Multilocus variable-number tandem repeat analysis: a helpful tool for subtyping French *Clostridium difficile* PCR ribotype 027 isolates

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The objective of this study was to evaluate the usefulness of multilocus variable-number tandem repeat analysis (MLVA) for typing and subtyping of *Clostridium difficile*. Sixty-eight strains were studied, including strains from PCR ribotypes 027, 078/126, 014/020/077, 017 and 023. The stability of variable-number tandem repeat (VNTR) loci was tested by comparing the MLVA results of two strains subcultured 11 times. After DNA extraction, seven tandem repeat loci (A6, B7, C6, E7, F3, G8, H9) from published MLVA schemes were amplified by PCR and sequenced. The distance between two strains was determined by calculating the summed tandem repeat difference. Genomic diversity was evaluated by using the minimum spanning tree (Bionumerics 5.1 software program; Applied Maths). Among the 68 *C. difficile* isolates examined, 65 unique MLVA types were identified, suggesting a high discriminatory power. An overall good agreement was observed between MLVA types and PCR ribotypes. The stability of VNTR loci was good. MLVA could separate isolates of the hypervirulent PCR ribotype 027 clone in several clusters; all 027 strains isolated within a hospital were grouped in a specific cluster or were placed very close to each other. Results of MLVA confirmed that strains from PCR ribotypes 078 and 126 were closely related although some were located in different branches of the tree. Similar results were observed for most strains from PCR ribotypes 014, 020 and 077. This highly discriminatory method is time-consuming and expensive, but is a valuable tool for subtyping of *C. difficile*, especially of 027 strains.

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INTRODUCTION

Clostridium difficile is an important spore-forming anaerobic enteropathogen found in soil and the intestinal tract of human and animals. This micro-organism is responsible for 10–25 % of antibiotic-associated diarrhoea cases and for virtually all cases of pseudomembranous colitis (Bartlett, 2010).

The incidence and severity of *C. difficile* infection (CDI) have risen worldwide over the last decade. These trends are assumed to be due in part to the emergence of a new hypervirulent and epidemic strain named 027. This strain has been responsible for extensive outbreaks in North America and Europe with a higher fatality rate and a poor clinical response to metronidazole (Kuijper *et al.*, 2007; Loo *et al.*, 2005; McDonald *et al.*, 2005). More complications (megacolon, septic shock) have been associated with

this strain (Pépin *et al.*, 2004). This clone is characterized by an 18 bp deletion and a single deletion at position 117 in the negative regulator *tcdC*, leading to the overproduction of toxins A and B. It also produces the binary toxin and is more resistant to antibiotics, particularly to erythromycin and newer fluoroquinolones (moxifloxacin, gatifloxacin).

France experienced its first 027 outbreak in early 2006, in northern France (Coignard *et al.*, 2006; Tachon *et al.*, 2006). This outbreak was detected, traced and investigated through the notification of severe cases or clusters and referral of strains to the *C. difficile* national reference laboratory for PCR ribotyping. PCR ribotyping is an amplification of variable intergenic regions between the 16S and 23S rRNA genes. This typing method is considered the gold standard for *C. difficile* typing in Europe although interpretation can be difficult when using gel electrophoresis (Indra *et al.*, 2008). Moreover, the discriminatory power is limited whenever a specific clone is predominant within a country or a hospital, such as 027, 014/020/077 or

Abbreviations: CDI, *Clostridium difficile* infection; MLVA, multilocus variable-number tandem repeat analysis; STRD, summed tandem repeat difference; VNTR, variable-number tandem repeat.

078/126 in France (Eckert *et al.*, 2010). Other genotyping methods are commonly used for epidemiological purposes, i.e. PFGE (PFGE of macrorestriction fragments of whole genome DNA) and restriction endonuclease analysis (restriction enzyme analysis is a restriction fragment pattern of whole genome DNA digested by *HindIII*). These methods are not discriminatory enough (PFGE) (Killgore *et al.*, 2008) or are sometimes difficult to interpret (restriction endonuclease analysis). An ideal typing method should be able to confirm a possible transmission from person to person especially when a clone of *C. difficile* is predominant within a hospital or a country (such as PCR ribotype 027). It should combine rapidity, high discriminatory power, ease of use and reproducibility. The MLVA [multilocus VNTR (variable-number tandem repeat) analysis] technique was first described for *C. difficile* by Marsh *et al.* (2006) and this technique has been improved by choosing new loci or deleting others (Bakker *et al.*, 2010; Marsh *et al.*, 2010; Tanner *et al.*, 2010; van den Berg *et al.*, 2007). This method has been shown to be more discriminatory than other typing methods (Killgore *et al.*, 2008; Marsh *et al.*, 2010) and has been successfully used for investigating *C. difficile* outbreaks (Killgore *et al.*, 2008). The objective of this study was to evaluate the usefulness of MLVA for typing and subtyping major PCR ribotypes isolated in France, such as 027, 078/126 and 014/020/077.

METHODS

Strains. A total of 68 *C. difficile* isolates were studied (Table 1). These strains included 18 strains of toxinotype III, PCR ribotype 027. Among these 027 strains, four were 'historical' PCR ribotype 027 fully susceptible to erythromycin and moxifloxacin and 14 were epidemic 027 isolates from sporadic cases ($n=4$) or from three different outbreaks ($n=10$) in northern France (Abbeville Hospital, Valenciennes Hospital and Draveil Hospital). Twenty-three strains of toxinotype V, PCR ribotype 078/126 were added. These strains included seven strains from three clinically documented outbreaks in three hospitals (Ivry sur Seine, Lille and Valenciennes) and 16 strains from unrelated CDI cases. Among the 23 strains, 10 were clearly identified as PCR ribotype 078 or 126 by a reference laboratory (J. Brazier, University Hospital of Wales, UK), whereas 13 could not be distinguished by our PCR ribotyping method and were classified as PCR ribotype 078/126 (Bidet *et al.*, 1999). Nine strains of PCR ribotypes 014 ($n=3$), 020 ($n=3$) and 077 ($n=3$) were also studied and compared to six strains of PCR ribotype 014/020/077 also indistinguishable by our PCR ribotyping method. Among the 014/020/077 strains, three strains originated from a possible transmission from a patient to a nurse in northern France. Five strains of toxinotype IV, PCR ribotype 023, isolated in France, and five strains of toxinotype VIII, PCR ribotype 017, isolated in three different countries, were included as well. Finally, reference strains CD630 (PCR ribotype 012) and VPI10463 (PCR ribotype 087) were also studied.

The stability of VNTR loci was tested using three different approaches. First, the typing results of two strains (CD630 and CD07-220) subcultured 11 times were compared. Second, five colonies from the same faecal specimen were also tested. Third, nine strains from three patients (A, B and C) suffering from multiple relapses (defined as a patient with a recurrence due to a strain

having the same PCR ribotype as the initial strain) were studied (Table 2).

PCR ribotyping and toxinotyping were performed as previously described by Bidet *et al.* (1999) and Rupnik *et al.* (1998), respectively.

DNA extraction. Isolates were subcultured on selective medium [brain–heart infusion agar supplemented with 5% (v/v) defibrinated horse blood, 0.1% (w/v) sodium taurocholate, 10 mg cefoxitin l^{-1} and 250 mg cycloserine l^{-1}] in an anaerobic atmosphere at 37 °C for 24 h. Isolates were stored at –80 °C in 1 ml brain–heart infusion broth containing 10% (v/v) glycerol. DNA was extracted using the InstaGene Matrix kit (Bio-Rad) according to the manufacturer's instructions.

MLVA typing. After DNA extraction, seven tandem repeat loci [A6, B7, E7, G8 (Marsh *et al.*, 2006; van den Berg *et al.*, 2007), C6, F3 and H9 (van den Berg *et al.*, 2007)] from published MLVA schemes were amplified individually by PCR with specific primers (Fawley *et al.*, 2008; van den Berg *et al.*, 2007; Zaiss *et al.*, 2009) as previously described. PCR products were purified and sequenced by MWG Biotech for repeat copy number determination. The number of tandem repeats at each locus was manually determined by sequence analysis (BioEdit; Ibis Therapeutics).

Data analysis. The distance between two strains was determined by calculating the STRD (summed tandem repeat difference) using the Manhattan coefficient. Genomic diversity was evaluated by using the minimum spanning tree (Bionumerics 5.1 software program; Applied Maths). The interpretation criteria were those described by Marsh *et al.* (2006) and Baines *et al.* (2008): isolates with an STRD ≤ 10 were defined as genetically related and clonal complexes were defined by an STRD ≤ 2 .

RESULTS AND DISCUSSION

Stability of VNTR loci

The stability of VNTR loci was studied by comparing MLVA patterns of strains CD630 (PCR ribotype 012) and CD07-220 (PCR ribotype 027), which were subcultured 11 times; different subcultures were tested (initial culture, 1st, 5th, 8th and 11th subcultures). Each isolate had the same MLVA type (STRD=0) (data not shown). These results indicate that loci are stable over time. They are in agreement with the results of van den Berg *et al.* (2007), who analysed two strains and showed that one strain was stable after 10 and 30 subcultures and the other one showed a difference of maximum one repeat unit after 10 and 30 subcultures; they concluded that a difference of one repeat unit between strains should not be interpreted as a different subtype (van den Berg *et al.*, 2007).

Furthermore, five colonies from the same faecal specimen were compared by MLVA and the same result was obtained for four isolates. An expansion of one tandem repeat (STRD=1) on locus A6 was observed for the last one (data not shown). A similar study was undertaken by Tanner *et al.* (2010). They investigated the presence of several subtypes of 027 strains in the same faecal sample. They found that 5 of 39 specimens yielded at least one isolate with a different MLVA profile (STRD ≥ 5) and showed that several subtypes of *C. difficile* 027 could coexist in the

Table 1. Description of the *C. difficile* isolates

A single type designation for PCR ribotypes 078, 126, 014, 020 and 077 was given when strains were clearly identified by a reference laboratory (J. Brazier, University Hospital of Wales, UK) and a group designation (014/020/77 or 078/126) was given by our laboratory.

Source	Strain	Toxinotype	PCR ribotype	A6*	B7	C6	E7	F3	G8	H9
Reference strain	CD630	0	012	39	17	15	7	7	6	4
Reference strain	VPI10463	0	087	27	8	34	5	6	11	2
Hungary	E IV 1	0	014	22	15	26	4	5	9	2
Belgium	A II 17	0	014	24	9	30	6	5	14	2
France	B I 9	0	014	38	14	23	7	5	9	2
Belgium	A III 22	0	020	27	10	23	5	5	11	2
Italy	F II 7	0	020	34	17	24	6	6	8	2
Hungary	E I 20	0	020	31	13	20	6	5	9	2
Hungary	E IV 7	0	077	34	17	38	8	5	7	2
Germany	C II 1	0	077	39	14	32	6	5	8	2
UK	Q I 8	0	077	30	10	38	5	6	8	2
Fresnes Hospital, France	CD09-133	0	014/020/077	40	18	41	6	5	8	2
Le Cateau Hospital, France	CD09-110	0	014/020/077	27	12	29	7	5	8	2
Lens Hospital, France	CD07-006	0	014/020/077	34	14	27	6	5	10	2
Lens Hospital, France	CD07-022	0	014/020/077	28	15	25	6	5	9	2
Lens Hospital, France	CD07-023	0	014/020/077	31	8	38	5	5	11	2
Paris Hospital, France	CD95-274	0	014/020/077	41	15	18	6	5	8	2
Reference strain	CD196	III	027 'historical'	14	11	49	9	5	9	2
Lyon Hospital, France	CD07-412	III	027 'historical'	18	14	41	9	5	17	2
Paris Hospital, France	CD06-Cha	III	027 'historical'	29	12	31	9	5	15	2
Caen Hospital, France	CD06-Por	III	027 'historical'	32	16	23	9	5	14	2
Abbeville Hospital, France	CD07-149	III	027	27	19	24	10	5	16	2
Abbeville Hospital, France	CD07-269	III	027	27	19	24	10	5	16	2
Abbeville Hospital, France	CD07-296	III	027	28	19	24	10	5	16	2
Belgium	A II 9	III	027	23	16	25	10	5	16	2
Belgium	A III 3	III	027	35	19	31	10	5	15	2
Belgium	A III 14	III	027	37	20	33	10	5	25	2
Paris Hospital, France	CD08-172	III	027	34	19	41	10	5	16	2
Draveil Hospital, France	CD09-037	III	027	13	15	10	9	5	15	2
Draveil Hospital, France	CD09-039	III	027	25	15	11	9	5	15	2
Draveil Hospital, France	CD09-040	III	027	13	15	10	9	5	15	2
Draveil Hospital, France	CD09-041	III	027	24	15	10	9	5	15	2
Valenciennes Hospital, France	CD07-214	III	027	39	19	40	10	5	16	2
Valenciennes Hospital, France	CD07-217	III	027	40	18	39	10	5	15	2
Valenciennes Hospital, France	CD07-220	III	027	40	19	41	10	5	16	2
Reference strain	Type 078	V	078	AA	25	41	8	5	13	2
France	B I 7	V	078	AA	20	25	10	5	9	2
Belgium	A II 33	V	078	AA	16	39	8	5	12	2
France	B II 8	V	078	AA	20	58	8	5	10	2
Reference strain	Type 126	V	126	AA	19	28	8	5	12	2
Germany	C II 8	V	126	AA	24	34	8	5	12	2
Germany	C I 1	V	126	AA	23	26	8	5	13	2
France	B II 5	V	126	AA	24	53	8	5	13	2
Spain	K III 2	V	126	AA	21	22	8	5	11	2
France	B II 2	V	126	AA	29	43	8	5	12	2
Ivry sur Seine Hospital, France	CD08-154	V	078/126	AA	36	36	8	5	12	2
Ivry sur Seine Hospital, France	CD08-156	V	078/126	AA	36	36	8	5	12	2
Lens Hospital, France	CD08-201	V	078/126	AA	25	46	8	5	11	2
Lille Hospital, France	CD08-096	V	078/126	AA	27	39	8	5	10	2
Lille Hospital, France	CD08-116	V	078/126	AA	27	38	8	5	10	2
Reference strain	CIP109238	V	078/126	AA	27	32	8	5	11	2
St Pol sur Ternoise Hospital, France	CD08-132	V	078/126	AA	24	29	8	5	11	2
Paris Hospital, France	CD08-329	V	078/126	AA	22	24	8	5	13	2

Table 1. cont.

Source	Strain	Toxinotype	PCR ribotype	A6*	B7	C6	E7	F3	G8	H9
Sambre Avesnois Hospital, France	CD08-135	V	078/126	AA	35	39	8	5	11	2
Valenciennes Hospital, France	CD07-069	V	078/126	AA	26	37	8	5	15	2
Valenciennes Hospital, France	CD07-070	V	078/126	AA	28	27	8	5	15	2
Valenciennes Hospital, France	CD07-076	V	078/126	AA	26	35	8	5	12	2
Valenciennes Hospital, France	CD07-212	V	078/126	AA	26	33	8	5	12	2
Abbeville Hospital, France	CD07-193	IV	023	AA	14	39	7	4	12	2
Maubeuge Hospital, France	CD08-202	IV	023	AA	17	24	7	4	19	2
Paris Hospital, France	CD08-141	IV	023	AA	14	32	8	4	20	2
Sambre Avesnois Hospital, France	CD09-029	IV	023	AA	15	26	7	4	18	2
Sambre Avesnois Hospital, France	CD08-248	IV	023	AA	26	23	8	4	22	2
Sweden	M I 7	VIII	017	AA	10	20	8	6	35	2
Poland	H I 1	VIII	017	AA	10	17	7	6	34	2
Arras Hospital, France	CD09-104	VIII	017	AA	23	21	9	6	30	2
Montfermeil Hospital, France	CD08-004	VIII	017	AA	10	28	8	6	31	2
Paris Hospital, France	CD08-223	VIII	017	AA	7	28	8	6	35	2

*AA, Absence of amplification.

same sample (Tanner *et al.*, 2010). We also studied the *in vivo* 'stability' of MLVA by comparing strains from recurrences in three patients. Table 2 presents the time elapsed between the CDI episodes and results of MLVA typing. Recurrences were caused by an identical isolate for patients A and C (STRD ≤ 1); for patient B, two episodes were caused by the same isolate (STRD=1). The third isolate was genetically related, with a difference at a single locus (STRD=3); however, this isolate corresponded to PCR ribotype 014/020/077, the most frequent PCR ribotype in France, and no conclusion can be drawn between a reinfection with a new strain belonging to the same PCR ribotype or a relapse (Eckert *et al.*, 2010). Interestingly, Goorhuis *et al.* (2009) studied seven patients suffering from recurrences with a PCR ribotype 017 strain and found that more than half of the recurrences (56 %) were actually caused by different isolates (STRD from 4 to 30) despite identification of an identical PCR ribotype.

MLVA subtyping

MLVA was performed on 68 arbitrarily selected *C. difficile* strains (Fig. 1). Amplification was obtained for the loci of all the strains tested except for locus A6, which was not amplified for the 33 strains of PCR ribotypes 023, 017, 078 and 126. This absence of amplification has already been described by several authors (Bakker *et al.*, 2010; Marsh *et al.*, 2006; Zaiss *et al.*, 2009). It can be explained by the absence of this locus as it was shown by sequence analysis for seven human type 078 strains and eight porcine type 078 strains (Bakker *et al.*, 2010). The absence of this locus does not have an impact on the numbers of genetically related clusters and clonal complexes for ribotype 027 and 017 strains as shown by Bakker *et al.* (2010).

A great diversity in the number of repetitions was noted for each locus except for loci F3 and H9 (Tables 1 and 3). Tanner *et al.* (2010) did not use these loci in their MLVA

Table 2. Application of MLVA to isolates from three patients suffering from recurrences of CDI

Patient	Sample no.	Date (d/m/y)	Differing locus*	STRD	PCR ribotype
A	CD94-687	30-03-1994	None	0	012
	CD94-875	28-04-1994			012
B	CD95-274	08-02-1995	A6	3	014/020/077
	CD95-404	01-03-1995			014/020/077
C	CD95-2526	20-10-1995	C6	1	014/020/077
	CD96-372	26-02-1996	None	0	Unknown†
	CD96-449	08-03-1996			Unknown†
	CD96-527	20-03-1996	B7	1	Unknown†
	CD96-581	27-03-1996	None	0	Unknown†

*Compared to the first isolate.

†PCR ribotype unknown but identical.

Fig. 1. Minimum-spanning tree analysis of MLVA data from 68 strains of *C. difficile*. The circles represent unique MLVA types (alphanumeric value); the small and large circles represent one and two isolates, respectively. PCR ribotypes (colour-coded) are indicated: epidemic PCR ribotype 027 (fuschia) or 027H (for historical) (pink), 078 (light-green), 126 (dark-green), 078* (for 078/126) (green), 023 (purple), 017 (brown), 014 (turquoise), 020 (light-blue), 077 (grey), 014* (dark-blue) for 014/020/077, 012 (fluorescent green) and 087 (olive). The numbers between the circles represent the STRDs between MLVA types. Coloured areas identify genetically related isolates defined by an STRD ≤ 10 . Outbreaks in hospital are enclosed.

typing scheme as they were invariant within PCR ribotype 027 isolates in accordance with our results. The loci A6, B7, C6 and G8 were most polymorphic with 20, 25, 32 and 21 alleles, respectively (Table 3). Unlike most previously published MLVA schemes using capillary electrophoresis (Baines *et al.*, 2008; Debast *et al.*, 2009; Fawley *et al.*, 2008; Goorhuis *et al.*, 2009; Killgore *et al.*, 2008; Marsh *et al.*, 2010; van den Berg *et al.*, 2007), an analysis of the number of VNTRs by sequencing was used because this method is more accurate for defining the number of tandem repetitions. Pasqualotto *et al.* (2007) showed that differences of up to 6 bases could be observed between the results of the two methods. Moreover, according to the sequence analysis, some changes in the tandem repeat

motif were seen in particular for PCR ribotype 078 and 126 strains (locus H9). As the method is based on the copy number of the same repetitions, these modifications were not taken into account in the analysis.

Among the *C. difficile* isolates examined, 65 unique MLVA types were identified, suggesting a high discriminatory power, while PCR ribotyping and toxinotyping identified 10 and 5 unique types, respectively. However, it was also shown by Tanner *et al.* (2010) that MLVA is perhaps too discriminatory as strains from a single faecal specimen could be differentiated by MLVA, suggesting that the results of epidemiological studies may vary depending on the colony picked up from the stool sample. Moreover, MLVA could be inappropriate for establishing a distant phylogenetic relationship (Zaiss *et al.*, 2009). In this study, 10 clusters (defined by an STRD ≤ 10) were identified including from 2 to 21 strains (Fig. 1). The largest complex consisted of 21 isolates corresponding to toxinotype V or PCR ribotype 078/126, which is one of the predominant PCR ribotypes in France.

An overall good agreement was observed between MLVA types and toxinotypes (Fig. 1), with the exception of two strains of toxinotype IV and V that were found to be genetically related (MLVA types BL and AK, light-pink cluster). Although strains of toxinotype IV and V are both characterized by the production of binary toxin and a deletion in the *tcdC* gene, strains exhibited different PCR

Table 3. Number of alleles and copy number for each locus based on analysis of 68 isolates

Locus	No. of alleles	Copy no. range
A6	20	0–41
B7	25	7–36
C6	32	10–58
E7	7	4–10
F3	4	4–7
G8	21	6–35
H9	2	2–4

ribotype patterns and differences on the PaLoc (Rupnik *et al.*, 1998). Isolates of different PCR ribotypes were located in different areas on the tree. Isolates of PCR ribotypes 078/126, 023 and 017 (toxintype V, IV and VIII, respectively) were well separated from all other strains.

MLVA results indicated that PCR ribotype 078 and 126 strains were closely related (STRD ≤ 10) for 21 strains whatever their PCR ribotypes: MLVA types AV and B1 corresponded to PCR ribotype 078 and MLVA types AT, AQ, AL, AR, AZ and AW corresponded to PCR ribotype 126. Moreover, our 078/126 strains, indistinguishable by our PCR ribotyping method, were also found in the same pink cluster. These results suggest that MLVA is not a helpful method to distinguish between these two PCR ribotypes. Interestingly, strains from each outbreak were clustered inside the pink cluster (STRD ≤ 5) (MLVA type AM, MLVA types AC and AH, MLVA types AB, AA and AX). These results are in accordance with previous studies showing that 078 or toxintype V isolates were genetically related but distinct from the rest of the isolates (Debast *et al.*, 2009; Marsh *et al.*, 2010). Another study showed that 85 % of the 078 strains from human or porcine origins were genetically related irrespective of the country of origin (Bakker *et al.*, 2010).

PCR ribotype 017 strains were closely related (brown circles) although three lineages could be distinguished. Strains from toxintype VIII were shown to be responsible for outbreaks, and country-specific clonal complexes were found in a previous study (Goorhuis *et al.*, 2009).

MLVA could separate isolates of the hypervirulent PCR ribotype 027 clone in several clusters; all 027 strains isolated within a hospital were grouped in a specific cluster or were placed very close to each other. The three strains from Valenciennes (MLVA types BT, AU and AE) are in the blue cluster and the three strains from Abbeville (MLVA types AF and AD) are in the yellow cluster. Two of the strains responsible for an outbreak in Draveil are in the grey cluster; the other two are close to this cluster (MLVA type AI, STRD=11). Interestingly, historical 027 strains (MLVA types BV, BU, BW and BX) are not closely related and are not related to the epidemic 027 strains. Loci A6 and B7 (formerly CDR4 and CDR49 in the study of Marsh *et al.*, 2006) were found to be highly variable and could differentiate the hospital-specific clones as described by Marsh *et al.* (2010). These results suggest that MLVA represents a helpful method for subtyping 027 strains.

We also included strains of PCR ribotype 014/020/077, the most prevalent PCR ribotype group in France, to compare with 014, 020 and 077 previously described as individual PCR ribotypes. These PCR ribotypes are usually difficult to distinguish by PCR ribotyping using gel electrophoresis of PCR amplicons. MLVA results indicated that there was not a specific lineage for 014, 020 or 077. These data suggest that these three PCR ribotypes are closely related although some strains are located in different branches of the tree. Interestingly, three strains with the same PCR ribotype (i.e.

014/020/077) were isolated from two patients and a nurse from the same ward over a period of 7 days, suggesting possible transmission from a patient to a nurse. MLVA typing indicated that strains from the first patient and the nurse clustered in the green area (MLVA types BA and BB), suggesting nosocomial transmission, whereas a strain from the second patient was not related (MLVA type BC).

Conclusion

MLVA has been shown to be highly discriminatory and was able to distinguish hospital-related clusters of PCR ribotype 027, which is endemic in some countries like in northern France. MLVA is also useful to investigate outbreaks at the local level and to understand the molecular evolution of *C. difficile*. However, strains from PCR ribotypes 014, 020 and 077 on one hand and strains from PCR ribotypes 078 and 126 on the other hand could not be clearly distinguished by MLVA. MLVA remains an interesting tool to confirm cross-transmission of *C. difficile*, although it is a time-consuming and expensive method.

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