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Comparative Evaluation of Conventional and Real-Time PCR Assays for Detecting *Bacteroides fragilis* in Clinical Samples

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A conventional PCR and a real-time PCR for detecting *Bacteroides fragilis* were evaluated against clinical specimens. Analytical sensitivities were 100 and 40 fg of DNA, respectively. Detection limits were 100 and 10 CFU/ml, respectively. A total of six culture-negative specimens were positive by PCR. Altering the gold standard from "positive culture" to "positive culture or both PCR assays positive" resulted in sensitivities of 91.7% and 100%, respectively, and in specificities of 100% and 98.6%, respectively.

Bacteroides fragilis is considered a common anaerobic pathogen (1–3), although its detection is limited by the lack of routine anaerobic culture performed in clinical laboratories (3). In that respect, molecular assays, given their fast turnaround times and high sensitivities, could facilitate a better diagnostic procedure. Studies on PCR-based detection of *B. fragilis* have focused on enterotoxigenic strains in fecal specimens (4–7) or as an indicator of water contamination (8–10). In contrast, detection of *B. fragilis* in general clinical practice, using either conventional PCR (C-PCR) (11, 12) or real-time PCR (RT-PCR) (13), is limited. In the present study, two newly designed PCR protocols were developed and evaluated using artificially spiked samples and clinical specimens.

The Gene Bank sequences CR626927.1, AP006841.1, and NC016776 were used as templates for primer/fluorogenic hydrolysis probe design (Table 1), targeting the single-copy beta-isopropylmalate dehydrogenase (*leuB*) gene (14).

DNA from a 48-h anaerobic culture of the *B. fragilis* ATCC 25285 isolate, extracted with the QIAamp DNA minikit (Qiagen GmbH, Hilden, Germany), was used for PCR optimization. C-PCR was performed in 50-μl reaction mixtures, using the GoTaq Hot Start Green Master Mix (Promega Corp., Madison, WI), 400 nM each of the Leu-1/2 primers, 5 μl of extracted DNA, and an annealing temperature of 58°C in a MyCycler thermal cycler (Bio-Rad Laboratories, Athens, Greece), with subsequent agarose gel separation. RT-PCR was performed in 20-μl reaction mixtures, using the KAPA Probe Fast qPCR Master Mix (Kapa Biosystems Inc., Woburn, MA), 400 nM each of the Leu-3/4 primers, 200 nM Leu-Prb, and 2.5 μl of extracted DNA. Reaction conditions consisted of 35 cycles of 15 s at 95°C and 60 s at 60°C in a StepOne Plus real-time PCR system (Applied Biosystems, Foster City, CA). All samples were tested neat and diluted 10^{-1} and 10^{-2} for inhibition detection.

The analytical sensitivities of the two PCR assays, as well as the standard curves for RT-PCR, were calculated using serial 2-fold dilutions of quantified DNA extracted from the *B. fragilis* ATCC 25285 strain. The specificities of the two PCR assays were evaluated using DNAs from various aerobic and anaerobic bacteria (see Table S1 in the supplemental material).

Whole EDTA-blood was collected from healthy volunteers and used to prepare serial 10-fold dilutions from a 0.5 McFarland turbidity suspension of the *B. fragilis* ATCC 25285 isolate. From each dilution, 200 μl was used for quantitative anaerobic culture on brucella agar plates supplemented with 5% (vol/vol) horse blood, vitamin K_1 (1 mg/liter), hemin (5 mg/liter), thioglycolate broth (produced in-house), and BBL *Bacteroides* bile esculin agar plates (Becton, Dickinson and Co., Sparks, MD) and incubated for 48 h under anaerobic conditions, and 200 μl was used for DNA extraction with the QIAamp DNA blood minikit (Qiagen). All reactions were performed in triplicate to access reproducibility.

A total of 86 intra-abdominal and pelvic clinical specimens, submitted for routine microbiological diagnosis, were collected from an equal number of patients. Inclusion criteria were sampling during surgery and transportation under anaerobic conditions (Port-A-Cul transport system; Becton, Dickinson), whereas exclusion criteria were sampling from drainages or open wounds or the use of inadequate anaerobic conditions during transportation. All samples were processed using standard methodology (3) and used for Gram staining and anaerobic culture as described previously and for DNA extraction with the QIAamp DNA minikit (Qiagen).

Five different approaches were considered to define the gold standard for *B. fragilis* infection and were used to calculate the sensitivities, specificities, positive predictive values (PPVs), and negative predictive values (NPVs) of the two PCR assays. These approaches were as follows: (i) a positive culture, (ii) both PCR assays positive, (iii) a positive culture or both PCR assays positive, (iv) a positive culture or any one PCR assay positive, and (v) a positive culture and any one PCR assay positive.

A detection signal of the positive control was obtained using both assays, whereas no amplification product was detected using extracted DNAs from all other isolates. The reproducible analytical sensitivities of C- and RT-PCR were 100 and 40 fg/ml DNA, respectively, whereas a positive detection signal (clinical detection limit) was obtained from approximately 100 and 10 CFU/ml, re-

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TABLE 1 Primers and probe for C-PCR and RT-PCR assays

PCR type	Primer or probe	Sequence	Amplicon size (bp)	Amplicon position[a]
C-PCR	Leu-1	5′-CGGATGCCATTGATAAAGTAGG-3′	448	481–929
	Leu-2	5′-CTGGAAGCAAGCACATTAGC-3′		
RT-PCR	Leu-3	5′-CACTTGACTGTTGTAGATAAAGC-3′	135	388–522
	Leu-4	5′-CATCTTCATTGCAGCATTATCC-3′		
	Leu-Prb[b]	5′-TGTGCTTGCTTCCAGTCGTCTATG-3′		

[a] Corresponding to the *leuB* gene of the *B. fragilis* NCTC9343 strain.
[b] 5′ 6-carboxyfluorescein (FAM) reporter and 3′ Black Hole quencher (BHQ).

spectively. A previously described assay (13), which was the only one available in the literature using RT-PCR, thus allowing for comparison, reported a lower detection limit of 1 CFU/ml. This difference may be explained by the fact that the multicopy 16S rRNA operon (5 to 6 copies in the *B. fragilis* bacterial genome) was used (15), compared to the single-copy *leuB* gene. Nevertheless, one should also be aware of the limitation of the 16S rRNA-based protocols in potentially generating cross amplifications with other, not-yet-characterized species, given the high similarity of the target gene (9). In addition, our study showed that although the analytical sensitivity of RT-PCR was approximately half of that of C-PCR, their differences regarding clinical detection limit were broader, indicating differences in clinical performance. It should be noted that a similar simultaneous comparison between C- and RT-PCR protocols has not been performed previously, and the only comparable data available are from two C-PCR studies (12, 13) with detection limits of 3,900 and 50 CFU/ml, respectively. Based on these data, it is safe to assume that the fluorogenic probe-based RT-PCR assays have better clinical performance than C-PCR ones, especially in samples with low target bacterial counts among heavy normal flora, although the latter can still be used as an alternative in laboratories lacking RT-PCR facilities.

Among the 86 clinical specimens, seven were culture positive for *B. fragilis*; six of these were also positive using both PCR assays, whereas the remaining single specimen was positive using only RT-PCR. Among the 79 culture-negative specimens, five were PCR positive with both assays, and a single one was additionally positive using RT-PCR. Two culture-positive specimens presented inhibition with both protocols; their positivity was confirmed only in the diluted reaction mixtures. The sensitivities, specificities, PPVs, and NPVs obtained using the five different approaches for the gold standard definition are shown in Table 2. In particular, using culture as the gold standard, both PCR assays resulted in low PPVs, which were attributed to the samples that were detected only by PCR and considered false positive. However, changing the gold standard definition for infection from "culture positive" to "culture positive or both PCR assays positive," the specificities and especially the PPVs increased considerably.

Positive detection by PCR of culture-negative specimens in intra-abdominal infections has been described previously (13) and has been attributed to the greater analytical sensitivity of the PCR. It should be noted that the targets of the two assays in the present study overlapped only marginally (Table 1), thus rendering cross contamination impossible. In that respect, it is safe to assume that these specimens that were culture negative but positive by both PCR assays were in fact positive and that conventional culture was not able to discriminate them.

One should always be aware, however, of the possibility that PCR may detect (i) nonviable bacteria, after antimicrobial chemotherapy initiation, or (ii) normal flora bacteria that do not participate in the specific infection, a common situation with culture also (given the polymicrobial nature of intra-abdominal infections within an environment heavy in normal flora). Nevertheless, as *B. fragilis* is a species with multiple pathogenic mechanisms, its detection in intra-abdominal specimens is usually considered clinically important.

Taking all these factors into account, our proposal is that of the five different approaches for gold standard definition, the third one (positive culture or both PCR assays positive) seems to be the one best reflecting true clinical conditions. Using this approach for comparison (Table 2), both assays proved to be almost equally specific and fast (with a maximum turnaround time of 6 h), while RT-PCR proved to be more sensitive. In contrast, using the tradi-

TABLE 2 Sensitivities, specificities, and positive and negative predictive values of the two PCR assays, using different approaches for the gold standard definition of *B. fragilis* infection

Gold standard definition	No. (%) of positive patients[a]	PCR assay	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Positive culture	7 (8.1)	RT-PCR	100.0	92.4	53.8	100.0
		C-PCR	87.5	93.7	54.5	98.7
Both PCR assays positive	11 (12.8)	RT-PCR	100.0	97.3	84.6	100.0
		C-PCR	100.0	100.0	100.0	100.0
Positive culture or both PCR assays positive	12 (14.0)	RT-PCR	100.0	98.6	92.3	100.0
		C-PCR	91.7	100.0	100.0	98.7
Positive culture or any one PCR assay positive	13 (15.1)	RT-PCR	100.0	100.0	100.0	100.0
		C-PCR	84.6	100.0	100.0	97.3
Positive culture and any one PCR assay positive	7 (8.1)	RT-PCR	100.0	92.4	53.8	100.0
		C-PCR	87.5	93.7	54.5	98.7

[a] Patients who were considered positive for an infection due to *B. fragilis*, using the different gold standard definitions.

tional culture-based gold standard, both assays exhibited low PPVs, an assumption that may not reflect the reality in clinical practice. The problem, however, of defining a new gold standard best reflecting the overlapping of conventional and molecular diagnosis of anaerobic infections needs more studies in order to be elucidated.

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