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Rapid Polymerase Chain Reaction Method for Specific Detection of Toxigenic *Clostridium difficile*

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A rapid polymerase chain reaction (PCR) method for detection of toxigenic *Clostridium difficile* directly from fecal samples by amplification of toxin A gene fragments was investigated. The technique was applied to monitor the spread of the microorganism in a long-term care ward with a relatively high incidence of overt episodes of diarrhea. The PCR approach has several advantages over traditional methods, rapidly allowing the specific detection of toxigenic *Clostridium difficile* strains from stool samples in both symptomatic and asymptomatic subjects with toxigenic strains. This PCR method allows early detection of toxigenic *Clostridium difficile* and could thus represent a powerful tool for the surveillance of epidemics.

Toxigenic *Clostridium difficile* is the major causative agent of pseudomembranous colitis and antibiotic-associated diarrhea (1, 2). Diagnosis is based upon isolation of the microorganism on selective media and detection of toxin(s), traditionally by demonstrating the cytotoxic effect on sensitive cell cultures (2, 3). More recently, new methods have been introduced to detect toxin A (4, 5) or B (6), but these methods sometimes provide conflicting results. The existence of nontoxigenic strains should be taken into account when the clinical relevance of *Clostridium difficile* isolation has to be established (7, 8).

Several studies have documented the nosocomial acquisition of *Clostridium difficile*. Some hospital wards have a high rate of colonization by this microorganism. Besides hospitalization, additional recognized risk factors are old age and a prolonged hospital stay (9). Thus, when an outbreak occurs, it is essential to monitor patients sharing the room or the ward with the index case, i.e. the patient in whom diarrhea first appears. For epidemiological reasons and to apply proper preven-

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tive measures, it is important to assess whether colonization by *Clostridium difficile* is due to a true nontoxigenic strain or to a potential pathogenic one, whose ability to produce enterotoxin is not expressed at the observation time. To distinguish between the two possibilities and in order to prevent epidemic spread of *Clostridium difficile* in the ward environment, a rapid and sensitive method to monitor asymptomatic patients at risk of developing overt disease is needed.

PCR has been investigated and proposed for detecting *Clostridium difficile* strains carrying toxin A and toxin B genes, either among isolated strains or in fecal samples. However, the original PCR method was unable to differentiate between toxigenic and nontoxigenic *Clostridium difficile* strains (10) because the genome region selected for amplification (16S rRNA) was inappropriate. Other methods shown to detect toxigenic strains involved laborious and time-consuming extraction of DNA from feces (11).

In this study, a rapid PCR technique was used to monitor the spread of toxigenic *Clostridium difficile* in a long-term care ward at our general hospital, in the period September 1992 to February 1993. The patients' mean hospital stay was longer than six months, and the ward was characterized by a relatively high incidence of overt episodes of diarrhea (12).

Materials and Methods. Feces (100 mg) were suspended in 2 ml of ultrapure water (MilliQ; Millipore, USA) and heated at 100°C for 10 min. After a short centrifugation (15,800 x *g*, 20°C, 5 min), the supernatant was treated with proteinase K (0.5 mg/ml; Sigma Chemicals, USA) and pronase E (0.5 mg/ml; Sigma) at 56°C for 90 min; samples were heated at 100°C for 5 min, centrifuged, and the supernatant was used as a template in the PCR reaction mixture. In the DNA extraction protocol, there is no need for further purification of the stool samples.

Amplifications were performed in a 100 µl final volume, containing 1 µl of template, 10 µl of 10-X enzyme dilution buffer (Perkin-Elmer Cetus, USA), 200 µM of each of dNTP, 2.5 mM of $MgCl_2$, 2.5 units of *Taq* polymerase (Perkin-Elmer Cetus) and 150 pM of each primer. Two sets of primers (NK1 + NK2 and NK3 + NK2) were used to perform a hemi-nested PCR (13). The sequence of the oligonucleotides was based on that described by Kato et al. (11) and was already proven to be specific and reliable for detection of *Clostridium difficile* toxin A gene. The target was a nonrepetitive region of that gene, ranging from 1960 to 2505 bp (NK1 + NK2; expected fragment size: 545 bp) and from 2254 to 2505 bp (NK3 + NK2; expected fragment size: 251 bp). Amplifications were carried out in a thermal cycler (Perkin-Elmer Cetus), which allows a PCR run to be completed in approximately 2 h and 30 min. The cycles were programmed as follows: 94°C for 5 min (initial denaturation step), 95°C for 15 sec, 50°C for 20 sec and 72°C for 40 sec (repeated 35 times). DNA from a toxigenic (VPI 10463) and a nontoxigenic (VPI 11186) *Clostridium difficile* strain were always included together with samples as controls.

Amplification products were fractionated on 2 % agarose gel containing ethidium bromide (50 µg/ml) and examined under UV light (transilluminator; Bio-Rad, USA) (Figure 1). The procedure could be completed within 6-8 h from the time of arrival of a sample at the laboratory. Results obtained by PCR were compared with those performed on the same specimens and obtained by the following traditional tests: isolation of *Clostridium difficile* on taurocholate cefoxitin cycloserine fructose agar (TCCFA) medium (14) and detection of toxin B by cytotoxic effect on McCoy cell culture (15).

To determine the sensitivity of the PCR technique, *Clostridium difficile* suspensions (VPI 10463 strain) containing 10 to 10^4 colony-forming units (cfu) were inoculated into 1 g of feces from a healthy volunteer and from a subject with diarrhea of viral origin. Samples were treated as described above, and the expected PCR products were detected starting from as few as 100 cfu. Both extraction and PCR runs were performed in triplicate.

Results and Discussion. Of the 89 patients examined, in 56 fecal samples were negative (63 %) and in 33 positive by traditional methods (37 %) (Table 1). Among the positive cohort, 23 subjects were symptomatic and 10 asymptomatic; this latter group had either a positive culture without detectable toxin B in the stool samples (8 patients) or lack of growth on TCCFA medium and a positive cytotoxic assays (2 patients).

As previously demonstrated (8), the PCR approach showed higher sensitivity in detecting colonized but asymptomatic subjects. In overt diarrhea, this difference in sensitivity is not relevant from a clinical standpoint (Table 1). In asymptomatic subjects, on the other hand, PCR helped in the assessment of the clinical situation. In fact, the technique allowed us to detect culture-negative, toxin B positive cases and to confirm the coloni-

Table 1: Detection of *Clostridium difficile* infection in fecal samples by traditional methods and a PCR method.

	TCCFA[a]		Toxin B assay[b]		PCRc	
	Positive	Negative	Positive	Negative	Positive	Negative
Symptomatic carriers (n = 23)	23	0	23	0	23	0
Asymptomatic carriers (n = 10)	8	2	2	8	10	0
Noncarriers (n = 56)	0	56	0	56	0	56

[a] Isolation of *Clostridium difficile* colonies on selective medium (TCCFA).
[b] Cytotoxic assay on McCoy cell culture.
[c] PCR results for amplification of *Clostridium difficile* toxin A gene target region.

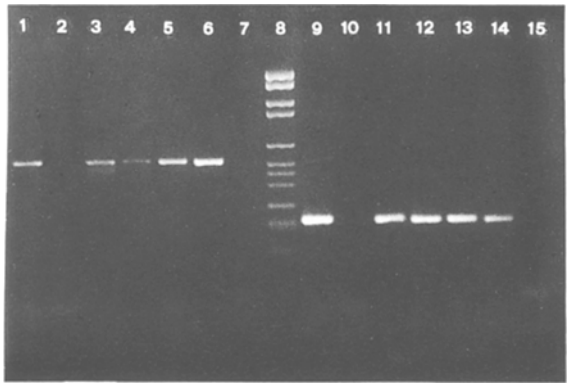

Figure 1: Amplification products for *Clostridium difficile* toxin A gene directly from heated fecal samples. Lanes 1–7: products of the first amplification with primers NK1-NK2. Lanes 9–15: results for the second amplification cycle with primer pair NK3-NK2. Lanes 1 and 9: positive control DNA from *Clostridium difficile* strain VPI 10463. Lanes 2 and 10: negative control DNA from *Clostridium difficile* strain VPI 11186. Lanes 3–6: positive samples in the first amplification. Lanes 11–14: the same samples in the second amplification cycle. Lane 8: pBR 328 molecular weight marker DNA (*Bgl* I and *Hinf* I digest) (Boehringer Mannheim, Germany). Lanes 7 and 15: blank control lanes.

zation of two subjects by a toxigenic *Clostridium difficile* strain. Moreover, discrimination between toxigenic and nontoxigenic *Clostridium difficile* infections was obtained only by using the PCR assay in eight culture-positive, cytotoxin-negative subjects in whom traditional methods were clearly inadequate.

On the basis of the results obtained, it appears that our protocol can be very helpful in the rapid and early detection of toxigenic *Clostridium difficile* from stool samples. This procedure allowed us to obtain reproducible results, without DNA purification steps. Furthermore, the consistency of the fecal material did not appear to interfere with the PCR results. However, freezing and thawing of fecal samples were proven to affect reproducibility of PCR results.

In our opinion, PCR should not to be used as a substitute for culture or toxin B assay in symptomatic patients. On the other hand, this technique could represent a very powerful tool for the surveillance of epidemics and for the evaluation of pre-emptive measures. Other authors have already demonstrated that, by using a similar PCR approach with amplification of toxin A and/or B gene sequences, toxigenic strains of *Clostridium difficile* can be distinguished from nontoxigenic strains in human stool samples (16, 17). Our PCR procedure has the advantage of allowing rapid and specific detection of toxigenic *Clostridium difficile* strains directly in fecal specimens, thus demonstrating in a single step both the presence of the microorganism and its toxigenic potential.

Acknowledgements

The experiments reported here were made possible by a grant from Progetto Finalizzato F.A.T.M.A., Consiglio Nazionale delle Ricerche, Rome, n 91.00092.PF41 and from Ministero dell' Università e della Ricerca Scientifica e Tecnologica.

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Evaluation of a Commercial Polymerase Chain Reaction Assay for *Chlamydia trachomatis* and Suggestions for Improving Sensitivity

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A commercial polymerase chain reaction (PCR) assay (Amplicor, Roche) for *Chlamydia trachomatis* was compared with a direct fluorescent antibody (DFA) test using urethral and cervical samples, many of which on the basis of prior testing by DFA contained small rather than large numbers of elementary bodies. Urine samples were collected from patients in a sequential unselected manner. Of 244 clinical specimens (138 male urethral and cervical; 106 male and female urine), 66 were positive by both DFA and PCR and 141 were negative by both tests. Nine samples were DFA negative and PCR positive, and 28 samples were DFA positive and PCR negative. However, 24 (86 %) of the latter samples contained fewer than ten elementary bodies. When serial dilutions of laboratory stock strains (serovars E and H) were tested, the DFA test detected *Chlamydia trachomatis* at a dilution tenfold greater than the PCR. Furthermore, of five DFA-positive clinical samples, three that were PCR negative when tested according to the manufacturer's instructions were positive when they were diluted less. A modification of the PCR assay along these lines might improve sensitivity.

Apart from culturing for *Chlamydia trachomatis*, a variety of methods has been described and used for the detection of chlamydial antigens (1, 2). We have assessed the sensitivity of several of these procedures by testing clinical specimens (3–5) and by an approach in which stock strains and clinical specimens are diluted in the laboratory (6). Our conclusion has been that a direct fluorescent antibody (DFA) test and a polymerase chain reaction (PCR)–based assay developed in our

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