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Original Research Article

Molecular typing of *Clostridioides difficile* from frozen stool samples to investigate cross-transmissions: A proof of concept

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ABSTRACT

Purpose: Toxigenic *Clostridioides difficile* is responsible for up to one third of post antibiotic diarrhea and for more than 95% of pseudomembranous colitis. Nowadays, diagnosis relies on the documentation of the presence of the toxin in stools by specific antigenic or PCR tests. Stool cultures have been mostly abandoned, leading to the absence of isolates for further epidemiological analyses.

Methods: Aliquots of stool samples, frozen for up to two years, were thawed and inoculated onto commercial *C. difficile* media. Eighteen stools were recovered from patients hospitalized in the pediatric ward where at that time a chain of transmission was suspected. Eleven stools were recovered from patients hospitalized in a medical ward over a three months period with no suspected transmission event. Up to 16 characteristic colonies were isolates per culture. PCR of toxins genes and molecular typing by Double Locus Sequence Typing (DLST) were performed on these colonies. Whole genome multi locus sequence typing (wgMLST) was performed on selected isolates.

Results: Among the 29 stool specimens, no growth was observed for four stools and only one colony grew for one stool. Except the latter, all 16 colonies of the 24 stools showed identical toxin genes profiles than the original stool. However, variant DLST genotypes was observed within 20% of investigated stools. The majority of variants were single locus variant due to an IN/DEL of the repeat in one of the two DLST locus.

Despite this variation, results of molecular typing overrule the putative transmission chain in the pediatric ward and revealed undetected chains of transmission in the medical ward. These results were confirmed with wgMLST.

Conclusions: The developed protocol allows prospective and retrospective molecular and genomic epidemiological investigation of *C. difficile* infections for infection control purpose.

1. Introduction

Clostridioides difficile is a strict anaerobe, spore-forming, Gram-positive bacillus responsible in humans for 15%–30% of post antibiotics diarrhea and more than 95% of pseudomembranous colitis [1]. It is the principal agent of nosocomial diarrhea in patients under antibiotic treatment. Between 4 and 15% of the general adult population is colonized by *C. difficile*, among which 6–70% are toxigenic strains [2].

C. difficile produces three toxins: toxins A (TcdA) and B (TcdB), and a binary toxin (Cdt). The production of toxins A and B, coded by the *tcdA* and *tcdB* genes, are the main virulence factors. Both toxins A and B can be responsible for *C. difficile* infection (CDI); however, toxin B is 100–1000 time more toxic for human cells than toxin A. Toxins A and B genes are located on the pathogenicity locus (PathLoc) [3]. The Pathloc contains five genes, *tcdA, tcdB, tcdC, tcdR*, and *tcdE*, responsible for the synthesis and regulation of both toxins. This locus is stable and conserved in strains of *C. difficile*. The gene *tcdC* is a negative regulator of the production of toxins and is mutated in hypervirulent strains. The binary toxin (Cdt) is found in 5–30% of clinical isolates of *C. difficile*. The genes, *cdtA* and *cdtB* coding for this binary toxin, are not located on the PathLoc, but on the *Cdt* locus. The role of this toxin in the disease is not clear, but is frequently found in hypervirulent strains such as the ribotype 027 [4,5].

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Table 1
Growth and isolation of *C. difficile* colonies and presence of adjacent flora (semi quantitative quantification: few to +++) of 10 stool samples on four different selective media: CLO (*C. difficile* agar, bioMérieux), CDIFF (*Clostridium difficile* Selective agar, Thermofisher), BBL (*Clostridium difficile* Selective Agar, Becton Dickinson) and chromID C. Difficile (bioMérieux).

Stool #	CLO		CDIFF		BBL		chromID C. Difficile	
	No of colonies	Adjacent flora	No of colonies	Adjacent flora	No of colonies	Adjacent flora	No of colonies	Adjacent flora
1	16	+	2	++	10	++	>20	0
2	0	0	8	few	10	++	3	0
3	12	few	2	+	8	+	>20	0
4	8	+	1	++	8	++	>20	0
5	3	few	8	++	0	++	1	0
6	4	few	8	0	0	+	>20	0
7	>20	+	8	+++	0	++	>20	0
8	8	+	5	++	0	++	>20	0
9	6	++	5	++	0	+++	>20	0
10	6	few	5	+	8	+	15	0

The diagnosis of CDI relies on the identification of *C. difficile* toxins in stools samples either by antigenic tests detecting the presence of both toxins A and B and the GDH, which is specific to *C. difficile*, or by PCR assays based on the amplification of the toxin B gene. Specific stool cultures have been mostly abandoned, leading to the absence of isolates for further analyses such as molecular typing for epidemiological purpose.

In our laboratory, a stool sample is systematically stored frozen when positive by PCR for the toxin B gene. The aim of this study was to setup and evaluate a procedure that included culture of frozen samples, isolation of single colonies on which toxin genes were detected by PCR and molecular typing using our in-house Double Locus Sequence Typing (DLST) method [6].

2. Material and methods

2.1. Culture from frozen stool samples

In our microbiology laboratory, presence of toxigenic *C. difficile* is diagnosed with nucleic acid amplification tests (NAAT, Xpert® *C. difficile* BT, Cepheid, Sunnyvale, CA, USA). An aliquot of each positive *tcdB* gene stool is stored at −20 °C in a 1.5 ml microtube for two years.

We first evaluated four selective media: chromID *C. Difficile* (bioMérieux, France), *C. difficile* CLO media (bioMérieux, France), CDIFF *Clostridium difficile* Selective agar (Thermofisher) and BBL *Clostridium difficile* Selective Agar (Becton Dickinson, Dublin, Ireland). Aliquots of 10 stool samples, frozen for 17–18 months, were thawed and, if needed, sterile water was added to obtain a fluid sample, which was then inoculated onto each media with a 10-µl loop. Incubation was performed in strict anaerobic conditions for 24–48 h at 37 °C as recommended by the manufacturer. The number of colonies presumptive of *C. difficile* were counted and the adjacent flora was semi-quantitatively evaluated.

To evaluate the genetic diversity of *C. difficile* within stool, 29 stool samples were inoculated on chromID C. Difficile media with a 10-µl loop and incubated for 24 h. Up to 16 colonies per stool were isolated for further analysis by DLST. The first 18 stools had been collected between July to December 2020 from patients hospitalized in the pediatric ward. At that time, a chain of transmission was suspected between the first seven patients. The remaining eleven stools were recovered from patients hospitalized in the same medical ward over a three months period (mid-December 2020 to mid-April 2021).

2.2. PCR amplification of toxin genes and DLST sequencing

Toxin PCR and DLST sequencing was performed as previously reported [6] with the following changes. A bacterial suspension was obtained by homogenizing a full 1-µl loop collected on one isolated colony in 25 µl of sterile water. Toxin PCR consisted in the amplification of the *tcdA, tcdB, cdtB* and *tpi* genes, using the multiplex PCR kit as recommended by the manufacturer (Qiagen, Hilden, Germany). Primers were those previously proposed by Silva et al. [7]. In brief, for one reaction, mix PCR was composed of 3.5 µl of H_2O, 5.5 µl of 2× multiplex PCR kit (Qiagen) and 1 µl of mix primers at an initial concentration of 2 µM each. One µl of the bacterial suspension was added to 10 µl of the mix PCR. PCR was performed with an initial denaturation of 15 min at 95 °C followed by 34 cycles of 30 s at 95 °C, 90 s at 60 °C, and 90 s at 72 °C; followed by a final elongation of 10 min at 72 °C. Sequencing of DLST genes (C6 and TR6) was performed as already described [6]. For one reaction, the PCR mix was prepared with 5.5 µl of GoTaq Green master mix 2× (Promega, Madison, USA), 4 µl of H_2O and 0.5 µl of mix primers, each at an initial concentration of 10 µM. One microliter of the bacterial suspension was added to 10 µl of the mix PCR. PCR was performed with 5 min at 95 °C followed by 30 cycles of 30 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C; followed by a final elongation of 2 min at 72 °C. All PCR and sequencing were performed in 96-well plates.

2.3. Whole genome sequencing

C. difficile isolates were sequenced using the Illumina MiSeq platform. Sequence reads were analyzed using BioNumerics™ (version 8.0, created by bioMérieux, available at http://www.applied-maths.com) with default setting, except the *de-novo* assembly, which was performed using the Unicycler pipeline. Multilocus sequence typing (MLST) was determined with the public MLST scheme available at https://pubmlst.org/organisms/clostridioides-difficile. Genome comparison was done with whole genome Multi Locus Sequence Typing (wgMLST) using a scheme developed by Applied Maths. Clustering was performed using the categorical-difference coefficient and the tree was built using the unweighted pair group method with arithmetic mean (UPGMA) method.

3. Results

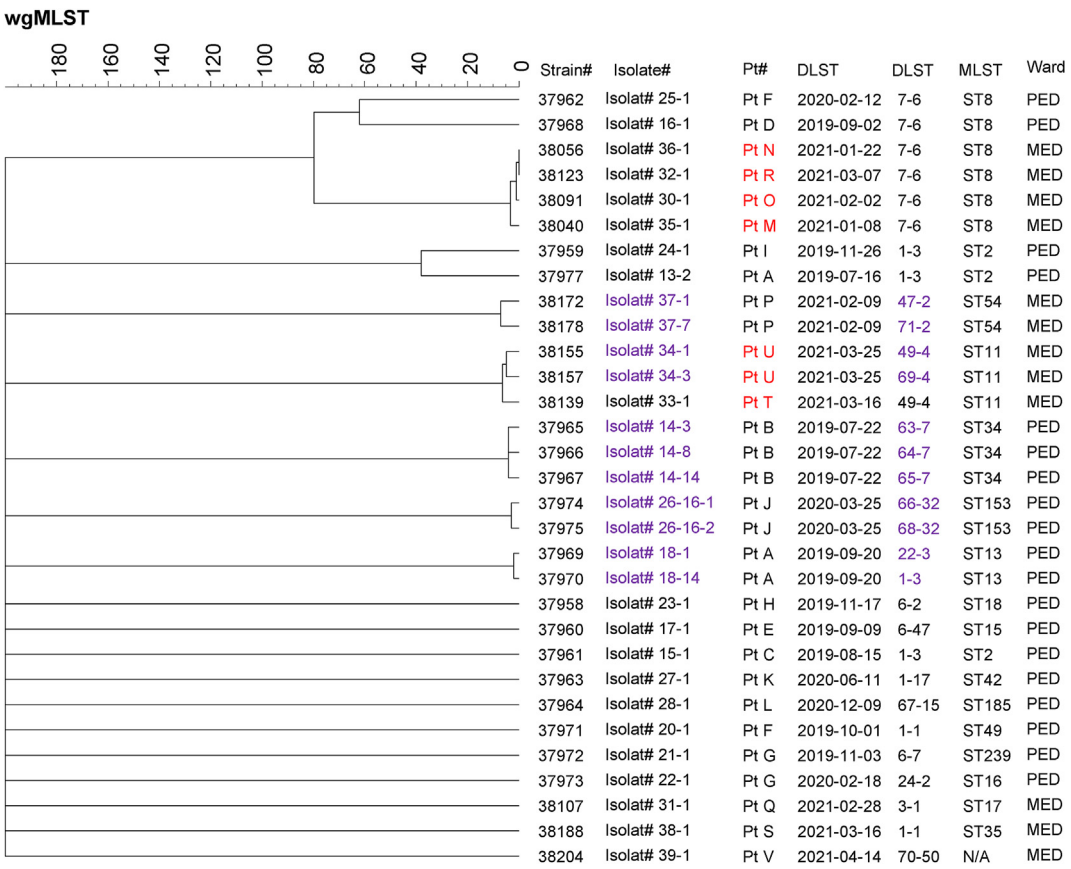
3.1. Evaluation of selective media

Ten frozen stool samples were cultured on the four selective media (#1 to 10). Numbers of isolated colonies showing a morphology characteristic of *C. difficile* (as described by the manufactory manual) and presence of adjacent flora were reported (Table 1). *C. difficile* morphologies were easily recognized on all media, except for the CDIFF media (Thermofisher) on which the growth of characteristic colonies other than *C. difficile* was observed. Identification was confirmed with Matrix Assisted Laser Desorption Ionization - Time of Flight (MALDI-TOF) and the amplification of the *tpi* gene by PCR. It is also on the CDIFF media that the adjacent flora was the most abundant. On the ChromID C.Difficile (bioMérieux), no adjacent flora was observed and *C. difficile* colonies were easy to recognize (black color with characteristic morphology after 24 h of incubation. According to these results, we used the ChromID C.Difficile (bioMérieux) for further analyses. Limitation of this media was

Table 2
Toxin PCR, DLST and MLST results from 385 colonies of *C. difficile* obtained from 29 stool specimens.

Stool #	Sample date	Original analysis on stool[a]					Culture	Toxin multiplex PCR profil				DLST[b]	MLST[c]
		CDBGX	CDBIGX	CD27GX	QTOXS	QAGS	Tested colonies	tpi+/A+/B+/bi−	tpi+/A+/B+/bi+	tpi+/A−/B−/bi−	tpi−/A−/B−/bi−		
11	July 11, 2019	pos	pos	neg	neg	pos	0	–	–	–	–	–	–
12	July 14, 2019	pos	pos	neg	neg	pos	0	–	–	–	–	–	–
13	July 16, 2019	pos	neg	neg	neg	pos	16	16	0	0	0	1-3 (n = 13) 62-2 (n = 3)	ST2
14	July 22, 2019	pos	neg	neg	pos	pos	16	16	0	0	0	63-7 (n = 13) 64-7 (n = 2) 65-7 (n = 1)	ST34 ST34 ST34
15	August 15, 2019	pos	neg	neg	pos	pos	16	16	0	0	0	1-3 (n = 16)	ST2
16	September 02, 2019	pos	neg	neg	neg	pos	16	16	0	0	0	7-6 (n = 16)	ST8
17	September 09, 2019	pos	neg	neg	neg	pos	1	0	0	1	0	6-47 (n = 1)	ST15
18	September 20, 2019	pos	neg	neg	neg	pos	16	16	0	0	0	22-3 (n = 14) 1-3 (n = 1)	ST13 ST13
19	September 20, 2019	pos	neg	neg	neg	pos	0	–	–	–	–	–	–
20	October 01, 2019	pos	neg	neg	neg	pos	16	16	0	0	0	1-1 (n = 16)	ST49
21	November 03, 2019	pos	neg	neg	neg	neg	16	16	0	0	0	6-7 (n = 16)	ST239
22	February 18, 2020	pos	neg	neg	pos	pos	16	16	0	0	0	24-2 (n = 16)	ST16
23	November 17, 2019	pos	neg	neg	neg	pos	16	16	0	0	0	6-2 (n = 16)	ST18
24	November 26, 2019	pos	neg	neg	neg	pos	16	16	0	0	0	1-3 (n = 16)	ST2
25	February 12, 2020	pos	neg	neg	pos	pos	16	16	0	0	0	7-6 (n = 16)	ST8
26	March 25, 2020	pos	neg	neg	pos	pos	16	16	0	0	0	66-32 (n = 15) 68-32 (n = 1)	ST153 ST153
27	June 11, 2020	pos	neg	neg	pos	pos	16	16	0	0	0	1-17 (n = 16)	ST42
28	December 09, 2020	pos	neg	neg	pos	pos	16	16	0	0	0	67-15 (n = 16)	ST185
29	January 15, 2021	pos	neg	neg	neg	neg	0	–	–	–	–	–	–
30	February 02, 2021	pos	neg	neg	pos	pos	16	16	0	0	0	7-6 (n = 16)	ST8
31	February 28, 2021	pos	neg	neg	pos	pos	16	16	0	0	0	3-1 (n = 16)	ST17
32	March 07, 2021	pos	neg	neg	neg	pos	16	16	0	0	0	7-6 (n = 16)	ST8
33	March 16, 2021	pos	pos	neg	pos	pos	16	0	16	0	0	49-4 (n = 16)	ST11
34	March 25, 2021	pos	pos	neg	neg	pos	16	0	16	0	0	49-4 (n = 15) 69-4 (n = 1)	ST11 ST11
35	January 08, 2021	pos	neg	neg	neg	neg	16	16	0	0	0	7-6 (n = 16)	ST8
36	January 22, 2021	pos	neg	neg	pos	pos	16	16	0	0	0	7-6 (n = 16)	ST8
37	February 09, 2021	pos	neg	neg	neg	pos	16	16	0	0	0	47-2 (n = 14) 71-2 (n = 2)	ST54 ST54
38	March 16, 2021	pos	neg	neg	pos	pos	16	16	0	0	0	1-1 (n = 16)	ST35
39	April 14, 2021	pos	pos	pos	pos	pos	16	0	16	0	0	70-50 (n = 16)	STNew

[a] GeneXpert Cdifficile: CDBGX, *tcdB* gene (toxin B); CDBIGX, gene *cdtA* (sub unit A of the binary toxin); CD27GX, specific deletion in the *tcdC* gene to ribotype 027. Antigenic assays: QTOXS, toxins A and B; QAGS, GDH.
[b] Double Locus Sequence Typing genotype; when SLV are present, the number of isolates per variant are indicated in parenthesis.
[c] Multi Locus Sequence Typing ST according to https://pubmlst.org/organisms/clostridioides-difficile.



Strain#	Isolate#	Pt#	DLST	DLST	MLST	Ward
37962	Isolat# 25-1	Pt F	2020-02-12	7-6	ST8	PED
37968	Isolat# 16-1	Pt D	2019-09-02	7-6	ST8	PED
38056	Isolat# 36-1	Pt N	2021-01-22	7-6	ST8	MED
38123	Isolat# 32-1	Pt R	2021-03-07	7-6	ST8	MED
38091	Isolat# 30-1	Pt O	2021-02-02	7-6	ST8	MED
38040	Isolat# 35-1	Pt M	2021-01-08	7-6	ST8	MED
37959	Isolat# 24-1	Pt I	2019-11-26	1-3	ST2	PED
37977	Isolat# 13-2	Pt A	2019-07-16	1-3	ST2	PED
38172	Isolat# 37-1	Pt P	2021-02-09	47-2	ST54	MED
38178	Isolat# 37-7	Pt P	2021-02-09	71-2	ST54	MED
38155	Isolat# 34-1	Pt U	2021-03-25	49-4	ST11	MED
38157	Isolat# 34-3	Pt U	2021-03-25	69-4	ST11	MED
38139	Isolat# 33-1	Pt T	2021-03-16	49-4	ST11	MED
37965	Isolat# 14-3	Pt B	2019-07-22	63-7	ST34	PED
37966	Isolat# 14-8	Pt B	2019-07-22	64-7	ST34	PED
37967	Isolat# 14-14	Pt B	2019-07-22	65-7	ST34	PED
37974	Isolat# 26-16-1	Pt J	2020-03-25	66-32	ST153	PED
37975	Isolat# 26-16-2	Pt J	2020-03-25	68-32	ST153	PED
37969	Isolat# 18-1	Pt A	2019-09-20	22-3	ST13	PED
37970	Isolat# 18-14	Pt A	2019-09-20	1-3	ST13	PED
37958	Isolat# 23-1	Pt H	2019-11-17	6-2	ST18	PED
37960	Isolat# 17-1	Pt E	2019-09-09	6-47	ST15	PED
37961	Isolat# 15-1	Pt C	2019-08-15	1-3	ST2	PED
37963	Isolat# 27-1	Pt K	2020-06-11	1-17	ST42	PED
37964	Isolat# 28-1	Pt L	2020-12-09	67-15	ST185	PED
37971	Isolat# 20-1	Pt F	2019-10-01	1-1	ST49	PED
37972	Isolat# 21-1	Pt G	2019-11-03	6-7	ST239	PED
37973	Isolat# 22-1	Pt G	2020-02-18	24-2	ST16	PED
38107	Isolat# 31-1	Pt Q	2021-02-28	3-1	ST17	MED
38188	Isolat# 38-1	Pt S	2021-03-16	1-1	ST35	MED
38204	Isolat# 39-1	Pt V	2021-04-14	70-50	N/A	MED

Fig. 1. UPGMA tree based on wgMLST data of *C. difficile* isolates (one isolate per DLST genotype and per stool). The scale of the tree represents the number of loci differences between isolates (max. 200). Isolate number is composed of the stool number and its isolate. Single locus variants found in the same stool are indicated in purple. Clusters of suspected transmission between patients are indicated in red.

later found during an extended investigation: 3/132 stools grew white-grey *C. difficile* colonies, all belonging to DLST 3–13 (MLST ST-5), and three other stools grew black colonies identified as *Hungatella* sp., a genus of obligate anaerobes present in the human gut microbiota and previously classified within the *Clostridium* genus).

3.2. Intra-stool genetic diversity of C. difficile

Twenty-nine frozen stool samples (#11 to 39, Table 2) were thawed and inoculated onto ChromID C. Difficile media. These samples were originally analyzed by NAAT and were all positive for the *tcdB* gene; three were positive for the binary toxin genes and none was positive for the ribotype 027 marker. Culture and molecular results are shown in Table 2. No growth was observed for four stools (#11, 12, 19 and 29), and only one colony grew for one stool (#17). From the remaining 24 cultures and in order to investigate the genetic diversity of *C. difficile* within each stool, 16 isolated colonies were analyzed with the toxin multiplex PCR and by DLST. Among the 385 isolated colonies, all were positive for *tcdA, tcdB,* and *tpi* genes, except the one for which a single colony could be recovered and it was found to be a non-toxigenic isolate. All 16 isolates from three stools (#33, 34, and 39) were positive for the binary toxin gene *cdtB*, of which two belonged to ST11 (ribotype 078). These results are congruent with NAAT results originally done on these stools (Table 2).

Among these 24 stool cultures with multiple isolates, 18 showed the presence of one DLST genotype and six cultures showed the presence of at least two genotypes, one of which being predominant (Table 2). Interestingly, for five of these six stools the mix DLST genotypes were single locus variant (SLV), e.g. DLST 63-7, 64-7 and 65-7 in stool #14. A deeper examination of these variant alleles showed either the difference was due to an IN/DEL of the GCAATA repeat in the locus TR6, or to an IN/DEL of the TAGCAA repeat in the C6 loci, which is one of the mechanism of diversification of these microsatellites loci. To verify if these mutations are important markers in the diversification of the strains, isolates from the five stools showing SLV (#14, 18, 26, 34 and 37; Table 2) were analyzed by wgMLST. With only 2 to 7 loci differences, SLV isolates were found to be genetically highly related (Fig. 1). This shows that in the same patient, variants of both DLST loci can be observed.

3.3. Epidemiological investigation

The first seven stool in Table 1 (#11 to 17) belonged to patients suspected to be part of a chain of transmission in the pediatric ward. Two stools could not be cultured. Among the remaining five patients, two harbored the same genotype DLST 1–3 and others were colonized with different genotypes. The last eleven stools from Table 2 (#29 to 39) belonged to patients hospitalized in the same medical ward over a three-month period. Four patients harbored the same genotype DLST 7-6 and two the DLST 49-4, but none were DLST 1–3.

One isolate per stool and per DLST genotype was selected (N = 31) to be analyzed by whole genome sequencing and wgMLST to further investigate their genetic relatedness. Results showed that isolates from the pediatric ward were all genetically distant from each other, including the two patients harboring DLST 1–3, which speaks against cross-transmission between these patients (Fig. 1). However, two clusters of respectively four and two patients with highly closely related isolates were observed in the medical ward. Isolates of the first cluster differed by

0–4 loci and those of the second cluster by 4–7 loci. These results suggested cross-transmissions. A retrospective epidemiological investigation pointed out possible contact between these patients (hospitalization in the same room/unit during overlapping periods).

4. Discussion

Our study showed that it is possible from frozen stool samples to grow *C. difficile* and perform a toxin genes detection by a multiplex PCR and molecular typing directly from isolated colonies without DNA extraction. Optimization of the multiplex PCR of toxin genes and sequencing of DLST loci yielded to a robust procedure: 100% of toxin PCRs and 97% of DLST sequences were obtained in the first assay.

Despite the small number of analyzed stool, our results showed that within the same stool sample, (i) a single toxigenic profile was observed, (ii) a single genotype was observed in 80% of the samples and (iii) when present, additional genotypes were in minority. Moreover, the great majority of these additional genotypes were single locus variant (SLV) of the predominant type and were genetically highly related, as shown by wgMLST. These results suggest a rapid diversification of both DLST allele in the same patient, what we did not suspected when we developed the DLST typing scheme. Thus, unlike previously reported [6], SLV should not be considered as possibly belonging to a different MLST sequence type. This preclude the use of this typing method as a first line approach for epidemiological surveillance or outbreak investigation of *C. difficile*. Further studies should be done to select more stable DLST markers.

Practically, using a 96-well plate, the analysis of 16 colonies per stool culture allowed the analysis of six samples in the same run. It is also possible to analyze height colonies from 12 samples or one isolate from 96 samples, with the risk of not detecting minority genotypes. The analysis of 96 colonies can be done in four working days and demands 8–10 h of technician work. We did not investigate if sequencing of both loci could be done directly on stool specimen as it was proposed for MLST [8]. The reason is that nowadays, further analysis with whole genome sequencing, such as wgMLST, is required to investigate outbreak, and this could be done only on isolated colonies. Interestingly, wgMLST revealed previously undetected chains of transmission, highlighting the value of this method and the need of a continuous surveillance for infection control purpose.

5. Conclusion

Provided that toxigenic stools are stored frozen for a defined period of time (one to two years), the developed protocol allows prospective and retrospective molecular and genomic epidemiological investigation for infection control purpose.

Ethical approval

The data were obtained during a quality enhancement project at our institution. According to national law, the performance and publishing of the results of such a project can be done without asking the permission of the competent research ethics committee.

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CRediT author statement

Dominique S. Blanc: Conceptualization, Methodology, Writing - Original Draft, Visualization, Supervision. **Fabrice Poncet:** Methodology, Investigation, Data Curation, Writing - Review & Editing. **Bruno Grandbastien:** Writing - Review & Editing. **Guy Prod'hom:** Writing - Review & Editing. **Gilbert Greub:** Writing - Review & Editing, Supervision. **Laurence Senn:** Methodology, Investigation, Writing - Original Draft & Review & Editing, Supervision.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

References

[1] Cohen SH, Gerding DN, Johnson S, Kelly CP, Loo VG, McDonald LC, et al. Clinical practice guidelines for *Clostridium difficile* infection in adults: 2010 update by the society for healthcare epidemiology of America (SHEA) and the infectious diseases society of America (IDSA). Infect Control Hosp Epidemiol 2010;31(5):431–55.

[2] Crobach MJT, Vernon JJ, Loo VG, Kong LY, Pechine S, Wilcox MH, et al. Understanding *Clostridium difficile* colonization. Clin Microbiol Rev 2018;31(2).

[3] Aktories K, Schwan C, Jank T. *Clostridium difficile* toxin biology. Annu Rev Microbiol 2017;71:281–307.

[4] Valiente E, Cairns MD, Wren BW. The *Clostridium difficile* PCR ribotype 027 lineage: a pathogen on the move. Clin Microbiol Infect 2014;20(5):396–404.

[5] Hunt JJ, Ballard JD. Variations in virulence and molecular biology among emerging strains of *Clostridium difficile*. Microbiol Mol Biol Rev 2013;77(4):567–81.

[6] Stojanov M, Magalhaes B, Terletsky V, Basset P, Prod'hom G, Greub G, et al. Development and evaluation of double locus sequence typing for molecular epidemiological investigations of *Clostridium difficile*. Eur J Clin Microbiol Infect Dis 2016;35(2):175–81.

[7] Silva RO, Santos RL, Pires PS, Pereira LC, Pereira ST, Duarte MC, et al. Detection of toxins A/B and isolation of *Clostridium difficile* and *Clostridium perfringens* from dogs in Minas Gerais, Brazil [publication of the Brazilian Society for Microbiology] Braz J Microbiol 2013;44(1):133–7.

[8] Griffiths D, Fawley W, Kachrimanidou M, Bowden R, Crook DW, Fung R, et al. Multilocus sequence typing of *Clostridium difficile*. J Clin Microbiol 2010;48(3):770–8.