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ORIGINAL PAPER

The use of PCR ribotyping for molecular typing of clinically significant *Clostridium difficile* Romanian isolates

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Abstract

Clostridium difficile infections (CDI) became a significant infectious pathology in Romania in 2010-2011, when first major outbreaks were reported. The increase of case numbers made molecular typing of C. difficile strains necessary for epidemiological purposes. In this study, 96 C. difficile strains, isolated from CDI patients, were identified using PCR ribotyping. The method was selected considering the context of efforts being made to standardise C. difficile typing in Europe, using PCR ribotyping as the method of choice, with a type nomenclature for cross-country comparison in CDI surveillance, but also taking into account technical accessibility. The study results showed a majority of isolates (n=63) with the toxigenic profile A+/B+/CDT+, most of them (n=61) identified as hyper virulent ribotype 027. Eighteen more ribotypes were identified, in lower proportions. Fourteen corresponded to ribotypes from the Brazier nomenclature: 002, 012, 014, 015, 017, 018, 031, 046, 053, 085, 087, 106, 115 and 140. Ribotype 014 was the most representative (7 isolates), followed by 002 (4 isolates). Three isolates were non-toxigenic: ribotypes 031, 085 and 140. Other four ribotypes remained unidentified and were named U.01, U.02, U.03 and U.04. Two isolates clustering as ribotype U.02 were A+/B+/CDT+. By selecting this typing method, a prevalent, highly epidemic ribotype and 18 other ribotypes were detected, providing information about the C. difficile type diversity of the analysed group of strains and proving its usefulness as an instrument in epidemiological studies.

Keywords: *Clostridium difficile*, diarrhoea, PCR ribotype, 027

1. Introduction

Clostridium difficile is a Gram positive anaerobic bacillus that has become a significant aetiological agent of human post-antibiotic infections in Romania, varying from mild diarrheal episodes to severe cases leading to pseudomembranous colitis or even death (11, 40, 41). The capacity to produce spores resistant to alcohol-based disinfectants, high temperatures or radiation allows *C. difficile* to persist on surfaces and is responsible for its epidemic potential (2,18). Evidence showed that outbreaks generated by highly pathogenic strains had occurred in hospitals around the country (7, 40, 41, 42). First major Romanian outbreaks occurred in 2010-2011 and the incidence remained high in the next years (40), forcing the need to investigate and type the strains causing these outbreaks. *C. difficile* typing has evolved from serological (8) to molecular methods, including toxinotyping or whole genome

sequencing of isolates (13, 24, 43). Different typing methods are selected based on their discriminatory resolution and reproducibility, allowing data comparison between laboratories in different geographic regions (19). Some of the methods, such as Multi Locus Variable-number tandem repeat Analysis (MLVA) (5, 12, 15), Restriction Endonuclease Analysis (REA) (51), or surface-layer protein A gene (*slp*A) sequence-based typing (21), have been developed to generate subtypes in the groups generated by less discriminatory methods like Pulsed Field Gel Electrophoresis (PFGE) (1, 36), Multi Locus Sequence Typing (MLST) (16, 32) or PCR ribotyping (23, 48). Methods used to distinguish between strains at the subtype level also serve as phylogenetic instruments (3, 10, 23, 26, 31). However, PCR ribotyping is the most popular *C. difficile* typing method used among European laboratories for epidemiological purposes. It is considered to be highly reproducible, allowing result comparisons between laboratories and comparative epidemiological analysis between countries. The method is based on PCR amplification of the DNA sequence corresponding to the Intergenic Spacer Region (ISR) between the 16S rRNA and 23S rRNA genes (17). Depending on the number of copies and distances between these two genes on the *C. difficile* chromosome, the PCR reaction generates a mixture of amplicons variable in length – 260-585 bp (37) – and number of copies. Through gel electrophoresis with high resolution agarose, these polymorphisms are rendered as band patterns specific to a type of *C. difficile*, named PCR ribotype (4, 47). Approximately 400 *C. difficile* PCR ribotypes have been identified and a large reference ribotype collection – the Brazier collection from the Reference Laboratory for Anaerobic Bacteria of Cardiff University in Wales – is currently being used for identifying new ribotypes (25, 52). The method was first developed by Kostman and his colleagues (1992) as an alternative to the traditional ribotyping method used to analyse *Pseudomonas cepacia* strains isolated from patients with cystic fibrosis (27). Gürtler and his group (1993) first applied it for typing *C. difficile* isolates*,* using two sets of specific primers to amplify fragments of the intergenic spacer region between the 16S rRNA gene and the 23S rRNA gene (17). Cartwright *et al.* (1995) also evaluated the potential of PCR ribotyping as an epidemiological tool for investigating nosocomial *C. difficile* isolates (6). They used negative images of the agarose gels to allow better analysis of the band patterns of different isolates. O'Neill and his colleagues (1996) modified PCR ribotyping by designing a more specific set of primers, that targeted the ISR flanked by shorter fragments of the 16S rRNA and 23S rRNA genes, to generate shorter amplicons (37). The method was further optimized by Bidet *et al.* (1999), who replaced O'Neill's 23S primer (initially designed with a *C. botulinum* sequence) with a primer specific to a *C. difficile* sequence, for a better interpretation of the band patterns (4). They also demonstrated the reproducibility of PCR ribotyping throughout experiments in which they used different PCR reaction parameters, different protocols for DNA extraction or *C. difficile* isolates that underwent multiple passages in the laboratory. Stubbs and his colleagues (1999) used a modified version of O'Neill's protocol to create a library of 116 ribotypes of *C. difficile* and they discovered that the predominant ribotype in the population of isolates that they studied was ribotype 001 (55%) (47). Some of the isolates identified as 001 were strains with high nuclease activity, difficult to type with PFGE (22). The results of this study underline one of the advantages provided by PCR ribotyping: it allows epidemiologists to compare prevalent types of *C. difficile* between countries or geographical regions. Presently, the Stubbs method of PCR ribotyping is widely used in European laboratories for *C. difficile* typing (50). It uses the O'Neill primers, considered to have a higher discriminatory resolution and it allows rapid interpretation and comparison of the results between laboratories, geographical regions or countries (25). Recently, a capillary gel electrophoresis-based version of PCR ribotyping (CE-ribotyping), with higher resolution and lower turnaround time, has been developed and is currently being standardized

internationally (14, 20). In this study, 96 *C. difficile* strains were analysed. The strains were isolated from stool samples of patients with clinically suspected or confirmed *Clostridium difficile* infection (CDI) between 2011 and the beginning of 2017, most of the isolates dating from 2013-2016. The toxigenic profiles of the isolates were determined, using a multiplex PCR method and PCR ribotypes were identified, to evaluate PCR ribotyping as a method of choice for rendering strain type diversity or detection of *C. difficile* prevalent types. The purpose of the analysis was to underline the practical usefulness of PCR ribotyping as a typing method applicable in local hospital laboratories for first-intent outbreak investigations or comparison of results between hospitals.

2. Materials and Methods

Toxigenic culture and isolation of *C. difficile* strains

The study analysed 96 *C. difficile* strains isolated from stool samples collected from patients with diarrheal symptoms, most of them confirmed as CDI patients by positive tests for the presence of toxins A/B or toxigenic *C. difficile* DNA in stool. The strains were isolated between 2011 and the beginning of 2017. Part of the strains analysed in the study were isolated in our laboratory using toxigenic culture as part of a two-step algorithm for CDI diagnosis: all the faecal samples received for testing the presence of toxigenic *C. difficile* were cultivated on selective *C. difficile* medium (CLO agar, bioMérieux, France), independently of the Enzyme Immunoassay (EIA) *C. difficile* screening test result. The plates were incubated in anaerobiosis (37°C, 48h) and typical *C. difficile* colonies grown on the selective agar were subsequently tested for A/B toxin production with a rapid immunochromatographic test (ImmunoCard Toxins A/B, Meridian Bioscience, USA), also used for the initial screening of faecal samples. The toxigenic *C. difficile* isolates were purified on Columbia blood agar (COS agar, bioMérieux, France) (48h, 37°C, anaerobiosis).

DNA samples

A resin-based kit (InstaGene Matrix, Bio-Rad, USA) was used to extract DNA from pure culture of each *C. difficile* isolate, following the manufacturer's instructions. The DNA samples obtained served as template for both molecular assays performed in this study.

Multiplex PCR for toxin gene detection

To determine the toxigenic profile of each isolate, we used a multiplex PCR protocol, designed to detect toxin genes *tcd*A and *tcd*B, located on the pathogenicity locus (*PaLoc*) and the *cdt*A and *cdt*B genes encoding for the subunits of binary toxin CDT (*Clostridium difficile* toxin), located in the *CdtLoc* locus on the *C. difficile* chromosome (39). For detection of toxin A (TcdA) – negative isolates, we used a previously published set of primers targeting a repetitive 3' end fragment of the *tcd*A gene, susceptible to deletions (33, 34). The PCR reaction mix also contained a set of primers for an internal amplification control (bacterial 16S rDNA) and another one targeting the gene *glu*D of *C. difficile* surface antigen glutamate dehydrogenase (GDH) (38). Primers used in this PCR assay are listed in Table 1. PCR was performed in a volume of 50 µl, containing the 7 sets of primers (2.5 – 15 pmol), a multiplex PCR mix (Qiagen Multiplex PCR Kit, USA) and 5 µl of DNA template. The amplification took place in an Eppendorf gradient thermal cycler, according to the following program: initial denaturation at 94°C for 10 minutes, 35 cycles as follows: 94°C/50 sec., 54°C/40 sec., 72°C/50 sec. and final elongation at72°C for 3 minutes, as previously described (39). The amplicons were verified by gel electrophoresis in a regular agarose gel (2%). The ethidium bromide stained gels were visualised and photographed with a G:BOX EF imaging system (SYNGENE, UK).

Table 1. Primers used in the multiplex PCR assay for toxigenic profile of *C. difficile* isolates

Primer	Primer sequence (5'→3')	Target	Reference
PS13	GAGGCAGCAGTGGGGAATA	Internal control (16S rDNA)	Persson *et al.*, 2008
PS14	TGACGGGCGGTGTGTACAAG		
tcdA-F3345	GCATGATAAGGCAACTTCAGTGGTA	*tcd*A – conservative fragment	Persson *et al.*, 2008
tcdA-R3969	AGTTCCTCCTGCTCCATCAAATG		
tcdB-F5670	CCAAARTGGAGTGTTACAAACAGGTG	*tcd*B	Persson *et al.*, 2008
tcdB-R6079A	GCATTTCTCCATTCTCAGCAAAGTA		
tcdB-R6079B	GCATTTCTCCGTTTTCAGCAAAGTA		
tcdA-FL	AGATTCCTATATTTACATGACAATAT	*tcd*A – repetitive fragment, susceptible to deletions	Lemée *et al.*, 2004b
tcdA-RL	GTATCAGGCATAAAGTAATATACTTT		
ctdB-F617	TTGACCCAAAGTTGATGTCTGATTG	*cdt*B	Persson *et al.*, 2008
cdtB-R878	CGGATCTCTTGCTTCAGTCTTTATAG		
cdtA-F739A	GGGAAGCACTATATTAAAGCAGAAGC	*cdt*A	Persson *et al.*, 2008
cdtA-F739B	GGGAAACATTATATTAAAGCAGAAGC		
cdtA-R958	CTGGGTTAGGATTATTTACTGGACCA		
908gluDs	GTCTTGGATGGTTGATGAGTAC	*glu*D	Paltansing *et al.*, 2007
909gluDas	TTCCTAATTTAGCAGCAGCTTC		

PCR Ribotyping of the *C. difficile* isolates

The *C. difficile* strains were typed with an optimized PCR ribotyping protocol previously described (47), using the O'Neill primers to amplify the ISR region: 5'CTGGGGTGAAGTCGTAACAAGG3' for 16S rDNA and 5'GCGCCCTTTGTAGCTTGACC3' for 23S rDNA (37), in a total volume of 25 µl of PCR reaction mix, containing 15 pmol of each primer, HotStar Taq polymerase (HotStarTaq Master Mix Kit, Qiagen, USA) and 5 µl of DNA template. The amplification parameters were set as follows: an initial denaturation at 95ºC**,** for 15 minutes and 35 cycles: 94°C/1 min., 57°C/1 min., 72°C/1 min., followed by a final elongation step at 72°C, for 8 minutes. The resulted amplicons were separated with conventional gel electrophoresis in a high resolution 3% agarose gel (Metaphor Agarose, Lonza, Switzerland), migrated at 70V/4h, for an efficient band separation. The resulted band patterns were viewed by staining the gel with ethidium bromide. Ribotypes were attributed by analysis with BioNumerics software v.6.6 (Applied Maths, USA), using the band profiles of the Brazier ECDC reference ribotypes in our collection (25, 52).

3. Results and discussion

Toxigenic profiles

In this study, 96 *C. difficile* isolates were tested using a multiplex PCR protocol that simultaneously detects: *tcd*A, *tcd*B, *cdt*A and *cdt*B genes, an internal control and *glu*D gene as *C. difficile* marker (38, 39). With this assay, we were able to identify the toxigenic types of our isolates, including the A−/B+/CDT− phenotype, by detecting deleted 3'end fragments of the *tcd*A gene with previously described primers (33). All the isolates analysed in the study were confirmed as *C. difficile* (*glu*D gene of the surface antigen GDH was detected in each DNA sample). Most of them were positive for the presence of toxin genes *tcd*A and *tcd*B in the pathogenicity locus (*PaLoc*), confirming the results of the rapid immunoenzyme screening

tests and the clinical suspicion of CDI. 93 (97%) of the studied isolates were toxigenic and three were non-toxigenic. 63 (68%) of the toxigenic strains were binary toxin positive, with A+/B+/CDT+ phenotype, suggesting the presence of ribotype 027 or other hyper virulent ribotypes among the isolates. 29 (30%) were A+/B+/CDT− (*cdt*A and *cdt*B genes were not detected). One clinical isolate had the TcdA negative phenotype: multiplex PCR showed a 259 bp deletion in a 3' end repetitive fragment of *tcd*A (369 bp), representing a marker for the cumulated deletion pattern (1.8 kb) of 1470-like strains (9, 33, 44). Isolates with this pattern have been proven to be associated with disease (45). The resulted toxigenic profiles of the isolates are presented in Table 2.

Table 2. Toxigenic profiles of *C. difficile* isolates analysed

Toxigenic profile	Pathogenic *C. difficile*	Toxigenic profiles (%)	*C. difficile* isolates
A+/B+/CDT+	Yes	65.63	63
A+/B+/CDT−	Yes	30.20	29
A−/B+/CDT−	Yes	1.04	1
NT (A−/B−/CDT−)	No	3.13	3
			96 isolates

A+/B+/CDT+ = TcdA positive, TcdB positive, CDT (binary toxin) positive;
A+/B+/CDT− = TcdA positive, TcdB positive, CDT (binary toxin) negative;
A−/B+/CDT− = TcdA negative, TcdB positive, CDT (binary toxin) negative;
NT (A−/B−/CDT−) = non-toxigenic.

PCR Ribotyping

Nineteen PCR ribotypes were detected among the *C. difficile* isolates analysed in this study, 15 of them corresponding to known ribotypes from the reference nomenclature: 002, 012, 014, 015, 017, 018, 027, 031, 046, 053, 085, 087, 106, 115 and 140. The other four patterns, detected in 7 isolates, did not match the reference ribotypes in our collection. Therefore they were designated codes U.01 (unknown), U.02, U.03 and U.04 by our laboratory. Approximately 64% of the studied isolates, representing the majority of the A+/B+/CDT+ toxigenic types, were identified as ribotype 027, considered to be a hyper virulent, multi-drug resistant type with high epidemic potential (28, 29, 30, 35, 46). It was identified as a prevalent aetiological agent of CDI outbreaks in Romanian hospitals since 2010-2011 (40, 42). Our ribotyping results are in accordance with the preliminary findings of a pilot surveillance study (2013-2014) showing most of the analysed CDI cases from different geographic regions of the country to be severe forms of infection with moxifloxacin-resistant ribotype 027 (40). Two other binary toxin producing strains clustered as a ribotype with an unidentifiable pattern by our reference types, named U.02. From the binary toxin-negative toxigenic group (n=30), the most frequent ribotype was 014, with 7 isolates, representing 7% of all *C. difficile* types and 24% of the A+/B+/CDT− toxigenic types (23% of the CDT− isolates). It was followed by ribotypes 002 (n=4), 087 (n=3) and U.03 (n=3). The other toxigenic ribotypes detected in our analysis were present in lower proportions, as shown in Table 3. The non-toxigenic isolates detected were ribotypes 031, 085 and 140. The presence of these clinically irrelevant *C. difficile* strains in the stool samples of patients with diarrheal symptoms suggests that these symptoms may have had other aetiologies.

Table 3. PCR Ribotypes of the *C. difficile* isolates analysed in the study

No. crt.	PCR Ribotypes	No. of *C. difficile* isolates	Toxigenicity profile
1.	027	61 (63.54%)	A+/B+/CDT+
2.	014	7 (7.29%)	A+/B+/CDT−
3.	002	4 (4.17%)	A+/B+/CDT−
4.	087	3 (3.13%)	A+/B+/CDT−
5.	053	2 (2.08%)	A+/B+/CDT−
6.	115	2 (2.08%)	A+/B+/CDT−
7.	046	2 (2.08%)	A+/B+/CDT−
8.	017	1 (1.04%)	A−/B+/CDT−
9.	018	1 (1.04%)	A+/B+/CDT−
10.	012	1 (1.04%)	A+/B+/CDT−
11.	106	1 (1.04%)	A+/B+/CDT−
12.	015	1 (1.04%)	A+/B+/CDT−
13.	085	1 (1.04%)	NT
14.	140	1 (1.04%)	NT
15.	031	1 (1.04%)	NT
16.	U.01	1 (1.04%)	A+/B+/CDT−
17.	U.02	2 (2.08%)	A+/B+/CDT+
18.	U.03	3 (3.13%)	A+/B+/CDT−
19.	U.04	1 (1.04%)	A+/B+/CDT−
Total:	**19**	**96**	**4**

A+/B+/CDT+ = TcdA positive, TcdB positive, CDT (binary toxin) positive;
A+/B+/CDT− = TcdA positive, TcdB positive, CDT (binary toxin) negative;
A−/B+/CDT− = TcdA negative, TcdB positive, CDT (binary toxin) negative;
NT (A−/B−/CDT−) = non-toxigenic;
U.01 – U.04 = unknown ribotypes.

Most of the isolates in our study dated from 2013-2016. The prevalence of ribotype 027 strains in this interval ranged from 40% to 80%, as shown in Table 4. We also included randomly selected *C. difficile* strains isolated from CDI patients in 2011 (n_{027}=1), 2012 (n_{027}=4) and one isolate from the beginning of 2017, identified as ribotype 115.

Table 4. Yearly distribution of *C. difficile* PCR ribotype 027 isolates

Year	PCR ribotype 027 per analysed (n)	PCR ribotype 027 isolates (%)
2011	1/3	33.33
2012	4/4	100
2013	21/32	65.63
2014	11/16	68.75
2015	16/20	80
2016	8/20	40
2017	0/1	0

The main goal of our study was to determine whether PCR ribotyping is an appropriate *C. difficile* typing method for our laboratory capacity and an efficient instrument for identifying predominant types of clinical strains generating CDI in our country. Our first

reason for choosing PCR ribotyping was its frequent use throughout European laboratories, allowing cross-country comparisons. The method was proved to be suitable for *C. difficile* typing in further epidemiological studies, due to its cost effectiveness, short turnaround time, basic infrastructure necessities and reproducibility.

4. Conclusion

In this study, we analysed the toxigenic potential and PCR ribotype diversity of a group of 96 *C. difficile* strains, isolated from patients with confirmed CDI, mostly between 2013 and 2016. We used the Stubbs PCR ribotyping protocol adapted in our laboratory for molecular typing. Our results revealed the prevalence of the hyper virulent ribotype 027, confirming its high epidemic potential. Other 14 known ribotypes – 002, 012, 014, 015, 017, 018, 031, 046, 053, 085, 087, 106, 115, and 140 – and 4 unidentified ribotypes – U.01, U.02, U.03, and U.04 – were found among our strains, in lower proportions. Of 63 A+/B+/CDT+ type isolates, 61 were identified as 027 and two others clustered as an unknown ribotype – U.02. Among the binary toxin negative ribotypes, the most representative was 014, with 7 isolates, followed by ribotype 002, with 4 isolates. Although the study was not set out as an epidemiological analysis, our results provide supplementary data for creating an image of the type diversity of Romanian *C. difficile* isolates. Through this work, we underline the importance and practical usefulness of this assay for molecular typing of *C. difficile*. We consider PCR ribotyping appropriate for first-intent outbreak investigations or inter-laboratory comparisons, due to its reproducibility and cost effectiveness. However, molecular methods with higher discriminatory resolution, like MLVA (15) or whole genome sequencing (13), need to be applied for further investigating hospital outbreaks, to evaluate their clonality or *C. difficile* transmission between patients.

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References

1. ALONSO R., MARTIN A., PELÁEZ T., MARÍN M., RODRÍGUEZ-CREIXÉMS M., BOUZA E. An improved protocol for pulsed-field gel electrophoresis typing of *Clostridium difficile*. *J. Med. Microbiol.,* 54: 155-157 (2005).
2. AWAD M.M., JOHANESEN P.A., CARTER G.P., ROSE E., LYRAS D. *Clostridium difficile* virulence factors: insights into an anaerobic spore-forming pathogen. *Gut Microbes*, 5(5): 579-593 (2014).
3. BAKKER D., CORVER J., HARMANUS C., GOORHUIS A., KEESSEN E.C., FAWLEY W.N., WILCOX M.H., KUIJPER E.J. Relatedness of human and animal *Clostridium difficile* PCR Ribotype 078 isolates based on multilocus variable-number tandem-repeat analysis and tetracycline resistance. *J. Clin. Microbiol.,* 48(10): 3744-9 (2010).
4. BIDET P., BARBUT F., LALANDE V., BURGHOFFER B., PETIT J.C. Development of a new PCR-ribotyping method for *Clostridium difficile* based on ribosomal RNA gene sequencing. *FEMS Microbiol. Lett.*, 175: 261-266 (1999).
5. BROUKHANSKI G., LOW D.E., PILLAI D.R. Modified multiple locus variable-number tandem-repeat analysis for rapid identification and typing of *Clostridium difficile* during institutional outbreaks. *J. Clin. Microbiol.*, 49(5): 1983-6 (2011).

6. CARTWRIGHT C.P., STOCK F., BEEKMANN S.E., WILLIAMS E.C., GILL V.J. PCR amplification of rRNA intergenic spacer regions as a method for epidemiologic typing of *Clostridium difficile*. *J. Clin. Microbiol.*, 33(1): 184–187 (1995).
7. DAVIES K.A., ASHWIN H., LONGSHAW C.M., BURNS D.A., DAVIS G.L., WILCOX M.H., EUCLID study group. Diversity of *Clostridium difficile* PCR ribotypes in Europe: results from the European, multicentre, prospective, biannual, point-prevalence study of *Clostridium difficile* infection in hospitalised patients with diarrhoea (EUCLID), 2012 and 2013. *Euro Surveill.,* 21(29): 37-47 (2016).
8. DELMÉE M., HOMEL M., WAUTERS G. Serogrouping of *Clostridium difficile* strains by slide agglutination. *J. Clin. Microbiol.*, 21(3): 323-327 (1985).
9. DEPITRE C., DELMÉE M., AVESANI V., L'HARIDON R., ROELS A., POPOFF M., CORTHIER G. Serogroup F strains of *Clostridium difficile* produce toxin B but not toxin A. *J. Med. Microbiol*., 38:434–441 (1993).
10. DINGLE K.E., ELLIOTT B., ROBINSON E., GRIFFITHS D., EYRE D.W., STOESSER N.,VAUGHAN A., GOLUBCHIK T., FAWLEY W.N., WILCOX M.H., PETO T.E., WALKER A.S., RILEY T.V., CROOK D.W., DIDELOT X.. Evolutionary history of the *Clostridium difficile* pathogenicity locus. *Genome Biol. Evol.*, 6: 36-52 (2014).
11. DUMITRU I.M., DUMITRU E., CERNAT R.C., PETCU A.E., ȘERBAN C.I., RUGINĂ S. *Clostridium difficile* colitis – a serious current problem. *BMC Inf. Dis.,* 13(Suppl 1): O20 (2013).
12. ECKERT C., VROMMAN F., HALKOVICH A., BARBUT F. Multilocus variable-number tandem repeat analysis: a helpful tool for subtyping French *Clostridium difficile* PCR ribotype 027 isolates. *J. Med. Microbiol.*, 60(Pt 8): 1088-94 (2011).
13. EYRE D.W., GOLUBCHIK T., GORDON N.C., BOWDEN R., PIAZZA P., BATTY E.M., IP C.L.C., WILSON D.J., DIDELOT X., O'CONNOR L., LAY R., BUCK D., KEARNS A.M., SHAW A., PAUL J., WILCOX M.H., DONNELLY P.J., PETO T.E.A., WALKER A.S., CROOK D.W. A pilot study of rapid benchtop sequencing of *Staphylococcus aureus* and *Clostridium difficile* for outbreak detection and surveillance. *BMJ Open*, 2: e001124 (2012).
14. FAWLEY W.N., KNETSCH C.W., MACCANNELL D.R., HARMANUS C., DU T., MULVEY M.R., PAULICK A., ANDERSON L., KUIJPER E.J., WILCOX M.H. Development and validation of an internationally-standardized, high-resolution capillary gel-based electrophoresis PCR-Ribotyping protocol for *Clostridium difficile*. *PLOS One* 10(2): e0118150 (2015).
15. FAWLEY W.N., WILCOX M.H. An enhanced DNA fingerprinting service to investigate potential *Clostridium difficile* infection case clusters sharing the same PCR ribotype. *J. Clin.Microbiol.*, 49(12): 4333-4337 (2011).
16. GRIFFITHS D., FAWLEY W., KACHRIMANIDOU M., BOWDEN R., CROOK D.W., FUNG R., GOLUBCHIK T., HARDING R.M., JEFFERY K.J., JOLLEY K.A., KIRTON R., PETO T.E., REES G., STOESSER N., VAUGHAN A., WALKER A.S., YOUNG B.C., WILCOX M., DINGLE K.E. Multilocus sequence typing of *Clostridium difficile*. *J. Clin. Microbiol.*, 48(3): 770-778 (2010).
17. GÜRTLER V. Typing of *Clostridium difficile* strains by PCR amplification of variable length 16S-23S rDNA spacer regions. *J. Gen. Microbiol.*, 139(12): 3089-3097 (1993).
18. HEINLEN L., BALLARD J.D. *Clostridium difficile* infection. *Am. J. Med. Sci*., 340(3): 247–252 (2010).
19. HUBER C.A., FOSTER N.F., RILEY T.V., PATERSON D.L. Challenges for standardization of *Clostridium difficile* typing methods. *J. Clin. Microbiol.*, 51(9): 2810-2814 (2013).
20. INDRA A., HUHULESCU S., SCHNEEWEIS M., HASENBERGER P., KERNBICHLER S., FIEDLER A., WEWALKA G., ALLERBERGER F., KUIJPER E.J. Characterisation of *Clostridium difficile* isolates using capillary gel electrophoresis-based PCR ribotyping. *J Med.Microbiol*., 57: 1377-1382 (2008).
21. KARJALAINEN T., SAUMIER N., BARC M.C., DELMÉE M., COLLIGNON A. *Clostridium difficile* genotyping based on *slp*A variable region in S-layer gene sequence: an alternative to serotyping. *J. Clin. Microbiol.,* 40(7): 2452-2458 (2002).
22. KATO H., KATO N., WATANABE K., UENO K., SAKATA Y., FUJITA K. Relapses or reinfections: analysis of a case of *Clostridium difficile*-associated colitis by two typing systems. *Current Microbiology,* 33: 220-223 (1996).
23. KILLGORE G., THOMPSON A., JOHNSON S., BRAZIER J., KUIJPER E., PEPIN J., FROST E.H., SAVELKOUL P., NICHOLSON B., VAN DEN BERG R.J., KATO H., SAMBOL S.P., ZUKOWSKI W., WOODS C., LIMBAGO B., GERDING D.N., McDONALD L.C. 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. *J. Clin. Microbiol.,* 46 (2): 431-437 (2008).

24. KÖSER C.U., ELLINGTON M.J., CARTWRIGHT E.J., GILLESPIE S.H., BROWN N.M., FARRINGTON M., HOLDEN M.T.G., DOUGAN G., BENTLEY S.D., PARKHILL J., PEACOCKET S.J. Routine use of microbial whole genome sequencing in diagnostic and public health microbiology. *PLoS Pathog.,* 8(8): e1002824 (2012).
25. KNETSCH C.W., LAWLEY T.D., HENSGENS M.P., CORVER J., WILCOX M.W., KUIJPER E.J. Current application and future perspectives of molecular typing methods to study *Clostridium difficile* infections. *Euro Surveill.,* 18(4): 20381 (2013).
26. KNETSCH C.W., TERVEER E.M., LAUBER C., GORBALENYA A.E., HARMANUS C., KUIJPER E.J., CORVER J., VAN LEEUWEN H.C. Comparative analysis of an expanded *Clostridium difficile* reference strain collection reveals genetic diversity and evolution through six lineages. *Infect. Genet. Evol.*, 12(7): 1577-1585 (2012).
27. KOSTMAN J.R., EDLIND T.D., LIPUMA J.J., STULL T.L. Molecular epidemiology of *Pseudomonas cepacia* determined by polymerase chain reaction ribotyping. *J. Clin. Microbiol.*, 30(8): 2084-2087(1992).
28. KUIJPER E.J., BARBUT F., BRAZIER J.S., KLEINKAUF N., ECKMANNS T., LAMBERT M.L., DRUDY D., FITZPATRICK F., WIUFF C., BROWN D.J., COIA J.E., PITUCH H., REICHERT P., EVEN J., MOSSONG J., WIDMER A.F., OLSEN K.E., ALLERBERGER F., NOTERMANS D.W., DELMÉE M., COIGNARD B., WILCOX M.H., PATEL B., FREI R., NAGY E., BOUZA E., MARÍN M., ÅKERLUND T., VIROLAINEN-JULKUNEN A., LYYTIKÄINEN O., KOTILA S., INGEBRETSEN A., SMYTH B., ROONEY P., POXTON I.R., MONNET D.L. Update of *Clostridium difficile* infection due to PCR ribotype 027 in Europe, 2008. *Euro Surveill.*, 13: 1-7 (2008).
29. KUIJPER E.J., COIGNARD B., TÜLL P., on behalf of the ESCMID Study Group for *Clostridium difficile* (ESGCD), EU Member States and the European Centre for Disease Prevention and Control (ECDC). Emergence of *Clostridium difficile*-associated disease in North America and Europe. *Clin. Microbiol. Inf.*, 12 (Suppl. 6): 2-18 (2006a).
30. KUIJPER E.J., DEBAST S.B., VAN KREGTEN E., VAESSEN N., NOTERMANS D.W., VAN DEN BROEK P.J. *Clostridium difficile* ribotype 027, toxinotype III in the Netherlands. *Emerg. Inf. Dis.*, 12(5): 827-830 (2006b).
31. LEMÉE L., BOURGEOIS I., RUFFIN E., COLLIGNON A., LEMELAND J.F., PONS J.L. Multilocus sequence analysis and comparative evolution of virulence-associated genes and housekeeping genes of *Clostridium difficile*. *Microbiology*, 151: 3171-3180 (2005).
32. LEMÉE L., DHALLUIN A., PESTEL-CARON M., LEMELAND J.F., PONS J.L. Multilocus sequence typing analysis of human and animal *Clostridium difficile* isolates of various toxigenic types. *J. Clin. Microbiol.*, 42: 2609-2617(2004a).
33. LEMÉEL., DHALLUIN A.,TESTELIN S., MATTRAT M.A., MAILLARD K., LEMELAND J.F., PONS J.L. Multiplex PCR targeting *tpi* (Triose Phosphate Isomerase), *tcdA* (Toxin A), and *tcdB* (Toxin B) genes for toxigenic culture of *Clostridium difficile. J. Clin. Microbiol.*, 42(12): 5710-5714 (2004b).
34. MARÍN M., MARTÍN A., ALCOLEA A., IGLESIAS C., ALCALÁ L., PELÁEZ T., SÁNCHEZ-SOMOLINOS M., BOUZA E. First case of autochthonous *Clostridium difficile* PCR ribotype 027 detected in Spain. *Enferm. Infecc. Microbiol. Clin*., 32(6): 355-358 (2014).
35. McDONALD L.C. Confronting *Clostridium difficile* in inpatient health care facilities. *Clin. Inf. Dis.*, 45: 1274-1276 (2007).
36. McDONALD L.C., KILLGORE G.E., THOMPSON A., OWENS R.C., KAZAKOVA S.V., SAMBOL S.P., JOHNSON S., GERDING D.N. An epidemic, toxin gene-variant strain of *Clostridium difficile*. *N. Engl. J. Med.*, 353: 2433-2441 (2005).
37. O'NEILL G.L., OGUNSOLA F.T., BRAZIER J.S., DUERDEN B.I. Modification of a PCR-ribotyping method for application as a routine typing scheme for *Clostridium difficile*. *Anaerobe*, 2(4): 205-209 (1996).
38. PALTANSING S., van den BERG R.J., GUSEINOVA R.A., VISSER C.E., van der VORM E.R.,, KUIJPER E.J. Characteristics and incidence of *Clostridium difficile*-associated disease in The Netherlands. *Clin. Microbiol. Infect.*, 13: 1058–1064 (2007).
39. PERSSON S., TORPDAHL M., OLSEN K. E. P. New multiplex PCR method for the detection of *Clostridium difficile* toxin A (*tcd*A) and toxin B (*tcd*B) and the binary toxin (*cdt*A/*cdt*B) genes applied to a Danish strain collection. *Clin. Microbiol. Infect.*, 14: 1057-1064 (2008).
40. POPESCU G.A., ȘERBAN R., PISTOL A., NICULCEA A., PREDA A., LEMENI D., MACOVEI I., TĂLĂPAN D., FLOREA D., RAFILA A. Clinical and microbiological characterization of *Clostridium difficile* infection in Romania (2013-2014); a hospital based study. *BMC Infectious Diseases,* 14(Suppl 7): O24 (2014a).

41. POPESCU G.A., FLOREA D., RAFILA A. *Clostridium difficile* is emerging in Romania: a story of 027 ribotype and excessive antibiotic consumption. *J. Gastrointestin. Liver Dis.,* 23(3): 342-343 (2014b).
42. RAFILA A., INDRA A., POPESCU G.A., WEWALKA G., ALLERBERGER F., BENEA Ș., BĂDICUȚ I., ASCHBACHER R., HUHULESCU S. Occurence of *Clostridium difficile* infections due to PCR ribotype 027 in Bucharest, Romania. *J. Infect. Dev. Ctries.*, 8(6): 694-698 (2014).
43. RUPNIK M., AVESANI V., JANC M., VON EICHEL-STREIBER C., DELMÉE M. A novel toxinotyping scheme and correlation of toxinotypes with serogroups of C*lostridium difficile* isolates. *J. Clin. Microbiol.*, 36(8): 2240–2247 (1998).
44. RUPNIK M., BRAUN V., SOEHN F., JANC M., HOFSTETTER M., LAUFENBERG-FELDMANN R., VON EICHEL-STREIBER C. Characterization of polymorphisms in the toxin A and B genes of C*lostridium difficile*. *FEMS Microbiology Letters*, 148: 197-202 (1997).
45. SAMBOL S.P., MERRIGAN M.M., LYERLY D., GERDING D.N., JOHNSON S. Toxin gene analysis of a variant strain of *Clostridium difficile* that causes human clinical disease. *Infect. Immun.*, 68: 5480-5487 (2000).
46. SMITH A. Outbreak of *Clostridium difficile* infection in an English hospital linked to hypertoxin-producing strains in Canada and the US. *Euro Surveill.*, 10: 050630 (2005).
47. STUBBS S.L.J., BRAZIER J.S., O'NEILL G.L., DUERDEN B.I. 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. *J. Clin. Microbiol.*, 37(2): 461-463 (1999).
48. TANNER H.E., HARDY K.J., HAWKEY P.M. Coexistence of multiple Multilocus Variable-Number Tandem-Repeat Analysis subtypes of *Clostridium difficile* PCR Ribotype 027 strains within fecal specimens. *J. Clin. Microbiol.*, 48, No. 3 985–987 (2010).
49. KUIJPER E.J. Typing and subtyping of *Clostridium difficile* isolates by using multiple-locus variable-number tandem-repeat analysis. *J. Clin. Microbiol.*, 45(3): 1024-1028 (2007).
50. VAN DORP S.M., KINROSS P., GASTMEIER P., BEHNKE M., KOLA A., DELMÉE M., PAVELKOVICH A., MENTULA S., BARBUT F., HAJDU A., INGEBRETSEN A., PITUCH H., MACOVEI I.S., JOVANOVIĆ M., WIUFF C., SCHMID D., OLSEN K.E., WILCOX M.H., SUETENS C., KUIJPER E.J. for the European *Clostridium difficile* Infection Surveillance Network (ECDIS-Net) on behalf of all participants. Standardised surveillance of *Clostridium difficile* infection in European acute care hospitals: a pilot study, 2013. *Euro Surveill.*, 21(29): 4 (2016).
51. WREN B.W., TABAQCHALI S. Restriction endonuclease DNA analysis of *Clostridium difficile*. *J. Clin. Microbiol.*, 25(12): 2402-2404 (1987).
52. http://www.ecdisnet.eu.