enomoto, T. Kobayashi, and R. Sakazaki. 1963. Application of teepol to isolation of *Vibrio parahaemolyticus*. Jpn. J. Bacteriol. 18:255-256.
2. Aoki, T., I. Hirono, T. de Castro, and T. Kitao. 1989. Rapid identification of *Vibrio anguillarum* by colony hybridization. J. Appl. Ichthyol. 5:67-73.
3. Aoki, T., T. Kitao, T. Itabashi, Y. Wada, and M. Sakai. 1981. Proteins and lipopolysaccharides in the membrane of *Vibrio anguillarum*. Dev. Biol. Stand. 49:225-232.
4. Austin, B., and D. A. Austin. 1987. Vibrios, p. 263-296. In B. Austin and D. A. Austin (ed.), Bacterial fish pathogens disease in farmed and wild fish. Ellis Horwood Limited, Chichester, United Kingdom.
5. Bolinches, J., J. L. Romalde, and A. E. Toranzo. 1988. Evaluation of selective media for isolation and enumeration of vibrios from estuarine waters. J. Microbiol. Methods 8:151-160.
6. Bryant, T. N., J. V. Lee, P. A. West, and R. R. Colwell. 1986. Numerical classification of species of *Vibrio* and related genera. J.

- Appl. Bacteriol. **61**:437-467.
7. **Chart, H., and T. J. Trust.** 1984. Characterization of the surface antigens of the marine fish pathogens *Vibrio anguillarum* and *Vibrio ordalii*. *Can. J. Microbiol.* **30**:703-710.
 8. **Kobayasi, T., S. Enomoto, R. Sakazaki, and S. Kuwahara.** 1963. A new selective medium for pathogenic vibrios, TCBS agar (modified Nakanishi's agar). *Nippon Saikingaku Zasshi* **18**:387-391.
 9. **Kourany, M.** 1983. Medium for isolation and differentiation of *Vibrio parahaemolyticus* and *Vibrio alginolyticus*. *Appl. Environ. Microbiol.* **45**:310-312.
 10. **Larsen, S. L.** 1984. *Vibrio anguillarum*: influence of temperature, pH, NaCl concentration and incubation time on growth. *J. Appl. Bacteriol.* **57**:237-246.
 11. **Martínez-Picado, J., A. R. Blanch, and J. Jofre.** 1994. Rapid detection and identification of *Vibrio anguillarum* by using a specific oligonucleotide probe complementary to 16S rRNA. *Appl. Environ. Microbiol.* **60**:732-737.
 12. **O'Brien, M., and R. Colwell.** 1985. Modified taurocholate-tellurite-gelatin agar for improved differentiation of *Vibrio* species. *J. Clin. Microbiol.* **22**:1011-1013.
 13. **Rehnstam, A.-S., A. Norqvist, H. Wolf-Watz, and Å. Hagström.** 1989. Identification of *Vibrio anguillarum* in fish by using partial 16S rRNA sequences and a specific 16S rRNA oligonucleotide probe. *Appl. Environ. Microbiol.* **55**:1907-1910.
 14. **Shimada, T., R. Sakazaki, S. Fujimura, K. Niwano, M. Mishina, and K. Takizawa.** 1990. A new selective, differential, agar medium for isolation of *Vibrio cholerae* O1; PMT (polymyxin-mannose-tellurite) agar. *Jpn. J. Med. Sci. Biol.* **43**:37-41.
 15. **Simidu, U., and K. Tsukamoto.** 1980. A method of the selective isolation and enumeration of marine Vibrionaceae. *Microb. Ecol.* **6**:181-184.