Original Research

Comparison of Five Different Enrichment Broth-Agar Combinations for the Isolation of *Campylobacter jejuni*

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Abstract

Campylobacter is a fastidious microaerophilic bacterium, it is a leading cause of bacterial gastroenteritis in humans globally. In the present study, the efficacy of five enrichment broths in combination with five selective agars for the isolation of Campylobacter jejuni was compared to identify a best possible combination of broth- agar for enrichment. Where, C. jejuni standard culture (ATCC®33560 TM) was enriched in five enrichment broths viz., Brucella, Preston enrichment, modified Charcoal Cefoperazone Deoxycholate broth (mCCDB), Park and Sanders, Bolton broths and consequently plated onto modified Charcoal Cefoperazone Deoxycholate agar (mCCDA), Karmali agar, Columbia blood agar, Campylobacter Cefex agar and Campylobacter agar base (CAB). Bolton broth and mCCD agar combination showed the maximum enrichment efficiency and the colony recovery by Bolton broth significantly differed when compared to the Park and Sanders and Brucella broths. The result of the current study shows the combination of enrichment broth and agar most appropriate for the isolation of C. jejuni.

Key words: Bolton, *C. jejuni*, Karmali, mCCDA, Preston Enrichment

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Introduction

Campylobacter is an emerging bacterial food borne pathogen of humans. It is believed that consumption of contaminated, poorly cooked poultry contributes significantly to Campylobacter infection (Keener *et al.*, 2004). It is the most frequent cause of human gastroenteritis in many countries. *Campylobacter* spp. are found in the intestinal tracts of warm-blooded animals. Due to Campylobacteriosis, one in 10 people become sick and 33 million healthy life years are lost annually. Campylobacter infections are usually mild

but can be fatal in very young children (under two years old), elderly and immunocompromised persons. Sequelae of long-term complications lead to Guillian-Barre syndrome (a severe paralytic condition), Miller Fisher syndrome (MFS) and Reiter syndrome or reactive arthritis (WHO 2015).

Campylobacter is a fastidious bacterium, which requires microaerophilic condition for growth (Humphrey *et al.,* 2007). When analyzing retail samples, which may be exposed to oxygen, transport stress and prolonged storage times may decrease the number of Campylobacter cells below the enumeration limit (Sproston *et al.,* 2014). *Escherichia coli* extended-spectrum beta-lactamase (ESBL), which is highly prevalent in poultry, is the most common competitive flora for the isolation of Campylobacter (Jasson *et al.,* 2009). In addition, Pseudomonas and *Proteus* spp. are also known to hinder the isolation of Campylobacter (Baylis *et al.,* 2000). Consequently, these competing organisms may lead to an underestimation of the presence of Campylobacter in the sample analyzed. Therefore, enrichment is necessary to reduce the background microflora and to demonstrate the best recovery of the organism (Sproston *et al.,* 2014).

Several enrichment media to improve the isolation of *Campylobacter jejuni* have been projected over the past few years. The selective media can be classified into two groups: media containing blood and media containing charcoal. The components of blood and charcoal dole out to reduce lethal oxygen derivatives. The antimicrobial agent used in the media determines its selectivity (Post 1995). Blood-based media: Brucella broth, Preston enrichment broth, Park and Sanders broth, Bolton broth, Columbia blood agar, Campylobacter cefex agar and Campylobacter agar base (CAB). Charcoal-based media: modified Charcoal Cefoperazone Deoxycholate broth (mCCDB), modified Charcoal Cefoperazone Deoxycholate agar (mCCDA) and Karmali agar. Upto date, there is no standard protocol which mentions the optimal enrichment media to isolate Campylobacter. Therefore, the current study compares the yield of the combination of five enrichment broths with five selective agars to determine an optimal broth and agar combination for the growth and revival of *C. jejuni*.

Materials and Methods

Bacterial Strain

Campylobacter jejuni strain (ATCC® 333560) was obtained from the American Type Culture Collection, United States. The lyophilized culture was rehydrated using six milliliters of Brucella broth. A loopful of this suspension was used to inoculate Tryptone Soybean Agar (TSA) plates with five per cent defibrinated sheep blood at 37°C for 24 to 48 h under microaerophilic conditions (three - five per cent oxygen and 10 per cent carbon dioxide) in a Steri-cycle® carbon dioxide incubator. The colonies in this medium were colourless, transparent, circular, whole, smooth, low convex, non-haemolytic, watery and dewdrops like

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swarms. Isolates were preserved in Brucella broth with 30 per cent glycerol by reviving at customary intervals. The glycerol broth tubes inoculated with the organism were stored at -80°C.

Enrichment Media

Five enrichment broths and five selective agars were prepared according to the instructions of their manufacturers - HIMEDIA, Mumbai. The basal components and antimicrobials used in the media preparations are listed in Table 1 and Table 2.

Table 1: Comparison of components and supplements of five enrichment broths

Enrichment Broth	Final Volume of the Broth (ml)	Peptic digest of animal tissue (g)	Yeast extract (g)	Sodium chloride (g)	Sodiummeta-Bisulphate (g)	Casein enzymatic hydrolysate (g)	Sodium Pyruvate (g)	Other ingridents (g)	Sepplements/Antibiotics
Buucella	500	10	2	5		10		Sodium-Bisulphate (0.1), dextrose (1)	Polymyxin B (1.250IU), Vancomycin (5mg), Trimethoprim (2.500mg), Amphotericin B (1mg), Cephalothin(7.500mg), Sodium pyruvate (0.125g), Sodium metabisulphite (0.125g) and Ferrous sulphate (0.125g)
Campylobacter enrichment broth(Preston)	470	10		5				Beef extract (10)	Polymyxin B sulphate (2500IU), Rifampicin (5mg), Trimethoprim (5mg) and Cycloheximide (50mg)
Bolton	500	10	5	5	0.5		0.5	Sodium carbonate (0.6), hemin (0.01), alpha-ketogularic acid (1), Lactalbumin hydrolysate (5)	Cefoperazone (10mg), Vancomycin (10mg), Trimethoprim (10mg) and Amphotericin B (5mg)
mCCDB	500	10		5		3	0.25	Beef extract(10), sodium deoxycholate (1), ferrous sulphate (0.25), charcoal bacteriological (4)	Cefoperazone (16mg) and Amphotericin B (5mg)
Park & Sander	940	10	2	5		10	0.25	Dextrose (1), sodium citrate (1), monohydrogen sodium sulphite (0.1)	Vancomycin (10mg), Trimethoprim (10mg), Cefoperazone (32mg) and Cycloheximide (100mg)

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Table 2: Comparison of components and supplements of five selective agars

Agar	Final volume of the agar (ml)	Peptone special	Agar (g)	Casein enzymatic hydrolysate (g)	Sodium chloride (g)	Sodium pyruvate (g)	Charcoal (g)	Other ingridents (g)	Sepplements/Antibiotics
Karmali	490	23	12		5		4	Corn starch (1)	Sodium pyruvate (50mg), Vancomycin (10mg), Cefoperazone (16mg), Cycloheximide (50mg) and Hemin (16mg)
mCCDA	500		12	3	5	0.25	4	Meat extract (10), Peptone (10), sodium deoxycholate (1), ferrous sulphate (0.25),	Cefoperazone (16mg), Cefoperazone (4mg), Teicoplanin (2mg) and Amphotericin B (5mg)
Campylobacter agar base	500		12		5			Proteose peptone (15), liver digest (2.5), yeast extract (5),	Polymyxin B (1.250IU), Vancomycin (5mg), Trimethoprim (2.500mg), Amphotericin B (1mg) and Cephalothin(7.500mg)
Columbia blood agar	1000	23	15		5			Corn starch (1)	Polymyxin B (1.250IU), Vancomycin (5mg), Trimethoprim (2.500mg), Amphotericin B (1mg), Cephalothin(7.500mg) and Sodium pyruvate (0.125g), Sodium metabisulphite (0.125g) and Ferrous sulphate (0.125g)
Campylobacter cefex agar	950		15	15	5	0.5		Peptic digest of animal tissue (10), yeast extract (2), gulcose (1), ferrous sulphate (0.5), sodium bisulphite (0.35)	Cefoperazone (32mg) and Cycloheximide (100mg)

Cell Suspension (Standard Culture)

The cultures in stock were enriched with the Brucella broth. One milliliter of cell suspension was inoculated to 9 ml of mCCD, Brucella, Bolton, Park and Sanders, and Preston enrichment broths. Except Bolton broth, all broths were incubated in microaerobic atmosphere for 48 h at 42°C. An initial pre-

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enrichment for Bolton broth was carried out for 4-6 h at 37°C which was followed by incubation for 44 ± 4 h at 42°C.

Enumeration of *Campylobacter jejuni*

One milliliter of each broth was serially diluted with 9 ml of each corresponding broths to obtain a ten-fold diluted culture. After serial dilution, 0.1 ml of the diluents in mCCDA, Karmali agar, Campylobacter cefex agar, Campylobacter agar base (CAB) and Columbia blood agar in duplicates were spread. The plates were incubated under microaerobic conditions for 48 h at 42°C. Number of typical colonies were counted and expressed as CFU / ml.

Results

Recovery of the bacteria after *C. jejuni* healthy cell suspension was inoculated into broths, namely Brucella, mCCD, Bolton, Park and Sanders and Preston enrichment broths with subsequent plating of each broth into five selective agars the mCCDA, Karmali agar, Campylobacter Cefex agar, Campylobacter agar base (CAB) and Columbia blood agar are shown in Figures 1 to 5.

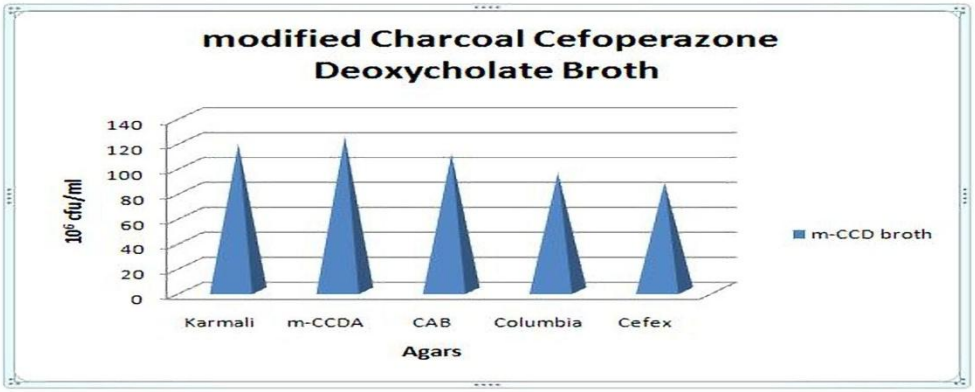

Fig. 1: Recovery of *C. jejuni* on five selective agars by enrichment in mCCD broth

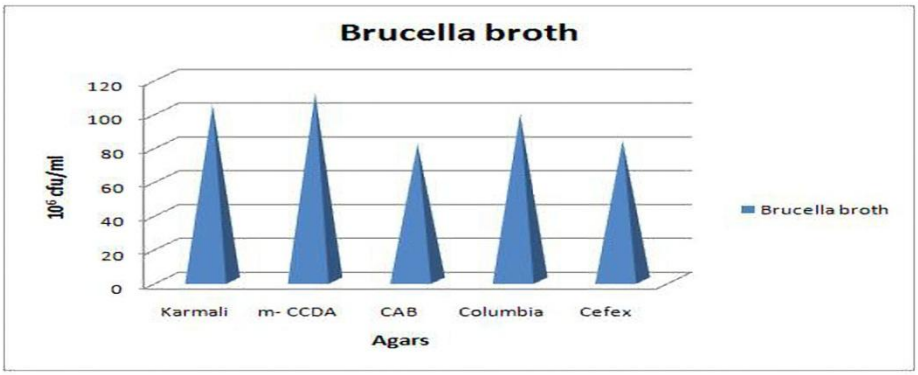

Fig. 2: Recovery of *C. jejuni* on five selective agars by enrichment in Brucella broth

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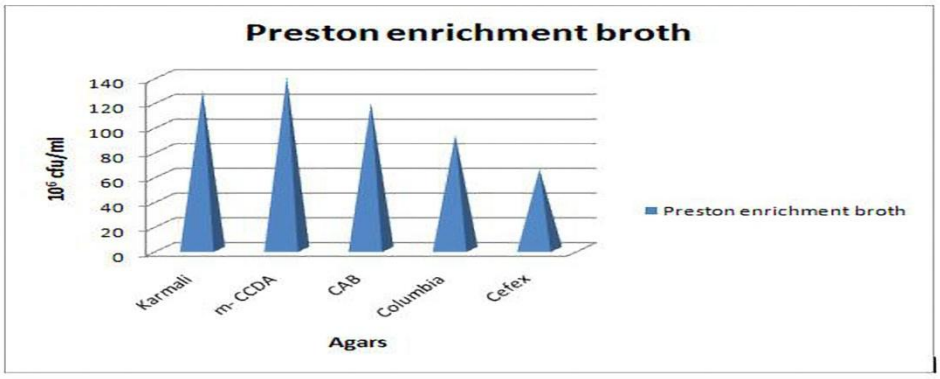

Fig. 3: Recovery of *C. jejuni* on five selective agars by enrichment in Preston enrichment broth

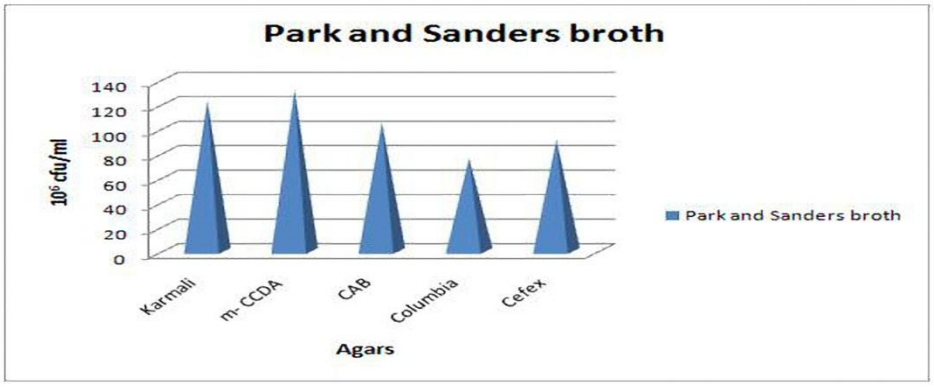

Fig. 4: Recovery of *C. jejuni* on five selective agars by enrichment in Park and Sanders broth

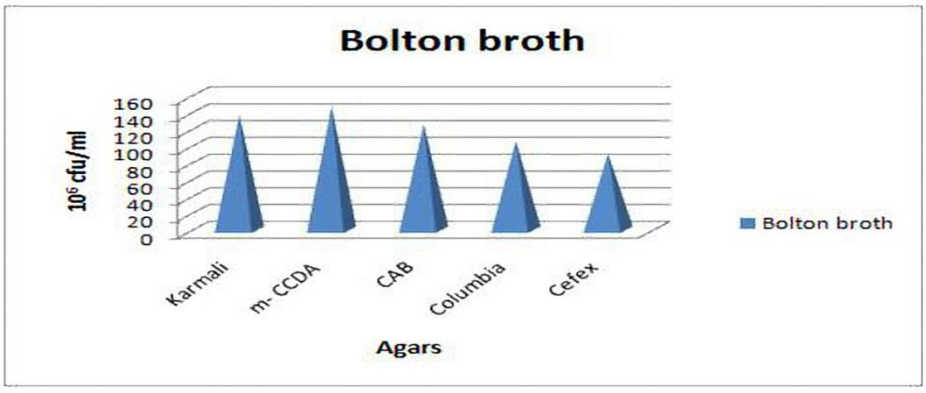

Fig. 5: Recovery of *C. jejuni* on five selective agars by enrichment in Bolton broth

Bolton's broth in combination with mCCDA showed the highest number of colonies followed by the Preston enrichment broth-mCCDA, Bolton- Karmali agar and Park and Sanders broth-mCCDA. The lowest number of colonies in mCCD broth - Campylobacter agar base and Preston enrichment broth - Cefex agar base were observed (Fig. 6).

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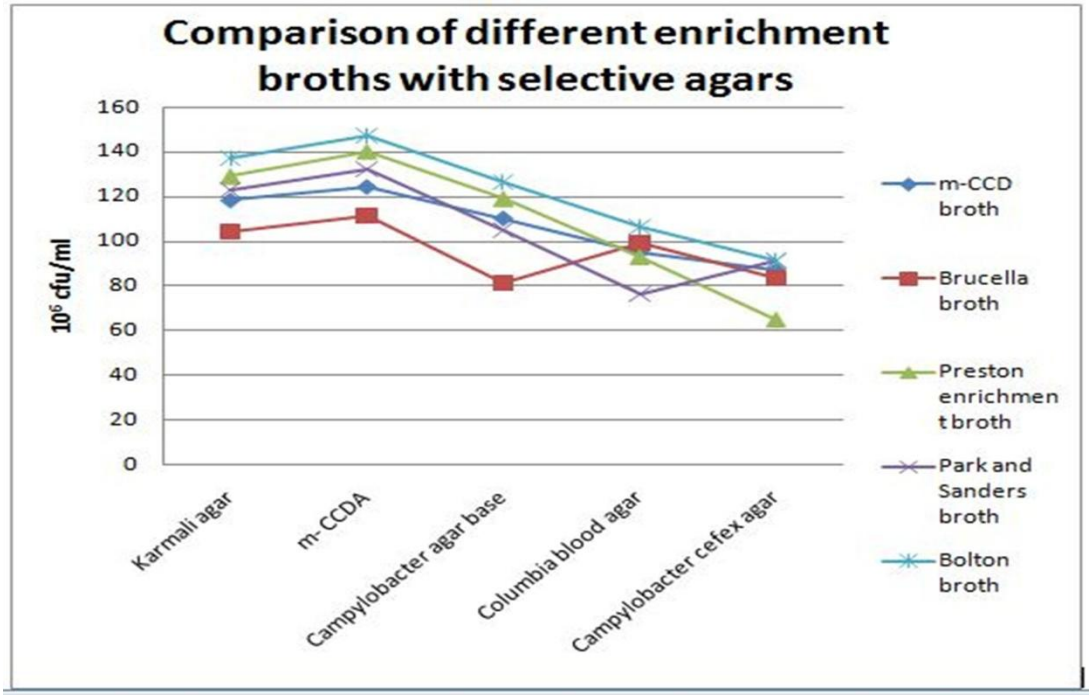

Fig. 6: Comparison of combination of different enrichment broths with selective agars for the recovery of *C. jejuni*

Bacterial recovery was highest in mCCDA, followed by Karmali agar and CAB. A lower number of colonies were observed on Campylobacter cefex agar followed by Columbia blood agar.

In the statistical analysis, the variance of the univariate analysis was used to know the efficiency of different broths and combinations of agar in the revitalization of number of colonies and it was found that, the broths had a significant effect on the revival of colonies. In addition, on performing the Duncan post hoc test, Bolton broth significantly differed with the Park and Sanders and Brucella broths, while the mCCD and Preston enrichment broths were similar with Park and Sanders and Brucella broths.

Discussion

Most of the Campylobacter media contain antimicrobials and peptones. Many contain blood (five - seven per cent (v / v) and few charcoal (three per cent), to overcome the adverse effects of lethal oxygen derivatives (e.g., hydrogen peroxide and superoxide) (Bolton and Robertson, 1982). Campylobacters are non fermenters of carbohydrates; therefore, peptones are included in the media as a source of nutrients. The Bolton broth has a nutrient formulation consisting of peptones, yeast extract and alpha-ketoglutaric acid stimulating best growth of organisms that is in agreement with the present study.

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Generally for the analysis of food, water and other environmental samples, it has been found that the incorporation of pre-enrichment procedures into laboratory protocols increases Campylobacter recovery (Bolton *et al.,* 1984). The normally used resuscitation procedure consists of 4 h of incubation at 37°C (Humphrey, 1989; Bolton, 2000), after which the pre-enrichment broths are incubated at 42°C. It is recommended that resuscitation be limited to 4 h to reduce overgrowth by contaminants (Goosens and Butzler, 1992). In the current study the pre enrichment procedure for the preparation of the Bolton broth was followed, which showed the greatest efficiency in the recovery of Campylobacter. In addition, Bolton broth includes components such as Sodium pyruvate and sodium metabisulfite which allow aerobic incubation, sodium carbonate provides carbon dioxide during growth (Post, 1995), which could be the reason for better performance. Antibiotics that inhibit yeasts and molds are generally included in Campylobacter media. Earlier, the most commonly used antimycotic agent was cycloheximide (Preston, Karamli, Campylobacter cefex and Park and sanders), however amphotericin B or natamycin are used as substitutes for cycloheximide because it was considered too toxic for inclusion in the media for microbiological conditions. This factor supports the results of the present study, where Bolton and mCCD broths, Columbia blood agar, mCCDA, CAB agars incorporate amphotericin B. It has been shown to be a satisfactory substitute (Martin *et al.*, 2002). One study evaluated the recovery of Campylobacter from 100 naturally contaminated samples. Bolton broth and Preston broth isolated Campylobacter from 66 and 53 samples, respectively. The results are in agreement with the present study. The authors speculated that the difference could be due to the peptones used in each broth are different. We therefore concluded that Bolton broth represents the best because of the overall compromise between growth of Campylobacter and inhibition of contaminants for the analysis of food samples (Baylis *et al.*, 2000). Another study demonstrated that mCCD, Preston, Park and Sanders broths had better enrichment efficiencies when compared to Brucella broth, which was significantly lower than the other broths (Kim *et al.*, 2009).

In a study, 483 naturally contaminated samples were analysed, where 84 and 90 percent of confirmatory rates were obtained for Campylobacter cefex agar and mCCDA respectively (Ahmed *et al.*, 2012), similar findings were observed in the current study. Campylobacter cefex agar showed lower rate of isolation and specificity due to extensive contamination with competitive microflora (Chon *et al.*, 2012).

Specific selective agars are recommended in different isolation protocols. In the ISO protocol (1995) subculture from Preston or Park and Sanders broth to Karmali agar along with subculture on any other agar chosen from the following: Butzler, Skirrow, CCDA or Preston agar was reported (ISO 1995). A proposed amendment to ISO 1995 applies to a single agar, either Karmali or mCCDA. These two agars are comparable in their sensitivity and productivity (Jacobs-Reitsma and Boer, 2001) The selective agents in mCCDA are cefoperazone and amphotericin B. While Karmali agar includes hematin instead of ferrous sulfate as one of the oxygen quenching agents and antibiotics vancomycin and cycloheximide. A

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modification of the antibiotics in mCCDA consisting of cefoperazone, teicoplanin and amphotericin B has been reported to improve the growth and isolation of *C. upsaliensis* from faecal samples, while recovering equal numbers of other *Campylobacter* species in mCCDA (Corry *et al.*, 1995). Many studies reported that CCDA is the best choice among all plate media and it is the most widely used selective medium worldwide (Bolton and Robertson, 1982; Bolton and Coates, 1983; Gharst *et al.*, 2013).

Extended Spectrum-beta-lactamase-producing *Escherichia coli* (ESBL) is a common competitive organism that hinders the growth and detection of Campylobacter (Jasson *et al.*, 2009). In addition to the occurrence of antimicrobial resistance, there is a growing prevalence of *E. coli* ESBL (Depoorter *et al.*, 2012), that hydrolyzes sodium cefoperazone, an important component of Bolton broth and mCCDA showing plentiful growth, which may hinder the Campylobacter colonies. Bolton's broth supplemented with triclosan (T-BB) and potassium clavulanate (C-BB) is known to improve the isolation rate of Campylobacter when compared to the non-supplemented Preston broth and Bolton broth (Seliwiorstow *et al.*, 2016). According to the FDA, the specified enrichment broth for all sample types is the Bolton broth and all protocols include a pre-enrichment procedure to increase recovery of the stressed and injured cells. Either of the agars for the selective isolation step following enrichment can be chosen. The agars are mCCD agar or Abeyta-Hunt-Bark agar. The mCCDA medium is included in international standard protocols (Hunt *et al.*, 2001), as it provides satisfactory results. Blood-free media (charcoal based) would be very convenient to prepare when compared to blood-based media. The black background of charcoal based agar helps to easily recognize and isolate Campylobacter colonies. As a result, in this study too it is confide for selective plating.

Conclusion

Campylobacter recovery from suspension using different enrichment media mainly depends on the components used i.e. peptones, oxygen quenching agents and antimicrobials. The pre-enrichment and incubation period also adds to the better recovery of cells. The result of the present study demonstrates Bolton broth and mCCD agar combination as the most appropriate enrichment broth and agar combination for isolation of *C. jejuni.*

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