

## New, Simple Medium for Selective Recovery of *Klebsiella pneumoniae* and *Klebsiella oxytoca* from Human Feces

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A culture medium was developed which selectively favored the growth of *Klebsiella pneumoniae* and *Klebsiella oxytoca* in *Escherichia coli*-rich fecal cultures, without the use of antibiotics. The discriminative capacity of this medium was based on the presence of only two carbon sources, citrate and inositol, which can be utilized by nearly all *K. pneumoniae* and *K. oxytoca* strains but not by *E. coli*. The medium consisted of Simmons citrate agar (SCA) with 1% inositol (SCAI). *Klebsiella* strains from fecal samples subcultured on SCAI grew unhampered as yellow, dome-shaped, often mucoid colonies, whereas *E. coli* appeared as tiny, watery colonies. Apart from some *Enterobacter* strains, no other types of bacteria were found to mimic the typical appearance of klebsiellae. Recovery experiments from stool samples revealed a limiting ratio of *Klebsiella* to *E. coli* of 1:10<sup>6</sup> or more when samples were plated on SCAI versus ratios of 1:10<sup>2</sup> to 1:10<sup>3</sup> on blood agar or MacConkey agar. Compared with an existing *Klebsiella* culture method, the combination of SCA and MacConkey-inositol-carbenicillin (MIC) agar, *Klebsiella* yields with SCAI were not lower than those with the combination of MIC and SCA. Furthermore, the efficiency of the SCAI method was twice that of the latter combination. The SCAI plate could be a valuable tool in studies on the epidemiology of *K. pneumoniae* and *K. oxytoca*, for example in nosocomial infections, especially those concerning immunocompromised patients.

*Klebsiella pneumoniae* and *Klebsiella oxytoca* play an important role in hospital-acquired infections (13). Moreover, they are a frequent cause of infections in immunocompromised patients (12, 16) and a potential hazard to patients with extensive burns (6). As potential members of the commensal flora of the human gut, *K. pneumoniae* and *K. oxytoca* are most frequently found in human feces. This does not account for the metabolically less active subspecies of *K. pneumoniae*: *K. ozaenae* and *Klebsiella rhinoscleromatis*, and, therefore, *Klebsiella* spp. refers only to *K. pneumoniae* and *K. oxytoca* in this article.

A reliable method to isolate *Klebsiella* spp. from stool samples would be an important contribution to epidemiological studies concerning nosocomial infections. Conventional isolation techniques, however, have the disadvantage of being rather insensitive for *Klebsiella* spp., due to the abundance of *Escherichia coli* strains in human feces, which mask the presence of smaller numbers of klebsiellae.

In 1970 Thom (14) developed a nutrient medium for the selective recovery of *Klebsiella* spp. from feces, the MacConkey-inositol-carbenicillin (MIC) agar. This medium was based on the MacConkey agar in which lactose was replaced by 1% inositol, with the addition of 100 mg of carbenicillin per liter. This medium owes its elective capacity to the fact that about 97 to 99% of *Klebsiella* spp. and only 0 to 1% of *E. coli* strains are capable of fermenting inositol (4, 5) and hence appear as red colonies. The selectivity of the medium is due to the presence of carbenicillin to which most *E. coli* strains are susceptible. Resistant *E. coli* strains will appear as pale colonies. Since about 10 to 15% of *Klebsiella* strains are also susceptible to this concentration of carbenicillin (11), these strains will be missed when this medium is used. Therefore, some investigators reduced the carbenicillin concentration to 10 mg/liter (1, 15).

Cooke et al. (1) used MIC agar simultaneously with Simmons citrate agar (SCA), the latter medium allowing the growth of only those types of bacteria that are capable of utilizing citrate as the only carbon source. On SCA, ca. 97 to 99% of *Klebsiella* strains appear as small blue colonies, whereas *E. coli* is unable to grow on this medium (4, 5). However, several other types of bacteria are capable of growing on citrate, thus impairing the detection of *Klebsiella* spp. So far, however, this method combined with two enrichment media yielded the highest isolation rates of *Klebsiella* spp.

To simplify the isolation procedure for *Klebsiella* spp. without loss of sensitivity, we developed a medium which contains citrate and inositol as the only carbon sources. No antibiotics were used, partly because of the risk of missing susceptible strains and partly because of the inconvenience of the short storage time at 4°C of media containing β-lactam antibiotics.

This medium was tested for its elective and selective properties with respect to the isolation of *Klebsiella* spp. from fecal samples and for its sensitivity compared with that of the media used by Cooke et al. (1).

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### MATERIALS AND METHODS

**Culture media.** The culture media used were blood agar (BA) (BBL 11043; BBL Microbiology Systems, Cockeysville, Md.), MacConkey agar (McC) (Oxoid CM7b; Oxoid Ltd., London, England), MIC (according to Cooke et al. [1]; BDH, Poole, England [inositol]; Beecham Pharma BV, Amstelveen, The Netherlands [carbenicillin]), SCA (Oxoid CM 155) and SCA with inositol (SCAI). The MIC agar was prepared as described by Cooke et al. (1), with a minor change, using bile salts (Oxoid L 55) instead of sodium taurocholate, which made no difference (R. Shinebaum,

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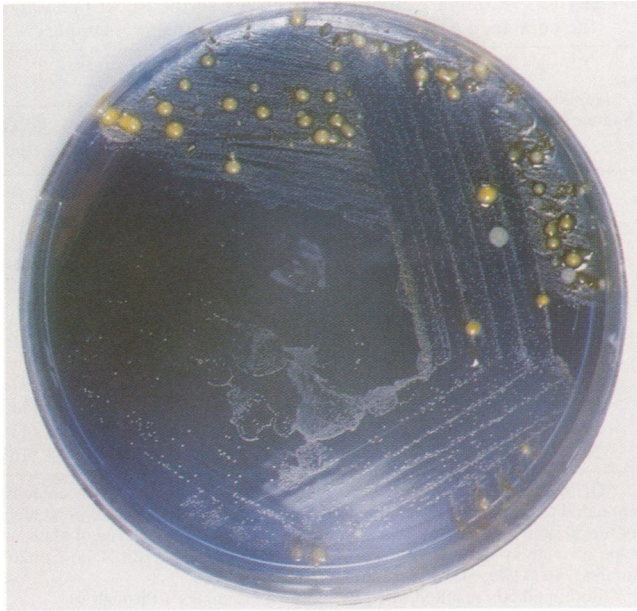


FIG. 1. Fecal culture on SCAI plate after 48 h of incubation at 37°C. Large, yellow, moist colonies are *Klebsiella* spp. Small grey-white colonies are *E. coli*.

personal communications). The combined use of MIC agar and SCA for isolating klebsiellae is further referred to as Cooke's method. For the SCAI medium, inositol (BDH 38044; 10 g/liter) was separately sterilized during 10 min at 120°C before it was added to the SCA, to avoid possible caramelizing of the sugar. In later studies, however, mixing inositol with SCA before steaming and sterilizing did not influence the qualities of the SCAI plate. In one experiment, Koser citrate broth (Oxoid CM 65) containing 1% inositol was used as diluent.

**Bacterial strains.** *Klebsiella* strains (102), consisting of 84 National Collection of Type Cultures strains and 18 clinical isolates from blood cultures, were used. These strains covered nearly all capsular serotypes of *Klebsiella* spp. For mixtures of *Klebsiella* spp. and *E. coli*, a clinical *E. coli* isolate, E825, was used. As sources of fecal bacteria, 109 fecal samples, 46 from patients in two hospitals and 63 from healthy hospital and laboratory personnel, were used in one

experiment, and 50 fecal samples, 29 from clinical patients and 21 from healthy hospital and laboratory personnel, were used in a second experiment. Furthermore, in an epidemiological study, 226 stool samples from outpatients of the rheumatology department of the Utrecht University Hospital were tested. All identifications of isolated bacterial strains were performed by the methods of Cowan and Steel (2) and the data given in the manuals of Krieg and Holt (7) and Lennette et al. (8).

**Enumeration of bacteria.** For counting colonies, the agar plates were inoculated with equal amounts from serial 1:10 dilutions of broth cultures by the technique of Miles and Misra (9) and incubated overnight or 48 h at 37°C.

**Fourfold streaks.** For comparison of sensitivity of SCAI and other media, fecal suspensions were plated with a standardized fourfold streak.

**Mixtures of *Klebsiella* isolates and feces.** Five different fecal samples were used. Two samples had been kept at -20°C for about 1 year and appeared to contain some *Enterococci* and *Bacillus* types but no members of the family *Enterobacteriaceae*. The other three samples were fresh and contained *E. coli* but no *Klebsiella* spp. The samples were suspended in saline and divided into four equal batches, each containing 1 g of feces. From a 3-h broth culture (37°C waterbath) of *Klebsiella* NCTC K28, serial dilutions were made in saline and added to the four batches of fecal suspension, resulting in final *Klebsiella* concentrations of 10<sup>6</sup>, 10<sup>5</sup>, 10<sup>4</sup>, and 10<sup>3</sup> CFU/g of feces. These mixtures were each subcultured by the method of Miles and Misra (9) on the three different media. Control samples were taken from the *Klebsiella* cultures diluted with saline only.

**RESULTS**

**Selective properties of SCAI.** The SCAI plate had the following properties. Inositol fermentation lowered the pH due to acid production, and the pH indicator bromothymol blue became yellow. Citrate utilization caused an elevation of the pH and turned the indicator blue. After incubation at 37°C for 48 h, most *Klebsiella* strains appeared on SCAI as large, moist, dome-shaped, often very mucoid, yellowish colonies caused by inositol fermentation (Fig. 1). The color varied from milk white to deep orange. Of 102 *Klebsiella* strains tested, 7 appeared as small blue colonies due to growth on citrate only. Although the percentage of inositol-negative klebsiellae was higher than the expected 1% (4), no inositol-negative strains were found among 49 klebsiellae present in 109 fecal samples from hospital patients and healthy hospital workers. Most of the non-*Klebsiella* strains

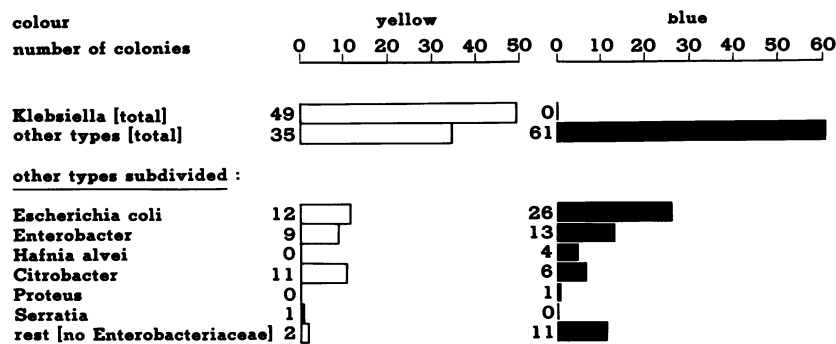


FIG. 2. Color on SCAI of 145 bacterial strains isolated from 109 fecal samples, 46 from hospital patients and 63 from healthy hospital workers. Open columns, numbers of yellow colonies; closed columns, numbers of blue colonies.

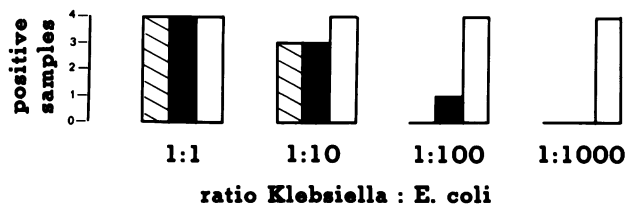


FIG. 3. Recovery of *Klebsiella* spp. added in different numbers to four fecal samples containing  $2 \times 10^6$  CFU of *E. coli* per g. Hatched columns, BA; closed columns, McC; open columns, SCAI.

found in these 109 stool cultures formed blue colonies (Fig. 2). More than half (58%) of the yellow colonies appeared to be klebsiellae. However, no blue klebsiellae were found. Twelve *E. coli* strains formed yellow colonies, but these and most of the other bacterial types could easily be distinguished from klebsiellae because of their appearance as flat or pyramidal colonies, which differed from the dome shape of the klebsiellae. Only some *Enterobacter* strains could mimic the appearance of *Klebsiella* colonies.

Usually after 48 h of incubation at 37°C, *E. coli* from feces appeared on SCAI as tiny, watery, colorless or pale-grey colonies spread over a large portion of the plate, probably growing for a short time on traces of nutrients from the feces (Fig. 1).

**Selective capacity of SCAI.** Broth cultures (3 h, 37°C waterbath) of two *Klebsiella* strains (NCTC K15 and K28) were each mixed with a broth culture of an *E. coli* strain (E825) in the relative proportions (*Klebsiella* to *E. coli*) of 1:10<sup>2</sup>, 1:10<sup>4</sup>, 1:10<sup>5</sup>, and 1:10<sup>6</sup>. From each mixture, 0.1 ml was streaked on SCAI and McC plates. No *Klebsiella* growth could be detected on the McC plates with the exception of the mixture containing the highest concentration of strain K15, in which a few klebsiellae were recognizable among many *E. coli* colonies. Strain K28 could not be recovered from any of the mixtures on McC. Both strains K15 and K28 were recovered from all mixtures cultured on SCAI, the lowest number of colonies found being 20 and 10, respectively, among about 10<sup>7</sup> CFU of *E. coli*.

**Recovery of *Klebsiella* isolates from feces.** The numbers of *Klebsiella* colonies from the different fecal samples were compared with those of the controls, and the recovery percentages were calculated. Fecal samples previously not containing *Enterobacteriaceae* did not interfere with the recovery percentages of *Klebsiella* spp. from feces-*Klebsiella* mixtures on each of the different culture media. But when *E. coli* was present, it significantly influenced the recovery of *Klebsiella* spp. from BA and McC but not from SCAI (Fig. 3). The limiting ratio of *Klebsiella* spp./*E. coli* on BA or McC appeared to be about 1:10<sup>2</sup> to 1:10<sup>3</sup>.

**Comparison of sensitivity and efficiency of the different media.** The sensitivity of the SCAI plate was compared with that of two other media (MIC agar and SCA). Fecal samples (50), 29 from clinical patients and 21 from healthy hospital and laboratory personnel, were screened for the presence of *Klebsiella* spp. From each sample, 1 g was suspended in 3 ml of saline, and the three different media were inoculated with 0.1 ml of the fecal suspension with a fourfold streak. After incubation overnight and for an additional 24 h at 37°C, colonies suspected of being klebsiellae were isolated and subcultured on McC, and lactose-positive strains were further identified. This test was partly blind. Apart from two or three samples, there were no significant differences in *Klebsiella* numbers resulting from growth on SCAI versus growth

TABLE 1. Comparison of the sensitivity of the SCAI plate and the combined MIC and SCA plates to 20 positive samples<sup>a</sup>

Sample number(s)	No. of <i>Klebsiella</i> -positive streaks (no. of <i>Klebsiella</i> colonies) by:		
	Cooke's method plate:		SCAI method
	MIC	SCA	
1-9 <sup>b</sup>			
10	2	3	4
11	1 (14)	2	1 (2)
12	1 (6)		1 (10)
13	1 (5)		1 (2)
14	1 (16)		3
15		1 (26)	2
16	1 (1)		
17			1 (1)
18			1 (1)
19			1 (1)
20			1 (16)

<sup>a</sup> Of 50 fecal samples tested, 20 (designated 1 through 20 [left column]) appeared to contain *Klebsiella* spp. The numbers of samples (and percentage of the 50 samples tested) found to contain *Klebsiella* spp. by type of plate and type of method were as follows: MIC, 15 (30%); SCA, 12 (24%); Cooke's method, 16 (32%); SCAI method, 19 (38%).

<sup>b</sup> Both methods resulted in equal scores for samples 1 through 9.

on the combined MIC and SCA. (Table 1), but as Cooke's method used two media (MIC and SCA), its chance to demonstrate *Klebsiella* spp. from paucibacillar samples was greater. Yet the total yield from SCAI was not lower than from the combined media used in Cooke's method, in which the MIC plate appeared to be slightly superior to the SCA plate. Comparison of the efficiency of both methods (Table 2) showed that with the combination of MIC and SCA, 84 isolations and 51 identifications were performed versus 45 isolations and 34 identifications with SCAI. Thus, the workload with the former method was nearly twice that of the SCAI method due to double isolations of the same strains from MIC and SCA. Yet the final *Klebsiella* score on MIC plus SCA was not higher than that on SCAI; therefore, the SCAI plate was more efficient than the combination of MIC and SCA.

**Application of the SCAI plate in epidemiology.** About 1 g of feces was taken from fecal samples from each of 226 outpatients of the rheumatology department. The feces from each sample was suspended in 10 ml of Koser citrate broth containing 1% inositol. SCAI plates were inoculated in fourfold streaks with 0.1 ml of the suspension immediately and after overnight incubation at 37°C. After 48 h of incuba-

TABLE 2. Efficiency of the SCAI plate compared with that of the MIC and SCA plates<sup>a</sup>

Plate	No. of isolated colonies	No. of identifications carried out	No. (%) <sup>b</sup> of strains identified as <i>Klebsiella</i> spp.	No. of samples found to contain <i>Klebsiella</i> spp. <sup>c</sup>
MIC	46	29	20 (43)	15
SCA	38	22	14 (37)	12
MIC + SCA	84	51	34 (40)	16
SCAI	45	34	21 (47)	19

<sup>a</sup> Of 50 fecal samples, 20 appeared to contain *Klebsiella* spp. Numbers in the table refer to the 20 positive samples.

<sup>b</sup> Based on the number of isolated colonies (column 2).

<sup>c</sup> From footnote a of Table 1.

tion at 37°C, the growth of klebsiellae on the SCAI plates was recorded. The results are presented in a cumulative form (Fig. 4) and show that klebsiellae were recovered from over 50% of the samples. Two or more streaks with klebsiellae were found in only 11.5% of all samples tested. In a separate test the margin of two streaks or more appeared to correspond with  $\geq 10^5$  CFU of *Klebsiella* isolates per g of feces (data not shown).

**DISCUSSION**

Of the *K. pneumoniae* and *K. oxytoca* strains, ca. 97 to 99% are capable of fermenting inositol versus only 0 to 1% of the *E. coli* strains (4, 5). Therefore, inositol initially was chosen as the sole carbon source for a selective *Klebsiella* medium. Ingredients of SCA, with the exception of citrate, were used as an electrolyte agar base. Addition of inositol to this agar base resulted in the growth of small colonies. It was observed, however, that *Klebsiella* colonies were larger when inositol was added to complete SCA containing sodium citrate. The mechanism of this phenomenon remains unclear. The advantage of using two carbon sources which both can be utilized by a high percentage of *Klebsiella* strains is that the chance of missing such strains is less. On the other hand, the additional presence of citrate probably does not favor the growth of more *E. coli* strains than does inositol alone because practically none of the *E. coli* strains are capable of utilizing citrate (4, 5). The general disadvantage of using two carbon sources is a decrease in selectivity resulting in the growth of a greater number of different bacterial strains. But although several types of bacteria grew on the SCAI medium, their colonies could easily be distin-

guished, by color and shape, from klebsiellae. The percentage of inositol-negative *Klebsiella* strains (7%) among the National Collection of Type Cultures strains and other stored strains was higher than that found earlier (4), whereas no inositol-negative *Klebsiella* colonies appeared from fresh fecal samples. This suggests that the inositol-fermenting capacity of some strains may be lost or suppressed upon long-term storage.

Based on the API 20E pattern (Fig. 5), several other types of *Enterobacteriaceae* were expected to grow on SCAI; the majority, however, were expected to grow as blue colonies due to citrate utilization. Indeed, most of the non-*Klebsiella* strains found in the 109 tested fecal samples formed blue colonies (Fig. 2). On the other hand, apart from some *Enterobacter* and *Citrobacter* strains, most of the other inositol-positive *Enterobacteriaceae* (Fig. 5) are relatively rare inhabitants of the gut and were, therefore, not found in the tested fecal samples (Fig. 2). The relatively high percentage of yellow *E. coli* strains found can at least partly be explained by the pH-lowering effect of large numbers of other inositol-fermenting strains (e.g., *Klebsiella* spp.) simultaneously present, which turn the whole medium yellow, including all bacterial colonies. But these *E. coli* strains and those that are capable of growing on inositol can clearly be distinguished from *Klebsiella* colonies and do not lead to confusion. So far, the only type found mimicking *Klebsiella* colonies was *Enterobacter aerogenes*, but this occurred rarely.

Two subtypes of *K. pneumoniae*: *K. ozaenae* and *K. rhinoscleromatis*, show lower percentages of inositol or citrate utilization. Therefore, the SCAI plate is not a reliable medium for epidemiological studies on these types. But as they are probably very rarely found in the human bowel, this will not influence studies on *Klebsiella* spp. in human feces.

Measuring the selective capacity of SCAI revealed that a single *Klebsiella* colony could easily be detected among  $10^6$  *E. coli* isolates, whereas on McC, the limiting ratio appeared to be 1:10<sup>2</sup>.

The SCAI plate was further compared with the two solid media used by Cooke et al. (1). Their complete method, however, included two liquid enrichment media, MIC broth and Koser citrate, which were in turn subcultured on MIC and SCA plates as described by R. Shinebaum and E. M. Cooke (in C. H. Collins and J. M. Grange, ed., Society of applied bacteriology. Technical series [no. 21], in press.) At present we are trying to develop an enrichment medium based on the same principle as the SCAI plate, but in some instances the low pH resulting from inositol fermentation causes autosterilization of the *Klebsiella* culture. This problem will be further investigated. From the results of the present comparative study, we concluded that the SCAI plate is probably as sensitive as the combination of MIC and SCA, but the SCAI method is about twice as efficient as Cooke's method.

Comparison with conventional media showed a different picture. In an epidemiological study (unpublished data), it was shown that in 53% of feces samples, *Klebsiella* could be demonstrated with the SCAI method (Fig. 4). As fecal samples contain an average of ca.  $10^7$  to  $10^8$  CFU of *E. coli* per g and the limiting ratio of *Klebsiella* spp./*E. coli* on BA or McC was shown to be 1:10<sup>2</sup> to 1:10<sup>3</sup>, the margin of the number of klebsiellae detectable on the latter two media would average  $10^5$  CFU/g of feces. Thus, as shown in Fig. 4, only about 11% of the total number of stools would have been positive with BA or McC. This is in accordance with the results of Degener et al. (3) who found 8 to 12%

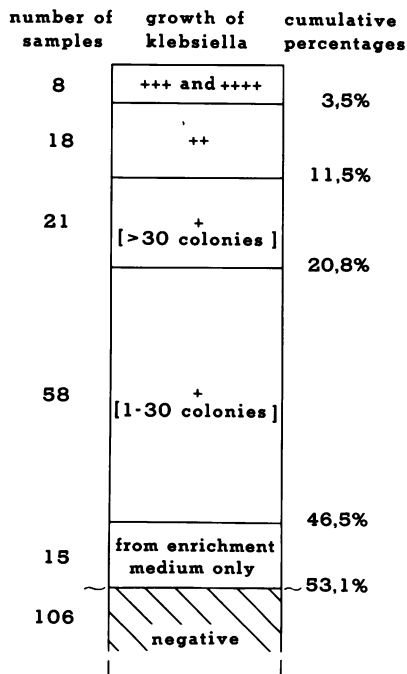


FIG. 4. Numbers and cumulative percentages of *Klebsiella*-positive fecal samples from 226 outpatients, arranged by the amount of growth of *Klebsiella* spp. on SCAI. Of 226 tested samples, 120 (53.1%) contained *Klebsiella* spp. One colony represents about 100 CFU per g of feces, and two streaks or more showing growth correspond to  $10^5$  CFU per g or more (see text). + to + + + +, Number of streaks showing growth of *Klebsiella* spp.

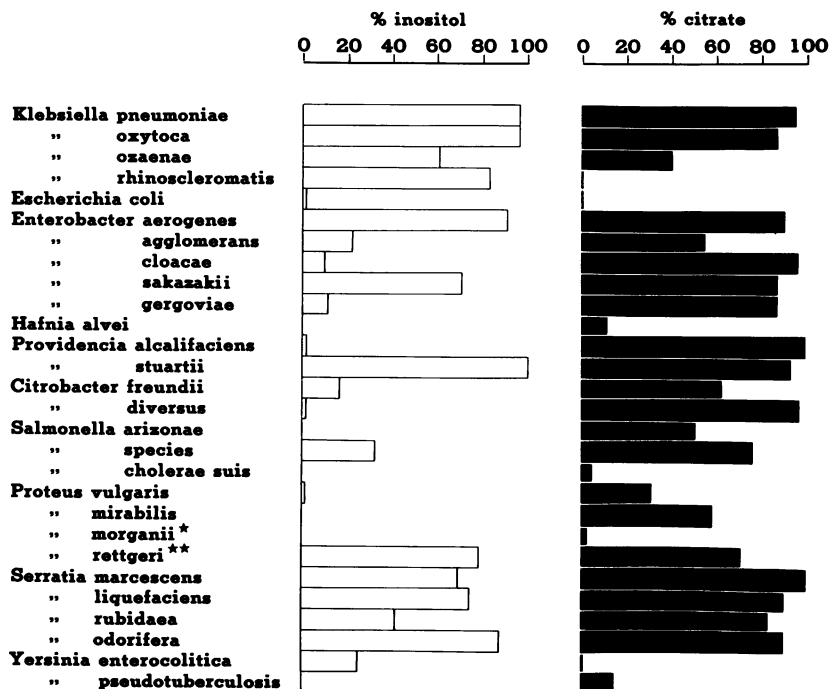


FIG. 5. Percentages of *Enterobacteriaceae* capable of fermenting inositol (open columns) or utilizing citrate as the sole carbon source (closed columns). Results are taken from API 20E. ★, More recently called *Morganella morganii* (7); ★★, more recently called *Providencia rettgeri* (7).

*Klebsiella*-positive fecal samples in similar age groups, using the conventional McC plates.

SCAI does not contain antibiotics like carbenicillin to inhibit the growth of *E. coli*. In an extensive comparative study, O'Brien et al. demonstrated considerable geographical differences in the carbenicillin resistance of *Klebsiella* strains, varying from 80 to 95% (10). Therefore, even with the relatively low carbenicillin concentration of 10 mg/liter, the chance of missing susceptible strains exists. Another disadvantage of  $\beta$ -lactam antibiotic-containing media is the short time they can be stored at 4°C due to degradation of the antibiotic. SCAI, however, can be stored at 4°C for at least 2 weeks without noticeable loss of its properties.

In conclusion, the SCAI plate combines the two selective carbon sources of both media of Cooke's method in one agar plate without additional carbon sources or antibiotics. The selectivity of the SCAI plate for *Klebsiella* spp. equals that of the combined MIC and SCA plates, and the SCAI medium is more efficient. These properties of the SCAI plate make it a valuable tool in studies on the epidemiology of *K. pneumoniae* and *K. oxytoca*, especially in those studies concerning hospital-acquired infections and prevention of infections in immunocompromised patients.

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