Evaluation of a Multiplex Real-Time PCR Assay for Detecting Major Bacterial Enteric Pathogens in Fecal Specimens: Intestinal Inflammation and Bacterial Load Are Correlated in *Campylobacter* Infections

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A total of 1,056 native or Cary-Blair-preserved stool specimens were simultaneously tested by conventional stool culturing and by enteric bacterial panel (EBP) multiplex real-time PCR for *Campylobacter jejuni*, *Campylobacter coli*, *Salmonella* spp., and shigellosis disease-causing agents (*Shigella* spp. and enteroinvasive *Escherichia coli* [EIEC]). Overall, 143 (13.5%) specimens tested positive by PCR for the targets named above; 3 coinfections and 109 (10.4%) *Campylobacter* spp., 17 (1.6%) *Salmonella* spp., and 20 (1.9%) *Shigella* spp./EIEC infections were detected. The respective positive stool culture rates were 75 (7.1%), 14 (1.3%), and 7 (0.7%). The median threshold cycle (C_T) values of culture-positive specimens were significantly lower than those of culture-negative ones (C_T values, 24.3 versus 28.7; $P < 0.001$), indicating that the relative bacterial load per fecal specimen was significantly associated with the culture results. In *Campylobacter* infections, the respective median fecal calprotectin concentrations in PCR-negative/culture-negative ($n = 40$), PCR-positive/culture-negative ($n = 14$), and PCR-positive/culture-positive ($n = 15$) specimens were 134 mg/kg (interquartile range [IQR], 30 to 1,374 mg/kg), 1,913 mg/kg (IQR, 165 to 3,813 mg/kg), and 5,327 mg/kg (IQR, 1,836 to 18,213 mg/kg). Significant differences were observed among the three groups ($P < 0.001$), and a significant linear trend was identified ($P < 0.001$). Furthermore, the fecal calprotectin concentrations and C_T values were found to be correlated ($r = -0.658$). Our results demonstrate that molecular screening of *Campylobacter* spp., *Salmonella* spp., and *Shigella* spp./EIEC using the BD Max EBP assay will result in timely diagnosis and improved sensitivity. The determination of inflammatory markers, such as calprotectin, in fecal specimens may aid in the interpretation of PCR results, particularly for enteric pathogens associated with mucosal damage and colonic inflammation.

Acute bacterial gastroenteritis is a common global health problem. Infections with *Salmonella* spp., *Campylobacter* spp., and Shiga toxin-producing *Escherichia coli* (STEC) are spread to humans from animal reservoirs. In contrast, humans are the primary reservoir of *Shigella* spp. These four microorganisms are the most commonly reported enteric bacterial pathogens responsible for community-acquired diarrhea (1, 2). Conventional bacterial stool culturing is considered the traditional gold standard for diagnosis of the most common bacterial pathogens (3, 4), and current guidelines recommend one to two culture specimens for adult patients and one specimen for pediatric patients (5). Fresh stool specimens should be transported to the laboratory and processed within 2 h of collection (6, 7). If the specimen cannot be processed within 2 h, it should be placed in a transport medium to preserve the viability of bacterial pathogens, particularly *Shigella* and *Campylobacter*. Antibiotics administered at the time of specimen collection may compromise the outcome of stool culturing (8).

Several novel molecular assays for detecting enteric bacterial pathogens in stool specimens have been developed. These assays are very specific, and their sensitivities are superior to those of conventional methods (3, 7, 9, 10). Among these tests, the BD Max enteric bacterial panel (EBP) is an FDA-cleared, multiplex PCR assay designed for the detection of *Salmonella* spp., *Shigella* spp./enteroinvasive *E. coli* (EIEC), *Campylobacter* spp. (*Campylobacter jejuni* and *Campylobacter coli*), and Shiga-like toxin genes (*stx*₁ and/or *stx*₂) in preserved and unpreserved stool specimens using the BD Max system (BD Diagnostics, Allschwil, Switzerland). The BD

Max system is a walkaway PCR instrument that can extract, amplify, and detect nucleic acids in batches of up to 24 samples within 3 h, requiring less than 2 min of hands-on time per sample (11).

In this study, we aimed to achieve the following: (i) to evaluate the performance of molecular testing compared with that of conventional culture methods in a privately owned clinical microbiology laboratory, currently serving approximately 2,200 hospital beds and several hundred physicians in private practice; (ii) to study the association between the relative bacterial load, as assessed using the threshold cycle (C_T) values of pathogen-specific PCR targets, and the respective culture results; and (iii) to explore the association between bacterial load, as assessed using C_T values, and the extent of intestinal inflammation, as assessed using stool calprotectin concentrations, in *Campylobacter* infections (*C. jejuni* and *C. coli*).

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FIG 1 Correlation between BD Max enteric bacterial panel C_T values and CFU counts for *Campylobacter jejuni* ATCC 33291 (A), *Salmonella enterica* subsp. *enterica* serovar Enteritidis ATCC 13076 (B), and *Shigella flexneri* DSM 4782 (C).

MATERIALS AND METHODS

Study design. A total of 1,056 native or Cary-Blair-preserved stool specimens received between 4 July and 26 August 2013 ($n = 262$) and between 12 November and 12 March 2014 ($n = 794$), respectively, were included in this study. The specimens were simultaneously tested by conventional stool culturing and EBP multiplex real-time PCR for *C. jejuni*, *C. coli*, *Salmonella* spp., and shigellosis disease-causing agents (*Shigella* spp. and EIEC). STEC was excluded from the study because in Switzerland general stool bacteriology only includes culturing of *Salmonella*, *Shigella*, and *Campylobacter*. The study protocol was approved by the ethics committee of the Canton of Bern, Switzerland.

Enteric bacterial panel. Ten microliters of each stool specimen was transferred with a loop to a sample buffer tube according to the manufacturer's instructions. Stool specimens were tested within 48 h of receipt and were stored at 2 to 8°C prior to testing. The fully automated process took less than 3 h.

Culture. Cary-Blair-preserved and native fecal specimens were processed on the day of arrival with a BD Kiestra Total Laboratory Automation System (Becton-Dickinson). The following media were inoculated: selenite broth, Hektoen enteric (HE) agar, xylose-lysine-deoxycholate (XLD) agar, and Campylosel agar (all from bioMérieux Suisse S.A., Geneva, Switzerland). Campylosel agar was incubated in a microaerobic atmosphere (5% O_2 , 10% CO_2 , and 85% N_2) at 42°C for 48 h, and results were read after 24 and 48 h. HE and XLD agar media were incubated aerobically for 48 h, and results were read after 24 and 48 h. Selenite broth was subcultured after 15 h of aerobic incubation onto HE and XLD agar. Potential pathogens were identified by matrix-assisted laser desorption/ionization–time of flight mass spectrometry (MALDI-TOF MS; Bruker Daltonics GmbH, Bremen, Germany) or Vitek-2 (bioMérieux Suisse S.A., Geneva, Switzerland). *Salmonella* and *Shigella* isolates were confirmed by serological testing (Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany; Sifin Diagnostics GmbH, Berlin, Germany).

BD Max EBP calibration curves. Privileged access to the C_T values generated by the BD Max system was obtained from the manufacturer for the duration of the study to establish a correlation between the BD Max C_T values and CFU counts. Serial 10-fold dilutions (1.5×10^8 cells ml^{-1} to 1.5×10^1 cells ml^{-1}) in 0.85% sodium chloride solution of *C. jejuni* ATCC 33291, *Salmonella enterica* subsp. *enterica* serovar Enteritidis ATCC 13076, and *Shigella flexneri* DSM 4782 were plated on the appropriate agar media, and colonies were counted after 24 h (*Salmonella* and *Shigella*) or 48 h (*Campylobacter*) of incubation. In parallel, 10 μl from each dilution step was tested in triplicate using the EBP. The C_T values and CFU counts per milliliter were compared.

Calprotectin. Calprotectin levels were determined in 69 (6.5%) stool specimens by a Ridascreen assay (R-Biopharm, Darmstadt, Germany) to assess the intestinal inflammatory response induced by *Campylobacter*, a classical agent of inflammatory diarrhea. Specimens were subdivided into three groups according to the respective *Campylobacter* culture and PCR results as follows: group 1 ($n = 40$), culture negative/PCR negative; group

2 ($n = 14$), culture negative/PCR positive; and group 3 ($n = 15$), culture positive/PCR positive.

Statistical analysis. A statistical software program (MedCalc, version 15.6; MedCalc, Ostend, Belgium) was used to conduct statistical analysis. To determine whether C_T values are related to bacterial counts, univariate linear regression analysis, with the C_T values as dependent variables and the CFU counts of the different pathogens as independent variables, was performed. A Mann-Whitney test was used to assess differences in the C_T values between two groups, and a Kruskal-Wallis test was used to assess differences in the calprotectin levels among three groups. The trends in the calprotectin concentration were analyzed using a Jonckheere-Terpstra trend test. P values of <0.05 were considered statistically significant. Correlations between the bacterial load, as determined according to the C_T value, and the calprotectin level in campylobacteriosis were determined using a Spearman rank correlation test.

RESULTS

A total of 1,056 stool specimens were included in the study. Of them, 913 (86.5%) were negative for *Campylobacter* spp., *Salmonella* spp., and *Shigella* spp./EIEC according to the EBP assay, and 143 (13.5%) were positive for *Campylobacter* spp., *Salmonella* spp., and *Shigella* spp./EIEC, including three coinfections, *Campylobacter* spp. along with *Salmonella* spp. ($n = 1$) or *Shigella* spp./EIEC ($n = 2$). In detail, molecular-based screening yielded 109 (10.4%) specimens positive for *Campylobacter* spp., 17 (1.6%) specimens positive for *Salmonella* spp., and 20 (1.9%) specimens positive for *Shigella* spp./EIEC.

In comparison, the overall positive stool culture rate for *C. jejuni*/*C. coli*, *Salmonella* spp., and *Shigella* spp. was 8.9%, i.e., 7.1% ($n = 75$) *C. jejuni*/*C. coli*, 1.3% ($n = 14$) *Salmonella* spp., and 0.7% ($n = 7$) *Shigella* spp. None of the culture-positive cases were missed by the EBP assay, and the assay's negative predictive value for *Campylobacter* spp., *Salmonella* spp., and *Shigella* spp. was as high as 100% (95% confidence interval [CI], 99 to 100%).

Privileged access to the C_T values generated by the BD Max system was obtained from the manufacturer for the duration of the study. The C_T values were strongly correlated with the CFU counts for the type strains of *C. jejuni* ($R^2 = 0.9809$), *S. enterica* subsp. *enterica* serovar Enteritidis ($R^2 = 0.9654$), and *S. flexneri* ($R^2 = 0.9205$) (Fig. 1).

To assess the effect of the relative bacterial load per specimen on the culture results, we compared the median C_T values of concordant (group 3) and discordant (group 2) specimens (Fig. 2). The median C_T values of the concordant specimens were significantly lower than those of the discordant ones (C_T values, 24.3 versus 28.7; $P < 0.001$). The pathogen-specific C_T values of the

FIG 2 Comparison of all C_T values of concordant (culture positive/PCR positive; group 3) and discordant (culture negative/PCR positive; group 2) specimens for *C. jejuni/C. coli*, *Salmonella* spp., and *Shigella* spp (A), *C. jejuni/C. coli* only (B), *Salmonella* spp. only (C), and *Shigella* spp. only (D). The median C_T values of the concordant specimens were lower than those of the discordant specimens; this difference was statistically significant (*C. jejuni/C. coli*, $P < 0.001$; *Salmonella* spp., $P = 0.02$; and *Shigella* spp., $P = 0.02$).

concordant and discordant specimens, respectively, were 23.7 and 27.8 ($P < 0.001$) for *C. jejuni/C. coli*, 26.9 and 37.7 ($P = 0.02$) for *Salmonella* spp., and 19.7 and 27.3 ($P = 0.02$) for *Shigella* spp.

To verify the discordant-positive *Campylobacter* PCR results, we determined the concentrations of fecal calprotectin, a surrogate marker of intestinal inflammation, in the concordant-negative (group 1), discordant-positive (group 2), and concordant-positive (group 3) stool specimens (Fig. 3). The median calprotectin concentrations in the concordant-negative, discordant-positive, and concordant-positive specimens were 134 mg/kg (interquartile range [IQR], 30 to 1,374), 1,913 mg/kg (IQR, 165 to 3,813), and 5,327 mg/kg (IQR, 1,836 to 18,213), respectively. Significant differences were observed among the three groups ($P < 0.001$), and a significant linear trend was detected ($P < 0.001$) (Fig. 3A). Furthermore, the calprotectin concentrations and C_T values were found to be correlated ($r = -0.658$) (Fig. 3B).

DISCUSSION

This study evaluated the feasibility and performance of a molecular-based screening method for detecting *C. jejuni/C. coli*, *Salmonella* spp., and *Shigella* spp. compared with conventional stool

culturing in a diagnostic laboratory. Furthermore, the association between the relative bacterial load and the respective culture results was examined, as well as the association between the bacterial load and the extent of intestinal inflammation in *Campylobacter* infections. The BD Max EBP assay was run on a fully automated BD Max system with minimal hands-on time and a short turn-around time.

In a previous study, *C. jejuni/C. coli* were the most frequently detected pathogens, similar to the results of our study (4). We observed an overall prevalence of 13.5% for *C. jejuni/C. coli*, *Salmonella* spp., and *Shigella* spp./EIEC by molecular testing. Earlier studies based on conventional stool cultures reported a prevalence of approximately 6% for *C. jejuni/C. coli*, *Salmonella* spp., and *Shigella* spp. (8, 9). In our investigation, the prevalence of *Campylobacter* spp. alone was 10.4%, that of *Salmonella* spp. was 1.6%, and that of *Shigella* spp./EIEC was 1.9%, as determined by PCR. Buchan et al. also identified an increased prevalence of gastroenteritis pathogens using a competitor molecular-based assay with the same targets as the EBP (9). Of note, in our study, we detected a prevalence of 1.9% *Shigella* spp./EIEC by PCR but only a prevalence of only 0.7% by culturing. The PCR target gene *ipaH* of

FIG 3 (A) Calprotectin concentrations were compared in stool specimens that were concordant negative (culture negative/PCR negative; group 1), discordant positive (culture negative/PCR positive; group 2), and concordant positive (culture positive/PCR positive; group 3) for *Campylobacter jejuni*/*C. coli*. The respective median calprotectin concentrations were 134 mg/kg (group 1), 1,913 mg/kg (group 2), and 5,327 mg/kg (group 3); the concentration was significantly higher in group 3 than in groups 1 and 2 ($P < 0.05$), revealing a significant trend across the three groups ($P < 0.001$). (B) Calprotectin concentrations and C_T values were correlated, with a Spearman r of -0.658 .

Shigella is shared by EIEC. Therefore, it is unclear whether the discrepant culture results were due to the higher than expected prevalence of EIEC or to the rather poor sensitivity for *Shigella* cultures, as has been reported by several authors (7, 9, 12–14). Difficulties with culturing *Shigella* were also illustrated in a case in our study. *S. flexneri* was cultured from one out of three stool specimens submitted, despite indications of relatively high bacterial loads in all three specimens (C_T values of 19.9, 18.6, and 22.0, respectively).

Inappropriate specimen storage and transportation and previous antibiotic treatment are among the known factors that compromise culture results by affecting the viability of bacterial pathogens. Jansen et al. (1) have demonstrated that culture-based methods miss a substantial proportion of *Campylobacter* infections. Although current guidelines restrict antibiotic therapy to patients at risk of severe disease, in Switzerland, more than 60% of laboratory-confirmed *Campylobacter* cases are treated with antibiotics (15).

In addition, we detected an association between the relative bacterial loads and the culture results, further corroborating the conclusion that PCR is more sensitive than culturing. This effect was the least marked for *Salmonella*, likely due to the standard broth enrichment step used to increase *Salmonella* culture sensitivity. In contrast, *Shigella* was rarely cultured at C_T values exceeding 24.3. *Campylobacter* was missed by culturing at both at high and low C_T values. This finding is suggestive of issues of reduced pathogen viability, which would directly affect culture results but would not affect results obtained using molecular-based methods (12).

As a pathogen, *Campylobacter* causes mucosal damage and inflammation of the colon. To quantify the inflammatory responses in the colons of patients with suspected *Campylobacter*-associated diarrhea, we measured the fecal calprotectin concentration (16, 17). Fecal calprotectin (18, 19), a protein dimer of the neutrophil cytosol, is a surrogate marker for distinguishing organic inflammatory diseases of the gastrointestinal tract, such as Crohn's disease and ulcerative colitis, from functional diseases, such as irritable bowel syndrome. A calprotectin level of >100 $\mu\text{g/g}$ stool is

indicative of active organic disease, whereas a level of <50 $\mu\text{g/g}$ stool is not indicative of inflammation in the gastrointestinal tract. Levels of between 50 and 100 $\mu\text{g/g}$ stool may indicate the presence of a variety of mild organic diseases, such as inflammation caused by nonsteroidal anti-inflammatory drugs (NSAIDs) and mild diverticulitis.

We found that the *Campylobacter* bacterial load, as assessed by the C_T value, and the respective intestinal inflammatory response, as determined by the fecal calprotectin concentration, were correlated (Fig. 3). Patients without *Campylobacter* infection (group 1) possessed a broad range of fecal calprotectin concentrations, suggesting that pathogens other than *Campylobacter* or noninfectious inflammatory bowel diseases, such as Crohn's disease or ulcerative colitis, might be responsible for the elevated calprotectin levels.

In the patients with *Campylobacter* infections (groups 2 and 3), we found a positive correlation between the bacterial load and the extent of the intestinal inflammatory response, as confirmed using the Jonckheere-Terpstra proportion test, which revealed significant trends among all groups (Fig. 3). However, in the absence of complementary clinical and epidemiological data that may assist in assessing confounding factors, cautious interpretation of the laboratory data is advisable.

In conclusion, our study demonstrated that the molecular-based screening of *Campylobacter* spp., *Salmonella* spp., and *Shigella* spp. using the BD Max EBP assay resulted in timely and sensitive detection of these major bacterial enteric pathogens. This assay is easy to perform; therefore, it is not restricted to molecular laboratories. Still, conventional stool culturing of PCR-positive specimens will remain crucial for antibiotic susceptibility testing and epidemiological purposes, such as serotype determination and subtyping in outbreaks (4).

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We declare that we have no conflicts of interest.

N.W., T.B., L.R., and M.R. conceived and designed the approach of the study. N.W. and S.T. were analysts. L.R. and N.W. were study statisticians. N.W., T.B., and L.R. wrote the paper.

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