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Nitrogen-deficient Medium in the Differential Isolation of *Klebsiella* and *Enterobacter* from Feces

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On a nitrogen-deficient agar medium, the tribe *Klebsielleae* formed large, glistening, mucoid colonies which were easily distinguished from other colony types. Of 113 *Klebsielleae* isolates from human feces which were characterized, *Klebsiella* accounted for 88% of the total; 75% were *K. pneumoniae*; *K. ozaenae* (13%) was isolated from one individual only. The remaining strains (12%) were identified as *Enterobacter cloacae*. Counts (for the tribe) ranged from 10^2 to 10^6, with a median of 10^4; 9 of 53 stool specimens were negative. *K. pneumoniae* was also isolated from 6 of 41 frozen foil-pack foods. Anaerobic studies at room temperature and 37 C revealed no appreciable differences from aerobic plates. The nitrogen-deficient medium appeared better than E M B for isolation of *Klebsielleae* when they were present in low numbers relative to other coliforms; slime production by *Klebsielleae* concomitant with minimal growth of other bacteria is involved.

Isolation of *Klebsiella* and *Enterobacter* from an environment such as fecal matter has routinely involved the use of E M B or MacConkey agars for primary isolation by the direct plating procedure (1). On the above media, these microorganisms may be masked because of the quantitatively greater numbers of *Escherichia coli.*

During an evaluation of media, for selective isolation of fecal bacteria, large, mucoid colonies were distinguishable on a medium that was essentially nitrogen-deficient. Identification of several isolates revealed them to be *Klebsiella* or *Enterobacter*. Results of a study of such isolates from a nitrogen-deficient medium are reported in this paper.

Materials and Methods

Isolation. A 10-g amount of feces was added to 90 ml of Difco Fluid Thioglycollate Medium, and the mixture was homogenized in a Waring Blendor for 2 to 3 min (5). Subsequent dilutions of 10^{-2} and 10^{-3} were made in tubes containing 9 ml of Fluid Thioglycollate Medium. By means of a bent glass rod, 0.1-ml amounts of the 10^{-2} and 10^{-3} dilutions were surface-streaked on duplicate plates of the nitrogen-deficient (N-deficient) medium (in a few instances the 10^{-1} dilution was also used). The composition of the N-deficient medium per liter of deionized water was: mannitol, 10 g; K_2HPO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; NaCl, 0.2 g; $MnSO_4 \cdot 4H_2O$, 0.005 g; $FeCl_3 \cdot 6H_2O$, 0.005 g; and Difco purified agar, 15 g. After sterilization at 121 C for 15 min, the medium was cooled to 50 to 55 C, and 10 ml of a 20% sterile suspension of $CaCO_3$ was added per liter. The *p*H was 7.6 to 7.7. The medium, which gave a negative ninhydrin test, is essentially that used frequently in the isolation of *Azotobacter* (10). Incubation for all media was aerobic at 37 C. The primary N-deficient plates were incubated for 24 hr. If growth of colony types (from pinpoint to 1 mm in size) other than the desired type was prevalent, an additional 24-hr incubation period was required for the latter type to stand out.

Study of isolates. In general, two to four isolates for each fecal sample were picked from the primary N-deficient plates for further study. These isolates were characterized as large (usually 2 to 7 mm), convex, glistening, translucent, mucoid colonies exhibiting a marked tendency toward coalescence. After staining with Hucker's modification of the Gram stain for the presence of gram-negative short rods, typical colonies were streaked to a second N-deficient plate and incubated for 48 hr. Colonies arising on the second N-deficient plate were generally much smaller, 0.5 to 1 mm in size. Selected colonies were streaked to Levine E M B agar (Difco) and incubated for 24 hr, followed by inoculation onto a slant of Simmons Citrate Agar (BBL). Growth on Simmons Citrate after 24 hr was used as the inoculum for three test media: M R-V P Medium (Difco), a motility medium and ornithine decarboxylase test medium with control [Difco Decarboxylase Medium Base plus 0.3% agar, with and without 2% DL-ornithine monochloride (Nutritional Biochemicals Corp., Cleveland, Ohio)]. These media were tubed in 3-ml amounts in sterile

tubes (12 by 75 mm), except for the M R-V P Medium which was used in 2-ml amounts. The inoculated M R-V P medium was incubated at least 48 hr. For the Voges-Proskauer (VP) test, 1 ml of a fresh 40% KOH solution was added to 1 ml of medium and read after 4 hr. No intensification of the color was found to be necessary. The motility medium was based on the improved motility medium of Ball and Sellers (2), in that it contained 0.1% agar and 3% gelatin; the medium contained in addition 1% tryptose (Difco), 0.5% NaCl, and 0.1% glucose. Motility was determined after incubation for 24 hr at 37 C; further incubation was carried out for 2 to 3 weeks at 24 C for the gelatin liquefaction test. The ornithine decarboxylase test medium was read after 24 hr and after 4 days. This semisolid decarboxylase test medium was used by Johnson et al. (12) in their identification procedure for enteric bacteria. For those *Klebsiella* which gave a positive methyl red (MR) test and negative VP test, indole production was determined by use of a 48-hr culture in 1% Trypticase water tested with Kovac's reagent.

In general, criteria for identification of the isolates were according to Edwards and Ewing (3) and Fife, Ewing, and Davis (8). For species differentiation of the *Enterobacter*, it was necessary to employ the lysine decarboxylase and arginine dihydrolase tests. Decarboxylase Base Moeller (Difco) plus 0.3% agar was used with 1% L-lysine monohydrochloride or 1% L-arginine hydrochloride (Aldrich Chemical Co., Milwaukee, Wis.).

RESULTS AND DISCUSSION

Identification and type distribution. Colonies of the tribe *Klebsielleae* were readily differentiated visually from other colonies on the N-deficient medium by larger size, more convex elevation, and gummy appearance (Fig. 1). There was no difference in colonial morphology between *Klebsiella* and *Enterobacter*. A total of 113 isolates of *Klebsiella* and *Enterobacter* were identified from 53 normal fecal specimens examined from 11 male adults during a 6-month period. The genus *Klebsiella* accounted for 88% of the total, or 99 strains, of which 84 (75%) were *K. pneumoniae*. Fifteen strains (13%) were *K. ozaenae*, identified principally on the basis of their MR-VP reactions. Several of the *K. ozaenae* isolates were found to produce the metallic sheen which is usually typical for *E. coli* on E M B agar. This property was consistent through five successive restreakings on E M B. Apparently, these MR-positive lactose-fermenters produce a sufficiently low *p*H, similar to *E. coli*, to precipitate the methylene blue eosinate, which dries in the presence of air to form a metallic sheen. The genus *Enterobacter* accounted for 14 strains, or 12% of the total, all being classified as *E. cloacae*. The results of the diagnostic tests are summarized in Table 1. Very few exceptions to "typical" reactions were noted. Only one strain of *K. pneumoniae* (nonmotile) was able to decarboxylate ornithine and liquefy gelatin. Although the ornithine decarboxylase test as performed in this study is not especially recommended for the *Klebsielleae* (3, 7), clear-cut reactions were consistently obtained. The motility and ornithine decarboxylase tests, which were used to differentiate the two major genera, corresponded in all cases except the one mentioned above. In this one case, it is possible that a nonmotile variant of a normally motile strain was obtained, since a positive ornithine decarboxylase test was shown by this isolate; however, *Klebsiella* occasionally do give a positive test for decarboxylation of this amino acid (3, 4). It is interesting to note that motility observed in the decarboxylase test medium correlated in each case with that seen in the recommended motility medium. One strain of *Enterobacter* gave a positive MR test and negative VP test; two strains failed to liquefy gelatin.

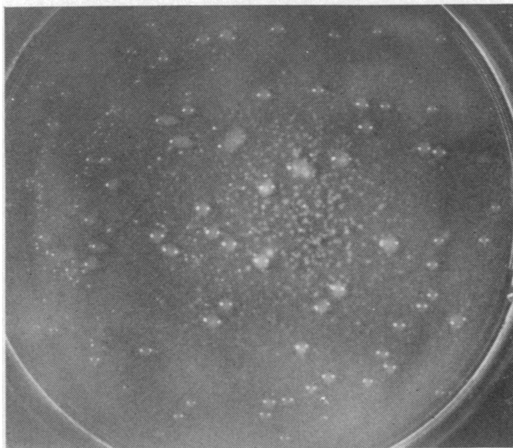

FIG. 1. *A primary plate of nitrogen-deficient agar showing large, glistening, mucoid Klebsielleae-type colonies from feces. Incubation aerobic at 37 C (48 hr).*

Incidence. *Klebsielleae*-type colonies were not observed on the N-deficient agar plates in 9 of the 53 stool samples. All three stool samples obtained from one individual were negative. Counts of *Klebsielleae* ranged from 10^2 to 10^6 with a median of 10^4. Since one subject consistently exhibited the presence of *K. pneumoniae*, 20 isolates obtained from one sample were identified as *K. pneumoniae*. However, *E. cloacae* was later found in the feces of this individual. Three subjects possessed both *K. pneumoniae* and *E. cloacae* in the same fecal specimen. Isolates of *K. ozaenae* were all from the feces of one individual on separate occasions.

TABLE 1. *Characteristics of 113 fecal isolates of the tribe Klebsielleae*[a]

Test or substrate	*Klebsiella pneumoniae*		*K. ozaenae*		*Enterobacter cloacae*	
	Reaction	No. positive	Reaction	No. positive	Reaction	No. positive
Lactose (E M B)	+	84	+	15	+	14
Methyl red, 37 C	−	0	+	15	−	1
Voges-Proskauer, 37 C	+	84	−	0	+	13
Simmons citrate	+	84	+	15	+	14
Motility	−	0	−	0	+	14
Gelatin	−	1	−	0	+	12
Ornithine decarboxylase	−	1	−	0	+	14
Lysine decarboxylase					−	0
Arginine dihydrolase					+	14
Indole			−	0		

[a] *K. pneumoniae*, 84; *K. ozaenae*, 15; *E. cloacae*, 14.

Eickhoff, Steinhauer, and Finland (4), during their study of the *Klebsielleae*, identified 17 strains from stools; 16 were *K. pneumoniae* and 1 was *E. cloacae*. Among the strains studied by Orskov (14), 76 belonging to the genus *Klebsiella* were obtained from feces. A few *E. cloacae* were also isolated. Of the 136 cultures reported by Fife, Ewing, and Davis (8) from stools, 49 were *K. pneumoniae*, 1 was *K. ozaenae*, 23 were *E. cloacae*, and the remainder were distributed among the rest of the members of the tribe.

Other fecal bacteria appearing on primary N-deficient agar. Since some combined nitrogen is carried over with the inoculum, colonies of bacteria other than *Klebsielleae* are able to grow out on the primary N-deficient medium. These colonies included gram-negative short rods, gram-positive sporeforming *Bacillus* species and gram-positive coccoid forms. Several of the colonies of gram-negative rods were tested on Citrate Mannitol (Difco) and Simmons Citrate agar slants according to the procedure described recently by Sellers (17) and were identified as *E. coli*. Although *E. coli* appeared to be the most frequent additional type on the primary plates and, in some cases, was extremely numerous, no difficulty whatsoever was encountered in picking the *Klebsielleae* colonies, because of their striking colonial morphology.

Isolation from frozen food. Of 41 frozen foil-pack foods (meals prepared for military use) examined, 6 yielded *K. pneumoniae* isolates (15 strains identified). Foods found positive were precooked frozen meats, including three samples of pot roast and one each of turkey, meat loaf, and veal cutlets. Eight samples of frozen vegetables were negative when tested by the direct plating method described. The food analysis was performed by adding 50 g to 200 ml of cold sterile distilled water with homogenization in a Waring Blendor for 2 to 3 min; 0.1 ml of this suspension was surface-streaked to plates of N-deficient agar. Incubation of media and identification of the food isolates were as described for feces. Most of the foods yielded no growth on the N-deficient medium; a few showed growth of *Bacillus* species.

Nitrogen fixation. Several workers have noted the nitrogen-fixing ability of *Aerobacter* (9, 11, 15, 16, 18). The motile strains of the former genus *Aerobacter* have recently been reallocated to the genus *Enterobacter* and the nonmotile strains to the genus *Klebsiella* (Judicial Commission of the International Nomenclature Committee, 1963; 6). Mahl et al. (13), in reexamining nitrogen-fixing rods previously classified as *Aerobacter aerogenes*, found these cultures to be *K. pneumoniae* serotypes. None of the *Enterobacter* strains tested fixed nitrogen. *K. pneumoniae* fixes relatively little nitrogen when grown aerobically in the presence of N_2 as the sole nitrogen source; when grown anaerobically, much larger amounts of nitrogen are fixed (9). Plates of streak-inoculated (feces) N-deficient agar were incubated in an atmosphere of 90% N_2 and 10% CO_2 in modified Brewer jars (the jars were flushed three times with 100% N_2 and after the fourth evacuation were filled with the mixture of gases). In addition, alkaline pyrogallol was used in modified Bray dishes and a methylene blue anaerobic indicator card (BBL) was placed in each jar. Incubation was carried out at 37 C for 72 hr, with duplicate plates incubated aerobically; no significant differences were observed. Jensen (11) reported the best yields of nitrogen fixation between 15 and 30 C. Accordingly, anaerobic studies were performed at room temperature; again, no appreciable differences were observed in comparison with aerobic plates. No claim is made for nitrogen fixation.

Comparison of E M B and N-deficient agars. In many studies of the microbial flora of fecal matter, the coliform count has been reported as a whole. The primary plating media used, such as E M B, MacConkey, Endo, and Deoxycholate agars, apparently in many instances may allow the lactose-fermenting organisms present to be quantitated only as a group. Fecal samples from four individuals were diluted 10^{-1} to 10^{-4} for determining relative ease of *Klebsielleae* differentiation on E M B and N-deficient agars. These subjects possessed a relatively low count of *Klebsielleae* (10^3 to 10^4) when compared with the total numbers of coliforms (10^6 to 10^7). The media appeared equal in recovery of the few numbers of the tribe at the 10^{-3} dilution; no *Klebsielleae* were observed at 10^{-4}. In contrast, the *Klebsielleae* appeared better differentiated on the N-deficient medium at the 10^{-1} and 10^{-2} dilutions, and a more accurate count could be obtained, particularly after 48 hr. At these dilutions on the N-deficient medium, the *Klebsielleae* continued to produce slime and became quite elevated, concomitant with minimal growth of other types; on E M B, the *Klebsielleae* remained much less distinct. Although a medium such as E M B would be more useful in studies involving all coliforms and lactose-nonfermenters, an N-deficient medium appears better for isolation of *Klebsielleae* when they are present in low numbers relative to other coliform bacteria.

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