Evaluation of a Novel Chromogenic Selective Medium for the Improved Detection of Campylobacter from Stool Samples

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BD mCCDA Clear-HT (CCHT; Nippon Becton Dickinson Company, Ltd.), a novel chromogenic selective medium was evaluated for its superior capacity to isolate Campylobacter jejuni/coli. When CCHT was assessed using 142 microbes including 42 Campylobacter jejuni/coli strains, all Campylobacter strains were found to form purple-colored colonies on CCHT whereas all the other microbes failed to grow. CCHT was then compared with commercially available selective media using 100 stool samples including 40 Campylobacter positive samples. CCHT detected Campylobacter jejuni/coli from 39 of 40 (97.5%) stool samples whereas it allowed competitive bacteria to grow as false positive colonies from 1 (1.0%) of 100 stool samples. The values of relative sensitivity (%) and specificity (%) for CCHT were 97.5 and 98.3 in this study. Our results demonstrated that CCHT had the highest detection ratio for Campylobacter jejuni/coli and the highest inhibition ratio against competitive bacteria among all selective media compared.

Key words : Campylobacter jejuni / Campylobacter coli / Chromogenic medium / Stool.

Campylobacter jejuni and C. coli are known worldwide as common causative agents of gastroenteritis (Humphrey et al., 2007). These Campylobacter species can not only lead to severe diarrhea but also trigger Guillain-Barré syndrome, an acute demyelinating polyneuropathy of the peripheral nervous system due to an autoimmune disorder (Speed et al., 1984). Hence, the accurate detection of these Campylobacter species is critical for the identification of causative agent and subsequent antimicrobial susceptibility tests.

For the detection of Campylobacter species, the charcoal cefoperazone deoxycholate agar (CCDA) and blood agar based medium such as Skirrow agar, are commonly used as conventional selective agar media worldwide (Bolton et al., 1984; Skirrow, 1977). However, these conventional selective media are opaque and stained due to the presence of charcoal or defibrinated blood. Thus, it is often hard to detect Campylobacter colonies on opaque and stained conventional media because Campylobacter forms colorless and stelliform colonies on these agar media. Also, the presence of antibiotic resistant bacteria has been commonly reported in stool samples (Wang et al., 2015). Because Campylobacter colonies have been often observed to be covered over by the growth of these antibiotic resistant bacteria in stool samples, selective media for Campylobacter should eliminate these competitive bacteria.

While several chromogenic media for the isolation of Campylobacter have been developed (Ahmed et al., 2012; Le Bars et al., 2011; Selwirowsk et al., 2014), BD mCCDA Clear-HT (CCHT; product code 252794, Nippon Becton Dickinson Company, Ltd., Tokyo, Japan), a new chromogenic selective agar medium with a superior capacity to isolate Campylobacter, has been developed according to the formulation reported by Teramura et al. (2015). This new medium comprises
the following ingredients per liter: Tryptone, 10.0 g; proteose peptone No.3, 10.0 g; yeast extract, 2.5 g; casamino acids, 3.0 g; agar, 15.0 g; sodium chloride, 5.0 g; sodium deoxycholate, 1.0 g; ferrous sulfate, 0.25 g; sodium pyruvate, 0.25 g; vancomycin hydrochloride, 10.0 mg; sodium cepoparzone, 32.0 mg; sodium cefoxitin, 4.0 mg; tetrazolium violet, 10.0 mg; and amphotericin B, 4.0 mg (pH 7.4). Campylobacter jejuni/coli can be isolated as purple-colored colonies on the transparent CHT owing to the reduction of tetrazolium violet, a redox indicator, after 48 h of incubation at 42°C under microaerobic conditions (Fig.1). This chromogenic medium was originally developed for improved Campylobacter detection from poultry samples, but not from stool samples (Teramura et al., 2015). The aim of this study was to evaluate the performance of CCHT in the efficient isolation of Campylobacter from stool samples.

The inclusivity and exclusivity of CCHT were assessed using 142 microbial strains. For inclusivity, Campylobacter jejuni ATCC 33560, 38 clinical isolates of C. jejuni, C. coli ATCC 33559 and 2 clinical isolates of C. coli (total 42 strains) were used. In addition, 65 gram-negative bacteria other than Campylobacter jejuni/coli, 33 gram-positive bacteria, and 2 yeasts were inoculated into CCHT for exclusivity tests as shown in Table 1. After Campylobacter strains tested were cultured on sheep blood agar (Nippon Becton Dickinson Company, Ltd.) at 35°C for 24 h under microaerobic conditions (Anaero Pack MicroAero system, Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan), the cultured Campylobacter was suspended into sterilized saline (0.85% NaCl) at McFarland #1 turbidity standard (McFarland, 1907). Similarly, after strains other than Campylobacter jejuni/coli were cultured on sheep blood agar at 35°C for 24 h under aerobic conditions, they were suspended in sterilized saline at McFarland #1 turbidity standard. A loopful of each microbe suspension was then streaked onto CCHT. The growth and colony color of the tested strains were read after 48 h of incubation at 42°C under microaerobic conditions.

In the inclusivity study, all 42 Campylobacter jejuni/coli strains in total grew and formed typical purple-colored colonies on CCHT in 48 h of incubation under microaerobic conditions. As for the 100 non-Campylobacter strains tested in the exclusivity study, all strains failed to grow on CCHT as shown in Table 1. These results showed that CCHT has both excellent selectivity and specificity in these preliminary studies.

CCHT was then compared with CCDA according to ISO 10272 (ISO, 2006) (Kanto chemical Co., Inc., Tokyo, Japan), and with CCDA-SEL (SEL; Kanto chemical Co., Inc.), and modified Skirrow EX agar (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan), which were commercially available selective media for clinical use in Japan using stool samples. After 100 stool samples were collected from June 2017 to July 2017 throughout Japan and were then tested using conventional Skirrow agar with 48 h of microaerobic incubation for diagnosis in BML Inc., 40 Campylobacter positive samples and 60 Campylobacter negative samples were prepared as blind-coded stool samples. The prepared stool samples were agitated and a loopful was directly streaked onto each medium. After 48 h of incubation at 42°C under microaerobic conditions, a typical colony on each medium was picked up and confirmed to be Campylobacter by Gram staining, cytochrome oxidase test strips (Nissui Pharmaceutical Co., Ltd.), and Api Helico identification strips (Shih, 2000; Sysmex bioMérieux Co., Ltd., Tokyo, Japan). Furthermore, all grown competitive microbes on all selective media were identified by Gram staining, cytochrome oxidase test strips, and Api 20E identification strips (Holmes et al., 1978; Sysmex bioMérieux Co., Ltd.). Simultaneously, the type of β-lactamase of grown competitive microbes was confirmed using the nitrocefin method (Hanaki et al., 2005; Cica beta test, Kanto chemical Co., Inc.). All data analysis was carried out using Microsoft Excel 2013. The analysis using Cohen’s kappa coefficients was performed for all media compared to determine the concordance with the final results at the significance level of 0.05.

Table 2 shows the results of the study comparing methods for the detection of Campylobacter using 100 stool samples. CCHT detected Campylobacter jejuni/coli as a specific purple-colored colony from 39 of 100 clinical stool samples. Similarly, the number of samples...
### TABLE 1. Exclusivity of CCHT

| Name of organism                        | No. of strains tested | No. of strains grown | Subtotal | Standard* |
|----------------------------------------|-----------------------|----------------------|----------|-----------|
| **Gram negative bacteria excluding Campylobacter spp.** |                       |                      |          |           |
| Achromobacter xylosoxidans            | 1N                    | 1                    | 0        |           |
| A. denitrificans                      | 1N                    | 1                    | 0        |           |
| Acinetobacter lwoffii                 | 1N                    | 1                    | 0        |           |
| Aeromonas hydrophila                  | 1J                    | 1                    | 0        |           |
| Burkholderia cepacia                  | 1N                    | 1                    | 0        |           |
| Cedecea lapagei                       | 1J                    | 1                    | 0        |           |
| Citrobacter amalonaticus              | 1N                    | 1                    | 0        |           |
| C. freundii                           | 1N                    | 1                    | 0        |           |
| Chryseobacter indologenes             | 1N                    | 1                    | 0        |           |
| Cronobacter sakazakii                 | 1J                    | 1                    | 0        |           |
| Edwardsiella tarda                    | 1N                    | 1                    | 0        |           |
| Enterobacter aerogenes                | 1N                    | 1                    | 0        |           |
| E. cloacae                            | 1J, 1N                | 2                    | 0        |           |
| E. gergoviae                          | 1N                    | 1                    | 0        |           |
| Escherichia coli                      | 12N                   | 12                   | 0        |           |
| E. coli O157                           | 2A                    | 2                    | 0        |           |
| E. fergusonii                          | 1N                    | 1                    | 0        |           |
| E. hermannii                           | 1N                    | 1                    | 0        |           |
| E. vulneris                           | 1N                    | 1                    | 0        |           |
| Hafnia alvei                           | 1J                    | 1                    | 0        |           |
| Klebsiella oxytoca                     | 1J                    | 1                    | 0        |           |
| K. pneumonieae                         | 1J                    | 1                    | 0        |           |
| Kluyvera ascorbata                     | 1J                    | 1                    | 0        |           |
| K. intermedia                          | 1J                    | 1                    | 0        |           |
| Leclercia adecarboxylycta              | 1N                    | 1                    | 0        |           |
| Morganella morganii                    | 1N                    | 1                    | 0        |           |
| Proteus mirabilis                      | 1J, 1N                | 2                    | 0        |           |
| P. vulgaris                           | 1N                    | 1                    | 0        |           |
| Providencia alcalifaciens             | 1N                    | 1                    | 0        |           |
| Pseudomonas aeruginosa                 | 3N                    | 3                    | 0        |           |
| P. putida                              | 1N                    | 1                    | 0        |           |
| P. stutzeri                            | 1N                    | 1                    | 0        |           |
| P. tolaasi                             | 1N                    | 1                    | 0        |           |
| Rahnelle aquatilis                     | 1N                    | 1                    | 0        |           |
| Raoultella planticola                  | 1N                    | 1                    | 0        |           |
| R. terrigena                           | 1N                    | 1                    | 0        |           |
| Salmonella enterica                    | 1J, 1N                | 2                    | 0        |           |
| Serratia marcescens                    | 1J                    | 1                    | 0        |           |
| S. rubidaea                            | 1N                    | 1                    | 0        |           |
| Stenotrophomonas ginsengisoi          | 1N                    | 1                    | 0        |           |
| S. maltophilia                         | 1N                    | 1                    | 0        |           |

| Gram positive bacteria                  |                      |                      |          |           |
| Bacillus cereus                         | 1N                    | 1                    | 0        |           |
| B. licheniformis                        | 1N                    | 1                    | 0        |           |
| B. subtilis                             | 1N                    | 1                    | 0        |           |
| Corynebacterium ammoniigenes           | 1N                    | 1                    | 0        |           |
| Enterococcus faecalis                   | 2J                    | 2                    | 0        |           |
| E. hirae                                | 1N                    | 1                    | 0        |           |
| Lactobacillus lactis                    | 1N                    | 1                    | 0        |           |
| L. plantarum                            | 1N                    | 1                    | 0        |           |
| Leuconostoc citrus                      | 1J                    | 1                    | 0        |           |
| L. mesenteroides                        | 1N                    | 1                    | 0        |           |
| Listeria ivanovi                        | 1J                    | 1                    | 0        |           |
| L. monocytogenes                        | 1J                    | 1                    | 0        |           |
| Micrococcus luteus                      | 1N                    | 1                    | 0        |           |
| Staphylococcus aureus                   | 3N                    | 3                    | 0        |           |
| S. aureus                               | 1A                    | 1                    | 0        |           |
| S. capitis                              | 1J                    | 1                    | 0        |           |
| S. caprae                               | 1J                    | 1                    | 0        |           |
| S. epidermidis                          | 2N                    | 2                    | 0        |           |
| S. haemolyticus                         | 1J                    | 1                    | 0        |           |
| S. hominis                              | 1J                    | 1                    | 0        |           |
| S. hyicus                               | 1J                    | 1                    | 0        |           |
| S. intermedius                          | 1A                    | 1                    | 0        |           |
| S. lentus                               | 1A                    | 1                    | 0        |           |
| S. saprophyticus                        | 1J                    | 1                    | 0        |           |
| S. schleiferi                           | 1A                    | 1                    | 0        |           |
| S. sciuri                               | 1A                    | 1                    | 0        |           |
| S. simulans                             | 1J                    | 1                    | 0        |           |
| S. xylosus                              | 1J                    | 1                    | 0        |           |
| Streptococcus thermophilus              | 1A                    | 1                    | 0        |           |
| Subtotal                                | 33                    | 33                   | 0        |           |

| Yeasts                                  |                      |                      |          |           |
| Candida albicans                        | 1N                    | 1                    | 0        |           |
| C. tropicalis                           | 1N                    | 1                    | 0        |           |
| Subtotal                                | 2                     | 2                    | 0        |           |
| Total                                   | 100                   | 100                  | 0        |           |

*Standard strains were from A, ATCC (American Type Culture Collection, VA, USA); J, JCM (Japan Collection of Microorganisms, Ibaraki, Japan); and N, NBRC (National Institute of Technology and Evolution Biological Resource Center, Chiba, Japan).*
from which Campylobacter was detected on CCDA, SEL, and modified Skirrow EX was 36, 37, and 39, respectively. In contrast, CCHT, CCDA, SEL, and modified Skirrow EX allowed competitive bacteria to grow as false positive colonies from 1, 18, 6, and 10 of 100 samples, respectively. Therefore, the values of relative sensitivity (%) for CCHT, CCDA, SEL, and modified Skirrow EX were 97.5, 90.0, 92.5, and 97.5, respectively. In addition, the values of relative specificity (%) for CCHT, CCDA, SEL, and modified Skirrow EX were 98.3, 70.0, 90.0, and 83.3, respectively. The order of Cohen’s kappa coefficient for the degree of concordance against the final results was CCHT (0.96) > SEL (0.82) > modified Skirrow EX (0.78) > CCDA (0.57).

As also shown in Table 2, there was one strain of Escherichia coli that grew on CCHT in 100 clinical stool samples. The results of the confirmation of β-lactamase type using the nitrocefin method showed this E. coli strain had an extended spectrum β-lactamase (ESBL). For CCDA, 4 strains of Acinetobacter baumannii, and one strain each of E. coli and Proteus mirabilis were isolated as competitive bacteria due to the presence of AmpC β-lactamase, whereas 10 strains of E. coli and 2 strains of Klyvera intermedia were isolated on due to ESBL. Although SEL allowed 6 strains to grow in 100 stool samples, there were 2 strains of A. baumannii, and one strain each of Enterobacter aerogenes, E. coli, K. intermedia, and P. mirabilis. Among these competitive bacteria, A. baumannii and P. mirabilis have AmpC β-lactamase, whereas E. aerogenes, E. coli, and K.

### TABLE 2. Summary of the detection of Campylobacter in 100 clinical stool samples by using various selective media

| Identification of grown competitive microbes in false positive samples | CCHT | CCDA | SEL | modified Skirrow EX |
|---|---|---|---|---|
| Escherichia coli (E) 1 | Acinetobacter baumannii (A) 4 | A. baumannii (A) 2 | A. baumannii (A) 1 | 
| E. coli (E) 10 | Enterobacter aerogenes (E) 1 | Citrobacter freundii (A) 1 | 
| E. coli (A) 1 | E. coli (E) 1 | E. coli (E) 5 | 
| Klyvera intermedia (E) 2 | K. intermedia (E) 1 | E. coli (A) 1 | 
| Proteus mirabilis (A) 1 | P. mirabilis (A) 1 | K. intermedia (E) 1 | 
| | | P. mirabilis (A) 1 | 

Numbers of samples from which Campylobacter was recovered by using various selective media in 100 blind-coded clinical stool samples are represented.

Forty positive and 60 negative samples were prepared as blind-coded clinical stool samples by BML Inc. Typical colonies were identified to be Campylobacter sp. by gram staining, the cytochrome oxidase test (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan), and Api Helico identification strip (Sysmex bioMérieux, Tokyo, Japan), respectively.

"Positive: number of samples from which typical colonies detected were confirmed to be Campylobacter sp."

"Negative: number of samples from which no typical colony was detected by using each medium."

"False positive: number of samples from which non-Campylobacter bacteria was detected."

"False negative: number of Campylobacter-positive samples from which no typical colony was detected."

"Cohen’s kappa coefficients were performed for all media compared. 0-0.20, slight; 0.21-0.40, fair; 0.41-0.60, moderate; 0.61-0.80, substantial; 0.81-1, almost perfect agreement."

"Isolated strains were identified by the Api 20E identification strip (Sysmex bioMérieux, Tokyo, Japan). Type of beta-lactamase was confirmed by the nitrocefin method (Cica beta test, Kanto Chemical Co., Inc., Tokyo, Japan)."

"Parentheses indicate the type of β-lactamase, E: Extended spectrum β-lactamase (ESBL), A: AmpC β-lactamase. The number after the parentheses indicates the number of strains isolated."
intermedia have ESBL. Of the 10 competitive bacteria that grew on modified Skirrow EX, one strain each of A. baumannii, Citrobacter freundii, E. coli, and P. mirabilis have AmpC β-lactamase and 5 strains of E. coli and one strain of K. intermedia have ESBL.

Because most Campylobacter have a resistance against β-lactam antibiotics (Lachance et al., 1991), cephem antibiotics have been used in most current isolation media for Campylobacter. Furthermore, there are several bacteria that have resistance against cephem antibiotics such as ESBL in stool samples. Hence, in case there are lots of cephem-resistant bacteria in stool samples, they may lead to false negative results due to the growth of competitive bacteria as reported by Chon et al. (2013). In this study, CCDA showed false negative results in 4 samples due to the masking of typical Campylobacter colonies by these competitive bacteria. Moreover, all competitive bacteria grown on CCDA have resistance against β-lactam antibiotics. Hence, it suggested that according to ISO 10272, CCDA has weaker selectivity against competitive microbes than the other 2 commercially available selective media, which have modified selectivity for stool samples.

On the contrary, the composition of selective agents in CCHT is similar to CCDA according to ISO 10272, except for the addition of a small amount of sodium cefoxitin. Although sodium cefoxitin is one of the cephamycins that is comparatively resistant to the hydrolytic activity of ESBL (Paterson et al., 2001), our in-house data showed that a surplus of sodium cefoxitin causes inhibitory action on Campylobacter jejuni/coli. Teramura et al. (2015) also reported that the concentration of sodium cefoxitin added to their new chromogenic media is an important factor in the effective detection of Campylobacter without inhibiting the growth of Campylobacter. Our results showed that addition of cefoxitin at an optimum concentration contributes to the effective inhibition of competitive bacteria derived from stool samples because CCHT has better selectivity and detectability than CCDA according to ISO10272. Moreover, although CCHT has a simple formulation based on conventional CCDA, both the detectability of Campylobacter jejuni/coli and selectivity of competitive bacteria is the same or better than those of other 3 commercially available selective media.

In conclusion, we evaluated BD mCCDA Clear-HT (CCHT), a novel chromogenic medium, for its superior capacity to isolate of Campylobacter jejuni/coli from clinical stool samples. Our results demonstrate that CCHT is not only excellent in both selectivity and detectability but also allow the Campylobacter colonies to be more clearly visible. Therefore, our study suggests that CCHT is an efficient alternative for the improved isolation of Campylobacter jejuni/coli from clinical stool samples.

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