Laboratory diagnosis of toxigenic *Clostridium difficile* by polymerase chain reaction:
Presence of toxin genes and their stable expression in toxigenic isolates from Japanese individuals.

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1 Title: Laboratory diagnosis of toxigenic *Clostridium*
2 *difficile* by polymerase chain reaction:
3 occurrence of the toxin genes and their stable
4 expression among toxigenic isolates from Japan
5

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20 A short title: Detection of toxigenic *C. difficile*

1 **Abstract:** *Clostridium difficile* causes pseudomembraneous
2 colitis and antibiotic-associated diarrhea. The definitive
3 diagnosis of *C. difficile* infection is finally accomplished
4 by the isolation of toxigenic *C. difficile*. However, only a
5 small number of Japanese clinical laboratories are able to
6 determine the definitive diagnosis of *C. difficile*
7 infection, probably owing to not being available for simple
8 and reliable assays for toxins of the isolates. In this
9 study, to identify toxigenic *C. difficile*, compatibility
10 between polymerase chain reaction (PCR) assay and tissue
11 culture assay was examined among toxigenic and nontoxigenic
12 *C. difficile* isolates from Japanese patients and healthy
13 carriers. Specificity of PCR primers were demonstrated by
14 restriction endonuclease digestion and seminested PCR in *C.*
15 *difficile* VPI 10463 strain. No PCR product was amplified in
16 other eight clostridial species. The detection limit was
17 10^3 cells. Both of the toxin A and B genes were detected in
18 58 toxigenic *C. difficile* isolates, which showed a variety
19 of cytotoxic activities in tissue culture assay. Neither of
20 the toxin genes were carried in 40 nontoxigenic strains.
21 The present study strongly suggests that the definitive
22 diagnosis of *C. difficile* infection is accomplished by PCR
23 detection of the toxin genes in place of tissue culture
24 assay of the isolates.

25

26 **Key words:** *Clostridium difficile*, PCR, cytotoxicity,
27 definitive diagnosis

1 **Introduction**

2

3

4 *Clostridium difficile* causes pseudomembraneous colitis and
5 antibiotic-associated diarrhea.^{1, 2} *C. difficile* infection
6 is one of the most important causes of nosocomial
7 infections.^{2, 3}

8 Toxins A and B are the major virulence factors of *C.*
9 *difficile*.¹ Both toxins are cytotoxic but toxin B is much
10 more potent than toxin A. Toxins A and B are encoded by two
11 separate genes located in close proximity on chromosome.⁴⁻⁶
12 The C-termini of both toxin genes contain similar repetitive
13 sequences, which seem to function as binding domains to
14 target cells. Both toxins are generally produced
15 simultaneously.

16 The definitive diagnosis of *C. difficile* infection is
17 accomplished by the isolation of toxigenic *C. difficile* from
18 stool with the toxins.¹ For detecting toxins in stool as
19 well as culture supernatants of the isolates, tissue culture
20 assay and/or immunological assay is used. However, tissue
21 culture assay requires specialized equipment and personnel.
22 Hence, commercial enzyme immunoassay kits are widely used.²
23 At present in Japan, two commercial enzyme immunoassay kits,
24 VIDAS assay kit CDA (bioMérieux-Viteck Japan Ltd., Tokyo)
25 and CD check D-1 (Mitsubishi Chemical Industries, Tokyo),
26 are only available as *in vitro* diagnostics for *C. difficile*
27 infection. Although the former uses a monoclonal antibody
28 to toxin A, it needs an automatic enzyme-linked fluorescent

1 immunoassay system, mini VIDAS (bioMérieux-Viteck Japan
2 Ltd.). The latter gives false-positive reactions to the
3 organisms other than *C. difficile* because of detecting
4 glutamate dehydrogenase as an antigen.⁷ Other commercial
5 enzyme immunoassay kits commonly used abroad for specific
6 detection of the toxins are not available in Japan.
7 Therefore, only a small number of Japanese clinical
8 laboratories, in which tissue culture assay, VIDAS assay kit
9 CDA, or originally developed enzyme immunoassays are
10 employed for detection of *C. difficile* toxins, are able to
11 determine the definitive diagnosis of *C. difficile*
12 infection. This is probably one of reasons why too small an
13 attention has been paid to *C. difficile* infection and there
14 are only a few epidemiological data of *C. difficile*
15 infection in Japan.

16 Polymerase chain reaction (PCR) is widely applied in
17 research to diagnose microbial infections by detecting
18 specific genes of causative agents. Now, this method is
19 coming into use in Japanese clinical laboratories and
20 several commercial PCR kits are available as *in vitro*
21 diagnostics. Being available for rapid and simple assays
22 like PCR to detect *C. difficile* toxins will advance Japanese
23 clinical studies on *C. difficile* infection.

24 In the present study, in order to examine compatibility
25 between PCR assay and tissue culture assay, distribution of
26 *C. difficile* toxin genes and cytotoxicity was investigated
27 among toxigenic and nontoxigenic isolates from Japanese
28 patients and healthy carriers.

1 **Methods**

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4 *Bacterial strains and cultivation*

5

6 A total of 98 strains of *C. difficile* isolated in this
7 laboratory from healthy adults and patients with antibiotic-
8 associated diarrhea⁸ were used together with *C. difficile*
9 VPI 10463 as reference. To check specificity of a PCR
10 assay, *Clostridium absonum* KZ 482, *Clostridium bifermentus*
11 KZ 48, *Clostridium botulinum* type A KZ 2, *Clostridium*
12 *chauvoei* KZ 378, *Clostridium histolyticum* KZ 20, *Clostridium*
13 *perfringens* KZ 221, *Clostridium sordellii* KZ 51 and
14 *Clostridium tetani* KZ 32 were used. A 0.1-ml portion of
15 liver-broth culture was inoculated into 10 ml of brain heart
16 infusion (Becton Dickinson Microbiology Systems,
17 Cockeysville, MD, USA) supplemented with 0.2% Na₂HPO₄ (m-
18 BHI) and incubated anaerobically at 37°C for 16 hr. The
19 culture was directly used for PCR. The filtrate of the 5-
20 day-old culture was used for a tissue culture assay.

21

22

23 *Quantitative determination of bacterial cells*

24

25 The total number of *C. difficile* vegetative cells and spores
26 were determined as described previously.⁹ Briefly, a 0.1-ml
27 amount of 10-fold diluted bacterial culture was pipetted
28 into 9-cm-diameter petri dishes and mixed with 20 ml of

1 brain heart infusion (Nissui Pharmaceutical Co., Ltd.,
2 Tokyo) supplemented with 0.8% glucose, 1.0% soluble starch,
3 0.05% L-cysteine·HCl, 0.1% taurocholic acid sodium salt (GR
4 grade, Nacalai tesque, Kyoto) and 1.3% agar. The inoculated
5 agar plates were incubated anaerobically at at 37°C for 3
6 days. The colony number was counted and estimated as the
7 number of both vegetative cells and spores.

8

9

10 *Tissue culture assay*

11

12 Cytotoxicity was examined by a microtitration assay using
13 baby hamster kidney cells according to Nakamura et al.⁸
14 After two-fold serial dilution of culture filtrates, 50 μ l
15 of each diluted sample were added to the cells. The
16 reciprocal of the highest dilution resulting in 100% cell
17 rounding after 24-hr incubation was used as the number of
18 cytotoxic units (CU)/50- μ l sample.

19

20

21 *PCR*

22

23 Sequences of PCR primers for the toxin A gene are based on
24 the data of Dove et al.¹⁰ (GenBank accession No. M30307):
25 KAG1, 5'-CTCGCATATAGCATTAGACCA-3' (position 34-54); KAG2,
26 5'-CATGGGATAGATATCAGGGC-3' (position 703-722); KAG4, 5'-
27 ATTTCCCAACGGTCTAGTCC-3' (position 919-938). Sequences of
28 PCR primers for the toxin B gene are based on the data of

1 Barroso et al.¹¹ (EMBL accession No. X53138): KAG8, 5'-
2 ATATCAGAGACTGATGAGGGA-3' (position 3063-3083); KAG9, 5'-
3 AACACCTTCCATTCTCCAG-3' (position 3905-3924); KAG10, 5'-
4 AAACCTGGTGTCCATCCTGT-3' (position 4098-4117). Sizes of
5 fragments to be amplified are: KAG1-KAG4, 905 bp; KAG8-
6 KAG10, 1055 bp; KAG2-KAG4, 236 bp; KAG8-KAG9, 862 bp. *Pst*I-
7 digestion of the KAG1-KAG4 product generates 764-bp and 141-
8 bp fragments, and *Hind*III-digestion of the KAG8-KAG10
9 product, 650-bp and 405-bp fragments. Amplification was
10 accomplished in 50- μ l volumes containing 2 μ l of bacterial
11 culture, 50 pmol of each primer, 0.2 mM each dNTP, 1.5 mM
12 MgCl₂, 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 0.001%(w/v)
13 gelatin, and 2.5 units of AmpliTaq[®] DNA polymerase (Perkin
14 Elmer, Foster City, CA, USA), by Omn-E thermal cycler
15 (Hybaid Ltd., Middlesex, UK). Amplification conditions were
16 30 cycles of 94°C for 1 min, 55°C for 1.5 min and 72°C for
17 1.5 min with KAG1-KAG4 and KAG8-KAG10, and 25 cycles of 94°C
18 for 1 min, 55°C for 1 min and 72°C for 1 min with KAG2-KAG4
19 and KAG8-KAG9. PCR products were separated on agarose gel
20 and visualized with ethidium bromide under UV light.

1 **Results**

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4 Bacterial culture was directly submitted to a PCR assay.

5 When a 5- μ l amount of the *C. difficile* VPI 10463 culture

6 sample was used, false-negative PCR results were

7 occasionally obtained, thus the sample volume was determined

8 as 2 μ l. This volume contained ca. 10^5 cells (vegetative

9 cells and spores). Specificity of the primers were

10 ascertained by using *C. difficile* VPI 10463. A single band

Fig1 → 11 of the expected size was amplified with KAG1-KAG4 (Fig. 1,

12 lane 2) and KAG8-KAG10 (Fig. 1, lane 4). *Pst*I-digestion of

13 the KAG1-KAG4 product and *Hind*III-digestion of the KAG8-

14 KAG10 product generated the expected fragments (Fig. 1,

15 lanes 3 and 5), respectively. Specificity of the primers

16 was further demonstrated by seminested PCR. When the KAG1-

17 KAG4 product was used as template, the expected fragment was

18 amplified with KAG2-KAG4 (Fig. 1, lane 6). Convincing

19 result was also obtained by seminested PCR with KAG8-KAG9

20 using the KAG8-KAG10 product as template (Fig. 1, lane 7).

21 When eight clostridial species other than *C. difficile* were

22 tested, no PCR product was amplified with KAG1-KAG4 nor

23 KAG8-KAG10.

24 Sensitivity of the PCR with KAG1-KAG4 and KAG8-KAG10

25 was examined by using *C. difficile* VPI 10463. A 2- μ l amount

26 of each 10-fold dilution of bacterial culture was added to

27 the reaction mixture directly. After 30 cycles of

28 amplification, 10^3 cells (vegetative cells and spores) could

1 be detected with both primer sets of KAG1-KAG4 and KAG8-
Fig 2 → 2 KAG10 (Fig. 2).

3 Ninety-eight *C. difficile* strains isolated in Japan was
4 examined by a tissue culture assay. Fifty-eight strains
5 showed cytotoxic activities which ranged from 2^4 to 2^{14}
6 CU/50- μ l sample, and the remaining 40 strains no cytotoxic
7 activity. The presence or absence of the *C. difficile* toxin
8 genes was examined by the PCR assay with the primer sets of
9 KAG1-KAG4 and KAG8-KAG10. The toxigenic strains carried
Table 1 → 10 both of the toxin A and B genes (Table 1). Size variation
11 of the two toxin gene fragments was not observed. There
12 were no strains carrying either the toxin A or B gene. The
13 nontoxigenic strains carried neither of the toxin genes
14 without exception.

1 **Discussion**

2

3

4 In this study, the result that nontoxigenic strains lack the
5 genes for both toxins while all toxigenic strains carried
6 both the toxin genes was obtained employing Japanese *C.*
7 *difficile* isolates. Full agreement between occurrence of
8 both toxin genes and cytotoxicity was also found in studies
9 in which *C. difficile* isolates from The Netherlands¹² and
10 from Denmark¹³ were employed. As a result from these
11 findings, both the toxin A and B genes, or at least either,
12 is considered to be stably expressed as long as contained in
13 the organism. This fact strongly suggest that the
14 definitive diagnosis of *C. difficile* infection is
15 accomplished by PCR detection of the toxin genes in place of
16 tissue culture assay or enzyme immunoassay of the isolates
17 from stool.

18 A wide variety of cytotoxic activities was shown with
19 the strains tested. This may be explained by transcription
20 levels of the toxin genes¹⁴⁻¹⁶. Hammond et al.¹⁴ showed that
21 quantitative differences in toxin-specific transcripts
22 (mRNA) could be correlated with the titer of toxin protein,
23 and that this transcriptional regulation was not due to DNA
24 sequence differences in the regulatory region (ORFtxe1), by
25 analyzing several strains. As a result, they suggested that
26 a trans-acting factor may be involved in the transcriptional
27 regulation. The regulation of *C. difficile* toxin production
28 should be further elucidated.

1 Several PCR assays for detecting toxigenic *C. difficile*
2 have been described.^{12, 13, 17-19} However, most of these
3 studies involve DNA extraction steps including boiling of
4 culture, which are laborious in handling a large number of
5 strains. In addition, polymorphisms in the toxin A and B
6 genes have been reported.²⁰⁻²² Characteristic differences
7 between strains are mainly focused to the 5' third of the
8 toxin B gene and/or the 3' third of the toxin A gene.²²
9 Therefore, PCR primers located in these regions are not
10 suitable for detecting the toxin genes except for particular
11 purposes such as molecular epidemiology. Our PCR method was
12 developed to meet these requirements.

13 Japanese carrier rate of toxigenic *C. difficile* is
14 estimated to be ca. 10%.⁸ This rate seems to be higher
15 compared with that of the study by George,²³ in which a
16 carrier rate of 3% was reported. The present study
17 indicates that the PCR assay is also useful for such
18 epidemiological studies concerning *C. difficile* infection
19 because of its simplicity and reliability.

20 Here we sincerely hope that this study will prompt
21 Japanese studies concerning *C. difficile* and its infection.

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1 **Table 1.** Distribution of the toxin genes and cytotoxicity
2 among 98 *C. difficile* isolates in Japan

Cytotoxicity*	Toxin gene**		Number of strains
	A	B	
+	+	+	58
+	+	-	0
+	-	+	0
+	-	-	0
-	+	+	0
-	+	-	0
-	-	+	0
-	-	-	40

19 **+, cytotoxicity-positive; -, cytotoxicity-negative.

20 **+, gene-positive; -, gene-negative.

1 A figure legend

2

3

4 **Fig. 1.** Agarose gel electrophoresis of PCR products
5 amplified with the primers specific for the toxin A and B
6 genes, and their restricted fragments. *C. difficile* VPI
7 10463 was used for PCR. Lane 1, molecular size marker
8 (AmpliSize[®], Bio-Rad Laboratories, Hercules, CA, USA); lane
9 2, the KAG1-KAG4 product (the toxin A gene fragment); lane
10 3, the *Pst*I-digested KAG1-KAG4 product together with the
11 non-digested product; lane 4, the KAG8-KAG10 product (the
12 toxin B gene fragment); lane 5, the *Hind*III-digested KAG8-
13 KAG10 product together with the non-digested product; lane
14 6, the seminested PCR product with KAG2-KAG4 using the KAG1-
15 KAG4 product as template; lane 7, the seminested PCR product
16 with KAG8-KAG9 using the KAG8-KAG10 product as template.

17

18 **Fig. 2.** Sensitivity of PCR with the primer sets of KAG1-
19 KAG4 and KAG8-KAG10 in detecting toxigenic *C. difficile*.
20 Serial 10-fold dilutions of *C. difficile* VPI 10463 culture
21 were used for PCR samples. The primer sets of KAG1-KAG4 and
22 KAG8-KAG10 were used for PCR amplification of the toxin A
23 and B genes, respectively. Lanes 1 through 5 and lanes 6
24 through 10, 10^5 , 10^4 , 10^3 , 10^2 and 10^1 cells (vegetative
25 cells and spores).

1 2 3 4 5 6 7

bp

1000 -

500 -

100 -

Toxin A

Toxin B