Development of nalidixic acid amphotericin B vancomycin (NAV) medium for the isolation of *Campylobacter ureolyticus* from the stools of patients presenting with acute gastroenteritis

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ABSTRACT

Recently, *Campylobacter ureolyticus* has been detected for the first time in the faeces of patients with acute gastroenteritis using polymerase chain reaction (PCR) techniques. Cultural isolation of *C. ureolyticusis* is not possible using the established selective methods for the isolation of thermophilic *Campylobacter* spp. from faeces. The aim of the current study is to develop a new selective medium capable of isolating *C. ureolyticus* from faecal samples. The newly-developed medium consists of Anaerobe Basal Agar with 10 g/L additional agar, 2 g/L sodium formate and 3 g/L sodium fumarate dibasic, to which 10 mg/L nalidixic acid, 10 mg/L amphotericin B and 20 mg/L vancomycin (NAV) are added as selective agents. Validation studies have shown that this experimental selective medium completely inhibits growth of *Candida* spp. and of *Enterococcus* spp. and permits reduced growth of selected coliforms and *Proteus* spp. Growth of *Campylobacter ureolyticus* on NAV medium is optimal in anaerobic and enriched hydrogen atmospheres. Additionally, an overnight enrichment step using Bolton broth to which 2 g/L sodium formate, 3 g/L sodium fumarate dibasic and the NAV supplement are added, in place of the commercial Bolton broth supplement, allows improved recovery of *C. ureolyticus* from patients' faeces.

KEY WORDS: Campylobacter ureolyticus.
Culture medium.
Gastroenteritis.

Introduction

Campylobacter ureolyticus is a recent addition to the *Campylobacter* genus, having previously been classified as *Bacteroides ureolyticus*.[1] *C. ureolyticus* has been detected in the faeces of patients with acute gastroenteritis using species-specific polymerase chain reaction (PCR) methods.[2]

C. ureolyticus has been detected in faecal specimens collected from children with Crohn's disease (CD) using genus-specific PCR,[3] and isolated from intestinal biopsies collected from children with CD.[4] Moreover, Mukhopadhya *et al*. detected a significantly higher prevalence of *C. ureolyticus* in colonic biopsy samples taken from adults with ulcerative colitis, compared with a control group, using a combination of *Campylobacter* genus-specific PCR and sequencing.[5] These reports suggest a previously unrecognised role for *C. ureolyticus* as an enteric pathogen.

Another recent study has shown that the bacterium is capable of adhering to human intestinal cell lines and of inducing cellular damage and microvillus degradation.[6] Reclassification of the organism has coincided with a re-evaluation of its pathogenesis.

While the prevalence of *C. ureolyticus* can be determined using molecular techniques, the organism will need to be isolated in order to study characteristics such as antimicrobial susceptibility, biochemistry and epidemiology. Established selective methods for the isolation of *Campylobacter* spp. from faeces[7,8] are unsuitable for the isolation of *C. ureolyticus* in terms of incubation temperature, atmosphere and the selective media used.[1,9] Additionally, there are no reports of recovery of *C. ureolyticus* from stools using filtration methods for the recovery of *Campylobacter* spp. such as the Cape Town protocol.[10,11]

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The aim of the current study is to promote recovery of *C. ureolyticus* from faecal samples by developing a selective medium for the purpose.

Materials and methods

Patient samples

The patient samples used in this study were received by the Department of Medical Microbiology, Cork University Hospital, from patients presenting with symptoms of acute gastroenteritis. All samples tested positive for *Campylobacter* spp. using EntericBio PCR (Serosep, Limerick, Ireland) but failed to grow in routine culture. A set of eight samples received during February and March 2012, having been identified as *C. ureolyticus* using species-specific PCR,[2] were

tested for the recovery of *C. ureolyticus*. All eight samples were then cultured using the improved pre-enrichment method.

Bacterial strains

The following control strains were used in this study: *C. ureolyticus* DSM 20703 (obtained from DSMZ, Germany) and *Campylobacter jejuni* NCTC 11322. In addition, well-characterised clinical isolates of a lactose-fermenting coliform, *Proteus* spp., *Enterobacter* spp., *Enterococcus* spp. and *Candida* spp. were included in this study.

Culture for* Campylobacter *spp.

Routine laboratory culture for *Campylobacter* spp. was performed using mCCDA (W11015, LIP Diagnostic Services, Galway, Ireland). The plates were incubated for 48 h in a microaerobic environment generated using CampyGen gas generating kits (CN025A; Oxoid, UK) and a 3.5 L gas jar (Oxoid). The presence of *Campylobacter* spp. was confirmed using conventional identification techniques including characteristic microscopic and colonial morphology. *Campylobacter jejuni* NCTC 11322 type strain was included with each batch of plates as a positive control.

Preparation of basal medium

Growth of *C. ureolyticus* DSM 20703 on Anaerobe Basal Agar (CM0972; Oxoid) with 10 g/L agar (Sigma-Aldrich, St. Louis, MO), Anaerobe Basal Agar with 10 g/L agar and 5% defibrinated horse blood (SR50; Oxoid) and Anaerobe Basal Agar with 10 g/L agar and 10% inactivated horse serum (TCS Biosciences, Buckingham, UK) was semi-quantitated according to a modified version of the ecometric method of Mossel *et al*.[12] The test plates were incubated at 37°C in an enriched hydrogen atmosphere (achieved using a gas generating kit [BR0038; Oxoid] and a 1 L gas jar for 48 h). This test was performed in triplicate.

The concentrations of sodium formate (Sigma-Aldrich) and sodium fumarate dibasic (Sigma-Aldrich) that permitted optimal microaerobic and anaerobic growth of *C. ureolyticus* was determined using Anaerobe Basal Agar with 10 g/L agar and the following concentrations of sodium formate (Sigma-Aldrich) and sodium fumarate dibasic (Sigma-Aldrich): 1 g/L sodium formate and 1.5 g/L sodium fumarate dibasic; 2 g/L sodium formate and 3 g/L sodium fumarate dibasic. Growth of *C. ureolyticus* DSM 20703 at these concentrations of sodium formate and sodium fumarate dibasic was semi-quantitated using the same modified version of the method of Mossel *et al*.[12] The test plates were incubated at 37°C in an enriched hydrogen atmosphere, anaerobically in an anaerobic cabinet and microaerobically for 48 h. This test was performed in triplicate.

Suitability studies of potential selective agents

Antimicrobial susceptibility testing was performed on *C. ureolyticus* DSM 20703 by disk diffusion with 21 of the most commonly used antimicrobial drugs (Table 1) on Anaerobe Basal Agar (Oxoid). Zone sizes were measured using Vernier callipers. The minimum inhibitory concentration (MIC) for *C. ureolyticus* DSM 20703 was also determined for trimethoprim/sulphonamide, cefixime and amphotericin B by Etest (AB Biodisk, Solna, Sweden), according to the manufacturer's instructions.

Based on the susceptibility testing results, the combination of nalidixic acid, amphotericin B and vancomycin was tested for possible antagonism by placing a nalidixic acid (30 µg) disk, a vancomycin (30 µg) disk and an amphotericin B Etest 15 mm apart on non-selective blood agar plates, lawned with Oxford *Staphylococcus* NCTC 6571 and *Escherichia coli* NCTC 10418 as controls. The inhibition zones were examined for radial symmetry.

Table 1. Concentrations of antimicrobial disks used in susceptibility testing of *C. ureolyticus* DSM 20703.

Antimicrobial agent	Content of disk (µg)
Ampicillin	10
Cefuroxime	30
Amoxicillin/clavulanic acid	30
Gentamicin	10
Ciprofloxacin	5
Amikacin	30
Ceftazidime	30
Meropenem	10
Ceftriaxone	30
Piperacillin/tazobactam	110
Aztreonam	30
Colistin	25
Rifampicin	5
Vancomycin	30
Teicoplanin	30
Trimethoprim	1.25
Nalidixic Acid	30
Nitrofurantoin	200
Cephradine	30
Cefoxitin	30
Cefepime	30
Ceftazidime	30

The possibility of using 2% salt as a selective agent was assessed and the suitability of a number of laboratory staining reagents for inhibition of competing faecal flora was also investigated. All studies of potential selective agents were performed in triplicate.

Validation of nalidixic acid amphotericin B vancomycin (NAV) medium

The growth of potentially overgrowing flora that might interfere with isolation of *C. ureolyticus* on the newly-developed nalidixic acid amphotericin B vancomycin (NAV) medium was evaluated by preparing a suspension equivalent to a 0.5 McFarland turbidity standard (0.5 suspension) of an *Enterococcus* spp., *Candida* spp. and a coliform separately in sterile distilled water. A 0.5 suspension of *Campylobacter ureolyticus* DSM 20703 was also prepared. Each suspension was diluted 10-fold in sterile distilled water and was streaked on a pre-reduced plate of NAV medium using a 1 µL inoculating loop.

The plates were incubated at 37°C in an enriched hydrogen atmosphere. Plates were examined for growth after 48-h incubation and growth of each organism was either expressed as a colony count, or, where colonies were too numerous to count, growth was graded as + (growth in

the primary inoculum only), ++ (growth in the primary and secondary inocula), +++ (growth including the tertiary inoculum) or ++++ (growth of all inocula). This test was performed in triplicate.

Growth of *C. jejuni* on NAV medium was assessed by plating the organism directly on NAV medium from a pure suspension. The inoculated plates were incubated anaerobically and microaerobically and examined after incubation for 48 h. This test was performed in triplicate.

***Growth of* C. ureolyticus**

Anaerobic conditions were achieved using an anaerobic cabinet (Concept 400 Anaerobic Workstation, Ruskinn Technology, Bridgend, UK). Growth on NAV medium was assessed by preparing a 0.5 suspension of *C. ureolyticus* DSM 20703 and a 10-fold dilution of this suspension, and by plating both suspensions on pre-reduced culture plates of NAV medium using a 1 µL loop after plates were pre-reduced for one hour. The inoculated plates were incubated anaerobically, microaerobically and in an enriched hydrogen atmosphere (achieved using an anaerobic gas generating kit [BR0038; Oxoid]) at 37˚C and were examined for growth of *C. ureolyticus* after 48-h incubation. Growth of *C. ureolyticus* DSM 20703 was graded as +, ++, +++ or ++++, as described above. This test was performed in triplicate.

The limit of detection on NAV medium for *C. ureolyticus* was also assessed by preparing various dilutions (1 in 10, 1 in 100, 1 in 1000 and 1 in 10,000) of a 0.5 suspension of *C. ureolyticus* DSM 20703 in sterile water and plating on NAV medium using a 1 µL inoculating loop. Plates were incubated in an enriched hydrogen atmosphere at 37˚C and examined for growth of *C. ureolyticus* after 48-h incubation. Growth of *C. ureolyticus* DSM 20703 was either expressed as a colony count or, where growth showed colonies too numerous to count, growth was graded as +, ++, +++ or ++++, as described previously. This test was performed in triplicate.

Use of NAV medium

Three patient stool samples that tested negative for gastrointestinal pathogens were spiked with *C. ureolyticus* DSM 20703. This was performed by preparing 0.5, 1.0, 1.5 and 2.0 (McFarland turbidity equivalent) suspensions of *C. ureolyticus* DSM 20703 in sterile water and by adding a 200 µL aliquot of each suspension to an 800 µL aliquot of faeces. Immediately after mixing, each spiked sample was inoculated on two plates of NAV medium (which had been pre-reduced in an anaerobic cabinet for an hour prior to inoculation) and spread in order to achieve good isolation of colonies. One inoculated plate was incubated in an enriched hydrogen atmosphere at 37˚C. A metronidazole disk (5 µg) was placed in the primary inoculum of the other inoculated plate to allow for distinction between aerobic and anaerobic organisms. This plate was then incubated anaerobically at 37˚C.

A total of eight patient faecal samples which tested positive for *C. ureolyticus* by PCR were also cultured on pre-reduced plates of NAV medium and incubated anaerobically and in an enriched hydrogen atmosphere at 37˚C. All faecal samples were tested in triplicate.

All inoculated NAV plates were examined for growth of *C. ureolyticus* at two, four and seven days. Colonies that were Gram-negative on staining and which were susceptible to metronidazole were tested for positive oxidase and positive urease tests, a formate-fumarate requirement for anaerobic growth, and an inability to grow aerobically at 37˚C.

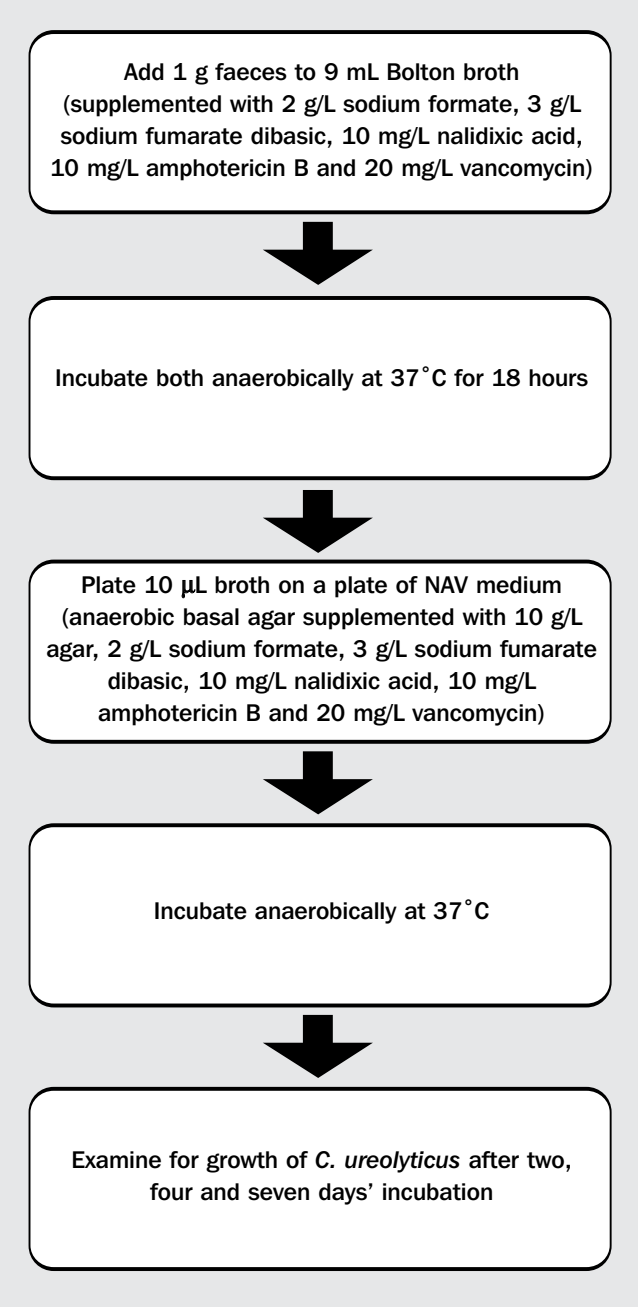

Fig. 1. Isolation of *C. ureolyticus* from faeces (including broth enrichment step).

Faecal enrichment

This enrichment method was developed to encourage growth of *C. ureolyticus* from faecal samples which had tested positive for *C. ureolyticus* by PCR, but for which direct culture had not yielded a positive result. In all cases, samples were stored at –20˚C and were tested in triplicate

Bolton broth (CM0983; Oxoid, UK) was combined with 2 g/L sodium formate, 3 g/L sodium fumarate dibasic and the NAV supplement in place of Bolton broth supplement, to the same concentrations as those used for the NAV medium. This was assessed for efficacy of overnight enrichment of

faecal samples before subculture on NAV medium, whereby 1 g faeces was added to 9 mL Bolton/NAV broth and incubated anaerobically overnight at 37°C, from which a 10 µL loopful was inoculated on NAV medium after incubation.

The validation method used for broth enrichment was as follows: a 0.5 suspension of *C. ureolyticus* DSM 20703 was prepared in sterile water, from which 10-fold dilutions were made. A 1 mL volume of each dilution was added to 9 mL Bolton/NAV broth and incubated as described above, before subculturing to NAV medium (Fig. 1).

Results

Determination of optimal composition of basal medium

The absolute growth index (AGI) of *C. ureolyticus* DSM 20703 on unsupplemented Anaerobe Basal Agar was equivalent to that achieved on Anaerobe Basal Agar with 5% defibrinated blood (100%) and greater than that on Anaerobe Basal Agar with 10% inactivated serum (73.3%). Therefore, supplementation of the basal medium with blood or serum was not deemed necessary.

When the AGI of *C. ureolyticus* DSM 20703 on batches of Anaerobe Basal Agar with varying concentrations of sodium formate and sodium fumarate dibasic were compared, the greatest microaerobic and anaerobic growth of *C. ureolyticus* occurred on Anaerobe Basal Agar with 2 g/L sodium formate and 3 g/L sodium fumarate dibasic. Hence, these were the concentrations of sodium formate and sodium fumarate dibasic used in the basal medium and with Bolton broth. The final compositions of NAV medium and NAV broth are shown in Tables 2 and 3.

Potential selective agents

C. ureolyticus DSM 20703 produced large zones of inhibition exceeding 60 mm in diameter to the majority of the antimicrobials tested, with the exception of nalidixic acid, teicoplanin, vancomycin, cephradine and cefoxitin. In the absence of Clinical Laboratory and Standards Institute (CLSI) guidelines, these large zones were interpreted as susceptible results. Surprisingly, the control strain showed a large zone of inhibition to trimethoprim (1.25 µg disk), and a trimethoprim/sulphonamide Etest MIC result of 0.016 µg/mL was observed.

C. ureolyticus DSM 20703 produced complete resistance (not exceeding 6 mm in diameter) to teicoplanin, vancomycin, cephradine and cefoxitin. The control strain produced a zone of inhibition of 12 mm diameter to nalidixic acid, which was interpreted as an intermediate result given the absence of official interpretive guidelines.

The addition of 2% salt proved non-inhibitory to *C. ureolyticus* DSM 20703 but was also non-inhibitory to the wild-type Enterobacteriaceae tested. In addition, swarming of *Proteus* spp. occurred on Anaerobe Basal Agar with 10 g/L agar and 2% salt but not on Anaerobe Basal Agar with 10 g/L agar. The only laboratory stain tested that was inhibitory to wild-type Enterobacteriaceae was malachite green. However, *C. ureolyticus* DSM 20703 failed to grow in the presence of malachite green, indicating the unsuitability of this as a selective agent.

The combination of nalidixic acid, amphotericin B and vancomycin showed no antagonism so that the inhibition zones produced by the nalidixic acid and vancomyin disks placed on plates lawned with Oxford *Staphylococcus* NCTC 6571 and *Escherichia coli* NCTC 10418 were radially symmetrical.

Table 2. Composition of NAV medium for cultivation of *C. ureolyticus* from faeces samples.

Component	Concentration (g/L)
Anaerobe basal agar	46
Additional agar	10
Sodium formate	2
Sodium fumarate dibasic	3
Nalidixic acid	0.01
Amphotericin B	0.01
Vancomycin	0.02

Validation of NAV medium

Growth of a wild-type coliform was reduced, but not completely inhibited, on Anaerobe Basal Agar with 10 mg/L nalidixic acid, 10 mg/L amphotericin B and 20 mg/L vancomycin, in comparison to growth on non-selective blood agar. The NAV medium completely inhibited growth of a randomly selected wild-type *Enterococcus* spp. and wild-type *Candida* spp. Growth of *Campylobacter ureolyticus* DSM 20703 on NAV medium was equivalent to that achieved on non-selective blood agar. The NAV medium did not support either microaerobic or anaerobic growth of *C. jejuni* NCTC 11322.

The NAV medium was capable of supporting microaerobic growth of *C. ureolyticus*, but microaerobic growth was reduced in comparison to growth in anaerobic or enriched hydrogen atmospheres. Growth of *C. ureolyticus* was equivalent both for anaerobic and enriched hydrogen atmospheres.

C. ureolyticus was recovered from dilutions of *C. ureolyticus* DSM 20703 to the 1 in 10,000 dilution of a 0.5 suspension from NAV medium. However, the limit of detection of the Bolton/NAV broth enriched control strain was $1x10^{-14}$ from a starting culture of a 0.5 suspension (Fig. 2).

Use of NAV medium

C. ureolyticus was recovered without difficulty from all of the spiked faecal samples using NAV medium after 48-h incubation both in anaerobic and enriched hydrogen atmospheres. However, this was accompanied by

Table 3. Composition of NAV broth for enrichment of *C. ureolyticus* from faeces samples.

Component	Concentration (g/L)
Bolton broth	27.6
Sodium formate	2
Sodium fumarate dibasic	3
Nalidixic acid	0.01
Amphotericin B	0.01
Vancomycin	0.02

considerable growth of Enterobacteriaceae from these samples. For the set of eight faeces samples tested, six of the eight PCR-detected (wild type) *C. ureolyticus* strains were recovered on culture only after enrichment. On NAV medium, *C. ureolyticus* colonies appeared as shiny, convex, translucent colonies 1–2 mm in diameter after 48-h incubation.

C. ureolyticus colonies did not exhibit pitting or corroding on NAV medium as they do on blood agar. After 72 h, some of the cultures exhibited a spreading morphology on NAV medium. On Gram staining, *C. ureolyticus* appeared as slender, pale-staining Gram-negative bacilli. *C. ureolyticus* cells ranged in length but were longer and wider than those of *C. jejuni*, and while some *C. ureolyticus* cells were curved, none exhibited the S shape or spiral shape characteristic of *C. jejuni* and *C. coli*. Identity was confirmed using colony PCR for *C. ureolyticus*.

Discussion

Bullman *et al*. detected *C. ureolyticus* in the stools of patients with gastroenteritis using PCR.[2] There are, however, no reports of isolation of *C. ureolyticus* from faeces. Conventional microbiological isolation of *Campylobacter* relies heavily on microscopy examination of colonies suspected to be *Campylobacter*. However, *C. ureolyticus* has a distinct appearance to that of *C. coli* or *C. jejuni*, appearing as longer, wider rods that may curve slightly. This, combined with the common use of selective *Campylobacter* media and temperatures selecting for thermophilic species by most clinical laboratories,[7,8] may have delayed the organism's identification from the faeces of patients presenting with gastroenteritis.[1,9,11] In the present study, a new selective medium was formulated to facilitate improved recovery of the organism from faeces.

The medium of Eley *et al*.[13] was used as a starting point for the development of a selective medium capable of isolating *C. ureolyticus* from faeces. Being similar in composition, but not requiring pH adjustment to 6.8, Anaerobe Basal Agar was used instead of Fastidious Anaerobe Agar.[13,14] Anaerobe Basal Agar with 10 g/L agar was determined to be sufficiently nutritious to support good growth of *C. ureolyticus* in an enriched hydrogen atmosphere without the need for supplementation with blood.

Although *C. ureolyticus* grows optimally in a hydrogen enriched microaerobic atmosphere, the organism is capable of microaerobic and anaerobic growth on media containing formate and fumarate.[1,15] While studies on *C. ureolyticus* differ with regard to the amount of sodium formate and sodium fumarate incorporated into the culture medium,[13,16–19] the authors found that the greatest anaerobic and microaerobic growth of *C. ureolyticus* was achieved using 2 g/L sodium formate and 3 g/L sodium fumarate dibasic.

The finding that microaerobic growth of *C. ureolyticus* on NAV medium was reduced in comparison to growth in anaerobic and enriched hydrogen atmospheres was somewhat surprising, given that *C. ureolyticus* is a microaerophile rather than an anaerobe, as previously reported.[15] The poor microaerobic growth exhibited by *C. ureolyticus* on NAV medium could be due to oxygen toxicity, which may also explain why this medium failed to support microaerobic growth of *C. jejuni*.[20]

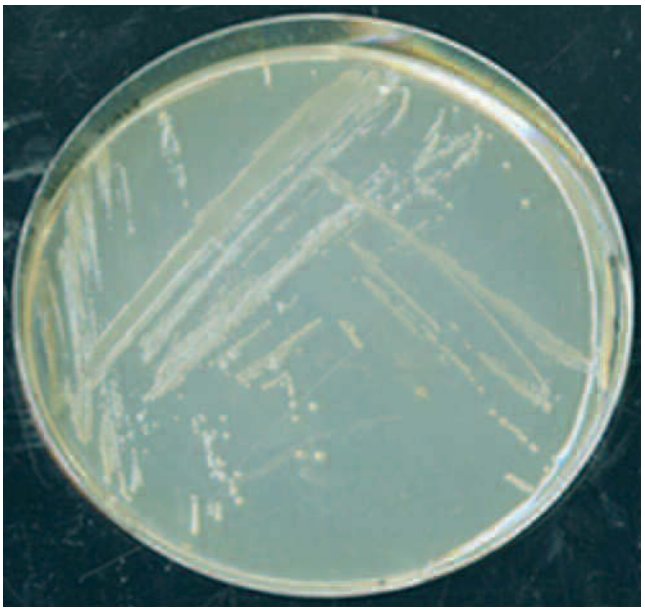

Fig. 2. Appearance of *C. ureolyticus* on NAV medium.

Although Anaerobe Basal Agar contains iron salts, pyruvate and cysteine, which act as reducing agents, it is likely that the addition of charcoal or blood to the medium would have reduced oxygen toxicity and improved microaerobic growth of *C. ureolyticus*.[14,20] However, it was feared that addition of blood or charcoal would facilitate increased growth of unwanted flora. This was of particular concern given that NAV medium contains only 10 mg/L nalidixic acid for the inhibition of Enterobacteriaceae.

Although microaerobic growth of *C. ureolyticus* on NAV medium was poor, anaerobic growth of *C. ureolyticus* was equivalent to growth in an enriched hydrogen atmosphere. The plates for *C. ureolyticus* culture were therefore incubated both in anaerobic and hydrogen enriched atmospheres, but not microaerobically.

In routine *Campylobacter* culture, the most commonly isolated non-enteropathogenic faecal flora are Enterobacteriaceae, enterococci and yeasts.[20–22] The antimicrobial agents included in NAV medium were chosen for their ability to inhibit these organisms while supporting growth of *C. ureolyticus*.

The NAV medium contained 10 mg/L amphotericin B as an antifungal agent, which is the same concentration as that used in mCCDA and modified Karmali selective medium.[14,20,23] This concentration of amphotericin B completely inhibited growth of the *Candida* spp. used in the validation study. Furthermore, no yeast isolates grew from any of the patient samples cultured, most likely due to the presence of amphotericin B[23] in the medium, combined with incubation in anaerobic and enriched hydrogen atmospheres, which are inhibitory to aerobic yeasts.[24]

The NAV medium also contained 20 mg/L vancomycin for the inhibition of Gram-positive organisms. This concentration of vancomycin (which is the same as that used by Karmali *et al*.[20]) completely inhibited growth of the *Enterococcus* spp. used in the validation study. However, a number of Gram-positive organisms did grow as discernable scanty colonies from the faecal samples cultured, including anaerobic cocci, streptococci, enterococci, *Bacillus* spp., *Clostridium* spp., *Lactobacillus* spp. and diphtheroid bacilli. However, any possible antagonism between vancomycin, amphotericin B

and nalidixic acid was ruled out prior to the use of this combination of antimicrobials.

The selection of an appropriate agent for the inhibition of Enterobacteriaceae (which comprise the major portion of unwanted faecal flora when isolating *Campylobacter*) was difficult. It was hoped that as all other species of *Campylobacter* are intrinsically resistant to trimethoprim,[25] this would also be true of *Campylobacter ureolyticus*. However, the apparent sensitivity of *C. ureolyticus* DSM 20703 to trimethoprim precluded incorporation of this agent into the medium.

In addition, *C. ureolyticus* DSM 20703 was susceptible to the majority of the cephalosporins tested, with the exception of cefoxitin and cephradine. Cefoxitin was deemed an unsuitable selective agent given reports of susceptibility among *C. ureolyticus* strains.[26,27] No information was available on the susceptibility of clinical isolates of *C. ureolyticus* to cephradine. However, in light of the reports of widespread susceptibility of *C. ureolyticus* to cephalosporins,[1,26,27] it was feared that clinical isolates of *C. ureolyticus* may be susceptible to cephradine, even though the control strain had tested resistant.

The possibility of using a stain to inhibit Enterobacteriaceae in place of an antibiotic was also investigated. However, the only stain that appeared inhibitory to the wild-type Enterobacteriaceae tested was malachite green, which is also inhibitory to the *C. ureolyticus* control strain. A 2% salt concentration was also an unsuitable selective agent, having no inhibitory effect on the Enterobacteriaceae tested and indeed had the added complication of promoting swarming by *Proteus* spp.

Given the problems associated with the use of cefoxitin and cephradine as selective agents, nalidixic acid at a concentration of 10 mg/L was chosen as the sole selective agent for inhibition of Enterobacteriaceae. Eley *et al*. successfully recovered *C. ureolyticus* from non-faecal clinical specimens using a medium containing 10 mg/L nalidixic acid.[13] In addition, Vandamme *et al*. reported the minimum inhibitory concentration of *C. ureolyticus* to nalidixic acid as 32 mg/L.[1]

In the present investigation, addition of 10 mg/L nalidixic acid to the basal medium was found to support growth of *C. ureolyticus* DSM 20703. The concentration of 10 mg/L nalidixic acid reduced growth of a wild-type coliform, although growth was not entirely inhibited. This was consistent with observation that the majority of contaminants grown from the patient faecal samples on NAV medium were Enterobacteriaceae. In spite of contamination, *C. ureolyticus* was successfully recovered from all of the spiked faecal samples, indicating that nalidixic acid at 10 mg/L sufficiently reduced growth of Enterobacteriaceae to allow for detection of any *C. ureolyticus* present.

Although a large number of Enterobacteriaceae have grown on NAV medium, swarming by *Proteus* does not occur. This is because NAV medium contains an additional 10 g/L agar in addition to that outlined for the preparation of anaerobic basal agar,[14] as described by Eley *et al*.[13] This ability of NAV medium to inhibit swarming by *Proteus* was important in enabling recovery of *C. ureolyticus*.

Validation studies of NAV medium were performed on a control strain, *C. ureolyticus* DSM 20703. Validation using wild-type faecal isolates would have been preferable but such isolates were unavailable, given that this is the first report of isolation of *C. ureolyticus* from faeces. This posed the potential problem that growth of wild-type faecal *C. ureolyticus* strains on NAV medium may differ from that of the control strain due to differences in antimicrobial susceptibilities and growth requirements.

In this study, wild-type strains were isolated from six out of eight *C. ureolyticus* PCR-positive samples using Bolton/NAV broth in combination with NAV medium. This finding differs from the validation study only in that *C. ureolyticus* DSM 20703 was recovered from faeces without the need for prior broth enrichment. The colonial morphology of these wild-type strains also did not resemble the control strain of *C. ureolyticus*. Studies of the limit of detection of NAV medium when combined with Bolton/NAV broth enrichment found this method capable of recovering *C. ureolyticus* from a $1x10^{-14}$ dilution of a 0.5 suspension of *C. ureolyticus*.

Despite the advantages of molecular methods, isolation of wild-type strains is necessary in order to study the pathogenesis of this organism in patients with gastroenteritis. The development of NAV medium permits the prospective study of *C. ureolyticus* using fresh stool samples. In addition, incubation of NAV medium in an anaerobic or enriched hydrogen environment, rather than a microaerobic environment, is recommended for the recovery of *C. ureolyticus* from stools. □

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