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Original Article

Molecular epidemiologic study of *Clostridium difficile* infections in university hospitals: Results of a nationwide study in Japan☆

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ABSTRACT

We conducted a nationwide molecular epidemiological study of *Clostridium difficile* infection (CDI) in Japan investigated the correlation between the presence of binary toxin genes and CDI severity. This is the first report on molecular epidemiological analyses for CDI in multiple university hospitals in Japan, to our knowledge. We examined 124,484 hospitalized patients in 25 national and public university hospitals in Japan between December 2013 and March 2014, investigating antimicrobial susceptibilities and toxin-related genes for *C. difficile* isolates from stools. Epidemiological genetic typing was performed by PCR-ribotyping and repetitive sequence-based (rep)-PCR to examine the genetic similarities. The results detected toxin A-positive, toxin B-positive, binary toxin-negative ($A^+B^+CDT^-$) detected from 135 isolates (80.8%) and toxin A-negative, toxin B-positive, binary toxin-negative ($A^-B^+CDT^-$) in 23 (13.8%). Toxin A-positive, toxin B-positive, and binary toxin-positive ($A^+B^+CDT^+$) were seen in 9 isolates (5.4%). Vancomycin (n = 81, 37.7%) or metronidazole (n = 88, 40.9%) therapies were undertaken in analyzed cases. Ribotypes detected from isolates were 017/subgroup 1, 070, 078, 126, 176, 449, 475/subgroup 1, 499, 451, 566 and newtypes. Rep-PCR classified 167 isolates into 28 cluster groups including 2–15 isolates. In addition, 2 pairs of strains isolated from different institutions belonged to the same clusters. Seven out of 9 (77.8%) of the patients with binary toxin producing strains had “mild to moderate” outcome in evaluated symptoms. In conclusion, we found that binary toxin did not show regional specificity and had no relevance to severity of CDI.

1. Introduction

Colitis due to *Clostridium difficile* infection (CDI) is considered to be involved in 15–39% of all antibiotic treatment-associated diarrheas [1]. *C. difficile* exists in the natural environment including soil, hay, and sand, and in enteron and stool in humans and animals [2]. Toxin A (*tcdA*) is a known enterotoxigenic causative substrate for diarrhea and toxin B (*tcdB*) is a known cytotoxin which damages cell action. The presence of *C. difficile* producing both toxin A and B

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correlated best with CDI occurrence but mutant strains producing only toxin B can be a causative factor for intestinal disease [3]. Binary toxin (CDT: *cdtA*/*cdtB*) was also found at a low rate. Binary toxin belongs to the ADP-ribosyltransferase family and induces depolymerization of actin in the cytoskeleton, resulting in increased bacterial adhesiveness [4].

Large-scale epidemiological studies of CDI in Japan are rare and details on morbidity, current diagnosis and treatments, pathophysiological analysis of severe disease and outcomes are not well-known. Some CDI cases with the restriction endonuclease analysis type BI, North American pulsed-field gel electrophoresis type 1 (NAP1), PCR ribotype 027 (BI/NAP1/027) strain which produces binary toxin have been reported in Japan, but nation-wide epidemiological analysis to understand the correlation of binary toxin and CDI severity have not been done [5–9]. Epidemiological study is an important tool to uncover the details of antimicrobial resistance needed for infection prevention and control [10].

Data from multicenter national and public university hospitals with comparable quality of care are required to evaluate the nationwide situation objectively. Such investigations provide vital details on the epidemiology, CDI severity and antibiotic resistance of CDI needed to treat or prevent CDI from both the clinical and bacteriological viewpoint.

2. Patients and methods

This study is a nationwide prospective surveillance of CDI in hospitalized patients 2 years old or older who were hospitalized for 24 h or more at national and public university hospitals in Japan. Twenty-eight university hospitals were enrolled in this study. Data were collected from December 2013 to March 2014. Patient information and *C. difficile* isolates were obtained from each institution by physicians and medical technologists.

2.1. Examination and definition of CDI

CDI symptoms were defined as follows: having loose stool more than 3 times in the past 24 h, or diarrhea for 24 h or longer [11]. CDI was diagnosed in patients with the above symptoms and positive *C. difficile* toxin was detected by enzyme linked immunosorbent assay (including immune-chromatography) or by PCR [12]. If the results were negative for toxin and only positive for *C. difficile* antigen, we confirmed positive *C. difficile* toxin from *C. difficile* culture. The CDI occurrence rate was calculated in all hospitalized patients.

2.2. Clinical investigation

Age, gender, underlying diseases, antibiotics used in the last 60 days and histories of CDI were investigated. In addition, date of emergence of CDI-related symptoms, clinical symptoms, clinical examination, therapies for CDI and the effectiveness of therapy and outcome (cured, ICU transmission or death) were also examined. CDI severity was defined as M: mild to moderate, S: severe or severe and complicated disease, according to the clinical practice guidelines of the Society of Healthcare Epidemiology of America and Infectious Disease Society of America [11].

2.3. Collection of isolates

C. difficile isolates were cultured from the stool of CDI patients at the clinical laboratory of each hospital. Isolates were stored in cooked meat medium (Becton, Dickinson and Company, Tokyo, Japan) and transferred to the clinical laboratory of Kobe University Hospital. The collected isolates were recovered onto cycloserine-cefoxitin-mannitol agar medium (Nissui Pharmaceutical Co Ltd., Tokyo, Japan) with anaerobic culture. Only positive bacterial isolates for antigen and toxic C. DIFF QUIK CHEK COMPLETE tests (Alere Medical, Tokyo, Japan) were used for analysis of toxin-producing genes, antibiotic susceptibility tests, and molecular-based epidemiologic analysis. Grown colonies were inoculated onto YT Broth (Life Technologies, Carlsbad, USA) and enriched.

2.4. DNA extraction and detection of toxin-producing genes

DNA extraction was performed by Ultra Clean Microbial DNA Isolation Kit (MO BIO Laboratories, Inc, Carlsbad, USA) according to the manufacturer's instructions. All the isolates were examined for 16s rRNA genes for confirmation of *C. difficile* [13]. PCR amplifications were performed to detect the presence of *tcdA*, *tcdB*, *cdtA*, *cdtB* and the 16S rRNA gene as done in previous studies [3,13,14].

2.5. Susceptibility tests

Antibiotic susceptibility tests were performed via minimal inhibitory concentration (MIC) plates (Kyokuto Pharmaceutical Industrial Co., Ltd, Tokyo, Japan) using the broth microdilution method for vancomycin (VCM), metronidazole (MNZ), levofloxacin (LVFX) and clindamycin (CLDM). MIC standards for antibiotic susceptibilities were as follows; VCM: European Committee on Antimicrobial Susceptibility Testing (EUCAST) [15]; MNZ and CLDM: CLSI; LVFX, CLSI was moxifloxacin criteria [16]. *C. difficile* ATCC 700057 was used as control.

2.6. PCR-ribotyping and repetitive sequence-based PCR (rep-PCR) for epidemiological analysis

To investigate the clonal relationship of *C. difficile* toxin-producing isolates, we performed PCR-ribotyping and repetitive sequence-based PCR (rep-PCR) as recommended by the CLSI guideline (MM11-A) for epidemiological analysis [17]. PCR-ribotyping was performed with primers for the 16S-23S rRNA gene as described by Stubbs et al. [18]. The fragment was analyzed using capillary electrophoresis and typed using the Webribo database (https://webribo.ages.at/) [19]. Rep-PCR was conducted using a DiversiLab Clostridium kit (bioMerieux, Grenoble, France) following the manufacturer's protocol. The PCR products were separated with Agilent Bioanalyzer 2100 (Agilent Technologies, Inc., Palo Alto, CA) using DNA LabChip reagents. The results were analyzed with the Pearson correlation method and the dendrogram was constructed by the unweighted pair group method with arithmetic mean (UPGMA) clustering method using DiversiLab software (ver.3.3.40). Isolates with rep-PCR profiles yielding a similarity of >95% were considered clonally related [20].

2.7. Statistical analysis

We conducted univariate analyses between positive toxin genes from the isolates and CDI severity in patients using SPSS statistical software ver. 24 (SPSS Japan Inc., Tokyo, Japan). All p values of 0.05 or less were considered to be statistically significant.