Use of the loop-mediated isothermal amplification method for identification of PCR ribotype 027 *Clostridium difficile*

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The loop-mediated isothermal amplification (LAMP) assay detecting the *slpA* gene of *slpA* sequence type gc8 (*slpA*-gc8) was established for the identification of a hypervirulent *Clostridium difficile* strain, PCR ribotype 027. Of 107 isolates examined, 27 belonging to PCR ribotype 027 were all positive for the LAMP assay. The remaining 80 isolates were typed into 47 different PCR ribotypes other than type 027, and were negative for the LAMP assay with the exception of two isolates. The sensitivity and specificity of the LAMP method for identification of PCR ribotype 027 were 100 % and 98 %, respectively. The LAMP assay detecting *slpA*-gc8 is a reliable tool for the identification of PCR ribotype 027 *C. difficile*. This simple and rapid method will contribute to detection of the hypervirulent strain.

INTRODUCTION

Clostridium difficile is well known as an important cause of health-care-associated infection. The emergence of a *C. difficile* strain characterized as restriction endonuclease analysis (REA) type BI, PFGE type NAP1 and PCR ribotype 027 has been reported to be responsible in part for the epidemic in North America and Europe in the past decade (Kuijper *et al.*, 2008; Loo *et al.*, 2005; MacCannell *et al.*, 2006; McDonald *et al.*, 2005; Warny *et al.*, 2005). In addition, it has been documented that patients infected with this strain are more likely to develop severe disease (Barbut *et al.*, 2007; Goorhuis *et al.*, 2007). Since the epidemic by PCR ribotype 027 threatens to extend to the rest of the world including Asia (Gerding, 2010), early recognition of the emergence of the strain is valuable (Goorhuis *et al.*, 2007), especially in the countries or areas where the strain is not currently epidemic.

In previous studies, a system for typing *C. difficile* by sequencing the gene encoding the surface-layer protein A (*slpA*) was established and evaluated (Kato *et al.*, 2010; Killgore *et al.*, 2008). These reports documented that the typing results exhibited a high concordance with those obtained by PCR ribotyping; *slpA* sequence type gc8 corresponded to PCR ribotype 027 (Kato *et al.*, 2010; Killgore *et al.*, 2008). In the present study, we established a simple method for identification of PCR ribotype 027 by

loop-mediated isothermal amplification (LAMP) detecting the *slpA* gene of *slpA* sequence type gc8 strains (*slpA*-gc8).

METHODS

Bacterial strain. A total of 107 *C. difficile* isolates were examined. The reference strains of serogroups A (ATCC 43594), C (ATCC 43596), F (ATCC 43598), G (ATCC 43599) and H (ATCC 43600) (Delmée *et al.*, 1986) were obtained from the American Type Culture Collection. The 59 clinical *C. difficile* isolates used in this study were isolated from various hospitals in Japan. A collection of 42 isolates used for a previous study including strain US42 (REA type BI/PFGE type NAP1/PCR ribotype 027; Killgore *et al.*, 2008) and strain UMG12(3) (PCR ribotype 078; Goorhuis *et al.*, 2008) were tested as well.

PCR detecting the toxin genes. The non-repeating and repeating sequences of the toxin A gene (*tcdA*) were amplified by PCR with primer sets NK3–NK2 (Kato *et al.*, 1991) and NK9–NK11–NKV011 (Kato *et al.*, 1998, 1999), respectively. The isolates were identified as toxin A-negative when PCR with the primer set NK3–NK2 was negative (Kato *et al.*, 1991). When PCR by the NK3–NK2 primer set was positive and PCR by the NK9–NK11–NKV011 primer set generated an amplification product of approximately 1200 bp in size, the isolates were identified as toxin A-positive. When PCR by NK3–NK2 was positive and the product by PCR with NK9–NK11–NKV011 was approximately 700 bp in size, the isolates were identified as toxin A-negative (Kato *et al.*, 1998, 1999). When the isolates were positive for PCR by NK3–NK2 but the PCR product generated by NK9–NK11–NKV011 was neither approximately 1200 bp nor 700 bp in size, toxin production by the isolates was determined. The presence of the non-repeating sequences of the toxin B gene (*tcdB*) was examined by PCR using primer set NK105–NK104 (Kato *et al.*, 1998). PCR detection of the gene encoding the binding component of binary toxin (CDT) was performed as previously described (Stubbs *et al.*, 2000).

Determination of toxin production by isolates. *C. difficile* was cultured anaerobically in brain–heart infusion broth (Becton

Abbreviations: CDT, binary toxin; LAMP, loop-mediated isothermal amplification; REA, restriction endonuclease analysis.

The GenBank/EMBL/DBJ accession numbers (*slpA* sequence type) for the *slpA* genes reported in this study are AB249986 (gc8-01), AB257285 (gc8-02), AB257286 (gc8-03), AB461839 (gc8-05) and AB461840 (gc8-06).

Dickinson) for 5 days. Toxin A was detected by an enzyme immunoassay kit, *C. difficile* toxin A test (Oxoid). Toxin B was detected by a culture assay using Vero cells and *C. difficile* goat anti-toxin B serum was used for the neutralization test (Kato *et al.*, 1998).

Typing of isolates. Typing of isolates by sequencing *slpA* was carried out as previously described (Kato *et al.*, 2010). Isolates were assigned to different major types when they had 20 or more amino acid differences, and to subtypes (01–06) when they had fewer than 20 such differences. PCR ribotyping was performed by the modified methods of Stubbs and others as previously described (Kato *et al.*, 2010; Stubbs *et al.*, 1999).

Detection of *slpA-gc8* by LAMP. For DNA extraction for LAMP, *C. difficile* was inoculated on Brucella agar supplemented with vitamin K₁ and haemin (Kyokuto Pharmaceutical) for 1–7 days anaerobically. A single colony was suspended in 50 µl TES (50 mM Tris hydrochloride, pH 8.0; 5 mM EDTA; and 50 mM NaCl). The suspension was heated at 95 °C for 15 min and then centrifuged at 15 000 g for 2 min, and the resultant supernatant was used as a template for the LAMP assay. The six primers used for the LAMP were derived from the *slpA* gene of strain US42, which was identified as REA type BI, PFGE type NAP1, PCR ribotype 027 and *slpA* sequence type gc8-01 (GenBank accession no. AB249986) (Kato *et al.*, 2010; Killgore *et al.*, 2008). The outer primers used were gc8002a-F3 (5'-GCTCTCCAGCAGAGGGAG-3') and gc8002-B3 (5'-AGTTCCA-TCAACTAAACCAAAC-3'); the inner primers were gc8002b-FIP (5'-TTGGAGCTGTATTTTGTCTCCGCAATAAAAGTAGTACAAG-TAG-3') and gc8002-BIP (5'-GTCTATGTCAGATGTATTGTATAC-ATAAGTTTCACAGCAGTTTCAGT-3'); and the loop primers were gc8002-FL (5'-TACCAGCATTTTCGACTTCACC-3') and gc8002-BL (5'-GCTTTTACAGATTCA-3'). The LAMP reaction was performed using the Loopamp DNA amplification kit (Eiken Chemical) according to the manufacturer's instructions. Two microlitres of DNA template was added to a total volume of 25 µl buffer consisting of 5 pM of each of the outer primers, 40 pM of each of the inner primers and 20 pM of each of the loop primers. Amplification was performed at 62 °C for 60 min. The increased turbidity was monitored by a real-time turbidimeter, LA-320C (Eiken Chemical). The turbidity was calculated based on the ratio of light intensity (the intensity of light received by the photodiode/the emitted light intensity). The threshold of LAMP positive for the turbidimeter was defined as 0.1.

Determination of the sequences of *tcdC*. The sequences of *tcdC* were determined by sequencing the PCR product with primer set C1–C2 as described previously (Spigaglia & Mastrantonio, 2002). The primer set NK3–Lok1 (Braun *et al.*, 1996; Kato *et al.*, 1991) was used for sequencing *tcdC* of isolate DJNS 7-18. The sequences obtained were compared to those of strain VPI 10463 and strain US42 as well as to the sequences previously described (MacCannell *et al.*, 2006) and those in the GenBank database. The comparative analysis was performed by GENETYX-MAC version 12.2.3.

RESULTS

Typing results

Sequencing of the *slpA* gene resulted in 24 major types and 54 subtypes for the 107 isolates tested. Of the 107 isolates, 29 were typed as *slpA* sequence type gc8, and five different subtypes (*slpA* sequence types gc8-01, gc8-02, gc8-03, gc8-05 and gc8-06) were found among these 29 isolates (Table 1). Types gc8-01, gc8-02, gc8-03 and gc8-06 differed from

each other by 1 nt, involving a single deduced amino acid. The *slpA* sequence of one isolate, DJNS 7-18, was found to differ from that of gc8-01 by eight deduced amino acids (*slpA* sequence type gc8-05). Among the 107 isolates, 48 different PCR ribotypes were identified. Of the 28 isolates belonging to gc8-01, gc8-02, gc8-03 or gc8-06, 27 were typed as PCR ribotype 027 (Fig. 1). One isolate of *slpA* sequence type gc8-01 (JND 10-61) was typed as a different PCR ribotype from PCR ribotype 027 (PCR ribotype 027r; Fig. 1), while its PCR banding pattern showed two band differences from that of 027. Isolate DJNS 7-18 and the remaining 78 isolates were typed into 46 different PCR ribotypes, banding patterns of which were distinct from that of PCR ribotype 027. The epidemiologically important PCR ribotypes 001, 002, 014, 017, 078, trf and smz (Barbut *et al.*, 2007; Goorhuis *et al.*, 2008; Kato *et al.*, 2010; Killgore *et al.*, 2008) were included in the 46 PCR ribotypes.

Detection of *slpA-gc8* by LAMP

slpA-gc8 was detected by LAMP in all 29 isolates of *slpA* sequence type gc8 including JND 10-61 and DJNS 7-18 (Fig. 2; Table 1). LAMP was performed on DNA extracted from strain US42, which was incubated for 1 or 7 days. The time for *C. difficile* culture to extract DNA did not affect the LAMP reaction. In a comparison of the LAMP results in 28 isolates identified as PCR ribotypes 027 and 027r (*slpA* sequence types gc8-01, gc8-02, gc8-03 and gc8-06), the amplification reaction in DJNS 7-18 (PCR ribotype tkm0718/*slpA* sequence type gc8-05) was slow (Fig. 2). LAMP was performed on 78 isolates of types other than *slpA* sequence type gc8 in a 90 min reaction, with negative results (Table 1). The sensitivity and specificity of the LAMP method for identification of PCR ribotype 027 were 100 % and 98 %, respectively.

slpA sequence type gc8 isolates recovered in Japan

Of seven *slpA* sequence type gc8 isolates recovered in Japan, six (subtypes gc8-01 and gc8-06) were toxin A-positive, toxin B-positive and CDT-positive. Sequencing analysis of the six isolates including JND 10-61 detected *tcdC* identical with that of strain US42 and published sequences (MacCannell *et al.*, 2006). Isolate DJNS 7-18 (subtype gc8-05) was toxin A-negative, toxin B-positive and CDT-positive. In the DJNS 7-18 isolate, PCR using the NK3–NK2 primer set was positive but no amplification was obtained by PCR using the NK9–NK11–NKV011 primer set. This isolate was tested by enzyme immunoassay for toxin A production, with a negative result, and toxin B was detected by a cell culture assay with a positive neutralization reaction. When the sequences of *tcdA* and *tcdC* were compared to those registered in the GenBank database, DJNS 7-18 had deletions in *tcdA* and *tcdC*; the deletions were the same in location and size as those of strain 8864 (GenBank accession no. AJ011301). Isolate DJNS 7-18 was

Table 1. Typing results and LAMP results for identification of PCR ribotype 027

<i>slpA</i> sequence major type	<i>slpA</i> sequence subtype	PCR ribotype	LAMP results	No. of isolates tested from:			Total no. of isolates studied
				Japan	North America and Europe*	ATCC	
gc8	gc8-01	027	+	4	16		20
	gc8-01	027r†	+	1			1
	gc8-02	027	+		2		2
	gc8-03	027	+		4		4
	gc8-05	tkm0718	+‡	1			1
	gc8-06	027	+	1			1
Other types§			–	52	21	5	78
Total				59	43	5	107

*A collection of 42 isolates used for a previous study (Killgore *et al.*, 2008) and strain UMCG12(3) (PCR ribotype 078; Goorhuis *et al.*, 2008).

†The banding pattern of JND 10-61 (PCR ribotype 027r) differed from that of type 027 by two bands.

‡The LAMP reaction in DJNS 7-18 was slow.

§A total of 78 isolates were typed into 23 *slpA* sequence major types, 49 *slpA* sequence subtypes and 45 PCR ribotypes.

recovered from a patient who suffered from pseudomembranous colitis in 2008.

DISCUSSION

In the present study, all PCR ribotype 027 isolates tested were successfully identified by LAMP assay detecting the *slpA*-gc8 gene. The isolates typed as 47 different PCR ribotypes other than type 027 were all negative for LAMP with the exception of two isolates, JND 10-61 and DJNS 7-18. In JND 10-61, which was typed as *slpA* sequence gc8-01, the PCR ribotype pattern differed from that of type 027 by two bands and its *tcdC* sequence was the same as that of

strain US42. More studies are needed to identify this isolate as a strain related to the hypervirulent strain. DJNS 7-18 was a toxin A-negative, toxin B-positive, CDT-positive isolate with the same deletions in *tcdA* and *tcdC* as those of strain 8864 (Lyerly *et al.*, 1992; Rupnik *et al.*, 1998). Since strain 8864 was not available in the present study, PCR ribotyping and *slpA* sequence typing results for strain 8864 could not be compared to those for DJNS 7-18. DJNS 7-18 was unique among our collection of clinical isolates in Japan. Additional studies using more clinical isolates are warranted to determine the significance of isolates typed as *slpA* sequence type gc8 but not as PCR ribotype 027.

PCR ribotype 027 has been reported to be responsible for multiple outbreaks and a major portion of the increase in *C. difficile* infection rates in North America and Europe (Kuijper *et al.*, 2008; Pépin *et al.*, 2004; Loo *et al.*, 2005; McDonald *et al.*, 2005). It has been documented that patients infected with the PCR ribotype 027 strain are more likely to have severe disease and to have been specifically treated with metronidazole or vancomycin (Barbut *et al.*, 2007). Goorhuis *et al.* (2007) demonstrated that clear trends were observed for more severe diarrhoea, higher attributable mortality and more recurrences in patients infected with PCR ribotype 027 than in those infected with other types (Goorhuis *et al.*, 2007). The significant pathogenicity of this strain, such as robust toxin production or significantly more spore formation (Merrigan *et al.*, 2010; Warny *et al.*, 2005), may contribute to the severity and wide spread of *C. difficile* infection caused by the strain. These reports indicate that PCR ribotype 027 is more likely to cause outbreaks with high morbidity and mortality. Particularly in the countries or areas where the strain is not currently predominant, such as in Japan (Kato *et al.*, 2007; Sawabe *et al.*, 2007), earlier recognition of outbreaks caused by the strain will provide beneficial information for public health centres, leading to alerts of

Fig. 1. PCR ribotype patterns of representative isolates typed as *slpA* sequence type gc8. Lanes: 1, DJNS 5-23 (*slpA* sequence type gc8-01); 2, JND 10-61 (gc8-01); 3, JND 8-64 (gc8-06); 4, DJNS 7-18 (gc8-05); 5, US42 (gc8-01); 6, negative control; M, 100 bp ladder as a molecular size marker. Strain US42 was used as the reference strain of PCR ribotype 027.

Fig. 2. Real-time turbidity of LAMP amplification products for representative isolates typed as *slpA* sequence type gc8. Strain US42 was used as a positive control.

the emergence of the strain to health-care facilities covered by the public centres. The LAMP assay detecting *slpA*-gc8 may be valuable for monitoring trends in the prevalence of *C. difficile* infection caused by PCR ribotype 027. Since the LAMP method is simple to perform and does not require any special equipment, such as a thermal cycler or electrophoresis system, it is accessible to even small laboratories of local public health centres as well as of health-care facilities.

The LAMP assay detecting *slpA*-gc8 proved to be a reliable tool for the identification of PCR ribotype 027 in the present study. The method may be applied to the direct detection of *slpA*-gc8 from stool specimens without a culture step. In the present study, one stool specimen from a patient who suffered from pseudomembranous colitis caused by PCR ribotype 027 was available (Kato *et al.*, 2007); detection of *slpA*-gc8 was performed on DNA extracted directly from the stool specimen as previously described (Kato *et al.*, 2005), with a positive result (data not shown). Further analysis using a large number of stool specimens is required to evaluate the method for the direct detection of PCR ribotype 027 strains from stool specimens.

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