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Paola Mastrantonio
Maja Rupnik *Editors*

Updates on *Clostridium difficile* in Europe

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Paola Mastrantonio • Maja Rupnik
Editors

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Editors
Paola Mastrantonio
Former Research Director in the
Department of Infectious Diseases
National Institute of Health (ISS)
Rome, Italy

Maja Rupnik
Department for Microbiological
Research
National Laboratory of Health,
Environment and Food (NLZOH)
Maribor, Slovenia

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Foreword

The chapters of this book were planned to cover the most important issues to be addressed in the study of infections due to *Clostridium difficile*, a microorganism still feared not only as the cause of nosocomial diarrhea related to protracted antibiotic administration but more and more frequently of diarrheal diseases unrelated to the hospital environment, including those affecting animals. In the last decades, a growing number of clinicians, microbiologists, and epidemiologists have investigated this topic, as evidenced by the large amount of scientific publications still in an upward trend over the years. In particular, this book has been focused on the clinical and experimental activities carried out in Europe for a better knowledge of this pathogen and its molecular characteristics, associated pathologies, and possible transmission routes, as well as to build up preventive and diagnostic strategies and efficacious therapeutic approaches for the treatment of *C. difficile* infection (CDI).

Thanks also to the foundation of the European Study Group on *C. difficile* (ESGCD) in 2000, in the framework of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID), European clinicians and researchers, together with experts from all over the world, were able to consolidate the already existing, positive collaboration that led in recent years to the establishment of a European network for the epidemiological surveillance, the molecular characterization, and the evaluation of the antibiotic resistance profile of the clinical isolates, with obvious advantages for the continuous updating of the treatment strategies of CDI. To emphasize the positive role of this study group in the fight against *C. difficile* infection, an invited chapter written by both the current and the past president of ESGCD has been included at the end of this book.

We are grateful to all the authors for their significant contributions to the book. In our view and intention, they ideally represent also the work of many other European experts in this field who did not get involved on this occasion for obvious limits of space.

Rome, Italy — Paola Mastrantonio
Maribor, Slovenia — Maja Rupnik

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The original version of this book was revised. Due to a different technical process the source lines were set differently in the original version and have now been corrected. An erratum to this book can be found at https://doi.org/10.1007/978-3-319-72799-8_15

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Economic Burden of *Clostridium difficile* Infection in European Countries

Elena Reigadas Ramírez and Emilio Santiago Bouza

Abstract

Clostridium difficile infection (CDI) remains a considerable challenge to health care systems worldwide. Although CDI represents a significant burden on healthcare systems in Europe, few studies have attempted to estimate the consumption of resources associated with CDI in Europe. The reported extra costs attributable to CDI vary widely according to the definitions, design, and methodologies used, making comparisons difficult to perform. In this chapter, the economic burden of healthcare facility–associated CDI in Europe will be assessed, as will other less explored areas such as the economic burden of recurrent CDI, community-acquired CDI, pediatric CDI, and CDI in outbreaks.

Keywords

C. difficile infection · Economic costs · Economic burden · Length of stay · Europe

1 Introduction

In this chapter, the economic burden of healthcare facility-associated *Clostridium difficile* infection (CDI) in Europe will be assessed, as will other less explored areas such as recurrent CDI (R-CDI), community-acquired CDI, pediatric CDI, and CDI in outbreaks.

Despite advances in the diagnosis and treatment of CDI and prevention efforts to reduce the incidence of CDI, the disease remains a significant challenge to health care systems worldwide

E. Reigadas Ramírez (✉)
Department of Clinical Microbiology and Infectious Diseases, Hospital General Universitario Gregorio Marañón, Madrid, Spain

Medicine Department, School of Medicine, Universidad Complutense de Madrid (UCM), Madrid, Spain

Instituto de Investigación Sanitaria Gregorio Marañón, Madrid, Spain
e-mail: helenrei@hotmail.com

E.S. Bouza (✉)
Department of Clinical Microbiology and Infectious Diseases, Hospital General Universitario Gregorio Marañón, Madrid, Spain

Medicine Department, School of Medicine, Universidad Complutense de Madrid (UCM), Madrid, Spain

Instituto de Investigación Sanitaria Gregorio Marañón, Madrid, Spain

CIBER de Enfermedades Respiratorias (CIBERES CB06/06/0058), Madrid, Spain
e-mail: emilio.bouza@gmail.com

(Dubberke and Olsen 2012; Bouza 2012). From an economic point of view, CDI increases patient healthcare costs as a result of extended length of hospital stay (LOS), re-admission, laboratory tests, and medication (Wiegand et al. 2012; Gabriel and Beriot-Mathiot 2014; Nanwa et al. 2015). *C. difficile* infection is costly, not only to third-party payers and hospitals, but also to society as a whole (McGlone et al. 2012).

Most of the existing literature is from the United States, where an in silico economic model, reported in 2012, suggested that the annual US economic burden of CDI would be $496 million from a hospital perspective, $547 million from a third-party payer perspective, and $796 million from a societal perspective (McGlone et al. 2012). Regrettably, few published studies have attempted to estimate the consumption of resources associated with CDI in Europe (Wiegand et al. 2012) and it has been estimated that the annual cost of CDI in Europe is €3 billion per year (Jones et al. 2013); consequently, approaches that can reduce CDI-associated resource use and costs are of interest.

Although antibiotics are a key component of therapy for CDI, they currently represent a minimal cost in the overall budget for CDI management, and the main extra associated cost reported in most studies is the extended LOS attributable to CDI (Wiegand et al. 2012; Asensio et al. 2013, 2015; Wilcox et al. 1996; Hubner et al. 2015).

CDI-related costs are also likely to increase as the population ages. In a systematic European meta-analysis on clinical and economic burden, the authors reported that the incremental cost of CDI may have increased by £1857–£4266 (27–93%) over a 12-year period (Wiegand et al. 2012). In a review by Kuijper et al., the potential cost of CDI was estimated to be €3 billion/year and is expected to almost double over the next four decades, assuming a European Union population of 457 million inhabitants (Kuijper et al. 2006).

The reported extra costs attributable to CDI vary widely according to the definitions, design, and methodologies used (Ghantoji et al. 2010; Wiegand et al. 2012). Most studies do not separate the costs of resources due to CDI from those generated by the underlying disease. Therefore, comparisons need to be made with caution and limited to results obtained in a similar manner.

A clearer understanding of the healthcare and economic burden of CDI is of value to hospital administrators, infection prevention teams, and persons involved in antimicrobial stewardship programs, who can use this key information to determine the appropriate degree of investment in infection control measures and in other priority areas.

Future studies should follow standard methodology, include other indirect cost perspectives such as societal and patient perspectives, and examine poorly explored populations, such as individuals with community-acquired CDI.

2 Economic Burden of Hospital-Acquired CDI in European Countries

A wide range of CDI costs in Europe have been reported, ranging from €5798–€11,202/episode (Wiegand et al. 2012). Data are only available from six European countries (Ireland, England, Wales, Germany, Spain, and Italy). Table 1 summarizes CDI costs by study and country.

2.1 Primary Episodes

The economic burden of primary episodes in Europe is reviewed below by region, as defined by EuroVoc. The most abundant literature comes from Western Europe, followed by Southern Europe.

2.1.1 Western Europe

A recent study conducted in a tertiary referral hospital in Ireland during August 2015 showed that the total incremental cost of CDI was €75,680, with a mean cost of €5820 per patient (Ryan et al. 2017).

Table 1 Summary of European *Clostridium difficile* infection costs by study and country

Author (year)	Country or region	CDI cases examined	Study population	Study period	Cost
Ryan et al. (2017)	Ireland	N = 13	Healthcare CDI	August 2015	€5820/CDI
Al-Eidan et al. (2000)	Ireland	N = 87	Healthcare CDI	1994–1995	£2860/CDI
Wilcox et al. (1996)	England	N = 50	Healthcare CDI	1994–1995	£4107/CDI
Wilcox et al. (2017)	United Kingdom	N = 128	Healthcare CDI, recurrences	2012–2014	£6294/CDI
					£7539/recurrent CDI
Vonberg et al. (2008)	Germany	N = 116	Healthcare CDI	2006	€7147/CDI
Hubner et al. (2015)	Germany	N = 43	Healthcare CDI	2010	€5262.96/CDI
Grube et al. (2015)	Germany	N = 2767	Healthcare CDI, recurrences	2011	€4132/CDI as primary diagnosis
					€19,381/CDI as secondary diagnosis
					€20,755/recurrent CDI
Le Monnier et al. (2015)	France	N = 1097	Healthcare CDI, recurrences	2011	€9575/CDI (€6056 CDI as primary diagnosis/€11,251 CDI as secondary diagnosis)
					€9625/recurrent CDI
Asensio et al. (2013)	Spain	N = 7601	Healthcare CDI, recurrences	2012	€3901/CDI
					€4875/first recurrent CDI
					€5916/second recurrent CDI
Asensio et al. (2015)	Spain and Italy	N = 232 (Spain)	Healthcare CDI, recurrences, children	2011–2013 (adults)	€4265/CDI case (Spain)
		N = 145 (Italy)		2006–2012 (pediatrics)	€14,936/adult CDI case (Italy)
					€17,714/recurrent CDI case (Italy)
					€3545/pediatric CDI case (Italy)

Another study conducted in Ireland established the mean cost per treated case of CDI in terms of bed occupancy, laboratory requests, and treatment to be £4577 (2010 GBP) (Al-Eidan et al. 2000).

It has been estimated that the cost for CDI is €5000–€15,000 per case in England (Kuijper et al. 2006). The earliest data on economic burden from England were communicated by Wilcox et al. (1996), who performed a study in geriatric wards. Cases and controls were matched for age, sex, and distribution of the main diagnoses. The total identifiable increased cost of CDI was £6986 in 2010 GBP.

A retrospective multicenter study analyzed a sample of 12 large, public, acute-care hospitals in France representing 5.82% of the cumulative annual number of patient-days spent in public acute-care hospitals in France in 2011 (Le Monnier et al. 2015). The costs of CDI incurred by public insurance and by the hospital itself (euros) were based on full unit cost per diagnosis-related group in hospitals at 2010 values. The annual incidence of CDI based on laboratory reporting was estimated at 3.74 cases per 10,000 patient-days. In cases where CDI was the primary diagnosis, the mean cost per stay was €6056 (median €4410) and the cumulative cost for the whole set of stays observed in 2011 for the 12 hospitals was €823,656. In patients where CDI was considered a secondary diagnosis, the mean extra cost adjusted for age, sex, and diagnosis-related groups in cases without CDI was €11,251 (median: €8822) per stay (Le Monnier et al. 2015). The extrapolated annual nationwide cost of CDI in 2011 in France was €163.1 million.

A single-center retrospective analysis of data from patients with nosocomial CDI carried out over a 1-year period at a teaching hospital in Germany showed an additional cost of €5262/case (Hubner et al. 2015).

Another single-center German study showed that costs for CDI patients were significantly higher than for their matched controls (median: €7147) (Vonberg et al. 2008). A large multicenter study conducted in 37 German hospitals based on data from the German DRG system analyzed 2767 CDI cases grouped according to whether CDI was a primary or secondary diagnosis (Grube et al. 2015). For comparison, non-CDI cases from the same hospitals during the same year were matched using propensity score matching.

Patients from the primary diagnosis group (n = 817) showed a mean cost per case of €4132 (€536 more than controls), while the secondary diagnosis group (n = 1840) had costs of €19,381 (€13,082 for controls) (Grube et al. 2015). The authors extrapolated their data and declared that CDI generates a yearly cost burden of €464 million for the German healthcare system.

2.1.2 Southern Europe

Evidence regarding the impact of CDI on healthcare resources in southern Europe is generally scarce. In the case of Spain, few studies have assessed the economic burden of CDI. An economic model analysis performed in 2012 by Asensio et al.(Asensio et al. 2013) assessed the cost of CDI in adult patients (≥18 years) treated with metronidazole or vancomycin from the perspective of the Spanish National Health System Service. The resources used in clinical practice were obtained through a Delphi panel of Spanish clinicians with expertise in CDI. Unit costs (€2012) were obtained from Spanish sources.

This study estimated that 7601 episodes of CDI occur annually in Spain (incidence of 17.1 episodes/year/10,000 hospital discharges) with an estimated annual cost to the Spanish National Health System Service of €32,157,093. The cost per episode of CDI was €3901 for initial or primary CDI episodes.

More recently, another study assessed the impact of CDI on hospital resources and costs in both Spain and Italy (Asensio et al. 2015). Each patient was matched with two randomly selected uninfected controls in the same institution. Data were collected for 232 adult infected patients and 426 matched non-infected patients in Spain (n = 106) and Italy (n = 126). CDI-associated costs were due to excess hospitalization. The difference in LOS between the two countries resulted in a significant variation in costs.

Hospitalization costs attributable to CDI in Spain were €4265 per patient for all patients, €2882/patient for patients aged ≤65 years, and €4885 for those aged >65 years (Asensio et al. 2015).

For Italy, the total cost attributable to CDI was €14,023 per patient for all patients. The cost was €15,668 for those aged ≤65 years and €13,862 for those aged >65 years, with the difference in cost being due to differences in LOS (21 vs. 19 days, respectively). The authors estimated a cost of CDI in Italy of €32,371 per 10,000 patient-days (Asensio et al. 2015).

A recent multicenter Italian cost analysis study has been performed in hospitalized patients from the hospital's perspective (Poli et al. 2015). This study showed that the mean total incremental cost for a patient with CDI was €3270 per case.

2.2 Recurrent Episodes

One of the first studies to assess the cost of recurrent CDI in an European country was a Spanish study in which the cost of the initial CDI episode was estimated to be €3901, the cost of the first recurrence was €4875, and that of the second recurrence was €5916 (Asensio et al. 2013).

In an Italian multicenter study including recurrences (Asensio et al. 2015), the cost attributable to recurrent CDI was €17,714 per patient,

while for patients with a single episode of CDI, the cost was €14,936. In this study, a total of 34 adult patients (12.5%) and 2 pediatric patients (10.5%) experienced a first recurrence of CDI. Three of the 34 adult patients and 1 of the 2 pediatric patients had an additional recurrence.

A French multicenter study estimated the median extra cost per stay with CDI to be €7514, i.e., approximately €9.5 million in 2011 for the 12 facilities included. The fraction of that total cost attributable to recurrences was 12.5% (Le Monnier et al. 2015). Recurrences occurring in acute-care settings were present in 12.0% of hospital stays with CDI. In addition, 9.3% (11/118) of recurrences were coded as the primary diagnosis and led to readmission of the patients, which resulted in prolonged LOS and additional medical costs.

Data from 37 German hospitals revealed high costs for recurrent CDI of €20,755 vs. €13,101 for matched controls from the same hospitals during the same year (Grube et al. 2015).

Wilcox et al. recently analyzed the impact of recurrent CDI in terms of hospital resource use and health-related quality of life associated with hospitalizations for recurrent CDI in six UK acute-care hospitals (Wilcox et al. 2017). The median cost per patient during a 28-day post-index period was £7539 for recurrent CDI and £6294 for first CDI episodes (Wilcox et al. 2017).

2.3 Length of Stay

In their review, Wiegand et al. (2012) estimated the average LOS in Europe to be 15 days. When examined by country, they found that Switzerland had the lowest LOS (12 days), followed by Belgium, France, Ireland (17 days), and Spain (18 days), while the highest LOS were observed for The Netherlands (21 days), Germany (27 days), and the UK (37 days) (Wiegand et al. 2012).

Even though LOS values are more reproducible between studies than costs, data on the excess LOS attributable to CDI are limited. Not many studies assess the attributable LOS, reporting only total LOS. It was recently suggested that, compared with newer statistical models, models that were previously used to determine the LOS attributable to CDI overestimated the additional LOS (Mitchell and Gardner 2012). Therefore, future studies must take this into account. Table 2 shows the European studies reporting LOS attributable to CDI; the mean incremental LOS attributable to CDI ranged from 4.2 to 20 days (Ryan et al. 2017).

As for recurrent CDI, the mean incremental LOS in Europe is 9.1–26 days (Asensio et al. 2013, 2015). Although data may vary, most studies agree that recurrent CDI presents longer LOS than primary episodes. In a recent study conducted in England, Wilcox et al. observed a median LOS of 21 days for recurrent CDI in contrast to 15.5 days for first episodes (Wilcox et al. 2017).

Few studies have assessed differences in extra costs between mild to moderate CDI cases and severe CDI cases. A study conducted by van Kleef et al. in a large English teaching hospital showed that severe cases had an average excess LOS which was twice that of the nonsevere cases (11.6 days [95% CI, 3.6–19.6] vs. approximately 5 days [95% CI: 1.1–9.5]) (van Kleef et al. 2014).

2.4 Distribution of Costs

The expense associated with CDI stems mainly from extended LOS. Various studies in Europe place the additional cost of LOS at 43.2–95.6% of the total extra costs of the CDI episode (Ryan et al. 2017; Asensio et al. 2013, 2015; Wilcox et al. 1996; Poli et al. 2015). Figure 1 represents the distribution of CDI costs of the above mentioned studies.

In contrast, cost for CDI antibiotics account for a low percentage of the total cost, ranging from 0.43% to 13.3% (Ryan et al. 2017; Asensio et al. 2013, 2015; Wilcox et al. 1996; Poli et al.

Table 2 Length of stay (LOS) attributable to *Clostridium difficile* by study and country

Author (year)	Country	CDI cases examined	Study population	Study period	LOS attributable to CDI (days)
Eckmann et al. (2013)	Netherlands	N = 270	Healthcare CDI	2008–2009	Mean 12.58
Ryan et al. (2017)	Ireland	N = 13	Healthcare CDI	August 2015	Mean 4.2
Al-Eidan et al. (2000)	Ireland	N = 87	Healthcare CDI	1994–1995	Mean 13
Eckmann et al. (2013)	England	N = 10,602	Healthcare CDI	2007–2009	Mean 16.09
van Kleef et al. (2014)	England	N = 157	Healthcare CDI	2012	Mean 7.2 (all CDI)
					Mean 11.6 (severe CDI)
					Mean 5.3 (non severe CDI)
Vonberg et al. (2008)	Germany	N = 116	Healthcare CDI	2006	Median 7
Eckmann et al. (2013)	Germany	N = 109,526	Healthcare CDI	2008–2010	Mean 15.47
Hubner et al. (2015)	Germany	N = 43	Healthcare CDI	2010	Mean 11.4
Le Monnier et al. (2015)	France	N = 1097	Healthcare CDI, recurrences	2011	Mean 8.9
Eckmann et al. (2013)	Spain	N = 830	Healthcare CDI	2008–2010	Mean 13.56
Asensio et al. (2013)	Spain	N = 7601	Healthcare CDI, recurrences	2012	Mean 7.4 (CDI)
					Mean 9.1 (first recurrent CDI)
					Mean 10.8 (second recurrent CDI)
Asensio et al. (2015)	Spain and Italy	N = 232 (Spain)	Healthcare CDI, recurrences, children	2011–2013 (adults)	Median 6.4 (Madrid)
		N = 145 (Italy)		2006–2012 (pediatrics)	Median 20.0 (Barcelona)
					Median 20.0 (Rome)
					Median 26.0 for first recurrent CDI case (Spain and Italy)
					Median 5.0 for pediatric case (Naples)

2015). Figure 2 illustrates the distribution of costs in patients with CDI antibiotics as percent of total cost per country. Most of these studies only include vancomycin and metronidazole as treatment for CDI, probably because they were conducted before fidaxomicin was licensed in those countries. Only one recent study conducted in Ireland included fidaxomicin as treatment for CDI.

Regarding distribution of costs for R-CDI, a recent study conducted in England observed that the cost of hospital admissions and emergency department visits accounted for more than 85%, similar to first-case CDI. The median cost for CDI-specific drugs was higher in R-CDI patients (£376/patient) than first-case CDI (£46/patient) (Wilcox et al. 2017).

3 Economic Burden of Community-Acquired CDI

Community-acquired CDI is a growing problem, and additional data are needed to accurately quantify the contribution of this subpopulation to the overall burden of CDI. Few studies provide insight on this understudied patient group (Kuntz et al. 2012; Sammons et al. 2013; Nanwa et al.

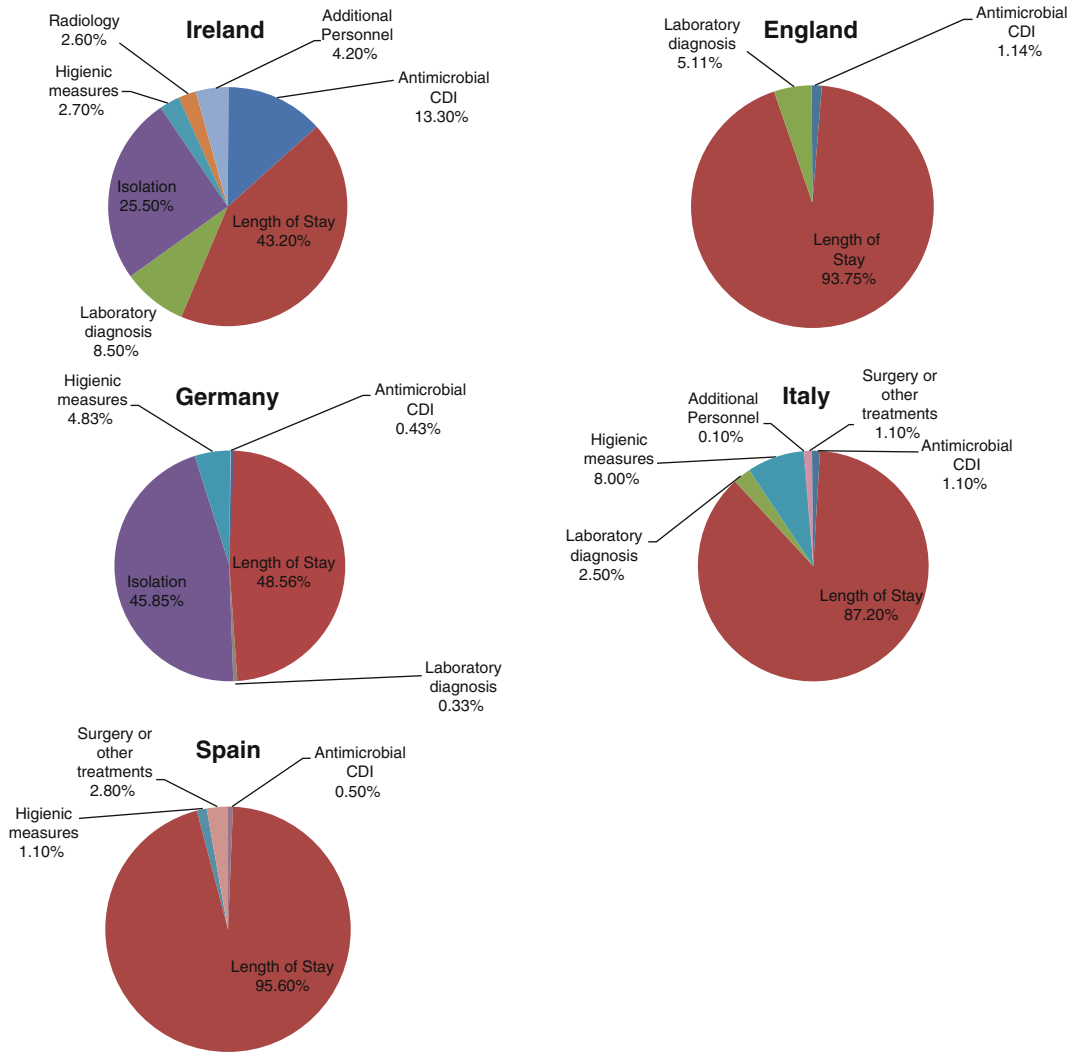

Fig. 1 Distribution of costs of *Clostridium difficile* infection

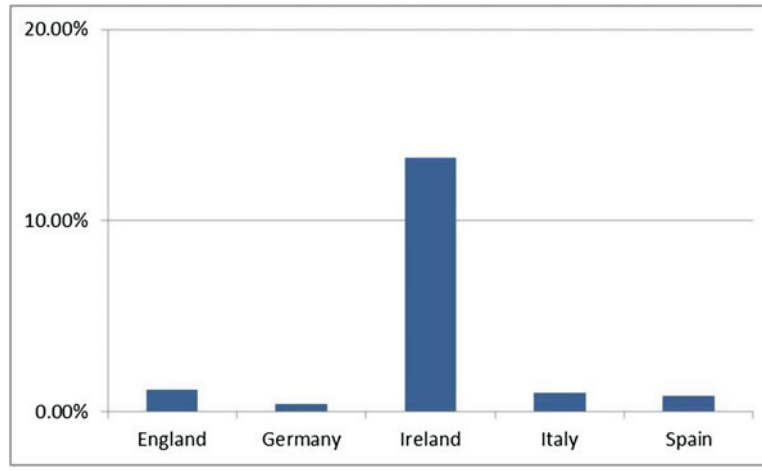

Fig. 2 Costs of antibiotics for CDI treatment as percentage of the total costs attributable to *Clostridium difficile* infection

2017), and none have been performed in European patients. In addition, across studies, the case definition of community-acquired CDI may differ depending on the time between a previous hospital admission and whether the case of CDI was an incident case (Kutty et al. 2010; Freeman et al. 2010).

The most recent and largest study is a population-based matched cohort study examining the mortality and costs of patients with community-onset CDI identified based on emergency department visits and hospital admissions in Ontario, Canada (Nanwa et al. 2017). In this study, Nanwa et al. studied 7950 subjects with community-onset CDI and found that up to 1 year after the index date, the disease was associated with 1.9- to 5.1-fold higher mean costs (CDN$10,700 in 2014) than in uninfected subjects. The largest cost components were hospitalizations and physician visits.

Differences in mortality and costs remained 3 years after index date, probably owing to recurrences of CDI, treatment failure, need for colectomy, and increased susceptibility to other conditions resulting from CDI. Mean attributable costs were higher among those aged >65 years, those infected in 2008 (year of an outbreak in Ontario caused by a particularly virulent CDI strain (Pillai et al. 2010)), and those who died within 1 year after the index date. However, this study had a major limitation, namely, the authors were not able to identify subjects whose only contact with the healthcare system was a visit to their family physician. Consequently, mortality and the economic burden of community-onset CDI per subject were likely overestimated, since all of the cases included were probably more severe.

Sammons et al. examined a cohort of children and performed a subanalysis on community-onset and hospital-onset CDI (Sammons et al. 2013). They found that patients with community-onset CDI comprised 54% of cases (2414 cases). Patients with hospital-onset CDI had significantly higher mortality rates and longer LOS than those with community-onset CDI, and mean differences in LOS and total standardized costs were 21.60 days and $93,600 for hospital-onset CDI and 5.55 days and $18,900 for community-onset CDI. Although mortality rates did not differ between those with community-onset CDI and matched unexposed subjects, community-onset CDI patients had significantly longer LOS and total hospital costs (Sammons et al. 2013).

Kuntz et al. performed a population-based study in which they identified 3067 CDIs and classified CDI by whether it was identified in the outpatient or inpatient healthcare setting (Kuntz et al. 2012). A total of 1712 (56%) were identified in the outpatient setting. These patients tended to be younger, with fewer comorbid conditions than patients with CDI identified in the inpatient setting. Eleven percent of patients with outpatient-identified CDI were hospitalized with a CDI-related diagnosis code during the follow-up period. These hospitalizations occurred, on average, 27 days after outpatient identification of CDI and lasted an average of 10 days.

As expected, the impact of CDI on healthcare utilization and cost was most notable in the setting in which the patient's infection had been identified. Outpatient care costs were higher among persons with CDI identified in the outpatient setting, with drugs representing the greatest percentage of these costs in both groups. Similarly, patients with inpatient-identified CDI had higher inpatient costs than patients with outpatient-identified CDI ($10,708.40 vs $837.40). Total costs for community-onset CDI were $1697 vs $11,315 of hospital-onset CDI (in US $2009 per patient) (Kuntz et al. 2012).

4 Pediatric Population

Data on the burden of CDI in children are even more limited and most of the literature on this topic comes from studies performed in the United States. In Italy, Asensio et al. (Asensio et al. 2015) reported separate data on the economic burden of CDI in children, although they found that the number of patients included was low (n = 19). Most cases of CDI in children were community-acquired as opposed to nosocomial.

Disease characteristics were generally comparable to those of adults, although the incidence of ulceration and bowel wall thickening was higher than in adults. The authors found that the median LOS attributable to CDI was lower than in adults (5 vs. 19 days in Rome), as was the frequency of isolation and admission to the ICU, probably because most cases were community-acquired. Therefore, although daily costs of care are higher for children than adults, the overall burden of CDI in the pediatric population in Italy is lower than in adults. The total cost attributable to CDI in pediatric patients in Naples was €3545 per patient (Asensio et al. 2015).

The only data on the economic burden of pediatric CDI in larger populations are from American studies. In their multicenter cohort study, Sammons et al. found that CDI was associated with worse outcomes among hospitalized children who were otherwise similar in the main demographic and clinical characteristics, although the difference was most pronounced in children with hospital onset disease. The presence of CDI was associated with >6-fold higher mortality rates among those with healthcare-onset CDI and resulted in significantly longer LOS and increased total hospital costs, corresponding to a mean difference in total standardized costs of $48,500 between matched exposed and unexposed patients (Sammons et al. 2013).

In another study performed in acute care hospitals in the Michigan Health and Hospital Association, children younger than 5 years of age had mean charges of $148,525, compared with $56,796 for discharges of patients who were aged ≥65 years, probably because of longer LOS: children younger than 5 years of age were hospitalized for a mean of more than 25 days per discharge vs 14.2 days for the remaining age groups reported (VerLee et al. 2012).

A large propensity score–matching analysis in 313,664 patients aged 1–18 years was performed to evaluate the influence of CDI on mortality, LOS, and costs in hospitalized surgical pediatric patients. The authors observed that after propensity score matching, the mean excess LOS and costs attributable to CDI were 5.8 days and $12,801 ($P < 0.001$), accounting for 8295 days spent in the hospital and $18.4 million (2012 USD) in annual expenditure (Kulaylat et al. 2017).

5 Economic Costs of CDI Outbreaks

Few data have been published on the costs derived from outbreaks. One of the few studies to assess this situation was that conducted in Ireland by Ryan et al. (2017). The authors collected data on LOS, diagnosis, diagnosis-related group codes at discharge, time in isolation because of CDI, additional measures because of CDI (medications, consultations, investigations, and procedures), unit costs (laboratory testing, personal protective equipment, single room accommodation, and cleaning/decontamination), and personnel time.

This study covered only a 1-month period (August 2015), during which they observed that the CDI outbreak resulted in additional costs of €46,967. The outbreak resulted in 58 bed days lost due to bed closures on the outbreak ward, with an estimated value of €34,585. Five outbreak control meetings were held, each with a mean duration of 47 min and supported by 15 h of administrative input. All meetings involved a consultant microbiologist, a senior laboratory scientist, a senior antimicrobial specialist pharmacist, an assistant director of nursing, multiple clinical nursing managers, and a number of other staff members. The mean personnel cost per meeting was €546, and the aggregate cost was €2728. The cost of outbreak-related cleaning/decontamination during August was €9654 (Ryan et al. 2017).

For the patients involved in the CDI outbreak, excluding the value of the 58 bed days lost (€34,585), costs were 30% higher (€7589 per patient) than those not involved in the outbreak during the same period (Ryan et al. 2017).

Van Beurden et al. assessed the costs of an outbreak of *C. difficile* ribotype 027 at the VU University medical center, a 750-bed tertiary care center in The Netherlands, from May 2013

to May 2014 (van Beurden et al. 2017). Several control measures were implemented, such as reinforcement of infection control, the introduction of hydrogen peroxide as disinfectant, extra cleaning, optimization of CDI diagnosis, optimization of CDI treatment, and antibiotic stewardship. Twelve meetings of the outbreak management team (consisting of five medical specialists, one infection prevention specialist, one care manager, and two co-workers from facility management) were held during the study period. Several beds had to be closed to ensure that every patient with suspected CDI was placed in contact isolation in a single room. After the implementation of these control measures, the incidence of CDI decreased to around 1.5 cases per 10,000 patient days in early 2014.

Missed revenue due to prolonged LOS among CDI patients, costs of the outbreak meetings, extra surveillance, contact isolation material (compared with the same period 1 year earlier and 1 year later), and additional microbiological diagnostics (compared with the same period 1 year earlier) were calculated directly from available data for the entire outbreak. Overall costs for additional cleaning, contact isolation, and missed revenue due to closed beds were extrapolated from the costs incurred during the previous 3 months of the outbreak. Attributable costs per item (in 2014 euros) were assessed over a 365-day period.

The total identifiable costs of this *C. difficile* outbreak were €1,222,376. Most costs (36%) stemmed from the loss of revenue resulting from decreased hospital capacity because of the increased LOS of CDI patients and the closure of multiple beds to ensure contact isolation of a single CDI patient. Twenty-five percent of the costs were from extra surveillance and the work of the department of infection control, 24% were for extra cleaning of the affected wards, 6% for extra microbiological diagnostic procedures, 3% for the outbreak meetings, and 3% for the use of extra gloves and aprons. Extra antibiotic treatment of CDI patients counted for 2% of the total costs (van Beurden et al. 2017).

As can be seen in both studies, the cost of one missed hospital admission due to closed beds or prolonged LOS is a major cost. The economic and healthcare impact of loss of revenue is very difficult to determine, and closed beds prevent inpatient accommodation, with the resultant morbidity and mortality (Singer et al. 2011). In addition, increased bed usage by medical specialties is associated with cancelled elective surgeries (Robb et al. 2004; Nasr et al. 2004).

Outbreak control generates extra work, which often relies on staff already overburdened with administrative tasks from patient care activities. Extra cleaning measures and multidisciplinary infection control teams are key elements for outbreak control (Barbut et al. 2011, 2015). Healthcare facilities should be able to assess the economic impact of an outbreak, and knowing the costs of additional measures will make it possible to establish a cost-efficient program for outbreak control, with adequate resource allocation.

It is obvious to state that accounting of LOS, cost of antimicrobial agents and other expenses of the healthcare system are unable to quantify the cost of pain, human suffering, and death.

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The Need for European Surveillance of CDI

Camilla Wiuff, A-Lan Banks, Fidelma Fitzpatrick, and Laura Cottom

For surveillance systems to be useful, they must adapt to the changing environment in which they operate and accommodate emerging public health requirements that were not conceived previously.

Joseph S. Lombardo and David L. Buckeridge

Abstract

Since the turn of the millennium, the epidemiology of *Clostridium difficile* infection (CDI) has continued to challenge. Over the last decade there has been a growing awareness that improvements to surveillance are needed. The increasing rate of CDI and emergence of ribotype 027 precipitated the implementation of mandatory national surveillance of CDI in the UK. Changes in clinical presentation, severity of disease, descriptions of new risk factors and the occurrence of outbreaks all emphasised the importance of early diagnosis and surveillance.

However a lack of consensus on case definitions, clinical guidelines and optimal laboratory diagnostics across Europe has lead to the underestimation of CDI and impeded comparison between countries. These inconsistencies have prevented the true burden of disease from being appreciated.

Acceptance that a multi-country surveillance programme and optimised diagnostic strategies are required not only to detect and control CDI in Europe, but for a better understanding of the epidemiology, has built the foundations for a more robust, unified surveillance. The concerted efforts of the European Centre for Disease Prevention and Control (ECDC) CDI networks, has lead to the development of an over-arching long-term CDI surveillance strategy for 2014–2020. Fulfilment of the ECDC priorities and targets will no doubt be challenging and will require significant investment however the hope is that both a national and Europe-wide picture of CDI will finally be realised.

C. Wiuff (✉) · A.-L. Banks
Strategic Lead Microbiology, NHS National Services Scotland, Health Protection Scotland, HAI & IC Section, Glasgow, UK
e-mail: camilla.wiuff@nhs.net; alan.banks@nhs.net

F. Fitzpatrick
Department of Clinical Microbiology, The Royal College of Surgeons in Ireland, Dublin, Ireland

Department of Clinical Microbiology, Beaumont Hospital, Dublin, Ireland
e-mail: fidelmafitzpatrick@rcsi.ie

L. Cottom
Department of Microbiology, Glasgow Royal Infirmary, Glasgow, UK
e-mail: lauracottom@gmail.com

Keywords
Surveillance · Epidemiology · Standardisation · Capacity building · Collaborative effort

1 Epidemiology of CDI in Europe

Since 1978, *Clostridium difficile* has been recognised as a leading infectious cause of antimicrobial-associated diarrhoea with symptoms ranging from mild or moderate diarrhoea to pseudomembranous colitis (PMC). Until the end of the millennium interest in this pathogen was primarily in relation to health care and impact on morbidity and mortality in the elderly. However, since 2000 there has been an explosion in reports on *C. difficile* infection (CDI) as a consequence of large increases in CDI cases (and incidence), significant changes in the clinical presentation of CDI including more severe disease, occurrence of outbreaks and descriptions of new risk factors (Freeman et al. 2010). The changes in the epidemiology of CDI leading to several outbreaks in North America and Europe have mainly been attributed to the emergence of a new hypervirulent strain PCR ribotype 027 (Kuijper et al. 2006), and to a lesser extent, PCR ribotype 078 (Goorhuis et al. 2008). Ribotype 027 was associated with more severe disease, higher mortality, increased risk of relapse and higher colectomy rates (Kuijper et al. 2006; Ricciardi et al. 2007; Warny et al. 2005). However, other ribotypes of *C. difficile* also caused outbreaks and contributed to the spread of this infection in Europe and worldwide (Bauer et al. 2011).

Outbreaks caused by PCR ribotype 027 were first reported in Europe in England and the Netherlands followed by a series of reports from several other European countries (Kuijper et al. 2007); and, by 2008 a total of 16 countries had reported this ribotype, including outbreaks in nine countries (Kuijper et al. 2008). However, the reasons for its emergence and rapid global spread remained unexplained until the genomes of a global collection of *C. difficile* 027 isolates from hospital patients between 1985 and 2010 was sequenced. Phylogenetic analysis showed that two separate lineages of 027, FQR1 and FQ2, had emerged in North America within a short period of time, after acquiring the same fluoroquinolone resistance mutation, of which one spread throughout the United States (US), South Korea and Switzerland and the other spread more widely across continents throughout Europe and Australia (He et al. 2013). Isolates obtained prior to the emergence of these two lineages were not associated with any hospital outbreaks, suggesting that they represented pre-epidemic lineages of ribotype 027. These findings highlighted the important role of selective pressure from flouroquinolone use in the evolution and spread of these two lineages in healthcare settings and highlighted the interconnectedness of the global healthcare systems due to human travel.

In the 2011/2012 European Centre for Disease Prevention and Control (ECDC) acute hospital point prevalence survey of hospital-acquired infection (HAI) *C. difficile* was the most frequently reported pathogen associated with healthcare associated gastrointestinal disease in European hospitals(accounting for 48% of all gastrointestinal disease) (ECDC 2013a). Based on this data it was estimated that 152,905 new cases of CDI occur every year in Europe with an incidence of 30 cases per 100,000 population. CDI was associated with considerable short or long term disability, and 8382 deaths per year (Cassini et al. 2016). In addition, CDI occurred increasingly in persons in the community without typical risk factors such as antimicrobial treatment and recent hospitalisation (Bauer et al. 2009; Wilcox et al. 2008) (Fig. 1).

2 Development of European CDI Guidance

In response to the changing epidemiology of CDI in Europe and North America, the European Study Group on *C. difficile* (ESGCD) published a standardised approach to detect, monitor and control CDI (Kuijper et al. 2006). In the following year, evidence-based guidance on infection prevention and control measures to limit the

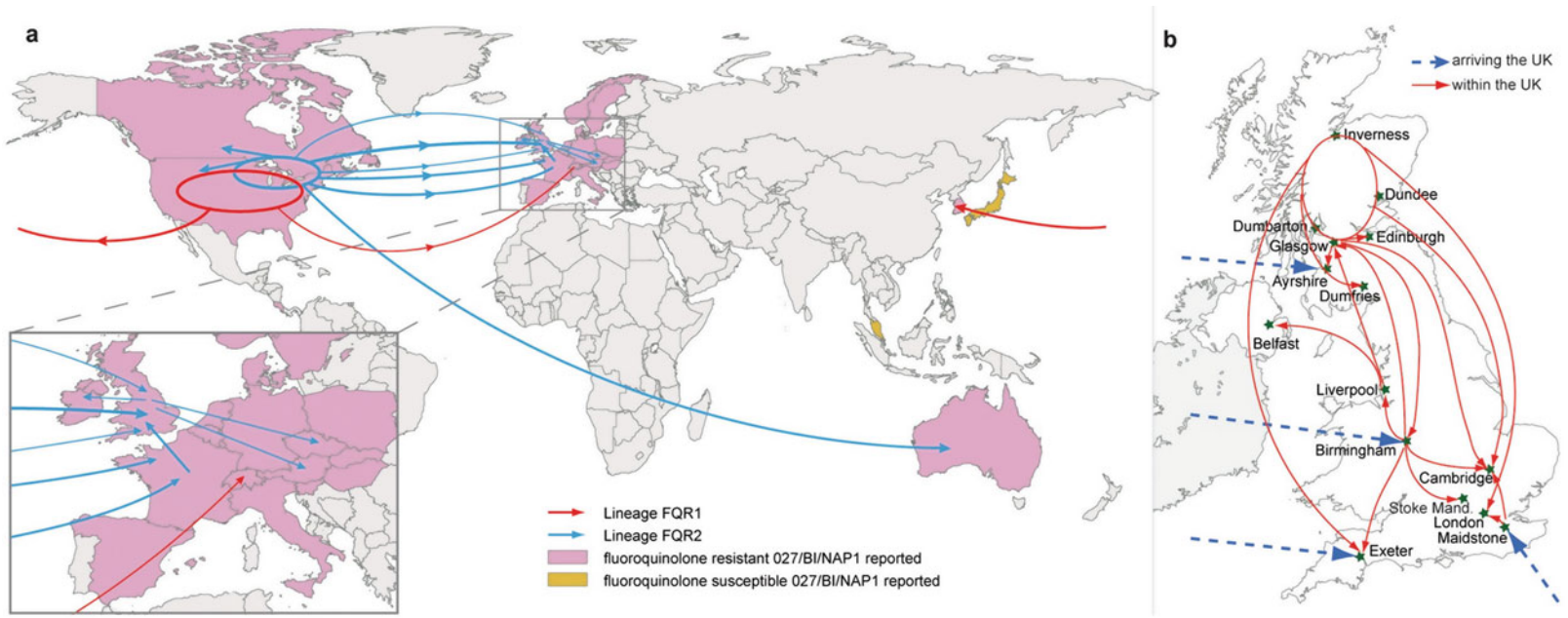

Fig. 1 Global transmission events of *C. difficile* PCR ribotype 027 (with permission from authors). Arrows indicate individual introductory transmission events of FQR1 and FQR2 (He et al. 2013)

spread of CDI was also developed. In this context CDI surveillance was identified as one of the ten most important measures in preventing and controlling CDI. Surveillance allows continuous monitoring and identification of increases in incidence and severity of disease at an early stage in order to implement changes in practice and monitor the impact of infection prevention and control efforts (Vonberg et al. 2008).

Timely feedback of surveillance data and its interpretations not only to the clinical and infection prevention and control teams but also to senior management, governing boards and administrators via the established communication system is considered essential to preventing and control CDI in hospitals (Commission. 2006). However, when CDI emerged as a serious threat to public health and patient safety at the start of this millennium, comparison of the burden of CDI between healthcare facilities and countries was problematic for a number of reasons. This included suboptimal case ascertainment and inconsistent patient sampling, inadequate laboratory diagnosis and use of non-standardised denominators for calculation of incidence rates. Standardisation of laboratory testing methodology and adherence to agreed surveillance definitions is needed for accurate monitoring of trends in hospitals and other healthcare settings (and for comparison between hospitals in their country and between countries). The suite of guidance documents on CDI diagnostics, infection prevention and control and treatment developed by ESGCD and supported by the European Society of Clinical Microbiology and Infectious Diseases (ESCMID), has provided the evidence platform for the development of European surveillance of CDI now undertaken and coordinated by ECDC (ECDC 2017) (Fig. 2).

3 Approaches to Diagnosing and Monitoring Cases of CDI

In the 1990s, a large number of diagnostic tests for *C. difficile* became commercially available, including faecal culture on selective media, detection of GDH (glutamate dehydrogenase) a non-specific antigen, direct detection of toxin A and B from stool using enzyme immune assay or cytotoxicity assay (Delmee 2001) but system-wide or national surveillance programmes remained rare. In 1993, a French multicentre point prevalence study identified *C. difficile* from 11.5% of 3921 diarrhoeal stool cultures sent to 11 microbiology laboratories (Barbut et al. 1996). Stool assays for toxin A and B became quickly the main clinical test for diagnosing CDI while stool cultures were used mainly for epidemiological investigations (Kelly and LaMont 1998). However, the majority of the available testing methods were associated with either low sensitivity or specificity, or both (see also Chap. 4), and some required culture facilities. Moreover, at that time there was no consensus across Europe in terms of diagnostic testing and surveillance due to the lack of guidance.

In 2002, the ESGCD carried out a survey of 212 laboratories in eight countries (B, DK, F, NL, G, I, SP, GB) to obtain an overview of diagnostic methods used and to estimate the average incidence of CDI across Europe (Barbut et al. 2003). A high proportion (87.7%) of laboratories performed *C. difficile* diagnosis on a routine basis, although laboratories in smaller hospitals often relied on sending samples to a bigger centre. Laboratory methods used in the surveyed hospitals included direct toxin detection (93%), culture (55%), glutamate dehydrogenase (GDH) (5.9%) but testing strategies (i.e. application of standalone tests vs. combination of tests) varied considerably between laboratories and between countries. Moreover, criteria for investigation for CDI

Vision, priorities and targets

By 2020, strong, harmonised and efficient European surveillance systems will serve the Member States, the European Commission and public health professionals by providing relevant data for the effective prevention and control of communicable diseases while minimising the burden on the Member States.

Consolidating surveillance, increasing its efficiency and enhancing the outputs and their impact

- Target 1: Critical evaluation of indicator-based EU surveillance.
- Target 2: Machine-to-machine reporting to TESSy in use by a majority of Member States.
- Target 3: Data processing is semi-automated while retaining a high quality, enabling ECDC routine surveillance outputs to be timelier, more easily available, user-customisable and thus perceived to be more useful by stakeholders.
- Target 4: The complementarity and synergy between indicator-based and event-based surveillance is improved.
- Target 5: ECDC and the Member State experts routinely communicate European surveillance findings through peer-reviewed scientific journals and other channels to better inform disease prevention and control as well as public health decision-making.

Developing standards, improving data quality and sharing best practice in surveillance

- Target 6: European surveillance standards agreed and implemented.
- Target 7: The European surveillance network culture promotes systematic learning from the example of the high-quality data providers.
- Target 8: Surveillance data quality assurance policies are in place at EU and Member State level.
- Target 9: The quality of European surveillance data has improved sufficiently to enable the routine application of time-series analysis, spatial analysis and other advanced statistical methods, where appropriate, to better monitor, understand and predict epidemiological trends of communicable diseases in Europe.

Promoting use of surveillance data

- Target 10: European event-based surveillance detects, assesses and monitors communicable disease threats to public health in near-real time.
- Target 11: European surveillance data are used to identify and monitor risk groups, where appropriate.
- Target 12: European surveillance data are used to monitor and evaluate prevention programmes against agreed indicators.
- Target 13: European surveillance data generate hypotheses for further scientific investigation and influence the EU research agenda.

Strengthening capacity in surveillance

- Target 14: ECDC works effectively with the European Commission, World Health Organization and other Agencies to promote the development of a European policy environment that supports maintenance and development of effective Member State surveillance systems and avoids overlap or duplication.

Controlling expansion

- Target 15: Routine molecular surveillance of selected pathogens is fully established at European level.
- Target 16: Relevant alternative data sources for surveillance and early threat detection have been fully explored (usefulness, data quality, potential for record linkage, etc.) and, if found to add value, are ready to be used.

Monitoring implementation of the strategy

- Target 17: The implementation of this long-term surveillance strategy is monitored and reported to the Member States on an annual basis.

Fig. 2 Targets in the ECDC long-term surveillance strategy for 2014–2020 (ECDC 2013b)

varied extensively with 58% of laboratories only testing for CDI if specifically requested by a physician while 40.7% of laboratories routinely tested for CDI when specific criteria (determined by the microbiologist) were met; most commonly loose or watery stools (40.3%), stools from patients with a history of antibiotic therapy (45.5%) and stools from nosocomial diarrhoea (57.1%). Within most countries the proportion of laboratories that used criteria for investigating CDI also varied between countries (ranging from 13% to 67%); only in the United Kingdom (UK), testing was routinely done according to specific criteria determined by the microbiologist (in 95% of laboratories). Ability to type *C. difficile* was infrequent with only 10.7% of laboratories reporting experience with typing. The inconsistent approach to diagnosing and typing CDI, including variation in the criteria for testing, laboratory methodology and strategy for testing and possible bias in the study (by inclusion of only the most responsive laboratories) raised concern of under-ascertainment due to un-diagnosed and mis-diagnosed cases and inaccurate estimates of the overall burden of disease and highlighted the need for international guidance.

The ESCGD review of the emergence of CDI in North America and Europe (Kuijper et al. 2006) specified for the first time a case definition for CDI, (including healthcare and community association), provided advice on optimal diagnostic testing and recommended that each member state should develop systematic and comprehensive surveillance systems in order to detect, monitor and respond to changes in the epidemiology of CDI, and in particular PCR ribotype 027 at both national and European levels. Following 2006 national surveillance systems were developed or expanded in countries across Europe.

In 2011, the European *C.difficile* Infection Surveillance Network (ECDIS-net) surveyed the national surveillance systems through a web-based questionnaire and reviewed extant surveillance protocols at the time. Fourteen countries were found to have a total of 18 surveillance systems in place (of which some had more than one data collection system) (Kola et al. 2016). The majority of the European surveillance systems were continuous and prospective; and 11/18 used mandatory reporting while seven used voluntary reporting. Key features of the surveillance systems varied widely with considerable variation in case definitions data collection methods, reporting and availability of reference typing. In total, 12/18 countries used the ECDC/ Centers for Disease Control and Prevention (CDC) case definitions, nine used the ECDC definitions for community associated and healthcare associated CDI while the remainder had different cut-off for healthcare association (including $\geq$48 h, $\geq$72 h, $>$3 or 4 $\geq$days after admission). Thirteen systems had a definition for severe disease while 11 had a definition for recurrence but both definitions varied between countries. Despite the increasingly recognised role of CDI in community settings only two countries (Austria and Scotland) engaged General Practitioners in their surveillance systems. Descriptive enhanced patient data were only collected in 6/18 systems and death within 30 days in five. Reference typing was performed routinely in 13/14 countries using various different criteria for submission including the presence of severe CDI, outbreaks or a more systematic periodic collection of a representative sample of cases. Finally, the reporting of the CDI burden varied widely with the use of a non-standardised denominators and stratification by geographical region, healthcare facility or laboratory making comparisons over time and between regions and facilities difficult (Kola et al. 2016).

Although, the overall capability and capacity for monitoring CDI has increased tremendously across Europe between 2003 and 2011, as of today there is still scope for improvement in diagnostic and surveillance setups in the majority of European countries.

4 Diagnostic Capability: A Pre-requisite for Surveillance

The attention given to diagnostic procedures and surveillance of CDI varied widely between countries. In 2008, with the support of ECDC a Europe-wide survey (involving 106 laboratories in 34 countries) assessed the epidemic preparedness and current CDI epidemiology aiming to ultimately build capacity for diagnosis and surveillance of CDI in each country (Bauer et al. 2011). The frequency of testing varied between countries from 3 to 141 CDI tests conducted per 10,000 patient days; and a correlation between testing rate and CDI incidence was identified (resulting in North European countries reporting the highest incidence rates). When a subset of *C. difficile* isolates were typed centrally (in Leiden, NL), a higher than expected diversity of PCR ribotypes was observed with ribotypes 001, 014/020, and 078 ribotype being the most prevalent and 027 being only the 6th most common type (4.8% of examined isolates).

Optimum laboratory diagnosis of CDI depends on testing patients at the correct time using appropriate testing methodology and strategy. A point prevalence study in multi-centre setting in Spain evaluated 988 unformed stools (from 897 patients) found 66% of CDI episodes were undiagnosed or misdiagnosed due to lack of clinical suspicion (48%) or due to using a non-sensitive test (19%) (Alcala et al. 2012). In the Europe-wide point prevalence study (EUCLID) 3800 unformed stools (from >450 hospitals in 20 countries) were tested using the recommended two-step diagnostic algorithm. In total, 25% of samples had not been tested due to lack of clinical suspicion and 23% of patients had been misdiagnosed due to using an inadequate laboratory test. It was estimated that on a single day 82 patients with diarrhoea due to *C. difficile* in hospitals across Europe were not diagnosed due to lack of suspicion (Davies et al. 2014). In addition, only 32% of participating hospitals used the optimum diagnostic method at the first measurement (in 2011–2012) whereas this had improved at the second measurement (in 2012–2013) when 48% used the optimum method.

These two recent studies highlighted again variation in awareness and capability and capacity to diagnose, sub-type, report, collect patient risk factor data and monitor CDI across Europe and as a consequence the true burden of CDI and distribution of ribotypes is unclear.

5 Benefits of Mandatory Surveillance: Experiences from United Kingdom

Prior to the year 2000, data on *C. difficile* was collected on a voluntary basis. In the UK, a steady increase in laboratory reports was observed during the 1990s (Department of Health and Health Protection Agency 2008; Health Protection Scotland 2006). In England, this was suggested to reflect a failure to implement guidelines published in 1994, as well as the result of increased testing and awareness of CDI, and an increase in community-associated CDI (Department of Health and Health Protection Agency 2008).

The increasing CDI rates and emergence of ribotype 027 precipitated the implementation of mandatory national surveillance of CDI by England, Wales and Northern Ireland in 2004, and by Scotland in 2006. Initially, the surveillance programmes included only those aged 65 years and above, but have since expanded to include all ages except the very young (Pearson 2009; Health Protection Scotland 2010). Between 2003 and 2007, several large hospital outbreaks of CDI occurred, involving ribotype 027 (two in England and one in Scotland), which brought CDI to the public attention (Healthcare Commission 2006; The Vale of Leven Hospital Inquiry 2014). Among the many key findings and recommendations contained within the critical reports that followed was an acknowledgement of a lack of appropriate surveillance mechanisms, both locally and nationally, that could have identified an outbreak, and the need for formal communication channels to be in place to allow information on CDI numbers and severity to be quickly disseminated. These major incidents were quickly followed by the setting of national targets within the UK to reduce CDI rates by 30% (Duerden 2011; Scottish Government 2012).

Around the same time as the UK was implementing national surveillance schemes, the ECDC and the U.S. CDC produced recommendations for surveillance of CDI (Kuijper et al. 2006; McDonald et al. 2007). The publication of these documents enabled a standardised surveillance case definition to be developed as well as definitions for severe CDI, recurrence, outbreaks and origin of infection that could be used as necessary within a surveillance programme. Shortly thereafter, evidence-based recommendations for infection prevention and control of CDI were published (Vonberg et al. 2008), with strong recommendations for the implementation of routine surveillance of CDI, including the setting of thresholds to identify outbreaks, emphasis on the importance of early diagnosis, and awareness of changes in incidence or severity of disease. The foundations were laid for the development of a range of tools and strategies to deal with the CDI epidemic (Department of Health and Health Protection Agency 2008; Health Protection Scotland 2009). Continuous and prospective surveillance at national level in healthcare and community settings was mandated by governments in England and Scotland; and real-time 'local surveillance' (by ward, unit or facility) to monitor the number of cases, disease severity, surgery and mortality rates with a duty for the multidisciplinary clinical and infection prevention control team to investigate the root cause of any anomalies or 'exceedances' identified at local level in order rectify deficiencies in patient care and/or infection control (Department of Health and Health Protection Agency 2008; Health Protection Scotland 2014). The heightened focus on local surveillance was a result of recommendations emerged from investigations of previous hospital outbreaks (The Vale of Leven Hospital Inquiry 2014; Healthcare Commission 2006).

CDI incidence rates in the UK peaked during 2007/2008, and have since been steadily declining (McDonald et al. 2007; Vonberg et al. 2008; Scotland 2014). In the 4-year period from 2007 to 2010, significant reductions in the incidence of CDI were observed in England (54%) and Scotland (72%) (Duerden 2011; Health Protection Scotland 2012); both downward trends that have continued to date.

In order to respond to the public health need and to provide more detailed epidemiological information on circulating strains of *C. difficile* a network of reference laboratories was established in England (the *Clostridium difficile* Ribotyping Network, CDRN) with collaborative links to a single reference laboratory in Scotland. Investigations and isolate typing criteria focussed on severe cases of CDI, clusters of cases and unexplained increases in incidence in both countries. In the first 3 years after establishing these laboratory services, the prevalence of ribotype 027 decreased markedly in England (from 55% to 21%). This change in distribution of ribotypes in England coincided with a 61% reduction in reports of CDI cases (from 36,095 in 2008–2009 to 21,698 in 2010–2011) and a decrease in reports of complications, including mortality (Wilcox et al. 2012). Likewise, the three major epidemic ribotypes 027, 001 and 106 were gradually replaced with other less prevalent ribotypes while rates of CDI were reducing in Scotland (Wiuff et al. 2011, 2014). It has been argued that the timely provision of ribotype information to infection prevention and control teams has enabled the targeting of interventions and resources on high incidence settings and in particular those with a high prevalence of 027 (Wilcox et al. 2012). However, there might also have been an additive effect of heightened awareness and an improved understanding of the need for clinical vigilance and aggressive intervention due to CDI caused by virulent strains such as 027.

The overall decrease in CDI can be attributed to a multi-disciplinary approach including evidence-based guidance for the treatment and management of CDI patients, restrictive antimicrobial stewardship policies, and, arguably due to the government targets for reducing CDI (Duerden 2011; Nathwani et al. 2011; Lawes et al. 2017). The establishment of mandatory surveillance systems across the UK driven by government policy was instrumental to the development of standardised, evidence based

diagnostic testing and expansion of national reference laboratory services. The success of the UK surveillance programmes has undoubtedly been due to the rapid and joined up development of diagnostic and surveillance capability and capacity with coverage of all healthcare settings.

Standardised national surveillance programmes are crucial to enable the monitoring of trends within and between countries, as well as facilitating the monitoring of interventions for improving care and outcomes of CDI patients. Central to all of this has been the adoption within the UK national surveillance programmes of standardised protocols for sampling, testing, typing of isolates, reporting and feeding back data in management structures. This has resulted in more solid reporting and accountability structures that lead to rapid responses to increases in CDI.

6 The Need for European Surveillance of CDI

Suboptimal laboratory diagnostics, a previous lack of consensus on optimal testing methodology for CDI and availability of typing across Europe has lead to under-diagnosis and impeded comparison between countries. Underestimation of CDI has also resulted from a deficiency in uniformity of case definitions, clinical algorithms and recognition amongst clinicians of when to suspect CDI. These inconsistencies have prevented the true burden of disease from being appreciated.

Acceptance that a multi-country surveillance program is required not only to detect and control CDI in Europe, but for a better understanding of the epidemiology, has paved the way forward. Deserved attention afforded to this infection and the concerted efforts to optimise diagnostic strategies have built the foundations for a more robust, unified surveillance. The hope now is that both a national and Europe-wide picture of CDI can be finally realised.

With funding from the ECDC, the ECDIS-Net a consortium of universities and government agencies from the Netherlands, Germany and the UK in collaboration with ECDC (that evolved from the earlier ECDIS study Group) was established in 2010 to support capacity building for surveillance at the European level. The formation of this multination surveillance collaboration was a result of heightened awareness in clinical communities across Europe following the culmination of a decade of published literature detailing the emerging challenges of CDI, with papers highlighting the changing epidemiology of CDI, the increased reporting of outbreaks and the identification of hypervirulent ribotypes such as 027.

One of the formative documents, which included collaboration with CDC, published, agreed CDI case definitions and issued recommendations regarding surveillance (Kuijper et al. 2006). Thereafter, the ECDIS Study Group survey of European hospitals (Bauer et al. 2011) identified considerable variation in methodology used in national surveillance programs; the significant variation in hospital procedures and the availability of typing which also limited comparison between countries. Perhaps the most startling finding was a CDI incidence of 70% higher than previously reported studies from 2005 (Barbut et al. 2007).

Importantly, the European Study Group (ESGCD) coordinated these developments in collaboration with an increasing number of national surveillance and laboratory coordinators from the participating countries and created a professional network that met and communicated frequently allowing extensive discussions of a wide range of aspects of CDI paving the way for achieving consensus on the current evidence base on CDI. A need for standardisation was further supported by a review of existing national CDI surveillance schemes which showed large variations in the surveillance definitions used, especially with regards to inclusion criteria for cases and tying, choice of denominator, and origin of infection of CDI (Kola et al. 2016). As a result of these Europe-wide collaborative efforts the first ECDIS-Net protocol, a precursor for a European protocol, was developed. A draft

protocol for CDI surveillance based on the above recommendations was piloted in 2013, with the results being published in 2016 and highlighted that the foundations for Europe-wide surveillance were in place. Furthermore, consideration was given to resource-poor countries that lacked facilities to perform internationally standardised PCR for ribotyping.

These foundations referred to by van Dorp et al., are the cumulation of the body of work supported by ECDC over the past decade in developing an evidence based consensus approach which could be applied across a myriad of healthcare and laboratory settings to allow a high-level surveillance system to come to fruition. In terms of progress, while a 2002 surveillance study of diagnostic methods and protocols, found that only 55% of laboratories were capable of culturing *C.difficile* (Barbut et al. 2003), in 2013, 95% performed CDI diagnostics (van Dorp et al. 2016a).

EDCD developed an over-arching long-term surveillance strategy for 2014–2020 (ECDC 2013b), in which the ECDC 'European Surveillance of *Clostridium difficile* infections – surveillance protocol 2.3' plays a disease specific role (Control ECDC 2017). The strategy has six priorities:

1. Consolidating surveillance, increasing its efficiency and enhancing the outputs and their impact
2. Developing standards, improving data quality and sharing best practices in surveillance
3. Promoting use of surveillance data
4. Strengthening capacity in surveillance
5. Controlling expansion
6. Monitoring the strategy

The overall strategic approach has been further subdivided into 17 individual targets to help guide the development of surveillance programmes.

Perhaps the most challenging of these for regional and national programmes looking to contribute to the Europe-wide level program are:

(a) Target 2 – Machine-to-machine reporting to The European Surveillance System (TESSy) in use by a majority of Member States.
(b) Target 3 – Data processing is semi-automated while retaining a high quality, enabling ECDC routine surveillance outputs to be timelier, more easily available, user-customisable and thus perceived to be more useful by stakeholders.
(c) Target 10 – European event-based surveillance detects, assesses and monitors communicable disease threats to public health in near-real time.
(d) Target 12: European surveillance data are used to monitor and evaluate prevention programmes against agreed indicators.

All of these targets require significant infrastructure investment/realignment to achieve, and run the risk of resource-poor member states being left behind. The EU carries a diverse mix of countries all with individual priorities and levels of expenditure. Most EU countries fall within the World Bank definition of a high-income country. Three Countries: Bulgaria, Croatia and Romania fall into the category of upper-middle income, and while being wealthy when compared to low income nations, their relative health expenditure is typically 20% of that of high income EU nations (World Bank 2017). Even amongst high income EU members GDP per capita can vary by as much as 400%. Priorities at an EU and national level will have to be decided to minimise the effect of expected budget cuts and regional variation on expenditure. Capital funding from central European organisations or the ECDC will not be available to fund member states public health infrastructure to address any imbalance. As such, nations with robust and successful surveillance networks will need to lead a concerted effort in sharing technical expertise and advice, to allow other nations to develop their own sustainable and integrated surveillance systems which can be integrated into an EU wide programme.

7 Conclusions

Significant reductions in CDI have been reported in countries across Europe, however, incidence rates vary widely between countries and their capacity for surveillance, diagnostics and epidemiological typing is highly variable (Kola et al. 2016; van Dorp et al. 2016b; Bauer et al. 2011). Therefore, there is a need to strengthen the capacity for surveillance of CDI within Europe; this is both feasible and manageable (Control 2017; van Dorp et al. 2016a).

A standardised approach to surveillance, diagnostics and typing would allow the estimation of the total burden of CDI in Europe (and its member states) and the continuous monitoring of incidence, severity, outcome and risk factors for developing CDI would lead to improved management and control of CDI.

In countries where large reductions in CDI incidence have been achieved comprehensive national surveillance programmes have been a key driver in the standardisation of diagnostic approach, sampling and reporting practices and in developing coordinated approaches and resources to infection prevention and management of CDI by highlighting the evolving epidemic of CDI.

Additional benefits of laboratory based surveillance could be achieved from the introduction of genetic finger printing using multi-locus variable-number tandem repeat (MLVA typing) and whole genome sequencing to investigate clusters and cross-transmission routes (Fawley et al. 2011; Eyre et al. 2013; Eyre and Walker 2013) and identify regional and inter-continental spread of new potentially epidemic and virulent lineages of *C. difficile* (He et al. 2013).

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Diagnostic Guidance for *C. difficile* Infections

Monique J. T. Crobach, Amoe Baktash, Nikolas Duszenko, and Ed J. Kuijper

Abstract

Diagnosis of *Clostridium difficile* infection (CDI) can be challenging. First of all, there has been debate on which of the two reference assays, cell cytotoxicity neutralization assay (CCNA) or toxigenic culture (TC) should be considered the gold standard for CDI detection. Although the CCNA suffers most from suboptimal storage conditions and subsequent toxin degradation, TC is reported to falsely increase CDI detection rates as it cannot differentiate CDI patients from patients asymptomatically colonised by toxigenic *C. difficile*. Several rapid assays are available for CDI detection and fall into three broad categories: (1) enzyme immunoassays for glutamate dehydrogenase, (2) enzyme immunoassays for toxins A/B and (3) nucleic acid amplification tests detecting toxin genes. All three categories have their own limitations, being suboptimal specificity and/or sensitivity or the inability to discern colonised patients from CDI patients. In light of these limitations, multi-step algorithmic testing has now been advocated by international guidelines in order to optimize diagnostic accuracy. Despite these recommendations, testing methods between hospitals vary widely, which impacts CDI incidence rates. CDI incidence rates are also influenced by sample selection criteria, as several studies have shown that if not all unformed stool samples are tested for CDI, many cases may be missed due to an absence of clinical suspicion. Since methods for diagnosing CDI remain imperfect, there has been a growing interest in alternative testing strategies like faecal biomarkers, immune modulating interleukins, cytokines and imaging methods. At the moment, these alternative methods might play an adjunctive role, but they are not suitable to replace conventional CDI testing strategies.

Keywords

Clostridium difficile · Diagnostics · Testing · Algorithmic testing

M. J. T. Crobach (✉) · A. Baktash · N. Duszenko
Department of Medical Microbiology, Centre for Infectious Diseases, Leiden University Medical Centre, Leiden, The Netherlands
e-mail: m.j.t.crobach@lumc.nl; A.Baktash.MM@lumc.nl; nduszenko@gmail.com

E. J. Kuijper
ESCMID Study Group for C.difficile (ESGCD), Basel, Switzerland

Department of Medical Microbiology, Center of Infectious Diseases, Leiden University Medical Center, Leiden, The Netherlands
e-mail: E.J.Kuijper@lumc.nl

1 Introduction

Diagnosis of *Clostridium difficile* infection (CDI) is challenging, as there is no optimal laboratory assay and even no universal reference test. Due to imperfect assays, combinations of assays to optimize their performance have been proposed. However, diverse testing strategies are applied across laboratories. These diverse testing strategies may impact CDI incidence rates. In addition to the conventional testing methods, alternative methods are sometimes applied either to diagnose CDI or as an aid to predict severity. Here, we will describe the diverse testing strategies with their advantages and limitations and clinical relevance.

2 Reference Tests

The diagnosis of CDI relies on one of two approaches: demonstrate the presence of toxins responsible for the clinical manifestations of CDI, or demonstrate the presence of *C. difficile* which is capable of producing toxins (so-called toxigenic *C. difficile*) (Planche and Wilcox 2011) (Table 1). The reference test for detection of toxins in stools is the cell cytotoxicity neutralisation assay (CCNA) (Planche and Wilcox 2011; Burnham and Carroll 2013). For CCNA, stool sample filtrate is inoculated onto an in vitro cell monolayer, using cell lines such as Vero cells, HeLa cells, human foreskin fibroblast cells or Hep-2 cells. At 24- and 48-h intervals, these cultures are evaluated for the characteristic rounding effect engendered by toxin B. Reversal of this effect by toxin B antitoxin demonstrates the specific role of toxin B in inducing the cytopathic effects observed, and thus its presence (Delmee 2001; Burnham and Carroll 2013). The reference test for detection of toxigenic *C. difficile* is toxigenic culture (TC) (Planche and Wilcox 2011; Burnham and Carroll 2013). For TC stool samples are inoculated onto selective media and incubated for at least 48 h (Hink et al. 2013). Colonies suspected of being *C. difficile*, by e.g. Gram staining, colony morphology, odour or more sophisticated techniques, are isolated. Their toxigenic potential is assessed by testing for *in vitro* toxin production via the aforementioned CCNA, by enzyme immunoassays (EIA) for toxins A/B, or by testing for toxin-producing genes via nucleic acid amplification tests (NAAT) (Burnham and Carroll 2013).

During the last years, there has been debate on which of these two reference tests represents true disease, as the CCNA detects in vivo toxins while TC detects in vitro toxin production (Planche and Wilcox 2011). There is a growing body of evidence demonstrating that toxigenic strains are often carried asymptomatically (Kyne et al. 2000; Loo et al. 2011). TC is not able to make a distinction between asymptomatic carriage of toxigenic *C. difficile* strains and true infection. Studies have shown that patients with

Table 1 Available assays for CDI detection

Type of assay	Target of detection	Detected condition
Culture	*C. difficile*	*C. difficile* colonisation, can be CDI
Glutamate dehydrogenase enzyme immunoassay (GDH EIA)	Glutamate dehydrogenase	*C. difficile* colonisation, can be CDI
Toxins A/B enzyme immunoassay (Tox A/B EIA)	Toxins A and B	CDI
Nucleic acid amplification test (NAAT)	*TcdB* and/or *TcdA* genes, sometimes *cdt* and deletion in *tcdC*	Toxigenic *C. difficile* colonisation, can be CDI
Cell cytotoxicity neutralization assay (CCNA)	Toxin B	CDI
Toxigenic culture (TC)	*C. difficile* and thereafter in vitro toxin production by Tox A/B EIA, NAAT or CCNA	Toxigenic *C. difficile* colonisation, can be CDI

CDI Clostridium difficile infection

positive CCNA or Tox A/B EIA have a worse prognosis than patients who test only positive in TC, indicating that this latter category may actually be colonised patients instead of patients with true CDI (Planche et al. 2013; Polage et al. 2015). Although CCNA may therefore better reflect true CDI, it is this reference test that suffers most from lack of standardization and suboptimal storage or collection conditions, thereby possibly generating false-negative results. Both reference tests are laborious and expensive and require trained personnel. Therefore, easy-to perform rapid assays have been developed. These include enzyme immunoassays for GDH, enzyme immunoassays for Toxins A/B and during the last decade, NAATs for toxin genes have become available. Given their ease of use and rapid turnaround time, these rapid tests have become the mainstays of CDI diagnosis in a clinical setting.

3 Rapid Assays

Reference methods are accurate, but the lengthy, laborious nature of such testing precludes its application in a clinical setting. Rapid tests are ideally suited for clinical use, but each suffers from its own shortcomings. Tox A/B EIAs directly detect free toxins in stools and are therefore believed to correlate to clinical symptoms (Polage et al. 2015). They are cheap and easy to use. However, sensitivity of Tox A/B EIAs is suboptimal. Compared to CCNA, pooled sensitivity of Tox A/B EIA was 83%. In comparison to toxigenic culture, pooled sensitivity of Tox A/B EIA was as low as 57%. Pooled specificity of Tox A/B EIAs was however reported to be as high as 99%, both compared to CCNA and TC (Crobach et al. 2016).

GDH EIAs are also easy to perform and cheap. They detect glutamate dehydrogenase, an enzyme that is produced by both toxigenic and non-toxigenic strains. GDH EIAs are sensitive (pooled sensitivity compared to CCNA and TC 94% and 96%, respectively) (Crobach et al. 2016). However, they cannot make a distinction between the presence of toxigenic or non-toxigenic strains and are thus less specific to detect true disease. This was demonstrated by a specificity of only 90% in comparison to CCNA (Crobach et al. 2016).

NAATs include PCR assays, helicase-dependent amplification assays and loop-mediated isothermal amplification assays. Most of these assays target conserved regions within the gene for toxin B (*tcdB*), although some target a highly conserved sequence of the toxin A gene (*tcdA*). Assays that detect the ribtoype 027/NAP1 strain (and related ribotypes) are also available, these detect the genes for binary toxin (*cdt*) and the deletion at nucleotide 117 on the regulatory gene *tcdC*.

NAATs are sensitive (sensitivity compared to CCNA and TC 96% and 95%, respectively) (Crobach et al. 2016). As they only detect toxigenic strains instead of all *C. difficile*, they are more specific than GDH EIA (specificity compared to CCNA and TC 94% and 98%, respectively) (Crobach et al. 2016). However, NAATs only detect the presence of toxin genes and hence the toxin producing capacity of *C. difficile*. Therefore, a major drawback of NAAT is that in addition to CDI cases, it will also detect asymptomatic carriers of toxigenic *C. difficile*.

4 Recommended Testing Algorithms

Although it would be the easiest to use one of the rapid assays for CDI detection in daily practice, this will falsely impact CDI detection rates. First of all, GDH EIA and NAAT results do not directly correlate with clinical symptoms possibly leading to over diagnosis of CDI. Second, all of these three tests, even the very specific Tox A/B EIAs, are not specific enough to be used as a

stand-alone test (Crobach et al. 2016). Namely, most of the samples submitted for CDI testing will not have the disease. Assuming a CDI prevalence rate of 5% among submitted samples, positive predictive values of the most specific assays (Tox A/B EIA) range from 69% to 81%, indicating that 19–31% of samples with a positive test result actually do not have the disease (Crobach et al. 2016).

In light of these limitations of the rapid assays, common guidelines for CDI diagnosis put forth by the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) and the Society for Healthcare Epidemiology of America/the Infectious Diseases Society of America (SHEA/IDSA) recommend the use of multi-step algorithmic testing to maximize diagnostic accuracy (Crobach et al. 2016; Cohen et al. 2010). The premise of this strategy is sequential testing that most efficiently uses molecular tests' different strengths. First, stool samples are screened by a sensitive test. According to the ESCMID guidelines this could either be either be GDH EIA or NAAT, while SHEA/IDSA guidelines recommend the use of GDH EIA as a first step (Cohen et al. 2010; Crobach et al. 2016). The high sensitivity of these tests provides them a high negative predictive value (NPV) with which to be reasonably confident that a negative test is in fact indicative of no CDI. In this manner, a large proportion of diarrheal cases can be quickly ruled out for CDI. If the first test is positive, reflex testing occurs by Tox A/B EIA (Crobach et al. 2016) or CCNA (Cohen et al. 2010), a test of high specificity with a correspondingly high positive predictive value (PPV) as it is now used in selected samples with a higher pre-test probability of CDI. Thus, a positive result on this second test is likely indicative of CDI. In the event of a positive first test and a negative second, the result is considered an ambiguous one in need of resolution by clinical evaluation or further testing, e.g. via TC. In the ESCMID guidelines, an alternative algorithm starting with both GDH and Tox A/B EIA in the first step, optionally followed by TC or NAAT in case of ambiguous results, is mentioned as a suitable equivalent (Crobach et al. 2016) (Fig. 1).

The gains in diagnostic accuracy achieved by such algorithmic testing are substantial. It was calculated that in a typical endemic setting of 5% CDI prevalence among submitted samples, PPV and NPV of the most accurate algorithm, NAAT followed by Tox A/B EIA, are 98.5% and 98.9%, respectively. In comparison, PPV and NPV of standalone NAAT are 45.7% and 99.8%, respectively; PPV and NPV of standalone Tox A/B EIA are 81.4% and 99.1%, respectively (Crobach et al. 2016).

Algorithmic testing does have its own drawback: increased turnaround time. While patients with a negative result can quickly be ruled out for CDI, actually establishing a CDI diagnosis requires two positive tests, inevitably requiring more time, especially if CCNA is used as the second test as recommended by IDSA/SHEA guidelines. This is a non-trivial drawback, as it has been shown that decreasing the time to diagnosis positively affects patient outcomes (Barbut et al. 2014). Numerous studies have found an association between low CT values and toxin presence or outcome (Chung and Lee 2017; Jazmati et al. 2016; Reigadas et al. 2016; Dionne et al. 2013; Leslie et al. 2012; Kaltsas et al. 2012). Efforts have been made to address the longer turnaround time of algorithms by examining whether quantitation of NAAT results by cycle threshold (CT), the point during a PCR when product begins being fluorescently detectable that serves as an indirect measure of the starting number of DNA copies in a sample, can be used by itself to establish a CDI diagnosis (Senchyna et al. 2017; Crobach et al. submitted). Although studies indicate that NAAT CT values can be used to predict the toxin status, the relationship between the two is not strong enough to negate the need for toxin testing by a second test at his moment (Senchyna et al. 2017; Crobach et al. submitted). For now, the increased

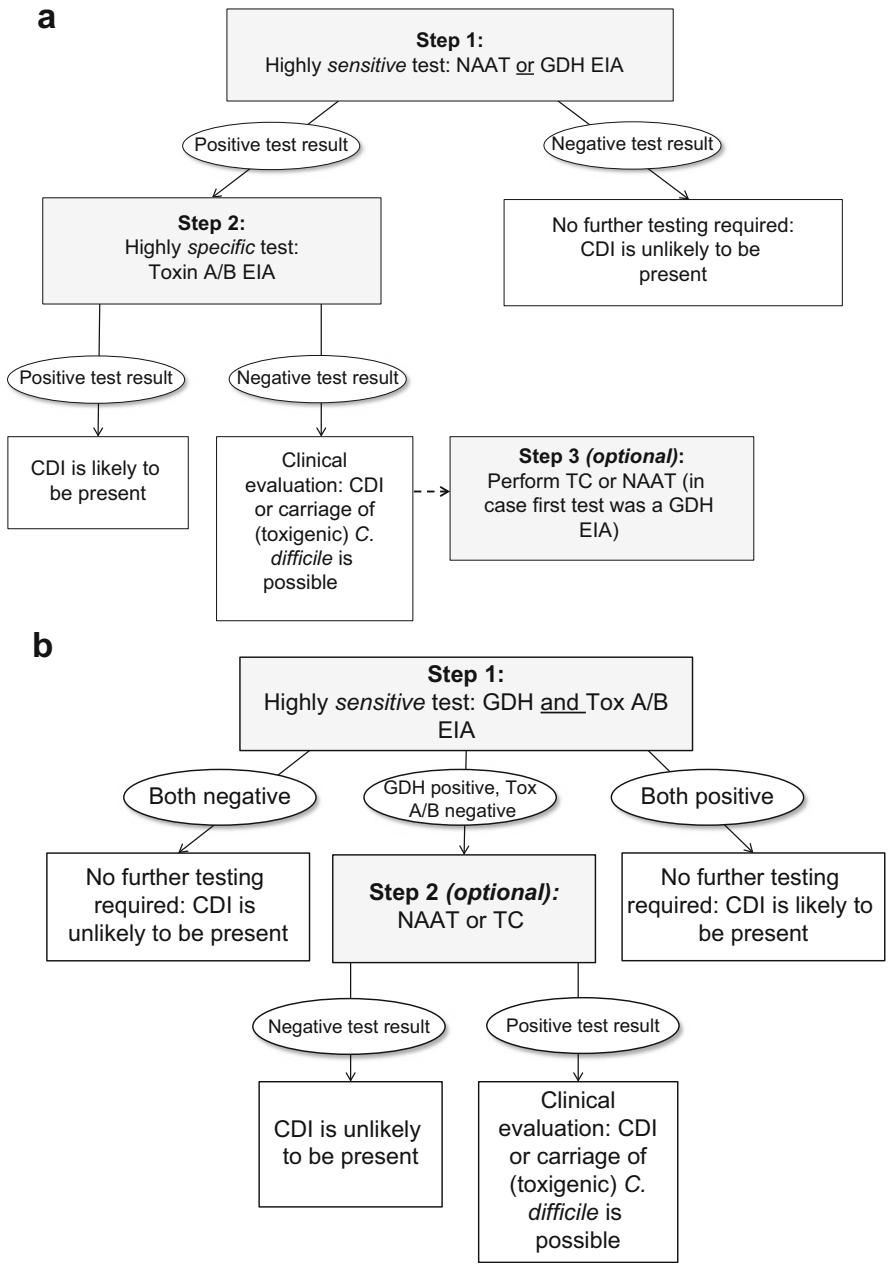

Fig. 1 Algorithms for CDI testing as recommended by ESCMID guidelines. (**a**) GDH or NAAT- Tox A/B algorithm, (**b**) GDH and Tox A/B – NAAT/TC algorithm. *CDI Clostridium difficile* infection, *GDH* glutamate dehydrogenase, *NAAT* nucleic acid amplification test, *TC* toxigenic culture, Tox A/B, toxin A/B; *EIA* enzyme immunoassay (Figure reprinted from Crobach et al. CMI 2016;22:S63, https://doi.org/10.1016/j.cmi.2016.03.010, available under a Creative Commons Attribution-NonCommercial-NoDerivates License (CC BY NC ND), https://creativecommons.org/licenses/by-nc-nd/3.0/legalcode)

turnaround time of algorithms must be accepted, as algorithms seem to represent the most accurate, clinically implementable testing strategy for CDI diagnosis.

There are still controversies concerning the use of NAATs in CDI diagnosis. ESCMID guidelines recommend against their use as stand-alone tests based on the limitations that we described above, but they do recommend their use as a first step in an algorithm (Crobach et al. 2016). The older SHEA/IDSA guidelines indicate that NAAT testing may ultimately address testing concerns although more data are needed before this methodology can be recommended for routine testing (Cohen et al. 2010). However, others prefer PCR testing over Tox A/B EIA testing and over GDH-Tox A/B algorithms, because of superior sensitivity (Surawicz et al. 2013). They do indicate that NAAT should only be applied in patients with diarrhoea, to overcome the problem of false positive results (Surawicz et al. 2013).

Although TC is not an efficient method for screening large numbers of diarrheal samples for potential CDI, it nevertheless remains an important technique for laboratories to be able to carry out. Isolating *C. difficile* by TC serves several post-diagnostic purposes. These include antimicrobial susceptibility testing and molecular typing of isolates. For molecular typing, pulsed-field gel electrophoresis (PFGE) was considered the standard method in North America, with the resulting banding patterns described as "North American pulse-field" (NAP) types (Killgore et al. 2008; Kristjansson et al. 1994). In Europe PCR ribotyping is most commonly applied, with the resulting patterns described as PCR ribotypes (Stubbs et al. 1999; Bidet et al. 1999). Recently, reference laboratories in Canada and the US have also applied PCR ribotyping, using a standardized protocol for capillary-electrophoresis PCR ribotyping (Fawley et al. 2015). While PFGE and PCR ribotyping are the methods of choice for surveillance purposes, additional typing methods like multilocus variable-number of tandem-repeat analysis (MLVA), multilocus sequence typing (MLST) and whole-genome sequencing (WGS) are mainly of use for outbreak investigations (Knetsch et al. 2013; van den Berg et al. 2007; Maiden et al. 1998). Furthermore, TC may be needed to resolve discrepant results of algorithmic testing where *C. difficile* is detected by GDH EIA or NAAT but toxin is not. A positive TC result rules out a false positive GDH EIA/NAAT result in these patients. In that case, clinical evaluation is needed; these patients can either be CDI patients with a false negative Tox A/B EIA result due to low toxin levels or degradation of toxins, or *C. difficile* carriers.

5 Selection of Stool Samples

Testing for CDI should only be performed on unformed stools as the presence of clinical symptoms is a prerequisite to diagnose CDI (Crobach et al. 2016; Cohen et al. 2010; Surawicz et al. 2013). However, it can be difficult to assess which unformed stools should be tested. A large study in 482 hospitals across 20 - European countries showed that 23% of samples positive for CDI were not diagnosed by the local hospital because of an absence of clinical suspicion (Davies et al. 2014). It was reported that mostly younger patients and patients who are not hospitalized or have been hospitalized for <3 days are inadvertently not tested for CDI (Davies et al. 2014; Alcala et al. 2012). In general practice, CDI is also often missed due to lack of suspicion, as was shown in a study among 12,714 unformed stool samples (Hensgens et al. 2014). In this study, general practitioners requested CDI testing in 7% of unformed stool samples, thereby detecting only 40% of all CDI cases (Hensgens et al. 2014). In light of these problems, testing of all submitted unformed stool samples is now endorsed by the ESCMID guidelines (Crobach et al. 2016). This approach has been shown to increase the diagnostic yield

(Davies et al. 2014; Reigadas et al. 2015). Restricting CDI testing to liquid samples instead of all unformed samples seems to be too stringent and may cause the diagnosis of CDI to be missed (Berrington and Settle 2007).

A special situation exists for patients with ileus due to CDI. In this case, formed stools or rectal swabs can be tested for CDI (McFarland et al. 1987; Rogers et al. 2013). Although perirectal swabs have also been proposed as suitable alternatives, their use may depend on the presence of faecal staining on the swab (Rogers et al. 2013; Kundrapu et al. 2012).

In young children, high *C. difficile* colonisation rates have been described (Enoch et al. 2011). Young children can also test positive for toxins, without clinical significant disease. On the other hand, the incidence of CDI among hospitalized children has been increasing (Schutze and Willoughby 2013). CDI testing is therefore burdensome in young children, and should always include clinical evaluation. Routine testing for CDI in children <1 year should be avoided, according to guidelines launched by the American Academy of Pediatrics (Schutze and Willoughby 2013). For children between 1 and 3 years of age with diarrhoea, CDI testing can be considered, but testing for other causes, particularly viral infections, is recommended first (Schutze and Willoughby 2013). For children above 3 years of age, normal testing procedures can be followed (Schutze and Willoughby 2013; Crobach et al. 2016).

6 Repeat Testing

Before the introduction of algorithms, lack of confidence in Tox A/B EIAs led to the submission of multiple stool samples during one diarrheal episode. Several studies sought to determine the yield of such repeat testing. Diagnostic yield can either be expressed in the percentage of first test negative samples converting to positive in a repeat test, or the percentage of positive samples that is detected by repeat testing. After a first negative Tox A/B EIA result, it was reported that 0.9–2.5% of samples test positive in a repeat sample submitted within 7 days (Aichinger et al. 2008; van Prehn et al. 2015). These samples constitute around 9% of all positive samples (Aichinger et al. 2008; van Prehn et al. 2015). Although the former studies were performed in endemic situations, a study performed during an outbreak situation demonstrated that there was a definite diagnostic yield of retesting in such a situation; of all samples submitted for repeat Tox A/B EIA testing, 8.2% tested positive. These samples constituted 5% of all positive CDI samples (Debast et al. 2008).

The utility of repeat NAAT testing has been evaluated in several studies, too. The percentages of samples that were positive within 7 days after a negative test range from 0.9% to 2.9% (Green et al. 2014; Luo and Banaei 2010; Khanna et al. 2012; Aichinger et al. 2008; van Prehn et al. 2015). The number of CDI cases detected by a repeat test range from 1.7% to 4.5% (Aichinger et al. 2008; van Prehn et al. 2015). The chance of turning positive was lower in the first 7 days after a negative NAAT result than in the 7–14 days period after the negative test result (Luo and Banaei 2010; Khanna et al. 2012). In one study, a history of CDI seemed to increase the risk of a positive repeat NAAT result within 7 days after a first negative test (Green et al. 2014).

The general consensus is that in a non-epidemic situation, the diagnostic yield of repeat testing by both Tox A/B EIA and NAAT is too low, and therefore, repeat testing should be discouraged (Cohen et al. 2010; Crobach et al. 2016; Surawicz et al. 2013). If an algorithm is used instead of stand-alone NAAT or Tox A/B EIA, the even higher predictive values make repeat testing redundant. However, in epidemic situations, or in patients with very high clinical suspicion, repeat testing may be of value (Crobach et al. 2016).

Sometimes, repeat samples are taken after CDI treatment as a test of cure. However, after resolution of diarrhoea, patients can still test positive for toxins (Wenisch et al. 1996). Furthermore, patients can become asymptomatic carriers after treatment for CDI: one small study showed that 1–4 weeks after treatment, 29/56

(56%) of patients were found to be asymptomatic carriers of *C. difficile* (Sethi et al. 2010). Testing for cure is therefore not recommended in current guidelines (Crobach et al. 2016; Cohen et al. 2010; Schutze and Willoughby 2013).

7 Consequences of Testing Strategy on CDI Incidence/ Reporting Rates

Despite the common recommendations of ESCMID and SHEA/IDSA advocating the use of algorithmic testing in CDI diagnosis, testing methods between hospitals vary widely. A large study across 60 European hospitals found that only 64% of hospitals use a recommended testing algorithm for CDI testing (Davies et al. 2016). Data from the US show, that in 2012, 51% of hospitals was still relying on stand-alone Tox A/B EIA (CDC 2012). Standalone use of Tox A/B EIAs decreased in response to recognition that low sensitivities were leading to CDI under-diagnosis, and consequently commercially available NAATs began emerging as viable replacements, particularly because the high sensitivity of NAATs would directly address the shortcoming of Tox A/B EIA. For example, in Canada, a cross-sectional study across Quebec showed that the number of hospitals detecting toxigenic *C. difficile* instead of *C. difficile* toxins increased significantly between 2010 and 2014, and in 2014 stand-alone NAAT was the most common applied assay (21% of hospitals) (Bogaty et al. 2017). But recent work has suggested that NAATs may now be causing CDI over-diagnosis, leading to an overestimation of CDI incidence in hospitals using NAAT rather than algorithmic testing.

In the study across 60 European hospitals, a 2.5-fold higher CDI positivity rate was demonstrated when stand-alone or GDH/NAAT were used instead of a recommended algorithm. This was reflected in the subsequent incidence rates; hospitals relying on NAAT or GDH/NAAT reported a mean incidence rate of 5.2 per 10,000 patient-days, while hospitals relying on an algorithm reported a lower mean incidence rate of 2.0 per 10,000 patient-days, despite similar testing frequencies (Davies et al. 2016).

These observations hold true when the same samples are concomitantly tested with both stand-alone NAAT and an algorithm. In one study of 1321 stool samples, the CDI positivity rate by NAAT was 6.4%, while the CDI positivity rate by a GDH and Tox A/B EIA – CCNA algorithm on the same samples was 4.2%. The overall incidence rates were 8.9 and 5.8 per 10,000 patient-days for stand-alone NAAT and the algorithm, respectively (Longtin et al. 2013). When stand-alone NAAT was compared to stand-alone Tox A/B EIA, higher CDI positivity rates and higher CDI incidence rates for NAAT compared to Tox A/B EIA were reported, too (Grein et al. 2014). Even so, hospitals that switch from non-molecular tests to stand-alone NAAT testing are reported to experience an increase in their CDI incidence rates (Moehring et al. 2013).

The implications of testing method-dependent CDI incidence rates are consequential. Besides the obvious effect of interfering with attempts to accurately monitor CDI for surveillance purposes, financially tangible effects also result. For instance, UK hospitals can be assessed financial penalties for excessive numbers of hospital-acquired CDI cases (Davies et al. 2016). Similarly, in the US, the Centres for Medicare and Medicaid Services (CMS) value-based purchasing program are affected by reported incidence rates (Marra et al. 2017). In the latter's case, an attempt to normalize rates by factoring in testing method has been made, although the study demonstrated the inadequacy of such normalization and stressed the need for refinement.

In conclusion, CDI incidence is clearly affected by testing method. Given the heterogeneity of such methods between institutions, and the importance of correctly ascertaining CDI incidence, it is necessary to somehow normalize incidence rates in a way that takes into consideration testing method.

8 Alternative Testing Strategies

Methods for diagnosing CDI remain imperfect, which naturally has spurned an interest in alternative testing strategies. Alternative testing strategies cannot only possibly aid in the

diagnosis of CDI, but might also be able to predict severity or prognosis of CDI. These testing methods include faecal biomarkers, immune modulating interleukins and cytokines and imaging methods. Their role is discussed below.

8.1 Calprotectin

Calprotectin, a calcium-and zinc-binding protein, is found predominantly in the cytosol of neutrophils (Usacheva et al. 2016; Popiel et al. 2015; Whitehead et al. 2014). In vitro studies have shown that it has bacteriostatic and fungostatic properties (Peretz et al. 2016). It is a marker of inflammation due to release into the gut lumen by neutrophils during infiltration and can be measured in stool (Popiel et al. 2015). However, infection cannot be differentiated from inflammation by this marker, since both give a rise in faecal calprotectin (FCP) levels (Usacheva et al. 2016). The role of calprotectin in evaluating disease severity has been well studied in IBD (Vrabie and Kane 2014). Several studies evaluated the role of FCP in CDI testing (Table 2). First, the usefulness of FCP testing to diagnose CDI was evaluated in several studies. In all studies, median FCP levels were found to be significantly higher in CDI patients than in diarrhoeal patients who tested negative for CDI

Table 2 Overview of studies evaluating the role of FCP in patients with CDI

Study	Type of study	Detection of CDI	Number of cases/controls	Results
Kim et al. Ann Lab Med (2016)	Retrospective cohort study	NAAT for toxin gene	30 pts. with severe CDI (group 1), 50 pts. with mild CDI (group 2) and 71 CDI neg healthy controls (group 3)	*CDI diagnosis* Median levels of FCP were significantly higher in group 1 than in group 2 and group 3, 1391.5 μg/g (170.0–2088.1 μg/g) vs 188.2 μg/g (41.4–188.2 μg/g) and 35.0 μg/g (10.7–108.9 μg/g) respectively Optimal cut-off value for CDI diagnosis 112.5 μg/g, ROC curve AUC 0.821 sens 75% and spec.79% *CDI severity* Median levels of FCP were significantly higher in group 1 than in group 2, 1391.5 μg/g (173.5–2075.9 μg/g) vs 188.2 μg/g (41.4–591.6 μg/g), respectively Optimal cut-off value for differentiating mild from severe CDI 729.8 μg/g, ROC curve, AUC 0.746, sens 70% and spec 80%
Peretz et al. BMC Infect Dis (2016)	Retrospective cohort study	NAAT for toxin gene and identification 027 strains	29 pts. with CDI (7 CDI ribotype 027, 22 other ribotype)	Overal mean levels of FCP 331.4 μg/g (21–932 μg/g) Mean levels of FCP were significantly higher in 027 pos group than in 027 neg group, 331.4 μg/g (21–932 μg/g) vs 249 μg/g (155–498 μg/g), respectively A trend was found between higher FCP levels and higher Clostridium severity score

(continued)

Table 2 (continued)

Study	Type of study	Detection of CDI	Number of cases/controls	Results
Popiel et al. JCM (2015)	Prospective exploratory observational study	NAAT for toxin gene	44 CD-PCR pos vs 20 CD-PCR neg	Median levels of higher-range assay of FCP (assay range, 100–1800 µg/g) were significantly higher in CD-PCR+ than in CD-PCR-, 983 µg/g (351- > 1800 µg/g) vs <100 µg/g (<100–194 µg/g) and also in the lower range assay of FCP (assay range, 30–300 µg/g) >300 µg/g (>300- >300 µg/g) vs 77.5 µg/g (30–238 µg/g) Optimal cut-off value 135 µg/g High range FCP ROC curve AUC 0.82 sens. 88.6% and spec. 75%
Whitehead et al. J Med Microbiol (2014)	Prospective cohort study	Phase 1: toxin EIA (N = 75) Phase 2: GDH EIA + NAAT for toxin gene (N = 45) Change of departmental *C. difficile* testing methodology during evaluation	75 pts. toxin EIA pos (group 1), 45 pts. GDH-EIA/NAAT pos (group 2), 99 pts. negative for *C. difficile* (group 3), group 3: 99 cases in CDI negative	Median levels of FCP were significantly higher in group 1 than in group 2, 336 µg/g (208–536 µg/g) vs 249 µg/g (155–498 µg/g), respectively. Both were significantly higher than in group 3, 106 (46–176 µg/g) Optimal cut-off value 176 µg/g and 169 µg/g, ROC curve AUC 0.84 and 0.80, sens 81% and 73%, spec 77% and 77% for group 1 and 2, respectively
Swale et al. PLOS One (2014)	Prospective cohort study	NAAT for toxin gene and toxin EIA	164 CDI cases vs 52 AAD controls 8 severe CDI cases vs 116 non-severe CDI cases *C. difficile* isolates recovered from 149 CDI cases 72 cases with ribotype 027 vs 77 non-ribotype 027	*CDI diagnosis* Median levels of FCP were significantly higher in CDI cases vs AAD, 684.8 µg/g (203.7–1581.0 µg/g) vs 66.5 µg/g (23.1–145.7 µg/g), respectively Optimal cut-off value 148 µg/g ROC curve AUC 0.86 4 sens 81,8% spec 76.9% PPV 91.5%, NPV 57.4% Sub-group analyses: *CDI severity* Median levels of FCP were not significantly higher in severe CDI cases vs non-severe CDI cases, 969.3 µg/g vs 512.7 µg/g), respectively. *Ribotype 027* Median levels of FCP were not significantly higher in ribotype 027 cases vs non-ribotype 027 cases, 1011 µg/g vs 658 µg/g), respectively

(continued)

Table 2 (continued)

Study	Type of study	Detection of CDI	Number of cases/controls	Results
Darkoh et al. Clin Vaccine Immunol (2014)	Prospective cohort study	AAD stools: CCNA, NAAT for toxin genes and toxin EIA Control stools: NAAT for toxin gene and toxin EIA	CDI-positive stools (N = 50), CDI- negative stools (N = 50) hospitalized patients without diarrhea (N = 45)	FCP concentration in CDI pos stools, 18 μg/g (2.8–70.2 μg/g) was 3-fold higher than in CDI-neg stools, 6.5 μg/g (2.0–31.0 μg/g) and 2-fold higher than of hospitalized pts. without diarrhea, 8.7 μg/g (1.8–33.2 μg/g) FCP levels of 80% of the CDI-pos stools and 30% of the CDI-neg stools higher than hospitalized pts. without diarrhea

AAD antibiotic-associated diarrhea, *CCNA* cell cytotoxicity neutralization assay, *CDI Clostridium difficile* infection, *FCP* fecal calprotectin, *NAAT* nucleic acid amplification test

and in non-diarrhoeal controls (Kim et al. 2017; Popiel et al. 2015; Darkoh et al. 2014; Swale et al. 2014; Whitehead et al. 2014). Studies who calculated optimal FCP cut-off points for distinguishing CDI from non-CDI samples reported sensitivities ranging from 77% to 88% and specificities ranging from 75% to 79% (Popiel et al. 2015; Kim et al. 2017; Swale et al. 2014; Whitehead et al. 2014). However, in two of these studies the discriminative power of FCP might have been attenuated as the group of CDI patients might have included CD carriers due to testing for CDI by NAAT only (Popiel et al. 2015; Kim et al. 2017). On the other hand, the use of healthy controls instead of patients suspected of CDI might have falsely increased the specificity in one study (Kim et al. 2017). Overall, the suboptimal sensitivity and specificity demonstrated in these observational studies, of which several with limitations or small sample sizes, does not provide enough evidence for the use of FCP to detect CDI.

Interestingly, besides the expected suboptimal specificity of FCP, sensitivity is also moderate. One study reported that in 20% of CDI patients, FCP levels were lower than in hospitalised patients without diarrhoea (Darkoh et al. 2014). Another study reported that from 120 CDI subjects only five had normal FCP levels (<50 μg/g) and speculated that these cases might represent mild disease (Whitehead et al. 2014). The correlation between FCP levels and CDI severity has also been evaluated in three small studies, but results were conflicting (Kim et al. 2017; Swale et al. 2014; Peretz et al. 2016). A correlation between CDI due to ribotype 027 and FCP levels was also evaluated in two studies (Peretz et al. 2016; Swale et al. 2014). Significantly higher FCP levels compared to non-027 CDI were found in one small study comprising 7 ribotype 027 cases and 22 - non-ribotype 027 cases (Peretz et al. 2016), the same trend was shown in a bit larger study, but results were not significant (Swale et al. 2014).

In conclusion, there is also insufficient evidence for the use of FCP levels to predict severity or presence of ribotype 027.

8.2 Lactoferrin

Lactoferrin is a glycoprotein and resides in neutrophils. It is released upon neutrophil activation. The faecal lactoferrin (FL) levels can be measured in stool and correlate with the number of infiltrated neutrophils. Multiple studies have proven that it can be an accurate marker of intestinal inflammation and useful in diagnosis of inflammatory diarrhoea (Usacheva et al. 2016).

The usefulness of FL to detect CDI was evaluated in a handful studies (Table 3). All studies report higher median FL levels in CDI samples

Table 3 Overview of studies evaluating the role of FL in patients with CDI

Study	Study type	Detection of CDI	Number of cases/controls	Results
Darkoh et al. Clin Vaccine Immunol (2014)	Prospective cohort study	AAD stools: CCNA, NAAT and toxin EIA control stools: NAAT and toxin EIA	50 pts. with CDI-positive stools, 50 pts. with CDI-negative stools, 45 hospitalized pts. without diarrhea	FL concentration in CDI- pos stools, 31.4 μg/g (3.0–155.2 μg/g) was significantly different and was 5-fold higher than in CDI-neg stools, 6.3 μg/g (0.6–140.3 μg/g) and 6-fold higher than of hospitalized pts. without diarrhea, 5.6 μg/g (0.5–35.0 μg/g) FL levels of 88% of the CDI-pos stools and 44% of the CDI-neg stools higher than in hospitalized pts. without diarrhea
Swale et al. PLOS One (2014)	Prospective cohort study	toxin EIA	164 CDI cases vs 52 AAD controls 8 severe CDI cases vs 116 non-severe CDI cases *C. difficile* isolates recovered from 149 CDI cases 72 cases with ribotype 027 vs 77 non-ribotype 027	Median levels of FL were significantly higher in CDI cases 57.9 μg/ml (11.4–177.5 μg/ml) vs AAD 2.7 μg/ml (0.7–7.8 μg/ml) Optimal cut-off value 8.06 ng/ml ROC curve AUC 0.859, Sens 81,7% Spec 76.9%, PPV 91.8%, NPV 57.1% Sub-group analysis: *CDI severity* Median levels of FL were significantly higher in severe CDI cases vs non-severe CDI cases, 104.6 μg/ml vs 40.1 μg/ml, respectively *Ribotype 027* Median levels of FL were not significantly higher in ribotype 027 cases vs non-ribotype 027 cases, 83.2 μg/ml vs 51.0 μg/ml, respectively
Archbald-Pannone, J Geriatr Paliat Care (2014)	Prospective cohort study	Not described	79 pts. with 41 severe CDI vs. 38 pts. with non-severe CDI	Overal mean concentration of FL in the cohort was 388.8 μg/ml Mean levels of LF in severe CDI pts. 580 μg/ml (sd 989.0 μg/ml) were significantly higher than in non-severe CDI pts. 181.7 μg/ml (sd 244.2 μg/ml)
Boone et al. Eur J Clin Microbiol Infect Dis (2014)	Prospective cohort study	NAAT and TC	N = 210 129 TC+&CCNA + (group 1), 62 TC+&CCNA- (group 2) and 19 TC – &CCNA- (group 3)	FL concentration in group 1 (90 μg/g) was significantly higher than in group 2 (24 μg/g) and group 3 (20 μg/g)
Boone et al. Eur J Clin Microbiol Infect Dis (2013)	Prospective cohort study	GDH Membrane based EIA and toxin EIA	N = 98 (85 toxigenic strains, 6 non-toxigenic, 6 neg for *C. difficile*, 1 mixed infection) 85 toxigenic (21 severe CDI, 57 moderate, 7 mild) 38 pts. had a 027 infection (45%)	96% of pts. with pos toxin stool had elevated LF and 59% of pts negative stool toxin had elevated levels Mean levels of severe CDI (961 μg/g, SE 303 μg/g) were significantly higher than in moderate CDI, (292 μg/g, SE 42 μg/g), and mild CDI (73 μg/g, SE 52 μg/g) There is a significant difference in LF between pts. with 027 and non-027

(continued)

Table 3 (continued)

Study	Study type	Detection of CDI	Number of cases/controls	Results
LaSala et al. J Clin Microbiol (2013)	Retrospective cohort study	GDH EIA, toxin EIA and NAAT	N = 112 43 GDH neg (group 1), 14 GDH pos/toxin neg/PCR neg (group 2), 25 GDH& toxin pos (group 3), 30 GDH pos/toxin neg/PCR pos (group 4)	Median levels of LF were significantly higher in group 3, 80 μg/ml (3–124 μg/ml) than in group 1, 13 μg/ml (3–143 μg/ml), group 2, 18 μg/ml (4–78 μg/ml) and group 4, 24 μg/ml (4–160 μg/ml)

AAD antibiotic-associated diarrhea, *CCNA* cell cytotoxicity neutralization assay, *CDI Clostridium difficile* infection, *EIA* enzyme immunoassay, *FL* fecal lactoferrin, *GDH* glutamate dehydrogenase, *NAAT* nucleid acid amplification test, *TC* toxigenic culture

than in control samples (either diarrheal samples without CDI or non-diarrheal samples) (Swale et al. 2014; Darkoh et al. 2014; Boone et al. 2014; LaSala et al. 2013). However, a substantial proportion of CDI-negative patients have elevated FL levels, too (Boone et al. 2014; Darkoh et al. 2014). This was also reflected in the suboptimal specificity of 77% that was found when an optimal cut-off point to distinguish CDI from patients with non-CDI antibiotic associated diarrhoea was determined (Swale et al. 2014).

Whether FL could be used as a marker for severe CDI was also evaluated in some studies. Severe CDI was found to be associated with higher median FL levels in two small studies (Archbald-Pannone 2014; Boone et al. 2013). In addition, higher FL levels were associated with a higher white blood cell count and decreased serum albumin (Boone et al. 2013), but no association with mortality was demonstrated (Archbald-Pannone 2014), possibly due to small cohorts. Furthermore, it was demonstrated that patients with CDI due to ribotype 027 and positive stool toxin had significantly higher FL levels and WBC counts than non-027 CDI patients (Boone et al. 2013, 2014). In patients with CDI due to ribotype 027, patients with positive stool toxin and elevated FL had a higher mortality risk (Boone et al. 2014).

To conclude, all of the studies report an association between elevated FL and CDI. However, the reported specificity is insufficient for implementation in the diagnosis of CDI. Furthermore, as the studies report different median FL levels, this would reduce predictive accuracy. Some parts may be ascribed to variation in disease severity, while other parts are due to laboratory handling and the volume of diluent. Another problem is that FL can be elevated due to co-morbidities, such as ulcerative colitis and Crohn's disease.

Some studies also report an association between elevated FL and CDI severity (Archbald-Pannone 2014; Boone et al. 2013). However these studies have had small sample sizes, and more research is needed. To our knowledge, there are no studies that observed that FL, on its own, is a predictor of severity or mortality. Therefore more research is needed to understand the role of lactoferrin.

8.3 Faecal Leukocyte Test

The faecal leukocyte test is performed on stool specimens, which are smeared on slides and Wright stained. The test takes approximately 1 h and samples are positive when >1 WBC/highfield are observed (Reddymasu et al. 2006). However, in a study evaluating 263 stool samples from patients suspected of CDI for the diagnosis of CDI, the faecal leukocyte test showed a sensitivity and specificity of 30% and 74.9% respectively compared to toxin EIA (Reddymasu et al. 2006). A larger study (n = 797 stool samples) observed a sensitivity and specificity of 14% and 90% respectively (Savola et al. 2001). Thus, faecal leukocyte testing is not a good test for CDI and a poor predictor of the toxin assay result.

8.4 Interleukins and Chemokines

IL 8 is a chemoattractant and recruits neutrophils to sites of infection. Activated dendritic cells and macrophages produce IL 23. This interleukin is involved in host defence against bacterial infections and the development of chronic inflammation. Darkoh and colleagues tested CDI stools, diarrheal non-CDI stools and non-diarrheal stools for interleukins both by a cytokine assay and by a quantitative EIA (Darkoh et al. 2014). Both IL-8 and IL-23 were detected in more CDI-positive stools than CDI-negative stools. The cytokine assay showed that the relative amount of IL-8 was higher in the 50 CDI-positive stools, compared to 50 CDI-negative stools. This in contrast with IL-23, were the relative amount was higher in the CDI-negative stools. When the findings were confirmed by EIA, they found that CDI-positive stools showed a significantly higher amount of IL 8 (mean 318.2 pg/ml) in stools compared to the CDI-negative stools (mean 84.7 pg/ml) and hospitalized patients without diarrhoea (mean 79.8 pg/ml). In contrast, IL 23 was significantly higher in CDI-negative stools and hospitalized patients without diarrhea than in the CDI positive stools, 946.7 pg/ml (185.5–2016 pg/ml), 1617 pg/ml (489.0–6810 pg/ml) and 722 pg/ml (110.0–7069 pg/ml), respectively. This study shows that IL-8 plays a role in CDI and that increased levels are associated with more severe forms of CDI. In contrast, IL-23 amounts during CDI may be inadequate to sustain sufficient cellular immunity. Therefore, lower concentrations of IL-23 may show a lack of immunological response in a proportion of CDI patients and may explain also recurrence (Darkoh et al. 2014).

CXCL-5 is a CXC chemokine and recruits and activates neutrophils. El Feghaly and colleagues studied the correlation between intestinal inflammation and disease severity in hospitalized patients with symptomatic CDI (El Feghaly et al. 2013). They found that faecal CXCL-5 mRNA and IL-8 mRNA were associated with diarrhoeal persistence and longer time to diarrhoea resolution. The levels were also higher in patients with CDI in the prior 90 days than in patients with no history of CDI (El Feghaly et al. 2013).

So, it seems that markers of inflammation play a role in CDI and may correlate to disease severity. However more research is needed to confirm these associations.

8.5 CT-Imaging

CT imaging can be useful in diagnosing fulminant CDI and pseudomembranous colitis (PMC). Several features are suggestive of advanced PMC such as colonic-wall thickening, pericolonic stranding, the accordion sign, the double-halo sign and ascites (Bartlett and Gerding 2008; Kirkpatrick and Greenberg 2001). The radiography is usually normal in absence of ileus or toxic megacolon.

Kirkpatrick et al. evaluated whether diagnosis of *C. difficile* colitis could be made with CT (Kirkpatrick and Greenberg 2001). They included 110 patients of which 54 had a positive stool assay and 56 patients a negative stool assay. The sensitivity at their centre was 52%, the specificity 93% and the positive and negative predictive values were respectively 88% and 67%. CT imaging is less sensitive when compared with NAAT or stool toxin tests but can be useful when there is a need for quick results (Bartlett and Gerding 2008).

8.6 Endoscopy

Nearly all cases of PMC are caused by CDI (Tang et al. 2016) though other causes are sometimes found, such as chemotherapy, toxin producing *Staphylococus aureus* and cytomegalovirus infection (Sundar and Chan 2003; Pressly et al. 2016). PMC is not very common and not all CDI will develop in PMC (Bartlett 2002). Therefore,

endoscopy is a relatively insensitive procedure. Furthermore, in one third of the patients PMC is missed by sigmoidoscopy because of involvement of the right colon; making colonoscopy the preferred endoscopic procedure. Endoscopy is an invasive procedure with perforation risks and is often expensive (Bartlett 2002; Bartlett and Gerding 2008). Endoscopy is therefore not recommended to diagnose CDI.

8.7 Histopathology

CDI is more likely when pseudomembranes are detected histologically. Pseudomembrane lesions are microscopically visualized as "mushroom" like and consist of pus, mucin and fibrin. Their reported sensitivity is 44% and the specificity is 89% (Wang et al. 2013). Biopsy is not needed for the diagnosis of CDI. However, histologic findings of pseudomembranes may suggest CDI and should stimulate stool testing.

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Ribotypes and New Virulent Strains Across Europe

Jeanne Couturier, Kerrie Davies, Cécile Gateau, and Frédéric Barbut

Abstract

Clostridium difficile is a major bacterial cause of post-antibiotic diarrhoea. The epidemiology of *C. difficile* infections (CDI) has dramatically changed since the early 2000s, with an increasing incidence and severity across Europe. This trend is partly due to the emergence and rapid worldwide spread of the hypervirulent and epidemic PCR ribotype 027. Profiles of patients with CDI have also evolved, with description of community-acquired (CA) infections in patients with no traditional risk factors for CDI. However, recent epidemiological studies indicated that some European countries have successfully controlled the dissemination of the 027 clone whereas other countries recently reported the emergence of other virulent or unusual strains. The aims of this review are to summarize the current European CDI epidemiology and to describe the new virulent *C. difficile* strains circulating in Europe, as well as other potential emerging strains described elsewhere. Standardized typing methods and surveillance programmes are mandatory for a better understanding and monitoring of CDI in Europe.

Keywords

C. difficile · PCR ribotypes · Emerging strains · European epidemiology · Binary toxin

1 Introduction

Clostridium difficile is the main bacterial cause of hospital-acquired diarrhoea; it is responsible for 15–25% of post-antibiotic diarrhoea and for virtually all cases of pseudomembranous colitis (Bartlett and Gerding 2008). *C. difficile* infection

J. Couturier (✉) · F. Barbut
National Reference Laboratory for C. difficile, Hôpital Saint-Antoine, Paris, France

Université Paris Descartes, Faculté de Pharmacie, Paris, France
e-mail: jeanne.couturier@aphp.fr; frederic.barbut@aphp.fr

K. Davies
Healthcare Associated Infections Research Group, Leeds Teaching Hospitals NHS Trust and University of Leeds, Leeds, UK
e-mail: kerrie.davies@nhs.net

C. Gateau
National Reference Laboratory for C. difficile, Hôpital Saint-Antoine, Paris, France
e-mail: cecile.gateau@aphp.fr

(CDI) epidemiology has dramatically changed in Europe since the beginning of the 2000s. The incidence has increased over the last 10 years from 2.45 cases per 10,000 patient-days in 2005 (Barbut et al. 2007), to 4.1 in 2008 (Bauer et al. 2011) and 7.0 in 2012–2013 (Davies et al. 2014). Nevertheless, the incidence of CDI varies widely across European countries from 0.7 to 28.7 per 10,000 patient-bed days per hospital. This trend is likely to result from a combination of several factors, including the level of awareness of CDI among physicians, the type of methods/algorithm for CDI diagnosis implemented in each country, and the global spread of the PCR ribotype (RT) 027 clone. A European study showed that there is still a substantial underdiagnosis of CDI coupled with large variations in testing policies among European countries (Davies et al. 2014).

In Europe, the hypervirulent epidemic RT 027 strain (or REA type BI/NAP1/toxinotype III) was first reported in England in 2005 (Smith 2005) and has since rapidly spread in other European countries. RT 027 is characterized by an 18 bp deletion and a deletion at position 117 in *tcdC* gene, resulting in the inactivation of the toxin repressor TcdC and higher amounts of toxin production (Warny et al. 2005), although the role of *tcdC* mutation in toxin overproduction is currently debated (Murray et al. 2009; Cartman et al. 2012). Moreover, epidemic 027 strains also produce an additional toxin, the binary toxin, and are resistant to erythromycin and moxifloxacin, which may have conferred a selective advantage. The same combination of genetic and phenotypic features can be found in other rare RT, such as RT 176 (Krutova et al. 2015; Drabek et al. 2015). RT 027-related CDI are associated with a higher rate of complications and recurrences (Sundram et al. 2009). The RT 027 has disseminated throughout Europe, with a clear shift in its regional repartition from United Kingdom and Ireland in 2008 (Bauer et al. 2011) to Eastern Europe in 2012–2013 (Davies et al. 2016b). Some countries have successfully controlled its spread and decreased its prevalence (Hensgens et al. 2009; Fawley et al. 2016), while other were recently hit by large outbreaks (Bouza et al. 2017). In addition, other virulent or unusual PCR ribotypes are emerging.

2 *C. difficile* Typing Methods

2.1 PCR Ribotyping

PCR ribotyping is the reference method for *C. difficile* typing in Europe. It relies on the presence of several alleles of the rRNA operon in the *C. difficile* genome. The length polymorphism of the intergenic spacer region between 16S and 23S rRNA genes results in RT-specific patterns after genomic amplification and migration (Bidet et al. 1999). PCR ribotyping was first developed using agarose gel electrophoresis, but the capillary gel-based electrophoresis method has now been widely adopted. The latter enables better standardization and easier comparison between laboratories and is recommended as the reference technique in Europe (Fawley et al. 2015).

Most European countries use a common nomenclature, but some laboratories developed their own local databases. An online database containing internationally recognised capillary electrophoresis RT profiles is available (WEBRIBO, https://webribo.ages.at/, Indra et al. 2008). However, there is no standardized protocol since several primer sets were published (Stubbs et al. 1999; Bidet et al. 1999), some of them enabling direct PCR ribotyping from stool samples (Janezic et al. 2011). Harmonization of the PCR ribotyping method and nomenclature is therefore essential and needs to be improved in Europe, in order to detect emergence of new unreferenced RT in a timely manner.

2.2 Other Methods Used for *C. difficile* Typing

Toxins A and B, which are considered as the main virulence factors of *C. difficile* (Pruitt and Lacy 2012), are encoded by *tcdA* and *tcdB* genes located within a locus of pathogenicity (PaLoc). The PaLoc also contains *tcdR* (positive regulator of toxin expression), *tcdE* (holin required for toxin secretion), and *tcdC* (potential negative regulator). The genetic polymorphism of the

PaLoc can be explored by toxinotyping, which is a PCR-restriction based method (Rupnik et al. 1998). Toxinotypes are defined according to differences in the PaLoc compared to the reference strain VPI 10463 (nonvariant toxinotype 0). To date, 34 toxinotypes have been described (Rupnik and Janezic 2016; http://www.mf.um.si/mf/tox/profile.html). Toxinotyping and PCR ribotyping are well correlated since most of the strains in a given RT have similar changes in the PaLoc and thus belong to a single toxinotype. The analysis of 123 strains showed that in a few cases, PCR ribotyping can be more discriminatory than toxinotyping, whereas RT include several toxinotypes less frequently (Rupnik et al. 2001). To avoid ambiguities, a revised toxinotyping nomenclature was recently published (Rupnik and Janezic 2016).

PFGE (Pulsed-field gel electrophoresis) is a genotype-based typing method developed in the 1980s and mostly used in North America. There is good concordance between results of PFGE and PCR ribotyping (Bidet et al. 2000). PFGE has a higher discriminatory power than PCR ribotyping (Killgore et al. 2008) but the interpretation of genetic relatedness is comparable between both typing methods. However, some strains are non-typeable with this method, and degradation of genomic DNA can hinder the analysis (Kristjánsson et al. 1994). PFGE is also very labour-intensive and the lack of standardisation makes inter-laboratory data comparison difficult.

The discriminatory power of PCR ribotyping is not sufficient to prove the nosocomial transmission of a strain, particularly when a RT is endemic at a regional or national level. In that case, another more discriminant typing method has to be used, such as multilocus variable-number tandem repeat (VNTR) analysis (MLVA). MLVA relies on the variability of the VNTR at different loci. The genetic relatedness of isolates is appreciated through the sum of tandem repeat number differences (STRD) (Marsh et al. 2006).

Whole genome sequencing (WGS) can distinguish between strains at the single nucleotide level, highly increasing the discriminatory power over other typing schemes. Given the transferability of data and the diversity of potential applications, such as comparative genome analysis and lineages analysis, this method is increasingly being used for *C. difficile* typing and could spread widely in the coming years (Knetsch 2013). WGS has successfully and rapidly identified transmission of healthcare-associated infection and could become a valuable tool in routine clinical practice (Eyre et al. 2012).

3 Global Distribution of *C. difficile* PCR Ribotypes in Europe

The European *C. difficile* infection study (Bauer et al. 2011) and the EUCLID study (Davies et al. 2014, 2016b) are two major European surveys aiming at describing the epidemiology of CDI including prevalence, diagnosis and RT distribution.

The first pan-European study on *C. difficile* was performed in 2008 in 106 laboratories from 34 countries (Bauer et al. 2011). The incidence of CDI and the RT distribution varied greatly between hospitals, as well as the density testing for CDI. The authors could differentiate 65 RT among 389 *C. difficile* isolates. One of the main findings of this study was that RT 027 was not predominant in 2008, representing only 5% of the isolates. The most common RT were 014/020 (16%), 001, (9%), and 078 (8%). Some RT seemed to spread regionally, such as RT 106 mostly described in UK and Ireland.

The EUCLID study (European, multicentre, prospective, biannual, point-prevalence study of CDI in hospitalized patients with diarrhoea) was conducted in 2012–2013 and included 482 hospitals from 19 European countries (Davies et al. 2016b). The objectives were to measure the underdiagnosis of CDI and to assess the diversity of RT repartition in Europe. During two sampling days (one in winter and one in summer), participating hospitals sent every diarrhoeal stool sample, irrespective of the request to test for *C. difficile* by the physician, to a national coordinating laboratory. The RT diversity was much higher than in the previous study, with 125 RT identified among 1196 isolates.

Interestingly, the most common RT was 027 (19%), highlighting the rapid spread of this strain at a global scale. An inverse correlation was noted between the rate of testing and prevalence of ribotype 027 across north, south, east, and west quadrants of Europe, which suggests that increased awareness of CDI and use of optimum testing methods and policies can reduce the dissemination of epidemic strains (Davies et al. 2014). The comparison with the 2008 data indicated a shift in the frequency of RT 027 from UK and Ireland (decreasing prevalence) to Eastern Europe countries (increasing prevalence). RT 001/072 (11%) and 014/020 (10%) were the second and third most prevalent RT, consistent with the 2008 results, however the prevalence of RT 078 dropped from 8% in 2008 to 3% in 2012–2013. The distribution of causative RT was country-specific as shown in the Fig. 1 (Davies et al. 2016b).

Besides these two large epidemiological studies, several other recent European studies analysed RT distribution at a national level. The results of these national studies are summarized in the Table 1.

A multicentre study characterized 3333 toxigenic strains isolated between 2010 and 2015 in 110 Belgian hospitals (Neely et al. 2017). RT 027 (4.2%) and 078 (7.0%) were associated with a higher rate of complications (unadjusted data) and higher levels of *in-vitro* toxin production from cultured isolates.

A study compared epidemiological data for community-associated (CA)-CDI and healthcare-associated (HA)-CDI in 113 laboratories across England between 2011 and 2013 (Fawley et al. 2016). A total of 703 *C. difficile* toxin-positive faecal samples from CA-CDI cases were analysed and the results were compared to HA-CDI data (n = 10,754) obtained from the *C. difficile* Ribotyping Network. RT distribution was similar in cases of CA- and HA-CDI, but RT 002 was more likely to cause CA-CDI, while RT 027 was more often associated with HA-CDI.

In Spain, Alcalá et al. performed *C. difficile* cultures on 807 unformed stool specimens sent to 118 Spanish microbiology laboratories on a single day, regardless of the prescription by the clinician (Alcalá et al. 2012). Among 42 toxigenic strains, RT 014/020, 001 and 078/126 were the most prevalent (20.5%, 18.2% and 18.2% respectively). RT 027 was not found.

The characterization of 498 clinical isolates from 20 hospitals in Portugal showed that RT 027 was predominant with 18.5% of all the strains, and 19.6% of HA-associated CDI. RT 014 was the second most frequent overall (9.4%) and the most frequent among CA-CDI (12%). The prevalence of RT 126 and 078 was low (3.8% and 2.8% respectively) (Santos et al. 2016). The authors described a great heterogeneity of the RT distribution through the country with a higher diversity in the north, where RT 027 was not predominant.

The geographic distribution of *C. difficile* genotypes in Germany was assessed using 393 isolates sent to the national advisory laboratory for diagnostic reason between 2011 and 2013 (von Müller et al. 2015). The typing method used was surface-layer protein A sequence typing, with strain assignment to RT for better comparison with international data. RT 001 (35%) and 078 (8%) were prevalent nationwide; RT 027 (26%) and 014/066 (9%) were detected in almost all regions.

In France, a multicentre study conducted in 2009 in 78 healthcare facilities showed that the most prevalent RT were 014/020/077 (18.7%), followed by 078/126 (12.1%) (Eckert et al. 2013). The prevalence of RT 027 strains remained low (3.1%), and they were only isolated in Northern France, where RT 027 emergence was first described in 2006 (Coignard et al. 2006; Birgand et al. 2010). These results are consistent with the more recent LuCID (Longitudinal European *Clostridium difficile* Infection Diagnosis) surveillance study (Davies et al. 2016a), during which RT 014/020/077 and 078/126 were the most prevalent in France (21.9% and 9.5% respectively) (Eckert et al. 2015).

In conclusion, RT 014/020 and 001/072 are endemic in almost all European countries while there is a national or regional specificity for other RT. Moreover, the RT diversity is significantly increasing across Europe.

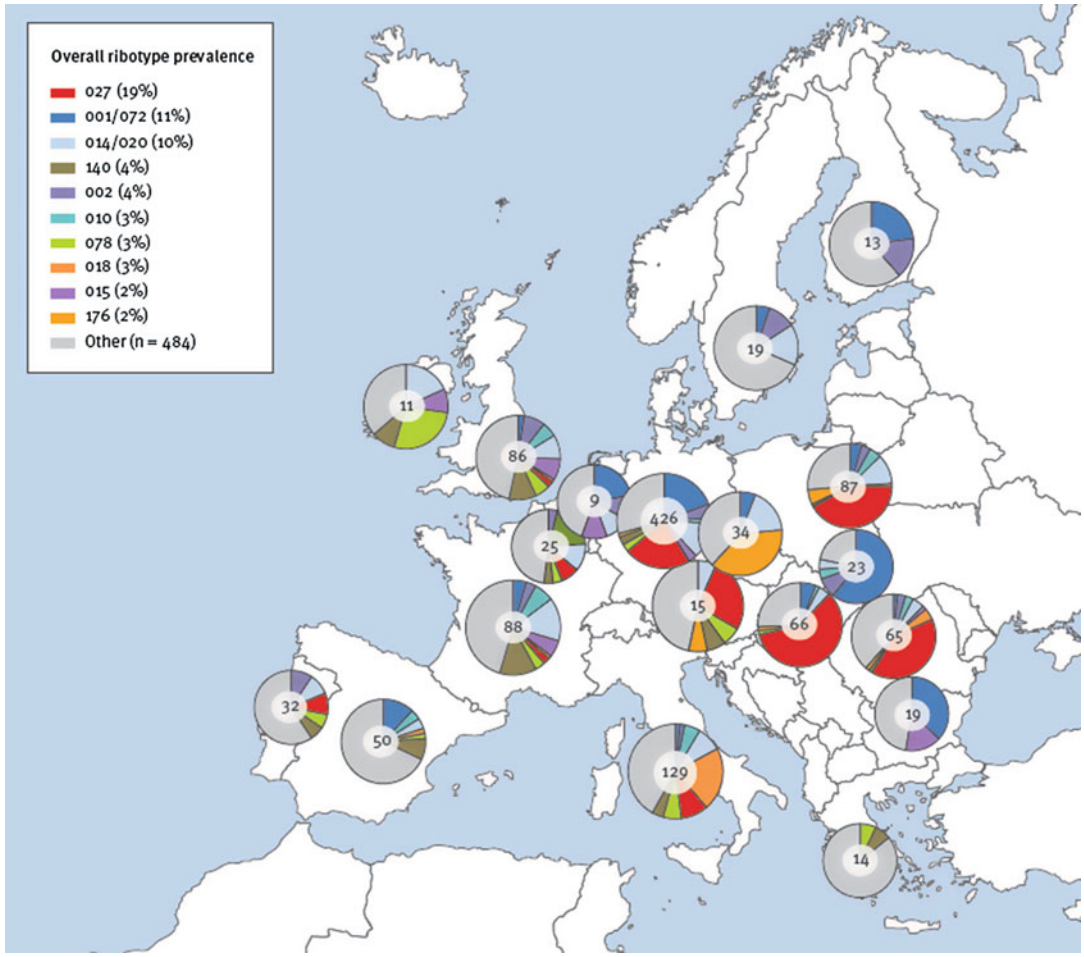

Fig. 1 Geographical distribution of *C. difficile* PCR ribotypes, by participating European country, EUCLID 2012–2013 and 2013 (n = 1196) (Reproduced with permission from Davies et al. 2016b) Pie charts show the proportion of the most common ribotypes per country and the number in the centre of the charts is the number of typed isolates in the country

4 Emerging PCR Ribotypes

4.1 PCR Ribotype 176

RT 176 strains are closely related to RT 027 (Stabler et al. 2006). They belong to toxinotype III, produce the binary toxin and bear a deletion at position 117 of the *tcdC* gene, leading to a potential RT 027 misidentification with commonly used molecular assays such as Xpert® *C. difficile* (Cepheid). Moreover, their similar banding pattern (only one band difference) after gel electrophoresis can be confusing for RT attribution (Valiente et al. 2012).

The first cases of RT 176-associated CDI were described in 2008 in Poland (Obuch-Woszczatyński et al. 2014), in 2009 in the Czech Republic (Nyč et al. 2011) and in 2015 in Croatia (Rupnik et al. 2016). The first RT 176-related outbreak was recently described in France (Couturier et al. 2017). Four strains isolated in two geographically close hospitals, previously identified as RT 027 with the agarose gel method, were reassigned as RT 176 by capillary gel-based electrophoresis. MLVA analysis showed that those four strains formed a clonal complex (STRD $\leq$2), and were genetically related to RT 027 strains (STRD $\leq$10).

Table 1 National epidemiological studies on *Clostridium difficile* PCR ribotypes repartition

Country	N strains	PCR ribotyping method	Most prevalent RT (%)	References
Belgium	3333	Agarose gel electrophoresis	014 (11.6), 020 (8.5), 002 (7.6), 078 (7.0), 027 (4.2), 005 (3.5), 106 (3.4)	Neely et al. (2017)
United Kingdom	11,457	Agarose gel electrophoresis	015 (10.2), 002 (9.1), 014 (9.1), 078 (8.0), 005 (7.4) and 027 (6.4)	Fawley et al. (2016)
Spain	42	Agarose gel electrophoresis	014/020 (20.5), 001 (18.2), 078/126 (18.2)	Alcalá et al. (2012)
Portugal	498	Capillary electrophoresis	027 (18.5), 014 (9.4), 020 (5.6), 017 (5.2)	Santos et al. (2016)
Germany	393	slpAST with assignment to RT	001 (35), 027 (26), 014/066 (9), 078 (8)	von Müller et al. (2015)
France	224	Agarose gel electrophoresis	014/020/077 (18.7), 078/126 (12.1), 015 (8.5), 002 (8), 005 (4.9)	Eckert et al. (2013)
Czech Republic	774	Capillary electrophoresis	176 (29), 001 (24)	Krutova et al. (2016)

slpAST surface-layer protein A sequence typing

The results of the EUCLID study showed a regional specificity of RT 176, isolated mostly in the Czech Republic where it accounted for 38% of the strains (Davies et al. 2016b). In 2014, a study among 18 Czech hospitals showed that 29% of *C. difficile* isolates belonged to RT 176, and 24% to RT 001 (Krutova et al. 2016, Table 1). Further typing analysis by MLVA, indicated that both RT formed clonal complexes in several hospitals, suggesting a rapid spread of these clones at a national level.

These results suggest a rapid nosocomial spread of RT 176 strains through Europe, stressing the need for a common data base for PCR ribotyping.

4.2 PCR Ribotype 078

RT 078 strains can produce toxins A and B, as well as the binary toxin and belong to toxinotype V. They are characterized by a 39 bp deletion in *tcdC*. RT 078 was reported as predominant in Greece in 2005 (Barbut et al. 2007), and was the third most common RT in the 2008 European study (Bauer et al. 2011). A recent study showed that RT 078 strains co-circulate with the hypervirulent 027 strains in Southern France (Cassir et al. 2017). While 027 strains are mostly responsible for outbreaks of HA-infections in the elderly, 078 strains are more frequently associated with CA-infections in a younger population. CA-CDI due to 078 strains were also described in England (Fawley et al. 2016) (see "*Clostridium difficile* infection in the community" below). Finally, RT 078 strains are frequently resistant to fluoroquinolones and erythromycin, partly explaining this epidemiological success (Baldan et al. 2015).

4.3 PCR Ribotype 126

RT 078 and 126 are highly related: they share similar banding patterns in agarose gel electrophoresis method, and can only be differentiated with the capillary gel-based electrophoresis. Consequently, they are often reported together as RT 078/126. Like RT 078 strains, RT 126 strains belong to toxinotype V and are considered as "hypervirulent" (Knetsch et al. 2011). They also produce the binary toxin and are characterized by a 39 bp deletion in *tcdC*.

The prevalence of RT 126 strains in animals in Germany is high, suggesting the potential zoonotic spread of this RT (Schneeberg et al. 2013). MLVA analysis showed that most of those strains are genetically related to RT 078 strains (STRD $\leq$10), and some of them belong to the

same clonal complex (STRD $\leq$2). RT 126 strains are also frequently resistant to antibiotics, including erythromycin, moxifloxacin and tetracyclin (Álvarez-Pérez et al. 2017).

4.4 PCR Ribotype 033/Toxinotype XI

PCR ribotype 033 strains belong to toxinotype XI. They are characterized by the absence of TcdA and TcdB expression and therefore cannot be detected by EIA (enzyme immunoassay) methods for toxins. These strains were first described in 2001 (Rupnik et al. 2001). In 2014, six symptomatic CDI cases due to toxinotype XI strains were reported by the French National Reference Laboratory for *C. difficile* (Eckert et al. 2014). In four cases, the patient was successfully treated by oral metronidazole. These strains were characterized by PCR ribotyping, amplification of *tcdA, tcdB, cdtA* and *cdtB* genes and toxinotyping. The six strains were defined as RT 033 (or 033-like) and were negative for TcdA and TcdB. The binary toxin genes were present and a 39 bp deletion was identified in the *tcdC* gene. The six strains were characterized by major deletions of the 5′ region of the PaLoc including *tcdB, tcdE* and *tcdR*; only a remnant part of *tcdA* (A2 and A3 fragments) and *tcdC* could be amplified.

The pathogenicity of toxinotype XI strains remains controversial. Studies on the role of the binary toxin as a virulence factor in animal models gave contradictory results. In the rabbit ileal loop model, an enterotoxic response was observed after inoculation of supernatants from culture of $A^-B^-CDT^+$ strains. However, despite colonization, no symptoms occurred in clindamycin-treated hamsters challenged with these strains (Geric et al. 2006). Although the prevalence of $A^-B^-CDT^+$ strains in Europe seems rather low (Barbut et al. 2007; Bauer et al. 2011), surveillance of this unusual strains is required. Indeed, the atypical genomic organization of the PaLoc can lead to a false negative diagnosis, more particularly when methods relying on the presence of toxin A and/or toxin B only are used. However, the increasing use of the Xpert® *C. difficile* assay, which detects binary toxin genes, will possibly enable a better identification of toxinotype XI strains.

4.5 PCR Ribotype 018

RT 018 has recently been reported as an emerging RT responsible for outbreaks in Italy, where RT 126 was previously predominant (Spigaglia et al. 2010). The EUCLID study (Davies et al. 2016b) showed that prevalence of RT 018 was high in Italy (22%), as opposed to other European countries. In addition, Baldan et al. characterized 312 *C. difficile* isolates from a large Italian teaching hospital between 2009 and 2013, and observed that RT 018 was predominant. After epidemiological investigation of the outbreaks, RT 018 represented 42% of index CDI cases and virtually all secondary cases (due to nosocomial transmission). The transmission index (number of secondary cases divided by number of index cases) of RT 018 was significantly higher than that of RT 078 (0.640 and 0.0606, respectively) (Baldan et al. 2015). Another study comparing RT 018, RT 126 and RT 078 demonstrated that RT 018 strains produced higher levels of toxins, showed increased adhesion to cells and became endemic in a short time (Barbanti and Spigaglia 2016). Moreover, RT 018 strains were all multidrug resistant (resistance to erythromycin, clindamycin and moxifloxacin). Together, these results suggest that RT 018 strains have phenotypic traits conferring an adaptive advantage and are able to spread widely. RT 018 strains were indeed reported in Southern Europe (Spain, Austria and Slovenia) and are associated with a higher rate of complicated infections (Bauer et al. 2011).

4.6 PCR Ribotype 017

RT 017 strains belong to toxinotype VIII and are part of *C. difficile* clade 4; they lack toxin A production and binary toxin genes (Cairns et al. 2012). The clinical relevance and the prevalence of this clone has been unclear for many years,

since it was mainly found in asymptomatic infants (Depitre et al. 1993; Kato et al. 1998). However, it has now been established that RT 017 strains are predominant in Asian countries such as Korea, China and Japan (Collins et al. 2013), and that they have spread worldwide. RT 017-related outbreaks have been reported in England (Cairns et al. 2015), The Netherlands (Kuijper et al. 2001), Poland (Pituch et al. 2001), and Ireland (Drudy et al. 2007). RT 017-related CA-CDI appear to be more likely to affect younger patients (Fawley et al. 2016). Severe RT 017-related CDI have been described in Germany, although RT 027 was the most prevalent strain in this study (Arvand et al. 2009).

4.7 Other Emerging PCR Ribotypes

RT 244 strains belong to the same hypervirulent clade as RT 027 (clade 2) (Lim et al. 2014). They produce the binary toxin and bear a single nucleotide deletion at position 117 in *tcdC*. Severe CA-CDI and outbreaks due to RT 244 strains were recently reported in Australia and New Zealand, where it was previously uncommon (De Almeida et al. 2013; Eyre et al. 2015). Eyre et al. showed that a strain isolated in a patient recently returned from Australia to the UK was phylogenetically related to their outbreak, highlighting the potential rapid spread of RT 244 via international travel.

The previously quoted French multicentre survey showed that among 224 toxigenic strains, 19 (8.5%) belonged to RT 015 which was the third most frequent RT (Eckert et al. 2013). Fawley et al. showed that RT 015 was also predominant in England (Fawley et al. 2016). Although RT 015 accounted for only 2% of the strains analysed in the EUCLID study, it seems that RT 015 strains can spread and become predominant at a national scale.

RT 106 strains represented 5% of all toxigenic isolates in the 2008 hospital-based European study, but their distribution showed a regional spread: among 20 strains, 13 were isolated in the United Kingdom and 5 in Ireland (Bauer et al. 2011). In a Southern England healthcare facility, 38% of *C. difficile* isolates (n = 97) were identified as RT 106, the second most prevalent RT after 027 (45%) (Sundram et al. 2009). Almost all of these RT 106 strains were resistant to ciprofloxacin and erythromycin. Moreover, in the Belgian multicentre study (Neely et al. 2017), recurrences were more frequent with RT 106-related CDI.

Other data reported the emergence of RT 001 strains with reduced susceptibility to metronidazole, raising concerns about the potential spread of these strains due to this selective advantage (Baines et al. 2008). In Southern Germany, the prevalence of RT 001 strains exhibiting resistance to erythromycin, ciprofloxacin and moxifloxacin is high in both in- and out-patients (Borgmann et al. 2008; Arvand et al. 2009).

Given their pathogenic and epidemic potential, the emergence of these RT should be closely followed in European countries.

The genetic and epidemiological features of the emerging RT described above are summarized in the Table 2.

4.8 Emerging Strains with a A+B-CDT- Unusual Profile

Recently, three clinical strains with an atypical PaLoc structure were described in France (Monot et al. 2015), including the first variant strain producing only toxin A ($A^+B^-CDT^-$). WGS analysis of this strain showed that its PaLoc only contained *tcdA* and *tcdR*. None of the three strains belonged to any of the most frequent RT. Moreover, the authors described variability in the sequence of the toxin genes, which may lead to potential false negative results with the most commonly used diagnostic methods (immunoenzymatic or molecular assays).

Table 2 Characteristics of currently circulating and emerging PCR ribotypes in Europe

RT	Toxinotype	Toxins A and B	Binary toxin	Deletion in *tcdC*	Main circulation area
027	III	+/+	+	−18 bp/ Δ117	Europe, mostly Eastern Europe Davies et al. (2016b)
176	III	+/+	+	−18 bp/ Δ117	Poland, Czech Republic Nyč et al. (2011), Obuch-Woszczatyński et al. (2014)
078	V	+/+	+	−39 bp/ A117T	Community-onset infections Eckert et al. (2011), Fawley et al. (2016)
126	V	+/+	+	−39 bp/ A117T	Eckert et al. (2011)
033	XIa/XIb	−/−	+	−39 bp	Low prevalence in Europe Eckert et al. (2014)
018	XIX	+/+	−	ND	Italy Spigaglia et al. (2010), Rupnik and Janezic (2016)
017	VIII	−/+	−	ND	Asia Collins et al. (2013), Ireland Drudy et al. (2007), England (Cairns et al. (2015), The Netherlands Kuijper et al. (2001), Poland Pituch et al. (2001), Germany
244	IXb	+/+	+	ND/ Δ117	Australia Lim et al. (2014), Rupnik and Janezic (2016)
015	NA	+/+	−	−18 bp or ND	France Eckert et al. (2013)
106	NA	+/+	−	−18 bp or ND	United Kingdom, Ireland Bauer et al. (2011)
001	XXIX	+/+	−	ND	Germany, multidrug resistant strains Borgmann et al. (2008), Rupnik and Janezic (2016)

ND not deleted, *NA* not available

5 *C. difficile* Infection in the Community

The epidemiology of CA-CDI is poorly known, since *C. difficile* testing is rarely requested in stool samples from community patients. However, recent data suggest that the incidence of CA-CDI is rising (Chitnis et al. 2013). In addition, CDI were recently described among young patients from community settings without the traditional risk factors (antibiotic exposure, recent hospitalization, co-morbidities) (Wilcox et al. 2008; Gupta and Khanna 2014).

Fawley *et al.* showed that RT 002, 020 and 056 were largely responsible for CA-CDI, whereas RT 027 was most associated with HA-CDI (Fawley et al. 2016). RT 078 strains have been reported in animals in the Netherlands (Goorhuis et al. 2008), and by using MLVA analysis, Debast *et al.* showed that RT 078 strains found in animals and in humans were genetically highly related, suggesting a foodborne interspecies transmission of *C. difficile* (Debast et al. 2009). In Canada, RT 078 epidemic strains (identified as pulsotype NAP7 by PFGE) were found in vegetables from grocery stores (Metcalf et al. 2010). RT 078 has also been described in the environment; it was the most frequently isolated RT in wastewater treatment plants in Switzerland (Romano et al. 2012). RT 078 was the commonest (19.0%) in 42 CA-CDI cases in a prospective study conducted in Scotland, followed by RT 014/020 (16.7%), 015 (14.3%) and 001 (11.9%) (Taori et al. 2014). However, in a US study of 984 CA-CDI cases, NAP1/RT 027 was the most frequent strain isolated (21.7%), while less than 7% of the isolates belonged to NAP7/RT 078 (Chitnis et al. 2013). In 2011, population- and laboratory-based surveillance for CDI was conducted in 10 US areas (Lessa et al. 2015). A total of 1364 strains were characterized. The most common strains were NAP1/RT 027 (18.8% of CA-CDI and 30.7% of HA-CDI), NAP4/RT 020 (11.4% and 10.3%) and NAP11/RT 106 (10.7% and 10.0%). Less than 4% of the strains in both settings belonged to NAP7/RT 078. These

results show a large overlapping of the RT distribution in HA- and CA-CDI, suggesting the existence of common reservoirs and multiple transmission routes between community and hospital settings.

6 Conclusion

In conclusion, there is a large diversity of RT across Europe, although some specific RT are able to disseminate at a regional or national level. A national and European clinical surveillance system, associated with microbiological characterization of strains, is essential in order to monitor the constantly changing epidemiology of CDI. A common European data base of the circulating RT would be very helpful to detect emergence of new virulent clones in a timely manner.

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Comparative Genomics of *Clostridium difficile*

Sandra Janezic, Julian R. Garneau, and Marc Monot

Abstract

Clostridium difficile, a gram-positive spore-forming anaerobic bacterium, has rapidly emerged as the leading cause of nosocomial diarrhoea in hospitals. The availability of genome sequences in large numbers, mainly due to the use of next-generation sequencing methods, have undoubtedly shown their immense advantages in the determination of the *C. difficile* population structure. The implementation of fine-scale comparative genomic approaches have paved the way to global transmission and recurrence studies, but also more targeted studies such as the PaLoc or the CRISPR/Cas systems. In this chapter, we provide an overview of the recent and significant findings on *C. difficile* using comparative genomics studies with implication for the epidemiology, infection control and understanding of the evolution of *C. difficile*.

Keywords

Genomics · Evolution · Transmission · Recurrence · CRISPR/Cas · Nontoxigenic strains · Epidemiology

1 Introduction

Clostridium difficile infection (CDI) is currently the most frequently occurring nosocomial diarrhoea in healthcare environments (Davies et al. 2016). This major pathogen synthesizes two toxins, encoded in a pathogenicity locus (PaLoc), that are generally recognised as its main virulence factors. Over the last decade, the incidence and severity of CDI have significantly increased mainly due to the emergence of new strain variants. Molecular typing methods were extensively used to understand its epidemiology, genetic diversity and

S. Janezic
National Laboratory for Health, Environment and Food (NLZOH), Maribor, Slovenia

University of Maribor, Faculty of Medicine, Maribor, Slovenia

J. R. Garneau
Département de Microbiologie, Institut Pasteur, Laboratoire Pathogenèse des bactéries anaérobies, Paris, France

Département de Microbiologie et d'infectiologie, Faculté de Médecine et des Sciences de la Santé, Université de Sherbrooke, Sherbrooke, QC, Canada

M. Monot (✉)
Département de Microbiologie, Institut Pasteur, Laboratoire Pathogenèse des bactéries anaérobies, Paris, France

Département de Microbiologie et d'infectiologie, Faculté de Médecine et des Sciences de la Santé, Université de Sherbrooke, Sherbrooke, QC, Canada

Université Paris Diderot, Sorbonne Paris Cité, Paris, France
e-mail: marc.monot@pasteur.fr

evolution. The *C. difficile* population structure contains hundreds of strain types organized in phylogenetic clades (Dingle et al. 2014; Elliott et al. 2014; Janezic et al. 2016).

The first complete genome sequence of a *C. difficile* strain was published in 2006 (Sebaihia et al. 2006) enabling the development of comparative genomics. Initially, microarray comparative genome hybridization (CGH) were used in global studies to estimate the diversity and evolution of strains (Table 1). However, many laboratories over the world can now afford the frequent and even the routine sequencing of *C. difficile* strains. The availability of genome sequences in large numbers, mainly due to the use of next-generation sequencing (NGS) methods, have now undoubtedly shown their immense advantages in the determination of the *C. difficile* population structure. The implementation of fine-scale comparative genomic approaches have paved the way to global transmission and recurrence studies, but also more targeted studies such as the PaLoc or the CRISPR/Cas systems (Table 1).

Here we provide an overview of the recent and significant findings on *C. difficile* using comparative genomics studies. Those researches shed new light on the epidemiology, the evolution and the clinical practice used for *C. difficile*.

2 Global Comparative Genomics

2.1 Population Structure of *C. difficile* Species

Lemee et al. (2004) conducted the first analysis of genetic relationship and population structure of *C. difficile* isolates using multilocus sequence typing (MLST). They identified 34 different MLST sequence types (MLST-ST) among the 72 isolates. The phylogenetic analysis demonstrated three distinct phylogenetic clades with no specific association between a particular clade and hosts or geographic origins. Furthermore, they showed that loci included in the MLST scheme were in linkage disequilibrium demonstrating a clonal population structure (i.e. mutational evolution) of *C. difficile* species (Lemee et al. 2004). The study by Griffiths et al. (2010) using a different MLST scheme (with different set of genes included) confirmed the clonal population structure of *C. difficile* and identified two additional lineages, one represented by the ST-22 (PCR ribotype 023, toxinotype IV) and the genetically distant outlier of ST-11 (PCR ribotype 078, toxinotype V). In 2012, Knetsch et al. (2012) described a putative sixth lineage, using MLST, represented by a single sequence type (ST-122; PCR ribotype 131). However phylogenetic analysis based on core genome comparison did not confirm the topology of the tree and placed this strain as an outlier within the clade 1, possibly a recombinant between clade 1 and clade 2 (Dingle et al. 2014). The population structure composed of clades was later confirmed by comparisons of whole genome sequences on more diverse collection of strains (He et al. 2010; Dingle et al. 2014; Janezic and Rupnik 2015; Knight et al. 2015). High concordance of MLST and core genome phylogeny demonstrated that MLST could be used as a good proxy to whole genome comparisons (Griffiths et al. 2010; Didelot et al. 2012; Dingle et al. 2014).

There are currently eight distinct phylogenetic clades described, which are designated from one to five and three cryptic clades C-I, -II, -III (Fig. 1) (Janezic et al. 2016). The cryptic clades represent highly divergent groups of strains, thus it is speculated that these groups of strains might even represent novel species or a subspecies (Dingle et al. 2014). These clades were initially associated only with non-toxigenic strains. However, in a recent publication toxigenic strains were characterized in one of these clades, i.e. the clade C-I (Monot et al. 2015).

The population structure composed of the first six clades (1–5 and C-I) was defined mainly on isolates originating from humans and in lesser extent from animals. Recently, a study on MLST analysis of isolates originating from the environment (mainly soil) demonstrated two highly divergent clades (C-II and C-III) comprising mainly

Table 1 *C. difficile* comparative genomic studies

Year	Strains	References	Topics	Summary
Hybridization: microArrays				
2006	8	Sebaihia et al. (2006)	Comparison	Core genome
–	75	Stabler et al. (2006)	Evolution	Phylogenomics
2009	73	Janvilisri et al. (2009)	Comparison	Core and divergence between host
2010	167	Scaria et al. (2010)	Comparison	Core genome
–	94	Marsden et al. (2010)	Comparison	UK and European ribotype 027
Sequencing: Sanger and NGS				
2009	2	Stabler et al. (2009)	Comparison	Historic and modern ribotype 027
2010	29	He et al. (2010)	Evolution	Short and long time scales
–	15	Scaria et al. (2010)	Comparison	Core genome
2011	14	Forgetta et al. (2011)	Comparison	Severe disease associated genomic markers
2012	15	Eyre et al. (2012)	Transmission	WGS for outbreak detection
–	486	Didelot et al. (2012)	Transmission	Micro-evolution
2013	151	He et al. (2013)	Evolution	Emergence and global spread of ribotype 027
–	1	Eyre et al. (2013d)	Evolution	Short-term stability of a single ribotype 027
–	1223	Eyre et al. (2013b)	Transmission	Identification of diverse source of infection
–	15	Eyre et al. (2013a)	Transmission	Detection of mixed infection
–	176	Eyre et al. (2013c)	Transmission	Role of asymptomatic carriage in transmission
2014	1693	Dingle et al. (2014)	Evolution	History of the pathogenicity locus
–	48	Kurka et al. (2014)	Typing	Ribotype and MLST correlation
–	185	Eyre et al. (2014)	Antibiotics	Fidaxomicin in relapse and reinfection
–	3	Moura et al. (2014)	Antibiotics	Metronidazole resistance
–	31[a]	Moura et al. (2014), Hargreaves et al. (2014)	CRISPR	Distribution and diversity
2015	53	Mac Aogain et al. (2015)	Recurrence	Discrimination between relapses and reinfections
–	18[a]	Boudry et al. (2015)	CRISPR	Mechanistic and physiology
–	3	Monot et al. (2015)	Evolution	Model of the pathogenicity locus evolution
2016	96	Quesada-Gomez et al. (2016)	Toxins	Specificity of hypervirulent Clade 2 TcdB proteins
–	5	Chowdhury et al. (2016)	Toxins	Toxin-negative strains in human and animals
–	108	Kumar et al. (2016)	Transmission	Relapse and reinfection of ribotype 027
2017	35	Sim et al. (2017)	Recurrence	Rate of relapses and reinfections
–	277	Cairns et al. (2017)	Evolution	Phylogeny of ribotype 017
–	265	Mawer et al. (2017)	Transmission	Symptomatic patients but fecal toxin negative
–	971	Eyre et al. (2017)	Transmission	WGS as hospital surveillance tools

[a]*C. difficile* phage or prophage

non-toxigenic isolates (Janezic et al. 2016). The topology of the MLST based tree was also confirmed by whole genome comparisons (Janezic and Rupnik, unpublished). It was hypothesized that, due to high abundance of isolates from these two clades in the environmental samples and only sporadic isolation from clinical samples, these strains could represent native environmental isolates, which are not primarily associated with humans and/or animals (Janezic et al. 2016).

The most heterogeneous clade, in terms of number of MLST-STs and PCR ribotypes, is clade

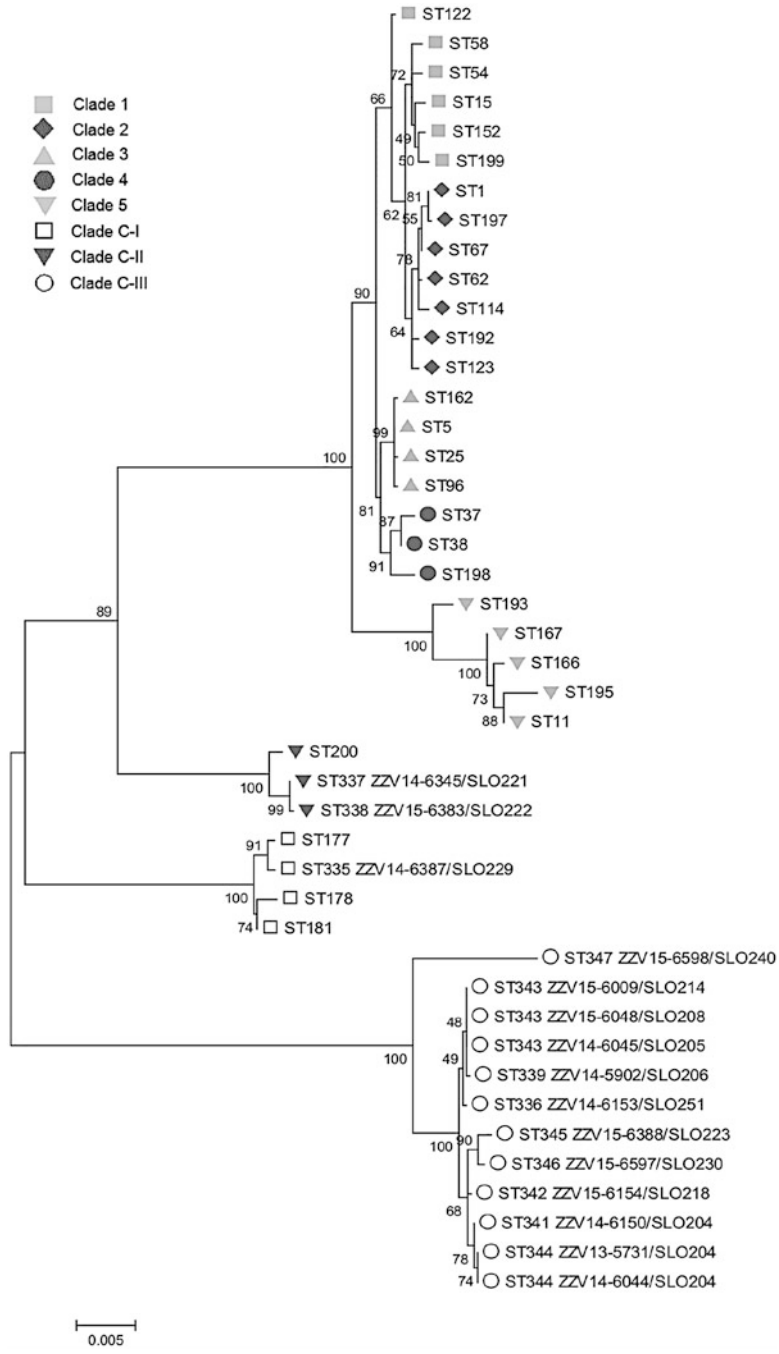

Fig. 1 Maximum likelihood phylogenetic tree showing the eight currently described *C. difficile* clades (Reproduced from Janezic et al. 2016)

1, where more than 200 different MLST-STs are present (data from PubMLST *C. difficile* database, accessed 21.7.2017) (Table 2). Many of the strains from this clade are of clinical significance, e.g. PCR ribotypes 014, 002, 001, 015, 018, which are among the ten most prevalent PCR ribotypes isolated from CDI (*C. difficile* infection) patients in Europe (Davies et al. 2016). Clade

Table 2 Overview of heterogeneity within clades and correlation between main MLST-ST and PCR ribotypes

Clade	Nr. MLST ST[a]	Most known PCR ribotypes/MLST-ST(s)[b]	
1	241	001	ST-3
		002	ST-8, ST-35, ST-48, ST-146
		012	ST-54
		014	ST-2, ST-13:14, ST-49:50, ST-132
		015	ST-10, ST-44
		018	ST-17
2	61	027	ST-1
		176	ST-1
		244	ST-41
3	13	023	ST-5, ST-22, ST-25
4	53	017	ST-37, ST-86
5	30	033	ST-11
		126	ST-11
		078	ST-11
C-I[c]	6	ND	ST-177:181
C-II[d]	3	ND	ST-200, ST-337:338
C-III[d]	9	ND	ST-336, ST-339, ST-341:347

[a]Data from PubMLST *C. difficile* database (accessed 21.7.2017)
[b]Data from Griffiths et al. 2010; Stabler et al. 2012; Knetsch et al. 2012; Dingle et al. 2014
[c]Data from Dingle et al. 2014
[d]Data from Janezic et al. 2016

2 is the second most heterogeneous clade, containing 61 different MLST-STs, including PCR ribotype 027 (ST-1), a well-known epidemic strain, and two emerging ribotypes 176 (ST-1) and 244 (ST-41) (Valiente et al. 2012; Lim et al. 2014). In the clade 3, 13 different STs are present and the most known representative is PCR ribotype 023 (represented with ST-5, ST-22 and ST-25), which is also often isolated from humans in European countries (Davies et al. 2016). The clade 4, composed of 53 STs, is also known as A-B+ clade due to its association with PCR ribotype 017 strains (A-B+CDT-). Despite the altered toxin expression this strain is widespread, especially in Asia (Shin et al. 2008; Collins et al. 2013). One of the best known representative of clade 5 is PCR ribotype 078, which has in recent years emerged in human CDI (Rupnik et al. 2008), while before it was thought to be primarily an animal pathogen (Jhung et al. 2008). Although in the first studies it seemed that this clade was more homogeneous, consisting primarily of ST-11 strains (Griffiths et al. 2010; Dingle et al. 2011; Knetsch et al. 2012), later studies (MLST and WGS) demonstrated the opposite, since there are currently 30 STs found in clade 5 (Table 2).

Large scale analyses of strains from diverse sources and geographic origins also revealed that significant microdiversity exist within clades and that *C. difficile* is continuously evolving (Table 2) (Griffiths et al. 2010; Dingle et al. 2011, 2014; Knetsch et al. 2012; Janezic et al. 2016).

2.2 Worldwide Evolution of Important *C. difficile* PCR Ribotypes

2.2.1 Epidemic *C. difficile* PCR Ribotype 027

C. difficile PCR ribotype 027 has in the last two decades gained much interest because of its rapid emergence worldwide. The strain has been associated with large CDI outbreaks and increased morbidity and mortality, which have first started to appear in USA and Canada (Pepin et al. 2004, 2005; Loo et al. 2005; McDonald et al. 2005). The strain was later also introduced

in Europe, with first outbreaks documented in the United Kingdom, and in following years also in continental Europe (Kuijper et al. 2008). Although the prevalence of PCR ribotype 027 declined markedly in Europe, the strain still remains one of the most common strains causing CDI (Bauer et al. 2011; Davies et al. 2016). To explore global population structure and genetic changes associated with its rapid emergence and global spread, He et al. (2013) sequenced genomes of 151 strains, representing the global population of ribotype 027 strains, collected between 1985 and 2010. They showed that ribotype 027 population consists of two genetically distinct fluoroquinolone resistant (FQR1 and FQR2) epidemic lineages. Both lineages have independently acquired the same mutation in DNA gyrase, conferring fluoroquinolone resistance, and a novel conjugative transposon (CTn5-like element, Tn6192). These were the only two genetic traits differentiating FQR1 and FQR2 lineages from the historic 027 isolates, and were most likely key genetic changes associated with the rapid emergence of ribotype 027. Also, low level of genomic diversity within the core genome of the 151 PCR ribotype 027 strains analysed was demonstrated, with only 536 SNPs identified. Only two of these SNPs (limited to a single isolate) were discovered in the PaLoc region of historic and epidemic isolates (He et al. 2013) which contrast with the earlier assumptions that genetic changes in the PaLoc were the cause of emergence of *C. difficile* 027 (McDonald et al. 2005; Warny et al. 2005).

Although both lineages emerged in North America, they showed different global spread and limited geographic clustering. FQR1 originated in Pittsburgh (Pennsylvania, USA), and was subsequently transmitted to Switzerland and South Korea. The FQR2 lineage which contains majority of epidemic strains was transmitted to continental Europe and United Kingdom on several different occasions, and a single introduction to Australia was demonstrated. The phylogenetic analysis of UK collection of epidemic FQR2 strains further demonstrated frequent long-range transmissions within the UK, some of them associated with large scale outbreaks that gained attention also in media (He et al. 2013).

2.2.2 Toxin Variant *C. difficile* PCR Ribotype 017

Another important strain that has gained much attention is PCR ribotype 017 (toxinotype VIII, MLST ST-37). Despite producing only one of the three *C. difficile* toxins (A-B+CDT-) PCR ribotype 017 strains are causing clinically significant infections worldwide (Drudy et al. 2007; Collins et al. 2013; Cairns et al. 2015). Initially, ribotype 017 strains have been identified in outbreaks in Asia where they were responsible for the majority of CDIs (Collins et al. 2013). It was hypothesized that this strain has spread from Asia throughout the world (Drudy et al. 2007; Collins et al. 2013; Cairns et al. 2015). To define population structure and patterns of global spread, Cairns et al. (2017) conducted a phylogenetic analysis on a global collection of PCR ribotype 017 strains. Two hundred and seventy seven PCR ribotype 017 strains, including human, animal and environmental isolates were obtained from all six continents and were isolated between 1990 and 2013. The phylogenetic analysis based of the core SNPs demonstrated presence of two genetically diverse lineages (SL1 and SL2) which are geographically and temporally widespread. In both lineages multiple clonal expansions were revealed. Phylogeographic analysis also revealed, contrary to current Asia-origin hypothesis, that origin of ribotype 017 is in North America, from where the strain has been introduced first to Europe and then from Europe to Asia and Australia and from there it was then spread worldwide. Further genetic analysis, based on the SNPs present in *gyr*A, *gyr*B and *rpo*B genes, predicted that ribotype 017 strains are commonly resistant to fluoroquinolone (76%) and rifampicin (35%) classes of antibiotics. Due to different clusters of genes inserted in the same genomic locations the authors also identified hot-spot regions for DNA uptake (Cairns et al. 2015).

2.3 *C. difficile* Transmissions and Epidemiology of Recurrent CDI

2.3.1 *C. difficile* Transmissions in Hospital Environment

In the past, assessment of genetic relatedness of *C. difficile* isolates has been hampered by the use of sub-optimal genotyping methods that do not have sufficient discriminatory power (e.g. PCR ribotyping, MLST) to distinguish closely related strains. Whole genome sequence analysis which enables comparison at the highest level of genetic resolution has been widely adopted for global and national *C. difficile* surveillances and has revealed some novel insights about transmissions dynamics and recurrent infections (Table 1).

Estimating the rates at which bacterial genomes evolve (e.g. within-host diversity and short-term evolution) is critical for understanding transmission patterns (Duchene et al. 2016). For *C. difficile*, rates of short-term evolution and within-host diversity have been explored in several studies, using serial samples from patients with recurrent or on-going CDI and in vitro gut model of CDI. In all these studies similar estimations of evolutionary rates were obtained, 1–2 SNPs/genome/year and within-host diversity 0.30 SNPs/genome/year (Didelot et al. 2012; Eyre et al. 2013b, d). By using these estimations two isolates obtained less than 124 days apart would be expected to have 0–2 SNPs differences and isolates obtained 124–364 days apart should exhibit 0–3 SNPs differences (Eyre et al. 2013b). This definition of genetically related isolates, i.e. isolates that are most probably a result of transmission, has now been widely adopted.

It is traditionally believed that most cases of *C. difficile* infections are acquired within hospital settings, where they are being transmitted from person to person (Vonberg et al. 2008; Khanna and Pardi 2012). Eyre and colleagues (Eyre et al. 2013b) compared genomic sequences of 1223 *C. difficile* isolates and demonstrated that only 35% of cases were acquired from another known case within a hospital setting and only a subset of these cases shared the same ward with at least one other case or had some sort of hospital contact, which is much lower than expected. Almost half (45%) of isolates were genetically unrelated ($\geq$10 SNPs) to any other previous case and could not be linked by transmission (direct or indirect), meaning that they were likely acquired from sources other than symptomatic patients. Identification of a rather diverse pool of *C. difficile* strains indicate existence of substantial reservoirs of *C. difficile* and that transmission routes other than those due to symptomatic CDI patients should be considered (e.g. asymptomatic patients and environment) (Eyre et al. 2013b).

Role of asymptomatic carriage of *C. difficile* in transmission was explored in a small study including 132 participants (Eyre et al. 2013c). The authors demonstrated that even though asymptomatic carriage is common, onward transmission from asymptomatic case is relatively rare. The same group has also described novel approach using WGS that enables assessment of the extent of infection transmission within healthcare institutions by measuring the proportion of cases that are acquired from a previous case (i.e. linked cases) (Eyre et al. 2017).

2.3.2 *C. difficile* Recurrence: Reinfections Versus Relapses

WGS has also been shown to be a valuable tool in understanding the epidemiology of CDI recurrences with greater accuracy, especially within hospital settings with endemic strains (Eyre et al. 2014; Mac Aogain et al. 2015; Kumar et al. 2016). Recurrent *C. difficile* infections occurs in up to 25% of patients after the first CDI episode and discriminating between reinfections (infection with newly acquired strain) and relapses (recurrent episode due to original strain) is important for CDI management; infection prevention and treatment, respectively (Kelly 2012).

Similar methodology that was used in transmission studies can also be applied in studies resolving the contribution of relapses and reinfections in recurrent CDI. Relapse is defined as a recurrent infection with an isolate differing $\leq$2 SNPs from the isolate from initial episode

and reinfection involving pairs of isolates differing ≥10 SNPs (Eyre et al. 2014). Mac Aogain et al. (2015) applied this methodology to 19 patients with recurrent CDI to resolve the nature of the recurrences and demonstrated that majority of recurrences (16 out of 19) were due to relapse with endogenous strain. Similar findings were also found in a study by Eyre et al. (2014) that used WGS to determine whether recurrences of CDI in 93 patients (28 were treated with fidaxomicin and 65 were treated with vancomycin) were due to reinfection or relapse. Overall 79.6% (74 of 93) recurrent CDIs were due to relapse. Reinfection accounted for just one fifth of recurrences.

2.4 Influence of SNPs on Virulence and Phenotype of CD630 Derivatives

The *C. difficile* strain CD630 was isolated in 1982 in Zurich, Switzerland from a patient with pseudomembranous colitis (Sebaihia et al. 2006). This is the first strain of *C. difficile* which genome has been sequenced and which derivatives were used as a model strain for generation of mutants in different studies exploring the importance of *C. difficile* toxins in pathogenesis. Two groups that used isogenic mutants (in which production of one of both toxins was ablated) of erythromycin sensitive derivatives (630E and 630Δ*erm)* from the strain CD630, got contradictory outcomes on virulence potential of toxin A (TcdA) (Collery et al. 2016). In a study by Lyras et al. (2009) the outcome was that *tcd*B mutant, producing only toxin TcdA (A+B-), was unable to cause disease in hamster model, whereas in a study by Kuehne et al. (2010), the authors demonstrated that both toxins, TcdA and TcdB, are capable of causing disease in a hamster model. Both strains possessed the same deletion of *erm*B gene and were isolated in two different laboratories by repeated sub-culturing of strain CD630 (Collery et al. 2016). Re-sequencing of both strains revealed that both strains had a number of SNPs, compared to the published genome of CD630, which were most likely accumulated during the sub-culturing. Genetic variations between the strains were found responsible for the phenotypic differences observed in both mutants (growth rate, motility, sporulation and virulence), explaining different outcomes of both studies. Since 630Δ*erm* strain more closely resembles the progenitor strain, the authors concluded that this strain should be favored over 630E and that re-sequencing of genomes of mutant strains should become a routine practice (Collery et al. 2016).

2.5 Comparative Genomic Analysis of Non-toxigenic Strains

Comparative genomic studies demonstrated that non-toxigenic *C. difficile* strains are represented in all clades, alongside toxigenic isolates (Dingle et al. 2014; Monot et al. 2015). Although toxin-negative *C. difficile* strains can be isolated from patients and animals suffering from gastrointestinal diseases, they are not considered to play a role in disease (Vedantam et al. 2012). Chowdhury et al. (2016) undertook a comparative genomic analysis of five toxin negative strains isolated from faeces from humans and animals with symptoms of gastrointestinal (GI) disease. Even though the authors stated that GI symptoms were likely due to non-toxigenic *C. difficile*, this could also be due to undetected co-infection with toxigenic *C. difficile* or to infection with a yet unknown or un-cultivable organism. Phylogenetic analysis demonstrated that all five isolates clustered with toxigenic isolates (belonging also to the same MLST-ST) and had also a similar virulence associated gene repertoire as those found in toxigenic strains (e.g. genes required for sporulation (*spo0A*) and adhesion (*groEL*, *fliC*), genes coding for surface proteins (*slpA* and *cwp*) necessary for colonization of the gut and different serine-proteases and metalloproteases).

Recently a transfer of the PaLoc from toxigenic to non-toxigenic strain, that was able to produce toxins after acquiring the PaLoc, has been demonstrated (Brouwer et al. 2013) further suggesting that non-toxigenic isolates could

represent reservoir for toxigenic strains, as was already suggested (Dingle et al. 2014). Current knowledge of the pathogenicity of non-toxigenic strains is still limited and therefore further research is required to explore potential of non-toxigenic strains to cause diseases as well as the PaLoc exchange between toxigenic and non-toxigenic strains (Chowdhury et al. 2016).

3 Targeted Comparative Genomics

3.1 Evolution of the *C. difficile* Pathogenicity Locus

The pathogenicity locus encodes the exotoxins TcdA and TcdB, the two main virulence factors involved in CDI. Bacterial strains completely lacking this genomic region are unable to cause the disease and its associated symptoms, so it appears of outmost importance to understand how this locus has been acquired and how it can evolve over time (Cohen et al. 2000). Comparative genomics, which is a very powerful approach to elucidate the evolutionary history of the PaLoc, have shown that this locus has undergone a very complex and intriguing eventful history (Dingle et al. 2014; Elliott et al. 2014; Monot et al. 2015). However, the conclusions drawn from such analyses are likely in constant evolution as they depend on the strains available.

3.1.1 PaLoc Acquisition and Exchange

Dingle et al. (2014) have estimated that the most recent acquisition of the PaLoc would have occurred some 500 years ago. The latest exchange of the PaLoc between *C. difficile* strains have been calculated to about 300 years and the most recent PaLoc loss from the genome would have happened in recent times (~30 years ago). Because of the very long genomic fragments concurrently swapped during those recent PaLoc losses and exchanges, it is thought that host-mediated homologous recombination is the mechanism by which those recent events have arisen. Those observations were made possible by plotting the distribution of indels and SNPs at the chromosomal scale and by analyzing in more details the SNP plots for the regions around the PaLoc. Distinctively, specific recombination mediated by an integrase supplied in *trans* appears to be the mechanism involved in the initial PaLoc acquisition. The reason for this is the absence of recombination signatures on DNA sequences distant from the PaLoc in non-toxigenic strains (Dingle et al. 2014).

Brouwer et al. (2013) demonstrated experimentally that non-toxigenic *C. difficile* strains could be converted into toxin producers by horizontal gene transfer and genetic recombination. It is worrying to think that different versions of the PaLoc can be acquired and transferred seemingly at any time by any strain because this makes all the non-toxigenic strains possible candidates for becoming toxin producers (Brouwer et al. 2013). The possible acquisition of the PaLoc by non-toxigenic strains that already exhibit high resistance to antibiotics widely used in clinics for the treatment of CDI, (e.g. strains belonging to ribotype 010 highly resistant to metronidazole Moura et al. 2013, 2014), is a very concerning scenario. All those recent findings concerning the PaLoc are of outmost importance and can have profound repercussions on the evolution of the disease in clinics. It is highly conceivable that the events reported here and the related mechanisms might be more prevalent than first thought and may be relevant to other commensal and pathogenic bacteria as well.

3.1.2 PaLoc Organization and Evolution

The evolutionary history of the PaLoc was first studied by performing comparative genomics on *C. difficile* genomes from a collection of 1693 toxigenic and non-toxigenic strains (Dingle et al. 2014). Thereafter, further studies have refined the established model by adding new PaLoc variants (Elliott et al. 2014; Janezic et al. 2015; Monot et al. 2015) leading to the actual known gene contents and organizations of the PaLoc detailed in Fig. 2a.

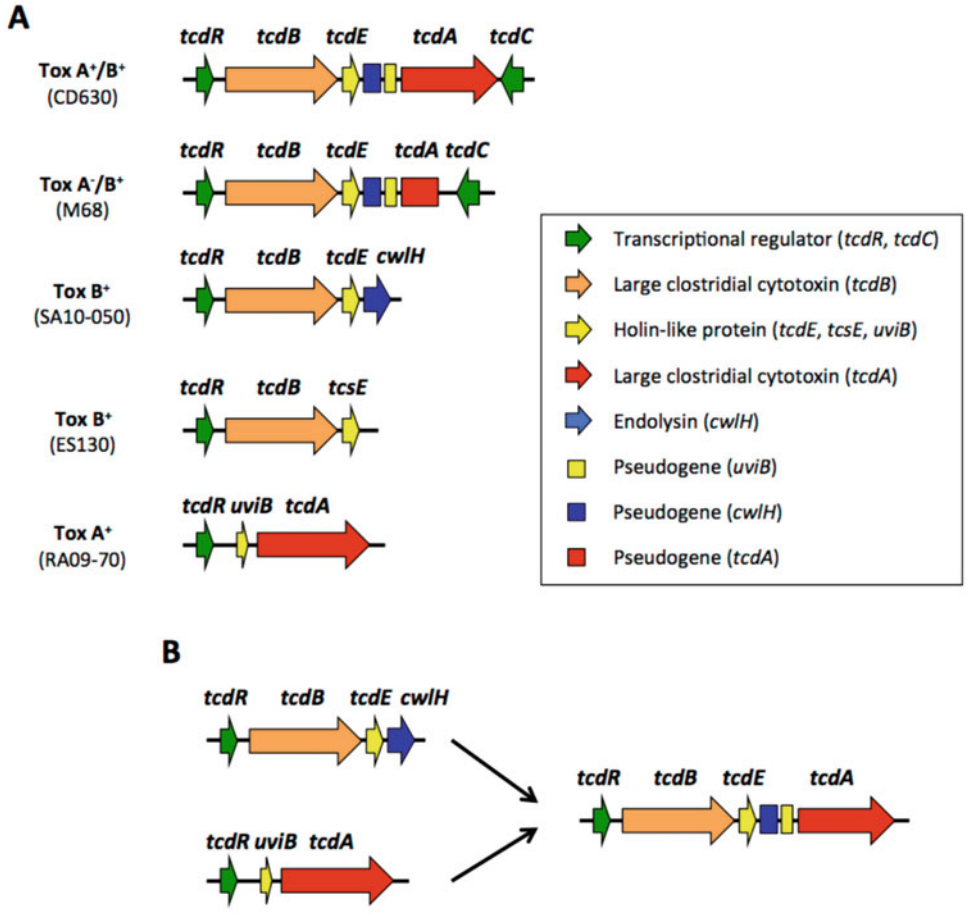

Fig. 2 PaLoc diversity and evolution. (**a**) Known types of *C. difficile* PaLoc and (**b**) Model of evolution from "Mono-Toxin" to "Bi-Toxin" PaLoc (Adapted from Fig. 6B and S7 of Monot et al. 2015)

Monot et al. (2015) found two types of genomic organization of the PaLoc that each contained only one of the two toxins (A+B- and A-B+). These two "Mono-Toxins PaLocs" were located at different positions in the *C. difficile* genome far from the usual PaLoc integration site, which was not described before. Based on sequences similarity analysis, the authors detected two gene remnants of these PaLoc variants in the classical PaLoc, *i.e.* "Bi-Toxin PaLoc". Altogether, this work supports a scenario in which the "Bi-Toxin PaLoc" was generated by a fusion of two "Mono-Toxin PaLoc" from ancestral *C. difficile* strains through multiple independent PaLoc acquisitions (Fig. 2b) (Monot et al. 2015).

The PaLoc could also be altered during evolution by insertion of mobile elements. These strains have been associated with milder clinical phenotypes and the presence of the transposable element Tn6218 is believably responsible for this change in the bacterial phenotype (Dingle et al. 2014). This specific genetic region has probably undergone many different exchanges or separate acquisition events, as many accessory genes were noticed in several variants widely spread in the *C. difficile* population. It is important to carefully study and follow this type of transposable region such as Tn6218, since it carries, among others, a set of genes providing high-level resistance to antibiotics used in clinical settings (Spigaglia et al. 2011; Kelly 2012; Deshpande et al. 2013).

Elements related to Tn6218 have been found in other various genomes such as *Bifidobacterium breve*, *Ruminococcus*, *Lachnospiraceae* and *Coprobacillus sp*., suggesting that the transfer of this element between different species is also highly probable and should undoubtedly be further investigated (Dingle et al. 2014).

First identified and described by Braun et al. in 2000, IStrons represent another type of mobile genetic element that has been shown to create variations inside the *C. difficile* genome and inside the PaLoc region (Rupnik 2008). It has been hypothesized that the original IStron (CdISt1-0) was the result of a fusion event between an insertion element (IS) and a group I intron, generating a novel class of chimeric ribozymes adapted to propagate in eubacterial genomes (Hasselmayer et al. 2004). Widely spread in *C. difficile* genomes, four variants of IStrons have now been identified, all exhibiting a self-splicing ribozyme activity and which transposition was found to be harmless for the interrupted gene (i.e. does not affect TcdA toxin production in *C. difficile*). Braun et al. (2000) have hypothesized that this particular chimeric element might be more efficient and more adapted, as the risk of mutation usually observed during transposition of an IS-elements is significantly reduced by the precise splicing activity provided by the group I intron (Braun et al. 2000).

The complex relationship between *C. difficile* and the PaLoc, and also the multiple ways by which it is able to evolve, can ostensibly lead to concrete repercussions on its virulence and epidemiology. This is illustrated by the characterization of a clinical strain RA09-70 exhibiting a new major variant of the PaLoc producing only the toxin A, the A+B- strain RA09-70 (Fig. 2a) (Eckert et al. 2013; Monot et al. 2015). This type of strain would go completely undetected by cytotoxicity assays, which successfully confirm CDIs only when TcdB is present. Dissemination of this type of strain could lead to a problematic under-diagnostic scenario, since this assay is commonly used as a sole method for the diagnosis of CDI (Monot et al. 2015).

3.2 Advances in CRISPR/Cas Systems and Phage-Host Interaction

Mobile genetic elements (MGE) and especially bacteriophages are major contributors and facilitators of genetic evolution in bacteria, including *C. difficile*. It has been suggested that *C. difficile* is exhibiting a complex, highly mobile and mosaic genome because it is striving in an environment where it is constantly being confronted to numerous interacting bacteria and phages also struggling to survive (Sebaihia et al. 2006). Therefore, *C. difficile* is incessantly incorporating favorable genetic material useful for its adaptation while simultaneously developing defense mechanisms in order to limit the incorporation and influence of harmful genetic material (Boudry et al. 2015). A myriad of defense mechanisms against foreign MGE and phages are now better known, but the CRISPR/Cas system has only recently been more actively explored in *C. difficile*. CRISPR/Cas systems have been defined in three majors types (I, II, III), further divided in 12 different subtypes (Makarova et al. 2011, 2013). *C. difficile* only harbors the subtype I-B, a system probably acquired by mean of horizontal gene transfer (HGT) from *Archaea* (Richter et al. 2012; Peng et al. 2014).

3.2.1 CRISPR Mechanism and Physiology

The analogy between the mammal acquired immunity and the bacterial CRISPR/Cas system is often used, since bacteria can become protected against genetically akin phages after exposition, in a fashion reminiscent of vaccination. The bacteria memorize previous unsuccessful infections by acquiring small sequences of the assailants and integrating them to its own genome, inside a specific region or array containing other similar protective sequences. Those sequences, called "spacers" in the CRISPR/Cas array system, are used by the bacteria to scan and recognize the identical or near identical sequences, called "protospacer", in the genome of a future potentially more lethal phage

invader. When the sequence is recognized, a functional CRISPR system is able to neutralize the infecting agent by cutting and digesting its DNA, interrupting the infection cycle, which may also result in the acquisition of additional protective sequences.

Recently, important findings have been made for this system in *C. difficile* using comparative genomics associated with laboratory procedures, such as transcriptome sequencing (RNA-Seq) and plasmid conjugation efficiency assays (Hargreaves et al. 2014; Boudry et al. 2015). Those analyses have allowed to conclude that the CRISPR/Cas system in *C. difficile* was functional and used in this species, since many genes and arrays coding for important components of the CRISPR arrays were actively transcribed. Nine different CRISPR arrays were found to be present and transcribed in the epidemic strain R20291, and reference strain 630 exhibited 12 expressed arrays (Boudry et al. 2015). The analysis of the targets for the identified spacers showed that a unique phage could be targeted by numerous different spacers, surely to increase the efficiency of phage neutralization by the system (Boudry et al. 2015). This could also be an indication that phage has the ability to evade the CRISPR system using a mutational process. Contrastively, a single spacer can have the ability to target conserved genes present in multiple related phages, thus bestowing them with an efficient and inexpensive defense against multiple potential invaders at once. Boudry et al. (2015) concluded that there is a good correlation between the real and predicted phage susceptibility according to the spacer content of the bacterial strains and the theoretical predicted phage targets. Remarkably, the spacer sequences found in *C. difficile* strain 630 were anticipated to target all known and isolated clostridial bacteriophages. Experimentally, this strain exhibited resistance to infection by all the phages that could be tested.

The CRISPR/Cas system seems particularly active and meaningful in *C. difficile* as numerous highly active CRISPR arrays are found, which moreover greatly contrast with what is observed in other bacteria such as *E. coli* and *Streptococcus pyogenes* in which the CRISPR loci are barely expressed or event completely silent (Pougach et al. 2010; Deltcheva et al. 2011).

3.2.2 CRISPR Distribution and Diversity

Hargreaves et al. (2014) determined the distribution and diversity of the CRISPR/Cas system in *C. difficile*. To do this, they examined the relationships between spacers and 31 *C. difficile* phages and prophage genomes. The spacer content is thought to bring a good perception of the predominant and relatively recent phage predation history (Diez-Villasenor et al. 2010). However, a large number of spacers match sequences of unknown nature, possibly targeting unknown *C. difficile* phages or even non-clostridial phages. They also found, in several strains of *C. difficile*, CRISPR arrays inside prophage genomes, which is considered an unusual situation for this system (Hargreaves et al. 2014; Boudry et al. 2015). Those phages carried spacers that were found to match sequences of other bacteriophages. Once they have successfully integrated the bacterial genome, prophages could plausibly use those spacers in order to give them an advantage over other phages by blocking their capacity to infect the same strain (Hargreaves et al. 2014). Prophages possessing CRISPR arrays are thought to rely on the bacterial host for the proper functioning of the system, since the *cas* operon containing the set of genes necessary to process the arrays were always absent (Boudry et al. 2015).

To obtain a global view of the distribution of the CRISPR/Cas system in *C. difficile*, Boudry et al. (2015) tested the presence of cas operons in 2207 *C. difficile* published and available genomes. Nearly 90% of them possessed a complete *cas* operon, making the CRISPR/Cas a common system in this bacteria.

4 Conclusions

The evolution of comparative genomics of *C. difficile* strains from molecular typing and microarrays to whole genome sequence enabled

significant improvements in the determination of the population structure of *C. difficile*. Beyond a deeper understanding of the diversity of strains, WGS also makes possible the emergence of new area of research such as transmission or reinfection studies.

Another aspect to be taken into account is the availability of massive sequence data allowing the analysis of specific loci. Due to its importance in virulence, the PaLoc was extensively explored and it has been concluded that this locus is in constant evolution.

This leads us to conclude that findings of comparative genomics highly depend on the strains available, thus making the availability of raw data in public database of primordial importance.

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Cellular Uptake and Mode-of-Action of *Clostridium difficile* Toxins

Panagiotis Papatheodorou, Holger Barth, Nigel Minton, and Klaus Aktories

Abstract

Research on the human gut pathogen *Clostridium difficile* and its toxins has gained much attention, particularly as a consequence of the increasing threat to human health presented by emerging hypervirulent strains. Toxin A (TcdA) and B (TcdB) are the two major virulence determinants of *C. difficile*. Both are single-chain proteins with a similar multidomain architecture. Certain hypervirulent *C. difficile* strains also produce a third toxin, namely binary toxin CDT (*Clostridium difficile transferase*). As *C. difficile* toxins are the causative agents of *C. difficile*-associated diseases (CDAD), such as antibiotics-associated diarrhea and pseudomembranous colitis, considerable efforts have been expended to unravel their molecular mode-of-action and the cellular mechanisms responsible for their uptake. Notably, a high proportion of studies on *C. difficile* toxins were performed in European laboratories. In this chapter we will highlight important recent advances in *C. difficile* toxins research.

Keywords

Clostridium difficile · Bacterial disease · Bacterial toxins · Toxin uptake · Toxin receptor

1 Introduction

The human gut pathogen *Clostridium difficile* is capable of producing at least three exotoxins, namely toxin A (TcdA), toxin B (TcdB) and the

P. Papatheodorou (✉)
Institute of Experimental and Clinical Pharmacology and Toxicology, Albert Ludwig University of Freiburg, Freiburg im Breisgau, Germany

Institute of Pharmacology and Toxicology, University of Ulm Medical Center, Ulm, Germany

Faculty of Natural Sciences, University of Ulm, Ulm, Germany
e-mail: panagiotis.papatheodorou@uni-ulm.de

H. Barth
Institute of Pharmacology and Toxicology, University of Ulm Medical Center, Ulm, Germany

N. Minton
BBSRC/EPSRC Synthetic Biology Research Centre, University of Nottingham, Nottingham, UK

K. Aktories
Institute of Experimental and Clinical Pharmacology and Toxicology, Albert Ludwig University of Freiburg, Freiburg im Breisgau, Germany

binary toxin CDT (*Clostridium difficile* transferase). The number of toxins and the quantities produced vary between different *C. difficile* strains. Certain hypervirulent strains release all three toxins during infection. Others produce strain-specific isoforms of toxin A and B (Rupnik and Janezic 2016). Toxin A and toxin B are related but they differ in structure and function from the binary toxin CDT. However, the three toxins share some fundamental similarities during the intoxication process. All three toxins are released by the bacteria and enter into host cells via receptor-mediated endocytosis. An enzymatically active portion of the toxins then escapes from acidified endosomes into the host cell cytosol in order to reach and modify its specific target proteins. In the case of toxin A and B, the enzyme portion is a glucosyltransferase that inactivates small GTPases of the Rho family. The enzyme portion of CDT is an ADP-ribosyltransferase that modifies monomeric G-actin. In the following sections, we will summarize the current knowledge about *C. difficile* toxins´ cellular uptake and mode-of-action which is fundamental for understanding their pathophysiological role in *C. difficile* infections (CDI). A model of *C. difficile* toxins´ uptake process and mode-of-action is depicted in Fig. 1.

2 Structure, Uptake and Mode-of-Action of *C. difficile* Toxin A and B

2.1 Modular Composition of *C. difficile* Toxin A and B

Toxin A and B are large, single-chain protein toxins that comprise several functional domains. The two toxins exhibit a high sequence homology (~50% amino acid identity) and an identical multidomain architecture indicating that a gene duplication event led to the existence of two nearly-identical toxins in *C. difficile* (Von Eichel-Streiber et al. 1992a). Both toxins are also highly similar to other large clostridial toxins (LCTs; also denoted as clostridial glucosylating toxins (CGTs)), such as *Clostridium sordellii* lethal toxin and hemorrhagic toxin, *Clostridium novyi* α-toxin and *Clostridium perfringens* TpeL toxin (Voth and Ballard 2005; Aktories et al. 2017). The large size of toxin A and B led quite early to the assumption that both toxins contain several domains with specific functions during the intoxication process. Eventually, a number of fundamental findings confirmed the modular composition of toxin A and B, which is also true for the other LCTs (Fig. 2).

2.1.1 The CROP Domain

At first, a region consisting of series of combined, repetitive oligopeptides (CROP) was identified and characterized in the C-terminal part of toxin A (Von Eichel-Streiber and Sauerborn 1990; Von Eichel-Streiber et al. 1992b). In toxin A, the CROP domain makes up nearly one-third of the complete protein and consists of 7 long repeats of 30 residues and 31 short repeats of 15–21 residues. In toxin B, the CROP domain contains 7 long repeats of 30 residues and only 21 short repeats of 20–23 residues and thus is significantly shorter than in toxin A. The number and length of the repeating CROP modules have been found to vary between toxins from different *C. difficile* isolates (Rupnik et al. 1998). Historically, the CROP domain was considered to start around residue 1849 of toxin A and residue 1852 of toxin B, respectively. However, according to more recent structural studies by Orth et al. (2014), the CROP domain starts at glycine-1832 for toxin A and at glycine-1834 for toxin B.

A series of studies including monoclonal antibodies or recombinant toxin fragments have provided evidence for a role of the CROP domain of toxin A in receptor binding (Frey and Wilkins 1992; Sauerborn et al. 1997; Frisch et al. 2003). In further studies, crystal structures of two C-terminal fragments (terminal 127 and 255 residues) of toxin A were obtained, thus providing new insights into the overall structure of the CROP domain (Ho et al. 2005; Greco et al. 2006). The CROP domain of toxin A adopts a

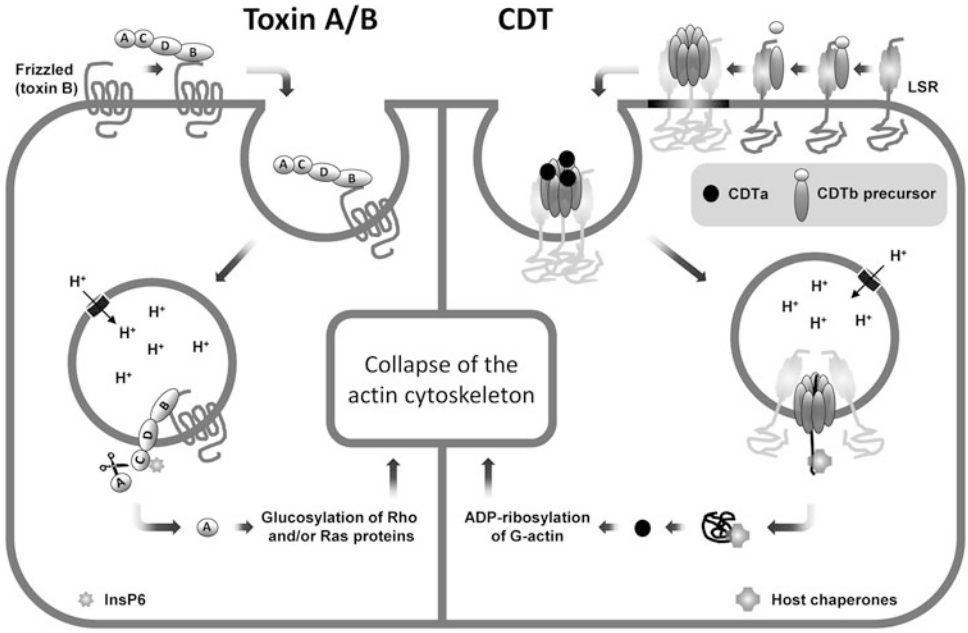

Fig. 1 Model of the uptake process and mode-of-action of toxin A/B and CDT. On the left part, the uptake and cellular action of toxin A and B are shown paradigmatically with the toxin B-specific receptor Frizzled. On the right part, the uptake and cellular action of CDT are shown. Both types of toxins bind to their specific receptors at the cell surface, are then taken up by receptor-mediated endocytosis, form pores in endosomes after acidification of the endosomal lumen and translocate an enzyme domain into the cytosol. The detailed mechanism for the uptake process and the mode-of-action is described in the main text of this review

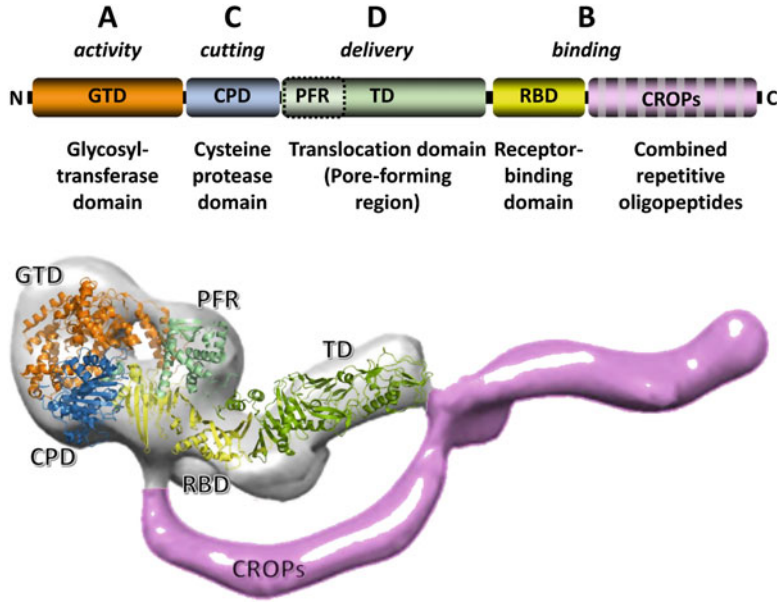

Fig. 2 Multidomain architecture of toxin A and B. Shown is a schematic representation of the multidomain architecture of toxin A and B and below a 3D model of toxin A obtained with negative stain electron microscopy (Pruitt et al. 2010) overlaid with the crystal structure of toxin A lacking the CROP domain (Chumbler et al. 2016). EM structure of toxin A was obtained with publisher's permission from the following original article: Pruitt et al. (2010)

solenoid-like (screw-like) fold (Greco et al. 2006; Ho et al. 2005; Jank and Aktories 2008). One of the two CROP structures was obtained by co-crystallization with the trisaccharide Galα1–3Galβ1–4GlcNAc, which was found to interact with toxin A in earlier reports (Greco et al. 2006; Krivan et al. 1986; Tucker and Wilkins 1991). However, this carbohydrate structure is not present on human cells and thus is unlikely to be part of intestinal receptors of toxin A in humans. The carbohydrate-binding properties of the CROP domain of toxin A were also supported by a study from Dingle et al. (2008). Notably, the CROP domain of toxin A and B is similar to certain saccharide-binding proteins from *Streptococcus downei* and *Streptococcus mutans* (Wren 1991).

2.1.2 The Glucosyltransferase Domain

In 1995, the group of Klaus Aktories (Freiburg, Germany) found that toxin A and B modify the small GTPase Rho and other members of the Rho subfamily via transfer of the glucose moiety from the co-substrate UDP-glucose to threonine-37 of the GTPase (Just et al. 1995a, b). Thus, it became apparent that toxin A and B are bacterial glucosyltransferases capable of inactivating small GTPases of host cells. Deletion analyses from Hofmann et al. with toxin B revealed glucosyltransferase activity in the N-terminal part of the toxin (Hofmann et al. 1997). In 2005, the crystal structure of the glucosyltransferase domain of toxin B in the presence of UDP-glucose and Mn^{2+} was determined (Reinert et al. 2005). It became obvious from the 3D structure that the glucosyltransferase domain of toxin B belongs to the glucosyltransferase type A family. Subsequent biochemical studies revealed important residues that are crucial for the enzymatic activity: residues 364–516 are important for substrate recognition (Hofmann et al. 1998); an essential and highly conserved DXD motif between amino acids 286 and 288 is involved in binding Mn^{2+} (Busch et al. 1998); residue tryptophan-102 is involved in UDP-glucose binding (Busch et al. 2000); isoleucine-383 and glutamine-385 are crucial residues for the co-substrate specificity (Jank et al. 2005); a four-helical-bundle subdomain at the N-terminus of the glucosyltransferase is required for the interaction with the inner plasma membrane (Geissler et al. 2010). Additional essential amino acids for substrate binding were identified by Jank et al. (2007). In 2012, D'Urzo and co-workers presented the crystal structure of the glucosyltransferase domain of toxin A bound to Mn^{2+} and UDP-glucose (D'Urzo et al. 2012). In the same year, Pruitt and colleagues succeeded in solving the structure of the glucosyltransferase domain of toxin A in the presence and absence of its co-substrate UDP-glucose (Pruitt et al. 2012). Very recently, Alvin and Lacy reported new crystal structures of the glucosyltransferase domains of toxin A and B in complex with a non-hydrolysable UDP-glucose analogue and an *apo*-like structure of the glucosyltransferase domain of toxin B (Alvin and Lacy 2017).

2.1.3 The Cysteine Protease Domain

In 2003, Barth and colleagues showed with toxin B that only the N-terminal glucosyltransferase domain reaches the cytosol after completion of the uptake process (Pfeifer et al. 2003). Thus, it was feasible that processing of toxin A and B is a prerequisite of the intoxication process. The cleavage site of toxin B was identified between leucine-543 and glycine-544 (Rupnik et al. 2005). Yet it was not clear whether the processing of toxin A and B occurs by a host protease or an internal domain of the toxins. Eventually, the group of Eichel-Streiber (Mainz, Germany) identified a small cytosolic compound, namely inositol hexakisphosphate (InsP6), which is capable of inducing autocatalytic processing of toxin A and B (Reineke et al. 2007). However, it was still not clear how processing occurred. This question was answered, when a cysteine protease domain, which is located adjacent to the glucosyltransferase domain, was identified by Egerer et al. (2007) in toxin A and B. A fragment of toxin B comprising only the glucosyltransferase and the cysteine protease domain is cleaved in the presence of InsP6, indicating that InsP6

induces autocatalytic processing of toxin A and B by activating the cysteine protease domain. Lysine-600 of the cysteine protease domain is essential for InsP6-binding, whereas cysteine-698, histidine-653, or aspartate-587 of toxin B represent the catalytic triad of the protease (Egerer et al. 2007, 2009). A first 3D structure of the cysteine protease domain (bound to InsP6) was provided for toxin A in 2009 by the group of Borden Lacy. The crystal structure uncovered a highly basic pocket that is required for InsP6-binding, which is separated from the active site by a beta-flap structure (Pruitt et al. 2009). Later, the 3D structure of the InsP6-bound cysteine protease domain of toxin B was presented either in the absence (Shen et al. 2011) or in the presence a specific small molecule inhibitor, respectively (Puri et al. 2010). It became apparent from these studies that InsP6-binding allosterically improves the access of the active site to its substrate. Very recently, a structural study from Chumbler et al. (2016) revealed the requirement for zinc in the mechanism of autoprocessing of toxin A and B.

2.1.4 The Translocation Domain

During cellular uptake, toxin A and B are trapped in endosomes and presumably form pores, which allow the translocation of the glucosyltransferase domain into the cytosol. A relatively large region between the cysteine protease domain and the CROP domain of toxin A and B, denoted as translocation domain, was initially suggested to be involved in these processes (Dove et al. 1990; Von Eichel-Streiber et al. 1992a; Barroso et al. 1994). In 2011, Genisyuerek et al. aimed to more precisely narrow down the pore-forming region and the translocation domain of toxin B. They found that a small segment reaching from amino acid residues 830–990 of toxin B is already sufficient for pore formation, at least in artificial lipid bilayers (Genisyuerek et al. 2011). In addition, the authors found that the residues glutamate-970 and glutamate-976 of toxin B were crucial for pore formation by acting as pH sensors for membrane insertion. Zhang et al. (2014) identified additional amino acids that are crucially involved in pore formation of toxin B, such as leucine-1106. By a series of C-terminal deletions of toxin B that were fused to the receptor-binding domain of the diphtheria toxin (DTRD), Genisyuerek et al. identified that amino acids 830–1550 of the toxin is sufficient for translocation of the enzyme portion into the cytosol, assuming that the region between amino acids 1551 and 1834 (start of the CROP domain) is not part of the translocation domain.

2.1.5 Additional Receptor-Binding Domains

Given the fact that the translocation domain of toxin A and B is much shorter than previously assumed, the question remains about the function of the remaining toxin segment between the translocation domain and the CROP domain. Recent discoveries suggest that this domain is involved in binding of toxin A and B to the cell surface (Gerhard 2016). Already in 1994, Barroso et al. tested various C-terminally truncated toxin B variants in intoxication assays and found that removal of the CROP domain did not fully diminish cytotoxicity (Barroso et al. 1994). In this study, the authors did not use purified proteins but lysates from *E. coli* that expressed the various toxin B variants. Later, Frisch et al. (2003) observed that an N-terminally extended CROP domain of toxin A competitively inhibited intoxication of cells by toxin A more efficiently than the CROP domain alone. Eventually, two German laboratories from Freiburg (Aktories and Papatheodorou) and Hanover (Just and Gerhard) confirmed in 2011 with purified recombinant proteins that the CROP domain is not absolutely required for binding and uptake of toxin A and B into host cells (Genisyuerek et al. 2011; Olling et al. 2011). The concept of CROP-independent binding and uptake of toxin A and B was further supported by the identification of the homologous TpeL toxin from *C. perfringens*, which is naturally devoid of a CROP domain (Amimoto et al. 2007). Schorch et al. (2014) substantiated that the C-terminus of TpeL represents its receptor-binding domain by identifying the LDL-related lipoprotein receptor 1 (LRP1) as host receptor for TpeL and by showing direct

binding between the TpeL C-terminus and an extracellular portion of LRP1. In the same study, the authors also proved independent cell surface-binding of a fragment of toxin B covering residues 1349–1811, which virtually corresponds to the proposed receptor-binding domain of TpeL. Furthermore, the authors were able to competitively inhibit cell binding of CROP-deficient toxin B by co-incubation with this fragment. These data argued strongly for a two-receptor model of toxin A and B, where the toxins independently bind host receptors via the CROP domain or the newly defined receptor-binding domain. Recently, Lambert and Baldwin provided additional direct evidence for dual receptor-binding sites in toxin A (Lambert and Baldwin 2016). Confusingly enough, experimental data from a recent work by Manse and Baldwin suggested at least three independent binding sites in toxin B (Manse and Baldwin 2015). Beside the CROP domain, the newly defined receptor-binding domain, which precedes the CROP domain, was shown to harbor two independent regions (residues 1372–1493 and 1493–1848) with cell binding-capability. However, it is not clear yet whether two independent binding sites are also present in the CROP-preceding receptor-binding domain of toxin A or other LCTs. Eventually, the recent identification of toxin B receptors that bind to the newly defined receptor-binding domain (described in a following section of this chapter) constitutes the strongest evidence for the existence of additional binding sites outside of the CROP domain.

2.1.6 Modular Structure (ABCD Model)

On the basis of the different domains of toxin A and B that have been described above, the modular composition of toxin A and B is best described with the so-called ABCD model already suggested by Jank and Aktories in 2008. In the ABCD model, A stands for *biological activity* (glucosyltransferase domain), B for *binding* (CROP domain and preceding additional binding sites), C for *cutting* (cysteine protease domain), and D for *delivery* (translocation domain) (Jank and Aktories 2008). The multidomain architecture of toxin A and B had already become evident in earlier attempts to obtain low resolution structures of the holotoxins by small-angle X-ray scattering (SAXS) and negative stain electron microscopy, respectively (Albesa-Jove et al. 2010; Pruitt et al. 2010). In 2016, the group of Borden Lacy reported the long-sought crystal structure of toxin A. Despite the fact that the structure of toxin A obtained in this study did not include the CROP domain, it showed for the first time how the other domains are organized within the holotoxin. In addition, the structure included additional domains of toxin A whose structure had not been solved so far, such as the translocation domain and the newly discovered, second receptor-binding domain (Chumbler et al. 2016).

2.2 Binding and Uptake of *C. difficile* Toxin A and B

2.2.1 Host Receptors of Toxin A and B

Toxin A was found to interact with different cell surface carbohydrate structures and with two proteins, namely the sucrase-isomaltase and the glycoprotein gp96 (Gerhard 2016). More recently, powerful genetic screens were established that finally allowed the discovery of host receptors of toxin B, such as CSPG4 (chondroitin sulphate proteoglycan-4), PVRL3 (poliovirus receptor-like 3) and members of the Wnt receptor frizzled family, such as FZD2 (Yuan et al. 2015; LaFrance et al. 2015; Tao et al. 2016). Recently, two binding sites were postulated within the newly defined receptor-binding domain of toxin B. Toxin B region 1372–1493 is bound by PVRL3 and toxin B region 1501–1830 by FZD proteins, respectively, whereas CSPG4 is a CROP-dependent receptor (Manse and Baldwin 2015; Tao et al. 2016).

2.2.2 Endocytic Pathways for the Cellular Uptake of Toxin A and B

Upon binding to a cell surface receptor, toxin A and B are taken up into host cells via receptor-mediated endocytosis. For many years, the exact

endocytic pathway for the uptake of toxin A and B remained unclear. At first glance, Kushnaryov and Sedmark provided evidence for endocytosis of *C. difficile* toxin A via coated pits, by visualizing colloidal gold labelled toxin A in CHO cells by electron microscopy (Kushnaryov and Sedmark 1989). In 2010, Papatheodorou et al. aimed to study the endocytic uptake of toxin A and B in more detail by the use of pharmacological and genetic inhibitors of distinct endocytic pathways (Papatheodorou et al. 2010). Their findings indicated that the endocytic uptake of toxin A and B involves a dynamin-dependent process that is mainly governed by clathrin (Papatheodorou et al. 2010). Gerhard and colleagues confirmed that clathrin and dynamin are substantially involved in endocytosis of toxin A and toxin $A_{1–1874}$ (lacking almost the entire CROP domain). However, as inhibition or knockdown of clathrin did not completely prevent uptake of toxin A and toxin $A_{1–1874}$, the authors suggested alternative endocytic routes for the toxin (Gerhard et al. 2013). Indeed, Chandrasekaran et al. (2016) reported very recently that the uptake of toxin A into CaCo-2 and MEF cells is clathrin-independent but requires dynamin and the Fer-CIP4 homology-BAR (F-BAR) domain-containing protein PACSIN2.

2.2.3 Delivery of the Glucosyltransferase Domain into the Cytosol

Toxin A and B are so-called 'short-trip' toxins, which deliver their enzymatic portion into the cytosol directly after reaching endosomal compartments via receptor-mediated endocytosis. The translocation of the glucosyltransferase domain across the endosomal membrane is by far the least understood step of the intoxication process of toxin A and B, respectively. This is mainly due to the lack of structural information of membrane-embedded conformations of the toxins, either prior or directly after the translocation event. Acidification of endosomal vesicles by vacuolar H^+-ATPases triggers conformational changes within toxin A and B, leading to the exposure of hydrophobic segments responsible for the insertion of the toxins into the endosomal membrane (Qa'Dan et al. 2000, 2001). Low pH-dependent pore formation of toxin A and B in cellular and artificial membranes was confirmed by the Aktories group (Barth et al. 2001; Giesemann et al. 2006). Formation of a pore in the endosomal membrane by the toxins´ translocation domain might be an essential step for the delivery of the glucosyltransferase domain into the cytosol. It is generally assumed that toxin A and B are able to form membrane pores as monomers and independent of host cell proteins. Pore formation of toxin A and B can be forced to occur also at the plasma membrane by artificially acidifying the extracellular medium of cultured cells (Barth et al. 2001; Giesemann et al. 2006; Qa'Dan et al. 2000). Giesemann et al. could show that the efficacy of pore formation by toxin A and B was dependent on membrane cholesterol (Giesemann et al. 2006). The glucosyltransferase domain is not required for pore formation of toxin A and B at the plasma membrane or in artificial lipid bilayers (Barth et al. 2001; Genisyuerek et al. 2011). Black lipid bilayer experiments with purified toxins revealed that the pores formed by toxin A and B are more of a temporary nature and, presumably, less stable than pores formed by other classical pore-forming bacterial toxins (Barth et al. 2001; Genisyuerek et al. 2011). Most likely, the glucosyltransferase domain of toxin A and B needs to be unfolded during the translocation process. However, it remains an open question as to how unfolding of the glucosyltransferase domain is initiated and whether the unfolded glucosyltransferase domain dips into the membrane pore via its N- or C-terminus. In addition, it is not clear if the glucosyltransferase domain translocates across the membrane pore alone or together with the adjacent cysteine protease domain.

2.3 Mode-of-Action of Toxin A and B

C. difficile toxin A and B were the first toxins to be shown to modify target proteins by glycosylation (Just et al. 1995a, b). Meanwhile, it is clear that this type of post-translational modification is used by many toxins to interfere with eukaryotic

cell functions, including various types of large clostridial glucosylating toxins (Just et al. 1996; Jank et al. 2015a; Jank and Aktories 2008) but also toxins from *Legionella* (Belyi et al. 2006), *Photorhabdus* (Jank et al. 2013), *Yersinia* (Jank et al. 2015b) and *E. coli* (EPEC) (Li et al. 2013) species. Toxin A and B catalyze the glucosylation of Rho GTPases by utilizing UDP-glucose as a co-substrate (Just et al. 1995a, b). Other related clostridial glycosyltransferases (e.g., *C. novyi* α-toxin and *C. perfringens* TpeL) prefer UDP-N-acetylglucosamine (UDP-GlcNAc) (Selzer et al. 1996; Guttenberg et al. 2012; Nagahama et al. 2011). Primary substrates of toxin A and B are RhoA,B, C, Rac1,2 and Cdc42 but also other isoforms of the Rho family such as TC10 and RhoG are modified. Secondary substrates are also some Ras proteins like Rap1,2, Ral, and Ras (Just and Gerhard 2004; Zeiser et al. 2013). Rho proteins are 21–25 kDa GTP-binding proteins and members of the Ras superfamily. The ~20 Rho family members are switch proteins governed by a GTPase cycle and act as master regulators of the actin cytoskeleton and of numerous cellular processes, such as cell migration, phagocytosis and intracellular traffic, cell cycle progression and apoptosis (Nobes and Hall 1994; Burridge and Wennerberg 2004; Jaffe and Hall 2005; Aktories 2011; Lemichez and Aktories 2013). Rho proteins are inactive in the GDP-bound state and become activated after nucleotide exchange and GTP-binding (Cherfils and Zeghouf 2013; Bishop and Hall 2000). This GDP/GTP exchange is mediated by numerous guanine nucleotide exchange factors (GEFs) (Garcia-Mata and Burridge 2007). Active Rho proteins interact with various effector proteins to elicit cellular functions (Bishop and Hall 2000; Burridge and Wennerberg 2004). This active state is blocked by GTP hydrolyses, which is stimulated by various GTPase-activating proteins (GAPs) (Tcherkezian and Lamarche-Vane 2007; Cherfils and Zeghouf 2013). Active GTP-bound Rho proteins are cell membrane associated, which is caused by N-terminal isoprenylation. Inactive, GDP-bound Rho proteins are extracted from membranes by GDIs (guanine nucleotide dissociation inhibitors) and are in a GDI-Rho complex in the cytosol.

C. difficile toxins glucosylate Rho proteins in threonine37, and Rac and Cdc42 in threonine35, which is the equivalent residue (Just et al. 1995a, b). This modification blocks the signal/switch functions of Rho proteins, because they are no longer able to interact with effectors. Glucosylation inhibits the activation of Rho GTPases by GEF proteins, and completely blocks the interaction with GAPs (Sehr et al. 1998). Moreover, glucosylation fixes Rho proteins in their inactive conformation (Vetter et al. 2000; Geyer et al. 2003). Additionally, it was shown that glucosylated Rho proteins remain attached to the cell membrane and are not extracted from membranes by GDI proteins (Genth et al. 1999).

Because glucosylation of Rho proteins blocks all functions of the switch proteins, *C. difficile* toxins A and B affect numerous cellular functions. Therefore, important questions are: How is the action of the toxins related to their pathophysiological effects? What kind of actions of toxins A and B result in diarrhea, inflammation and enterocolitis, which are the major symptoms of *C. difficile* infection?

Cytopathological effects of toxins A and B are characterized by gross changes in cell morphology, redistribution of the actin cytoskeleton, loss of stress fibers and retraction of the cell body with remaining irregular cell extensions, a process, which was called arborisation (Fiorentini and Thelestam 1991; Ottlinger and Lin 1988). All these effects can be referred to inhibition of Rho protein functions. Especially, glucosylation of Rac appears to be essential for the cytopathic effects of toxins A and B (Halabi-Cabezon et al. 2008). The RacQ61L mutant, which is hardly modified by the toxins, prevents cytopathic effects. The toxins alter cell-cell contacts and cell adhesion, which also depend on Rho proteins, thereby barrier functions of enterocytes are disabled (Hecht et al. 1988, 1992; Nusrat et al. 2001; Nusrat et al. 1995). The functional

consequences are paracellular fluxes as a consequence of alteration of tight junctions, which depend on Rho and actin (Nusrat et al. 1995; Hirase et al. 2001).

Toxin A and B were shown to induce apoptosis in several types of cells (Mahida et al. 1996; Brito et al. 2002; Qa'Dan et al. 2005; Fiorentini et al. 1998). Induction of apoptosis (at least at low and moderate toxin concentrations) essentially depends on the glucosyltransferase activity of the toxins (Brito et al. 2002; Gerhard et al. 2008).

Ng and coworkers reported that toxin A and B induce inflammasome activation in an ASC (apoptosis-associated speck-like protein)-dependent manner, thereby causing the release of IL-1β (Ng et al. 2010). More recently, the group of Feng Shao showed that Pyrin, which is encoded by the Mediterranean fever gene MEFV, acts as an intracellular "sensor" for toxin-modified RhoA-dependent inflammasome activation (Xu et al. 2014). Pyrin associates with the ASC adaptor protein thereby activating pro-caspase 1 (Lu and Wu 2015). Caspase-1 is a key enzyme to activate IL-1β and IL-18, the final common path of inflammasome activation. Inflammasome formation appears to be regulated by phosphorylation of Pyrin and binding to 14-3-3 proteins that keeps Pyrin in an inactive state (Gao et al. 2016). Moreover, it was reported that Pyrin is phosphorylated by Rho effector protein kinase N (PKN), resulting in binding to 14-3-3 proteins and inhibition of inflammasome activation (Park et al. 2016). Toxin-induced activation and release of IL-1β can induce release of IL-6, interferon-γ (IFN-γ) and IL-8, respectively. IL-8 is a highly potent neutrophil attractant. This is in line with the strong neutrophil invasion into colon mucosa that occurs during *C. difficile* infection and which is probably essentially involved in mucosal damage (Linevsky et al. 1997; Warny et al. 2000; Ishida et al. 2004; Jafari et al. 2013; Steiner et al. 1997; Mahida et al. 1996). An additional recent finding is of interest, where it was shown that the pyrin inflammasome triggers pyroptosis (Russo et al. 2016). Pyroptosis is featured by cell swelling followed by cell lysis with massive release of cellular content that can induce strong inflammation (Miao et al. 2010; Jorgensen and Miao 2015).

While the above mentioned toxin actions depend on the glucosyltransferase activity of toxin A and B, toxin effects have been described which reportedly occur with "glucosyltransferase-dead" toxins. For example it has been reported that toxin-induced production of reactive oxygen species (ROS) participate enteritis and necrosis caused by *C. difficile* toxins (Qiu et al. 1999; Farrow et al. 2013; Wohlan et al. 2014; Donald et al. 2013). However, these toxin effects occurred at very high concentrations of toxins (often 100–1000 times higher than that necessary for cytopathic effects). Therefore, the pathophysiological relevance is not clear.

2.4 Relative Importance of Toxin A and B in *Clostridium difficile* Infection

Historically, symptoms of CDI were mainly attributed to the action of toxin A, due to the fact that only purified toxin A but not toxin B was able to cause disease symptoms in hamsters when applied intragastrically (Lyerly et al. 1988). However, *C. difficile* strains have been isolated from symptomatic patients that produce only toxin B (Lyerly et al. 1992; Kim et al. 2012). Thus, two previous studies from the laboratories of Nigel Minton (Nottingham, UK) and Julian Rood (Melbourne, Australia) have attempted to more precisely determine in the hamster infection model the *in vivo* relevance of toxin A and B. To this end, both laboratories generated isogenic *C. difficile* mutants in the same strain (*C. difficile* 630) defective in the production of either toxin A or toxin B. Whereas both studies showed that toxin B alone causes disease symptoms in hamsters, contradictory results were obtained in terms of the importance of toxin A. Whereas a toxin B mutant created in the Rood group and which was capable of

producing only toxin A did not cause disease in hamsters (Lyras et al. 2009), the equivalent mutant from the Minton group remained virulent (Kuehne et al. 2010). Compelling evidence has been provided recently by the Minton group that the reason for the observed contradiction resides in the use of two different erythromycin-sensitive derivatives of strain 630 for mutagenesis, which are genetically and phenotypically distinct. Unique Single Nucleotide Polymorphisms (SNPs) were identified in both strains that dramatically affected certain phenotypes, as well having marked effects on the transcriptome, which most likely impact on virulence (Collery et al. 2017). The recent isolation of a toxin A-positive, toxin B-negative *C. difficile* strain from a clinical case of CDI further supports the *in vivo* relevance of toxin A (Monot et al. 2015).

3 Structure, Uptake and Mode-of-Action of CDT (*C. difficile* Transferase)

3.1 Bipartite Composition of CDT

In contrast to toxin A and B, CDT is an AB-type binary toxin composed of a binding and translocation component (CDTb) and a separate enzyme component (CDTa). CDTb mediates binding to the host cell surface, internalization of CDTa into endocytic vesicles and pore formation in endosomes for the translocation of CDTa into the cytosol of host cells. Pore formation of CDTb is accomplished by oligomerization of CDTb into heptamers that are capable of integrating into the endosomal membrane. CDTa is an ADP-ribosyltransferase that is specific for monomeric G-actin.

3.1.1 The Binding Component of CDT

CDT is most similar to other clostridial binary toxins, such as *Clostridium perfringens* iota-toxin and *Clostridium spiroforme* toxin CST, and more distantly related to *Clostridium botulinum* C2 toxin. All those toxins are actin ADP-ribosyltransferases that resemble anthrax toxin of *Bacillus anthracis* with respect to their binding components. For instance, CDTb exhibits a 36% identity to protective antigen (PA), the binding component of anthrax toxin (Young and Collier 2007). Much that we know about the structure-to-function relationship of CDTb was learned from previous extensive studies on the binding components of the anthrax toxin (PA) and, in part, the C2 toxin (C2II). From the already available structures of PA (Schleberger et al. 2006; Petosa et al. 1997), it was possible to deduce that CDTb consists of four domains (I to IV) with distinct functions. Domain I at the N-terminus forms the activation domain and is followed by Domain II, which is involved in membrane insertion and pore formation. Domain III is responsible for pore formation and oligomerization. The C-terminal Domain IV corresponds to the receptor-binding domain of CDTb (Barth et al. 2004). Domain IV is highly similar among the binding components of CDT (CDTb), CST (CSTb) and iota toxin (Ib). Interestingly, binding and enzymatic components are mutually interchangeable among CDT, CST and iota-toxin, but not among the latter toxins and the C2 or anthrax toxin (Considine and Simpson 1991; Popoff and Boquet 1988).

CDTb is expressed as a precursor protein of 876 amino acids (~90 kD) including an N-terminal signal peptide. Serine-type proteases activate the CDTb precursor by removal of a 20 kD peptide from the N-terminus (Perelle et al. 1997). The activated binding component has as a size of ~75 kD and is now able to form heptamers. It is unclear, whether the activation and oligomerization process occurs prior or after binding of the CDTb precursor to host cells (Gerding et al. 2014).

3.1.2 The Enzyme Component of CDT

The enzyme component of CDT (CDTa) has a size of ~53 kD and consists of 463 amino acids, including an N-terminal signal sequence of 43 amino acids, which is probably cleaved by proteolysis (Perelle et al. 1997). The mature

CDTa finally has a size of ~48 kD (420 amino acids) and is most similar to the enzyme components of iota-toxin (Ia; 84% sequence identity) and CST (CSTa; 82% sequence identity). CDTa consists of two domains with similar folding, which might originate from a duplication process of an ancient ADP-ribosyltransferase gene (Han et al. 1999). Amino acids 1–215 of mature CDTa are probably involved in the interaction with CDTb, whereas amino acids 224–420 harbour the catalytically active ADP-ribosyltransferase portion. CDTa belongs to the R-S-E class of ADP-ribosyltransferases, which are characterized by the presence of a typical arginine residue (R), an STS motif (S) and an EXE motif (E). So far, the enzyme component of the iota-toxin has been crystallized either in the presence of a stable NAD^+ analogue (Tsuge et al. 2008) or in complex with actin (Tsurumura et al. 2013). Recently, NMR assignments were reported for the CDTb-interacting and the active portion of CDTa (Roth et al. 2016a, b).

3.2 Binding and Uptake of CDT

3.2.1 The Lipolysis-Stimulated Lipoprotein Receptor

As for toxin A and B, binding to a specific structure at the cell surface of host cells is a prerequisite of the intoxication process of CDT. CDT belongs to the iota-like toxins, a subfamily of the family of clostridial, binary actin ADP-ribosylating toxins. It was already known from a previous study that iota-like toxins use a proteinaceous receptor for cell entry (Stiles et al. 2000). Eventually, in 2011, the LSR (lipolysis-stimulated lipoprotein receptor) was identified as host receptor for iota-like toxins by the help of a novel genetic screen (haploid genetic screen), which is based on the human haploid cell line Hap1 (Papatheodorou et al. 2011). Interestingly, it turned out that LSR is the host receptor also for the CDT-related *C. perfringens* iota-toxin and *C. spiroforme* toxin but not for the more distantly related *C. botulinum* C2 toxin (Papatheodorou et al. 2011, 2012). LSR acts as a lipoprotein receptor in the liver for the clearance of chylomicron remnants from the blood, but is also expressed in various other tissues, including the intestine (Yen et al. 1994, 1999; Mesli et al. 2004). Later studies identified a role of LSR in the formation of tricellular tight junctions (Masuda et al. 2011; Furuse et al. 2012; Czulkies et al. 2017). Another recent study found that LSR is critically required for proper blood-brain barrier formation (Sohet et al. 2015). Eventually, several studies found a role of LSR in cancer progression and metastasis (Papatheodorou and Aktories 2016). As shown by Hemmasi et al. (2015), amino acids 757–866 at the C-terminal end of CDTb interact with an immunoglobulin (Ig)-like, V-type domain of LSR present in its N-terminal, extracellular part.

3.2.2 Endocytic Pathways for the Cellular Uptake of CDT

Until now, the endocytic route of CDT (and other iota-like toxins) has not been entirely clarified. However, it was shown by the group of Michel Popoff (Paris, France) that dynamin, but not clathrin, is required for cellular uptake of iota-toxin (Gibert et al. 2011). In this study, colocalisation of iota-toxin with the interleukin-2 receptor in endocytic vesicles was observed, indicating a similar endocytic route for both proteins (Gibert et al. 2011). The endocytic uptake of the interleukin-2 receptor is negatively regulated by RhoGDI (RhoGDP-dissociation inhibitor) (Lamaze et al. 2001). Strikingly, iota-toxin entry into Cos-1 cells was inhibited upon overexpression of RhoGDI (Gibert et al. 2011). Endocytic uptake of CDT and other iota-like toxins might involve lipid rafts, since oligomers of the binding components have been identified in detergent-resistant, cholesterol-rich membrane microdomains (Nagahama et al. 2004; Hale et al. 2004). Importantly, Papatheodorou and colleagues observed clustering of LSR into lipid rafts after binding of CDTb (Papatheodorou et al. 2013). LSR-clustering into lipid rafts occurred also after binding of the RBD of CDTb, which is not able to oligomerize by itself (Papatheodorou et al. 2013). Wigelsworth et al.

found that the lipid rafts-protein CD44 (cluster of differentiation 44) is required for cellular uptake of CDT (Wigelsworth et al. 2012). Interestingly, CD44 was found in lipid rafts from Ib-treated Vero cells (Blonder et al. 2005). It might be possible that CD44 interacts with LSR-CDT complexes in lipid rafts, thus facilitating the endocytic uptake of the toxin.

3.2.3 Role of Chaperones During the Cellular Uptake of CDT

The delivery of CDTa into the host cell cytosol depends on CDTb, which under acidic conditions likely forms pores in endosomal membranes that serve as translocation channels for the trans-membrane transport of CDTa (Roeder et al. 2014; Ernst et al. 2016). The pH-driven transport of CDTa across endosomal membranes requires the activities of certain host cell chaperones (Roeder et al. 2014). *In vitro*, CDTa directly and specifically binds to the heat shock proteins Hsp90 and Hsp70, as well as to some peptidyl-prolyl cis/trans isomerases (PPIases) of the cyclophilin (Cyp) and FK506 binding protein (FKBP) families (Kaiser et al. 2011; Ernst et al. 2015, 2017). The current model suggests that these host cell factors specifically and selectively facilitate the intracellular trans-membrane transport of ADP-ribosylating toxins by interacting with the ADP-ribosyltransferase domain of the A subunits. These findings were mainly obtained by the group of Holger Barth (Ulm, Germany) and contribute to a better understanding of the cellular uptake of CDT into human cells and to the development of novel pharmacological strategies against infections with hypervirulent, CDT-producing *C. difficile* strains. Host cytosolic factors that might assist during refolding of the translocated glucosyltransferase domain of toxin A and B have yet to be described.

3.3 Mode-of-Action of CDT

CDT ADP-ribosylates monomeric G- actin in arginine-177. Thus, modification of actin occurs at the same residue of actin that is also modified by other binary actin-ADP-ribosylating toxins, including *C. botulinum* C2 toxin and *C. perfringens* iota toxin (Vandekerckhove et al. 1987, 1988). In contrast to monomeric G-actin, polymerized F-actin is not a substrate of CDT and of any other related binary toxin, because arginine-177 is not available for modification in the double helix of F-actin (Holmes et al. 1990; Margarit et al. 2006). Essential for actin functions is the ability of the microfilament protein to reversibly polymerize from G- to F-actin, a process that is tightly regulated by numerous actin binding proteins (Dominguez and Holmes 2011). Early studies obtained with *C. botulinum* C2 toxin and *C. perfringens* iota toxin showed that modification of actin in arginine-177 inhibits actin polymerization (Aktories et al. 1986; Schering et al. 1988). This holds also true for CDT-induced ADP-ribosylation of actin. Moreover, all previous results obtained with other types of binary actin-ADP-ribosylating toxins that modify arginine177 of actin can be reliably referred to the action of CDT. This includes the early finding that ADP-ribosylated actin binds to plus ends of F-actin filaments and acts as a capping protein to block F-actin elongation by inhibition of the binding of non-ADP-ribosylated actin (Aktories and Wegner 1989; Perieteanu et al. 2010; Weigt et al. 1989; Wegner and Aktories 1988). Also the interaction of actin with actin binding proteins (for example gelsolin) that is largely affected by toxin-induced ADP-ribosylation (Wille et al. 1992), is similarly relevant for CDT.

Binary toxin-induced F-actin depolymerization has typical cytotoxic effects in cell culture (Wiegers et al. 1991), resulting in rounding-up of cells and loss of cell adherence followed by apoptosis (Heine et al. 2008). Notably, not only the actin cytoskeleton but also microtubules are affected by binary actin-depolymerizing toxins. CDT and other actin-depolymerizing toxins induce long microtubule-based protrusions (Schwan et al. 2009). These cell membrane protrusions form a network of long tentacle-like structures on the surface of epithelial cells. Microtubule-based protrusions are dynamic structures. They grow

and retract. CDT-induced depolymerization causes the mislocalization of capture proteins like ACF7 and Clasp2, which are involved in stabilization of growing microtubules at the actin cell cortex (Kodama et al. 2003; Drabek et al. 2006). Without appropriate capture proteins at the cell membrane, microtubule growth is no longer stopped, resulting in protrusion formation (Schwan et al. 2009). More recent studies indicate that septins, which are GTP-binding proteins that can reversibly oligomerize (Mostowy and Cossart 2012), are crucially involved in toxin-induced protrusion formation (Nolke et al. 2016). Moreover, these findings also show that septin-dependent protrusion formation is regulated by the Rho protein family member Cdc42 and its effectors Borg (binder of Rho GTPases) (Nolke et al. 2016).

CDT-induced partial depolymerization of F-actin disturbs re-cycling of vesicles at the basolateral side of epithelial cells. Thereby, the vesicles, which contain extracellular matrix (ECM) proteins like fibronectin and vitronectin, are re-routed from the basolateral side to the apical membrane, where microtubules form protrusions. Here, fibronectin and other ECM proteins are released (Schwan et al. 2014).

3.4 Role of CDT During *C. difficile* Infection

Although CDT is a very potent and efficient cytotoxin, its role in *C. difficile* infection is not well understood. Only in extremely few cases *C. difficile*-dependent enterocolitis could be traced back to CDT in the absence of *C. difficile* toxins A and B. What is then is its role in disease? The group of Nigel Minton (Nottingham, UK) assessed the virulence of all possible combinations of isogenic *C. difficile* toxin mutants in the hamster infection model and found that CDT is a factor that increases the virulence of *C. difficile* in the presence of toxins A and B (Kuehne et al. 2014). Several mechanisms are discussed. First, CDT may increase the adherence of bacteria due to the formation of a network of microtubule-based protrusions, which facilitates adherence of *C. difficile* bacteria. In the same direction points the finding that CDT causes the redistribution of fibronectin from the basolateral membrane of epithelial cells to the apical side, where it acts as a receptor for *C. difficile*. Moreover, it is of interest that CDT was shown to efficiently induce apoptosis of protective colonic eosinophils in a TLR2-dependent manner (Cowardin et al. 2016). Moreover, it should be considered that actin and microtubules play a crucial role in activation of the inflammasome (Gao et al. 2016). Also this could be an important functional connection eventually leading to increase in virulence of *C. difficile* in the presence of CDT, and toxins A and B.

4 Conclusions

It is well-accepted that *C. difficile* diseases are mainly governed by the production of protein toxins, including *C. difficile* toxins A (TcdA) and B (TcdB). The third toxin, CDT, appears to be an important enhancing virulence factor. Therefore, recent progress in our knowledge about the mode-of-actions of these toxins is key for the understanding of the pathophysiology of *C. difficile* infections and the development of novel therapeutic strategies against the diseases caused by the pathogen. However, many open questions remain. In respect to TcdA and TcdB, the membrane translocation of these toxins into target cells is still largely enigmatic. Moreover, *C. difficile* enterocolitis is characterized by severe inflammation and cell necrosis. The precise pathophysiological pathways caused by the toxins leading to inflammation and necrosis are still not satisfactorily understood and explained. The great success of fecal transplantation in therapy of *C. difficile* diseases indicate that the microbiome is crucially involved in the pathogenesis of *C. difficile* infections. This also indicates a pivotal role of the immune system of the host. Therefore, the actions of *C. difficile* toxins on various types of immune cells in context of intestinal tissue should be studied in detail.

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Clostridium difficile Biofilm

Claudia Vuotto, Gianfranco Donelli, Anthony Buckley, and Caroline Chilton

Abstract

Clostridium difficile infection (CDI) is an important healthcare-associated disease worldwide, mainly occurring after antimicrobial therapy. Antibiotics administered to treat a number of infections can promote *C. difficile* colonization of the gastrointestinal tract and, thus, CDI. A rise in multidrug resistant clinical isolates to multiple antibiotics and their reduced susceptibility to the most commonly used antibiotic molecules have made the treatment of CDI more complicated, allowing the persistence of *C. difficile* in the intestinal environment.

Gut colonization and biofilm formation have been suggested to contribute to the pathogenesis and persistence of *C. difficile*. In fact, biofilm growth is considered as a serious threat because of the related increase in bacterial resistance that makes antibiotic therapy often ineffective. However, although the involvement of the *C. difficile* biofilm in the pathogenesis and recurrence of CDI is attracting more and more interest, the mechanisms underlying biofilm formation of *C. difficile* as well as the role of biofilm in CDI have not been extensively described.

Findings on *C. difficile* biofilm, possible implications in CDI pathogenesis and treatment, efficacy of currently available antibiotics in treating biofilm-forming *C. difficile* strains, and some antimicrobial alternatives under investigation will be discussed here.

Keywords

Biofilm · *Clostridium difficile* · Genetic factors · EPS matrix · Adhesion

1 Introduction

Microbial biofilms are considered as the 'true' habitat for many causative agents of infection and disease. These microbial communities growing on biotic and abiotic surfaces are embedded in a matrix of extracellular polymeric substances (EPS) (Heydorn et al. 2000), offering to microorganisms an efficacious protection from antibiotics (Goldberg 2002) and disinfectants (Peng et al. 2002), as well as the possibility to survive in conditions of nutrient deficiency (Koch et al. 2001). Biofilm formation is characterised by several phases, starting from reversible and irreversible attachment to the

C. Vuotto (✉) · G. Donelli
Microbial Biofilm Laboratory, IRCCS Fondazione Santa Lucia, Rome, Italy
e-mail: c.vuotto@hsantalucia.it

A. Buckley · C. Chilton
Healthcare Associated Infection Research Group, Section of Molecular Gastroenterology, Institute for Biomedical and Clinical Sciences, University of Leeds, Leeds, UK

surface, passing through the development of a single-species community or a polymicrobial one, and then ending with the dispersion of cells from the biofilm (Percival et al. 2015). The architecture of a mature biofilm can vary depending on the microorganisms that constitute it, forming flat or mushroom-shaped structures (Klausen et al. 2003), with the latter generally observed within in vitro biofilms only.

Intracellular and intercellular communication within a biofilm is supported by signals released when cell density reaches a critical level, a phenomenon known as quorum sensing (QS) (Lindsay and Von Holy 2006; Li and Tian 2012). QS is cell density-dependent gene regulation through the production of signalling molecules, termed autoinducers (AI), that activate the maturation and disassembly of the biofilm in a coordinate manner, with dispersal of microbial cells into the surrounding environment increasing the dissemination risk and the colonisation of new niches (Donelli 2006). This "lifestyle" allows pathogenic microorganisms to acquire numerous advantages in terms of survivability and spread in hostile environments (Hall-Stoodley et al. 2004).

The human gut is a clear example of a rich and diverse microbial ecosystem, consisting of a huge number of microbial species that play a crucial role in maintaining metabolic and immunologic homeostasis (Cummings et al. 2004). Despite this, few studies have been published on microbial biofilms growing in the gut, where different bacterial species coexist in association with the mucosal membrane as well as the intestinal luminal particles (Macfarlane and Dillon 2007). These mucosal communities show different fermentation profiles (Macfarlane and Macfarlane 2006), that may be important in modulating the host's immune system and contributing to some inflammatory bowel diseases (ulcerative colitis, Crohn's disease), due to their proximity to the epithelial surface (Macfarlane et al. 2011). Single species biofilms of gut pathogens, such as *Escherichia coli*, *Salmonella*, and *Vibrio* spp., are the most studied as their extremely adhesive and invasive features can modify the dynamics of the gut and cause infections (Azriel et al. 2015; Sengupta et al. 2016; Rossi et al. 2017; Owrangi et al. 2017). Furthermore, other intestinal isolates, belonging to the anaerobic species *Bacteroides*, *Clostridium*, *Fusobacterium*, *Finegoldia*, *Prevotella*, and *Veillonella*, have been demonstrated to be able to develop as in vitro mono-species biofilms, and to interact with each other by forming dual-species biofilms (Donelli et al. 2012).

A growing interest in the potential biofilm growth of *C. difficile* has been recorded in recent years, due to the prominence of this microorganism as etiologic agent of nosocomial diarrhoea worldwide.

CDI is one of the principal threats to hospitalized and immunocompromised patients, mainly when antibiotics are administered to them in order to treat a number of infections. In fact, antibiotic molecules, by disrupting the protective intestinal microbiota, can promote *C. difficile* colonization of the gastrointestinal tract and, thus, CDI. The resistance of an increased number of clinical isolates to multiple antibiotics, such as clindamycin and fluoroquinolones, and the reduced susceptibility to antibiotics commonly used against milder cases of CDI, e.g. metronidazole (Dupont 2013), allow *C. difficile* to persist after treatment. The selective advantage for their dissemination is mainly gained through the acquisition of mobile genetic elements involved in antibiotic resistance and alterations of the antibiotic target sites (Spigaglia 2016).

Even if the two main *C. difficile* virulence factors, toxin A and toxin B (Carter et al. 2012), and the actin-ADP-ribosylating toxin, play the major role in clinical manifestation of CDI, also adherence and motility have to be taken into account. In fact, the surface layer proteins (SLPs) coded by *slpA* are involved in adherence and inflammatory stimulation, the extracellular matrix-binding domain, the surface anchor protein needed for covalent attachment to peptidoglycan, the fimbriae, and the extracellular polysaccharides must be all considered as additional factors involved in *C. difficile* pathogenesis (Sebaihia et al. 2006).

The importance of adhesive properties as key virulence factor lies in the fact that adherence is the first and most essential step of the biofilm

growth cycle (Römling and Balsalobre 2012; Percival et al. 2015).

In this framework, the complex multifactorial process leading to the *C. difficile* biofilm formation (Dawson et al. 2012; Đapa et al. 2013; Dapa and Unnikrishnan 2013) should be taken into due consideration and interventions should be also focused on this mode of infection, mainly in light of the recurrent CDI in ~20% of patients (Barbut et al. 2000). A better understanding of the process of *C. difficile* biofilm formation as well as its contribution to CDI recurrence could significantly improve disease prevention and treatment.

Findings on *C. difficile* biofilm, possible implications of biofilm formation in CDI pathogenesis, treatment efficacy of currently available antibiotics, and some antimicrobial alternatives under investigation will be here discussed.

2 Main Features of *C. difficile* Biofilm

The mechanisms underlying biofilm formation in *Clostridium* species, particularly *C. difficile* (Pantaléon et al. 2014), as well as the role of biofilm in CDI have not been extensively analysed with respect to other bacterial species (Hall-Stoodley and Stoodley 2009). However, *C. difficile* biofilm may develop either associated with intestinal microbiota or during gut infections, by growing as mono-species or being part of a complex multi-species biofilm. Therefore, biofilm mode of growth may play a key role in the gut colonization and bacterial survival of *C. difficile*, affecting its pathogenesis and persistence, and possibly contributing to the recurrence of CDI.

For this reason, research on the ability of *C. difficile* to form a biofilm has attracted considerable interest, with a number of in vitro studies being carried out in this regard. Donelli and co-workers, by using crystal violet staining and Field Emission Scanning Electron Microscopy (FESEM), first showed that a clinical isolate of *C. difficile* (CdiBs21) formed a moderate biofilm on flat bottomed plastic tissue culture plates (Donelli et al. 2012). Afterwards, the hypervirulent strain R20291 was revealed to be a strong biofilm producer, identifying a link between sporulation and biofilm formation with a biofilm reduction in a *spo0A* mutant (Dawson et al. 2012). Further analysis, by Đapa and co-workers on the massive biofilm formation of R20291 strain, confirmed the involvement of virulence-associated proteins, Cwp84, flagella, and a putative quorum-sensing regulator, LuxS. In the same conditions, the strain 630 formed a weak biofilm (Đapa et al. 2013).

Biofilm formation by hypervirulent and other *C. difficile* strains showed differences in terms of ability to form weak, moderate or strongly adherent biofilms, with the hypervirulent strains always producing greater biofilms (Hammond et al. 2014; Mathur et al. 2016; Piotrowski et al. 2017).

Biofilm structure is supported by the EPS matrix, mainly composed of proteins, extracellular DNA (eDNA) and polysaccharides, that provides the scaffold by which bacteria adhere to each other and to surfaces. EPS matrix is responsible for the impenetrability of bacterial biofilms, thus contributing to the antibiotic resistance in vivo as well as to the escape from immune responses during the infection. Specifically, *C. difficile* biofilm is composed of a multi-component matrix (Fig. 1) made of proteins, extracellular DNA and polysaccharide II (PSII) (Dawson et al. 2012; Đapa et al. 2013). The latter is an antigen commonly found on the surface of all *C. difficile* species (Ganeshapillai et al. 2008) and detected in the matrix of several *C. difficile* strains (Đapa et al. 2013; Semenyuk et al. 2014). Semenyuk and colleagues found, in the *C. difficile* biofilm matrix extract and in the whole cell extracts, six proteins involved in metabolism: formate-tetrahydrofolate ligase, acetyl-CoA acetyltransferase, 2-hydroxyisocaproate CoA-transferase, NAD-specific glutamate dehydrogenase, 3-hydroxybutyryl-CoA dehydrogenase, fructose-bisphosphate aldolase. On the contrary, cell wall-associated proteins were revealed in cell-surface extracts only, the matrix proteins not arising from the cell surface. These proteins, possibly

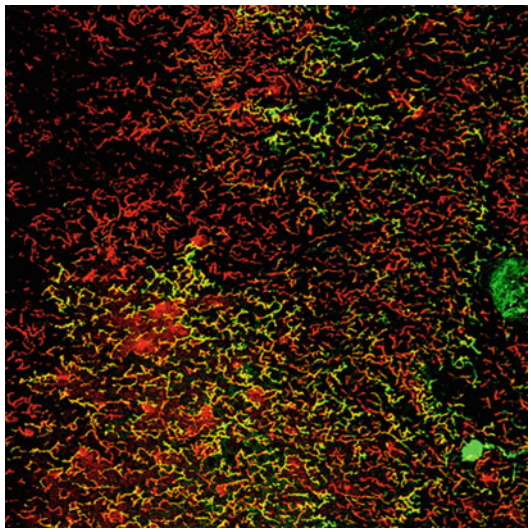

Fig. 1 CLSM analysis of *C. difficile* in vitro biofilm after 48 h. The red-fluorescent propidium iodide stain labels bacteria, while the lectin Concanavalin A binds to residues of the exopolysaccharides matrix

originated from the cell lysis, most likely contribute in some way to biofilm formation (Semenyuk et al. 2014).

Other biofilm forming Gram-positive bacteria display heterogeneity within biofilms, where vegetative cells, sporulating cells and matrix-producing cells coexist with different spatial localisation (Vlamakis et al. 2008). These heterogeneous populations imply differential gene expression, and genetic regulation occurs within a biofilm. Electron micrographs of *C. difficile* biofilms show the composition to be vegetative cells, sporulating cells and cell debris (Donelli et al. 2012; Dawson et al. 2012). eDNA is an essential component of the *C. difficile* biofilm matrix, as incubation with DNase I reduces the biofilm biomass produced (Dawson et al. 2012; Đapa et al. 2013; Semenyuk et al. 2014). One way to explain the presence of eDNA and cell debris seen within *C. difficile* biofilms could be through the differential expression of toxin-antitoxin (TA) systems. TA systems comprise a stable toxin, which is intracellular and only affects an essential cellular process, and an unstable antitoxin, which sequesters the effect of the toxin (Wen et al. 2014). The cell death and lysis caused by TA systems in a small percentage of the bacterial cells could contribute to the assembly of the matrix during biofilm formation for the 'greater good' of the population (Gil et al. 2015). The *C. difficile* genome encodes a number of putative TA systems (Gil et al. 2015) with the MazE-MazFTA system best described (Rothenbacher et al. 2012). However their contribution towards biofilm formation has not been determined.

Additionally, toxins and spores were discovered in the biofilm matrix embedding toxigenic *C. difficile* cells (Semenyuk et al. 2014). Interestingly, toxins resulted to be at low concentrations in biofilms after 24 h and at higher level in 3 day-old biofilms, while spores have reduced germination efficiency in mature biofilms, thus presumably facilitating the preservation of a dormant population ready to cause recurrent infections (Semenyuk et al. 2014). Remarkably, by indirect immunofluorescence analysis, the presence of two exosporium proteins (i.e., CdeC and the N-terminal domain of BclA1) have been detected on spores in *C. difficile* biofilms (Pizarro-Guajardo et al. 2016a). By transmission electron microscopy, it has been also demonstrated that two exosporium morphotypes, one with a thick outermost exosporium layer and another with a thin outermost exosporium layer, were formed during biofilm development (Pizarro-Guajardo et al. 2016b). Dormant spores located within biofilms were detected for the duration of the experiment within a triple-stage chemostat gut model inoculated with indigenous gut microbiota and *C. difficile* cells (Crowther et al. 2014a, b). Sessile spores displaying increased recalcitrance to germination may be compared to superdormant spores of Bacillus spp. (Ghosh et al. 2009), resulting persister cells.

The complex biofilm architecture of *C. difficile* strains has been analysed in different in vitro studies by FESEM (Fig. 2) and Confocal Laser Scanning Microscopy (CLSM). FESEM micrographs of *C. difficile* grown on glass coverslips revealed wide mats of rod-shaped vegetative cells, spores, and sporulating cells interconnected by a network of extracellular material constituted by cell debris and string-

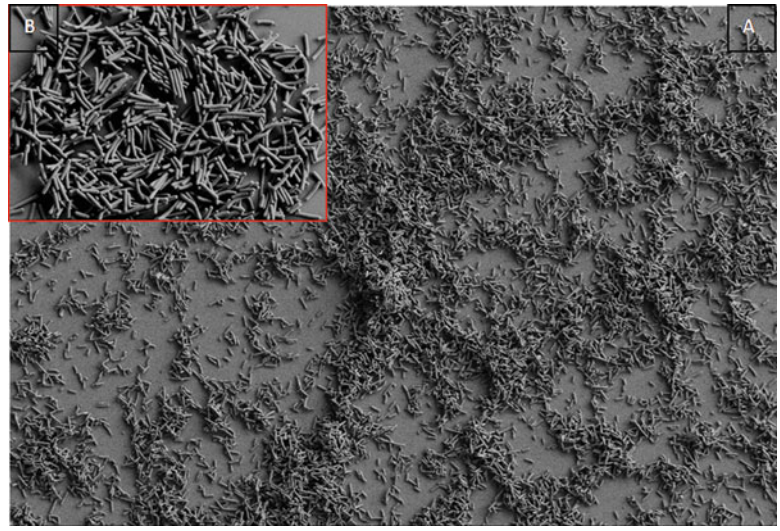

Fig. 2 FESEM analysis of *C. difficile* biofilm formed in vitro after 48 h. Biofilms micrographs were obtained at an accelerating voltage of 2 kV with magnifications of 1000× (**a**) and 5000× (**b**)

like material connecting the cells (Fig. 3). The appearance seems to be consistent with their being biofilms and with other SEM observations on plastics (Dawson et al. 2012; Semenyuk et al. 2014) or agar (Lipovsek et al. 2013).

CLSM analysis describes more accurately the biofilm architecture, allowing one to define the thickness and to visualize cells inside the biofilm (Fig. 4). Semenyuk and colleagues explored the evolution of biofilm structure and composition over the time, identifying, after 24 h, regions with a high concentration of apparently proliferating cells and cell debris as well as small colonies, distant from the main biofilm colony, interpreted as sites of new growth formed by cells migrated from the larger colony edge. After 3 days, together with rod-shaped cells and apparent cell debris, authors detected ovoid cells in the biofilm that were identified as spores by phase contrast microscopy. At 6 days, most of the cells in the biofilm had become spores with isolated regions of vegetative cells (Semenyuk et al. 2014).

As already demonstrated for other bacterial species, the *C. difficile* biofilm thickness tends to increase every day, even if the depth varied according to the areas (Dawson et al. 2012; Maldarelli et al. 2016), and also the amount of matrix constituting biofilm increases proportionally (Dapa and Unnikrishnan 2013).

Whilst the so far reported in vitro evidence on the ability of *C. difficile* to form a biofilm, in vivo confirmation needs further investigation. *C. difficile* adhesion to epithelial mucosa of animal models, including mice and hamsters, has been demonstrated (Borriello et al. 1988; Spigaglia et al. 2013), but scarce and conflicting proofs exist on *C. difficile* adherence to human gut tissues (Borriello 1979; Lyra et al. 2012). More specifically, regarding *C. difficile* biofilm formation in vivo, clumps of *C. difficile* cells have been observed in a mouse model associated with damaged tissue (Lawley et al. 2009), while aggregation or clusters of *C. difficile* cells were observed in hamster and monoxenic mouse, respectively (Spencer et al. 2014; Soavelomandroso et al. 2015). More recently, multispecies communities associated with the mucus of the cecum and colon have been detected, with *C. difficile* present as a minority member of communities in the outer mucus layer (Semenyuk et al. 2015).

Although the in vivo data at our disposal are limited and results obtained in vitro might not

Fig. 3 FESEM analysis of *C. difficile* biofilm formed on glass coverslips after 5 days; mushroom-like structures formed by rod-shaped vegetative cells, spores, sporulating cells and cell debris. Biofilms micrographs were obtained at an accelerating voltage of 2 kV with magnifications of 5000×

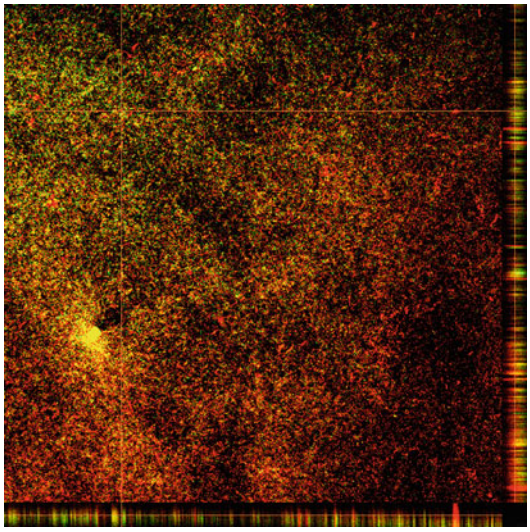

Fig. 4 Three-dimensional CLSM image of *C. difficile* biofilm grown in vitro for 5 days. The red-fluorescent propidium iodide stain labels bacteria, while the lectin Concanavalin A binds to residues of the exopolysaccharides

reflect the in vivo situation, it is likely that the presence of large microcolonies of *C. difficile,* or biofilm communities including this species, play a pivotal role in its gut colonization and survival, biofilm formation in vivo possibly being another factor contributing to recurrence of CDI.

3 Genetic Factors Behind *C. difficile* Biofilm Formation

The formation of *C. difficile* biofilms is a multifactorial process involving many virulence-associated proteins and potentially several complex networks to regulate biofilm formation. The cell surface of *C. difficile* plays a pivotal role throughout the whole biofilm process, from the initial adherence of a cell to the dispersal of biofilm. Thus, structures directly involved in biofilm formation have been identified by investigating proteins and macromolecules present on the cell surface. Flagella, Type IV pili (T4P) and the S-layers are all implicated in *C. difficile* biofilm formation.

In the closely related bacterium, *Clostridium perfringens*, T4P plays an important role in twitching motility, biofilm formation and disease pathogenesis (Varga et al. 2006). The T4P filament in *Clostridium* spp. is typically made up of a major pilin subunit, PilA, and minor pilin subunits, PilJ, with further genes putatively involved in the retraction of the pilus to provides the twitching motility (Varga et al. 2006; Piepenbrink et al. 2014, 2015; Melville and Craig 2013). T4P were once thought only to be

present in Gram negative bacteria, but Varga et al. (2006) first identified several putative pilin genes within the genome of *C. difficile* strain 630, and Goulding et al. (2009) used immunogold labelling to show that pili structures are present on the cell surface of *C. difficile* during infection in hamsters. Analysis of pilin gene transcripts from in vitro *C. difficile* biofilm cultures, shows an upregulation of *pilA1* transcripts compared to planktonic cultures (Maldarelli et al. 2016), which is even more prominent in *C. difficile* strain R20291 compared to strain 630 (Purcell et al. 2016). The importance of T4P in *C. perfringens* can be seen in mutants that are defective in T4P formation as these mutants display abnormal biofilm formation compared to the wild-type strain (Varga et al. 2008). In *C. difficile*, mutants that have a disrupted *pilA1* gene lack T4P structures on the cell surface under laboratory conditions (Bordeleau et al. 2015). Interestingly, T4P play an important role in the early stages of *C. difficile* biofilm formation, as mutants with a *pilA1* disruption show a reduced biofilm biomass compared to wild-type (Maldarelli et al. 2016; Purcell et al. 2016). However, T4P seem to play little role in the maturation of a biofilm, as these mutants showed no difference in biofilm biomass compared to wild-type when grown over 7 days (Maldarelli et al. 2016). Up to nine putative pilin-like proteins are encoded on the *C. difficile* genome (Melville and Craig 2013; Maldarelli et al. 2014), three of these being designated as major pilin subunits (*pilA1–3*). The biological function for each of these pilin genes remains unclear, with current hypotheses suggesting T4P made from these different pilin subunits could perform different functions, or pilin switching could be a mechanism for immune evasion, or, as many of these are not located in T4P operons, these could be non-functional. In other bacteria, T4P plays a pivotal role in biofilm formation and disease pathogenesis; T4P is essential for passage of *Neisseria meningitidis* to cross the blood-brain barrier (Nassif et al. 1994), whilst T4P-mediated motility is important for *Pseudomonas aeruginosa* early biofilm development (Klausen et al. 2003). The role of T4P during *C. difficile* colonisation and persistence remains to be investigated.

Recent work on how T4P is regulated in *C. difficile* has identified the bacterial secondary messenger molecule Bis-(3′-5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) as a key component to the regulatory pathway. In Gram negative bacteria, c-di-GMP modulates virulence attributes, such as biofilm formation in *Vibrio cholerae* (Tischler and Camilli 2005) and *P. aeruginosa* (Kulasakara et al. 2006), decreased flagella-mediated motility in *Escherichia coli*, and cell differentiation in *Caulobacter crescentus* (Aldridge et al. 2003). Two enzymes, diguanylatecyclases (DGCs) and phosphodiesterases (PDEs) that either synthesise or degrade c-di-GMP (Römling and Amikam 2006), tightly control the intracellular levels of c-di-GMP. *C. difficile* is unusual among Gram positive organisms by the number of DGCs & PDEs encoded on the genome; strain 630 has 37 putative c-di-GMP metabolising enzymes. Ectopic expression of 31 of these enzymes in the surrogate organism, *V. cholerae*, confirmed these genes as either having DGC or PDE activity (Bordeleau et al. 2011). Interestingly, heterologous and homologous expression of *C. difficile* 630 CD1420 (*dccA*) in either *V. cholerae* or *C. difficile*, respectively, increased cellular levels of c-di-GMP and induced biofilm formation (Bordeleau et al. 2011; Purcell et al. 2012). Through overexpression of *dccA*, high intracellular levels of c-di-GMP resulted in increased expression of the genes in the T4P operon and a greater number of pili observed on the cell surface (Bordeleau et al. 2015). In other bacteria, c-di-GMP controls the transcription and translation of many genes by direct binding to c-di-GMP riboswitches (Sudarsan et al. 2008). Riboswitches are mRNA molecules that bind small molecules (such as c-di-GMP) resulting in the transcription of downstream genes (Winkler and Breaker 2005). In this way, the same small molecule can coordinate multiple

genetic pathways. RNA-seq experiments first identified a Type II c-di-GMP riboswitch located up stream of the start of the *C. difficile* major T4P operon (*pilA1*) (Soutourina et al. 2013), called Cdi2_4, which is switched 'ON' via a conformational change upon binding c-di-GMP to the riboswitch to relieve a predicted Rho-independent transcription terminator (Bordeleau et al. 2015). Between different strains of *C. difficile* there appears to be subtle variations in *pilA1* expression patterns during biofilm formation with strains 630 and R20291 (Purcell et al. 2016), which could be due to differences in the total c-di-GMP levels.

Research on other regulatory proteins within *C. difficile* suggests that its pathogenesis is intimately linked to the metabolic state of the bacterium (Bouillaut et al. 2015). CodY is a pleotropic regulator involved in the adaptive response of Gram-positive bacteria to low nutrient levels, and in *C. difficile*, an estimated 52 genes are directly regulated by CodY (Dineen et al. 2010; Bouillaut et al. 2015). One of these genes is *pdcA* (*CD1515*), which is a PDE enzyme that affects the regulation of flagella biosynthesis by influencing c-di-GMP levels (Purcell et al. 2012; Purcell et al. 2017). Thus, through this regulatory pathway, *C. difficile* biofilm formation is connected to the nutrient availability of the bacterium. c-di-GMP acts as a signalling molecule coordinating the transition from a planktonic, motile lifestyle to a sessile, biofilm lifestyle in many bacterial pathogens. In *C. difficile*, high c-di-GMP levels directly repress the major flagella operon *flgB* through a Type I c-di-GMP riboswitch, Cdi1_3, located 496 bp upstream of the *flgB* start codon (Sudarsan et al. 2008; Soutourina et al. 2013). Through the two types of riboswitches, one family of signalling molecules can regulate the expression of T4P and flagella biosynthesis during *C. difficile* biofilm formation.

A decrease in flagella transcripts would indicate a limited role for flagella during biofilm formation, and targeted disruption of *fliC* gene in strains 630 or R20291 had no effect on biofilm formation compared to the wild-type strains (Faulds-Pain et al. 2014; Valiente et al. 2016). However, one report has shown a *fliC* mutant had significantly less biofilm biomass compared to wild-type (Đapa et al. 2013). The genetic organisation of the *C. difficile* flagella operon can be split into three parts, however the F2 locus is the most divergent between the genomes of different *C. difficile* strains (Stabler et al. 2009; Stevenson et al. 2015). The F2 locus encodes genes involved in glycosylation of the flagella with sugar moieties, and the disruption of these genes resulted in the production of flagella on the cell surface even though most of these mutants were non-motile (Twine et al. 2009; Faulds-Pain et al. 2014; Valiente et al. 2016). Interestingly, these mutants produced more biofilm biomass compared to the wild-type strain (Faulds-Pain et al. 2014; Valiente et al. 2016). In the closely related bacterium, *Bacillus subtilis*, inhibition of flagella rotation acts as a mechanical trigger to activate the DegS-DegU two-component signal transduction system, which regulates biofilm formation and matrix production (Cairns et al. 2013, 2014). Although no DegS/DegU homologues have been identified in *C. difficile*, this could be why these flagellate, non-motile mutants produced more biofilm biomass, although more work is needed to understand the regulatory mechanisms behind this phenotype.

Using riboswitches is one way *C. difficile* regulates the change from motility to biofilm, however other regulatory RNA molecules appear to play a role. Small non-coding RNAs (sRNAs) act by base pairing with their target mRNAs, leading to modulation of mRNA stability or translation (Chao and Vogel 2010; Soutourina 2017). Some sRNAs require an RNA chaperone protein called Hfq to help the base pair binding of the sRNA and mRNA molecules. In other bacteria, mutating Hfq has pleotropic effects on cell physiology, ranging from increased sensitivity to external stresses (detergents, iron limitation and oxidative stress), to increased biofilm formation, or reduced virulence (Chao and Vogel 2010). The creation of a *C. difficile hfq* gene disruption has been unsuccessful to date, so Boudry et al. (2014), used a knockdown approach to decrease Hfq protein levels fivefold compared to wild-type to determine its contribution toward cell

physiology. Using this approach, the authors observed an increase in biofilm formation in the Hfq depleted strain, indicating that sRNAs play a role in negatively regulating biofilm formation. Alongside this, the authors observed a decrease in flagella present on the cell surface and increased expression of cell wall/membrane proteins, all of which could have contributed to the increase in biofilm formation (Boudry et al. 2014).

Another cell surface organelle that has been implicated in *C. difficile* biofilm formation is the S-layer. The *C. difficile* S-layer (Cerquetti et al. 2000) is a two-dimensional paracrystalline protein array coating the cell and is made up of SlpA subunits that are post-translational cleaved by another protein called Cwp84 (de la Riva et al. 2011; Fagan and Fairweather 2014). The S-layer harbours up to 28 different cell wall proteins that are anchored to the cell wall by CWB2 protein domains (Fagan and Fairweather 2014; Willing et al. 2015). Disruption of *cwp84* results in uncleaved SlpA in the cell wall, which in turn results in aberrant retention of other cell wall proteins at the cell surface (Kirby et al. 2009; de la Riva et al. 2011). The effect of *cwp84* disruption on biofilm formation was dependent on the strain background. In strain R20291 a *cwp84* mutant showed reduced biofilm formation (Đapa et al. 2013), whereas in strain 630 this mutant showed an increase in biofilm formation (Pantaléon et al. 2015). As these strains encode a different array of proteins that are predicted to associate with the S-layer (Biazzo et al. 2013), an immature S-layer may contain different surface-associated proteins between the two strains. Whether the S-layer *per se* is involved in biofilm formation or if this effect is due to the proteins associated with the S-layer remains uncertain.

Regarding quorum sensing, bacteria detect a threshold level of autoinducer (AI) molecules and activate a signal cascade that leads to altered gene expression. The AI-2 molecule is synthesised by LuxS and is produced by Gram-positive and Gram-negative bacteria. Due to the number of bacteria that can produce and detect AI-2 molecules, this quorum signalling mechanism is thought to function as an intra- and interspecies communication molecule. *C. difficile* encodes a *luxS* homologue and produces a chemically active AI-2 molecule that can induce homologous and heterologous gene expression (Carter et al. 2005; Lee and Song 2005). Biofilm formation in a *C. difficile luxS* mutant was severely diminished compared to wild-type strain, where not even a bacterial monolayer was able to form (Đapa et al. 2013; Slater and Unnkrishnan 2015).The regulatory pathway behind AI-2 induced biofilm formation is currently unknown. In the gut mucosa, *C. difficile* interacts with members of the sessile community (Lawley et al. 2009; Buckley et al. 2011; Donelli et al. 2012; Crowther et al. 2014a, b; Semenyuk et al. 2015), where such interspecies signalling could play an important role for disease progression.

Based on current research, it seems clear that the genetic regulation behind *C. difficile* biofilm formation is extremely complex and several different global regulators that link various metabolic pathways influence it. The *C. difficile* sporulation master regulator, *spo0A*, besides coordinating sporulation by undergoing post-translational phosphorylation (Spo0A-P) in order to activate the sigma factor cascade (Pettit et al. 2014; Al-Hinai et al. 2015), also plays a role in biofilm formation. Disrupting the *spo0A* gene resulted in a reduced biofilm phenotype that could be restored by complementation (Dawson et al. 2012; Đapa et al. 2013). In *Bacillus* spp., the intracellular concentration of Spo0A-P is critical to determining if the cell proceeds down either the sporulation pathway (high Spo0A-P levels), or biofilm pathway (low Spo0A-P levels) (Mhatre et al. 2014). During the early stages of biofilm formation Spo0A-P induces the expression of *sinI*, which inhibits a protein that represses the biofilm matrix genes, SinR (Vlamakis et al. 2013; Cairns et al. 2014). *C. difficile* encodes homologues of *sinI* and *sinR* (Edwards et al. 2014), however their role in regulating biofilms, and the regulon of SinR, are unknown and deserves further investigation.

It has also been demonstrated that biofilm formation in *C. difficile* may be stress-inducible; exposure of cells to sub-inhibitory

concentrations of antibiotics, such as metronidazole and vancomycin, induce biofilm formation (Đapa et al. 2013; Vuotto et al. 2016). In other bacteria, this stress-induced biofilm formation is induced by the SOS regulatory network in response to DNA damage, through activation of the transcriptional repressor, LexA, by the recombinase protein, RecA (Butala et al. 2009). Mutation of *lexA* in *C. difficile* caused pleotropic effects to the cell: elongated cell morphology, decreased sporulation and motility and increased biofilm formation (Walter et al. 2015). In silico analysis of predicted LexA binding sites within the *C. difficile* genome suggests LexA could regulate up to 29 loci (Walter et al. 2014). How this regulatory pathway contributes to *C. difficile* biofilm formation is unclear and warrants further investigation.

4 In Vitro and In Vivo Models to Study the Interactions of Sessile Microorganisms

The mammalian intestinal mucosa is home to a complex mixture of microbial communities, which can aggregate to form mats or biofilm structures over the epithelial cells. *C. difficile* cells can associate with these microbial communities during CDI (Lawley et al. 2009; Goulding et al. 2009; Spencer et al. 2014; Semenyuk et al. 2015). The interactions between microbial species within a biofilm can vary depending on the associated microbial species, and this can affect the spatial organisation of cells within biofilm. Sessile microbes can form synergistic, exploitive or competitive relationships with other biofilm-forming microorganisms (Liu et al. 2016). Since understanding the interactions between *C. difficile* and the sessile community could be key to designing defined microbial treatments for recurrent CDI, several in vitro and in vivo models have been developed to study these kind of interactions. The use of in vitro models allows researchers to manipulate and control certain factors and/or conditions, thus providing a valuable tool for biofilm research. Systems can generally be defined as 'closed' or 'open'. Closed (or static) biofilm models, such as the popular microtiter tray based models, are based on batch culture, in which there is limited nutrient availability and aeration, as well as a build-up of metabolic products. Open (or dynamic) biofilm model systems are based on continuous flow models, whereby fresh media replace metabolic products and waste constantly. Whilst open biofilm models may be better able to simulate sheer forces and flow, they often require more technical expertise and complex equipment than closed systems, and so are less amenable to high throughput workloads.

The use of microtiter trays is one of the simplest methods used to investigate both mono- and poly-microbial species biofilm formation. This method has been used to determine some of the genetic mechanisms behind *C. difficile* biofilm formation, as well as its interaction with other gut microbiota. Donelli et al. (2012) found that several gastrointestinal residing bacteria were able to cooperatively form a biofilm when co-cultured together and in addition, highlighting a positive interaction between *C. difficile* and *Finegoldia magna*. This method has been also used to characterise the inter-kingdom interactions between *C. difficile* and *Candida albicans*. Biofilm formation of *C. albicans* was reduced when co-incubated with filter sterilized *C. difficile* growth media, which was attributed to the production of *p*-cresol by *C. difficile* (van Leeuwen et al. 2016), although the direct interaction was not reported. The interactions between fungi and other intestinal microflora are probably more complex than we assume and others have found a correlation between CDI and the presence of *Candida* spp. (Raponi et al. 2014). The inhibition of *C. difficile* grown in a planktonic culture by probiotic strains *Lactobacillus* and *Bifidobacteria* has been documented previously (Plummer et al. 2004; Trejo et al. 2010), but recent unpublished studies by Normington and coworkers have shown these probiotic organisms inhibit *C. difficile* biofilm formation (Normington et al. 2017).

Regarding the open (or dynamic) biofilm model systems, Crowther et al. (2014b)

developed a modified version of the continuous triple chemostat system (Macfarlane et al. 1998) to monitor the sessile populations, by using glass rods suspended from the lid. During simulated CDI, the authors observed consistent sessile populations formed upon the different rods sampled at the same time. The composition of the sessile communities in these experiments was *Bacteroides* spp., *Bifidobacteria* spp., *Lactobacillus* spp. and *Enterococcus* spp., however as determined by total viable counts, many more bacterial species must be associated within the biofilms (Crowther et al. 2014a). Currently, Buckley and colleagues are using bacterial 16S rRNA sequencing analysis to identify the composition of the sessile community from these rods, and in addition, they recently identified fungal species within the biofilm structure (Unpublished results). Upon instillation of *C. difficile* spores, these spores became associated with the biofilm, and both sessile spore and vegetative populations were isolated during the CDI phase (Crowther et al. 2014a, b; Unpublished results). The interactions between *C. difficile* on the other sessile populations are currently under investigation (Fig. 5).

In vivo models of CDI have been used to specifically identify the bacterial populations associated with the mucus layer during disease. Using paraffin embedded sections, to preserve the mucus layer, and fluorescent *in situ* hybridisation (FISH), Semenyuk et al. (2015) identified *C. difficile* vegetative cells within the outer mucus layer. Microbial taxonomy analysis from 16S rRNA sequences recognized other bacterial genera residing within the mucus layer, from several families belonging to *Bacteroidetes* and *Firmicutes* (*Lactobacillaceae*, *Lachnospiraceae* and *Clostridium* cluster XVII and XIV). Those microbial species that directly interact with *C. difficile* in vivo are still unknown. Interestingly, during the early phase of CDI an increase in *Enterbacteriaceae* was observed within the mucosal populations (Semenyuk et al. 2015). Whether such an increase enhances *C. difficile* recruitment into the biofilm, or allows extra-intestinal invasion (Ng et al. 2013), as seen by (Goulding et al. 2009; Lawley et al. 2009), remains to be determined.

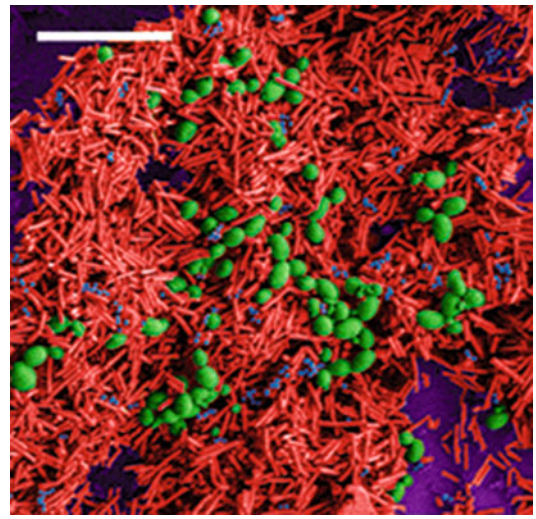

Fig. 5 Scanning electron micrograph of an in vitro polymicrobial biofilm. A biofilm containing *C. difficile* (red cells), *Candida spp.* (green cells) and *Staphylococcus spp.* (blue cells) was grown anaerobically for 3 days. White scale bar indicates 20 μm (SEM image taken from Normington et al. 2017)

5 Effects of Antibiotics on *C. difficile* Biofilm

Biofilm formation has been demonstrated to be an important factor enhancing the antimicrobial resistance (Ciofu et al. 2017). In fact, during infection the biofilm mode of growth protects cells from antibiotic treatment, their resistance often increasing from 10- to 1000-fold compared with the same cells growing planktonically (Mah and O'Toole 2001; Hoiby et al. 2010). Several mechanisms can contribute to antibiotic resistance in biofilm; including the biofilm matrix, acting as a physical barrier that affects penetration of antimicrobial agents (Flemming and Wingender 2010), the presence of persister cells

(Shah et al. 2006) and the genetic mutations occurring within bacteria in biofilm (Tyerman et al. 2013).

Tolerance mechanisms have been proposed in *C. difficile* biofilm (Đapa et al. 2013), so the effect of antibiotics most commonly used to treat CDI, such as metronidazole and vancomycin (Peng et al. 2017), has been assessed against biofilm-growing cells and pre-formed biofilms.

Semenyuk and colleagues determined that 630 and VPI 10463 *C. difficile* cells grown as biofilm for 20 h had greater resistance to metronidazole than planktonic cells, with 1 μg/ml of antibiotic inhibiting liquid cell growth by about 100-fold and 100 μg/ml reducing only about a tenfold of the sessile cells. These data demonstrated that biofilms conferred a 100-fold increase in metronidazole resistance (Semenyuk et al. 2014).

In addition to being ineffective to counteract in vitro *C. difficile* biofilm, it has been demonstrated that, at sub-inhibitory concentrations, metronidazole can even enhance biofilm formation in specific cases. In particular, three clinical strains belonging to PCR-ribotype 010, non-toxigenic and showing different metronidazole susceptibility profiles, exhibited variation in biofilm-forming ability. In the presence of metronidazole, a susceptible strain and a strain with reduced-susceptibility revealed a significant increase in biofilm biomass, due to a more abundant EPS matrix production, while the biofilm-forming ability of the stable-resistant strain was not affected by the antibiotic pressure (Vuotto et al. 2016). This study highlights the possibility that the exposure of *C. difficile* to low concentrations of antibiotic present in the gut at the beginning or end of antibiotic therapy for CDI could serve as stress signal and, thus, stimulate biofilm production, with severe clinical implications in the treatment failure and recurrence of CDI. When similar experiments were carried out by using *Bacteroides fragilis*, opposed results were obtained. In fact, sub- inhibitory concentrations of metronidazole were able to inhibit biofilm formation (Silva et al. 2014).

Aside from *C. difficile* biofilms, metronidazole efficacy has been evaluated on biofilm-related bacterial vaginosis (BV). Monotherapy with metronidazole resulted to be able to only temporarily suppress *Gardnerella vaginalis* biofilms but not completely eradicate it, in most cases rapidly regaining activity after treatment ending (Swidsinski et al. 2008, 2014). Another study also showed that 30 BV-associated biofilm-forming bacteria were resistant to metronidazole (Alves et al. 2014).

Vancomycin, compared to metronidazole, demonstrates a higher clinical cure rate in adults with severe CDI and a similar clinical cure rate in moderate CDI cases, thus becoming the recommended therapy for more severe cases (Ofosu 2016). However, regarding its ability to act against mature biofilms, a number of papers have been published on staphylococcal species (Meeker et al. 2016; Ozturk et al. 2016; Hashem et al. 2017; Jimi et al. 2017) but limited and not encouraging data are so far available for *C. difficile*.

Đapa and co-workers first analysed the influence of vancomycin on biofilms of a *C. difficile* strain belonging to the PCR-ribotype 027, by examining the effects of different concentrations of antibiotic. High concentrations of vancomycin (20 μg/mL) failed to kill bacteria within biofilms while sub-inhibitory and inhibitory concentrations of vancomycin (0.25 μg/mL and 0.5 μg/mL, respectively) induced *C. difficile* biofilm formation. This suggests that increased antibiotic resistance in *C. difficile* may be mediated by the thick biofilm matrix and/or by the physiological state of bacteria within biofilms (Đapa et al. 2013). These results were corroborated by Mathur et al. (2016), whom observed low efficacy of vancomycin against various PCR-ribotypes.

Using a triple-stage human gut model, Crowther and colleagues simulated CDI and determined the effect of vancomycin on the motile and sessile *C. difficile* populations. Vancomycin exposure reduced the *C. difficile* planktonic populations to below the limit of detection, however the sessile populations were unaffected. This could be due to the levels of vancomycin that were detected within the biofilms [mean 40.4 mg/L (range 38.7–43.4 mg/L)] compared to those (54.7 mg/L) of the vessel lumen

(Crowther et al. 2014a). A reduced level of vancomycin within the biofilm could prevent a critical level of vancomycin from being achieved, or even further enhance matrix production. We clearly observed a differential response of sessile bacteria to antimicrobial administration, with *C. difficile* spores being largely unresponsive either to clindamycin instillation.

The effect of tigecycline, teicoplanin, rifampicin and nitazoxanide was also evaluated on the biofilm of five different *C. difficile* strains, noting that the sensitivities of these biofilms to different antimicrobials were strain-dependent, regardless of the produced biomass (Mathur et al. 2016).

6 Alternatives to Counteract Biofilm-Growing *C. difficile*

Antibiotic administration, although carried out at higher doses over a prolonged period, often fails to counteract biofilm-related infections. In addition, antibiotic overuse and misuse are key factors contributing to the global increase of antibiotic resistance. Alternative therapeutic agents with antibacterial properties that prevent, disrupt, weaken or kill the microbial community within a biofilm, are becoming increasingly attractive. In particular, anti-biofilm compounds: (i) may prevent biofilm formation by killing planktonic cells or blocking bacterial adhesion; (ii) may counteract mature biofilms by destabilising the matrix or by making the microbial cells susceptible to antimicrobial and/or host defence mechanisms; (iii) may undo virulence factors involved in biofilm formation or may affect quorum sensing; (iv) may have a bactericidal effect on biofilm-growing cells (Roy et al. 2017).

Efforts to fight these microbial communities include the use of different compounds, alone or in combination, to target different phases of biofilm, drug repurposing, peptides, nanomaterials, and medical device coatings refractory to microbial adhesion or functionalised with anti-biofilm compounds (Ribeiro et al. 2016).

The conventional antibiotics used in CDI therapy are often unsuccessful and recurrent infections may occur, perhaps due to its ability to grow as a biofilm thus impairing antimicrobial activity. Different approaches, which are an alternative to the use of antibiotics, have been proposed to decrease *C. difficile* biofilm formation or disrupt mature biofilm.

Among the huge number of antimicrobial compounds today at our disposal, relatively few have been tested so far against *C. difficile* biofilm. The first one tested was Manuka honey, its anti-biofilm properties on other species being already demonstrated (Badet and Quero 2011). Biofilms formed by two *C. difficile* strains, a ribotype 027 strain and a ribotype 106 strain, were used to test the effect of Manuka honey at varying concentrations of 1–50% (w/v). A dose-dependent response was observed for both test strains, with the optimum Manuka honey activity obtained at 40–50% (v/v) (Hammond et al. 2014). Consistent results were also obtained by evaluating its efficacy on clinical *C. difficile* strains belonging to four prominent PCR ribotypes (R017, R023, R027 and R046) (Piotrowski et al. 2017).

The antimicrobial agent thuricin CD, a sactibiotic produced by a bacterial strain derived from a human faecal sample, was also assessed against biofilms of R027, Liv022 R106 and DPC6350, alone or in combination with some antibiotics commonly used to treat CDI. Results underlined the effectiveness of thuricin CD against all the tested strains and its ability to significantly potentiate the efficacy of the antibiotics rifampicin, tigecycline, vancomycin and teicoplanin against R027 biofilms (Mathur et al. 2016).

More innovative proposals to avoid treatment failure and recurrent CDI infection have been sort through the use of bacteriophages and photodynamic therapy.

It has been demonstrated that some bacteriophages have good activity against biofilms of different species by invading it and significantly

reducing the viable numbers of cells. Accordingly, bacteriophages appear to be a highly promising therapeutic option for eradicating CDI by replacing antibiotics or supplementing them (Azeredo and Sutherland 2008). Nale and colleagues evaluated the impact of a four-phage cocktail on *C. difficile* ribotype 014/020 biofilm, in vitro alone or in combination with vancomycin treatment in *Galleria mellonella* larva CDI model. Phages were able to prevent in vitro biofilm formation, to penetrate established biofilms, and also to reduce colonization and/or prevent disease in the *Galleria mellonella* model, when used alone or in combination with vancomycin (Nale et al. 2016).

Photodynamic therapy, more frequently applied to determine its usefulness to treat periodontal (Sculean et al. 2015) and wound (Percival et al. 2014) infections, has also been tested against planktonic and sessile-growing *C. difficile* strains. This approach exploits the ability of light-activated photosensitisers (PS) to produce reactive oxygen species (ROS) lethal to cells. Three of thirteen PS screened were able to kill 99.9% of the tested *C. difficile* strains both in planktonic and biofilm states, after exposure to red laser light (0.2 J/cm^2) (De Sordi et al. 2015). Although PS are an interesting perspective for biofilm eradication, as they work by producing free radical species, their use in the human gastrointestinal tract remains limited without further development of the technology.

Recent discoveries of alternative *C. difficile* treatments include rhodanine derivatives (AbdelKhalek et al. 2016) and acyldespiptides (Gil and Paredes-Sabja 2016), that exhibit in vitro activity against planktonic populations, while their efficacy against the sessile populations remains to be evaluated.

In addition to the antimicrobial compounds already tested and the other approaches above mentioned, further possibilities to interfere with *C. difficile* biofilm could presumably come from the discovery of novel compounds that bind c-di-GMP riboswitches (Furukawa et al. 2012), from the use of DNase as enhancer of the effect of metronidazole (Machado et al. 2015) or by employing specific QS inhibitors able to interfere with biofilm maturation (Đapa et al. 2013).

7 Conclusions

Biofilms are the most representative form of bacterial growth in the large intestine, with biofilm formation being known to influence the ability of pathogens to colonize and establish during infection.

Clinically relevant strains of *C. difficile* have been proven to be able to form biofilms in vitro that appear as complex cellular processes involving an array of different regulating proteins, intracellular chemical signals and effector proteins, all having a role in different aspects of bacterial physiology. Although much has been done to understand the regulatory signals governing biofilm formation, this picture is still incomplete and the details of the precise function and regulation of each of these proteins/pathways remain to be studied. This intertwinement is likely to allow an accurate modulation of the differentiation pathways for motility, biofilm formation or sporulation at a spatio-temporal manner.

Even if the *C. difficile* colonization in vivo has yet to be analysed in deep, the demonstrated ability to form a mature biofilm in vitro seems to be predictive of the in vivo colonization mode. In CDI, the establishment of persistent biofilms in vivo, in addition to the formation of spores, could potentially explain the occurrence of recurrent infections. Thus, a potential infection model involving the colonization of the colon by *C. difficile* through the formation of microcolonies or biofilms, which is followed by toxin production. This in vivo biofilm mode of growth possibly protects the bacterium from the cellular immune responses triggered by the toxins and from the antibiotic treatment. In light of the above, a deeper knowledge of the factors involved in the *C. difficile* biofilm development during infection might provide an advanced understanding of the role of biofilm in CDI.

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European Practice for CDI Treatment

Fidelma Fitzpatrick, Mairead Skally, Melissa Brady, Karen Burns, Christopher Rooney, and Mark H. Wilcox

Abstract

Clostridium difficile infection (CDI) remains a significant cause of morbidity and mortality worldwide. Historically, two antibiotics (metronidazole and vancomycin) and a recent third (fidaxomicin) have been used routinely for CDI treatment; convincing data are now available showing that metronidazole is the least efficacious agent. The European Society of Clinical Microbiology and Infectious Diseases CDI treatment guidelines outline the treatment options for a variety of CDI clinical scenarios, including use of the more traditional anti-CDI therapies (e.g., metronidazole, vancomycin), the role of newer anti-CDI agents (e.g., fidaxomicin), indications for surgical intervention and for non-antimicrobial management (e.g., faecal microbiota transplantation, FMT). A 2017 survey of 20 European countries found that while the majority (n = 14) have national CDI guidelines that provide a variety of recommendations for CDI treatment, only five have audited guideline implementation. A variety of restrictions are in place in 13 (65%) countries prior to use of new anti-CDI treatments, including committee/infection specialist approval or economic review/restrictions. Novel anti-CDI agents are being evaluated in Phase III trials; it is not yet clear what will be the roles of these agents. Prophylaxis is an optimum approach to reduce the

F. Fitzpatrick (✉)
Department of Clinical Microbiology, The Royal College of Surgeons in Ireland, Dublin, Ireland

Department of Clinical Microbiology, Beaumont Hospital, Dublin, Ireland
e-mail: fidelmafitzpatrick@rcsi.ie

M. Skally · M. Brady
Department of Clinical Microbiology, Beaumont Hospital, Dublin, Ireland
e-mail: maireadskally@beaumont.ie; melissabrady@beaumont.ie

K. Burns
Department of Clinical Microbiology, Beaumont Hospital, Dublin, Ireland

Health Protection Surveillance Centre, Dublin, Ireland
e-mail: karenburns@beaumont.ie

C. Rooney
Microbiology, Leeds Teaching Hospitals and University of Leeds, Leeds, UK
e-mail: christopherrooney@nhs.net

M. H. Wilcox (✉)
Microbiology, Leeds Teaching Hospitals and University of Leeds, Leeds, UK

Leeds Teaching Hospitals and University of Leeds, Leeds, UK
e-mail: mark.wilcox@nhs.net

impact of CDI especially in high-risk populations; monoclonal antibodies, antibiotic blocking approaches and multiple vaccines are currently in advanced clinical trials. The treatment of recurrent CDI is particularly troublesome, and several different live bio therapeutics are being developed, in addition to FMT.

Keywords

C. difficile treatment · Anti-CDI agents · CDI guidelines · Novel *C. difficile* agents · *C. difficile* prophylaxis

1 Introduction

The European Society of Clinical Microbiology and Infectious Diseases (ESCMID) first published guidelines for *Clostridium difficile* infection (CDI) treatment in 2009, which were revised in 2014 (Debast et al. 2014). These evidence-based guidelines outline the treatment options for a variety of CDI clinical scenarios, including recommendations for use of the more traditional anti-CDI therapies (e.g., metronidazole, vancomycin), the role of newer anti-CDI agents (e.g., fidaxomicin), indications for surgical intervention and for non-antimicrobial management (e.g., faecal microbiota transplantation, FMT). Many European countries have published their own national CDI treatment guidelines, which are broadly similar to the ESCMID guidelines, though contextualised to the local setting (ECDC 2017).

When discussing European practice for CDI treatment, variability between countries is inevitable for a number of reasons. Treatment of patients with CDI begins with making the diagnosis, specifically having a high index of clinical suspicion if a patient has a combination of signs and symptoms and/or CDI risk factors and thereafter confirmation by microbiological testing or colonoscopic/histopathological findings. Clinician awareness of CDI as part of the differential diagnosis and access to timely laboratory diagnostics is therefore crucial for appropriate patient management. However, there remains considerable variability across countries with an estimated 40,000 inpatients potentially undiagnosed annually in European hospitals (Davies et al. 2014). Mnemonic checklists can be useful tools to reduce clinician error and promote awareness (Chew et al. 2016). Albeit potentially more useful when English is the commonly spoken language, the SIGHT mnemonic is a useful aide memoire for clinicians when managing patients with suspected potentially infectious diarrhoea (Fig. 1) (Public Health England 2013).

Once CDI is diagnosed, variability in anti-CDI treatment practices may be due to individual judgement and/or knowledge, individualised patient factors and national regulatory or economic issues, e.g., the availability of newer (more expensive) anti-CDI agents. Lastly, the ESCMID (and national) guidelines recommend a number of potential treatment options for similar CDI clinical scenarios, so individual clinician preference will likely be a potential cause of variability. This variability in anti-CDI treatment

S	Suspect that a case may be infective where there is no clear alternative cause for diarrhoea.
I	Isolate the patient/resident. Consult with the infection prevention and control team where available while determining the cause of the diarrhoea.
G	Gloves and aprons must be used for all contacts with the patient/resident and their environment.
H	Hand washing with soap and water should be carried out after each contact with the patient/resident and the patient/resident's environment.
T	Test the stool for *C. difficile* toxin, by sending a specimen immediately.

Fig. 1 SIGHT Mnemonic protocol (Adapted with permission from SIGHT Mnemonic UK protocol (DH and HPA 2008))

preferences has previously been described in Ireland (Prior et al. 2017). In the United States (US) almost half of patients with severe CDI were treated with metronidazole, despite vancomycin being recommended in national guidelines at that time (Stevens et al. 2017).

In this chapter, we firstly review the ESCMID CDI guideline recommendations and include an update as relevant of subsequent publications, present the findings of a 2017 survey of European CDI national experts regarding CDI guidelines and their implementation and lastly look to the future as we summarise promising new therapies for CDI treatment.

2 ESCMID Guidelines for CDI Treatment

The ESCMID guidelines provide a number of definitions to guide clinical management of patients with CDI, including diagnosis, treatment response, severity and recurrence (Debast et al. 2014). A number of CDI scenarios are considered including the initial management of CDI in addition to the management of recurrent and severe CDI. (Table 1) For all scenarios the timely implementation of appropriate infection prevention and control measures to prevent further cross-infection is highlighted, in addition to the discontinuation of antimicrobial therapy (if clinically indicated), fluid and electrolyte replacement, review of proton pump inhibitor use and avoidance of anti-motility medications.

2.1 Non-severe CDI

Three potential options are recommended for treatment of non-severe CDI, namely metronidazole, vancomycin or fidaxomicin. Metronidazole, which is a relatively safe and inexpensive antimicrobial is the treatment of choice (grade A–I), once there is no contraindications for its use. However, adverse effects such as metallic taste and nausea may limit its use/compliance in certain patient populations. Another recommended option for treatment of non-severe CDI is fidaxomicin (Grade B–I). Fidaxomicin is also recommended later in the guidelines for treatment of severe/complicated and first recurrent CDI (Grade B–I) and multiple recurrent CDI (Grade B–II). The non-inferiority of fidaxomicin to vancomycin for treatment of CDI with lower recurrences rate and superior sustained clinical response has been reported, though patients with severe CDI were not evaluated (Louie et al. 2011; Cornely et al. 2012). Subsequently, superiority of fidaxomicin to vancomycin in patients with non-NAP1/BI/027 strains was reported (Crook et al. 2012). However, in patients infected with the NAP1/B1/027 strain, there was no significant difference in recurrence rates between the two drugs. What implication this particular finding has for clinical practice in Europe will depend on the current prevalence rate of this strain in a country. However fidaxomicin is considerably more expensive than metronidazole or vancomycin, therefore economic factors may come into play in European countries regarding its availability and use (Nelson et al. 2017).

Since publication of the ESCMID guidelines, the superiority of vancomycin over metronidazole for treatment of mild-to-moderate primary or recurrent CDI has been reported. (Johnson et al. 2014) and numerous publications have examined the benefits of fidaxomicin in a number of patient populations. A recent Cochrane review evaluated anti-CDI treatment options and reported that vancomycin is superior to metronidazole and fidaxomicin is superior to vancomycin for achieving symptomatic cure (Nelson et al. 2017). The authors noted that the lack of any 'no treatment' control studies does not allow for any conclusions regarding the need for specific anti-CDI treatment in patients with mild CDI and pointed to the economic advantage of metronidazole.

2.2 Definition and Treatment of Severe CDI

Classification of CDI by severity can be problematic, as patients with severe ileus may not

have diarrhoea. In practice, the clinical spectrum of severe CDI varies considerably and the diagnosis is usually reached using a combination of findings. The ESCMID guidelines summarise the range of patient, laboratory, endoscopic and radiological factors associated with severity of CDI colitis and recommend three unfavourable prognostic factors, namely raised leukocyte count >15 $\times 10^9$/L, decreased albumin <30 g/L and rise in serum creatinine level (>1.5 times the premorbid level or >133 μM) (Debast et al. 2014). A recently validated clinical prediction rule to identify patients at risk of severe outcomes (age ≥60 years, peak serum creatinine ≥1.5 mg/dL and peak leukocyte count of ≥20,000 cells/μL) may be useful for clinicians to identify high-risk patients likely to benefit from more aggressive therapy (e.g., early administration of oral vancomycin).(Na et al. 2015).

The recommended treatment of choice for severe CDI in the ESCMID guidelines is oral vancomycin (Grade A–I) which achieves high intracolonic concentrations with minimal systemic adverse effects (Debast et al. 2014). Intravenous metronidazole combined with vancomycin retention enema or oral/NG vancomycin at the higher 500 mg dose is provided as an alternative (Grade B–III). A recent retrospective study comparing vancomycin and metronidazole reported superiority of vancomycin for

Table 1 Overview of ESCMID recommendations for CDI treatment (Debast et al. 2014)

Clinical scenario	Oral antibiotic treatment	Oral treatment not possible	Non-antibiotic treatment	Not recommended
Non-severe CDI	**Metronidazole 500 mg TDS (A–I)**	IV Metronidazole 500 mg TDS 10 days (A–II)	Stop inducing antibiotic (s) and 48 h clinical observation (C-II)	Probiotics (D–I)
	Or Vancomycin 125 mg QDS (B–I)			Toxin binding (D–I)
	Or Fidaxomicin 200 mg BD (B–I)			
	All 10 days			
First recurrence	Fidaxomicin 200 mg BD (B-I)			
	Or Vancomycin 125 mg QDS (B–I)			
	Or Metronidazole 500 mg TDS (C–I)			
	All 10 days			
Multiple recurrences	Fidaxomicin 200 mg BD: 10 days (B-II)	**Faecal transplantation in combination with oral antibiotic treatment (A–I)**		Metronidazole 500 mg TDS (D-II)
	Or Vancomycin 125 mg QDS: 10 days followed by pulse or taper strategy (B-II)			Probiotics (D–I)
				Passive immunotherapy with immune whey (D–I)
Severe CDI or complicated course	**Vancomycin 125 mg QDS (A–I)** *Consider increasing to 500 mg QDS (B-III)*	**IV Metronidazole 500 mg TDS 10 days (A-II)** combined with either	**Surgery**: Total colectomy and ileostomy	Metronidazole 500 mg TDS (D–I)
	Or Fidaxomicin 200 mg BD (B–I)	Vancomycin retention enema (500 mg in 100 mL normal saline QDS intracolonic)		Fidaxomicin (D-III)
	All 10 days	*or* Vancomycin 500 mg QDS by oral/nasogastric tube for 10 days (B-III)		

PO oral, *IV* intravenous, *BD* twice daily, *TDS* three times daily, *QDS* four times daily

severe CDI, though no difference in CDI recurrence rates (Stevens et al. 2017). At the time of publication of ESCMID guidelines, it was noted that there was insufficient data available for fidaxomicin. While there have been subsequent reports of fidaxomicin use in critical care patients with CDI and case reports of salvage use after failure of standard therapy, (Penziner et al. 2015; Arends et al. 2017), as most studies exclude patients with severe CDI the role of fidaxomicin in these patients has yet to be fully elucidated. (Nelson et al. 2017).

The precise role of surgical management in severe CDI is a topic of debate (Fitzpatrick 2008). There are no clear guidelines or protocols to guide the timing of surgical intervention. Certainly, the decision that surgical management is required for CDI should be taken by the multidisciplinary team, surgeons consulted at an 'early' stage (though there is no clear definition as to when this is) and an interdisciplinary risk/benefit analysis of surgery individualised for that patient. The ESCMID guidelines recommend total colectomy, 'before colitis becomes very severe', if colonic perforation or if there is systemic inflammation and the patient's condition has deteriorated and is not responding to anti-CDI therapy (Table 1) (Debast et al. 2014). Because of the morbidity (and mortality) associated with colectomy in a systemically unwell patient, there is increased interest in evaluating options that avoid colon resection (Kautza and Zuckerbraun 2016; Sartelli et al. 2015). The potential role of FMT as an alternative to emergency bowel surgery has also been recently highlighted (van Beurden et al. 2017).

2.3 Recurrent CDI

Recurrent CDI itself is a significant risk factor with the risk of recurrence increasing significantly with each episode of recurrence. Predicting which patients will develop recurrent CDI would enable clinicians to minimise recurrence risk (e.g., avoid concomitant antimicrobials) and also by heightening awareness, facilitates prompt diagnosis and treatment of recurrences (Hu et al. 2009). The ESCMID guidelines recommendation for the first recurrence of non-severe CDI is either vancomycin or fidaxomicin (both B–I recommendations). For subsequent recurrences, while a variety of strategies are recommended (Table 1), FMT is allocated an A–I recommendation.

Recent surveys have highlighted the interest of European clinicians in FMT as a therapeutic option for patients with CDI; though note its potential underutilisation (Porter and Fogg 2015; Prior et al. 2017). Since publication of the ESCMID guidelines, a recent two-centred randomized controlled trial of FMT via colonoscopy for recurrent CDI reported a 91% cure rate with donor FMT (63% with autologous FMT – though this varied significantly between the two centres at 43% and 90% cure rates respectively) (Kelly et al. 2016). Notably patients with recurrence after autologous FMT resolved after a subsequent donor FMT. Severe and severe-complicated indication, inpatient status during FMT, and the number of previous CDI-related hospitalizations are strongly associated with early failure of a single FMT for CDI (Fischer et al. 2016).

3 Survey of European CDI Experts on CDI Treatment

Though European and National CDI guidelines exist and variability in practice for treatment of patients with CDI is likely as previously discussed, to our knowledge there has been no recent assessment of CDI treatment guideline recommendations and their implementation in European countries. We designed an interactive online survey in this regard using Demographix® software (57 Chestnut Road, London SE27 9EZ UK). The purpose was to describe the practice for CDI management and treatment in Europe. National experts from European countries were invited by email to complete the online survey, during the period 07th June 2017 to 28th July 2017. Data was analysed using an Excel® database (Microsoft Corp., Redmond, WA, USA).

Eighty-three CDI experts from 35 European countries were invited to take the survey with 34 respondents, representing 20 (57%) countries. Respondents included experts in the fields of microbiology, public health and infection prevention and control, who were working in hospitals (n = 10), laboratories (n = 2), health protection, public health or infectious diseases agencies (n = 4) or other organisations (n = 4). To avoid study bias arising from multiple respondents from the same country, data from one respondent per country was included in the analysis.

National guidelines for managing patients with CDI were available in 14 (70%) countries with guideline revisions undertaken during the last 5 years (n = 7), 1 year (n = 2), or were presently under revision (n = 1). Revisions had not been undertaken in four countries with these guidelines published in 2007, 2011 (n = 2) and 2013. Of the six countries that did not have national guidelines, guidance was sought from the ESCMID CDI guidelines (n = 5) or local guidelines (n = 1). The recommendations provided in national guidelines varied by country, as outlined in Table 2. Of the options provided in the survey, the commonest recommendation was treatment of patients with CDI (93%; n = 13) and the least common were CDI key performance indicators (KPIs) and audit of guideline implementation (21%; n = 3). Other recommendations were provided in national guidelines of 36% (n = 5) countries, including: essential elements of a CDI prevention programme, use of tools such as checklists, *C. difficile* reference laboratory requirements, access to infection specialists in the non-acute sector, healthcare facility infrastructure requirements, environmental and equipment decontamination, epidemiology, clinical diagnosis of CDI, antimicrobial stewardship, FMT and defining roles and responsibilities to support the implementation of the guidance.

In total, 36% (n = 5) of countries previously surveyed or audited some (but not all) aspects of the implementation of national CDI guidelines though the majority, 64% (n = 9), had not. Of the five surveys/audits conducted:

- Two were conducted in the past 5 years and three more than 5 years ago. No surveys were conducted in the last year.
- CDI treatment was included in one national survey only.
- Facilities surveyed included hospitals only (n = 4) or diagnostic microbiology laboratories only (n = 1)

Of the six countries that did not have national guidelines, a previous survey or audit of some (but not all) aspects of local CDI guidelines was conducted for one and five (83%) did not previously conduct a survey or audit. For the survey that was conducted, CDI treatment was not included and facilities surveyed included hospitals only. Information on when the survey took place was not provided.

Severe CDI was defined as a variable combination of factors, as outlined in Fig. 2. The commonest being leucocytosis of $\geq$15,000 cells per μL (n = 17; 85%). A variety of anti-CDI regimens were recommended as summarised in Table 3. In addition, a number of other factors were reported to influence choice of the recommended anti-CDI therapy including:

- *C. difficile* ribotype.
- Patient factors:
  - Risk factors for recurrence.
  - Patient tolerance/ability to take oral medications/response to treatment.
- Fidaxomicin use:
  - Approval required from microbiology/infectious diseases for use.
  - Economic considerations because of high cost.
  - Reservations about its use as lack of survival benefit.
- FMT:
  - Availability of facilities for a FMT service.
  - Use as an option for severe CDI when surgery is not possible.
- Immunoglobulin therapy recommended in case of severe protein loss.

Table 2 Recommendations for CDI management in 14 European countries with national CDI guidelines

Recommendation	Included in guideline	Not included in guideline
	Number (n) and percentage (%) of countries	
Surveillance of CDI, n (%)	11 (79)	3 (21)
Laboratory diagnosis of CDI, n (%)	12 (86)	2 (14)
Treatment of patients with CDI, n (%)	13 (93)	1 (7)
Management of outbreaks and clusters of CDI, n (%)	11 (79)	3 (21)
CDI key performance indicators (KPIs), n (%)	3 (21)	11 (79)
Audit of guideline implementation, n (%)	3 (21)	11 (79)
Other recommendations, n (%)	5 (36)	9 (64)

One country with local guidelines is not included as applicable data was not available for this country

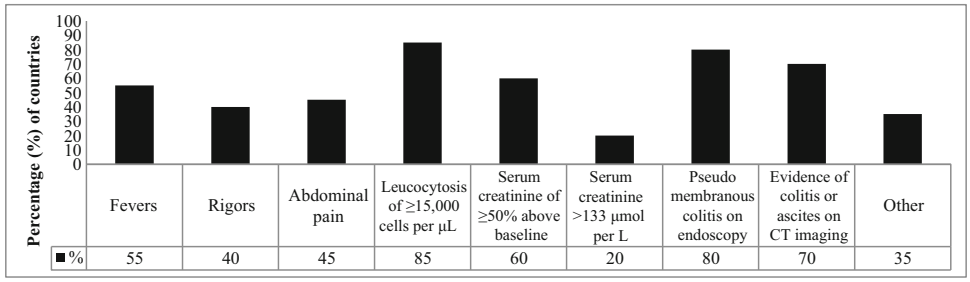

Fig. 2 Definition of severe CDI in 20 European Countries as a percentage (%) of countries surveyed 'Other' defining factors were included for 35% (n = 7 countries), and were a combination of: toxic megacolon, ileus, colonic dilation in CT scan >6 cm, immunosuppression, shock, hypotension, admission to hospital for treatment of CDI acquired outside the hospital, admission to the ICU for treatment of CDI, colectomy due to CDI, mortality within 30 days of diagnosis of CDI, suspicion of pseudomembranous colitis, diarrhoea, positive stool test, hemodynamic instability, signs of septic shock, signs of peritonitis, decreased bowel sounds, vomiting, lack of bowel movements, left shift, hypoproteinemia, anaemia and increased serum lactate

Table 3 Recommendations for CDI treatment in 15 European countries with national (n = 14) or local (n = 1) CDI guideline

	MTZ	Vancomycin	Fidaxomicin	Tapering vancomycin regimen	Immunoglobulin therapy	FMT
	Number (n) and percentage (%) of countries surveyed					
New CDI, n (%)	13 (87)	9 (60)	3 (20)	0 (0)	0 (0)	0 (0)
Recurrence (1st), n (%)	4 (27)	13 (87)	6 (40)	1 (7)	0 (0)	1 (7)
Recurrence (2nd), n (%)	0 (0)	8 (53)	7 (47)	6 (40)	1 (7)	3 (20)
Three or more recurrences, n (%)	0 (0)	6 (40)	4 (27)	9 (60)	3 (20)	12 (80)
Severe CDI, n (%)	3 (20)	11 (73)	4 (27)	1 (7)	1 (7)	1 (7)
Other, n (%)	3 (20)	2 (13)	1 (7)	0 (0)	1 (7)	1 (7)

MTZ metronidazole, *FMT* faecal microbiota transplantation

Of the 20 countries, a variety of restrictions were in place in 13 (65%) countries before new anti-CDI therapies could be used including:

- Reimbursement restrictions (n = 1).
- Health technology assessment (n = 1).
- Pharmacoeconomic review (n = 3).
- Committee approval either national (n = 6) or local (n = 4).
- Microbiology or infectious diseases approval (n = 2).
- CEO/financial director approval (n = 2).
- Cost and access issues re monoclonal therapy (n = 1).
- Antimicrobial resistance (n = 1).

4 *Clostridium difficile* Pipeline Prophylactic and Therapeutic Agents

The four current approved therapeutic agents for CDI vary markedly in efficacy. Whilst metronidazole has historically been the most commonly used option for treating CDI, as previously discussed, it is now known that this antibiotic is inferior to vancomycin (Johnson et al. 2014; Nelson et al. 2017). Concern regarding treatment failures with metronidazole remains (Vardakas et al. 2012). Metronidazole achieves poor intraluminal colonic concentrations, especially as mucosal inflammation subsides, such that the antibiotic may be undetectable as diarrhoea resolves. Also, some *C. difficile* isolates show reduced susceptibility to metronidazole, which may be relevant given the sub-optimal pharmacokinetics for this antibiotic in CDI. Laboratory detection of reduced metronidazole susceptibility is itself problematic with variations in methodology and MIC interpretation limiting analysis of trends and comparisons with published data (Moura et al. 2013).

Fidaxomicin and bezlotoxumab, a monoclonal anti-toxin B antibody and the most recently approved therapeutic agent, have been shown to reduce the risk of recurrent CDI by 40–50% in comparison with vancomycin alone (Wilcox et al. 2017; Cornely et al. 2012; Crook et al. 2012). High acquisition cost of fidaxomicin has inhibited uptake in some settings and was observed in our survey of European countries as outlined above. However, a recent real world study suggested a reduction in mortality associated with fidaxomicin use and that this was therapy was cost-effective (Goldenberg et al. 2016). In the phase 3 trials, bezlotoxumab was associated with a significant reduction in CDI readmissions.

The ideal antimicrobial agent for CDI should reduce vegetative *C. difficile* cells, toxins and spores in the host gut lumen without perturbation of the host microbiota, both to avoid creating an environment that is conducive to *C. difficile* expansion or to select for resistant potential pathogens (e.g. vancomycin resistant enterococci [VRE] or multi-resistant Gram-negative bacilli) (Chang et al. 2008). This is a very challenging profile for an antibiotic and indeed recent 'failures' of two antimicrobial agents in late-stage clinical trials emphasise how difficult it is to improve on current CDI therapies.

4.1 Surotomycin and Cadazolid

Surotomycin, an oral lipopeptide derivative of daptomycin, was examined in two phase 3 trials (NCT01598311 and NCT01597505) but did not demonstrate non-inferiority compared with vancomycin (Boix et al. 2017). Notably, surotomycin dosing caused an overgrowth of Gram-negative bacilli in both in mice and in a gut model of CDI that is highly predictive of human disease; recurrent CDI was also seen in the latter model (Deshpande et al. 2016; Chilton et al. 2014b). Most recently, a press release announced that cadazolid (Actelion), which is a novel hybrid oxazolidinone-fluroquinolone antibiotic that inhibits *C. difficile* protein synthesis and, to a lesser extent, DNA synthesis, did not meet its primary endpoint in comparison with vancomycin in one of two phase 3 trials (ActelionLtd. 2017; Gehin et al. 2015; Chilton et al. 2014a; Baldoni et al. 2014). It is too early to determine why this result was obtained, but may

relate to activity of cadazolid on the gut microbiome in vivo, and/or persistence of *C. difficile* spores (Chilton et al. 2014a).

4.2 Ridinilazole

Ridinilazole (SMT19969) is a novel, non-absorbable, very narrow-spectrum antimicrobial with minimal activity against host gut microbiota (Goldstein et al. 2013). While its mode of action has not been fully determined, it does not appear to act through classical antibiotic pathways, such as inhibition of cell wall, protein, lipid, RNA or DNA synthesis (Vickers et al. 2016). Bassiere et al. described the effects of ridinilazole on *C. difficile* cell morphology, as visualised by scanning electron microscopy and confocal microscopy (Basseres et al. 2016). Following exposure to sub-lethal concentrations of ridinilazole, bacterial cell division was halted and there was an absence of septum formation; this resulted in marked cell elongation. It has not been confirmed whether these observations are a direct effect of ridinilazole, or a downstream response to the antibiotic. Ridinilazole has good activity against some but not all clostridia; it is 7- to 17-fold more active in vitro than metronidazole and vancomycin and has similar potency to fidaxomicin against *C. difficile* (Baines et al. 2015; Weiss et al. 2014; Sattar et al. 2015; Corbett et al. 2015). Notably, *in vitro, in vivo* and gut model data confirm that ridinilazole has little antimicrobial activity against indigenous gut microflora groups, except selected clostridia (Freeman et al. 2015; Goldstein et al. 2013; Baines et al. 2015; Corbett et al. 2015; Chang et al. 2016).

Safety and tolerability of ridinilazole was established in healthy subjects and in a recently reported phase II randomised double-blind trial (CoDIFy) (Vickers et al. 2015; Vickers et al. 2017). CoDIFy was designed as a non-inferiority study and compared 10 days therapy of either oral ridinilazole 200 mg BD or oral vancomycin 125 mg QDS. Sustained clinical response rates were 67% and 42%, respectively (n = 69 mITT population); CDI recurrence occurred in 14% of ridinilazole recipients compared with 35% of vancomycin subjects; this difference meant that ridinilazole achieved a sustained response rate of 66.7% vs. 42.4% for vancomycin, which met pre-set statistical superiority criteria (Vickers et al. 2017). Microbiome analyses of faecal samples from subjects in this phase 2 study showed that vancomycin recipients had a marked loss of diversity and replacement of the predominant phyla of healthy stool (*Bacteroides* and *Firmicutes*) by *Enterobacteriaceae*. These disruptions were still present 2 weeks after the end of treatment, even in subjects who had not had a recurrence at that point. By contrast, ridinilazole, had a minimal effect on gut microbiota (Chang et al. 2016).

4.3 CDI Prophylaxis

4.3.1 Ribaxamase

Ribaxamase (SYN-004, synthetic biologics) is a recombinant beta-lactamase that has been formulated to be administered orally in patient receiving beta-lactam antibiotic therapy (Kaleko et al. 2016; Connelly et al. 2015). Ribaxamase degrades unmetabolised antibiotic in the colon to reduce the deleterious effects on the gut microbiota (Roberts et al. 2016). Animal studies have demonstrated safety, and notably no reduction in the systemic concentration of co-administered ceftriaxone (Connelly et al. 2015). A phase 2 double-blind placebo-controlled study has examined the potential of ribaxamase to prevent CDI, antibiotic-associated diarrhoea and the emergence of antimicrobial resistant potential pathogens in patients hospitalized with a lower respiratory tract infection treated with IV ceftriaxone (Synthetic Biologics 2017). Patients who received ribaxamase had a 71.4% relative risk reduction for CDI ($p = 0.045$). There was also a significant reduction in new colonisation by VRE in

ribaxamase versus placebo recipients ($p = 0.0002$). Adverse events were similar in active and placebo patients.

4.3.2 DAV132

Another novel approach to CDI prophylaxis is DAV132 (DaVolterra), which is an activated charcoal based product that is administered as an enteric coated capsule. DAV132 irreversibly captures antibiotics in the intestine whilst avoiding interruption of antibiotic absorption. DAV132 has been examined in a proof-of-concept study involving 18 healthy subjects who had received DAV132, uncoated formulated activated charcoal (FAC) or water 16 and 8 h before, alongside the probe drugs, and 8 h thereafter. The AUC0-96 h of amoxicillin was reduced by more than 70% when it was taken with FAC, but was not adversely affected when taken with water or DAV132. By contrast, the AUC0-96 h of sulfapyridine was reduced by >90% when administered with either FAC or DAV132 in comparison with water. Hence, DAV132 can selectively adsorb drugs in the proximal colon, without interfering with their absorption.

A further healthy volunteer trial examined the efficacy of DAV132 to protects the gut microbiome and prevent CDI during moxifloxacin (MOX) treatment (de Gunzburg et al. 2015). DAV132 decreased free faecal MOX concentration by >99% compared with MOX alone, but MOX plasma PK did not change significantly. Alterations of the faecal microbiome observed with MOX were prevented by co-administration of DAV132. In a human gut model DAV132 protected the microbiota and prevented *C. difficile* overgrowth and toxin production (de Gunzburg et al. 2015). Hamsters were also fully protected by DAV132 against MOX-induced CDI (de Gunzburg et al. 2015). Such results warrant further clinical development of DAV132 to protect the lower gut microbiota, and so prevent CDI associated with antibiotic administration.

4.4 Active *C. difficile* Immunisation

Vaccination to boost host antibody-mediated immunity is an attractive strategy to prevent CDI. The relative importance of *C. difficile* toxins A and B to human infection remains controversial, but host immune response to these toxins likely influences the likelihood of infection, clinical severity and outcome of CDI (Solomon et al. 2013; Kuehne et al. 2010). Higher serum IgG levels to toxin A have been shown in patients with asymptomatic colonisation compared with those with CDI, and recurrent infection is associated with poor IgG and IgM responses (Kyne et al. 2000, 2001). Interestingly, the effectiveness of the anti-toxin B monoclonal antibody bezlotoxumab at reducing the risk of CDI recurrence was not enhanced by the addition of an anti-toxin A monoclonal antibody, actoxumab; also, actoxumab alone was not efficacious at preventing recurrence. Nevertheless, it remains logical to design a vaccine around the augmentation of the host response to both toxins A and B (Kuehne et al. 2010). Other *C. difficile* antigens may also be important, noting for example that antibodies to surface proteins are greater in colonised versus infected patients (Pechine et al. 2005).

Three vaccines that use *C. difficile* toxin targets have progressed to phase 2 or 3 clinical development. The first to reach a phase 3 clinical trial is a formalin-inactivated toxoid-based vaccine developed by Sanofi Pasteur (Foglia et al. 2012). Following vaccination, seroconversion to toxin A was more pronounced than to toxin B (but took up to 70 days) and notably was less common in elderly subjects; three vaccine doses were required to achieve an adequate neutralising-antibody response (Foglia et al. 2012; Kotloff et al. 2001). A 100 μg dose (given with an AlOH adjuvant) was found to yield the best immunogenic response, and a phase 3 trial of this vaccine in the prevention of primary CDI in at-risk subjects aged >50 years commenced in 2013 (NCT01887912). Another formalin-inactivated toxoid based vaccine, but with alterations in both toxins A and B to reduce

toxigenicity, has recently commenced a phase 3 primary CDI prevention trial (Pfizer; NCT03090191), also based on a three dose strategy (Donald et al. 2013; Sheldon et al. 2016). A third *C. difficile* vaccine candidate (VLA84, Valneva) has completed a phase II trial with 500 subjects (Valneva 2016). VLA84 uses a different antigen approach to either of the two toxoid-based vaccines that are currently undergoing phase 3 evaluation. VLA84 is a single recombinant fusion protein consisting of portions of the C-terminal cell binding domains of toxins A and B. The developers claim that production and characterization of VLA84 could be simpler and less costly compared with toxoid-based vaccines. The phase 2 study of VLA84 met its primary endpoint in terms of identifying the dose and formulation with the highest seroconversion rate against both toxins A and B (subjects were followed up to day 210) and confirmed the favourable safety profile that was seen in Phase I. A phase 3 programme for VLA84 is being planned.

4.5 Microbiome Based Therapeutics

4.5.1 Faecal Microbiota Transplantation (FMT)

The evidence base concerning the effectiveness of FMT continues to grow, but it remains a non-regulated product, with many different versions reported. FMT comprises the administration of a complex live faeces-derived mixture of micro-organisms, including some of uncertain significance (some beneficial, others possibly harmful or neither) and so (particularly longer term) safety remains unproven. Of particular concern here is the increasing use of FMT when licensed CDI therapeutics has not been tried. Hence, different regulatory authorities have taken varied stances on FMT to safeguard patient interests. Requirements for consenting subjects, screening of donors and recipients, faecal material preparation and delivery via either rectal or nasogastro/duodenal routes, mean that there are intensive endeavours to develop alternatives to FMT that can still harness the restorative and protective effectiveness of specific components of the gut microbiota, but possibly with greater reassurance on safety. In the US, Openbiome is aiming to overcome some of the practical barriers to FMT, and safety concerns, by facilitating access to screened faecal transplant material and by collecting longer term follow up data. (http://www.openbiome.org/impact/).

The first randomised (sham procedure controlled) trial of FMT to treat recurrent CDI demonstrated an intention-to treat (ITT) efficacy rate of 81% to prevent further recurrences; notably, however, the study contained only 16 patients in the FMT arm (van Nood et al. 2013). In a randomised but non-blinded clinical trial, 39 subjects with recurrent CDI were given FMT (preceded by vancomycin 125 mg QDS for 3 days), comprising at least one infusion of faeces via colonoscopy, or vancomycin 125 mg QDS for 10 days and then 125–500 mg/day every 2–3 days for at least 3 weeks. The primary end point was the resolution of diarrhoea related to CDI at week 10; surprisingly, a positive *C. difficile* test was not required to define recurrence post-study treatment (Cammarota et al. 2015). The study was stopped after a 1-year interim analysis, at which point 18/20 (90%) vs. 5/19 (26%) patients in the FMT vs. vancomycin treatment groups, respectively had resolution of *C. difficile* diarrhoea ($P < 0.0001$). There were no significant adverse events in either of the study groups.

Adults with recurrent or refractory CDI were enrolled in a randomised, double-blind, non-inferiority study in six Canadian centres of free-thawed ($n = 114$) vs. fresh ($n = 118$) FMT via enema. Clinical resolution without recurrence up to 13 weeks did not differ significantly in the per-protocol (83.5% vs. 85.1%) and mITT (75.0% vs. 70.3%) populations (Lee et al. 2016). These results suggest that using freeze-thawed faecal material is a practicable alternative to fresh donor material. All patients received suppressive antibiotics for the most recent episode of CDI, and these were discontinued 24–48 h before FMT; this probably explains why only 38% of the subjects were positive for toxin or toxin gene immediately prior to FMT administration.

Notably, about one third of FMT recipients in both groups, who were ultimately, classified as resolved, required two FMTs, which is a relatively common observation. A non-blinded, non-randomised study of encapsulated (and freeze-thawed) faeces was performed in 20 subjects with at least three episodes of mild-to-moderate CDI and failure of a 6- to 8-weeks of vancomycin therapy, or ≥2 episodes of severe CDI requiring hospitalization (Youngster et al. 2014). Diarrhoea resolution occurred in 14 patients (70%; 95% CI, 47%–85%) after a single capsule-based FMT; 4/6 re-treated non-responders had resolution of diarrhoea, giving an overall 90% (95% CI, 68%–98%) response rate. No serious adverse events were attributed to FMT.

The six randomised controlled trials of FMT have been recently reviewed; three that compared FMT to antibiotic management; the remainder compared FMT to various 'types' of FMT in terms of preparation, source and delivery (Johnson and Gerding 2017). It is important to note that, unlike prior uncontrolled studies that reported FMT efficacy rates of at least 90%, efficacy (for one FMT) in these RCTs was 44–91%, with four recording success rates of ≤65%. These include a randomized controlled trial of FMT versus a 6-week vancomycin tapering regimen (VAN-TP) (Hota et al. 2017). VAN-TP was stopped early for futility; 56% of patients randomized to FMT by enema developed recurrent CDI, compared with 42% VAN-TP recipients.

There are many important factors for European clinicians to consider when establishing or using a FMT service. Factors that should be taken into account at an institutional level when commencing an FMT service are the national regulatory frameworks that FMT falls under (i.e. as a drug or biological material), donor selection and screening practices, stool preparation techniques and long term safety of microbiome manipulation in these patients. Concerns regarding the long term safety of FMT are not unfounded, especially in patients with inflammatory bowel disease. Reports of peripheral neuropathy, Sjögren syndrome, idiopathic thrombocytopenic purpura, microscopic colitis, contact dermatitis, rheumatoid arthritis, obesity, bacteraemia, and ulcerative colitis flare after FMT (Tariq et al. 2016; De Leon et al. 2013; Quera et al. 2014; Alang and Kelly 2015). Institutions need to ensure they are working within their national and European frameworks and regulations. Where national regulations are absent, comparisons should be made to international standards to ensure the highest level of safety. In Europe, the regulation of FMT is currently at the discretion of the EU member states, though in many countries no such national regulation exists. Future planned EU regulation of FMT donor material may hinder its widespread use, depending on whether it is regulated as a drug or bodily tissue. A recent European Consensus paper provided recommendations on a number of areas pertinent to FMT implementation, including regulatory, administrative and laboratory guidelines (Cammarota et al. 2017).

4.5.2 Live Bio Therapeutic Microbiota Preparations

RBX2660

RBX2660 is a live bio therapeutic microbiota suspension that aims to harness the effectiveness of FMT, but within a standardised, regulated product, for the treatment of recurrent CDI. It has been studied in three phase 2 clinical trials. PUNCH CD (NCT01925417) was a safety focussed, prospective multi-centre, open-label study; 34 subjects (with ≥2 recurrent CDI episodes or ≥2 severe episodes resulting in hospitalization) received at ≥1 dose of RBX2660 and 31 completed 6 months follow up (Orenstein et al. 2016). Following a 10–14 day course of anti-CDI antibiotics and a 24–48 h washout period, RBX2660 was administered as a single dose via enema. Further recurrent CDI occurred in 48% of subjects after one dose of RBX2660, with 15/31 patients receiving a second enema; of these, 78.6% were considered to be treatment successes, contributing to an overall success rate of 27/31 (87.1%). No serious adverse events were related to RBX2660.

PUNCH CD 2 (NCT02299570) was a phase 2b multi-centre randomized double-blind, placebo-controlled trial with 2 year follow-up (Dubberke et al. 2016). The primary efficacy objective was assessment of response (defined as no CDI recurrence) to RBX2660 versus placebo at 8 weeks. A total of 127 patients formed the ITT population (enrolled at 21 sites in the U.S. and Canada); patients were randomized into three treatment arms: two doses of RBX2660 (Group A, n = 41); two doses of placebo (Group B, n = 44); or one dose of RBX2660 and one dose of placebo (Group C, n = 42) via enema with doses 7 days apart. Efficacy for Group A was 61% vs. 45.5% for Group B, P = 0.152. Efficacy for Group C was 66.7% compared with Group B (45.5%), P = 0.048; efficacy of Group A and C (63.9%) vs. B (45.5%), P = 0.046. For subjects who developed recurrent CDI after receipt of study drug, open-label treatment success was Group A (68.8%, 11/16); Group B (87.5%, 21/24); Group C (71.4%; 10/14) for an overall open label success rate of 77.8%. Adverse events at 56 days were primarily gastrointestinal, with no significant difference in the proportion of adverse or serious adverse events among the treatment groups. As the two doses of RBX2660 treatment arm was not superior to two doses of placebo, the primary efficacy endpoint was not met.

The third phase 2 study, PUNCH Open Label (NCT02589847) had 31 active treatment sites and four control sites in the US and Canada. One hundred thirty-two RBX2660 and 110 historical control subjects were included; follow up results at 8 weeks have been reported, although there is a 2-year assessment point also (Rebiotix Inc 2017). RBX2660 met its primary efficacy endpoint at 8 weeks, preventing CDI recurrence, with a success rate of 78.8% compared with 51.8% in historical controls treated with antibiotics alone ($p < 0.0001$). No new safety concerns were identified. Analyses of faecal microbiomes shows that these became more diverse and aligned to a 'healthy' microbiome after treatment with RBX2660 (Blount et al. 2017; Ray et al. 2017). 16S rRNA sequencing was also performed on stool samples collected from 42 subjects treated with RBX2660 treatment arm and for 19 RBX2660 drug lots. The RBX2660 microbial profiles had similar taxonomic distributions, with a group mean that was highly divergent and significantly different from those of patients at baseline. However, after RBX2660 treatment, patients' microbiomes progressively resembled those of RBX2660.

SER-109

SER-109 (Seres) is also a live biotheraputic that comprises an encapsulated mixture of purified *Firmicutes* spores, obtained from the faeces of healthy humans, which were effective at preventing CDI in animal models. The resilience of the spores means that an ethanol based purification process can be applied to reduce the risk that transmissible infectious agents contaminate the therapeutic product. Also, resistance to gastric acid facilitates oral dosing. Two phase 2 studies of SER-109 have been completed. The first was a non-comparative study in patients with ≥3 CDI episodes during 12 months (Khanna et al. 2016). Following standard of care CDI antibiotic treatment, patients received SER-109 either on two consecutive days (geometric mean dose, 1.7×10^9 spores), or on 1 day (geometric mean dose, 1.1×10^8 spores). The primary end point was absence of *C. difficile*-positive diarrhoea during 8 weeks of follow-up. In total, 26/30 patients (86.7%) across the two dosing groups met the primary efficacy end point. Three patients with early, self-limiting *C. difficile*-positive diarrhoea did not require antibiotic treatment, and were *C. difficile*-negative on re-testing at 8 weeks; thus, 29/30 (96.7%) were considered to have achieved clinical resolution. Notably, gut microbiome analyses showed that baseline loss of microbiota diversity was rapidly reversed after receipt of SER-109, with persistence of *Firmicutes* spores. There were no safety concerns in the study.

A recently completed, phase 2 (ECOSPORE) study of SER-109 enrolled 89 subjects with ≥3 recurrences who were randomized (2:1 ratio) in a placebo-controlled, double-blind, 24-week trial (Trucksis et al. 2017). SER-109 was administered orally as a single dose (1×10^8

bacterial spores), after CDI antibiotic treatment. Recurrence was defined as diarrhoea for ≥2 consecutive days, a positive CDI test, and the need for antibiotic treatment. The study's primary endpoint of reducing the relative risk of CDI recurrence at 8 weeks was not achieved, despite a (non-significant) reduction in the relative risk of CDI recurrence. In the ITT population, recurrence occurred in 44% (26/59) vs. 53% (16/30) of subjects who received SER-109 vs. placebo, respectively. A pre-specified sub-group analysis showed that the lack of efficacy of SER-109 to prevent recurrence occurred in subjects aged <65 years old. However, in subjects aged ≥65 years old, CDI recurrence occurred in 45% of SER-109 (14 of 31) recipients, and in 80% of those who received placebo (12 of 15). A re-analysis showed that the disappointing results may be because cases were included and recurrences diagnosed without the most stringent requirement for free faecal toxin to be present. Also, while SER-109 was biologically active, a higher dose may be necessary. Further clinical trials are now in progress.

Non-toxigenic *C. difficile*

Non-toxigenic *C. difficile* (NTCD) strains are avirulent. Theoretically, it may be possible to displace toxigenic strains in colonised (or infected) individuals. A randomized,

Table 4 Anti-CDI agents in the pipeline agents that have completed at least a phase 2 clinical trial for treatment or prevention of CDI

Clinical trial phase	Drug/product (developer)	Indication Notes
Phase III	*C. difficile* vaccine (Sanofi Pasteur)	Primary prevention of CDI
		NCT01887912: efficacy of vaccine (3 doses) containing toxin A and B toxoids
	C. difficile vaccine (Pfizer)	Primary prevention of CDI
		Vaccine containing toxoids of toxin A and B. 3 doses
		NCT03090191: efficacy of vaccine (3 doses) containing toxin A and B toxoids
	SER-109 (Seres)	Treatment of recurrent CDI
		Oral microbiome therapeutic (mixture of bacterial spores) tested in a single-arm, open-label clinical trial
		NCT03183128: Is SER-109 superior vs placebo to reduce recurrence of CDI?
Phase II	Ridinilazole (SMT 19969, Summit)	Treatment of CDI
		Ridinilazole is a novel, small molecule, highly selective antibiotic. Successful phase 2 trial completed; phase 3 initiation expected 2018
	RBX2660 (Rebiotix)	Treatment of recurrent CDI
		Microbiota Suspension. 3 completed phase 2 trials
		Expected to enter Phase 3 in 2017/18
	SYN-004 (Synthetic Biologics)	Prevention of CDI. SYN-004 is a class A b-lactamase
		Successful phase 2 trial completed; phase 3 initiation expected 2017/18
	VLA84 (Valneva)	Primary prevention of CDI
		Vaccine consisting of a fusion protein with portions of toxins A and B
		Successful phase 2 trial completed in 2016
	Non-toxigenic *C. difficile* (Viropharma)	Prevention of recurrent CDI
		Biological therapy. Completed successful phase 2 trial in 2013
	Ramoplanin (Nanotherapeutics)	Treatment of CDI
		No new clinical efficacy data published since a phase 2 study was completed in 2004
		Development plans/potential is therefore unclear. No clinical studies listed in clinicaltrials.gov

double-blind, placebo-controlled, dose-ranging study examined the efficacy of a NTCD strain to prevent recurrent CDI in patients with either primary (>80%) or recurrent CDI who had completed treatment with metronidazole, vancomycin, or both (Gerding et al. 2015). Approximately two thirds (69%) of recipients became colonised by NTCD. CDI recurrence rates were 2% in colonized subjects, compared with 31% (similar to placebo) in those not colonised ($p < 0.001$), highlighting the correlation between engraftment and clinical efficacy. Interestingly, no subjects who were colonised at week six remained so at week 26. It remains unclear whether this successful proof of concept phase 2 clinical trial will lead to commercial development of the NTCD strain.

In summary, there are varied approaches in advanced clinical trials for the primary prevention, treatment and/or secondary prevention of CDI (Table 4). Unfortunately, however, recent experience shows us that developing new management options for CDI is very challenging. Well-designed trials with clearly defined patient populations are key to delivering new therapeutic and preventative options.

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Antibiotic Resistances of *Clostridium difficile*

Patrizia Spigaglia, Paola Mastrantonio, and Fabrizio Barbanti

Abstract

The rapid evolution of antibiotic resistance in *Clostridium difficile* and the consequent effects on prevention and treatment of *C. difficile* infections (CDIs) are matter of concern for public health. Antibiotic resistance plays an important role in driving *C. difficile* epidemiology. Emergence of new types is often associated with the emergence of new resistances and most of epidemic *C. difficile* clinical isolates is currently resistant to multiple antibiotics. In particular, it is to worth to note the recent identification of strains with reduced susceptibility to the first-line antibiotics for CDI treatment and/or for relapsing infections. Antibiotic resistance in *C. difficile* has a multifactorial nature. Acquisition of genetic elements and alterations of the antibiotic target sites, as well as other factors, such as variations in the metabolic pathways and biofilm production, contribute to the survival of this pathogen in the presence of antibiotics. Different transfer mechanisms facilitate the spread of mobile elements among *C. difficile* strains and between *C. difficile* and other species. Furthermore, recent data indicate that both genetic elements and alterations in the antibiotic targets can be maintained in *C. difficile* regardless of the burden imposed on fitness, and therefore resistances may persist in *C. difficile* population in absence of antibiotic selective pressure.

Keywords

C. difficile · Antibiotic susceptibility methods · Mechanisms of resistance · Multidrug resistance (MDR)

1 Introduction

Clostridium difficile is recognized as the major cause of healthcare antibiotic-associated diarrhea (Lessa et al. 2015; European Centre for Disease Prevention and Control (ECDC) 2013). Potentially, all antibiotic classes may promote *C. difficile* infection (CDI) by disrupting intestinal microflora and allowing *C. difficile*, ingested or resident, to proliferate, colonize the gastrointestinal tract, and infect the host. Therefore, resistance to multiple agents represents a selective advantage for *C. difficile* strains to enhance their survival and spread.

An alarming increase in incidence of CDI has been observed worldwide over the last 15 years,

P. Spigaglia (✉) · P. Mastrantonio · F. Barbanti
Department of Infectious Diseases, Istituto Superiore di Sanità, Rome, Italy
e-mail: patrizia.spigaglia@iss.it

with a significant financial burden on the healthcare system (Redelings et al. 2007; Burckhardt et al. 2008; Bauer et al. 2011; Gravel et al. 2009; Miller et al. 2011a; Dubberke and Olsen 2012; Lessa et al. 2012). The increased number of infections has been mainly associated with the emergence of highly virulent *C. difficile* strains. In particular, strains PCR-ribotype (RT) 027/North American pulsed field gel electrophoresis type I (NAPI)/restriction endonuclease analysis group B1, have been recognized responsible for severe CDI, characterized by high rate of recurrences, mortality and refractory to traditional therapy (Pépin et al. 2004; 2005b; McDonald et al. 2005; Muto et al. 2005; Goorhuis et al. 2007; Clements et al. 2010). Despite the wide diffusion of RT 027, recent European surveillances indicated the emergence of highly virulent RTs different from RT 027 (Davies et al. 2014; Freeman et al. 2015a). Several types, such as RT 014/020, RT 001/072 and RT078 are endemic in almost all European countries, whereas others RTs have a regional spread (Freeman et al. 2015a).

Antibiotic have a central role in driving the emergence of new *C. difficile* types. The global spread of *C. difficile* RT 027 has been associated with the massive use of fluoroquinolones (FQs) and the acquisition of resistance to these antibiotics by strains of this type (He et al. 2013). Actually, the majority of epidemic and emergent strains, RT 027 or not, show resistance to multiple antibiotics (Spigaglia et al. 2011).

Genetic analysis have demonstrated that *C. difficile* has a versatile genome content, with a wide range of mobile elements, many of them encoding for predicted antibiotic resistances (Sebaihia et al. 2006; He et al. 2010, 2013). Besides horizontal gene transfer, other mechanisms may contribute to promote antibiotic resistance in *C. difficile*, which appears to be a multifactorial phenomenon.

In this chapter, antibiotic resistances of *C. difficile* will be discussed taking in consideration the most recent published data.

2 *C. difficile* Antibiotic Susceptibility

C. difficile susceptibility is usually evaluated for antibiotics known to be significantly associated to CDI or used for CDI treatment. Among the first group, clindamycin (CLI) and cephalosporins (CFs) are historically recognized as high-risk agents for CDI (Bartlett et al. 1977; Bignardi 1998). Although a decreased number of infections has been observed in the hospitals that have curtailed the use of these antibiotics (de Lalla et al. 1989; Khan and Cheesbrough 2003; Wistrom et al. 2001), the risk of hospital acquired-CDI remains high after CLI or CFs therapy, so their importance as promoting agents should not be minimized. More recently, a rise in the FQs-associated CDI has been observed in concomitant with the increasing incidence of *C. difficile* RT 027. Current strains RT 027 show high-level resistance to FQs, never observed in historical isolates of the same type (McDonald et al. 2005). Infection control procedures and antimicrobial stewardship have led to a significant reduction in the incidence of infections caused by RT 027 but this type is still globally widespread (Muto et al. 2007; Lessa et al. 2015; Freeman et al. 2015a). Furthermore, resistance to FQs has become very common also in strains belonging to other epidemic types (Freeman et al. 2015a, b; Spigaglia et al. 2011).

Standard CDI therapies include metronidazole (MTZ) and vancomycin (VAN) as first choice for mild and severe CDI, respectively (Debast et al. 2014; Jarrad et al. 2015; Lyras and Cooper 2015). In addition, rifamycins (RFs), in particular rifaximin (RFX), have recently been prosed as "chaser therapy" for treatment of relapsing CDI (Iv et al. 2014), while fidaxomicin (FDX), a bactericidal new narrow spectrum macrocyclic antibiotic, is used for the management of CDI with high risk for recurrences (Chaparro-Rojas and Mullane 2013).

2.1 Antibiotics Associated to CDI

Although rates of antibiotic resistance varies considerably depending on the geographic regions and local/national antibiotic policy, data extrapolated from studies recently published indicate that the majority of *C. difficile* clinical isolates are resistant to CFs, FQs, ERY and CLI (Table 1). In recent studies, performed on a large number of *C. difficile* strains, is reported that resistance to CFs of second generation is more commonly observed compared to resistance to CFs of third generation (95% vs. 38%) (Dong et al. 2013; Pirs et al. 2013; Norman et al. 2014; Oka et al. 2012; Karlowsky et al. 2012; Buchler et al. 2014; Kuwata et al. 2015; Knight et al. 2015; Knight and Riley 2016). Similarly, resistance to ciprofloxacin (CIP), a FQ of second generation, is very common in *C. difficile* (99%) (Rodriguez-Pardo et al. 2013; Lee et al. 2014; Norman et al. 2014; Lachowicz et al. 2015; Kuwata et al. 2015; Shayganmehr et al. 2015), while resistance to FQs of fourth generation such as moxifloxacin (MXF) and gatifloxacin (GAT) has been detected in 36% and 68% of the strains analyzed, respectively (Karlowsky et al. 2012; Tenover et al. 2012; Eckert et al. 2013; Rodriguez-Pardo et al. 2013; Lee et al. 2014; Kim et al. 2012; Liao et al. 2012; Terhes et al. 2014; Weber et al. 2013; Pirs et al. 2013; Varshney et al. 2014; Freeman et al. 2015a, b; Senoh et al. 2015; Adler et al. 2015; Kociolek et al. 2016; Putsahit et al. 2017; Gao et al. 2016; Santos et al. 2016; Knight et al. 2015; Kullin et al. 2017).

2.2 Antibiotics for CDI Treatment

2.2.1 Metronidazole

Although percentage of *C. difficile* strains resistant to MTZ is low (Table 1), several studies have reported high rate of treatment failures in patients that received this antibiotic (Musher et al. 2005; Pépin et al. 2005a; Vardakas et al. 2012). Furthermore, it has recently been observed an increase in the geometric mean of MICs for isolates RT 027 (1.1–1.42 mg/L), RT 001/072 (0.65 mg/L), RT 106 (0.65 mg/L), RT 356 (0.61 mg/L) and in the non-toxigenic RT 010 (1.5 mg/L), compared to other RTs (0.13–0.41 mg/L) (Moura et al. 2013; Freeman et al. 2015a, b). In addition, several recent papers have reported the isolation of strains with MICs >2 mg/L, the EUCAST epidemiological cut-off (ECOFF) for MTZ (http://www.eucast.org/clinical_breakpoints) (Table 2). Although the clinical relevance of strains with reduced susceptibility to MTZ is still unclear, it has been suggested a potential impact of strains RT 027 with reduced susceptibility to MTZ on the pathophysiology of recurrent CDIs (Richardson et al. 2015). In addition, strains RT 027 with this characteristic have recently been identified as cause of severe infections in Israel (Adler et al. 2015; Miller-Roll et al. 2016). In particular, a wide outbreak caused by a strain RT 027 with high MIC values for MTZ has been reported in Jerusalem in 2013 (Adler et al. 2015). Besides RT 027, reduced susceptibility to MTZ has also been observed in other important epidemic types, such as RT 078 and RT 126 (Table 2).

C. difficile colonies with increased MICs to MTZ can be isolated in presence of sub-inhibitory concentrations of antibiotic (Peláez et al. 2008; Moura et al. 2013). Heteroresistance, that is the capacity of a part of bacterial population to acquire resistance and grow in presence of an antibiotic, could be considered a pre-resistance stage in *C. difficile* (Falagas et al. 2008; Peláez et al. 2008). Mean concentrations of MTZ in the feces of treated patients are not so high (from 0.8 to 24.2 μg/g) (Bolton and Culshaw 1986), therefore it is possible that the concentrations achieved in the colon may be insufficient for the treatment of infections due to strains with higher MIC values for MTZ (Brazier et al. 2001; Baines et al. 2008; Moura et al. 2013).

2.2.2 Vancomycin

Reduced susceptibility to VAN in *C. difficile* is not largely diffused as in Enterococci and

Table 1 Antibiotic susceptibility of *C. difficile* clinical isolates as reported in 46 papers published between 2012 and 2017

Antibiotic[a]	Number of strains analyzed		Number of resistant strains	% of resistance
CFs				
	CTT	212	24	11.2
	FOX	423	404	95.5
	CRO	1252	393	31.4
	CTX	95	95	100
	CAZ	86	65	76.0
MLS_B				
	ERY	2316	1138	49.1
	CLI	5839	2982	51.1
FQ_S				
	CIP	1326	1312	99.0
	MXF	6053	2161	35.7
	GAT	199	136	68.3
MTZ		6724	114	1.7
VAN		5760	134	2.3
RIF		3450	525	15.2

[a]*CFs* cephalosporins, *CTT* cefotetan, *FOX* cefoxitin, *CRO* ceftriaxone, *CTX* cefotaxime, *CAZ* ceftazidime, MLS_B macrolide-lincosamide-streptogramin B, *ERY* erythromycin, *CLI* clindamycin, *FQs* fluoroquinolones, *CIP* ciprofloxacin, *MXF* moxifloxacin, *GAT* gatifloxacin, *MTZ* metronidazole, *VAN* vancomycin, *RIF* rifampin

References: Karlowsky et al. (2012), Liao et al. (2012), Reil et al. (2012), Kim et al. (2012), Oka et al. (2012), Tenover et al. (2012), Dong et al. (2013), Gouderzi et al. (2013), Pirs et al. (2013), Eckert et al. (2013), Obuch-Woszczatynski et al. (2013, 2014), Rodriguez-Pardo et al. (2013), Weber et al. (2013), Lee et al. (2014), Simango and Uladi (2014), Buchler et al. (2014), Norman et al. (2014), Novak et al. (2014), Terhes et al. (2014), Varshney et al. (2014), Zhou et al. (2014), Kuwata et al. (2015), Shayganmehr et al. (2015), Mackin et al. (2015), Freeman et al. (2015a), Knight et al. (2015), Knight and Riley (2016), Adler et al. (2015), Eitel et al. (2015), Krutova et al. (2015), Lachowicz et al. (2015), Senoh et al. (2015), Seugendo et al. (2015), Kociolek et al. (2016), Gao et al. (2016), Kouzegaran et al. (2016), López-Ureña et al. (2016), Santos et al. (2016), Jamal and Rotimi (2016), Kullin et al. (2016, 2017), Putsahit et al. (2017), Alvarez-Perez et al. (2017), Nyc et al. (2017), and Ramírez-Vargas et al. (2017)

Staphylococci, although an increased number of strains with higher MICs to this antibiotic (MICs range >2-16 mg/L) have recently been isolated (Tables 1 and 2). The clinical significance of strains with reduced susceptibility to VAN remains to be determined due to the high concentrations that this antibiotic reaches in the gastrointestinal tract (Young et al. 1985). Anyway, it is noteworthy that reduced susceptibilities to VAN and to MTZ are reported in several RTs, including RT 027, RT 001, RT 017, RT 078 and RT 356/607 (Chia et al. 2013; Goudarzi et al. 2013; Adler et al. 2015; Freeman et al. 2015a, b; Miller-Roll et al. 2016). Strains with these characteristics could represent a potentially serious problem for first-line treatment of CDI in the future.

2.2.3 Rifamycins

Recent data indicate that 15% of *C. difficile* clinical isolates are resistant to rifampin (RIF) (Table 1) and the rate of overall resistance appears to be rising (Huang et al. 2013; Rodríguez-Pardo et al. 2013; Eitel et al. 2015; Terhes et al. 2014). *C. difficile* strains resistant to rifamycines (RFs) have been detected in almost all the countries (17/22) participating in a recent *C. difficile* pan-European surveillance and, in particular, higher percentages of resistance, ranging from 57% to 64%, have been reported in Italy, Czech Republic, Denmark and Hungary (Freeman et al. 2015a, b). Selective pressure after exposure to antibiotic seems to have a role in selecting *C. difficile* colonies resistant to RFs

Table 2 *C. difficile* susceptibility to metronidazole and vancomycin as reported in 14 papers published between 2012 and 2017

Antibiotic[a]	Year of publication	Number of strains analyzed	% of resistance	MIC values (n. of strains)	Susceptibility method[b]	Prevalent PCR-ribotype	References
MTZ	2013	110	3.6	>2(4)	AD	nd[c]	Chia et al. (2013)
	2013	75	5.3	32(3); ≥64(1)	AD	nd	Gouderzi et al. (2013)
	2014	271	13.3	≥32 (36)	ET	nd	Norman et al. (2014)
	2015	916	0.1	8(1)	AI	106	Freeman et al. (2015a)
	2015	208	18.3	>2 (38)	ET	027	Adler et al. (2015)
	2015	86	4.7	32(4)	AD	nd	Shayganmehr et al. (2015)
	2016	35	28.6	>5 (10)	DD	nd	Kouzegaran et al. (2016)
	2016	457	3.5	>2(16)	ET	027, 126,203,651	Santos et al. (2016)
	2016	146	2.6	≥8	ET	nd	Jamal and Rotimi (2016)
	2016	166	4.2	>2(7)	ET	027	Miller-Roll et al. (2016)
	2017	50	4.0	≥256 (2)	ET	078, 126	Alvarez-Perez (2017)
VAN	2012	403	0.5	4(2)	AD	nd	Liao et al. (2012)
	2013	110	9.1	>2(10)	AD	nd	Chia et al. (2013)
	2013	75	8.0	2 (4); 4 (2)	AD	nd	Gouderzi et al. (2013)
	2014	86	1.2	8(1)	ET	nd	Buchler et al. (2014)
	2015	918	0.9	>8(8)	AI	001/072, 018, 027, 126, 356	Freeman et al. (2015a)
	2015	208	47.1	>2 (98)	ET	017, 027	Adler et al. (2015)
	2016	196	3.1	4(6)	AD	nd	Kociolek et al. (2016)
	2016	35	20.0	>5(7)	DD	nd	Kouzegaran et al. (2016)
	2016	457	0.4	3(2)	ET	001	Santos et al. (2016)
	2016	166	15	>2 (25)	ET	027	Miller-Roll et al. (2016)

[a]*MTZ* metronidazole, *VAN* vancomycin
[b]*AD* agar dilution, *ET* epsilometer test, *AI* agar incorporation, *DD* disk diffusion
[c]*nd* not determined

(Curry et al. 2009; Miller et al. 2011b). Therefore, resistant *C. difficile* strains might emerge even during therapy (Johnson et al. 2009; Carman et al. 2012). RFs are commonly used as anti-tuberculosis (TB) agents. Interestingly, in Poland, all strains belonging to the emergent RT 046 isolated from patients affected by TB and treated with prolonged RIF therapy, showed high MICs to these antibiotics (Obuch-Woszczatyński et al. 2013). Susceptibility to RIF correlated completely with susceptibility to RFX (Miller et al. 2011b). Thus, susceptibility of the rifamycin class in *C. difficile* can be assessed by testing susceptibility to RIF.

3 Multi-drug Resistance (MDR) in *C. difficile*

Many of the most common epidemic RTs, including the high virulent RT 027 and RT 078, are associated to MDR (Table 3). The first European prospective survey of *C. difficile* infections in 2005 showed that 55% of resistant clinical isolates were MDR (Spigaglia et al. 2011). Data from papers published in the last 6 years, indicate that about 60% of the analyzed strains are MDR and the MDR patterns mainly include resistance to CLI, FQs, ERY and CFs (Table 3). Resistance to other antibiotic classes, such as tetracycline (TET), chloramphenicol (CHL), imipenem (IMP) is less commonly detected in MDR *C. difficile* isolates. In general, percentage of TET-resistant strains ranged between 2.4% and 41.7% (Dong et al. 2013; Pirš et al. 2013; Lachowicz et al. 2015; Norman et al. 2014; Simango and Uladi 2014; Zhou et al. 2014), while resistance to CHL and IMP is found in about 3% and 7.1% of the European clinical isolates (Freeman et al. 2015a, b).

Interestingly, resistance to multiple antibiotics characterized recently emerged epidemic RTs. In particular, strains RT 176, a type closely related to RT 027, recently circulating in Poland and the Czech Republic, are characterized by resistance to ERY, MXF, CIP and RIF (Obuch-Woszczatyński et al. 2014; Krutova et al. 2015). Resistant to CLI, ERY, MXF and RIF characterized most of the strains belonging to RT 356/607 and RT 018, two genetically correlated types recently emerged in Italy (Spigaglia et al. 2010, 2015). Interestingly, RT 018 strains isolated in Korea and Japan show resistance only to CLI, ERY and MXF (Kim et al. 2012; Senoh et al. 2015). The 20-years of use of RIFs in Italy (Salix Pharmaceuticals, Ltd. 10 December 2003, posting date), could explain the spread of this resistance in Italian *C. difficile* isolates. Strains RT 018 are highly virulent and transmissible, with a transmission index that has been demonstrated tenfold higher compared to that of strains RT 078 (Baldan et al. 2015). Old age ($\geq$65 years), severe pulmonary comorbidity, previous use of FQs, and infection by RT 018 have been associated as significant risk factors for complicated infections (Bauer et al. 2011).

4 *C. difficile* Antibiotic Susceptibility Methods

Susceptibility testing is usually performed by clinical microbiology laboratories to determine antimicrobial resistance profiles of *C. difficile* isolates recovered from patients, but it is also used to monitor resistant patterns of strains isolated during epidemiological studies and surveillance networks.

The most common antibiotic susceptibility methods used for *C. difficile* are the agar dilution (AD) and the epsilometer test, a commercially available gradient diffusion system for quantitative antibiotic susceptibility testing (Fig. 1).

The AD is indicated as the reference method for *C. difficile* by the Clinical and Laboratory Standards Institute (CLSI) (Clinical and Laboratory Standards Institute 2012). The AD assay shows some advantages for epidemiological studies because it is an accurate method, the choice of antibiotics to be tested is flexible and can be modified according to investigational necessity and, finally, it is suitable for large number of isolates. The disadvantages of the AD approach are the laborious, time-consuming steps required to prepare testing plates, particularly when the

Table 3 Antibiotic susceptibility patterns most frequently observed in MDR *C. difficile* clinical isolates as reported in 19 papers published between 2012 and 2017

Year of publication	Number of strains analyzed	% of MDR strains	Main antibiotic susceptibility patterns (n. of strains)[a]										PCR-ribotype	References
2012	80	28	CLI	MXF	RIF							(22)	027	Tenover et al. (2012)
2013	145	60	ERY	CLI	CTX							(30)	nd[b]	Obuch-Woszczatyński et al. (2013), Goudarzi et al. (2013) and Dong et al. (2013)
			CLI	CIP	FOX							(14)	DTM[c]	
			CLI	MXF	CIP	TET	FOX					(13)	DTM	
			CLI	CIP	TET	FOX						(9)	DTM	
			ERY	CLI	MXF	RIF						(7)	046	
			CLI	MXF	CIP	FOX						(4)	DTM	
2014	183	77	ERY	CLI	CIP							(94)	001, 017, 018	Lee et al. (2014), Obuch-Woszczatyński et al. (2014), Novak et al. (2014) and Simango and Uladi (2014)
			ERY	CLI	CIP	CTX						(23)	nd	
			ERY	CLI	MXF	CIP	GAT					(17)	027, 176	
			ERY	CLI	MXF	CIP	GAT	LVX				(7)	001	
2015	525	66	ERY	CLI	MXF	GAT						(85)	018, 369	Lachowicz et al. (2015), Senoh et al. (2015), Kuwata et al. (2015), Spigaglia et al. (2015), Krutova et al. (2015) and Shayganmehr et al. (2015)
			CLI	CIP	CRO							(51)	DTM	
			ERY	CLI	MXF	RIF						(48)	018, 027, 356/607	
			ERY	MXF	CIP	IMP						(34)	027	
			CIP	CAZ	IMP	AMK						(25)	nd	
			ERY	CLI	MXF	CIP	IMP					(20)	176	
			ERY	MXF	RIF							(15)	027	
			CIP	CAZ	IMP							(14)	nd	
			ERY	MXF	CIP	RIF						(13)	176	
			ERY	CLI	GAT							(11)	018, 369	
			ERY	CLI	MXF							(11)	046, 078, 126	
			CIP	CAZ	AMK							(10)	nd	

(continued)

Table 3 (continued)

Year of publication	Number of strains analyzed	% of MDR strains	Main antibiotic susceptibility patterns (n. of strains)[a]										PCR-ribotype	References
2016	159	35	CLI	MXF	RIF	CIP						(32)	012, 017	Knight and Riley (2016) and López-Ureña et al. (2016)
			ERY	CLI	MXF							(23)	078, 126	
2017	276	62	ERY	MXF	RIF							(81)	017	Alvarez-Perez et al. (2017), Kullin et al. (2017) and Ramírez-Vargas et al. (2017)
			CLI	MXF	CIP	LVX	RIF	TET	CHL	TGC	LZD	(12)	012	
			CLI	MXF	CIP	LVX	RIF					(12)	012	
			ERY	MXF	LVX	TET						(5)	078, 126	
			ERY	LVX	TET							(5)	078, 126	
			CLI	MXF	CIP	LVX	TET	CHL	LZD			(4)	012	
			MXF	LVX	TET							(4)	078, 126	
			TET	LVX	ETP							(4)	126	

[a]*ERY* erythromycin, *CLI* clindamycin, *MXF* moxifloxacin, *CIP* ciprofloxacin, *LVX* levofloxacin, *GAT* gatifloxacin, *RIF* rifampin, *MTZ* metronidazole, *VAN* vancomycin, *TET* tetracycline, *CHL* chloramphenicol, *CTX* cefotaxime, *FOX* cefoxitin, *IMP* imipenem, *ETP* ertapenem, *CAZ* ceftazidime, *AMK* amikacin, *CRO* ceftriaxone, *LZD* linezolid, *TGC* tigecycline

[b]*nd* not determined

[c]*DTM* different typing method

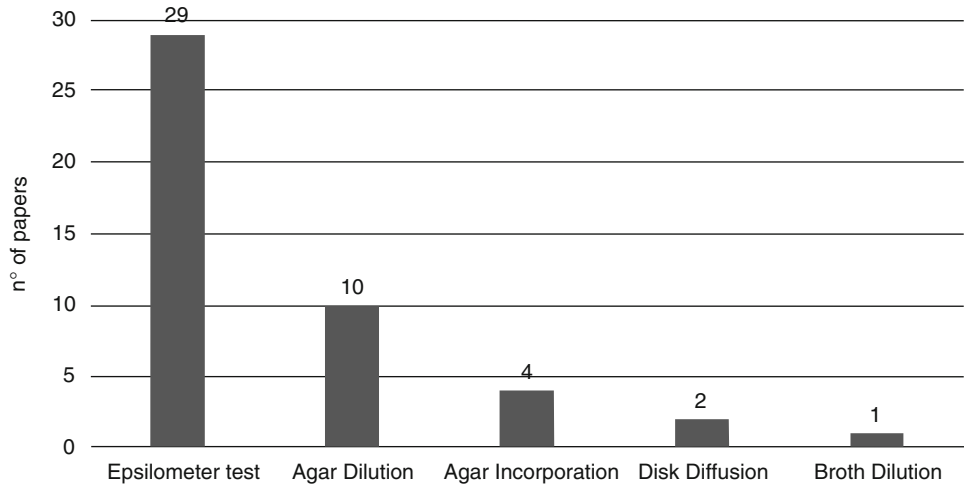

Fig. 1 Antibiotic susceptibility methods most frequently used for *C. difficile* analysis as reported in 46 papers published between 2012 and 2017
Papers: Karlowsky et al. (2012), Liao et al. (2012), Reil et al. (2012), Kim et al. (2012), Oka et al. (2012), Tenover et al. (2012), Dong et al. (2013), Gouderzi et al. (2013), Pirs et al. (2013), Eckert et al. (2013), Obuch-Woszczatynski et al. (2013, 2014), Rodriguez-Pardo et al. (2013), Weber et al. (2013), Lee et al. (2014), Simango and Uladi (2014), Buchler et al. (2014), Norman et al. (2014), Novak et al. (2014), Terhes et al. (2014), Varshney et al. (2014), Zhou et al. (2014), Kuwata et al. (2015), Shayganmehr et al. (2015), Mackin et al. (2015), Freeman et al. (2015a), Knight et al. (2015), Knight and Riley (2016), Adler et al. (2015), Eitel et al. (2015), Krutova et al. (2015), Lachowicz et al. (2015), Senoh et al. (2015), Seugendo et al. (2015), Kociolek et al. (2016), Gao et al. (2016), Kouzegaran et al. (2016), López-Ureña et al. (2016), Santos et al. (2016), Jamal and Rotimi (2016), Kullin et al. (2016, 2017), Putsahit et al. (2017), Alvarez-Perez et al. (2017), Nyc et al. (2017), and Ramírez-Vargas et al. (2017)

number of compounds to be tested is high and/or when only a limited number of strains are to be analyzed, and the need of skilled and experienced technologists to properly perform it. For these reasons, most laboratories use the epsilometer test, more flexible and simple, for routine. Although there were differences in MIC values between AD and epsilometer test, high categorical agreement between these methods has been demonstrated (Moura et al. 2013; Baines et al. 2008; Poilane et al. 2000). In addition, the epsilometer test allows analysis of susceptibility to multiple antibiotics for numerous strains at the same time. Despite these advantages, the high cost hinders the extensive use of this method in clinical laboratories and epidemiological studies.

Detection of strains with reduced susceptibility to MTZ poses problems in choosing the more proper antibiotic susceptibility method to test them. In fact, resistance to MTZ is often unstable and laboratory manipulation of strains frequently results in MIC decrease towards susceptibility range (Peláez et al. 2008; Lynch et al. 2013). Recent studies suggest the agar incorporation (AI) as the method of choice to detect strains with reduced susceptibility to MTZ compared to the AD (Freeman et al. 2005; Moura et al. 2013). Differences in the media used (Schaedlers broth and Wilkins-Chalgren agar for AI and Brucella broth/agar for AD) and in the pre-cultured period (24 h for AD and 48 h for AI) seem to affect MIC determination (Baines et al. 2008; Moura et al. 2013). The CLSI and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints for MTZ are not equivalent: the first is defined $\geq$32 mg/L, the second >2 mg/L (Clinical and Laboratory Standards Institute 2015; http://www.eucast.org/clinical_breakpoints/). Since methodological differences and different interpretation categories may cause discrepancies in results, influencing therapeutic decision and comparison of data, international committees are currently

co-operating with the intention of harmonizing susceptibility testing and breakpoints for this antibiotic.

Disk diffusion testing is not recommended by CLSI for *C. difficile* but some recent papers suggest that it could be an option for antimicrobial susceptibility testing of this pathogen. A study carried out in Denmark on 211 isolates, showed that an excellent agreement was found between MIC results when the epsilometer test and disk diffusion were used to test *C. difficile* strains susceptibility to VAN, MXF, and MTZ (Erikstrup et al. 2012). Furthermore, two studies, performed in Denmark and Brazil, respectively, successfully used disk diffusion to test *C. difficile* isolates with reduced susceptibility to MTZ and VAN (Holt et al. 2015; Fraga et al. 2016). Despite these results, an exact zone diameter for breakpoints is still not determined either by CLSI or by EUCAST, therefore the debate about disk diffusion, as qualified antibiotic susceptibly testing method for *C. difficile*, is still open.

Although in some paper *C. difficile* MIC values have been obtained using broth microdilution (Genzel et al. 2014; Lim et al. 2016), CLSI recommends this method only to test Bacteroides species (Clinical Laboratory Standards Institute 2012). Furthermore, a recent study of Hastey et al. has demonstrated a negative bias for the broth microdilution when compared to the AD for *C. difficile* (Hastey et al. 2017). In this study, the MIC values obtained using the broth microdilution were lower than those obtained with AD. Furthermore, the reproducibility with broth microdilution was variable, probably dependent on the antibiotics tested. Therefore, in accordance with the CLSI guideline (Clinical and Laboratory Standards Institute 2012), the results indicate that the broth microdilution method is not equivalent with AD for *C. difficile* antimicrobial susceptibility testing.

The phenotypic tests are traditional methods to evaluate antibiotic susceptibility of *C. difficile* but they need time (almost 1 week to get the results) and the isolation of *C. difficile* from patient stools. Since resistance to several antibiotics has been correlated with resistance genes and alteration in antibiotic targets, molecular analysis may be considered to investigate *C. difficile* resistance beside phenotypic tests. The decreased cost of these technologies will allow their introduction on a large scale as tool for infection control in the future, as suggested by very recent studies that demonstrate the importance of molecular analysis and comparative genomics in the epidemiological surveillance of *C. difficile* (Ramírez-Vargas et al. 2017; Cairns et al. 2017).

5 *C. difficile* Mechanisms of Resistance

Several mechanisms responsible for antibiotic resistance have been identified in *C. difficile*, including chromosomal resistance genes, mobile genetic elements (MGEs), alterations in the antibiotic targets and/or in metabolic pathways, and biofilm formation (Table 4). Furthermore, recent evidences support that *C. difficile* resistance to some antibiotics may be complex and multifactorial.

5.1 Antibiotics Associated to CDI

5.1.1 Cephalosporins

C. difficile is usually resistant to CFs and several studies report *C. difficile* overgrowth after CFs therapy (Ambrose et al. 1985; de Lalla et al. 1989; Impallomeni et al. 1995). Although *C. difficile* is described as "constitutively resistant" to CFs, the mechanism of resistance to these antibiotics is still not completely characterized. The variable MICs values observed for the different CFs suggest that resistance may be strain-dependent. Antibiotic-degrading enzymes, β-lactamases, and modification of target sites, penicillin-binding proteins (PBPs), are the mainly mechanisms involved in resistance to these antibiotics. A number of coding DNA sequences (CDSs) potentially involved

Table 4 *C. difficile* antibiotic mechanisms of resistance

Antibiotics[a]	Mechanism of resistance	Genetic element	Target/protein/gene	References
CFs	Antibiotic enzymatic destruction; Altered target		Putative β-lactamases and PBPs (25 CDSs potentially involved identified in *C. difficile* 630)	Spigaglia (2016)
MLS_B	23 S RNA methylases; RNA methyl transferase	Tn*5398* and Tn*5398* -like	*erm* B	Farrow et al. (2001), Brouwer et al. (2011), Spigaglia et al. (2005, 2011)
		Tn*6194*	*erm* B	Wasels et al. (2013), He et al. (2010, 2013)
		Tn*6215*	*erm* B	Goh et al. (2013), Wasels et al. (2015b)
		Tn*6218*	*erm* AB/*cfr*	Dingle et al. (2014)
			cfr B/*cfr C*	Hansen and Vester (2015), Marin et al. (2015), Candela et al. (2017)
FQs	Altered target		*gyr* A/*gyr* B	Ackermann et al. (2001), Carman et al. (2009), Dridi et al. (2002), Walkty et al. (2010) Huang et al. (2009), Mac Aogáin et al. (2015), Spigaglia et al. (2008b, 2011), Kuwata et al. (2015), Liao et al. (2012)
MTZ	Metabolic pathways alterations; biofilm formation			Chong et al. (2014), Moura et al. (2014), Vuotto et al. (2016)
VAN	Altered target; biofilm formation		*mur* G	Leeds et al. (2014), Dapa et al. (2013)
RFs	Altered target		*rpo* B	Cairns et al. (2017), Carman et al. (2009), Curry et al. (2009), Pecavar et al. (2012), O'Connor et al. (2008), Spigaglia et al. (2011), Huang et al. (2009), Liao et al. (2012), Miller et al. (2011b), Walkty et al. (2010)
TET	Ribosomal protection	Tn*6397*	*tet* M	Roberts et al. (2001, 2011)
		Tn*916*-like	*tet* M	Sebaihia et al. (2006), Brouwer et al. (2011, 2012), Spigaglia et al. (2005, 2007)
		Tn*6164*	*tet* 44	Corver et al. (2012)
CHL	Chloramphenicol acetyltransferase	Tn*4453*a and Tn*4453*b	*cat* D	Wren et al. (1988, 1989)

[a]*CFs* cephalosporins, MLS_B Macrolide-lincosamide-streptograminB, *FQs* fluoroquinolones, *MTZ* metronidazole, *VAN* vancomycin, *RFs* rifamycins, *TET* tetracycline, *CHL* chloramphenicol

has been identified in *C. difficile* 630 genome and in other *C. difficile* strains (identity between 73% and 100%) (Spigaglia 2016). Anyway, further genomic and functional analyses will be necessary to elucidate the role of these potential beta-lactam interacting genes.

5.1.2 Macrolide-Lincosamide-StreptograminB (MLS_B)

In *C. difficile*, resistance to the macrolide-lincosamide-streptogramin B (MLS_B) family is usually conferred by ribosomal methylation. Erythromycin ribosomal methylases (*erm*) genes of class B are the most widespread in *C. difficile* population, even if other *erm* genes have rarely been detected (Roberts et al. 1994; Spigaglia et al. 2005; Schmidt et al. 2007). In *C. difficile*, *erm*B is usually located on mobilisable genetic elements and Tn*5398,* a mobilisable non conjugative element of 9.6 kb in length, is the best known among these elements (Farrow et al. 2001). Tn*5398* contains two copies of *erm*B gene and it is able to transfer in vitro from *C. difficile* to *Staphylococcus aureus* and to *Bacillus subtilis* (Hächler et al. 1987; Mullany et al. 1995). Integration/excision functions to transfer Tn*5398* from the donor to the recipient strain are provided by other conjugative transposons present in the donor genome, because Tn*5398* does not have genes encoding a recombinase (Mullany et al. 2015). Integration into the recipient chromosome occurred either by homologous recombination or by using a site-specific recombinase of the recipient. It is also possible, as recently suggested, that a portion of the donor genome containing Tn*5398* integrates by homologous recombination into the recipient (Wasels et al. 2015b).

The majority of *C. difficile* strains resistant to MLS_B show *erm*B-containing elements with a different genetic organizations compared to Tn*5398* (Farrow et al. 2001; Spigaglia et al. 2005, 2011). Seventeen organizations (E1-E17) have been identified by a PCR-mapping method and the E4 was identified as the most frequent among European *C. difficile* clinical isolates (Spigaglia et al. 2011). Elements E4 are related to the conjugative transposon Tn*6194* identified in *C. difficile* 2007855 (He et al. 2010, 2013; Wasels et al. 2013). Tn*6194* has a conjugative region related to that of Tn*916*, a large family of conjugative elements widely spread in both Gram-positive and Gram-negative bacteria, and an accessory region related to Tn*5398,* and it is able to *in vitro* transfer from *C. difficile* to *Enterococcus faecalis* (Wasels et al. 2014).

Tn*6215* is a peculiar mobilisable transposon of about 13 kb in length found in *C. difficile* CD80 (Goh et al. 2013). Noteworthy, conjugation-like mechanism or phage ΦC2 transduction can be involved in the transfer of this element between *C. difficile* strain to another. Furthermore, it has recently been suggested that a transformation-like mechanism can be responsible for the transfer of Tn*6215* and Tn*5398* when *C. difficile* CD13 is used as recipient strain (Wasels et al. 2015b).

Although *erm*B-containing elements have a cost on the *C. difficile* fitness *in vitro* (Wasels et al. 2013), these elements are common in *C. difficile* population suggesting that, regardless of the burden on fitness, other factors (i.e. the capability of transfer and the intrinsic genetic characteristics of strains) are involved in their successful spread.

Resistance to both ERY and CLI or only to ERY have been observed also in *C. difficile* strains negative for *erm* genes (Spigaglia and Mastrantonio 2004; Pituch et al. 2006; Ratnayake et al. 2011; Spigaglia et al. 2011). Although alterations in the 23S rDNA or ribosomal proteins (L4 or L22) have been found in some of these strains, the same changes were also observed in susceptible isolates and, therefore, their role in resistance has been excluded (Spigaglia et al. 2011). Furthermore, treatment of resistant *erm*-negative strains with two pump inhibitors (reserpine and carbonyl cyanide m-chlorophenyl hydrazone – CCCP), did not determine any reduction in MICs, suggesting that resistance is not mediated by efflux-mechanisms (Spigaglia et al. 2011). Recently, other determinants that could have a role in *C. difficile* resistance to MLS_B in the absence of *erm* genes have been identified. In particular,

*cfr*B or *cfr*C, which encode a 23S rRNA methyltransferase and confer resistance to PhLOPSA (phenicols, lincosamides, oxazolidinones, pleuromutilins, and streptogramin A), have been found in several *C. difficile* strains resistant to linezolid and other clinically relevant antibiotics (Hansen and Vester 2015; Marin et al. 2015; Candela et al. 2017). A *cfr* gene has been identified in a non-conjugative element, denominated Tn*6218*, which is related to Tn*916* (Dingle et al. 2014).

5.1.3 Fluoroquinolones

Alterations in the quinolone-resistance determining region (QRDR) of GyrA and/or GyrB are responsible for resistance to FQs in *C. difficile* (Ackermann et al. 2001, 2003; Dridi et al. 2002; Drudy et al. 2006, 2007). Several amino acidic substitutions have been identified in the DNA gyrase subunits (Table 5), but the most common in *C. difficile* FQs-resistant strains is the substitution Thr82Ile in GyrA (Ackermann et al. 2001; Dridi et al. 2002; Spigaglia et al. 2008b, 2011; Kuwata et al. 2015). Interestingly, Thr82Ile in GyrA has not a detectable cost on the fitness of *C. difficile in vitro*, suggesting that this substitution can be maintained in the bacterial population even in the absence of antibiotic selective pressure (Wasels et al. 2015a).

Resistant mutants to FQs can be obtained with high frequency after exposure of *C. difficile* susceptible strains to MXF and levofloxacin (LVX) (Spigaglia et al. 2009). Since the concentration of this drug in the human intestine, during the early stage of treatment, is not inhibitory, it is possible for a sub-population of bacteria to acquire mutations conferring resistance to FQs.

5.2 Antibiotics for CDI Treatment

5.2.1 Metronidazole

Metronidazole is a nitro-aromatic pro-drug that need the reduction of the 5-nitro group of the imidazole ring to become cytotoxic to bacterial cells (Goldman 1982). In *Helicobacter pylori* and *Bacteroides fragilis*, resistance to MTZ is usually conferred by nitroimidazole (*nim*) genes (Gal and Brazier 2004), but these genes have not been identified in *C. difficile* (Moura et al. 2014). Although it is not completely understood, data obtained in recent studies on strains RT 027 and RT 010 suggest that *C. difficile* resistance to MTZ is a multifactorial process that involves alterations in metabolic pathways, such as activity of nitroreductases, iron uptake and DNA repair (Chong et al. 2014; Moura et al. 2014). In addition, biofilm formation seems to play a role in *C. difficile* MTZ-resistance (Vuotto et al. 2016). How biofilm growth could contribute to increase *C. difficile* resistance to MTZ is still unclear. However, it can be hypothesized that biofilm matrix can act as a protective barrier, inducing, at the same time, an alteration of the physiological state of the bacteria within the biofilm that determines a higher level of resistance to antibiotics.

5.2.2 Vancomycin

Vancomycin is the first-line antibiotic for moderate to severe CDI (Debast et al. 2014; Jarrad et al. 2015). This antibiotic, which consists of a glycosylated hexapeptide chain and cross linked aromatic rings by aryl ether bonds, inhibits the biosynthesis of peptidoglycan, an essential component of the bacterial cell wall envelope, and it is poorly absorbed by the gastrointestinal tract (Perkins and Nieto 1974; Yu and Sun 2013). The mechanism of resistance in *C. difficile* is still unclear. Although Tn*1549*-like elements have been found in several strains (Brouwer et al. 2011, 2012), these elements, differently from the original Tn*1549* element described in *E. faecalis*, do not have a functional *van*B operon. Interestingly, a *van*G-like gene cluster homologous to that found in *E. faecalis* have also been described in *C. difficile* but it seems not able to promote resistance to VAN (Ammam et al. 2012, 2013; Ramírez-Vargas et al. 2017). Recently, VAN-resistant mutants, showing the amino acid change Pro108Leu in the MurG, have been obtained *in vitro* (Leeds et al. 2014). Since MurG is involved in the membrane-bound

Table 5 Amino acid substitutions detected in *C. difficile* isolates resistant to fluoroquinolones or rifamycins

Antibiotic[a]	Target	Amino acid position	Original residue	Resistance substitution	References
FQs	GyrA	43	Val	Asp	Carman et al. (2009)
		71	Asp	Val	Dridi et al. (2002), Walkty et al. (2010), Liao et al. (2012)
		81	Asp	Asn	Huang et al. (2009), Liao et al. (2012)
		82	Thr	Ile or Val	Ackermann et al. (2001), Dridi et al. (2002), Spigaglia et al. (2008b), Kuwata et al. (2015), Liao et al. (2012)
		118	Ala	Thr	Dridi et al. (2002)
		384	Ala	Asp	Mac Aogáin et al. (2015)
	GyrB	377	Arg	Gly	Liao et al. (2012)
		416	Ser	Ala	Liao et al. (2012)
		426	Asp	Asn or Val	Dridi et al. (2002), Spigaglia et al. (2008b), Liao et al. (2012)
		447	Arg	Lys	Walkty et al. (2010), Liao et al. (2012)
		466	Glu	Val	Liao et al. (2012)
	GyrA/ GyrB	82/366	Thr/Ser	Ile/Ala	Huang et al. (2009), Kuwata et al. (2015)
		82/366 and 426	Thr/Ser and Asp	Ile/Ala and Val	Walkty et al. (2010), Kuwata et al. (2015)
		82/366 and 434	Thr/Ser and Gln	Ala/Ala and Lys	Kuwata et al. (2015)
		82/416	Thr/Ser	Ile/Ala	Spigaglia et al. (2008b), Liao et al. (2012)
		82/426	Thr/Asp	Ile/Asn	Walkty et al. (2010), Kuwata et al. (2015)
		82/426	Thr/Asp	Ile/Val	Spigaglia et al. (2011)
		82/426	Thr/Asp	Val/Val	Huang et al. (2009), Liao et al. (2012)
		82/444	Thr/Leu	Ile/Phe	Walkty et al. (2010)
RFs	RpoB	485	Ser	Phe	Cairns et al. (2017)
		492	Asp	Asn or Val	Pecavar et al. (2012)
		502	His	Arg or Asn or Leu or Tyr	O'Connor et al. (2008), Pecavar et al. (2012), Miller et al. (2011b)
		505	Arg	Lys	O'Connor et al. (2008), Curry et al. (2009), Miller et al. (2011b), Spigaglia et al. (2011), Pecavar et al. (2012)
		550	Ser	Phe or Tyr	Pecavar et al. (2012)
		448; 505	Ser; Arg	Thr; Lys	O'Connor et al. (2008), Curry et al. (2009)
		487; 502	Leu; His	Phe; Tyr	Pecavar et al. (2012)
		492; 505	Asp; Arg	Asn; Lys	O'Connor et al. (2008)
		498; 505	Ser; Arg	Thr; Lys	Curry et al. (2009), Miller et al. (2011b)
		502; 496	His; Pro	Tyr; Ser	Carman et al. (2009)
		502; 505	His; Arg	Asn; Lys	O'Connor et al. (2008), Curry et al. (2009), Miller et al. (2011b), Spigaglia et al. (2011), Pecavar et al. (2012)
		505; 548	Arg; Ile	Lys; Met	O'Connor et al. (2008), Curry et al. (2009), Pecavar et al. (2012)

[a]*FQs* Fluoroquinolones, *RFs* rifamycins

stage of peptidoglycan biosynthesis, this substitution may affect VAN activity. In addition, biofilm formation has been found to probably have a role in VAN-resistance. In fact, *C. difficile* within biofilms resulted more resistant to high concentrations of VAN (20 mg/L) and sub-inhibitory and inhibitory concentrations of the antibiotic seems to induce biofilm formation (Dapa et al. 2013).

5.2.3 Rifamycins

Treatment failures and recurrence of infection rates associated with MTZ and VAN treatments have increased in the last years (Vardakas et al. 2012) therefore other therapy options for CDI have been proposed.

RFs, in particular RFX, have recently been proposed as "chaser therapy" for treatment of relapsing CDI (Iv et al. 2014), while fidaxomicin (FDX) is a bactericidal new narrow spectrum macrocyclic antibiotic that is used for the management of CDI with high risk for recurrences (Chaparro-Rojas and Mullane 2013). Both RFs and FDX are inhibitors of bacterial transcription but they have different RNA polymerase (RNAP) target sites. FDX binds to the 'switch region' of RNAP, a target site that is adjacent to the RIF target but does not overlap (Mullane and Gorbach 2011; Srivastava et al. 2011).

Different amino acid substitutions have been identified within the β-subunit of the RNA polymerase (*rpo*B) of strains resistant to RFs (Table 5). Among the amino acid substitutions identified, Arg505Lys is the most common, particularly in strains RT027 (Miller et al. 2011b; Spigaglia et al. 2011; Carman et al. 2012; Pecavar et al. 2012).

5.2.4 Fidaxomicin

This antibiotic provides cure rates not inferior to VAN and is associated with a significantly lower rate of CDI recurrence caused by strains non-RT 027 (Louie et al. 2011). Furthermore, it has a minimal impact on the composition of indigenous fecal microbiota, in particular on Bacteroides species (Tannock et al. 2010; Louie et al. 2012), with a high local concentration in the gut and feces (1225.1 μg/g after 10 days of therapy) (Goldstein et al. 2012; Sears et al. 2012). Reduced susceptibility to FDX is very rare and only one *C. difficile* clinical isolate with a MIC = 16 mg/L has been described (Goldstein et al. 2011). Mutations in *rpo*B or CD22120, encoding for a homologue to the multidrug resistance-associated transcriptional regulator MarR, have been observed in *C. difficile* mutants resistant to FDX obtained *in vitro* (Leeds et al. 2014). Since mutations causing resistance to FDX arise in *rpo*B gene at distinct loci compared to those causing resistance to RFs, FDX retains activity against strains resistant to RFs (Anti-Infective Drugs Advisory Committee Briefing Document, Optimer Pharmaceuticals, Inc.).

5.3 Other Antibiotics

5.3.1 Tetracycline

In *C. difficile*, resistance to TET is due by *tet* genes (Table 4). The most widespread *tet* class is *tet*M, usually carried by conjugative Tn*916*-like elements (Spigaglia et al. 2005; Mullany et al. 2012; Dong et al. 2014). This family of transposon is responsible for the spread of antibiotic resistance (usually referred to TET but also to MLS_B and other antibiotics) to many important pathogens (Roberts and Mullany 2011). The best-known *C. difficile* element of this family is Tn*5397*, which is a 21 kb element able to transfer *in vitro* between *C. difficile* and *B. subtilis* or *E. faecalis* (Mullany et al. 1990; Jasni et al. 2010). A group II intron and a different excision/insertion module differentiate Tn*5397* from Tn*916*. In fact, Tn*5397* has a *tnd*X gene that encodes a large serine recombinase, while Tn*916* contains two genes, *xis*Tn and *int*Tn, encoding an excisionase and a tyrosine integrase (Roberts et al. 2001). Furthermore, Tn*916* inserts into multiple regions of the *C. difficile* genome (Mullany et al. 2012), while Tn*5397* inserts DNA predicted filamentation processes induced by cAMP (Fic) domain (Wang et al. 2006).

Different genetic organizations of Tn*916*-like elements and different *tet*M alleles have been identified in *C. difficile* (Spigaglia et al. 2005, 2006). In particular, the Tn*916*-element detected in the clinical isolate CD1911 contains both *tet*M and *erm*B, (Spigaglia et al. 2007). This element is non-conjugative and probably originated from the combination of one or more plasmids and a Tn*916*-like element.

Albeit more rarely, other *tet* genes have been identified in *C. difficile*. In particular, the co-presence of both *tet*M and *tet*W have been described in *C. difficile* isolates from humans and animals (Spigaglia et al. 2008a; Fry et al. 2012).

Interestingly, an element of 106 kb, the Tn*6164*, has been identified in *C. difficile* strain M120, a RT 078 isolate (Corver et al. 2012). This transposon is composed by parts of other elements from different bacteria, particularly from *Thermoanaerobacter sp*. and *Streptococcus pneumoniae* and it contains *tet*(44) and *ant*(6)-Ib, predicted to confer resistance to TET and streptomycin, respectively. Since strain M120 is susceptible to these antibiotics, Tn*6164* does not seem involved in resistance, but it seems to be associated to higher virulence of strains RT 078, in fact an analysis of data from patients indicate that mortality was more common in patients infected with strains RT 078 containing Tn*6164* compared with those infected with strains without this element.

5.3.2 Chloramphenicol

C. difficile resistance to CHL is usually conferred by a CHL acetyltransferase encoded by a *cat*D gene (Wren et al. 1988, 1989) (Table 4). In *C. difficile*, the *cat*D gene is located on the transposons Tn*4453*a and Tn*4453*b, which are strictly related to the *Clostridium perfringens* mobilisable element Tn*4451* (Lyras et al. 1998). Recently, a conjugative transposon designed Tn*6104*, has been described (Brouwer et al. 2011). This transposon contains genetic elements closely related to Tn*4453*ab and Tn*4451* but instead of a *cat*D gene it shows genes predicted to encode for transcriptional regulator, a two component regulatory system, an ABC transporter, three sigma factors and a putative toxin-antitoxin system. The role of these genes is not clear and remains to be determined.

6 Conclusions

C. difficile infection (CDI) is a growing concern for global public health. An increased CDI incidence, morbidity and mortality have been reported in the last decades in association with the emergence and spread of *C. difficile* highly virulent types. *C. difficile* adaptive capability and genome plasticity has determined an increase of strains resistant to multiple antibiotics and, currently, most of epidemic clinical isolates are MDR. A wide range of mobile elements and alterations of antibiotic targets mediate resistance to several antibiotics, including the MLS_B family and FQs, which are significantly associated to CDI. Furthermore, a decreased susceptibility to the first-line antibiotics used for CDI therapy, in particular MTZ and VAN, and to those used for recurrences, such as RFs, may have a role in the low rate of response to treatment reported over the last years. Antibiotics resistances seem to be maintained in this pathogen regardless of the burden imposed by the acquisition of genetic elements/mutations conferring resistance and the decrease of antibiotics pressure. This feature may explain the persistence of "old" resistances and the rapid diffusion of "new" resistances in *C. difficile* population. The multifactorial nature of antibiotic resistances and the rapid evolution of *C. difficile* epidemiology, emphasizing the need for effective antimicrobial stewardships, implementation of infection control programs, and development of alternative therapies to prevent and contain the spread of resistant strains and to ensure an efficacious therapy for CDI.

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Probiotics for Prevention and Treatment of *Clostridium difficile* Infection

Lorena Valdés-Varela, Miguel Gueimonde, and Patricia Ruas-Madiedo

Abstract

Probiotics have been claimed as a valuable tool to restore the balance in the intestinal microbiota following a dysbiosis caused by, among other factors, antibiotic therapy. This perturbed environment could favor the overgrowth of *Clostridium difficile* and, in fact, the occurrence of *C. difficile*-associated infections (CDI) is being increasing in recent years. In spite of the high number of probiotics able to in vitro inhibit the growth and/or toxicity of this pathogen, its application for treatment or prevention of CDI is still scarce since there are not enough well-defined clinical studies supporting efficacy. Only a few strains, such as *Lactobacillus rhamnosus* GG and *Saccharomyces boulardii* have been studied in more extent. The increasing knowledge about the probiotic mechanisms of action against *C. difficile*, some of them reviewed here, makes promising the application of these live biotherapeutic agents against CDI. Nevertheless, more effort must be paid to standardize the clinical studied conducted to evaluate probiotic products, in combination with antibiotics, in order to select the best candidate for *C. difficile* infections.

Keywords

Probiotic · *C. difficile* · Clinical study · Mechanism of action · Antagonism

1 Introduction

The gut microbiota is a complex and diverse microbial community that has co-evolved with humans in a commensal way (Donaldson et al. 2016). In a healthy state, this collection of microorganisms protects the host by inhibiting colonization and growth of pathogens. However, antibiotic exposure strongly perturbs the intestinal microbiota, producing a decrease in microbial abundance and species diversity, as well as a suppression of the innate immune system disrupting the gut barrier and frequently causing antibiotic-associated diarrhea. In some cases, the intestinal dysbiosis followed after antibiotic treatment allows the overgrowth of *Clostridium difficile* given that this perturbed

L. Valdés-Varela · M. Gueimonde · P. Ruas-Madiedo (✉)
Department of Microbiology and Biochemistry of Dairy Products, Instituto de Productos Lácteos de Asturias – Consejo Superior de Investigaciones Científicas (IPLA-CSIC), Villaviciosa, Asturias, Spain
e-mail: lvaldes@ipla.csic.es; mgueimonde@ipla.csic.es; ruas-madiedo@ipla.csic.es

environment has a low abundance of short chain fatty acids, a high abundance of primary bile acids, a high carbohydrate availability and a immunosuppressed host in the absence of microbial competitors in the gut (Lawley and Walker 2013).

C. difficile can be found in the gut microbiota of both, healthy infants and adults, the occurrence being higher in infant (70%) than in the adult (17%) population (Ozaki et al. 2004; Jangi and Lamont 2010). In these healthy carriers the presence of this microorganism does not seem to cause any disease. However, at the same time *C. difficile* is the main causative agent of antibiotic-associated diarrhea in nosocomial environments (Leffler and Lamont 2015). As previously indicated the antimicrobial therapy affects the endogenous gut microbiota diminishing colonization resistance, allowing the overgrowth of this pathogen and causing *C. difficile*-associated diarrhea (CDAD). This problem has been traditionally linked to elderly and institutionalized/hospitalized persons under antibiotic therapy (Rupnik et al. 2009); however, the occurrence of *C. difficile*-associated infections (CDI) seems to be increasing also in traditionally considered low-risk populations (Carter et al. 2012). This change in the epidemiology of CDI has been related to the worldwide distribution of hyper virulent strains (Yakob et al. 2015); besides, foods and animals have been found to act as carriers of this pathogen pointing at *C. difficile* as a zoonotic agent and suggesting potential food-borne transmission (Rodriguez et al. 2016). A range of virulent factors are the cause of colitis during CDI course, the main ones being several toxins, encoded in pathogenicity loci, and the flagella, which are factors allowing mobility and adherence of the pathogen (Abt et al. 2016). Pathogenesis was initially attributed to the production of toxins A (TcdA) and B (TcdB), belonging to the large clostridial toxin (LCT) family, which act as intracellular glycosyl-transferases that inactivate Rho family GTPases, thus blocking downstream cellular events (Carter et al. 2012). More recently, strains producing a third toxin, the binary toxin (CDT), have been associated with an increase in the CDI severity; this toxin has two components the CDTa, which acts as an ADP-ribosyltransferase targeting actin, and CDTb that is able to binds to the cell and translocate the first component to the cytosol (Gerding et al. 2014). In spite of recent advances in the identification of processes involved on receptor binding and entry into mammalian cells, the mode-of-action of clostridial toxins remains to be totally elucidated (Orrell et al. 2017).

The standard treatment for *C. difficile* infection is the administration of antibiotics, mainly metronidazole, vancomycin or fidaxomicin, but unfortunately the recurrence rate of the disease is very high and this treatment becomes less effective. Indeed, it has been described that some *C. difficile* subpopulations (ribotypes) have a reduced susceptibility to metronidazole (Moura et al. 2013). In case of multiple recurrent CDI, fecal microbiota transplantation (FMT) is being more frequently used as the ultimate therapy, although the selection of the appropriate donor is a critical issue (Woodworth et al. 2017). These facts have prompted researchers to look for alternative therapeutic options (Fig. 1) which have been recently reviewed by different authors (Mathur et al. 2014; Hussack and Tanha 2016; Kachrimanidou et al. 2016; Kociolek and Gerding 2016; Martin and Wilcox 2016; McFarland 2016; Ofosu 2016; Padua and Pothoulakis 2016; Ünal and Steinert 2016). Among them, probiotics have been proposed as a potential tool for preventing the dysbiosis of microbiota, caused by the administration of antibiotics, and for assisting in the microbiota restoration after antibiotics or infection (Reid et al. 2011); thus, they have also been evaluated for prevention and treatment of CDI (Na and Kelly 2011).

Probiotics were defined in 2001 by a group of experts joined by FAO/WHO as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host"; this definition was recently revised, and accepted after minor grammatical modifications, by members of the International Scientific Association for Probiotics and Prebiotics (ISAPP) which also propose an overall framework for use of this term, encompassing diverse end uses (Hill et al. 2014). In next sections we will review the current available data about the efficacy of

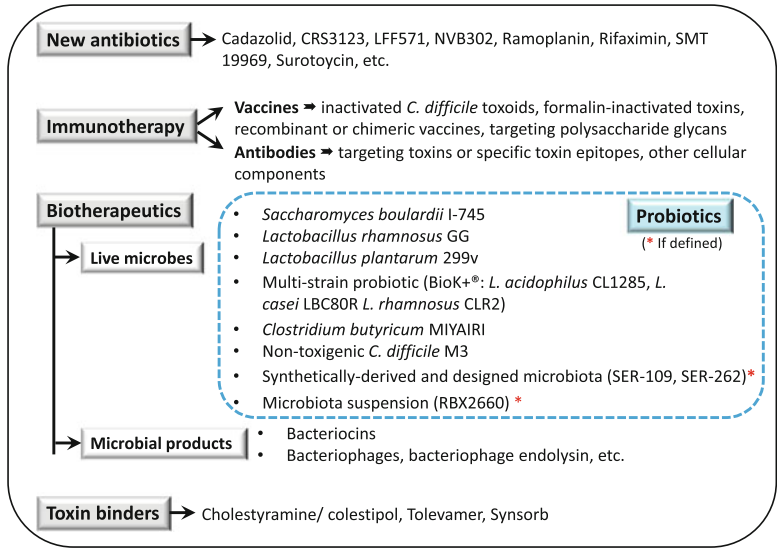

Fig. 1 Some therapeutic options currently under study for the prevention and treatment of *Clostridium difficile* infection

probiotics in prevention and therapy for CDI, as well as some putative mechanisms involved in this anti-*C. difficile* effect.

2 Clinical Studies Evaluating Probiotic Efficacy

The ability of probiotics for inhibiting the growth of *C. difficile* has been characterized by using different experimental approaches (Auclair et al. 2015; Forssten et al. 2015; Valdes-Varela et al. 2016b; Fredua-Agyeman et al. 2017). This use of probiotic microorganisms has long been considered a potential option to combat CDI. However, despise the large number of in vitro studies performed for the selection of probiotic strains with activity against *C. difficile* and for their use for CDI prevention or treatment, the evidence from human clinical trials is still limited. Different probiotic strains have been reported to increase the colonization resistance against *C. difficile* (Hopkins and Macfarlane 2003; Kondepudi et al. 2014; Auclair et al. 2015; Forssten et al. 2015). Certain strains of bifidobacteria and lactobacilli have been found to reduce the adhesion of *C. difficile* to intestinal epithelial cells or intestinal mucus (Collado et al. 2005; Banerjee et al. 2009) or to be able to inhibit its growth (Lee et al. 2013; Schoster et al. 2013; Valdes-Varela et al. 2016b). Moreover, animal studies seem to confirm a potential benefit of probiotics on the inhibition of *C. difficile* colonization (Mansour et al. 2017). Nevertheless, to date most of the clinical studies have focused on prevention and there is a lack of data on the potential use of probiotics on the treatment of *C. difficile* infection.

During the last couple of decades several studies have evaluated the usefulness of different probiotic strains in the prevention of CDAD. However, in spite of the large number of strains screened in vitro, most of the evidence from clinical trials regards only a few bacterial strains and, most often, the studies have focused on the prevention of antibiotic-associated diarrhea, without further confirmation of *C. difficile* etiology. Among the assessed strains the effect of *Lactobacillus rhamnosus* strain GG (Arvola et al. 1999; Vanderhoof et al. 1999), or the yeast species *Saccharomyces boulardii* (Kotowska et al. 2005; Can et al. 2006), in the prevention of antibiotic associated diarrhea has been widely recognized. Although not so extensively studied, other probiotic strains and probiotic mixes have also been evaluated around the world with positive results

(Wullt et al. 2003; Maziade et al. 2015). The availability of a large number of clinical studies focusing on antibiotic-associated diarrhea has provided enough data for carrying out systematic reviews and meta-analysis studies, either considering probiotics as a group, which shows important limitations due to inter-strain and/or inter-product variability, or meta-analyses focused on specific strains. The meta-analysis studies on the general use of probiotics for the prevention of antibiotic-associated diarrhea have consistently provided evidence for a beneficial role, especially in children (Cremonini et al. 2002; D'Souza et al. 2002; Sazawal et al. 2006; Johnston et al. 2007; Hempel et al. 2012; Goldenberg et al. 2015). Moreover, meta-analyses conducted for some specific probiotics, such as *S. boulardii* or *L. rhamnosus* GG, have further confirmed the beneficial effect of these strains in the prevention of antibiotic-associated diarrhea (McFarland 2006; Szajewska et al. 2007a, b). This has resulted in recommendations issued by the ESPGHAN (European Society for Paediatric Gastroenterology Hepatology and Nutrition) with regard to the use of probiotics for the prevention of antibiotic-associated diarrhea in children (Szajewska et al. 2016).

Furthermore, some studies have specifically focused in confirmed *C. difficile*-associated diarrhea and these have also provided positive results for primary prevention (Wullt et al. 2003; Gao et al. 2010; Sampalis et al. 2010; Allen et al. 2013; Dietrich et al. 2014; Maziade et al. 2015). Some practical examples exist as well, such as that of the "Pierre-Le Gardeur" Hospital in Canada, that after a *C. difficile* outbreak begun to administer a probiotic mix (BioK+®) together with any antibiotic prescriptions, achieving a significant reduction on the number of *C. difficile* disease cases (Maziade et al. 2015). Recent meta-analyses and systematic reviews have assessed the effects of probiotic administration, most of them administering the strains together with the antibiotic treatment, on the primary prevention of CDAD in different population groups (Table 1). In general the data support a beneficial effect of probiotics on the primary prevention of CDAD. However, the high heterogeneity among the available clinical studies makes difficult defining the best probiotic to be used, its dose, and the administration regime.

Regarding the prevention of the recurrence of the disease, the available data are more limited than in the case of primary prevention. Some clinical intervention studies have been conducted with variable results (McFarland et al. 1994; Surawicz et al. 2000), with reviews and meta-analyses indicating that there is only limited evidence on the benefit of probiotics in secondary prevention of CDI (Allen et al. 2013; O'Horo et al. 2014; McFarland 2015). The limited data available on secondary prevention underlines the need for more clinical intervention trials to be conducted in this topic.

Table 1 Recent meta-analyses and systematic reviews on the use of probiotics in primary prevention of *C. difficile* infection

Target population	Probiotic	N° eligible RCTs[a]	N° volunteers included	Conclusion	References
Elderly	Any	5	>3400	No significant effect	Vernaya et al. (2017)
Adults	Any	19	>6200	Significant reduction	Shen et al. (2017)
Adults	*Lactobacillus* (any)	10	>4800	Inconclusive evidence	Sinclair et al. (2016)
Adults and children	Any	26	>7900	Significant reduction	Lau and Chamberlain (2016)
Adults and children	Any (and by species)	21	>3700	Significant reduction	McFarland (2015)
Adults and children	Any	31	>4200	Significant reduction	Goldenberg et al. (2013)

[a]*RCT* randomized controlled trial

To sum up, the available evidence strongly suggests that probiotics are helpful for primary prevention with only moderate evidence of a role in avoiding disease relapse. However, the potential role of probiotics in the treatment during the active phase of the disease remains largely unknown. Perhaps the major criticism that can be done to the available data is that there has not been a serious standardization effort for the probiotic products, doses, antibiotics and therapeutic protocols to be used. Moreover, analyses of the cost-effectiveness of probiotic use on the prevention of *C. difficile* disease have not been performed until recently, with variable results, indicating the need for further studies conducted under different healthcare systems (Leal et al. 2016; Starn et al. 2016).

3 Models to Study Probiotics Against *C. difficile*

Different experimental models have been developed in order to study the interaction of *C. difficile* with the host (recently reviewed by Young 2017); additionally, these models can be used in the search for new therapeutic alternatives and adjuvant strategies for preventing or treating CDI (Table 2). Investigations using in vitro models of bacterial cultures are valuable systems for the screening of potential probiotics against *C. difficile* but, as disadvantage, they have the lack of feedback mechanisms with host and/or host-microbe interactions (Best et al. 2012). However, these microbial culturing models can be combined with cell culture systems to better mimic the interaction *C. difficile*- probiotic- host (Venema and van den Abbeele 2013). Co-cultures of toxigenic *C. difficile* strains with probiotic candidates have been carried out to determine the potential of the latter for reducing the germination of spores and outgrowth into vegetative toxin-producing cells of the pathogen (Table 2). Models of gut microbiota have been assayed to in vitro evaluate the potential of probiotic candidates for decreasing the growth of *C. difficile* in this complex microbial ecosystem. These models range from simple batch fermentations to complex multi-compartmental continuous systems (Venema and van den Abbeele 2013). Static batch cultures, containing fecal suspensions, have been used to observe the influence of probiotics on the survival of *C. difficile* (Tejero-Sariñena et al. 2013). Continuous culture systems (human "colonic" model) allow the study of the pathogen in an environment closer to the reality, over considerably longer periods than in static batch cultures (Best et al. 2012; Le Lay et al. 2015). Currently, most of the colonic simulators consists of four different units (glass vessels) continuously

Table 2 Summary of some in vitro models used to study potential probiotics against *Clostridium difficile*

In vitro experimental models			References
Microbial cultivation	*vs.* probiotic	Co-cultures of *C. difficile* with probiotic candidates	Trejo et al. (2010), Best et al. (2012), Kolling et al. (2012), Lee et al. (2013), Schoster et al. (2013), Kondepudi et al. (2014), Yun et al. (2014), Ambalam et al. (2015), Andersen et al. (2016), Spinler et al. (2016), and Rätsep et al. (2017)
	vs. microbiota/ probiotic	Static-batch system	Tejero-Sariñena et al. (2013)
		Semi-continuous system	Le Lay et al. (2015)
		"Colonic" model	Forssten et al. (2015)
Intestinal cell lines	Adhesion/ exclusion	HT29-MTX cell	Zivkovic et al. (2015)
		Immobilized intestinal mucus	Collado et al. (2005), Banerjee et al. (2009), and Ferreira et al. (2011)
	Cytotoxicity	Label-based endpoint methods	Barnerjee et al. (2009), Trejo et al. (2010, 2013), and Valdés-Varela et al. (2016a)
		Label-free, RTCA method	Valdés et al. (2015) and Valdés-Varela et al. (2016a, b)

connected, having different pH and flow rates, thus representing the ascending, transverse, descending and distal colon (Forssten et al. 2015).

Several in vitro studies investigated the effect of probiotic treatment on the interaction of *C. difficile* with components of the intestinal mucosa, such as mucus or epithelial cells (Table 2). The cytotoxicity of clostridial cell-free supernatants (obtained from co-cultures of probiotic vs. *C. difficile*) or of caecum contents (collected from animals infected with *C. difficile* and treated with potential probiotics) has been evaluated upon cell lines using classic label-based, endpoint methods (Barnerjee et al. 2009; Trejo et al. 2010, 2013; Valdés-Varela et al. 2016a). However, label-free technologies are currently been available and being used in drug development processes, which are non-invasive techniques that allow the continuous (real time) monitoring of the status of live cells (Xi et al. 2008). Indeed the label-free, impedance-based RTCA (real time cell analyzer) technology has been applied to develop methods allowing the clinical diagnosis of toxigenic *C. difficile* in different biological samples (Yu et al. 2015). Recently, this RTCA technology was also used in our group to develop a model to test the cytotoxicity of *C. difficile* supernatants upon the intestinal epithelial cell lines HT29 and Caco-2 (Valdés et al. 2015). Moreover, this model was used to search for potential probiotic strains able to counteract the toxic effect of *C. difficile* supernatants upon HT29 (Valdés-Varela et al. 2016a) as well as to evaluate the toxicity of *C. difficile* co-cultured with some of these probiotics (Valdés-Varela et al. 2016b).

On the other hand, several models have been used to assess the ability of probiotic candidates to modify the adhesion *C. difficile* to the intestinal mucosa, such as those using immobilized (human) intestinal mucus which showed a good correlation with data obtained with a enterocyte-like (Caco-2) model (Collado et al. 2005; Banerjee et al. 2009; Ferreira et al. 2011). The ability of potential probiotic strains to inhibit the adhesion of *C. difficile* has also been evaluated using intestinal cell lines, such as HT29-MTX which is a derivative from HT29 (adapted to methotrexate) thus synthesizing higher amounts of mucus (Zivkovic et al. 2015). A study has suggested that this cell model may be more suitable for studying cell-pathogen interactions, as well as effectiveness of antimicrobial treatments, as compared to Caco-2 or HT29 models which do not have Goblet cells or do not constitutively secrete mucus, respectively (Gagnon et al. 2013).

In an step forward, several authors have evaluated the protective effect of selected probiotic candidates against CDI in animal models (Best et al. 2012; Kolling et al. 2012; Trejo et al. 2013; Kondepudi et al. 2014; Yun et al. 2014; Andersen et al. 2016; Arruda et al. 2016; Spinler et al. 2016; Rätsep et al. 2017). This infection has been studied in different models, including mice, hamsters, rats, rabbits, hares, guinea pigs, prairie dogs, quails, foals, piglets and monkeys. Moreover, zebrafish embryos have been described as suitable models for identification of in vivo targets of *C. difficile* toxins and evaluation of novel candidate therapeutics; zebrafish possess many of the major organs present in humans and, due to the transparency of the embryo, damage by toxins can be visualized by standard light microscopy (Best et al. 2012). Each of the *C. difficile* animal models has inherent advantages and disadvantages. The hamster model has been widely used to study pseudomembranous colitis in human because of extreme sensitivity to infection following antibiotic administration, using clindamycin as agent of choice; however, this model does not represent the usual course and spectrum of CDI in humans. Recently, new mouse and piglet CDI models have been developed which appear to mimic many of disease symptoms observed in humans (Sun et al. 2011; Best et al. 2012; Hutton et al. 2014).

4 Mechanisms of Probiotic Action

As pointed in previous sections, probiotics are gaining more and more interest as preventive and co-adjuvant therapies for treatment of antibiotic-associated dysbiosis. However, their modes of

action are poorly understood and vary between probiotic microorganisms. Indeed the effects of any probiotic are strain-specific and, therefore, beneficial effects cannot be extrapolated to other species or strains (Hickson 2011). It has been described that probiotics could have diverse positive actions on the host by: (i) modulating the intestinal microbiota and inhibiting pathogenic microorganisms at the intestinal luminal environment, (ii) enhancing of intestinal barrier function at the intestinal epithelium, and (iii) modulating the immune response, among others (Ng et al. 2009). Several mechanisms have been proposed for explaining the potential role of probiotics against *C. difficile*. Some of these effects, such as the production of antimicrobial factors (Corr et al. 2007), competitive inhibition of the pathogen (Collado et al. 2005) or the ability to degrade and to reduce the toxicity of *C. difficile* (Castagliuolo et al. 1999; Valdes-Varela et al. 2016a), could be of help not only in prevention but also in the treatment of CDI.

4.1 Microbial Antagonism: Interaction Probiotics vs. *C. difficile*

The restoration of intestinal microbiota after dysbiosis, caused by any etiological agent, is the main way of action of any treatment against intestinal pathogens including *C. difficile* (Gareau et al. 2010; Reid et al. 2011). This was evidenced, for example, in an in vivo study with a murine CDI model of antibiotic-induced dysbiosis, in which the gut microbiota was restored after treatment with a multi-strain probiotic supplement (*Lactobacillus plantarum* F44, *Lactobacillus paracasei* F8, *Bifidobacterium breve* 46, *Bifidobacterium animalis* subsp. *lactis* 8:8) (Kondepudi et al. 2014). There are several mechanisms by which probiotics can help the restoration of the intestinal microbiota, some of them being related with typical bacterial antagonism (Ng et al. 2009); however, little is known about those mechanisms acting specifically in the context of CDI (Parkes et al. 2009; Ollech et al. 2016).

Some probiotic strains are able to compete with pathogenic bacteria for the adhesion sites, i. e. competitive exclusion, thus providing a "physical" barrier that increases the colonization resistance (Fig. 2a). In vitro studies showed the ability of selected *Bifidobacterium* and *Lactobacillus* strains to modify the adhesion of *C. difficile* to intestinal epithelial cells, or intestinal mucus, the effect being strain-dependent (Collado et al. 2005; Zivkovic et al. 2015). A reduction from 60% to 3% in the adhesion of *C. difficile* to gingival epithelial cell cultures (obtained from healthy horses) was reported when *Lactobacillus reuteri* Lr1 was added; additionally, it was detected that this strain was able to co-aggregate with the pathogen (Dicks et al. 2015). In this regard, it has been suggested that the aggregation capability between lactobacilli and *C. difficile* could be a way to reduce the adhesion of the pathogen to the intestinal mucosa (Ferreira et al. 2011). *S. boulardii* is also able to reduce the adhesion of *C. difficile* to epithelial cells and the same effect was detected using extracts obtained from the cell-wall of this yeast (Tasteyre et al. 2002). Similarly, it has been proved that cell-free supernatants obtained from *Lactobacillus delbrueckii* ssp. *bulgaricus* B-30892 (Banerjee et al. 2009) and different bifidobacterial strains (Trejo et al. 2006) were able to reduce the adhesion of *C. difficile* to intestinal epithelial Caco-2 cells. Different treatments of the bifidobacterial supernatants showed that the factors related to the anti-clostridial adhesion were no heat-resistant, non-related with acids (active at neutral pH) and were not affected by proteinases, but its nature remains unknown (Trejo et al. 2006). Indirect evidence suggests that exopolysaccharides covering the surface of some probiotics could be involved in the inhibition of the binding capability of some pathogens, including *C. difficile*, by probiotics (Ruas-Madiedo et al. 2006). Thus, altogether, these studies suggest that different surface molecules and/or secreted

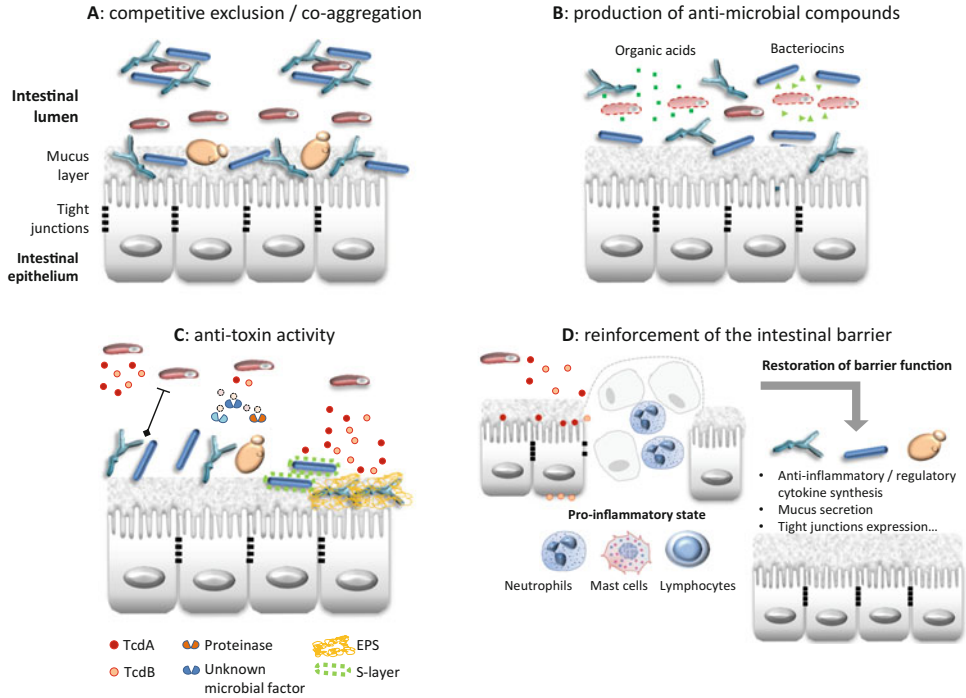

Fig. 2 Potential mechanisms of action proposed for probiotics against *Clostridium difficile*. (**a**) competitive exclusion/co-aggregation. (**b**) production of anti-microbial compounds. (**c**) anti-toxin activity. (**d**) reinforcement of the intestinal barrier

factors might be implicated in the interference of probiotics against *C. difficile* adhesion to the intestinal mucosa.

Another mechanism of probiotic action is the inhibition of the pathogen growth through the competition for the limiting nutritional sources and/or by the production of antimicrobial factors, such as organic acids and bacteriocins (Fig. 2b). In a study carried out with a CDI animal model it was shown that mice treated with *Streptococcus thermophilus* LMD-9 exhibited less pathology, and lower detectable toxin levels in cecal contents, compared with untreated controls; an inverse correlation was observed between the levels of luminal lactate and the abundance of *C. difficile*, suggesting that the anti-clostridial effect was due to the production of this organic acid (Kolling et al. 2012). Similarly, the lactic acid synthesized by *Lactobacillus acidophilus* GP1B had an inhibitory effect on *C. difficile* growth in a CDI mouse model, which may be related to a reduction in pH as a result of organic acids produced by the probiotic bacterium (Yun et al. 2014). Several in vitro studies have investigated the activity of probiotics to inhibit *C. difficile* growth; using a fecal, pH-controlled (between 6.7 and 6.9), anaerobic batch model it was found that *Lactobacillus casei* NCIMB30185 and *B. breve* NCIMB30180 were able to reduce the numbers of *C. difficile* in this complex microbial ecosystem (Tejero-Sariñena et al. 2013). Co-cultivation of *C. difficile* with cell-free supernatants from different commercial probiotics highlighted that the mechanism of inhibition was pH-dependent; thus, the production of organic acids, mainly lactic and acetic acids, are the inhibition factors controlling the growth of *C. difficile* (Schoster et al. 2013). In another in vitro study, the co-incubation of *C. difficile* with *L. rhamnosus* LR5, *Lactococcus*

lactis SL3, *B. breve* BR3 and *B. animalis* subsp. *lactis* BL3 demonstrated their potential to decrease *C. difficile* numbers, mainly mediated by the organic acid production. However, among those strains, SL3 appeared to have the strongest activity which seems to be pH-independent and likely could be mediated through the action of a bacteriocin (Lee et al. 2013). Similar pH-dependent and pH-independent effects against *C. difficile* were also reported using cell-free supernatants from other commercially available probiotics (Fredua-Agyeman et al. 2017). With respect to the competition for nutrients, some studies have been carried out using "synbiotic" combinations, which are mixtures of probiotics and prebiotic substrates that (theoretically) will improve the performance of probiotics or other beneficial microbes in the gut. In a mice (C57BI/6) model of CDI, the feeding with a synbiotic formulation, consisting of four strains (*L. plantarum* F44, *L. paracasei* F8, *B. breve* 46, *B. animalis* subsp. *lactis* 8:8) and three prebiotics (galacto-oligosaccharides, isomalto-oligosaccharides and resistant starch), conferred protection against this pathogen (Kondepudi et al. 2014). Some studies have suggested that the growth inhibition of *C. difficile* by probiotics is strain but also carbon source specific. Ambalam et al. reported the ability of cell-free supernatants from *L. paracasei* F8 and *L. plantarum* F44 to inhibit the growth of *C. difficile* strains when they grew on glucose, due to the production of organic acids and heat-stable antimicrobial proteins, whilst the effect was only pH-dependent when growing on prebiotics (Ambalam et al. 2015). Our workgroup recently analyzed the influence of carbon sources upon *C. difficile* growth and toxicity when co-cultured with *Bifidobacterium longum* IPLA20022 or *B. breve* IPLA20006 in the presence of short-chain fructo-oligosaccharides (scFOS) or inulin. The use of scFOS reduced the growth of the pathogen, as well as the toxicity of the co-culture supernatants, which was not observed with inulin (Valdés-Varela et al. 2016b).

4.2 Probiotics Against *C. difficile* Toxin Activity

The toxins produced by *C. difficile* are responsible for the clinical profile of the CDI. Therefore, therapeutic agents that reduce toxin-induced damage could be valuable tools to alleviate the severity of symptoms and to improve the course of the disease. Some authors have reported that probiotics are able to reduce the activity of *C. difficile* toxins but, in most cases, the specific mechanisms of action by which probiotics exert the protective effect in this infection is unknown (Fig. 2c). In a hamster model of enterocolitis induced by *C. difficile*, *Bifidobacterium bifidum* CIDCA5310 protected the animals, and avoided mortality, when compared with the control (infected) group; besides, the supernatants obtained from caecum contents were less toxics upon Vero (cells from monkey's kidney) cultures in animals fed with the bifidobacteria suggesting that this strain is able to in vivo counteract the effect of clostridial toxins (Trejo et al. 2013).

Co-culture of toxigenic strains of *C. difficile* with different strains of bifidobacteria and lactobacilli leads to a reduction of the cytotoxic effects of spent-culture supernatants on cultured Vero cells, which correlates with a diminution of clostridial toxins present in these supernatants (Trejo et al. 2010). However, the growth of clostridial strains in BHI medium with different concentrations of cell-free supernatants from bifidobacteria or lactobacilli cultures did not decrease the toxic effect of pathogens; taking into account these results, authors hypothesized that co-culture of clostridia with lactobacilli or bifidobacteria leads to the modification of the environment, thus leading to the repression of toxin synthesis/secretion pathway. Similarly, a cell extract from *L. acidophilus* GP1B was able to decrease the pathogenicity of *C. difficile* by inhibiting quorum sensing signaling, probably by lowering the expression of quorum sensing-regulated toxin genes (Yun et al. 2014).

On the other hand, it was observed that some microorganisms release metabolites that are able to inhibit the harmful effects of toxins. A

bacterial cell-free supernatant obtained from *L. delbrueckii* subsp. *bulgaricus* LDB B-30892 reduced cytotoxic effects of *C. difficile* ATCC9689 upon the human intestinal epithelial cell line Caco-2 (Banerjee et al. 2009). These authors suggested that bioactive components, of unknown nature, were released by this strain which were the probable causative agents of inhibition of the clostridial toxins. Similarly, bacterial cell-free supernatants obtained from *L. lactis* CIDCA8221 contained heat-sensitive metabolites, higher than 10 kDa, that were not affected by treatment with different proteases or proteases-inhibitors, which were able to inhibit cytotoxic effects of *C. difficile* toxins upon epithelial Vero cells (Bolla et al. 2013). These results suggest that the protective effect of *L. lactis* CIDCA8221 supernatant could be owing to a non-covalent interaction between molecules present in the lactococcal supernatant and toxins. In this regard, surface components of the bacterial cell envelope, such as exopolysaccharides which can be released to the environment, have been proposed to in vitro inhibit of adverse effect of pathogenic toxins (Ruas-Madiedo et al. 2010). A study showed the ability of the outermost (proteinaceous) S-layer from *Lactobacillus kefir* strains to inhibit the damage induced by supernatants obtained from *C. difficile* upon Vero cells; the protective effect was not affected by inhibitors of proteases or heat treatment, while pre-incubation with specific anti-S-layer antibodies reduced the inhibitory effect of these proteins (Carasi et al. 2012). From this study it was concluded that the capability for reducing the toxigenic effect of *C. difficile* could be attributed to an interaction between its toxins and the *L. kefir* S-layer protein (Carasi et al. 2012). Recently, our workgroup analyzed the capability of *Bifidobacterium* and *Lactobacillus* strains to reduce the toxic effect of supernatants obtained from *C. difficile* LMG21717 ($TcdA^+$, $TcdB^+$) culture upon the human intestinal epithelial cell line HT29. For this purpose, the probiotic candidates were incubated together with a toxigenic supernatant of *C. difficile* and the analyzed strains from *B. longum* and *B. breve* species were able to reduce the toxic effect of the pathogen; more specifically, the strain *B. longum* IPLA20022, in a viable state, showed the highest ability to reduce the levels of both clostridial toxins and to counteract the cytotoxic effect upon HT29 (Valdés-Varela et al. 2016a). Furthermore, the incubation of supernatant from *B. longum* IPLA20022 with the toxigenic *C. difficile* supernatant showed similar effect on the cell line than that obtained with the bifidobacterial biomass. The treatment of the clostridial supernatant with this probiotic strain prevented the rounding of HT29 cells, detected in cells treated only with *C. difficile* supernatant, thus keeping a monolayer structure resembling that of the control (non-treated HT29) (Fig. 3). Taking into account these results we hypothesize that the adsorption of toxins to the bifidobacterial surface and/or the secretion of molecules able to reduce the cytotoxic effect by degrading the toxins are both probable mechanisms of action (Valdés-Varela et al. 2016a). In this regard, 20 years ago it had been reported that *S. boulardii* inhibited *C. difficile* TcdA effects in the rat ileum by releasing a 54-kDa serine protease which hydrolyzed toxin A and its intestinal receptor (Castagliuolo et al. 1996); this could be the mechanism behind the effectiveness of this yeast in both, the prevention and the treatment of antibiotic-associated colitis in humans (Castagliuolo et al. 1999). More recently it was observed that a protease secreted by *Bacillus clausii* O/C is able to inhibit the cytotoxic effect of *C. difficile*, thus this enzyme could be involved in the protective effect of this bacilli in antibiotic-associated diarrhea (Ripert et al. 2016). A similar phenomenon may be taking place with the above mentioned *Bifidobacterium* strains (Valdés-Varela et al. 2016a).

4.3 Other Mechanisms of Action

The intestinal barrier function given, among other factors, by the presence of an intact intestinal epithelium enabling the absorption of nutrients and the exclusion of harmful substances can be compromised by the activity of enteric pathogens including *C. difficile* (Barreau and

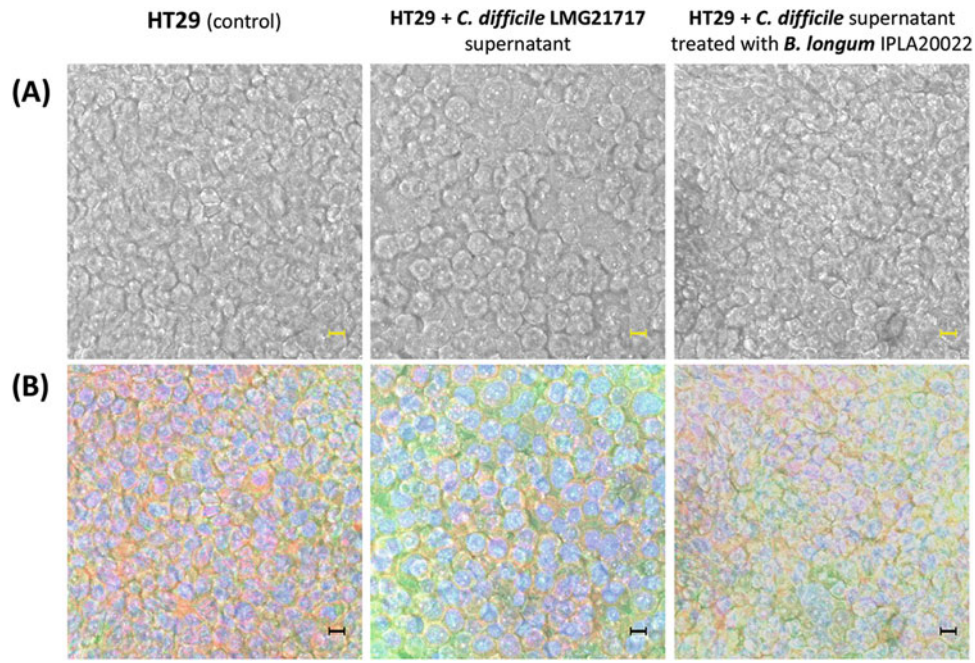

Fig. 3 CSLM (Leica TCSAOBS SP8 X confocal microscopy) images obtained, after 20 h incubation, for HT29 cells submitted to different treatments. (**a**) panel shows transmission (visible) images and (**b**) panel shows Z-projection snapshots resulting from a combination of the transmission image with the "blue" image, captured with the violet laser diode (excited at 405 nm, showing DAPI stained nucleus), the "red" image, captured with the white laser (excited at 578 nm, showing phalloidind-alexa-fluor-568 stained F-actin), and the "green" image resulting from the auto-fluorescence emitted by the intracellular components of HT29. The 63x/1.4 oil objective was used; bars 10 μm. Individual images of stained nucleus and/or F-actin were included in the reference Valdes-Varela et al. (2016a)

Hugot 2014). In fact, internalized clostridial toxins induce changes in the F-actin cytoskeleton and a breakdown of the tight junctions, thus contributing to the disruption of the epithelial barrier function; the increase in the permeability of this barrier ends with an inflammatory process due to the infiltration of neutrophils, production of chemokines and pro-inflammatory cytokines, and activation of mast cells and lymphocytes among other events (Voth and Ballard 2005; Rupnik et al. 2009; Abt et al. 2016). Thus some probiotics have been claimed to be able to reinforce the intestinal barrier function, although there is not much information in the context of CDI (Fig. 2d). In a hamster model of CDI, the oral administration of live *S. boulardii* 5-days before the infection significantly reduced cecal tissue damage, NF-κB phosphorylation and TNFα protein expression caused by different *C. difficile* ribotypes, thus indicating that this probiotic can prevent intestinal damage and inflammation (Koon et al. 2016). In fact, after a literature search conducted by Stier and Bischoff (2016) they found that mechanisms of *S. boulardii* action involve not only a direct effect on the pathogen or its toxins, but also impact on the innate and adaptive immune response of the host induced after CDI. Regarding probiotic bacteria, it has been shown that *L. rhamnosus* L34 and *L. casei* L39 are able to modulate, by different ways, the inflammation caused by *C. difficile*, thus making suitable the use of these vancomycin-resistant lactobacilli for treating CDI (Boonma et al. 2014). In our research group we have detected that lactobacilli strains are able to increase the synthesis of interleukin (IL)-8 and mucins by HT29-MTX monolayers challenged with *C. difficile*, thus helping to the reinforcement of the innate immune defense

(Zivkovic et al. 2015). More recently, a combination of *Lactobacillus helveticus* BGRA43, *Lactobacillus fermentum* BGHI14 and *S. thermophilus* BGVLJ1–44 was in vitro tested against *C. difficile* in a Caco-2 model and results showed an increase in the release of transforming growth factor (TGF)-β, thus resulting in a promising probiotic candidate to be further evaluated against CDI (Golic et al. 2017).

Finally, recombinant lactobacilli, although they cannot be considered as probiotics, could be suitable vehicles for the *in situ* production and delivery of therapeutic molecules in the intestine. In a recent study, it was explored the basis for an oral anti-toxin strategy based on engineered *Lactobacillus* strains expressing TcdB-neutralizing antibody fragments in the gastrointestinal tract; the results showed that only lactobacilli displaying the anti-TcdB variable domain of the heavy chain antibody can inhibit the cytotoxic effect of TcdB in the gastrointestinal tract of a hamster model (Andersen et al. 2016).

5 Conclusion and Future Trends

The search for probiotics with anti-*C. difficile* activity has been an active area of research for more than two decades. However, in spite of the abundance of in vitro studies, the in vivo evidence is less conclusive. The role of probiotics in preventing antibiotics-associated diarrhea is well established by several clinical intervention studies and meta-analyses. Good evidence is also available regarding the benefit of certain probiotics in the prevention of specific *C. difficile* diarrhea, being still necessary to define the best conditions for maximizing the efficacy. However, the studies on the use of probiotics in the treatment of CDI are still scarce; this is in spite of the several potential mechanisms of action that would be of interest in the case of *C. difficile* infection. Among them, the ability of certain strains to inhibit the growth of *C. difficile*, or to promote the restoration of the normal gut microbiota, represent two very direct potentially beneficial mechanisms of action. Moreover, specific probiotic strains have been found to be able to reduce the toxicity of this pathogen and/or to degrade the produced toxins. This inhibition of *C. difficile* toxicity may constitute an interesting strategy for the treatment of CDI by probiotics; first by eliminating the toxins from the intestine and, secondly, by the promotion of the microbiota restoration by the use of selected probiotic strains with both properties.

The existing clinical interest of CDI together with the successful application of FMT, allow foreseeing that the interest in the use for probiotic therapies, likely using defined combinations of strains, will continue rising during the next years. In this regard the development of products, based on the combination of strains with different properties and anti-*C. difficile* mechanisms of action, promises to allow the development of highly efficacy products for both prevention and treatment of CDI.

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Faecal Microbiota Transplantation as Emerging Treatment in European Countries

Marcello Maida, James Mcilroy, Gianluca Ianiro, and Giovanni Cammarota

Abstract

Clostridium difficile infection (CDI) is one of the most common healthcare-associated infections in the world and is a leading cause of morbidity and mortality in hospitalized patients.

Although several antibiotics effectively treat CDI, some individuals do not respond to these drugs and may be cured by transplanting stool from healthy donors. This procedure, termed Faecal Microbiota Transplantation (FMT), has demonstrated remarkable efficacy as a treatment for recurrent CDI.

FMT has also been investigated in other diseases and disorders where perturbations to the gut microbiota have been theorized to play a causative role in pathogenesis and severity, such as inflammatory bowel disease (IBD). Although FMT is currently not recommended to cure IBD patients in clinical practice, several studies have recently been carried out with promising results. The aim of future research is therefore to standardize protocols and develop FMT as a therapeutic option for these patients.

This review summarizes data on the use of FMT as a treatment for CDI and IBD, with special attention given to studies conducted in European countries.

Keywords

Clostridium difficile · European · Faecal microbiota transplantation · Fecal · Inflammatory bowel disease

1 Introduction

Gut microbiota is critical to health and functions and therefore emerges as a "virtual" organ with a level of complexity comparable to that of any other organ system. Fecal microbiota transplantation (FMT) is a medical treatment that aims to restore the normal gut microbiota in diseases or infections associated with bacterial imbalances. FMT has the

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M. Maida
Section of Gastroenterology, S.Elia – Raimondi Hospital, Caltanissetta, Italy

J. Mcilroy
School of Medicine, Medical Sciences and Nutrition, University of Aberdeen, Aberdeen, UK

G. Ianiro · G. Cammarota (✉)
Gastroenterological Area, Fondazione Policlinico Universitario Gemelli, Università Cattolica del Sacro Cuore, Rome, Italy
e-mail: giovanni.cammarota@unicatt.it

potential to compete with powerful antibiotics as a treatment strategy in several gastrointestinal disorders. *Clostridium difficile* infection (CDI) is one of the most common healthcare-associated infections in the world and is a leading cause of morbidity and mortality in hospitalized patients. Although several antibiotics effectively treat CDI, some individuals do not respond to these drugs and may be cured by FMT, which has demonstrated extraordinary efficacy for the cure of recurrent CDI (rCDI). FMT has also been investigated in other diseases and disorders where perturbations to the gut microbiota have been theorized to play a causative role in pathogenesis and severity, such as inflammatory bowel disease (IBD) (Ianiro et al. 2014; Cammarota et al. 2015a). The current therapeutic options for IBD have limitations with regards to cost, safety profile and the onset of drug resistance and dependence. There is therefore a need to develop novel therapeutic avenues that are both safe and effective to control the disease. Although FMT is currently not recommended to cure IBD patients in clinical practice (Cammarota et al. 2017), several studies have recently been carried out with promising results. The aim of future research is therefore to standardize protocols and develop FMT as a therapeutic option for these patients. This review summarizes data on the use of FMT for the treatment of both CDI and IBD, with special attention given to studies carried out in European countries.

2 Faecal Microbiota Transplantation for *Clostridium difficile* Infection

2.1 The Burden of *C. difficile*

CDI is the most common cause of hospital associated diarrhoea in the western world and is one of the leading causes of morbidity and mortality in hospitalized patients globally (Bagdasarian et al. 2015). CDI is highly prevalent in North America and Europe. A population-based study performed in the United States reported that there were 453,000 incidences of CDI in 2011 (Lessa et al. 2015) CDIs, 83,000 cases of first recurrences and an estimated number of deaths of 29,300 only in 2011 (Lessa et al. 2015). In Europe the extent of CDI is less clear. The burden of healthcare-associated CDIs in acute care hospitals has been estimated at 123,997 cases annually with a mortality of 3700 per year (European Surveillance of CDI 2015). A prospective study conducted in 2005 in 38 hospitals in 14 different European countries reported a mean incidence of nosocomial CDI of 2.45 per 10,000 patient-days (range 0.1–7.1) (Barbut et al. 2007). Beside this, a more recent and larger hospital-based survey performed through a network of 97 hospitals from 34 European countries, reported a higher CDI incidence of 4.1 per 10,000 patient days (Bauer et al. 2011).

Similar epidemiological data are observed in the eastern countries. A meta-analysis of 51 studies, showed similar rates of CDI in Asia compared to Europe and North America (Borren et al. 2017).

Beside this, epidemiological trends show that the incidence of CD has increased over recent decades. In the United States, reported cases of CDI doubled from 2000 to 2010 and are expected to increase further (Lessa et al. 2015). A recent retrospective cohort study that analysed more than 38 billion commercially insured patients in the United States showed that between the years of 2001 and 2012, the annual incidence of CDI and multiply recurrent CDI (mrCDI) per 1000 person-years increased by 42.7% (from 0.4408 to 0.6289 case) and 188.8% (from 0.0107 to 0.0309 case) respectively (Ma et al. 2017). However, it should be noted that these results may be biased by the selection of the only insured patients.

This raising in incidence and virulence of CD can been explained, at least in part, by inappropriate antibiotic usage, outbreaks of CDI in healthcare facilities, and the diffusion of fluoroquinolone-resistant strains belonging to the PCR-ribotype 027 (Warny et al. 2005; McDonald et al. 2005).

CDI infection is also palaces a significant economic burden on the health services. A recent

analysis of health-care associated infections in the United States ranked CDI fourth in terms of attributable costs and length of hospital stay (Zimlichman et al. 2013).

The bacterium *Clostridium difficile* (CD) is spread via the faecal-oral route. CDI generally requires two things: the presence (endogenous infection) or acquisition (exogenous infection) of CD and an altered composition of gut microbiota. Risk factors facilitating infection are older age, hospitalization, recent use of antibiotics, long-term therapy with proton pump inhibitors and chronic kidney disease (Asha et al. 2006; Mullane et al. 2013; Stevens et al. 2011).

Once the bacterium is present in the large intestine it proliferates, taking advantage of an impaired gut microbiota. The production of toxins create its main virulence factors. Toxin A (TcdA) and B (TcdB) induce mucosal inflammation, disruption of colonic epithelium with pseudomembrane formation resulting in lower abdominal pain, fever and diarrhea. Clinical pictures of CDI are variable and range widely from mild colitis to fulminant disease with associated toxic megacolon and death.

Diagnosis of CDI is established by the presence of (1) diarrhoea ($\geq$3 loose stools in 24 h), (2) ileus or toxic megacolon (3) confirmation of infection thought a stool test positive for CD or for A and B toxins, and/or endoscopic or histopathological picture of pseudomembranous colitis (Bagdasarian et al. 2015).

According to current guidelines (Surawicz et al. 2013; Debast et al. 2014), first line treatment of CDI includes rehydration and removing the inciting antibiotic. Following this, therapy with metronidazole, vancomycin or fidaxomycin should be considered. Unfortunately, despite administration of antibiotics, up to 60% of patients experience a recurrence (Cohen et al. 2010). A recently published study by Ma et al. (2017) has demonstrated the increasing incidence of multiply rCDI in the United States.

2.2 Faecal Microbiota Transplantation and *C. difficile*

In recent decades, FMT has been trialed as a treatment for rCDI and, over the years, a considerable body of evidence has emerged in support of its effectiveness. Consequently, FMT is recommended as a treatment option for rCDI in guidelines produced by the European Society for Microbiology and Infectious Disease and the American College of Gastroenterology (Surawicz et al. 2013; Debast et al. 2014). Furthermore, a recent European consensus conference on FMT was held with the aim of standardizing FMT guidance across Europe. According to the statements of the conference, FMT is recommended as treatment option for both mild and severe rCDI (Cammarota et al. 2017).

Three randomized controlled trials (RCTs) have been performed, to date, with the aim to assess the effectiveness of FMT compared to conventional therapy, two of the RCT's were conducted in European countries and one in Canada (Table 1). The first RCT was conducted in the Netherlands by van Nood et al. (2013). The group randomised 43 patients with rCDI to receive one of the following therapies: (1) vancomycin (500 mg orally four times per day for 4 days), followed by bowel lavage and subsequent FMT through a nasoduodenal tube; (2) vancomycin regimen with bowel lavage; (3) vancomycin regimen alone. The study was interrupted after the interim analysis. Among the first group 15/16 patients (94.1%) had a resolution of CDI, 13 patients after one infusion and 2 patients after multiple infusions. In contrast, resolution of CDI occurred in 4/13 patients (31%) receiving vancomycin alone and in 3/13 patients (23%) receiving vancomycin with bowel lavage ($p < 0.001$). There were no differences in adverse events among the three study groups.

In a second open-label RCT conducted in Italy, Cammarota et al. (2015c) randomised 39 patients to (1) FMT (short regimen of

Table 1 Characteristics of main studies assessing FMT for *C. difficile* infection in European and extra European countries

Author	Study level	Single/ multiple center study	Area	Sample	Age (mean or median)	Route of delivery	Frozen/fresh material	Fecal dosage (g/ml)	Follow-up (weeks)	Overall resolution rate after minimum follow-up
Studies from European countries										
MacConnachie et al. (2009)	RCS	Single	UK	15	81.5 (68–95)	Nasogastric tube	Fresh	30 g/150 ml	16	11/15
Garborg et al. (2010)	RCS	Single	Norway	40	75 (53–94)	Gastroscopy and colonoscopy	Fresh	50–100 g/ 250 ml	11	33/40
Polak et al. (2011)	PCS	Single	Czech Republic	15	82 (NR)	Nasojejunal tube	Fresh	20–30/NR	12	13/15
Mattila et al. (2012)	RCS	Single	Finland	70	73 (22–90)	Colonoscopy	Fresh	20–30 ml feces/ 100–200 ml water	12	66/70
Jorup-Ronstrom et al. (2012)	RCS	Single	Sweden	32	75 (27–94)	Colonoscopy and enema	Fresh	NR/30 ml	104	22/32
van Nood et al. (2013)	RCT	Single	Netherlands	16	73 (60–86)	Nasojejunal tube	Fresh	≥150/500 ml	10	15/16
Cammarota et al. (2015c)	RCT	Single	Italy	20	73 (29–89)	Colonoscopy	Fresh	152 ± 32 g/ 500 ml	10	18/20
Satokari et al. (2015)	RCS	Single	Finland	49	56.8 (20–88)	Colonoscopy or enema	Fresh and frozen	30 g/150 mL	12	47/49
Hagel et al. (2016)	RCS	Multiple	Germany	92	75 (59–81)	Gastroscopy, duodenal route, colonoscopy, capsule	Fresh, frozen (no separated efficacy data available)	NR	20	79/92
Ianiro et al. (2017)	PCS	Single	Italy	64	74 (29–94)	Colonoscopy	Fresh, frozen	120–180 g for fresh feces, 50 g for frozen feces/ 500 ml	8	62/64

Studies from extra European countries										
Hamilton et al. (2012)	PCS	Single	USA	43	58 (39–68)	Colonoscopy	Frozen	50 g/250 cc	8	41/43
Kassam et al. (2012)	RCS	Single	Canada	27	69.4 (26–87)	Enema	Fresh	150 g/300 cc	61	24/26
Brandt et al. (2012)	RCS	Single	USA	77	65 (22–87)	Colonoscopy	Fresh	300–700 mL	17	66/77
Kelly et al. (2014)	RCS	Multiple	USA	80	50 (6–88)	Upper and lower route (no data)	NR	NR	12	70/80
Khan et al. (2014)	RCS	Single	USA	20	66 (50–86)	Colonoscopy	Fresh	50 g/200 cc	24	20/20
Lee et al. (2014)	RCS	Single	Canada	94	72 (24–95)	Enema	Fresh	150 g/300 ml	24–96	81/94
Youngster et al. (2014b)	RCT	Single	USA	20	54.5 ± 24.2	Nasogastric tube and colonoscopy	Frozen	NR	8	18/20
Dutta et al. (2014)	PCS	Single	USA	27	64.5 (18–89)	Enteroscopy and colonoscopy	Fresh	25–30 g/180 mL 20.6 (enteroscopy) or 270 mL (colonoscopy)	80	27/27
Zainah et al. (2014)	RCS	Single	USA	14	73.4 (52–92)	Nasogastric tube and colonoscopy	Fresh	30–50 g stool. Total: 20–180 mL (NGT), 300–500 mL (colonoscopy)	14	11/14
Costello et al. (2015)	PCS	Single	Australia	20	64 (31–90)	Colonoscopy and push enteroscopy	Frozen	50 g/150 ml	12	20/20
Hirsch et al. (2015)	RCS	Single	USA	19	61 (26–92)	Capsule	Frozen	18–27 g/350 ml/ 8–12 capsules	12	17/19
Lee et al. (2016)	RCT	Multiple	Canada	178	72 (56–88)	Enema	Fresh, frozen	100 g/300 ml	13	171/178
Mandalia et al. (2016)	RCS	Single	USA	95	NR	Upper GI route, colonoscopy	NR	NR	12	93/95

(continued)

Table 1 (continued)

Author	Study level	Single/ multiple center study	Area	Sample	Age (mean or median)	Route of delivery	Frozen/fresh material	Fecal dosage (g/ml)	Follow-up (weeks)	Overall resolution rate after minimum follow-up
Meighani et al. (2016)	RCS	Single	USA	201	67 (49–85)	Nasogastric, enema, colonoscopy	NR	NR	12	176/201
Millan et al. (2016)	PCS	Single	Canada	20	68 (35–85)	Colonoscopy	Fresh, frozen (no separated efficacy data available)	NR	12 weeks	20/20
Tauxe et al. (2016)	RCS	Single	USA	28	77 (65–96)	Colonoscopy, nasogastric, nasoduodenal and nasojejunal tube, PEG	NR	NR	8–96 (mean 36)	27/28
Youngster et al. (2016)	PCS	Single	USA	180	64 (7–95)	Capsule	Frozen	48 g/30 capsules	8–24	168/180
Hota et al. (2017)	RCT	Single	Canada	30	75.7 ± 14.5	Enema	Fresh	50 g/500 ml	17	7/12
Staley et al. (2017)	RCS	Single	USA	49	62.3 ± 17.1	Capsules	Freeze-dried	~1 × 1011 cells/capsule	8	43/49

RCT randomized controlled trial, *PCS* prospective case series, *RCS* retrospective case series, *NR* not reported

vancomycin, 125 mg four times a day for 3 days, followed by one or more infusions of feces via colonoscopy) or (2) vancomycin (vancomycin 125 mg four times daily for 10 days, followed by 125–500 mg/day every 2–3 days for at least 3 weeks). As with Van Nood et al. this study was stopped at 1-year after interim analysis. The authors reported CDI resolution in 90% (18/20) of patients in the FMT arm compared to 26% (5/9) of patients in the vancomycin arm ($p < 0.0001$). There were no serious adverse events reported.

These RCTs show that FMT is safe, well tolerated and overperforms conventional antibiotic therapy. However, there are limitations to these studies that should be considered. These include small sample sizes and the early interruption of both the trials after interim analysis. In this regard, it is well known that RCTs stopped early for benefit can overestimate the magnitude of the treatment effect and underestimate the incidence of adverse events (Bassler et al. 2010).

The third RCT was conducted on a sample of 30 patients with rCDI that were randomly assigned in a 1:1 ratio to (1) a 14 day course of oral vancomycin followed by an FMT enema or (2) a 6-week oral vancomycin therapy. Resolution of infection within 120 days was reported in 7/16 (43.8%) patients receiving FMT and 7/12 (58.3%) receiving vancomycin, without significant differences in adverse events. The study was interrupted due to a futility analysis. In contrast to Van Nood et al. and Cammarota et al., a single FMT delivered by enema was not more efficacious than oral vancomycin as a treatment for rCDI (Hota et al. 2017). Weakness of this study include a small sample size and early interruption because of a futility analysis. In addition, the protocol did not include retreatment in the case of failure after first infusion, and this represents a limitation in assessing the overall effectiveness of FMT.

Finally, one of the most important issues to be raised is that all three studies evaluated FMT through three different routes of delivery (nasojejunal tube, colonoscopy and enema). This makes the studies challenging to compare and may explain, at least in part, the variability of results, since the route of administration may affect the treatment outcome.

To assimilate these data, a recent meta-analysis of 18 observational studies assessing FMT for CDI on a total sample of 611 patients, reported a primary cure rate of 91.2% (95% CI 86.7–94.8%), and an overall recurrence rate of 5.5% (95% CI 2.2–10.3%). Interestingly, a sub-analysis comparing the efficacy of lower vs. upper gastrointestinal delivery showed a greater primary cure rate for lower (93.2–95% CI, 88.7–96.7%) compared to upper gastrointestinal delivery (81.8–95% CI, 71.9–90.0%) ($p = 0.015$) (Li et al. 2016).

In line with this, a long-term retrospective multicenter observational study by the 'German Clinical Microbiome Study Group' (GCMSG), has been performed on a large sample of 133 rCDI with the aim to assess effectiveness of FMT performed trough different routes of delivery in Germany (Hagel et al. 2016). Patients receiving FMT by application into the rectum/colon/terminal ileum experienced a primary response of 89.6% on day 30 ($n = 43/48$) and 83.3% ($n = 25/30$) on day 90. For patients receiving FMT by application through gastroscopy, nasojejunal tube or capsule, the cure rates were 81% ($n = 60/74$) and 76.5% ($n = 49/64$) respectively. Despite inherent limitation deriving from the retrospective design, this study confirmed a trend towards higher response rates with FMT through the lower GI administrations.

Between lower routes, colonoscopy appears to be the most effective route of administration. Hamilton et al. (2012) reported a prospective analysis of 43 consecutive patients with rCDI, treated with frozen FMT by colonoscopy, showing an overall resolution rate of 95% (41/43 patients) after one or more infusions. Interesting, 30% of patients had underlying inflammatory bowel disease and FMT was equally effective in both groups.

A similar single-center prospective study performed by Cammarota et al. on a sample of 64 patients with rCDI reported that FMT delivered by colonoscopy was effective in 97%

(62/64) of patients after one or more infusion. The authors reported that only 30% of patients were cured after a single infusion, which highlights the importance of repeating infusions in the case of failure after first treatment. Multivariate analysis revealed that severe CDI (OR 24.66; 95% CI 4.44–242.08; p 0.001) and inadequate bowel preparation (OR 11.53; 95% CI 1.71–115.51; p 0.019) were found to be independent predictors of failure after single infusion (Ianiro et al. 2017).

Moreover, a retrospective analysis by Khan et al. (2014) reported a cure rate of 100% on a group of 20 patients with community and hospital-acquired relapsing and refractory CDI treated with FMT administered via colonoscopy. Finally, a retrospective analysis showed that the frequency of surgery in patients with CDI decreased after implementing FMT through colonoscopy for treatment of severe CDI (Cammarota et al. 2015b). Taken together, these studies support colonoscopy as an effective route of delivery for FMT without reporting any adverse events secondary to endoscopic technique or transplantation itself.

FMT can also be administered by enema, although this route appears to be inferior when compared to colonoscopy, especially if FMT is administered as a single infusion.

A retrospective study assessing 94 patients with recurrent or refractory CDI treated with FMT via enema reported that the primary resolution after a single infusion was 47.9% (45/94 patients) and 86.2% (81/94 patients) after multiple infusions (Lee et al. 2014). Similarly, another retrospective study of 26 cases of refractory CDI showed that 81% of patients (21/26) cleared the infection after first infusion and 92% (24/26) after multiple infusions (Kassam et al. 2012).

Despite the literature suggesting that lower GI administration may be superior, upper GI delivery of FMT is common worldwide. In a retrospective analysis of 40 patients with rCDI mainly treated with FMT administered by gastroscopy, by Garborg et al. (2010) reported a resolution rate of 82.5% (33/40 patients) within 80 days after the procedure. FMT by nasoduodenal tube has been tested in the previously cited RCT by van Nood et al. (2013), showing a resolution rate of rCDI in 94.1% of cases (16/17 patients). Furthermore, a randomized, open-label, 20 patient pilot study in patients with relapsing/refractory CDI, reported primary resolution of 60% (6/10 patients) by nasoduodenal tube and of 80% (8/10 patients) by colonoscopy after a single infusion, with an overall resolution rate after retreatment of 80% and 100%, respectively (Youngster et al. 2014b).

These data support the effectiveness of FMT. The observed variability of efficacy may be due to, at least in part, the methodical differences between studies (Table 1). Despite promising results, these routes of administration are still burdened by procedure-related risks and their invasive nature.

One innovative and non-invasive method of administration is through orally delivered FMT capsules. A retrospective analysis by Hirsch et al. (2015) assessed effectiveness of FMT by capsule on a sample of 19 patients with rCDI. Thirteen patients (68%) had resolution after a single instance of FMT treatment. Of six patients that did not respond to the initial treatment, four achieved cure after a subsequent infusion, resulting in a cumulative resolution rate of 89%. These results are similar to those reported from invasive transplantation procedures.

Similarly, an open-label, single-arm preliminary feasibility study (n = 20) was performed in order to evaluate the effectiveness and safety of frozen FMT capsules for the treatment of relapsing or rCDI. Healthy volunteers were screened as potential donors and FMT capsules were generated and stored at −80 °C. Patients received 15 capsules on two consecutive days, resulting in a overall 90% (95% CI, 68–98%) rate of clinical resolution after a 6 months follow-up, with no reported serious adverse events (Youngster et al. 2016a).

Similar results have been reached with encapsulated FMT using a freeze-dried preparation of microbiota resistant to a wide range of temperatures. Staley et al. (2017) tested this new delivery system on a group of 49 patients with rCDI showing a resolution rate of 88% (43/49 patients) after a 2 month follow-up. These

lyophilized preparations confer additional advantages over the standard encapsulated FMT. Namely, the preservation of viability and diversity of the taxonomic spectrum of microbiota and physicochemical properties that enable consistent encapsulation.

Taken collectively, these studies suggest that capsule delivered FMT is a non-invasive, safe and effective. However, larger prospective studies are needed to confirm these data.

A further point of consideration is how donations are prepared. Most of FMT's are performed with fresh stool, but there are logistical challenges associated with this method. On the contrary, frozen preparations offer several advantages, such as the immediate availability of FMT, the possibility of administering FMT at centers that cannot collect and process samples, a reduction in number and frequency of donor screenings and reductions in cost.

In previous years, some studies supported the use of frozen FMT for rCDI. However, no study included a direct comparison of frozen vs. fresh transplantation (Hamilton et al. 2012; Youngster et al. 2014a; Satokari et al. 2015) (Table 1).

To solve this problem, a recent RCT by Lee et al. (2016) was conducted with the aim of comparing frozen vs. fresh FMT. A large cohort of 232 adults with recurrent or refractory CDI was randomly assigned to receive frozen (n = 114) or fresh (n = 118) FMT by enema. The proportion of patients with clinical resolution was 83.5% for the frozen FMT group and 85.1% for the fresh FMT group by per-protocol analysis, (difference, −1.6% [95% CI, −10.5% to ∞]; $p = 0.01$ for non-inferiority). In the intention-to-treat analysis, the clinical resolution rate was 75.0% for the frozen FMT group and 70.3% for the fresh FMT group (difference, 4.7% [95% CI, −5.2% to ∞]; $P < 0.001$ for non-inferiority). There was no statistically significant differences in adverse events. This study confirms the non-inferiority of frozen as opposite to fresh FMT in terms of efficacy and safety.

In addition to these data, a recent meta-analysis of six studies showed that frozen FMT was as effective as fresh FMT, both after single infusion (65.0–95% CI 57.0%, 73.0% vs. 65.0–95% CI 57.0%, 73.0%, p = 0.962) and after multiple infusions (95.0–95% CI 91.0%, 99.0% vs. 95.0–95% CI 92.0%, 99.0%, p = 0.880) (Tang et al. 2017).

Based on these data, it appears that frozen and fresh FMT are equally effective and when considering the potential logistical and economic advantages, frozen FMT appears to be preferable.

In conclusion, faecal microbiota transplantation is a highly efficacious treatment for rCDI and is increasingly being used in Europe in accordance with recommendations from international practice guidelines (Surawicz et al. 2013; Debast et al. 2014).

Although a deal of evidence supports its effectiveness and safety, current FMT protocols differ in several aspects, including route of delivery, timing and number of infusions, dosage and methods of preparation (fresh or frozen) (Table 1). To date, no clear evidence supports the superiority of any individual protocol for the treatment of rCDI.

Latest literature suggests that lower administration via colonoscopy outperforms upper delivery routes. However, the recent introduction of FMT by oral capsules have proven to be effective and non-invasive. Capsules may expand the access to FMT in the future. As with routes of delivery, the method of preparation should be considered. Based on available evidence, the efficacy of frozen and fresh FMT is equivocal. However, in consideration of the potential logistical and economic advantages, frozen FMT is preferable.

Despite a wide availability of data from prospective and retrospective studies, future RCTs should compare the effectiveness of different routes of delivery and fresh vs. frozen FMT.

Moreover, it must be pointed out that many of the studies performed in the field of FMT suffer from methodological gaps. A systematic review of 85 studies assessing FMT showed that key components of FMT interventional studies, which are necessary to replicate and understand efficacy and safety results, are often poorly

reported (Bafeta et al. 2017). For example, 47% of studies did not report eligibility criteria for donors, 96% omitted materials and methods for the collection of stools, 76% did not clearly indicate methods used for the preparation and storage of stools, and 67% of studies did not specify the weight of stools used. These methodological gaps affect the interpretation and reproducibility of results.

Notwithstanding the above, a recent consensus conference standardised the modalities of FMT across European countries (Cammarota et al. 2017). This consensus report provides guidance on technical, regulatory, administrative and laboratory requirements for FMT. Nevertheless, future research must focus on the standardization of donor screening, processing and delivery techniques. This, coupled with strict monitoring by regulatory authorities, will be critical in improving efficacy and safety of FMT in Europe and beyond.

3 Faecal Microbiota Transplantation for Inflammatory Bowel Disease

The first reported use of FMT as a treatment intervention for inflammatory bowel disease (IBD) was published in 1989 by Bennet and Brinkman (1989). Bennet, who was both a patient and a clinician, reported clinical resolution of symptoms after a week of self-administered enemas. Despite these encouraging results, research into FMT and IBD was sparse for over two decades, with only scattered case reports and case series being published in the literature (Borody et al. 1989, 2001, 2003, 2011a, b). These studies were limited by small numbers of patients, vague methods of FMT preparation and poorly defined and inconsistent outcomes. Indeed, a systematic review of the available evidence published in 2012 consisted of only nine retrospective reports, which was deemed by the authors to be insufficient to perform a meta-analysis (Anderson et al. 2012). However, the landmark paper published by Van Nood et al. (2013) reporting FMT's efficacy in recurrent CDI galvanised the scientific and medical community to evaluate FMT's therapeutic potential in several other diseases and disorders associated with imbalances of bacteria within the intestinal tract, such as IBD, where the prospect of modulating the microbiota is supported by logical scientific reasoning and is conceptually appealing for patients seeking alternatives to immunomodulatory and immunosuppressive drugs.

There is now a large body of controlled and non-controlled evidence on the role of FMT in the IBD subtypes of Crohn's disease (CD) and Ulcerative Colitis (UC) and pouchitis. In comparison to the available data for CD and UC, the evidence for pouchitis is meagre and consists of two case reports (Fang et al. 2016; Schmid et al. 2017) that describe conflicting outcomes and two uncontrolled cohort studies (Landy et al. 2015; Stallmach et al. 2016) Each of the cohort studies have differing methodologies, endpoints and outcomes, which make the results challenging to integrate into the pouchitis treatment paradigm. Notably, in the only study that allowed for multiple FMT infusions, five out of five patients achieved a clinical response and four out of five achieved clinical remission (Stallmach et al. 2016). This suggests that more frequent dosing may be required to achieve the desired endpoint in pouchitis.

In Crohn's disease (CD), the quality of the available evidence is low, with the available literature consisting of case reports (Borody et al. 1989; Swaminath 2014; Gordon and Harbord 2014; Kao et al. 2014; Bak et al. 2017), or small cohort studies (Kahn et al. 2014; Cui et al. 2015a; Suskind et al. 2015; Vermeire et al. 2016; Wei et al. 2015; Vaughn et al. 2016; Goyal et al. 2016). Nevertheless, a recent systematic review and meta-analysis conducted by Paramsothy et al. (2017b) reported that 52% of the pooled proportion of CD patients achieved clinical remission during follow-up, which is in keeping with results published in a previous meta-analysis by Colman and Rubin (2014). Taken together, these results suggest that FMT could benefit patients suffering from

CD. However, limited sample sizes and significant differences in methodology between studies may have inflated the pooled effect size in the meta-analysis and therefore these results should be interpreted with caution.

The strongest evidence for FMT in IBD comes from four randomised controlled trials (RCTs) and a significant body of controlled and non-controlled cohort studies in patients (Table 2). The first cohort studies took place in Austria led by Angelberger et al. (2013) and Kump et al. (2013). The only European RCT was conducted in the Netherlands by Rossen et al. (2015), who randomised 50 adult patients suffering from active UC to undergo FMT from either a healthy donor or a patient's own stool (autologous FMT) as a placebo. The primary endpoint was clinical remission (simple clinical colitis activity index scores ≤ 2) combined with ≥ 1-point decrease in the Mayo endoscopic score at week 12. FMT was administered once through nasoduodenal tube at baseline and week 3. The authors reported that there was no statistically significant difference in clinical and endoscopic remission between the treatment arm and the autologous placebo arm of the study.

Moayyedi et al. (2015) randomised 75 adult patients suffering from active UC to receive weekly FMT or water enemas for 6 weeks and evaluated responses at week 7. The primary endpoint of the study was clinical remission, defined as Mayo Score of ≤ 2 with an endoscopic Mayo score of 0 at week 7. In contrast to Rossen et al. the faecal microbiota was frozen before use. FMT was found to induce remission in a statistically greater percentage of patients than placebo (24% vs. 5%; $p = 0.03$). Interestingly, the authors reported that stool from one donor (donor B) induced remission in 39% of patients, which was remarkably higher than that of the other donors (10%). This suggests that donor characteristics may influence the efficacy of FMT in UC, which gives rise to the alluring prospect of matching donors to recipients.

In the largest RCT to date, Paramsothy et al. (2017a) allocated 81 adult patients with active UC to receive to FMT or isotonic saline with added brown food colourant and odorant placebo. Study participants initially received a colonoscopic infusion as baseline followed by self-administered enemas five times per week for 8 week (a total of 40 FMTs). The primary end point of steroid-free clinical remission together with endoscopic remission (total Mayo score ≤ 2 points) was met in 11 of 41 (27%) of patients receiving FMT vs. 3 of 40 (8%) of patients receiving placebo ($p = 0.02$). In contrast to the previous two RCTs, each FMT was prepared using a mixture of faecal microbiota from three to seven unrelated donors. The authors noted that this approach was implemented in an attempt to maximise the microbial diversity of each FMT and the validity of this approach was confirmed using 16S rRNA phylogenetic analyses. However, in implementing this approach, practitioners increase the risk of infection transmission between donors and patients. Furthermore, combining donor samples masks any donor patient compatibility effect, and increases the complexity of tracking microbial colonisation post FMT.

Costello et al. (2017b) and colleagues allocated 73 adult patients with active UC to receive FMT prepared from a mixture of faecal microbiota from three to four healthy donors or autologous FMT (placebo). FMT was administered by colonoscopy at baseline followed by two enemas by day 7. The primary endpoint was steroid-free remission of UC as defined by a total Mayo score of ≤ 2 with an endoscopic Mayo score of ≤ 1 at week 8. In the intention to treat (ITT) analysis, 12/38 (32%) patients who received pooled donor FMT achieved the primary end point of steroid-free remission, as compared to 3/35 (9%) who received autologous FMT ($p = 0.02$). In contrast to the previous studies, the faecal microbiota was prepared in anaerobic conditions. Further research is required to establish if this is the optimal method of preparation for FMT in IBD. However, as the majority of the human gut microbiota are known to be strict anaerobes that die in the presence of oxygen, anaerobic methods of production may positivity influence bacterial viability (Chu et al. 2017). Seminal work published by Sokol et al. demonstrated that

Table 2 Characteristics of main studies assessing FMT for IBD

Study type	Author	FMT route	Sample	Frequency	Control/ comparison	Frozen/ vs. fresh	Definition of clinical remission or primary end point (RCT)	Clinical remission	Definition of clinical response or primary end point (RCT)	Clinical response
Cohort	Kunde et al. (2013)	Enema	10 (paediatric)	Once a day for 5 days	N/A	Fresh	PUCAI <10	3/9 (33%) at 1 week	PUCAI decrease of >15	7/9 (78%) at 1 week
Cohort	Cui et al. (2015b)	Endoscope to distal duodenum	15	1–2	N/A	Frozen	Montreal score 0	4/14 (29%)	Montreal improvement ≥1 and discontinuation of steroids	8/14 (57%)
Cohort	Kump et al. (2015)	Colonoscopy	17 (10 controls)	Once every 2 weeks for 5 weeks	Triple antibiotic therapy for 10 days	NR	Mayo ≤2	4/17 (24%)	Mayo drop ≥3	10/17 (59%)
Cohort	Wei et al. (2015)	Colonoscopy	11	1	N/A	Fresh	IBDQ >170, Mayo <2	6/11 (55%)	IBDQ increase >16, decrease in Mayo by >1	11/11 (100%)
Cohort	Karakan et al. (2016)	Colonoscopy	14	1–6	N/A	NR	NR	6/14 (43%)	NR	11/14 (78.5%)
Cohort	Zhang et al. (2016)	Endoscope	19	1	N/A	Fresh	Mayo ≤2 with no individual sub score ≥1	2/19 (11%)	Mayo drop ≥3 or ≥30% along with drop in bleeding sub score ≥1 or bleeding subscore ≤1	11/19 (58%)
Cohort	Wei et al. (2016)	Colonoscopy	20	1	FMT + oral pectin (5 days)	Fresh	Mayo ≤2	3/10 (33%) FMT, 4/10 FMT + Pectin 2	Reduction in the total Mayo score of >30% from baseline, a 1-point improvement in tarry stools, or an increase of >16 points in IBDQ criteria at week 12	7/10 (70%) FMT, 6/10 FMT + Pectin
Cohort	Ishikawa et al. (2017)	Colonoscopy	17	Single	19 FMT + triple antibiotic therapy	Fresh	Reduction in CAI ≥3 and CAI <10	14/19 (74%)	CAI < +3	6/19 (32%)

Cohort	Jacob et al. (2016)	Colonoscopy	20	12 (bi weekly for 6 weeks)	N/A	Frozen	Mayo ≤2 with no individual sub score >1	3/20 (15%)	Mayo drop ≥3 and a bleeding sub score of ≤1	7/20 (35%)
Cohort	Nishida et al. (2017)	Colonoscopy	41	1	N/A	Fresh	Mayo ≤2 with no individual sub score of 1 point or more	0/41 (0%)	Mayo </= 2 Reduction in Mayo ≥3 or reduction of Mayo ≥2 decrease in recal bleeding subscore of 1	11/41 (27%)
RCT	Rossen et al. (2015)	Nasoduodenal	23 (25 placebo)	One at baseline one at week 3	Autologous FMT	Fresh	Clinical remission and endoscopic improvement SCCAI ≤2 in combination with ≥1 point drop in combined Mayo endoscopic score	7/23 FMT (30%) vs. 5/25 (20%) placebo	≥1.5 point reduction in SCCAI	11/23 (48%) FMT vs. 13/25 (52%) placebo
RCT	Moayyedi et al. (2015)	Enema		Six enemas over 6 weeks	Saline	Frozen	Clinical and endoscopic remission Mayo <3 with endoscopic Mayo 0	9/38 (24%) FMT vs. 2/37 (5%) placebo	≥3 point reduction in Mayo score	15/38 FMT (39%) vs. 9/37 (24%)
RCT	Paramsothy et al. (2017a)	Colonscopy at baseline followed by enemas	41 (40 placebo)	Colonscopy at baseline followed by self administered enemas for 8 weeks (n = 40)	Discoloured and odoured water	Frozen	Total Mayo score ≤2, with all subscores ≤1, and ≥1 point reduction from baseline in endoscopy subscore	11/41 (27%) FMT vs. 3/40 (8%) placebo	Steroid- free drop in combined Mayo subscore for bleeding and stool frequency of ≥3	22/41 (54%) FMT vs. 9/40 (23%)Placebo

(continued)

Table 2 (continued)

Study type	Author	FMT route	Sample	Frequency	Control/ comparison	Frozen/ vs. fresh	Definition of clinical remission or primary end point (RCT)	Clinical remission	Definition of clinical response or primary end point (RCT)	Clinical response
RCT	Costello et al. (2017b)	Colonscopy at baseline followed by enemas	38 (35 placebo)	Colonscopy at baseline followed by one enema a week for 2 weeks	Autologous	Frozen	Total Mayo $\leq$2 with subscores of $\leq$1 for rectal bleeding, stool frequency and endoscopic appearance; and a $\geq$ 1 point reduction in endoscopic subscore	11/41 (27%) vs. 3/40 (8%) $P = 0.02$	$\geq$3 point reduction in Mayo score *or* $\geq$50% reduction from baseline in combined rectal bleeding plus stool frequency sub-scores	22/41 (54%) vs. 9/40 (23%) $P < 0.01$

RCT randomized controlled trial, *PCS* prospective case series, *RCS* retrospective case series, *NR* not reported

administering the anti-inflammatory commensal bacterium *Faecalibacterium prausnitzii* attenuates colitis in animal models (Sokol et al. 2008). *Faecalibacterium prausnitzii* is known to be highly oxygen sensitive. Therefore, it can be hypothesised that maintaining the viability of anaerobic bacteria during sample preparation may positivity influence the efficacy of FMT in IBD.

To integrate these data, Costello et al. (2017a) undertook a systematic review and meta-analysis of the four published RCT's. The authors reported that overall, remission was achieved in 39/140 patients (28%) in donor FMT recipients compared with 13/137 (9%) in placebo groups (OR: 3.67 95% CI: 1.82–7.39; $P < 0.01$). Interestingly, despite fundamental differences in the design of each trial, a robust microbial trend appears to emerge in responders. All authors report that faecal microbiota rich in butyrate-producing species from *Clostridium* cluster XIVa is associated with clinical remission.

Paramsothy et al. (2017b) performed a comprehensive systematic review assessing the efficacy and safety of FMT in IBD. The authors found that overall, FMT is a safe intervention in the short term, with the majority of adverse events being mild self-limiting gastrointestinal complaints. However, serious adverse events such as disease flares and *C. difficile* infection requiring colectomy have been reported (Cui et al. 2015a, b; Scaldaferri et al. 2015; Costello et al. 2017a, b). A case of aspiration pneumonia in a patient that received FMT through the nasogastric route was reported in one study (Vermeire et al. 2016). Mortality due to toxic megacolon and sepsis has also been reported (Grewal et al. 2016).

It is clear that FMT is effective at inducing remission in patients with active UC with few serious adverse events. There is however, currently insufficient data on long-term risks and efficacy. Each trial has several methodical differences that make the results challenging to integrate into clinical practice. Further research is required to optimise and standardised protocols. Furthermore, as FMT's mechanism of action in IBD has yet to be elucidated, it is incumbent on researchers to investigate the mechanistic underpinnings of this procedure through microbial analysis of donors and patients. The evidence for FMT in CD and pouchitis is less convincing and further research through RCTs is required to draw definitive conclusions. As of July 2017, there are active clinical trials of FMT in IBD ongoing in Finland, Czech Republic, France, Italy, Poland and Spain. These trials will play an integral role in shaping clinical guidelines and policy in Europe for this highly promising yet relatively unrefined medical treatment.

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Immunization Strategies Against *Clostridium difficile*

Jean-François Bruxelle, Séverine Péchiné, and Anne Collignon

Abstract

C. difficile infection (CDI) is an important healthcare- but also community-associated disease. CDI is considered a public health threat and an economic burden. A major problem is the high rate of recurrences. Besides classical antibiotic treatments, new therapeutic strategies are needed to prevent infection, to treat patients and prevent recurrences. If fecal transplantation has been recommended to treat recurrences, another key approach is to restore immunity against *C. difficile* and its virulence factors. Here, after a summary concerning the virulence factors, the host immune response against *C. difficile* and its role in the outcome of disease, we review the different approaches of passive immunotherapies and vaccines developed against CDI. Passive immunization strategies are designed in function of the target antigen, the antibody-based product and its administration route. Similarly, for active immunization strategies, vaccine antigens can target toxins or surface proteins and immunization can be performed by parenteral or mucosal routes. For passive immunization and vaccination as well, we first present immunization assays performed in animal models and second in humans and associated clinical trials. The different studies are presented according to the mode of administration either parenteral or mucosal and the target antigens, either toxins or colonization factors.

Keywords

C. difficile · Toxins · Colonization factors · Passive immunizations · Vaccines

1 Introduction

Clostridium difficile, recently reclassified as *Clostridioides difficile* (Lawson et al. 2016) is an anaerobic spore forming intestinal pathogen responsible for post-antibiotic diarrhea and pseudomembranous colitis (PMC) (Lawson et al. 2016). *C. difficile* infection (CDI) is characterized by a large spectrum of clinical signs from asymptomatic carriage to fulminant colitis. CDI is an important healthcare- but also community-associated disease causing almost half a million infections each year in the USA

J.-F. Bruxelle · S. Péchiné · A. Collignon (✉)
EA4043 Unité Bactéries Pathogènes et Santé (UBaPS), Univ. Paris-Sud, Université Paris-Saclay, Châtenay-Malabry Cedex, France
e-mail: jean-francois.bruxelle@u-psud.fr; severine.pechine@u-psud.fr; anne.collignon@u-psud.fr

(Lessa et al. 2015). Increased morbidity and mortality have been associated with the emergence of hypervirulent epidemic strains such as BI/NAP1/027 strains. Even if a decrease in prevalence of 027 strains in some European countries has been observed, CDI remains poorly controlled and 027 and other epidemic strains are still prevalent (van Dorp et al. 2016). A major problem is the high rate of recurrences, 20–30% after a first episode and up to 60% after a first recurrence (Shields et al. 2015). Thus, CDI is considered a public health threat and an economic burden.

CDI is most commonly triggered by disruption of the intestinal microbiota by antibiotics and subsequent intestinal colonization. *C. difficile* highly resistant spores serve the transmission agent. After contamination of the host, spores germinate in response to bile acids and glycine and resume vegetative growth. Then, vegetative forms colonize the gut thanks to several colonization factors. Finally, the toxins are released and led to diarrhea and colitis. Initial colonization is influenced by the intestinal microbiota, and *C. difficile* persistence in the gut is dependent on the microbiota and the host immune response (Péchiné and Collignon 2016). Persistence of spores in the gut associated with an altered microbiota and a poor immune response could be responsible for recurrences.

Guidelines for CDI treatment have been recently updated in America and in Europe (Cohen et al. 2010; Debast et al. 2014). The treatment of a first episode is well defined and is based on antibiotherapy, such as metronidazole, vancomycin or fidaxomicin depending on the severity of the episode. In case of recurrences, variable guidelines have been recommended and there is no firm consensus on optimal treatment. Novel therapeutic strategies are needed to prevent infection, to treat patients and prevent recurrences. If fecal transplantation has been recommended to treat recurrences, another key approach is to restore immunity against *C. difficile* and its virulence factors.

C. difficile studies are mainly performed in vivo in two different animal models, the hamster and mouse models. Hamsters are extremely susceptible to *C. difficile* and are used as virulence and protection model. In mice, several models have been described either in germ free or conventional animals. Mouse models are used to monitor intestinal colonization by *C. difficile* and also in virulence and protection assays (Best et al. 2012).

Here, after a summary concerning the virulence factors, the host immune response against *C. difficile* and its role in the outcome of disease, we review the different approaches of passive immunotherapies and vaccines developed to treat and prevent CDI.

2 Virulence Factors and Host Immune Response

The main *C. difficile* virulence factors are the toxins, especially TcdA and TcdB. However, surface proteins involved in the colonization process participate also to pathogenesis (Janoir 2016).

2.1 Surface Proteins and Colonization Factors

The first interaction between *C. difficile* and the host involves bacterial surface components. Some have been identified in *C. difficile* and shown to be involved in the colonization process.

2.1.1 Cell-Wall Proteins

The two S-layer proteins (SLPs) are the main components of the bacterial surface and form a crystalline array over the entire cell surface. The low molecular weight (LMW)-SLP, is surface exposed, involved in cell adherence and highly variable between strains (Eidhin et al. 2006). The high molecular weight (HMW)-SLP is anchored in the cell wall, involved in adherence to intestinal tissue and extra cellular matrix proteins and is conserved between strains (Karjalainen et al. 2001; Calabi et al. 2002). Ryan et al. have shown that SLPs interact with Toll-like receptor 4 (TLR 4) and induce a pro-inflammatory response (Ryan et al. 2011).

The Cwp66 protein also serves adhesin function (Waligora et al. 2001) with its surface exposed, highly variable and highly immunogenic C-terminal domain. The cystein protease Cwp84 is also surface exposed (Janoir et al. 2007; Chapetón Montes et al. 2013) and anchored in the cell wall through its C-terminal domain. The N-terminal domain contains the proteolytic site responsible for the cleavage of the SlpA precursor into the two SLPs (Kirby et al. 2009; Dang et al. 2010) and for the degradation of extracellular matrix components facilitating therefore bacterial spread (Janoir et al. 2007). Interestingly, the Cwp84 protease is conserved among *C. difficile* strains and has been shown to be immunogenic in humans (Péchiné et al. 2005b).

2.1.2 Flagellar Proteins

Flagellated and non-flagellated *C. difficile* strains have been described. Flagella are involved in motility, adherence to host cells and host signalling through TLR5 (Stevenson et al. 2015).

Tasteyre et al. have shown that naturally occurring non-flagellated strains are less adherent to mouse caecum than flagellated strains and that the flagellin FliC and cap protein FliD are able to bind to murin mucus (Tasteyre et al. 2000, 2001). More recently, it has been shown that *fliC* and *fliD* mutants in the 630Δerm strain displayed increased adherence to Caco2 cells compared to the parenteral strain. Thus, in 630Δerm genetic background, flagella do not seem to play a role in adherence (Dingle et al. 2011). In contrast, *fliC* and *fliD* mutants in *C. difficile* 027 strain R20291 displayed decreased adherence to Caco2 cells and mouse caeca suggesting a role of flagella in cell adherence and colonization (Baban et al. 2013). *C. difficile* flagellin FliC has been shown to activate an innate immune response via its interaction with TLR5 and activation of NF-κB signalling (Yoshino et al. 2013; Batah et al. 2016). Interestingly, Batah et al. demonstrated, in an animal model, a synergic effect of flagella and toxins in eliciting an inflammatory mucosal response (Batah et al. 2017).

In addition, toxin and flagellar genes are co-regulated in strains such as the 630 and not in others such as the R20291 (Baban et al. 2013). Thus, contribution of flagella to the pathogenic process is complex and could be different according to genetic background.

2.1.3 Other Surface Components

Other colonization factors have been characterized. The surface exposed fibronectin binding protein FbpA, (Hennequin et al. 2003; Barketi-Klai et al. 2011), highly conserved between *C. difficile* isolates interacts with fibronectin in host tissues. The heat shock protein GroEL, highly conserved, also serves adhesin function (Hennequin et al. 2001). The collagen binding protein CbpA with a N-terminal collagen-binding domain is surface-localized (Tulli et al. 2013); the lipoprotein CD0873, part of an ABC transporter, is surface-associated, displays significant adhesive properties and is immunogenic in patients (Kovacs-Simon et al. 2014); a secreted-zinc metalloprotease is able to cleave several host proteins such as IgA2, fibrinogen or fibronectin (Cafardi et al. 2013; Hensbergen et al. 2014). Other surface components include polysaccharides (PS) such as PS-I, PS-II. However, only PS-II is common to all strains of *C. difficile* (Ganeshapillai et al. 2008).

2.2 Toxins

2.2.1 TcdA and TcdB

Both toxins have the same ABCD domain structure: the binding, cutting and delivery domains acting sequentially to deliver the N-terminal glucosyltransferase domain (GTD) in the cytosol of enterocytes (Jank and Aktories 2008). This N-terminal domain glucosylates and inactivates the Rho-GTPases leading to actin cytoskeleton disruption, cell death and epithelial barrier disruption (Voth and Ballard 2005; Popoff and Geny 2011). The receptor binding C-terminal domain (RBD) is composed of combined repetitive oligopeptides (CROPs) that are responsible for binding to cell receptors (Dingle et al. 2008).

TcdA and TcdB, despite their similar structure, are immunologically distinct. Antibodies directed to TcdA are able to neutralize TcdA but fail to neutralize TcdB, and the opposite is true for antibodies directed against TcdB (Libby and Wilkins 1982). The two toxins display high variability especially in the C-terminal domain (Leuzzi et al. 2013).

Different recombinant fragments derived from TcdA and TcdB have been identified for the generation of neutralizing antibodies (Leuzzi et al. 2013; Maynard-Smith et al. 2014). The RBD of both TcdA and TcdB was first identified as an important antigenic motif (Lyerly et al. 1990; Sauerborn et al. 1997; Belyi and Varfolomeeva 2003). In contrast, the TcdA GTD induces low antibody responses (Leuzzi et al. 2013; Maynard-Smith et al. 2014). Several regions of TcdB induce neutralizing antibodies: the central region domain (Maynard-Smith et al. 2014), the RBD (Kink and Williams 1998) and the GTD (Libby and Wilkins 1982; Leuzzi et al. 2013).

The respective role of TcdA and TcdB in pathogenesis is a key question. One group concluded that TcdB is essential for virulence (Lyras et al. 2009; Carter et al. 2015). For the other group, both toxins are responsible for disease. Interestingly the full virulence of *tcdB* mutant was restored when it expressed the binary toxin in addition to TcdA (Kuehne et al. 2010, 2014). It seems wise to take into account both toxins TcdA and TcdB for immunization strategies.

2.2.2 Binary Toxin

An additional toxin, the binary toxin or *C. difficile* transferase (CDT) is produced by some strains (Perelle et al. 1997) such as the epidemic/hypervirulent BI/NAP1/027 strains. The CdtB component is involved in toxin binding to host cells. The CdtA catalytic component ADP ribosylates actin and leads to inhibition of actin polymerization, depolymerization of actin filaments and cell rounding. In addition, formation of microtubule-based protusions leads to enhanced adherence (Schwan et al. 2009; Papatheodorou et al. 2011; Schwan et al. 2014). Of note, there are naturally occurring TcdA-TcdB- CDT+ strains, which can be responsible for diarrhea in humans (Eckert et al. 2015).

2.3 Host Humoral Immune Response Against *C. difficile*

Several authors assessed the immune response to *C. difficile* surface components and toxins (Péchiné and Collignon 2016).

Regarding surface proteins, SLPs are highly immunogenic. The LMW-SLP is an immunodominant antigen, as demonstrated by the presence of antibodies against this protein in sera of patients infected by *C. difficile* (Cerquetti et al. 1992; Wright et al. 2008). Drudy et al. found that antibody levels to SLPs were similar in patients with CDI, asymptomatic carriers and controls. However, patients with recurrences failed to mount an efficient IgM immune response to SLPs compared to patients with a single episode of CDI (Drudy et al. 2004).

The adhesin Cwp66, the protease Cwp84, the flagellar proteins FliC and FliD and the Fbp protein were found to be expressed during the course of infection and to be immunogenic. Most patients with CDI developed antibodies to FliC, FliD, Cwp84 and Cwp66 C-terminal domain, confirming the expression of these surface proteins during the course of the disease (Péchiné et al. 2005a). In another study, serum antibody levels were compared in a CDI patient group with a control group. For the adhesins Cwp66 and FbpA, the protease Cwp84, and the FliC and FliD flagellar proteins, the mean level of total antibodies were statistically lower in the CDI group than in the control group suggesting a role of these antibodies in CDI occurrence (Péchiné et al. 2005b).

Concerning *C. difficile* PS, two studies in CDI patients have reported a humoral immune response specific to *C. difficile* PS. Oberli et al. detected PS-II specific IgA in CDI patient stools and Martin et al. detected PS-I specific IgA and IgG in CDI patient stools and sera respectively (Oberli et al. 2011; Martin et al. 2013).

Regarding toxins, TcdA and TcdB have been shown to be immunogenic. In some studies, a correlation was found between anti-TcdB antibodies and asymptomatic carriage or absence of recurrence. Whereas in others studies, anti-TcdA antibody levels were shown to be more significant. Viscidi et al. found that antibody levels to TcdB were higher in sera of convalescent CDI patients than in sera of controls (Viscidi et al. 1983). Another study showed a correlation between clinical recovery without relapse, high TcdB IgG titers, and/or neutralizing antibodies (Aronsson et al. 1985). Kyne et al. monitored antibody response to *C. difficile* toxins and non-toxin antigens over time in hospitalized patients (Kyne et al. 2000). Although 15–31% of high-risk hospitalized patients were colonized with *C. difficile*, only a minority developed symptomatic infection. The asymptomatic carriers had significantly higher serum IgG antibody levels to TcdA within 3 days of colonization than those who developed diarrhea. So, after contamination by *C. difficile* a rise in IgG antibody to TcdA resulted in asymptomatic colonization rather than symptomatic infection. Interestingly, serum IgG levels against TcdB and non-toxin antigens were also higher in asymptomatic carriers, but the difference was not statistically significant. The same group also observed that patients with a single episode of CDI had significantly higher levels of IgM against TcdA, TcdB and non-toxin antigens by day 3 of illness compared to patients who later developed recurrent CDI. These patients had also significantly higher levels of circulating IgG against TcdA by day 12. After adjusting for other risk factors, patients with CDI and a low level of seric IgG against TcdA had a 48-fold greater risk of recurrence (Kyne et al. 2001).

Besides circulating antibodies, neutralizing anti-TcdA IgA in stools have been detected (Kelly et al. 1992). Warny et al. showed that fecal anti-TcdA IgA titers were significantly higher in patients who suffered a single episode compared to those relapsing (Warny et al. 1994). Jonhson et al. found that anti TcdA secretory IgA (sIgA) titers were higher in the intestinal secretions of CDI convalescent patients compared to non carrier subjects (Johnson et al. 1992). Anti-TcdA sIgA could inhibit toxin binding to intestinal receptors (Kelly et al. 1992; Warny et al. 1994). Low levels of fecal IgA and reduction in colonic IgA-producing cells associated with the gut mucosa have been shown to be associated with prolonged CDI and recurrences of infection (Johal et al. 2004). For Islam et al. in the early course of CDI (<72 h), low specific sIgA titers against TcdB but not TcdA were associated with susceptibility to disease (Islam et al. 2014). The mucosal immunity to TcdB may be particularly important in the early stages of infection.

All these results demonstrated that the adaptative host immune response plays a role in disease presentation and outcome.

A better knowledge of *C. difficile* pathogenesis and the host response has paved the way to the development of several antibody-based products (AP) and passive and active immunization strategies have been developed for the prevention and/or treatment of CDI (Mizrahi et al. 2014).

3 Passive Immunization Strategies with Antibody-Based Products

Passive immunization strategies are designed in function of the target antigen (*C. difficile* toxins or surface proteins), the antibody-based product (AP) and its administration route (oral or parenteral).

Toxins, as key virulence factors, represent the first studied target for passive immunization. However, such a strategy does not act on the bacterial clearance and consequently neither on colonization nor dissemination of *C. difficile* in the environment. Another rational strategy is to target the whole bacterium or its surface proteins.

In passive immunization strategies, AP must be present in the intestinal lumen in order to act directly against *C. difficile*. If administered via a parenteral route the AP should have a low immunogenicity, a good bioavailability and should be transferred from the systemic circulation to the intestinal lumen. If directly administered via the

oral route, the AP faces digestion process. In both cases, the main issue of passive immunization strategy resides in pharmacokinetic properties of the AP.

Currently, the progress on antibody engineering enables to design a variety of AP ranging from polyclonal antibodies through monoclonal antibodies and various antibody fragments such as heavy-chain single domain antibodies (V_HH). Therefore, the concomitant choices of the target, the administration route, and the variety of AP explain the diversity of studies dealing with passive immunization strategies against *C. difficile*.

3.1 Assays in Animal Models

3.1.1 Parenteral Administration of Antibody-Based Products in Animal Models

Polyclonal Antibodies Against Toxins

First, polyclonal antibodies were used in passive immunotherapy against *C. difficile*. In 1982, Libby and Wilkins were the first to demonstrate that passive immunization of mice with specific rabbit antiserum against toxins protected mice against the homologous toxin but not the heterologous toxin (Libby and Wilkins 1982). In another animal model, Giannasca et al. showed that intraperitoneally (i.p.) injection of mouse antitoxin antibodies before challenge protected hamsters in a dose-dependent manner against *C. difficile* (Giannasca et al. 1999). Robert et al. produced polyvalent anti-toxin antibodies in sheep, the i.p. administration of this anti-serum to hamsters after challenge with different strains of *C. difficile* was protective in a dose-dependent manner (Roberts et al. 2012).

Monoclonal Antibodies Against Toxins

Then, monoclonal antibodies (MAbs) have been produced and tested in different models. Due to their high specificity and homogeneity, MAbs target a specific epitope. Corthier et al. developed a passive immunotherapy targeting specifically *C. difficile* toxins in monoxenic mice with MAbs (Corthier et al. 1991). Intravenous (i.v.) administration of a mouse monoclonal IgG targeting TcdA C-terminal repeating units was able to protect mice against lethal *C. difficile* infection. After administration, the MAb titer remained high for at least 8 days, and mice were fully protected against *C. difficile* while no mouse survived in the control group. Of note, no impact on *C. difficile* colonization in passively immunized mice was observed.

In order to develop passive immunotherapy in humans and decrease toxicity, monoclonal antibodies have been humanized (HuMAbs).

First, fully HuMAbs directed against either TcdA or TcdB were produced and studied by Babcock et al. (2006). These HuMAbs recognized the RBD of TcdA and TcdB respectively. After characterization, anti-TcdA CDA1 and anti-TcdB MDX1388 were selected for protection assays in animal models. In a classic infection model, hamsters were treated i.p. with CDA1, MDX1388 alone or in combination for 4 days before challenge with *C. difficile* spores. In a relapse model, treatment with CDA1 and/or MDX1388 was associated with a vancomycin treatment. CDA1 alone led to early partial protection compared to controls without treatment. However, this protection did not persist. MDX1388 administered alone did not lead to protection. Interestingly, compared to controls, combination therapy with CDA1 and MDX1388, leading to neutralization of RBD of both toxins, provided better and prolonged protection in both models. These antibodies were shown to neutralize the toxin effects of diverse and clinically relevant strains of *C. difficile*, including multiple isolates of the BI/NAP1/027 and BK/NAP7/078 strains (Hernandez et al. 2015). In addition, Babcock et al. reported that levels of circulating HuMAbs in hamsters were much lower than anticipated and that 10% of the hamsters had no detectable circulating antibodies after a total of 200 mg of antibody i.p.-administered. They suggested that it could be due to the inefficient transport of human antibodies from the peritoneum into the bloodstream, or that some hamsters developed an immune response to the human antibody leading to their rapid clearance.

Then, others produced HuMAbs targeting toxins based on the sequence of CDA1 and MDX1388 (Péchiné et al. 2017). For instance, Davies et al., have developed a mixture of three humanized IgG1 MAbs (UCB MAbs), of which one neutralized TcdA and two TcdB (Davies et al. 2013). The UCB MAbs showed high potency in a variety of in vitro binding and neutralization assays. Compared to CDA1 and MDX1388, UCB MAbs led to higher levels of protection in their hamster model of CDI, and displayed higher valencies of toxin binding. Interestingly, pharmacokinetic and biodistribution assays of i.p. administered humanized IgG1 in non-infected hamsters showed that antibody half-life in serum was about 6 days. MAbs were detectable in healthy hamster colon (about 28 ng/ml per cm of mucosa ±17) 7 days after i.p. administration of about 2 mg of humanized IgG1. This persistence may likely explain the levels of protection provided by these UCB MAbs. Qiu et al. developed anti-TcdA and anti-TcdB HuMAbs from murine MAbs candidates. Administered parenterally, they were able to protect animals in a dose dependent manner against mortality (85% of hamster survival after *C. difficile* challenge) and to reduce the severity and duration of diarrhea associated to several *C. difficile* clinical strains (Qiu et al. 2016).

These RBD specific MAbs block toxin activity by inhibiting receptor binding and subsequently internalization in epithelial cells. Another way to block toxin activity is to target the N-terminal domain either the translocation domain (TD) or the glucosyltransferase domain (GTD). Indeed, these domains are more conserved between *C. difficile* strains and therefore represent targets for AP against a broader range of clinical strains. For Anosova et al., the combination of three fully HuMAbs, one specific to the RBD of TcdA, and two specific for the GTD of TcdB protected hamsters from CDI (Anosova et al. 2015).

These studies showed that by targeting both toxins protection can be increased. However in these animal models MAbs were administered with several doses a few days before challenge, therefore mimicking either a prophylactic strategy or an established circulating antibody response against toxins. Circulating antibodies may reach the intestinal mucosa either via passive transudation from the blood, or via the FcRn-mediated antibody transport. Moreover, toxin-mediated epithelium damages facilitate antibody transfer.

Monoclonal Antibody Subunits Against Toxins

With the development of antibody engineering, various MAb fragments have been produced and tested (Péchiné et al. 2017).

In particular, V_HH fragments or nanobodies, which correspond to the N-terminal region of a single variable (VH) domain from camel heavy chain antibody appear promising.

Yang et al. and Schmidt et al. developed two neutralizing, tetravalent, antibodies composed of V_HHs targeting both TcdA and TcdB (designated ABA and VNA2-Tcd) (Yang et al. 2014; Schmidt et al. 2016). ABA and VNA2-Tcd, two chimeric multivalent APs were composed of two V_HHs recognizing the GTD and translocation domain (TD) of TcdA respectively, and two V_HHs recognizing the GTD of TcdB. After i.p. administration, both protected against CDI in different animal models (mice and gnotobiotic piglets but not hamsters). Moreover, ABA was able to neutralize toxins from a panel of genotypically diverse $TcdA^+$ $TcdB^+$ clinical isolates, including some BI/NAP1/027 strains. However, to increase protective efficacy of parenterally administered V_HH, its serum half-life has been improved by developing a replication-deficient recombinant adenovirus expressing the heteromultimeric V_HH-based agents (ABA and VNA2-Tcd). This strategy to optimize delivery has shown its efficacy to neutralize toxins and to prevent CDI.

Of note, V_HHs against the two fragments of CDT have been constructed but neutralization

properties have only been studied in vitro (Unger et al. 2015).

Polyclonal and Fragments Antibodies Against Surface Proteins

Another approach is to target colonization factors, which may help to eliminate colonizing bacteria. Few studies have tested parenteral passive immunization targeting colonization factors with polyclonal antibodies. For instance, Malderelli et al. targeted *C. difficile* pilin with anti-PilW serum but did not obtain protection in mice against *C. difficile* (Maldarelli et al. 2016). Ghose et al. produced polyclonal antibodies targeting the *C. difficile* flagellin FliC (Ghose et al. 2016b). Passive immunization of mice via i.p. route with anti-FliC hyper-immune serum was able to protect 80% of treated mice against *C. difficile* after lethal challenge. Since FliC plays a key role in the pathogenesis ranging from bacterial colonization through immunomodulatory effects and gene regulation, protection elicited by anti-FliC antibody may involved various mechanisms. Kandalaft et al. described the production of V_HHs targeting the SLPs (Kandalaft et al. 2015). They were only studied in vitro but surprisingly, a combination of three V_HHs targeting the LMW-SLP inhibited motility.

3.1.2 Mucosal Administration of Antibody-Based Products in Animal Models

Lyerly et al. used a bovine immunoglobulin G (IgG) concentrate (BIC) from gestating cow's colostrum vaccinated with *C. difficile* formalin inactivated culture filtrate to orally passively immunize hamsters (Lyerly et al. 1991). BIC contained high levels of neutralizing IgG specific to both toxins and probably to other antigens. Treated hamsters were completely protected from the disease during treatment period compared to controls. However, treated hamsters developed diarrhea and died after treatment cessation. These results showed for the first time that passive immunization by oral route against *C. difficile* targeting mainly toxins can protect against toxin toxicity.

Van Dissel et al. used in the hamster model an immune whey protein concentrate (Immune WPC-40; Mucomilk) containing high concentration of sIgA antibodies against the whole bacterial cell and TcdA and TcdB (van Dissel et al. 2005). Immune WPC-40 conferred 80–90% protection in hamsters challenged with a toxigenic *C. difficile* strain. In contrast to Lyerly et al., the protection was maintained in surviving hamsters after treatment cessation for at least 28 days. These authors suggest that sIgA directed against the whole bacterial cell may reduce *C. difficile* gut colonization and promote bacterial clearance.

Otherwise, Kink et al. tested in therapeutic or prophylactic strategy, neutralizing avian antitoxin antibodies (IgY) directed against the C-terminal domain of TcdA or TcdB administered orally to hamsters (Kink and Williams 1998). Prophylactic treatment before challenge with anti-TcdA alone was efficient to protect hamsters from CDI. However, for therapeutic treatment, co-administration of anti-TcdA and anti-TcdB after *C. difficile* challenge was necessary to fully protect hamsters.

Targeting the colonization factors may protect against early stage of *C. difficile* infection. O'Brien et al. showed that anti-serum directed against SLP administered orally to hamsters was able to delay mortality after lethal challenge with *C. difficile* compared to untreated hamsters (O'Brien et al. 2005).

The main issue of passive immunization by oral route concerns the AP stability in the digestive environment. Immunoglobulins (Igs) have to resist to acidity in the stomach, and to pancreatic enzymes in the small intestine. However antibody sensitivity to digestive enzymes depends on antibody isotype. For instance, IgG1 are more susceptible to hydrolysis by pepsin than IgG2. Trypsin, preferentially digests bovine IgG1 and IgG2 than IgM, whereas chymotrypsin preferentially hydrolyzes IgM than IgG (de Rham and Isliker 1977; Brock et al. 1977). Notably, sIgA are more resistant than IgG to degradation in the stomach and intestine

(Fagarasan and Honjo 2003). This emphasizes the importance of isotype selection for oral passive immunization. Nevertheless, to improve orally administrated AP half-life, a specific formulation and/or vectorization may be recommended to maintain activity and to target *C. difficile* in the colonic infection site.

To extend life time of orally AP against *C. difficile* toxins, Andersen et al. engineered a *Lactobacillus* strain in order to express cell wall-anchored TcdB-neutralizing antibody fragments (V_HH) (Andersen et al. 2015). In a prophylactic treatment in a hamster model, oral administration of a combination of two *L. paracasei* strains expressing two different V_HHs conferred a partial (50%) protection against lethal *C. difficile* challenge. Hamsters showed either no damage or limited inflammation of the colonic mucosa after 4 days of *C. difficile* infection although they were colonized by *C. difficile*.

3.2 Assays in Humans and Clinical Trials

In humans, passive immunotherapy against *C. difficile* has been considered mainly to prevent recurrences.

3.2.1 Parenteral Administration of Antibody-Based Products in Humans

Treatment with Polyvalent Immunoglobulins

In 1991, passive immunotherapy administered to humans against CDI consisted of human polyvalent gamma globulins (Leung et al. 1991). Children with chronic recurrent CDI presented a deficient level of anti-TcdA IgG and IgA, and i.v. administration of gamma globulins (IVGG), every 3 weeks (400 mg/kg) was able to increase anti-TcdA IgG level in serum and was associated with resolution of clinical symptoms and clearance of toxins in stools. It was then confirmed in two adults with severe PMC receiving either 300 mg/kg or 200 mg/kg of IVGG; both patients presented a rapid resolution of diarrhea, pain and fever within 36 h. The efficacy of IVGG was correlated with the presence of anti-TcdA neutralizing IgG. The mechanism of action of IVGG in treatment for CDI is most likely explained by exudation of serum proteins across an already inflamed colonic mucosa. Then, IVGG were tested in many patients with CDI with various results (Diraviyam et al. 2016). Recently, Negm et al., in 17 CDI patients, observed a therapeutic response to polyvalent i.v. immunoglobulins (IVIg) in 41% (10/17) of the patients (Negm et al. 2017). In addition, they observed differences in TcdA neutralizing efficacy between three commercial IVIg preparations as well as differences of level of specific IgG isotypes against *C. difficile* antigens. These results emphasize the diversity of polyvalent immunoglobulins either regarding isotype or specificity.

Clinical Trials with Monoclonal Antibodies

After successful assays in animal models, CDA1 and MDX1388 targeting TcdA and TcdB RBD respectively, were selected and tested in clinical trials (Table 1).

A phase I with CDA1 in healthy volunteers was completed (Taylor et al. 2008). Single injection of CDA1 at different doses did not lead to serious adverse events nor anti-human antibodies production. The half life of CDA1 ranged from 25 to 31 days. Two phase II clinical trials were performed. In the first phase II, CDA1 was tested in patients receiving standard-of-care (SOC) treatment for CDI and compared to a placebo group. The recurrence rate was not significantly different between the two groups (Leav et al. 2010). A second phase II trial (sponsored by University of Massachusetts and Medarex Inc.) tested the efficacy of the combination of CDA1 (actoxumab, MK-3415) and MDX1388 (bezlotoxumab, MK-6072) in patients with symptomatic CDI with SOC antibiotics (NCT00350298) (Lowy et al. 2010). The recurrence rate was significantly lower in the patient group treated with this combination (7%) compared to placebo group (25%). Two phase III trials have been completed under Merck licence (MODIFY I NCT01241552 and MODIFY II NCT01513239). They were randomized, double-blind, placebo-controlled trials

Table 1 Passive immunization strategies: antibody-based products in clinical development

AP	Clinical trial/ sponsor	Mode of administration	Outcome measures	Results	References ClinicalTrials.gov
Fully HuMAbs	Phase I CDA1	Single i.v. injection different doses: 0.3, 1, 5, 10 and 20 mg/kg	Safety	No serious adverse effect	Taylor et al. (2008)
	Healthy subjects		PK	No HAHA	
				Half-life 25.3–31.8 days	
CDA1 (MK-3415, actoxumab): anti RBD of TcdA MDX1388 (MK-6072, bezlotoxumab, Zinplava™): anti RBD of TcdA	Phase II CDA1	Single i.v. injection (10 mg/kg) with SOC in CDI patients vs placebo	Recurrence	No significant difference between the two groups	Leav et al. (2010)
	CDI patients				
	Phase II CDA1, MDX1388. CDI patients	Single i.v. injection of CDA1 + MDX1388 (10 mg/kg each) with SOC in CDI patients vs placebo	Recurrence	Significant lower recurrence rate in treated group vs placebo (7% vs 25%)	NCT00350298
	Univ of Massachusetts-Medarex				Lowy et al. (2010)
	Phase III MODIFY I[a] CDI patients ≥18 years	Four arm study in CDI patients with SOC	Recurrence	Significant lower recurrence rate vs placebo with:	NCT01241552
	MK-3415 (CDA1)	CDA1 (10 mg/kg)		MDX1388 (17% vs 28%)	
	MK-6072 (MDX1388)	MDX1388 (10 mg/kg)		CDA1+MDX1388 (16% vs 28%	
	Merck Sharp&Dohme (MSD)	CDA1+MDX1388 (10 mg/kg each) vs placebo			
	Phase III MODIFY II (completed). CDI patients ≥18 years	Three arm study in CDI patients with SOC	Recurrence	Significant lower recurrence rate vs placebo with:	NCT01513239
		MDX1388 (10 mg/kg)		MDX1388 (16% vs 26%)	Wilcox et al. (2017)
	MK-3415 (CDA1)	CDA1+MDX1388 (10 mg/kg each) vs Placebo		CDA1+MDX1388 (15% vs 26%). Addition of CDA1 did not improve efficacy	
	MK-6072 (MDX1388)				
	MSD				
	Phase III MODIFY III (not yet recruiting)	Single infusion MK-6072	Safety		NCT03182907
		Children with CDI 1–17 years with SOC vs placebo	Tolerability		
	MK-6072 MSD		PK		

AP antibody-based product, *SOC* standard of care antibiotic treatment, *PK* pharmacokinetics, *HAHA* anti human human antibody

[a]Discontinued for CDA1

conducted at 322 sites in 30 countries involving 2655 adults receiving SOC antibiotics for primary or recurrent CDI. The primary end point was recurrent infection. MODIFY I was a four-arm study: patients were randomly assigned to receive a single dose of bezlotoxumab (10 mg/kg), actoxumab (10 mg/kg), actoxumab plus bezlotoxumab (10 mg/kg each), placebo. Interestingly, this clinical trial did not show a significant efficacy of actoxumab alone on recurrence

rate. Consequently, actoxumab alone was discontinued after an interim analysis and not evaluated alone in MODIFY II. Thus MODIFY II is a three-arm study: bezlotoxumab (10 mg/kg), actoxumab plus bezlotoxumab (10 mg/kg each), placebo. In both trials, the recurrence rate was significantly lower with bezlotoxumab alone or combined with actoxumab than with placebo. However, addition of actoxumab to bezlotoxumab did not improve efficacy, which may attest a major role of TcdB in the pathogenesis (Wilcox et al. 2017). The rate of adverse events was similar among the three groups; the most common events were diarrhea and nausea. The use of a single dose was supported by the long half-life of the MAbs (approximately 19 days). To date, bezlotoxumab (Zinplava ™) is the only AP against *C. difficile* i.v. administered approved for clinical use by the Food and Drug Administration and the European Medicines Agency. It is indicated to reduce recurrence of CDI in patients 18 years of age or older who received SOC antibiotic treatment for CDI and are at high risk for recurrence. Another phase III trial in children (1–17 years of age) (MODIFY III) is ongoing but not yet recruiting.

Systemically administered AP targeting the toxins can protect from recurrent CDI. However, it does not confer a locally oriented and a long lasting protection against *C. difficile*. To date, no clinical trial has evaluated parenteral passive immunization targeting *C. difficile* colonization factor. Circulating antibodies are poorly transferred through a healthy intestinal mucosa and are most likely to encounter *C. difficile* after epithelium disruption. So, investigators have assessed the protective efficacy of orally administered AP to target toxin and *C. difficile* colonization in the intestinal tract.

3.2.2 Mucosal Administration of Antibody-Based Products in Humans and Clinical Trials

Van Dissel et al. tested WPC-40 orally in 16 patients with confirmed CDI, among them 7 had a first episode of CDI treated with SOC antibiotics and 9 had a history of relapses (van Dissel et al. 2005). No adverse effect was observed during the follow-up. In addition, no toxin was detected in feces after treatment in 14 out of 15 patients and *C. difficile* could no longer be cultured from the stools in 9 out of 15 subjects. Interestingly, none of the patients experienced another episode of CDI after treatment during the follow-up. In accordance with this study a larger cohort was conducted in 101 patients with CDI (median age 74 years). After completion of at least 10 days of antibiotic treatment, patients received orally WPC for 2 weeks and were followed during 60 days. Interestingly, only 10% relapsed within the follow-up. A phase 2 clinical trial has been performed and completed but the results are not posted (NCT00177775).

After a phase I, Mattila et al. performed a double-blind phase II study comparing another *C. difficile* immune whey IgG concentrate (CDIW) with metronidazole for recurrent CDI (Mattila et al. 2008). CDIW was produced from colostrum of cows immunized with formalin inactivated *C. difficile*. Patients included in the study were adults who experienced at least two episodes of CDI. No statistically significant differences were observed between the two treatment groups. At the end of study 8 patients out of 18 experienced a relapse in CDIW (44%) treated group and 9 out of 20 in Metronidazole group (45%). These authors suggest that partial failure may probably be explained by a weak neutralization of colonization and toxin activity in vivo. These results emphasize the need of a well-engineered AP with high specificity and neutralizing activity in the infection site.

To conclude, the development of passive immunization strategies against *C. difficile* has led to many interesting AP targeting toxins but only a few targeting colonization factors. APs are developed mainly for the prevention of recurrences associated with SOC antibiotic treatment against CDI. Currently, the most efficient strategy to prevent recurrences is to target and neutralize toxins systemically. Even well tolerated, these treatments are costly and do not confer a long lasting protection. In addition passive immunization strategies targeting toxins alone do not influence intestinal colonization

nor prevent epidemic burden. To obtain a long term protection vaccines have been developed.

4 Active Immunization Strategies: Vaccines

Active immunization strategies are characterized by the type of the induced antibody response and depend on the targeted antigen, the administration route and regimen.

Vaccine candidates must be immunogenic to induce an antibody response. Obviously this antibody response must be protective against *C. difficile*. The targeted antigen must be specific to *C. difficile* and conserved among diverse clinical strains. It must be biologically accessible to the antibody produced, restricting vaccine candidates to surface and released or secreted antigens. Anti-toxin antibodies are associated with protection against CDI and recurrences. Thus, as in passive immunization strategies, toxins represent the first target studied for vaccination against *C. difficile*. However, to prevent colonization, and therefore to limit dissemination of bacteria in the environment, surface antigens represent an interesting alternative.

Protective antibody response to *C. difficile* can be either systemic or mucosal. The immune response depends on the administration route and the adjuvant (Zhang et al. 2015; Savelkoul et al. 2015). Here, we discuss the different vaccination strategies against *C. difficile* targeting toxins or colonization factors tested in animal models and in humans and clinical trials after vaccination by parenteral or mucosal routes.

4.1 Vaccines Targeting Toxins

4.1.1 In Animal Models

Parenteral Immunization in Animal Models

Usually, both toxins are simultaneously used as vaccine antigens. The first generation of vaccine was composed of formalin-inactivated toxins (toxoids) from culture filtrates, then toxoids were purified. Libby et al. and Fernie et al. obtained a full protection of hamsters against *C. difficile* after immunization with inactivated culture filtrate with Freund adjuvant (Libby et al. 1982; Fernie et al. 1983). In contrast, other authors (Kim et al. 1987) observed that toxoid A was sufficient to protect hamsters against *C. difficile*. However, differences in vaccination regimen, antigen purity and *C. difficile* strain could explain the discrepancies between studies. Recently, a highly purified toxoid vaccine targeting TcdA and TcdB, adjuvanted with alum, has been developed and first tested in animal models (Anosova et al. 2013). Intramuscular (i.m.) immunization of hamsters was protective against mortality and disease in a dose-dependent manner, with 90% of protection with the highest dose tested. The protection was correlated with a neutralizing toxin-specific IgG response. These promising results in animal models using toxoids in parenteral vaccination against *C. difficile* have led to the development in clinical trials, which will be further discussed.

In a second generation of vaccine, alternatives to toxoids were investigated with vaccines based on non-toxic recombinant fragments of *C. difficile* toxins such as the antigenic C-terminal RBD. Sauerborn et al. first used recombinant *C. difficile* TcdA C-terminal domain in a subcutaneaous (s.c.) immunization assay in mice with Freund's adjuvant (Sauerborn et al. 1997). Seven out of ten immunized mice were protected against a lethal dose of TcdA, correlated with production of anti-TcdA antibodies. Then, different combinations of recombinant toxin fragments have been used in parenteral immunizations. For instance, hamsters were immunized via i.p. route with a combination of fragments adjuvanted with MF29, the RBD of TcdA associated either with TcdB GTD or TcdB RBD fragment (Leuzzi et al. 2013; Spencer et al. 2014). These combinations induced systemic IgGs, which neutralized both toxins and protected vaccinated hamsters from a lethal challenge of various *C. difficile* ribotypes. Karczewski et al. obtained full protection of hamsters after i.m. immunization with toxoid A and B adjuvanted with aluminum

hydroxyphosphate sulfate and ISCOMATRIX™. Only partial protection was obtained with full length toxoid A combined with different fragments of TcdB (the enzymatic domain + different fragments of the C-terminal domain) (Karczewski et al. 2014). To reduce the antigenic cocktail, fragments can be fused and the larger fusion protein may benefit of an increased immunogenicity. Tian et al. constructed a recombinant fusion protein composed of TcdA and TcdB RBD fragments (Tian et al. 2012). This fusion protein was shown to be immunogenic in mice after i.m immunization and the produced antibodies were able to neutralize toxin cytotoxicity. In addition, in the hamster model full protection against *C. difficile* challenge was observed. Wang et al. constructed a chimeric atoxic toxin constituted of inactivated GTD and TD of TcdB and the RBD of TcdA (cTxAB) (Wang et al. 2012). A rapid and potent neutralizing antibody response against both toxins was induced after parenteral immunization of mice with cTxAB adjuvanted with alum. cTxAB parenteral immunizations protected mice from a primary infection and relapses as well, thus conferring a long lasting protection against *C. difficile*.

With the emergence of hypervirulent strains such as BI/NAP1/027 strains producing the binary toxin, it could be of interest to broaden vaccine protective efficacy. Secore et al. (2017) recently described a tetravalent vaccine composed of recombinant inactivated TcdA and TcdB and binary toxin components CDTa and CDTb adjuvanted with ISCOMATRIX i.m-administered in hamsters. The addition of CDT to TcdA and TcdB significantly improved vaccine efficacy against BI/NAP1/027 strains. Interestingly, they observed that this tetravalent vaccine was able to elicit neutralizing antibodies against the three toxins in hamsters and in Rhesus macaques. Another group targeted TcdB variants, TcdA and CDT (Tian et al. 2017). They produced two fusion proteins, a trivalent one with CDTb/$TcdB_{(003)}$/TcdA (T-toxin) and a quadravalent one with CTDb/ $TcdB_{(003)}$/TcdA/ $TcdB_{(027)}$ (Q-toxin). They i.m. immunized mice and hamsters with T-toxin or Q-toxin with alum as adjuvant and showed that these vaccinations induced toxin neutralizing antibodies to each of the toxins and a broad protection in hamsters against *C. difficile* 630 (ribotype 003) and *C. difficile* ribotype 027 strains. Taken together these studies showed the interest of multivalent fusion proteins as vaccine antigens.

To increase antigen immunogenicity, polypeptides with immunoadjuvant properties can be fused to the targeted antigen. For instance, Ghose et al. constructed a fusion protein constituted of the *Salmonella enterica* serovar Typhimurium flagellin subunit D1 as an innate immune agonist and the RBDs of TcdA or TcdB as targeted antigen (Ghose et al. 2013). After i.p immunization, mice displayed more anti-TcdA IgA and the same level of anti TcdB IgA in stools than mice immunized with unfused antigens adjuvanted with alum or heat-labile enterotoxin. Concerning the circulating IgG response, immunization with the fusion protein induced a higher anti-TcdA response than non-adjuvanted unfused antigens. However, the same level of anti-TcdA response was observed with the fusion protein and adjuvanted unfused antigens.

Parenteral vaccination with non-toxic recombinant vaccines showed promising results in animal models. Of note, these fragment vaccines have several advantages compared to toxoid vaccine, such as elimination of the potential risk of incomplete toxoid inactivation, large-scale production in a cost effective way and decrease of batch-to-batch variations (Wang et al. 2015).

In the third generation of vaccine, vectorized antigens and DNA vaccines targeting *C. difficile* were investigated. DNA vaccines are versatile, stable and easy to produce. DNA vaccines facilitate antigen presentation and enable proper protein folding for correct epitope presentation. In addition, DNA vaccines have the ability to induce both humoral and cellular immune response and a good immune priming (Saade and Petrovsky 2012). Gardiner et al. were the first to test DNA vaccination against *C. difficile* (Gardiner et al. 2009). A synthetic gene TxA-RBD optimized for expression in human cells was constructed. Mice were inoculated by

electroporation with the TxA-RBD expressing plasmid and then challenged with a lethal dose of purified TcdA. Treated mice were fully protected and presented a strong serum anti-TcdA IgG antibody response. Jin et al. (2013) screened the immunogenicity of various toxin fragments by DNA vaccination. Antibody response was elicited by two DNA vaccines, one expressing fragment of the TcdA RBD (TcdA-C), the other expressing the TcdB catalytic N-terminal domain (TcdB-N). Passive transfer in mice of immune serum elicited with both TcdA-C and TcdB-N fully protected mice against a lethal dose of *C. difficile* concentrated culture filtrate. Baliban et al. constructed an optimized DNA vaccine encoding the RBD of TcdA and TcdB and showed that i.m followed by electroporation in mice and non-human primates was able to promote a strong serum IgG but not IgA response associated with neutralizing IgG antibodies to both toxins in blood and interestingly in stools (Baliban et al. 2014). In addition, immunized mice were significantly protected against *C. difficile*. Seregin et al. designed an adenovirus-based vaccine targeting TcdA (Seregin et al. 2012). This vaccine induced a rapid and strong antibody response and a T cell response against TcdA, which led to full protection in mice after *C. difficile* challenge. DNA vaccines benefit from several advantages but also display disadvantages such as poor immunogenicity in humans and need to be further optimized to be used in clinical trials (Saade and Petrovsky 2012; Khan 2013).

All these three generations of parenteral vaccines targeting toxins aim to induce a systemic response. However these studies did not report the induction of a parallel mucosal immune response. Even if, anti-toxin circulating IgG antibodies against *C. difficile* can be protective they are less likely effective locally on the early step of infection. In contrast, mucosal IgA response would be more likely able to rapidly act locally on the infection site.

Mucosal Immunization in Animal Models

Torres et al. to identify the best route of immunization for a protective vaccine against *C. difficile* in hamsters, compared mucosal (intra-nasal i.n., intra-rectal i.r., intra-gastric i.g.), parenteral (s.c, i.p) and a combination of mucosal and parenteral routes (i.n. and i.p.) with formalin-inactivated culture filtrate of toxigenic *C. difficile* with CT as adjuvant for mucosal immunizations and RIBI for parenteral immunizations (Torres et al. 1995). Immunizations via i.n., s.c. and i.p. routes led to full protection. Similarly, Giannasca et al. in a vaccination strategy targeting toxins tested several immunization routes. The optimal protection was obtained with combined i.m and i.r routes (Giannasca et al. 1999).

Inducing mucosal immunity via the mucosal route encounters many difficulties. Antigens have to cross mucosal surface to be uptaken by immune cells. In addition, the mucosal immune system is closely interacting with the intestinal microbiota resulting in important regulation and immune tolerance (Chen and Cerutti 2010; Xiong and Hu 2015). Adjuvants and vectorization are key factors to modulate the mucosal immune system and develop a mucosal vaccine (Lavelle 2005).

Even if after i.n immunization the intestinal mucosa can be stimulated through mucosal homing, it is not the optimal route to induce an intestinal antibody response compared to the oral route. Antigen vectorization is recommended for oral immunizations. Ryan et al. used a live attenuated bacterial vector for oral immunization (Ryan et al. 1997). A live attenuated *V. cholerae* strain was used, expressing a fusion protein consisting of the C-terminal RBD of TcdA fused to the secretion signal of *E. coli* hemolysin A as secretion system, co-administered with CT as adjuvant to orally immunize rabbits. Vaccination induced an anti-TcdA IgG response but did not significantly induce an IgA response. However, in an ileal loop challenge assay, this vaccination was protective against TcdA. Permpoonpattana et al. used *Bacillus subtilis* spores as vehicle to orally deliver the carboxy-terminal repeat domains of TcdA alone (amino acids 2388–2706) or with TcdB (amino acids 2137–2366) in mouse and hamster vaccination assays (Permpoonpattana et al. 2011). Such a strategy was able to induce

a neutralizing and protective mucosal IgA and systemic IgG response. Of note, neutralizing sIgA antibodies to the TcdA repeat domain were shown to be cross-reactive with the analogous domain of TcdB. In this study, antibodies against TcdA provide protection against challenge with A+/B+ toxigenic *C. difficile* strains. Recently, Hong et al. expressed in *Bacillus subtilis* spores the same carboxy terminal domain of TcdA (A26–39) and immunized hamsters by oral and sublingual routes (Hong et al. 2017). Hamsters were protected after challenge with *C. difficile* strain 630. In addition, protection was associated with the absence of *C. difficile* toxins and spores in fecal samples and high level of anti $TcdA_{26–39}$ fecal IgA and circulating IgG. The authors suggest that antibodies induced by $TcdA_{26–39}$ cross-react with seemingly unrelated proteins expressed on the vegetative cell surface or spore coat of *C. difficile*. Another approach developed by Guo et al. used a *Lactococcus lactis* strain to express the TcdA and TcdB RBD (Guo et al. 2015). After oral administration in mice, animals were partially protected against *C. difficile* challenge correlated with an IgG and sIgA specific response in immunized mice.

Live vaccines benefit from the ideal features of an effective mucosal delivery system such as antigen protection from degradation, antigen delivery to mucosal surface, antigen uptake by target cells, and expression of potent immunostimulatory molecules.

4.1.2 In Humans and Clinical Trials

The positive results of animal vaccination assays constitute a proof of concept for the development of human vaccines (Table 2).

Parenteral Immunization in Humans and Clinical Trials

Safety, immunogenicity and dose response of the highly purified formalin inactivated toxoid A and B vaccine, previously tested in hamsters, have been assessed in human volunteers. After i.m injection with alum as adjuvant, vaccination was well tolerated and volunteers developed neutralizing anti-toxin antibodies in serum (Kotloff et al. 2001). Then, Sougioultzis et al. tested this vaccine in three patients with recurrent CDI (Sougioultzis et al. 2005). After vaccination, the patients discontinued treatment with oral vancomycin without any further recurrence.

First, this toxoid vaccine was developed by Acambis (ACAM-CDIFF™) and then by Sanofi-Pasteur (CDIFFENSE™). Four phases I clinical trials have been completed (NCT00127803, NCT00214461, NCT00772954, NCT01896830) (Greenberg et al. 2012). Safety and tolerability were observed associated with a good immunogenicity.

A phase II trial for therapeutic use assessed safety and efficacy against recurrences. The objective was to compare the event rate of CDI in groups assigned to the vaccine versus placebo in the 9-weeks period after the third dose of the study vaccine in subjects with first episode of CDI receiving antibiotics SOC (NCT00772343). A phase II trial for prophylactic use assessed safety and efficacy against CDI. The objective was to evaluate in adults at risk of CDI in all dose groups of the vaccine versus placebo, the safety and the immune response (NCT01230957) (de Bruyn et al. 2016). No safety issue was observed. In addition, vaccination induced a strong neutralizing IgG response specific to both TcdA and TcdB. The best antibody response was induced after 3 immunizations with the highest dose (100 μg) adjuvanted with alum. This formulation elicited this immune response at least for 180 days. A phase III clinical trial is ongoing. It is recruiting 10,000 adults (≥50 years of age) at risk for CDI to assess the efficacy to prevent primary symptomatic episode (NCT01887912). However, on December 1, 2017, a press release indicated that Sanofi decided to end the CDIFFENSE vaccine clinical development.

In parallel, Pfizer (USA) develops a genetically modified full length TcdA and TcdB toxoid vaccine. A Phase I clinical trial in healthy adults (50–85 years of age) has been completed and assessed safety, immunogenicity by testing a three-dose vaccination regimen by i.m route with one of three dose levels of *C. difficile* vaccine with or without alum as an adjuvant (NCT01706367). Only mild to moderate local reactions and systemic events could be observed. No clear dose-level response pattern was

Table 2 Vaccines in clinical development

Vaccine sponsor	Clinical trial	Mode of administration	Outcome measures	Results	References ClinicalTrials.gov
	Phase I (completed)	i.m. injection (Days 0, 28 and 56 or 0, 28) of different formulations of vaccine (low, medium, high-doses) alum adjuvanted vs placebo vaccine	Safety immunogenicity	No safety concerns	NCT00127803
	Healthy adult volunteers (18–55 years) and healthy elderly subjects (≥65 years)			100% seroconversion in volunteers 18–55 years for TcdA	NCT00214461
					NCT00772954
					NCT01896830
			Tolerability	Lower seroconversion rate for TcdB	Greenberg et al. (2012)
Highly purified formalin inactivated full length TcdA and TcdB, alum adjuvanted	Phase II for therapeutic use (completed)	i.m. injection (Days 0, 28 and 56 or 0, 28) of different formulations of CDIFF (low and high dose with adjuvant, high dose without adjuvant) vs placebo (4 arms)	Recurrence	No results posted	NCT00772343
	116 Subjects with first CDI treated SOC (18–85 years)		Safety immunogenicity		
ACAM-CDIFF™	Phase II for prophylactic use (completed)	i.m. 3 dose injection of either one of 4 different formulations (with and without adjuvant) vs placebo on one of 3 different schedules (7 arms)	Safety	No safety concerns	NCT01230957
Acambis CDIFFENSE™	Subjects (40–75 years) at risk of CDI		Immunogenicity	Good immunogenicity	de Bruyn et al. (2016)
			Efficacy against primary CDI		
Sanofi-Pasteur	Phase III (recruiting)	i.m. 3 dose injection (Days 0, 7 30) vs placebo	Efficacy against primary CDI	No results posted	NCT01887912
	Subjects at risk of CDI ≥50 years		Immunogenicity, safety		
Genetically modified full lenght TcdA and TcdB toxoids	Phase I (completed)	i.m. 3 dose injection different doses with/without adjuvant (alum) vs placebo	Safety	Generally safe and well tolerated	NCT01706367
	Healthy adult volunteers (50–85 years)		Immunogenicity	No clear dose response. Good immunogenicity	NCT02052726
					Sheldon et al. (2016)
Pfizer	Phase II (completed)	i.m. 3 doses (Day 1, 8, 30) high dose, low dose vs placebo (3 arms)	Safety	Generally safe and well tolerated	NCT02117570
	Healthy adult volunteers (50–85 years)		Tolerability		
			Immunogenicity		
	Phase II (ongoing but not recruiting participants)	i.m. 3 doses on 1 of 2 schedules vs placebo (6 arms)	Safety	No results posted	NCT02561195
			Tolerability		
	Healthy adults 65–85 years		Immune response		
	Phase III (recruiting participants) Adults ≥50 years	Vaccine vs placebo (2 arms)	Efficacy: CDI and recurrence	No results posted	NCT03090191

(continued)

Table 2 (continued)

Vaccine sponsor	Clinical trial	Mode of administration	Outcome measures	Results	References ClinicalTrials.gov
Recombinant fusion protein consisting of truncated TcdA and TcdB	Phase Ia/Ib (completed)	i.m. 4 injections (Day 0, 7, 28, 56) of two different doses with or without adjuvant (alum)	Safety, immunogenicity,	Good safety and tolerability	NCT01296386
	Ia healthy subjects 18–65 years		Dose response	Highly immunogenic for TcdA and TcdB	Bézay et al. (2016)
	Ib elderly ≥65 years				
VLA84 (formerly IC84 intercell) Valneva Austria	Phase II (completed)	i.m. injections (Day 0, 7, 28) different doses with or without alum vs placebo	Dose confirmation, immunogenicity, safety	Results of seroconversion rates and neutralization antibodies	NCT02316470
	500 healthy adults ≥50 years				
CDVAX Inactivated Bacillus spores expressing a toxoid antigen and a spore colonization factor	Phase I (terminated)	Oral vaccine	Safety, mucosal and systemic immunogenicity	No results posted	NCT02991417
Royal Holloway Univ. Cutting S.M.	Healthy adults 18–50 years				

SOC standard of care antibiotic treatment

detected. Vaccination induced potent antitoxin neutralizing immune response in all groups, still evident in immunized subjects at month 12 (Sheldon et al. 2016). A phase II trial assessed the safety and tolerability of the three doses vaccine in healthy adults aged 50–85 years (NCT02117570). Another phase II trial is ongoing to study the safety, tolerability and the subjects' immune response to the vaccine. Two different vaccine schedules will be compared (low dose and high dose). Each subject will initially receive three doses of vaccine or placebo, then 1 year after the third dose subjects that did not receive placebo will be randomized to receive a fourth dose. Subjects will be followed for up to 4 years after their third vaccination (NCT02561195). A phase III is currently recruiting (NCT03090191) and will assess efficacy against primary CDI and recurrence.

Valneva (Austria) announced positive Phase I results for its *C. difficile* vaccine candidate VLA84 (formerly IC84), a recombinant fusion protein consisting of truncated forms of TcdA and TcdB. Phase Ia/Ib trials showed good safety and tolerability profile of the vaccine (Bézay et al. 2016). VLA84 was highly immunogenic and was able to induce similar immune responses to TcdA and TcdB in adults and elderly subjects (NCT01296386). A dose-confirmation, immunogenicity and safety study in 500 healthy adults (≥50 years) in a phase II trial has been completed (NCT02316470).

All these three vaccines aim to induce a systemic antibody response against both toxins and showed promising results and could elicit long lasting protection. However today, no vaccine has been approved for clinical use.

Mucosal Immunization in Humans and Clinical Trials

Currently, only one trial was performed and aimed to elicit both mucosal and systemic

immune response to *C. difficile*. A phase I clinical trial under the supervision of Cutting S.M. (Royal Holloway University) and funded by the European Union 7th Framework Programme assessed the safety and immunogenicity of an oral vaccine against *C. difficile* in healthy adults (CDVAX). Their approach was a novel mucosal vaccine delivery system based on the use of inactivated *Bacillus subtilis* spores that express two different recombinant *C. difficile* antigens on their surface, a toxoid antigen and a unique spore colonization factor. Adverse events were monitored and specific mucosal and systemic immunity (sIgA, circulating IgA and IgG) was evaluated in this trial (NCT02991417).

4.2 Vaccines Targeting Surface Components

Several studies rationalized the concept of targeting surface components to develop vaccines (Mizrahi et al. 2014). To induce a specific immune response against *C. difficile* and limit cross-reactivity, vaccines target specific *C. difficile* surface components conserved among strains. Presently, all these vaccines are in pre-clinical development in animal models.

4.2.1 Parenteral Immunization in Animal Models

Several antigen candidates have been investigated for parenteral vaccination targeting *C. difficile* surface components.

After s.c mouse immunization with nontoxigenic *C. difficile* membrane fraction adjuvanted in an oil emulsion (TiterMax Gold adjuvant), Senoh et al. induced a specific IgG and IgA response in sera and intestinal fluids, respectively. Interestingly, hyperimmune sera and intestinal fluids were able to inhibit *C. difficile* adhesion in vitro to human intestinal Caco2 cells (Senoh et al. 2015).

One approach aims to target *C. difficile* surface proteins involved in bacterial gut colonization and participating to *C. difficile* pathogenesis. For instance, Ní Eidhin et al. i.p immunized hamsters with crude SLPs from a clinical *C. difficile* strain (PCR ribotype 001) with either alum or RIBI as adjuvant (Ní Eidhin et al. 2008). Hamsters immunized with alum mounted a strong IgG response whereas hamsters immunized with RIBI mounted a weak IgG response. However, all immunized hamsters developed diarrhea and died after *C. difficile* challenge. Recently, another study successfully tested *C. difficile* flagellin FliC in i.p vaccination assay (Ghose et al. 2016b). Ghose et al. i.p immunized mice and hamsters with recombinant FliC adjuvanted with alum. As expected, immunization induced a high systemic anti-FliC IgG response in mice. In addition, immunized mice were fully protected against a clinical epidemic 027 strain (UK1) whereas immunized hamsters were partially protected against strain 630Δerm. Surface spore proteins have also been tested as vaccine antigen. Indeed, spores may play an essential role in persistence of *C. difficile* in the intestinal tract. Ghose et al. tested several spore proteins and i.p immunized mice with the exosporium CdeC or CdeM proteins with alum as adjuvant. They observed a full protection in mice against the 027, UK1 strain. This protection was correlated with circulating specific IgG and a significant decrease of the level of spore shedding compared to controls (Ghose et al. 2016a). In addition, these spore proteins were also able to afford a 80% protection against 630Δerm in vaccinated hamsters.

Another approach is to target highly specific antigens abundantly present on *C. difficile* surface, such as cell wall polysaccharides (Monteiro et al. 2013, 2016). Oberli et al. (2011) and Romano et al. (2014) demonstrated PS-II immunogenicity in mice with respectively diphtheria toxoid (CRM_{197}) or recombinant TcdA and TcdB fragments as carrier protein, to make PS immunogenic. PS-I was shown immunogenic in CDI patients. Interestingly, patients with CDI presented anti-PS-I IgG in serum and anti-PS-I IgA in stools. Mice immunized with synthetic PS-I-CRM_{197} conjugate adjuvanted with either alum or Freund's adjuvant produced specific anti PS-I IgG, IgM and IgA (Martin et al. 2013). In addition, Martin et al. identified a

minimal epitope in PS-I, which is the disaccharide RhA(1–3)-Glc. Then, the same group (Broecker et al. 2016a) constructed a pentavalent glycoconjugate based on the PS-I minimal epitope with an increased antigenicity. Of note, this synthetic pentavalent vaccine candidate elicited a weak but highly specific IgG response to native PS-I glycan in mice, but vaccine efficacy was not tested.

C. difficile LTA (also named PS-III) has been shown to be conserved in *C. difficile* strains. Cox et al. (2013) constructed different glycoconjugates, and immunization of mice induced a specific IgG response. Rabbit and mouse hyperimmune anti-sera recognized *C. difficile* live vegetative cells and spores. Broecker et al. (2016b) constructed a semi synthetic LTA-CRM_{197} glycoconjugate that elicited anti-LTA IgG in mice with or without alum adjuvant. Anti-LTA antibodies recognized *C. difficile* surface and significantly limited bacterial mouse gut colonization 5 days post challenge.

4.2.2 Mucosal Immunization in Animal Models

A mucosal immunization targeting surface proteins compared to parenteral immunization aims to induce locally an immune response against *C. difficile* correlated to a decrease of bacterial gut colonization. Several vaccine candidates have been tested via mucosal routes in animal models.

After i.r immunization in a human microbiota-associated mouse model with a *C. difficile* toxin free cell wall extract adjuvanted with CT, after *C. difficile* challenge, a significant decrease of bacterial gut colonization in immunized mice compared to controls was observed (Péchiné et al. 2007). Pechiné et al. evaluated *C. difficile* surface proteins as mucosal vaccine candidates in this mouse model (Péchiné et al. 2007). First, the flagellar cap protein FliD was used to determine the best mucosal route of immunization between i.r, i.g and i.n. The best immune response was induced with FliD and CT as adjuvant via the i.r route, leading to systemic anti-FliD IgG and mucosal sIgA response. Then, to prevent *C. difficile* gut colonization, mice were i.r immunized with CT as adjuvant with flagellar preparation containing FliC and FliD or an association of Cwp84 and FliD as antigens. All immunized groups showed a significant decrease of intestinal colonization from day 13 after challenge. In another study aiming to identify surface antigens in mucosal vaccination, Péchiné et al. (2013) i.r immunized hamsters with a cell wall extract of a non-toxigenic *C. difficile* strain adjuvanted with CT. A partial protection of hamsters (33%) against a lethal dose of *C. difficile* was observed. Using a comparative proteomic analysis between sera from protected immunized animals and sera from the control group, three proteins have been identified as key factors leading to production of protective antibodies: the chaperon protein DnaK, the heat shock protein GroEL and the S-layer protein precursor SlpA. GroEL was used as antigen to i.n. immunize mice with CT as adjuvant and was able to induce a systemic anti-GroEL IgG response associated with a significant decrease of bacterial colonization from day 8 after *C. difficile* challenge. Recently, Bruxelle et al. tested the SlpA precursor as vaccine antigen (Bruxelle et al. 2016). Immunization (i.r.) of mice with SlpA as antigen and CT as adjuvant induced a systemic anti-SlpA IgG and a mucosal sIgA response. Furthermore, this regimen induced a trend in decrease of *C. difficile* intestinal colonization significant at day 10 after challenge. In the hamster model, this immunization regimen led to a partial and non-lasting protection against *C. difficile*. In comparison, Ní Eidhin et al. (2008) immunized hamsters with crude SLP extract. They tested different vaccination regimens with different antigen doses, different adjuvants and different routes of immunization (i.p prime with RIBI adjuvant, i.n prime with CT, i.n boost with CT). The best protection was obtained in hamsters immunized with the i.p prime and i.n boost combination, two hamsters out of three survived after a lethal challenge. In mice, this regimen induced a strong circulating anti-SLP IgG and IgA response.

Immunization (i.r, i.n. or s.c) of hamsters with Cwp84 as antigen and CT or Freund complete as adjuvant has been performed (Péchiné et al.

2011). The best protection was obtained with the rectal route and CT as adjuvant (40% greater survival in the i.r. immunized group compared to a control group). Surprisingly, this protection was not correlated with circulating anti-Cwp84 antibodies. Then, oral immunization was performed in hamsters with Cwp84 encapsulated in pectin beads for colonic delivery. A similar partial protection (40%) was obtained, with no correlation to systemic antibody response. These results in the hamster model support the role of other mechanisms of protection in parallel of the circulating antibody response such as innate immunity and mucosal immune response.

C. difficile colonization is multifactorial and combination of several surface components in vaccine is likely necessary to obtain a full protection against colonization. A better knowledge of the colonization process and the mucosal immune response against *C. difficile* will improve mucosal vaccine development.

5 Conclusion

Highly specific and conferring protection, the humoral response is closely associated with the outcome of the infection and the control of recurrences. Several strategies have been developed to prevent or treat CDI (Fig. 1).

One strategy is based on passive immunizations with various APs, another is to develop vaccines. In both passive and active immunizations, the key virulence factors TcdA and TcdB were the first antigens. Then, surface components of the vegetative cells and the spores were studied as antigens. The first assays have

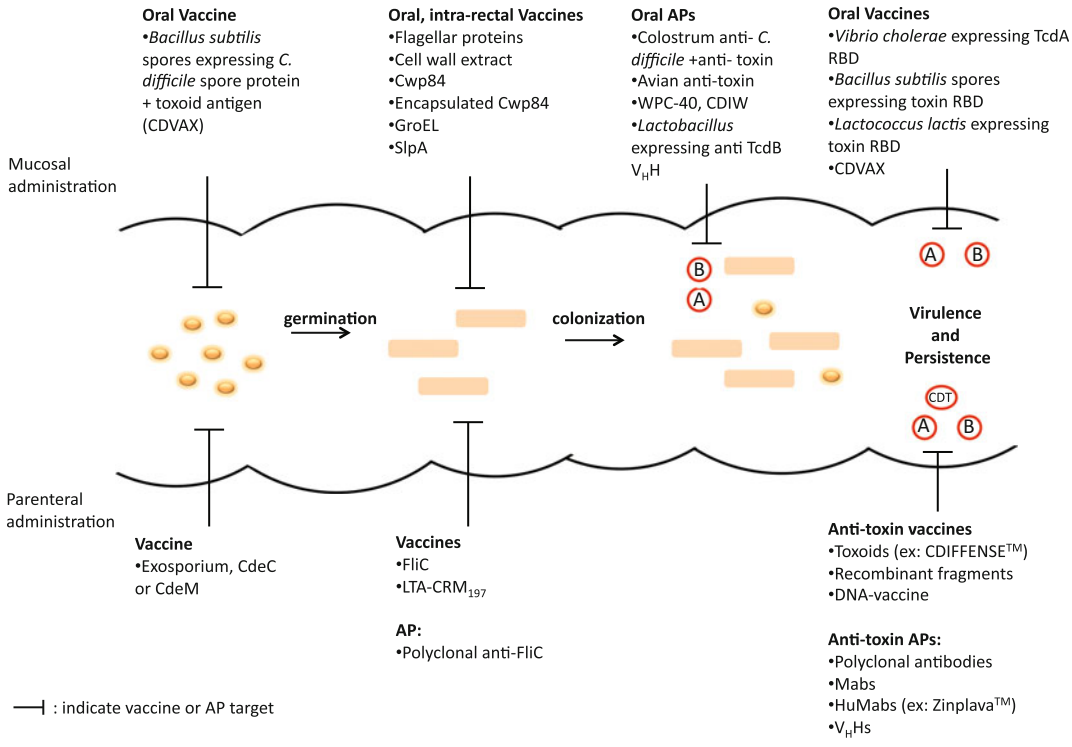

Fig. 1 Mucosal and parenteral immunization strategies against *C. difficile*

been performed in animal models and have paved the way to development in humans.

Today, bezlotoxumab (Zinplava ™) is the only AP against *C. difficile* i.v. administered approved for clinical use and indicated to reduce recurrence of CDI in patients who received SOC antibiotic treatment for CDI and are at high risk of recurrences. The main advantage of passive immunization with specific MAbs consists in its rapidity of protection capable to complement the poor host immune response. However, this strategy is expensive and does not confer a long-term protection. Vaccines display advantages such as long-term protection but depend on the faculty of the host to develop an immune response. It is well known that immune response is impaired in elderly who are particularly at risk of CDI. Several parenteral vaccines targeting both toxins TcdA and TcdB are tested in clinical trials (2 in phase III). It is likely that parenteral toxin-based vaccines will be approved soon for human use.

The mucosal and systemic immune responses have both their role in the protection against CDI. Mucosal immunization displays advantages such as the local induction of an innate and adaptive immune response. Several assays have been performed in animal models either with toxin antigens or colonization factor antigens. A phase I clinical trial has been completed with bacillus spores expressing a toxin fragment and a spore protein. These promising assays should be confirmed. Surface components of the vegetative cells also led to promising results in animal models. However, a combination of various colonization factors seems necessary to reach full protection. In addition, the combination of toxin antigens with colonization factors antigens has the advantage to inhibit the two steps of the pathogenic process, colonization and toxin release. Future research should focus on development of novel immunologic strategies including systemic and mucosal vaccines targeting both virulence and colonization factors.

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Non-human *C. difficile* Reservoirs and Sources: Animals, Food, Environment

Cristina Rodriguez Diaz, Christian Seyboldt, and Maja Rupnik

Abstract

Clostridium difficile is ubiquitous and is found in humans, animals and in variety of environments. The substantial overlap of ribotypes between all three main reservoirs suggests the extensive transmissions. Here we give the overview of European studies investigating farm, companion and wild animals, food and environments including water, soil, sediment, waste water treatment plants, biogas plants, air and households. Studies in Europe are more numerous especially in last couple of years, but are still fragmented in terms of countries, animal species or type of environment covered. Soil seem to be the habitat of divergent unusual lineages of *C. difficile*. But the most important aspect of animals and environment is their role in *C. difficile* transmissions and their potential as a source for human infection is discussed.

Keywords

Farm animals · Pets · Water · Soil · Environment · Food · Transmission

1 Introduction

Clostridium (*Clostridioides*) *difficile* is regarded mainly as an important human pathogen. Because it can colonize his natural niche, the gut, only in the absence of established gut microbiota, it seem that his natural multiplying hosts are young animals and children. As an anaerobic sporeforming bacterium it will be transmitted from the gut into different environments. *C. difficile* is hence ubiquitous and can be found in humans, animals and the environment with a great variety of transmission routes between them.

Several recent or older reviews have covered different aspects of non-human reservoirs (Rodriguez et al. 2016; Warriner et al. 2016;

C. Rodriguez Diaz (✉)
Faculty of Veterinary Medicine, Department of Food Science, FARAH, University of Liège, Liège, Belgium
e-mail: kriszsi@hotmail.com

C. Seyboldt
Institute of Bacterial Infections and Zoonoses, Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Jena, Germany
e-mail: Christian.Seyboldt@fli.de

M. Rupnik
Environment and Food, National Laboratory for Health, NLZOH, Maribor, Slovenia

Faculty of Medicine, University of Maribor, Maribor, Slovenia
e-mail: maja.rupnik@nlzoh.si

Bauer and Kuijper 2015; Rodriguez-Palacios et al. 2013; Hensgens et al. 2012; Squire and Riley 2013; Otten et al. 2010; Gould and Limbago 2010; Weese 2010; Rupnik 2007, 2010). Here we give the overview of studies performed to date in Europe.

2 *C. difficile* in Farm Animals: European Studies

Looking back to the early research on *C. difficile*, the presence of these bacteria in farm animals first gained attention in the 1970s. The first reference in the literature describing *C. difficile* in farm animals (rabbit, horse and cow dung) and in the environment (hay, sand, and river mud) in Europe dates from 1974 (Hafiz 1974). Thereafter, other authors in different European geographic areas also confirmed the presence of *C. difficile* and infection in hares (France) (Dabard et al. 1979), pigs (UK) (Lysons et al. 1980; Jones and Hunter 1983), goats (UK) (Hunter et al. 1981; Borriello et al. 1983), ducks, geese, rabbits and chickens (UK) (Borriello et al. 1983). The first report of *C. difficile* in cattle in Europe was published in 2008 in which bacterial toxins were found in biological samples from calves (Pirs et al. 2008).

Over the last 20 years, several studies have investigated not only the presence and the prevalence of *C. difficile* in different farm animal species but also the pathogenic potential of the bacterium in these animals. In addition to the interest in *C. difficile* as an infectious agent in livestock animals and the economic losses that it can generate, the main objective of research groups worldwide has been to demonstrate the existence of an animal reservoir and to elucidate the relationships between potential reservoirs and *C. difficile* infection in humans. Hence, many studies also report the typing of animal strains (Table 1).

2.1 *C. difficile* in Pigs and Cattle

Pigs are the farm animals that have been most commonly studied in Europe in the context of infection by *C. difficile,* followed by cattle (Fig. 1). In cattle, the described prevalence (up to 22%) is much lower than that in pigs (up to 96%) and studies have reported between 90% and 100% toxigenic strains circulating in both types of animal farms. In cattle, several studies have addressed the possibility of a breeding effect on *C. difficile* colonisation in animals and therefore different types of production systems have been investigated, including production farms, fattening farms or dairy farms (Koene et al. 2012; Romano et al. 2012a, b; Zidaric et al. 2012). However, in pigs these possible differences between types of breed have not been addressed in the literature. Only a single study reports the prevalence of *C. difficile* on free-range pigs, but the results of the study revealed the *C. difficile* prevalence in this population similar to the prevalence found in intensively raised animals (Alvarez-Perez et al. 2013).

2.2 *C. difficile* in Other Less Commonly Studied Farm Animals in Europe

Poultry seem to be a natural host as colonized birds are asymptomatic, the prevalence in young animals is very high and the diversity of ribotypes within a farm is very high. Still, not many studies in Europe have explored this species. Also goats and sheep were only recently studied in the respect to *C. difficile*. A mean prevalence of 8.6% was reported in sheep, 5.8% in goats and 33.1% in poultry.

As interest has increased regarding the possible zoonotic transmission of *C. difficile* in recent years, new studies have investigated

Table 1 Overview of recent European studies on *C. difficile* in animals

Species	References	Reported prevalence and the most prevalent ribotypes
Pigs	Pirs et al. (2008), Avbersek et al. (2009), Alvarez-Perez et al. (2009), Indra et al. (2009), Hoffer et al. (2010), Hopman et al. (2011), Keessen et al. (2011b), Koene et al. (2012), Rodriguez et al. (2012, 2013), Alvarez-Perez et al. (2013), Schneeberg et al. (2013a), Noren et al. (2014)	22.6–96% (neonates);
		0–36% (adults)
		002, 005, 014, 013, 015, 023, 046, 066, 078,126
Cattle	Pirs et al. (2008), Avbersek et al. (2009), Hoffer et al. (2010), Koene et al. (2012), Rodriguez et al. (2012), (2013), Romano et al. (2012a), Zidaric et al. (2012), Schneeberg et al. (2013a), Schmid et al. (2013)	1.8–22.2% (neonates);
		0–9.9% (adults);
		002, 003, 012, 014, 015, 029, 033, 038, 045, 066, 070, 077, 078, 081, 126, 137
Goat and ship	Koene et al. (2012), Romano et al. (2012a), Avbersek et al. (2014), Rieu-Lesme and Fonty (1999)	Goats 0–10.1%
		001, 010, 014, 020, 045, 066
		Sheep 0–18.2%
		015, 056, 061, 097
Poultry	Zidaric et al. (2008), Indra et al. (2009), Koene et al. (2012)	0–100%
		001, 010, 014, 023, 446
Horses	Avbersek et al. (2009), Ossiprandi et al. (2010), Koene et al. (2012), Rodriguez et al. (2014a, b, 2015)	3.7–33.3%
		005, 006, 010, 012, 014, 023, 033, 035, 039, 042, 045, 051, 078, 126
Cats	Koene et al. (2012) and Schneeberg et al. (2012)	3.7–15.7%
		009, 010, 039, 014/020, 045
Dogs	Schneeberg et al. (2012), Koene et al. (2012), Wetterwik et al. (2013), Pirs et al. (2013), Álvarez-Pérez et al. (2015, 2017), Orden et al. (2017a), Spigaglia et al. (2015)	0–100% (neonates);
		4.8–25% (adults);
		009, 010, 012, 014/020, 021, 027, 031, 039, 045, 056, 078, 106, 107, 154, 213, 430
Rabbits (farm)	Drigo et al. (2015)	3%
		002, 014, 020, 078, 012, 205
Wild animals	Burt et al. (2012), Bandelj et al. (2016), Andres-Lasheras et al. (2017)	0–100%
		078, 033, 045, 126

the prevalence and epidemiology of the bacterium in animal production types that are less commonly addressed than cattle, pigs or poultry. An investigation conducted in Italy reported a *C. difficile* prevalence of 3% for rabbits raised in industrial holdings for food production (Drigo et al. 2015).

2.3 Factors Associated with *C. difficile* Colonization in Farm Animals

Several factors, including animal species, age, microbiota, breeding effect and seasonality have been associated with *C. difficile* colonisation in farm animals (Fig. 2) and likely apply also for other animals. It is possible that *C. difficile* is better adapted to some animal hosts than to others. The reported prevalence varies strongly between different species and studies (Rodriguez et al. 2016; Table 1).

Age is the best studied among factors associated with *C. difficile* carriage in farm animals. All of the studies conducted in various European countries (Alvarez-Perez et al. 2009; Schneeberg et al. 2013a have shown high colonization rates in newborn animals that are either considerably reduced or eliminated in adult animals. This reduction in infection prevalence with age has two important consequences. First, the risk of foodborne transmission from

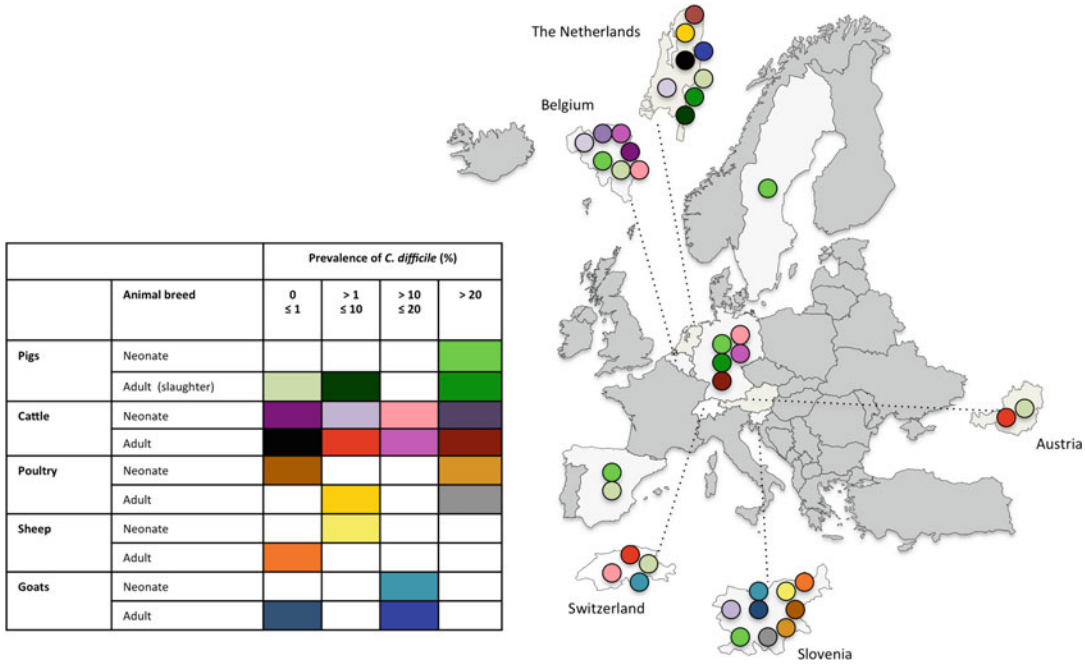
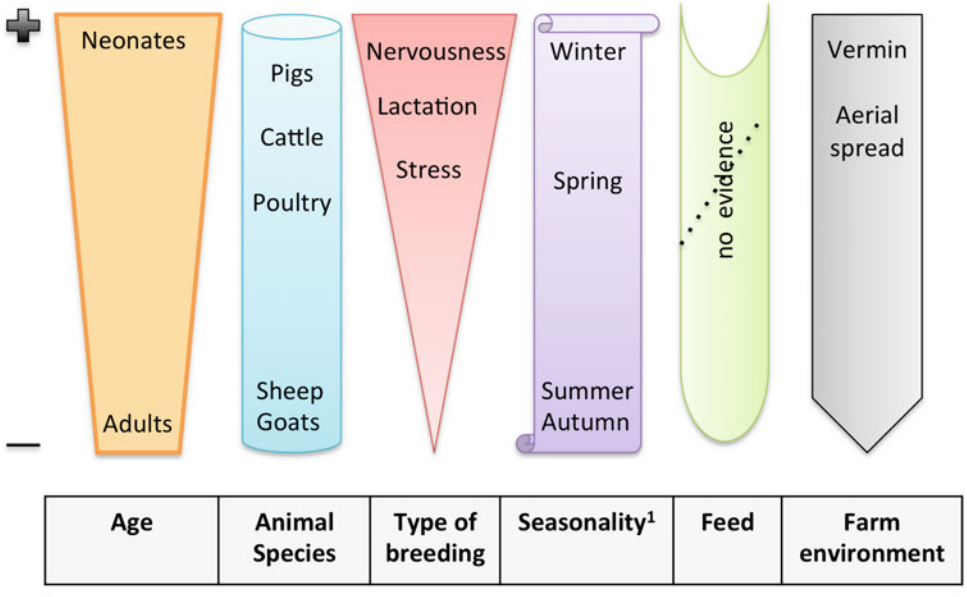


Fig. 1 Prevalence of *C. difficile* in farm animals in Europe



1 Data from meats and humans, but no data regarding farm animals

Fig. 2 Factors associated with the presence of *C. difficile* in livestock animals in Europe

contaminated animal products during harvest is greatly reduced. Second, CDI in adult animals is very rare; therefore, *C. difficile* is currently not considered a common health problem in adult farm animals.

Regarding gut microbiota composition, in Europe only one study have evaluated changes in the intestinal microbiota with *C. difficile* colonization in poultry (Skraban et al. 2013). Differences in the presence of *Enterococcus cecorum*, *Lactobacillus gallinarum, Moniliella* sp. and *Trichosporon asahii* were detected among *C. difficile* positive and negative animals. Interestingly, *Acidaminococcus intestini*, identified for the first time as a part of the poultry intestinal microbiota in this study, was detected in high abundance in animals not colonized by *C. difficile*. Further studies may lead to the identification of several bacterial populations that can potentially protect hosts from CDI.

2.4 Infection vs. Carriage of *C. difficile* in Farm Animals

In farms, *C. difficile* shows a similar prevalence among animals with or without diarrhoea (Pirs et al. 2008; Alvarez-Perez et al. 2009; Koene et al. 2012; Schneeberg et al. 2013a, which may indicate that the bacterium is not the main causal agent of disease, but instead, an opportunistic pathogen that worsens the clinical status and outcome of affected animals. In piglets, *C. difficile* causes important economic losses in farms due to both, diarrhoea and premature death as well as delays in growth and reduced weight gain (Songer 2000; Squire and Riley 2013). There are a few reports of *C. difficile* infection in pigs in Europe, including one study that reported an outbreak in periparturient sows in a large outdoor production unit in Croatia (Kiss and Bilkei 2005) and one case-report study of typhlocolitis and diarrhoea in piglets in Ireland (McElroy et al. 2016). In calves and poultry, *C. difficile* has also been proposed as a possible cause of diarrhoea, enteritis and death (Hammitt et al. 2008; Cooper et al. 2013), although there is no evidence of outbreaks due to the bacterium in these animal species. A review of these data indicates that the incidence, clinical relevance and pathogenesis of CDI in farm animals in Europe has not yet been elucidated.

2.5 Farm Animals and Colonization with Different *C. difficile* PCR Ribotypes

A great variety of *C. difficile* PCR ribotypes has been reported in different farm animals in Europe. Comparative international study with 12 participating European and non European countries that included 112 strains from 13 species including farm animals has distributed strains into 50 PCR ribotypes. Some ribotypes were found across all tested species (014, 078) while some others are more likely to be associated with a given animal species (033 with cattle) (Janezic et al. 2012).

An interesting aspect is also ribotype variability within the farm. At pig farms a single PCR ribotype will be present. In cattle the variability will be greater although the number of detected types is still modest. In contrast, in poultry and rabbit farms the reported variability is very high and from 12 to16 PCR ribotypes are found per single farm (Zidaric et al. 2008; Drigo et al. 2015).

PCR ribotype 078 is the only one that has been repeatedly reported in swine throughout different European countries and is described in several studies as the dominant type irrespective of age or diarrhoeal status (Koene et al. 2012; Rodriguez et al. 2012; Schneeberg et al. 2013a; McElroy et al. 2016). The remaining PCR ribotypes isolated from pig farms constitute a long list and include ribotypes 002, 014, 015 and 023; however, they have only been reported in specific studies (Avbersek et al. 2009; Hopman et al. 2011; Keessen et al. 2011b; Koene et al. 2012; Rodriguez et al. 2012; Schneeberg et al. 2013a; Noren et al. 2014; McElroy et al. 2016).

In cattle, an even greater variety of PCR ribotypes has been isolated. PCR ribotype 078 has also been commonly detected in cattle farms in different countries in Europe (Hoffer

et al. 2010; Rodriguez et al. 2012; Zidaric et al. 2012; Schneeberg et al. 2013b). In contrast to pig farms, where isolates within the farm are clonal, at least one study on veal calves farm did not detect clonal dissemination (Zidaric et al. 2012). Calves were mostly colonized already upon the arrival to farm and two of all detected ribotypes (078 and 126) were persisting from the beginning to the last stages of the production cycle. Another PCR ribotype, 033, seem to be cattle-associated and has been described in five different studies conducted in Belgium, Germany, Switzerland and Slovenia. Other PCR ribotypes frequently associated with these animals are types 012 and 002, which were described in Belgium, The Netherlands and Slovenia (Avbersek et al. 2009; Koene et al. 2012; Rodriguez et al. 2012; Zidaric et al. 2012). The percentage of toxigenic strains in cattle varies between 70% and 100%, but no association between diarrhoeal status and colonization with specific PCR ribotypes has been established.

For other small ruminants such as goats and sheep, as well as poultry, the presence of specific PCR ribotypes has not been described in part because there are only a few studies in Europe describing the presence of *C. difficile* in these animal species, and the few available studies describe a large variety composed of different types (Zidaric et al. 2008; Indra et al. 2009; Koene et al. 2012; Romano et al. 2012a; Avbersek et al. 2014)(Table 1).

2.6 Antimicrobial Susceptibility of *C. difficile* Isolates Isolated from Farm Animals

Drug resistance in *C. difficile* strains is usually associated with specific antibiotics, especially quinolones, erythromycin and clindamycin, and with specific PCR ribotypes. In pig and cattle production, different studies have reported resistances to fluoroquinoles, ciprofloxacin and erythromycin, especially among isolates of PCR-ribotype 078 (Keessen et al. 2013; Pelaez et al. 2013), but also among PCR-ribotypes 012 and 033 (Bandelj et al. 2017). In pork and cattle industry, the use of fluoroquinolones has also been related with the isolation of multiple antibiotic-resistant strains (Zidaric et al. 2012).

For *C. difficile* isolates from small ruminants, the limited available data in the literature reported antibiotic susceptibility to vancomycin, metronidazole and moxifloxacin of all isolates obtained from goats and sheep and a possible relationship between PCR ribotype 045 and resistance to fluoroquinolones, beta-lactams, lincosamides and macrolides (Avbersek et al. 2014).

Susceptibility to several other drugs, including antibiotics typically used for the treatment of CDI in humans like metronidazole, vancomycin or rifampicin, completely inhibited *C. difficile* growth (Pirs et al. 2013), which reflects no major differences in antibiotic susceptibilities between animal and human strains.

3 *C. difficile* in Companion Animals in Europe

Dogs and cats are the most studied companion animals. Taking the European studies involving dogs and cats together, the overall prevalence for *C. difficile* in cats is slightly lower than in dogs, but studies including cats are scarce.

In six European studies including cats from veterinary clinics or shelters, the *C. difficile* prevalence ranged from 0% to 30% (2%; Al Saif and Brazier 1996, 15.7%; Koene et al. 2012, 3.7%; Schneeberg et al. 2012; 8%; Weber et al. 1989 (Table 1). Both studies marking this prevalence borders included only a small number of 37 and 20 cats respectively (Alvarez-Perez et al. 2017; Borriello et al. 1983). A so far unpublished study conducted in Germany investigated 407 cats in household settings and observed a prevalence of 2.46% (10 of 407) (D. Rabold, personal communication).

Little more information is available in respect to dogs in Europe. The reported prevalence rates in the different studies range from 1.45% in dogs of a control group (1 of 74) up to 100% in puppies of one litter at certain time-points (Perrin et al. 1993; Alvarez-Perez et al. 2015). Other reports describe *C. difficile* carriage rates of

4.8–25% for dogs in different study settings (Table 1). An unpublished Germany study investigated 444 dogs in household settings and detected a prevalence of 3.4% (15 of 444) (D. Rabold, personal communication).

Eight European studies reported PCR ribotypes in dogs and only three considered cats. Ribotypes 009, 014, and 039 are common in dogs and cats across Europe. The most frequently reported ribotypes in cats are 039 or 039/2 (7), 014 or 014/020 (8) and 010 (10) (Koene et al. 2012; Schneeberg et al. 2012; Alvarez-Perez et al. 2017). The most frequently described ribotypes in dogs are 010 (58), 014/020 (28), 056 (22), 078 (8), 039 (7), 009 (6), 012 (4), 106 (4) (Alvarez-Perez et al. 2015, 2017; Orden et al. 2017a; Spigaglia et al. 2015; Wetterwik et al. 2013; Pirs et al. 2013; Schneeberg et al. 2012; Koene et al. 2012).

Factors most likely associated with *C. difficile* colonization in dogs and cats are age, enteric disease, antibiotic treatment and hospitalisation.

A plausible association of age and carriage rate in dogs (puppies and older animals) was reported. In puppies high prevalence up to 100% was noted in the time from 2 to 6 weeks after birth. The carriage rate in puppies markedly decreased with age and reached 3.1% and 0% at the end of the observation time (Perrin et al. 1993; Álvarez-Pérez et al. 2015). Additionally, Alvarez-Perez et al. (2017) reported that carriage was significantly linked with age over 7 years investigating 105 dogs from 17 veterinary clinics.

A tendency for higher *C. difficile* prevalence in cats was described in connection with antibiotic/corticosteroid medication and hospitalisation. Regarding the available data from Europe it seems obvious that *C. difficile* does not cause disease in dogs and cats and similar percentages are isolated from symptomatic and healthy animals (Weber et al. 1989; Wetterwik et al. 2013). However, dogs and cats can harbour *C. difficile* strains with virulence potential and with exception of the longitudinal studies conducted in puppies (Table 1) the duration of *C. difficile* shedding was scarcely addressed. It is not clear weather a *C. difficile* carriage can be a result of a longer lasting colonisation or is just connected with a short transient passage.

In the respect to antibiotic resistance, metronidazole resistant *C. difficile* strains, rarely observed in general, were isolated from dogs with recorded application of metronidazole (Wetterwik et al. 2013; Orden et al. 2017a) or suspected metronidazole treatment as it is commonly used for *Giardia* spp. infections in Italian dogs (Spigaglia et al. 2015). Therefore it seems plausible that this resistance was acquired during replication over a longer period in the dogs' digestive tract.

4 *C. difficile* in Horses in Europe

In contrast to other companion animals, horses are reported to develop *C. difficile* enteric disease. Foals and adult horses could be affected and outbreaks as well as sporadic cases were described. Antibiotic treatment and hospitalization have been depicted as important risk factors. *C. difficile* rates in horses with enteric disease were 5–63% in different studies. Healthy horses may harbor *C. difficile* as well; reported prevalence was ranging between 0% and 10% (reviewed in Diab et al. 2013). A Swedish study found higher carriage rates of 29% in healthy foals younger than 14 days. Additionally soil samples from stud farms contained *C. difficile* more frequent than soil samples from farms with mature horses. It was concluded that strains from the environment and healthy foals can serve as reservoir (Baverud et al. 2003). Recent European studies indicating *C. difficile* in horses to be rare. Reports from Slovenia, Italy, the Netherlands and Belgium stated *C. difficile* carriage rates from 3.7% to 33.3% (Table 1) with a remarkably high diversity of detected ribotypes, just ribotype 014 was detected in three of the five studies (Avbersek et al. 2009; Koene et al. 2012; Ossiprandi et al. 2010; Rodriguez et al. 2014a, 2015). Only two of these studies contain information on antibiotic resistance. In the first study conducted in Sweden, the resistance of 52 strains isolated from horses and their close environments were investigated for 10 different antibiotics. All of these strains were resistant to trimethoprim/sulphamethoxazole and bacitracin, but susceptible to metronidazole and fusidic acid.

A total of 14 *C. difficile* strains, all of them isolated from hospitalised horses, were resistant to erythromycin and rifampicin (Baverud et al. 2003). As all of these strains were isolated from horses previously treated with erythromycin alone or in combination with rifampicin, authors suggest that erythromycin treatment probably selects the spread of this resistant pattern (Baverud et al. 2004). In a further study conducted in Belgium, antibiotic resistance was tested from ten strains isolated from hospitalized horses. All isolates displayed resistance to clindamycin and ceftiofur. Ceftiofur is one of the most commonly used antibiotics in some equine clinics (Rodriguez et al. 2014a).

5 *C. difficile* in Wild Animals in Europe

Limited data are available in Europe regarding the presence of *C. difficile* in wild animals outside of their direct or indirect relationships with livestock. In Slovenia, a study found *C. difficile* in barn swallows in an area identified as a barn swallow congregation point during the autumn migration of the species across Europe. The authors found an overall prevalence of 4% (4.6% (7/152) in juvenile birds and 0/23 in adults). PCR ribotypes 078, 002 and 014 were identified among a large variety of new types. The conclusions of this study focus on the possible role of barn swallows in the national and international dissemination of the bacterium (Bandelj et al. 2014). Another study also conducted in Slovenia investigated the carriage of *C. difficile* in migrating passerine birds by sampling cloacal specimens from animals during migration (Bandelj et al. 2011). However, in this study, none of the samples yielded a positive result for the presence of the bacterium.

In Spain, the faecal shedding of *C. difficile* by 40 zoo animal species was investigated (Alvarez-Perez et al. 2014). The bacterium was found with an infection prevalence of 3.5% in samples from the chimpanzee (*Pan troglodytes troglodytes*), dwarf goat (*Capra hircus*), Iberian ibex (*Capra pyrenaica hispanica*) and plains zebra. All isolates displayed resistance to the fluoroquinolones ciprofloxacin, enrofloxacin and levofloxacin and belonged to PCR ribotypes 078, 039 and 110. The distribution of these PCR ribotypes typically found in farm or companion animals and humans may be explained by the close contact of zoo animals with humans and their environment as well as by continuous contact between these animals and droppings of other wild animals such as birds, which may aid in the dissemination of these common *C. difficile* strains.

In a clinical case study conducted in a zoo in Denmark, *C. difficile* was reported as a cause of Asian elephant enterocolitis. Molecular differences between the isolates obtained from three different elephants were not detected; thus, it was suggested that the same clone caused the outbreak. The origin of the contamination was not elucidated. The elephants were fed large quantities of broccoli, and authors hypothesized that sulforaphane, which is present in this vegetable, could have caused dysbiosis and subsequently led to CDI (Bojesen et al. 2006). However, because the same clone was present in all of the affected elephants, it is also possible that the broccoli itself was contaminated with toxigenic *C. difficile*; therefore, the broccoli could have been the source of contamination.

C. difficile was also investigated in zooplankton populations and associated environments at five sampling stations in the Gulf of Naples, Italy. The bacterium was detected in zooplankton samples but not in marine sediments. Many types were characterized including PCR ribotypes 009 and 066. These results demonstrated for the first time that *C. difficile* is also well adapted to aquatic marine populations that were not previously studied, which suggests that the bacterium could be transmitted through the ingestion of raw or undercooked seafood (Pasquale et al. 2011).

6 Transmissions Between Animals and Environment

Clostridium difficile colonizes the intestinal tract of animals, which then excrete the bacterial spores in the faeces. In this way animals can serve as source of environmental contamination

or as vectors in direct and indirect transmission. Environmental contamination will include manure and farm waste recycling (as fertilizers or biogas substrates), soil contamination (pastures), water contamination or aerial contamination and some examples will be described in Sect. 7.

To assess the direct or indirect transmission of *C. difficile* by vermin in pig farms, samples of house mice, drain flies, lesser houseflies, yellow mealworms, house sparrows and bird droppings were investigated. *C. difficile* prevalence ranging between 4% and 100% was reported, and PCR-ribotype 078 was identified in each type of sampling. The authors concluded that vermin could be important sources of *C. difficile* contamination in farms (Burt et al. 2012). Similarly, a recent study conducted in north-eastern Spain reported the presence of *C. difficile* in pest species including rodents and pigeons in pig farms and the associated environment. Most of the characterized isolates were identified as the susceptible metronidazole and vancomycin strains, PCR-ribotype 078 and 126, which were also isolated from pigs. This study also confirmed the cross-transmission of bacterium between wild animals and production animals in farms, although the impact of this phenomenon on the epidemiology of *C. difficile* was not well established (Andres-Lasheras et al. 2017). C. *difficile* was also detected in flies at dairy farms (Bandelj et al. 2016).

In respect of dogs and cats and their role in transmission of *C. difficile* between companion animals and environment in Europe nearly nothing is known, but two studies comprise interesting information. Occurrence of the same strain (MLVA and ribotype) in dogs and a cat indicating direct or indirect transmission were described in animal shelters in Germany (Schneeberg et al. 2012). Orden et al. (2017b) investigated recreational sandboxes for children and dogs within the Madrid region (Spain). Two of the most frequent ribotypes (009 and 106) were also reported in independent study in Madrid dogs (Alvarez-Perez et al. 2017).

7 *C. difficile* in Food in Europe

Foodborne zoonotic pathogens are transmitted via the consumption of contaminated food and drinking water. The possible foodborne transmission of *C. difficile* was reported for the first time in 1983 in in Europe (Borriello et al. 1983). However, currently, the importance of *C. difficile* as a zoonotic disease remains largely unknown.

Food contamination routes can be various. Apparently healthy animals can carry *C. difficile* spores through the slaughter stage and introduce a potential risk of meat contamination during processing. Vegetables would be contaminated by manure spread or irrigation with contaminated water. Root vegetables could carry *C. difficile* spores often present in soil irrespective of fertilizing.

7.1 Detection of Contaminated Meats in Retail Markets

The evidence that carcass contamination occurs inside the slaughterhouse reinforces the hypothesis of the potential risk of foodborne infections linked to the ingestion of foods contaminated with *C. difficile* spores. In Europe, meats have been found contaminated with *C. difficile* with a frequency ranging from 2.3% to 4.7%, and the main PCR ribotypes identified were 078, 014, 045, 012 and 053 (Bouttier et al. 2010; Jobstl et al. 2010; De Boer et al. 2009; Rodriguez et al. 2014b) (Table 2). Nevertheless, other surveys have failed to find *C. difficile* in meat samples (Indra et al. 2009; Hoffer et al. 2010; De Boer et al. 2009). The reason for the lower variety of PCR ribotypes in meat samples is not clear considering the high variety of types found in farm animal faecal samples. One possible explanation is that there are differences in the sporulation frequencies and susceptibilities to external agents among the different PCR ribotypes

Table 2 Overview of recent European studies on *C. difficile* in foods

Food	References	Reported prevalence and detected ribotypes
Meats	Indra et al. (2009), Von Abercon et al. (2009), Bouttier et al. (2010), De Boer et al. (2009), Hoffer et al. (2010), Jobstl et al. (2010), Rodriguez et al. (2014b)	0–6.3%
		001, 003, 012, 014, 045, 053, 071, 078, 087
Seafood	Pasquale et al. (2011, 2012)	49–75%
		001, 002, 003, 005, 010, 012, 014, 020, 045, 066, 078, 106
Vegetables	Eckert et al. (2013)	2.9–4.5%
		001, 014, 015, 020, 077

(Zidaric et al. 2012). This feature may contribute to the survival of only some PCR ribotypes to the final stages of the meat supply chain (i.e., distribution in retail markets). Furthermore, it is noteworthy that animals may not be the sole origin of *C. difficile* contamination via meat and that other sources could involve contamination during processing or in retail markets.

7.2 *C. difficile* in Foods Other Than Meats in Europe

In Europe, only a couple of studies have addressed the presence of *C. difficile* in foods other than meat, such as seafood and vegetables. The prevalence reported for seafood appears to be high with more than 50% of samples showing positive results (Pasquale et al. 2011, 2012); however, the prevalence described for vegetables is similar to that described for meat (2.9%) (Eckert et al. 2013). Several PCR ribotypes have been detected in these types of samples including PCR ribotypes 014, 078, 001 and 015, among others, and most of these PCR ribotypes have also been associated with CDI in humans in European hospitals (Bauer et al. 2011).

8 Studies on *C. difficile* in Environment in European Countries

Although the first large study including samples from non-hospital environment was done in Europe (Al Saif and Brazier 1996), the reports to date on *C. difficile* in environmental sources in European countries are scarce (Table 3). Tested environments include water, soil, waste water treatment plants (WWTP), biogas plants, air, sediment, manure, silage/hay, sandboxes and surfaces in public places and households. Few environments have been described by more than one report and even is such cases the sample numbers are low.

Unsurprisingly, WWTPs seem to be the environment with the highest positivity rate and *C. difficile* is usually detected in all tested samples either from inlet water, sewage, of effluent (Kotila et al. 2013; Steyer et al. 2015; Romano et al. 2012b). Only a single study, using non-culturing method, reported positivity rate lower than 100% (Romanazzi et al. 2016). Rivers and estuarine sediments also have high yield of *C. difficile* positive samples (Al Saif and Brazier 1996; Zidaric et al. 2010; Hargreaves et al. 2013).

Prevalence of *C. difficile* seem to be somewhat lower in soil than water but this depends on soil type. As an example, the overall prevalence in more than 500 soil samples in Sweden was 4%. While soil from public environments (parks, playgrounds, gardens, cultivated fields) showed the 4% positivity, samples from pastures and paddocks in stables with only mature horses were positive only in 1% and in stud farms at 11% (Baverud et al. 2003). Sandboxes, here specified as environments different than soil, also showed slightly different positivity rate if they were used by children (9 positive of 20) or designated for dogs (12 positive of 20) (Orden et al. 2017b).

Table 3 Overview of studies on *C. difficile* in environment in different European countries

Different types of water samples	WWTP	Soil	Other types of samples	Typing	ABR	Country and reference
Tap water	Single sample1/1			Yes	No	Finland
1 positive/unspecified total number						Kotila et al. (2013)
28 CFU/100 ml						
			Biogas plants (n = 8)	No	No	Germany
			69/154 44,8%			Froschle et al. (2015)
	1WWTP inlet, sewage, effluent positivity <100%			No	No	Italy
						Romanazzi et al. (2016)
Seawater 2/5; 40%			Sediments 0/5	Yes	No	Italy
						Pasquale et al. (2011)
			Air inside pig farm	Yes	No	Netherlands
			2–625 CFU/m^3			
			Air at exhausters			
			6–120 CFU/m^3			
			Air at 20 m distance			
			2/4 positive			Keessen et al. (2011a, b)
	1 WWTP effluent 12/12			Yes	No	Slovenia
						Steyer et al. (2015)
River (n = 25) 42/69; 60,9%				Yes	No	Slovenia
						Zidaric et al. (2010)
Water from drinking bowls at dairy farm 3/80; 3,75%		28/80; 35%	Manure; dairy farms	Yes	No	Slovenia
			23/80; 28,7%			Bandelj et al. (2016)
			Silage/hay			
			3/80; 3,75%			
Puddle water 15/104; 14,4%		28/78; 36,7%	Organic garbage pile 1/1	Yes	Yes	Slovenia
						Janezic et al. (2016)
			Sandboxes (for dogs or children)	Yes	Yes	Spain
			21/40; 52,5%			Orden et al. (2017b)
		25/598, 4%	Surfaces at public places	No	Yes	Sweden
			0/95			Baverud et al. (2003)
	9 WWTPs inlet and effluent 18/18			Yes	No	Switzerland
						Romano et al. (2012a, b)

(continued)

Table 3 (continued)

Different types of water samples	WWTP	Soil	Other types of samples	Typing	ABR	Country and reference
Rivers (n = 4)		22/104; 21,2%	Private houses	Yes[a]	No	UK
14/16; 87,5%; 1–5 CFU/100 ml			550 samples; 2,2% positive			Al Saif and Brazier (1996)
Seawater						
7/15; 46,7%; 3–6 CFU/100 ml						
Lake						
7/15; 46,7%; 1–5 CFU/100 ml						
Inland drainage						
7/26; 27%						
Swimming pool						
4/8; 25%; 1–3 CFU/100 ml						
Tap water						
1/18; 5,5%; 1–3 CFU/100 ml						
Seawater			Estuarine sediments in 2009	Yes	Yes	UK
0/4			11/18; 61,1%			Hargreaves et al. (2013)
			Estuarine sediments in 2010			
			13/21; 61,9%			
			Foam			
			1/1			

WWTP waste water treatment plant, *ABR* antibiotic resistance
[a]Typing published in separate publication (Al-Saif et al. 1998)

Another example of unequal distribution within the environment are biogas plants. In Germany, eight plants with different substrate use (single predominate substrate which was either grass silage or cattle manure) were sampled (Froschle et al. 2015). *C. difficile* that was most frequently detected of all clostridia tested (44, 8% of samples), followed by *C. novyi* (3,9% of samples); other tested species were not detected (*C. botulinum, C. chauvoei, C. haemolyticum, C. septicum*). Animal substrates were more likely to contain *C. difficile* than plant substrates (10/17, 58,8% vs. 2/44; 4,5%). Because all settings use mixed substrates (animal and plant, with predominance of one) the positivity of digested sludge was 22 of 42 samples (52,4%) and in digestion products 35 of 51 samples (68,6%).

A single study has investigated airborne spore transmission within and around a pig production farm with known high *C. difficile* prevalence (Keessen et al. 2011a). *C. difficile* was detected in all farm units except in the pregnant sow unit. The detected airborne *C. difficile* colony counts ranged from 2 to 625 CFU/m^3. At farrowing unit pens with piglets of different age were sampled and the *C. difficile* spores detected in the air decreased with piglet age being highest in pens with neonatal and up to 2 weeks old piglets. Air exhausts at roofs of four different units resulted in spore counts from 6 to120 CFU/m^3, two of four air samples at 20 m distance downwind were positive while air samples up to 140 m distance were all negative.

Strain typing was done in most of the studies (Table 3). Variety of detected ribotypes within a single environment is very large, but PCR ribotypes detected almost in every study were 014 and 010. Soil, in particular in rural but not

urban areas, was shown to be natural environment for very distinctive and divergent lineages of *C. difficile* strains (Janezic et al. 2016).

Antibiotic resistance was tested only in four studies (Table 3; Janezic et al. 2016) and mainly to only few selected antibiotics. Environmental isolates are resistant to similar antibiotics as human isolates. Interestingly, nontoxic environmental strains could be more resistant than toxigenic environmental strains (Janezic et al. 2016).

9 Importance of Animals, Food and Environment for Human Infection

The transmission of *C. difficile* from animal and environmental source occurs via the faecal-oral route through either direct or indirect contact with contaminated surfaces (e.g., water, foods or faeces) or when spores are ingested. Furthermore, close contact with colonized animals may also be involved in the epidemiology of *C. difficile* in humans.

A certain proportion of *C. difficile* strains is very likely constantly transmitted between humans, animals and the environment as partial overlap of ribotypes isolated from humans to those found in food, animals or environment is well documented. A comparison of PCR ribotypes isolated in a single country during 3 years period from humans, animals and environment showed that 11 of total 90 PCR ribotypes were shared between all three reservoirs (Janezic et al. 2012). Strains within a given ribotype still represent very heterogeneous group and whole genome sequence level is needed for identity confirmation. This was so far done only in two studies, one on ribotype 078 strains in Netherlands and other on ribotype 014 strains in Australia (Knight et al. 2016; Knetsch et al. 2014). Although in both identity between pig and human strains was proven, the proportion of such shared strains within the studied ribotype was very low.

To date, no direct infection originating from food, animal or environmental source was described. Single study in Finland aimed at linking environmental samples from sewage and tap water to a large gastroenteritis outbreak associated with sewage contaminated drinking water (Kotila et al. 2013). Authors claimed to report for the first time that 'waterborne transmission of *C. difficile* spores was possible and a potential cause of CDI during outbreak.' However, only limited number of samples was obtained either from environment or from patients (9 strains from 19 CDI patients). Only one patient and one tap water isolate showed same PCR ribotype (014). As this is the one of the most prevalent PCR ribotypes in humans, some animals and most environments only whole genome sequencing could confirm the true association and identity of both strains.

Impact and prevention of *C. difficile* foodborne transmission is an emerging issue in *C. difficile* field. The verified presence of *C. difficile* in food begets the question about the risks for consumers. If the gut microbiota is normal, intestinal colonization may be transient (i.e., in the sense that shedding can result from short-term successful bacterial colonization or from intestinal passage of the ingested dormant spores) and can occur without associated pathology. Even if the spore numbers in foods are typically low, ingestion of a small dose in combination with an altered gut microbiota may be able to trigger infection.

The spores of *C. difficile* are heat resistant and can survive gentle cooking of foods (70 °C) but cannot survive the same range of high temperatures as the spores of other clostridial species (Rodriguez-Palacios and Lejeune 2011). Therefore, thermal treatment (85 °C for 10 min) may be the best strategy for reducing the risk of foodborne transmission. Furthermore, thermal treatment is an easy household practice that should be emphasized because it is also useful for eliminating other pathogens present in foods. Under this scenario, special attention must be given to the presence of *C. difficile* in raw foods consumed directly (e.g., raw meats or fish consumed without thermal treatment), biological products (e.g., fruits or vegetables, normally grown with the help of organic fertilizers), or traditional food products in developing countries

that are sometimes prepared without the appropriate hygienic procedures. In these cases, the prevalence and counts of spores may have greater importance than is currently recognized and may present an important potential risk of foodborne infection, especially in populations with gastrointestinal perturbations.

10 Conclusions

C. difficile reservoirs other than humans and hospitals are becoming increasingly recognized. Studies in Europe and elsewhere are more numerous especially in last couple of years, but are still fragmented in terms of animal species or type of environment covered. Broader application of environmental, food and veterinary studies in combination with application of whole genome sequencing will definitely provide new insights in *C. difficile* biology and epidemiology in years to come.

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The ESCMID Study Group for *Clostridium difficile*: History, Role and Perspectives

John E. Coia and Ed J. Kuijper

Abstract

C. difficile is a major nosocomial pathogen, but is also increasingly recognised as an important diarrhoeal pathogen in the community, not always associated with antibiotics. The European Society of Clinical Microbiology and Infectious Diseases (ESCMID) Study Group for *Clostridium difficile* (ESGCD) is a group of clinicians and scientists from many European countries and further afield, who share a common interest in *C. difficile*. The aims of the Study Group are centred around raising the profile of CDI in humans and animals, fostering collaboration amongst centres in different European countries and providing a forum for discussing and disseminating information. One of the principal aims of the Study Group is to raise awareness of *C. difficile* infections in European hospitals. ESGCD has a particular interest in the development and dissemination of European guidance on prevention, diagnosis and treatment of CDI. This chapter will discuss the organisation of ESGCD within the ESCMID Study Group structure, the origins of the Study Group, the aims and objectives of the group, and will highlight some of the past and present activities of ESGCD in relation to these.

Keywords

C. difficile research · ESCMID · Research projects · *C. difficile* guidelines

1 Introduction

C. difficile is a major nosocomial pathogen, but is also increasingly recognised as an important diarrhoeal pathogen in the community, not always associated with antibiotics. The European Society of Clinical Microbiology and Infectious Diseases (ESCMID) Study Group for *Clostridium difficile* (ESGCD) is a group of clinicians and scientists from many European countries and further afield, who share a common interest in *C. difficile*. The aims of the Study Group are centred around raising the profile of CDI in humans and animals, fostering collaboration amongst centres in different European countries and providing a forum for discussing

J. E. Coia (✉)
Scottish Microbiology Reference Laboratories, Glasgow, UK

ESCMID Study Group for C.difficile (ESGCD), Basel, Switzerland
e-mail: john.coia@nhs.net

E. J. Kuijper
ESCMID Study Group for C.difficile (ESGCD), Basel, Switzerland

Department of Medical Microbiology, Center of Infectious Diseases, Leiden University Medical Center, Leiden, The Netherlands
e-mail: E.J.Kuijper@lumc.nl

and disseminating information. One of the principal aims of the Study Group is to raise awareness of *C. difficile* infections in European hospitals. ESGCD has a particular interest in the development and dissemination of European guidance on prevention, diagnosis and treatment of CDI. This chapter will discuss the organisation of ESGCD within the ESCMID Study Group structure, the origins of the Study Group, the aims and objectives of the group, and will highlight some of the past and present activities of ESGCD in relation to these.

ESCMID and ESGCD

The organisation that we know today as ESCMID was originally founded in 1983 as the European Society of Clinical Microbiology (ESCM), with an initial membership of 41 people. In 1990, with the approval of 83% of the membership, the name of the society was formally changed to the European Society of Clinical Microbiology and Infectious Diseases. By this time, the membership had grown to 971 (Phillips 2008). In the intervening years, ESCMID has flourished to become Europe's leading society for clinical microbiology and infectious diseases with members from all European countries and all continents, and with more than 33,000 individual and affiliated members around the world. The Society's annual scientific meeting, the European Congress of Clinical Microbiology and Infectious Diseases (ECCMID), is now regarded as one of the premier meetings in the field. The 27th ECCMID, held in Vienna in April 2017, attracted 12,500 delegates from 126 countries (ESCMID 2017a).

ESCMID is a non-profit organization whose mission is to improve the diagnosis, treatment and prevention of infection-related diseases. This is achieved by promoting and supporting research, education, training, and good medical practice. The promotion of research as a core activity of the Society has been a feature virtually since the inception of ESCM. The idea of Study Groups and Working Parties as a means to support this key aim arose from Katherine Dornbusch's proposal in 1985 that the Society should associate itself with her existing international study of antibiotic resistance. The concept of such Study Groups was strongly supported by Jacques Acar during his presidency of the Society (Phillips 2008). The success of the Study Group approach is exemplified by the European Study Group on Antibiotic Breakpoints (ESGAB), which was established in 1988, and would subsequently go on to become EUCAST in 1997. The work of this group has been a major driver of standardisation and harmonisation of clinically-focussed antimicrobial sensitivity testing in Europe. A key difference between Working Parties and Study Groups was that the former were expected to have a limited single-objective-based lifetime, while the latter would be semi-permanent as long as the topic remained of significant relevance. Thus, the Study Groups' main objectives were to bring together human and veterinary researchers, both from academia and industry, to collaborate in multi-centre studies, to address scientific issues in position papers or practice guidance, and to mount educational meetings. This is still reflected in the Study Group statutes, which state that "The Study Group shall devote itself to the promotion of research and education in diagnosis and therapy in its defined field(s) of expertise" (ESCMID 2017b).

At the time of writing, ESCMID supports 28 Study Groups engaged in advancing scientific knowledge and/or disseminating professional guidelines in the field of clinical microbiology and infectious diseases (ESCMID 2017b). The Study Groups are overseen by the Scientific Affairs Subcommittee of ESCMID, and their performance is annually evaluated against a number of criteria to ensure that the required standards of scientific and professional outputs is maintained. In the 2017 evaluation ESGCD was ranked as the second best performing Study Group, and has consistently been one of the top five performing Study Groups in the preceding 3-year period. In the 5-year period 2012–2016 ESGCD presented 8 symposia and 13 other communications at ECCMID meetings, and 29 communications at other scientific meetings. In the same period the study group and its members published 31 articles (including several

medical guidelines as described below), and supported 14 research projects, two educational events and four scientific meetings outwith ECCMID.

2 The History and Origins of ESGCD

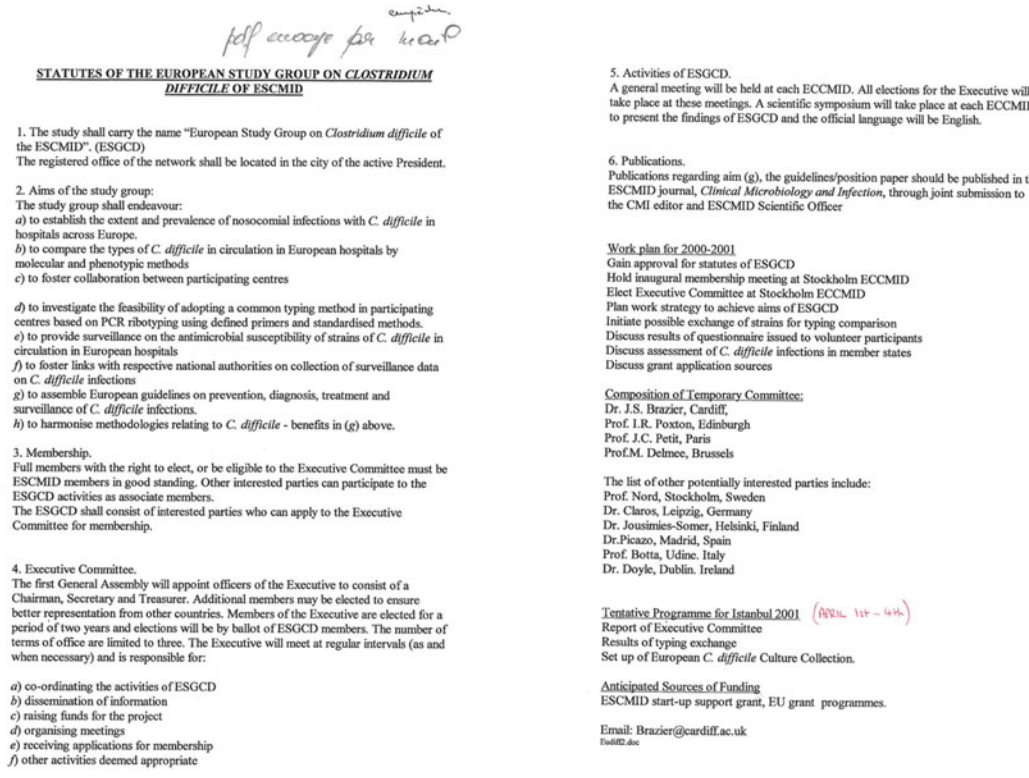
The aetiological role of *C. difficile* in PMC has been known for 40 years (Larson et al. 1978). However, it was the emergence and rapid spread in North America and Europe of the hypervirulent PCR ribotype 027 strain (Warny et al. 2005) at the dawning of the new millennium (Honda and Dubberke 2014), which was the catalyst for a resurgence of interest in CDI. ESGCD played an important role in the recognition of Type 027 in Europe, since Canadian researchers presented their data at the 10th ECCMID in Stockholm (2000) to ESCGD members and subsequently sent strains to the UK anaerobic reference laboratory under the directorship of Dr. John Brazier, who subsequently supported other European laboratories to recognize this new emerging type. This emergence of a new hypervirulent type was a stimulus for a group of scientists and clinicians with existing research and clinical interest in *Clostridium difficile* to expand the activities of the Study Group under the auspices of ESCMID.

The inaugural gathering of the nascent ESGCD was held on Sunday 28th May 2000 during the 10th ECCMID in the International Fairs Building in Stockholm, Sweden. At this time temporary officers of ESGCD were elected, with Dr. JS Brazier (UK) as the first Chairperson, and with Dr. M Claros (Germany) and Professor M. Delmeé (Belgium) acting as interim Secretary and Treasurer respectively. The original statutes of the ESGCD, which were tabled at the inaugural meeting, are shown in Fig. 1.

The first full meeting of ESGCD took place on 3 April 2001 at the 11th ECCMID in Istanbul. At this meeting Jon Brazier was confirmed as Chairperson of the group, with Michel Delmée assuming the role of treasurer, and Dr. Maja Rupnik (Slovenia) taking on the position of secretary of the group. The original aims of the Study Group were to:

- Establish the extent and prevalence of nosocomial infections with *C.difficile* in hospitals across Europe
- Compare the types of *C.difficile* in circulation in European hospitals by molecular and phenotypic methods
- Undertake a survey of *C. difficile* in animals
- Foster collaboration between participating centres worldwide on human CDI and animal CDI
- Investigate the feasibility of adopting a common typing method based on PCR ribotyping using defined primers and standardised methods
- Provide surveillance on the antimicrobial susceptibility of strains of *C.difficile* in circulation in European hospitals
- Foster links with respective national authorities on collection of surveillance data on *C.difficile* infections.
- Assemble European guidelines on prevention, diagnosis, treatment and surveillance of *C. difficile* infections, and by this process to harmonise methodologies relating to CDI.
- Collaborate with commercial entities developing treatments for CDI (vaccines, new antibiotics, immunotherapies)

In the intervening 16 years since those initial meetings in Stockholm and Istanbul, ESGCD has continued to hold an annual business meeting at each successive ECCMID. The members of the Executive committee also met every 6 months in Brussels or Edinburgh to discuss the progress of ESGCD activities. There have been a further three Chairpersons of ESGCD, and the full list of these, and the current executive committee, is provided in Tables 1 and 2 respectively. Veterinary and human clinical microbiologists have always participated in the executive committees. The Group has been a prolific and consistent contributor to the scientific programme of the ECCMID meetings through a wide range of



STATUTES OF THE EUROPEAN STUDY GROUP ON *CLOSTRIDIUM DIFFICILE* OF ESCMID

1. The study shall carry the name "European Study Group on *Clostridium difficile* of the ESCMID". (ESGCD)
The registered office of the network shall be located in the city of the active President.

2. Aims of the study group:
The study group shall endeavour:
a) to establish the extent and prevalence of nosocomial infections with *C. difficile* in hospitals across Europe.
b) to compare the types of *C. difficile* in circulation in European hospitals by molecular and phenotypic methods
c) to foster collaboration between participating centres

d) to investigate the feasibility of adopting a common typing method in participating centres based on PCR ribotyping using defined primers and standardised methods.
e) to provide surveillance on the antimicrobial susceptibility of strains of *C. difficile* in circulation in European hospitals
f) to foster links with respective national authorities on collection of surveillance data on *C. difficile* infections
g) to assemble European guidelines on prevention, diagnosis, treatment and surveillance of *C. difficile* infections.
h) to harmonise methodologies relating to *C. difficile* - benefits in (*g*) above.

3. Membership.
Full members with the right to elect, or be eligible to the Executive Committee must be ESCMID members in good standing. Other interested parties can participate to the ESGCD activities as associate members.
The ESGCD shall consist of interested parties who can apply to the Executive Committee for membership.

4. Executive Committee.
The first General Assembly will appoint officers of the Executive to consist of a Chairman, Secretary and Treasurer. Additional members may be elected to ensure better representation from other countries. Members of the Executive are elected for a period of two years and elections will be by ballot of ESGCD members. The number of terms of office are limited to three. The Executive will meet at regular intervals (as and when necessary) and is responsible for:

a) co-ordinating the activities of ESGCD
b) dissemination of information
c) raising funds for the project
d) organising meetings
e) receiving applications for membership
f) other activities deemed appropriate

5. Activities of ESGCD.
A general meeting will be held at each ECCMID. All elections for the Executive will take place at these meetings. A scientific symposium will take place at each ECCMID to present the findings of ESGCD and the official language will be English.

6. Publications.
Publications regarding aim (g), the guidelines/position paper should be published in the ESCMID journal, *Clinical Microbiology and Infection*, through joint submission to the CMI editor and ESCMID Scientific Officer

Work plan for 2000-2001
Gain approval for statutes of ESGCD
Hold inaugural membership meeting at Stockholm ECCMID
Elect Executive Committee at Stockholm ECCMID
Plan work strategy to achieve aims of ESGCD
Initiate possible exchange of strains for typing comparison
Discuss results of questionnaire issued to volunteer participants
Discuss assessment of *C. difficile* infections in member states
Discuss grant application sources

Composition of Temporary Committee:
Dr. J.S. Brazier, Cardiff,
Prof. I.R. Poxton, Edinburgh
Prof. J.C. Petit, Paris
Prof.M. Delmee, Brussels

The list of other potentially interested parties include:
Prof. Nord, Stockholm, Sweden
Dr. Claros, Leipzig, Germany
Dr. Jousimies-Somer, Helsinki, Finland
Dr.Picazo, Madrid, Spain
Prof. Botta, Udine. Italy
Dr. Doyle, Dublin. Ireland

Tentative Programme for Istanbul 2001 (APRIL 1st – 4th)
Report of Executive Committee
Results of typing exchange
Set up of European *C. difficile* Culture Collection.

Anticipated Sources of Funding
ESCMID start-up support grant, EU grant programmes.

Email: Brazier@cardiff.ac.uk
Esdiff2.doc

Fig. 1 Original statutes, workplan and composition of the committee of ESGCD

Table 1 List of previous ESGCD chairpersons

ESGCD chairperson	
2000–2005	Jon Brazier, Cardiff, UK
2005–2008	Ian Poxton, Edinburgh, UK
2008–2016	Ed Kuijper, Leiden, Netherlands

Table 2 Current ESGCD executive committee (2016–present)

Role	Name
Chairperson	John Coia, Glasgow, UK
Secretary	Sarah Tschudin Sutter, Basel, Switzerland
Treasurer	Karen Burns, Dublin, Ireland
Member	Ed Kuijper, Leiden, Netherlands
Member	Bente Olesen, Herlev, Denmark
Member	Elena Reigadas, Madrid, Spain
Member	Lutz von Müller, Coesfeld, Germany
Member	Marcela Krutova, Prague, Czech Republic
Member	Alban Le Monnier, Paris, France

symposia, workshops, presentations and posters. ESGCD has also contributed to many of the international Clospath meetings and to all the International *C.difficile* Symposia (ICDS; www.icds.si) in Slovenia, both by financial support and scientific presentations. However, most importantly, over this time the Study Group has evolved and grown to become a hub for research, and for the development and promulgation of standards for surveillance, diagnostics, infection prevention and control, and therapeutics for CDI. Although the focus has been on Europe, the fostering of collaborations with colleagues outwith Europe, particularly in North America, has ensured that the activities of ESGCD have helped to shape and influence the understanding and management of CDI globally.

3 Activities and Achievements of ESGCD

The aims of the Study Group are addressed through the support and promotion of a range of activities by Study Group members, often in collaboration with other groups and institutions. These activities include:

- Scientific and clinical projects and publications initiated by the Study Group
- Scientific and clinical projects and publications initiated in collaboration with other groups and institutions, including industrial partners
- Scientific and clinical projects and publications to which Study Group members have contributed, or which have benefitted from the professional support of ESGCD
- Proposals for scientific and educational sessions at ECCMID, or under the auspices of ESCMID
- Presentations at ECCMID and other scientific meetings
- ECCMID postgraduate workshops
- Promotion and support of scientific meetings and workshops and educational activities outside of ESCMID
- Funded research projects

Rather than provide an exhaustive list of all the activities and outputs of ESGCD, the remainder of this section will focus on the achievements in three key domains which are central to the aims and objectives of the Study Group. These are laboratory investigation of CDI (including diagnosis and typing), epidemiology and surveillance of CDI in Europe, and management of CDI (including infection prevention and control, and treatment). Activities in each of these areas has provided the basis for, and encouraged the development of, the collaboration amongst key stakeholders (individual clinical and research groups, organisations and institutions) at the national and international level. A common cross-cutting element of this approach has been the role of ESGCD in development and promotion of comprehensive, evidence-based guidance in each of these areas. A key overall achievement of these activities is that CDI is now recognised as a very significant clinical disease entity that requires to be controlled and managed in its own right, rather than being viewed as a troublesome complication of other medical interventions.

3.1 Laboratory Investigation of CDI

Accurate diagnosis is a cornerstone of any laboratory-based surveillance system. Moreover, even where there is the laboratory capability to undertake accurate diagnostic testing, the comparability of resulting surveillance data is crucially dependent upon the criteria employed for sampling and testing. The absence of specific guidelines which would help to facilitate reliable diagnosis and the accurate comparison of the incidence and the epidemiology of CDI from one hospital to another or from one country to another, was a key early concern of ESGCD. This was reflected in the minutes of the first meeting of the Study Group, where it was noted that a survey of diagnostic methods and testing protocols for CDI in Europe should be undertaken. This was one of the first major activities of ESGCD, and established a baseline measurement of the marked discrepancies between laboratories and between countries regarding the criteria by which *C.difficile* was investigated for, and the methods and strategies that were used for the diagnosis of CDI (Barbut et al. 2003). This lack of specific guidance was addressed by the publication in 2009 of ESCMID recommendations for the diagnosis of CDI developed by ESGCD (Crobach et al. 2009). A recent review of this guidance, with evaluation of the current evidence, led to the publication of updated ESGCD guidelines in 2016 (Crobach et al. 2016).

As has already been noted above, the emergence of CDI as a major pathogen in the early part of this century was associated with particular strains of *C.difficile*, and our current understanding of the epidemiology of CDI and ability to investigate and control outbreaks of infection

with this organism remains reliant upon the development and availability of robust typing methodologies. The importance of typing in elucidating the emergence and spread of novel subtypes was highlighted in a review in 2006 (Kuijper et al. 2006), which summarised the outputs of a series of meetings organised by the ECDC with experts in the field of CDI, including ESGCD and the US CDC. ESGCD has played an important role in promoting the development, standardisation and adoption of molecular subtyping (particularly PCR ribotyping) of *C.difficile* in Europe. In order to obtain an overview of the phenotypic and genotypic features of clinical isolates of *C. difficile*, during 2005 the Study Group undertook a 2-month prospective study of Clostridium difficile infections in 38 hospitals from 14 different European countries (Barbut et al. 2007). Further measures to develop and promulgate standardised typing methodologies for *C. difficile* have been closely linked to activities to develop surveillance of CDI in Europe, and are considered in the next section.

3.2 Epidemiology and Surveillance of CDI in Europe

Following the recognition of the arrival of the new hypervirulent *C. difficile* strain, PCR ribotype 027, in 2005 in Europe, ESGCD contacted ECDC and a range of stakeholders and partners to consider how recognition and awareness of CDI could be increased, and how surveillance in Europe could be improved. As part of this, the background review document on CDI (Kuijper et al. 2006), which has been referred to above was produced. This initiative was also the catalyst for the first pan-European surveillance study, the "European Clostridium difficile Infection Survey (ECDIS), supported by ECDC. This was performed in 2008–2009 and was subsequently published in the Lancet (Bauer et al. 2011). Based on the results of the ECDIS study, it was decided to provide support for further capacity building for surveillance of CDI across Europe. This resulted in the ECDIS-net project (ECDIS-net 2017), in which ESGCD and its members played a key role. ECDIS-net comprised a consortium of experts in the field of CDI including microbiologists, epidemiologists and molecular biologists, who were all in close contact with or were part of their respective National Institutes of Health, and who were active in surveillance studies of *C. difficile*. The project aimed to enhance laboratory diagnostic capacity, standardise approaches and build capacity for molecular subtyping (particularly PCR ribotyping), and to develop a European CDI surveillance protocol. As part of this work, surveys of diagnostic and typing capacity (van Dorp et al. 2016b), and of CDI surveillance systems (Kola et al. 2016), in Europe were undertaken. Following the development of the surveillance protocol, a pilot study of standardised surveillance of *Clostridium difficile* infection in European acute care hospitals was undertaken (van Dorp et al. 2016a). The protocol developed now forms the basis of the ECDC protocol for surveillance of CDI in Europe (ECDC 2017). ESGCD has subsequently partnered with ECDC in a joint project consortium on Microbiological support to European surveillance of CDI (see below).

3.3 Management of CDI

As part of its activities ESGCD has also been active in initiatives to improve the management of CDI in Europe. Again, in keeping with the original aims and objectives of the Study Group, a particular focus has been on the development and promotion of evidence-based guidance. A number of group members were involved in the production of Infection control measures to limit the spread of *C. difficile* produced on behalf of the European *C.difficile* Infection Control Group and the ECDC which were published in 2008 (Vonberg et al. 2008). This evidence-based guidance has recently been reviewed as part of the current activities of ESGCD (see below). Guidance for treatment of CDI was developed and published by Study Group members in 2009

as the ESCMID treatment guidance document for CDI (Bauer et al. 2009). An evidence-based update of this guidance was published in 2014 by Debast and colleagues (2014).

4 Current Activities of ESGCD

As can be seen from the most recent annual report (ESGCD 2017), ESGCD continues to be one of the most active ESCMID Study Groups. Nine publications were authored by, or had significant contributions to or support from, ESGCD members. These comprised production of revised European diagnostic guidance for CDI (Crobach et al. 2016), two publications from the ECDIS-Net project on diagnostic and typing capacity in Europe (van Dorp et al. 2016b) and piloting of a standardised European CDI surveillance system (van Dorp et al. 2016a), a publication from the EUCLID project on diversity of PCR-ribotypes in Europe (Davies et al. 2016), three research studies on CDI in the Czech Republic (Krutova et al. 2016a, 2016b; Nyc et al. 2017), a comprehensive review of CDI (Smits et al. 2016), and a study of transmissibility of *C. difficile* without contact isolation (Widmer et al. 2017). Updated evidence-based ESCMID guidance on Prevention and Control of CDI in acute care hospitals has now been developed by ESGCD and has been submitted for publication.

At ECCMID 2017 in Vienna an Educational Workshop on Prevention of CDI in acute care hospitals was jointly organised by ESGCD and the ESCMID Study Group for Nosocomial Infections (ESGNI), and a Meet-the-Expert session on safety, ethical and regulatory issues in Faecal microbial transplantation (FMT) was jointly organised by ESGCD and United European Gastroenterology (UEG). A joint ESGCD/UEG symposium on FMT is part of the programme of the 25th UEG week in Barcelona in October 2017, and this will provide a focus for further discussion and collaboration on standardization of FMT for treatment of patients with multiple recurrences of CDI. Guidance on how to establish a donor feces bank has also recently been published (Terveer et al. 2017). ESGCD members are also participating as expert panel members on the ESCMID Study Groups' competencies in antimicrobial prescribing and stewardship (ESCAPS) to explore a consensus for antimicrobial prescribing and stewardship competencies.

ESGCD is also a contributor to a joint project supported by ECDC in a consortium led by Professor Ed Kuijper (Netherlands) on Microbiological support to European surveillance of CDI. Several ESGCD members participated in a Train-the-Trainer workshop on diagnostic and molecular typing techniques for *C.difficile* held by the consortium in Vienna in May 2017. Members of ESGCD will also form part of the consortium being led by Prof Mark Wilcox (UK) and Prof Marc Bonten (Netherlands) "Addressing the clinical burden of CDI: Evaluation of the burden, current practices and set-up of a European research platform", which is part of the Innovative Medicines Initiative 2 (IMI 2) programme. ESGCD members are involved in an Astellas sponsored study undertaking retrospective data collection on patients with samples received during the European, multi-centre, prospective bi-annual point prevalence study of *Clostridium difficile* Infection in hospitalised patients with diarrhoea (EUCLID2).

Current Study Group plans for 2018 include jointly organising an Educational Workshop on controversies in CDI infection control with ESGNI at ECCMID 2018 in Madrid. At the same meeting ESGCD is also jointly organising, with the ESCMID Study Groups for Genomic and Molecular Diagnostics (ESGMD) and Anaerobic Infections (ESGAI), a symposium on Whole-genome sequencing and anaerobes. Outwith ESCMID, ESGCD will be represented at the Austrian Agency for Health and Food Safety (AGES) 7th Next Generation Sequencing Workshop in Vienna in March 2018, and will provide support for the planned 6th International *Clostridium difficile* Symposium (ICDS) in Slovenia later in 2018.

5 Perspectives and the Future

Since its establishment at the start of the current millennium, ESGCD has been one of the most consistently active and productive ESCMID Study Groups, and has achieved considerable success in attaining the aims and objectives outlined in the original statutes. This success has been generated by a combination of approaches reflecting not only research projects and other activities undertaken by members of ESGCD itself, but also through wider collaborations. These partnerships have served to add further value to the activities of ESGCD, and have encompassed specific formal research projects in combination with other national and international partners e.g. the European Centre for Disease Prevention and Control (ECDC), as well as less structured ad-hoc interactions between groups of individual scientists and clinicians. Importantly, industrial partners also approached ESGCD for advice and participation in their projects, using the knowledge and experiences of ESGCD members. All of these activities have been underpinned by a common underlying goal of mitigating the impact of CDI, and a significant proportion of the material in the chapters of this book reflect the outcomes of some of this work.

There can be little doubt that considerable progress has been made in understanding the epidemiology of CDI in Europe, and in developing comprehensive guidance for the surveillance, diagnosis, prevention, control and management of this major nosocomial pathogen. However, despite these successes CDI remains a very significant infection challenge in many parts of Europe and beyond. CDI is also an important disease in animals and the recent emergence of Type 078 in human CDI coincided with the finding of this type in diarrhoeal piglets. CDI fits in a "One Health" approach, since whole genome sequencing has revealed genetic identity between human and animal isolates for at least two ribotypes (078, Knetsch et al. 2014; 014, Knight et al. 2016). Many unresolved issues remain, and even as our knowledge advances, fresh questions arise. How can surveillance of CDI be extended in resource-poor settings? What are the virulence mechanisms of the "hypervirulent" strains? What is the precise role of the intestinal microbiota in defence against CDI? What are the relative contributions of different control measures in prevention of nosocomially-acquired CDI? What is the role of asymptomatic carriage? How can diagnostic testing be improved and simplified? What is the best approach to deal with recurrent disease or severe disease? What fresh insights will the application of whole-genome sequencing, which has already challenged our existing paradigm of CDI, bring to our understanding and management of CDI? What is the variety of reservoirs contributing to hospital and community CDI? Clearly the work of, and need for, the activities of ESGCD is far from complete.

Perhaps the most enduring legacy of ESGCD will be the establishment and support of a collaborative multidisciplinary network (European Reference Network, ERN) of academic researchers and healthcare professionals that have a shared interest in addressing the existing, and emerging, unanswered questions that remain. It is also important to include patient organisations, since their contribution to ERN is very much appreciated and warranted. ESGCD, driven by the continued enthusiasm of its members and working in partnership with other networks and national and international institutions, can and should provide a key focal point for clinical and research activities and initiatives in our ongoing efforts to tackle the continuing challenge of CDI in Europe.

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Paola Mastrantonio and Maja Rupnik

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