We are IntechOpen, the world's leading publisher of Open Access books Built by scientists, for scientists

6,900
Open access books available

185,000
International authors and editors

200M
Downloads

Our authors are among the

154
Countries delivered to

TOP 1%
most cited scientists

12.2%
Contributors from top 500 universities



WEB OF SCIENCE™

Selection of our books indexed in the Book Citation Index in Web of Science™ Core Collection (BKCI)

Interested in publishing with us?
Contact book.department@intechopen.com

Numbers displayed above are based on latest data collected.
For more information visit www.intechopen.com



Chapter 4

Clostridium difficile Infection Diagnosis by Biological Molecular Methods

Luminița Smaranda Iancu, Andrei Florin Cârlan and Ramona Gabriela Ursu

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.68692

Abstract

In the past 15 years, the incidence of *Clostridium difficile* infection has emerged especially because of the new highly virulent strains. The classical diagnosis methods used to diagnose *C. difficile* infection take time and the enzyme immunoassay (EIA) test has demonstrated the lack of sensitivity. Even though new modern molecular methods have become available, the diagnosis of *C. difficile* in patients or healthy carriers remains a big challenge for both clinicians and laboratory staff. In the present chapter, we will list the main genotyping methods, stressing their advantages and disadvantages, as well. A brief presentation of the most useful kit (principle, sensitivity, specificity, benefits and disadvantages) to assess the impact of molecular methods in comparison with classical methods will offer support for future research in the present context of an increasing prevalence of *C. difficile* infection that represents worldwide, a real public health problem. To improve the patients' quality of life, to limit hospital transmission, and to save money, we have tried to identify the best diagnosis algorithm as tool in *C. difficile* diagnosis and surveillance. This algorithm may differ depending on the capacities of the laboratories and on the socioeconomic level of the countries in question.

Keywords: *C. difficile*, molecular method, PCR, RT-PCR, REA, PFGE, MLVA, VNRT, MLST, typing, diagnosis algorithm

1. Introduction

In the last years, especially starting with the early 2000s, the *Clostridium difficile* infection (CDI) has become a top subject in the medical field all over the world because of both high prevalence of healthcare-associated infection (HAI) and community infection. The causes of these spectra, including colonization and the diversity of clinical pictures, are topics of different

INTECH
open science | open minds

© 2017 The Author(s). Licensee InTech. This chapter is distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/3.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. CC BY

chapters and, for this reason, we approach the subject directly. For a better understanding of the biological methods, the readers must look first at the genetics chapter.

The growing incidence of the infection with *C. difficile* around the globe, especially because of the highly virulent strains, has changed the approach of laboratory diagnosis and imposed the drafting of numerous guides, which include preventive measures to stop their circulation [1]. Different expert groups, published in the last years large studies on the national programs for *C. difficile* surveillance, coming to the conclusion that many countries do not have the capacity to quickly diagnose the *C. difficile* infections, and that hospital outbreaks led to a continuous spread of the strains, including the highly virulent ones [2]. In countries as Romania, Bulgaria, and Greece, in which the Eurosurveillance studies (run by EUCLID) concluded the low-level capacity of laboratories, in the last years, at national level, the experts have initiated the implementation of a new guide to improve the surveillance of *C. difficile* [2, 3]. The apparent low incidence of CDI in some countries, such as China, is the consequence of the limited laboratory diagnosis capacities [4].

The overuse of antibiotics and the long and repeated hospitalization, especially in the case of patients over 65, are the main causes of this increase [5]. This change is mainly related to the high diversity of virulent strains and to the new approach of the antibiotic therapeutic scheme [6]. A long list of antibiotics, including the large broad spectrum, may be the cause of the dysbiosis that lead to CDI: clindamycin, cephalosporins (e.g., cefaclor, cefotaxime), β-lactams (e.g., penicillins, ampicillin, amoxicillin-clavulanic acid), fluoroquinolones, including last generations of gatifloxacin, levofloxacin, and moxifloxacin [5, 7, 8]. Not all authors accept the whole list; some of them consider cephalosporins to be certainly involved, whereas others consider fluoroquinolones (cited by Daniel and Rapose [9]). CDI was noticed even after short-term antibiotic courses, inclusively as a preventive measure [5]. Since 2002, the most virulent strains of *C. difficile*, described in literature as toxinotype IIINAP1/027, were isolated as resistant to fluoroquinolones [7]. The 027 toxinotype nomination is related to laboratory methods, pulsed field gel electrophoresis (PFGE), and to the place of isolation (North American PFGE type 1 (NAP1), respectively). This resistance pattern is associated with the ability to produce A and B toxins in larger quantities, including binary toxin production, and has a greater capacity to spread endemically [6].

The Center for Disease Control and Prevention (USA, Atlanta) consider CDI as the first cause of healthcare-associated infection [10]. All authors agree that CDIs are related to *C. difficile* strains, which produce toxins (A, B, and binary toxin, and many other virulence factors) [11, 12].

With the new virulence strains that have emerged, the incidence, morbidity, and mortality through CDI have increased all over the world, especially in the developed countries, starting with the early 2000s [13]. In USA, CDC estimated that a quarter million people need hospitalization and around 5.6% die from CDI each year; more than 90% of deaths occur in people over 65 years, but almost 50% of the infections were noted in patients younger than 65 [10]. In parallel with the overuse of antibiotics, many old patients have co-morbidities that increase the risk of severe evolution; on the other hand, the need for quick, sensitive,

and specific diagnosis have led to new laboratory techniques, including high-level biological and molecular ones that improved the positivity rate, decreasing the false negative one. All these factors, together with the previously mentioned and, probably, with other factors (e.g., pump protons inhibitors) made that, only from 2000 to 2007, the death number related to CDI to increase with 400% [10]. In the intensive care unit (ICU), the prevalence of CDI is the highest because it cumulates many risk factors. An excellent meta-analysis, from 2015, concluded that the prevalence of CDI among diarrheic ICU patients is more than five times higher in comparison with ICU patients with risk for pseudomembranous colitis, after approximately 10.5 days. The mortality rate was also higher: 32 versus 24%. This higher morbidity and mortality rate from ICU requires additional measures in order to prevent the spread of infections and more expensive regimens [14].

Even though the number of studies conducted to find out the costs of medical burden have increased, only a few of them have considered all factors that influence healthcare-associated costs; briefly, the CDC considered "at least $1 billion in excess medical costs per year" [10], and Kyne et al. estimated that *C. difficile* diarrhea cost/case was around $4600 and, using the appropriate statistics analysis, concluded that, in the USA, the total annual cost for diarrhea treatment exceeds $1.1 billion [15]. Some authors have considered different algorithms for diagnosis using phenotypic and biological molecular methods, concluding that the modern methods are less expensive than the traditional diagnostic ones recommended by some guides [16]. The risk of rapid spread in healthcare facilities of highly virulent strains, including those resistant to antibiotics, has led to improved laboratory techniques that have become able to quickly highlight CDI, with the possibility of applying the most appropriate preventive measures and modified therapeutic schemes [17, 18].

2. Arguments for the rapid diagnosis of *C. difficile* infection/colonization by molecular methods

The rapid diagnosis by molecular methods is costly at first sight, when compared to the rapid detection of toxins by enzyme immunoassay (EIA) or glutamate dehydrogenase (GDH) detection (an antigen produced in high amounts by all *C. difficile* strains, including the non-toxigenic one). Even though the EIA is a cheap test and despite the different commercial variants available, the specificity varied from 40 to 100% [13], with no major consequences regarding the clinical impact because a positive NAAT cannot differentiate between infection and colonization (**Table 1**). For this reason, it is necessary to also test the biomarkers that suggest the active infection. Different biomarkers were used, e.g., fecal lactoferrin and calprotectin, or cytokine analysis, but they did not demonstrate an efficient support in this differentiation [13].

In the modern era, when molecular platforms play an important role in patient diagnosis and management cases, a clear approach must be taken into consideration in order to use this tool in the best way, to reach as soon as possible a sensitive and specific diagnosis for patients and to reveal the most useful epidemiological markers, to initiate appropriate preventive

measures. To reach this goal, may become difficult because many years after the emergence of the new virulent strain (027 type) in parallel with others, which are eventually antibiotic-resistant (e.g., moxifloxacin-resistant, ribotype 012, 017, and 046 isolated from Sweden) [17], some authors highlight the lack of consensus regarding case definition, sampling, and diagnosis step algorithms [18, 19].

2.1. Direct detection of *C. difficile* in clinical specimens by molecular platforms

Different protocol algorithms use two or three steps to improve diagnosis sensitivity and specificity, looking to the lowest cost as well. In two-step algorithm that includes as first test the GDH detection, the negative test proves the absence of *C. difficile*. For the positive test, the EIA and/or the nucleic acid amplification test (NAAT) can be used as confirmatory tests. In the two-step algorithm, the NAAT is used after the GDH detection, and the positive test will impose the patient's isolation and the preventive methods to stop the transmission. The two-step algorithm may fail in CDI diagnosis, the GDH detection being related to the test sensitivity (79–98%) (Shetty et al., cited by Tenover et al. [20]); low sensitivity is related to different nontype 027 strains [20].

For the three-step variant, the EIA is used as confirmatory test and for negative results, and the NAAT is necessary to confirm or note the negative results (**Figure 1**).

Using directly molecular methods, the *C. difficile* diagnosis rates become twofold higher in comparison with EIA alone. Soon after that, the incidence of *C. difficile* increased but decreased afterward, probably because the experts used new case definitions, and better preventive methods can explain the transmission decline in hospitals. After many years of using in parallel different diagnosis algorithms, there are no sufficient data to support a clear conclusion regarding the clinical benefit and hospital costs. Larson et al.'s study concludes that PCR alone can save around 200,000 USA dollars annually, mainly by removing several tests [21]. Burnham and Carroll clearly mentioned that the need of future cost-efficient studies relates to NAAT testing alone [22, 23].

Phenotypical methods	Toxin A/B tested by EIAs
	Glutamate dehydrogenase antigen (GDH)
	EIA screen—GDH/toxin
	Lateral flow, Membrane assay
	Cell culture cytotoxin neutralization assays (CCCNA)
	Toxigenic culture (Culture + Cytotoxin assay) "Gold standard"
Molecular methods	Molecular strains typing
	PCR amplification
	Sequence-based

Table 1. Main techniques for the detection of *C. difficile* [13].

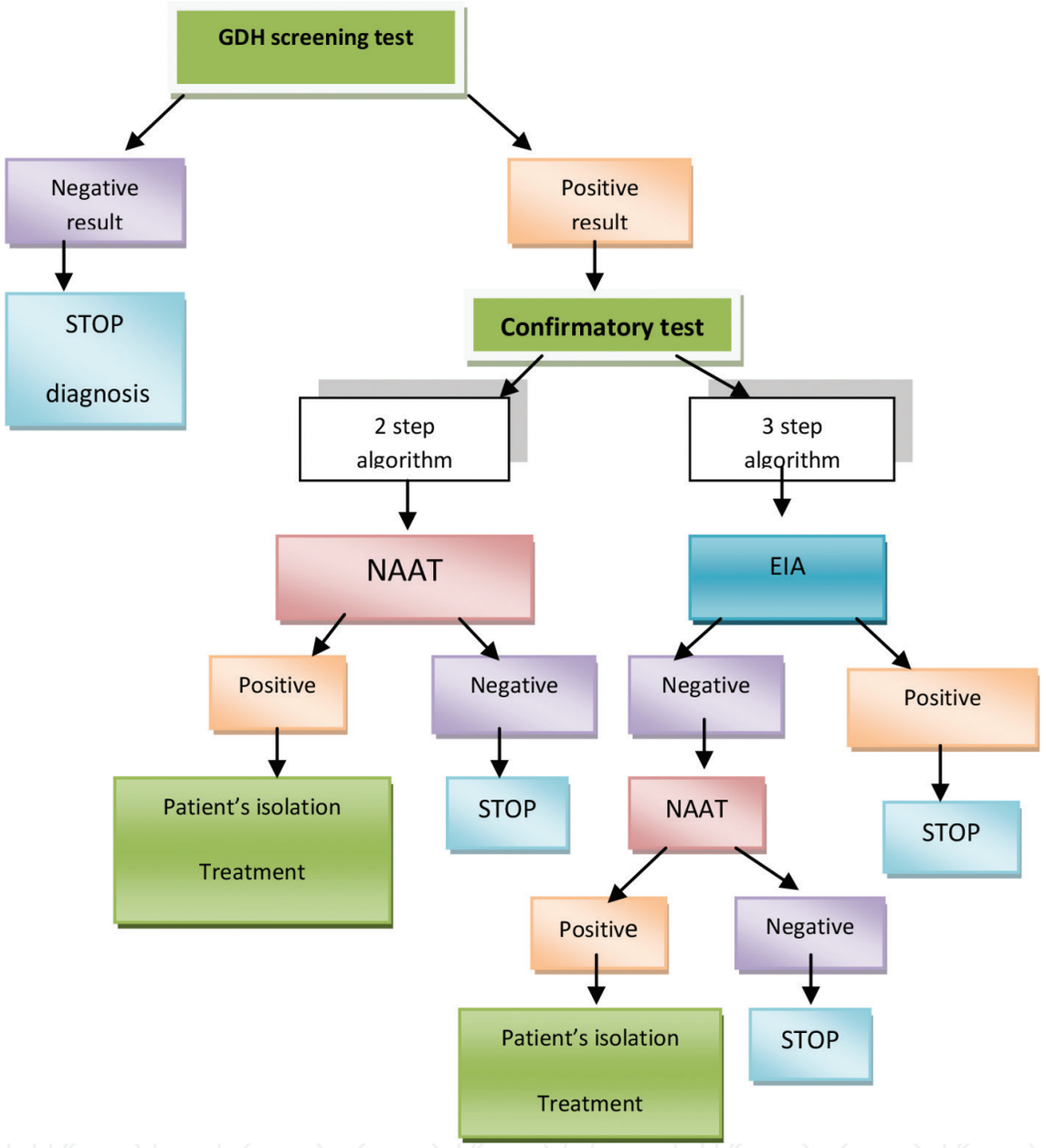


Figure 1. Algorithm steps for *C. difficile* diagnosis—different laboratory approaches.

3. Molecular methods applicable to *C. difficile* diagnosis

Until now, it has been clearly stated that molecular tests improve the detection of *C. difficile* in samples (stools, respectively), but that gene detection is not always a proof of their phenotypic expression. A few studies have tried to correlate gene detection with toxin expression using sequence analysis: some of them have found a major advantage for amplification methods in comparison with the EIA and GDH detection for the 027 isolates, but the small number of ribotypes belonging to the non-027 strains seems to be the main cause for test similarity results for this category [13] (**Table 2**).

Method	Comments	References
AP-PCR	Advantages: It is a reproducible, rapid, and simple technique It is sensitive and accurate, comparable with immunoblotting and REA, for the identification of *C. difficile* Applicability: In clinical laboratory, for strain identification Approximated time: 36–48 h; it is cost-efficient, especially for healthcare facility outbreak investigations Cost: $150–200 per specimen, when duplicate reactions with two sets of primers are used Disadvantages: Low reproducibility	[26–28, 36]
PCR ribotyping	Two sets of primers were proposed (USA *versus* UK) It is the reference standard method for *C. difficile* strain typing in Europe Advantages: Discriminatory power Applicability: Laboratory with moderate equipment Disadvantages: Moderate typability, reproducibility, ease of interpretation, and transportability	[10, 28, 33, 37, 54]
Repetitive extragenic palindromic sequence-based PCR (rep-PCR)	It uses heterogenous PCR primers that target noncoding repetitive sequences from the *C. difficile* genome Advantages: It is highly reproducible and more discriminatory than PCR ribotyping Applicability: *C. difficile* ribotyping Disadvantages: Even though the results for ribotypes 027 and 001 were excellent correlated with the PFGE and PCR ribotyping, for other types, it showed a reduced concordance. In the same studies, the method failed to separate between these two ribotypes Semiautomated variant (DiversiLab): Better standardization and reproducibility than the manual rep-PCR	[33, 37]
REA	It uses the *HindIII* enzyme in most of the protocols; a large number of bands can be separated by classical gel electrophoresis Applicability: It has been proved to be a very useful method for epidemiological studies, with an excellent discriminatory capacity and reproducibility Disadvantages: It is difficult to interpret band patterns, and protocols are difficult to compare between laboratories	[33, 35]
PFGE	The most frequent method used for *C. difficile* strain typing, as support for outbreak investigations Advantages: It is the "gold standard" method for *C. difficile* typing, as well as for other bacterium strains, and it is as comparative method between laboratories; it is a discriminatory and reproducible method, even though some strains are not typified In a modified protocol, the number of nontypeable strains has become 0, and both advantages were similar It is the reference standard in the USA Applicability: control of epidemic outbreaks Disadvantages: Needs longer time, it is more expensive, and it is designated for expertise laboratories because of the difficulties in the inter-laboratory comparison	[33–35, 37, 38]
Toxin-typing RFLP	It is based on the capacity of the *C. difficile* genome to encode a synthesis of minimum two toxins (A and B) (pathogenicity locus-PaLoc), and it is defined "as a group of strains with identical changes in the PaLoc when compared with the other strains," additional toxins (*tcd C*, *tcdR*, *tcdE*) and, for some strains, binary toxin too. There are 32 toxinotypes (some of them described as "minor," and 0 type) (from reference strain VPI10463) The current method for toxinotyping is restriction length polymorphism (RFLP) Advantages: Highly reproducible Applicability: In well-equipped laboratories for epidemiological studies. Toxinotypes III, IV, V, VIII, IX, and XII, the most frequent isolated from humans (VIII and IIIb [BI/NAP1/027 strains], are associated with disease severity and have been isolated worldwide) Disadvantages: Lack of consensus standards, difficult interpretation	[33, 39–42, 45]

Table 2. The main biological molecular methods for *C. difficile* infection diagnosis.

3.1. Amplification methods

For many years, the nucleic acid amplification tests (NAATs) have been used to detect *C. difficile* in fecal samples as an efficient tool to replace nonmolecular methods such as EIA and GDH; the first publication appeared in the early 1990s and the conventional PCR methods had as target the different genes (e.g., *tcdA, tcdB*, and *16S rRNA* genes, respectively) [13, 22].

The emergence of the new *C. difficile* virulent strains and the low sensitivity and specificity of the phenotypic methods have imposed their replacement with molecular methods, starting with genotyping and strain typing, respectively. In a very well-documented presentation of these methods, Dingle and MacCannel [24] proposed a list of typing methods. Briefly, these methods are restriction fragment methods (restriction endonuclease analysis [REA], pulsed field gel electrophoresis [PFGE], toxinotyping, PCR amplification methods [PCR ribotyping], repetitive extragenic palindromic PCR [rep-PCR], multilocus variable-number tandem repeat analysis [MLVA], arbitrarily primed PCR [AP-PCR], and sequence-based methods, including the whole genome sequencing [WGS], target sequencing approaches, multilocus sequence typing [MLST], surface layer protein A sequencing [slpA], tandem repeat sequence typing [TRST], single nucleotide polymorphism typing [SNPT], whole genome multilocus sequence typing, and the Kmer-based comparison).

From these methods, we are presenting the one with the highest clinical applicability, and for the most important of them, we have conducted a synthesis of recent scientific literature (PFGE, ribotyping, MLVA, and sequence-based methods). We are concluding this presentation of methods for *C. difficile* detection by presenting the FDA commercially approved tests, which have the advantage of accuracy, reproducibility, and clinical validation by comparing different laboratories from different countries.

Finally, we will list the main contribution of the molecular methods regarding the antibiotic resistance surveillance, the comparison between the biological molecular methods and a comparison between the classical rapid detection tests (EIA, DGH) versus the modern biological molecular methods.

3.1.1. The arbitrarily primed PCR or AP-PCR

It is a simple and rapid method to detect different bacteria outbreaks [25]. The AP-PCR belongs to the early stages of biological molecular era and uses a selection of primers that, at low annealing temperatures, produced a variety of bands. Details regarding the patent of this technique (*Arbitrarily primed polymerase chain reaction method for fingerprinting genomes*; United States Patent 6696277) can be found in (http://www.freepatentsonline.com/6696277.html) [26].

Advantages—AP-PCR provides a rapid and sensitive screen for the determination of clonal relationships among *C. difficile* strains [27]. The comparative studies using PCR ribotyping and AP-PCR from the cited authors (e.g., McMillin, 1992; Van Belkum, 1993, 1994) used previous study primers (Van Belkum, 1994) and demonstrated that for samples in which the DNA was severely affected and the RFLP was unable to recognize fragments, the AP-PCR was able to provide clear typing results [28].

Disadvantages—It is less reproducible than the PCR ribotyping and, for this reason, it is recommended as the best typing method, even though it has concordant results with the AP-PCR [27]. The AP/PCR and the RAPD (random amplified DNA, that uses short primers) were not accepted later as comparative interlaboratory methods because of their difficulties in control and standardization (Colliers et al., cited by Gürtler and Grando [29]).

3.1.2. Restriction endonuclease analysis (REA)

In one of the first articles (1987) that related to the REA, Wren and Tabaqchali used *HindIII* as cutting enzyme and concluded that analyzing the DNA with it is a very sensitive method for *C. difficile* strain differentiation and a tool for epidemiological studies. Other restriction enzymes, such as *BamHI, EcoRI, SalI,* and *SmaI,* were less useful in a clear separation of bands [30]. In a very well cited paper [31], two extraction methods were used, and the 206 REA types were classified into 75 groups; they concluded that the REA is a rapid, very sensitive, discriminatory, and reproducible method for *C. difficile* typing, and recommended its use in large epidemiological studies. Using this efficient tool, different authors have also identified outbreaks from North America in USA and in Canada, respectively [7, 32]. Using the biological molecular methods, different study groups have clearly noted that the main strain that is spread over the world (Europe, USA, etc.) is known as BI/NAPI/027 because of different methods used to identify it (e.g., type BI, using REA; "NAP1," from the NA, North America, and P, from pulse-field type 1, using as method the PFGE; and 027 by PCR-ribotyping) [7, 31]. The REA is a relatively simple method for analyzing the total genomic DNA and has been successfully applied to several bacterial species, including *C. difficile*. Among the major advantages, we can note its good discriminatory capacity, but the main concern is related to the numerous restriction fragments that are generated, especially fragments smaller than 11 kb [33]. It is difficult to compare the method between laboratories, and its high-level protocol technicity required a high-level expertise [24].

3.1.3. Pulse field gel electrophoresis (PFGE)

For years, the epidemiology and clinical case management were imposed on *C. difficile* strain typing. Even though the PFGE was one of the first used methods in some regions as North America, it is still considered as being a standard method. In short, the PFGE supposed DNA digestion with restriction enzymes (e.g., *Smal*) and agarose gel electrophoresis for DNA fragment separation and characterization. After that, an electric field is being changed repeatedly, using three directions. The large DNA fragments that cannot be clearly separated by classical gel electrophoresis are separated by an electric field that changes directions many times in 12–24 h. In literature, this clearer band pattern is known as the "North-American pulsed-field," or as the NAP types. One pulsotype is defined as that with more than 80% similarity, in interpreting such bands, we must bear in mind the subjectivity of interpretation and the fact that, sometimes, we cannot differentiate between the studied strain and the reference strains that we used for comparison [13]. The PFGE was the method used by many researchers, some of them looking at the comparability degree, using different methods (e.g., MLVA, REA, and PFGE); for example, Killgore et al. find that the D value (discriminatory index score) for PFGE is 0.843; in the cited study, the D varied between 0.964 and 0.631, and they recommend as reference methods, the REA and the MLVA, for their discriminatory capacity [32]. Even though it is considered

as a "gold standard," the method has changed the protocol over the years and many protocols have been suggested (Corkill et al., 2000; Herschleb et al., 2007) [34, 35].

From the PubMed database, using the next keyword—*PFGE C. difficile human infections*—we have selected 48 articles, published between 1994 and 2017. In time, the PFGE has been used by researchers in different clinical and laboratory applications.

Starting with the 1990s, many authors have compared the PFGE with other molecular methods used to analyze *C. difficile* [33, 36, 37]. Some of them have concluded that the PFGE had comparable discriminatory powers for epidemiologic typing of *C. difficile* isolates and that the ribotyping is appreciably less discriminatory [33], whereas others have found that the PCR-based methods were easier and quicker to perform, but their fingerprints were more difficult to interpret than those of the PFGE [36].

Pasanen et al. concluded that PFGE and classical PCR ribotyping can be used as reference methods in epidemiological studies to confirm the results obtained with other methods. Because of the discrepancies between studies (e.g., some authors recommend REA and MLVA [32] for *C. difficile* typing, while others recommend PFGE and conventional PCR [37]), it is obvious that further studies and long periods of time are needed in different large geographical areas for the results to be statistically significant.

Another research team has detected *C. difficile* in infections other than the very well-known antibiotic-associated diarrhea, like the infection of a prosthetic joint [38] in association with inflammatory bowel disease [39] in which, interestingly enough, the nonclonal distribution of distinct strains was further demonstrated by the PFGE genomic fingerprinting. A team from Ohio, USA, published two cases of *C. difficile* bacteremia, which is a rare event. In one of the cases, the bacteremia was caused by the North American pulsed field gel electrophoresis (PFGE) type 1 (NAP-1) strain [40].

In the natural history of *C. difficile* infection, it is important to establish if the infection is a singular or a recurrent one of relapse or re-infection [41, 42]. The same method (PFGE) has been used to identify host and bacterial factors associated with healthcare-associated acquisition of *C. difficile* infection and colonization. Among patients with healthcare-associated *C. difficile* infection and those with colonization, 62.7 and 36.1%, respectively, had the North American PFGE type 1 (NAP1) strain [43].

In 2006, Kuijper et al. [44] concluded that the increased virulence of C. *difficile* is probably related to the association with antibiotic resistance because the fluoroquinolone-resistant strains are also highly toxigenic; these strains belonging to the ribotype 027 (toxinotype III) have been isolated in the last years from many countries, from hospitals (e.g., in England and The Netherlands, from 75 and 16 hospitals, respectively), and from healthcare facilities (e.g., Belgium and France) [44]. After this ECDC report, other authors have detected the rapid spread of *C. difficile* NAP1/027 in Brazil [45], Korea [46], Hong Kong [47], Latin America (Chile) [48], and Romania [49].

If the previously mentioned authors have associated the *C. difficile* strain NAP1/027 with recent outbreaks in North America and Europe, characterized by more severe disease symptoms, higher mortality rates, and greater risk of relapse, Sirard et al. [51] studied, in a nonoutbreak situation, whether specific strains, such as NAP1/027, were associated with more severe disease symptoms, higher toxin production, and/or greater sporulation *in vitro*. Their results

suggested that some NAPI/027 strains can be isolated from less severe cases, and even though those strains produce large toxin amounts, probably other factors explain better the patients' evolution, and that future studies are necessary to clarify this discrepancy [50].

3.1.4. PCR ribotyping

The strain typing method must be used for epidemiological reasons; in Europe, the ribotyping is the most used method in *C. difficile* identification. For amplification, this method uses one fragment from the most constant region, from 16S to 23S RNA genes, the so-called intergenic spacer. In this operon, there are many copies from the genome of *C. difficile* and, using a single primer pair, we can obtain different bands with sizes from 200 to 700 bp [13, 37, 52]. For their evidence, viewing and comparing, we can use the migration in agarose gel, with commercial kits or their analysis with a dedicated software [32, 36, 51, 52].

The definition of Gürtler and Grando [29] seems to briefly and completely describe one of the most useful method in *C. difficile* typing, first introduced by Gürtler (1993), when an original primer set, given to large fragments, was very difficult to differentiate in agarose gel electrophoresis; later, different authors have tried to overcome these difficulties, and, finally, Bidet et al. (1999) used the appropriate primers (located nearest to ITS1). When the gel electrophoresis was used to separate the amplified bands, their patterns were difficult to interpret. Despite these disadvantages, the low price, the high sensitivity, and specificity were listed as the main positive arguments for the simple gel electrophoresis [29].

One major disadvantage of this method is the difficulty to compare the results from different laboratories and the lack of unique software analysis. In order to make this method applicable in all laboratories and increase the reproducibility, the high-resolution capillary gel electrophoresis was called on [53]. To standardize the method in countries like the UK, different laboratories have started using the unique protocol [13], including the differentiation of some types (such as the 014 and the 020) that cannot be distinguished using the conventional agarose gel-based PCR ribotyping. To increase the discriminative capacity, the capillary gel electrophoresis can be used, including for the subtyping of the 014 type characterization [54], variant that also needs standardization.

CDI is a major issue of concern in Europe and USA. For surveillance studies, even though reporting the CDI is not mandatory in all EU countries, the ECDC, as CDC Atlanta advises, initiates large surveillance programs to decrease the incidence and severity of CDI [55].

In a comprehensive review, with the permission of Public Health England, the authors published the prevalence of *C. difficile* ribotypes, detected from 2007 to 2011. The decreasing order of ribotypes was 27, 001, 106, 015, 002, 078, 014, 005, 023, 016, 014/020, 020, 017, 026, 017, 026 [13]. Since then, many authors have published their results, specific for their countries: PCR—ribotype 018 (Italy) [57], ribotype 176 (the Czech Republic) [58], the first two *C. difficile* ribotype 027/ST1 isolates being identified in Beijing [59], the PCR-ribotype 176 in Prague [60], another four PCR-ribotypes (027, 033, 078, and 126) in Italy [61], ribotype 258 (Qatar) [62], the first Polish ribotype 027 [63], ribotype 126 in Southern Taiwan [64], and ribotype 244— Australia [57, 65]. The prevalence of CDI with different ribotypes worldwide underscores the importance of local surveillance in detecting and controlling *C. difficile* infection.

Few countries are performing follow-up studies on *C. difficile* ribotype's recurrence and relapses [66]. In Sweden, 29 patients were positive in at least one of the follow-up tests; 16 had the same ribotype in follow-up tests, i.e., relapse, and 13 patients with a different ribotype, i.e., re-infection [67]. In a recent study (USA) [68], conducted in a small study group, 25 patients respectively, 5 of them were colonized and only 3 were classified as recurrent cases (12%). All eight patients had risk factor healthcare exposure, and no other risk factors were identified (e.g., antibiotics or proton pump inhibitor treatment); neither of them was diagnosed with the 027 ribotype in the follow-up period. By comparison, Komar et al. (2016, UK) has monitored patients with *C. difficile* infection over a 2-year period, and they found that the epidemic *C. difficile* 027/ST1 caused the majority of infections during the sampling period [69].

Another application of ribotyping is testing the susceptibility of *C. difficile* to antibiotics, according to the known virulence of specific ribotypes [69, 70]. Moreover, a team from the UK has implemented the antimicrobial stewardship, which is a key component in the reduction of healthcare-associated infections, particularly of the *C. difficile* infection (CDI). They have successfully restricted the use of cephalosporins and, subsequently, of fluoroquinolones. From an endemically high level of >280 cases per year in 2007–2008, the number of CDIs decreased to 72 cases in 2011–2012 [71]. The same antimicrobial stewardship program was used in Austria, where the ribotype 027 is prevalent. The reduction of moxifloxacin use, combined with provided structured information on CDI, was associated with an immediate decrease of CDI rates in this large community teaching hospital [72].

3.1.5. Multilocus variable-number tandem repeat analysis (MLVA)

A different typing method had no high discriminatory capacity, and new techniques were in place to overcome this disadvantage bearing in mind that, in the epidemiological investigation of different outbreaks, it is essential to identify the source of infection/etiological agent and its connection with secondary cases. The MLVA based on the amplification of different size fragments that can be easily seen using the capillary gel electrophoresis and the automated analysis of amplified fragments is one of them [55, 56]. In this large study from Europe that included laboratories from different countries, the MLVA was used to trace the 027 ribotype. Using the MLVA's capacity to discriminate and to monitor the transmission events in the hospital or in healthcare facility settings, many research groups used the MLVA [13, 54, 55, 73–78]. Different studies have concluded that despite its high discriminatory capacity, the MLVA is not a standardized method and that, for future inter-laboratory comparability, further large studies must be run across the world, using an identical protocol.

In the recent years, numerous teams have used the MLVA in many approaches. A research group from the Netherlands described the clinical and the microbiological characteristics of CDI among hospitalized children, using the MLVA. They have concluded that the *C. difficile* PCR ribotype 265 was most prevalent in children, this strain being rarely found in other countries, except for Belgium. The MLVA showed genetic relatedness between three-fourths of pediatric and adult ribotype 265 strains, without a clear epidemiological link [79].

Using the same MLVA Assay, Krutova et al. have performed a survey in 18 hospitals in the Czech Republic. They have found the spread of two *C. difficile* PCR ribotypes within 18 hospitals, underlying the importance of standardizing the CDI testing protocols and implementing the mandatory CDI surveillance in the country [80].

Kullin et al. have found that identical MLVA types occurred in different wards over time and that several patients were infected with identical strains. The patient-to-patient transfer and the unique infection events might cause the predominance of ribotype 017 strains in the cohort. The multidrug-resistant strains are a potential reservoir for future infections [81].

Usui et al. have analyzed the *C. difficile* prevalence among piglets in Japan to clarify the infection origin and the extent of the associated risk by using molecular and microbiological methods for both swine and human clinical and foreign isolates; the MLVA was able to connect the European 078 ribotype source, spread in Japan by imported pigs [82].

The MLVA was also used to identify the first case of *C. difficile* RT027 infection in the Czech Republic (CZ), the patient having previously been hospitalized in Germany, prior to moving to CZ [83].

A team from the UK (Oxford) has run a comparison between the MLVA and the WGS, and they have found that both methods were very similar despite the fact that they have analyzed different parts of the bacterial genome. With improvements in the WGS technology, it is likely that the MLVA locus data will be available from the WGS in the near future [84].

Another application of the MLVA has been put into practice in the Netherlands, where the authors have encountered an outbreak because of these two types occurring simultaneously in a 980-bed teaching hospital. The clonal dissemination has been investigated by the MLVA that showed persistent clonal dissemination of types 017 and 027 despite the appropriate infection control measures [77].

Manzoor et al. have developed in the UK an eMLVA (extended) scheme, which provides insight into the genetic diversity of the *C. difficile* population at both global and cross-infection clusters in patient levels, with the possibility of replacing the PCR ribotyping. This eMLVA scheme could discriminate clinically significant clusters, while maintaining a good concordance with the PCR ribotyping. The typing schemes containing only seven loci showed, in contrast, poor association with the PCR ribotyping [85].

3.1.6. Multiplex nucleic acids test

Sometimes, the *C. difficile* strain diversity makes the classic PCR fail in identifying different types of isolates (some producing both toxins encoded by their genes, *tcdA, tcdB*, and some not, including binary toxin, mediated by the *cdtA* gene). The real-time PCR multiplex type has been put into practice by different researchers [86] or a real-time variant, fluorescence-based multiplex PCR, to simultaneously detect *tcdA* and *tcdB* genes in the patients' stools [87].

The *C. difficile* genome imposed the simultaneous detection of several genes: *tcdA, tcdB,* binary toxin, and Δ117 (single pair deletion at 117nt in *tcdC* gene), for the detection of the 027 ribotype (known as the epidemic strain 027/NAP1/PI, respectively). In the last decade, the FDA have approved or cleared many NAATs, including the multiplex type. Some of them

are loop-mediated isothermal amplification assays—LAMP—(e.g., Illumigene, Meridian Bioscience, Inc., that detect the *tcdA* gene from the conserved region), helicase-based amplification (e.g., Portrait Cdiff Assay, Great Basin; AmpliVue Cdiff Assay—Quidel Corporation), or based on array technology (e.g., Verigene Cdiff Assay, Nanosphere, a multiplex one, that uses PCR-amplified DNA in a nanoparticle-based assay, able to detect the *tcdA* and *tcdB* genes, the binary toxin gene, and the Δ117) [88]

The Verigene *C. difficile* Nucleic Acid Assay has a high sensitivity (98.7%), and a relatively low specificity (87.5%) compared to direct cultivation as the gold standard method, and it is recommended to be used as a test for the 027 ribotype identification, having been able to detect the previously listed markers [88].

3.2. The main sequence-based method for *C. difficile*

3.2.1. Multilocus sequence typing

In the last years, the MLST has become one of the most accurate methods, especially for the identification of different pathogens, including *C. difficile*, using the internal fragments of seven genes. The higher accuracy is related to these allele fragments that have different lengths (from 300 to 600 bp) and are easy to arrange precisely with the support of the automated DNA sequencer, in both strands. For each housekeeping gene, the different sequence present in each species genome will be given as distinct allele, for each isolate [13, 89, 90]. MLST generates high-throughput sequence data that can be uploaded from laboratories worldwide to a common web database [55].

C. difficile is theoretically well suited to MLST, as the species are relatively genetically heterogeneous. In 2004, MLST was introduced to study the population structure and global epidemiology of *C. difficile* (*aroE*, *ddl*, *dutA*, *tpi*, *recA*, *gmk*, and *sodA*), in order to analyze a group of *C. difficile* isolates [13, 90, 91, 92]. A major advantage of sequence-based typing methods like MLST is the ease of interpretation of the generated data. The sequence data are unambiguous and, therefore, objective, highly reproducible and easily exchangeable between laboratories. A practical disadvantage of MLST remains the relatively high cost of sequencing multiple targets, which could partially explain why MLST has not replaced the conventional PCR ribotyping in many European laboratories [55].

The analysis of recent scientific literature has revealed the main clinical applications of MLST. It is very important to first analyze the spread of epidemic strains, including the hypervirulent ones. This has been done, e.g., by researchers from Latin America, who have found that, in Chile, the most prevalent subtype (near to 80%) is subtype 1, related to the hypervirulent strain NAP1/027/ST1. The MLST analysis was capable of describing a high similarity (73%, respectively) of this subtype with nine different other subtypes, characterized by a similar 117 bp deletion (in the *tcdC* gene) [49]. The spread of epidemic strains of *C. difficile* has been also studied on Czech isolates, using the MLST [80]. Kuwata et al. have claimed that in their first study from Japan, based on MLST, both toxigenic and nontoxigenic *C. difficile* strains showed high genetic variation and that drug resistance was more likely related to toxigenic strains [93]. Some authors have used the MLST and have found that the ICU-acquired toxigenic *C. difficile* was not linked to those detected on admission. The active screening for toxigenic *C. difficile* was not considered to be a resource-efficient measure

in settings with a low prevalence of colonization [86]. Another application of the MLST usage was to evaluate the dissemination of clones in hospitals and breeding-farms or a contamination in the slaughter-house, and the probability of interspecies transmission [94].

3.2.2. Whole genome sequencing (WGS)

Whole genome sequencing (WGS) is another sequence-based assay besides MLST, which has emerged as a promising sequence-based technique since it allows the detection of variations between *C. difficile* strains by, for example, single nucleotide polymorphisms (SNPs) analysis [95]. A study performed in England, using the WGS technologies to identify SNPs, identified three sublineages of *C. difficile* RT017 circulating in London. Like the notorious RT027 lineage, which has caused global outbreaks of *C. difficile* infection since 2001, the lineage of toxin-defective RT017 strains appears to be continually evolving [96]. Even more, a team from Switzerland has developed a double locus sequence typing (DLST) scheme as a tool to analyze *C. difficile* isolates. The results of DLST were compared with the ones from MLST: DLST had a higher discriminatory power compared with MLST and successfully identified all isolates of the study. The main advantage of DLST is including the absence of DNA extraction (polymerase chain reaction PCR is performed on colonies), no specific instrumentation, the low cost, and the unambiguous definition of types [97].

4. The new epidemiology of *C. difficile* spread

In the past 20 years, the use of antibiotics and, in many countries, their overuse are the main causes of the extended spread of hyper virulent and multidrug-resistant strains of *C. difficile*, related to the 027 ribotype, as well as to many others. All methods try to rapidly identify the infected strain, but there are no solid arguments in differentiating between infection and colonization (positive *C. difficile* diagnosis, in the lack of symptoms). An excellent synthesis regarding colonization (carriage) and its role as a source of infection was published recently by Furuya-Kanamory et al.; the need of colonized patient identification is obvious: their prevalence is higher than in symptomatic cases; they can become a dangerous source of infection, and preventive methods must target them [98]. The cited study also made a vast synthesis of prevalence colonization rate over the world, which varied in large limits: 0–15% for healthy adults, almost 30% in colonized patients with nontoxigenic strains, and 0–51% in elderly residents of healthcare institutions [98].

Clinical and epidemiological data must be taken into consideration in this differentiation. The new rapid phenotypical and genotypical methods were a real support for the laboratory diagnosis that, in recent years, was able to rapidly identify the etiology of CDI; the need for rapid identification is related to the patients' treatment and to the preventive measures. The alarming increase of cases is related to the highly virulent strain circulation growth and to these new sensitive and specific methods that increased the rate of positive diagnosis. All these factors have led to declaring CDI a public health problem [13, 23, 99]. The prevalence of CDI increased especially since 2003 (in the Quebec study, from Canada, this was fourfold times higher than before) [23]. Later, new reports from North America and Europe [7, 55] have claimed the spread of ribotype 027 strains, highly virulent and resistant to fluoroquinolones,

as the main cause of this increasing prevalence. If medical care-associated infections were, from the beginning, the main cause of these alarming phenomena, other studies have clearly demonstrated the common origin for about one quarter of the cases (Lessa et al., cited by Burnham and Carroll [13]).

5. Commercially available real-time PCR test

In the last two decades, the laboratories have focused on using commercial kits, which have many advantages when compared to "in house" methods. We have chosen the real-time PCR tests, approved by the FDA, and they seem to be the most commonly used around the world, due to their performances.

The commercially available, real-time PCR tests for *C. difficile* diagnosis include the BD GeneOhm Cdiff (BD Diagnostics; San Diego, CA, USA) (target *tcdB* gene), Prodesse ProGastro Cd (Gen-Probe Inc.; San Diego, CA, USA) (target *tcdB* gene), Xpert *C. difficile* (Cepheid; Sunnyvale, CA, USA) (target *tcdB* gene and binary toxin), and Illumigene *C. difficile* (Meridian Biosciences; Cincinnati, OH, USA) (target *tcdA* gene), Real-Time PCR tests. Compared to the Cytotoxicity Neutralization Assay Culture (CNAC) and/or to the toxigenic culture, the PCR assays have been reported with sensitivities and specificities ranging from 77 to 100% and 93 to 100%, respectively [20, 86]. These tests have been demonstrated as being similar or even more efficient when compared with CCNA for children stool samples [86].

The FDA approved a list of nucleic acid-based tests, which analyze variations in the sequence, structure, or the expression of the deoxyribonucleic acid (DNA) in order to diagnose infection with an identifiable pathogen, including *C. difficile*. (http://www.fda.gov/medicaldevices/productsandmedical procedures/invitrodiagnostics/ucm330711.htm) [96].

The consultation of PubMed has allowed us to select 114 articles: 3 articles that used IMDx *C. difficile* for Abbott m2000 test, 2 with BD Diagnostics BD MAX Cdiff Assay, 2 with Quidel Molecular Direct *C. difficile* Assay, 3 with Verigene *C. difficile* Nucleic acid Test, 4—Portrait Toxigenic *C. difficile* Assay, 3—Simplexa *C. difficile* Universal Direct Assay, 8—Xpert *C. difficile*/Epi, 27—Illumigene *C. difficile* Assay, 54—Xpert *C. difficile*, 3—ProGastro Cd Assay, and 5—BD GeneOhm C. diff Assay.

For this short presentation, we have chosen the latest references that offered the most eloquent data regarding sensitivity, specificity, and, if available, positive predictive value and negative predictive value (**Table 3**) [101–111].

One feature of these articles is that they compare different platforms and analyze samples from different medical centers, and this action favors the evaluation of the test's accuracy and reproducibility. The sensitivity and the rapidity of the NAATs are excellent and fast (e.g., 90 min using Portrait Toxigenic test, or 65 min GeneXpert for 80 samples, simultaneously) making them reliable methods for the direct detection of *tcdA* and/or *tcdB* in stool specimens, compared with the toxigenic culture [100, 101]. Some differences in the sensitivity of the NAATs may partly depend on the number of toxigenic *C. difficile* in stool specimens. Considering the rapidity and the high specificity of the real-time PCR assays compared to the toxigenic culture, they

References	Country	Method	Sensitivity	Specificity
[101]	Korea	Xpert	90.0%	92.9%
		Max	86.3%	89.3%
		IMDx	84.3%	94.4%
		Illumigene	82.4%	93.7%
[106]	Korea	Xpert	82.8%	98.8%
		BD MAX	81.6%	95.8%
		IMDx	62.1%	99.4%
[103]	New York, USA	BD Max Cdiff assay (Max)	96.9%	95%
		Abbott m2000 assay (IMDx)	92.8%	100%
[104]	Washington, Seattle	Verigene CDF	95.2%	99.4%
		Simplexa Universal Direct assay	87%	100%
		BD MAX	87%	98.8%
[107]	Heidelberg, Germany	BD MAX Cdiff assay	90.5%	97.9%
		Xpert *C. difficile* test	97.3%	97.9%
[108]	USA	Lyra assay on the SmartCycler II	82.1%	98.8%
		ABI 7500 Fast DX	96.9%	85.7%
		ABI QuantStudio DX	89.3%	99.0%
[109]	California, USA	Simplexa Universal Direct	98%	96%
		AmpliVue assays	100%	100%
[105]	Japan	Verigene CDF test	96.7%	97.4%
[102]	USA	Portrait toxigenic *Clostridium difficile* assay	98.2%	92.8%
[110]	California, USA	Early prototype core molecular mirroring nuclear magnetic resonance detection platform (Mentor-100)	88.4%	87.0%
[111]	Chicago, IL, USA	Wampole toxin A/B EIA	85.4%	90.9%
		CdQCC (GDH antigen)	95.8%	89.6%
		BD GeneOhm	100%	100 %
		Progastro Cd RT-PCR	93.8%	99.3%

Table 3. Characteristics of the FDA-approved tests for the detection of *Clostridium difficile*.

can be used as the first test method for *C. difficile* infection/colonization. However, additional efforts should focus on the discrimination between infection and colonization. According to the overall performance of these assays, these results support the routine use of the said platforms for the detection of the toxigenic *C. difficile* in the clinical laboratories, a fact that will have a positive impact on patient care. Moreover, the Verigene CDF test is a novel nucleic acid microarray

that reliably detects both *C. difficile* toxins A and B in unformed stool (liquid) specimens and appears to adequately identify the ribotype 027 isolates [93, 104]. Another advantage is that sample processing is minimal, these tests having proved to be simple, cost-efficient, and with broad applicability to panel-based approaches, potentially simplifying the workflow [105].

Another application of these assays is the possible reporting of the DNA load of toxigenic *C. difficile* in the stool sample, which may represent a solution. Using the Xpert platform, most samples with discrepant results had *C. difficile* concentrations below the illumigene limit of detection. The significance of the low-level *C. difficile* detection needs to be further investigated [105]. The estimated cost avoidance provided by a more rapid molecular diagnosis can be outweighed by the cost of isolating and treating PCR-positive/cytotoxin-negative patients [103, 104]. The costs, the clinical consequences, and the impact on the nosocomial transmission of treating and/or isolating patients—positive for toxigenic *C. difficile* by PCR but negative for *in vivo* toxin production—worth further study. Diagnostic algorithms combining immunoassays and NAATs could also improve the specificity and reduce the global cost of this analysis [108–110].

In a recent study from 2014, Deak et al. [109] summarized the efficiency of two test cleared by the FDA (Simplexa Universal Direct and AmpliVue *C. difficile* Assay) having as references the Meridian Illuminigene Assay and the toxigenic *C. difficile* culture. In short, the best results were obtained with Simplexa (sensitivity 98%; limits: 88 ÷ 99.9), with short specimen preparation time, as AmpliVue (3 min, respectively), a small difference regarding the total time of the procedure (73 min *versus* 68 min for Illuminigene, and 91 min for AmpliVue), and only 8 min for hands-on time (*versus* 18, and 11 min for Illuminigene and AmpliVue, respectively). Both evaluated tests showed the same specificity (100%; limits: 96.9 ÷ 100). In conclusion, better parameters regarding the handling time, the higher sensitivity, and the possibility to use the Simplexa platform for other tests from Focus Diagnostics, recommend the kit for large laboratories with high sample numbers; the good performances of the AmpliVue can also be taken into consideration, when rapid and sensitive methods must be used for the *C. difficile* infection diagnosis [109].

6. Biological molecular methods for the characterization of *C. difficile* multidrug-resistant (MDR) strains

The antibiotic resistance explains CDI and changes the therapeutic scheme of these cases. New patterns of *C. difficile* strains encourage the spread and persistence of these strains in healthcare facility settings; for this reason, the surveillance of resistance and the molecular characterization of these mechanisms are very important in CDI control and prevention [111]. Both conjunction [112] and translocation of the genetic mobile element CTn5 [113] were described. The genetic mechanisms were much better understood and easier to study after 630 strain genome sequences [114] and resequencing studies. In a recent study, van Eijk et al. [114] analyzed the *C. difficile* laboratory strain 630Δerm and demonstrated, for the first time, the analysis of major methylation patterns for any *C. difficile* strain. In this research, the authors revealed that in addition to insertions, deletions, and SNPs, the CTn5 element moved

from its original location within the CD1844 to the *rumA* gene, in its isolate. This molecular genetic study highlighted that the major rearrangement has important implications for the redistribution of strains with highly mobile genomes and that, even though it is not related to direct studies regarding the antibiotic resistance of *C. difficile*, it argues for the complete re-sequencing of common lab strains in each laboratory [113].

The complete genome sequences of *C. difficile* have made it possible to study the mobile genetic elements (transposons or transposable elements). This analysis describes that about 11% of the *C. difficile* genome consists of mobile genetic elements, the majority being conjugative transposons; their presence is at the origin of virulence, antibiotic resistance, synthesis of different surface protein, and microorganism-host regulation-binding capacities [114]. The horizontal genetic transfer of these transferable elements between species or between strains of *C. difficile* is the main cause of such successful spreading, especially in healthcare facilities, as well as within the community.

The interest in such studies started with the worldwide spread of the highly virulent RT027 and of other ribotypes, which explain the CDI treatment failure. Generally speaking, the resistance phenomena are the consequences of antibiotic treatment, especially clindamycin (CLI), cephalosporins (CFS), and fluoroquinolones (FQs), even though all antibiotics are theoretically capable to be the cause of CDI, in parallel with other risk factors (previously listed). The first antibiotics associated with CDI was CLI, a large broad spectrum antibiotics, with bacteriostatic activity, for which *C. difficile* demonstrated a high resistance level (more than 90%) [60]. The relative risk (RRs) for *C. difficile* acquisition is 9.0 for CLI, varying in large limits for CFs (from 7.8 for cefaclor, to 36.2 for cefotaxime), with lower risks for β-lactams (2.0 for penicillin, to 22.1 for ampicillin and amoxicillin-clavulanic acid association) [5]. Even though the FQs have been used since 1988, their implication in CDI was described in the last 10 years (Sunenschine et al., cited by Bartlett and Gerding [5]). The most studied strains that belong to the RT027 demonstrated a high-level resistance to the FQs [7]. An excellent synthesis regarding the *C. difficile* antibiotic resistance and associated RTs was recently published by Spigaglia, in 2016 [61]. Although for the antibiotic susceptibility studies, the most frequent methods are the agar dilution (AD) and the epsilometer test (Etest), ribotyping is a supportive method that classified the strains, to connect infection/microorganism's source/sources with secondary cases, in order to establish and implement the most appropriate prevention methods. Other methods can be used to study the genetic mechanisms of antibiotic resistance and their genetic horizontal transfer (by conjunction, or conjugative-like processes), or with the support of bacteriophages in transduction, including mobile genetic elements, as support for pathogenicity, antibiotic resistance, and environmental survival capacity [116–118]. Recent protocols were tested for the genetic manipulation of *C. difficile* genome and future researches will be able to continue this new approach [119].

The rate of antibiotic resistance varied between different studies depending, most probably, on the antibiotics policy and the spread of different *C. difficile* strains, in various regions. A large synthesis of 30 studies dating from 2012 to 2015 [115] revealed that resistance to CLI and CFs is higher than 50% (55 and 51%, respectively), with a similar percent for ERY and FQs (47%). One of the most used classes of antibiotics, the cephalosporins (CFs), is the source of many cases of CDI; the new CFs, with a wider antibiotic spectrum and more recently introduced in therapy, will induce the resistance phenomenon after several years of use. Thus,

the second-generation cephalosporin (cefotetan/CTT and cefoxitin/FOX) level of resistance is about 80% and, for the third generation, recently introduced to the market (e.g., ceftriaxone/CRO and cefotaxime/CTX), the level of resistance has not yet reached 40% [115]. Although the phenomenon was highlighted by numerous strains resistant to CFs, the resistance mechanism is not completely understood. The strains belonging to different RTs (027 and to others ribotypes as it follows: 001, NAP1, 2, 4, and 6 [identified by PFGE], 002, 014, 017, 018, 053, 078, etc.) and *C. difficile* seem to be "constitutively resistant" to CFs, even though the main cases of CDI were described after CFs therapy, having different minimum inhibitory concentrations (MICs) to different CFs. These different MIC values are probably due to drug-resistant strains. The WGS 630 strain analysis shows 25 coding sequence (CSs); their presence is not constant in all strains, the percentage of identity ranging between 73 and 100% [115].

Not infrequently, the growing alarming statistic indicators (incidence, prevalence, etc.) are the starting point of some research that explains the epidemiological phenomenon using molecular biology techniques. Looking to CDI incidence dynamics, Clabots et al. explained the increased incidence of CDI cases in 1985, at the Minneapolis Veterans Administration Medical center (from 7.1 to 17.3/month), by highlighting a cryptic plasmid of 3.1 kilobases (kb), present exclusively in clindamycin-resistant strains and absent in sensitive isolates. The identification of this plasmid was carried out by restricting endonuclease digestion and Southern blot hybridization, but the authors failed in their purpose to identify the source of these strains and plasmid mechanism acquisition [120].

A large pan-European longitudinal surveillance of antibiotic resistance, conducted by Freeman J, et al. [122] enrolled around 1000 strains from 22 countries and 39 sites. Nine hundred and fifty-three strains were tested for PCR ribotyping, toxins, and antibiotic susceptibility for metronidazole (MTZ), vancomycin (VAN), fidaxomicin (FDX), rifampicin (RIF), moxifloxacin (MOX), clindamycin (CLI), imipenem (IM), chloramphenicol (CH), and tigecycline (TIG). CL, MOX, and RIF resistance levels varied (50, 40, and 13%, respectively) and were evident in many RTs. The most frequent RTs registered were 027 (12%), 001/072 (9%), 078, and 014 (8% each). MTZ, VAN, and TG were active in almost all cases, with a very low resistance level (2.18, 3.16, and 0.44%, respectively). One strain from UK (belonging to RT106) had a MIC for MTZ, 8 mg/L, and 20 strains demonstrated a reduced MTZ susceptibility, while 11 of them were RT 027. The reduced VAN susceptibility was very rare; four countries (the Czech Republic, Ireland, Latvia, and Poland) sent strains with MIC of 4 mg/L, Italy and Spain submitted different RTs with VAN reduce susceptibility, including resistant strains, belonging to RTs 027, 126, 356, and 001/007 [121]. The highest MIC values for VAN were determined for Rt 018 and 356.

Because the rates of treatment failure and recurrences have increased after MTZ and VAN cure, their replacement with different antibiotics was suggested. Rifamycins, like rifaximin (RFX) and fidaxomicin (FDX), a new bactericidal narrow spectrum macrocyclic antibiotic can be alternative therapies. Using antibiotic susceptibility test interpretative criteria, scientists proposed the use of rifampin (RIF) as an antibiotic related to RFX. The extrapolated literature data (from 2008 to 2012), from six relevant studies [115], show that 11% of *C. difficile* strains isolated from different clinical cases demonstrated resistance to RIF, mainly because this antibiotic is the drug of choice for tuberculosis treatment in many countries. All strains from Italy belonged to RT046, and the stud-

ied strains were isolated from TB patients with long RIF treatment [115]. In a study from Poland, from 2014, that characterized the pattern of susceptibility and ribotype association [121] for 83 strains, the majority belonged to RT027 (57.8%), 25.3% to RT176, and 16.9% to others. The majority of strains (85.5%) were resistant to erythromycin (ERY), more than a quarter percent (27.7%), resistant to CLI, with high MIC (greater than 256 mg/L). A high percent (83.1%) from all 83 strains was resistant to MOX, 87.9% resistant to imipenem (IMP), and only 2.4% to tetracycline. MTZ and VAN seem to continue to be efficient, having a low MIC_{90} value (0.75 mg/L) for both antibiotics. The most resistant strains belonged to RT027 and 176, with a MIC for ERT higher than 256 mg/L; the majority (95.2%) of RT176 strains were co-resistant to ERY and CLI. All strains resistant to ERY, MOX, and RIF belong to RT027 (18% strains respectively), and MDR strains (defined as strains resistant to at least three classes of antibiotics) were established for 71 strains (85.5%) [119] a percent that is an alarming phenomenon.

It is obvious that all articles from previous years that have studied the resistance patterns of *C. difficile* strains from clinical samples, animal, and environmental sources [108, 109, 111, 112, 120–131] from Europe, North America, and South-East Asia were designated not only to describe these patterns and their dynamics but also to classify them as RTs, to have a complete description of spread and risk for CDI, for higher virulent and MDR strains, too. Different studies have tried to describe, by different biological molecular methods, the *C. difficile* strain resistance mechanisms for cephalosporins, macrolide-lincosamide-streptogramin B (MLS_B) family, fluoroquinolones, including for antibiotics useful in the treatment of CDI t (e.g., metronidazole, vancomycin, rifamycins, and fidaxomicin); excellent syntheses regarding these mechanisms were recently published [61]. Having such a vast view on the evolution of antibiotic resistance to classical and new antibiotics, new strategies to limit this phenomenon could be designed and implemented to reduce hospitalization days, healthcare costs, and, not least, the emergence of new cases, potentially fatal.

7. Comparison of molecular biological methods

Nowadays, the revolution in the biological molecular field has generated a long list of methods and commercial kits, all these being found in numerous studies. Patient studies on different risk groups, reviews of literature, including meta-analyses and book chapters have been published in recent years, regarding the new phenomenon, CDI. The increasing incidence, the morbidity, and mortality by CDI in the world stimulate scientists to find the cause and solutions. Starting from years ago and even today, EIA and GDH for toxins and antigen detection were largely used but the lack of sensitivity of these methods imposed the search for new solutions. In the last 30 years, the PCR-based methods have found numerous practical applications for diagnosis, disease surveillance and, last but not least, for the study of antibiotic resistance, including for the highly virulent *C. difficile* strains. The main criteria to reassess variants of molecular biology tests are discriminatory power, reproducibility, technical difficulty, time needed to perform them, their cost-benefit ratio, the ease of interpreting the results, and data inter-comparison between laboratories [24]. Starting from such criteria, Dingle and MacCallenn established that the restriction fragment techniques (REA, PFGE, and toxinotyping) have very

good reproducibility and discriminatory capacities, with less power for the toxinotyping, for the second criteria [33]. PFGE, which needs a longer time, is more expensive and it is designated for laboratory expertise because of the difficulties in inter-laboratory comparison [13, 33].

For years, different studies have concluded that REA and PGFE present a comparable discriminatory capacity, useful in epidemiological large studies regarding *C. difficile* typing isolates and that ribotyping has a less discriminatory capacity [33].

Second major laboratory methods are based on PCR amplification (ribotyping, REP-PCR, MLVA, and AP-PCR). From this list, ribotyping seems to fulfill all criteria and, close to it, MLVA, even though it needs a longer time [33].

In the future, sequence-based methods (MLST, slpA, TRST, and WGS) will probably become the key to answer difficult questions, such as strains trace, outbreaks sources, and so on, even though, currently, these are mainly dedicated to research laboratories since they are characterized by high technicity, high cost, and time, as well as by difficulties relating to the inter-comparison between laboratories. One question in the daily activity has to do with the selection of the appropriate genotyping method. To answer it, it is necessary to list their reference characteristics such as validity (to be applicable to all studied strains), discriminatory capacity (to be able to make the difference between unrelated strains), and reproducibility (this must be applicable between and within laboratories). Other criteria are related to difficulties and the step number of the method, rapidity, and to the cost-efficiency ratio. With this choice, we must take into consideration the purpose of genotyping: in the rapid tracking of local outbreaks, MLVA seems to be the most useful method and, in long epidemiological studies, MLST, PFGE, and WGS [35].

WGS will soon be the mandatory method to carry out quality multicenter studies, as ECDC has proposed minimum four major infections as follows: carbapeneme- resistant *Enterobacteriaceae*, *Neisseria gonorrhoeae, C. difficile,* and MRSA (methicillin-resistant *S. aureus*). According to the ECDC's experts, WGS "will improve the accuracy and effectiveness of disease surveillance, outbreak investigation and evaluation of prevention policies by enhanced assessment of disease and drug resistance transmission dynamics" [130]. Even though WGS can offer data (e.g., "in silico" design microrestriction profile for enzymes used in different techniques) for sequence-based and nonsequence-based genotyping methods, it remains a cumbersome test, expensive and dedicated only to high expertise laboratories [35].

8. Comparison between classical and modern methods

After the FDA has approved the standardized methods/tests, many authors have tried to find answers to different questions: Are the methods of molecular biology a better variant and more cost-effective than the phenotypic methods? In many countries, even with a good technical infrastructure and highly qualified personnel, EIA and GDH are the most useful methods for rapid CDI diagnosis. These tests and others, based on the phenotypic characterization (e.g., slide agglutination for serotyping, antibiotic susceptibility pattern), have some disadvantages such as a low reproducibility and the incapacity to differentiate between large

numbers of isolates. Different study groups underline that EIA for A and B toxin detection has a low sensitivity and poor specificity [22, 131]. As we know, the efficiency of the test depends on the capacity of test itself to trace the smallest amounts of antigens in samples with higher specificity (these are dependent on the antibody clonality, on the binding power to EIA support, etc.) and on the difference in strain circulation in different geographical areas. For some EIA kits, sensitivity was about 15% for some ribotypes [127]. One very useful test is DGH that, in comparison with the toxigenic culture, showed a sensitivity similar to the real-time PCR for RT027, but the lowest sensitivity for non-027 infections; the sensitivity of GDH determination may be 70%, in comparison with the Gene-Xepert Assay [127]. GDH positive results must be confirmed with a different method (e.g., culture cytotoxicity assays, EIA, or NAATs) because this antigen can provide such results for both toxigenic and nontoxigenic strains.

Different authors, including professional society experts, recommend the two-step diagnosis, having GDH as a screening test and a follow-up test to confirm toxin presence. A recent meta-analysis (Shetty et al., cited by Carroll and Loeffelholz [23]) has concluded that GHD is highly sensitive, with high negative predictive value and that it has conducted to the best results in the two-step algorithms. To avoid this second test for toxin detection, a new immunochromatographic test (ICT) was proposed for both simultaneous detections. This ITC variant (Cdiff Quik Chek Complete; TechLab, Blacksburg, VA) has a good sensitivity for GDH, but a less good one for toxin detection (from 61 to 78%) and, for this reason, different laboratories use it in three-step diagnosis algorithm: GDH positive sample and toxin negative need to be retested by one NAAT.

On the other hand, different expert groups from Europe and USA [22, 131] strongly recommend replacing EIA with more sensitive assays, in order to quickly identify the *C. difficile* infection (or colonization) and to rapidly implement the appropriate preventive measures. Caroll and Loeffelholtz [23] support this approach having as arguments the final cost, which is the lowest for laboratories that use more sensitive tests, and the reduced healthcare associated infection, as consequence of rapid and efficient preventive methods.

Nowadays, in many laboratories, EIA and PCR-based methods used for *C. difficile* diagnosis replaced cultivation—the best way to study antibiotic susceptibility from pure culture—as source of future studies, including sequence analysis, but the toxigenic culture remains impractical, in relation to the time needed for end results.

As we have already briefly presented, the FDA has approved many NAATs, and different studies have concluded that such platforms have the highest sensitivity and that results are comparable between them. Contrary to this statement, we must note that the NAATs can detect conserved regions of A and/or B toxin genes, and not the toxin itself. For this reason, these tests cannot differentiate between infection and colonization and, moreover, they require complex infrastructure and highly qualified staff, both of which are more expensive, and that future researches are necessary for a clear conclusion regarding their utility for diagnosing the infection in children [23].

Even though the laboratory methods applicable for the identification of *C. difficile* are diverse, their various disadvantages have imposed the use of new techniques, including strain identification by MALDI-TOF (matrix-assisted laser desorption/ionization-time-flight mass

spectrometry) [13, 132–135]. The method has been recently introduced in the clinical laboratory practice to identify the bacteria and to describe the resistance profile to antibiotics [13]. MALDI-TOF is based on the identification of some molecules, on calculating flight time in a vacuum tube, after the first sample was co-crystallized on a matrix and irradiated with laser. Flight duration allows the calculation of the weight/load ratio for the detected ions, generating a spectrum that will be compared with a database, to identify the bacterium in the sample. Whole bacteria, as well as brute extracts, may be used as samples [132, 133].

Identification is based on comparing the profile of some unique proteins present in the bacterial wall, thus becoming specific biomarkers, useful including in strain typing. Initially, Reil et al. have used the method to identify a limited number of ribotypes, including the highly virulent and the multidrug-resistant one (027 ribotype, respectively), based on the molecular weight of the selected proteins (between 2 and 20 kDa). Subsequently, Razarrdi and Akerlund have improved the method by selecting an initial wider range for the molecular weight of the selected proteins from 30 to 50 kDA followed by an analysis within the range used in the initial experiments (2–20 kDa), using ribotyping as a reference test by PCR. This way, for epidemiological purposes, typing *C. difficile* strains based on high molecular weight (HMW) might be combined with PCR ribotyping. In addition, by analyzing some surface proteins, involved in the attachment to specific receptors, MALDI-TOF would provide information related to the virulence of the infecting strain, including the immune response toward them and diseases severity [133].

By using MALDI-TOF, after obtaining the culture on chromogenic media, the diagnosis is more sensitive (from 94.7 to 100%) and more specific (from 79.7 to 100%), thus with significant statistical differences ($p < 0.001$). Furthermore, the technique allows the exclusion of false-positive results obtained by using the chromogenic media, hence the role of MALDI-TOF as confirmatory test. The rapid detection (10 min) and the very low cost make the test more useful as an identification test, compared with molecular biology methods (e.g., 16S rRNA gene sequencing), including the control of nosocomial infections associated with antibiotics therapy [134, 135].

To draw a general conclusion, we can state that the best algorithm starts with GDH as a screening test, a confirmation of toxin presence using a highly sensitive test, ideally a NAATs variant, bearing in mind that GDH efficiency is related to the strain types from different geographical areas [13, 20, 23].

9. Future directions

The prevention of CDI involves many factors, starting with the correct diagnosis, but we should also think about prevention (contact/isolation), antimicrobial stewardship, and fecal bacteriotherapy [136].

In a recent report of the ECDC, the importance of defining CDI, recurrent CDI cases, and CDI case origin were mentioned. Also, ESCMID recommended the following possible algorithms for the CDI diagnosis:

- Screening with NAAT, confirmation with toxin A/B EIA
- Screening with both GDH and A/B EIA toxin, optional confirmation with NAAT or toxigenic culture
- Screening with GDH EIA, confirmation with A/B EIA toxin, optional second confirmation with NAAT or toxigenic culture [137].

In recent years, quick methods—such as immunochromatography, which require less than 30 min, thus being useful as a screening test—have been proposed for the diagnosis of CDI; however, future clinical studies are necessary. New technologies—such as multicapillary column gas chromatography, a quick method but with sensitivity and specificity values less than 90%, a fact that requires the improvement of the method to increase accuracy—have been proposed. Another proposal aims at associating the use of selective media with the Fluorescent In Situ Hybridization (FISH) test, which, during the same day, can identify the strain by typing, determine the resistance profile, and detect toxins, therefore, having a higher clinical relevance, compared to other methods [135].

Like in other clinical situations, in which the diagnosis methods have switched to automatization, there is also a need for standardization of *C. difficile* detection. The clinical validation of the assays on many samples, to easily compare and to rely on the results from different countries, is very useful. The surveillance protocol established by the ECDC will improve the case management and the preventive measures [137–139].

Author details

Luminiţa Smaranda Iancu*, Andrei Florin Cârlan and Ramona Gabriela Ursu

*Address all correspondence to: luminita.iancu@umfiasi.ro

"Grigore T. Popa" University of Medicine and Pharmacy Iaşi, Iaşi, Romania

References

[1] Guide to Preventing *Clostridium difficile* Infections. Association for Professionals in Infection Control and Epidemiology USA. 2013. DOI: 1-933013-54-0. http://apic.org/Resource_/EliminationGuideForm/59397fc6-3f90-43d1-9325-e8be75d86888/File/2013CDiffFinal.pdf

[2] Davies KA, Longshaw CM, Davis GL, Bouza E, Barbut F, Barna Z, Delmee M, Fitzpatrick F, Ivanova K, Kuijper EJ, Macovei IS, Mentula S, Mastrantonio P, Von Muller L, Oleastro M, Petinaki E, Pituch H, Noren T, Novakova E, Nyč O, Rupnik M, Schmid D, Wilcox MH. Underdiagnosis of *Clostridium difficile* across Europe: The European, multicentre, prospective, biannual, point-prevalence study of *Clostridium difficile* infection in hospitalised patients with diarrhoea (EUCLID). Lancet Infectious Diseases. 2014;**14**:1208-1219. DOI: 10.1016/S1473-3099(14)70991-0

[3] Popescu GA, Szekely E, Codiţă I, Tălăpan D, Şerban R, Ruja G. Ghid de diagnostic, tratament şi prevenire a infecţiilor determinate de *Clostridium difficile*. 2016. DOI: 978-973-0-022285-2http://www.cnscbt.ro/index.php/ghiduri-si-protocoale/520-ghid-diagnostic-tratament-si-prevenire-clostridium-difficile/file

[4] Cheng JW, Xiao M, Kudinha T, Xu ZP, Sun LY, Hou X, Zhang L, Fan X, Kong F, Xu YC. The role of glutamate dehydrogenase (GDH) testing assay in the diagnosis of *Clostridium difficile* infections: A high sensitive screening test and an essential step in the proposed laboratory diagnosis workflow for developing countries like China. Plos one 10(12): e0144604. https://doi.org/10.1371/ journal. pone.0144604

[5] Bartlett JG, Gerding DN. Clinical recognition and diagnosis of *Clostridium difficile* infection. Clinical Infectious Diseases. 2008;**46**:S12–S18. DOI: https://doi.org/10.1086/521863

[6] Carter GP, Rood JI, Lyras D. The role of toxin A and toxin B in *Clostridium difficile*—Associated disease. Past and present perspective. Gut Microbes. 2010;**1**(1):58-64. DOI: 10.1038/nature07822

[7] McDonald LC, Killgore GE, Thompson A, Qwens RC, Kazakova SV, Sambol SP, Kohnson S, Gerding DA. An epidemic, toxin gene-variant strain of *Clostridium difficile*. The New England Journal of Medicine. 2005;**353**:2433-2441. DOI: 10.1056/NEJMoa051590

[8] Khan FY, Elzouki AN. *Clostridium difficile* infection: A review of the literature. Asian Pacific Journal of Tropical Medicine. 2014;**7**:6-13. DOI: 10.1016/S1995-7645(14)60197-8

[9] Daniel A, Rapose A. The evaluation of *Clostridium difficile* infection (CDI) in a community hospital. The Journal of Infection and Public Health. 2015;**8**:155-160. DOI: 10.1016/j.jiph.2014.08.002

[10] Center for Diseases Control and Prevention (CDC). Antibiotic Resistance. Threats in the United States. 2013, Atlanta 2013. Microorganisms with a Threat Level of Urgent. Clostridium difficile. p 51. https://www.cdc.gov/drugresistance/pdf/ar-threats-2013-508.pdf

[11] Collins DA, Elliott B, Riley TV. Molecular methods for detecting and typing of *Clostridium difficile*. Pathology. 2015;**47**:211-218. DOI: 10.1097/PAT.0000000000000238

[12] Goudarzi M, Seyedjavadi SS, Goudarzi H, Aghdam EM, Nazeri S. *Clostridium difficile* infection: Epidemiology, pathogenesis, risk factors and therapeutic options. Scientifica. 2014;**2014**:916826. DOI: 10.1155/2014/916826

[13] Burnham CA, Carroll KC. Diagnosis of *Clostridium difficile* infection: An ongoing conundrum for clinicians and for clinical laboratories. Clinical Microbiology Reviews. 2013;**26**(3):604-630. DOI: 10.1128/CMR.00016-13

[14] Karanika S, Paudel S, Zervou FN, Grigoras C, Zacharioudakis IM, Mylonakis E. Prevalence and clinical outcomes of *Clostridium difficile* infection in the intensive care unit: A systematic review and meta-analysis. Open Forum Infectious Diseases. 2015;**3**(1):ofv186. DOI: 10.1093/ofid/ofv186. eCollection 2016

[15] Kyne L, Hamel MB, Polavaram R, Kelly CP. Health care costs and mortality associated with nosocomial diarrhea due to *Clostridium difficile*. Clinical Infectious Diseases. 2002;**34**(3):346-353

[16] Schroeder LF, Robilotti E, Peterson LR, Banaei N, Dowdyd DW. Economic evaluation of laboratory testing strategies for hospital-associated *Clostridium difficile* infection. Journal of Clinical Microbiology. 2014;**52**(2):489-496. DOI: 10.1128/JCM.02777-13

[17] Akerlund T, Alefjord I, Dohnhammar U, Struew I, Norén T, Tegmark-Wisell K, The Swedish Study Group. Geographical clustering of cases of infection with moxifloxacin-resistant *Clostridium difficile* PCR ribotypes *012*, *017* and *046* in Sweden, 2008 and 2009. Euro Surveillance. 2011;**16**(10): 1-7. http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19813

[18] Khanafer N, Barbut F, Eckert C, Perraud M, Demont C, Luxemburger C, Vanhems P. Factors predictive of severe *Clostridium difficile* infection depend on the definition used. Anaerobe. 2016;**37**:43-48. DOI: 10.1016/j.anaerobe.2015.08.002

[19] Korman TM. Diagnosis and management of *Clostridium difficile* infection. Seminars in Respiratory and Critical Care Medicine 2015;**36**(01):031-043. DOI: 10.1055/s-0034-1398741

[20] Tenover FC, Baron EJ, Peterson LR, Persing DH. Laboratory diagnosis of *Clostridium difficile* infection. The Journal of Molecular Diagnostics. 2011;**13**(6):573-582. DOI: 10.1016/j.jmoldx.2011.06.001

[21] Larson AM, Fung AM, Fang FC. Evaluation of *tcdB* real-time PCR in a three step diagnostic algorithm for detection of toxigenic *Clostridium difficile.* Journal of Clinical Microbiology. 2010;**48**(1):124-130. DOI: 10.1128/JCM.00734-09

[22] Cohen SH, Gerding DN, Johnson S, Kelly CP, Loo VG, McDonald LC, Pepin J, Wilcox MH. Clinical practice guidelines for *Clostridium difficile* infection in adults: 2010 Update by the Society for Healthcare Epidemiology of America (SHEA) and the Infectious Diseases Society of America (IDSA). Infection Control and Hospital Epidemiology. 2010;**31**(5):431-455. DOI: 10.1086/651706

[23] Carroll KC, Loeffelholz M. Conventional versus molecular methods for the detection of *Clostridium difficile*. Journal of Clinical Microbiology. 2011;**49**:49-52. DOI: 10.1128/JCM.00700-11

[24] Dingle TC, MacCanell DR. Molecular strain typing and characterization of toxigenic *Clostridium difficile*, In: Sails A, Tang Y-W, editors. Current and Emerging Technologies for the Diagnosis of Microbial Infections. 2015;**42:**329-357. Methods in Microbiology. Elsevier, ISSN: 0580-9517. DOI: 10.1016/bs.mim.2015.07.001

[25] Lemann F, Chambon C, Barbut F, Gardin C, Brière J, Lamber-Zechovsky N, Branger C. Arbitrary primed PCR rules out *Clostridium difficile* cross-infection among patients in a haematology unit. The Journal of Hospital Infection. 1997;**35**(2):107-115

[26] Arbitrarily primed polymerase chain reaction method for fingerprinting genomes. United States Patent 6696277. Available from: http://www.freepatents online.com/6696277.html

[27] Martirosian G, Kuipers S, Verbrugh H, Van Belkum A, Meisel-Mikolajczyk F. PCR ribotyping and arbitrarily primed PCR for typing strains of *Clostridium difficile* from a Polish Maternity Hospital. Journal of Clinical Microbiology. 1995;**33**(8):2016-2021

[28] Sabat AJ, Budimir A, Nashev D, Sá-Leão R, Van Dijl JM, Laurent F, Grundmann H, Friedrich AW, on behalf of the ESCMID Study Group of Epidemiological Markers (ESGEM). Overview of molecular typing methods for outbreak detection and epidemiological surveillance. Euro Surveillance. 2013;**18**(4):20380

[29] Gürtler V, Grando D. Reprint of new opportunities for improved ribotyping of *C. difficile* clinical isolates by exploring their genomes. The Journal of Microbiological Methods. 2013;**95**(3):425-440. DOI: 10.1016/j.mimet.2013.09.009

[30] Wren B, Tabaqchali S. Restriction endonuclease DNA analysis of *Clostridium difficile*. Journal of Clinical Microbiology. 1987;**25**(12):2402-2404

[31] Clabots CR, Johnson S, Bettin KM, Mathie PA, Mulligan ME, Schaberg DR, Peterson LR, Gerding DN. Development of a rapid and efficient restriction endonuclease analysis typing system for *Clostridium difficile* and correlation with other typing systems. Journal of Clinical Microbiology. 1993;**31**(7):1870-1875

[32] Killgore G, Thompson A, Johnson S, Brazier J, Kuijper E, Pepin J, Frost EH, Savelkoul P, Nicholson B, Van den Berg RJ, Kato H, Sambol SP, Zukowski W, Woods C, Limbago B, Gerding DN, McDonald LC. 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):431-437

[33] Kristjánsson M, Samore MH, Gerding DA, DeGirolami PC, Bettin KM, Karchmer AW, Arbeit RD. Comparison of restriction endonuclease analysis, ribotyping, and pulsed-field gel electrophoresis for molecular differentiation of *Clostridium difficile* strains. Journal of Clinical Microbiology. 1994;**32**(8):1963-1969. DOI: 0095-1137/94/$04.00+0

[34] Alonso R, Martin A, Pelaez T, Marin M, Rodriguez-Creixems M, Bouza E. An improved protocol for pulsed-field gel electrophoresis typing of *Clostridium difficile*. Journal of Medical Microbiology. 2005;**54**(Pt 2):155-157

[35] Li W, Raoult D, Fournier PE. Bacterial strain typing in the genomic era. FEMS Microbiology Ecology. 2009;**33**:892-916. DOI: 10.111/j.1574-6976.2009.00182.x

[36] Bidet P, Lalande V, Salauze B, Burghoffer B, Avesani V, Delmée M, Rossier A, Barbut F, Petit JC. Comparison of PCR-ribotyping, arbitrarily primed PCR, and pulsed-field gel electrophoresis for typing *Clostridium difficile*. Journal of Clinical Microbiology. 2000;**38**(7):2484-2487

[37] Pasanen T, Kotila SM, Horsma J, Virolainen A, Jalava J, Ibrahem S, Antikainen J, Mero S, Tarkka E, Vaara M, Tissari P. Comparison of repetitive extragenic palindromic sequence-based PCR with PCR ribotyping and pulsed-field gel electrophoresis in studying the clonality of *Clostridium difficile*. Clinical Microbiology and Infection. 2011;**17**(2):166-175. DOI: 10.1111/j.1469-0691.2010.03221.x

[38] McCarthy J, Stingemore N. *Clostridium difficile* infection of a prosthetic joint presenting 12 months after antibiotic-associated diarrhoea. Journal of Infection. 1999;**39**(1):94-96

[39] Clayton EM, Rea MC, Shanahan F, Quigley EM, Kiely B, Hill C, Ross RP. The vexed relationship between *Clostridium difficile* and inflammatory bowel disease: An assessment of carriage in an outpatient setting among patients in remission. American Journal of Gastroenterology. 2009;**104**(5):1162-1169. DOI: 10.1038/ajg.2009.4

[40] Hemminger J, Balada-Llasat JM, Raczkowski M, Buckosh M, Pancholi P. Two case reports of *Clostridium difficile* bacteremia, one with the epidemic NAP-1 strain. Infection. 2011;**39**(4):371-373. DOI: 10.1007/s15010-011-0115-7.

[41] 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. 1996;**33**(4):220-223

[42] Oka K, Osaki T, Hanawa T, Kurata S, Okazaki M, Manzoku T, Takahashi M, Tanaka M, Taguchi H, Watanabe T, Inamatsu T, Kamiya S. Molecular and microbiological characterization of *Clostridium difficile* isolates from single, relapse, and reinfection cases. Journal of Clinical Microbiology. 2012;**50**(3):915-921. DOI: 10.1128/JCM.05588-11

[43] Loo VG, Bourgault AM, Poirier L, Lamothe F, Michaud S, Turgeon N, Toye B, Beaudoin A, Frost EH, Gilca R, Brassard P, Dendukuri N, Béliveau C, Oughton M, Brukner I, Dascal A. Host and pathogen factors for *Clostridium difficile* infection and colonization. New England Journal of Medicine. 2011;**365**(18):1693-1703. DOI: 10.1056/NEJMoa1012413

[44] Kuijper EJ, Coignard B, Tüll P; ESCMID Study Group for *Clostridium difficile*; EU Member States; European Centre for Disease Prevention and Control. Emergence of *Clostridium difficile*-associated disease in North America and Europe. Clinical Microbiology and Infection. 2006;**12**(Suppl 6):2-18

[45] Balassiano IT, Miranda KR, Boente RF, Pauer H, Oliveira IC, Santos-Filho J, Amorim EL, Caniné GA, Souza CF, Gomes MZ, Ferreira EO, Brazier JS, Domingues RM. Characterization of *Clostridium difficile* strains isolated from immunosuppressed inpatients in a hospital in Rio de Janeiro, Brazil. Anaerobe. 2009;**15**(3):61-64. DOI: 10.1016/j.anaerobe.2008.12.007

[46] Kim H, Lee Y, Moon HW, Lim CS, Lee K, Chong Y. Emergence of *Clostridium difficile* ribotype *027* in Korea. Korean Journal of Laboratory Medicine. 2011 July;**31**(3):191-196. DOI: 10.3343/kjlm.2011.31.3.191

[47] Baldan R, Trovato A, Bianchini V, Biancardi A, Cichero P, Mazzotti M, Nizzero P, Moro M, Ossi C, Scarpellini P, Cirillo DM. *Clostridium difficile* PCR ribotype *018*, a successful epidemic genotype. Journal of Clinical Microbiology. 2015;**53**(8):2575-2580. DOI: 10.1128/JCM.00533-15

[48] Cheng VC, Yam WC, Chan JF, To KK, Ho PL, Yuen KY. *Clostridium difficile* ribotype *027* arrives in Hong Kong. The International Journal of Antimicrobial Agents. 2009;**34**(5):492-493. DOI: 10.1016/j.ijantimicag.2009.04.004

[49] Aguayo C, Flores R, Lévesque S, Araya P, Ulloa S, Hormazabal JC, Tognarelli J, Ibáñez D, Pidal P, Duery O, Olivares B, Fernández J. Rapid spread of *Clostridium difficile*

NAP1/027/ST1 in Chile confirms the emergence of the epidemic strain in Latin America. Epidemiology and Infection. 2015;**143**(14):3069-3073. DOI: 10.1017/S0950268815000023

[50] Popescu GA, Florea D, Rafila A. *Clostridium difficile* is emerging in Romania: A story of *027* ribotype and excessive antibiotic consumption. Journal of Gastrointestinal and Liver Diseases. 2014;**23**(3):342-343

[51] Sirard S, Valiquette L, Fortier LC. Lack of association between clinical outcome of *Clostridium difficile* infections, strain type, and virulence-associated phenotypes. Journal of Clinical Microbiology. 2011;**49**(12):4040-4046. DOI: 10.1128/JCM.05053-11

[52] 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):461-463

[53] Westblade LF, Chamberland RR, MacCannell D, Collins R, Dubberke ER, Dunne WM, Jr, Burnham CA. Development and evaluation of a novel, semiautomated *Clostridium difficile* typing platform. Journal of Clinical Microbiology. 2013;**51**(2):621-624. DOI: 10.1128/JCM.02627-12

[54] Indra A, Huhulescu S, Schneeweis M, Hasenberger P, Kernbichler S, Fiedler A, Wewalka G, Allerberger F, Kuijper EJ. Characterization of *Clostridium difficile* isolates using capillary gel electrophoresis-based PCR ribotyping. Journal of Medical Microbiology. 2008;**57**(Pt 11):1377-1382. DOI: 10.1099/jmm.0.47714-0

[55] Knetsch CW, Lawley TD, Hensgens MP, Corver J, Wilcox MW, Kuijper EJ. Current application and future perspectives of molecular typing methods to study *Clostridium difficile* infections. Euro Surveillance. 2013;**24**(4):20381

[56] European *Clostridium difficile* Infection Study Network (ECDISNET). Europe: Supporting capacity building for surveillance of *Clostridium difficile*. Leiden: ECDIS-NET.Version 1.2., 2013. Available from: http://www.ecdisnet.eu/ [Accessed 16 January 2017]

[57] Barbanti F, Spigaglia P. Characterization of *Clostridium difficile* PCR-ribotype *018*: A problematic emerging type. Anaerobe. 2016;**42**:123-129. DOI: 10.1016/j.anaerobe.2016.10.003

[58] Polivkova S, Krutova M, Petrlova K, Benes J, Nyc O. *Clostridium difficile* ribotype *176*—A predictor for high mortality and risk of nosocomial spread? Anaerobe. 2016;**40**:35-40. DOI: 10.1016/j.anaerobe.2016.05.002

[59] Cheng JW, Xiao M, Kudinha T, Xu ZP, Hou X, Sun LY, Zhang L, Fan X, Kong F, Xu YC. The first two *Clostridium difficile* ribotype *027/ST1* isolates identified in Beijing, China—An emerging problem or a neglected threat? Scientific Reports. 2016;**6**:18834. DOI: 10.1038/srep18834

[60] Drabek J, Nyc O, Krutova M, Stovicek J, Matejkova J, Keil R. Clinical features and characteristics of *Clostridium difficile* PCR-ribotype *176* infection: Results from a 1-year university hospital internal ward study. Annals of Clinical Microbiology and Antimicrobials. 2015;**14**:55. DOI: 10.1186/s12941-015-0114-0

[61] Spigaglia P, Barbanti F, Morandi M, Moro ML, Mastrantonio P. Diagnostic testing for *Clostridium difficile* in Italian microbiological laboratories. Anaerobe. 2016;**37**:29-33. DOI: 10.1016/j.anaerobe.2015.11.002

[62] Al-Thani AA, Hamdi WS, Al-Ansari NA, Doiphode SH, Wilson GJ. Polymerase chain reaction ribotyping of *Clostridium difficile* isolates in Qatar: A hospital-based study. BMC Infectious Diseases. 2014;**14**:502. DOI: 10.1186/1471-2334-14-502

[63] Lachowicz D, Szulencka G, Obuch-Woszczatyński P, Van Belkum A, Pituch H. First Polish outbreak of *Clostridium difficile* ribotype *027* infections among dialysis patients. European Journal of Clinical Microbiology & Infectious Diseases. 2015;**34**(1):63-67. DOI: 10.1007/s10096-014-2204-x

[64] Hung YP, Lin HJ, Tsai BY, Liu HC, Liu HC, Lee JC, Wu YH, Wilcox MH, Fawley WN, Hsueh PR, Tsai PJ, Ko WC. *Clostridium difficile* ribotype *126* in southern Taiwan: A cluster of three symptomatic cases. Anaerobe. 2014;**30**:188-192. DOI: 10.1016/j.anaerobe.2014.06.005

[65] Lim SK, Stuart RL, Mackin KE, Carter GP, Kotsanas D, Francis MJ, Easton M, Dimovski K, Elliott B, Riley TV, Hogg G, Paul E, Korman TM, Seemann T, Stinear TP, Lyras D, Jenkin GA. Emergence of a ribotype *244* strain of *Clostridium difficile* associated with severe disease and related to the epidemic ribotype *027* strain. Clinical Infectious Diseases. 2014;**58**(12):1723-1730. DOI: 10.1093/cid/ciu203

[66] 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. Enfermedades Infecciosas Y Microbiologia Clinica. 2014;**32**(6):355-358. DOI: 10.1016/j.eimc.2013.07.004

[67] Sandell S, Rashid MU, Jorup-Rönström C, Ellström K, Nord CE, Weintraub A. *Clostridium difficile* recurrences in Stockholm. Anaerobe. 2016;**38**:97-102. DOI: 10.1016/j.anaerobe.2016.01.005

[68] Thabit AK, Housman ST, Burnham CD, Nicolau DP. Association of healthcare exposure with acquisition of different *Clostridium difficile* strain types in patients with recurrent infection or colonization after clinical resolution of initial infection. The Journal of Hospital Infection. 2016;**92**(2):167-172. DOI: 10.1016/j.jhin.2015.11.009

[69] Kumar N, Miyajima F, He M, Roberts P, Swale A, Ellison L, Pickard D, Smith G, Molyneux R, Dougan G, Parkhill J, Wren BW, Parry CM, Pirmohamed M, Lawley TD. Genome-based infection tracking reveals dynamics of *Clostridium difficile* transmission and disease recurrence. Clinical Infectious Diseases. 2016;**62**(6):746-752. DOI: 10.1093/cid/civ1031

[70] Gerding DN, Hecht DW, Louie T, Nord CE, Talbot GH, Cornely OA, Buitrago M, Best E, Sambol S, Osmolski JR, Kracker H, Locher HH, Charef P, Wilcox M. Susceptibility of *Clostridium difficile* isolates from a Phase 2 clinical trial of cadazolid and vancomycin in *C. difficile* infection. The Journal of Antimicrobial Chemotherapy. 2016;**71**(1):213-219. DOI: 10.1093/jac/dkv300

[71] 71.Holt HM, Danielsen TK, Justesen US. Routine disc diffusion antimicrobial susceptibility testing of *Clostridium difficile* and association with PCR ribotype *027*. European Journal of Clinical Microbiology & Infectious Diseases. 2015;**34**(11):2243-2246. DOI: 10.1007/s10096-015-2475-x

[72] Sarma JB, Marshall B, Cleeve V, Tate D, Oswald T, Woolfrey S. Effects of fluoroquinolone restriction (from 2007 to 2012) on *Clostridium difficile* infections: Interrupted time-series analysis. The Journal of Hospital Infection. 2015;**91**(1):74-80. DOI: 10.1016/j.jhin.2015.05.013

[73] Wenisch JM, Equiluz-Bruck S, Fudel M, Reiter I, Schmid A, Singer E, Chott A. Decreasing *Clostridium difficile* infections by an antimicrobial stewardship program that reduces moxifloxacin use. Antimicrobial Agents and Chemotherapy. 2014;**58**(9):5079-5083. DOI: 10.1128/AAC.03006-14

[74] Van den Berg RJ, Schaap I, Templeton KE, Klaassen CH, Kuijper EJ. Typing and subtyping of *Clostridium difficile* isolates by using multiple-locus variable-number tandem-repeat analysis. Journal of Clinical Microbiology. 2007;**45**(3):1024-1028

[75] Marsh JW, O'Leary MM, Shutt KA, Pasculle AW, Johnson S, Gerding DN. Multilocus variable-number tandem-repeat analysis for investigation of *Clostridium difficile* transmission in Hospitals. Journal of Clinical Microbiology. 2006;**44**(7):2558-2566

[76] 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. Journal of Medical Microbiology. 2011;**60**(Pt 8):1088-1094

[77] Goorhuis A, Debast SB, Dutilh JC, van Kinschot CM, Harmanus C, Cannegieter SC. Type-specific risk factors and outcome in an outbreak with 2 different *Clostridium difficile* types simultaneously in 1 hospital. Clinical Infectious Diseases. 2011;**53**(9):860-869. DOI: 10.1093/cid/cir549

[78] Fawley WN, Wilcox MH. *Clostridium difficile* ribotyping network for England and Northern Ireland. An enhanced DNA fingerprinting service to investigate potential *Clostridium difficile* infection case clusters sharing the same PCR ribotype. Journal of Clinical Microbiology. 2011;**49**(12):4333-4337

[79] Van Dorp SM, Smajlović E, Knetsch CW, Notermans DW, De Greeff SC, Kuijper EJ. Clinical and microbiological characteristics of *Clostridium difficile* infection among hospitalized children in the Netherlands. Clinical Infectious Diseases. 2017;**64**(2):192-198. DOI: 10.1093/cid/ciw699

[80] Krutova M, Matejkova J, Kuijper EJ, Drevinek P, Nyc O. Czech *Clostridium difficile* study group: *Clostridium difficile* PCR ribotypes *001* and *176*—The common denominator of *C. difficile* infection epidemiology in the Czech Republic, 2014. Euro Surveillance. 2016;**21**(29): 1-11. DOI: 10.2807/1560 7917.ES.2016.21.29.30296

[81] Kullin B, Brock T, Rajabally N, Anwar F, Vedantam G, Reid S, Abratt V. Characterisation of *Clostridium difficile* strains isolated from Groote Schuur Hospital, Cape Town, South

Africa. European Journal of Clinical Microbiology & Infectious Diseases. 2016;**35**(10):1709-1718. DOI: 10.1007/s10096-016-2717-6

[82] Usui M, Nanbu Y, Oka K, Takahashi M, Inamatsu T, Asai T, Kamiya S, Tamura Y. Genetic relatedness between Japanese and European isolates of *Clostridium difficile* originating from piglets and their risk associated with human health. Frontiers in Microbiology. 2014;**5**:513. DOI: 10.3389/fmicb.2014.00513. eCollection 2014

[83] Krutova M, Nyc O, Kuijper EJ, Geigerova L, Matejkova J, Bergerova T, Arvand M. A case of imported *Clostridium difficile* PCR-ribotype 027 infection within the Czech Republic which has a high prevalence of *C. difficile* ribotype 176. Anaerobe. 2014;**30**:153-155. DOI: 10.1016/j.anaerobe.2014.09.020

[84] Eyre DW, Fawley WN, Best EL, Griffiths D, Stoesser NE, Crook DW, Peto TE, Walker AS, Wilcox MH. Comparison of multilocus variable-number tandem-repeat analysis and whole-genome sequencing for investigation of *Clostridium difficile* transmission. Journal of Clinical Microbiology. 2013;**51**(12):4141-4149. DOI: 10.1128/JCM.01095-13

[85] Manzoor SE, Tanner HE, Marriott CL, Brazier JS, Hardy KJ, Platt S, Hawkey PM. Extended multilocus variable-number tandem-repeat analysis of *Clostridium difficile* correlates exactly with ribotyping and enables identification of hospital transmission. Journal of Clinical Microbiology. 2011;**49**(10):3523-3530. DOI: 10.1128/JCM.00546-11

[86] Kilic A, Alam MJ, Tisdel NL, Shah DN, Yapar M, Lasco TM, Garey KW. Multiplex real-time PCR method for simultaneous identification and toxigenic type characterization of *Clostridium difficile* from stool samples. Clinical Microbiology. 2015;**35**:306-313. DOI: 10.3343/alm.2015.35.3.306

[87] Belanger SD, Boissinot M, Clairoux N, Picard FJ, Bergeron MG. Rapid detection of *Clostridium difficile* in feces by real-time PCR. Journal of Clinical Microbiology. 2003;**41**:730-734. DOI: 10.1128/JCM.41.2.730-734.2003

[88] Carroll KC, Buchan BW, Tan S, Stamper PD, Riebe KM, Pancholi P, Kelly C, Rao A, Fader R, Cavagnolo R, Watson W, Goering RV, Trevino EA, Weissfeld AS. Multicenter evaluation of the *Verigene Clostridium difficile Nucleic Acid Assay*. Journal of Clinical Microbiology. 2013;**51**:4120-4125. DOI: 10.1128/JCM.01690-13

[89] Maiden MC. Multilocus sequence typing of bacteria. Annual Review of Microbiology. 2006;**60**:561-588

[90] Maiden MC, Bygraves JA, Feil E, Morelli G, Russell JE, Urwin R, Zhang Q, Zhou J, Zurth K, Caugant DA, Feavers IM, Achtman M, Spratt BG. Multilocus sequence typing: A portable approach to the identification of clones within populations of pathogenic microorganisms. Proceedings of the National Academy of Sciences of the United States of America. 1998;**95**(6):3140-3145

[91] Lemee L, Dhalluin A, Pestel-Caron M, Lemeland JF, Pons JL. Multilocus sequence typing analysis of human and animal *Clostridium difficile* isolates of various toxigenic types. Journal of Clinical Microbiology. 2004;**42**(6):2609-2617

[92] Lemee L, Bourgeois I, Ruffin E, Collignon A, Lemeland JF, Pons JL. Multilocus sequence analysis and comparative evolution of virulence-associated genes and housekeeping genes of *Clostridium difficile*. Microbiology. 2005;151(Pt 10):3171-3180

[93] Kuwata Y, Tanimoto S, Sawabe E, Shima M, Takahashi Y, Ushizawa H, Fujie T, Koike R, Tojo N, Kubota T, Saito R. Molecular epidemiology and antimicrobial susceptibility of *Clostridium difficile* isolated from a university teaching hospital in Japan. European Journal of Clinical Microbiology & Infectious Diseases. 2015;**34**(4):763-772. DOI: 10.1007/s10096-014-2290-9

[94] Zhang X, Wang X, Yang J, Liu X, Cai L, Zong Z. Colonization of toxigenic *Clostridium difficile* among ICU patients: A prospective study. BMC Infectious Diseases. 2016;**16**:397. DOI: 10.1186/s12879-016-1729-2

[95] Rodriguez C, Avesani V, Taminiau B, Van Broeck J, Brévers B, Delmée M, Daube G. Investigation of *Clostridium difficile* interspecies relatedness using multilocus sequence typing, multilocus variable-number tandem-repeat analysis and antimicrobial susceptibility testing. The Veterinary Journal. 2015;**206**(3):349-355. DOI: 10.1016/j.tvjl.2015.09.001

[96] Cairns MD, Preston MD, Lawley TD, Clark TG, Stabler RA, Wren BW. Genomic epidemiology of a protracted hospital outbreak caused by a toxin A-negative *Clostridium difficile* sublineage PCR ribotype *017* strain in London, England. Journal of Clinical Microbiology. 2015;**53**(10):3141-3147. DOI: 10.1128/JCM.00648

[97] Stojanov M, Magalhaes B, Terletsky V, Basset P, Prod'hom G, Greub G, Senn L, Blanc DS. Development and evaluation of double locus sequence typing for molecular epidemiological investigations of *Clostridium difficile*. European Journal of Clinical Microbiology & Infectious Diseases. 2016;**35**(2):175-181. DOI: 10.1007/s10096-015-2525-4

[98] Furoya-Kanamory L, Marquess J, Yakob L, Riley TV, Paterson DL, Foster NF, Huber CA, Clements CA. Asymptomatic *Clostridium difficile* colonization: Epidemiology and clinical implications. BMC Infectious Diseases. 2015;**15**;516-526. DOI: 10.1186/s12879-015-1258-4

[99] Vindigni SM, Surawicz CM. *C. difficile* infection: Changing epidemiology and management paradigms. Clinical and Translational Gastroenterology. 2015;**6**:e99. DOI:10.1038/ctg.2015.24

[100] U.S. Food and Drug Administration [Internet]. 2017. Available from: http://www.fda.gov/medicaldevices/productsandmedicalprocedures/invitrodiagnostics/ucm330711.htm [Accessed 16 January 2017]

[101] Shin BM, Yoo SM, Shin WC. Evaluation of Xpert *C. difficile*, BD MAX Cdiff, IMDx *C. difficile* for Abbott m2000, and Illumigene *C. difficile* assays for direct detection of toxigenic *Clostridium difficile* in stool specimens. The Annals of Laboratory Medicine. 2016;**36**(2):131-137. DOI: 10.3343/alm.2016.36.2.131

[102] Denys GA. Portrait toxigenic *Clostridium difficile* assay, an isothermal amplification assay detects toxigenic *C. difficile* in clinical stool specimens. Expert Review of Molecular Diagnostics. 2014;**14**(1):17-26. DOI: 10.1586/14737159.2014.864239

[103] Stellrecht KA, Espino AA, Maceira VP, Nattanmai SM, Butt SA, Wroblewski D, Hannett GE, Musser KA. Premarket evaluations of the IMDx *C. difficile* for Abbott m2000 Assay and the BD Max Cdiff Assay. Journal of Clinical Microbiology. 2014;**52**(5):1423-1428. DOI: 10.1128/JCM.03293-13

[104] Gilbreath JJ, Verma P, Abbott AN, Butler-Wu SM. Comparison of the Verigene *Clostridium difficile*, Simplexa *C. difficile* Universal Direct, BD MAX Cdiff, and Xpert *C. difficile* assays for the detection of toxigenic *C. difficile*. Diagnostic Microbiology and Infectious Disease. 2014;**80**(1):13-18. DOI: 10.1016/j.diagmicrobio.2014.06.001.v

[105] Tojo M, Nagamatsu M, Hayakawa K, Mezaki K, Kirikae T, Ohmagari N. Evaluation of an automated rapid diagnostic test for detection of *Clostridium difficile*. PLoS One. 2014;**9**(8):e106102. DOI: 10.1371/journal.pone.0106102. eCollection 2014

[106] Yoo J, Lee H, Park KG, Lee GD, Park YG, Park YJ. Evaluation of 3 automated real-time PCR (Xpert *C. difficile* Assay, BD MAX Cdiff, and IMDx *C. difficile* for Abbott m2000 Assay) for detecting *Clostridium difficile* toxin gene compared to toxigenic culture in stool specimens. Diagnostic Microbiology and Infectious Disease. 2015;**83**(1):7-10. DOI: 10.1016/j. diagmicrobio.2015.05.005

[107] Dalpke AH, Hofko M, Zorn M, Zimmermann S. Evaluation of the fully automated *BD MAX Cdiff* and *Xpert C. difficile* assays for direct detection of *Clostridium difficile* in stool specimens. Journal of Clinical Microbiology. 2013;**51**(6):1906-1908. DOI: 10.1128/JCM.00344-13

[108] Beck ET, Buchan BW, Riebe KM, Alkins BR, Pancholi P, Granato PA, Ledeboer NA. Multicenter evaluation of the Quidel Lyra Direct *C. difficile* nucleic acid amplification assay. Journal of Clinical Microbiology. 2014;**52**(6):1998-2002. DOI: 10.1128/JCM.03089-13

[109] Deak E, Miller SA, Humphries RM. Comparison of *Illumigene*, *Simplexa*, and *AmpliVue Clostridium difficile* molecular assays for diagnosis of *C. difficile* infection. Journal of Clinical Microbiology. 2014;**52**(3):960-963. DOI: 10.1128/JCM.02354-13

[110] Yang P, Hash S, Park K, Wong C, Doraisamy L, Petterson J, Petti CA, Ward PM, Lee SH, Menon S, She RC. Application of nuclear magnetic resonance to detect toxigenic *Clostridium difficile* from stool specimens: A proof of concept. The Journal of Molecular Diagnostics. 2017;**9**: 230-235. pii: S1525-1578(16)30258-6. DOI: 10.1016/j.jmoldx.2016.09.012

[111] Vasoo S, Stevens J, Portillo L, Barza R, Schejbal D, Wu MM, Chancey C, Singh K. Cost-effectiveness of a modified two-step algorithm using a combined glutamate dehydrogenase/toxin enzyme immunoassay and real-time PCR for the diagnosis of *Clostridium difficile* infection. Journal of Microbiology, Immunology and Infection. 2014;**47**(1):75-78. DOI: 10.1016/j.jmii.2012.07.008

[112] Solomon K, Fanning S, McDermott S, Murray S, Scott L, Martin A, Skally M, Burns K, Kuijper EJ, Fitzpatrick F, Fenelon L, Kyne L. PCR ribotype prevalence and molecular basis of macrolide–lincosamide–streptogramin B (MLSB) and fluoroquinolone

resistance in Irish clinical *Clostridium difficile* isolates. The Journal of Antimicrobial Chemotherapy. 2011;**66**:1976-1982. DOI: 10.1093/jac/dkr275

[113] Wasels W, Monot M, Spigaglia P, Barbanti F, Ma L, Bouchier C, Dupuy B, Mastrantonio P. Inter- and intraspecies transfer of a *Clostridium difficile* conjugative transposon conferring resistance to MLSB. Microbial Drug Resistance. 2014;**20**:555-560. DOI: 10.1089/mdr.2014.0015

[114] Van Eijk E, Anvar SY, Browne HP, Leung WY, Frank J, Schmitz AM, Roberts AP, Smits WK. Complete genome sequence of the *Clostridium difficile* laboratory strain *630Δerm* reveals differences from strain *630*, including translocation of the mobile element *CTn5*. BMC Genomics. 2015;**16**:31. DOI: 10.1186/s12864-015-1252-7

[115] Sebaihia M, Wren BW, Mullany P. The multidrug-resistant human pathogen *Clostridium difficile* has a highly mobile, mosaic genome. Nature Genetics. 2006;**38**:779-786. DOI: 10.1038/ng1830

[116] Spigaglia P. Recent advances in the understanding of antibiotic resistance in *Clostridium difficile* infection. Therapeutic Advances in Infectious Disease. 2016;**3**:23-42. DOI: 10.1177/2049936115622891

[117] Smith CJ, Markowitz SM, Macrina F. Transferable tetracycline resistance in *Clostridium difficile*. Antimicrobial Agents and Chemotherapy. 1981;**19**(6):997-1003. DOI: 0066-4804/81/060997-07$02.00/0

[118] Mullany P, Allan E, Roberts AP. Mobile genetic elements in *Clostridium difficile* and their role in genome Function. Research in Microbiology. 2015;**166**(4):361-367. DOI: 10.1016/j.resmic.2014.12.005

[119] Roberts AP, Allan E, Mullany P. The impact of horizontal gene transfer on the biology of *Clostridium difficile*. Advances in Microbial Physiology. 2014;**65**:63-82. DOI: 10.1016/bs.ampbs.2014.08.002

[120] Bouillaut L, McBride SM, Sorg JA. Genetic manipulation of *Clostridium difficile*. Current Protocols in Microbiology. 2011;**Chapter 9**:Unit 9A.2. DOI: 10.1002/9780471729259.mc09a02s20

[121] Clabots CR, Peterson LR, Gerding DN. Characterization of a nosocomial *Clostridium difficile* outbreak by using plasmid profile typing and clindamycin susceptibility testing. Journal of Infectious Diseases. 1988;**158**(4):731-736

[122] Freeman J, Vernon J, Morris K, Nicholson S, Todhunter S, Longshaw C, Wilcox MH. Pan-European longitudinal surveillance of antibiotic resistance among prevalent *Clostridium difficile* ribotypes. Clinical Microbiology and Infection. 2015;**21**:248-248. DOI: /10.1016/j.cmi.2014.09.017

[123] Lachowicz D, Pituch H, Obuch-Woszczatynski P. Antimicrobial susceptibility patterns of *Clostridium difficile* strains belonging to different polymerase chain reaction ribotypes isolated in Poland in 2012. Anaerobe. 2015;**31**:37-41. DOI: 10.1016/j.anaerobe.2014.09.004

[124] Drudy D, Goorhuis B, Bakker D, Kyne L, Van der Berg R. Clindamycin resistant clone of *C. difficile* PCR ribotype *027* in Europe. Emerging Infectious Diseases. 2008;**14**:1485-1487. DOI: 0.3201/eid1409.071346

[125] Baines SD, Wilcox MH. Antimicrobial resistance and reduced susceptibility in *Clostridium difficile*: Potential consequences for induction, treatment, and recurrence of *C. difficile* infection. Antibiotics. 2015;**4**:267-298. DOI: 10.3390/antibiotics4030267

[126] Ackermann G, Tang Y J, Kueper R, Heisig P, Rodloff A, Silva J, Cohen SH. Resistance to moxifloxacin in toxigenic *Clostridium difficile* isolates Is associated with mutations in *gyrA*. Antimicrobial Agents and Chemotherapy. 2001;**45**(8):2348-2353

[127] Costa MC, Stampfli HR, Arroyo LG, Pearl DL, Weese JS. Epidemiology of *Clostridium difficile* on a veal farm: Prevalence, molecular characterization and tetracycline resistance. Veterinary Microbiology. 2011;**152**(3-4):379-384. DOI: 10.1016/j. vetmic.2011.05.014

[128] Spigaglia P, Barbanti F, Dionisi AM, Mastrantonio P. *Clostridium difficile* isolates resistant to fluoroquinolones in Italy: Emergence of PCR ribotype *018*. Journal of Clinical Microbiology. 2010;**48**:2892-2896. DOI: 10.1128/JCM.02482-09

[129] Spigaglia P, Barbanti F, Mastrantonio P. Multidrug resistance in European *Clostridium difficile* clinical isolates. The Journal of Antimicrobial Chemotherapy. 2011;**66**:2227-2234. DOI: 10.1093/jac/dkr292

[130] Tenover FC, Novak-Weekley S, Woods CW, Peterson LR, Davis T, Schreckenberger P, Fang FC, Dascal A, Gerding DN, Nomura JH, Goering RV, Akerlund T, Weissfeld AS, Baron EJ, Wong E, Marlowe EM, Whitmore J, Persing DH. Impact of strain type on detection of toxigenic *Clostridium difficile*: Comparison of molecular diagnostic and enzyme immunoassay approaches. Journal of Clinical Microbiology. 2010;**48**:3719-3724. DOI: 10.1128/JCM.00427-10

[131] Tenover FC, Tickler IA, Persing DH. Antimicrobial-resistant strains of *Clostridium difficile* from North America. Antimicrobial Agents and Chemotherapy. 2012;56:2929-2932. DOI: 10.1128/AAC.00220-12

[132] Pulidot MR, Garçia-Quitanillat M, Martin-Peňa R, Cisneros JM, McConnell J. Progress on the development of rapid methods for antimicrobial susceptibility testing. The Journal of Antimicrobial Chemotherapy, 2013;**68**:2710-2717. DOI: 10.1093/jac/dkt253

[133] Rizzardi K, Åkerlund T., High molecular weight typing with MALDI-TOF MS—A novel method for rapid typing of *Clostridium difficile*. PLoS One. 2015 Apr 29;**10**(4):e0122457. DOI: 10.1371/journal.pone.0122457. eCollection 2015

[134] Chen JH, Cheng VC, Wong OY, Wong SC, So SY, Yam WC, Yuen KY. The importance of matrix-assisted laser desorption ionization-time of flight mass spectrometry for correct identification of *Clostridium difficile* isolated from chromID *C. difficile* chromogenic agar. Journal of Microbiology, Immunology and Infection (2016), http://dx.doi.org/10.1016/j. jmii.2015.12.002

[135] Plüddemann A, Onakpoya I, Harrison S, Shinkins B, Tompson A, Davis R, Price CP, Heneghan C. Position Paper on Anti-Microbial Resistance Diagnostics. University of Oxford: Centre for Evidence-Based Medicine; 2015. Available from: http://www.cebm.net/wp-content/uploads/2015/07/AMR-Diagnostic-technologies_10-June-2015.pdf

[136] Tian TT, Zhao JH, Yang J, Qiang CX, Li ZR, Chen J, Xu KY, Ciu QQ, Li RX. Molecular characterization of *Clostridium difficile* Isolates from human subjects and the environment. PLoS One. 2016;**11**(3): e0151964. DOI: 10.1371/journal. pone.0151964

[137] CDC Technical document European surveillance of *Clostridium difficile* infections Surveillance protocol version 2.2 [Internet]. 2017. Available from: http: //ecdc.europa.eu/en/publications/publications/european-surveillance-clostridium-difficile.v2final.pdf [Accessed 17 January 2017]

[138] Sartelli M, Malangoni MA, Abu-Zidan FM. WSES guidelines for management of *Clostridium difficile* infection in surgical patients. World Journal of Emergency Surgery. 2015;**10**:38. DOI: 10.1186/s13017-015-0033-6

[139] Crobach MJ, Planche T, Eckert C, Barbut F, Terveer EM, Dekkers OM, Wilcox MH, Kuijper EJ. European society of clinical microbiology and infectious diseases: Update of the diagnostic guidance document for *Clostridium difficile* infection. Clinical Microbiology and Infection. 2016;**4**:63-81. DOI: 10.1016/j.cmi.2016.03.010

IntechOpen

IntechOpen

IntechOpen