PERFORMANCE OF CORE GENOME MULTILOCUS SEQUENCE TYPING COMPARED TO CAPILLARY-ELECTROPHORESIS PCR RIBOTYPING AND SNP ANALYSIS OF *Clostridioides difficile*

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ABSTRACT *Clostridioides difficile* is the most common cause of antibiotic-associated gastrointestinal infections. Capillary-electrophoresis (CE)-PCR ribotyping is currently the gold standard for *C. difficile* typing but lacks discriminatory power to study transmission and outbreaks in detail. New molecular methods have the capacity to differentiate better, but backward compatibility with CE-PCR ribotyping must be assessed. Using a well-characterized collection of diverse strains (N=630; 100 unique ribotypes [RTs]), we aimed to investigate PCR ribotyping prediction from core genome multilocus sequence typing (cgMLST). Additionally, we compared the discriminatory power of cgMLST (SeqSphere & EnteroBase) and whole genome MLST (wgMLST) (EnteroBase) with single nucleotide polymorphism (SNP) analysis). A unique cgMLST profile (>6 allele differences) was observed in 82/100 ribotypes, indicating sufficient backward compatibility. Intra-RT allele difference varied per ribotype and MLST clade. Application of cg/wgMLST and SNP analysis in two outbreak settings with ribotypes RT078 and RT181 (known with a low intra-ribotype

allele difference) showed no distinction between outbreak- and non-outbreak strains, in contrast to wgMLST and SNP analysis. We conclude that cgMLST has the potential to be an alternative to CE-PCR ribotyping. The method is reproducible, easy to standardize and offers higher discrimination. However, in some ribotype complexes adjusted cut-off thresholds and epidemiological data are necessary to recognize outbreaks. We propose to decrease the current threshold of 6 to 3 alleles to better identify outbreaks.

KEYWORDS *Clostridioides difficile, whole-genome sequencing, typing methods, core genome MLST, whole genome MLST*

INTRODUCTION

Clostridioides difficile is a Gram-positive anaerobic bacterium that is associated with nosocomial gastrointestinal infection (1) (2). It is estimated that there were almost 500,000 patients with *C. difficile* infection (CDI) and around 29,000 deaths in the United States in 2011 (2). Individuals with *C. difficile* infection (CDI) are an important source of *C. difficile* transmission in healthcare settings (2). Typing of *C. difficile* is necessary for infection control, epidemiology and evaluation of treatment. Several methods are used for typing *C. difficile*, including capillary electrophoresis (CE) PCR ribotyping (3) (4) and multilocus sequence typing (MLST) (5). CE-PCR ribotyping is currently the gold standard. However, it does not provide sufficient discriminatory power to distinguish related strains (6). Furthermore, for CE-PCR ribotyping, standardization and interlaboratory comparisons are difficult to establish (7), whereas for MLST this is relatively simple. In the case of a suspected outbreak CE-PCR ribotyping can be used in combination with multilocus variable-number tandem repeat (VNTR) analysis (MLVA) for subtyping of strains belonging to one PCR ribotype (8). This combination of methods is usually sufficient to type strains and understand transmission events. However, these methods do not provide sufficient information about strain characteristics (e.g. possession of virulence and resistance genes) and possible treatment failures (relapse vs. reinfection). The techniques are also less suitable to study transmission and to determine the role of symptomatic and asymptomatic patients in hospital acquired CDI (9). Therefore, typing methods

with more discriminatory power and preferably based on better standardized whole genome sequencing (WGS) are urgently needed.

There are two commonly applied methods to identify genomic variations using WGS. Single nucleotide polymorphism (SNP) analysis usually uses a reference genome and detects SNPs between the reference genome and the studied genome (10). SNP analysis provides the highest resolution, but it is relatively slow, requires extensive bioinformatic tools, is difficult to standardize and typing nomenclature is missing (11), (12), (9). The second approach is based on gene-by-gene allelic profiling of the core genome (cgMLST) or whole genome (wgMLST) (13). cgMLST provides high discriminatory power, is more rapid than SNP analysis, offers reasonably accurate reproducibility (11) and could be used as a typing method since the scheme is maintained by a centralized database (14).

Currently there are several cg/wgMLST schemes available for *C. difficile*, both commercially and publicly. The first commercial platform is SeqSphere+ software [Ridom GmbH, Germany] comprising of a scheme (the cgMLST.org Nomenclature Server) using up to 2147 core genes and 1357 accessory genes out of 3756 genes present in strain 630 (14). The second is BioNumerics [bioMérieux, France] with the cgMLST/wgMLST scheme developed by Applied-Maths, comprising 1999 core genes and 6713 accessory genes and several other genes associated with virulence, antimicrobial resistance and others from different *C. difficile* strains (15). Besides these 2 commercial platforms, there is a publicly available cg/wgMLST scheme from EnteroBase [University of Warwick, UK] consisting of 2556 genes for the cgMLST scheme and up to 13763 genes for the wgMLST scheme (16). The cgMLST scheme of EnteroBase (EB cgMLST) is also available through the Center for Genomic Epidemiology (cgMLSTFinder 1.1; https://cge.cbs.dtu.dk/services/cgMLSTFinder/).

Several studies have been published on the application of cgMLST (14), (11), (15), (16). Most studies show that cgMLST is backward compatible with CE-PCR ribotyping but only a restricted number of different ribotypes were analysed and outbreaks were not included. Recently, Seth-Smith and colleagues showed that cgMLST predicted 36 ribotypes using nearly 300 well characterised clinical strains

from Switzerland. However, some ribotypes complexes (RT 078/126) has a low genomic difference, whereas other ribotypes (e.g., RT 023) were very disperse (17). Our study builds upon previous work by assessing backward compatibility more in depth, using 100 unique ribotypes and changing thresholds to determine optimal differentiation between ribotypes. Furthermore, we analyse the performance of CE-PCR ribotyping, cgMLST, wgMLST and SNP analysis by using multiple software programs (SeqSphere & EnteroBase) and applied the methods on two outbreaks. Importantly, our study shows that a threshold of ≤ 3 targets/alleles is needed for *C. difficile* isolates that are likely to belong to the same clone in an outbreak setting.

MATERIALS AND METHODS

Sequence data. The NCBI database was searched at the start of this study for sequenced closed *C. difficile* genomes, this resulted in 4845 available genomes. Only sequence data generated on Illumina sequencing platform and representing known ribotypes were selected. A random selection of overrepresented strains (e.g. RT027 and RT078) were included. This approach resulted in 609 complete genome sequences that were analysed. Besides downloaded strains from the NCBI database we included also 21 recently sequenced strains at Leiden University Medical Center (LUMC). This comprised fifteen Greek RT181 CDI outbreak strains that were already sequenced for a previous study (PRJEB36956, Table S1, (18) and 6 strains from a Dutch CDI outbreak due to RT078. For sequencing of strains, total DNA was isolated from cultured bacteria. A few colonies were emulsified in Tris/EDTA (TE) buffer and heated at 100° C for 10 minutes according to the Griffiths *et al.* protocol (5). DNA was sequenced at Genome Scan B.V., Leiden, The Netherlands, on an Illumina NovaSeq 6000 after preparation with the NebNext Ultra II DNA library prep kit for Illumina. This produced on average 3 million paired-end reads (read size 150bp) per sample, with a minimum of 90% reads with a quality of ≥30.

Ridom cgMLST. Ridom® SeqSphere+ (version 6.0.2; Ridom GmbH, Münster, Germany) was run with default settings for quality trimming, *de novo* assembly and allele calling on a Microsoft Windows operating system. Quality trimming occurred at both 5'-ends and 3'-end until an average base quality of 30 was reached (length of 20 bases and a 120-fold coverage) (14), (13). *De novo* assembly was performed using the SKESA assembler version 2.3.0 (19) integrated in SeqSphere+ (20) using

default settings for SKESA. SeqSphere$^+$ scanned for the defined genes using BLAST (21) with criteria described previously (22), (13). For further analysis, distance matrices, minimum spanning trees and neighbour joining trees were constructed using the integrated features within SeqSphere$^+$ with "pairwise ignoring missing values" option turned on.

EnteroBase cgMLST and wgMLST. cgMLST was performed using cgMLST Finder 1.1, available through the Center for Genomic Epidemiology (cgMLSTFinder 1.1; https://cge.cbs.dtu.dk/services/cgMLSTFinder/). Genomic data was processed using automated pipelines inside EnteroBase, as described in detail previously (23). In short, *de novo* assembly of Illumina sequence reads was performed using Spades v3.10 (24). In order to pass quality control, assemblies were needed to comply with criteria described previously (16). BLASTn and UBLASTP were used to align assemblies to alleles. EnteroBase module MLSType was used to assess allele numbers and cluster types (23). cgMLST Finder 1.1 provides a distance matrix for analysis. Distance matrices were used to calculate the mean intra- and inter-allelic distance between different CE-PCR ribotypes. For wgMLST analysis, an *ad hoc* scheme was used based on the wgMLST scheme from EnteroBase (EB wgMLST) (16), (25) . This *ad hoc* scheme was integrated in Ridom® SeqSphere (14). *De novo* assembly, allele calling and further analysis were carried out as mentioned previously (under Ridom cgMLST).

SNP analysis. SNPs were identified as previously described (26) using the webtool at the following address: http://cge.cbs.dtu.dk/services/CSIPhylogeny/. Default settings were used for the SNP analysis. *C. difficile* strain 630 (NC_009089) was used as the reference genome for all analyses. In short, reads were mapped to the reference sequence using BWA (version 0.7.2) (27). Depth at each position was calculated using genomeCoverageBed, which is a component of BEDTools (version 2.16.2) (28). SNPs were called using mpileup, which is a component of SAMTools (version 0.1.18) (29). Mapping quality (minimum of 25 reads) and SNP quality (SNPs were filtered out if quality was below 30 or if they were called within the vicinity of 10 bp of another SNP) were calculated by BWA and SAMTools, respectively. CSIPhylogeny 1.4 provides a distance matrix for analysis. Distance matrices (based

on pairwise comparison, missing data were excluded) were used to calculate the mean intra- and inter-RT SNP distance between different CE-PCR ribotypes.

Mean intra-ribotype allele difference. Mean intra-ribotype allele difference was determined for 19 ribotypes using distance matrices produced with cgMLST and wgMLST schemes and SNP analysis. From each ribotype, 3 to 13 strains were included. To prevent inclusion of related strains, e.g. from outbreak reports, we selected ribotypes with at least 3 strains from different geographic locations and/or from different collection years.

Data availability. All own genome sequence data generated as part of this study were submitted to the NCBI/ENA under study number PRJEB46469. Sequence Read Archive (SRA) accession numbers for other analyzed genomes are provided in Table S1.

RESULTS

Ridom cgMLST is backward compatible with CE-PCR ribotyping.

To test the backward compatibility of cgMLST (SeqSphere) with CE-PCR ribotyping, we compared cgMLST and CE-PCR ribotyping using a selection of sequenced *C. difficile* strains with known ribotypes (Janezic & Rupnik, 2019). Figure 1 depicts a neighbour joining tree based on the Ridom SeqSphere cgMLST scheme (SqSp cgMLST) including 100 different PCR ribotypes from all 5 MLST Clades. Most ribotypes show a different allelic profile in cgMLST in comparison with other ribotypes. However, there are ribotypes within every MLST clade that show low allele difference (<6 alleles) in comparison with other ribotypes.

When all included strains (n=630 strains) from 100 unique ribotypes were analysed (shown in Table 1), 82 ribotypes were distinguishable, i.e., the strains within these ribotypes differed by >6 alleles from strains within other ribotypes. Eighteen ribotypes (18%) clustered together with 1-3 other ribotypes from the same clade and had ≤ 6 allelic differences. This was observed in Clades 1, 2 and 5. In figure 2 we show the ribotypes in each cluster and how these clusters vary at different thresholds (0-6 allelic differences). When the threshold was lowered from 6 to 0, the number of different ribotypes that clustered decreased from 13 to 2 (RT045 and RT127). The

amount of clusters decreased from 14 to 1. Even at a threshold of 0 allele difference, these ribotypes showed clustering, demonstrating the limitation of short-read sequencing and cgMLST.

Intra-ribotype allele difference varies per ribotype and per MLST clade

We determined the mean allelic difference between strains from the same ribotype and tested if intra-ribotype allele differences vary between MLST clades and ribotypes. We also compared the mean intra-ribotype allele or SNP differences with cgMLST, wgMLST and SNP analysis. Mean intra-ribotype allele difference varied between ribotypes (Figure 3A). The method with the smallest scheme (SqSp cgMLST) showed the lowest intra-ribotype allele difference average (mean range of 5-376 alleles) whereas SNP analysis showed the highest average (mean range of 67-2563 alleles). As a comparison, the mean and median of the inter-ribotype allele difference were 1742 and 2131 alleles with SqSp cgMLST, respectively. Figure 3 A also shows that RT027 had an intra ribotype allele difference of 8.4 (SqSp cgMLST), 10.7 (EB cgMLST), 18.1 (EB wgMLST) and 100.7 (SNP). Another complex ribotype, RT078 showed 13.2, 15.5, 29.3 and 139.4, respectively. The most frequently found ribotype in Europe, RT014 showed 148.1, 173, 258.8 and 855.7 respectively. RT023 (clade 3) showed 108.7, 121.3, 157.5 and 1014.7, respectively. RT017 (clade 4) showed 22.3, 23.5, 63.7 and 129.3, respectively. EB wgMLST and SNP analysis showed similar results as cgMLST, but showed much higher average intra-ribotype allele and SNP difference. The ribotype with lowest intra-ribotype allele difference for clade 1 was again RT002 (64 alleles and 140 SNPs) and the highest was RT056 (650 alleles and 2563 SNPs). The ribotype with the lowest intra-ribotype difference from clade 2 was RT181 (11 alleles and 67 SNPs), whereas the highest was RT036 (39 alleles and 120 SNPs). RT023 from clade 3 showed an average of 158 intra-ribotype allele difference and 1015 SNP difference. RT017 from clade 4 showed 64 allele and 129 SNP difference. Lastly, RT126 from clade 5 showed the lowest difference (18 allele and 130 SNP differences) and RT127 the highest (379 allele and 592 SNP differences). SNP analysis showed the highest resolution and often >2 times difference in comparison with wgMLST.

The mean intra-ribotype allele difference per clade was also calculated for clades 1, 2 and 5 by combining the averages per ribotype within a clade (figure 3B). Clade 1 had the highest average allele difference for SqSp cgMLST, EB cgMLST, EB

wgMLST and SNP analysis (114, 136, 171 allele difference and 685 SNPs, respectively). Followed by clade 5 with 39,49, 66 allele differences and 177 SNPs, respectively. Clade 2 had the lowest average intra-ribotype allele difference (9, 12, 18 allele differences and 100 SNPs, respectively).

Clade 1 had the highest mean intra-ribotype allele difference for wgMLST and SNP analysis (171 alleles and 685 SNPs), followed by clade 5 with 66 alleles and 177 SNPs. Clade 2 had again the lowest mean intra-ribotype allele difference (18 alleles and 100 SNPs).

WGS based typing methods cannot distinguish outbreak strains from non-outbreak strains in ribotypes with a low intra-ribotype allele difference

CE-PCR ribotyping has a low resolution in comparison with whole genome-based typing for outbreak analysis. However, even with the increased resolution of WGS based typing, it remains crucial to understand what defines an outbreak. Bletz *et al.* proposed a threshold of ≤ 6 alleles for cgMLST for isolates that are expected to belong to the same clone (14). In order to guide the interpretation of Bletz *et al.* we compared cgMLST, wgMLST and SNP analysis in 2 suspected outbreak settings. We selected outbreak strains from MLST clades 2 (RT 181) and 5 (RT 078), since both clades have a lower average allele difference. Confirmed outbreak strains were defined as having an epidemiological link (e.g. nursed in the same ward) combined with ≤ 6 allele differences. Control strains belonged to similar PCR ribotypes as the outbreaks strains or to other PCR ribotypes from the same clade.

Next, we analysed the distance matrices of two clusters containing confirmed outbreaks and non-outbreak strains with cgMLST, wgMLST and SNP analysis. The strains within each cluster were either labelled as outbreak strain or control strain. These distance matrices of both clusters were visualized in graphs (Figure 5A and B) with each data point representing a distance in alleles or SNPs between 2 strains. We calculated the range of allele or SNP difference of outbreak strains (Range O) and compared it with the range of allele or SNP difference of non-outbreak strains (Range NO). The area between the upper limit of range O and the lower limit of range NO determines the area where adjustment of the threshold is possible, provided that outbreak strains and non-outbreak strains do not overlap. The larger the area, the better the method can discriminate between outbreak and non-outbreak strains.

The first CDI suspected outbreak we analysed was due to RT078 (clade 5) in a Dutch general hospital, involving 6 patients in the Gastroenterology ward between October-December 2018 (figure 4A). Three patients had an hospital-onset of CDI and 3 had a community-onset (including the index case). The first case (patient A) was admitted on 1st of November and had a community-onset of CDI since the 25th of October. The second case (patient B) was admitted on the 2nd of November and developed hospital-onset of CDI on the 5th. The 3rd case (patient C) was admitted on the 12th of November and developed hospital-onset of CDI on the 16th. One patient (patient D) was transferred from another hospital on the 24th of November and had CDI since the 13th, this patient did not belong to the outbreak. Two other patients (patient E & F) had a community onset of CDI and were admitted both on the 4th of December and had CDI since the 28th of November and 1st of December, respectively. Three isolates from 3 different patients showed a clustering and had 0 allele differences (patients A, B and C), the other 3 patients (patients D, E and F) did not belong to this cluster and had >6 allele differences. Twelve additional control samples from Clade 5 were added to this collection. These included five Leeds-Leiden reference strains (RT033, RT045, RT066, RT078 and RT126) and 7 other strains (RT045, RT066, RT126, RT127 and RT078 (N=3)). Figure 4A depicts the minimum-spanning tree (based on SqSp cgMLST) of the studied isolates of clade 5 (N=18). This resulted in three clusters (≤ 6 alleles), each comprising of epidemiologically related and unrelated strains of which cluster 1 is the largest, involving three strains of the confirmed RT078 outbreak (3 cases [patient A, B and C] and 1 non-case [patient E]) and three control strains (RT066, RT078 and RT126). The second outbreak (18) occurred in a Greek 180-bed rehabilitation clinic involving 15 CDI patients infected with RT181 (clade 2) at the orthopaedics and neurological wards between March and April 2019 (Figure 4B). All 15 patient isolates showed allele differences between 0-2 alleles. Seven control samples from Clade 2 were added to this collection, including Leeds-Leiden reference strains of RT016, RT027, RT198, 1 strain of RT036 and RT176 and 2 strains of RT181. Figure 4B shows the minimum-spanning tree based on SqSp cgMLST. Two clusters could be recognized, each comprising epidemiologically related and unrelated strains. Cluster 1 contained both confirmed outbreak strains (RT181, N=15) and control strains (RT181, N=2). Therefore, the current threshold of ≤6 alleles is not suitable to recognise an outbreak of RT 181.

Figure 5A shows that all WGS method could distinguish between confirmed outbreak and non-outbreak RT 078 strains, since there is no overlap between range O and range NO. SNP analysis had the best discriminatory power, followed by EB wgMLST and cgMLST, which showed the lowest discriminatory power. Figure 5B shows that wgMLST is the only method that could discriminate between outbreak and non-outbreak RT 181 strains, whereas cgMLST and SNP analysis show overlap in their ranges. Ranges O and NO are shown in Table 2 for both clusters and all applied typing methods. No overlap was seen between Range O and Range NO from Cluster 1 from the RT078 CDI outbreak. For SqSp cgMLST and EB cgMLST cluster 1 showed a difference of 3 alleles and 2 alleles between the Range O and Range NO, respectively. Furthermore, the difference between Range O and Range NO was for wgMLST and SNP analysis 6 alleles and 8 SNPs, indicating that the threshold could be lowered. However, Cluster 1 from the RT181 CDI outbreak showed overlap between Range O and Range NO in cgMLST and SNP analysis, but not in wgMLST, suggesting that the threshold only could be adjusted in wgMLST.

DISCUSSION

We tested the backward compatibility between SqSp cgMLST and CE-PCR ribotyping and found 82 of 100 different PCR ribotypes had a unique cgMLST profile using a cut-off of ≤6 alleles differences. Assessing the performance of cgMLST, wgMLST and SNP typing in comparison with CE-PCR ribotyping revealed that intra-ribotype alleles difference varied per ribotype and per MLST clade. Application of cg/wgMLST and SNP analysis in outbreak settings of RT078 and RT181 showed that these methods can only distinguish outbreak strains from non-outbreak strains when a cut-off threshold of 3 alleles is used.

We show that SqSp cgMLST is backward compatible with CE-PCR -ribotyping, but there are certain ribotypes that are indistinguishable by SqSp cgMLST. These data are consistent with Seth-Smith *et al*. who found different PCR ribotypes (RT 078-126, RT 106-RT500) clustering with maximum of 9 allelic difference. In agreement with the findings of Seth-Smith et al., we found ribotypes from clade 2 and 5 with the lowest mean intra-ribotype allele difference. We applied in our study the average allele differences, contrary to the study of Seth-Smith who used the maximum allelic difference. When we analysed for the maximum allelic difference, we found higher differences in all studied ribotypes than Seth-Smith *et al.* (e.g. RT027: 12 allelic

difference vs. 16 allelic difference in our study; RT078: 9 vs. 28, respectively; RT023: 52 vs. 199, respectively). This may have been caused by the selection of samples, since we excluded samples from outbreaks by selecting strains separated in time and space.

Our results are also consistent with another study (30) that used SNP analysis to investigate the diversity within a ribotype. The study showed that MLST ST1 (correlates with ribotype 027) was genetically less diverse with a lower SNP distance range between isolates than ST2 (correlates with ribotype 014). Finally, Frentrup *et al*, observed clustering of several ribotypes (e.g. RT001/RT241, RT106/RT500 and RT078/RT126) from MLST clades 1 and 5 (16), also in agreement with our observations.

Interestingly, decreasing the threshold from 6 to 0 allele difference will still result in clustering of certain ribotypes. The clustering between two strains of RT045 and two strains of RT127 at a threshold of 0 alleles in SqSp cgMLST was verified with EB cgMLST and SNP analysis. With EB cgMLST one clustering pair of RT045 and RT127 showed 1 allele difference, whereas the other remained at 0 allele difference. Verification with SNP analysis showed 2 and 7 SNP differences. This observation impairs the backward compatibility of cgMLST with CE-PCR ribotyping and excludes studying the epidemiological links of some strains belonging to RT045 and RT127.

Our results demonstrate that the mean allele differences between strains from the same PCR ribotype with SqSp cgMLST and EB cgMLST are lower in comparison with EB wgMLST and SNP analysis, with the latter showing the highest resolution. Similar results were seen in the studied RT078 CDI outbreak, where EB wgMLST and SNP analysis showed more discriminatory power in comparison with cgMLST. Interestingly, EB wgMLST was the only WGS based method that could discriminate between outbreak strains and non-outbreak strains in RT181 CDI outbreak. A reason could be that EB wgMLST uses a pangenome as a scheme consisting of several *C. difficile* genomes, in contrast with SNP analysis, which used strain 630 as the reference genome. Ribotypes from clades (e.g. clade 2) that have emerged relatively recently will have lower mean intra-ribotype allele differences as strains from these ribotypes look genetically more similar. Therefore, it may be challenging to distinguish which strains are involved in an outbreak. Another problem with these recently emerged ribotypes (e.g. RT181) is that we have limited data to assess the intra-ribotype allele difference more accurately.

Based on our observations in two CDI outbreaks, we conclude that WGS based methods cannot discriminate between outbreak and non-outbreak strains in MLST clades with low intra-ribotype allele difference. It remains unknown why some clades are less diverse. It is possible that they have emerged relatively recently and therefore are less diverse. Alternatively, the strains in these clades could have a lower mutation rate resulting in less diversity and therefore a lower intra-ribotype allele difference (31), (32). For outbreaks caused by PCR ribotypes belonging to other clades than 2 and 5, the performance of cgMLST is comparable with SNP analysis. Our results are consistent with other studies showing a comparable performance of cgMLST with SNP analysis (14), (33). Based upon the Oxfordshire data set (31), Frentrup *et al.* had a similar conclusion regarding cgMLST and SNP analysis (16). They showed that *C. difficile* genomes that differ by ≤2 alleles generally also differ by 2 ≤SNPs, using a logistic regression model, and concluded that cgMLST is equivalent to SNP analysis for identifying transmission chains between patients. Bletz *et al.* showed similar results between cgMLST and SNP analysis in detecting clusters when an outbreak due to ST1 was investigated (14). The main strength of our study is that we compared the performance of several typing methods, in contrast to previous studies (14), (11), (15), (16). We also expanded the collection of *C. difficile* strains and tested more than 600 sequenced strains belonging to 100 unique ribotypes. Our study has also some limitations. The lack of sufficient available genome sequences from strains belonging to clades 3 and 4 limits the generalizability of our findings. Though the backward compatibility was not tested for EB wgMLST, the results can be extrapolated from SqSp cgMLST, EB cgMLST and SNP analysis, since the discriminatory power of EB wgMLST lies between the latter two. We could not verify the correctness of the strain PCR ribotypes, as we had only access to the information as deposited by the researchers. There are a few ribotypes that have similar banding pattern and could be misidentified. The best example is the similarity of RT014 with RT020; they have an almost identical PCR banding pattern, but they differ substantially from each other by cgMLST. Though we only studied two outbreaks, we carefully selected the outbreaks by choosing PCR ribotypes with low intra-ribotype alleles variation. Finally, we have not tested long read sequencing from which theoretically in silico PCR ribotyping can also be obtained.

We propose to decrease the current threshold of 6 alleles (14) to 3 alleles when using cgMLST in outbreak situations. We found a difference of 2 and 3 alleles between controls and outbreak strains with EB cgMLST and SqSp cgMLST, respectively. In the study by Eyre *et al.* the evolutionary rate of *C. difficile* was estimated to be 0.74 SNVs (95% confidence interval, 0.22-1.40) per genome per year (34). They expected 0-2 SNPs to occur when isolates are obtained <124 days apart and 3 SNPs when isolates were obtained 124-364 days apart. However, only vegetative *C. difficile* isolates obtained from patients were analyzed. According to Weller & Wu sporulation reduces the evolutionary rate of *Firmicutes* (35). Therefore, we expect that the evolutionary rate of *C. difficile* is lower during CDI transmission than during CDI within a patient, since the spores need time to transmit to another patient and otherwise lie dormant in the surroundings in a healthcare facility or in the environment for a long period. Accordingly, we expect that outbreak strains will generally fall within 0-2 alleles.

Nevertheless, we recommend a threshold of 3 alleles to compensate for any assembly artifacts when less conservative pipelines are used and for outbreaks that last longer than 124 days (36).

A concern with application of cgMLST is the availability of various cgMLST schemes and software programs. The centralized databases need resources to maintain their databases of sequentially numbered alleles. To tackle the problem of the need for a centralized database and to rapidly identify related genomes against a background of thousands of other identified genomes, Hash-Based cgMLST has been developed (11). It is based on cgMLST, but converts alleles in a unique hash or short string of letters. Furthermore, if every software provider uses its own cgMLST scheme, inter-laboratory comparison is delayed and understanding of epidemiology is hampered. As Werner *et al.* proposed, it is favourable that a fixed cgMLST scheme is constructed (33). Furthermore, there are logistical and cost considerations for routine implementation of cgMLST. Reference laboratory are needed with a good infrastructure to sequence strains on a routine basis while keeping the costs in mind as well.

In summary, cgMLST has the potential to replace CE-PCR ribotyping for *C. difficile*. The method provides similar differentiation of strains, is easy to standardize, is reproducible and shows a high discriminatory power. Several cgMLST based typing

methods have emerged with all their specific advantages and disadvantages (11), (14), (16). For the time being, it remains unclear whether one method will get the preference over other methods or that every center will use its own method. However, it is important to ensure that local and international strains can be compared regardless of the use of different methods either by exchange of raw data or via a centralized multi-national database with a fixed cgMLST scheme where every center contributes to. A consensus group could be assembled to harmonize these efforts as has been done previously for CE-PCR ribotyping (4).

Conflict of Interest statements

AB; None. JC: None. CH: None. WKS: None. WF: None. MHW: None. NK: None. DWE: Lecture fees from Gilead, outside the submitted work. AM: None. EK: unrestricted research grant from Vedanta Bioscience, Boston.

Acknowledgments

We thank B.V.H. Hornung for his assistance with data collection.

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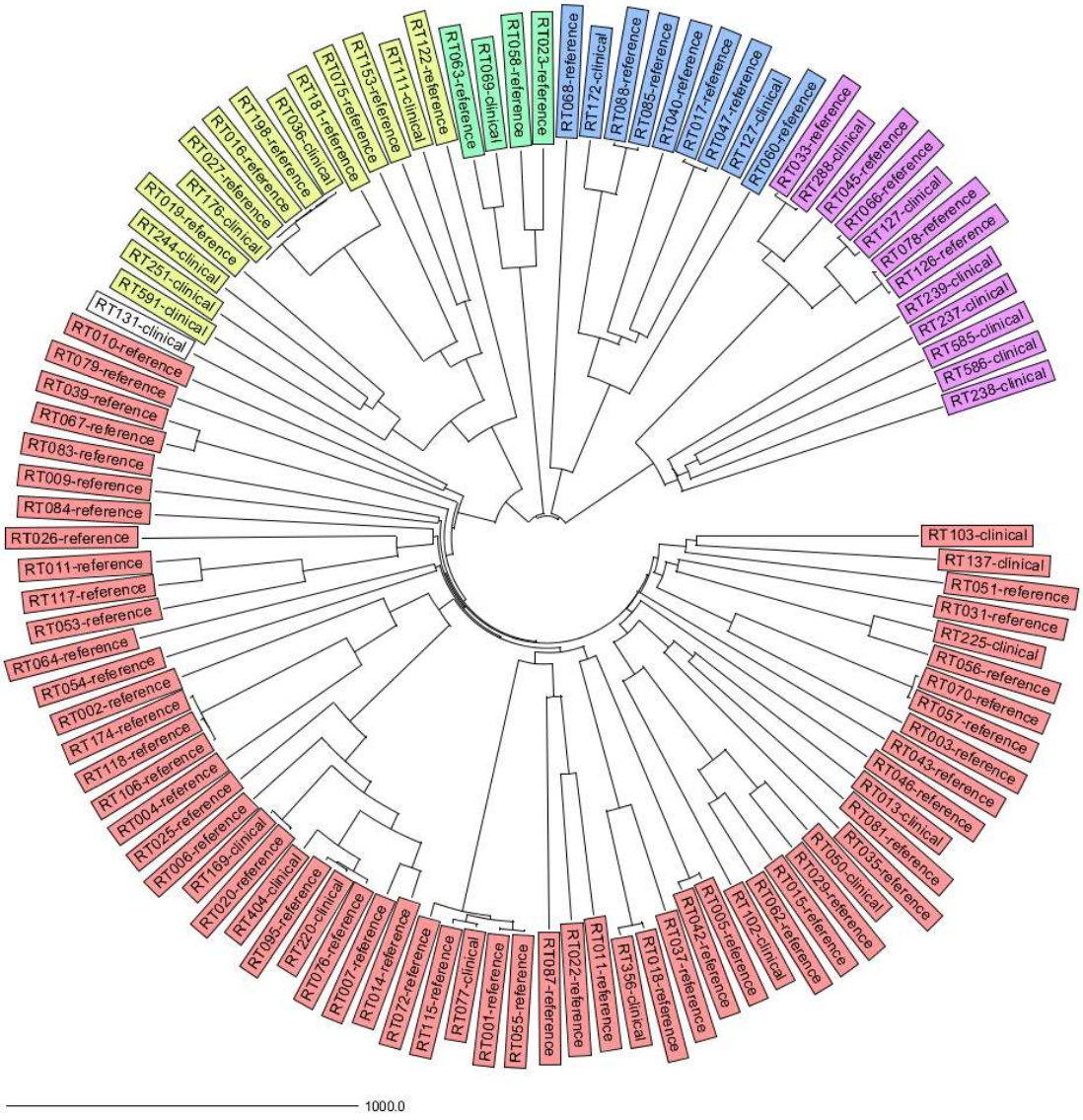


Figure 1: Neighbor joining tree from 100 unique ribotypes based on SqSp cgMLST allele difference. Each ribotype is depicted with “RTn” followed by “reference” (belonging to the Leeds-Leiden collection) or clinical (non-Leeds-Leiden strain). Ribotypes from MLST Clade 1, 2, 3, 4 ,5 are colored red, yellow, green, blue and purple, respectively. RT131 stated as CD131-01, 131, has no designated MLST Clade and is shown in white. The distance is given in absolute allelic difference.

Table 1: Clustering between ribotypes at different thresholds based on SqSp cgMLST

Threshold (in alleles)	RT [a]	amount of strains [b]	RT [c, d]	amount of strains [b]	Clade	Threshold (in alleles)	RT [a]	amount of strains [b]	RT [c, d]	amount of strains [b]	Clade
6	020	1/20	076	1/2	1	4	016	1/1	027	1/23	
	016	1/1	027	5/23	2		027	6/23	036	1/4	2
			036	1/4				9/23	176	5/16	
			176	4/16			033	2/46	288	2/2	
	027	3/23	036	2/4	2		045	2/15	078	5/58	
		10/23	176	13/16				2/15	126	4/29	
		2/23	198	1/2				3/15	127	3/17	
	036	1/4	176	1/16	2		066	1/2	078	1/58	
	033	2/46	288	2/2	5		078	25/58	126	15/29	
	045	2/15	078	16/58	5	3	018	1/18	356	3/13	1
		2/15	126	7/29			027	3/23	036	1/4	2
	066	1/2	078	3/58	5			6/23	176	3/16	2
		1/2	126	1/29			045	1/15	078	1/58	
	078	39/58	126	23/29	5			1/15	126	1/29	
5	018	1/18	356	1/13	1			3/15	127	3/17	
	016	1/1	027	2/23	2		078	18/58	126	13/29	
			176	1/16		2	001	1/14	055	1/1	1
			198	1/2			018	1/18	356	3/13	1
	027	4/23	036	1/4	2		016	1/1	027	1/23	
		10/23	176	6/16			027	3/23	176	2/16	
		2/23	198	1/2			045	2/15	126	2/29	
	036	1/4	176	2/16	2			1/15	127	2/17	
	033	1/46	288	1/2	5		078	8/58	126	4/29	
	045	2/15	078	7/58	5	1	018	1/18	356	6/13	1
		2/15	126	4/29			045	1/15	127	1/17	
		3/15	127	2/17		0	045	2/15	127	2/17	
	066	1/2	078	4/58	5						
		1/2	126	2/29							
	078	31/58	126	21/29	5						

a) Studied PCR ribotype

b) Amount of strains that cluster with another PCR ribotype

c) The comparison between PCR ribotypes is depicted only once per threshold (e.g. comparison between RT016 and RT027 at threshold 6 is only shown in the RT016 row and is not again depicted in the RT027 row)

d) Matching other PCR ribotype strain

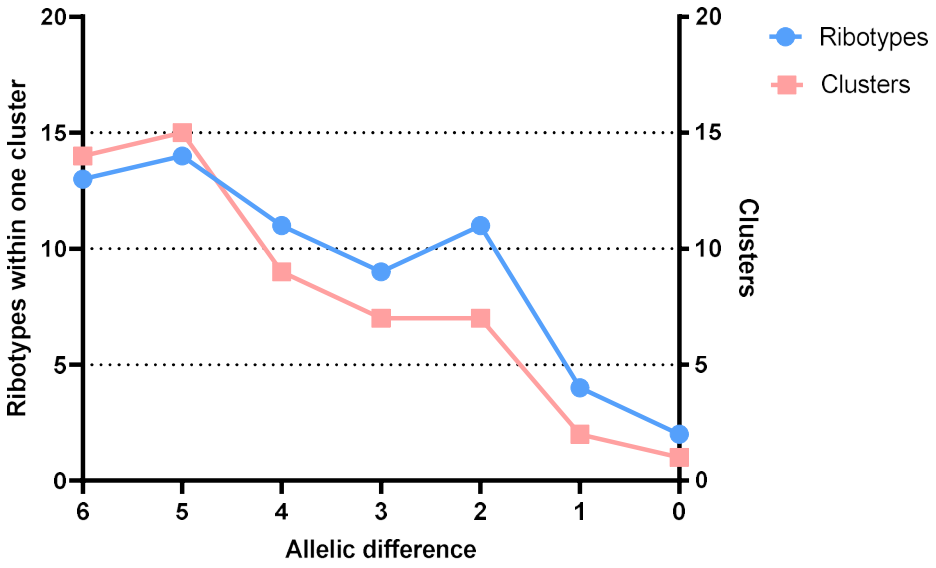
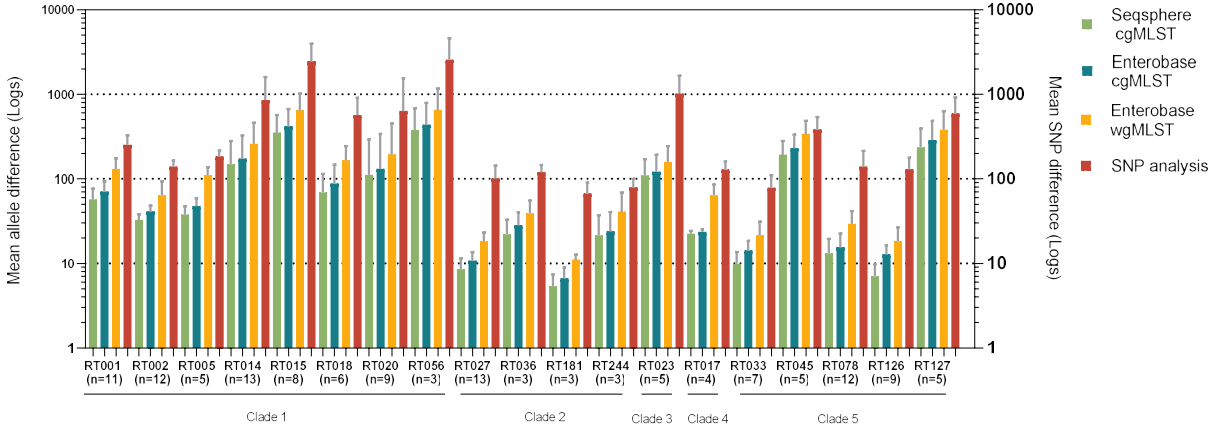


Figure 2: Clustering of different PCR ribotypes at different thresholds (0-6 allelic difference). The number of clustering PCR ribotypes is shown in blue and the amount of clusters at every threshold is shown in pink.

3 A


Mean allele difference (Logs)
Mean SNP difference (Logs)
10000
1000
100
10
1
Seqsphere cgMLST
Enterobase cgMLST
Enterobase wgMLST
SNP analysis
RT001 (n=11)
RT002 (n=12)
RT005 (n=5)
RT014 (n=13)
RT015 (n=8)
RT018 (n=6)
RT020 (n=9)
RT056 (n=3)
RT027 (n=13)
RT036 (n=3)
RT181 (n=3)
RT244 (n=3)
RT023 (n=5)
RT017 (n=4)
RT033 (n=7)
RT045 (n=5)
RT078 (n=12)
RT126 (n=9)
RT127 (n=5)
Clade 1
Clade 2
Clade 3
Clade 4
Clade 5

3 B

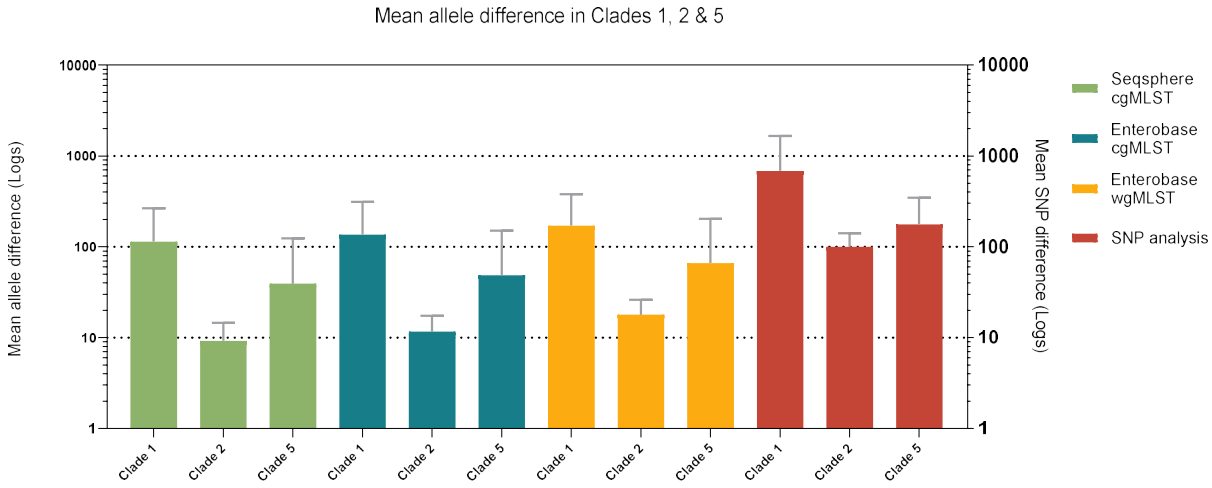
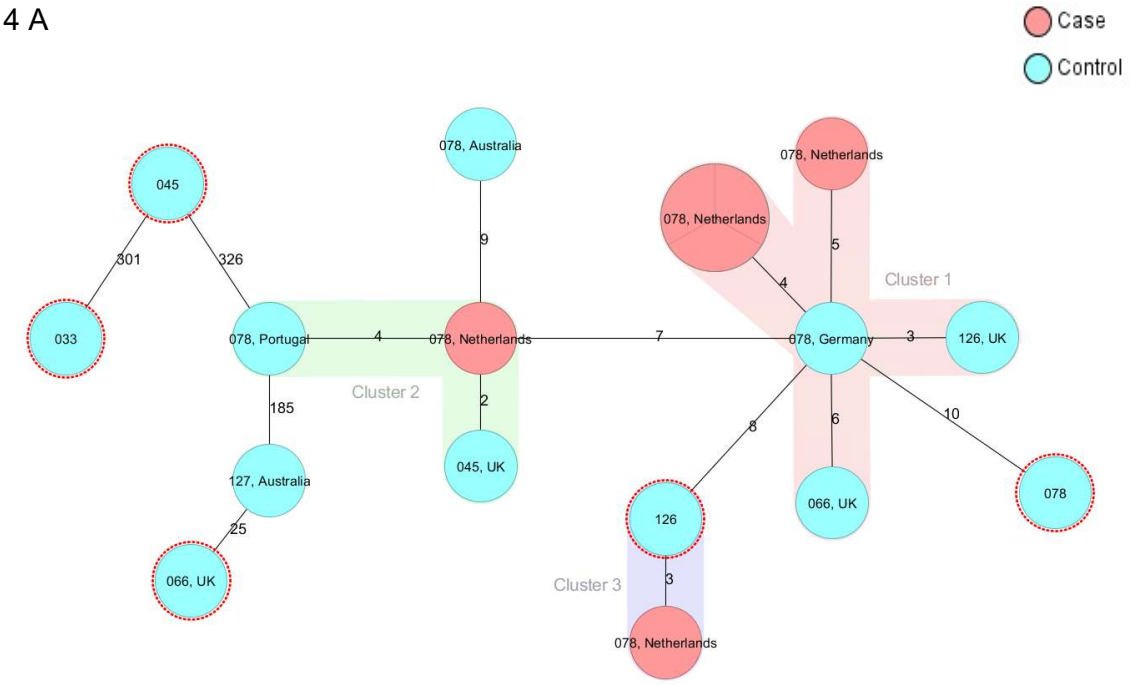


Figure 3: A) Mean intra-ribotype allele and SNP difference shown for ribotypes from MLST Clade 1 (RT001-RT056), Clade 2 (RT027-RT244), Clade 3 (RT023), Clade 4 (RT017) and Clade 5 (RT033-RT127). Mean intra-ribotype allele difference per ribotype is shown in light green, turquois and orange for SqSp cgMLST , EB cgMLST and EB wgMLST, respectively. Mean intra-ribotype SNP difference per ribotype is shown in red. B) Mean intra-ribotype allele and SNP difference shown for MLST Clade 1, Clade 2 and Clade 5. Mean intra-ribotype allele difference per Clade is shown in light green, turquois and orange for SqSp cgMLST, EB cgMLST and EB wgMLST, respectively. Mean intra-ribotype SNP difference per Clade is shown in red.

4 A


Case
Control
078, Australia
045
078, Netherlands
078, Netherlands
301
326
9
5
4
Cluster 1
033
078, Portugal
4
078, Netherlands
7
078, Germany
3
126, UK
Cluster 2
185
2
8
6
10
127, Australia
045, UK
126
066, UK
078
25
066, UK
Cluster 3
3
078, Netherlands

B

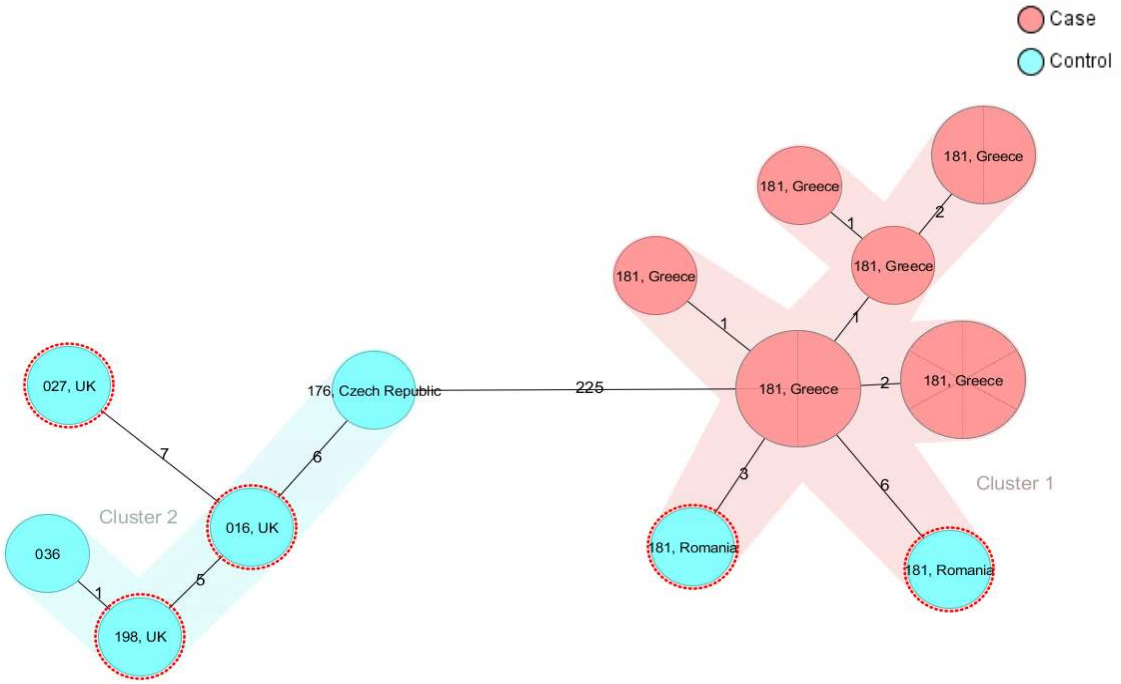
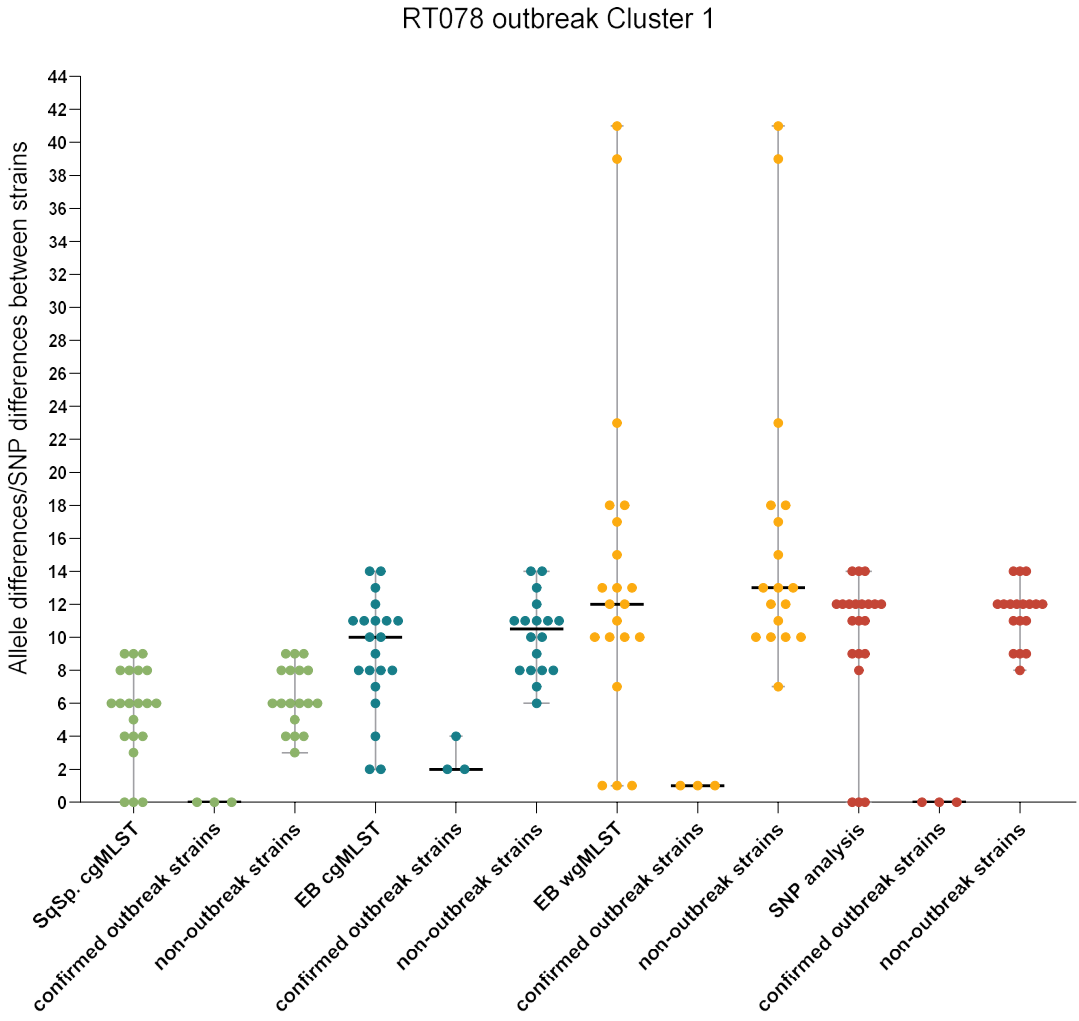

Case
Control
181, Greece
181, Greece
2
1
181, Greece
181, Greece
1
1
027, UK
176, Czech Republic
225
181, Greece
2
181, Greece
7
6
Cluster 1
Cluster 2
3
6
036
016, UK
181, Romania
181, Romania
1
5
198, UK

Figure 4: SqSP cgMLST analysis with minimum- spanning trees of 2 suspected CDI outbreaks of RT078 and RT181. A) Minimum-spanning tree of PCR-ribotype 078 (clade 5) CDI suspected outbreak with 6 cases (RT078, shown in red), confirmed outbreak with 3 cases (RT078, shown in largest red circle) and added control strains of ribotypes belonging to clade 5 (reference strains of RT033, RT045, RT066, RT078, RT126 shown in blue with red circles and non-reference strains of RT045, RT066, RT078, RT126 and RT127 shown in blue). B) Minimum-spanning tree of PCR-ribotype 181 (clade 2) CDI suspected outbreak with 15 suspected and confirmed cases (RT181, shown in red) and control strains of ribotypes of clade 2 (reference strains of RT016, RT027, RT181 and RT198 shown in blue with red circles and non-reference strains of RT036 and RT176 shown in blue.
The size and septation of the circle in the minimum-spanning trees corresponds to the number of included strains. The numbers between each circle correspond to the number of different alleles between the strains. The coloured shadowing of circles represents a cluster with <= 6 allele differences that are genetically related. One or more strains inside a circle means that these strains have 0 allele difference.

5A


RT078 outbreak Cluster 1
Allele differences/SNP differences between strains
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40
42
44
SqSp. cgMLST
confirmed outbreak strains
non-outbreak strains
EB cgMLST
confirmed outbreak strains
non-outbreak strains
EB wgMLST
confirmed outbreak strains
non-outbreak strains
SNP analysis
confirmed outbreak strains
non-outbreak strains

B

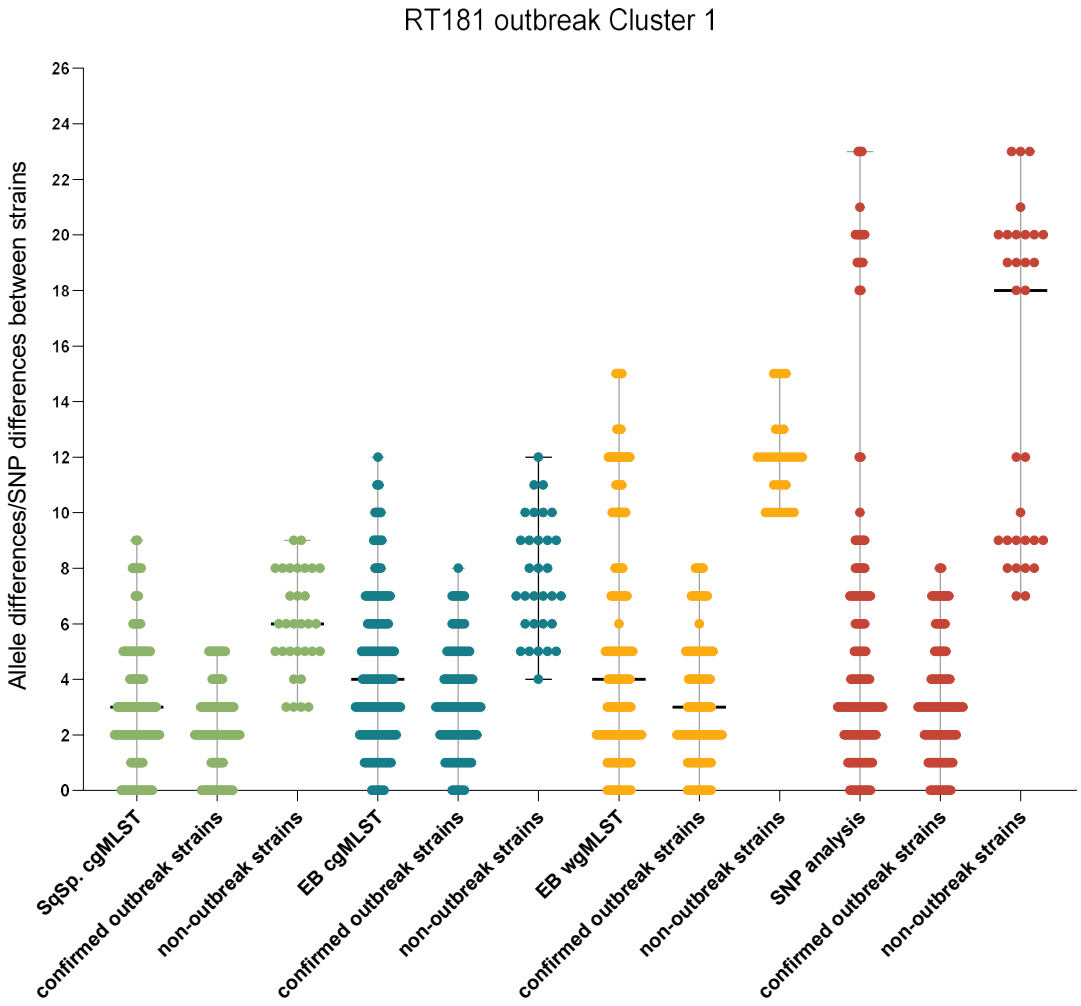


Figure 5: Visualised distance matrices of strain pairs based on cgMLST, wgMLST and SNP analysis of isolates of cluster 1 as described in figure 4A & 4B. A) Visualised distance matrix of strain pairs belonging to cluster 1 of RT078. B) Visualised distance matrix of strain pairs belonging to cluster 1 of RT181. Allele difference per pair of strains is shown in light green, turquois and orange for cgMLST in SeqSphere, cgMLST and wgMLST in EnteroBase, respectively. SNP difference per pair of strains is shown in red.

Table 2: comparison in range between outbreak and non-outbreak strains of RT078 and RT181

Typing method	Strains	Range (alleles or SNPs)	Difference between Range O & Range NO [a]
SqSp cgMLST	078 confirmed outbreak	0	3
	non-outbreak	3-9	
	181 confirmed outbreak	0-5	overlap
	non-outbreak	3-9	
EB cgMLST	078 confirmed outbreak	2-4	2
	non-outbreak	6-14	
	181 confirmed outbreak	0-8	overlap
	non-outbreak	4-12	
EB wgMLST	078 confirmed outbreak	1	6
	non-outbreak	7-41	
	181 confirmed outbreak	0-8	2
	non-outbreak	10-15	
SNP analysis	078 confirmed outbreak	0	8
	non-outbreak	8-14	
	181 confirmed outbreak	0-9	overlap
	non-outbreak	7-23	

a) Range O is the range in allele or SNP difference between all outbreak strains. Range NO is the range in allele or SNP difference of non-outbreak strains compared with themselves and compared with outbreak strains.

Table S1: included WGS strains of *C. difficile* in this study.

Sample ID	Ribotype	Collection Date	Country of Isolation	ST	CC	SRA accession no
SRR7308630-001	001	2013	UK	3	1	SRR7308630
SRR1519369-LL-001	001	?	?	3	1	SRR1519369
SRR7309226-001	001	2013	Netherlands	3	1	SRR7309226
SRR7308692-001	001	2013	Italy	3	1	SRR7308692
SRR7308732-001	001	2013	Finland	3	1	SRR7308732
SRR7308761-001	001	2013	Sweden	3	1	SRR7308761
SRR7308773-001	001	2013	Slovakia	3	1	SRR7308773
SRR7308776-001	001	2013	Slovakia	3	1	SRR7308776
SRR7308804-001	001	2013	Spain	3	1	SRR7308804
SRR7308833-001	001	2013	France	3	1	SRR7308833
SRR7308981-001	001	2013	Italy	3	1	SRR7308981
SRR7309099-001	001	2013	Germany	3	1	SRR7309099
SRR7309176-001	001	?	Bulgaria	3	1	SRR7309176
SRR7308836-001	001	?	Netherlands	3	1	SRR7308836
SRR7308645-002	002	2013	Portugal	8	1	SRR7308645
SRR7308677-002	002	2013	Netherlands	8	1	SRR7308677
SRR7309212-002	002	2013	Belgium	8	1	SRR7309212
SRR7308752-002	002	2013	France	8	1	SRR7308752
SRR7309014-002	002	2013	Sweden	8	1	SRR7309014
SRR7309032-002	002	2013	Finland	8	1	SRR7309032
SRR7309128-002	002	2013	Romania	8	1	SRR7309128
SRR7309141-002	002	2013	Portugal	8	1	SRR7309141
SRR7309152-002	002	2013	Romania	8	1	SRR7309152
SRR7309217-002	002	2013	Italy	8	1	SRR7309217
SRR7309219-002	002	2013	UK	8	1	SRR7309219
SRR1519370-LL-002	002	?	?	8	1	SRR1519370
SRR6042346-002	002	2010	USA	55	1	SRR6042346
SRR7308785-002	002	2013	Poland	8	1	SRR7308785
SRR7308659-002	002	2013	Germany	8	1	SRR7308659
SRR7308698-002	002	2013	UK	8	1	SRR7308698
SRR7308733-002	002	2013	Italy	8	1	SRR7308733
SRR7308704-002	002	2013	France	8	1	SRR7308704
SRR1519371-LL-003	003	2005	UK	12	1	SRR1519371
SRR7852176-003	003	?	UK	12	1	SRR7852176
SRR1519372-LL-004	004	1995	UK	115	1	SRR1519372
SRR7852181-005	005	?	UK	6	1	SRR7852181
SRR7852186-005	005	?	UK	6	1	SRR7852186
SRR7852185-005	005	?	UK	?	?	SRR7852185
SRR6042365-005	005	2010	USA	6	1	SRR6042365
ERR833662-005	005	?	UK	6	1	ERR833662
SRR6042356-005	005	2010	USA	6	1	SRR6042356
SRR7852187-005	005	?	UK	6	1	SRR7852187
SRR7852208-005	005	?	UK	6	1	SRR7852208

SRR6042370-005	005	2010	USA	6	1	SRR6042370
SRR1519373-LL-005	005	2005	UK	6	1	SRR1519373
SRR1519374-LL-006	006	1995	UK	2	1	SRR1519374
SRR1519375-LL-007	007	1995	UK	49	1	SRR1519375
SRR7852197-007	007	?	UK	49	1	SRR7852197
SRR7852191-009	009	?	UK	3	1	SRR7852191
SRR1519376-LL-009	009	2007	UK	3	1	SRR1519376
SRR1519377-LL-010	010	?	UK	15	1	SRR1519377
ERR833666-010	010	?	UK	15	1	ERR833666
ERR833672-010	010	?	UK	15	1	ERR833672
SRR1519378-LL-011	011	2005	UK	36	1	SRR1519378
ERR833660-012	012	?	UK	54	1	ERR833660
SRR1519379-LL-012	012	?	Belgiium	54	1	SRR1519379
SRR593175-013	013	?	USA	45	1	SRR593175
ERR1307016-014	014	?	Australia	2	1	ERR1307016
SRR7308710-014	014	2013	Hungary	13	1	SRR7308710
SRR7308735-014	014	2013	Sweden	49	1	SRR7308735
SRR7308765-014	014	2013	Czech Republic	2	1	SRR7308765
SRR7308801-014	014	2013	Italy	2	1	SRR7308801
SRR7309218-014	014	2013	Ireland	2	1	SRR7309218
SRR7308852-014	014	2013	UK	13	1	SRR7308852
SRR7308907-014	014	2013	Italy	2	1	SRR7308907
SRR7308925-014	014	2013	Czech Republic	2	1	SRR7308925
SRR7308936-014	014	2013	Slovakia	13	1	SRR7308936
SRR7309040-014	014	2013	Spain	2	1	SRR7309040
SRR7309087-014	014	2013	Romania	? (unknown)	?	SRR7309087
SRR7309095-014	014	2013	Sweden	? (unknown)	?	SRR7309095
SRR7309127-014	014	2013	Germany	2	1	SRR7309127
SRR7309213-014	014	2013	UK	14	1	SRR7309213
SRR7308784-014	014	2013	Hungary	2	1	SRR7308784
SRR1519380-LL-014	014	?	?	2	1	SRR1519380
SRR7309104-014	014	2013	France	2	1	SRR7309104
SRR7309188-014	014	2013	Poland	2	1	SRR7309188
SRR7308861-014	014	2013	UK	2	1	SRR7308861
SRR7309062-015	015	2013	UK	44	1	SRR7309062
SRR7309143-015	015	2013	UK	44	1	SRR7309143
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SRR7308689-020	020	2013	Germany	2	1	SRR7308689
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ERR125913-Sang-023	023	2008	UK	5	3	ERR125913
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ERR126268-023	023	2007	UK	22	3	ERR126268
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ERR044843-027	027	2006	Netherlands	1	2	ERR044843
ERR044846-027	027	2009	Poland	1	2	ERR044846
ERR030295-027	027	2010	UK	1	2	ERR030295
ERR044842-027	027	2006	Netherlands	1	2	ERR044842
ERR044841-027	027	2005	Netherlands	1	2	ERR044841
ERR044830-027	027	2006	Germany	1	2	ERR044830
ERR044838-027	027	2006	Netherlands	1	2	ERR044838
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ERR044848-027	027	2008	Switzerland	1	2	ERR044848
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ERR044833-027	027	2008	Ireland	1	2	ERR044833
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ERR247107-Sang-033	033	1982	Australia	11	5	ERR247107
ERR247106-Sang-033	033	1980	Australia	11	5	ERR247106
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ERR171349-078	078	2009	Netherlands	11	5	ERR171349
ERR257066-078-Farm-P7	078	2011	Netherlands	11	5	ERR257066
ERR257064-078-Pig-P7	078	2011	Netherlands	11	5	ERR257064
ERR171310-078	078	2006	Netherlands	11	5	ERR171310
ERR171312-078	078	2007	Netherlands	11	5	ERR171312
ERR171314-078	078	2007	Netherlands	11	5	ERR171314
ERR171321-078	078	2008	Netherlands	11	5	ERR171321

ERR171327-078	078	2009	Netherlands	11	5	ERR171327
ERR403717-078	078	2008	Netherlands	11	5	ERR403717
ERR257072-078-Farm-P5	078	2011	Netherlands	11	5	ERR257072
ERR257071-78-Pig-P5	078	2011	Netherlands	11	5	ERR257071
ERR257069-078-Farm-P6	078	2011	Netherlands	11	5	ERR257069
ERR257056-078-Farm-P10	078	2011	Netherlands	11	5	ERR257056
ERR257055-078-Pig-P8	078	2011	Netherlands	11	5	ERR257055
ERR257054-078-Farm-UP	078	2011	Netherlands	11	5	ERR257054
ERR171356-078-Farm-P1	078	2011	Netherlands	11	5	ERR171356
ERR257047-078-Farm-P11	078	2011	Netherlands	11	5	ERR257047
ERR257060-078-Farm-P9	078	2011	Netherlands	11	5	ERR257060
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SRR7308912-078	078	2013	Ireland	11	5	SRR7308912
SRR7308920-078	078	2013	Spain	11	5	SRR7308920
SRR7309151-078	078	2013	Germany	11	5	SRR7309151
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SRR7308895-078	078	2013	Germany	11	5	SRR7308895
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ERR171315-078	078	2007	Netherlands	11	5	ERR171315
ERR171318-078	078	2007	Netherlands	11	5	ERR171318
ERR171334-078	078	2009	Netherlands	11	5	ERR171334
ERR171336-078	078	2002	Netherlands	11	5	ERR171336
ERR171347-078-Pig-UP	078	2002	Netherlands	11	5	ERR171347
ERR171348-078-Pig-UP	078	2009	Netherlands	11	5	ERR171348
SRR7308809-078	078	2013	Germany	11	5	SRR7308809
SRR7308838-078	078	2013	Portugal	11	5	SRR7308838
ERR171357-078-Pig-P1	078	2011	Netherlands	11	5	ERR171357
SRR7308822-078	078	2013	Italy	11	5	SRR7308822
SRR7309025-078	078	2013	Italy	11	5	SRR7309025
SRR7309049-078	078	2013	Portugal	11	5	SRR7309049
SRR1519424-LL-079	079	1994	?	126	1	SRR1519424
SRR1519425-LL-081	081	1996	UK	9	1	SRR1519425
SRR7852189-081	081	?	UK	9	1	SRR7852189
SRR7852192-081	081	?	UK	9	1	SRR7852192
SRR1519426-LL-083	083	1997	UK	59	1	SRR1519426
SRR1519427-LL-084	084	1995	UK	48	1	SRR1519427
SRR593202-084	084	?	USA	17	1	SRR593202
ERR125915-Sang-085	085	2009	UK	39	4	ERR125915
SRR1519428-LL-085	085	?	?	39	4	SRR1519428
ERR2216001-087	087	?	?	46	1	ERR2216001
SRR1519429-LL-087	087	?	?	46	1	SRR1519429
SRR1519430-LL-088	088	1997	UK	39	4	SRR1519430
SRR1519431-LL-095	095	1995	?	2	1	SRR1519431
ERR2215983-102	102	?	?	24	1	ERR2215983

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ERR3276441-106	106	?	Spain	42	1	ERR3276441
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ERR3278163-106	106	?	Spain	42	1	ERR3278163
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ERR3288336-106	106	?	Spain	42	1	ERR3288336
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SRR6042371-106/174	106/174	2010	USA	42	1	SRR6042371
SRR593379-111	111	?	USA	123	2	SRR593379
SRR1519433-LL-115	115	1997	UK	3	1	SRR1519433
SRR1519434-LL-117	117	1996	Poland	54	1	SRR1519434
SRR1519435-LL-118	118	2007	UK	42	1	SRR1519435
SRR1519436-LL-122	122	?	?	116	2	SRR1519436
ERR247101-Sang-126	126	?	Australia	258	5	ERR247101
ERR256918-Sang-126	126	2011	UK	11	5	ERR256918
ERR247083-Sang-126	126	2009	Australia	258	5	ERR247083
ERR2898771-126	126	2002	Italy	11	5	ERR2898771
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ERR257019-Sang-126	126	2010	Belgium	11	5	ERR257019
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ERR256916-Sang-126	126	2010	UK	11	5	ERR256916
ERR2898818-127	127	2008	Australia	11	5	ERR2898818
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ERR2898911-127	127	2013	Australia	11	5	ERR2898911
ERR2898801-127	127	2012	Australia	11	5	ERR2898801
ERR2898802-127	127	2014	Australia	11	5	ERR2898802
ERR247118-Sang-127	127	2005	Australia	11	5	ERR247118
ERR2898820-127	127	2005	Australia	11	5	ERR2898820
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ERR2898799-127	127	2011	Australia	11	5	ERR2898799
ERR2898823-127	127	2010	Japan	11	5	ERR2898823
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ERR2898824-127	127	2010	Japan	11	5	ERR2898824
ERR2898828-127	127	2011	Taiwan	11	5	ERR2898828
ERR2898829-127	127	2012	Taiwan	11	5	ERR2898829
ERR2898898-127	127	2009	Auastralia	11	5	ERR2898898
ERR171360-Sang-130	130	2010	UK	158	4	ERR171360
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SRR593375-137	137	?	USA	4	1	SRR593375
SRR1519439-LL-153	153	2003	Netherlands	32	2	SRR1519439
SRR1519440-169	169	2005	UK	13	1	SRR1519440
ERR171361-Sang-172	172	2010	UK	159	4	ERR171361
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SRR7852188-174	174	?	UK	6	1	SRR7852188

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SRR7308660-176	176	2013	Czech Republic	1	2	SRR7308660
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SRR7309148-176	176	2013	Czech Republic	1	2	SRR7309148
SRR7309197-176	176	2013	Czech Republic	1	2	SRR7309197
SRR7308825-176	176	2013	Czech Republic	1	2	SRR7308825
SRR7308625-176	176	2013	Czech Republic	1	2	SRR7308625
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SRR7308771-176	176	2013	Czech republic	1	2	SRR7308771
SRR7308985-176	176	2013	Germany	1	2	SRR7308985
SRR7308631-176	176	2013	Germany	1	2	SRR7308631
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HNFKWDSXX-103786-001-038-181	181	2019	Greece	1	2	ERR3981067
HNFKWDSXX-103786-001-039-181	181	2019	Greece	1	2	ERR3981068
HNFKWDSXX-103786-001-040-181	181	2019	Greece	1	2	ERR3981069
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HNFKWDSXX-103786-001-044-181	181	2019	Greece	1	2	ERR3981071
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HNFKWDSXX-103786-001-046-181	181	2019	Greece	1	2	ERR3981073
HNFKWDSXX-103786-001-047-181	181	2019	Greece	1	2	ERR3981074
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Isolate1-Leeds	181	?	Romania	1	2	ERR3981079
Isolate2-Leeds	181	?	Romania	1	2	ERR3981080
SRR1519442-LL-198	198	2006	UK	1	2	SRR1519442
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ERR833670-220	220	?	UK	2	1	ERR833670
ERR833669-220	220	?	UK	2	1	ERR833669
SRR7852190-225	225	?	UK	58	1	SRR7852190
ERR247085-Sang-237	237	2009	Australia	167	5	ERR247085
ERR247082-Sang-237	237	2008	Australia	167	5	ERR247082
ERR1854833-238	238	2007	Australia	169	5	ERR1854833
ERR1854841-239	239	2005	Australia	168	5	ERR1854841
SRR2751309-244	244	?	Australia	41	2	SRR2751309

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SRR2751302-Sang-244	244	?	Australia	41	2	SRR2751302
SRR2751305-244	244	?	Australia	41	2	SRR2751305
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SRR2751293-244	244	?	Australia	41	2	SRR2751293
ERR2215996-Clade2-244	244	?	?	41	2	ERR2215996
ERR2215977-Clade2-251	251	?	?	231	2	ERR2215977
ERR1347091-251	251	?	Australia	231	2	ERR1347091
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ERR1347089-251	251	?	Australia	231	2	ERR1347089
ERR1347088-251	251	?	Australia	231	2	ERR1347088
ERR2898847-288	288	2006	Australia	11	5	ERR2898847
ERR2898924-288	288	2013	Australia	11	5	ERR2898924
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SRR7309022-356	356	2013	Italy	17	1	SRR7309022
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ERR3288469-404	404	?	Spain	110	1	ERR3288469
ERR3288324-404	404	?	Spain	110	1	ERR3288324
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ERR3278162-404	404	?	Spain	110	1	ERR3278162
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ERR1854837-585	585	1998	?	164	5	ERR1854837
ERR1854838-586	586	2007	Australia	167	5	ERR1854838