Comparative evaluation of three chromogenic agars for detection and rapid identification of aerobic Gram-negative bacteria in the normal intestinal microflora

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Objective To compare three different chromogenic agars and MacConkey agar for the detection of aerobic Gram-negative bacteria in the normal intestinal microflora and to assess the accuracy of the chromogenic agars for the direct identification of *Escherichia coli*.

Methods A total of 164 Gram-negative clinical isolates (*E. coli*, *Proteus*, *Klebsiella*, *Enterobacter*, *Morganella* and *Pseudomonas* species) and 30 stool specimens were inoculated in parallel on four media: Chromagar *E. coli*/Coliform, Chromogenic urinary tract infection UTI medium, CHROMagar Orientation and MacConkey agar. All colonies that differed by color and/or morphology were selected for further identification by VITEK 1 and/or API 20E from each medium.

Results On *E. coli*/Coliform agar five out of 32 (16%) *E. coli* strains failed to produce the color as described by the manufacturer. No remarkable discrepancies were found for the other clinical isolates. There was no significant difference in detection rate (DR) of aerobic Gram-negative bacteria in stool specimens between the different chromogenic agars and MacConkey agar. The overall DR was about 84%, and varied from 100% for monomicrobial specimens to 33% for polymicrobial specimens. The positive predictive values (PPV) for the direct identification of *E. coli* on Chromagar *E. coli*/Coliform, Chromogenic UTI medium and CHROMagar Orientation were 1.00, 0.93 and 0.93, respectively. The negative predictive values (NPV) were 0.53, 0.68 and 0.69, respectively.

Conclusion Chromogenic UTI medium and CHROMagar Orientation are the preferred media because of the higher NPV. The high PPV of these agars allows accurate and rapid identification of *E. coli*.

Keywords Chromogenic media, normal intestinal microflora, stool specimens, Gram-negative bacteria, *Escherichia coli*, identification, detection

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INTRODUCTION

MacConkey agar is the most widely used medium in the microbiological laboratory for the isolation and differentiation of *Enterobacteriaceae*. This medium differentiates aerobic Gram-negative bacteria by detecting lactose utilization with a neutral red indicator. As a result of the absence of any differential genus-specific indicator property in the MacConkey agar, mixed Gram-negative cultures are not always detected. Several chromogenic agars, which have been available for some years, allow the presumptive identification of aerobic Gram-negative bacteria on the basis of colony morphology and distinctive color patterns [1–6].

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By means of chromogenic substrates incorporated into the agar, chromogenic agar plates reveal genus- or species-specific enzyme activities of micro-organisms. Thus, these media may allow better discrimination of the different species in mixed cultures, resulting in greater sensitivity of detection compared to MacConkey agar.

The present study was conducted in the course of a large epidemiological study of antibiotic resistance among bacteria present in stool specimens of hospitalized patients. The purpose of this study was to compare three different chromogenic agars and MacConkey agar for the detection of aerobic Gram-negative bacteria in the normal intestinal microflora. As *E. coli* is the dominant aerobic Gram-negative species in the gut, the accuracy of the chromogenic agars for the direct identification of *E. coli* was assessed.

MATERIALS AND METHODS

Culture media

Three chromogenic culture media (Chromagar *E. coli*/Coliform (Oxoid, Basingstoke, UK), Chromogenic UTI medium (Oxoid) and CHROMagar Orientation (Becton Dickinson, Heidelberg, Germany) were compared. Chromagar *E. coli*/Coliform and Chromogenic UTI medium were prepared from the dehydrated media according to the manufacturers' instructions. CHROMagar Orientation was received as ready-made agar plates. MacConkey agar (Becton Dickinson) served as the reference medium.

Determination of colony color

The identification of micro-organisms is based on chromogenic substrates incorporated into the media to detect certain bacterial enzymes. The respective organisms react with the chromogenic agents in such a way that the colonies take on a characteristic color and colony morphology. Chromogenic *E. coli*/Coliform medium is supplemented with two different chromogenic agents: one allows for the detection of β-glucuronidase and releases a blue dye, whereas the other chromogen detects the presence of β-galactosidase and releases a pink to red dye. Chromogenic UTI medium and CHROMagar Orientation are also supplemented with two chromogenic agents which are cleaved by the enzymes β-galactosidase (pink to red dye) and β-glucosidase (blue to blue-green dye). Additionally, tryptophan deaminase, an enzyme characteristically found in the Proteus-Morganella-Providencia (PMP) group of organisms is detected on the latter two media by the production of a brownish, diffuse pigment that stains the medium around the respective colonies.

Bacterial strains

A total of 164 isolates (*E. coli* ($n = 32$), *Klebsiella* spp. ($n = 28$), *Enterobacter* spp. ($n = 27$), *M. morganii* ($n = 26$), *Proteus* spp. ($n = 21$) and *P. aeruginosa* ($n = 30$)) were screened for their colony colors on the three chromogenic agars. These clinical isolates, obtained from blood cultures from hospitalized patients, were identified according to local standard operating procedures and stored at −80 °C. After subculturing on Colombia blood agar, the isolates were inoculated in parallel on the three chromogenic media by the same technician and incubated aerobically at 35 ± 2 °C. The color of the isolates was determined after 18–24 h and compared with the color catalog of the manufacturers.

Stool specimens

Thirty stool specimens were used for the evaluation of the three chromogenic media. The stool specimens were collected from surgical patients on admission to the hospital. The stool specimens were inoculated in parallel on the three chromogenic media and on the MacConkey agar. All four plates were inoculated by the same technician, with 50 μL of 10^{-2} and 10^{-4} dilutions of the stool specimens in physiological saline. The plates were incubated aerobically at 35 ± 2 °C and examined after 18–24 h for the colony characteristics. Dilutions with the highest variety of different colonies were included in the final analysis. From each medium, all colonies that differed by color and/or morphology were selected for further identification. A description of colony appearance was recorded. At least three colonies were selected from the MacConkey medium. When no differences in color and/or morphology were observed these three colonies were randomly selected. After microscopic examination (Gram-stain) all colonies were identified by VITEK 1 (Bio-Mérieux, Marcy l'Etoile, France) or API 20E system (Bio-Mérieux). The accuracy of the presumptive identification of

E. coli on the basis of colony color is described in terms of positive and negative predictive values.

RESULTS

Bacterial strains

Table 1 demonstrates the color reactions for the 164 clinical isolates on the three different chromogenic agars. On *E. coli*/Coliform agar five out of 32 *E. coli* strains failed to produce a purple color. These five isolates produced colorless colonies and were β-glucuronidase negative as tested with the API 20E system. On both the Chromogenic UTI and the CHROMagar Orientation medium, the *Proteus vulgaris* strains produced blue colonies, except for two strains that did not grow or yield colorless/beige colonies. These two strains also did not grow on the *E. coli*/Coliform agar. Compared to the *E. coli*/Coliform medium, the Chromogenic UTI and CHROMagar Orientation have the advantage of inhibiting swarming of *Proteus* spp. No major discrepancies with the color catalogues of the manufacturers were found for the other clinical isolates.

Stool specimens

The total number of Gram-positive and negative strains isolated from the Chromagar *E. coli*/Coliform, the Chromogenic UTI medium, the CHROMagar Orientation and the MacConkey agar were 84, 83, 91 and 93, respectively. After identification tests were performed for all isolates, the total number of different Gram-negative micro-organisms was determined for each of the 30 stool specimens. One specimen yielded no growth of Gram-negative micro-organisms on all four media and 29 specimens yielded growth on at least one of the four media. Fifteen stool specimens yielded pure cultures of Gram-negative bacteria and 14 mixed cultures.

Table 2 shows the distribution of Gram-negative micro-organisms. *E. coli* was the dominant isolate. A total of 47 Gram-negative micro-organisms were isolated on at least one medium. Two stool cultures yielded *Acinetobacter* spp. For one specimen this micro-organism was only detected on MacConkey; from the other specimen the species were detected both on the MacConkey agar and the Chromagar *E. coli*/Coliform. No significant differences in detection rate (DR) of Enterobacteriaceae in stool specimens were found between the chromogenic agars and the MacConkey agar. The overall DR was 84% (Table 2), and varied from 100% for monomicrobial specimens to 33% for polymicrobial specimens (Table 3).

A total of 25 Gram-positive micro-organisms were isolated on at least one medium. The DR of Gram-positive micro-organisms on the Chromagar *E. coli*/Coliform, the Chromogenic UTI medium, the CHROMagar Orientation and the MacConkey agar were 48%, 64%, 76% and 44%, respectively.

Table 1 Evaluation of color reactions of 164 clinical Gram-negative isolates on three chromogenic media

	Number of isolates with colors as described[a]/ total number of isolates[b]		
Micro-organism	**Chromagar *E. coli*/Coliform**	**Chromogenic UTI medium**	**CHROMagar Orientation**
Enterobacter aerogenes	13/13	13/13	13/13
Enterobacter cloacae	13/14	14/14	14/14
Escherichia coli	27/32	32/32	32/32
Klebsiella pneumoniae	28/28	28/28	28/28
Morganella morganii	26/26	26/26	26/26
Proteus mirabilis	13/13	13/13	12/13
Proteus vulgaris	6/8	6/8	6/8
Pseudomonas aeruginosa	30/30	30/30	30/30
Total	156/164 (95%)	162/164 (99%)	163/164 (99%)

[a]The colors as described by the manufacturers of Chromagar *E. coli*/Coliform, Chromogenic UTI medium and CHROMagar Orientation, respectively, were as follows: *Enterobacter* spp., pink/blue/blue; *E. coli*, purple/pink/pink; *K. pneumoniae*, pink/blue/blue; *M. morganii*, colorless/brown/brown; *Proteus mirabilis*, colorless/brown/brown; *Proteus vulgaris*, colorless/blue/blue; *P. aeruginosa*, straw/fluoresce/fluoresce.
[b]After 18–24 h of incubation at 37 °C.

Table 2 Distribution of Gram-negative bacteria recovered from 30 stool specimens on different media

	Detected on any medium[a]	Chromagar *E. coli*/Coliform[b]	Chromogenic UTI medium[b]	CHROMagar Orientation[b]	MacConkey[b] agar
Acinetobacter spp.	2	1	–	–	2
Citrobacter spp.	4	4	1	3	2
Escherichia coli	27	26	27	27	26
Enterobacter spp.	3	1	2	1	3
Hafnia alvei	2	–	1	–	1
Klebsiella spp.	7	6	6	6	5
Morganella morganii	1	1	1	–	–
Serratia spp.	1	1	1	1	1
Total	47	40	39	38	40
Detection rate		85%	83%	81%	85%

[a]Total number of strains detected on at least one of the four media.
[b]No significant difference (Chi-square; $P = 0.4$).

Presumptive identification of *E. coli* on the basis of colony color

After 18–24 h incubation, it was easy to discriminate between Gram-positive cocci and all other micro-organisms on the basis of morphology alone. Gram-positive cocci grew as very small colonies and were excluded from the analysis of the accuracy of the identification of *E. coli* based on colony color. The remaining number of micro-organisms isolated from Chromagar *E. coli*/Coliform, Chromogenic UTI medium and CHROMagar Orientation were 58, 47 and 54, respectively. The accuracy of the three chromogenic media to identify *E. coli* by means of purple or pink colonies is reported in Table 4. On the *E. coli*/Coliform medium 15 of the 41 isolated *E. coli* strains did not produce a purple color. On the Chromogenic UTI medium and CHROMagar Orientation the false-negative rates were six out of 32 strains and eight out of 34 strains, respectively. The false positive rates were very low for the three chromogenic media. The positive predictive values (PPV) for the direct identification of *E. coli* on Chromagar *E. coli*/Coliform, UTI and Orientation were 1, 0.93 and 0.93, respectively. The negative predictive values (NPV) were 0.53, 0.68 and 0.69, respectively.

DISCUSSION

Infections caused by Gram-negative bacteria continue to be a major problem for hospitalized patients. According to the data from the National Nosocomial Infections Surveillance System, Gram-negative bacteria are the leading cause of nosocomial infections in the United States [7]. The increase in antimicrobial resistance of these micro-organisms demands effective surveillance programs. Screening for resistant bacteria in the fecal flora seems relevant for two reasons. Firstly, the gastrointestinal tract may be the reservoir of bacteria that cause clinical infections [8]; secondly, fecal bacteria, whether they cause infections or not, might act as a reservoir of mobile resistance genes [9]. Different methodologies have been used for the screening of stool specimens for antimicrobial resistant strains. Österblad *et al*. [10] compared two frequently applied methods: (1) the replica plating method, i.e. the comparison of growth on antibiotic-free and antibiotic containing plates,

Table 3 Detection rates of aerobic Gram-negative bacteria in mono- and polymicrobial stool specimens

	Number of stool specimens (detection rate)		
	1 micro-organism ($n = 15$)	**2 micro-organisms ($n = 8$)**	**3 micro-organisms ($n = 6$)**
Chromagar *E. coli*/Coliform	15 (100%)	4 (50%)	1 (17%)
Chromogenic UTI medium	15 (100%)	3 (38%)	1 (17%)
CHROMagar Orientation	15 (100%)	2 (25%)	2 (33%)
MacConkey agar	15 (100%)	4 (50%)	1 (17%)

Table 4 Accuracy of presumptive identification of *E. coli* on the basis of colony color

	Number of *E. coli* strains		
	Chromagar *E. coli*/Coliform	**Chromogenic UTI medium**	**CHROMagar Orientation**
True positive strains[a]	26	26	26
False-negative strains	15	6	8
White colonies	4	3	6
Pink colonies	11	–	–
Transparent colonies	–	1	2
Blue colonies	–	2	–
False-positive strains	–	2	2
Citrobacter freundii	–	1	–
Hafnia alvei	–	1	–
Gram-positive rod	–	–	2
True negative strains	17	13	18
Positive predictive value	1	0.93	0.93
Negative predictive value	0.53	0.68	0.69

[a]The colors as described by the manufacturers' of Chromagar *E. coli*/Coliform, Chromogenic UTI medium and CHROMagar Orientation, respectively, were purple, pink and pink.

and (2) the selection of five colonies with different appearances from the MacConkey medium which are subsequently tested for resistance by an agar-dilution method. The rate of resistance detection by these two methods did not differ statistically for any of the antibiotics tested. In most studies, fecal isolates were only classified on the basis of colony morphology and Gram stain [11,12]. Other studies focused only on *E. coli* as the indicator bacterium of the intestinal flora [13,14]. As the prevalence of resistance varies between species, it is essential to discriminate between the different Gram-negative bacteria [15]. In the present study three chromogenic agars were compared with the MacConkey agar for the detection of aerobic Gram-negative fecal cultures. No significant differences in overall detection rate (DR) of Enterobacteriaceae in stool specimens were found between the chromogenic agars and the MacConkey agar. To our knowledge DR of Enterobacteriaceae in stool specimens are not known from other studies using chromogenic media. The DR corresponded well, however, to the rates of urinary tract pathogens described in previous studies using these media [1,2]. Hengstler *et al.* did not find any differences in the recovery of Gram-negative bacteria from urine specimens between the two chromogenic media and the MacConkey agar [2]. In another study five chromogenic media were compared for the detection of urinary tract pathogens. The DR in monomicrobial specimens varied from 94 to 100% (361 samples), in polymicrobial specimens from 90 to 96% (94 samples) [1]. In our study on stool specimens the DR for monomicrobial specimens was 100% and for polymicrobial specimens only 17–50% (Table 3). Although the sample size in our pilot study was small it appears that in these polymicrobial samples the dominant bacteria were always detected on the MacConkey agar and on the three chromogenic media. Micro-organisms that were not detected on either one of the four media were often present at very low colony counts. Therefore, chromogenic media and the MacConkey agar are equally accurate when the objective is to study the dominant micro-organisms in the fecal flora.

Although no difference in detection rate was found, the easy recognition of simultaneous growth of multiple organisms on colony color can be advantageous as can be explained by the following example. In a study of antimicrobial resistance using the '5 colony picking method' [10], one can define an algorithm for the selection of colonies from the agar. If, for example, 80 pink colonies and 20 blue colonies grow on a chromogenic agar, the proposed algorithm would result in selecting four pink and one blue colony for the antimicrobial resistance testing. This method leads to a proportional sampling of the dominant flora that is not possible with MacConkey agar.

In previous studies on antibiotic resistance about 60% of the isolated Gram-negative bacteria from stool specimens were identified as *E. coli* [4]. Accurate identification of *E. coli* on colony color

may considerably reduce time and costs of fecal screening programs. In our study the Chromagar *E. coli*/Coliform showed a high rate of false negative strains; 15/41 (37%) strains did not react with the chromogenic substrate. From other studies it is reported that about 95–98% of *E. coli* isolates produce β-glucuronidase [16,17]. Carricajo and Hengstler found percentages from 84 to 91% [1,2]. Chromogenic media supplemented with a chromogenic agent that interacts with β-glucuronidase, as in the Chromagar *E. coli*/Coliform, are therefore not accurate for rapid identification of *E. coli* owing to the low sensitivity. However, the high sensitivity of the other media combined with a high PPV often leads to a rapid and correct detection of *E. coli*.

Standard inocula for antimicrobial susceptibility testing are usually prepared by subculturing colonies from the chromogenic media and MacConkey agar to blood-based media or Mueller–Hinton agar. Samra *et al*. compared the accuracy of antimicrobial susceptibility testing by picking isolates from the CHROMagar Orientation with the technique of picking isolates from MacConkey agar [5]. The antimicrobial susceptibilities of these isolates were determined by the disk diffusion technique according to the National Committee for Clinical Laboratory Standards (NCCLS). The numbers of susceptible isolates were identical. These investigators did not observe significant differences between the numbers of intermediate and resistant Gram-negative isolates. From the study of Samra *et al*. we conclude that the chromogenic substances do not interfere with the susceptibility test results.

In summary, chromogenic agars are reliable media for the detection of aerobic Gram-negative bacteria in the normal intestinal microflora. The easier recognition of different colonies on these media in particular, is a major advantage. The Chromogenic UTI medium and the CHROMagar Orientation are the preferred media because of higher negative predictive values. The high positive predictive values of these chromogenic agars allows accurate and rapid identification of *E. coli*.

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