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Evaluation of Chromocult coliform agar for the detection and enumeration of Enterobacteriaceae from faecal samples from healthy subjects

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Abstract

The purpose of this study was to examine the use of Chromocult agar medium for isolation and enumeration of Enterobacteriaceae from human faecal samples, to compare it to MacConkey agar and to evaluate its usefulness as a possible alternative selective medium in human faecal studies. The medium was shown to be effective in identifying *Escherichia coli* and coliforms in faeces without the need for extensive accompanying biochemical tests for confirmation of identity. A positive correlation ($r = 0.86$) was found between the recovery of Enterobacteriaceae on the two media, and no significant difference ($P > 0.05$) between overall mean bacterial counts for the whole study group or at different intervals of faecal collection were observed. Chromocult agar is an effective replacement for MacConkey agar in human faecal studies and has the advantage of differentiating *E. coli* from other coliforms.

Keywords: Chromocult; Coliforms; *Escherichia coli*; Enumeration; Enterobacteriaceae; Faecal flora

1. Introduction

The microflora in the human large intestine is thought to comprise more than 400 different bacterial species (Finegold et al., 1974; Moore and Holdeman, 1974), with approximately 10^{11}–10^{12} bacteria present for every gram of gut contents (Cummings and Macfarlane, 1991). Intestinal bacteria may be broadly divided into species that are either harmful or beneficial towards the health of the host (Gibson and Roberfroid, 1995). Members of the Enterobacteriaceae family form a very large group of morphologically and physiologically similar bacteria (Cowan and Steel, 1993). The Enterobacteriaceae, particularly *Escherichia coli*, are considered to be characteristic intestinal bacteria although they are not major members of the human intestinal flora. (Dufour, 1977). Other members of the group include *Citrobacter* spp., *Enterobacter* spp. and *Klebsiella* spp., and together they form part of the facultatively anaerobic bacterial flora. Although the majority are nonpathogenic (Sal-

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minen et al., 1995), some are of great clinical significance as important pathogens for man and cause intestinal and other infections.

Interest in the intestinal microflora of humans has been stimulated in recent years by the development and marketing of preparations of living microbial cells and food ingredients that, when consumed, are believed to influence the composition of the intestinal microflora and to have a beneficial effect on the health of the host. These preparations are known as "prebiotics" and "probiotics", respectively (Fuller, 1989). Obtaining positive scientific validation of the use of these preparations requires the use of culture techniques to identify and enumerate intestinal species of interest from faecal or intestinal material (O'Sullivan, 1999). The bacteria present in the large distal bowel are often inferred from the bacteria present in faecal material hence studies of human intestinal microflora usually involve analyses of the microflora in faecal samples (Moore et al., 1978).

MacConkey agar has been widely used in the last 20–30 years for counting and isolation of Enterobacteriaceae and coliforms, and it is still the medium of choice in many microbiology laboratories (de Boer, 1998; O'Sullivan, 1999; Tannock, 1999). Lactose and the pH indicator neutral red are incorporated into the medium to allow differentiation between lactose-fermenting and non-lactose-fermenting Enterobacteriaceae, whilst Gram-positive cocci and non-enteric organisms are inhibited by crystal violet and bile salts. Chromocult agar was developed for the simultaneous detection of total coliforms and *E. coli* due to the inclusion of two chromogenic substrates: Salmon-GAL for detection of total coliforms by production of β-galactosidase and X-glucuronide for the detection of *E. coli* by the production of β-glucuronidase. Gram-positive organisms and some non-enteric bacteria are inhibited by Tergitol-7. Additional confirmation of isolation of *E. coli* can be made by the detection of indole due of the inclusion of trytophan in the medium, thereby increasing the reliability of identification of *E. coli*.

The purpose of this study was to examine the use of Chromocult agar medium for isolation and enumeration of Enterobacteriaceae from human faecal samples and to evaluate its use as a possible alternative selective medium to MacConkey agar in human faecal studies.

2. Material and methods

2.1. Media

Chromocult® coliform agar (Chromocult; Merck, UK) and MacConkey agar No. 3 (MacConkey; Oxoid-Unipath, UK) were prepared in accordance with the manufacturers instructions.

2.2. Faecal samples and preparation

Faecal samples from 75 subjects were obtained once weekly for 3 weeks (225 faecal samples in total). Subjects were healthy, nonvegetarian, with no history of either gastrointestinal or metabolic disorders, and were not subject to any dietary restrictions, prescribed diets or supplementary fibre intake. Subjects had not received antibiotics, steroids or any drugs for 6 months prior to the study. Faecal samples were collected into sterile bags (GENbag anaer™, Bio Mérieux, France) and an anaerobic sachet was added to the bag which was then sealed with a clip. Samples were then brought immediately into the laboratory. Upon receipt, faecal samples were transferred into an anaerobic cabinet (Compact M, Don Whitley Scientific, UK) supplied with 80% nitrogen, 10% carbon dioxide, 10% hydrogen (BOC, UK). All subsequent procedures were performed under anaerobic conditions.

Faeces (1.0 g) were weighed, transferred into a universal containing 9.0 cm^3 sterile, pre-reduced buffered peptone water (RBP; Oxoid-Unipath) and homogenized using a sterile swab. Serial dilutions of the homogenized samples were prepared using RBP. Media were then inoculated in triplicate with 0.1 cm^3 appropriately diluted homogenized faecal sample. Media were incubated aerobically for 24 h at 37 °C.

2.3. Identification and enumeration

Organisms were characterized by colony morphology, Gram stain, indole production (Chromocult agar only) and oxidase activity. For colonies on Chromocult agar, the following criteria were used for identification: dark blue- to violet-coloured colonies were considered to be *E. coli*, salmon-coloured colonies were considered to be Enterobacteriaceae classified as coliforms, colourless colonies were considered Enterobacteriaceae not classified as coliforms. For colonies on

MacConkey agar, the following criteria were used for identification: Enterobacteriaceae classified as coliforms were considered to be pink-coloured colonies (lactose fermenters; LF), Enterobacteriaceae not classified as coliforms were considered to be colourless colonies (non-lactose fermenters; NLF). Following preliminary identification based on the above criteria, 151 isolates from both media were randomly picked and confirmation of their identity was established by use of biochemical identification using the Vitex Gram-negative identification system (Bio Mérieux). Mean numbers of colonies confirmed as Enterobacteriaceae grown on both media were calculated for all 225 samples and results expressed as colony-forming units (g wet weight faeces)$^{-1}$. Oxidase positive colourless colonies recovered on both media were considered to be *Pseudomonas* spp. and were not counted.

2.4. Statistical analysis

The numbers of Enterobacteriaceae were converted to $\log_{10}$ values and expressed as $\log_{10}$ CFU (g wet weight faeces)$^{-1}$. Results were analyzed by linear regression to verify the linearity of the relationship between numbers of Enterobacteriaceae recovered on both media. The Student's *t*-test was used to compare the overall mean values on both media. One-way analysis of variance (ANOVA) was performed to compare means on both media over the 2-week study period. Statistical significance was taken as $P < 0.05$.

3. Results

One hundred and fifty-one colonies were selected from Chromocult and MacConkey agars and identified. The results (Table 1) showed that 143/151 (94.70%) of isolates were coliforms (*Citrobacter* spp., 7; *Escherichia* spp., 94; *Enterobacter* spp., 19; and *Klebsiella* spp., 23), 6/151 (4.0%) were non-coliforms (*Hafnia* spp., 2; *Kluyvera* spp., 1; *Morganella* spp., 2; and *Salmonella* spp., 1) and 2/151 (1.30%) were non-Enterobacteriaceae (*Pseudomonas* spp., 2). Of the 143 coliforms, 94 (65.7%) were identified as *E. coli* and 49 (34.3%) were non-*E. coli*.

Table 1
Faecal Enterobacteriaceae identified on Chromocult and MacConkey agar media

Organisms	Number	%	*E. coli*	Non-*E. coli*
Faecal coliforms	143	94.7	65.7	34.3
Non-coliform Enterobacteriaceae	6	4.0	–	–
Non-Enterobacteriaceae	2	1.3	–	–

One hundred and fifty-one colonies from both media were identified and the proportions of each group are represented as percentage values.

Table 2
Accuracy of identification of faecal Enterobacteriaceae on Chromocult and MacConkey agar

	Chromocult agar number (%)	MacConkey agar number (%)
Positive for coliforms	71 (88.75)	62 (87)
False positive	7 (8.75)	9 (13)
False negative	2 (2.5)	ND

Following preliminary identification on their respective media, colony identity was confirmed for the 151 isolates using a biochemical profile system. Correct preliminary and confirmed identity resulted in a positive result. Discrepancies in confirmed results and preliminary identification resulted in either a false positive or false negative result. ND—false negatives were not determined for MacConkey agar.

False positive and negative identification was based upon discrepancies in preliminary identification (based on preliminary identification of colony colour, indole and oxidase reaction) followed by confirmed biochemical identification (Table 2). Of the 80 isolates on Chromocult agar, 71 (88.75%) were correctly identified, 2 (2.5%) of the isolates recovered were false positive for *E. coli* (violet/blue-coloured *Citrobacter* spp., 1, and *Enterobacter* spp., 1) and 7 (8.75%) of the isolates recovered were false negative for non-*E. coli* (salmon-coloured *E. coli*.). From the 71 isolates recovered on MacConkey agar, 62 (87%) were correctly identified and 9 (13%) of the recovered isolates were false negative for coliforms (NLF *Enterobacter* spp., 2; *E. coli*, 4; and *Klebsiella* spp., 3).

From the 225 faecal samples, mean $\log_{10}$ CFU (g wet weight faeces)$^{-1}$ were obtained from triplicate assays on each sample for both media. A regression analysis of the mean counts of Enterobacteriaceae on Chromocult and MacConkey agar is shown in Fig. 1. There was a strong linear relationship between the two media (slope = 1.0, $r = 0.86$). There was no significant difference between mean values of Enterobacteriaceae on both media (Student's *t*-test, $P > 0.05$).

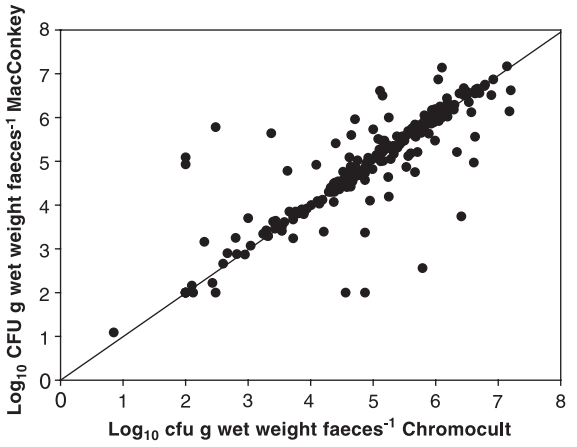

Fig. 1. Relationship of number of faecal Enterobacteriaceae on Chromocult and MacConkey agar. Faecal Enterobacteriaceae were determined on Chromocult and MacConkey agars on faecal samples from 75 individuals taken at weekly intervals for 2 weeks ($n=225$). Mean counts are represented as $\log_{10}$ CFU (g wet weigh faeces)$^{-1}$. Linear regression analysis was used to determine the relationship between mean counts on both media. There was no significant difference between mean counts on the two media (Student's t-test, $P<0.05$).

The mean ± S.E.M. $\log_{10}$ CFU (g wet weight faeces)$^{-1}$ obtained for faecal Enterobacteriaceae isolated on Chromocult and MacConkey agar media for the three sample days are shown in Fig. 2. Changes in mean values for both media between sample days occurred over the study period and the mean values increased. However, increases occurred on both media and the results of ANOVA showed no significant difference ($P>0.05$) in mean values obtained on the two media over the study period.

4. Discussion

The results of this study indicate the suitability of Chromocult agar as an alternative to MacConkey agar for the identification and enumeration of human faecal Enterobacteriaceae without the need for further biochemical tests for confirmation of identity. There was comparable recovery of bacterial numbers from the 225 faecal samples assayed and there was no significant difference ($P<0.05$) between numbers recovered on the two media. The results showed considerable variations in mean bacterial numbers recovered from the subjects on different days of the study. This observation confirms that the concentration of faecal bacterial groups can vary between individuals within a given group and that the faecal flora varies even in the same individual (Moore et al., 1978). However, mean numbers of Enterobacteriaceae obtained were within previously reported ranges obtained from human faeces (Drasar et al., 1969; Simon and Gorbach, 1984). The results of identification of randomly selected isolates were consistent with previous reports that *Escherichia*, together with *Citrobacter*, *Enterobacter* and *Klebsiella* at much lower densities, are the main genera of faecal coliforms (Dufour, 1977; Grant, 1997; Alonso et al., 1998).

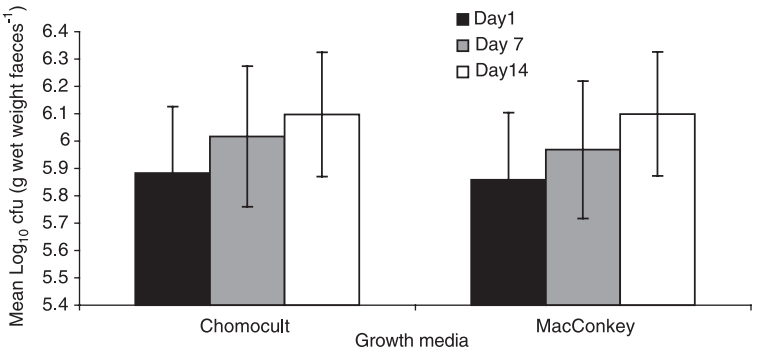

Fig. 2. Mean ± S.E.M. faecal Enterobacteriaceae isolated on Chromocult and MacConkey agar over a 2-week period. Faecal coliforms content of faecal samples from healthy volunteers were determined using Chromocult coliform and MacConkey agars. Mean $\log_{10}$ CFU g (wet weight faeces)$^{-1}$ from both media are represented on the respective sample days ($n=75$ per day). There were no significant differences between mean counts on both media for the respective sample days (ANOVA, $P>0.05$).

A relatively small number (11.25%) of the colonies that had been preliminary identified were shown to be either false positive for β-glucuronidase activity or false negative for β-galactosidase activity on Chromocult agar. Two non-*E. coli* strains (*Citrobacter* spp., 1, and *Enterobacter* spp., 1) exhibited β-glucuronidase production and appeared as violet/blue colonies on Chromocult agar. Other authors have also reported β-glucuronidase activity in small numbers of non-*E. coli* coliforms (Pérez et al., 1986; Sarhan and Foster, 1990; Gauthier et al., 1991; Alonso et al., 1996, 1999; Geissler et al., 2000). During the study, 8.75% of *E. coli* (7 strains) were shown to be false negative exhibiting no β-glucuronidase activity on the Chromocult agar, which is in agreement with most previous reports.

On MacConkey agar, 13% of the isolated colonies were confirmed as false negative following identification (NLF *E. coli*, 4; *Enterobacter*, 2; and *Klebsiella*, 3). Dufour (1977) reported that some strains of *E. coli* were unable to ferment lactose, resulting in a false negative reaction. Other authors have reported lactose-negative *E. coli* strains incubated at 41 °C, however, when re-cultured at 37 °C were shown to be lactose-positive (Alonso et al., 1999). Although lactose-negative coliforms are infrequent in clinical and environmental samples, their potential presence must be considered when employing a medium containing no other selective substrates to distinguish them.

One clear advantage of the use of Chromocult medium during this study was the ability to easily distinguish *E. coli* from total coliforms and non-coliform by colony colour. Although *E. coli* colonies appeared flat and pink whereas other coliforms (*Citrobacter* spp., *Enterobacter* spp. and *Klebsiella* spp.) had pink mucoid colonies on MacConkey agar (results not shown), this distinction was subjective and not considered a reliable enough distinguishing characteristic. The spot-indole test performed on colonies from MacConkey agar may give false negative results (Miller and Wright, 1982). For this reason, lactose-fermenting colonies on MacConkey agar could not be reliably confirmed as *E. coli* using this method. The selectivity of the two media was demonstrated throughout this study with the only non-Enterobacteriaceae isolated being *Pseudomonas* spp., which, although not present in large numbers, could easily be distinguished by a positive oxidase reaction. This differentiation procedure was easy to perform and gave an immediate result which then allowed the rapid and accurate enumeration of faecal Enterobacteriaceae to be made.

Chromocult agar allowed the rapid enumeration of faecal Enterobacteriaceae and had the advantage over MacConkey medium of separately enumerating faecal *E. coli*, total coliforms and non-coliforms.

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