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ORIGINAL ARTICLE

Antigen Detection and PCR *versus* Conventional Culture for Diagnosis of Campylobacter Infections in Pediatric Gastroenteritis

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ABSTRACT

Key words:
Campylobacteriosis, ELISA, and PCR

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Background: *Campylobacter is one of the leading pathogens which causes bacterial gastroenteritis among children worldwide, especially in developing countries. Several laboratory methods have been used to diagnose campylobacteriosis including culture, ELISA, and PCR.* ***Objectives:*** *The aim of this study was to compare PCR and antigen detection by ELISA with culture for the detection of Campylobacter.* ***Methodology:*** *The present study was conducted on 160 stool samples that were collected from pediatric patients complaining of acute gastroenteritis. All samples were cultured on Modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA) and suspected colonies were consequently identified. Detection of Campylobacter antigen (PEB1) in stools was done by ELISA. Molecular detection of virulence genes: cadF, hipO, and asp in stools was done by multiplex PCR.* ***Results:*** *Thirty-five samples (21.9%) were found to be positive for Campylobacter by culture. Campylobacter antigen was detected in 50 samples (31.3%) by ELISA. cadF gene was detected in 47 samples (29.4%) by PCR, 39 of which were positive for hipO gene and thus identified as Campylobacter jejuni, while asp gene was not detected in any sample.* ***Conclusion****: Alternative diagnostic tests for campylobacteriosis that do not rely on culture have become increasingly important. Nucleic acid-based techniques can detect the presence of Campylobacter infection and even distinguish between different Campylobacter species.*

INTRODUCTION

Campylobacter species are fastidious Gram-negative bacteria commonly found in nature, particularly in the digestive tracts of both wild and domesticated birds and mammals. The primary mode of transmission to humans is through the handling and consumption of chicken products that have been contaminated with this zoonotic pathogen[1].

Campylobacter species are a significant source of bacterial gastroenteritis globally, affecting people in both developing and developed nations[2]. They can lead to various health problems in humans, including diarrhea, abdominal cramps, as well as extra-intestinal illnesses such as endocarditis, meningitis, bacteremia, and Guillain-Barré syndrome[1].

Although Campylobacter gastroenteritis is often self-limiting, inadequate treatment may lead to complications as bacteremia. Therefore, it is essential to identify the presence of Campylobacter in stool samples and initiate timely and effective antimicrobial therapy to minimize the severity and duration of the infection[3].

Stool culture has been considered the standard diagnostic technique for Campylobacter infections, yet, it is a troublesome and time-consuming process that requires specialized selective media and microaerophilic conditions. Incubation for 48 to 72 hours at 37°C and/or 42°C is also necessary, making culture an inconvenient and costly approach[4].

Culture-independent tests provide alternative approaches for identifying the presence of Campylobacter in stool samples. This has significant implications for patient care and public health surveillance programs[5]. Various techniques have been developed and made commercially available, including enzyme immunoassay for Campylobacter antigen detection and PCR-based methods[6].

The aim of the present study was to compare PCR and antigen detection by ELISA with culture for the detection of Campylobacter from the stools of pediatric patients suffering from gastroenteritis.

METHODOLOGY

Sample collection and transport:

The study population included 160 pediatric patients aged from 2 to 10 years attending the Gastroenterology clinic at Abo El-Reesh Hospital, one of Cairo

University Hospitals complaining of acute watery diarrhea. Patients were subjected to history taking through their parents (name, age, sex, and symptoms). Patients with chronic diarrhea or receiving recent antibiotic therapy within the previous week were not included in our study. The research protocol received approval from the Ethical Committee of the Faculty of Medicine at Cairo University (16/4/2019) and informed consent was taken from children's guardians contributing to our study.

Stool specimens were collected in clean containers, and part of each specimen was introduced into screw-capped bottles containing sterile Cary-Blair transport medium **(Oxoid, UK)** and transported to the laboratory for culture within 2 hours. The rest of the specimen was stored at -80°C in Eppendorf tubes to be tested by ELISA and PCR.

Diagnosis of campylobacteriosis:

Stool culture on Modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA)

- Stool specimens were immediately cultured on Modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA) plates **(Oxoid, UK)** in which blood is substituted with charcoal, ferrous sulfate, and sodium pyruvate. This encourages the majority of *Campylobacter* species to flourish. For 48 to 72 hours, plates were incubated in microaerophilic conditions at 42 °C. CampyGen CN0025 **(Oxoid, UK)** was used to create the microaerophilic condition.
- Gram-negative, curved, or spiral rod-shaped, motile, and oxidase-positive isolates were identified as Campylobacter genus.

Detection of Campylobacter antigen (PEB1) in specimens using ELISA

According to the manufacturer's instructions, a sandwich ELISA kit **(Sunlong Biotech, China)** was used to detect the Campylobacter antigen (PEB1) as follows: 40 µl of sample dilution buffer and 10 µl ml of sample were introduced to sample wells (dilution factor is 5). Mixing was done with gentle shaking. At 37°C, the plate was incubated for 30 minutes. After washing, 50 µl of Horseradish PEB1 (HRP)-conjugate reagent was added to each well (except the blank control well) . At 37°C, the plate was incubated for 30 minutes. Next, after washing, 50 µl of both chromogen solutions A and B were added to each well, mixing was done by gentle shaking and the plate was then incubated at 37 °C for 15 minutes. To stop the reaction, fifty µl of stop solution was added to each well. Using a spectrophotometer, the absorbance optical density was read at 450 nm.

Molecular detection of Campylobacter genes

- *DNA extraction*: Using the QIAamp DNA stool mini kit **(Qiagen, Germany)**, the DNA was extracted from stool samples in accordance with the manufacturer's instructions.
- *Multiplex PCR*: Three genes were targeted: *cadF* (a Campylobacter virulence gene that is genus-specific), *asp* (aspartokinase gene for *Campylobacter coli*), *hipO* (hippuricase gene for *Campylobacter jejuni*). The primer sets **(Promega, USA)** were used for gene amplification **(table 1)**. A previously identified *Campylobacter jejuni* strain was used as a positive control, and distilled water as a negative control. PCR was carried out in the BIO-RAD, T100™ Thermal Cycler using Taq Green PCR Master Mix (2X) **(Promega, USA)** according to **Zaghloul *et al.*[7]**.
- *Agarose gel electrophoresis (2%):* After staining with ethidium bromide, the PCR-amplified products were visualized using a UV transilluminator **(Promega, USA)**. After comparing the PCR products with a 100 bp DNA molecular marker **(Promega, USA)**, the sizes of the products were assessed (400 bp for *cadF*, 500 bp for *asp,* and 735 bp for *hipO*) **(table 1)**.

Table 1: Primer sequences and the size of PCR products

Target gene	Prime sequence (5'-3')	Product size (bp)	Reference
cadF	F-TTGAAGGTAATTTAGATATG R-CTAATACCTAAAGTTGAAAC	400	8
asp	F-GGTATGATTTCTACAAAGCGAGA R-ATAAAAGACTATCGTCGCGTG	500	9
hipO	F-GAAGAGGGTTTGGGTGGT R-AGCTAGCTTCGCATAATAACTTG	735	9

Statistical analysis:

The Statistical Package for Social Sciences (SPSS Inc., Chicago, IL) version 24 of IBM SPSS advanced statistics was used to analyze the data. Numbers and percentages were used to describe qualitative data. The suitable method for examining the relationship between qualitative variables was the chi-square (Fisher's exact) test. Calculations were made for the sensitivity, specificity, total accuracy, positive predictive value, negative predictive value, and 95% confidence interval. The Pearson correlation method was used for correlation analysis. A *P-value* of 0.05 or lower was regarded as statistically significant. All tests were two-tailed.

RESULTS

This study included 160 pediatric patients who were suffering from acute watery diarrhoea attending the Gastroenterology Clinic at Abo El-Reesh Hospital, Cairo University Hospitals. Diagnosis of Campylobacter was done by culture of stool specimens on mCCDA medium, and by rapid detection methods which included detection of Campylobacter antigen (PEB1) directly in stool specimens using ELISA and molecular detection of Campylobacter in stool specimens by PCR.

Demographic data of patients

The age of the patients ranged between 2 and 10 years. The highest prevalence was observed in children between 2-3 years (51.4%). Higher rates of Campylobacter isolation were observed in males than in females, 24 (68.6%) *versus* 11 (31.4%), with no significant difference statistically (*P-value*= 0.699).

Clinical presentations

All cases were presented clinically with diarrhea, followed by fever (66.3%). Vomiting and abdominal cramps were observed in 59.4% and 21.9% of cases respectively. Passage of bloody stools was the least presenting symptom which was observed in 4 cases (2.5%) only. Patients with a campylobacter infection had more fever and abdominal cramps compared to campylobacter-negative cases with a statistically significant difference (*P-value* $\leq$ 0.05).

Diagnosis of campylobacteriosis

1) Stool culture on Modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA)

Campylobacter culture was positive in 35 out of 160 samples (21.9%) **(figure 1)**.

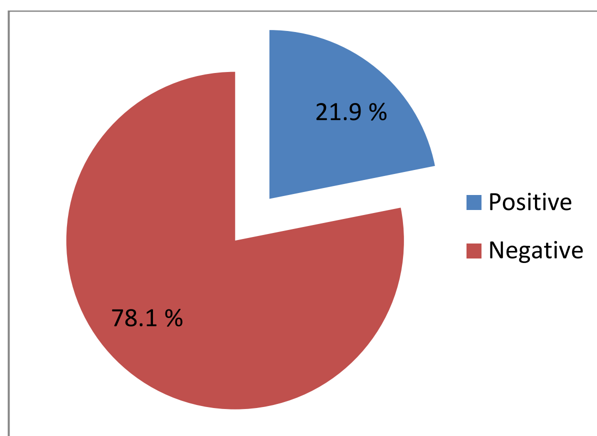

Fig. 1: Campylobacter isolation rate among the studied group

2) Detection of Campylobacter antigen (PEB1) in specimens using ELISA

Campylobacter antigen was detected in 50 samples (31.3%) out of the total 160 samples. Among these positive samples, 35 were also positive by culture (true positive), while 15 were negative by culture **(table 2)**. Compared to culture using mCCDA agar, the sensitivity of ELISA was 100%, while the specificity, positive predictive value (PPV), and negative predictive value (NPV) were 88%, 70%, and 100% respectively **(table 3)**.

There was a statistically significant moderate agreement between culture (the gold standard) and ELISA results (kappa value = 0.77, *P-value* < 0.001).

Table 2: ELISA and PCR results in relation to culture results:

			Culture (gold standard)	
			Positive	**Negative**
ELISA	**Positive (50)**		**35** **TP**	**15** **FP**
	Negative (110)		**0** **FN**	**110** **TN**
PCR	**Positive (47)**	***C. jejuni* (39)**	**35** **TP**	**12** **FP**
		***Non C. jejuni/coli* (8)**		
	Negative (113)		**0** **FN**	**113** **TN**
Total	**160**		**35**	**125**

TP (True positive), FP (False positive), FN (False negative), TN (True negative)

Table 3: Sensitivity, specificity, PPV, NPV of ELISA and PCR results

	ELISA (%)	PCR (%)	95% Confidence Interval (CI)
Sensitivity	100	100	90.0% to 100%
Specificity	88	90.4	83.8% to 94.9%
Positive Predictive Value (PPV)	70	74.4	57.9% to 84.2%
Negative Predictive Value (NPV)	100	100	90.0% to 100%
Total Accuracy	90.6	92.5	84.8% to 94. 6%

3) Molecular detection of Campylobacter genes

Detection of Campylobacter directly in stool specimens by multiplex PCR was done for the 160 stool samples using different target genes (*cadF, asp,* and *hipO*).

cadF gene was positive in 47 samples (29.4%) and negative in 113 samples (70.6%). *hipO* gene was positive in 39 samples of these 47 positive samples (24.4%) so, they were classified as *Campylobacter jejuni*, while *asp* gene was not detected in any sample, so, 8 samples out of 47 positive samples were classified as *Campylobacter* species other than *jejuni* and *coli* **(figure 2, table 2)**.

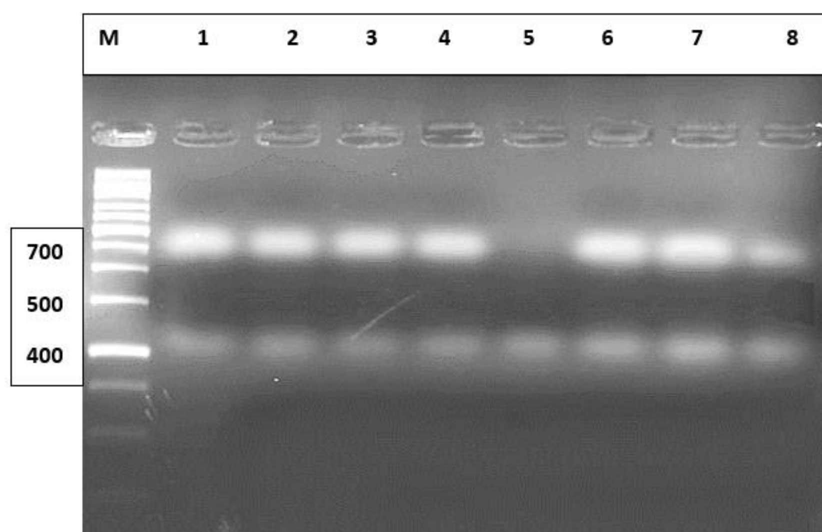

Fig. 2: Agarose gel electrophoresis showing DNA marker, *cadF* and *hipO* genes
Lane 1: A *Campylobacter jejuni* positive control strain showing both *cadF* (400bp) and *hipO* (735bp) genes.
Lanes 2, 3, 4, 6, 7, and 8 showing *Campylobacter jejuni* strains with both *cadF* and *hipO* genes.
Lane 5: A Campylobacter strain other than *jejuni* and *coli* with *cadF* gene only.
Lane (M): 100 bp DNA molecular marker

Results of PCR for Campylobacter isolation in relation to culture results

cadF gene was detected in 47 samples (29.4%) out of the total 160 samples. Among these positive samples, 35 were also positive by culture, while 12 were negative by culture. All culture-positive samples were positive for the *cadF* gene by PCR **(Table 2)**. Sensitivity, specificity, PPV, and NPV were 100%, 90.4%, 74.4%, and 100% respectively **(Table 3)**. There was a statistically significant substantial agreement between culture (gold standard) and PCR results (kappa value = 0.81, *P-value* < 0.001).

DISCUSSION

The most typical manifestation of Campylobacter infection is acute enteritis, and this condition is not differentiated from those caused by other microorganisms. Although there is a good prognosis for Campylobacter enteritis, the severity and duration of illness can be reduced by giving the appropriate antibiotic therapy which can also reduce complications including Guillain–Barré syndrome[10].

For the isolation of Campylobacter from stool samples, several selective media, both with and without blood, have been used. Even while acquiring cultures of the organism from stool samples continues to be the gold standard to determine the causative agent, The diagnosis occurs too late to allow for effective chemotherapy. So, rapid non-culture techniques for Campylobacter detection would therefore be helpful in diagnosis. Obtaining results on the same day would enable patients to receive early treatment and improve the accuracy of triaging patients[11].

This study was designed to compare different methods used in the diagnosis of Campylobacteriosis. The study included 160 pediatric patients aged 2-10 years suffering from acute watery diarrhea. *Campylobacter* species were isolated from 35 cases (21.9%) by culture method.

This result was close to what was reported by other authors. In Egypt, Abushahba *et al.*[12] reported that 27.5% of 80 stool samples in Assiut obtained from

infants under the age of 12 months were positive for *Campylobacter species* by culture using mCCDA. However, Abo Elazem and Emam[13] recorded 11.25% Campylobacter isolation rate out of 80 children presenting with gastroenteritis in Benha using mCCDA.

Other studies, however, reported a much lower prevalence among studied cases. In a large retrospective study at Abbassia Fever Hospital in Cairo, Wasfy *et al.*[14] detected 146 (2.3%) of 6278 patients who visited the hospital suffering from gastroenteritis due to *Campylobacter species*. Awadallah *et al.*[15] in Zagazig detected *Campylobacter* species among only 2.7% of 110 cases of gastroenteritis. In Iran, Mazaheri *et al.*[16] found that *Campylobacter* species were isolated from 8.6% out of 419 Iranian children aged 6-12 years with acute gastroenteritis.

Internationally, studies reported various results regarding the Campylobacter isolation rate among children with gastroenteritis. In Ethiopia, Chala *et al.*[17] isolated *Campylobacter species* from 10 stool specimens (10.1%) out of 99, collected from children with gastroenteritis. In a rural region in Romania, Chiriac *et al.*[18] investigated the cause of gastroenteritis as healthcare-associated infections in pediatric wards. The authors found that among 615 cases aged 2-6 years, 482 (69.59%) were due to *Campylobacter* species.

Differences observed between countries in the mentioned studies are mainly attributed to differences in proper sanitary conditions which may be deficient in developing countries including Egypt, as well as the close contact with animals. All of which make it simple and common to contract any gastrointestinal pathogen, including Campylobacter.

In the present study, Campylobacter infection was found in 20 (17.1%) of children less than 3 years, and in 15 (34.9%) of those older than 3 years, with a significant difference in isolation rate among the two groups being higher among older children. However, most positive cases were below 3 years of age. This may be because most of the patients enrolled in the study were in this age group.

A study done by Rathaur *et al.*[19] revealed that the age group 1-3 years made up 52.9% of patients with Campylobacter diarrhea. Similarly, a 10-year study was conducted in Germany revealed that the majority of patients with Campylobacter infection were children aged from 1 to 4 years[20]. Another study in Ghana revealed that children aged 2-5 years were the most common group affected with Campylobacter diarrhea[21].

Any age group is liable to become infected with Campylobacter, but infection is more frequent in children younger than 5 years. This is because they can get the infection easily through eating with unclean hands, consumption of contaminated food, especially undercooked chicken and unpasteurized milk, and contact with household pets, most often puppies, cats, and birds[22].

In our study, higher rates were observed in males in comparison to females (68.6% *versus* 31.4%, respectively), which was statistically insignificant. Similarly, a study conducted in Egypt showed that infection with Campylobacter is more common in male children than in females, 6 *versus* 3 respectively[13]. In a study performed by Chiriac *et al.*[18], gender distribution showed a slight predominance of boys with no statistical significance.

Relying on culture methods to detect *Campylobacter* species in stool samples continues to be a major challenge for the diagnosis of campylobacteriosis. Campylobacter grows slowly, taking 48–72 hours, and requires a specific culture medium and condition for microaerophilic development[23]. However, culture is still necessary for epidemiological purposes and testing for antibiotic resistance[24]. Given the difficulties faced during culturing stools for detecting *Campylobacter* species, other detection methods seem mandating.

In this study, the culture method (the gold standard method of diagnosis) was compared with detection of specific antigen (PEB1) by ELISA and Campylobacter virulence genes (*cadF* , *asp, and hipO*) by PCR.

Campylobacter antigen was detected in 50 samples (31.3%) out of the total 160 samples. All culture-positive samples were ELISA-positive as well, in addition to 15 other samples. Compared to culture using mCCDA agar, the sensitivity, specificity, PPV, and NPV of ELISA were 100%, 88%, 70%, and 100%, respectively. There was a statistically significant moderate agreement between culture and ELISA results.

Stool antigen assays to detect Campylobacter directly in stool samples are quick tests and produce results on the same day, but their sensitivity, specificity, and positive predictive value have been found to be significantly varied[25, 26, 27].

A comparable study conducted in Egypt by Abo Elazem and Emam[13] reported that sensitivity and specificity for ELISA in the diagnosis of campylobacteriosis were 100% and 97%, respectively, where out of 80 cases, 9 cases were culture-positive and 11 cases were ELISA-positive. Regnath and Ignatius[28] stated that among 533 fecal specimens, 38 *Campylobacter* species were isolated. Samples were retested by ELISA. Considerable agreement between ELISA and culture results were obtained as sensitivity and specificity were 96.8% and 97.2%, respectively. In comparison to the sensitivity discovered for the other techniques, Veras *et al.*[29] discovered that ELISA had a 100% sensitivity, which was greater than what was discovered by the other methods, its specificity and PPV, however, were significantly diminished, falling to 80% and 24%, respectively. On the other hand, Patrick *et al.* [30] found that out of 2,767 fecal samples tested for *Campylobacter species*, 95 were positive by culture. All specimens were tested by four different ELISA assays. The sensitivity and specificity of the different methods

ranged from 79.6% to 87.6% and 95.9 to 99.5%, respectively.

Amin and Gerges[31] revealed that out of a total of 343 stool samples and rectal swabs, 5.7% of the patients and 0.7% of the controls had campylobacter isolated by culture on 2 selective media. Enzyme immunoassay achieved 89.7% specificity and 91.7% sensitivity when compared to culture.

In this study, PCR was investigated as a method for diagnosis of Campylobacter infection. Unlike culture methods, a PCR approach has the advantage of detecting and identifying Campylobacter up to the species level on the same day[32].

Campylobacter, as identified by the presence of *cadF* gene which is genus-specific, was detected among 47 (29.4 %) out of the total 160 samples. Among these positive samples, 35 were also positive by culture. Of these 47 isolates, 39 isolates were identified as *Campylobacter jejuni* (*C. jejuni*) by detection of *hipO* gene. None was identified as *Campylobacter coli* (*C. coli*) as *asp* gene was not detected in any sample, and 8 were classified as non-*jejuni*/*coli*. The sensitivity, specificity, PPV, and NPV of PCR were 100%, 90.4%, 74.4%, and 100%, respectively. There was a statistically significant substantial agreement between the results of culture and PCR.

In a study by Veras *et al.*[29], the effectiveness of PCR and ELISA as alternative methods to culture for detecting *Campylobacter* species in stool samples was investigated. Culture detected only 13.07% of positive samples, whereas ELISA detected 37.9%. All the positive samples identified by culture were also detected by ELISA. PCR identified 20.3% of positive samples, but not all *Campylobacter* species were detected by culture due to its low sensitivity. The authors concluded that the true number of Campylobacter infections may be underestimated by culture and that PCR and ELISA could be good alternatives for diagnosis. However, culture remains the only method that can provide 100% specificity and PPV. Amin and Gerges[31] detected 12 cases that tested positive for culture, 11 (91.7%) also tested positive for PCR, while one isolate tested negative for PCR. The test had 100% specificity, 91.7% sensitivity, 100% PPV, 98.6% NPV, and no positive cases were found among the culture-negative patients.

As previously mentioned, the detected *Campylobacter* species in our study were 39 *C. jejuni,* whereas 8 samples tested positive for *Campylobacter* species other than *jejuni* and *coli. C. jejuni* causes infections more than other *Campylobacter* species which complies with our results[33]. Regnath and Ignatius[28] reported that among 38 isolated *Campylobacter* species from 533 fecal specimens with culture and enzyme immunoassay, 36 were *Campylobacter jejuni* and one was *Campylobacter coli*. In a study by Ashraf *et al.* [34] in Zagazig, mCCDA and sheep blood agar were used to test stool samples from 246 patients with gastroenteritis, and 13 (5.3%) specimens tested positive for *Campylobacter* species. Using the Na hippurate hydrolysis test for phenotypic and biochemical identification, out of the 13 isolates, 10 isolates were identified as *C. jejuni* and 3 were *C. coli*. PCR targeting the *hipO* gene confirmed all the biochemically suspected *Campylobacter jejuni* isolates.

In South Africa, Reddy and Zishiri [35] screened 83 Campylobacter isolates for the presence of *cadF*, *asp,* and *hipO* genes. Sixty-nine isolates (83%) were *Campylobacter jejuni* as the predominant species, while 14 isolates (17%) were *Campylobacter coli*. Chala *et al.*[17] found 10 out of 99 stool samples were positive for *Campylobacter* species, 5 of the 10 Campylobacter isolates were *C. jejuni*, one was *C. coli*, and the other four species were unidentified.

In our study, we couldn't decide whether inconsistent results (positive stool antigen assay and/or PCR, negative culture) were actual infections that were missed by culture, or whether they were merely false positives. The accuracy of culture-based methods is limited by the fragility of Campylobacter, which can die during sample handling, as well as the challenge of identifying small colonies of Campylobacter among the complex mixture of fecal flora present in fecal samples[36]. The use of transport medium is believed to enhance the survival of Campylobacter in specimens, but the exact duration of successful storage is not well-established[24]. Additionally, Campylobacter has the ability to enter a viable but non-cultivable state, which allows it to overcome various stresses. Traditional culture methods are unable to detect Campylobacter in this non-cultivable state[37]. Also, selective culture is relatively good for detecting *C. jejuni* and *C. coli* which were the two predominant species, However, the high concentration of cefoperazone present in the selective medium can inhibit the growth of less common *Campylobacter* species such as C. *upsaliensis, C. fetus*, and *C. lari* [24].

According to Bessède *et al.*[26], stool samples were classified as positive for *Campylobacter* species using two criteria. First, if the culture method yielded a positive result. Second, if the culture method was negative, but both the molecular and enzyme immunoassay methods provided positive results. Based on these criteria, 12 of the culture-negative samples in this study that were positive by both ELISA and PCR could be considered positive for Campylobacter infection.

CONCLUSION & RECOMMENDATIONS

The current study revealed that selective culture on mCCDA was a cost-effective and practical approach which allows isolating and identifying Campylobacter strains within 48-72 hours. ELISA and PCR were equal in sensitivity to culture method, but PCR was more

specific than ELISA. PCR is a faster detection method compared to culture and has the capability to identify and differentiate Campylobacter at the species level.

Conflict of interest:

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article none.

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Availability of data and material:

Data are available upon request.

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