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Original Article

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—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 diarrhoea and PCR-ribotype 027/NAP1/B1 is the most notorious "hypervirulent" ribotype.[1] 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.[2]

CE-ribotyping is currently the recommended standard for characterization of *C. difficile* isolates.[3] Based on slight variations in ribotyping banding patterns, three RTs (176, 198 and 244) have been determined as closely related to RT027.[4] Interestingly, RT176 is associated with outbreaks in the Czech Republic[5] and Poland,[6] whereas RT244 emerged in Australia.[7] Recently, other RT027-like ribotypes (016, 036) have been reported that belong to the same multilocus sequence type (ST1) as RT027.[8]

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).[9] 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,[10] 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[3] with Bidet primers.[11] 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-Leeds-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/)[12] 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[13] with visualization of amplified products by agarose-gel electrophoresis. The *tcdC* gene fragment was amplified and sequenced with primers C1 and C2,[14] 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)[15] with a change of reverse primer for G8Cd loci, as described elsewhere.[16] 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 $\leq$ 2.[15]

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.[17] 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/.[17]

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 4 mg/L for moxifloxacin[18] was applied. The fragment of the *gyrA* gene was amplified and sequenced with primers *gyrA*1 and *gyrA*2[19] 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 *C. difficile* strain dataset. In contrast, one CE-ribotyping profile was recognized by the WEBRIBO database as a WRT475, and by the ECDC-Leeds-Leiden database as RT034. Two CE-ribotyping profile were only recognized by the ECDC-Leeds-Leiden database as RT153 and 328. Two CE-ribotyping profiles were only identified by the WEBRIBO database as WRTs AI-78 and 411. The remaining three CE-ribotyping profiles did not match any type in both databases and were designated as F1 (n = 1), F2 (n = 1), and F3 (n = 9). CE-ribotyping profiles and fragment sizes of new CE-ribotyping profiles are shown in Fig. 1. Geographical distribution of isolates with specific CE-ribotyping profiles are depicted in Fig. 2.

The presence of toxin genes and *tcdC* sequencing

All 39 isolates (twenty-eight RT027-like isolates and eleven RT027 controls) contained *tcdA*, *tcdB* and *cdtA*, *cdtB* genes for production of toxins A, B and binary, respectively. Sequencing of the *tcdC* gene fragment confirmed the presence of 18 bp deletion (at position 330–347) in all 39 *C. difficile* isolates. The presence of the single base deletion at position 117 in the *tcdC* gene was observed in 33 isolates (RTs 016, 176, 027, 080, 328, WRTAI-78 and F1, F2, F3). In contrast, RTs 034, 075, 153 and WRT411 carried substitution A > G at position 117 in the *tcdC* gene.

Multi-locus variable tandem-repeats analysis (MLVA)

MLVA revealed six clonal complexes in RTs 016, 027, 176 and F3 (Fig. 3). No clonal complexes were found between isolates from different RTs. CC1 included nine isolates of F3 profile (three hospitals, the same region and the same year of culture (2015)). Nine control isolates belonging to RT027 formed three clonal complexes (CC2, CC4 and CC6). CC2 included four isolates from three hospitals collected in the time period 2008–2011. CC4 included three isolates from two hospitals cultured in 2013 and CC6 included two RT027 isolates from two hospitals collected in the time period 2011 and 2012. CC3 included two RT016 isolates from the same hospital and different year of culture (2011, 2012). CC5 included 3 isolates of RT176 from two hospitals and the same year of culture (2008).

Genetic relatedness (defined as STRD $\leq$ 10), between isolates belonging to the same RT where observed between isolates of RT027 in CC2 (n = 4) and CC4 (n = 3) STRD = 6, between isolates of RT176 (isolate no. 2688 and three isolates in CC5, STRD = 3) and between isolates of RT016 (isolate no. 2296 and two isolates in CC3, STRD = 6).

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), ST67 (RT153), ST95 (RT075), ST191 (WRTAI-78), ST192 (RT080), ST223 (RT034), ST229 (RT328) and ST264 (F2) and new ST (RT075, n = 2). The alleles profile of new ST in both RT075 isolates was: *adk* = 1, *atpA* = 5, *dxr* = 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 ($\geq$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 *tcdC* 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 (WRTs 411 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.

DNA	Year	Sex	Age	City	CE-ribotyping profile	*tcdC* *p.* 117	GyrA	MOX mg/L	MLVA							MLST
									A6Cd	B7Cd	C6Cd	E7Cd	F3Cd	G8Cd	H9Cd	ST/clade
2656	2011	M	81	Hamina	016	del A	Thr82Ile	≥32	50	19	40	10	5	13	2	1/2
2657	2012	M	77	Hamina	016	del A	Thr82Ile	≥32	50	19	39	10	5	14	2	–
2296	2011	F	70	Hamina	016	del A	Thr82Ile	≥32	51	18	35	10	5	14	2	1/2
2689	2008	F	93	Karhula	027	del A	Thr82Ile	≥32	41	19	37	10	5	14	2	1/2
2692	2009	M	88	Kotka	027	del A	Thr82Ile	≥32	41	19	38	10	5	14	2	–
2694	2010	M	81	Pyhtää	027	del A	Thr82Ile	≥32	42	19	39	10	5	14	2	–
2695	2011	M	72	Jokela	027	del A	Thr82Ile	≥32	29	8	33	10	5	14	2	1/2
2697	2012	M	77	Hyvinkää	027	del A	Thr82Ile	≥32	29	9	33	10	5	14	2	–
2298	2013	M	87	Hamina	027	del A	Thr82Ile	≥32	47	19	39	10	5	15	2	1/2
2690	2008	M	84	Pori	027	del A	Thr82Ile	≥32	30	8	40	10	5	14	2	1/2
2696	2011	M	65	Kotka	027	del A	Thr82Ile	≥32	42	20	38	10	5	14	2	–
2698	2012	M	77	Seinäjoki	027	del A	Thr82Ile	≥32	19	12	25	10	5	15	2	1/2
2699	2013	N	83	Hamina	027	del A	Thr82Ile	≥32	47	19	39	10	5	15	2	–
2700	2013	M	72	Kotka	027	del A	Thr82Ile	≥32	47	19	39	10	5	15	2	–
2307	2015	F	67	Helsinki	034 = WRT475	A > G	WT	0.016	31	11	22	7	6	15	2	223/2
2669	2008	N	20	Oulu	075	A > G	WT	1	28	8	40	7	6	24	2	New ST
2670	2010	N	66	Oulu	075	A > G	WT	1.5	29	9	19	7	6	13	2	New ST
2301	2012	M	64	Kemi	075	A > G	WT	0.016	29	21	30	9	5	9	2	95/2
2302	2013	F	52	Muhos	080	del A	WT	0.016	18	29	36	12	6	10	2	192/2
2682	2009	M	82	Seinäjoki	153	A > G	WT	1.5	33	17	31	8	6	8	2	67/2
2686	2008	M	64	Uusikaupunki	176	del A	Thr82Ile	≥32	29	8	37	10	5	12	2	1/2
2687	2008	F	87	Salo	176	del A	Thr82Ile	≥32	29	8	37	10	5	12	2	–
2688	2008	N	96	Turku	176	del A	Thr82Ile	≥32	29	8	34	10	5	12	2	1/2
2701	2008	F	87	Salo	176	del A	Thr82Ile	≥32	29	8	37	10	5	12	2	–
2305	2009	M	70	Helsinki	176	del A	Thr82Ile	≥32	29	9	36	10	5	14	2	1/2
2299	2008	F	88	Turku	328	del A	WT	0.016	47	19	43	10	5	15	2	229/2
2658	2015	N	90	Espoo	New F3	del A	WT	1.5	32	14	27	8	6	13	2	41/2
2659	2015	M	77	Espoo	New F3	del A	WT	1	32	14	27	8	6	13	2	–
2660	2015	N	94	Helsinki	New F3	del A	WT	1	32	14	27	8	5	13	2	–
2661	2015	M	40	Helsinki	New F3	del A	WT	1.5	33	14	27	8	5	13	2	–
2662	2015	N	62	Helsinki	New F3	del A	WT	1	31	14	27	8	6	13	2	–
2663	2015	M	81	Vantaa	New F3	del A	WT	1	32	15	27	8	6	13	2	–
2665	2015	N	90	Espoo	New F3	del A	WT	1.5	33	14	27	8	6	13	2	–
2666	2015	N	85	Espoo	New F3	del A	WT	1.5	32	14	27	8	6	13	2	–
2667	2015	N	90	Espoo	New F3	del A	WT	1	32	14	27	8	6	13	2	–
2297	2013	M	74	Seinäjoki	New F1	del A	WT	0.016	29	9	29	10	5	14	2	41/2
2306	2009	M	82	Kempele	New F2	del A	WT	0.016	21	15	27	2	5	16	2	264/2
2668	2008	N	25	Oulu	WRT411	A > G	WT	1.5	33	17	11	5	6	13	2	47/2
2671	2008	M	60	Helsinki	WRTAI-78	del A	WT	1	32	16	21	7	6	12	2	191/1

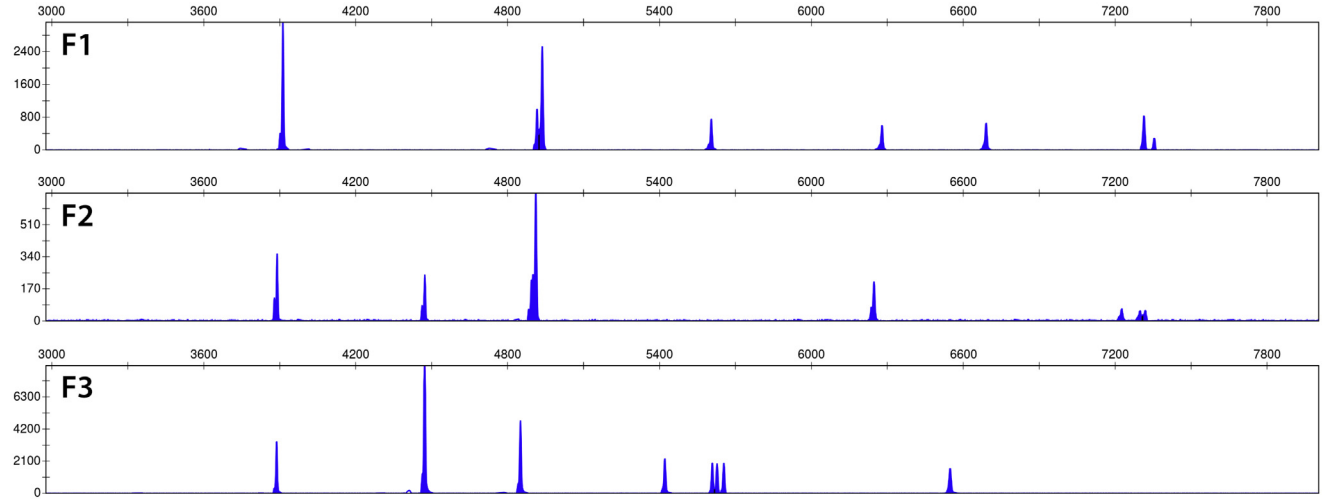

Fig. 1. New CE-ribotyping profiles identified in the study. Band sizes in base pairs are following: F1 — 232, 322, 324, 383, 444, 482, 541, 545; F2 — 232, 284, 324, 444, 536, 543, 545; F3 — 262, 323, 362, 422, 441, 443, 446, 543.

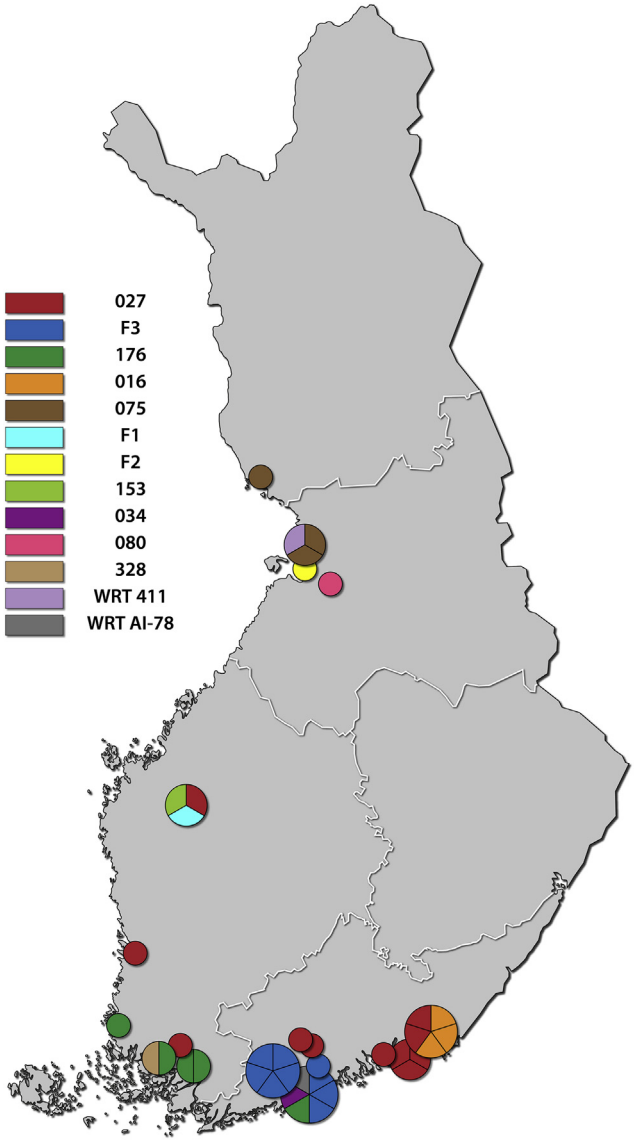

Fig. 2. Geographic distribution of *C. difficile* isolates in the study. Each CE-ribotyping profile is represented by different colour.

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[20] and from Poland where it persists with RT027.[6,21] The clinical relevance of RT176 has been studied in both countries.[21–23] 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.[24]

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.[25–27] 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.[7] 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

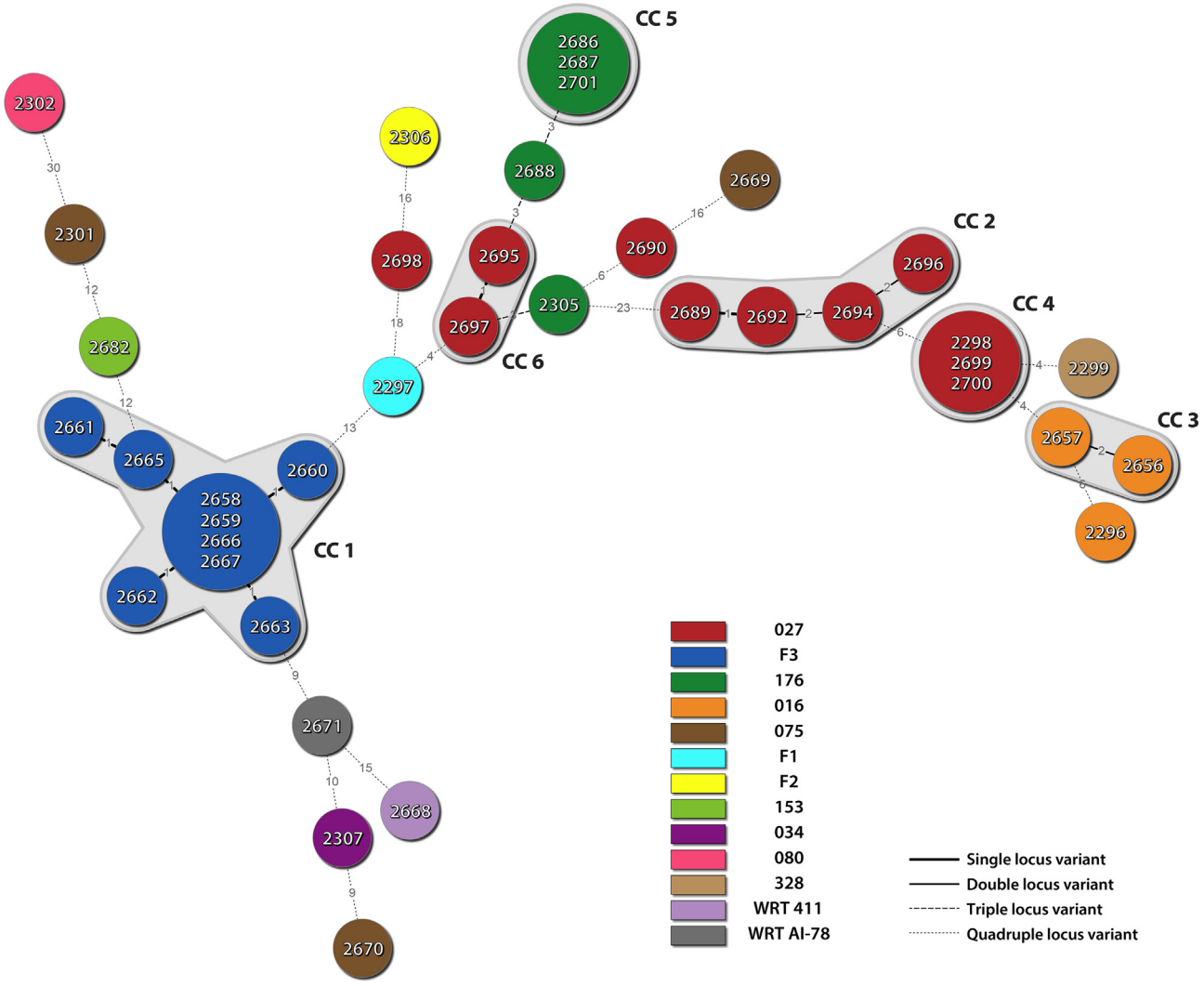

Fig. 3. Minimum spanning tree of Finnish *C. difficile* isolates in the study. Each CE-ribotyping profile is represented by different colour. The numbers in the circles represent DNA number of isolate. If the more than one number is present in one circle, it represents isolates with STRD = 0 (i.e. 100% 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.

method in isolates belonging to the same RT.[15,28] MLVA was firstly used for subtyping of *C. difficile* RT176 isolates in the study Nyc et al.[29] 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[22,23] and in eleven Czech hospitals from eighteen hospitals involved in the study in 2014.[20] In *C. difficile* RT027 isolates, the MLVA was applied in several studies to determine the genetic relatedness of isolates.[5,28–34]

MLST of seven housekeeping genes revealed eleven different STs. Three RTs (016, 027 and 176) had an identical ST1 as reported earlier.[8] Likewise, RT075 was reported as ST95 and clade 2.[8] 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[8] and in RTs 106, 194, 321 together with presence of similar deletions (18 bp and Δ117) in the *tcdC* gene.[35] ST67 identified in RT153 isolate has been reported in RT019[8,35] and also with wild type genotype at position 117 in the *tcdC* gene.[35] 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.

Only isolates of RTs 027, 016 and 176 were moxifloxacin resistant and carried amino acid substitution Thr82Ile in the GyrA, which has been associated with fluoroquinolone resistance.[19,36,37]

In conclusion, the molecular characterisation of twenty-eight *C. difficile* 027-like isolates 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

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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