

## Quantification of *Clostridium difficile* in Antibiotic-Associated-Diarrhea Patients<sup>▽</sup>

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**Comparing culture- and non-culture-based methods for quantifying *Clostridium difficile* in antibiotic-associated-diarrhea patients, we found that the real-time PCR method correlated well with quantitative culture and was more sensitive. A positive association between the population levels of *C. difficile* and the presence of its toxins was found.**

*Clostridium difficile* is a common nosocomial pathogen that primarily colonizes or infects hospitalized patients after treatment with antibiotics. Approximately 16 to 35% of hospital patients are colonized with *C. difficile* (2). Most of these patients remain asymptomatic carriers, while some develop infections that vary from mild, watery diarrhea to fatal pseudomembranous colitis (9). The causes of this high variation in clinical presentation are not fully understood. Although *C. difficile* toxin levels in feces may correlate with the severity of disease, more data are needed to support this relationship (1).

The diagnosis of *C. difficile* infection is usually based on the detection of the microbe in feces by culture and the detection of its toxin(s) by enzyme immunoassays. Recently introduced molecular methods, such as real-time PCR, have greater sensitivity than rapid toxin tests and thus are able to detect significantly more positive cases. However, these methods could also increase the detection of colonization in healthy people, up to 55.5% of which are colonized (3, 7).

The detection of *C. difficile* and its toxins in routine diagnostics and major clinical studies remains qualitative (4). Population levels of *C. difficile* have been determined in only few studies on the development of the intestinal microbiota in infants (11, 12). The comparisons between different methods (classical culture-based and molecular culture-independent methods) have been qualitative (positive or negative) without quantifying the microorganism or its products. However, a quantitative approach to measuring *C. difficile* infection may have some advantages in investigating relationships within the microbial microecosystem or in distinguishing between low-level colonization and clinical infection.

The aims of our study were to establish *C. difficile* population levels in feces of antibiotic-associated-diarrhea (AAD) patients by comparing the culture and real-time PCR methods

and to compare the *C. difficile* populations in toxin-positive and -negative AAD cases.

**Experimental procedures.** Consecutive fecal samples were sent for routine *C. difficile* diagnostics in Estonia (four hospitals) and Norway (Stavanger University Hospital) during 2008. All samples were initially tested for *C. difficile* toxin A/B (Oxoid, United Kingdom), cultured on selective agar (cycloserine cefoxitin fructose agar [CCFA], Oxoid, United Kingdom) in local laboratories, and stored at  $-80^{\circ}\text{C}$ . In total, 74 samples randomly selected from *C. difficile* screening-positive and -negative AAD patients were included in the study. AAD was defined as clinically diagnosed diarrhea in patients currently or recently (within 1 week) treated with antibiotics. Children less than 2 years of age were excluded. The ages of 44 female and 30 male AAD patients ranged from 3 to 89 years (median, 72 years).

For quantitative culture of *C. difficile*, serial dilutions of feces were seeded on Brazier's CCEY agar supplemented with cefoxitin and cycloserine (LabM, United Kingdom) and incubated in an anaerobic cabinet (Concept, United Kingdom). CFU per gram (CFU  $\log_{10}/\text{g}$ ) were calculated. The detection limit of quantitative culture was 2 CFU  $\log_{10}/\text{g}$ .

Bacterial DNA from fecal samples was extracted by using a QIAamp DNA stool minikit (QIAGEN, Hilden, Germany). Real-time PCR targeting the small-subunit rRNA genes was used to quantify *C. difficile* in fecal samples (13). Real-time PCR was performed with the ABI Prism 7500 HT sequence detection system (Applied Biosystems) by using primers (forward, 5'-TTGAGCGATTTACTTCGGTAAAGA-3'; reverse, 5'-CCATCCTGTACTGGCTCACCT-3') and conditions described previously (13). Standard curves were constructed using plasmids containing 16S rRNA gene fragments amplified with corresponding primers, and the cell equivalents were calculated (8, 13, 15).

**Results and conclusions.** Forty-two *C. difficile*-positive cases were found by culture, with counts ranging from 5.0 to 7.9 CFU  $\log_{10}/\text{g}$  (median, 6.58 CFU  $\log_{10}/\text{g}$ ). PCR detected 59 positive cases, with counts ranging from 5.57 to 11.2 cell equivalents  $\log_{10}/\text{g}$  (median, 7.88 cell equivalents  $\log_{10}/\text{g}$ ). Thus, PCR detected *C. difficile* in an additional 20 culture-negative cases

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TABLE 1. Identification of *C. difficile* in samples from AAD patients by culture and PCR

Culture result	No. of cases with PCR result	
	Positive	Negative
Positive	39	3
Negative	20	12

(Table 1), and three confirmed culture-positive cases appeared to be negative by PCR.

This report is the first published study that quantitatively evaluated the *C. difficile* population in AAD patients and compared the results of culture and molecular methods. Although quantitative real-time PCR of *C. difficile* has been previously used to detect microbial contamination in hospitals and to evaluate its population level in the intestinal tracts of healthy infants, this particular method for the detection of *C. difficile* counts in cases of AAD has not been described yet (10, 11, 12).

In total, 62 presumably positive *C. difficile* cases (culture and/or PCR positive) were detected (Table 1). The *C. difficile* counts found by PCR were significantly higher than those by culture in these cases (median values, 6.59 versus 5.54 log<sub>10</sub>/g;  $P < 0.001$  [Wilcoxon signed-rank test]). A comparison of counts of *C. difficile* by the culture and PCR methods is shown in Fig. 1. However, there was a significant positive correlation between the counts found by these methods ( $r = 0.59$ ,  $P < 0.001$  [Spearman's rank correlation test]), and the counts

TABLE 2. Detection of toxin in cases positive for *C. difficile* by culture, PCR, or culture and/or PCR

Method(s)	<i>C. difficile</i> detection	No. of cases	
		Toxin positive	Toxin negative
Culture	Positive	26	16
	Negative	1	31
PCR	Positive	26	33
	Negative	1	14
PCR and/or culture	Positive	27	35
	Negative	0	12

found by PCR in culture-negative samples were lower than those in culture-positive samples (6.26 versus 8.77 cell equivalents log<sub>10</sub>/g;  $P < 0.001$  [Mann-Whitney rank sum test]). Neither the presence nor levels of *C. difficile* were related to patient age.

Although previous studies have reported high analytical sensitivity of PCR, quantitative real-time PCR has not been compared with quantitative culture (4). In our study, the quantitative PCR results correlated well with the quantitative culture results. Moreover, PCR detected *C. difficile* in several culture-negative cases but usually with lower counts than in culture-positive cases. The higher sensitivity and significantly higher counts found by PCR were expected due to the detection of noncultivable microbial cells by PCR. In our study, all patients were treated with antibiotics, which can significantly alter the viability of *C. difficile* cells and reduce culture counts. It is therefore not possible to estimate the amount of false-positive cases by PCR in comparison with culture methods. Hence, we cannot describe the specificity of this method.

In total, 27 samples were positive for *C. difficile* toxins. All toxin-positive cases were also culture and/or PCR positive (Table 2). Among the presumed *C. difficile*-positive cases (culture and/or PCR positive), the *C. difficile* counts found in toxin-positive samples were higher than those in toxin-negative samples for both the culture (median values, 7.0 versus <2 CFU log<sub>10</sub>/g;  $P < 0.001$  [Mann-Whitney rank sum test]) and the PCR (median values, 9.28 versus 6.32 cell equivalents log<sub>10</sub>/g;  $P < 0.001$  [Mann-Whitney rank sum test]) methods.

We found that toxin-positive samples contained significantly higher counts of *C. difficile* cells than *C. difficile*-positive but toxin-negative samples. Although this result is reasonable, no other studies have correlated *C. difficile* population levels with the presence of toxin in AAD patients.

To understand the pathogenesis of *C. difficile* infection and to evaluate the usefulness of diagnostic tests, it is important to understand the relationship between *C. difficile* population levels and the clinical severity of the disease. *C. difficile* can asymptotically colonize large populations of hospitalized patients (and probably the healthy outpatient population). At the same time, the majority of AAD cases are not caused by *C. difficile* (5). Thus, colonization with *C. difficile* and diarrhea caused by other mechanisms or pathogens may frequently coincide. Additional tests that have been proposed to differentiate *C. difficile* colonization and infection, e.g., the fecal lactoferrin assay, lack sensitivity and specificity (6, 14, 16, 17). The

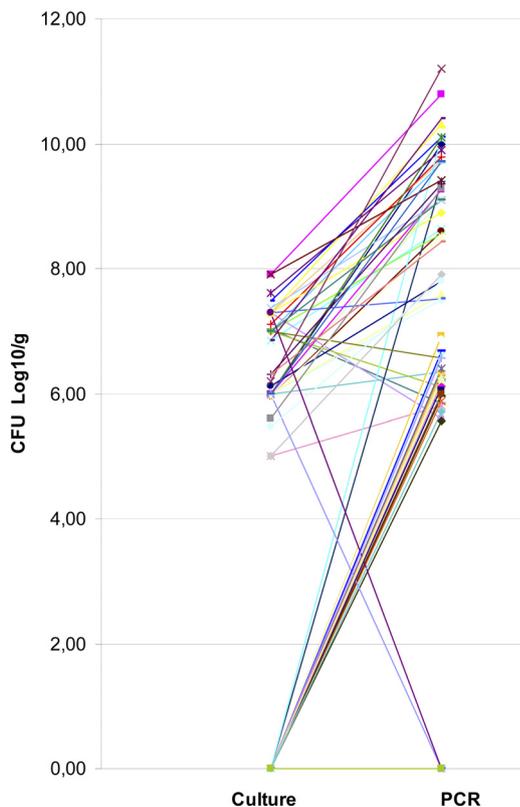


FIG. 1. Counts of *C. difficile* in AAD patients by the culture and PCR methods.

introduction of increasingly sensitive methods for the detection of *C. difficile* in clinical practice heightens the need to differentiate infection and colonization and may challenge the usefulness of the quantification of *C. difficile*. Further studies are needed to correlate the quantitative *C. difficile* counts with toxin levels and the clinical severity of the disease.

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