Biolog identification of non-sorbitol fermenting bacteria isolated on *E. coli* O157 selective CT-SMAC agar

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Abstract

E. coli O157:H7 is recognised as an important human pathogen world-wide and has been associated with diseases such as haemorrhagic colitis (HC), haemolytic uraemic syndrome (HUS) and thrombotic thrombocytopaenic purpura (TTP). Accurate laboratory detection of *E. coli* O157:H7 is important for diagnostic purposes and to justify epidemiological data on *E. coli* O157:H7. A well-known phenotypic characteristic of *E. coli* O157:H7 bacteria is their inability to ferment sorbitol. This characteristic is often used to isolate these organisms from food and water using selective agar medium such as SMAC. However, the high number of false positive results obtained by a number of researchers when selectively screening for *E. coli* O157:H7 on CT-SMAC has prompted an investigation to determine which other sorbitol-negative bacteria also grow on CT-SMAC. The agar medium used for the investigation consisted of Sorbitol MacConkey agar (SMAC) supplemented with Cefixime-tellurite (CT). All sorbitol-negative colonies obtained from CT-SMAC, after selective enrichment and IMS were identified using the Biolog microbial identification system. The majority of sorbitol-negative isolates identified were *Burkholderia*, *Pseudomonas*, *Vibrio* and *Aeromonas* spp. Only two *E. coli* O157:H7 isolates were identified with Biolog and confirmed with a polymerase chain reaction (PCR) specific for the shiga toxin 1 (Stx1) genes and with O157 and H7 antisera. The inability of the CT-SMAC agar medium to specifically select for *E.coli* O157:H7 was confirmed by the results of this study. These observations call for further improvement of affordable methods for the selective isolation of *E. coli* O157:H7 in the presence of large numbers of interfering bacteria capable of growing on CT-SMAC.

Keywords: *Escherichia coli* O157:H7, Sorbitol-MacConkey agar, Biolog bacterial identification

Introduction

Escherichia coli (*E. coli*) is the species most commonly isolated from human faecal samples and is part of the normal intestinal flora of healthy individuals (Nataro and Kaper, 1998). *E. coli* O157:H7 is the classical serotype linked to serious outbreaks and sporadic cases of enterohaemorrhagic diseases such as haemorrhagic colitis (HC) and haemolytic uraemic syndrome (HUS) (Nataro and Kaper, 1998; Verweyen et al., 2000). Animals such as cattle are the main reservoir for *E. coli* O157:H7 but they do occur in other animal species such as sheep, goats, pigs, cats, dogs, chickens and gulls (Johnson et al., 1990; Griffin and Tauxe, 1991; Beutin et al., 1994; Wallace et al., 1997). Water-borne transmission of *E. coli* O157:H7 has been well documented and reported from both recreational water and contaminated drinking water (Swerdlow et al., 1992; Keene et al., 1994; O'Connor, 2002). These strains of *E. coli* produce shiga toxins (Stx), which are potent cytotoxins that are similar to the toxin produced by *Shigella dysenteriae* Type 1 (Scotland et al., 1983; O'Brien et al., 1984).

Immunomagnetic separation (IMS) has been proposed as a sensitive method for the isolation of *E. coli* O157:H7 bacteria from clinical, food and environmental samples (Wright et al., 1994; Bennet et al., 1996; Chapman and Siddons, 1996; Cubbon et al., 1996; Vernozy-Rozand et al., 1997; Tomoyasu, 1998; Müller et al., 2003). A variety of selective and differential plating media have been developed for the isolation of *E. coli* O157:H7 (Nataro and Kaper, 1998). The most common *E. coli* O157 isolation media are Sorbitol MacConkey (SMAC) agar supplemented with Cefixime and potassium-tellurite (CT-SMAC) (March and Ratnam, 1986; Nataro and Kaper, 1998; Müller et al., 2001; Fujisawa et al., 2002; Müller et al., 2002). SMAC contains sorbitol, which replaces the lactose of the standard MacConkey agar medium (SMAC Oxoid product brochure, 2003). Unlike typical *E. coli*, *E. coli* O157:H7 do not ferment or produce acid from D-sorbitol within 24 h and lack glucoronidase activity (Manafi and Kremsmaier, 2001). *E. coli* O157:H7 bacteria present as colourless colonies on SMAC media (Manafi and Kremsmaier, 2001). SMAC agar is not suited for the isolation of non-O157 EHEC bacteria because there is no genetic link between Stx production and sorbitol fermentation (Nataro and Kaper, 1998). However, some non-O157 EHEC strains have proven to be sorbitol-negative (Ojeda et al., 1995). The addition of Cefixime and tellurite to SMAC agar permits the selective growth of *E. coli* O157:H7 and *Shigella sonnei* strains but inhibits the growth of most of the other *E. coli* strains (Zadik et al., 1993). *Pseudomonas* spp. do not ferment sorbitol and also present as colourless colonies on CT-SMAC (Cousin, 2000). Except for the addition of Cefixime and potassium-tellurite (CT) to SMAC (Zadik et al., 1993), researchers have modified SMAC agar by adding other agents to increase the selectivity of the media such as rhamnose and chromogenic enzyme substrates (Szabo et al., 1986; Chapman et al., 1991; Fujisawa et al., 2000; Okrend et al., 2000). Müller et al. (2001) found CT-SMAC to lack in selectivity and suggested further research to improve available methods for the selective cultivation of *E. coli* O157:H7 in the presence of large numbers of wild type *E. coli* and other bacteria capable of growing on the selective media. Resistance to the antibiotics used for the suppression of bacteria other than *E. coli* O157:H7 may largely be accountable for the problem. One solution may be to find an-

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timicrobial agents, which more efficiently suppress background growth and strains of *E. coli* other than *E. coli* O157:H7 (Szabo et al., 1986; Okrend et al., 1990; Chapman et al., 1991).

Automated microbial identification systems, such as the MicroLog/Biolog identification system (Biolog Inc., Hayward, Calif.), have become widely used in both food, clinical and research laboratories (Odumeru et al., 1999). The analysis of carbon-source utilisation is a simple and rapid method to identify bacterial isolates from various sources (Odumeru et al., 1999). This system establishes identifications based on the exchange of electrons generated during bacterial respiration (Truu et al., 1999).

In this study Biolog identification was used to determine the efficacy of CT-SMAC agar as a selective agar medium for the presumptive isolation of sorbitol-negative *E. coli* O157:H7.

Materials and methods

Samples

Settled sewage samples (28) were obtained from 13 sewage purification plants (SPPs) in the Gauteng region of South Africa. Additional isolates (7) were obtained from 5 river water sampling points.

Bacterial control cultures

The bacterial control cultures used as references in this study included the following:
E. coli O157:H7 (ATCC 43889), *Klebsiella pneumoniae* (clinical isolate), *Aeromonas hydrophila* (clinical isolate), *Staphylococcus aureus* (ATCC 25923), *Streptococcus pyogenes* (clinical isolate) and *Streptococcus pneumoniae* (ATCC 49619). Reference isolates were confirmed by using the API identification kit (bioMérieux Vitek) according to the manufacturer's instructions. *Escherichia coli* O157:H7 (ATCC 43889) were confirmed positive with O157 and H7 antisera as well as with a polymerase chain reaction (PCR) for the detection of shiga toxins 1 and 2 (Stx1 and Stx2) (Müller et al., 2001).

Selective enrichment

One hundred microlitres (100 µℓ) of each sewage sample were inoculated in 50 mℓ of buffered peptone-saline water (BPSW) (Oxoid, CM509) supplemented with Vancomycin (8 mg·ℓ$^{-1}$), Cefixime (0.05 8 mg·ℓ$^{-1}$) and Cefsulodin (10 8 mg·ℓ$^{-1}$) (VCC) antibiotic solution (MAST® Diagnostics) to inhibit the growth of most interfering bacteria. The suspensions were incubated in a shaking incubator (LSI, Labcon) for 6 h at 37°C while rotating at 150 r·min^{-1}. River water samples (100 mℓ) were filtered through 0.45 µm Cellulose Nitrate filter membranes (Sartorius AG, Germany) and incubated as described above.

Immunomagnetic separation

Twenty microlitres of the Dynabead® suspensions were incubated in 1.5 mℓ Eppendorf tubes with 1 mℓ aliquots of the pre-enriched samples at room temperature for 10 min with continuous mixing by hand. This step was performed to allow the O157-specific antibodies coated onto the beads to bind to the target bacteria. The bead-bacteria complexes were separated using a magnetic particle concentrator, Dynal MPC-M for 3 min (Dynal, Oslo). After discarding the supernatants and washing the bead-particles with PBS-Tween (Sigma), the complete immunomagnetic separation and washing procedure was repeated 4 times (Dynal® product brochure, 1995). The final bead-bacteria complexes were re-suspended in 100 µℓ washing buffer (PBS-Tween). After IMS, 10 µℓ of the IMS concentrates were transferred to *E. coli* O157 selective media by the spread plate method.

Escherichia coli O157 selective media

The media used in this study were Cefixime-tellurite Sorbitol-MacConkey agar (CT-SMAC) (Oxoid). *E. coli* O157:H7 strains produced typical colourless colonies on CT-SMAC after 24 h of incubation at 37°C. Suspect *E. coli* O157:H7 colonies were individually tested for agglutination using a commercial *E. coli* O157 slide agglutination kit with antisera against *E. coli* O157 (Mast Assure, Mono Factor O157, code: M12030). All *E. coli* colonies were biochemically confirmed by their ability to produce indole from tryptophan using Kovac's reagent (ISO, 2001).

Biolog bacterial identification

Gram-stains were performed on all the sorbitol-negative isolates to determine which Biolog micro-plate (GN2/GP2) (Biolog, Hayward, Calif.) to use in the bacterial identification procedure. Bacterial cell morphology was also documented (results not shown). Biolog GN2 and GP2 micro-plates were respectively used to identify Gram-negative and Gram-positive bacterial isolates. Appropriate cell densities (OD = ± 0.65 at 560 nm) (Spectro 22, Digital Spectrophotometer, Labomed, USA) of sorbitol-negative microbial suspensions were inoculated into the micro-well plates as described in the GN2/GP2 Biolog MicroPlate system manuals and incubated at 37°C for 24 h. Colour development was automatically recorded using a micro-plate reader (ICN Flow Titertek® Multiscan Plus, Version 2.03, LabSystems, Finland) with a 590 nm wavelength filter. A similarity index of 0.500 or higher was used for all micro-plates after 24 h. The results were interpreted with the Biolog Microlog 3.70 database and software (Biolog, Hayward, Calif.).

Bacterial DNA extraction

Suspect colonies were suspended in distilled water and heated (99°C) in a thermocycler (Pharmacia LKB Thermocycler) in 0.5 mℓ Eppendorf tubes for 10 min. The samples were centrifuged at 10 000 x *g* (Eppendorf Centrifuge 5402) for 1 min and 10 µℓ volumes of the supernatant containing genomic DNA particles were used for DNA amplification without further treatment. Suspect *E. coli* O157:H7 colonies were examined for the presence of the phage-integrated genes coding for Stx1 and Stx2.

PCR- DNA amplification for Stx1 and Stx2

PCR amplification of genes specific for Stx1 and Stx2 was performed on the eluted DNA suspensions after DNA extractions. Oligonucleotide primers (synthesised by Sigma-Genosys Ltd.) specific for Stx1 (VT1) and Stx2 (VT2) were used in the polymerase chain reaction (PCR) as described by Pollard et al. (1990). The primers for Stx1 were VT1a: 5'- GAAGAGTCCGTGGGATTACG-3' and VT1b: 5'-AGCGATGCAGCTATTAATAA-3'. The primers for Stx2 were VT2a: 5'-TTAACCACACCCACGGCAGT-3' and VT2b: 5'-GCTCTGGATGCATCTCTGGT-3'. The PCR cycle consisted of an initial 5 min DNA denaturation cycle at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C (Pharmacia LKB Thermocycler). The amplicons (20 µℓ aliquots from each amplification) were detect-

ed by gel electrophoresis using a 2% agarose (SeaKem® LE) gel suspension stained with ethidium bromide (Sigma). The amplified products were visualised by UV-transillumination (UVP -Transilluminator) and the image was captured using the UVP Image store 5 000 gel documentation system with 100 base-pairing DNA (Promega) as molecular size marker.

Results and discussion

A high number of bacterial isolates, which included both sorbitol-positive and sorbitol-negative isolates, were detected in 10 µℓ of IMS-concentrate, which were plated on CT-SMAC. This observation could be due to non-specific binding of other bacteria to the O157-antibody-coated dynabeads or possibly on account of bacteria remaining in suspension during washing of the dynabead suspensions. These bacteria were resistant to the cefixime-tellurite antibiotic solution present in the SMAC media. Gram-staining of 81 Gram-negative, sorbitol-negative isolates revealed that the majority of the bacteria were bacilli (82.5%) followed by 17.5% with cocco-bacilli features. CT-SMAC was able to suppress the growth of all Gram-positive sorbitol-negative bacteria.

The Biolog bacterial identification system was used to identify the 81 Gram-negative isolates (using GN2 micro-plates) from 33 samples isolated after IMS and CT-SMAC. A number of reference strains (Gram-negative and Gram-positive) were included as controls and identified using GN2 and GP2 microplates to determine the specificity of the Microlog/Biolog microbial identification system. All reference strains were correctly identified with Biolog at genotype level and species level except *Streptococcus pyogenes*, which was identified as species *S. intestinalis*. *E. coli* O157:H7 was identified up to *E. coli* species level.

According to the results obtained from analysing environmental samples, Biolog identification revealed a variety of different bacterial species. These species embodied a number of well-known sorbitol-negative bacteria which included: *Burkholderia glumae* (19.5% of all isolates), *Aeromonas veronii* (4.9%), *Vibrio tubiashii* (3.7%), *Flavobacterium ferrugineum* (3.7%), *Aeromonas hydrophila* (2.4%), *Aeromonas media* (2.4%), *Aeromonas caviae* (2.4%), *Vibrio mediteranei* (2.4%), *Vibrio furnishii* (2.4%), *Vibrio fluvialis* (2.4%), *Vibrio vulnificus* (1.2%), *Vibrio cholerae* (1.2%), *Vibrio campbelli* (1.2%), *Vibrio splendidus* (1.2%), *Vibrio metschnikovii* (1.2%), *Vibrio cincinnatiensis* (1.2%), *Actinobacillus pleuropneumoniae* (1.2%) and *Yokenella regensburgei* (1.2%). The majority of bacteria isolated on CT-SMAC were identified as *Burkholderia glumae* (19.5% of isolates and 50% of samples). *Burkholderia* species are Gram-negative bacilli (0.5 to 0.7 µm x 1.5 to 2.5 µm, motile by means of 2 to 4 polar flagella) and classified as plant pathogens. *Vibrio* spp. were identified in 56% of the samples (18.3% of all isolates) and are 0.5 to 0.8 µm x 1.4 to 2.4 µm, facultatively anaerobic, Gram-negative, curved or straight bacilli. *Vibrio* spp. are mainly aquatic species and pathogenic for both human and animals (Bergey's Manual of Systematic Bacteriology, 1984). *Pseudomonas* spp. were identified in 23% of samples and 6.1% of all isolates. Some of the *Pseudomonas* spp. included species such as *P. taetrolens*, *P. syringae*, *P. fluorescens* and *P. savastonoi*. *Pseudomonas* spp. are widely distributed in nature. Some of these *Pseudomonas* species have been recognised as opportunistic pathogens of humans, animals and plants (*Bergey's Manual of Systematic Bacteriology,* 1984).

A number of sorbitol-negative colonies were identified with Biolog as bacteria, which are exclusively or predominantly sorbitol-positive. *P. syringiae* were sorbitol-positive in 100/100 of cases, *P. savastonoi* (88/100), *P. fluorescens* (50/50), *Kluyvera ascorbata* (40/100), *Rahnella aquatilis* (94/100), *Klebsiella pneumoniae* (96/100) and *Kluyvera cryocrescens* (38/100) (*Bergey's Manual of Systematic Bacteriology,* 1984).

Shortcomings of media for the selective cultivation of *E. coli* O157:H7 in test samples with heavy background growth such as sewage and river water have been reported by other researchers (Bettelheim, 1998). False-positive laboratory reports can result in delayed consideration of alternative diagnoses or therapeutic interventions. Resistance of non-O157 bacteria to the antibiotics used in SMAC may largely be accountable for the problem. It could be beneficial to incorporate other antimicrobial agents into the current CT-SMAC protocol, which could more efficiently suppress other bacteria in the VCC-BPSW and background growth on CT-SMAC. Another solution may be to develop affordable alternative media based on the chromogenic/fluorogenic properties of *E. coli* O157.

Only two strains isolated from 2 different sewage samples were positively identified as *E. coli* O157 (2.47% of 81 isolates) using Biolog. These two strains agglutinated with O157 antisera and possessed the gene coding for Stx1 according to the PCR results. This implies a false positive *E. coli* O157 detection rate of 97.68%. Other researchers have observed false positive detection rates for *E. coli* O157:H7 of between 37% and 57% in beef and milk samples using CT-SMAC agar (Manafi and Kremsmaier, 1999; Manafi and Kremsmaier, 2001).

A total of 13/81 (16%) of all sorbitol-negative isolates was unidentifiable with the Biolog microbial identification system. A possible explanation is that the isolates from this study were isolated from environmental samples still unknown and not included in the Biolog database. All the sorbitol-negative bacteria identified were able to multiply in VCC-BPSW and grow on CT-SMAC. Additional or alternative antibiotics might be useful in improving the selective enrichment of *E. coli* O157:H7 in BPSW and SMAC. The addition of Cefixime-tellurite was not sufficient in suppressing all interfering bacteria. Other researchers have suggested adding Cefixime-rhamnose (CR-SMAC), 5-bromo-4-chloro-3-indoxyl-ß-D-glucuronide (BCIG-SMAC) or 4-methylumbelliferyl-ß-D-glucuronide (MSA-MUG) to SMAC agar to improve selectivity (Szabo et al., 1986; Okrend et al. 1990; Chapman et al., 1991). Stein and Bochner (1998) suggested adding novobiocin (10 $mg.\ell^{-1}$) in selective agar for the inhibition of background flora. Non-specific binding and/or bacterial clumping of other bacteria to the O157-antibody-coated dynabeads during IMS posed another problem when aiming to selectively isolate *E. coli* O157:H7 from sources such as sewage and polluted water. Multiple washing steps (4 or more washing steps) of the IMS solutions with PBS-Tween and careful resuspension and/or slow vortexing after washing may increase the sensitivity of the method. It is thus important to consider all these problems to improve the selective capabilities of the IMS / CT-SMAC method.

Conclusions

The selective media used in this study had shortcomings with regard to the selective cultivation of *E. coli* O157 bacteria. Consequently the isolation of *E. coli* O157:H7 among suspect colonies was rather low (2.4%). It is therefore possible that *E. coli* O157:H7 colonies were missed, and that the actual prevalence is higher than reflected by the two isolates obtained from two different sewage samples. However, it can also be possible that the prevalence of *E. coli* O157:H7 in the samples investigated in this

study was indeed low. Unfortunately it was difficult to assess the potential presence of these pathogens in the environmental water concerned because no meaningful data are available on the prevalence of *E coli* O157:H7 infections in the communities from which these wastes originated. In addition, there were no meaningful data in the rest of the world to which these results can be compared. Although *E. coli* O157:H7 has been recovered from sewage in countries such as Germany (Höller et al., 1999), no quantitative data on the prevalence of the pathogens in the samples under investigation were available. The apparently low prevalence of *E. coli* O157:H7 in the environmental water investigated may be explained by data, which suggested that the prevalence of *E coli* O157:H7 infections is lower among humans in the Southern Hemisphere, such as South Africa, than in countries of the Northern Hemisphere (WHO, 1997).

According to the results in this study it is clear that the presence of these interfering bacteria obstructs the isolation of *E. coli* O157:H7 from sources with high bacterial counts. New selective media have been developed to compensate for the number of false positive results of CT-SMAC and to increase the effectiveness of *E. coli* O157:H7 isolation (Manafi and Kremsmaier, 2001). Other *E. coli* O157:H7 isolation media include Rainbow agar O157 (Biolog, Hayward, USA), CHROMagar O157 (CHROMagar, Paris, France), Biosynth Culture media O157:H7 (Biosynth, Staad, Switzerland) and Fluorocult *E. coli* O157:H7 (Merck, Darmstadt, Germany). A major consideration when using alternative selective media is the cost. These *E. coli* O157 selective media are too expensive for the routine analyses of contaminated water samples. Sorbitol-MacConkey agar with the addition of alternative antimicrobial, chromogenic or fluorogenic agents will still be the most cost-effective *E. coli* O157 selective media available on market. Therefore, the modification of the selective CT-SMAC *E. coli* O157:H7 cultivation media is recommended with regards to *E. coli* O157 isolation from environmental samples with high bacterial content.

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