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Evaluation of Double Violet Agar in the Isolation of *Klebsiella pneumoniae* from River Water

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A field evaluation of double violet agar for the isolation and presumptive identification of *Klebsiella pneumoniae* from water has been performed. Water from the North Oconee River, Clarke County, Ga., was cultured for presence of klebsiellae using the membrane filter technique. Colonies were presumptively identified as *K. pneumoniae* on the basis of their appearance on double violet agar. Such identifications were evaluated using appropriate biochemical tests. Once investigators have become familiar with cultural reactions on the medium, double violet agar can be used to indicate presence of *K. pneumoniae* in water with greater than 80% accuracy.

Fung and Miller (6) described the selectivity of methyl violet-containing media for *Enterobacter* spp. and indicated that further studies might be undertaken to determine the dye's usefulness in sanitary microbiology. Campbell and Roth (3) reported that incorporation of methyl violet 2B into violet red bile agar would inhibit growth of *Escherichia coli* and allow for differentiation of *Klebsiella pneumoniae* from *Enterobacter aerogenes* on the basis of colonial morphology. They indicated that the medium, double violet agar, or modifications of it might prove to be important in both aquatic and clinical bacteriology.

Pure culture techniques are necessary for the development of selective media, but field testing is essential in determining efficiency in practical use. Double violet agar was developed to aid in isolation of *K. pneumoniae* from water systems without using additional tests which are expensive and time consuming. This paper reports the results of a field study on the efficacy of the selectivity of the medium and the ability to differentiate klebsiellae from other isolates, such that additional confirmatory tests would be unnecessary.

The membrane filter method (MF) for the detection of fecal coliforms in water has gained general acceptance since 1971 when it was published in *Standard Methods for Examination of Water and Wastewater* (1). MF represents a highly reproducible and rapid means of testing water, although some questions have been raised as to its applicability to some samples, notably chlorinated wastewater (10). Use of the method in this study meant that several samples could be processed quickly, and there was no appreciable difference in results obtained from MF and spread plates (unpublished data).

MATERIALS AND METHODS

Field evaluation of double violet agar was conducted on the North Oconee River, Clarke County, Ga., a slow-moving stream which travels through urban and rural agricultural areas. Surface samples were taken as prescribed in *Standard Methods* and processed by the MF technique for fecal coliforms onto solid media (1). Samples were collected in sterile 150-ml screw-capped bottles and transported to the laboratory immediately. Usual lag time between collection and filtration was less than 30 min. Water samples were filtered through sterile Gelman (64194) 0.45-μm pore size gridded membrane filters. Samples were collected daily from the Oconee River, except when rain precluded sampling, from the two extreme downstream sites (D and E) described by Hendricks (7).

Double violet agar has been described by Campbell and Roth (3). The medium was prepared and poured into petri dishes (60 by 15 mm) and allowed to dry before inoculating. Inoculated filters were placed on the hardened agar and incubated for 24 h at 35 C before picking colonies to confirmatory media.

Colonies suspected to be *Klebsiella* sp. were picked for testing in triple sugar iron agar, motility-indol-ornithine decarboxylase, and urea agar (Christensen), and then incubated for 24 h at 35 C. Triple sugar iron agar results were read at 24 h for slant and butt reaction, formation of gas, and reduction of sulfur to H_2S. Motility and ornithine decarboxylation were read before performing the indol test by using Kovacs reagent (5). Breakdown of urea by urease was determined by color change in the indicator incorporated in the medium. Results were read 18 to 24 h after inoculation.

RESULTS

Preliminary work with double violet agar using pure cultures of *E. aerogenes* and *K. pneumoniae* showed marked differences in colonial morphology between the two species (3). Other species that are frequently isolated from water systems and could be confused with *Klebsiella* spp., i.e., *E. coli* or mucoid aquatic pseudomonads, either did not grow at all or grew sparsely on double violet agar.

In our field studies, samples were taken daily, weather permitting, from two sites on the North Oconee River below an unchlorinated, treated sewage treatment plant outfall (7). In the period between 23 June and 22 August 1975, *Klebsiella* counts averaged 325 to 350 per 100 ml at each station, with counts ranging from 70 colony-forming units per 100 ml to 770 colony-forming units per 100 ml.

Colonies picked to triple sugar iron agar, motility-indole-ornithine decarboxylase agar, and urea for generic verification represented three types: (i) mucoid, pale lavender, (ii) mucoid, dark purple, and (iii) tiny (<2 mm) nonmucoid, dark purple. Lavender colonies were found to be *K. pneumoniae*. Purple mucoid colonies were *Enterobacter cloacae*, and the drier purple colonies were *E. aerogenes*. Accuracy in correctly picking *K. pneumoniae* that were later confirmed by biochemical tests averaged 85.4% (Table 1).

TABLE 1. *Percentage of confirmed Klebsiella pneumoniae isolated from water using double violet agar*

Date of sample	Colonies confirmed/ colonies suspect	Confirmed (%)
6/26	3/4	75.0
7/3	6/6	100.0
7/6	4/6	66.67
7/7	5/6	83.33
7/8	4/5	80.0
7/11	5/6	83.33
7/18	4/5	80.0
7/24	5/6	83.33
7/25	5/7	71.4
7/29	2/2	100.0
8/1	6/6	100.0
8/7	7/9	77.8
8/13	7/7	100.0
8/14	6/6	100.0
8/14	9/10	90.0
8/15	4/5	80.0
8/18	4/4	100.0
8/19	3/4	75.0
8/20	5/6	85.7
8/21	4/5	80.0
8/22	7/8	86.67

DISCUSSION

K. pneumoniae is an enteric organism that has been isolated from a variety of sources, both clinical and environmental (3). Its clinical significance has been well established, but its potential as a public health problem in the aquatic environment is uncertain. There has been some question as to potential pathogenicity of strains isolated from aquatic systems, particularly pulp mill effluent. In studying cultural reactions and deoxyribonucleic acid homology of *K. pneumoniae*, Seidler et al. (11) found that most isolates from water and pulp mill effluent were indistinguishable from human strains. Although they did not perform virulence tests, they nonetheless felt that presence of *K. pneumoniae* in the environment indicated deterioration of water quality. Virulent strains of *K. pneumoniae* have been reported isolated from natural receiving waters, an uninhabited watershed, paper and pulpmill effluents, fresh produce, sugarcane, a textile finishing plant effluent, and intestines of animals and man (3). Matsen et al., in utilizing mouse virulence tests, found that aquatic strains and antibiotic-resistant human isolates were essentially the same (9). Knittel (8) has reported that *K. pneumoniae* may not be as ubiquitous as previously assumed (2) and indicated that there is a correlation between isolation of *K. pneumoniae* and *E. coli*. Duncan and Razzell (4) found that *K. pneumoniae* could be isolated from a variety of sources and considered it to be of little importance in public health. While the significance of *K. pneumoniae* in the environment is not clear, double violet agar should prove useful in monitoring the organism in aquatic systems.

Double violet agar is a differential medium for distinguishing between *K. pneumoniae* and *E. aerogenes*, which are often confused with one another. Three tests have been useful in differentiating the two organisms, motility, ornithine decarboxylase, and urea; however, none has thus far been practical for incorporation into a plating medium. On double violet agar *K. pneumoniae* is uniformly mucoid and pale lavender in color, whereas *E. aerogenes* is nonmucoid and dark purple and *E. cloacae* is mucoid and dark purple. We previously reported that a laboratory strain of *E. cloacae* produced a large colony on double violet agar, but with nonmucoid morphology (3). We attribute this difference to strain specificity or to the fact that the one isolate used in the earlier study had been maintained in culture for several months. In this study, we have found that fresh isolates of *E. cloacae* may cause some confusion until the investigator

becomes accustomed to distinguishing its mucoid, but dark purple, colony from the paler colonies of *K. pneumoniae*.

Double violet agar, which uses methyl violet 2B as its primary selective agent, inhibited all gram-positive bacteria, *E. coli*, and many other gram-negative bacteria during this field investigation. This reduces the total number of bacteria normally isolated from water sources and should be considered when diluting a sample.

A disadvantage encountered with the medium primarily involved the subtle difference between colonial morphology of *K. pneumoniae* and *E. cloacae*. The color difference is more marked as investigators become familiar with the medium and cultural reactions on it. This could account for the 85.4% accuracy encountered in picking colonies later confirmed biochemically to be *K. pneumoniae*. We have noticed increased accuracy in choosing suspect colonies as experience with the medium is gained.

Preliminary work in clinical situations has indicated that double violet agar also may be useful as an adjunct to current methods in that field. This is particularly true in culturing of contaminated body fluids in which *K. pneumoniae* is the suspected etiological agent and isolation is difficult or impossible because of overgrowths of gram-positive bacteria or rapidly growing gram-negative bacteria.

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