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Original Article

Culture and culture-independent diagnostic tests in *Campylobacter* enteritis

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Abstract

Introduction: *Campylobacter* infections are among the most common causes of bacterial enteritis. This study aims to determine the sensitivity, specificity and positive predictive values (PPV) of culture and culture-independent tests for the diagnosis of *Campylobacter* enteritis.
Methodology: A total of 400 stool samples were included in the study. BD MAX enteric bacterial panel (BD Diagnostics, Franklin Lakes, NJ, USA) and EntericBio Gastro Panel II (Serosep, Limerick, Ireland) were used as commercial molecular tests. RIDA®QUICK *Campylobacter* (R-Biopharm, Darmstadt,, Germany) and CerTest (Biotec, Zaragoza, Spain) were used to detect *Campylobacter* antigens. Samples were cultured in CCDA media and subjected to bacterial identification by mass spectrometry.
Results: Among the 400 specimens, 41 (10.2%) were evaluated as *Campylobacter* positive; 21 were culture-positive and 20 were detected as positive by both PCR methods. Of the 21 isolates grown in culture, 16 (76.2%) were identified as *C. jejuni* and 5 (23.8%) as *C. coli.* While all 21 culture-positive specimens were detected as positive by both molecular tests, 18 of the specimens were found positive by RidaQuick, and 16 by Certest ICA. Of the 20 culture-negative *Campylobacter* cases, 18 were positive by RidaQuick and 12 by Certest ICA. Sensitivities of culture, ICA-RidaQuick and ICA-CerTest were 51.2%, 87.8 and 68.3, respectively. The specificities of all tests were in the range of 90-100 %. PPV of molecular tests, ICA-RidaQuick and ICA-CerTest were > 95%, 72 % and 48.3 %, respectively.
Conclusions: Molecular tests were superior to culture and ICA in terms of sensitivity, specificity, and positive predictive value.

Key words: *Campylobacter*, gastroenteritis, multiplex PCR, immunochromatography.

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Introduction

Campylobacter is a gram-negative, curved or spiral-shaped genus of bacteria, belonging to the family Campylobacteriacae [1]. Despite being among the most common causes of bacterial enteritis worldwide, it is overlooked due to difficulties in diagnosis [2–5]. *Campylobacter* species are fastidious microorganisms requiring a microaerobic environment to grow. *Campylobacter* culture requires implantation of the stool sample into a selective medium and incubation at 42 °C for about 72 hours under microaerobic conditions [1]. Final identification of the grown bacteria by biochemical tests is also time-consuming. Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) saves time by identifying isolated microorganisms within half an hour [6,7]. Although the identification time for the isolated bacteria is shortened by MALDI-TOF MS, the extended growth period of culture continues to be a problem for early diagnosis.

The finalization of stool cultures may take 3-4 days if there is no growth and up to 7 days when enteropathogenic bacteria grow. A significant proportion of *Campylobacter* infections were reported to be overlooked in culture-based methods [8–10].Therefore, culture-independent diagnostic tests (CIDT) based on the detection of bacterial DNA or specific antigens have been developed and these have attracted increasing attention in routine laboratory applications [10–16]. Immuno-chromatographic assays (ICA) detect specific antigens in stool samples with the help of monoclonal antibodies. Such lateral flow assays produce results within a few minutes and are very easy to perform but their sensitivity and specificities may vary. Syndromic panels are combined molecular assays that can detect multiple agents causing a clinical syndrome such as gastroenteritis, pneumonia, or meningitis. Syndromic panel assays are capable of detecting nucleic acids of microorganisms with high sensitivity and specificity. Our aim was to determine

the sensitivities, specificities, and positive predictive values of culture and CIDT for *Campylobacter* diagnosis from stool samples.

Methodology

Materials

This prospective cohort study was conducted at the Medical Microbiology Department of Dicle University Hospital, a tertiary hospital in southeastern Turkey, between December 2016 and January 2018. The research was conducted in accordance with the Helsinki Declaration 2013 and was approved by the Non-interventional Clinical Research Ethics Committee of Dicle University on May 14th, 2015 (No: 258).

A total of 400 unpreserved stool samples obtained from adult and pediatric patients suspected of bacterial gastroenteritis or colitis were included. Stool specimens without pus and specimens of patients hospitalized for more than 48 hours were excluded. As soon as the samples were brought to the laboratory, they were transferred to Cary-Blair transport medium (Sterilin, Newport, England) and kept at + 4 °C to be inoculated into culture media in 12 hours. After the ICA tests and BD Max (BD Diagnostics, Franklin Lakes, NJ, USA) enteric panel tests were performed, the remaining samples were transferred to 2 mL tubes and stored at -80 °C until the EntericBio (Serosep, Limerick, Ireland) assay was run.

Culture and Identification

Stool samples preserved in Cary Blair transport medium were inoculated on the modified charcoal cefoperazone deoxycholate agar –(mCCDA) (Himedia, Mumbai, India) supplemented with cefoperazone, amphotericin B, and teicoplanin (CAT) (Himedia, Mumbai, India). The mCCDA plates were incubated in a microaerobic environment provided by the Anoxomat™ MARK II system (MART Microbiology BV, Drachten, Netherlands) at 42 °C for 48-72 h. Mass spectrometer failed to identify *Campylobacter* species growing on selective medium. Therefore, *Campylobacter* suspected colonies were checked by Gram stain, sub-cultured to 5% sheep blood agar medium and incubated for 48 h. The sub-cultured isolates were identified by the MALDI TOF MS system using MALDI Biotyper version 3.1 (Bruker Daltonics, Billerica, USA). *C. jejuni* (ATCC 37291) was used as a quality control strain of the culturing processes.

Immuno-chromatographic tests

The fresh stools were analyzed by two commercial ICA: RIDA®QUICK *Campylobacter* (R-Biopharm, Darmstadt, Germany) and CerTest *Campylobacter* (Biotec, Zaragoza, Spain). Both assays were performed and interpreted according the manufacturers' instructions. Stool specimens were thoroughly mixed for homogeneous distribution of the antigens prior to any use.

The RIDA®QUICK *Campylobacter* (R-Biopharm, Darmstadt, Germany) test kit included two different reagents; Reagent A and Reagent B. The CerTest *Campylobacter* (Biotec, Zaragoza, Spain) included a collection tube with diluent. It took less than half an hour to perform each assay. C-line was the control line; assays were repeated for samples without the C-line. The presence of T-line was evaluated as *Campylobacter*-positive.

Molecular assay by Serosep EntericBio

The assay was performed and interpreted according to the manufacturer's instructions. Specimens stored at -80 °C were thawed before EntericBio assay. A swab was dipped into the tube, lightly covered with the thawed stool sample and resuspended in a stool preparation solution (SPS) tube. SPS tubes were placed in a heat block at 103 °C for 30 minutes to release the DNA. The heat-treated specimens were placed in the EntericBio workstation in which the processed specimens were automatically transferred to the reaction wells. Finally, the wells were capped and transferred to the real-time polymerase chain reaction (PCR) instrument for automatic amplification, detection, and analysis with the EntericBio Gastro Panel 2 program. The test results were interpreted after 3 h. The presence of *Campylobacter* spp., *Salmonella* spp., *Shigella* spp., Verotoxigenic *E. coli*, *Cryptosporidium*, and *Giardia* DNA in the specimen was interpreted based on the presence of a line blot at each of the six locations.

BD Max Enteric Bacterial Panel (EBP)

A total of 200 specimens studied with BD Max EBP included fresh stool specimens, while the remaining 200 were kept at -80 °C and studied after thawing. The BD MAX EBP (BD Diagnostics, Franklin Lakes, NJ, USA) is a commercial in vitro molecular assay for the detection of enteric bacterial pathogens; *Campylobacter* spp., *Shigella* spp., *Salmonella* spp. and Verotoxigenic *E. coli* in stool samples. Following the manufacturer's instructions, the stool specimens were placed in sample buffer tubes (SBT), vortexed and loaded onto the BD MAX instrument (BD Diagnostics, USA) along with the BD MAX EBP reagent strip. A fully automated process including sample preparation,

lysis, DNA extraction, and multiplex PCR was run. Samples with indeterminate results due to BD MAX system failure were analyzed for a second time.

Definition of Campylobacter-positivity

Campylobacter positivity was defined as either bacterial growth in culture or positivity of both molecular tests.

Results

Over a period of thirteen months, 2186 suspected gastroenteritis stool samples were sent to the microbiology laboratory; 400 of them fulfilled the inclusion criteria of the study. The majority of the patients were from pediatric clinics (71.3%); the percentage of patients included from gastroenterology, infectious diseases and other clinics were 14.5%, 9.8% and 4.4%, respectively. The patients were between 0-90 years of age, with a mean age of 29.42 ± 23.97 years. A total of 41 specimens (10.2%) were evaluated as *Campylobacter* positive; 21 were culture-positive and 20 were positive by both PCR methods. The culturing process, including initial incubation and identification after sub-culturing, took 4 to 5 days. Of the 21 isolates grown in culture, 16 (76.2%) were identified as *C. jejuni* and 5 (23.8%) as *C. coli* by MALDI-TOF MS.

While all 21 culture-positive specimens were detected as positive by both molecular tests, 18 of the specimens were found positive by RidaQuick and 16 by Certest ICA. Of the 20 culture-negative *Campylobacter* cases, 18 were positive by RidaQuick and 12 by Certest ICA. Sensitivities of culture, ICA-RidaQuick and ICA-CerTest were 51.2%, 87.8 and 68.3, respectively. The specificities of all five methods were in the range of 90-100% (Figure 1). Positive predictive values (PPV) of molecular tests were above 95%, while PPV of ICA-RidaQuick and ICA-CerTest were 72 % and 48.3 % respectively (Figure 2).

Of the 42 enteric bio-positive specimens; 21 were culture and BD Max EBP-positive and 20 were BD Max EBP-positive. One specimen was reported as very weakly positive by Enteric Bio and was found negative in other tests. Of the 43 BD Max EBP positive specimens; 21 were culture and Enteric Bio positive and 20 were Enteric Bio –positive. Two specimens with weak positivity were detected as negative by other assays. All methods (culture, PCR tests and ICAs) were positive in 16 cases. The test results obtained with different diagnostic methods are summarized in Table 1. A total of 312 specimens were identified as negative by all diagnostic tests for *Campylobacter*.

Discussion

Until recent years, culture methods were considered the gold standard for detecting *Campylobacter*. However, *Campylobacter* species cannot be cultured from samples containing a small number of bacteria or viable but non-culturable bacteria. Molecular methods can be used in such cases [8,17]. In the current study, a total of 21 *Campylobacter* culture isolations were made from the 400 specimens, all of which were detected with two commercial molecular assays. However, the multiplex PCR panels – EntericBio and BD Max EBP - detected 20 additional samples with positive results. Our findings were compatible with previous studies. In a large multicenter study, 1552 stool specimens were tested by traditional culture and the Food and Drug Administration (FDA)-cleared immunoassay method.

Figure 1. Sensitivity and specificities of diagnostic tests for Campylobacter detection.

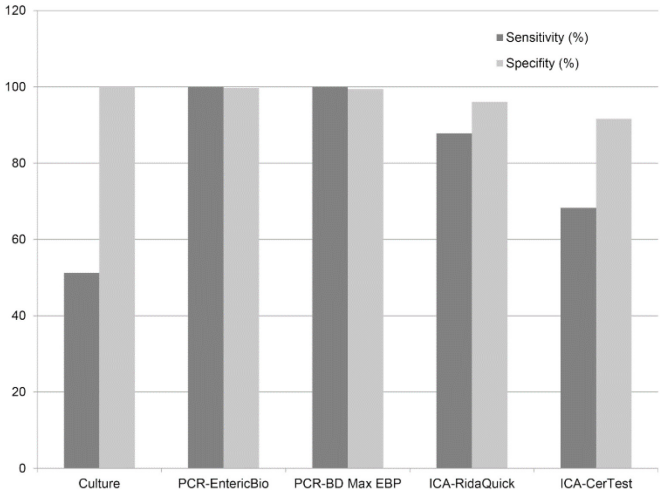
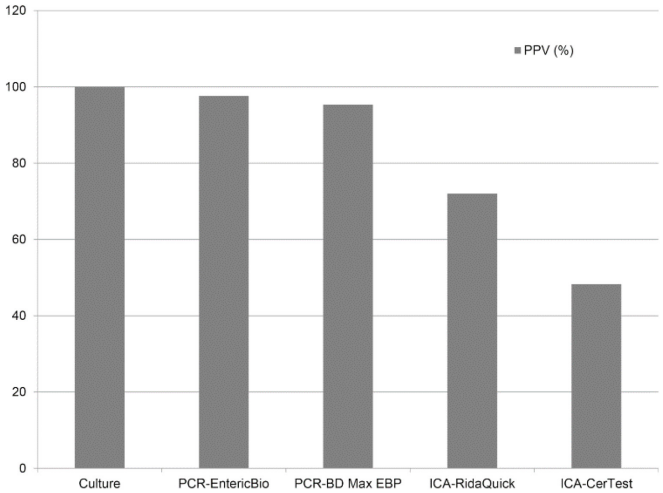


Figure 2. Positive predictive values of diagnostic tests for Campylobacter detection.



Any positive result by culture or EIA was tested by four molecular methods; 16S rRNA qPCR, an FDA-cleared multiplex PCR assay, species-specific PCR assays and sequencing. All culture-independent methods showed complete agreement while culture couldn't detect 13 of 47 CIDT-positive samples [9]. The Global Enteric Multicenter Study (GEMS) investigated 32 entero-pathogens in stool samples of children younger than 5 years in Africa and Asia by quantitative real-time PCR (qPCR) and original microbiological methods, including culture. The pathogen-specific attributable incidences of *Campylobacter* with qPCR were twice that of the original microbiological methods [18]. Valledor *et al*. compared five different real-time PCR kits with culture methods to identify the most common enteropathogenic bacteria - *Campylobacter* spp., *Salmonella* spp., and *Yersinia enterocolitica* - in stool samples. They reported that the culture showed the lowest positive predictive agreement value as it could not detect *Campylobacter* species other than *C. jejuni* and *C. coli*, and even failed to detect 3 *C. jejuni* and 3 *C. coli* positive samples. The authors noted that sometimes even *C. jejuni* and *C. coli* could have a poor growth rate in selective media [19]. In a study comparing EntericBio molecular system with culture, O'Leary *et al*. detected 12 additional *Campylobacter*-positive specimen by EntericBio system to culture-positive 30 specimen [14]. In a study evaluating detection of *Campylobacter* species by molecular assays and culture methods, 16S/23S PCR/DNA probe assay detected *Campylobacter* DNA in 41 of 109 culture-negative specimens. Among 16S/23S PCR/DNA-positive specimens, 35 were confirmed by 16S PCR/DNA probe assay [20]. Another study on a PCR-based molecular screening method (MSM) for the detection of *C. jejuni* revealed that the sensitivity ranges of MSM and culture were 98 to 100 % and 77.8 to 86.8 %, respectively. The "gold standard" of the study was assessed as all culture-positive and all MSM-positive specimens, which were confirmed by a secondary PCR of a different target gene [21]. Similarly, the "gold standard" of the current study was determined as either bacterial growth in culture or positivity of both molecular tests. The disagreement between culture and PCR positivity may be due to the number of bacteria in the samples. Samples with a high number of bacteria can be detected by both culture and PCR, while samples with a small number of bacteria may not grow in culture. Knabl *et al*., in their study comparing the culture method with BD Max EBP, reported the detection rate of *Campylobacter* species at a concentration of 10 colony forming units (CFU)/mL as 100% and 43.8% for BD Max EBP and culture, respectively [16]. Anderson *et al*. compared the sensitivities of culture and BD Max EBP by organism concentration and revealed that the sensitivity rates dropped as the concentration of organism dropped. The sensitivities of culture were measured as 100%, 63.8%, 43.8% and 18.8% for *Campylobacter* concentrations of 10^5, 10^4, 10^3 and 10^2 CFU/mL, respectively. The BD Max EBP had a sensitivity of 100% at 10^3-10^6 CFU/mL concentrations and 68.8% at 10^2 CFU/mL [22].

The sensitivity and specificity of different commercial stool antigen assays may vary depending on the structure of the kit or antibodies used. Previous studies suggested that these tests should not be used as a stand-alone diagnostic test [10,13,15]. In the current study, the sensitivities of two commercial stool antigen assays -ICA-Rida Quick and ICA-CerTest- were found as 87.8 and 68.3, respectively. PPV was 72% for ICA-Rida Quick and 48.3% for ICA-CerTest. In a study about the accuracy of *Campylobacter* antigen detection

Table 1. Distribution of the test positivity obtained with different diagnostic methods used for Campylobacter detection.

Number of specimens	Diagnostic tests				
	Culture	PCR-Enteric Bio	PCR- BD Max EBP	ICA-Rida Quick	ICA- CerTest
Culture positive (no: 21)					
16	+	+	+	+	+
2	+	+	+	+	-
3	+	+	+	-	-
PCR positive (no:23)					
12	-	+	+	+	+
6	-	+	+	+	-
2	-	+	+	-	-
2	-	-	+*		
1	-	+*	-		
ICA positive (no: 44)					
30	-	-	-	-	+
14	-	-	-	+	-

+: positive; -: negative; *: The amplification curves of these samples were not typical logarithmic curves, they were considered weak positive.

methods, Regnath and Ignatius revealed that ICA-Rida Quick (R-Biopharm, Germany) was positive for 24/25 culture-positive and 14/508 culture-negative stool specimens [10]. The same ICA was positive for 37/38 frozen *Campylobacter* isolates [10]. A multicenter study, led by the CDC, was conducted on the performance of four commercial stool antigen tests - two lateral flow assays and two microplate assays - for the detection of *Campylobacter*. A total of 95 specimens (3.4%) were positive among 2767 stool specimens. The sensitivity, specificity and positive predictive ranges of stool antigen assays were 79.6% to 87.6%, 95.9% to 99.5% and 41.3 to 84.3%, respectively [15]. A study conducted in Bulgaria compared CerTest *Campylobacter* (Biotec, Spain) ICA, The Eva Green real-time mPCR and culture for detection of *Campylobacter* in stool samples. The study reported that 20 out of 40 ICA-positive stool samples were confirmed by both culture and PCR, while 20 were evaluated as false negative [23].

Conclusions

Timely diagnosis of gastroenteritis is important in terms of early and proper treatment as well as preventing transmission to other people. The findings of our study revealed that the culture method was time-consuming and overlooked about half of the *Campylobacter enteritis* cases. Mass spectrometry could not shorten the turn around times sufficiently, as it could not identify *Campylobacter* species from selective media without subculture. Contents of the selective media severely reduce the identification performance of mass spectrometer. Bacteria can only be identified by mass spectrometry when taken from a nutrient medium such as blood agar. In addition, the ICA tests performed in the study lacked adequate sensitivity and specificity to be used as stand-alone tests. Molecular methods were found superior to both culture and ICA in terms of sensitivity, specificity, and positive predictive value. Supporting routine microbiology laboratory with molecular tests will be beneficial for early and accurate diagnosis, appropriate treatment and disease control.

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Authors' Contributions

NÖ and FB conceived and designed the study. FÇ, NG and AB performed the tests. NÖ analyzed the data and wrote the manuscript. TD supervised the study. All authors read, provided feedback and approved the final manuscript.

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