Selective Medium for the Isolation and Enumeration of *Klebsiella* spp.

S. H. WONG,¹ D. R. CULLIMORE,²* and D. L. BRUCE³

Department of Biology¹ and Regina Water Research Institute,² University of Regina, Regina, Saskatchewan, Canada S4S 0A2

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A highly selective medium for the enumeration and isolation of *Klebsiella pneumoniae* and *Klebsiella oxytoca* was developed in which the typical colonies were convex and 1 to 2 mm in diameter. Their pigment was either a mucoid pink-red color or a more watery pale red with a dark red center. Relatively little colonial growth occurred for any other bacterial genera, and where such colonies did grow, they could be easily differentiated since the form was atypical. The medium already appears to have potential value as a means of assessing the efficiency of treating sewage and monitoring the microbiological quality of vegetables.

*Klebsiella* spp. are widely recognized as opportunistic, antibiotic-resistant pathogens often acting as agents of bacteremias and respiratory and genitourinary infections, particularly in patients under stress. Their significance in the soil and water environments has not been so completely documented. *Klebsiella pneumoniae* and *Klebsiella oxytoca*, although opportunistic pathogens (5), are also widely distributed in nature (17) and can fix atmospheric nitrogen under some conditions (14). Reports indicate that these two species have been isolated from lakes (4), rivers (17), seawater (6), sewage and various waste waters (7, 12), soil (12), animals including humans (1, 8–10, 13, 18), forest and farm products (3, 15, 18), and drinking water (2). Although the colony characteristics of *Klebsiella* spp. have been well documented when grown on various selective media designed for the enumeration and characterization of members within family 1, the *Enterobacteriaceae*, relatively little attention has been directed towards the development of selective media specifically for this genus. Different methods have been reported with M-FC medium (1), double-violet agar (4), MCIC (2), and M-C medium (7).

This paper describes a highly selective medium for *Klebsiella* spp. which is based upon the use of a minimal medium (5) containing sodium nitrate instead of ammonium sulfate as first described in 1980 by Wong (S. H. Wong, M.Sc. thesis, Acadia University, Wolfville, Nova Scotia, Canada, 1980).

It was discovered that this simple modification allowed contaminated cultures of *K. pneumoniae* to be easily purified.

In the medium used in our experiments, the specific nitrogen source was changed to potassium nitrate. The modified medium of Wong contained the following (grams per liter of triple-distilled deionized water): lactose, 5; sodium deoxycholate, 1; Na₃PO₄, 0.7; KNO₃, 1.08; NaH₂PO₄, 0.3; MgSO₄ · H₂O, 0.2; MnSO₄, 0.1; FeCl₃, 0.005; ZnCl₂, 0.005; CoCl₂, 0.005; Mo(OH)₄, 0.005; neutral red, 0.03; crystal violet, 0.004; Noble agar, 15.0. All of the chemicals were analytical grade and were supplied by J. T. Baker Chemical Co., Phillipsburg, N.J. The exceptions were lactose and agar (Difco Laboratories, Detroit, Mich.), neutral red (Fisher Scientific Co., Winnipeg, Manitoba, Canada), and crystal violet (Sigma Chemical Co., St. Louis, Mo.).

To prepare the medium, all of the chemicals except the agar, neutral red, and crystal violet were dissolved in hot water to 80% of the final volume. A slight precipitate formed on cooling and was filtered with a Whatman no. 2 filter paper. Once clarified, the pH of the medium was adjusted to 6.8 (with 1 N NaOH), and the agar and dyes were added. After being boiled to dissolve the agar, the medium was autoclaved (15 lb/in² for 15 min).

To determine the effectiveness of the modified medium of Wong in selectively culturing *Klebsiella* spp., various fecal, sewage, vegetable, soil, and water samples were examined. To enumerate or isolate *Klebsiella* spp., samples were diluted in a 10-fold dilution series in a sterile standard phosphate buffer at a pH of 6.8. A 1-ml amount of each dilution was added to 20-ml samples of molten agar to make a seeded pour plate, or 0.1 ml was streaked over the surface to form a lawn plate. Incubation of plates was at 35°C for 36 h. Both surface and submerged colonies appeared as pink to red with intensified red centers. Surface colonies were also mucoid. Discrete colonies were restreaked onto the same medium to determine purity and then identified by using a standard identification procedure and the Minitek system (BBL Microbiology Systems, Cockeysville, Md.).

Two major colony types were observed during the studies. These were either a 1- to 2-mm-diameter convex rather mucoid pink-to-red colony or a somewhat larger colony type which was more watery and pale red with a dark red center.

The medium had high specificity for *Klebsiella*, revealing relatively large populations of this genus in the various samples as follows: alfalfa sprouts, 6.5 × 10⁷ CFU/g (wet weight); bean sprouts, 2.7 × 10⁶ CFU/g (wet weight); carrots, 3 × 10⁸ CFU/ml (washings from surface); raw sewage, 2 × 10⁸ CFU/ml; primary sewage, 1.4 × 10⁷ CFU/ml; secondary sewage, 3 × 10⁶ CFU/ml; tertiary sewage, 2 × 10⁷ CFU/ml (Table 1).

Although the medium was also selective for *K. pneumoniae* and *K. oxytoca* with soil and water samples, a wider diversity in population was noted. In all cases, *Klebsiella* spp. colonies dominated the media, with very little occurrence of other atypical colony types. This high degree of selectivity was also demonstrated by the fact that of the 26 human fecal samples streaked by loop onto Wong-modified medium, only 4 exhibited any typical *Klebsiella* colonial growth.

From the 4 positive human fecal specimens (Pasqua Hospital, Regina, Saskatchewan, Canada), 6 alfalfa sprout samples, 1 bean sprout sample, 1 carrot sample (these 8

* Corresponding author.
samples were from various supermarkets in Regina), 4 treated sewage samples (Regina sewage treatment plant), 2 soil samples, and 50 water samples, 693 isolates were obtained which exhibited the typical colony characteristics (mucoid, pink-to-red discrete colonies with intensified centers). A total of 349 (50.3%) of the colonies were identified as *K. pneumoniae*, and 343 (49.5%) were identified as *K. oxytoca*. The single remaining typical colony (0.2%) was *Enterobacter cloacae*

Only four occurrences of atypical (colorless) colonies were observed. In each case, a different causant species was identified (*Pseudomonas* spp., *Enterobacter agglomerans*, *Serratia rubidaea*, and *Yersinia enterocolitica*). Of all the colonies examined on this medium (697), only 4 (0.6%) were of an atypical type.

The results of the addition of potassium nitrate in the selective efficiency of the modified medium remains uncertain. Indeed, the relationship between nitrate reduction and nitrogen assimilation by members of the *Enterobacteriaceae* is not known. Since both *K. pneumoniae* and *K. oxytoca* do grow in this nitrate-based medium, it could be that these species possess this assimilative ability. Apart from the nitrate, the only other possible sources of assimilable nitrogen are the crystal violet (3 N atoms per molecule), neutral red (4 N atoms per molecule), and Noble agar (Difco Laboratories, Detroit, Mich.). The manufacturer states in the ninth edition of the Difco Manual that this agar was "carefully washed agar, free from all impurities which would interfere with its efficiency when employed in the preparation of Noble cyanide citrate agar." With no nitrate added to the medium, visible colonies did appear after 48 h when plates were incubated under identical conditions, but colonies were small and otherwise atypical.

Although this does not indicate that the agar is free of nitrogen, it would support the hypothesis that most of the available forms of this element would be in the nitrate fraction. Selective growth could occur as a result of one or a combination of two or more of the following assimilative routes; denitrogen fixation, nitrate reduction and assimilation, direct nitrate uptake and utilization, possible degradation or uptake or both of the nitrogenous compounds in Noble agar. Thus, although the mechanism by which the medium functions needs to be resolved at least in part by acetylene fixation and nitrate reductase (16) studies, the medium does appear to be highly selective for some *Klebsiella* strains.

Studies are presently under way to determine the precise mechanism by which the medium is selective. *Klebsiella ozaenae* (ATCC 11296) and *Klebsiella rhinoscleromatis* (supplied from stock culture, Microbiology Department, University of Alberta, Alberta, Canada) both failed to grow, indicating that the selectivity for *Klebsiella* spp. may be at the interspecies level.

To assess the sensitivity of recovery of Wong modified medium, dilutions of randomly selected strains of *Klebsiella* spp. were made in normal saline and seeded to plate count agar as well as to various selective media. All plates were incubated at 35°C for 24 h (Table 2). It can be seen that the lowest recovery on Wong modified medium was at least 80% of the CFU found on plate count agar, with most strains showing 100% or better. This compares very favorably with MCIC medium, on which sensitivity of recovery is much higher.

**TABLE 2. Comparative recovery sensitivity**

*Klebsiella* strain tested 

<table>
<thead>
<tr>
<th>Medium</th>
<th>MacConkey</th>
<th>EMB</th>
<th>ENDO</th>
<th>MCIC</th>
<th>Wong</th>
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<tr>
<td><em>K. oxytoca</em></td>
<td></td>
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<tr>
<td>21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.9</td>
<td>1.0</td>
<td>1.0</td>
<td>0.8</td>
<td>0.9</td>
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<tr>
<td>22&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.7</td>
<td>0.7</td>
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<tr>
<td>27&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>165&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>0.8</td>
<td>1.0</td>
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<tr>
<td><em>K. pneumoniae</em></td>
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<td>21&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.9</td>
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<tr>
<td>165&lt;sup&gt;e&lt;/sup&gt;</td>
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<tr>
<td>696&lt;sup&gt;e&lt;/sup&gt;</td>
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<td>1.0</td>
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<tr>
<td>ATCC 29665</td>
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<td>1.0</td>
<td>1.2</td>
<td>0</td>
<td>0.9</td>
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</table>

<sup>a</sup> Cultures were diluted in normal saline and plated on the selective media indicated as well as on plate count agar. Growth on the various media is expressed relative to numbers of colonies on plate count agar, which is taken as unity.

<sup>b</sup> Isolation from clinical sources.

<sup>c</sup> Isolation from soil or vegetable sources.
lower, with K. pneumoniae strains 168 and ATCC 29665 not yielding any growth at all. It can be concluded that the sensitivity for the recovery of K. oxytoca and K. pneumoniae on Wong modified medium is excellent. Other Klebsiella species, however, were never isolated on this medium.

In addition to this medium allowing the easy purification of Klebsiella spp. cultures, it has a number of other potentially valuable uses. From the initial studies, the enumeration of Klebsiella spp. on Wong modified medium in raw and treated sewage would allow the efficiency of a treatment system to be easily monitored. In the samples of sewage subjected to various progressive forms of treatment, the counts were reduced from the raw sewage by 30, 85, and 99.5% at the primary, secondary, and tertiary stages, respectively. Another use would be the monitoring of microbial loadings in vegetables, particularly sprouted legumes. The medical significance of this medium remains to be determined.

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LITERATURE CITED