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Dye-Containing Buffered Charcoal-Yeast Extract Medium for Differentiation of Members of the Family *Legionellaceae*

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The addition of 0.001% bromocresol purple and 0.001% bromothymol blue to buffered charcoal-yeast extract agar allowed differentiation between members of the family *Legionellaceae*. On this medium, *Legionella pneumophila* grew as relatively flat, pale green colonies, whereas *Tatlockia micdadei* (gen. nov., comb. nov., Pittsburgh pneumonia agent) produced blue-gray colonies. *Fluoribacter* spp. (gen. nov., atypical Legionalla-like organisms) developed glistening colonies which were brighter green than those of *L. pneumophila*.

Legionellaceae are fastidious, gram-negative organisms which require cysteine and iron for growth and do not grow on bacteriological media routinely used for the clinical identification of pathogens. *Legionella pneumophila*, *Tatlockia micdadei* (gen. nov., comb. nov., Garrity et al. [3], Pittsburgh pneumonia agent, *Legionella micdadei* [4], *Legionella pittsburghensis* [5]), and *Fluoribacter* spp. (gen. nov., Garrity et al. [3]; atypical legionella-like organisms) are unable to oxidize or ferment common carbohydrates, and are difficult to visually distinguish from each other. By adding bromocresol purple and bromothymol blue to the primary isolation medium developed by Feeley et al. (2), a successful differential agar was developed. This medium allowed the rapid identification of *L. pneumophila* and *T. micdadei* from clinical samples as well as conveniently enabled the simultaneous isolation of these two agents from an environmental sample.

MATERIALS AND METHODS

Buffered charcoal-yeast extract (CYE) agar was prepared as previously described (5), and contained 10 g of yeast extract, 0.4 g of L-cysteine HCL·$2H_2O$, 17 g of agar, 2.0 g of activated charcoal (Norite A), 0.25 g of ferric pyrophosphate, and 10 g of *N*(2-acetamido)-2 aminoethane sulfonic acid per liter of medium. The medium was adjusted with sterile 1 N KOH to yield a pH of 6.9 when solidified.

Stock solutions of bromocresol purple (Difco) and bromothymol blue (Difco Laboratories) were separately prepared by dissolving, for each, 1.0 g in 100 ml of 0.1 N KOH; these mixtures were sterilized by membrane filtration. A 1-ml portion of each dye solution was added to 1 liter of the complete sterile base medium before pouring. The medium was then dispensed into petri dishes (15 by 100 mm) at 20 ml per plate. Buffered CYE agar was also prepared without dyes to allow comparison of growth on dye-free and on dye-containing plates.

Inoculum preparation. Subcultures of isolates (1 to 2 days old), maintained on buffered CYE agar and incubated at 37°C in air, were used for inoculation of the experimental medium. Each isolate was suspended in 2.0 ml of sterile 0.85% saline, and the turbidity was adjusted to match a standard having an opacity of 1.5 IU (0.05 ml of 1% barium chloride added to 9.95 ml of 1% H_2SO_4). Twenty-three strains of *L. pneumophila* (16), *T. micdadei* (8), and *Fluoribacter* (5), were used (Table 1). Several mixtures were also prepared by combining equal volumes of saline suspensions of representative *L. pneumophila*, *T. micdadei*, and *Fluoribacter* strains.

Test procedure. All 32 isolates, as well as various mixtures of them, were streaked onto CYE agar plates, with and without the added dyes, by using a calibrated 0.001-ml platinum loop to allow the development of individual colonies. Plates were incubated at 35°C in air and examined for colony size, shape, color, and degree of growth at 3, 5, and 7 days.

RESULTS

All strains of *L. pneumophila* produced white colonies with a barely discernable green coloration on the dye-containing medium. Colonies were round and shiny at 3 days. After 5 to 7 days of incubation, the colonies grew larger and became flat, dull, and opaque; in addition, their green color became more distinct.

In contrast, *T. micdadei* yielded round, shiny, blue-gray colonies. Continued incubation resulted in an increase in the size of the colonies, as well as in the intensity of the blue color. Similar growth was observed on dye-free control plates; however, the colonies were gray-white and could not be differentiated from *L. pneumophila*.

The colonies of *Fluoribacter* were round, distinctly green, and shiny after incubation for 3 days. Continued incubation for 5 to 7 days re-

TABLE 1. *Bacterial strains used in this study*

Laboratory designation	Group classification[a]	Strain	Source[b]
LDB-1	I	Philadelphia 1 (ATCC 33152)	CDC
LDB-2	I	Bellingham	CDC
LDB-3	I	PER	CDC
LDB-4	I	Knoxville 1 (ATCC 33153)	CDC
LDB-5	I	Pontiac	CDC
LDB-7	I	Togus 1 (ATCC 33154)	CDC
LDB-8	I	Bloomington 2 (ATCC 33155)	CDC
LDB-9	I	Los Angeles 1 (ATCC 33156)	CDC
LDB-10	I	Chicago 2 (ATCC 33215)	CDC
LDB-11	I	Houston 2	CDC
LDB-12	I	COS	PUH
LDB-13	I	PCL	PUH
LDB-14	I	VAMC-WAL	VAMC
LDB-15	I	VAMC-EVA	VAMC
LDB-16	I	VAMC-L7WA	VAMC
LDB-17	I	VAMC-L7WB	VAMC
TAT	II	TATLOCK (ATCC 33218)	CDC
PPA 1	II	EK (ATCC 33204)	PUH
PPA 2	II	LR	PUH
PPA 3	II	PGH-12 (ATCC 33346)	CDC-VAMC
PPA 4	II	CRE	PUH
PPA 5	II	LAU	PUH
PPA 6	II	LAF	PUH
PPA 7	II	VAMC-MCC (ATCC 33344)	VAMC
ALLO 1	IIIA	WIGA (ATCC 33217)	PUH
ALLO 2	IIIA	MI-15	PUH
ALLO 3	IIIB	LS-13 (ATCC 33342)	PUH
ALLO 4	IIIC	NY-23 (ATCC 33279)	PUH
ALLO 5	IIIC	TEX-KL (ATCC 33343)	PUH

[a] I, *Legionella pneumophila*; II, *Tatlockia micdadei*; IIIA, *Fluoribacter bozemanae*; IIIB, *Fluoribacter gormanii*; IIIC, *Fluoribacter dumoffii*.

[b] CDC, Center for Disease Control, Atlanta, Georgia; VAMC, Veterans Administration Medical Center, Pittsburgh, Pennsylvania; PUH, A. W. Pasculle, Presbyterian-University Hospital, Pittsburgh, Pennsylvania; CDC-VAMC, Center for Disease Control and Veterans Administration Medical Center.

sulted in a more intense green color with colonies remaining shiny. In the absence of dyes, colonies could not be easily differentiated from *Legionella* or *Tatlockia*. When both dye-containing and dye-free plates were exposed to long-wave (366 μM) ultraviolet light in a darkened room, the emitted blue-white fluorescence (1) of the colonies was equal.

Several suspensions containing one strain of each of the three groups of bacteria were inoculated onto the differential CYE (dye-containing) medium to enable a side-by-side comparison. After 3 days of incubation, colonies of *Legionella* and *Fluoribacter* were similar in color (pale green) and appearance, whereas *Tatlockia* colonies were gray-blue. Continued incubation for 5 to 7 days allowed clear differentiation of the three groups by color and colonial morphology (Fig. 1). Although the degrees of growth and proportion of each isolate on dye-free control plates were equal to those on the dye-containing medium, visual differentiation of the three organisms without the dyes was difficult. Exposure of both dye-containing and dye-free plates to ultraviolet light clearly demonstrated the *Fluoribacter* colonies in the mixtures.

DISCUSSION

Currently, the direct immunofluorescence test is the primary method for the differentiation of *Legionella*, *Tatlockia*, and *Fluoribacter*. The necessary reagents are not commercially available, and only those reagents for identifying *Legionella* are readily available from the Center for Disease Control. Although cellular fatty acid analysis is useful in differentiating these organisms, it is beyond the capability of most clinical microbiology laboratories. Thus, most clinical microbiology laboratories do not have the capability of differentiating these organisms except by the previously described fluorescence of *Fluoribacter* colonies (1). Our findings show that presumptive identification can be made on the described differential medium, for which ingredients are easily obtainable. After 3 days of incubation, *Tatlockia* can be identified easily by its characteristic gray-blue colonies. The differentiation of *Legionella* and *Fluoribacter* is more difficult after 3 days of incubation, since both genera produce pale green colonies. Although there are subtle differences in degree of coloration, which, with experience, can suggest the identity of the colony, fluorescence under ultraviolet light can be used at this time to confirm this identification. With further incubation (5 to 7 days), these two genera are easily differentiated because of their marked difference in green coloration and colonial morphology.

To the inexperienced observer, organisms which may resemble *Legionella* or *Fluoribacter* on this medium include those faintly chromogenic environmental organisms such as some isolates of *Xanthomonas* or *Flavobacterium* spp. which we tested. When using this medium, we recommend that the following criteria be used for the presumptive identification of *Legionellaceae*: (i) suspicious colonies must not only have the characteristic color but must also have the characteristic colony size and morphology (colonies should also be examined under a dissecting microscope with illumination from

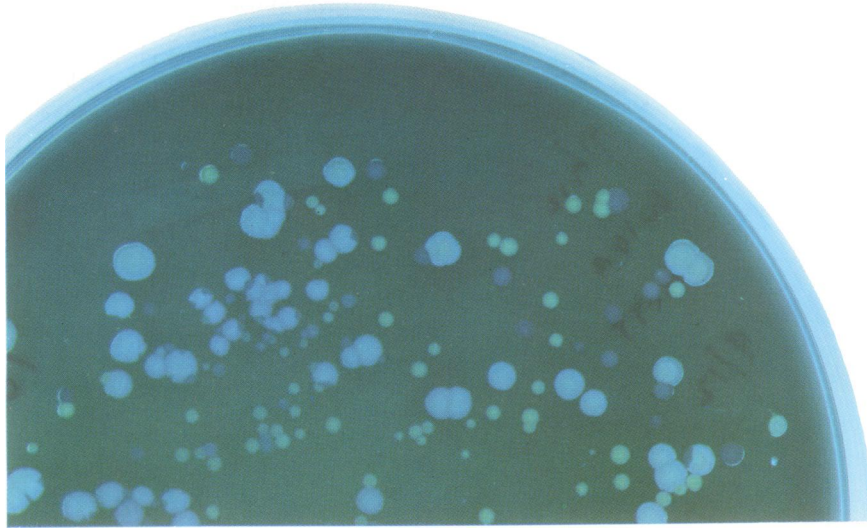

FIG. 1. *Mixture of Legionellaceae on the dye-containing CYE medium. A mixture of L. pneumophila, T. micdadei, and Fluoribacter bozemanae was streaked onto a plate of differential CYE agar with a 0.001-ml platinum loop. After 5 days of incubation at 37°C in air, L. pneumophila produced large (≈2.5-mm) flat colonies that were white to pale green in color. Fluoribacter species grew as small (≈1.2- to 1.5-mm) colonies that were characteristically convex, shiny, and distinctly green. T. micdadei produced small (≈1.0- to 1.5-mm) round, shiny blue-gray colonies. Note: there is a slightly bluish cast to the figure, a result of the printing process; the colors are therefore not a totally accurate representation of the actual colors of the colonies.*

above) (3), (ii) the isolate should not grow on sheep blood agar, and (iii) the isolate should yield the characteristic cell morphology when Gram stained (3). Isolates fulfilling these criteria should be subjected to serotyping, cellular fatty acid analysis, or DNA homology determination for definitive confirmation.

Differential CYE medium has been used successfully for the isolation and presumptive identification of *Legionella* and *Tatlockia* from a limited number of clinical and environmental specimens (unpublished data). This identification was subsequently confirmed serologically and by DNA hybridization.

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