Research article

Establishing *Campylobacter* culture methods in a clinical diagnostic laboratory and the first report of *Campylobacter* species isolation in northern Sri Lanka

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Abstract

Introduction: The Enteric Reference Laboratory of the Medical Research Institute (ERL/MRI), Colombo is the only public sector laboratory in Sri Lanka that performs *Campylobacter* cultures. Due to logistic limitations involving specimen transport from distant sites, efforts were taken to establish *Campylobacter* culture facilities in our local clinical microbiology laboratory.

Methods: A blood-free charcoal-based selective agar medium (Karmali medium) was chosen based on performance characteristics and quality control (QC)/verification performed at the ERL/MRI. A suitable incubating method was assessed and chosen, and QC was performed in our laboratory. A technical staff member of our local laboratory received capacity building training at the ERL/MRI.

Results: The quality control/verification process of the Karmali medium was satisfactory. The variable atmospheric incubator was chosen as the incubating method as it was shown to be more economical in the long-term given the anticipated work load and the QC was satisfactory. Following a satisfactory verification process, *Campylobacter* culture method was introduced in our laboratory. Five *C. jejuni* and one hippurate-negative *C. jejuni/C. coli* was detected in faecal specimens of six paediatric patients between May-December 2018. The isolation rate was 2.25% (6/267). Ciprofloxacin resistance was detected in four out of five *C. jejuni* isolates.

Conclusion: Establishing *Campylobacter* culture methods in a routine clinical diagnostic laboratory will be beneficial in regions with high prevalence of diarrhoeal disease and with logistic limitations for specimen transport to the central reference laboratory. This is the first report of

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isolation and antimicrobial susceptibility of *Campylobacter* species from patients in northern Sri Lanka.

Introduction

Campylobacter species was identified as a significant enteric pathogen following the development of selective culture media by Butzler et al and Skirrow.[1] It is now recognized as a major cause of gastroenteritis worldwide, especially in younger children. A wide range of other gastrointestinal and extragastrointestinal manifestations have also been reported. The main causative species identified in human disease is *Campylobacter jejuni* followed by *Campylobacter coli*. Over the past decade the incidence and prevalence has increased worldwide. It is endemic in developing countries with reported isolation rates between 4-20%.[2]

The Enteric Reference Laboratory of the Medical Research Institute (ERL/MRI), Colombo is the only public sector laboratory in Sri Lanka that performs *Campylobacter* cultures. However there are many logistic limitations involving specimen transport from distant sites to the central reference laboratory. We detail the efforts taken to establish *Campylobacter* culture facilities in a clinical microbiology laboratory in a tertiary care hospital in Sri Lanka.

Methods

Identification
C. jejuni and *C. coli* are thermophilic, highly motile, Gram negative, curved, spiral or S-shaped bacilli requiring microaerobic atmospheric (5% O_2, 10% CO_2 and 85% N_2) conditions and 42 °C incubation temperature for optimum growth. Recommended duration of incubation is 48-72 hours.[3] Some authors report that 48-hour incubation is adequate for plates incubated in variable atmospheric incubators and 72-hour incubation may increase isolation rates if plates are incubated in jars.[4] Depending on the growth media used, grey, flat, irregular or spreading colonies are produced. Preliminary identification is by Gram stain, motility, oxidase test and catalase test. Oxidase positive colonies isolated on selective media incubated at 42 °C under microaerophilic conditions showing characteristic Gram stain morphology can be reliably identified as *Campylobacter* spp. Further identification tests include hippurate hydrolysis, indoxyl acetate hydrolysis, H_2S production, urease production, H_2 requirement, aryl sulfatase production, selenite reduction, growth in 1% glycine, growth at 25 °C and aero-tolerance. Hippurate hydrolysis can differentiate the most common species *C jejuni* (positive) from other *Campylobacter* spp. (negative) and no other tests are necessary for routine clinical purposes. *C. jejuni* and *C. coli* are biochemically identical with the exception of hippurate hydrolysis activity. Differentiation between rare hippurate hydrolysis negative *C. jejuni* strains and *C. coli* requires molecular methods; if unavailable, such isolates should be reported as hippurate-negative *C jejuni/C coli*.[3] (Table.1)

Table.1 Phenotypic properties of commonly encountered *Campylobacter* species of known or presumed clinical importance.

Organism	Catalase	H₂ required	Urease	H₂S (TSI)	Hippurate hydrolysis	Indoxyl acetate	Aryl sulfatase	Selenite reduction	Growth in 1%	Growth at 25 °C	Aerobic growth
C. jejuni subsp. *jejuni*	+	-	-	-	+	+	V	V	+	-	-
C. jejuni subsp. *doylei*	V	-	-	-	V	+	-	-	+	-	-
C. coli	+	-	-	V	-	+	-	+	+	-	-
C. fetus subsp. *fetus*	+	-	-	-	-	-	-	V	+	+	-
C. lari subsp. *lari/C. lari* subsp. *concheus*	+	-	V	-	-	-	-	V	+	-	-
C. upsaliensis	-	-	-	-	-	+	-	+	+	-	-

Adapted from "Useful phenotypic properties of *Campylobacter* and *Arcobacter* species"[3]

Selection of culture medium

Blood-based and charcoal-based selective agar media are used for *Campylobacter* isolation.[3] Blood-free, charcoal-based selective agars such as CSM (charcoal selective medium) and CCDA (cefoperazone charcoal deoxycholate agar) have better isolation rates and selectivity compared to the more commonly used blood-based selective agars such as Skirrow's medium and Butzler's medium. Of the available charcoal-based selective agars, Karmali's medium had the additional advantage of better differentiation.[5] Therefore Karmali medium (Oxoid, England) was chosen as the culture medium to be used in our laboratory.

Selection of incubation method

Microaerobic incubation methods for *Campylobacter* isolation include commercial gas generator kits used in jars, gas-jar evacuations followed by atmosphere replacement with bottled gasses and variable atmosphere incubators.[3] The cost of using gas generating sachets in a sealed jar was compared with the cost of using a variable atmosphere incubator based on estimates provided by local agents. The incubator method was selected over the gas-jar evacuation method based on the availability of local agents and long-term maintenance factors. Apart from the capital cost of the incubator, the projected cost for estimated daily usage of CO_2 and N_2 gas cylinders per month was calculated. Based on these findings the CO_2 incubator with the option to adjust O_2 control ranges to create hypoxic or hyperoxic culture conditions (HERAcell 150i,Thermo Scientific,Germany) was selected for use in our laboratory.

Technical training

A technical staff member of the local laboratory received capacity building training in enteric bacteriological diagnostics at the ERL/MRI, with special focus on *Campylobacter* diagnosis .

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Quality control/verification of Karmali selective medium prior to introduction
A verification (quality control/QC) process was performed by testing the Karmali selective medium in parallel with the selective medium (Butzler's) used in the ERL/MRI for *Campylobacter* isolation and a non-selective blood agar control medium containing blood agar base and 5% sheep blood (5% SBA). Karmali medium was prepared using Karmali campylobacter agar base (Oxoid, England) and modified Karmali selective supplement (Oxoid, England) according to the manufacturers' recommendations. Testing protocols for QC were modified based on a method described by Gun-Munro et al. and included dilutions of control cultures, simulated faeces specimens and clinical specimens.[5]

The first phase consisted of testing a control strain of *C. jejuni* ATCC 33291 and a confirmed clinical isolate of *C. coli* by direct culture and by simulated positive faecal specimens. *C. jejuni* ATCC 33291 and *C. coli* available at the MRI are maintained at minus 70 °C in Tryptic Soy Broth containing 20% glycerol. These strains were subcultured on 5% SBA and incubated micro aerobically at 42 °C for 48 hours in sealed jars containing gas generating sachets to create an atmosphere containing 5% O_2, 10% CO_2 and 85% N_2 to obtain a pure growth. 0.5 McFarland suspensions of these cultures was made from both *C. jejuni* and *C. coli* in phosphate buffered saline (PBS) and serial 10 fold dilutions were prepared from each of these working solutions. Faecal samples that were previously confirmed as negative for pathogenic enteric bacteria were emulsified in PBS and 0.5ml of neat and 10 fold diluted solutions of both *C. jejuni* and *C. coli* were inoculated to obtain homogenous suspensions. 20µl each of the neat and the 10-fold diluted solutions of the control cultures and the simulated faeces suspensions were then inoculated in parallel in Butzler's medium, Karmali medium and 5% SBA control in triplicate. All plates were incubated micro aerobically at 42 °C for 48 hours in sealed jars containing gas generating sachets to create an atmosphere containing 5%O_2, 10%CO_2 and 85%N_2.

The second phase consisted of culturing routine clinical specimens received by the national enteric reference laboratory in parallel on both Butzler's medium and Karmali medium.

Quality control/verification of the variable atmospheric incubator
C. jejuni ATCC 33291 and *C. coli* isolates received by our laboratory from the ERL/MRI were subcultured on 5% SBA by incubating at 42 °C for 48 hours in sealed jars containing gas generating sachets to create an atmosphere containing 5% O_2, 10% CO_2 and 85% N_2 . Each culture of *C. jejuni* and *C. coli* were inoculated as two sets in triplicate on Butzler's medium, Karmali medium and 5% SBA plate (control). One triplicate set of Butzler's, Karmali and 5% SBA plates were incubated in sealed jars containing gas generating sachets (CampyGen CN35, Oxoid, England) to create an atmosphere containing 5% O_2, 10% CO_2 and 85% N_2. The other triplicate set was incubated in parallel in the variable atmospheric incubator available in our laboratory (Thermo Scientific, HERAcell 150i) with adjustment of the atmosphere to create 5% O_2, 10% CO_2 and 85% N_2. All plates were incubated at 42 °C for 48 hours.

Introduction to the routine clinical bench
Following the satisfactory verification process, Karmali medium was introduced to the stool (faeces) culture bench in May 2018. Routine inoculation of faecal specimens from paediatric and

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adult patients was done. Plates were incubated in the variable atmospheric incubator at 42 °C in microaerophilic conditions for 48 hours. *Campylobacter* isolates were identified by Gram stain, oxidase test, catalase test and motility testing. Hippurate hydrolysis was used to confirm *C. jejuni*. Antibiotic susceptibility testing was performed by disc diffusion method according to Clinical Laboratory Standards Institute (CLSI) guidelines (M-45, 3rd edition 2016)[6] which have been validated only for erythromycin, ciprofloxacin and tetracycline.

Results

The capital expenditure for the variable atmospheric incubator was 2,497,500.00 Sri Lankan rupees (including VAT). Table.2 is a comparison of the cost using gas generating sachets in sealed jars and the use of the variable atmosphere incubator using carbon dioxide and nitrogen cylinders. The estimated cost is calculated for a period of one month based on market prices at the time of writing.

Table 2: Cost comparison of using gas generating sachets in sealed jars and variable atmosphere incubator using carbon dioxide and nitrogen cylinders.

Item	Price	Unit price	Estimated usage per month	Cost per month*
CampyGen CN 35 (3.5L) Oxoid	6,730.00 (10 sachets/box)	673.00	30 sachets	20,190.00
Total cost for gas generating sachet/jar method				**20,190.00**
CO_2 gas cylinder (medical grade; 3Kg) (Ceylon Oxygen Ltd)	2,174,00	2174.00	Quarter cylinder	543.50
N_2 gas cylinder (4.5 grade; 47L 150 Bar) (Ceylon Oxygen Ltd)	2,890.50	2890.50	1 cylinder	2,890.50
Total cost for variable incubator method				**3,434.00**

*The estimated cost is calculated for a period of one month based on current market prices.

Quality control of Karmali selective medium

C. jejuni ATCC 33291 and *C. coli* isolation comparisons between Karmali selective medium, Butzler's selective medium and 5% SBA control are shown in Table 3. In the first phase of evaluation, growth of control cultures on Karmali selective medium was similar to the growth on the 5% SBA control. Isolation rates on Karmali selective medium were greater at higher organism dilutions when compared with Butzler's medium. In simulated faecal specimens, faecal flora suppression was greater, colonies showed typical appearance, and isolation rates of *Campylobacter* colonies were higher in Karmali medium compared to Butzler's medium. During the time period of this evaluation neither *C. jejuni* nor *C. coli* were isolated from any clinical specimens received by the enteric reference laboratory.

Table.3: *C. jejuni* ATCC 33291 and *C. coli* isolation comparison between Karmali selective medium, Butzler's selective medium and 5% SBA control

Suspension	Growth on 5% sheep blood agar control		Growth on Butzler's medium		Growth on Karmali medium	
	C jejuni ATCC 33291	*C coli*	*C jejuni* ATCC 33291	*C coli*	*C jejuni* ATCC 33291	*C coli*
Neat (0.5 McFarland)	+	+	+	+	+	+
10^{-1} dilution	+	+	+	+	+	+
10^{-2} dilution	+	+	+	+	+	+
10^{-3} dilution	+	+	+	+	+	+
10^{-4} dilution	+	+	+	+	+	+
10^{-5} dilution	+	+	-	-	+	+

Quality control of the variable atmospheric incubator

C. jejuni and *C. coli* isolation was comparable in the selective medium (Karmali and Butzler's) and growth on 5% SBA control medium was satisfactory on all plates that were incubated in parallel in the sealed jars containing gas generating sachets (CampyGen CN35, Oxoid, England) and the variable atmospheric incubator available in our laboratory (HERAcell 150i,Thermo Scientific, Germany).

Results following introduction to the routine clinical bench

Five *C. jejuni* and one hippurate-negative *C. jejuni/C. coli* were detected in faecal specimens from six paediatric patients between May-December 2018. The isolation rate was 2.25% (6/267). All isolates were sent for re-confirmation to the ERL/MRI.

Colonies on Karmali medium were round, grey, moist and semi-translucent. Faecal flora was significantly suppressed, and *Campylobacter* colonies were easily identifiable. All six isolates demonstrated typical 'S' shaped/spiral Gram stain appearance and motility. Five isolates were oxidase-positive and catalase-positive. These five isolates were confirmed as *C. jejuni* by hippurate hydrolysis and all were sensitive to erythromycin and tetracycline. Four *C. jejuni* were resistant and one was sensitive to ciprofloxacin. ERL/MRI confirmed the sixth isolate as oxidase-positive, catalase-negative and indoxyl-acetate-positive. This was reported as hippurate-negative *C. jejuni/C. coli* which was sensitive to all three antibiotics tested. (Figures 1and 2)

All patients were ≤ 2 years of age. One patient had a mixed infection with *Salmonella* Chester. *Salmonella* Chester was isolated in both faeces and blood of this patient.
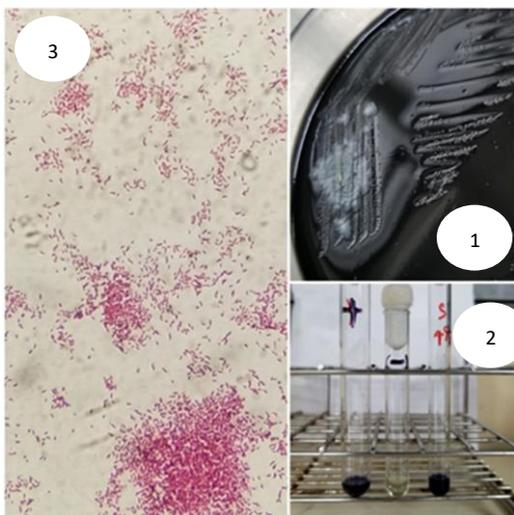
Figure 1
1.*Campylobacter* species colonies isolated from a faecal specimen on Karmali selective medium
2.Hippurate hydrolysis test
3.Gram stain
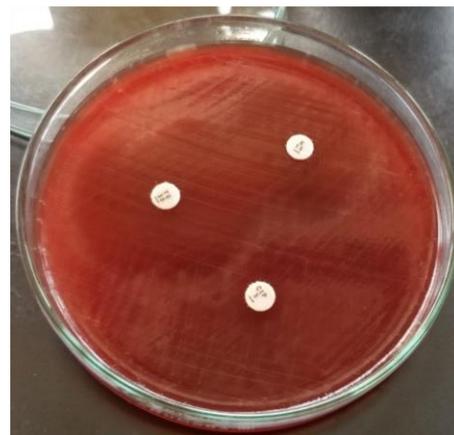
Figure2
Campylobacter ABST on Mueller-Hinton agar with 5% sheep blood

Discussion

The first report of *Campylobacter* isolation in Sri Lanka was by Palasuntheram et al in 1982 and all three isolates were identified as *C. jejuni*.[7] The same authors reported a total of nine isolates of *C. jejuni* by the year 1984.[8] Studies by Pathirage[9], Cooray et al[10], and Udugama[11] and Vidyarathne et al[12] reported isolation rates of *Campylobacter* species as 9.36%, 2.5-6.7%, 3.8% and 8.82% respectively. *C. jejuni* and *C. coli* were the only species identified and the predominant species was *C. jejuni*. The overall ciprofloxacin resistance rate reported by Pathirage[9], Cooray et al[10], Udugama[11] and Vidyarathne et al[12] was 43.9%, 35.2%, 100% and 83.3% respectively.

Our laboratory received 2092 faecal specimens between June-December 2018. Significant *Shigella* species isolation has been noted over the years.[13] However a large number of faecal specimens were culture-negative for other common enteric pathogens such as *Salmonella* spp, *Vibrio* spp. and pathogenic *E.coli*.[13] Detection of *Aeromonas*, *Plesiomonas* and *Yersinia* cannot be commented upon as they are not included in the current routine diagnostic workup. It was considered important to introduce routine *Campylobacter* culture methods in our clinical laboratory due to the high prevalence of diarrhoeal diseases in the region and the logistic limitations of being situated more 400km away from the central reference laboratory.

Early studies recommend the use of more than one selective culture medium to optimize isolation rates of *C. jejuni* and *C. coli*.[1,14,15]   Based on studies done by Karmali et al[1] and Gun-Munro et al[5], a charcoal-based selective medium is recommended for use as a single medium, especially in

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resource-limited developing countries. Satisfactory isolation rates, along with greater suppression of normal faecal flora and enhanced recognition of *Campylobacter* colonies were observed in charcoal-based media and the medium described by Karmali et al showed optimum performance. As shown by our cost estimations, the use of a variable atmospheric incubator was more economical in the long-term despite the initial capital cost. This is especially useful for regional laboratories that process a large number of specimens. Our clinical microbiology laboratory is based in the only tertiary care hospital of the Northern Province and also serves as a regional laboratory for four main peripheral hospitals within the Province. Consideration of the projected workload and logistic problems related to distance from ERL/MRI, led to the investment in a variable atmospheric incubator. We chose Karmali charcoal-based selective medium as the single culture medium based on published performance criteria[1,5] and the ease of preparation of a non-blood based medium in a routine clinical laboratory.

Despite *Campylobacter* species not being isolated from any clinical specimens (in both Butzler and Karmali media) during the second phase of evaluation at ERL/MRI, the satisfactory performance of Karmali medium during the first phase was considered adequate for an introductory trial in our routine diagnostic service. The isolation of six *Campylobacter* isolates (five *C. jejuni* and one hippurate-negative *C. jejuni/C. coli*) during a six month period indicates the success of the methods introduced and prevalence of the disease. Although all isolates were sensitive to erythromycin and tetracycline, ciprofloxacin resistance was detected in 4 of the 5 *C. jejuni* isolates. Fluoroquinolone-resistant *Campylobacter* spp. has been identified as one of the high priority pathogens in the WHO priority pathogens list for R&D of new antibiotics.[16] This highlights the importance of isolation and determination of antimicrobial susceptibility of *Campylobacter* spp. for treatment and surveillance purposes. This is the first report of isolation and antimicrobial susceptibility of *Campylobacter* species from patients in northern Sri Lanka.

Future approach
Studies by Endtz et al and Besse`de E et al indicated that the true incidence of *Campylobacter* enteritis could be underestimated if antibiotic containing selective culture media are used as the only method of diagnosis as this will inhibit *Campylobacter* species with variable antibiotic susceptibilities.[2,3,17,18] Incubating conditions (temperatures and gas composition) will also affect the isolation rates of various thermophilic and non-thermophilic *Campylobacter* species and those species that require H_2 for primary isolation.[2,3] Currently both culture-dependent and/or culture-independent (serological and molecular) methods are being used worldwide for laboratory diagnosis of *Campylobacter* species.[2,3] There is no gold standard method that will enable the detection of all probable *Campylobacter* human pathogens due to limitations of each method.[2] Membrane filtration techniques, enzyme-immuno assays (EIA-stool antigen tests) and molecular diagnostic methods have detected a wide-range of *Campylobacter* species as causative agents in human disease.[17,18] A South African study in children with diarrhoea using the membrane filtration culture technique showed that only 40% of *Campylobacter* species isolated were *C. jejuni*.[19] The Centres for Disease Control and Prevention (CDC) defines a confirmed case as a culture positive case and a probable case as detection of *Campylobacter* species using a culture-independent diagnostic test (CIDT) such as PCR and recommends culture confirmation (knowns as reflex culture) for these cases.[20] Quantitative PCR alone or PCR and EIA have been suggested as

alternatives for culture.[21] The use of CIDT as stand-alone tests are still being evaluated as the sensitivity, specificity and positive predictive value of these assays are reported to be variable.[20] M'ikanatha et al showed a variation between laboratories in testing methods used for *Campylobacter* detection in human stool samples and has recommended that laboratory practice guidelines for *Campylobacter* testing should be developed to ensure uniformity between laboratories and optimal case detection rates.[22] However isolation of *Campylobacter* will remain important for antimicrobial susceptibility testing and precise identification.[20] In countries with endemic high antimicrobial resistance rates this is essential for surveillance and treatment.[18,20] Therefore while culture techniques will continue to remain a main diagnostic feature, the introduction of CIDT for better case detection rates will need to be considered in the future.

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