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1 **Local** outbreak of *Clostridioides difficile* PCR-Ribotype 018 investigated by Multi Locus
2 Variable Number Tandem Repeat Analysis, Whole Genome Multi Locus Sequence Typing and
3 core genome Single Nucleotide Polymorphism **typing**.

4

5 Cécile Gateau ^{a,c}, Stéphanie Deboscker ^b, Jeanne Couturier ^{a,c}, Thomas Vogel ^d, Elise Schmitt
6 ^d, Joris Muller ^b, Céline Ménard ^e, Béatrice Turcan ^b, Rabab Syed Zaidi ^a, Anlyata Youssouf ^a,
7 Thierry Lavigne ^b, Frédéric Barbut ^{a,c}

8 Corresponding author: cecile.gateau@aphp.fr

9 ^a National reference Laboratory for *Clostridium difficile*, Hôpital Saint-Antoine, 34, rue
10 Crozatier, Paris, France

11 ^b Equipe Opérationnelle d'Hygiène, CHU de Strasbourg, 1, place de l'Hôpital, 67091
12 Strasbourg, France

13 ^c Université Paris Descartes, Faculté de Pharmacie, Inserm UMR S-1139, 4 Avenue de
14 l'Observatoire, 75006 Paris, France

15 ^d Service de Soins de Suite et de Réadaptation Gériatrique, CHU de Strasbourg, 1, place de
16 l'Hôpital, 67091 Strasbourg, France

17 ^e Laboratoire de bactériologie, CHU de Strasbourg, 1, place de l'Hôpital, 67091 Strasbourg,
18 France

19

20

21 **Abstract**

22 The prevalence of *Clostridioides difficile* PCR-ribotype (RT) 018 is low in Europe but variations
23 are observed across countries. We report here the first RT 018-related outbreak in France
24 that took place in 4 geriatric units (GU) in Strasbourg, France. From January to December
25 2017, 38 patients were diagnosed with *C. difficile* infection (CDI). Strains were first
26 characterized by PCR ribotyping: 19 out of 38 (50%) strains belonged to RT 018. These strains
27 as well as 12 RT 018 isolated in other French healthcare facilities and 2 strains of RT 018
28 isolated in the GU in 2015 were characterized by multi locus variable-number tandem repeat
29 (VNTR) analysis (MLVA), whole genome multi locus sequence typing (wgMLST) and core
30 genome single nucleotide polymorphism typing (cgSNP). The MLVA indicated that 15 out of
31 19 epidemic strains of RT 018 were included in 2 Clonal Complexes (CC). Four RT 018 strains
32 from the outbreak did not belong to the CC. The wgMLST and cgSNP typing analysis revealed
33 a single CC that included 19 strains from the geriatric unit (17 from GU in 2017 and 2 from
34 GU in 2015) and 4 strains (33%) from other healthcare facilities (HCF). Our results suggest
35 that a specific RT 018 clone has spread in the geriatric unit and has evolved slowly over time.
36 MLVA, wgMLST and cgSNP typing results provided fairly consistent information but wgMLST
37 and cgSNP typing better separated epidemic strains from non-epidemic strains. Compared to
38 wgMLST, the cgSNP typing did not provide additional information.

39

40 **Key words:** *C. difficile*, Outbreak, PCR-ribotyping, MLVA, wgMLST, cgSNP

41

42 Introduction

43 *Clostridioides* (formerly *Clostridium*) *difficile* (*C. difficile*) is an anaerobic Gram-positive spore-
44 forming bacillus responsible for a wide spectrum of clinical symptoms ranging from mild self-
45 limited diarrhea to pseudomembranous colitis (PMC), toxic megacolon, septic shock and
46 possible death [1,2]. Previous antibiotic treatment, hospitalization, advanced age and severe
47 underlying diseases represent major risk factors for *C. difficile* infection (CDI) [3]. Recent data
48 have indicated that *C. difficile* is the leading cause of healthcare-associated infections with
49 more than 453,000 CDI-cases per year, leading to 29,600 deaths in the U.S.A [4]. In Europe,
50 the estimated number of healthcare-associated CDI cases in 2013 was 126,000 per year with
51 a 3% attributable mortality [5]. The incidence of CDI has been increasing worldwide partly
52 due to the emergence of an epidemic strain (NAP1/027/BI) responsible for large outbreaks
53 of severe CDI in the last few years [6,7]. Recent European surveillance has shown that
54 NAP1/027/BI is still predominant and represents 19% of all isolates but a wide variation of
55 ribotype distribution is observed across countries [8]. For example, prevalence of PCR-
56 ribotype (RT) 018 is high in Italy (22%), as opposed to other European countries (< 3%). *C.*
57 *difficile* RT 018 has been reported as an emerging RT associated to severe CDI and outbreaks
58 in Italy and more recently in Germany, Korea and Japan [9–12]. A study comparing RT 018,
59 RT 126 and RT 078 indicated that RT 018 isolates produce higher levels of toxins and have an
60 increased adhesion to cells [9]. Moreover, all RT 018 strains were multidrug-resistant
61 (resistance to erythromycin, clindamycin and moxifloxacin). These results suggest that RT
62 018 strains have phenotypic traits conferring an adaptive advantage and are able to spread
63 widely.

64

65 We report here the first RT 018-related outbreak in geriatric units in France that was
66 investigated by different molecular typing techniques including MLVA (**multi locus** VNTR
67 [variable number-tandem repeat] analysis), wgMLST (whole genome multi locus sequence
68 typing) and cgSNP **typing** (core genome single nucleotide polymorphism).

69

70 **Materials and methods**

71 **Setting**

72 The outbreak took place in 2 geriatric wards from a single university-affiliated hospital in
73 Strasbourg, France. The first ward is composed of 3 different units (A, B, C). The second ward
74 is composed of 1 unit (D). These different geriatric units (GU) (A, B, C, D) are located on 4
75 floors of the same building. Each unit has between 24-28 beds in single or double rooms. In
76 2017, the first ward performed 4,774 admissions with a total of 32,900 patient-days (PD) and
77 the second ward performed 816 admissions with a total of 8,622 patient-days. **The incidence**
78 **increased from 3.24/10000 PD in 2016 to 9.15/10000 PD in 2017.**

79 **CDI diagnosis**

80 A CDI case was defined as a patient with a clinical diarrhea (≥ 3 diarrheal stools/day) and a
81 positive test for *C. difficile* in stools. **CDI testing was performed upon physician's request and**
82 **systematically in case of nosocomial diarrhea.** CDI testing was based on a two-step algorithm
83 using glutamate dehydrogenase (GDH) **detection** by enzyme immunoassay (EIA) (C. Diff Quik
84 Chek, Alere) as a screening method followed by PCR targeting toxin genes (GenXpert *C.*
85 *difficile*, Cepheid). In addition, for all CDI cases, stools were cultured on ChromID plates
86 (bioMérieux, Marcy l'Etoile, France) and *C. difficile* isolates were sent to the National
87 Reference Laboratory for *C. difficile* (Paris) for molecular typing. CDI onset after 3 days of
88 hospitalization or community-onset CDI in a patient with an overnight hospital stay within
89 the previous 12 weeks was classified as hospital-associated CDI; others were community-
90 associated CDI [13]. Patient information and medical history of all CDI cases during this
91 outbreak were collected from the electronic medical records.

92 **Strain characterization**

93 ***Antibiotic susceptibility***

94 Susceptibility to erythromycin (ERY), clindamycin (CM) moxifloxacin (MXF), metronidazole
95 (MZ), vancomycin (VA) and tetracycline (TET) was determined using the disk diffusion
96 method according to the CA-SFM 2013 (Comité de l'Antibiogramme de la Société Française
97 de Microbiologie)[14]. Briefly, strain dilution (10^8 CFU/ml) was inoculated on Brucella Agar
98 (Becton Dickinson) supplemented with vitamin K1 (1mg/ml) (Emprove, Merck), hemin (5
99 mg/l) (Applichem) and defibrinated horse blood (5%) (Oxoid). Plates were incubated 48h at
100 35-37°C and diameters were interpreted according to the criteria for anaerobic bacteria
101 given by the CA-SFM 2013. Resistance breakpoints were set as follows: ERY (15 UI) < 15 mm;
102 CM (2 UI) < 15 mm; MXF (5 µg) < 18 mm; MZ (5 µg) < 21 mm; VA (30 µg) < 17 mm; TET (30
103 µg) < 19 mm. *C. difficile* ATCC 700057 was used as quality control.

104 ***PCR-ribotyping (European CDI Surveillance Network: ECDIS-net protocol)***

105 *C. difficile* isolates were characterized by high-resolution capillary gel-based electrophoresis
106 PCR-Ribotyping [15] on the ABI 3500 sequencer (Applied Biosystems), using primers
107 described by Bidet *et al.* [16]. The PCR ribotypes (RT) were determined using the freely
108 available WEBRIBO database (<https://webribo.ages.at/>) [17].

109 ***Toxin gene profiles***

110 Genes encoding toxin A (*tcdA*), toxin B (*tcdB*), and the two components of binary toxin (*cdtA*
111 and *cdtB*) were detected from colonies by multiplex PCR, as previously described [18].

112

113

114 **MLST (Multi locus sequence typing)**

115 MLST was performed according to Griffith 's method [19]. Seven housekeeping genes (i.e.
116 *adk*, *atpA*, *dxr*, *glyA*, *recA*, *sodA* and *tpi*) were amplified by PCR and sequenced as previously
117 described [19]. Finally, the amplified sequences were used to query the database
118 (<http://pubmlst.org/cdifficile/>) to obtain the sequence type (ST).

119 **MLVA [Multi locus VNTR (variable number-tandem repeat) analysis] typing**

120 Seven VNTR (A6, B7, C6, E7, F3, G8, H9) were amplified for MLVA analysis, using forward
121 primers labeled at the 5' end with a fluorochrome, as described elsewhere [20]. The number
122 of tandem repeats was determined for each strain by capillary gel-based electrophoresis and
123 concatenated to generate a MLVA type, using BioNumerics software (Applied Maths NV,
124 Sint-Martens-Latem, Belgium). The genetic distance between two strains was determined by
125 calculating the STRD (summed tandem repeat difference), corresponding to the differences
126 in total number of repetitions for all loci. Genomic diversity was represented by the
127 minimum spanning tree (MST) using the Manhattan coefficient. Isolates with an STRD ≤ 10
128 were defined as genetically related and clonal complexes were defined by an STRD ≤ 2 [21].

129 **Whole genome sequencing (WGS)**

130 **We performed WGS on 31 strains.** *C. difficile* isolates were subcultured on Columbia agar
131 (bioMérieux) for 48 h in anaerobic atmosphere. DNA was extracted using UltraClean
132 Microbial DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA). Then, the DNA library
133 was performed using Nextera XT DNA library preparation and Nextera XT index kits (Illumina,
134 San Diego, CA, USA). Finally, we generated 2x150 base paired-end reads using MiSeq
135 Reagent kit v3 on a MiSeq instrument (Illumina). The quality of reads was evaluated with

136 BioNumerics 7.6.3 (Applied Maths NV, Sint-Martens-Latem, Belgium): isolates with average
137 quality below 30 and estimated coverage below 30 were excluded from subsequent analysis.

138

139 ***Whole genome multi locus sequence typing (wgMLST) analyses***

140 wgMLST analyses were performed using BioNumerics 7.6.3. Briefly, alleles were identified by
141 the combination of a kmer frequency approach using raw reads and assembly-based BLAST
142 approach using SPAdes [22] assembled genomes against a pan-genome locus scheme of *C.*
143 *difficile* developed by Applied Maths. This wgMLST scheme contains 8745 coding loci,
144 representing a pan-genome of *C. difficile* identified from 259 previously published genomes
145 [23]. Once all alleles were assigned to each genome, a minimum spanning tree (MST) was
146 constructed. Loci with no allele calls and loci with less than 80% identity were ignored in the
147 pairwise comparison during the tree construction. WgMLST scheme is more detailed in the
148 supplementary data. The genetic relationship between two isolates was assessed by
149 calculating the number of different alleles for wgMLST. We defined 2 isolates as genetically
150 related or belonging to the same complex clonal (CC) when they had an allelic difference \leq
151 200 or ≤ 20 , respectively.

152 ***Core-genome Single-Nucleotide polymorphism typing (cgSNP) analyses***

153 Once the genomes were assembled (described above), one outbreak isolate was selected
154 based on the quality of the assembly (lowest number of contigs, highest N50). This full
155 genome was used as the reference genome for mapping and SNP calls after removal of
156 contigs smaller than 1000 bp. Mapping was performed with Bowtie2 [24]. The genetic
157 relationship between two isolates was assessed by calculating the number of SNP. SNPs
158 were kept for analysis if the region was mapped in all genomes, if the coverage was

159 sufficient (5 reads in total, at least 1 read forward and 1 read reverse), if none of the
160 genomes contained ambiguous bases or gaps at this position and if no other SNP occurred
161 within 12 bp. SNPs were visualized on the reference genome to identify hotspots of SNPs
162 that could be the result of recombination and no evidence for recombination events were
163 seen.

164 We defined 2 isolates as genetically related when they had a SNP difference ≤ 100 and in the
165 same clonal complex ≤ 10 .

166 **Statistical analysis**

167 Statistical analyses were performed using the R software, version 3.3.3 (R Core Team - 2017
168 R Foundation for Statistical Computing, Vienna, Austria). Patient demographic characteristics
169 were described by their mean and standard deviation for quantitative variables and by the
170 frequency for categorical variables. Quantitative and categorical variables were compared
171 between patients infected with RT 018 and those infected with another RT 018 by Mann-
172 Whitney-Wilcoxon and Fisher exact tests. A p value of < 0.05 was considered significant.

173 **Results and discussion**

174 **Fig. 1 shows an increase in the number of CDI cases in 2017 (38) compared to 2016 (13) and**
175 **2018 (24). (Fig.2)**

176 Most of the patients with CDI (18/38, 47%) were located in Unit B on the 4th floor (**Fig. 2**).

177 The peak of the outbreak occurred during the first 4 months of 2017 (22/38; 58%).

178 Among the 38 CDI cases, 33 strains (isolated from 32 patients) were sent to the national
179 reference laboratory for characterization. Nineteen strains (57.6%) belong to RT 018
180 whereas the remaining 14 belong to RT 020 (9.1%), RT 106 (9.1%), RT 015 (6.1%), RT 014

181 (3%), RT 012 (3%), RT 005 (3%), RT 002 (3%), RT 076 (3%) and RT AI-60 (3%). All epidemic RT
182 018 isolates were positive for toxins A and B genes and had pseudogenes for binary toxin.
183 They all belong to ST 17, **MLST clade 1**.

184 We compared the clinical features of patients infected with RT 018 (n = 19), to those
185 infected with other RT (n = 13) (**Table 1**). No significant difference was observed for age,
186 gender, leukocyte count > 15,000 cells/mm³, incontinence, antibiotic treatment in the
187 previous 90 days, and origin of CDI. Nevertheless, patients infected with RT 018 strains had
188 a higher 90-day all-cause mortality rate compared to those infected by other RT. These data
189 are consistent with the higher virulence of RT 018 which has been associated with a higher
190 toxin production, increased adhesion to cells and an enhanced sporulation rate [9]. These
191 microbiological features could also in part explain the rapid local spreading and interpatient
192 transmissibility observed for strains RT 018 in the hospital environment.

193 **Table 2** compares antimicrobial susceptibility testing of 19 RT 018 strains and 14 other RT
194 strains. All 33 strains were susceptible to metronidazole and vancomycin but resistant to
195 clindamycin. However, resistance rate to erythromycin and moxifloxacin was significantly
196 higher for RT 018 compared to the other RT (p < 0.001). Erythromycin and moxifloxacin
197 resistance has been described in RT 018 outbreak strains from Korea, Germany and Japan
198 [12,25,26]. In Italy, an analysis was performed on a large number of strains and showed that
199 100% of RT 018 were erythromycin, moxifloxacin and clindamycin resistant compared to
200 only 75% for RT 027 [27]. The high-level of fluoroquinolone resistance is a very common trait
201 for epidemic RT and may have conferred a selective advantage to strains RT 018 in patients
202 treated by antibiotics [28].

203 Recent studies have shown that WGS has a superior discriminatory power compared to
204 other molecular methods [29,30]. The accuracy of WGS allows different approaches to
205 assess genomic variations: **nucleotide polymorphisms** and allelic profiles (wgMLST or
206 cgMLST) [31]. **Recently, a study comparing a set of different *C. difficile* strains showed that the
207 cgMLST was as discriminatory as the SNP analysis** [32]. In the present study, we determined the
208 genetic relationship between RT 018 by cgSNP **typing** and wgMLST method and we
209 compared the results with MLVA. Indeed, MLVA has been previously used to successfully
210 subtype strains from the same PCR-ribotype [20].

211 We performed MLVA and WGS on 31 strains: 17 RT 018 strains (CD17-039, CD17-041, CD17-
212 042, CD17-043, CD17-124, CD17-125, CD17-126, CD17-127, CD17-128, CD17-132, CD17-133,
213 CD17-134, CD17-136, CD17-244, CD17-245, CD17-248, CD17-313) isolated from the GU in
214 2017 and 2 RT 018 strains isolated in the same GU in 2015 (CD15-230, CD15-235). We also
215 included 12 strains isolated outside of the hospital in Strasbourg: 9 epidemiologically
216 unrelated RT 018 strains isolated in Paris (SA11-109, SA11-123, Lu15-003), Caen (Lu14-078),
217 Montélimar (CD14-251), Cholet (CD17-161), Toulon (CD15-058), and unknown origin
218 (Co2566), and 3 RT 018 strains isolated from a local HCF in Strasbourg (CD17-179, CD17-180,
219 CD17-200). Among these 3 strains, 2 strains (CD17-179, CD17-200) were isolated from
220 recurrences in a patient who presented a first episode of CDI (CD17-041) at a Strasbourg
221 hospital 6 months before.

222 The minimum spanning tree analysis by MLVA is presented in **Fig.3**. The analysis indicated
223 that among the 31 RT 018 strains, 19 were included in 2 close CC separated by 5 STRD on 2
224 loci. The first one (cluster 1) comprised 9 strains (53%), all isolated in patients from the GU
225 and the second one (cluster 2) included 10 strains (6 strains (35%) from the GU and 4 strains

226 (36%) from other HCF (CD15-058, CD17-179, CD17-180, CD17-200). Four strains (21%) of the
227 GU did not belong to the CC including two epidemic strains isolated in 2017 and 2 strains
228 isolated in 2015. These results suggest that MLVA can successfully subtype strains belonging
229 to the same RT, as previously shown for RT027, RT0021 and RT076 [20,33].

230 The minimum spanning tree analysis of wgMLST and cgSNP typing are showed in **Fig.4** and
231 **Fig.5**.

232 wgMLST and cgSNP typing gave consistent information. A recent study investigating
233 *Staphylococcus aureus* outbreaks has shown that these two methods provide comparable
234 epidemiological discrimination, but that they vary in their overall suitability for
235 epidemiologic or infection control settings [34]. All the RT 018 isolates from the outbreak
236 clustered in the same CC that also included 4 strains (36%) from other HCF (CD15-058, CD17-
237 179, CD17-180, CD17-200). Among these 4 strains, 1 (CD17-180) was isolated in another HCF
238 in Strasbourg and 1 (CD15-058) was isolated in a city far from Strasbourg. This strain was
239 also present in one of the 2 CC generated by MLVA. We failed to collect information about
240 these patients and their medical history but we can hypothesize that they likely had
241 connections with the present outbreak **or were exposed to a common source**. The 2 other
242 strains present in the CC (CD17-179 and CD17-200) came from the same patient who had
243 presented a first episode of CDI (CD17-041) in a Strasbourg hospital, 6 months before.
244 However, the strains CD17-179 and CD17-200 were different from CD17-041 by 1 allele as
245 showed by wgMLST. **In contrast to whole genome SNP analysis, wgMLST is based on the**
246 **concept of allelic variation, meaning that recombinations, deletions or insertions of multiple**
247 **positions are indeed counted as a single evolutionary event. This approach might be**
248 **biologically more relevant than approaches that consider only point mutations, as it both**

249 captures isolated point mutations as well as close-by point mutations or gene exchanges
250 that occur as one event in the DNA. So, this variation suggests a slight evolution over time.
251 wgMLST and cgSNP typing analyses also indicated that the 2 RT 018 strains isolated in 2015
252 in the geriatric unit belonged to the same CC, suggesting that this clone was already present
253 in the ward, either in the environment or in asymptomatic carriers, and emerged again in
254 2017. The 2 cases of CDI in 2015 appeared 2 months apart. However, in Strasbourg hospital,
255 specific hygiene measures are implemented only when 2 cases of CDI occur in the same unit
256 within 15 days. These 2 cases were therefore not considered linked, and no specific hygiene
257 measure were implemented, most likely contributing to dissemination of the strain in 2017.
258 During the CDI outbreak in 2017, the hygiene measures were reinforced by a daily
259 cleaning/disinfection using a sporicidal product (Oxyfloor, Anios).

260 Considering that the outbreak was well documented and occurred in a short period of time
261 and in a restricted space, we were expecting a cluster of epidemic isolates. Our results
262 showed that wgMLST and cgSNP typing gave more relevant results than MLVA from an
263 epidemiological prospective. A recent study showed that, compared to the WGS, the
264 absolute numbers of STRD obtained by MLVA can over- or under-estimate direct relatedness
265 between epidemiologic isolates due to method-related reasons (low number of loci
266 explored, high variability of the genetic targets) [12]. Zhou *et al.* compared cgSNP typing with
267 MLST and highlighted the ability of cgSNP typing to differentiate several lineages within the
268 same MLST clade [35].

269 WGS-based methods seem to be the most accurate methods for investigating outbreaks
270 and will likely outweigh very shortly MLST and MLVA which are currently widely used in
271 microbiological laboratories [35,36] [37].

272 **Conclusion**

273 This first outbreak of *C. difficile* RT 018 in France associated with a high 90-day all-cause
274 mortality rate confirmed the potential of dissemination and the high virulence of this
275 emerging ribotype. MLVA, wgMLST and cgSNP typing analysis of the isolates gave quite
276 consistent information but the wgMLST and the cgSNP typing better separated epidemic
277 strains from non-epidemic strains.

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279

280

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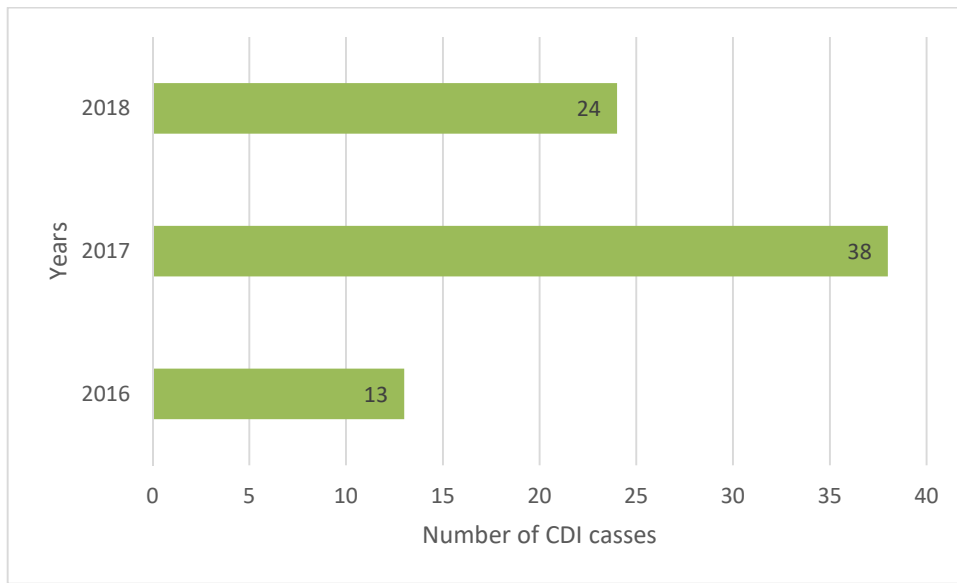
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1 **Fig. 1.** Evolution of the number of CDI cases per year during the years 2016, 2017 and 2018.



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3 **Fig. 2.** Epidemic curve of CDI cases per month (January 2017- December 2017). Each color
 4 represents a geriatric unit localized on 4 floors.

5 *Asterisk: strains not sent to the national reference laboratory for *C. difficile* (*unavailable RT*)

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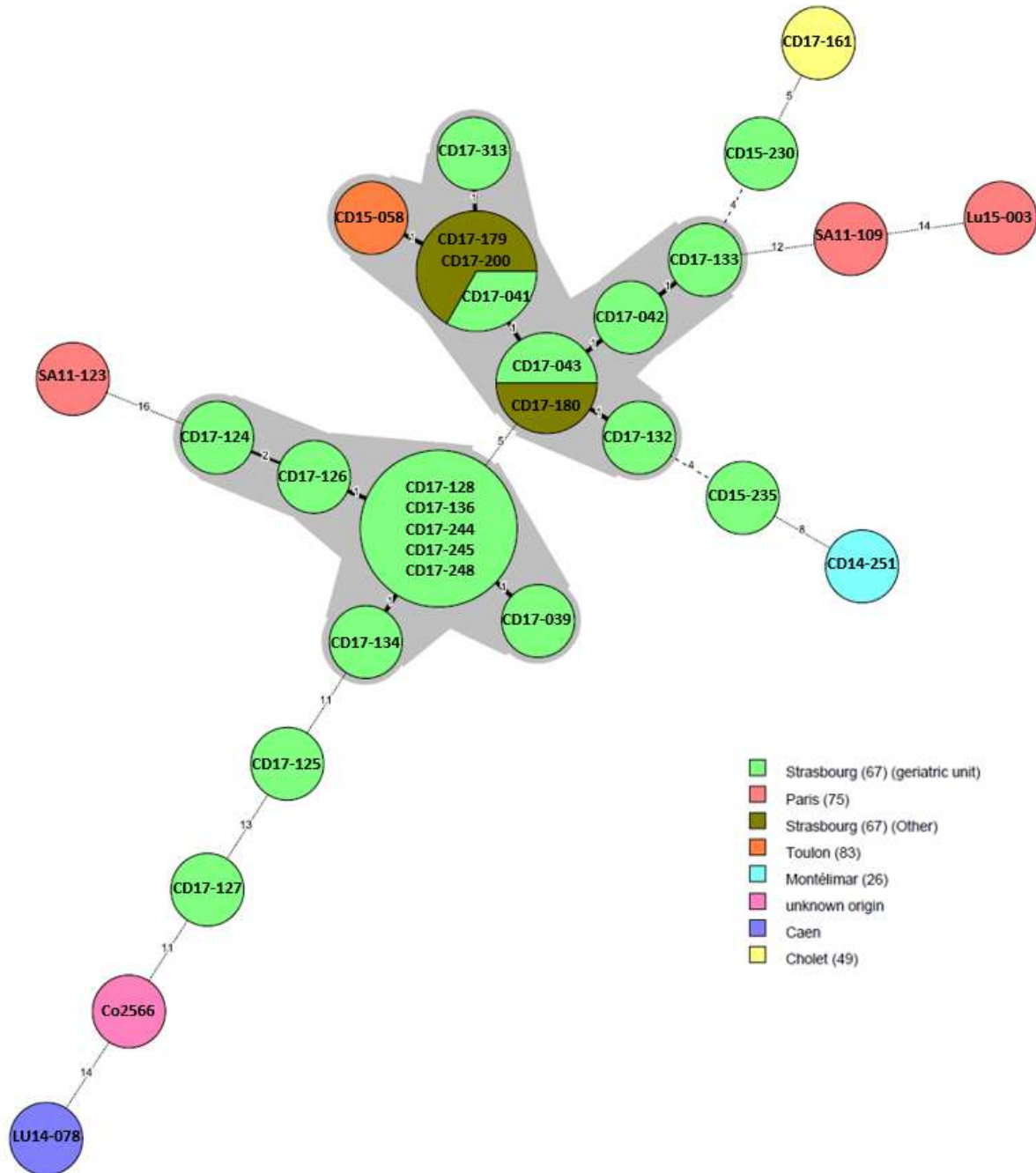
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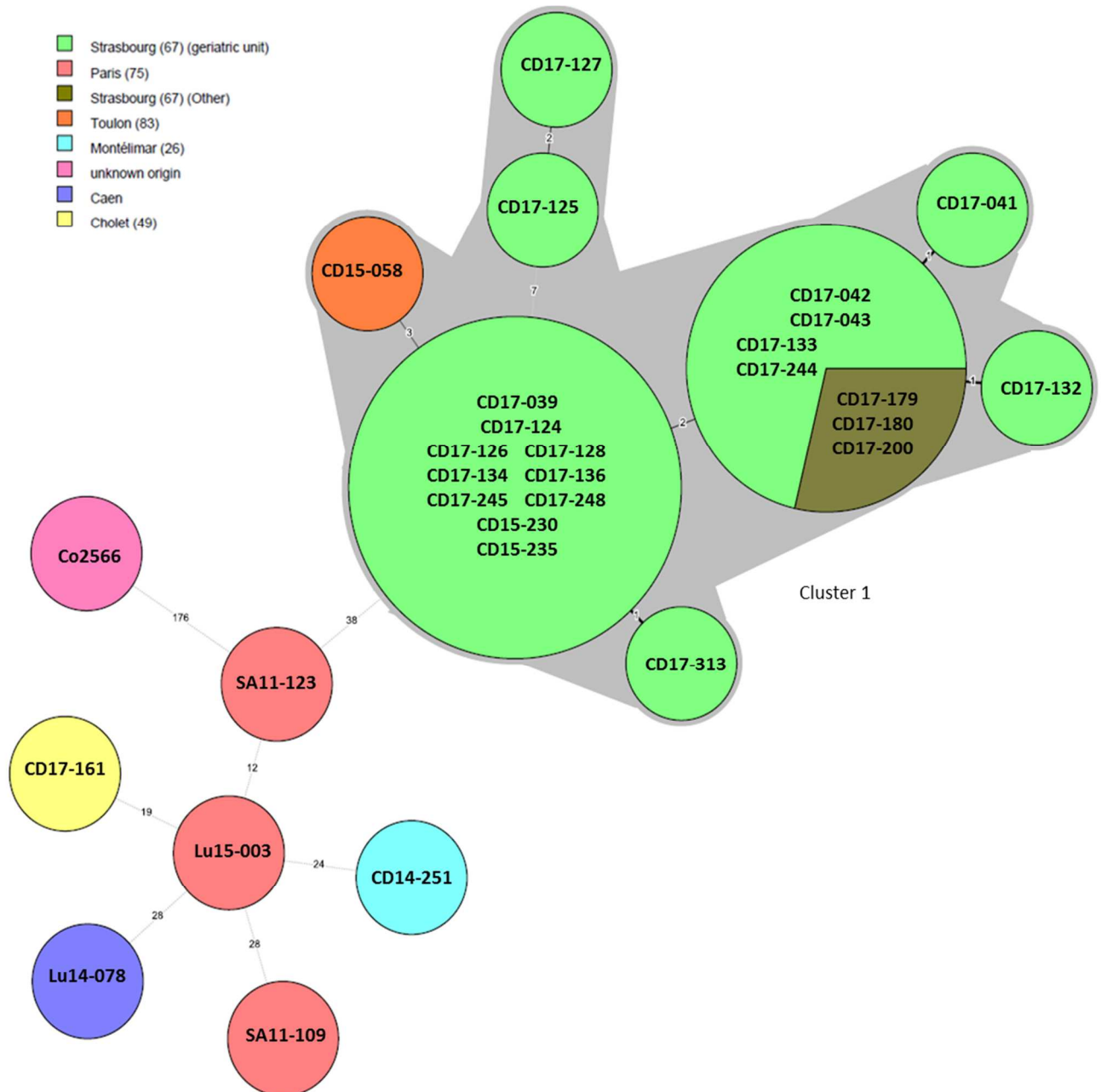
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12 **Fig. 3.** MLVA analysis with minimum spanning tree of PCR-ribotype 018 strains. A different
 13 color is assigned to each HCF. Each circle represents a single MLVA-type, the size of the circle
 14 being proportional to the number of isolates included. The numbers between the circles
 15 correspond to the number of STRD between the MLVA-types. MLVA-types differing on 1 or 2
 16 loci are connected by solid lines, those differing on 3 or more loci are connected by a dashed
 17 line. The gray areas represent clusters, grouping the genetically linked strains.



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22 **Fig. 4.** wgMLST analysis with minimum spanning tree of PCR-ribotype 018 strains. A different
 23 color is assigned to each HCF. Each circle represents a single wgMLST-type, the size of the
 24 circle being proportional to the number of isolates included. The numbers between the
 25 circles correspond to the number of allele between the wgMLST types. wgMLST types
 26 differing on 1 to 3 loci are connected by solid lines, those differing on 4 or more loci are
 27 connected by a dashed line. The shaded areas represent clusters, grouping the genetically
 28 linked strains.



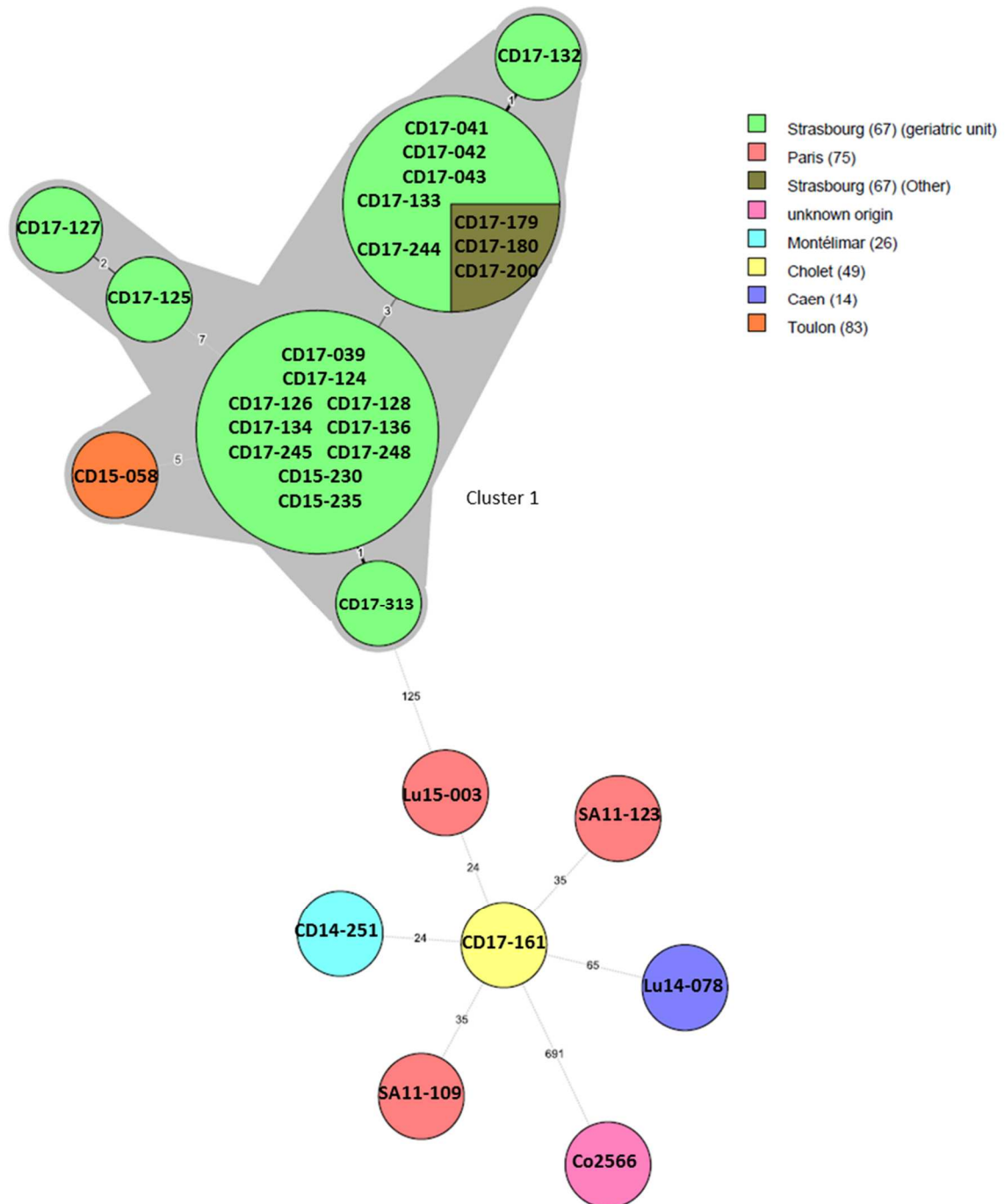
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32 **Fig. 5.** cgSNP typing analysis with minimum spanning tree of PCR-ribotype 018 strains. A
 33 different color is assigned to each HCF. Each circle represents a single cgSNP type, the size of
 34 the circle being proportional to the number of isolates included. The numbers between the
 35 circles correspond to the number of SNP between the cgSNP types. cgSNP types differing on
 36 1 to 3 loci are connected by solid lines, those differing on 4 or more loci are connected by a
 37 dashed line. The shaded areas represent clusters, grouping the genetically linked strains.

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1 **Table 1.** Clinical features of 32 CDI patients

Variables	RT 018	Other RT	<i>p</i> ^a
Age (mean ± SD), years	86 ± 8	88 ± 6	0.324
Gender (Male), n/N (%)	8/19 (42)	4/13 (31)	0.713
Leukocyte count (> 15 000 cells/mm ³), n/N (%)	6/18 (33)	2/11 (18)	0.671
Incontinence, n/N (%)	9/16 (56)	9/12 (75)	0.434
Antibiotic (in the previous 90 days), n/N (%)	18/19 (95)	13/13 (100)	1
Hospital-associated CDI , n/N (%)	18/19 (95)	12/13 (92)	1
90-days all causes mortality, n/N (%)	12/19 (63)	3/13 (23)	0.036

^a *p* values were calculated using Mann-Whitney Wilcoxon or Fisher's exact test.

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15 **Table 2.** Comparison of antimicrobial resistance patterns of RT 018 and other ribotype
16 strains.

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	Resistance n/N (%)		<i>p</i> ^a
	RT 018	Other RT	
Erythromycin (ERY),	19/19 (100)	3/14 (21)	< 0.001
Clindamycin (CM),	19/19 (100)	14/14 (100)	1
Moxifloxacin (MXF),	19/19 (100)	0/14 (0)	< 0.001
Metronidazole (MZ),	0/19 (0)	0/14 (0)	1
Vancomycin (VA),	0/19 (0)	0/14 (0)	1
Tetracycline (TET),	0/19 (0)	0/14 (0)	1

^a *p* values were calculated using Fisher's exact test

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