New developments in chromogenic and fluorogenic culture media

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Abstract

This review describes some recent developments in chromogenic and fluorogenic culture media in microbiological diagnostic. The detection of β-D-glucuronidase (GUD) activity for enumeration of Escherichia coli is well known. E. coli O157:H7 strains are usually GUD-negative and do not ferment sorbitol. These characteristics are used in selective media for these organisms and new chromogenic media are available. Some of the new chromogenic media make the Salmonella diagnostic easier and faster. The use of chromogenic and fluorogenic substrates for detection of β-D-glucosidase (β-GLU) activity to differentiate enterococci has received considerable attention and new media are described. Rapid detection of Clostridium perfringens, Listeria monocytogenes, Bacillus cereus and Staphylococcus aureus are other application of enzyme detection methods in food and water microbiology. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

The ability to detect the presence of a specific and exclusive enzyme using suitable substrates, in particular fluorogenic or chromogenic enzyme substrates, has led to the development of a great number of methods for the identification of microorganisms even in primary isolation media. The incorporation of such substrates into a selective medium can eliminate the need for subculture and further biochemical tests to establish the identity of certain microorganisms (Manafi et al., 1991; Manafi, 1996). This review describes recent developments in fluorogenic and chromogenic media for specific detection of microorganisms.

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indolyl (salmon) showing no diffusion on the agar plate.

2. The application of chromogenic and fluorogenic media in food and water microbiology

2.1. Enterobacteriaceae

2.1.1. Escherichia coli

The new generation of media uses β-D-glucuronidase (GUD) as indicator for E. coli. GUD is present in 94–96% of E. coli strains, but also some Salmonella, Shigella and Yersinia spp. (Hartman, 1989). Some authors found GUD-positive strains of Citrobacter freundii (Gauthier et al., 1991) or some strains of Klebsiella oxytoca, Serratia fonticola and Yersinia intermedia (Alonso et al., 1996). GUD test is used increasingly for detection of E. coli in water and food microbiology as E. coli is an important indicator of fecal contamination in samples from the food processing and water purification plants. Other Escherichia spp. do not produce this enzyme as described by Rice et al. (1991). Some pathogenic strains of E. coli such as typical E. coli O157:H7 however, do not posses GUD either (Frampton and Restaino, 1993).

GUD activity is measured by using different chromogenic and fluorogenic substrates such as p-nitrophenol-β-D-glucuronide (PNPG), or 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-GLUC) and 4-methylumbelliferyl-β-D-glucuronide (MUG). MUG is hydrolyzed by GUD yielding 4-MU, which shows blue fluorescence when irradiated with long-wave UV light (366 nm). MUG has been incorporated into both liquid media including lauryl sulfate broth, m-Endo broth, EC broth, Brila-broth, DEV-lactose-pepton-broth and LMX broth. The solid media to which MUG was added, include violet red bile agar, ECD-agar, MacConkey-agar and m-FC agar and are already described earlier (Manafi, 1996). Fluorocult media (Merck) are used for determination of E. coli, e.g. in drugs (Huang et al., 1994), in foods of animal origin (Bredie and de Boer, 1992), for the isolation of diarrhea-causing E. coli (Muto et al., 1991), clinical samples (Heizmann et al., 1988; Mori et al., 1991), milk and milk products (Hahn and Wittrock, 1991) and bathing water (Havemeister, 1991). The minimum concentration sufficient for distinct indication was 50 μg/ml. However, the concentration necessary for acceptable detection of E. coli depends on the other constituents of the media. As the fluorescence of 4-MU is pH dependent, the pH of growth media containing MUG should be slightly alkaline; otherwise alkaline solution needs to be added to reveal fluorescence. MUG can be sterilized together with other medium ingredients without loss of activity; furthermore, no inhibitory effect on E. coli growth has hitherto been observed. The disadvantage of incorporating MUG into solid media is that fluorescence diffuses rapidly from the colonies into the surrounding agar. MUG methods are cited, e.g. in DIN 10 110 ‘Determination on E. coli in meat and meat derivates’, DIN 10 183, part 3 ‘Determination of E. coli in milk, milk derivates, icecream, baby food based on milk or ISO 11866-2 ‘Enumeration of presumptive E. coli – Part 2: most probable number technique using MUG’.

A new chromogenic selective agar for the detection of E. coli is TBX Agar. TBX is a modification of Tryptone bile agar to which the substrate 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-GLUC or BCIG) is added. GUD cleaves the chromogenic substrate BCIG and the released chromophore causes distinct and easy to read blue-green coloured colonies of E. coli. Furthermore TBX Agar complies with the ISO/DIS Standard 16649 for the enumeration of E. coli in food and animal feeding stuffs.

On a solid medium containing 8-hydroxyquinoline-β-D-glucuronide (HQG) (200 mg/l) and a ferric salt, β-glucuronidase positive strains grow as black colonies. The pigment is located only in the colony mass. The product of hydrolysis is 8-hydroxyquinoline, which forms black complexes with ferrous and ferric ions. The commercially available medium Uricult-Trio (Orion Diagnostica) contains HQG. Of 324 E. coli strains isolated from urine samples, 92% grew black-brown colonies on dip-slide, thus being β-glucuronidase positive (Larinkari and Rautio, 1995).

2.1.2. E. coli O157:H7

E. coli O157:H7 is an important food-borne pathogen and can cause diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (HUS). The agar medium most commonly used for the isolation of E.
coli O157:H7 is Sorbitol-MacConkey agar (SMAC, March and Ratnam, 1986). Strains of E. coli O157:H7, unlike the majority of E. coli strains, do not ferment D-sorbitol within 24 h and are GUD- from milk and raw meat. It was found, that RB agar inhibits the Gram positive bac- teria. d-desoxycholat inhibits the Gram positive bac- teria tested were CT-SMAC, BCM O157 and O157 strains were colourless on this medium. SMAC agar is not generally useful for Shiga-like-toxin (Stx)-producing E. coli strains of serotypes other than O157:H7 because no known genetic linkage exists between Stx production and sorbitol fermentation. E. coli O157:H7 also does not ferment rhamnose on agar plates, in contrast to the majority of other sorbitol-nonfermenting strains. The selectivity of SMAC agar has been improved with the addition of cefixime-rhamnose (CR-SMAC, Chapman et al., 1991), cefixime-tellurite (CT-SMAC agar, Zadik et al., 1993), MSA-MUG (Szabo et al., 1986) and BCIG-SMAC (Okrend et al.,1990). Recently, new selective media have been developed to increase the effectiveness of E. coli O157:H7 isolation, including Rainbow agar O157 (RB, Biolog Inc., Hayward, USA), BCM O157:H7 (BCM, Biosynth AG, Staad, Switzerland), Fluorocult E. coli O157:H7 (Merck, Darmstadt, Germany) and CHROMagar O157 (CHROMagar, Paris, France).

Fluorocult E. coli O157:H7 is a selective agar for the isolation and differentiation of E. coli O157:H7 from food samples and from clinical material. Sodium desoxycholat inhibits the Gram positive bacteria. E. coli O157:H7 strains grow as greenish colonies and show no fluorescence under UV light.

On RB O157, E. coli strains grow, yielding colonies ranging in colour through various shades of red, magenta, purple, violet, blue, and black. The typical glucuronidase negative strains form distinctive charcoal gray or black colonies. Other glucuronidase positive strains gave red or magenta coloured colonies (Bochner, 1995). RB agar shows good results for isolation when the background flora is low, or when E. coli O157 is present as a high proportion (e.g. 5%). Bettelheim (1998b) has evaluated and compared RB agar with SMAC agar. Some other EHEC also stand out as blue-black, whereas O113 and some other EHEC strains were mauve, red or pink. Manafi and Kremsmair (1999) have found that strains of E. coli O157:H7 could be readily isolated and recognized by typical black colonies on RB agar. The mixture tellurite/novobiocin (2.5 mg tellurite and 10 mg novobiocin/l according to Stein and Bochner, 1998) had inhibited the major background-flora except Hafnia alvei strains isolated from milk and raw meat. It was found, that RB agar had a sensitivity and a specificity of 91.1% and 91.6%, respectively using pure cultures and only 2.1% false positive results (H. alvei) analyzing the food samples.

On BCM agar E. coli O157:H7 strains produce convex colonies 1.5–2.5 mm in diameter surrounded by distinct blue/black precipitates. Sorbitol positive isolates were blue to turquoise. A comparison of BCM O157:H7 and SMAC-BCIG agars using naturally contaminated beef samples was made using presumptively positive enrichment broths identified by various rapid methods. The percent sensitivity and specificity values were 90.0 and 78.5 for BCM O157:H7 and 7.0 and 46.4 for SMAC-BCIG. Thus, BCM O157:H7 (+) medium displayed greater sensitivity and specificity than MSA-BCIG for detecting E. coli O157:H7 using artificially and naturally contaminated beef products (Restaino et al., 1999b). On CHROMagar O157 (Chromagar, Paris, France) E. coli O157 strains produce pink colonies (Wallace and Jones, 1996; Bettelheim, 1998a). A collaborative evaluation of detection methods for E. coli O157:H7 from radish sprouts and ground beef using plating and immunological methods is described by Önoue et al. (1999). The plating media tested were CT-SMAC, BCM O157 and CHROMagar O157. E. coli O157:H7 was recovered well from ground beef by all of the methods except direct plating with SMAC. For radish sprouts, the IMS-plating methods with CT-SMAC, BCM O157 and CHROMagar O157 were most efficient.

A study was done by Taormina et al. (1998) to recover E. coli O157:H7 cells from unheated and heated ground beef, and to compare the ability of five enrichment broths to recover E. coli O157:H7 cells from heated ground beef. Eight selective agars and two non-selective agars were evaluated for their ability to support colony formation by E. coli O157:H7 surviving heat treatment. Of the selective media tested, modified eosin methylene blue agar (MEMB) and RB agar O157 supported recovery of significantly (P < 0.05) higher numbers of heat-stressed cells of E. coli O157:H7, regardless of heating time. Chromagar O157, CT-SMAC and CR-SMAC performed poorly, even in unheated samples.
SMAC and BCM O157:H7 agar were similar to CT-SMAC and CR-SMAC in their ability to recover *E. coli* O157:H7 from heated beef. TBX agar performed significantly better than these media, but was inferior to MEMB agar and RB agar O157:H7. Enrichment using tryptone soya broth with novobiocin or a procedure using brain heart infusion and tryptone phosphate broths recovered the highest population of heat-stressed *E. coli* O157:H7.

A new medium ‘EOH-agar’ was developed for identification of *E. coli* O157:H7 by Kang and Fung (1999) and was compared with SMAC agar. Indigo carmine (0.03 g/l) and phenol red (0.036 g/l) were found as the best combination for differentiation between *E. coli* O157:H7 and other *E. coli* and added to the basal agar medium (SMAC medium excluding neutral red and crystal violet). On the dark blue EOH medium, *E. coli* produced a yellow colour with clear zone, whereas *E. coli* O157:H7 produced a red colour without clear zone. The recovery numbers of *E. coli* O157:H7 from inoculated ground beef, pork, and turkey and of heat- and cold-injured *E. coli* O157:H7 also were not significantly different between SMAC and EOH media (P > 0.05).

### 2.1.3. Media for simultaneous detection of *E. coli* and coliforms

As both *E. coli* and coliforms are important indicators of water pollution, there arises the necessity to create media which are able to simultaneously detect both bacteria. This would then guarantee a better performance of microbiological quality control. The new enzymatic definition of coliforms, which is not method related, is the possession of β-d-galactosidase gene which is responsible for the cleavage of lactose into glucose and galactose by the enzyme β-d-galactosidase. The determination of β-galactosidase is accomplished by using *o*-nitrophenyl-β-d-galactopyranoside (ONPG), *p*-nitrophenyl-β-d-galactopyranoside (PNPG), 6-bromo-3-indolyl-β-d-galactopyranoside (Salmon-Gal), 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (XGal) or 6-bromo-2-naphthyl-β-d-galactopyranoside (BNGal), 8-hydroxychinoline-β-d-galactoside, cyclohexenoesculetin-β-d-galactoside and fluorogenic 4-methylumbelliferyl-β-d-galactopyranoside (MUGal). Attempts were made to enhance the coliform assay response by adding 1-isopropyl-β-d-thiogalactopyranoside (IPTG) to the media (Manafi, 1995) or sodium dodecyl sulfate (SDS) (Berg and Fiksdal, 1988) increasing the β-d-galactosidase activity by improving the transfer of the substrate and/or enzyme across the outer membrane.

Commercially available media have been developed which permit rapid simultaneous detection of *E. coli* and coliforms in water (Table 1). These media contain a variety of enzyme substrates for detection of β-d-galactosidase (presence of coliforms) and β-d-glucuronidase (presence of *E. coli*), and have previously been reviewed (Manafi et al., 1991; Manafi, 1996).

#### 2.1.3.1. Liquid commercially available media for the detection of *E. coli* and coliforms

The Colilert system, containing ONPG/MUG, simultaneously detects the presence of both total coliforms and *E. coli*. After incubation, the formula becomes yellow if total coliforms are present and fluorescent under long-wavelength (366 nm) ultraviolet light if *E. coli* is in the same sample. Schets et al. (1993) compared Colilert with Dutch standard enumeration methods for *E. coli* and coliforms in water and have found, that Colilert gave false-negative results in samples with low numbers of *E. coli* or total coliforms, indicating that the Colilert does not always support the growth of environmental *E. coli* or coliforms. They regarded the Colilert as unsuitable for monitoring water samples in comparison to Dutch standard method. Another study by Landre et al. (1998) reported the false positive coliform reaction mediated by *Aeromonas* spp. in the Colilert system. Data obtained clearly demonstrate that *A. hydrophila* can elicit a positive coliform type reaction at very low densities. Cell suspensions as low as 1 cfu/ml were observed to yield a positive reaction. Similar results were obtained with other members from the mesophilic group of aeromonads. Use of Colilert for monitoring water quality will lead to overestimation of coliforms as *Aeromonas* spp. are known to be present in treated drinking water supplies. Development of blue-green color in an initially light yellow coloured solution, indicate the presence of coliforms using LMX\textsuperscript® broth or Readycult\textsuperscript® Coliforms, containing Xgal/MUG. Fluorescence at 366 nm in the same vessel denoted the presence of *E. coli*. An evaluation of a number of presence/absence (P/A) tests for coliforms and *E. coli*, including LMX broth (Merck) and Colilert
Table 1
Commercially available media for the detection of E. coli and coliforms (updated from Mana®, 1996)*

<table>
<thead>
<tr>
<th>Medium</th>
<th>Substrate/colour</th>
<th>Manufacturer</th>
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<tbody>
<tr>
<td></td>
<td>Coliforms</td>
<td>E. coli</td>
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<tr>
<td><strong>Liquid media</strong></td>
<td></td>
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<tr>
<td>Fluorocult® LMX broth</td>
<td>XGAL/blue-green</td>
<td>MUG/blue fluorescence</td>
</tr>
<tr>
<td>Readycult coliforms</td>
<td>XGAL/MUG</td>
<td>MUG/blue fluorescence</td>
</tr>
<tr>
<td>ColiLert</td>
<td>ONPG/Yellow</td>
<td>MUG/blue fluorescence</td>
</tr>
<tr>
<td>Coliquick</td>
<td>ONPG/Yellow</td>
<td>MUG/blue fluorescence</td>
</tr>
<tr>
<td>Colisure</td>
<td>CPRG/red</td>
<td>MUG/blue fluorescence</td>
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<tr>
<td><strong>Solid media</strong></td>
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<tr>
<td>Fluorocult® agars</td>
<td>–</td>
<td>MUG/blue fluorescence</td>
</tr>
<tr>
<td>TBX-agar</td>
<td>–</td>
<td>BCIG/blue</td>
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<tr>
<td>Uricult Trio</td>
<td>–</td>
<td>HOQ/black</td>
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<tr>
<td>EMX-agar</td>
<td>XGAL/blue</td>
<td>MUG/blue fluorescence</td>
</tr>
<tr>
<td>C-EC-MF-agar</td>
<td>XGAL/blue</td>
<td>MUG/blue fluorescence</td>
</tr>
<tr>
<td>Chromocult</td>
<td>SalmonGal/red</td>
<td>XGLUC/blue-violet</td>
</tr>
<tr>
<td>Coli ID</td>
<td>XGAL/blue</td>
<td>SalmonGlu/Rose-violet</td>
</tr>
<tr>
<td>CHROMagar ECC</td>
<td>SalmonGal/red</td>
<td>XGLUC/purple</td>
</tr>
<tr>
<td>Rapid® E. coli 2</td>
<td>XGAL/blue</td>
<td>SalmonGlu/purple</td>
</tr>
<tr>
<td>E. coli/coliforms</td>
<td>SalmonGal/red</td>
<td>XGLUC/purple</td>
</tr>
<tr>
<td>ColiScan</td>
<td>SalmonGal/red</td>
<td>XGLUC/purple</td>
</tr>
<tr>
<td>MI-agar</td>
<td>MUGal/blue fluorescence</td>
<td>Indoxyl/blue</td>
</tr>
<tr>
<td>HiCrome ECC</td>
<td>SalmonGal/red</td>
<td>XGLUC/blue</td>
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<tr>
<td><strong>Other systems</strong></td>
<td></td>
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<tr>
<td>ColiComplete</td>
<td>XGAL/blue</td>
<td>MUG/blue fluorescence</td>
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<tr>
<td>ColiBag/Water check</td>
<td>XGAL/blue-green</td>
<td>MUG/blue fluorescence</td>
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<td>Pathogel</td>
<td>XGAL/blue</td>
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<td>E. coli</td>
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<tr>
<td>m-Coliblue</td>
<td>TTC/red</td>
<td>XGLUC/blue</td>
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</table>

* Abbreviations: ONPG, o-nitrophenyl-β-D-galactosidase; Salmon-GAL, 6-bromo-3-indolyl-β-D-galactopyranoside; XGAL, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; CPRG, chlorophenol red β-D-galactopyranoside; XGLUC, 5-bromo-4-chloro-3-indolyl-β-D-glucuronide; MUG, 4-methylumbelliferyl-β-D-glucuronide; TTC, triphenyl tetrazolium chloride; HOQ, hydroxyquinoline-β-D-glucuronide.

(Idexx) has been published under the Department of the Environment series in the UK (Lee et al., 1995). They compared four P/A tests with UK Standard methods and found that more coliforms were detected than with membrane filtration technique; in addition, it was shown that results in LMX broth were the easiest to interpret. The study concludes that there is no P/A test that is best at all locations for both coliforms and E. coli, and as there can be marked ecological differences between sources it is important that particular P/A tests are validated in each geographical area before use. The efficacy and rapidity of detectable reactions make LMX® broth or Readycult® coliforms a very useful tool in routine water and food microbiology (Hahn and Wittrock, 1991; Betts et al., 1994; Lee et al., 1995; Manafi, 1995; Manafi and Rosmann, 1998). Only a few non-coliform bacteria such as the strains of Serratia spp., H. alvei, Vibrio metchnikovii, V. vulnificus, A. hydrophila and A. sobria gave a false positive reaction with Xgal (Manafi, 1995; Fricker and Fricker, 1996). This is in accordance with Ley et al. (1993), who found that Xgal medium, in addition to providing a rapid test for coliforms, also detected β-galactosidase-positive aeromonads and nonsheen-forming members of the Enterobacteriaceae on m-Endo agar. They reported the low sensitivity of m-Endo for detecting Aeromonas spp. which are considered ubiquitous waterborne organisms and should not be present in drinking water (Moyer, 1987). It is suggested adding cefsulodin at 5–10 μg/ml to culture media inhibiting the growth of Aeromonas and Flavobacterium species (Brenner et al., 1993; Alonso et al., 1996; Geissler et al., 2000).
Results of these studies suggest that cefsulodin may be a useful selective agent against Aeromonas spp. which should be included in coliform chromogenic media when high levels of accompanying flora are expected.

2.1.3.2. Solid commercially available media for the detection of E. coli and coliforms

Chromocult coliforms agar (CCA) contains chromogenic Salmon-GAL and X-GLUC, the growth of coliforms even sublethal damaged cells is granted due to use of peptone, pyruvate, sorbit and a buffer of phosphate. Gram positive and some Gram negative bacteria are inhibited by Tergitol 7. On the CCA, non-E. coli fecal coliforms (Klebsiella, Enterobacter and Citrobacter) (KEC) were identified by the production of a salmon to red colour from β-galactosidase cleavage of the substrate Salmon-GAL, while E. coli colonies were detected by the blue/violet colour, produced by the cleavage of X-glucuronide by β-D-glucuronidase. CCA was compared with the Standard Methods membrane filtration fecal coliform (mFC) medium for fecal coliform detection and enumeration (Alonso et al., 1998). Statistically, there were no significant differences between fecal coliform counts obtained with the two media (CCA and mFC agar) and two incubation procedures (2 h at 37°C plus 22 h at 44.5°C, and 44.5°C) as determined by variance analysis. In this study E. coli represented, on average 70.5–92.5% of the fecal coliform population. A high incidence of false negative KEC (19.5%) and E. coli (29.6%) colonies was detected at 44.5°C. The physiological condition of the fecal coliform isolates could be responsible for the nonexpression of β-galactosidase and β-glucuronidase activities at 44.5°C. Byamukama et al. (2000) described the quantification of E. coli contamination with CC agar from different polluted sites in a tropical environment. It proved to be efficient and feasible for determining fecal pollutions in the investigated area within 24 h.

Geissler et al. (2000) compared the performance of LMX® broth, Chromocult Coliform®-agar (CC) and Chromocult Coliform®-agar plus cefsulodin (10 μg/ml) (CC-CFS), with Standard Methods multiple tube fermentation (MTF), for the enumeration of total coliforms (TC) and E. coli from marine recreational waters. Data from the analysis of variance showed significant differences (P ≤ 0.05) between TC counts on CC-CFS and LMX. Disagreement between the CC-CFS agar and the two commercial enzyme media, was primarily due to the false positive results. Background interference was reduced on CC-CFS and the TC counts that were obtained reflected more accurately the number of TCs. The results of this study support the validity of the LMX and CC media for the enumeration of E. coli in marine waters. Background interference was reduced on CC-CFS and the TC counts that were obtained reflected more accurately the number of TCs. Some authors evaluated agar media incorporating Xgal (Ley et al., 1993; Jermini et al., 1994). They have found that coliform strains produced sharp blue colonies on the agar plate because of insolubility of the indigo dye, which does not alter the viability of the colonies. The MI agar method (Brenner et al., 1996) containing indoxyl-β-D-glucuronide (E. coli) and 4-methylumbelliferyl-β-D-galactopyranoside (coliforms), was compared with the approved method by the use of wastewater-spiked tap water samples. Overall, weighted analysis of variance (significance level, 0.05) showed that the new medium recoveries of total coliforms and E. coli were significantly higher than those of mEndo agar and nutrient agar plus MUG, respectively, and the background counts were significantly lower than those of mEndo agar (<5%). Brenner et al. (1996) made a comparison of the recoveries of E. coli and total coliforms from drinking water by the MI agar method and the USEPA-approved membrane filter method. The current USEPA-approved membrane filter method for E. coli requires two media, an MF transfer, and a total incubation time of 28 h. Since December 1999, MI agar method is approved for detecting total coliforms and E. coli under the total coliforms Rule and for enumerating total coliforms under the Surface water Treatment Rule in USA.

On COLI ID agar (bioMérieux) coliforms are blue colour colonies and E. coli are rose colour with a rose zone around the colonies. Other Gram negative bacteria are on COLI ID bright rose, small and have no surrounding zone, Gram positives and yeasts are inhibited. Similar to the Chromocult Coliform agar, on Coliscan and on CHROMagar ECC, E. coli colonies are blue-violet, other coliforms are red colonies. There are two approaches to the Coliscan method, Coliscan Easygel and Coliscan-membrane filters. The sample can be added directly into the
bottle of Coliscan Easygel, swirled, poured into a pretreated petri dish and incubated.

Alonso et al. (1999) compared the performance of CHROMagarEC1 (CECC), and CECC supplemented with sodium pyruvate (CECCP) with the membrane filtration lauryl sulfate-based medium (mLSA) for enumeration of E. coli and non-E. coli thermotolerant coliforms such as Klebsiella, Enterobacter and Citrobacter. To establish that they could recover the maximum coliforms and E. coli population, they compared two incubation temperature regimens, 41 and 44.5°C. Statistical analysis by the Fisher test of data did not demonstrate any statistically significant differences (P = 0.05) in the enumeration of E. coli for the different media (CECC and CECCP) and incubation temperatures. Variance analysis of data performed on Klebsiella, Enterobacter and Citrobacter counts showed significant differences (P = 0.01) between Klebsiella, Enterobacter and Citrobacter counts at 41 and 44.5°C on both CECC and CECCP. Analysis of variance demonstrated statistically significant differences (P = 0.05) in the enumeration of total thermotolerant coliforms (TTCs) on CECC and CECCP compared with mLSA. Target colonies were confirmed to be E. coli at a rate of 91.5% and Klebsiella, Enterobacter and Citrobacter of likely fecal origin at a rate of 77.4% when using CECCP incubated at 41°C. The results of this study showed that CECCP agar incubated at 41°C is efficient for the simultaneous enumeration of E. coli and Klebsiella, Enterobacter and Citrobacter from river and marine waters.

2.1.4. Other systems for detection of E. coli and coliforms

Grant (1997) described a membrane filtration medium (m-ColiBlue) which detects total coliforms (red colonies) and E. coli (blue colonies) simultaneously. Recovery of total coliforms and E. coli on this membrane filtration (MF) medium was evaluated with 25 water samples and the testing of the m-ColiBlue24 broth, was conducted according to a US Environmental Protection Agency (USEPA) protocol. For comparison, this same protocol was used to measure recovery of total coliforms and E. coli with two standard MF media, m-Endo broth and mTEC broth. E. coli recovery on the new medium was also compared to recovery on nutrient agar supplemented with MUG. Comparison of specificity, sensitivity, false positive error, undetected target error, and overall agreement indicated E. coli recovery on m-ColiBlue24 was superior to recovery on mTEC for all five parameters. Recovery of total coliforms on this medium was comparable to recovery on m-Endo.

ColiComplete is a paper disc contain MUG and Xgal and can be used with lauryl tryptose broth in the MPN test. One paper disc is added to each tube and after incubation at 35°C for 24 h, the tubes are observed under long-wave UV for the confirmation of E. coli (blue fluorescence). Blue colouration in the broth indicates the presence of total coliforms and/or E. coli (Feldsine et al., 1994).

ColiBag (a pre-sterilized plastic bag), E.colite, ColiGel and Pathogel are media containing enzyme substrates Xgal and MUG. ColiGel and Pathogel contain a gelling agent, which solidifies the sample; coliforms gave blue colonies and E. coli gave blue and fluorescent colonies under UV light. ColiPAD offers a multiple tube fermentation procedure for the determination of E. coli and coliforms in water and wastewater.

Japanese and US Food and Drug Administration standard methods (USDA), as well as two agar plate methods, were compared with the three commercial kits using enzyme substrates (Venkateswaran et al., 1996). The levels of detection of coliforms were high with the commercial kits (78–98%) compared with the levels of detection with the standard methods (80–83%) and the agar plate methods (56–83%). Among the kits tested, the Colilert kit had highest level of recovery of coliforms (98%), and the level of recovery of E. coli as determined by β-glucuronidase activity with the Colilert kit (83%) was comparable to the level of recovery obtained by the USDA method (87%). Levine’s eosine methylene blue agar was compared with MUG-supplemented agar for isolation of E. coli. Only 47% of the E. coli was detected when eosine methylene blue agar was used; however, when violet red bile (VRB)-MUG agar was used, the E. coli detection rate was twice as high.

2.1.5. Salmonella

The conventional media for the detection of Salmonella have a very poor specificity creating an abundance of false positives (such as Citrobacter, Proteus) among the rare real positive Salmonella.
The workload for unnecessary examination of suspect colonies is so high that the real positive *Salmonella* colonies might often be missed in routine testing. The use of new chromogenic and fluorogenic media make the *Salmonella* diagnostic easier and faster.

2.1.5.1. SM-ID agar (bioMérieux, France)

On SM-ID agar *Salmonella* colonies are detected by their distinctive red colouration, while coliforms appear blue, violet, or colourless. The biochemical characteristics used with SM-ID medium are acid formation from glucuronate combined with a neutral red indicator. Two chromogenic substrates for β-galactosidase (Xgal) and β-glucosidase (Xglu) allow differentiation of *Salmonella* (negative) from other enterobacteria acidifying glucuronate (positive: blue to purple). The selective agents are bile salts and brilliant green (Dusch and Altwegg, 1995).

2.1.5.2. Rambach agar (Merck, Germany)

Rambach agar is composed of propylene glycol, peptone, yeast extract, sodium deoxycholate, neutral red, and Xgal. After incubation of plates at 37°C for 24 h, the formation of acid from propylene glycol causes precipitation of the neutral red in *Salmonella* colonies yielding a red colour (Rambach, 1990). *Salmonella* strains show a bright red colour, coliforms blue (β-α-galactosidase activity) or violet (the formation of acid from propylene glycol and β-α-galactosidase activity) and *Proteus* spp. remain colourless. Sodium deoxycholate inhibits the accompanying Gram-positive flora. The main disadvantage of Rambach agar is that it does not detect *S. typhi*, *S. paratyphi* nor some rare strains such as *S. moscow* and *S. wassenaar*. Furthermore, *Salmonella* strains which are able to produce β-α-galactosidase such as *S. arizona* or, in particular, subspecies IIIa, IIIb, and VI, show blue-violet colonies on both media (Kühn et al., 1994). Pignato et al. (1995) have observed that the total analysis time for salmonellae in foods can be reduced to 48 h by using the combination of Salmosyst broth (Merck) as a liquid medium and Rambach agar as isolation plate.

2.1.5.3. MUCAP-test (Biolife, Italy)

MUCAP is a confirmation test for *Salmonella* species based on the rapid detection of caprylate esterase, using fluorogenic 4-methylumbelliferyl-caprylate. In the presence of C₈ esterase the substrate is cleaved with the release of 4-methylumbelliferone (4-MU). One drop of MUCAP has to be added to each colony tested on Columbia agar and observed under UV light (366 nm) for 1–5 min. Strong bluish fluorescence indicates the presence of *Salmonella* spp. The MUCAP test was found to be very sensitive, rapid and easy to perform but not very specific, giving many false-positive results. The combination of the MUCAP test and selective media was more specific. Since most of the false-positive strains are oxidase positive, the combination of MUCAP and the oxidase test is recommended (Humbert et al., 1989).

2.1.5.4. CHROMagar Salmonella (CHROMagar, France)

A comparison of CHROMagar *Salmonella* medium (CAS), based on the esterase activity, and Hektoen enteric agar (HEA) for isolation of *Salmonella* have been done by Gaillot et al. (1999) with *Salmonella* isolates and stool samples. All stock cultures, including cultures of H₂S-negative isolates, yielded typical mauve colonies on CAS while 99% isolates produced typical lactose-negative, black-centered colonies on HEA. Sensitivities for primary plating and after enrichment were 95 and 100%, respectively, for CAS and 80 and 100%, respectively, for HEA. The specificity of CAS (88.9%) was significantly higher than that of HEA (78.5%; P < 0.0001).

2.1.5.5. Rainbow Salmonella agar (Biolog, USA)

The growth of distinctive black colonies of *Salmonella* on the clear Rainbow Agar makes it easier to detect *Salmonella* in mixed cultures. The advantages of using Rainbow Agar *Salmonella* versus traditional media is the sensitivity and specificity for *Salmonella* species. Even some newer *Salmonella* media such as XLT4 utilize additives such as Tergitol 4 that are inhibitory to *S. typhi* and *S. choleraesuis*.

2.1.5.6. Chromogenic Salmonella esterase agar (PPR Diagnostics Ltd, UK)

Cooke et al. (1999) described a novel agar medium, chromogenic *Salmonella* esterase (CSE) agar. The agar contains peptones and nutrient extracts together with the following 4-[2-(4-
octanoyloxy-3,5-dimethoxyphenyl)-vinyl]-quinolinium-1-(propan-3-yl-carboxylic-acid)-bromide (SLPA-octanoate bromide form). SLPA-octanoate is a newly synthesized ester formed from a C₈ fatty acid and a phenolic chromophore. In CSE agar, the ester is hydrolyzed by *Salmonella* spp. to yield a brightly coloured phenol which remains tightly bound within colonies. The typical *Salmonella* strains were burgundy coloured on a transparent yellow background, whereas non-*Salmonella* spp. were white, cream, yellow or transparent. The sensitivity (93.1%) of CSE agar for non-typhi *Salmonella*ae compared favorably with those of Rambach (82.8%), xylose-lysine-deoxycholate (XLD; 91.4%), Hektoen-enteric (89.7%), and SM ID (91.4%) agars. The specificity (93.9%) was also comparable to those of other *Salmonella* medias (SM ID agar, 95.9%; Rambach agar, 91.8%; XLD agar, 91.8%; Hektoen-enteric agar, 87.8%). Strains of *Citrobacter freundii*and *Proteus* spp. giving false-positive reactions with other media gave a negative colour reaction on CSE agar. CSE agar enabled the detection of >30 *Salmonella* serotypes, including *agona, anatum, enteritidis, hadar, heidelberg, infantis, montevideo, thompson, typhimurium*, and *virchow*.

### 2.1.5.7. Compass Salmonella agar (Biokar diagnostics, France)

Perry and Quiring (1997) described an agar medium based on the detection of C₈-esterase activity using 5-bromo-6-chloro-3-indolyl-caprylate. Magenta coloured colonies indicating the presence of *Salmonella* ssp.

### 2.1.5.8. Chromogenic ABC medium (Lab M. Ltd., UK)

Perry et al. (1999) described a new chromogenic agar medium, ABC medium (αβ-chromogenic medium), which includes two substrates, 3,4-cyclohexenoesculetin-β-D-galactoside (CHE) and 5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside (X-αGal), to facilitate the selective isolation of *Salmonella* ssp. CHE-Galactoside is utilised by most Enterobacteriaceae, yielding black colonies. The X-5-Gal is hydrolysed by *Salmonella* ssp. to produce characteristic, easy to distinguish, green colonies. This medium exploits the fact that *Salmonella* ssp. may be distinguished from other members of the family Enterobacteriaceae by the presence of α-galactosidase activity in the absence of β-galactosidase activity. A total of 1022 strains of *Salmonella* spp. and 300 other Gram-negative strains were inoculated onto this medium. Of these, 99.7% strains8 agar, the ester is hydrolyzed by *Salmonella* spp. to yield a brightly coloured phenol which remains tightly bound within colonies. The typical *Salmonella* strains were burgundy coloured on a transparent yellow background, whereas non-*Salmonella* ssp. produced a characteristic green colony, whereas only one strain of non-*Salmonella* produced a green colony.

### 2.2. Enterococci

The use of chromogenic and fluorogenic substrates for detection of β-D-glucosidase activity to differentiate enterococci has received considerable attention (Dufour, 1980; Littel and Hartman, 1983).

#### 2.2.1. Enterolert and Microtiter plate MUST

4-methylumbelliferyl-β-D-glucoside (MUD), when hydrolyzed by enterococcal β-D-glucosidase, releases 4-methylumbelliferone, which exhibits fluorescence under a UV lamp (366 nm). Enterolert (IDEXX Laboratories Inc., Westbrook, Maine), and a Microtiter plate MUST (Sano®, France), containing MUD detecting β-D-glucosidase. Niemi and Ahtiainen (1995) described the evaluation of MTP method with *Salmonella* spp., in particular in natural contaminated water samples. Budnick et al. (1996) evaluated the Enterolert for enumeration of enterococci in recreational bathing water samples. No statistical significant difference and a strong linear correlation were found between the two methods. Culturing of 501 Enterolert test wells resulted in false-positive and false-negative rates of 5.1 and 0.4%, respectively.

#### 2.2.2. mEI agar

Dufour (1980) described a medium (mEI agar) for use in a single-step, 24-h MF procedure to enumerate...
enterococci in marine water and freshwater. The medium contained nalidixic acid, cycloheximide, triphenyltetrazolium chloride (TTC) and indoxyl-β-D-glucoside. Enterococci strains produce an insoluble indigo blue complex, which diffuses into the surrounding media, forming a blue halo around the colony. Rhodes and Kator (1997) evaluated mEI agar with respect to specificity and recovery of enterococci from environmental waters. Extending incubation from 24 to 48 h improved enterococci recovery but 77% of the colonies classified as non-target were confirmed as enterococci. Decreasing the concentration of or eliminating indoxyl-β-D-glucoside from mE did not significantly affect recovery of purified isolates. Messer and Dufour (1998) recently modified the medium by reducing the TTC from 0.15 to 0.02 g/l and adding 0.75 g of indoxyl-β-D-glucoside per liter. The new MF medium, mEI medium, detected levels of enterococci in 24 h comparable to those detected by the original mE medium in 48 h, with the same level of statistical confidence. The comparative recovery studies, specificity determinations, and multilaboratory evaluation indicated that mEI medium has analytical performance characteristics equivalent to those of mE medium. The simplicity of use and decreased incubation time with mEI medium will facilitate the detection and quantification of enterococci in fresh and marine recreational waters.

2.2.3. Chromocult enterococci broth (CEB) and Readycult enterococci

Using chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-glucopyranoside (XGLU), Chromocult Enterococci broth (CEB) (Merck) and Readycult Enterococci (Merck) utilises the β-D-glucosidase reaction as an indicator of the presence of enterococci. XGLU is liberated and rapidly oxidized to bromochloroindigo which produces blue colour in Chromocult broth as well as blue coloured enterococci colonies in Chromocult Enterococci agar. Other β-D-glucosidase-producing organisms being suppressed by the sodium azide content of the broth (Manafi and Windhager, 1997). The results obtained with pure cultures showed that 97% of the strains, which gave positive results, were identified as enterococci (E. faecalis, E. faecium, E. durans, E. casseliflavus and E. avium). The false-positive strains (3%) were Leucononostoc, Lactococcus lactis and Aerococcus spp. No false-negative results were found. This medium was used to enumerate enterococci in water using a presence/absence procedure and gave still positive results with 10 cfu/100 ml water samples after 24 h incubation.

2.2.4. RAPID’ enterococcus agar

RAPID’ enterococcus agar (Sanofi, France) is a new medium using chromogenic Xglu for the detection of enterococci and a selective mix for the inhibition of Gram negative and other β-glucosidase positive bacteria other than enterococci.

2.2.5. Comparative studies

In most literature, Slanetz and Bartley (1957) agar is treated as a suitable MF medium to detect fecal enterococci from a water sample. Amorós and Alonso (1996) presented a study including a comparison of Slanetz–Bartley agar and Enterococci agar (with XGLU) using samples of irrigation channels and seawater. They found no statistical differences between both media in seawater, although the specificity of the Enterococci agar decreased in marine water and there was a considerable number of false-positives.

2.3. Staphylococcus aureus

On the new chromogenic medium, CHROMagar™ S. aureus medium (CHROMagar, France), colonies of S. aureus are typical pink coloured. In supplementing CHROMagar S. aureus with an appropriate antibiotic, for instance methicillin, one can obtain information related to some methicillin resistant S. aureus strains. This medium has been evaluated by using pure cultures and clinical specimens. Out of the 181 S. aureus and 81 coagulase negative Staphylococcus strains belonging to 18 different species, S. aureus, S. epidermidis, S. caprae and S. schleiferi strains yielded pink colonies (Freydiere et al., 2000).

2.4. Clostridium perfringens

The identification of C. perfringens is possible using Fluorocult TSC-agar (Merck, Germany) using a fluorogenic enzyme substrate. n-Cycloserine inhibits the accompanying bacterial flora and causes the colonies to remain smaller. C. perfringens colonies can be detected using 4-MU-phosphate. Acid phos-
phatase is a highly specific indicator for *C. perfringens* which shows a light blue fluorescence on this medium (Baumgart et al., 1990).

2.5. *Bifidobacteria and lactic acid bacteria*

The use of chromogenic X-α-d-galactoside for differential enumeration of *Bifidobacterium* spp. and lactic acid bacteria was described by Chevalier et al. (1991). The X-α-d-galactoside-based medium is useful to identify bifidobacteria among *Lactobacillus* or *Streptococcus* strains. *Bifidobacteria* shows blue coloured colonies on the agar plate.

2.6. *Listeria monocytogenes*

*L. monocytogenes* is a human and animal pathogen that is widespread in nature. The organism is a transient constituent of the intestinal flora excreted by 1–10% of healthy humans. It is an extremely hardy organism and can survive for many years in the cold in naturally infected sources. *L. monocytogenes* has been isolated from a wide variety of foods, including dairy products, meats, and fish. Although most of the foodborne listeriosis outbreaks have been linked to the consumption of dairy products, recent sporadic cases have been associated with meats, as well as other foods. All strains of *L. monocytogenes* are pathogenic by definition although some appear to be more virulent than others. Cultural methodology for isolating the organism from foods has been in a state of flux since 1985.

2.6.1. *BCM L. monocytogenes detection system*

The new BCM *L. monocytogenes* detection system (BCM-LMDS, Biosynth, Switzerland) consists of a selective pre-enrichment broth, selective enrichment broth, selective/differential plating medium, and identification on a confirmatory plating medium. The BCM pre-enrichment broth, allowed the growth of *Listeria* and resuscitation of heat-injured *L. monocytogenes*, which contains the fluorogenic substrate 4-methylumbelliferyl-myo-inositol-1-phosphate and detects phosphatidylinositol phospholipase C (PI-PLC) activity, provided a presumptive positive test for the presence of pathogenic *L. monocytogenes* and *L. ivanovii* after 24 h at 35°C. On BCM-plating medium, *L. monocytogenes* and *L. ivanovii* were the two *Listeria* species forming turquoise convex colonies (1.0–2.5 mm in diameter) from PI-PLC activity on the chromogenic substrate, 5-bromo-4-chloro-3-indoxyl-myo-inositol-1-phosphate. *L. monocytogenes* was distinguished from *L. ivanovii* by either its fluorescence on BCM confirmatory plating medium or acid production from rhamnose.

2.6.2. Rapid L’MONO agar

Rapid L’MONO agar (Sanofi, France) is based on the detection of phosphatidylinositol phospholipase C and Xylose fermentation. In this way, the colonies of *L. ivanovii* appear blue surrounded by a yellow halo (Xylose positive) whilst the colonies of *L. monocytogenes* are blue without the halo (Xylose-negative). The colonies of other *Listeria* spp. remain white because they do not posses phosphatidylinositol phospholipase C activity. Further evaluation of these media is still needed.

2.7. *Bacillus cereus*

BCM *B. cereus* and *B. thuringiensis* plating medium (Biosynth, Switzerland) is a differential and selective medium which allows *B. cereus* and *B. thuringiensis* to grow, but inhibits growth of many potential false positives including other *Bacillus* species. Based on the detection of phosphatidylinositol-specific phospholipase C (PI-PLC) activity by *Bacillus* species, *B. cereus* and *B. thuringiensis* strains produce turquoise flat dull colonies with and without turquoise halos to the enzymatic reaction of PI-PLC with 5-bromo-4-chloro-3-indoxyl-myo-inositol-1-phosphate.
3. Conclusions

Rapid detection and identification of microorganisms is of high importance in a diverse array of applied and research fields. By incorporation of synthetic enzyme substrates into primary isolation media, enumeration and detection can be performed directly on the isolation plate. These enzyme substrates have proved to be a powerful tool, utilizing specific enzymatic activities of certain microorganisms. Many of them are offering enhanced accuracy and performance to the microbiologist. The incorporation of such substrates into a selective medium can eliminate the need for subculture and further biochemical tests to establish the identity of certain microorganisms. These enzymatic assays may constitute an alternative method for enumerating microorganisms in water and foods, which is specific, sensitive and rapid.

4. Uncited reference

Okrend et al., 1990.

References

of Staphylococcus aureus. Abstr C 230, 100th American Society for Microbiology, Los Angeles, USA.