The recognition and characterisation of Finnish Clostridium difficile isolates resembling PCR-ribotype 027

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Abstract Purpose: To characterise and compare twenty-eight Finnish Clostridium difficile RT027-like isolates, selected based on the presence of 18 bp deletion in the tcdC gene and toxin gene profile (A, B, binary), with eleven RT027 isolates from different Finnish geographical areas and time periods.
Methods: Twenty-eight C. difficile RT027-like isolates and 11 RT027 comparative strains were characterised by capillary-electrophoresis (CE) ribotyping, multi-locus variable tandem-repeats analysis (MLVA), multi-locus sequence typing (MLST), and sequencing of tcdC and gyrA gene fragments. Susceptibility to moxifloxacin was determined by E-test.
Results: Of 28 RT027-like isolates, seven RTs (016, 034, 075, 080, 153, 176 and 328), three WEBRIBO types (411, 475, AI-78) and three new profiles (F1 e F3) were identified. MLVA revealed six clonal complexes (RTs 016, 027, 176 and F3). MLST showed eleven sequence types (1, 41, 47, 67, 95, 191, 192, 223, 229, 264 and new ST). Twenty-two isolates (RTs 016, 080, 176, 328, F1, F2, F3 and WRTAI-78) carried Δ117 in the tcdC gene. Isolates of RTs 016, 027 and 176 were moxifloxacin resistant and harboured Thr82Ile in the GyrA.
Conclusion: Our results show a high diversity within 28 Finnish RT027-like C. difficile isolates, with twelve CE-ribotyping profiles and eleven STs. MLVA revealed the regional spread of RTs 016, 027, 176 and F3. The presence of Δ117 in the tcdC gene in eight non-027 RTs highlights the importance of careful interpretation of the results from molecular systems targeting this

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**Introduction**

*Clostridium difficile* is the leading pathogen of hospital acquired diarrhea and PCR-ribotype 027/NAP1/B1 is the most notorious “hypervirulent” ribotype. Its route of global spread was traced by phylogeographic analysis of whole genome sequencing data indicating that two distinct epidemic lineages of *C. difficile* RT027 (FQR1 and FQR2) emerged in North America and separately acquired fluoroquinolone resistance and a conjugative transposon. The FQR2 lineage spread to the UK, continental Europe and Australia.

CE-ribotyping is currently the recommended standard for characterization of *C. difficile* isolates. Based on slight variations in ribotyping banding patterns, three RTs (176, 198 and 244) have been determined as closely related to RT027. Interestingly, RT176 is associated with outbreaks in the Czech Republic and Poland, whereas RT244 emerged in Australia. Recently, other RT027-like ribotypes (016, 036) have been reported that belong to the same multilocus sequence type (ST1) as RT027.

Finland is a country with a high CDI testing frequency (124.3 tests in 2011–2012 and 223.3 tests per 10,000 patient bed-days in 2012–2013) and corresponding high CDI incidence (14.9 cases in 2011–2012 and 28.7 cases per 10,000 patient bed-days in 2012–2013). In Finland, a national free of charge ribotyping service has been available for clinical microbiology laboratories since 2008. This service is mainly for *C. difficile* strains from patients with severe course of *C. difficile* infection (CDI) and for supporting the management of local CDI outbreaks. During 2008–2015, a total of 1771 isolates, representing 4.1% of notified CDI cases (0.2-5.9% range by year) were sent to the Finnish national reference laboratory for molecular characterisation. Of 1771 typed *C. difficile* isolates representing 146 different RTs by gel-based ribotyping, 662 (37%) tested positive for binary toxin genes belonged to 28 different RTs. Of these 662 isolates, 344 were RT027 (harboured 18 bp deletion), 253 had 39/54 bp deletion, 37 had no deletion, and 28 isolates showed similar molecular characteristics to RT027 (contained genes for toxins A, B and binary toxin and had an 18 bp deletion in the tcdC gene).

The aim of the study was to characterise and compare twenty-eight Finnish RT027-like *C. difficile* isolates with eleven RT027 isolates from different Finnish geographical areas and time periods.

**Material and methods**

**Strain collection**

Twenty-eight tcdA (toxin A), tcdB (toxin B) and cdtA, cdtB (binary toxin) positive *C. difficile* isolates also containing 18 bp deletion in the tcdC gene were identified at the Finnish national reference laboratory. Eleven Finnish *C. difficile* RT027 isolates from different Finnish geographical areas and time periods were included as comparative strains. CE-ribotyping, confirmation of toxin gene profiles, MLVA, MLST and susceptibility testing were performed at the Department of Medical Microbiology, Motol University Hospital, Czech Republic.

**Capillary electrophoresis-ribotyping**

DNA was extracted using Chelex-100 resin (Bio-Rad). Amplification of the 16S–23S intergenic spacer regions was performed according to the consensus CE-ribotyping protocol with Bidet primers. Fragment analysis was carried out using ABI 3130 with default settings for POP7 and 36 cm capillary length. LIZ 1200 was used as a size standard. The electrophoretic profiles were compared with the ECDC-Leiden *C. difficile* reference dataset in the Leiden University Medical Centre, the Netherlands. The raw data obtained (*fsa files) were also uploaded to the freely available WEBRIBO database (<https://webribo.ages.at/>) to compare with profiles present in the database.

**The presence of toxin genes and tcdC gene fragment sequencing**

The presence of genes (tcdA, tcdB, cdtA, cdtB) for toxin production (A, B and binary) was investigated by multiplex PCR with visualization of amplified products by agarose-gel electrophoresis. The tcdC gene fragment was amplified and sequenced with primers C1 and C2, and obtained sequences were compared with NCBI reference sequence NC_009089.1.

**Multi-locus variable tandem-repeats analysis**

MLVA was performed by sequencing of seven regions with short tandem repeats (A6Cd, B7Cd, C6Cd, E7Cd, F3Cd, G8Cd, H9Cd) with a change of reverse primer for G8Cd loci, as described elsewhere. Minimum spanning tree was created using BioNumerics v5.1 (Applied Maths) by using a Manhattan coefficient to calculate the summed tandem repeat difference (STRD). A clonal complex was defined as an STRD ≤ 2.

**Multi-locus sequence typing**

MLST was performed in 22 non-clonal related isolates (based on results of MLVA) by amplification and sequencing of seven housekeeping genes (adk, atpA, dxr, glyA, recA, sodA and tpi) previously described. The sequence type (ST) was determined as a combination of alleles identified...
by comparing obtained sequences with sequences available in the *C. difficile* MLST database available at: [http://pubmlst.org/cdifficile/].

**Testing of susceptibility to moxifloxacin**

Susceptibility to moxifloxacin was determined by E-test strips (Liofilchem, Italy) with gradient antibiotic range from 0.016 to 32 mg/L on Wilkins Chalgren agar. A breakpoint of 4 mg/L for moxifloxacin was applied. The fragment of the *gyrA* gene was amplified and sequenced with primers *gyrA* and *gyrA*2 and obtained sequences were compared with NCBI reference sequence NC_009089.1.

**Results**

The summary of results of molecular characterisation of 39 Finnish *C. difficile* isolates (28 RT027-like and 11 RT027) is shown in Table 1.

**Capillary electrophoresis-ribotyping**

Twelve different CE-ribotyping profiles in twenty-eight RT027-like isolates were observed. Four of these, RTs 016 (n = 3), 075 (n = 3), 080 (n = 1), 176 (n = 5), were identically identified by the WEBRIBO database and by the ECDC-Leeds-Leiden reference strain dataset. In contrast, one CE-ribotyping profile was recognized by the WEBRIBO database as a WRT475, and by the ECDC-Leeds-Leiden reference strain as RT034. Two CE-ribotyping profile were identically identified by the WEBRIBO database and by the NCBI reference sequence NC_009089.1.

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

MLST of seven housekeeping genes, performed in 22 non-clonal related *C. difficile* isolates belonging to thirteen different CE-ribotyping profiles, revealed eleven different sequence types (STs), of which, ten clustered to clade 2 and one (ST191) to clade 1. RTs 016 (n = 2), 027 (n = 5) and 176 (n = 3) belonged to ST1. Other found STs were: ST41 (F1, F3), ST47 (WRT411), ST42 (RT153), ST45 (RT027), ST91 (WRT417), ST192 (RT080), ST223 (RT343), ST229 (RT328) and ST264 (F2) and new ST (RT027, n = 2). The alleles profile of new ST in both RT027 isolates was: *adk* = 1, *atpA* = 5, *dxt* = 11, *glyA* = 17, *recA* = 1, *sodA* = 22 and *tpi* = 2.

**Testing of susceptibility to moxifloxacin**

Nineteen *C. difficile* isolates belonging to RTs 016 (n = 3), 027 (n = 11), 176 (n = 5) were moxifloxacin resistant (≥32 mg/L) and also carried amino acid substitution Thr82Ile in the GyrA. Other *C. difficile* isolates (n = 20) were moxifloxacin susceptible (MICs = 0.016–1.5 mg/L) and were wild types in the sequenced *gyrA* gene fragment.

**Discussion**

Of 1771 genotyped Finnish *C. difficile* isolates, 662 (37.3%) carried binary toxin genes. Of these, 372 (21.0%) also had an 18 bp deletion in the *tdcC* gene. A majority of the isolates (n = 344, 19.4%) were RT027. The remaining 28 isolates were considered as RT027-like and represented 1.6% of the Finnish *C. difficile* collection. These twenty-eight RT027-like *C. difficile* isolates belonged to twelve different ribotyping profiles. Among these twelve CE-ribotyping profiles, one was incorrectly identified (RT034 as WRT475) in the WEBRIBO database, two were only typed by ECDC-Leeds-Leiden database (RT328 and 153) two were identified only in WEBRIBO database (WRT411 and AI-78) and three CE-ribotyping profiles were completely new (F1–F3), not present in both databases.

Several CE-ribotyping profiles in the study revealed closer fragment peaks similarity to ribotypes present in the ECDC-Leeds-Leiden database. The strains F3 has a closest match to RT016, WRT411 to RT375 and WRTAI-78 to RT046.
Table 1: Molecular characteristic of Finnish *C. difficile* isolates in the study. WT — wild type, ST — sequence type, MOX — moxifloxacin, MLVA — Multi-Locus variable tandem-repeats analysis, MLST — multi-locus sequence typing, WRT — WEBRIBO type.

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The minor differences of the CE-ribotyping profiles with split of peaks suggest genetic relatedness, but additional differences were also found, such as a different ST (RT016 = ST1 and F3 = ST41) or different toxin genes profile (RT046 harbour only tcdA and tcdB) indicating more rearrangements in the C. difficile genomes. RTs 080, 034, 153, 328; WRTs AI-78, 411 and new CE-ribotyping profiles F1, F2 are represented by only one isolate in the collection and their distribution in Finland and Europe remains unclear.

The occurrence of RT176 has been reported from Czech Republic where it belongs to the prevailing ribotypes and from Poland where it persists with RT027. The clinical relevance of RT176 has been studied in both countries. Interestingly, four out of five Finnish RT176 isolates in this study were sent for molecular typing because of a severe course of CDI. Three RT027-like isolates in the study belonged to RT016. The increased occurrence of RT016 was identified in North East England in 2009-2010.

Twenty-two (78.6%) C. difficile RT027-like isolates (RTs 016, 176, 080, 328, WRTAI-78 and F1, F2, F3) had Δ117 in the tcdC gene. The Δ117 is used as a target site for differentiating RT027 from other ribotypes, and the presence of Δ117 in the tcdC gene in non-RT027 isolates leads to the incorrect RT027 identification by molecular methods. This highlights the need of molecular characterisation of C. difficile isolates by ribotyping for CDI surveillance purposes. Interestingly, the ECDC-Leeds-Leiden reference strain RT080 included for confirmation of MLST result also revealed substitution A > G at position 117 in the tcdC gene, which differs from Finnish RT080 C. difficile isolate.

As was recently published by Eyre et al., RT244 isolates (n = 25) also harboured Δ117 but in the absence of other deletions in the tcdC gene. Because the presence of 18 bp deletion was one of inclusion criteria for isolates in this study, we might not have recognized isolates with only Δ117 in the tcdC gene in the Finnish strain collection.

MLVA revealed six clonal complexes in RTs 016, 027, 176 and F3. Isolates revealing STRD ≤ 10 but belonging to different RTs were not considered as genetically related because the MLVA is suitable as subtyping molecular...
method in isolates belonging to the same RT. MLVA was firstly used for subtyping of C. difficile RT176 isolates in the study Nyc et al. where genetically as well as clonal relatedness in ten Czech (n = 10) and in eleven Polish isolates was confirmed. Clonal spread of RT176 was also found in two Czech single center studies in 2013 and in eleven Czech hospitals from eighteen hospitals involved in the study in 2014. In C. difficile RT027 isolates, the MLVA was applied in several studies to determine the genetic relatedness of isolates. MLST of seven housekeeping genes revealed eleven different STs. Three RTs (016, 027 and 176) had an identical ST1 as reported earlier. Likewise, RT075 was reported as ST95 and clade 2. Surprisingly, two other RT075 isolates in our study revealed new ST, with nearest match with STs 47, 61, and 95. Both isolates derived from the same hospital but were identified in the different years (2008 and 2010). ST41 and clade 2, identified in one isolate with new CE-ribotyping profile F1, has also been reported in RTs 156, 208 and in RTs 106, 194, 321 together with presence of similar deletions (18 bp and Δ117) in the tcdC gene. ST67 identified in RT153 isolate has been reported in RT0198, and also with wild type genotype at position 117 in the tcdC gene. Additionally, we performed MLST in the ECDC-Leeds-Leiden reference strain RT080 and the same ST192 was observed in both the ECDC-Leeds-Leiden and Finnish RT080 C. difficile isolates.

In conclusion, the molecular characterisation of twenty-eight C. difficile isolates resembling PCR-ribotype 027 revealed seven known ribotypes, three WEBRIBO types, three new CE-ribotyping profiles and eleven different sequence types. MLVA revealed outbreaks and regional spread of RTs 016, 027, 176 and F3. Twenty-two non-RT027 C. difficile isolates of eight ribotypes showed the presence of Δ117 in the tcdC gene, a

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**Fig. 3.** Minimum spanning tree of Finnish C. difficile isolates in the study. Each CE-ribotyping profile is represented by different colour. The numbers in the circles represent DNA number of isolate. If the more than one number is present in one circle, it represents isolates with STRD = 0 (i.e. 100% identical in seven variable-number tandem repeat loci). The numbers on the lines represent STRD between isolates. CC — clonal complex, STRD — sum of tandem repeat differences, WRT — WEBRIBO type.
target site for detection of RT027 by commercial molecular methods that could result in an incorrect identification. These results highlight the importance of careful interpretation of the results from commercial systems targeting this site in the genome of *C. difficile* and the need of strain typing for epidemiological purposes.

**Conflicts of interest**

The authors declare that they have no conflicts of interest.

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