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Increasing Prevalence of Toxin A-Negative, Toxin B-Positive Isolates of *Clostridium difficile* in Korea: Impact on Laboratory Diagnosis[▽]

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Of 462 Korean *Clostridium difficile* isolates, 77.5% were toxin B positive but 21.4% were toxin A negative (A^- B^+). The binary toxin gene was detected in nine isolates. A higher fluoroquinolone resistance of A^- B^+ strains may contribute to the increase of these strains. Toxin A detection alone may underdiagnose *C. difficile*-associated disease.

Clostridium difficile-associated disease (CDAD) is due to strains producing toxins A (enterotoxin) and B (cytotoxin), which are encoded by *tcdA* and *tcdB*, respectively (4, 5). Toxin A-negative, toxin B-positive (A^- B^+) strains of *C. difficile*, described in the early 1990s (3), have been increasingly reported in some parts of the world (6, 15). A^- B^+ strains fail to produce toxin A due to deletion of the repetitive domain of the *tcdA* gene but can cause CDAD, including fatal pseudomembranous colitis (15). Some *C. difficile* strains also produce binary toxin (actin-specific ADP-ribosyltransferase [CDT]), which contributes to CDAD. Two genes, *cdtA* and *cdtB*, encode the enzymatic and binding components of the toxin (14).

Clindamycin in the 1970s and cephalosporins in the late 1980s and through the 1990s were the antimicrobial agents associated with the highest relative risk of CDAD (7). However, more recently, outbreaks of CDAD due to a new binary toxin-producing (CDT^+) strain (pulsed-field gel electrophoresis type NAP1, PCR ribotype 027) with high morbidity and mortality have been reported in Canada, the United States, and Europe. This epidemic strain showed increased resistance to fluoroquinolones (11), suggesting that fluoroquinolone use was a risk factor in these outbreaks.

Laboratory diagnosis of CDAD includes detecting cytotoxin and/or toxin A and toxin B proteins (1). Besides direct toxin assay from stool specimens, toxigenic *C. difficile* culture is recommended to improve the diagnosis. The presence of A^- B^+ strains may profoundly affect the diagnosis of CDAD, depending on the kinds of tests used, but the prevalence of this type of strain in Korea is not well known.

The aim of this study was to determine the prevalence of A^- B^+ isolates and the presence of CDT^+ strains of *C. difficile* in Korea. The susceptibility to fluoroquinolones was also determined.

The *C. difficile* strains were isolated between 1980 and 2006 from stool specimens of suspected CDAD patients at a tertiary care hospital in Korea. Cycloserine-cefoxitin-fructose agar was used for the isolation (1), and the isolates were identified by using conventional tests and the ATB 32A system (bioMerieux, Marcy-l'Etoile, France). The control *C. difficile* strains, VPI 10463 (A^+ B^+), 3608/03 (A^- B^-), 1470 (A^- B^+), and SE844 (CDT^+), were obtained from Maja Rupnik in Slovenia. Strain NAP1/027 was provided by one of the authors of the present report (T. V. Riley).

C. difficile toxin genes were detected by PCR as described previously (17). The primer pairs used were NK2-NK3 for *tcdA*, NK9-NK11 for the repetitive domain of *tcdA*, NK104-NK105 for *tcdB*, cdtA pos-cdtA rev for *cdtA*, and cdtB pos-cdtB rev for *cdtB*. PCR ribotyping was performed as described previously (13).

The antimicrobial susceptibilities were determined by the National Committee for Clinical Laboratory Standards-recommended agar dilution method (12), using norfloxacin, ciprofloxacin, ofloxacin, and levofloxacin (Sigma-Aldridge, St. Louis, MO), gatifloxacin (Grunenthal, Aachen, Germany), and moxifloxacin (Bayer, Wuppertal, Germany).

Of the 462 isolates tested, 358 (77.5%) were either A^+ B^+ (259; 56.1%) or A^- B^+ (99; 21.4%). A^- B^+ strains, which were first detected in 1995 in samples from two patients, steadily

TABLE 1. Toxigenic status of Korean *C. difficile* strains by year of isolation

Year of isolation (no. of isolates tested)	No. (%) of isolates with toxin status			
	A^+ B^+ CDT^-	A^+ B^+ CDT^+	A^- B^+ CDT^-	A^- B^- CDT^-
1980 (3)	2 (66.7)	0 (0.0)	0 (0.0)	1 (33.4)
1990 (12)	9 (75.0)	0 (0.0)	0 (0.0)	3 (25.0)
1995 (48)	34 (70.8)	1 (2.1)	2 (4.2)	11 (22.9)
2002 (46)	28 (60.9)	0 (0.0)	6 (13.0)	12 (26.1)
2003 (105)	67 (63.8)	0 (0.0)	16 (15.2)	22 (21.0)
2004 (53)	22 (41.5)	1 (1.9)	21 (39.6)	9 (17.0)
2005 (40)	15 (37.5)	1 (2.5)	12 (30.0)	12 (30.0)
2006 (155)	73 (47.1)	6 (3.9)	42 (27.1)	34 (21.9)
Total (462)	250 (54.1)	9 (2.0)	99 (21.4)	104 (22.5)

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TABLE 2. In vitro activities of fluoroquinolones against 187 Korean *C. difficile* isolates according to toxigenic status

Toxin status (no. of isolates tested)	Antimicrobial agent	MIC (μg/ml)		
		Range	MIC_{50}	MIC_{90}
$A^+ B^+$ (115)	Norfloxacin	16–256	64	128
	Ciprofloxacin	4–128	16	32
	Ofloxacin	8–256	64	128
	Levofloxacin	2–128	32	64
	Gatifloxacin	1–32	8	8
	Moxifloxacin	1–16	8	8
$A^- B^+$ (31)	Norfloxacin	32–128	64	128
	Ciprofloxacin	8–64	32	32
	Ofloxacin	8–256	128	256
	Levofloxacin	4–128	64	128
	Gatifloxacin	1–32	32	32
	Moxifloxacin	1–32	32	32
$A^- B^-$ (41)	Norfloxacin	32–128	32	64
	Ciprofloxacin	4–32	8	8
	Ofloxacin	8–256	8	8
	Levofloxacin	4–128	4	4
	Gatifloxacin	1–32	1	2
	Moxifloxacin	1–16	1	2

increased thereafter (Table 1). All our PCR detection of the repetitive domains of *tcdA* and of *tcdB* was accurate compared with the results of *C. difficile* Tox-A enzyme-linked immunosorbent assay (TechLab, Blacksburg, VA) and a Vero cell cytotoxicity assay with antitoxin (TechLab).

In another Korean study in 2004, the proportion of $A^- B^+$ strains was even higher, i.e., 45.7% of 81 isolates (16). These results documented that $A^- B^+$ strains are much more prevalent in Korea than in other countries, i.e., 0% to 12.5% (2, 10, 17), and indicated that there is a potential for underdiagnosis of CDAD when the toxin A test alone is used for the diagnosis.

Because of a significant increase in $A^- B^+$ strains in 2002, 187 strains isolated between August 2002 and May 2004 were tested for PCR ribotype and for antimicrobial susceptibility. Overall, 39 PCR ribotypes were identified: 115 $A^+ B^+$ isolates comprised 22 ribotypes, and the most-common type accounted for 62 (33%) isolates; all 31 $A^- B^+$ strains showed the same pattern, which was identical to that of strain 1470 (ribotype 017). The majority of $A^- B^+$ strains gave this distinct ribotype pattern in many studies, suggesting their clonal spread worldwide (2, 6, 15).

Only nine of our isolates (2.0%) were CDT^+, i.e., PCR positive for the *cdtA* and *cdtB* genes. However, six of nine CDT^+ strains were isolated in 2006, suggesting a gradual increase of this toxin type for which continuous study is required. The prevalence of CDT^+ strains has been reported to be 1.6% in Asia (15), 5.8% in the United States (8), and 6% in France (9). The nine CDT^+ strains revealed four ribotypes which were different from that of the epidemic PCR ribotype 027 strain.

Overall, the MICs of fluoroquinolones were slightly lower for the nontoxigenic strains and slightly higher for $A^- B^+$ strains in comparison to their MICs in $A^+ B^+$ strains (Table 2). The MICs of gatifloxacin and moxifloxacin were higher for the $A^- B^+$ strains than for the $A^+ B^+$ strains. Drudy et al. (6) reported that $A^- B^+$ strains isolated during an outbreak showed high-level resistance to fluoroquinolones and considered that this may be a factor promoting outbreaks in hospitals. We require a further study to determine if the risk factor for increasing CDAD due to $A^- B^+$ strains is indeed the use of fluoroquinolones.

In conclusion, testing for both toxin A and toxin B became very important for the accurate laboratory diagnosis and epidemiologic study of CDAD with the increasing prevalence of $A^- B^+$ strains in Korea. CDT^+ strains have emerged in Korea, although the ribotype 027 strain was not found.

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