Simultaneous detection and ribotyping of Clostridioides difficile, and toxin gene detection directly on fecal samples

Tessel Meike van Rossen (t.vanrossen@amsterdamumc.nl)
Amsterdam UMC location VUmc https://orcid.org/0000-0001-8945-134X
Joffrey van Prehn
Leiden University Medical Center
Alex G. Koek
Amsterdam UMC
Marcel Jonges
Amsterdam UMC
Robin van Houdt
Amsterdam UMC
Rosa van Mansfeld
Amsterdam UMC
Ed J. Kuijper
Leiden University Medical Center
Christina M.J.E. Vandenbroucke-Grauls
Amsterdam UMC
Andries E. Budding
inBiome B.V.

Research

Keywords: Clostridioides difficile, PCR ribotyping, toxin genes, infection control, hospital epidemiology

Posted Date: January 7th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-41133/v2

Version of Record: A version of this preprint was published at Antimicrobial Resistance and Infection Control on January 29th, 2021. See the published version at https://doi.org/10.1186/s13756-020-00881-

9.

Simultaneous detection and ribotyping of *Clostridioides difficile,* and toxin gene detection directly on fecal samples – original article

Tessel M. van Rossen [a]#, Joffrey van Prehn [b], Alex Koek [a], Marcel Jonges [a], Robin van Houdt [a], Rosa van Mansfeld [a], Ed J. Kuijper [b], Christina M.J.E. Vandenbroucke-Grauls [a], Andries E. Budding [c]

[a]Amsterdam UMC, Vrije Universiteit Amsterdam, department of Medical Microbiology & Infection Control, Amsterdam Infection & Immunity Institute, De Boelelaan 1117, Amsterdam, the Netherlands

[b]Leiden University Medical Center, Center for Infectious Diseases, department of Medical Microbiology, Albinusdreef 2, Leiden, the Netherlands

[c]inBiome B.V., Science park 106, Amsterdam, the Netherlands

#Address correspondence to Tessel M. van Rossen, Amsterdam UMC locatie VUmc, PK 2X132, De Boelelaan 1117, Amsterdam, the Netherlands,
t.vanrossen@amsterdamumc.nl, telephone number: 0031-204440799.

Abstract

Background: *Clostridioides difficile* is the most common cause of nosocomial diarrhea. Ribotyping of cultured strains by a PCR-based test is used to study potential transmission between patients. We aimed to develop a rapid test that can be applied directly on fecal samples for simultaneous detection and ribotyping of *C. difficile,* as well as detection of toxin genes.

Methods: We developed a highly specific and sensitive primer set for simultaneous detection and ribotyping of *C. difficile* directly on total fecal DNA. Toxin genes were detected with primers adapted from Persson et al. (CMI, 2008). Our study set comprised 130 fecal samples: 65 samples with positive qPCR for *C. difficile* toxin A/B genes and 65 *C. difficile* qPCR negative samples. PCR products were analyzed by capillary gel electrophoresis.

Results: Ribosomal DNA fragment peak profiles and toxin genes were detected in all 65 *C. difficile* positive fecal samples and in none of the 65 *C. difficile* negative samples. The 65 samples were assigned to 27 ribotypes by the Dutch reference laboratory. Our peak profiles corresponded to these ribotypes, except for two samples. During a *C. difficile* outbreak, patients were correctly allocated to the outbreak-cluster based on the results of direct fecal ribotyping, before *C. difficile* isolates were cultured and conventionally typed.

Conclusion: *C. difficile* ribotyping directly on fecal DNA is feasible, with sensitivity and specificity comparable to that of diagnostic toxin gene qPCR and with ribotype assignment similar to that obtained by conventional typing on DNA from cultured isolates. This supports simultaneous diagnosis and typing to recognize an outbreak.

Key words: *Clostridioides difficile, PCR ribotyping, toxin genes, infection control, hospital epidemiology*

Background

Clostridioides difficile is an anaerobic, spore-forming bacterium and the most common cause of hospital-associated diarrhea. In severe cases, CDI can lead to pseudomembranous colitis, toxic megacolon and bowel perforation. The attributable mortality of *C. difficile* infection in an endemic setting is approximately 5% (1, 2). Hospital outbreaks of *C. difficile* occur often, presumably due to the large numbers of bacterial spores that can be excreted by symptomatic patients. Outbreaks threaten patient safety, and pose a substantial financial burden to healthcare. Incremental costs per CDI are estimated to be approximately €5000 ($5700), but in outbreak-settings these can increase to €7000-16000 ($7.900-18.100) per patient (3, 4).

To detect *C. difficile* transmission between patients, bacterial strains need to be characterized beyond the species level. Furthermore, early recognition of hypervirulent strains is important for prompting institution of strict infection control measures, since these strains are associated with higher mortality and transmission rates (5-7). A commonly used typing technique for *C. difficile,* which is recommended for surveillance purposes, is PCR ribotyping (8). This method is based on strain-specific differences in number and length of the ribosomal 16-23S interspace regions (IS-regions). A drawback of the currently used PCR ribotyping methods is that they can only be performed on cultured *C. difficile* isolates (9-14). This delays the time to results and eventual

implementation of infection control measures. Therefore, Janezic et al. designed new primers and tested these directly on total fecal DNA; they obtained a specificity of 100% and a sensitivity of 84.8% (15).

Our objective was to develop ribotyping primers which could also be applied directly on fecal DNA but with higher sensitivity, while retaining specificity. Ideally, direct ribotyping on feces should be as sensitive as qPCR, as this would make it possible to use it as first-line diagnostic assay. We assessed this new method during a suspected outbreak of *C. difficile* in our hospital. Thereafter, we validated our primers and our optimized protocol in a larger sample set of *C. difficile*-positive and -negative stools to assess sensitivity, specificity, and typing performance.

Methods

Primers

Primers were designed with AliView (Uppsala University, Uppsala, Sweden) based on alignment of 20 downloaded *C. difficile* sequences from the Silva database (Max Planck Institute for Marine Microbiology and Jacobs University, Bremen, Germany) (16). Specificity was assessed by comparison to *C. difficile* closest phylogenetic relatives, *C. sordellii* and *C. bifermentans*. Primers were targeted to the 16S-23S ribosomal DNA interspace regions (IS-regions). Since we aimed to perform ribotyping directly in fecal samples comprising high loads of non-*C. difficile* bacteria, we attempted to improve specificity for *C. difficile* by shifting the primers from the more conserved 16S region towards the IS-region. We observed that different primers were needed for amplification

of short (<400 nucleotides) and long (>400 nucleotides). This resulted in the following four primers:

CdiffISf1: CTGTTTAATTTTGAGGGTTCGTTTTTACG,

CdiffISf2a: CCTACTGTTTAATTTTGAAAGTTCTTTACG,

CdiffISf2b: CCTACTGTTTAATTTTGAAAGTTCTTTATG (forward) and

ClosR: AGGCATCCGCCCTGCACCCT (reverse).

Using BLAST, we observed a 100%/100% match with the 20 *C. difficile* sequences and no cross reactivity with *C. sordellii* and *C. bifermentans,* which are taxonomically closest to *C. difficile*. Forward primers were FAM-labeled for analysis with ABI Prism 3500 GeneticAnalyzer (Applied Biosystems, Foster City, California, USA). For detection of toxin A (*tcdA*), toxin B (*tcdB*), binary toxin (*cdtA*, *cdtB*) genes, we used the primers designed by Persson et al. (17). Forward primers were HEX-labeled.

Fecal samples and *C. difficile* strains

During the optimization phase of our direct ribotyping technique, a *C. difficile* outbreak was suspected in the Intensive Care Unit (ICU) of our institution. To assess the clinical applicability of our method, we applied this new technique directly to the fecal samples of eleven patients with positive *C. difficile* tests. Thereafter, we validated our method in a larger study set of 130 fecal samples: 65 samples with positive qPCR for *C. difficile* toxin A/B genes (the standard diagnostic test for *C. difficile* detection in our laboratory) and 65 *C. difficile* qPCR negative samples. In addition to the eleven *C. difficile* positive fecal samples from the *C. difficile* outbreak, we randomly selected 54 fecal samples with positive qPCR for *C. difficile* toxin A and/or B genes. For control, *C. difficile* strains were

cultured from all 65 fecal samples with positive qPCR for *C. difficile* toxin A and/or B genes. Culture was performed anaerobically on selective *C. difficile* agar plates (bioMérieux, Marcy l'Etoile, France) according to standard protocol of our diagnostic microbiological laboratory. All 65 *C. difficile* strains were also sent to the Dutch National Reference Laboratory at Leiden University Medical Center (LUMC) for conventional ribotyping using a standardized protocol (20). These ribotypes served as reference. As control samples we randomly selected 65 fecal samples with negative qPCR's for *C. difficile* from the routine diagnostic microbiology laboratory. Of these *C. difficile* negative samples, three were positive in PCR for *Salmonella* spp., six for *Campylobacter* spp., one for *Shigella* spp., one for both *Campylobacter* spp. and *Shigella* spp., one for parechovirus, one for norovirus and one for enterovirus. To assess potential cross-reactivity *in vitro*, we also performed direct ribotyping on *C. difficile's* closest taxonomically relatives, *C. sordellii* and *C. bifermentans*. All fecal samples were obtained from hospitalized patients with diarrhea, admitted to Amsterdam UMC, location VUmc, between 2016 and 2018 (Supplementary Table 1).

DNA isolation from fecal samples and *C. difficile* strains

DNA isolation was performed according to standard protocol of our diagnostic microbiological laboratory. Within 36 hours after arrival at the laboratory, fecal samples were stored at -80°C. For this study, samples were thawed and a pea-sized amount of feces (100-400mg) was collected with a swab. In case of liquid feces, swabs were immersed halfway into the liquid. Swabs were placed in Eppendorf tubes, vortexed and incubated in 1 ml S.T.A.R. buffer (Roche, Basel, Switzerland) at -80°C for 1 hour or

overnight. Subsequently, tubes were heated at 100°C for 10 min. After centrifugation for 10 seconds at 4000 rpm, 300 µl of the supernatant fraction was suspended in 300 µl lysis buffer for DNA isolation with MagNaPure96 (Roche, Basel, Switzerland). *C. difficile* strains were collected with a 1 µl inoculation loop and stored in 200 µl TE-lysis buffer (Tris-HCl, EDTA, pH 8.0, Promega V6231) at -20°C. After thawing, suspensions were vortexed for 15 seconds and centrifuged for 3 minutes at 12000 rpm. The supernatant was diluted 1:10 (1 µl supernatant and 9 µl nuclease free water and 15 µl Mastermix was added for the PCR reaction.

Amplification and analysis

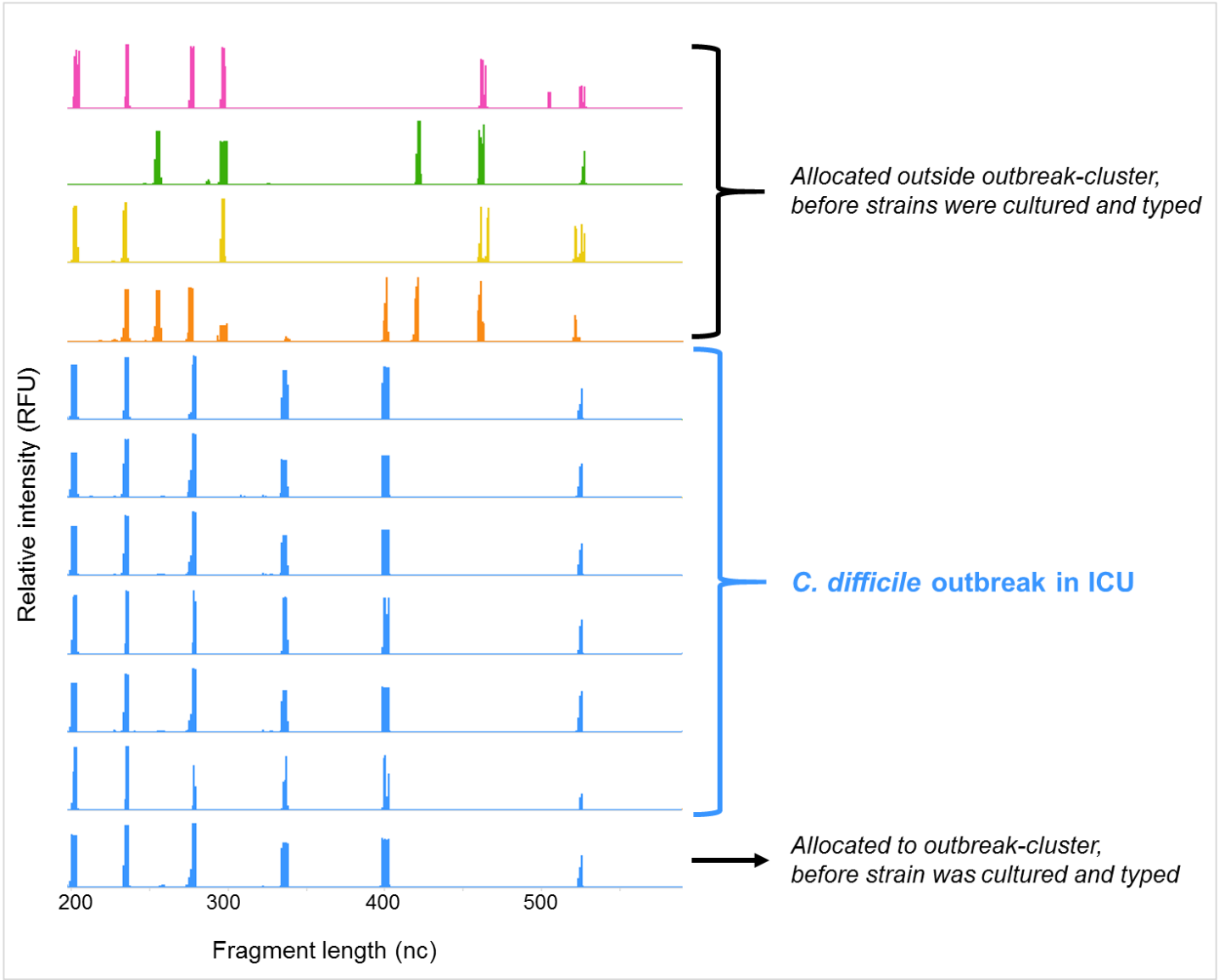
For both PCR reactions of direct ribotyping and toxin gene detection, extracted DNA of cultured strains was diluted 1:10; DNA of fecal samples was used undiluted. When inhibition of the PCR reaction was suspected (no peaks or primer-dimer signal detected), the reaction was repeated with total fecal DNA diluted 1:5 to identify a possible false negative result due to inhibition. PCR mixtures for ribotyping, with a final volume of 25 µl consisted of 10 µl DNA and 15 µl of IS-pro mastermix (inBiome bv) with 0.13 µM of each primer. PCR mixtures for toxin gene detection, with a final volume of 25 µl consisted of 10 µl DNA and 15 µl IS-pro mastermix (inBiome bv) with 0.6 µM of each *tcdA*-primer, 0.4 µM *tcdB*-F primer, 0.2 µM of each *tcdB*-R primer, 0.05 of each *cdtA*-F primer, 0.1 µM *cdtA*-R primer and 0.1 µM of each *cdtB*-primer. PCR mixtures for ribotyping and toxin gene detection were placed in separate wells. Amplifications were carried out with GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA, USA). Cycling conditions for both ribotyping and toxin gene detection PCRs were 95°C

for 10 min, 12 cycles (with 0.7°C decrements at every cycle) of 94°C for 30 s, 65°C for 30 s and 72°C for 1 min and 23 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 1 min, followed by extension at 72°C for 11 min. Afterwards PCR product was stored at 4°C. Within 12 hours, 5 µl PCR product and 20 µl formamide with custom size marker (eMix, InBiome, Amsterdam, the Netherlands) was pipetted in a 96-wells plate, heated at 94°C for 3 min and cooled down to 4°C. DNA fragment analysis was performed with ABI Prism 3500 GeneticAnalyzer (Applied Biosystems, Foster City, California, USA) in separate capillaries for direct ribotyping (FAM-labeled primers) and toxin gene detection (HEX-labeled primers). DNA fragment lengths including intensity were visualized and analyzed with TIBCO Spotfire (TIBCO Software Inc., Palo Alto, California, USA). To standardize the amount of bacterial DNA, we calculated relative intensity of each ribosomal DNA fragment peak by dividing the absolute intensity of each peak of a sample by the absolute intensity of the highest peak of that sample. Clustering of fecal samples based on ribotype DNA fragment peak profile similarity was performed by UPGMA (Unweighted Pair Group Method with Arithmetic Mean), with cosine correlation as distance measure and average value as ordering weight. Toxin gene-specific peaks were defined according to DNA fragment sizes described by Persson et al.: *tcdA* (629bp), *tcdB* (410bp), *cdtA* (221bp) and *cdtB* (262bp). Presence or absence of toxin gene peaks was scored binarily using an intensity cutoff of 3000 RFU.

Results

Application of direct ribotyping during an outbreak with *C. difficile*

During the optimization phase of our direct ribotyping technique, a *C. difficile* outbreak was suspected in the ICU. In our institution, the standard typing technique for *C. difficile* is Amplified Fragment Length Polymorphism (AFLP) on cultured strains. The suspected outbreak cluster involved six patients with the same *C. difficile* AFLP-type. During this outbreak, samples of five other patients became positive for *C. difficile* by qPCR or toxin enzyme immune assay (EIA). We performed direct ribotyping on total fecal DNA of all eleven patients. The six patients with the same *C. difficile* AFLP-type had identical ribotype peak profiles (figure 1). In the five other patients that became positive for *C. difficile* during the outbreak, direct ribotyping enabled us to allocate 1 of the 5 patients to the outbreak-cluster and 4/5 patients outside the outbreak-cluster (figure 1). Importantly, results of direct fecal ribotyping were obtained before strains were cultured and conventionally typed by AFLP.



199 **Figure 1** – Ribosomal DNA fragment profiles in fecal samples of eleven patients with

200 positive *C. difficile* toxin A and/or B genes qPCR. Bacterial transmission was suspected

201 in seven patients with identical peak profiles (blue). *nc = nucleotides, RFU = relative*

202 *fluorescence units*

203

204 ***C. difficile* PCR ribotyping and toxin gene detection**

205 After our first experience with direct ribotyping during the outbreak, we aimed to validate

206 our technique with a larger sample set of 130 fecal samples (including the 11 samples

207 collected during the outbreak). With the ribotype primers we amplified DNA of a total of
208 65 fecal samples that were previously proven to contain *C. difficile* by qPCR for *C.*
209 *difficile* toxin A and/or B genes (the standard diagnostic test for *C. difficile* detection in
210 our laboratory). Mean Cp value of *C. difficile* toxin gene qPCR was 33 (range 27-40
211 cycles, Supplementary Table 1). DNA fragment peak profiles were obtained from all 65
212 fecal samples (3 after 1:5 dilution because of inhibition) and from all 65 cultured strains.
213 Hence, the sensitivity of the new primers set for toxigenic *C. difficile* detection was 100%
214 (n=65, 95% Confidence Interval (CI) 94.5-100%).
215
216 We observed DNA fragment peaks ranging in size from approximately 200 to 590
217 nucleotides, consistent with published studies when corrected for differences in primer
218 binding sites (11, 12, 14, 15). Also the number of DNA fragments was in agreement with
219 previously described ribotype profiles, and varied between 5 and 10 (11, 12, 14, 15).
220
221 To examine the specificity of our primers for *C. difficile* detection, we applied the
222 primers to total DNA obtained from *C. bifermentans* and *C. sordellii* strains and 65 fecal
223 samples with negative qPCR for *C. difficile* toxin genes. Of these samples, fourteen were
224 positive by diagnostic PCR's for other bacterial species and viruses that are well-known
225 causes of diarrhea such as *Campylobacter spp.*, *Salmonella spp.* and norovirus. No DNA
226 fragment peak profiles were detected in these samples, indicating a diagnostic specificity
227 of 100% (n=65, 95% CI 94.5-100%).
228

To assess reproducibility, DNA isolation and direct ribotyping was performed *in duplicate* on a subset of 40 fecal samples with a positive qPCR for *C. difficile* toxin A and/or B genes. DNA fragment peak profiles were observed in 40/40 paired fecal samples. Profiles of 36/40 paired fecal samples were 100% identical (90%). All discrepancies were found in larger DNA fragments (>400) in low load samples (*C. difficile* toxin A and/or B genes qPCR Cp values 35-39)

To examine possible technical issues of ribotyping directly on feces – for example decreased intensity of DNA fragment peaks due to PCR inhibition or appearance of nonspecific peaks due to an excess of fecal DNA – the peak profile of each fecal sample was compared with that of its corresponding cultured strain, see figure 2 for example. Peak profiles of 61/65 paired fecal samples and strains were completely identical (94%). In 3/65 samples we observed 1 peak difference. These samples had a low bacterial load in qPCR (Cp values 35-39); and it was one of the larger DNA fragment peaks (>400 nucleotides) that was missing. In 1/65 samples we observed that the three largest DNA fragments in the strain profile were missing in the profile of the fecal sample (Cp value 29).

For detection of toxin A (*tcdA*), toxin B (*tcdB*) and binary toxin (*cdtA*, *cdtB*) genes directly on total fecal DNA we used primers designed by Persson et al. and added these in our study set (figure 3) (17). All *C. difficile* positive fecal samples showed at least one toxin gene peak, whereas no peaks were observed in the *C. difficile* negative fecal samples. The presence of toxin genes specific for different ribotypes was consistent with

literature (11, 21-23). In one sample with RT190, toxin A, B and binary toxin B genes were detected but not binary toxin A gene. This could be due to non-specificity of our assay; however, *C. difficile* strains with presence of binary toxin B but not binary toxin A gene have been described (24, 25). Also, we detected both toxin A and B gene peaks in RT017 samples, while this ribotype is known to produce only toxin B (26-28). This was observed previously by Persson et al.: some strains that lack toxin A expression can still harbor parts of toxin A gene which can be detected by PCR (17).

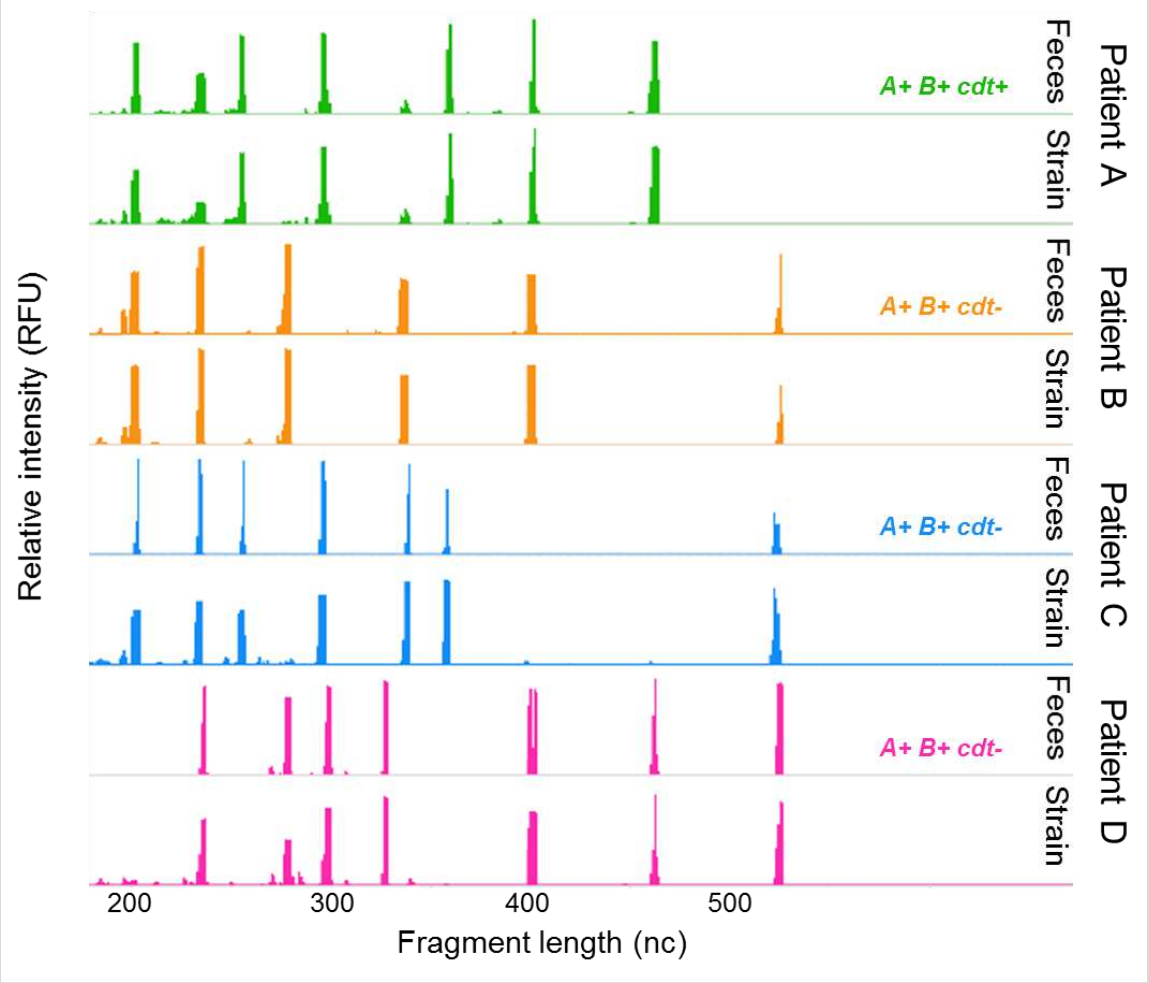


Figure 2 – Examples of DNA fragment peak profiles from four fecal samples and their corresponding cultured strains. *nc = nucleotides, RFU = relative fluorescence units, A = toxin A gene, B = toxin B gene, cdt = binary toxin genes.*

Reference ribotypes obtained by conventional ribotyping of strains

Conventional ribotyping of all 65 *C. difficile* strains that were cultured from the 65 fecal samples was performed by the Dutch National Reference Laboratory. These ribotyping results served as reference. The Reference Laboratory could not determine the ribotype of 2/65 strains and determined a probable ribotype in 5/65 strains. Overall, 63/65 strains of our study set were assigned to 27 different reference ribotypes.

Clustering of fecal samples based on peak profile similarity

We assessed if direct ribotyping on fecal samples was feasible as first screening tool for detection of a clonally related *C. difficile* cluster by performing cluster analysis based on ribosomal DNA fragment profile similarity. A heat map and dendrogram were created based on peak profiles of all 65 fecal samples with positive qPCR for *C. difficile* toxin A/B genes (figure 2). The resulting clusters consisted of fecal samples containing the same *C. difficile* ribotypes as determined by the Reference Laboratory (for example, one cluster consisted of four fecal samples that all contained RT002), except for two samples: one with RT002 and one with RT050. The ribotyping patterns in both samples lacked the larger DNA fragment peaks when compared to profiles of samples with the same reference ribotype.

In conventional ribotyping, a pattern with a single band difference is usually considered as a different ribotype. Using this definition, we assessed the performance of direct ribotyping on feces for ribotype assignment by comparing peak profiles of samples with

the same reference ribotype. We observed identical peak profiles in 42/48 (90%) fecal samples containing identical ribotypes (RT001: 4 out of 5 profiles were identical, RT002: n=3/4, RT012: n=3/3, RT014: n=4/4, RT015: 2/2, RT017: n=10/10, RT026: n=5/5, RT050 n=0/2, RT078: n=5/6, RT190: n=2/2, RT258: n=3/3, RT626: n=2/2).

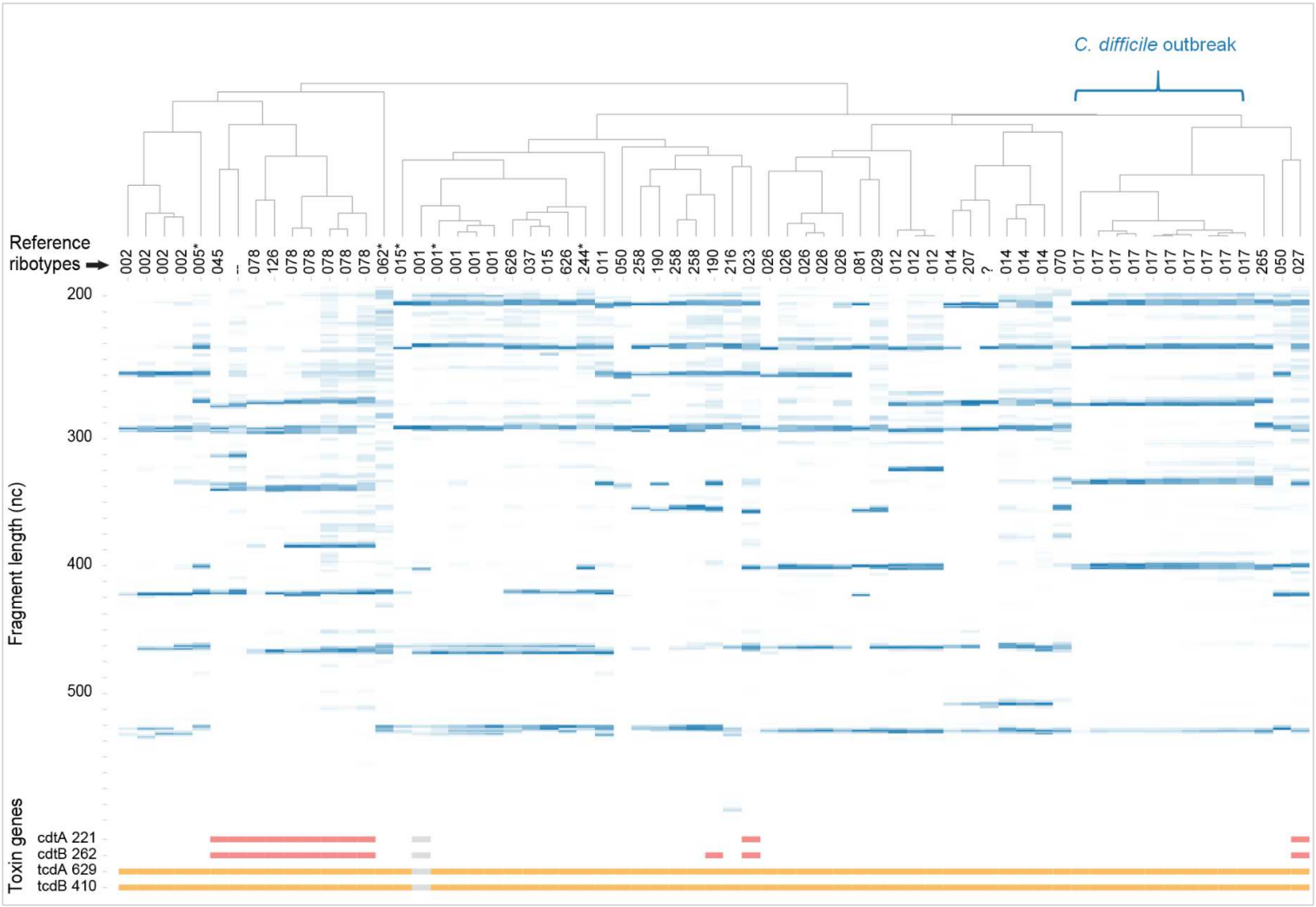


Figure 3 - Heat map and dendrogram based on ribosomal DNA fragment peak profiles of all fecal samples. Each column represents one sample. Numbers on the X-axis correspond to the reference ribotype assigned to the corresponding strain by conventional ribotyping by the Dutch National Reference Laboratory. Numbers on the Y-axis correspond to the DNA fragment length of the detected ribotype DNA fragment peaks/bands (in blue)

and/or *C. difficile* toxin genes (red/orange) per sample. Toxin gene detection could not be performed in one sample due to insufficient DNA material (grey).

'' = probable reference ribotype; '?' = unknown reference ribotype; '-' = no reference ribotype; tcdA = C. difficile toxin A; tcdB = C. difficile toxin B; cdtA = C. difficile binary toxin A; cdtB = C. difficile binary toxin B, nc = nucleotides.*

Discussion

We developed a highly sensitive and specific set of PCR primers for *C. difficile* ribotyping that can be applied directly on fecal samples. Samples containing identical strains clustered together based on ribotype peak profile similarity. During an outbreak of *C. difficile* RT017 in our institution, patients were correctly allocated to- or outside the outbreak-cluster before *C. difficile* isolates were cultured and conventionally typed.

To the best of our knowledge, this is the second study on *C. difficile* ribotyping directly on fecal samples. Janezic et al. were the first to describe this method in 2011 using agarose gel electrophoresis (15). They detected DNA fragments in 86 out of 99 *C. difficile* positive samples, resulting in a sensitivity for *C. difficile* detection of 86.9%. With our primers set, we detected DNA fragments in all 65 *C. difficile* positive samples, resulting in a sensitivity of 100% (95% CI 94.5-100%). The specificity of our PCR was 100% (n=65, 95% CI 94.5-100%); this was the same specificity as obtained by Janezic et al (15).

315 Previous studies showed that in 5-10% of patients with CDI, two or more *C. difficile*
316 strains can be found in the stool of these patients (29). To find evidence for a mixed
317 infection, we compared peak profiles of paired fecal samples and strains and observed 1-
318 3 peaks difference in 4/65 paired samples. However, 3 of these profiles showed (an) extra
319 peak(s) in the strain sample, while only one sample had one extra peak in the fecal
320 sample. This could be an indication of a mixed infection in 1/65 samples (1.5%), this is
321 lower than the expected percentage of mixed infections described in the literature.
322
323 A major advantage of the technique we describe is the use of high-resolution capillary
324 gel-based electrophoresis (CE-ribotyping) instead of the conventional agarose gel-based
325 technique (15). With CE-ribotyping it is possible to obtain digital data and reach high
326 levels of discrimination and reproducibility, which improves standardization of *C.*
327 *difficile* ribotyping (20, 30).
328
329 A limitation of our study is the relatively small number of samples that we tested and the
330 relatively higher number of samples with RT017, due to an outbreak. However, our set
331 contains all major ribotypes circulating in the Netherlands, which we consider sufficient
332 to demonstrate that direct ribotyping on fecal material is possible and accurate (18, 19).
333
334 The approach described here still shows some variation in banding patterns. In low load
335 samples, one or two bands from longer fragments may be lost. As current ribotyping
336 definitions consider a single band difference as a difference in ribotype, definitive

assignment to ribotypes is not feasible yet. However, by using profile-based clustering the essential information for detection of *C. difficile* outbreaks can be provided.

Currently, ribotyping is still the most frequently used typing technique for general epidemiological surveys on CDI, though whole genome (or core genome) MLST (MultiLocus Sequence Typing) is increasingly used to study transmission of *C. difficile* (31, 32). However, most of these techniques are more costly and time-consuming. At this moment, the whole process from submitting a feces sample and determining a PCR ribotype takes approximately 6 days. Our test is a PCR that can be applied directly on total fecal DNA and provides direct information on both the presence and the type of *C. difficile*. Many local diagnostic clinical microbiological laboratories nowadays only perform fecal *C. difficile* toxin gene PCR for diagnostics and hence do not culture strains for downstream molecular analysis. Since the number of laboratories with DNA sequencing devices is increasing, our technique might also become available to many local diagnostic laboratories in the near future. *C. difficile* ribotyping directly on feces could allow accelerated screening for bacterial transmission in an outbreak setting. If more detailed typing is desired, strains can be sent to a *C. difficile* reference laboratory for conventional ribotyping or MLST.

Conclusions

We showed that *C. difficile* ribotyping and simultaneous toxin gene detection directly on fecal samples is feasible, with equal sensitivity as qPCR. This application allows for detection of *C. difficile* infection with concomitant rapid screening for bacterial

transmission between patients. This may result in more timely application of infection control measures and could therefore help in limiting *C. difficile* outbreaks.

Declarations

Ethics approval and consent to participate

The Medical Ethics Committee of Amsterdam UMC waived the need for ethics approval and the need to obtain consent for the collection, analysis and publication of the retrospectively obtained and anonymized data for this non-interventional study.

Consent for publication

Not applicable

Availability of data and materials

Details of the sample set used in the current study are shown in Supplementary Table 1. The datasets used and analyzed during this study are available from the corresponding author on reasonable request.

Competing interests

A.E.B. is co-founder and owner of inBiome BV, a spin-off company of Amsterdam UMC, Vrije Universiteit Amsterdam, that provided the e-Mix.

Funding

T.M.R. was supported by Netherlands Organization for Health Research and

Development (ZonMw) grant Goed Gebruik Geneesmiddelen, project number 848016009. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

Authors' contributions

J.P., C.M.J.E.V. and A.E.B. conceived the study. T.M.R, J.P., C.M.J.E.V. and A.E.B. designed the study. T.M.R., J.P. and A.E.B. analyzed the data. A.K., M.J. and R.H acquired the data. T.M.R. drafted the manuscript. E.K. collaborated on behalf of the Dutch National Reference Laboratory for *C. difficile*. All authors critically revised the manuscript and approved the final version.

Acknowledgments

Reference ribotypes were kindly provided by the Dutch National Reference Laboratory for *C. difficile* (LUMC, Leiden, The Netherlands). Parts of this work have been presented at ASM Microbe (Boston, Massachusetts, USA, 2016), European Congress of Clinical Microbiology and Infectious Diseases (Madrid, Spain, 2018; Amsterdam, The Netherlands, 2019), Scientific Spring Meetings of the *Nederlandse Vereniging voor Medische Microbiologie* meetings (Papendal, The Netherlands, 2016 and 2018) and Clostpath11 (Leiden, The Netherlands, 2019).

406 **References**

407 1. Kwon JH, Olsen MA, Dubberke ER. The morbidity, mortality, and costs
408 associated with Clostridium difficile infection. Infect Dis Clin North Am.
409 2015;29(1):123-34.
410 2. Smits WK, Lyras D, Lacy DB, Wilcox MH, Kuijper EJ. Clostridium difficile
411 infection. Nat Rev Dis Primers. 2016;2:16020.
412 3. Ryan P, Skally M, Duffy F, Farrelly M, Gaughan L, Flood P, et al. Evaluation of
413 fixed and variable hospital costs due to Clostridium difficile infection: institutional
414 incentives and directions for future research. The Journal of hospital infection.
415 2017;95(4):415-20.
416 4. van Beurden YH, Bomers MK, van der Werff SD, Pompe E, Spiering S,
417 Vandenbroucke-Grauls C, et al. Cost analysis of an outbreak of Clostridium difficile
418 infection ribotype 027 in a Dutch tertiary care centre. The Journal of hospital infection.
419 2017;95(4):421-5.
420 5. Merrigan M, Venugopal A, Mallozzi M, Roxas B, Viswanathan VK, Johnson S,
421 et al. Human hypervirulent Clostridium difficile strains exhibit increased sporulation as
422 well as robust toxin production. Journal of bacteriology. 2010;192(19):4904-11.
423 6. Hubert B, Loo VG, Bourgault AM, Poirier L, Dascal A, Fortin E, et al. A portrait
424 of the geographic dissemination of the Clostridium difficile North American pulsed-field
425 type 1 strain and the epidemiology of C. difficile-associated disease in Quebec. Clinical
426 infectious diseases : an official publication of the Infectious Diseases Society of America.
427 2007;44(2):238-44.

7. Kuijper EJ, Coignard B, Tull P. Emergence of Clostridium difficile-associated disease in North America and Europe. Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases. 2006;12 Suppl 6:2-18.

8. Krutova M, Kinross P, Barbut F, Hajdu A, Wilcox MH, Kuijper EJ, et al. How to: Surveillance of Clostridium difficile infections. Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases. 2018;24(5):469-75.

9. Gurtler V. Typing of Clostridium difficile strains by PCR-amplification of variable length 16S-23S rDNA spacer regions. Journal of general microbiology. 1993;139(12):3089-97.

10. O'Neill GL, Ogunsola FT, Brazier JS, Duerden BI. Modification of a PCR Ribotyping Method for Application as a Routine Typing Scheme forClostridium difficile. Anaerobe. 1996;2(4):205-9.

11. Stubbs SL, Brazier JS, O'Neill GL, Duerden BI. PCR targeted to the 16S-23S rRNA gene intergenic spacer region of Clostridium difficile and construction of a library consisting of 116 different PCR ribotypes. Journal of clinical microbiology. 1999;37(2):461-3.

12. Bidet P, Barbut F, Lalande V, Burghoffer B, Petit JC. Development of a new PCR-ribotyping method for Clostridium difficile based on ribosomal RNA gene sequencing. FEMS microbiology letters. 1999;175(2):261-6.

13. Bidet P, Lalande V, Salauze B, Burghoffer B, Avesani V, Delmee M, et al. Comparison of PCR-ribotyping, arbitrarily primed PCR, and pulsed-field gel

electrophoresis for typing Clostridium difficile. Journal of clinical microbiology. 2000;38(7):2484-7.

14. Indra A, Huhulescu S, Schneeweis M, Hasenberger P, Kernbichler S, Fiedler A, et al. Characterization of Clostridium difficile isolates using capillary gel electrophoresis-based PCR ribotyping. Journal of medical microbiology. 2008;57(Pt 11):1377-82.

15. Janezic S, Strumbelj I, Rupnik M. Use of modified PCR ribotyping for direct detection of Clostridium difficile ribotypes in stool samples. Journal of clinical microbiology. 2011;49(8):3024-5.

16. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 2013;41(Database issue):D590-6.

17. Persson S, Torpdahl M, Olsen KE. New multiplex PCR method for the detection of Clostridium difficile toxin A (tcdA) and toxin B (tcdB) and the binary toxin (cdtA/cdtB) genes applied to a Danish strain collection. Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases. 2008;14(11):1057-64.

18. *difficile* DNRLf*C*. Tenth Annual Report of the National Reference Laboratory for *Clostridium difficile* and results of the sentinel surveillance, May 2015 - May 2016. Website of the Dutch National Institute for Public Health and the Environment (Rijksinstituut voor Volksgezondheid en Milieu); 2016.

19. *difficile* DNRLf*C*. Eleventh Annual Report of the National Reference Laboratory for *Clostridium difficile* and results of the sentinel surveillance, May 2016 - May 2017.

473 Website of the Dutch National Institute for Public Health and the Environment
474 (Rijksinstituut voor Volksgezondheid en Milieu); 2017.
475 20. Fawley WN, Knetsch CW, MacCannell DR, Harmanus C, Du T, Mulvey MR, et
476 al. Development and validation of an internationally-standardized, high-resolution
477 capillary gel-based electrophoresis PCR-ribotyping protocol for Clostridium difficile.
478 PloS one. 2015;10(2):e0118150.
479 21. Rupnik M. Heterogeneity of large clostridial toxins: importance of Clostridium
480 difficile toxinotypes. FEMS microbiology reviews. 2008;32(3):541-55.
481 22. Stubbs S, Rupnik M, Gibert M, Brazier J, Duerden B, Popoff M. Production of
482 actin-specific ADP-ribosyltransferase (binary toxin) by strains of Clostridium difficile.
483 FEMS microbiology letters. 2000;186(2):307-12.
484 23. Gerding DN, Johnson S, Rupnik M, Aktories K. Clostridium difficile binary toxin
485 CDT: mechanism, epidemiology, and potential clinical importance. Gut microbes.
486 2014;5(1):15-27.
487 24. Perelle S, Gibert M, Bourlioux P, Corthier G, Popoff MR. Production of a
488 complete binary toxin (actin-specific ADP-ribosyltransferase) by Clostridium difficile
489 CD196. Infect Immun. 1997;65(4):1402-7.
490 25. Azimirad M, Naderi Noukabadi F, Lahmi F, Yadegar A. Prevalence of binary-
491 toxin genes (cdtA and cdtB) among clinical strains of Clostridium difficile isolated from
492 diarrheal patients in Iran. Gastroenterol Hepatol Bed Bench. 2018;11(Suppl 1):59-65.
493 26. Kim J, Kim Y, Pai H. Clinical Characteristics and Treatment Outcomes of
494 Clostridium difficile Infections by PCR Ribotype 017 and 018 Strains. PloS one.
495 2016;11(12):e0168849.

27. Cairns MD, Preston MD, Hall CL, Gerding DN, Hawkey PM, Kato H, et al. Comparative Genome Analysis and Global Phylogeny of the Toxin Variant Clostridium difficile PCR Ribotype 017 Reveals the Evolution of Two Independent Sublineages. Journal of clinical microbiology. 2017;55(3):865-76.

28. Seugendo M, Janssen I, Lang V, Hasibuan I, Bohne W, Cooper P, et al. Prevalence and Strain Characterization of Clostridioides (Clostridium) difficile in Representative Regions of Germany, Ghana, Tanzania and Indonesia - A Comparative Multi-Center Cross-Sectional Study. Frontiers in microbiology. 2018;9:1843.

29. Dayananda P, Wilcox MH. A Review of Mixed Strain Clostridium difficile Colonization and Infection. Frontiers in microbiology. 2019;10:692.

30. Killgore G, Thompson A, Johnson S, Brazier J, Kuijper E, Pepin J, et al. Comparison of seven techniques for typing international epidemic strains of Clostridium difficile: restriction endonuclease analysis, pulsed-field gel electrophoresis, PCR-ribotyping, multilocus sequence typing, multilocus variable-number tandem-repeat analysis, amplified fragment length polymorphism, and surface layer protein A gene sequence typing. Journal of clinical microbiology. 2008;46(2):431-7.

31. Janezic S, Rupnik M. Development and Implementation of Whole Genome Sequencing-Based Typing Schemes for Clostridioides difficile. Front Public Health. 2019;7:309.

32. Griffiths D, Fawley W, Kachrimanidou M, Bowden R, Crook DW, Fung R, et al. Multilocus sequence typing of Clostridium difficile. Journal of clinical microbiology. 2010;48(3):770-8.

519 **Supplementary material**

Sample no.	Reference ribotype identified in cultured strain	Toxin genes detected in fecal sample	Cp value of *C. difficile* toxin A and/or B genes qPCR on fecal sample
1	002	*cdtA- / cdtB- / tcdA+ / tcdB+*	37.6
2	002	*cdtA- / cdtB- / tcdA+ / tcdB+*	30.5
3	002	*cdtA- / cdtB- / tcdA+ / tcdB+*	37.1
4	002	*cdtA- / cdtB- / tcdA+ / tcdB+*	35.9
5	005*	*cdtA- / cdtB- / tcdA+ / tcdB+*	29.0
6	045	*cdtA+ / cdtB+ / tcdA+ / tcdB+*	36.4
7	--	*cdtA+ / cdtB+ / tcdA+ / tcdB+*	30.0
8	078	*cdtA+ / cdtB+ / tcdA+ / tcdB+*	34.5
9	126	*cdtA+ / cdtB+ / tcdA+ / tcdB+*	36.6
10	078	*cdtA+ / cdtB+ / tcdA+ / tcdB+*	27.9
11	078	*cdtA+ / cdtB+ / tcdA+ / tcdB+*	28.8
12	078	*cdtA+ / cdtB+ / tcdA+ / tcdB+*	32.0
13	078	*cdtA+ / cdtB+ / tcdA+ / tcdB+*	32.9
14	078	*cdtA+ / cdtB+ / tcdA+ / tcdB+*	27.2
15	062*	*cdtA- / cdtB- / tcdA+ / tcdB+*	33.5
16	015*	*cdtA- / cdtB- / tcdA+ / tcdB+*	38.3
17	001	insufficient material	35.6
18	001*	*cdtA- / cdtB- / tcdA+ / tcdB+*	29.2
19	001	*cdtA- / cdtB- / tcdA+ / tcdB+*	34.2
20	001	*cdtA- / cdtB- / tcdA+ / tcdB+*	33.1
21	001	*cdtA- / cdtB- / tcdA+ / tcdB+*	33.3
22	626	*cdtA- / cdtB- / tcdA+ / tcdB+*	31.4
23	037	*cdtA- / cdtB- / tcdA+ / tcdB+*	32.6
24	015	*cdtA- / cdtB- / tcdA+ / tcdB+*	35.7
25	626	*cdtA- / cdtB- / tcdA+ / tcdB+*	36.7
26	244*	*cdtA- / cdtB- / tcdA+ / tcdB+*	35.1
27	011	*cdtA- / cdtB- / tcdA+ / tcdB+*	37.3
28	050	*cdtA- / cdtB- / tcdA+ / tcdB+*	29.3
29	258	*cdtA- / cdtB- / tcdA+ / tcdB+*	35.8
30	190	*cdtA- / cdtB- / tcdA+ / tcdB+*	36.7
31	258	*cdtA- / cdtB- / tcdA+ / tcdB+*	37.2
32	258	*cdtA- / cdtB- / tcdA+ / tcdB+*	30.9
33	190	*cdtA- / cdtB+ / tcdA+ / tcdB+*	30.8
34	216	*cdtA- / cdtB- / tcdA+ / tcdB+*	29.9

35	023	*cdtA+ / cdtB+ / tcdA+ / tcdB+*	29.6
36	026	*cdtA- / cdtB- / tcdA+ / tcdB+*	36.5
37	026	*cdtA- / cdtB- / tcdA+ / tcdB+*	31.5
38	026	*cdtA- / cdtB- / tcdA+ / tcdB+*	28.8
39	026	*cdtA- / cdtB- / tcdA+ / tcdB+*	35.3
40	026	*cdtA- / cdtB- / tcdA+ / tcdB+*	37.1
41	081	*cdtA- / cdtB- / tcdA+ / tcdB+*	40.0
42	029	*cdtA- / cdtB- / tcdA+ / tcdB+*	31.6
43	012	*cdtA- / cdtB- / tcdA+ / tcdB+*	37.7
44	012	*cdtA- / cdtB- / tcdA+ / tcdB+*	28.3
45	012	*cdtA- / cdtB- / tcdA+ / tcdB+*	31.7
46	014	*cdtA- / cdtB- / tcdA+ / tcdB+*	38.9
47	207	*cdtA- / cdtB- / tcdA+ / tcdB+*	38.9
48	?	*cdtA- / cdtB- / tcdA+ / tcdB+*	32.5
49	014	*cdtA- / cdtB- / tcdA+ / tcdB+*	29.1
50	014	*cdtA- / cdtB- / tcdA+ / tcdB+*	32.7
51	014	*cdtA- / cdtB- / tcdA+ / tcdB+*	34.5
52	070	*cdtA- / cdtB- / tcdA+ / tcdB+*	31.9
53	017	*cdtA- / cdtB- / tcdA+ / tcdB+*	33.7
54	017	*cdtA- / cdtB- / tcdA+ / tcdB+*	36.7
55	017	*cdtA- / cdtB- / tcdA+ / tcdB+*	34.6
56	017	*cdtA- / cdtB- / tcdA+ / tcdB+*	35.3
57	017	*cdtA- / cdtB- / tcdA+ / tcdB+*	30.6
58	017	*cdtA- / cdtB- / tcdA+ / tcdB+*	30.9
59	017	*cdtA- / cdtB- / tcdA+ / tcdB+*	31.1
60	017	*cdtA- / cdtB- / tcdA+ / tcdB+*	30.1
61	017	*cdtA- / cdtB- / tcdA+ / tcdB+*	29.8
62	017	*cdtA- / cdtB- / tcdA+ / tcdB+*	31.6
63	265	*cdtA- / cdtB- / tcdA+ / tcdB+*	30.1
64	050	*cdtA- / cdtB- / tcdA+ / tcdB+*	28.5
65	027	*cdtA+ / cdtB+ / tcdA+ / tcdB+*	29.7

Supplementary Table 1 – Details of all 65 fecal samples with positive qPCR for *C. difficile* toxin A and/or B genes and their 65 corresponding cultured strains. Samples are ordered and numbered according to their order on the X-axis in figure 2 (from left to right). All *C. difficile* fecal samples studied were also cultured and these strains were sent to the Dutch National Reference Laboratory for *C. difficile* at Leiden University Medical

Center (LUMC) for conventional ribotyping using a standardized protocol (20). Toxin genes were detected in fecal samples by using toxin gene primers described by Persson et al (17). Cp values of *C. difficile* toxin A and/or B qPCR on fecal samples (the standard diagnostic test for *C. difficile* detection in our laboratory) indicates the *C. difficile* bacterial load in the studied fecal samples. *'*' = probable reference ribotype; '?' = unknown reference ribotype; '-' = no reference ribotype; tcdA = C. difficile toxin A; tcdB = C. difficile toxin B; cdtA = C. difficile binary toxin A; cdtB = C. difficile binary toxin B*

Figures

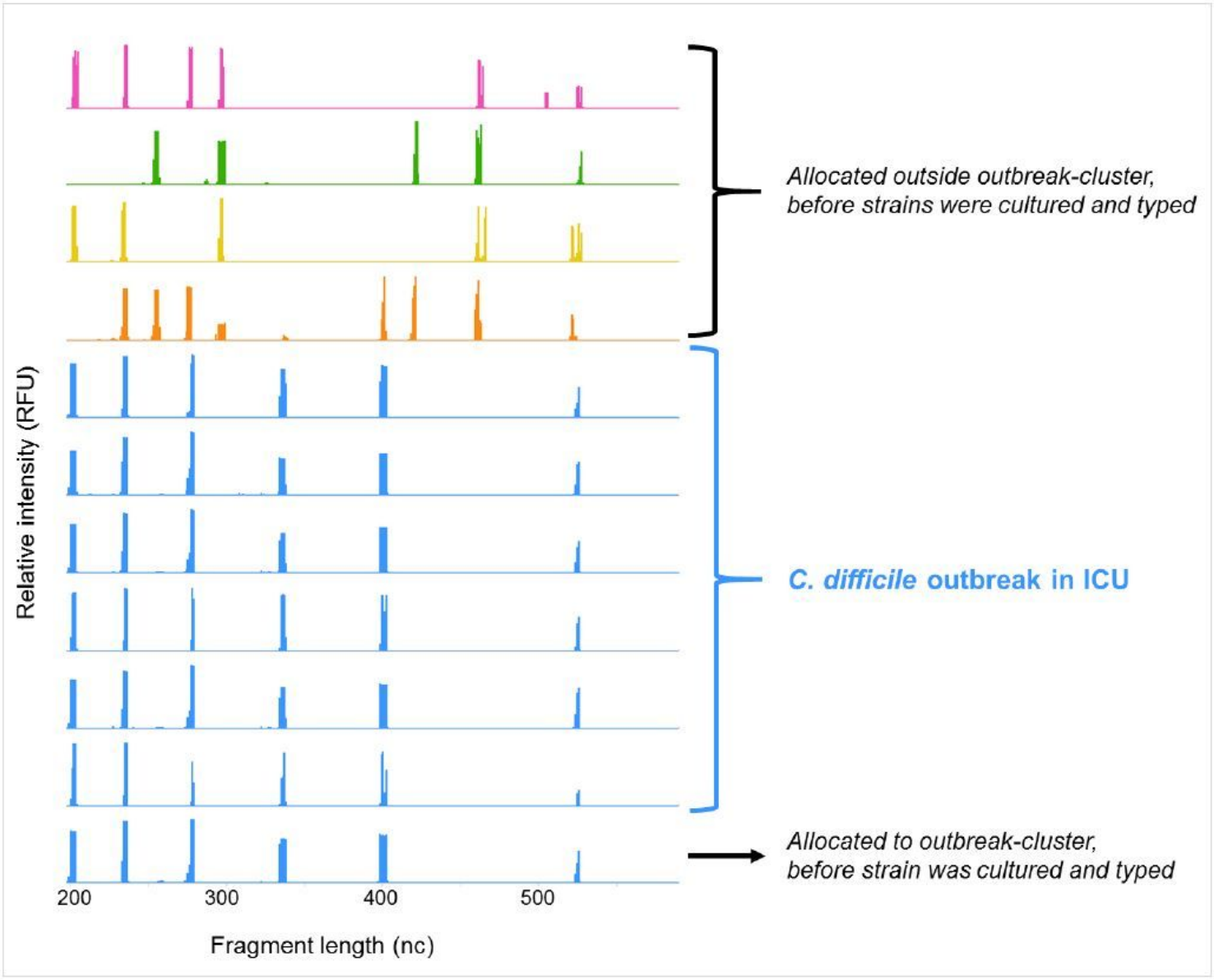


Figure 1

Ribosomal DNA fragment profiles in fecal samples of eleven patients with positive C. difficile toxin A and/or B genes qPCR. Bacterial transmission was suspected in seven patients with identical peak profiles (blue). nc = nucleotides, RFU = relative fluorescence units.

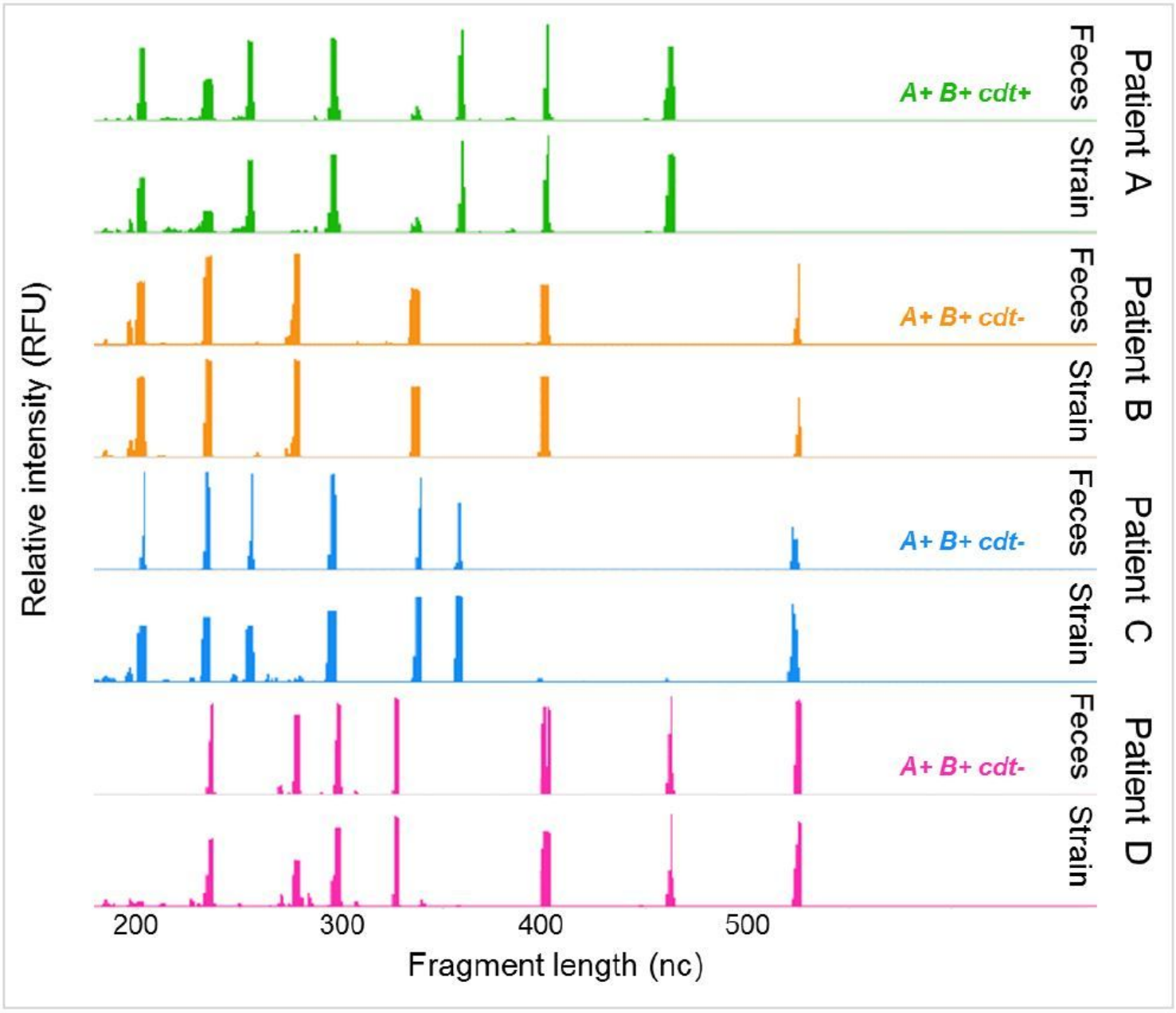


Figure 2

Examples of DNA fragment peak profiles from four fecal samples and their corresponding cultured strains. nc = nucleotides, RFU = relative fluorescence units, A = toxin A gene, B = toxin B gene, cdt = binary toxin genes.

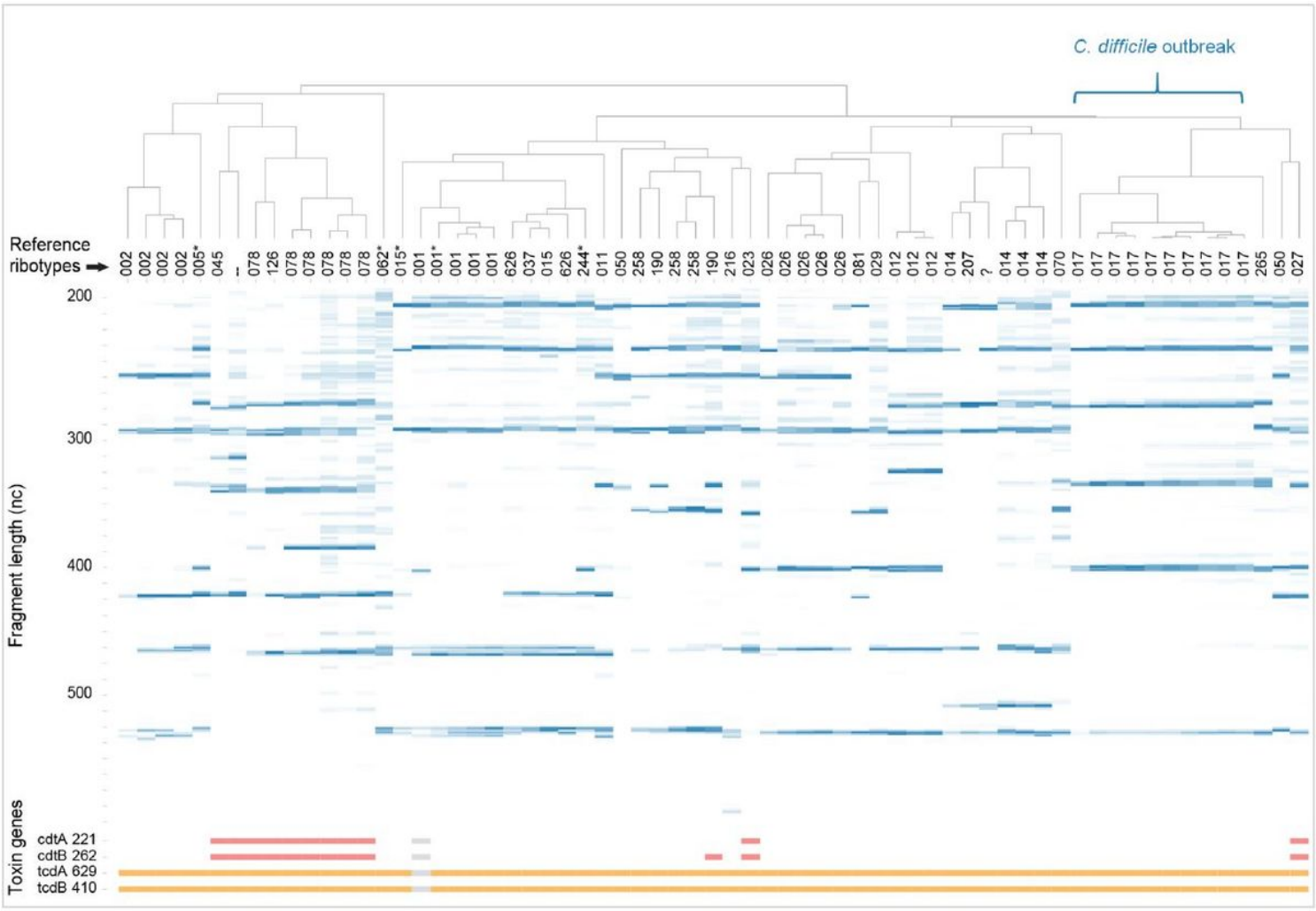


Figure 3

Heat map and dendrogram based on ribosomal DNA fragment peak profiles of all fecal samples. Each column represents one sample. Numbers on the X-axis correspond to the reference ribotype assigned to the corresponding strain by conventional ribotyping by the Dutch National Reference Laboratory. Numbers on the Y-axis correspond to the DNA fragment length of the detected ribotype DNA fragment peaks/bands (in blue) and/or C. difficile toxin genes (red/orange) per sample. Toxin gene detection could not be performed in one sample due to insufficient DNA material (grey). '*' = probable reference ribotype; '?' = unknown reference ribotype; '-' = no reference ribotype; tcdA = C. difficile toxin A; tcdB = C. difficile toxin B; cdtA = C. difficile binary toxin A; cdtB = C. difficile binary toxin B, nc = nucleotides.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryFiles.pdf