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NOTE

Nonselective nature of Coomassie Brilliant Blue agar for the presumptive identification of *Aeromonas salmonicida* in clinical specimens

Jeffrey D. Teska, Rocco C. Cipriano

U.S. Fish and Wildlife Service, National Fish Health Research Laboratory, Box 700, Kearneysville, West Virginia 25430, USA

ABSTRACT: To demonstrate the nonselective and nondifferential nature of Coomassie Brilliant Blue (CBB) agar for the presumptive diagnosis of furunculosis, bacteria isolated from kidney and mucus samples of lake trout *Salvelinus namaycush* and Atlantic salmon *Salmo salar* were studied. Three populations of salmonids with enzootic furunculosis were screened for *Aeromonas salmonicida*. All blue '*A. salmonicida*-like' colonies appearing on CBB agar were subcultured and identified by biochemical characterization using standard microbiological methods. Bacteria belonging to one of 4 groups were cultured as blue colonies on CBB agar, including non-Enterobacteriaceae glucose fermenters (3 species), pseudomonads (9 species), nonpseudomonad glucose nonfermenters (6 species), and Gram-positive organisms (3 species). Results indicated that the screening of colonies as potential *A. salmonicida* can be accelerated using CBB agar, but the medium alone cannot differentiate nor select for *A. salmonicida*. It is recommended that when CBB agar is used as a primary plating medium, all blue colonies should be subcultured and characterized minimally with triple sugar iron (TSI) agar, cytochrome oxidase, ornithine decarboxylase, motility, and gelatin before an identification is made. Presumptive identification of *A. salmonicida* using colony color on CBB agar alone was not validated by this study.

Many bacterial fish pathogens can be readily cultured from clinical specimens on routine plating media such as tryptic soy agar (TSA) or brain heart infusion agar (BHIA). These media are nonselective and nondifferential because they support growth of many bacteria. A pathogen routinely cultured on these media is *Aeromonas salmonicida*, the cause of furunculosis in salmonids. Following primary isolation of this pathogen from clinical specimens, individual colonies must be subcultured onto additional media to ensure bacterial purity, and then further characterized by standard microbiological tests to obtain a confirmatory diagnosis. Serodiagnostic assays are often performed to shorten the time required for a presumptive diagnosis; however, the serodiagnostic approach does not provide a definitive identification.

Virulent isolates of *Aeromonas salmonicida* contain a protein coat exterior to the cell membrane called the A-layer. This protein layer is associated with virulence and accounts for the autoaggregation of *A. salmonicida* cells. When Coomassie Brilliant Blue (CBB), a protein-specific dye, is incorporated into TSA at a concentration of 0.01 %, the dye binds to the A-layer of *A. salmonicida*. As a result, colonies from A-layer-positive phenotypes appear blue when grown on CBB agar whereas A-layer-negative phenotypes appear white. This agar has provided a powerful laboratory method for screening for the A-layer (Udey 1982) and for selecting isolates of *A. salmonicida* that are likely to be virulent (Cipriano & Bertolini 1988). The medium is also used as a primary plating medium for detection of *A. salmonicida* in salmonid populations (Cipriano et al. 1992), and Markwardt et al. (1989) have suggested that the visualization of blue colonies from clinical specimens can constitute a presumptive diagnosis for furunculosis. When CBB agar is used in this manner, however, there is potential for misidentification. This study was therefore carried out to correct any mistaken impressions that the Markwardt et al. study may have given rise to about the selective and differential properties of the medium. CBB agar is currently being employed for the purpose of primary isolation of *A. salmonicida* by several State and Federal laboratories in the Great Lakes and Northeast regions of the United States. This study was conducted to demonstrate the nonselective nature of CBB agar when used as a primary isolation medium and to demonstrate that it cannot be relied on for consistently valid presumptive diagnoses for *A. salmonicida*.

Materials and methods. ***Sample populations:*** Three populations of hatchery-reared salmonids in which *Aeromonas salmonicida* was enzootic were sampled. Samples were taken from 2 populations of lake trout *Salvelinus namaycush* at a state fish hatchery (Rome, New York, USA) and 1 population of Atlantic salmon *Salmo salar* smolts at the White River National Fish Hatchery (Bethel, Vermont, USA). Mucus and kidney samples of 100 fish from each population were cultured on CBB agar [i.e. tryptic soy agar (TSA; Difco Laboratories, Detroit, MI, USA) containing 0.1 % Coomassie Brilliant Blue R250 (Bio-Rad Laboratories, Richmond, CA, USA)]. Homogenized and diluted samples were plated onto CBB agar according to the procedures of Cipriano et al. (1992). Culture plates were incubated at ambient temperature (about 25 °C) for 48 h before subculturing blue colonies on TSA for further biochemical characterization.

Biochemical characterization: A biochemical profile was obtained for each isolate. It consisted of reactions on with the following: triple sugar iron (TSI) agar; Gram and flagella stains; cytochrome oxidase; motility; citrate (Simmons); DNase; pigment production of TSA, Flo agar, and Tech agar; gelatin liquefaction; indole; methyl red; Voges-Proskauer; arginine dihydrolase (Moeller); lysine decarboxylase (Moeller); ornithine decarboxylase (Moeller); nitrate reduction; O/F glucose; phenylalanine diaminase; urea; growth on MacConkey, SS, and cetrimide agars; and acid production (1 % carbohydrate in OF basal medium) from adonitol, arabinose, arabitol, cellibiose, dulcitol, erythritol, fructose, galactose, glucose, inositol, lactose, levulose, maltose, mannitol, mannose, melibiose, raffinose, rhamnose, salicin, sorbose, sorbitol, sucrose, trehalose, and xylose.

All media were prepared according to the manufacturer's instructions and incubated at 25 °C, and results were recorded according to standard methods (Koneman et al. 1983). Substrate utilization tests (i.e. carbohydrates and amino acids) were examined 1, 2, 3, 7, and 10 d postinoculation. Terminal end-product tests (i.e. methyl red, phenylalanine, nitrate) were evaluated on Day 10 postinoculation.

Identification of isolates to the genus and species level was done with the aid of 3 identification schemes: members of the family Enterobacteriaceae were identified according to Farmer et al. (1985); Gram-positive rods and cocci were identified according to Hollis & Weaver (1981), and nonfermenters and unusual Gram-negative fermenters were identified according to King (1975).

Results. Bacteria appearing on CBB agar after 48 h ranged in colonial size from pinpoint to several millimeters in diameter. Colony coloration ranged from clear translucent or opaque white to various shades of blue. Medium to dark blue colonies were selected for further characterization (Table 1). The bacterium *Aeromonas salmonicida* was isolated only from Vermont Atlantic salmon (White River NFH) and made up 2.6 % of the bacterial isolates from these fish. The pathogen was detected in the mucus of 20 (37 %) and the kidneys of 2 (3.7 %) of the 54 Atlantic salmon sampled. No *A. salmonicida* was detected in either of the New York lake trout populations. Many non-*A. salmonicida* bacteria grew as blue colonies on CBB agar from all 3 populations. These bacteria were placed in 5 classification groupings based primarily on the Gram stain and glucose metabolism: Enterobacteriaceae, non-Enterobacteriaceae glucose fermenters, pseudo-

Table 1. Identity of isolates yielding medium to dark blue colonies on CBB agar and frequency of occurrence of these isolates in mucus and kidney samples from fish populations. LT1: NY lake trout population #1; LT2: NY lake trout population #2; AS: VT Atlantic salmon

	LT1	LT2	AS
Enterobacteriaceae	0.0	0.0	0.0
Non-Enterobacteriaceae glucose fermenters	25.6	8.8	2.6
Aeromonas hydrophila	23.3	8.8	0.0
Aeromonas salmonicida	0.0	0.0	2.6
Pasteurella multocida	2.3	0.0	0.0
Pseudomonads	62.8	75.9	65.8
Ps. cepacia	0.0	1.1	0.0
Ps. diminuta	0.0	1.1	0.0
Ps. fluorescens	41.9	3.3	57.9
Ps. mallei-like	0.0	23.1	0.0
Ps. mesophilica[a]	9.3	11.0	5.3
Ps. pseudoalcaligenes	0.0	2.2	0.0
Ps. pseudomallei	11.6	0.0	2.6
Ps. testosteroni	0.0	31.9	0.0
Ps. vesicularis	0.0	2.2	0.0
Nonpseudomonad glucose nonfermenters	9.4	7.7	18.5
Achromobacter xylosoxidans	0.0	1.1	0.0
Acinetobacter calcoaceticus lwoffi	0.0	0.0	13.2
Alcaligenes denitrificans	0.0	2.2	0.0
Alcaligenes faecalis	4.7	0.0	0.0
Flavobacterium sp. (MF-4)	0.0	4.4	0.0
Moraxella nonliquefaciens	4.7	0.0	5.3
Gram-positive	2.3	7.7	13.2
Corynebacterium aquaticum	0.0	1.1	7.9
Corynebacterium pseudodiphtheriticum	2.3	0.0	0.0
Staphylococcus lentus	0.0	6.6	5.3

[a] *P. mesophilica* is now *Methylobacterium mesophillicum*

monads, nonpseudomonad glucose nonfermenters, and Gram-positive organisms.

Species belonging to the genus *Pseudomonas* were the most prevalent organisms in the 3 fish populations examined (62.8 to 75.9 %). The second most prevalent group of bacteria was the cytochrome oxidase-positive fermenters which included 2 species of *Aeromonas* and *Pasteurella multocida*. Less consistency among the 3 populations was observed for this group of organisms compared with *Pseudomonas* species. Individual species with high prevalence (greater than 10 % of total isolates) in 1 or more populations included *Acinetobacter calcoaceticus* var. *lwoffi*, *Aeromonas hydrophila*, *Methylobacterium mesophillicum* (*Ps. mesophilica*), *Ps. fluorescens*, *Ps. mallei*-like, *Ps. pseudomallei*, and *Ps. testosteroni*. Gram-positive organisms composed a low percentage of the isolates from the 3 fish populations, ranging from 2.3 to 13.2 % per population. No representatives of the family Enterobacteriaceae were identified among the blue colonies from any of the fish populations examined.

Discussion. Our results indicate that CBB agar is nonselective and nondifferential. The medium will support the growth of a wide spectrum of Gram-negative and Gram-positive bacteria, including fermentative and nonfermentative organisms. The only indicator system in CBB agar that could make the medium differential is protein-specific staining by the Coomassie Brilliant Blue. Bacteria possessing outer protein coats will incorporate the stain and appear as blue colonies on the medium. Outer protein coats, however, are not unique to *Aeromonas salmonicida*. Many species of Gram-negative and Gram-positive bacteria possess outer protein components that will stain with Coomassie Brilliant Blue. Therefore, CBB agar can be considered a differential medium with regard to specific protein staining (i.e. blue vs white colonies), but it cannot be expected to identify bacteria with blue-staining colonies to the species level. Contrary to Markwardt et al. (1989), *A. salmonicida* was not the only bacterium that produced dark blue colonies on CBB agar. Based on our findings CBB agar is neither selective nor differential for *A. salmonicida*, and the isolation of blue colonies from clinical specimens is not a sufficient criterion to warrant a presumptive diagnosis of furunculosis.

The use of CBB agar as a primary plating medium can, however, significantly reduce the numbers of bacteria that must be screened to ensure definitive identification. Confirmatory identification can only be ensured after a blue colony is shown to minimally consist of nonmotile, Gram-negative bacilli that are cytochrome oxidase-positive and indole-negative, and that ferment glucose and liquify gelatin. The production of a brown pigment on TSA can be used as an additional criterion for presumptive identification, but certain strains of *Aeromonas salmonicida* do not produce pigment. Also, *Pseudomonas dimunita* and *Xanthomonas* (*Pseudomonas*) *maltophilia* are common water and soil microorganisms that can form blue colonies on CBB agar and produce brown pigment on TSA. Other species of *Aeromonas* (Ross 1962, Paterson 1974) and *Pseudomonas* (Ogunnariwo & Hamilton-Miller 1975, Frerichs & Holliman 1991) have also been reported to produce brown pigmentation.

A diagnostician must often be quick about arriving at a presumptive diagnosis so that effective treatments can be prescribed. Such a diagnosis should be based on a minimal number of descriptive characteristics that provide confident identification of a pathogen. Although CBB agar offers excellent advantages over routine media such as TSA or BHIA, we reiterate that the growth of blue colonies on CBB agar, in itself, does not constitute a valid presumptive identification of *Aeromonas salmonicida*.

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