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A new chromogenic medium for isolation of *Bacteroides fragilis* suitable for screening for strains with antimicrobial resistance

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ABSTRACT

There have been an increasing number of reports describing the acquisition of antimicrobial resistance by *Bacteroides fragilis* including the occurrence of strains with resistance to multiple antimicrobials that are relied upon for treatment of infections. The aim of this study was to design a chromogenic selective medium for isolation of *B. fragilis* that could be adapted for specific isolation of antimicrobial-resistant strains. *Bacteroides* chromogenic agar (BCA) was the result of this endeavour and allowed growth of *Bacteroides* spp. as black colonies and the efficient inhibition of almost all other genera tested. The medium also allowed some differentiation of *B. fragilis* from other members of the *B. fragilis* group. When compared with an adaptation of *Bacteroides* bile-esculin agar (BBE) for the isolation of *B. fragilis* from 100 stool samples, 30 isolates of *B. fragilis* were recovered on BCA compared with 19 isolates recovered on BBE ($P = 0.022$). When supplemented with meropenem (4 μg/ml) or metronidazole (2 μg/ml), BCA could be used to select for the growth of *B. fragilis* isolates with resistance to these agents. We conclude that BCA is a useful research tool for surveillance studies to assess the prevalence of *B. fragilis* and, in particular, the occurrence of antimicrobial-resistant strains.

1. Introduction

Bacteroides fragilis is a common inhabitant of the normal gut flora but also an important opportunistic pathogen frequently associated with polymicrobial infections. Such infections include abscesses, intra-abdominal infections and bacteraemia. There have been an increasing number of reports describing the acquisition of antimicrobial resistance by *B. fragilis* and closely related species of the *B. fragilis* group [1–9]. On rare occasions, multidrug resistant (MDR) isolates have been encountered. For example, in 2013 the United States Centers for Disease Control and Prevention (CDC) reported the isolation of a MDR-*B. fragilis* in a patient who had recently been hospitalized in India [10]. The isolate showed resistance to a number of agents relied upon for the treatment of *B. fragilis* including clindamycin, imipenem, metronidazole and piperacillin/tazobactam. Resistance to ampicillin/sulbactam, cefotetan and moxifloxacin also was observed. MDR-*B. fragilis* has also been encountered in Europe [11,12]. The spectre of MDR-*B. fragilis* and the possibility that such strains will be increasingly encountered, prompted us to design a new selective chromogenic medium with the aim of detecting *B. fragilis* with high sensitivity and specificity. Such a medium might prove to be a useful tool for assessing the prevalence of antimicrobial-resistant *B. fragilis* in various populations.

2. Materials and methods

2.1. Materials

Unless otherwise stated all antimicrobials, culture media, chemicals and materials for PCR assays were obtained from Sigma-Aldrich (Poole, UK). 3,4-cyclohexenoesculetin-ß-D-glucoside was kindly synthesized by Professor Arthur James of Northumbria University (Newcastle upon Tyne, UK) as previously described [13]. Meropenem was supplied by the hospital pharmacy departments of Freeman Hospital, UK and the University Hospital of Wales, UK. Horse blood and sheep blood were obtained from TCS Biosciences (Buckingham, UK). Columbia agar was obtained from Oxoid

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(Basingstoke, UK). Fastidious anaerobe agar was obtained from E&O Laboratories (Bonnybridge, UK). For matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) we used the Bruker Biotyper (Bruker, Coventry, UK). Microbial isolates were obtained from the National Collection of Type Cultures (NCTC, London, UK) or the National Collection of Pathogenic Fungi (NCPF, London, UK) or the American Type Culture Collection (ATCC, Manassas, USA). Clinical isolates were obtained from the culture collections of the Freeman Hospital, UK or the UK Anaerobe Reference Unit, Cardiff, UK. Microbank beads used for storage of isolates were obtained from Pro-Lab Diagnostics (Bromborough, UK).

2.2. Preparation of Bacteroides chromogenic agar (BCA)

The new culture medium, designated *Bacteroides* chromogenic agar (BCA), was based on the principles used in the design of *Bacteroides fragilis* bile-esculin agar (BBE) as reported by Livingston et al. [14]. Twenty six media formulations were evaluated (data not shown) before arriving at the final formulation of BCA. In the final formulation, 3,4-cyclohexenoesculetin-ß-D-glucoside (CHE-GLU) [12] was used in place of esculin to target the β-glucosidase activity of *B. fragilis*. In the presence of iron salts, CHE-GLU is hydrolysed by *B. fragilis* to generate an insoluble black precipitate that shows little diffusion through agar, thus offering superior differentiation of *B. fragilis* colonies from other species within polymicrobial cultures. As with esculin, exposure to air is not required for generation of black colonies. Bile salts were not required. Initial trials showed that black colonies may not be generated in the presence of fermentable carbohydrates and this led to the inclusion of L-arabinose and L-rhamnose, which are fermented by other members of the *B. fragilis* group but not by *B. fragilis* [15]. This was done in an attempt to increase specificity for *B. fragilis* rather than targeting all members of the *B. fragilis* group. Gentamicin was retained as a selective agent, as used by Livingston et al. [14], but whole blood was used instead of hemin as this was found to be beneficial to the colony size produced by *B. fragilis*.

The final medium was produced as follows: 39 g of Columbia agar was suspended in 946 ml of deionized water and supplemented with 0.3 g CHE-GLU, 0.5 g of ferric ammonium citrate, 10 g of L-arabinose and 10 g of L-rhamnose before autoclaving at 116 °C for 20 min. The medium was allowed to cool to 50 °C in a waterbath before being supplemented aseptically with 50 ml defibrinated horse blood, 100 μg/ml gentamicin, 100 μg/ml fosfomycin, 25 μg/ml glucose-6-phosphate and 4 μg/ml amphotericin to give a final volume of 1 L. The resulting culture medium was dispensed into 90 mm Petri dishes.

2.3. Preparation of Bacteroides bile-esculin agar (BBE)

Bacteroides bile-esculin agar (Fluka Ref: 48300) was obtained from Sigma (Poole, UK) as a dehydrated medium and 64.5 g was suspended in 1 L of deionized water and briefly heated at 100 °C with frequent stirring as recommended by the manufacturer. The medium was allowed to cool to 50 °C in a waterbath. A 2 ml volume of a sterile solution of 5 mg/ml hemin was then added along with 2.5 ml of a stock solution of 40 mg/ml gentamicin and the pH was adjusted to 7.0. The resulting culture medium was dispensed into 90 mm Petri dishes. BBE obtained from Fluka is distinct from the classical formulation of BBE described by Livingston et al. [14].

2.4. Evaluation of Bacteroides chromogenic agar (BCA) with various species

BCA was challenged with pure cultures of 53 clinical isolates of *B. fragilis* that had been previously recovered from pathological samples at the Freeman Hospital Microbiology Department (Newcastle upon Tyne, UK) from distinct patients. Identification of *B. fragilis* isolates was achieved using MALDI-TOF MS. Forty-four isolates of other anaerobic or facultative anaerobic species obtained from various culture collections were also inoculated onto the medium. The other (non-*Bacteroides*) species included *Citrobacter freundii* (NCTC 9750), *Enterococcus aerogenes* (NCTC 10006), *Enterobacter cloacae* (NCTC 11936), *Escherichia coli* (NCTC 12241, 13476), *Hafnia alvei* (NCTC 6578), *Klebsiella pneumoniae* (NCTC 8172, 13438, 13439, 13440), *Morganella morganii* (NCTC 232), *Proteus mirabilis* (NCTC 10975), *Providencia rettgeri* (NCTC 7475), *Raoultella planticola* (NCTC 9528), *Salmonella typhimurium* (NCTC 74), *Serratia marcescens* (NCTC 10211), *Shigella sonnei* (NCTC 9774), *Yersinia enterocolitica* (NCTC 11176), *Enterococcus durans* (NCTC 662), *Enterococcus faecium* (NCTC 7171, 12952), *Enterococcus faecalis* (NCTC 775), *Enterococcus gallinarum* (NCTC 11428), *Streptococcus agalactiae* (NCTC 8181), *Streptococcus anginosus* (NCTC 10713), *Streptococcus pyogenes* (NCTC 8306), *Staphylococcus aureus* (NCTC 6571 11939), *Staphylococcus epidermidis* (NCTC 11047), *Lactobacillus acidophilus* (NCTC 2949), *Listeria monocytogenes* (NCTC 11994), *Candida albicans* (ATCC 90028), *Candida glabrata* (NCPF 3943), *Clostridium chauvoei* (NCTC 8070), *Clostridium histolyticum* (NCTC 503), *Clostridium perfringens* (NCTC 6719, 8449, 3110, 8081), *Clostridium septicum* (NCTC 547), *Clostridium sordellii* (NCTC 4708), *Clostridium sporogenes* (NCTC 532), *Clostridium difficile* (NCTC 11209) and *Peptostreptococcus anaerobius* (NCTC 11460). A few of these isolates were chosen due to their possession of acquired mechanisms of antimicrobial resistance, for example, *E. faecium* (NCTC 12952) has resistance to glycopeptides and other antimicrobials whereas *E. coli* (NCTC 13476) and *K. pneumoniae* (NCTC 13438, 13439, 13440) possess a range of antimicrobial resistance mechanisms including carbapenemase-production.

From a fresh subculture on Columbia blood agar, each isolate was suspended in sterile saline (0.85%) to a density equivalent to 0.5 McFarland units using a densitometer. A 1/10 dilution was then performed in sterile saline and an inoculum of 1 μL (approximately 10 000 CFU) was delivered onto BCA and Columbia blood agar (growth control) using a multipoint inoculator. For the 53 isolates of *B. fragilis*, a 1/1000 dilution was also performed on the initial (0.5 McFarland) suspensions and 1 μL of this was inoculated onto BCA and Columbia blood agar to give an inoculum of approximately 100 CFU/spot. All plates were incubated at 35–37 °C in an anaerobic workstation for 48 h and all tests were performed in duplicate.

2.5. Comparison of Bacteroides chromogenic agar (BCA) with Bacteroides bile-esculin agar (BBE) for isolation of B. fragilis from stool samples

One hundred anonymized stool samples from distinct patients that had been routinely submitted for culture for enteric pathogens at the Freeman Hospital Microbiology Department (Newcastle upon Tyne, UK) were used to evaluate BCA. One gram (or 1 ml) of each stool sample was suspended in 2 ml of 0.85% sterile saline and 10 μL of this suspension was inoculated onto individual plates of BCA and BBE. The inoculum was then spread to obtain isolated colonies. All plates were incubated at 35–37 °C in an anaerobic workstation for 48 h. After incubation, each colony type (irrespective of colour) recovered on either of the two media was identified using MALDI-TOF MS. Differences between BCA and BBE for isolation of *B. fragilis* from stool samples were compared using McNemar's test with the continuity correction applied. Statistical significance was taken as $P < 0.05$.