The use of loop-mediated isothermal amplification method for identification of *Clostridium difficile* PCR ribotype 027

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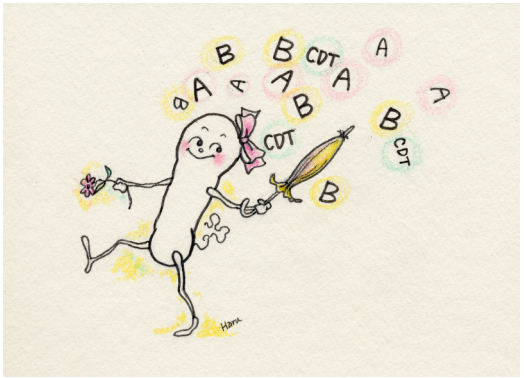


Objectives:

A number of outbreaks caused by *Clostridium difficile* PCR ribotype 027 have been documented in North America and Europe. The emerging strain was reported to cause more severe disease. Earlier recognition of PCR ribotype 027 might be beneficial in countries where the type is epidemic as well as in countries where the type is not currently predominant. We established and evaluated a loop-mediated isothermal amplification (LAMP) method for identification of PCR ribotype 027.

Materials and methods:

A total of 102 toxin B-positive *C. difficile* isolates were tested.

Of these, 53 isolates were recovered from patients with *C. difficile* infection (CDI) in Japan.

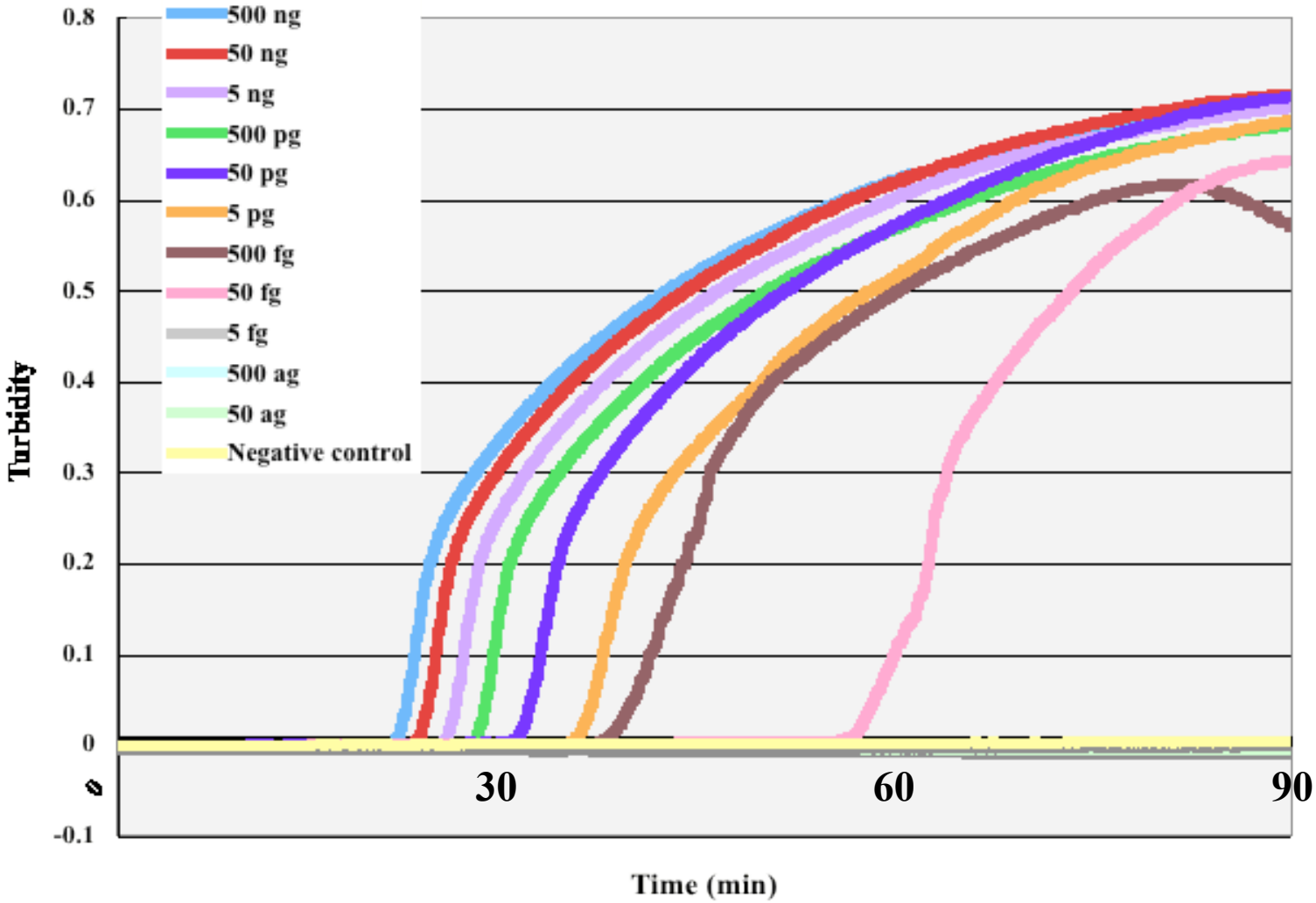
Included were 42 isolates used for the previous study (Killgore G *et al*. 2008. J Clin Microbiol 46:431-437) and one PCR ribotype 078 strain (UMCG12(3)) recovered from CDI patient in the Netherlands. The reference strains of serogroups A, C, F, G, H, and K were obtained from ATCC.

PCR ribotyping and *slpA* sequence typing were performed as previously described (Stubbs, SL *et al.* 1999. J Clin Microbiol 37: 461-463, Kato, H *et al.* 2010. J Med Microbiol 59: 556-562). Isolates were assigned to different *slpA* sequence major types when they had 20 or more amino acid differences.

LAMP method for identification of PCR ribotype 027:

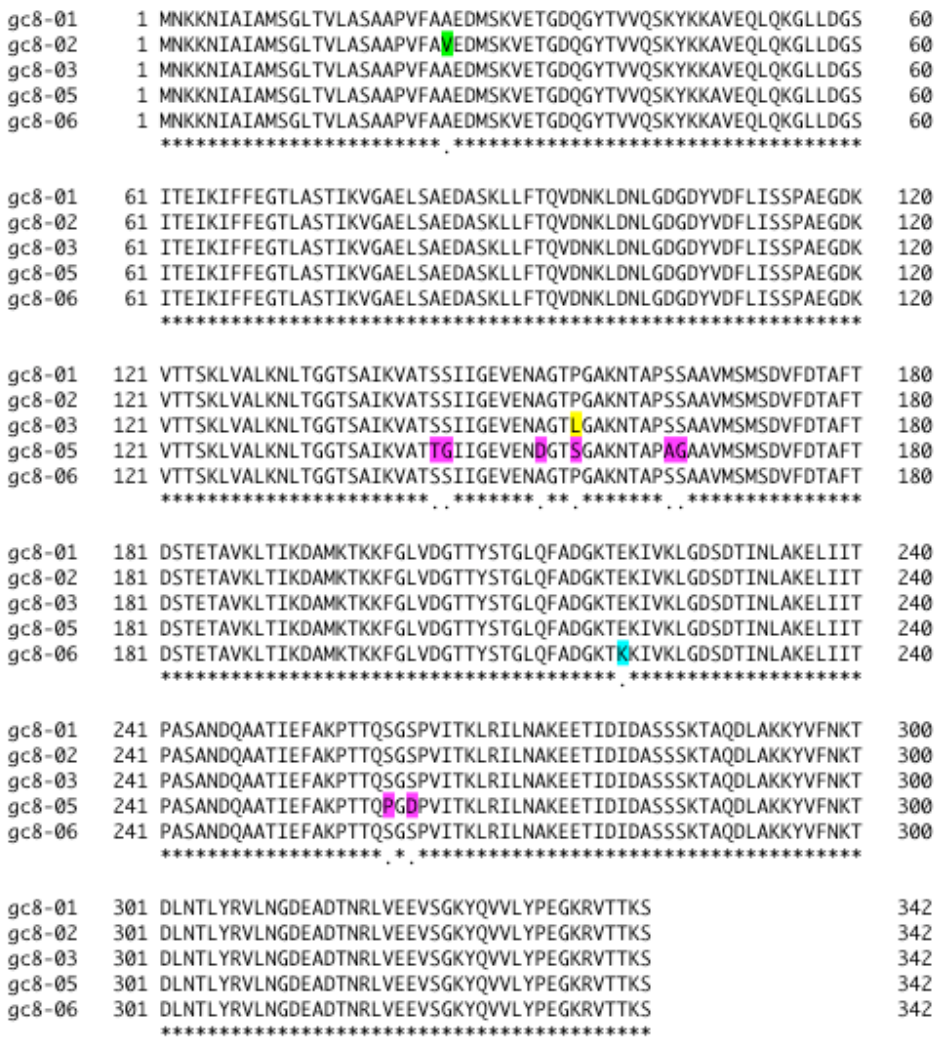
- **Six primers were derived from the *slpA* sequences of US42 strain (PCR ribotype 027/*slpA* sequence type gc8-01).**
- **Amplification was performed at 62℃ and the increased turbidity was measured by a real-time turbidimeter.**

MICs of moxifloxacin and gatifloxacin were determined by Etest method.



Real-time detection of turbidity of amplification products by LAMP. The template extracted from US42 strain (*slpA* sequence subtype gc8-01) in 10-fold serial dilutions was added.

Amino acid sequences of *slpA* in major type gc8 (subtypes gc8-01, 02, 03, 05 and 06)



Of 102 isolates tested, 29 were typed as *slpA* sequence major type gc8. Among the 29 isolates, different 5 *slpA* sequence subtypes were found.

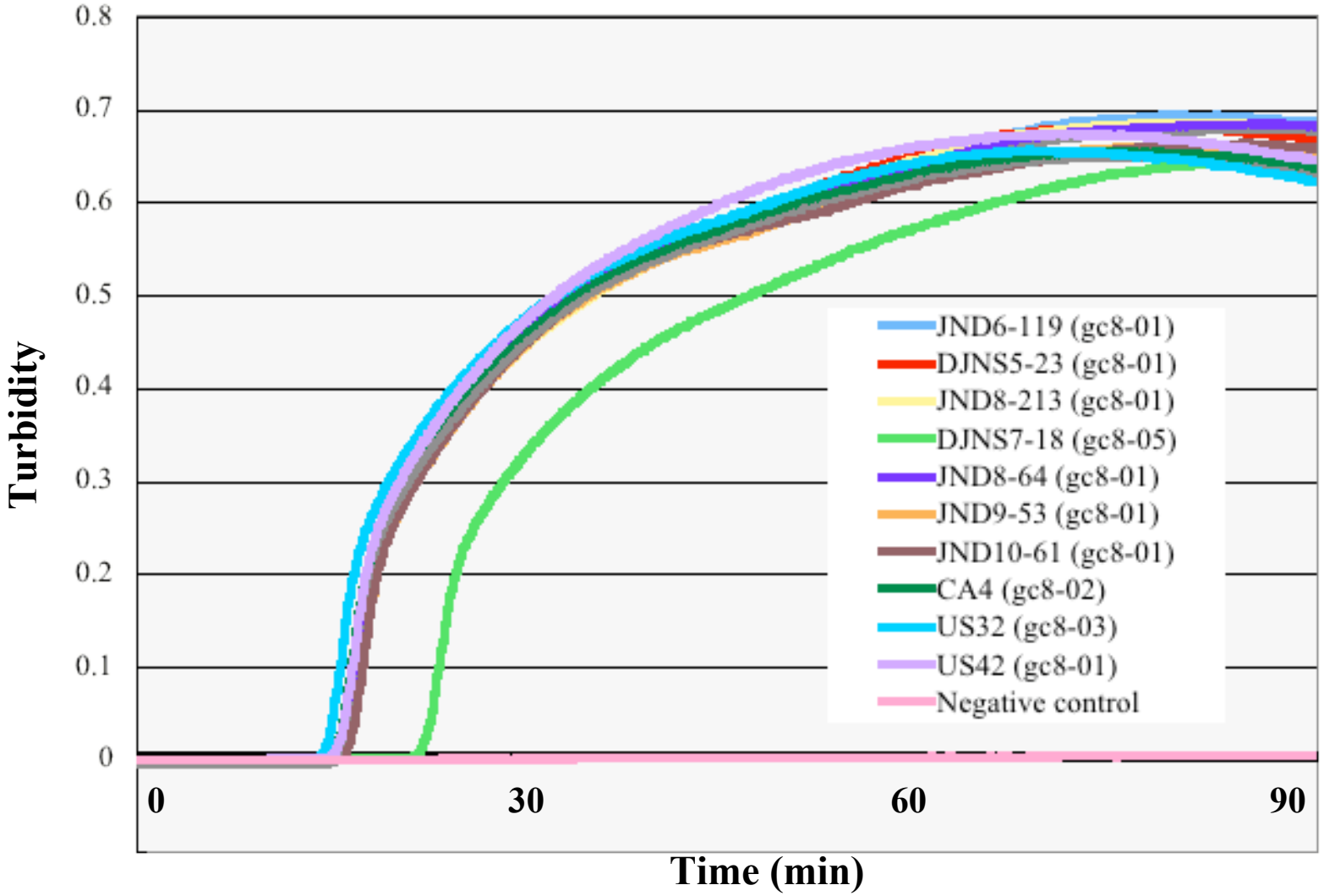
Isolates used in this study and results of detection of *slpAgc8* by LAMP

slpA sequence major type	*slpA* sequence subtype	LAMP results	Isolates from		
			Japan	North America and Europe	ATCC
gc8	gc8-01	+	5[a]	16	
	gc8-02	+		2	
	gc8-03	+		4	
	gc8-05	+[b]	1		
	gc8-06	+	1		
Other types[c]		-	46	21	6
Total			53	43	6

[a] Number of isolates tested.

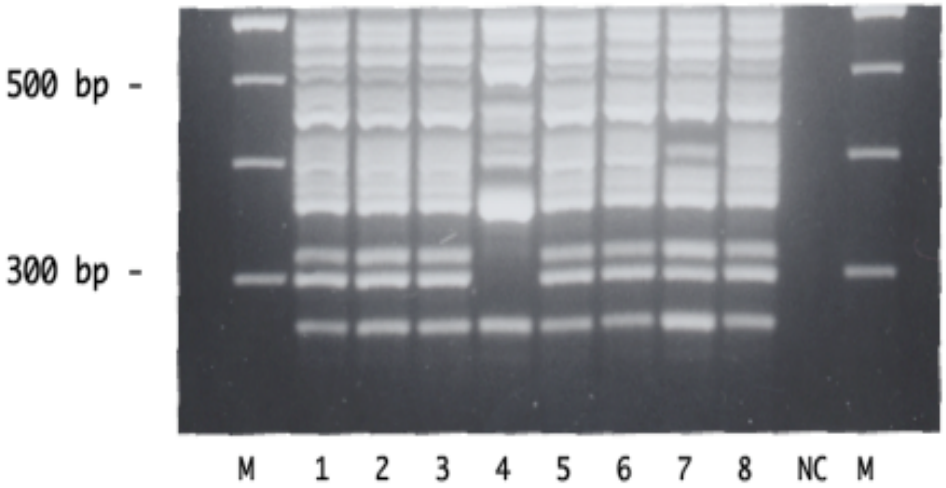
[b] Amplification reaction was slower.

[c] A total of 73 isolates were typed into different 23 *slpA* sequence major types and further 54 *slpA* sequence subtypes.



Real-time detection of turbidity of amplification products by LAMP in *slpA* sequence type gc8 isolates. Amplification reaction in DJNS 7-18 isolate (*slpA* sequence subtype gc8-5) was slower than that of other subtypes.

PCR ribotype patterns of *slpA* sequence type gc8 isolates clinically recovered from CDI in Japan



Lane	Isolate No.	*slpA* sequence type	PCR ribotype
1	JND 6-119	gc8-01	027
2	DJNS 5-23	gc8-01	027
3	JND 8-213	gc8-01	027
4	DJNS 7-18	gc8-05	tkm0718
5	JND 8-64	gc8-06	027
6	JND 9-53	gc8-01	027
7	JND 10-61	gc8-01	cc1061[a]
8	US42	gc8-01	027

NC Negative control
M Molecular size marker (100 bp ladder)

[a] PCR ribotype pattern of JND 10-61 differed from that of PCR ribotype 027 by two bands (lane 7).

Characteristics of isolates typed as *slpA* sequence type gc8

Isolate No.	*slpA* sequence type	Differences of *slpA* sequences (aa) from those of gc8-01	PCR ribotype	PCR results *tcdA/tcdB/cdtB*	MIC (mg/L) of Moxifloxacin	Gatifloxacin
JND 6-119	gc8-01	0	027	+ / + / +	0.75	0.75
DJNS 5-23	gc8-01	0	027	+ / + / +	0.5	0.5
JND 8-213	gc8-01	0	027	+ / + / +	0.75	0.75
DJNS 7-18	gc8-05	8	tkm0718	- / + / + [a]	>32	>32
JND 8-64	gc8-06	1	027	+ / + / +	0.5	0.5
JND 9-53	gc8-01	0	027	+ / + / +	0.75	0.5
JND 10-61	gc8-01	0	cc1061[b]	+ / + / +	1.0	1.0
US42	gc8-01	0	027	+ / + / +	>32	>32
CA4	gc8-02	1	027	+ / + / +	>32	>32
US32	gc8-03	1	027	+ / + / +	>32	>32

[a] DJNS 7-18 isolate had deletions in the *tcdA* and *tcdC* genes similar to those in 8864 strain (GenBank accession number AJ011301)

[b] The PCR ribotype pattern of JND 10-61 differed from that of PCR ribotype 027 by two bands.

Demographic information of patients of CDI caused by *slpA* sequence type gc8 *C. difficile*

Isolate No.	*slpA* sequence type	Age, year	Prefecture	Date of specimen collection	CDI onset and exposure [a]
JND 6-119	gc8-01	31	Tokyo	2001 June	CA-CDI
DJNS 5-23	gc8-01	30	Gifu	2005 April	HO-HCFA
JND 8-213	gc8-01	72	Hyogo	2005 Sept	HO-HCFA
DJNS 7-18	gc8-05	62	Aichi[b]	2007 May	CA-CDI
JND 8-64	gc8-06	58	Chiba[c]	2008 May	CO-HCFA
JND 9-53	gc8-01	65	Aichi[b]	2008 Sept	CA-CDI
JND 10-61	gc8-01	58	Chiba[c]	2008 Sept	Intermediate

[a] CA-CDI, community-associated CDI; HO-HCFA, healthcare facility-onset, healthcare facility-associated CDI; CO-HCFA, community-onset, healthcare facility-associated CDI.

[b]Two patients visited different hospitals located at different cities in Aichi.

[c]Two patients were hospitalized at the same hospital in Chiba.

Results:

1. **Among 102 *C. difficile* isolates, different 24 *slpA* sequence major types and 56 subtypes were found. These 102 isolates were typed into different 46 PCR ribotypes.**
2. **The *slpA* gene of *slpA* sequence type gc8 (*slpAgc8*) was detected by LAMP in all 29 isolates of *slpA* sequence type gc8. Of 29 isolates, 27 isolates were typed as PCR ribotype 027.**
3. **All of 73 isolates of types other than PCR ribotype 027 were negative by LAMP detecting *slpAgc8***

Conclusion:

The LAMP assay detecting *slpA* gene of *slpA* sequence type gc8 appears to be a valuable tool for the quick and simple identification of PCR ribotype 027

ACKNOWLEDGMENTS:

The authors would like to thank G. E. Killgore, B. Limbago, A. Thompson, L. C. McDonald (Centers for Disease Control and Prevention, US), S. Johnson, W. Zukowski, S. P. Sambol, D. N. Gerding (Hines VA Hospital, US), J. Brazier (University Hospital of Wales, UK), E. J. Kuijper, R. J. van den Berg (Leiden University Medical Center, The Netherlands), J. Pepin, E. H. Frost (University of Sherbrooke, Canada), B. Nicholson, C. Woods (Duke University School of Medicine, US), and P. Savelkoul (VU University Medical Center, The Netherlands) for providing the 42 *Clostridium difficile* isolates and isolate information, E. J. Kuijper and D. Bakker (Leiden University Medical Center, The Netherlands) for providing UMCG12(3) strain, Y. Ito (Gifu Red Cross Hospital, Japan), H. Kato, A. Nakamura, Y. Iwashima (Nagoya City University Hospital, Japan), T. Akahane (Azumino Red Cross Hospital, Japan) , C. Sakai, H. Satomura (Chiba Cancer Center, Japan), S. Nakamura, S. Senda (Tokoname City Hospital, Japan), E. Sawabe (Tokyo Medical and Dental University Hospital, Japan) for their assistance in the collection of isolates and specimens.