

## Methyl Violet: a Selective Agent for Differentiation of *Klebsiella pneumoniae* from *Enterobacter aerogenes* and Other Gram-Negative Organisms

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Three selective media for differentiation of *Klebsiella pneumoniae* from *Enterobacter aerogenes* on the basis of colonial morphology were evaluated. Using methyl violet 2B as a selective agent, strains of *K. pneumoniae* isolated from urine, fresh water, and fresh produce were tested against other members of *Enterobacteriaceae* in addition to strains of *Aeromonas hydrophila* and *Pseudomonas aeruginosa*. Comparison of colonial morphology showed *K. pneumoniae* produced larger, smoother colonies than other bacteria tested. These media were developed to aid in presumptive separation of *K. pneumoniae* from *E. aerogenes* in the monitoring of bacterial quality of water.

The importance of *Klebsiella pneumoniae* as a frequent contaminant of water supplies has been discussed by Bordner and Carroll (2). Virulent *Klebsiella* isolates have been reported in high numbers from natural receiving waters (12), an uninhabited watershed, paper and pulpmill effluent, fresh produce (5), sugar cane (13), and textile finishing plant effluent (A. P. Dufour, V. J. Cabelli, and M. A. Levin, Abstr. Annu. Meet. Am. Soc. Microbiol., 1973, E16, p. 3). The organism is also a frequent inhabitant of the intestines of animals and man (3, 12, 14).

*K. pneumoniae* isolates are frequently confused with strains of *Enterobacter aerogenes* (Table 1). Ewing (8) states that most cultures submitted to his laboratory labeled as *E. aerogenes* were actually *K. pneumoniae*. This may occur because some workers isolating a lactose-positive coliform with IMViC pattern --++ automatically identify it as *E. aerogenes*. At the present time, *K. pneumoniae* is differentiated from *E. aerogenes* on the basis of motility and ornithine decarboxylase (3, 8), and urea, arginine dihydrolase, and lysine decarboxylase are helpful in speciation.

The use of dyes in differential bacteriological media was described by Endo (7) in the isolation of coliforms. Modification of his medium and development of others containing inhibitory dyes have been important in the advancement of aquatic, food, and sanitary microbiological studies. Many of the dye-containing differential media that have been developed are used in the isolation of enterococci and mem-

bers of *Enterobacteriaceae*. Fung and Miller (9) reported that methyl violet B at a dilution of 1:1000 would inhibit growth of *Escherichia coli* while permitting growth of *Enterobacter* and *Serratia* species. Methyl violet 2B is a basic dye similar to crystal violet (4, 11) in that both have a triphenylmethane structure.

Because of the ubiquitous distribution and the pathogenicity of *Klebsiella*, workers have become increasingly aware of the potential health hazard and the need for monitoring the organism in environmental sources. Present methods for monitoring coliforms are inadequate for determining the presence of *Klebsiella*. This study was undertaken to develop solid media that select for *K. pneumoniae* and that allow for presumptive separation and differentiation from *E. aerogenes* based on colonial morphology and growth characteristics.

### MATERIALS AND METHODS

**Cultures.** Organisms were isolated from fecal specimens, fresh water, fresh produce, urine specimens, and wound exudates. Lactose-positive organisms belonging to *Enterobacteriaceae* were characterized as *E. coli*, *E. aerogenes*, or *K. pneumoniae*, using motility, indol, methyl red, Voges-Proskauer, citrate (Simmons), urea (Christensen), lysine decarboxylase, and ornithine decarboxylase. Isolates of *Aeromonas hydrophila* were from fresh water. Four cultures of *Pseudomonas aeruginosa* and 10 cultures of *Pseudomonas* spp. of aquatic origin were tested on methyl violet-containing media. The aquatic pseudomonads were all mucoid in appearance on standard plate count agar. Pure cultures of the following

TABLE 1. Biochemical characteristics used in the identification of *E. aerogenes*, *E. coli*, and *K. pneumoniae*

Test	Reaction to test		
	<i>E. aerogenes</i>	<i>E. coli</i>	<i>K. pneumoniae</i>
Lactose fermentation	+	+	+
Ornithine decarboxylase	+	d <sup>a</sup>	+
Lysine decarboxylase	+	d	-
Motility	+	+	-
Urease	-	-	+
Indol	-	+	±

<sup>a</sup> d, Delayed.

organisms were obtained from American Type Culture Collection (Rockville, Md.): *Arizona arizonae* 13314 (*Salmonella arizonae* [3]), *Salmonella choleraesuis* 13312, *Proteus vulgaris* 13315, and *Citrobacter freundii* 8090. A culture of *Enterobacter cloacae* was obtained from E. H. Pauley, Curator, V.P.I. culture collection (Blacksburg, Va.). Isolates were maintained on nutrient agar slants.

**Identification.** All media were incubated at 37 C. SIM (Difco) was used for motility and indol tests. Motility was read after 24 h, and indol formation was determined by addition of a few drops of Kovacs reagent after 48 h. Isolates were grown for 96 h in MR-VP medium (BBL) for methyl red and Voges-Proskauer tests, which were run according to methods of Edwards and Ewing (6). Use of citrate as a sole carbon source was determined by light inoculation to a Simmons citrate agar slant (BBL) and incubation for 1 to 4 days. Urease activity was determined by change in pH indicator of Christensen urea agar after 24 h.

Decarboxylase reactions were performed using lysine decarboxylase broth (Difco) and decarboxylase base Moeller (Difco) plus 1% L-ornithine (Sigma). Tubes were incubated for 4 days before being declared negative.

**Media.** Three media were chosen from experimental variations for having potential for practical application. (i) Methyl violet lactose (MV-2) contained per liter of deionized water: nutrient agar (Difco), 23.0 g; methyl violet 2B (Allied Chemical Corp.), 1.0 g; and lactose (Fisher), 10.0 g. (ii) Double violet agar (MV-17) contained per liter of deionized water: violet red bile agar (Difco), 41.5 g; and methyl violet 2B (Allied Chemical Corp.), 2.0 g. (iii) Double violet penicillin agar (MV-22) contained per liter of deionized water: violet red bile agar (Difco), 41.5 g; methyl violet 2B (Allied Chemical Corp.), 2.0 g; and penicillin G (benzylpenicillin sodium salt) (Sigma), 50 µg/ml. Components of MV-2 and MV-17 were heated together to boiling and cooled to 50 before pouring into sterile petri plates. With the MV-22 medium, the first two components were heated together to boiling and cooled to 50 C before adding 10 ml of a 100× stock solution of aqueous penicillin G. After mixing, the medium was poured into sterile petri plates. None of

the media was autoclaved. Quality control was checked by allowing the media to remain at room temperature for up to two weeks.

**Experimental procedure.** Only cultures of lactose-positive organisms that followed typical IMViC reaction patterns for each species were used for pure culture studies. Other pure cultures that were used were selected because of their frequent isolation from fresh water. Plates of each medium were made and allowed to dry. Pure cultures were streaked with an inoculating loop to each medium and incubated at 37 C for 24 h before ascertaining growth. Plates were then reincubated for 72 h more and checked for growth at 24-h intervals.

## RESULTS

Methyl violet lactose agar, the simplest in composition of the three media, was the least inhibitory. *K. pneumoniae* isolated from such diverse sources as fresh produce, human feces, and a freshwater lake showed no differences in colonial morphology. *K. pneumoniae* grew as a large, glistening colony, generally about 3 to 5 mm in diameter, and appeared as "gumdrops." At times a gold, metallic sheen was produced, but this characteristic appeared to vary from strain to strain. Colonies were a pale-medium purple (lavender) in color and appeared very wet. *E. aerogenes* also grew well on the medium, but colonies never produced a metallic sheen and were usually smaller (1 to 2 mm), dry and dark purple in color. *E. coli* did not grow after 24 h of incubation, but pinpoint colonies (<1 mm diameter) did develop after a total of 48 h. The test strains of *P. aeruginosa* and aquatic pseudomonads grew only sparsely as pinpoint colonies after 24 h or not at all. *A. hydrophila*, an organism that is commonly isolated from polluted waters, did not grow even after incubation for 72 h. *C. freundii*, *S. cholerae-suis*, and *S. arizonae* all produced dry, dark purple colonies 1 mm or less in diameter. *E. cloacae* grew only slightly larger colonies (<1 mm to 2 mm), with morphology similar to other *Enterobacteriaceae*. *Proteus vulgaris* exhibited no growth even after incubation for 72 h.

Double violet agar, containing twice as much methyl violet as methyl violet lactose, as well as crystal violet and bile salts as inhibitors, was more inhibitory than methyl violet lactose agar. Growth of *K. pneumoniae* was heavy and glistening, but no metallic sheen was observed. Colony diameter was 3 to 5 mm. *E. aerogenes* grew sparsely with colonies 1 to 2 mm in diameter. *E. coli* was completely inhibited, even after incubation for up to 72 h. *P. aeruginosa* grew very sparsely, with only a few pinpoint colonies appearing after 24 h, and aquatic pseudomonads did not grow. *A. hydrophila* did

not grow even after incubation for 72 h. *C. freundii*, *S. cholerae-suis*, and *S. arizonae* all were similar to growth patterns exhibited on methyl violet lactose medium, i.e., dry, dark purple colonies 1 mm or less in diameter. *E. cloacae* had larger (3 to 4 mm) dry, flat, rough colonies that were dark purple in color. *P. vulgaris* did not grow.

Double violet penicillin agar was the most inhibitory medium tested. In addition to the components of double violet agar, 50 µg of penicillin G per ml was added before pouring the plates. *K. pneumoniae* had heavy, almost confluent growth with large (3 to 5 mm) colonies. *E. aerogenes* grew only sparsely, forming only a few moderate-sized colonies (3 to 4 mm). *E. coli* did not grow, even after incubation for 72 h. *P. aeruginosa* grew sparsely with pinpoint colonies (<1 mm) appearing after 24 h. Aquatic pseudomonads did not grow. *A. hydrophila*, *S. cholerae-suis*, *S. arizonae*, and *P. vulgaris* exhibited no growth at 24 h or at 72 h. *C. freundii* produced colonies that were small (1 mm or less), dry and dark purple. Colonies of *E. cloacae* were larger (3 to 4 mm) and were dry and dark purple, but growth was poor, with fewer than 20 colonies appearing on the plate.

A summary of growth patterns of test organisms on the different media is seen in Table 2.

## DISCUSSION

Due to the biochemical similarities between *Klebsiella* and *Enterobacter*, members of the genera are frequently confused and incorrectly identified (8). Because of the clinical significance of *Klebsiella* and recent work revealing its widespread distribution in nature, it is important to be able to distinguish between the two genera in water quality determinations.

The selective media described in this paper should prove practical particularly in field or clinical applications. None of the media required heat sterilization. Poured, uninoculated plates were incubated at room temperature for two weeks without any sign of bacterial or fungal contamination. Fung and Miller (9) reported that many common gram-positive organisms, including sporeformers, were inhibited completely at a 1:1000 dilution of methyl violet. They further reported that only a limited number of genera of gram-negative organisms will grow in the presence of this inhibitor. We demonstrated that the colonial size and morphology of *K. pneumoniae* is distinctive when compared with the other gram-negative organisms tested. *Klebsiella* incubated on inverted plates produced unique elongated gumdrop colonies that were especially pronounced when

TABLE 2. Growth characteristics of test organisms on methyl violet lactose, double violet and double violet penicillin media

Organism	Growth pattern		
	Methyl violet lactose	Double violet	Double violet penicillin
<i>K. pneumoniae</i>	Glistening lavender colonies, 3 to 5 mm, abundant growth	Glistening lavender colonies, 3 to 5 mm, abundant growth	Glistening lavender colonies, 3 to 5 mm, abundant growth
<i>E. aerogenes</i>	Dry purple colonies, 1 to 2 mm, abundant growth	Dry purple colonies, 1 to 2 mm, sparse growth	Dry purple colonies, 3 to 4 mm, sparse growth
<i>E. cloacae</i>	Dry, round dark purple <1 to 2 mm	Dry, flat, rough dark purple colonies 3 to 4 mm	Dry, flat, rough dark purple colonies, 3 to 4 mm
<i>E. coli</i>	No growth (24 h)	No growth (24 h)	No growth (24 h)
<i>P. aeruginosa</i>	Dry purple colonies, <1 mm, sparse growth	Dry purple colonies, <1 mm, sparse growth	Dry purple colonies, <1 mm, sparse growth
Aquatic pseudomonads	No growth	No growth	No growth
<i>A. hydrophila</i>	No growth	No growth	No growth
<i>P. vulgaris</i>	No growth	No growth	No growth
<i>S. cholerae-suis</i>	Dry, round dark purple colonies, <1 mm, abundant growth	Dry, round dark purple colonies, <1 mm, abundant growth	No growth
<i>S. arizonae</i>	Dry, round dark purple colonies, 1 mm or less	Dry, round dark purple colonies, <1 mm	No growth
<i>C. freundii</i>	Dry, round dark purple colonies, 1 mm	Dry, round dark purple colonies, <1 mm	Dry, round dark purple colonies, 1 mm or less

colonies were well isolated. This gumdrop morphology is produced on inverted plates because the colony has such a fluid consistency that it will eventually drop material from the colony onto the petri plate cover. Relatively untrained laboratory personnel could prepare, inoculate, and read plates with little orientation, and no sophisticated equipment is necessary.

Results obtained on these media also indicate that bacteria commonly isolated from polluted water sources would not be readily confused with *K. pneumoniae*. Aquatic pseudomonads that produce mucoid colonies on standard plate count agar and could be easily confused with *K. pneumoniae* on basis of colonial morphology did not grow on any of the methyl violet-containing media. The results from attempts to grow ten aquatic pseudomonads on the methyl violet media seem to indicate little possibility of false-positives for *K. pneumoniae* from waters polluted with high numbers of mucoid pseudomonads. The genera tested included *Aeromonas*, which is associated with pathogenesis in cold-blooded vertebrates (10), and several members of *Enterobacteriaceae* that have been encountered in water (3).

Preliminary field studies have indicated that use of the media in conjunction with membrane filtration technique (1) is promising in determining the presence of *Klebsiella* in water supplies. Field studies are continuing at this time.

With previous techniques, tests requiring from 1 to 4 days after isolation are necessary to distinguish accurately between *Klebsiella* and *Enterobacter*. In addition, an important test, motility, is difficult to read and often inaccurately done, so a medium which can inhibit *E. coli* and presumptively distinguish between *Klebsiella* and *Enterobacter* on the basis of colonial morphology will be useful in both the clinical laboratory and in the field determination of water quality from the bacteriological standpoint.

In our judgement, double violet agar is the most adequate medium tested for differentiating between pure cultures of *Klebsiella* and

*Enterobacter*. This is based on differences in colonial morphology. Further tests in progress will evaluate its efficiency in water quality studies and its usefulness in field work.

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