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Clostridium difficile infection: Early history, diagnosis and molecular strain typing methods

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ABSTRACT

Recognised as the leading cause of nosocomial antibiotic-associated diarrhoea, the incidence of *Clostridium difficile* infection (CDI) remains high despite efforts to improve prevention and reduce the spread of the bacterium in healthcare settings. In the last decade, many studies have focused on the epidemiology and rapid diagnosis of CDI. In addition, different typing methods have been developed for epidemiological studies. This review explores the history of *C. difficile* and the current scope of the infection. The variety of available laboratory tests for CDI diagnosis and strain typing methods are also examined.

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Contents

1. Introduction

Clostridium difficile is one of the most important nosocomial pathogens in humans. It is responsible for outbreaks of hospital-acquired infection, with symptoms including serious diarrhoea and, in several cases, pseudomembranous colitis and even death. Although the principal risk factors in patients are a history of antibiotic treatment, an age of over 65 years, and prolonged hospitalisation [1,2], in recent years, studies have described the bacterium spreading further into the community [3] and an increase in the incidence and severity of nosocomial *C. difficile* infection (CDI) in North America and Europe [4]. This rise has been attributed to the emergence of new hypervirulent strains, including PCR-ribotype 027 [5] and PCR-ribotype 078 [6], which has been associated with antimicrobial exposure. Furthermore, a significant

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correlation between the lack of PCR-ribotype diversity in healthcare settings and greater antimicrobial resistance has been observed [7].

In the past years, several studies and guidelines have been published to compare CDI incidence among different clinical settings, to increase the awareness of *C. difficile* and to improve the diagnosis and management of the infection [8]. This review is intended to describe the history of *C. difficile*, starting from the first descriptions up to the present, including the current knowledge regarding the detection, typing methods, and laboratory diagnosis of CDI.

2. *Clostridium difficile* discovery and its early history in humans

C. difficile was first identified by Hall and O'Toole in 1935, in a study of the daily microbial changes in the faeces of ten normal breast-fed infants up to the tenth day, when they left the hospital. The bacterium was described as a strict anaerobe with subterminal, non-bulging, elongate spores. In recognition of the difficulty of its isolation and study, it was originally named *Bacillus difficilis* [9]. Another remarkable property was its pathogenicity. Some strains were capable of producing toxins and caused respiratory death, with marked edema in the subcutaneous tissues of guinea pigs, rabbits, cats, dogs, rats and pigeons and convulsions in guinea pigs similar to those of tetanus. Its toxin was thermolabile, being inactivated in 5 min at 60 °C, but was not absorbed from the intestinal tract of the guinea pig, rat and dog: it acted only upon injection into the tissues [10]. In 1938, the bacterium *B. difficilis* was reclassified into the genus *Clostridium* [11] and *C. difficile* nomenclature was adopted by the Approved List of Bacterial Names [12].

Between 1940 and 1962, only two studies in the literature refer to *C. difficile* in humans [13,14]. However, there was no evidence in these cases that *C. difficile* was pathogenic. In the 1970s, a number of reports focused on the isolation of *C. difficile* from different hospitalised cases [15–21], but there did not seem to be an obvious pathogenic role in these cases, and *C. difficile* was still considered to be part of the normal faecal flora of humans. During this period, the first studies in animal models were published [22,23]. One of these studies [23] reported a cytopathic toxin in tissue-cultured cells and suggested the activation of an uncultivated virus. However, in retrospect, these findings could represent a description of the cytopathic effect of *C. difficile* induced by its toxins [24].

Pseudomembranous colitis (PMC) was first described in 1893 [25], prior to the availability of antibiotics, as a post-operative complication of gastrojejunostomy for an obstructive peptic ulcer in a young woman. Ten days after surgery, the patient developed haemorrhagic diarrhoea and died. After autopsy, the disease was identified as diphtheric colitis [26]. In subsequent years, many other early cases of PMC were recorded after surgical operations, in particular for patients with obstructive colorectal carcinoma [27] or under antimicrobial therapy [28–30]; however, while many studies showed important clues, its association with *C. difficile* would not occur until 1978 [31–36]. The finding was reported by three studies that were published in the literature almost simultaneously. In March 1978, one study [37] suggested that *C. difficile* was the causative agent of PMC. The authors found high titres of toxin in the faeces of all patients with PMC studied and hypothesised that the bacterium might be present in small quantities in the intestines of healthy adults and that under the appropriate conditions, it was able to multiply and cause postoperative diarrhoea or PMC due to its potential for toxin production. In April 1978, a second study [38] reported the isolation of *C. difficile* from the faeces of a patient with clindamycin-associated PMC and demonstrated both the presence of a faecal toxin and the toxigenicity of the isolate using a tissue-culture assay. In May 1978, a third study [39] reported that *C. difficile* was responsible for PMC and that previous antibiotic therapy produces susceptibility to infection, presumably as a result of a change in the bacterial flora. Finally, in late 1978, it was demonstrated that vancomycin eliminates toxin-producing *C. difficile* from the colon and is associated with rapid clinical improvement in patients with pseudomembranous colitis [40]. Fig. 1 summarises the early history of *C. difficile* in humans.

Since then, the number of reports documenting *C. difficile* infection in hospitals increased, and it became the pathogen of the 90s [41]. In the early 2000s, a rise in the incidence, severity and mortality rate of CDI was reported in Europe and North America, associated with the emergence of a new hypervirulent strain, PCR-ribotype 027 [5]. *C. difficile* is now a worldwide public health concern, as it is considered the major cause of antibiotic-associated infections in healthcare settings. Three previous reviews have addressed the recent epidemiology of CDI in hospitals, nursing homes and in the community as well as the principal outbreaks reported [2,42,43].

In recent years, with the availability of next-generation sequencing technologies, it has been demonstrated that *C. difficile* is closely related to the *Peptostreptococcaceae* family. It has therefore been suggested that *C. difficile* should be attributed to a new *Peptoclostridium* genus, renaming *C. difficile* to *Peptoclostridium difficile*. The newly proposed genus, *Peptoclostridium*, are Gram-positive, motile, spore-forming obligate anaerobes. All strains are mesophilic or thermophilic, grow in a neutral to alkaline pH and are oxidase- and catalase-negative. The G + C content of the genomic DNA ranged from 25 to 32 mol % [44].

3. The scope of CDI

C. difficile intestinal colonisation can be asymptomatic or produce disease. The clinical manifestations of CDI range from mild or moderate diarrhoea to fulminant pseudomembranous colitis [8]. Other symptoms described are malaise, fever, nausea, anorexia, the presence of mucus or blood in the stool, cramping, abdominal discomfort and peripheral leucocytosis. Extraintestinal manifestations (arthritis or bacteraemia) have been described but are rare. Severe disease can present colonic ileus or toxic dilatation and distension with little or no diarrhoea. The worst outcome of CDI is sepsis and death [8], which is estimated to occur in 17% of cases; however, this percentage is higher among older people [45].

Antibiotic treatment [1] and advanced age have classically been associated with *C. difficile* infection and related to an increased mortality rate [46]. A recent review regarding CDI cost-of-illness describes a mean cost ranging from 8911 to 30,049 USD for hospitalised patients (per patient/admission/episode/infection) in the USA [47]. In Europe, the annual economic burden is estimated to be approximately 3000 million euro [48]. However, it is necessary to note that the diagnostic strategy remains suboptimal in a large number of healthcare facilities, and a significant proportion of infections may remain undiagnosed [49].

Colonisation by non-toxigenic *C. difficile* has also been described, with a prevalence ranging between 0.4% and 6.9% [50], although this prevalence is lower than the estimated asymptomatic colonisation by toxigenic strains, which is between 7% and 51% [51,52]. Furthermore, it has been hypothesised that asymptomatic carriers can be colonised by both types of strains (toxigenic and non-toxigenic) for long periods of time without developing the disease [53]. However, these asymptomatic carriers could play an important role in transmission as a source for many unexplained cases [54]. It has been suggested that the presence of non-toxigenic *C. difficile* in the intestinal tract protects against CDI, although there is no clear evidence to explain how these avirulent strains reduce

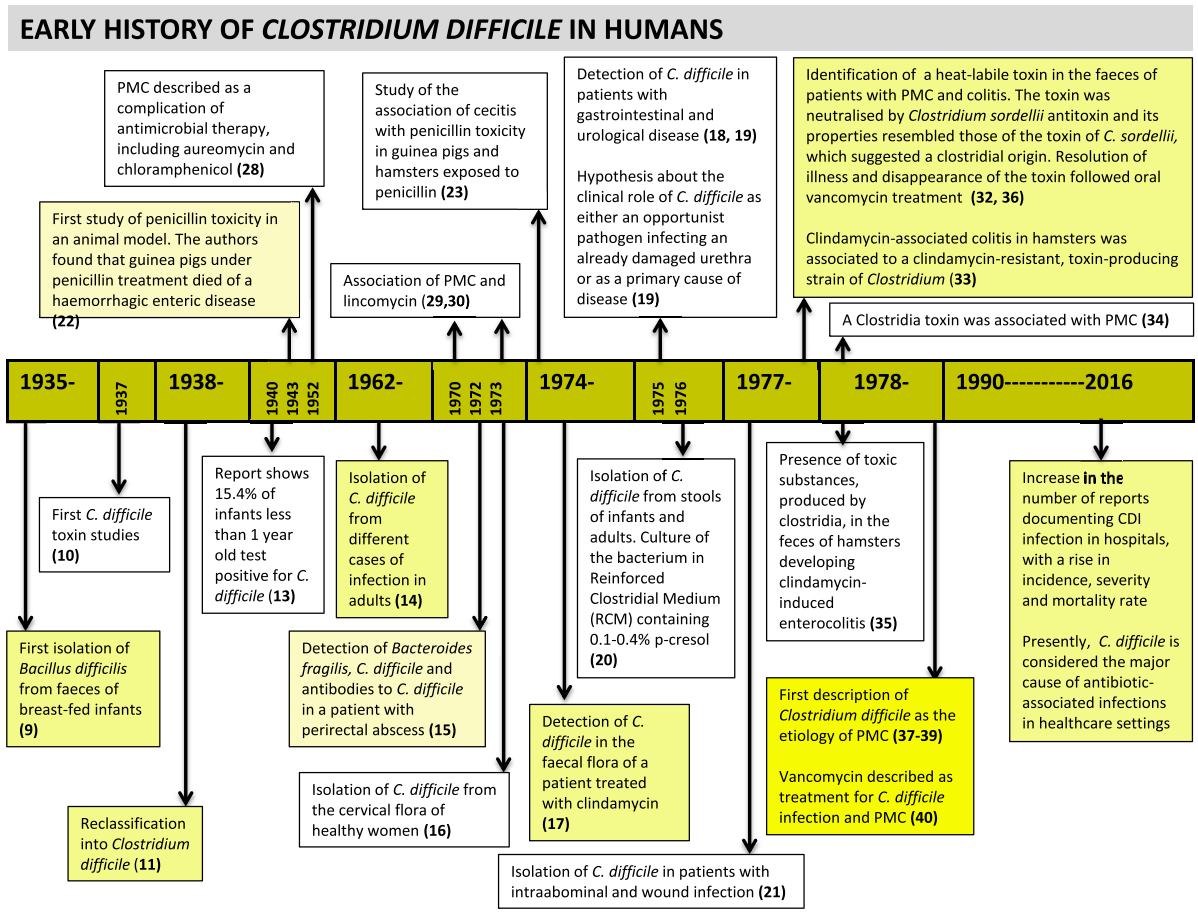

Fig. 1. *Clostridium difficile* history in humans PMC: pseudomembranous colitis.

the risk of developing an infection [50]. Simple competition for a niche in the gastrointestinal tract or other complex effects on mucosal immunity and nutrient acquisition have been hypothesised [50]. A variation in *C. difficile* non-toxigenic colonisation with age has been described, ranging from 6.9% for patients aged 60 years or more [55] to 22.8% for patients younger than 20 years of age [56], and up to 53–96% in neonatal units [57,58], supporting the hypothesis that these strains are more prevalent in younger patients and infants [50].

4. *C. difficile* outside Europe and North America

As previously cited, *C. difficile* is the most frequent bacteria associated with nosocomial diarrhoea in Europe and North America. However, little information is available regarding the extent of the infection in other regions or developing countries. In Zimbabwe, a study conducted in a healthcare centre reported a prevalence of 8.6% in a total of 268 diarrhoeal stool samples. Further characterisation of the isolates showed that all were susceptible to metronidazole and vancomycin, but approximately 70% were resistant to co-trimoxazole, which is an antibiotic widely used in this region as prophylaxis against infections in HIV/AIDS patients [59]. In a study of the gut microbiota of 6-month-old Kenyan infants consuming home-fortified maize porridge daily for 4 months and receiving micronutrient powder containing 2.5 ng of iron, *C. difficile* was detected with a high prevalence (56.5%). The results obtained showed that iron fortification in infants adversely affected the gut microbiota, with an increase in the proportion of some pathogenic bacteria, including *Escherichia coli*, *Salmonella*, *Clostridium perfringens* and *C. difficile* [60]. A review [61] on the epidemiology of *C. difficile* in Asia shows that infection occurred at similar rates to other areas but with a predominance of variant toxin A and toxin B positive strains, including PCR-ribotypes 017 and 018. In contrast with the situation in America and Europe, PCR-ribotypes 027 and 078 have rarely been reported in Asia. The unregulated use of antibiotics in some Asian regions and the lack of surveillance raise concerns over the risk of bacterial mutation and infection [61]. An additional review describes the situation in Thailand in detail. A lack of data regarding *C. difficile* epidemiology is reported along with a high level of indiscriminate use of antimicrobials. *C. difficile* strains isolated from Thai patients showed a high degree of resistance for a wide range of antibiotics, including clindamycin, cefoxitin and erythromycin. Nevertheless, the strains were fully susceptible to metronidazole and vancomycin. In the same review, the authors concluded with the recommendation for a monitoring plan for *C. difficile* infections in hospital and community settings in Thailand and other Asian countries [62]. The same observation has been made for Latin America, where little data are available regarding the epidemiology of *C. difficile* in hospitals, and increased awareness and vigilance among healthcare professionals and the general public seem essential [63]. In an epidemiological study of *C. difficile*-associated diarrhoea in a Peruvian hospital, the reported overall incidence per 1000 admissions was 12.9. As the presence of another patient with CDI in the same room was significantly associated with the development of diarrhoea, the authors concluded that *C. difficile* transmission commonly occurred in this healthcare setting and highlighted the need for implementing adequate hygiene programmes [64] (Table 1).

Table 1
C. difficile infection in Asia, Africa and South America.

Continent	Country	Patients enrolled in the study/type of samples	CDI cases	Main PCR-ribotypes	Date of study[a]	Reference
Africa	Zimbabwe	Diarrhoeal stools of outpatients over 2 years of age presenting at healthcare centres	8.6% (23/268)	–	2014	[59]
	Kenya	6 month-old Kenyan infants consuming home fortified maize porridge and 2.5 ng of iron daily for 4 months	56.5% (65/115)[b]	–	2015	[60]
	Nigeria	HIV-positive inpatients of a University Teaching Hospital	43.5% (10/23)	–	2008–2009	[67]
		HIV positive outpatients a University Teaching Hospital	14% (10/71)			
Asia	Japan	–	–	018/014/002/001	2013	[61]
	Korea	Adult patients from 17 tertiary hospitals with a diagnosis of CDI	2.7/1000[c]	–	2004–2008	
	Malaysia	Stool samples from hospitalised inpatients with antibiotic associated disease	13.7% (24/175)	–	2008	
	India	Stool samples from hospitalised patients with antibiotic associated disease	22.6% (21/93)	_	1983–1984	
		Hospitalised patients with diarrhoea over 1 year	11.1% (38/341)	–	1991	
		Diarrheal hospitalised patients	16.7% (26/156)	–	1999	
		Hospitalised patients suspected of suffering CDI	17.2% (17/99)	–	2006–2008	
		Children with acute diarrhoea in hospitals	7-11%	–	1991/2001/2005	
		HIV seropositive adult subjects with diarrhoea	18.08%	–	2008–2011	[66]
	Bangladesh	Children admitted to hospital with diarrhoea	1.6% (13/814)	–	1999	[61]
	China	Patients from a 1216 bed hospital in Shanghai	17.1/10,000[d]	017/012/046	2007–2008	
	Taiwan	Stools samples from patients with CDI at all high-risk units	0.45/1000[d]	–	2010	
			7.9/1000[e]			
	Hong-Kong	Stools samples from patients suspected of CDI collected at a university-affiliated teaching hospital	5.1% (37/723)	027[f]	2008	[137]
	Singapore	Samples from patients of tertiary and secondary general hospitals	5.16/10,000[h]	–	2006	[138]
			2.99/10,000[h]		2008	
	Indonesia	Patients with diarrhoea in community and hospital settings	1.3% (2/154)	–	1997–1999	[140]
	Thailand	Hospitalised patients of all ages	52.2% (106/203)	–	1990	[62]
		Diarrheal stools of patients between 0 and 3 years	84.8%			
		Patients over 15 years	10% (20/140)	–	1991_1994	
		Antimicrobial treated group	1.4% (2/140)			
		Control group				
		Immunocompromised patients	4.8%–52.2%	–	1998	
		Febrile neutropenia paediatric oncology	36.7% (11/30)			
		Human immunodeficiency virus HIV positive cohort	58.8% (20/34)			
		Diarrheal patients	36.5% (99/271)			
		Non diarrheal patients	15; 6% (16/102)			
		Acquired immunodeficiency syndrome in HIV positive patients				
		Patients of all ages	41.7% (20/48)	–	2001	
		Treated with antimicrobials	15.5% (13/84)			
		Non-treated with antimicrobials				
		Hospitalised patients	18.6% (107/574)	–	2000–2001	
		Hospitalised patients over 15 years	26.9% (47/175)	–	2012	
South America	Chile	Hospitalised patients suspected of having CDI	20.6% (81/392)	012 (14.8%) 027 (12.3%) 046 (12.3%) 012/020 (9.9%)	2011–2012	[139]
	Argentina	Faecal specimens from hospitalised and ambulatory patients	6.5% (16/245)	–	1998–1999	[63]
		Diarrheal stool samples from hospitalised patients	36.8% (32/87)	–	2000–2001	
		Hospitalised patients	37/10,000[d]	017 (90.8%)/001 (5.3%)/014 (3.1%)/031 (0.7%)	2000	
			84/10,000[d]		2001	
			67/10,000[d]		2002	
			43/10,000[d]		2003	
			48/10,000[d]		2004	
			42/10,000[d]		2005	
	Brazil	Faeces of children over 1 year with acute diarrhoea	5.5% (10/181)	–	2000–2001	
		Faeces of children aged between 3 months and 7 years	6.7% (14/210)	–	2003	

	Patients with diarrhoea in the intensive care unit	44.9% (22/49)	–	2002	
	Faecal samples from children between 3 months and 7 years old	–	001/015/031/043/046/131/132/133/134/135/136/142/143	2007	
	Adult hospitalised patients	28.5% (6/21)[i]	014/106	2008–2009	
	Hospitalised patients suspected of having CDI	3.3/1000[d,j]	–	2002–2003	
	Patients of a medical surgical intensive care unit presenting nosocomial diarrhoea	19.7% (43/218)[k]	038/135	2006–2009	
	Immunosuppressed adult patients receiving antimicrobial treatment before an episode of nosocomial diarrhoea	21.7% (19/70)	010/020/133/233	2008–2009	
Chile	Hospitalised patients at a university tertiary hospital suspected of having CDI	28.2% (26/92)	–	2001	
	Hospitalised patients suspected of having CDI	0.53/100[l] 7/100[m]	–	2000–2001	
	Inpatients of a health unit	15.9% (112/706)	–	2003–2008	
Costa Rica	Patients presenting diarrhoea and receiving antimicrobial drugs	30% (31/104)	–	2008	
	Patients with CDI in a Costa Rica hospital	–	027 (54%)	2010	
Jamaica	Patients with and without immunosuppressive treatment and patients under radiotherapy	14.1% (16/113)	–	2009	
Mexico	Hospitalised patients at a tertiary hospital	5.04/1000[e]	–	2003–2007	
Puerto Rico	Hospitalised patients with diarrhoea	10.3%	–	2005	
Peru	Hospitalised patients at a tertiary hospital	156/4264 (35.5%)	–	2005–2006	[64]

[a] Study period if available or date of publication.
[b] Detection by 16S pyrosequencing and targeted real-time PCR.
[c] Incidence of CDI for adult admission.
[d] Incidence of CDI for admission.
[e] Incidence of CDI in medical care units.
[f] Only one strain identified as PCR-ribotype 027.
[h] Incidence of CDI cases per 100,000 inpatient-days.
[i] *C. difficile* was isolated from 4/6 of the patients with CDI.
[j] *C. difficile* was isolated from 16/138 stool samples of patients with CDI.
[k] Mean incidence of CDI 1.8/1000 patient days. Highest incidence between December 2007 and August 2008 (5.5/1000 patient days).
[l] Global incidence of CDI per year in the hospital.
[m] Incidence in the nephrology unit.

One of the most serious human health problems in developing regions is the microbial contamination of drinking water and foods, leading to severe gastrointestinal diseases that are exacerbated by under-nutrition and the lack of medical treatment in these regions. Water-, sanitation- and hygiene-related deaths occur almost exclusively in developing countries (99.8%), of which 90% are the deaths of children [65]. Indeed, children are the most at-risk group, especially in the first year of life. *C. difficile* was identified among a large number of bacteria associated with diarrhoea in this population. However, the source of contamination (water, food or environment) by the enteropathogens identified in diarrheic children was not elucidated [65].

Another issue of concern is CDI in immuno-compromised patients in developing countries. In a study conducted to assess the microbial aetiologies of diarrhoea in adults infected with human immunodeficiency virus (HIV) in India, *C. difficile* was the most common bacterial pathogen identified, with a reported prevalence of 18% [66]. Consistent with this study, HIV-positive inpatients and outpatients in Nigeria were shown to be *C. difficile*-positive in 43% and 14% of cases, respectively [67] (Table 1). Both studies show the importance of establishing controlled and regulated access to antibiotics in developing countries, as well as the importance of the early diagnosis of intestinal pathogens to reduce morbidity and mortality rates, especially among HIV-positive people. In a further study evaluating CDI in travellers, infection was reported to be more commonly acquired in low- and middle-income countries. Furthermore, CDI was often acquired in the community by young patients and associated with the empirical use of antimicrobials, frequently fluoroquinolones [68].

5. *C. difficile* is found everywhere

C. difficile is ubiquitous in the environment, and the bacterium has the capacity to persist on inanimate surfaces for as long as several months [69]. These contaminated areas can contribute towards *C. difficile* transmission in healthcare settings. Bed frames, floors or bedside tables have been described as the most commonly contaminated areas in rooms used to isolate patients with *C. difficile* diarrhoea [70], even after detergent-based cleaning [71]. Table 2 summarises the available studies in the literature regarding the dissemination of *C. difficile* spores in healthcare settings and related environments. However, the difference in prevalence among studies may be due to the sampling and culture methods used [70] and in the cleaning programmes used to control the spread of *C. difficile*. In this context, a previous study reported that unbuffered hypochlorite (500 ppm) was less effective than phosphate buffered hypochlorite (1600 ppm) for surface decontamination [72]. In addition to the patient room environment, the bacterium was isolated from the hands and stools of asymptomatic hospital staff and from the home of a patient suffering CDI. Furthermore, *C. difficile* inoculated onto a surface (floor) has been shown to persist there for five months [73]. In an intensive care unit, an outbreak of pseudomembranous colitis was attributed to the cross-contamination of inanimate environmental sources with persistence in the hospital for several weeks [77]. Regarding the medical equipment, two previous studies have reported that the replacement of electronic thermometers with single-use disposables significantly reduced the incidence of *C. difficile*-associated diarrhoea in both acute care and skilled nursing care facilities [78,79]. However, it has also been reported that with the use of disposable or electronic thermometers, there was no effect on either the overall rate of nosocomial diarrhoea or the rate of nosocomial infections [79]. A further study also describes how the use of tympanic thermometers reduces the risk of acquiring vancomycin-resistant *Enterococcus* and CDI by 60% and 40%, respectively [80].

Increased interest in the transmission of *C. difficile* has led to new studies in the literature reporting the presence of spores in other areas never studied before. Medical staff has increasingly used mobile technology devices in hospitals, such as iPads, to access electronic patient information. A recent study [82] evaluated the contamination of 20 iPads by *C. difficile* spores in a healthcare setting. Although with the number of samples tested, there was not sufficient data to estimate the prevalence, and in addition, there was no *C. difficile* recovery, the study also reported the effect of different agents on iPad disinfection. The results showed that bleach wipes were able to remove the inoculated spores completely from the screen surface, while a microfibre cloth was more effective than alcohol wipes. As there are no existing medical guidelines specific to electronic devices, and the manufacturer recommends avoiding the use of chemicals or abrasives to clean the device, the authors emphasised the importance of reducing the tablets' environmental contact in rooms housing patients suffering from CDI.

There are few studies describing the presence of *C. difficile* in the natural environment and in the environment in the community (Table 3). The prevalence of *C. difficile* was recently studied in retail baskets, trolleys, conveyor belts and plastic bags in 17 different supermarkets from 2 cities in Saudi Arabia. The study reported a *C. difficile* prevalence of 0.75% on sampled surfaces, with the highest level of contamination in baskets and trolleys, which could suggest the need for the implementation of planned disinfection in supermarkets to control community-acquired CDI [83]. In the natural environment, the bacterium was detected in seawater, zooplankton [84], tropical soils [85] and rivers [86]. In the rural environment, *C. difficile* was recovered from homestead soils, household-stored water [87] and soils of stud farms with mature horses [88]. In this last study, *C. difficile* was inoculated in equine faeces and the bacterium was found to survive at least 4 years (no later time points were tested) when kept at room temperature and outdoors at an ambient temperature over the year.

While *C. difficile* is also known as an enteric pathogen in some food-producing and companion animal species, there are several reports describing the presence of the bacterium in the intestinal contents of apparently healthy animals (Table 3). Moreover, recently published data suggests that animals are an important source of human CDI that can spread disease through environmental contamination, direct or indirect contact, or food contamination, including carcass and meat contamination at slaughter or, in the case of crops, through the use of organic animal manure [129]. Table 3 summarises the prevalence of *C. difficile* reported in pets (dogs and cats), food animals (pigs and cattle), horses and wild animals. Despite the large number of studies describing the presence of human epidemic PCR-ribotypes in these animals, *C. difficile* has not been confirmed as a zoonotic agent, but it seems evident that there is a potential risk of transmission, especially in people with close contact with contaminated animals and their environment.

6. *C. difficile* characteristics and its toxins

Since its discovery in 1935, the characteristics of *C. difficile* growth, sporulation and virulence have been documented in detail. The fundamental aspects of the bacterium are summarised in Table 4. One of these characteristics is that *C. difficile* has no protease, phospholipase C or lipase, but it is among the few bacteria able to ferment tyrosine to *p*-cresol, which is a phenolic compound that inhibits the growth of other anaerobic bacteria [134,20]. Dawson et al. (2008) [134] found that *Clostridium sordellii* tolerated *p*-cresol but did not produce it. Therefore, the authors suggested that the mechanism of tolerance might not be linked to the production of this organic compound. Furthermore, the increased

Table 2
C. difficile spores in the environment of healthcare settings.

Environment	Positive surfaces (number or percentage of positive surfaces/patient rooms)	Study conditions	Main PCR-ribotypes (% of toxigenic strains)	Reference
Hospital rooms previously accommodating CDI patients	Patient-helper trapeze (5) Call button (3) Bed table (4) Bedrail (6) Tap (3) Toilet (7) Inner door handle (1) Shackle of hand disinfectant (1) Door handle facing to outer sluice (1) Stethoscope (1) Rail at foot-end (2)	Surface sampling before being cleaned	PCR-ribotype 012 PCR-ribotype 020C PCR-ribotype SE121 PCR-ribotype 023	[70]
	Patient-helper trapeze (1) Bedrail (2) Toilet (3)	Surface sampling after being cleaned		
Hospital side rooms used for the isolation of patients with symptomatic CDI	Floor (45%) Light (35%) Bed (9%) Sink/table (8%) Window (3%)	Surface sampling after detergent-based cleaning	PCR-ribotype 1 (93% of toxigenic strains)	[71]
Hospital with an outbreak of antibiotic-associated colitis	Environmental cultures obtained on the ward (31.4%)	Surface sampling before ward disinfection	–	[72]
	Environmental cultures obtained on the ward (21%)	Surface sampling after ward disinfection with unbuffered hypochlorite[a]		
Environment and contacts of hospitalised patients carrying *C. difficile* in their stools	Floors and other surfaces (9.3%)	Areas where carriers had diarrhoea	(100% of toxigenic strains)	[73]
	Floors (2.6%)	Areas without *C. difficile* carriers		
Different areas of hospitals with and without positive patients for *C. difficile*	Environmental cultures (32.5%)	Case-related environments[b]	–	[74]
	Environmental cultures (1.3%)	Control sites[c]		
	Highest counts of *C. difficile* from toilet seats, toilet bowl rims, bathroom handrails and bathroom floors	Ambulatory patients		
	Highest counts of *C. difficile* from bed handrails and near the beds	Non ambulatory patients		
Two elderly medicine wards	Environmental cultures (35%)	Two different types of cleaning which included hypochlorite or neutral liquid detergent[d]	–	[75]
Samples of the inanimate ward environment on two elderly medicine hospital wards (A and B)	Environmental cultures (34%). Highest counts of *C. difficile* (sorted in descending order) from: sluice floor, commodes, toilet floors, ward floors, radiators and air vents	Environmental samples for ward A	–	[76]
	Environmental cultures (36%). Highest counts of *C. difficile* (sorted in descending order) from: commodes, toilet floors, ward floors/air vents/ sluice floor and radiators	Environmental samples for ward A		
Samples from surfaces of a variety of areas in a nursing home	Environmental cultures (kitchen, kitchen-staff locker room and bathroom, resident's rooms, private bathrooms, residence hall, lifts and staircase railings (0%)	Sampling before and after cleaning routine	–	[81]

[a] If disinfection with phosphate buffered hypochlorite, 98% of reduction in surface contamination.
[b] Hospital bedrooms and bathrooms used by eight patients with faecal cultures positive for *C. difficile*.
[c] Hospital bedrooms and bathrooms where there were no known cases of diarrhoea.
[d] Decrease of CDI incidence on one ward when it was disinfected with hypochlorite.

production and *p*-cresol tolerance of some strains, including PCR-ribotype 027 strains, has led to hypotheses regarding its contribution towards *C. difficile* hypervirulence [134].

C. difficile is able to produce two major toxins, toxin A (308 kDa) and toxin B (270 kDa), as well as the binary cytolethal distending toxin (CDT). Toxins A and B belong to the group of Large Clostridial Toxins (LCT). Only strains that produce at least one of the three toxins cause disease. Toxin A is considered to be an enterotoxin because it causes fluid accumulation in the bowel. Toxin B does not cause fluid accumulation but is extremely cytopathic for tissue-cultured cells [130]. These toxins are encoded by two genes, *tcdA* and *tcdB*, mapping to a 19.6 kb pathogenicity locus (PaLoc) and containing 3 additional regulatory genes, *tcdC*, *tcdR*, and *tcdE*, that are responsible for the synthesis and regulation of toxins A and B [135]. Deletions, insertions, or polymorphic restriction sites in one or more of the PaLoc genes can result in toxin variant strains that produce either toxin A or toxin B [135]. While the power of purified toxin A to produce pathology *in vitro* has been widely described, a study [136] reported that toxin B, but not toxin A, was essential for virulence. Finally, in 2011, it was shown that both mutant variants, toxin A+ toxin B− and toxin A− toxin B+, can cause disease [137]. It is worth noting that toxin A + B− isolates of *C. difficile* have not been described in nature. Toxin A− toxin B+ strains have been widely reported in human cases [138] but also in animals suffering from diarrhoea [139]. A previous study reported that toxin A− toxin B+ strains caused the same spectrum of disease that is associated with toxin A+ toxin B+ strains, ranging from asymptomatic colonisation to fulminant colitis, with outbreaks in hospitals and other

Table 3
C. difficile spores in the natural environment, in the environment in the community and in animals.

Environment/animals	Prevalence (%)	Samples	Main PCR-ribotypes	Reference
17 supermarkets from two cities in Saudi Arabia	20/1600 (0.75%)	Retail baskets and trolleys (4/400) Conveyor belts (1/400) Plastic bags (3/400)	PCR-ribotype 027 4 different PCR-ribotypes (with NIN)	[83]
Five sampling stations along the coastline of the Gulf of Naples	9/21 (42.9%)	Sea water (2/5) Sediment (0/5) Zooplankton (3/5) Shellfish (4/6)	PCR-ribotype 003 PCR-ribotype 005 PCR-ribotype 009 PCR-ribotype 010 PCR-ribotype 056 PCR-ribotype 060	[84]
Samples from grassland, non-agricultural soils from five different regions of Costa Rica	3/117 (3%)	Central Plateau/Dry Pacific/North	–	[85]
Water samples (n = 69) from 25 different rivers in Slovenia	42/69 (60.9%) 17/25 (68%)	Water samples Rivers	34 different PCR-ribotypes PCR-ribotype 014 predominant (16.2% of all isolates)	[86]
Samples from a rural community in Zimbabwe	54/146 (37%) 14/234 (6%) 20/115 (17.4%) 7/161 (4.3%)	Soil samples Water samples Chicken faces samples Faecal samples of other animals	–	[87]
Samples from studfarms and farms with horses and samples from faecal samples of horses	14/132 (11%) 2/220 (1%)	Outdoor soil samples Soil samples from farms with mature horses	–	[87]
	4/72 (6%) 18/43 (42%)	Diarrhoeic mature horses Faecal samples of horses with colitis	–	[88]
	(5%–63%)	Foals and adults horses with gastrointestinal disease	–	[111]
	5/134 (3.7%)	Faecal samples of horses at hospital admission	PCR-ribotype 014 4 additional PCR-ribotypes with NIN	[112]
	10/73 (13.7%)	Faecal samples of hospitalised horses	PCR-ribotype 014 6 additional PCR-ribotypes with NIN	[113]
	4/82 (4.8%)	Faecal samples of horses at hospital admission	17 different PCR-ribotypes	[114]
	14/62 (23%)	Faecal samples from diarrheic horses	6 different PCR-ribotypes	[115]
Wild animals	2 fatal cases of elephants with enterocolitis	Samples of intestinal contents of two Asian Elephants	PCR-ribotype I	[89]
	1 clinical case of an ocelot with diarrhoea	Stool sample of a male Ocelot	–	[90]
	11/30 (36.7%)	Pooled faecal samples of captive white-tailed deer	PCR-ribotype 078	[91]
	2/34 (5.9%)	Non-diarrheic maned wolf and a diarrheic ocelot	–	[92]
	7/175 (4%)	Droppings from barn Swallows	PCR-ribotype SB3, PCR-ribotype SB159 PCR-ribotype S166	[93]
	0/465 50%)	Cloacal samples of migrating passerine birds	–	[94]
	7/200 (3.5%)	Faecal samples from zoo animals (chimpanzee, dwarf goat, Iberian ibex and plains zebra)	PCR-ribotype 078 PCR-ribotype 039 PCR-ribotype 110	[95]
	15 cases of enterocolitis in harbor seals	Faecal samples of juvenile harbor seals (Phoca vitulina)	–	[96]
	3/46 (6.5%)	Stool samples from free-living South America coati	PCR-ribotype 014/020 PCR-ribotype 106 PCR-ribotype 013	[97]
	5/109 (4.6%)	Faecal samples of free-living South America coatis	–	[98]
	95/724(13.1%)	Faecal samples of black and Noway rats	35 different PCR-ribotypes	[99]
	7/161 (4.4%)	Faecal samples of feral swine	–	[100]
	41/60 (25%)	Faecal samples of Iberian pigs (free range system)	PCR-ribotype 078	[101]
Pets (dogs and cats)	14/139 (10%)	Faecal samples of dogs	PCR-ribotype 001 3 other PCR-ribotypes (with NIN)	[102]
	62/204 (30%)	Faecal samples of dogs	29 PCR-ribotypes	[103]
	16/117 (13.6%)	Faecal samples of dogs		[104]

			PCR-ribotype 012	
			PCR-ribotype 014	
			PCR-ribotype 046	
	(33.3%–100%)	Faecal samples from 18 puppies aged between 7 and 55 days	PCR-ribotype 056	[105]
			PCR-ribotype 010	
			PCR-ribotype 078	
			PCR-ribotype 213	
			PCR-ribotype 009	
			PCR-ribotype 020	
	2/50 (4%)	Faeces from healthy dogs	PCR-ribotype 009	[106]
			PCR-ribotype 010	
	2/20 (10%)	Faeces from dogs with diarrhoea	PCR-ribotype 014	
	48/93 (52%◦	Faces from dogs	–	[107]
	9/165 (5.5%)	Faeces from dogs	PCR-ribotype 010	[108]
	5/135 (3.7%)	Faeces from cats	PCR-ribotype 014/020	
			PCR-ribotype 039	
			PCR-ribotype 045	
			PCR-ribotype with NIN	
	Two cats with acute diarrhoea	Faecal samples of two adult cats	–	[109]
	23/245 (9.4%)	Faecal samples of cats in a veterinary hospital	–	[110]
Pigs (piglets and adult pigs at slaughter	1/100 (1%)	Faecal samples from pigs at slaughter (5–6 months)	PCR-ribotype 078	[116]
	45/67 (67.1%)	Faecal samples from neonatal piglets	PCR-ribotype 046	[117]
	2/61 (3.3)	Faecal samples from pigs at slaughter	–	[118]
	58/677 (8.6%)	Faecal samples from pigs at slaughter	PCR-ribotype 078	[119]
			PCR-ribotype 014	
			PCR-ribotype 013	
	0/165 (0%)	Faecal samples from pigs at slaughter	–	[120]
	241/513 (47%)	Faecal samples from neonatal piglets	–	[121]
	30/436 (6.9%)	Faecal samples from pigs at slaughter	PCR-ribotype 078	[122]
	103/174 (59.2%)	Faecal samples from neonatal diarrhoeic piglets	PCR-ribotype 273	[123]
	11/11 (100%)	Faecal samples from neonatal non diarrhoeic piglets		
	2/250 (0.8)	Faecal samples of finishing pigs at farm (13–27 weeks)	–	[124]
Cattle (calves and adult cattle)	4/42 (9.5%)	Faecal samples of calves (<12 weeks)	PCR-ribotype 077	[125]
			PCR-ribotype 038	
			PCR-ribotype 002	
	10/101 (9.9%)	Faecal samples of cattle at slaughter (15–56 months)	PCR-ribotype 078	[116]
			PCR-ribotype 029	
	3/67 (4.5%)	Faecal samples of cattle at slaughter	–	[118]
	176/999 (17.6%)	Faecal samples of calves	PCR-ribotype 078	[126]
			PCR-ribotype 033	
			PCR-ribotype 045	
	90/150 (60%)	Faecal samples of calves aged between 3 and 25 days	–	[127]
	2/330 (0.61)	Faecal samples of dairy and beef cow	PCR-ribotype 027	[128]

With NIN: with non international nomenclature.

Table 4
Fundamental aspects of the bacterium.

C. difficile characteristics		Data	Reference
Cells	Gram	Gram-positive	[130]
	Motility	Motile in broth cultures	
	Ciliature	Peritrichous	
	Size	0.5–1.9 μm wide 3.0–1.9 μm long	
	Chains	Some strains produce chains of 2–6 cells aligned end-to-end	
Spores	Shape and position	Oval, subterminal (rarely terminal) and swell the cell	
	Cogerminants for spores	Bile salts (cholate, taurocholate) Glycine, histidine	[131]
Colonies	Morphology	Circular, occasionally rhizoid	[10,130]
	Size	2–5 mm	
	Colour	Opaque, greyish, whitish, with a matt-to-glossy surface[a] Yellow fluorescence[b]	[133]
	Other characteristics	Non-haemolytic. They produce a characteristic odour, described as smelling like cow manure, a barnyard or horse stables	[132]
Growth temperature	Optimum	30–37 °C	[10,130]
	Range of growth	25–45 °C	
pH		5 (minimum)	
Water activity		0.969 (minimum)	
Atmosphere		Anaerobic conditions	
Cultures in PYG after 5 days of incubation[c]	pH	5.0–5.5	[130]
	Products in this medium	Acetic, isobutyric, butyric, isovaleric, valeric, isocaproic, formic and lactic acids[d]	
	Other characteristics	Cultures are turbid with smooth sediments	

[a] Colonies on blood agar.
[b] After 24 h of incubation on cycloserine cefoxitin fructose agar (CCFA) or after 48 h of incubation on blood agar under long-wavelength ultraviolet light.
[c] Peptone yeast glucose (PYG) broth is a liquid non-selective medium used to identify metabolic products of anaerobic bacteria.
[d] When lactate is not used, pyruvate is converted to acetate and butyrate, and threonine is converted to propionate.

healthcare settings worldwide [135]. It must be noted that toxin A− toxin B+ strains are sometimes reported solely on the basis of the lack of *tcdA* amplification; however, there are some A+ variant strains with a partially deleted *tcdA* fragment [140].

Both toxins A and B translocate to the cytosol of target cells and inactivate small GTP-binding proteins. By glycosylating small GTP-ases, the two toxins cause actin condensation and cell rounding, which is followed by cell death. Toxin A acts primarily within the intestinal epithelium, while toxin B has broader cell tropism [141]. Both toxins induce the production of tumour necrosis factor alpha and pro-inflammatory interleukins, which induce the inflammatory cascade and the pseudomembrane formation in the colon. The endoscopy of *C. difficile* colitis shows a colonic mucosa with multiple whitish plaques, usually raised and adherent, of a size varying from a few millimetres to 1–2 cm; the cells can even be confluent in severe disease. The intervening colonic mucosa can be oedematous, granular, hyperaemic or completely normal [142].

The genes *cdtA* and *cdtB*, encoding the CDT, which belongs to the group of clostridial binary toxins, are not found on the PaLoc. This toxin is encoded on a separate region of the chromosome (CdtLoc). It has been described that all strains with *cdtA* and *cdtB* genes are variant strains (with changes in the *tcdA* and *tcdB* genes) [143]. In contrast, most types not producing binary toxin have toxin genes very similar to the reference strain, VPI 10463.[1] These CDT + strains represent up to 6% of the toxigenic isolates from hospitalised patients [143]. The production of CDT is frequently associated with hypervirulent strains. CDT has been described as causing the collapse of the actin cytoskeleton and cell death. The lipolysis-stimulated lipoprotein receptor (LSR) is known to be the host receptor for the *C. difficile* CDT toxin [144]. Furthermore, the CDT toxin also induces the formation of microtubule-based protrusions and increases the adherence of the bacterium [145]. While CDT is still being investigated, some studies have already reported data regarding the clinical relevance of this toxin. Bacci et al. (2011) [146] associated the presence of CDT in patients with higher case-facility (death) rates. Other authors also found that CDT was a marker for more virulent *C. difficile* strains or that it contributed directly to strain virulence. Tagashira et al. (2013) [147] described two cases of fulminant colitis due to CDT + strains in the same ward of a hospital in Japan occurring within ten weeks of each other. A further study suggested that CDT was a predictor of recurrent infection, and its presence may require longer antibiotic treatments [148]. CDT + *C. difficile* strains that do not produce toxins A and B have been described in independent cases of patients with diarrhoea suspected of having CDI [149].

[1] http://www.mf.uni-mb.si/mikro/tox/.

7. Laboratory diagnosis of CDI

To aid in the surveillance of CDI and to increase comparability between clinical settings, standardised case definitions have been proposed (Fig. 2) [8]. A laboratory diagnosis of CDI must be based on the detection of *C. difficile* toxins or on the isolation of toxigenic *C. difficile* strains from stool samples [150]. However, these results should be combined with the clinical findings to diagnose the disease. The clinical manifestation includes diarrhoea with the passage of 3 or more unformed stools in 24 or fewer consecutive hours [8]. In this context, only unformed and fresh stools should be tested for diagnostic purposes (the specimen should be loose enough to take the shape of the container). The cytotoxic activity is lost very quickly, meaning if the analysis of fresh specimens is not possible, the samples should be stored at 4 °C or below. However, cultures are not affected by temporal conditions due to sporulation [150]. Formed stools only must be tested if they come from patients with ileus or potential toxic megacolon or in the case of epidemiological studies [150].

A recent guide to the utilisation of the microbiology laboratory for the diagnosis of infectious diseases [151] highlights the importance of the collection device, temperature and transport time because the interpretation of the results will depend on the quality of the specimens received for analysis. Specifically, regarding *C. difficile*, the recommendations are that the stool

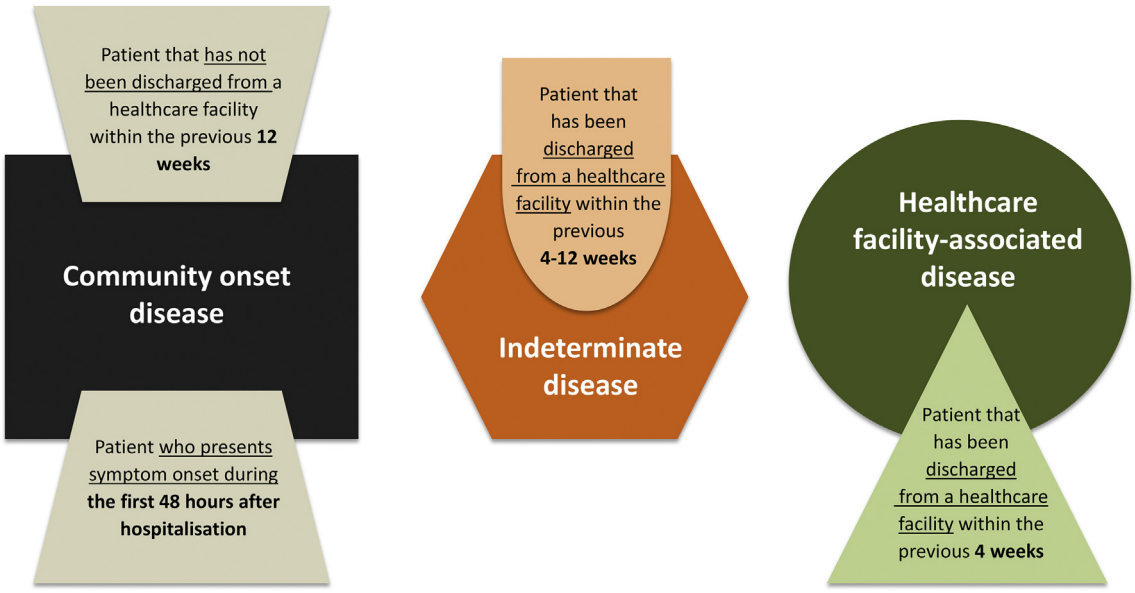

Fig. 2. Community onset CDI and healthcare facility-associated CDI definitions.

samples must be received in sterile close containers and kept at room temperature for a maximum of 2 h, and therefore, specimens of dubious quality must be rejected [151].

The culture of samples is recognised as the most sensitive method for the detection of *C. difficile*, but its specificity for CDI is low because the rate of asymptomatic carriage of *C. difficile* among hospitalised patients is so high. This method is not clinically practical for routine diagnosis because it does not distinguish between toxigenic and non-toxigenic isolates and requires 24–48 h to obtain the first results [150]. However, stool culture testing permits the molecular typing of the isolated strains and antibiotic susceptibility determination, making it essential for epidemiological surveys [8,150]. Therefore, stool culture testing can be coupled with a cell cytotoxicity assay or EIA (enzyme immunoassay) to detect toxin-producing *C. difficile* strains (known as toxigenic cultures), resulting in increased specificity [150].

Since it was first proposed by George et al. in 1979 [133], cycloserine-cefoxitin fructose (CCF) has been the most commonly used medium for *C. difficile* isolation. The original formulation has been extensively modified, including the replacement of egg yolk by blood [150]. The addition of 1 g/L of taurocholate, desoxycholate or cholate has also been shown to induce the germination of *C. difficile* spores when they are incorporated in CCF [152,153]. Sodium salt of cholic acid is more inexpensive than pure taurocholate but just as effective [150]. The concentration of the selective agents has also varied among studies, from 250 mg/L to 500 mg/L for cycloserine and from 8 mg/L to 16 mg/L for cefoxitin. Other modifications to improve this media have been proposed. Delmée et al. [154] included cefotaxime instead of cefoxitin, which increased the sensitivity and specificity of the medium. *C. difficile* colonies are easily recognised in this media when observed under the microscope: with an appearance similar to ground glass, they release an odour akin to horse manure and reveal a yellow-green fluorescence under ultraviolet illumination. Early identification of *C. difficile* colonies in this primary selective culture can be performed using an antigen latex agglutination assay. Latex particles are coated with IgG antibodies specific to *C. difficile* cell wall antigens. When the bacterium is present, the latex particles agglutinate into large visible clumps within 2 min. However, cross-reactions have been described, including with *C. sordellii*, *Clostridium glycollicum* and *Clostridium bifermentans* [155].

Presently, several commercial selective media are available for the detection of *C. difficile* from stool specimens. The new chromogenic media seem to be effective as well as more rapid and sensitive than the classic selective media and have been shown to aid in the diagnosis of CDI [156–159]. However, pre-made agars are expensive and unaffordable for many research groups. Furthermore, they are used for the clinical recovery of *C. difficile* from faecal samples and not for the semi-quantification of viable spores [160].

Pre-treatment of samples with ethanol shock has been associated with an increase in sensitivity [161–164]. However, in the different studies conducted in our laboratory (unpublished data), ethanol shock or pre-heat treatment of samples does not improve the recovery of *C. difficile* from faecal or food samples. Rather, it seems that an increase in the time of enrichment is best for improving the sensitivity of the method. A bacterial competition in the enrichment broth has been observed [164]. In a previous study on carcasses and faecal samples [116], after 30 days of enrichment, different *C. difficile* types were identified, and colonies other than *C. difficile* were rarely present in the plate. However, the enrichment of samples is a time-consuming technique for laboratory purposes and might not be worth the slight increase in sensitivity observed.

Toxin detection is the most important clinical test [8]. It can be performed using cell lines to examine a stool filtrate (cell cytotoxicity assay) or by EIA [150]. Cell cytotoxicity is often considered the best standard test for identifying toxigenic *C. difficile* as it can detect toxins at picogram levels and is recognised as the most sensitive available test for the detection of toxin B [165]. A laboratory cell line (Vero, Hep2, fibroblasts, CHO or HeLa cells) is exposed to a filtrate of a stool suspension. If *C. difficile* toxins are present, a cytopathic effect is observed after 24–48 h (cell rounding via cytoskeleton disruption). The effect is mainly due to toxin B, which is more cytotoxic than toxin A. The presence of toxigenic *C. difficile* can be confirmed if a specific antiserum (added later) reverses the effect on the cells. This method is very sensitive and specific but is relatively slow and requires the maintenance of cell lines. If the antiserum does not neutralise the cytopathic effect, which is observed in 21% of cases, the results are inconclusive [150].

EIA is rapid but less sensitive than the cell cytotoxicity assay [8], missing 40% of diagnoses [165]. However, it is easy to perform and

does not require technical training or special equipment. EIA can detect both toxin A and toxin B and may also detect glutamate dehydrogenase (GDH), a specific enzyme of *C. difficile* found in toxigenic and non-toxigenic isolates. *C. difficile* constitutively produces GDH in easily detectable levels, so tests based on GDH detection have good sensitivity (96%–100%). As GDH only identifies the bacterium and not the presence of toxins, the method is comparable with stool culture. Furthermore, it only takes 15–45 min and the cost is low (estimated at 8 USD); it can also be combined with a cell cytotoxicity assay, EIA for toxins, or culture with further toxin characterisation of the strains [165]. However, the results from GDH seem to differ based on the commercial kit used, and therefore, for some authors, this approach remains an interim recommendation [8]. EIA may be useful in laboratories without tissue culture facilities, but it must be combined with a positive culture. If the EIA results are negative and the culture test results are positive, it is recommended to isolate the strain from the plate and repeat EIA testing to determine if it is toxigenic [150]. Table 5 summarises the most-used tests for the diagnosis of *C. difficile* infection.

Delmée et al. (2001) [150] proposed the following scheme for the routine bacteriological diagnosis of CDI in humans. First, culture and toxin detection (by cytotoxicity assay or by EIA) should be performed directly from the stool specimen. If both tests are negative, a diagnosis of CDI is excluded. In contrast, if both are positive, the patient is diagnosed with CDI and requires immediate treatment. When the culture is positive but toxin detection is negative, an EIA test should be performed on several colonies removed from the culture plate. If the test is still negative, treatment is not necessary. If the test is positive, the patient should be considered positive for CDI. Finally, when the culture is negative but toxin detection is positive, a control specimen is requested and the culture must be repeated, which results in a patient testing positive for *C. difficile* in most cases. Repeat testing of patients who were previously positive as a "test of cure" is not appropriate [150].

Many studies have developed different real-time PCR (RT-PCR) methods for the detection of *C. difficile* toxin genes directly from stool samples, not only from humans [166,167] but also from animals [168], and for the quantitative detection of *C. difficile* in hospital environmental samples [169]. Various automated RT-PCR systems are commercially available, intended as diagnostic tools for CDI. These systems include BD GeneOhm™ Cdiff (Becton Dickenson)[2] and Xpert® *C. difficile* (Cepheid).[3] These commercial RT-PCRs have been shown to be rapid (<4 h for a result), sensitive and specific. Therefore, they have been largely proposed for the laboratory diagnosis of CDI [170–172]. In addition, a recent guide for the management of *C. difficile* infection in surgical patients suggests that PCR testing of perirectal swabs may be an efficient method for toxigenic *C. difficile* detection [173].

There are several other molecular genetic test systems commercially available for the identification of *C. difficile* from stool and culture samples. One example is Genotype Cdiff (Hain Lifescience), which is based on DNA strip technology.[4] This system is based on DNA amplification, hybridisation and visualisation using the enzyme alkaline phosphatase. The results are visible in a colorimetric reaction. The test is rapid in detecting *C. difficile*, its toxins, deletions in the *tcdC* gene, and mutations in the *gyrA* gene that are associated with moxifloxacin resistance. However, these new technologies require considerable capital equipment, costly cartridges and experienced laboratory personnel. Furthermore, the results reported have not shown any significant improvement when compared with classic methods [150]. Therefore, some laboratories use these procedures to verify dubious results observed after rapid screening with other methods or to further process the samples for epidemiological purposes.

8. *C. difficile* typing methods

To characterise and compare the circulating strains and to identify emerging strains and those responsible for outbreaks worldwide, several typing methods have been applied. Table 6 summarises the available typing methods and their advantages and disadvantages. Lemée et al. (2004) [140] designed a multiplex PCR for the simultaneous identification and toxigenic type characterisation of *C. difficile* isolates. Several other studies have proposed different multiplex PCR primers and protocols not only to detect the genes encoding the major toxins A and B but also to detect binary toxin genes (*cdtA* and *cdtB*) and other deletions in the Paloc genes [174].

Pulsed-field gel electrophoresis (PFGE[5]) and restriction enzyme analysis (REA) are widely used in the United States and Canada. PFGE was one of the first molecular methods used for *C. difficile* and other food-borne pathogens in North America [175]. The method uses restriction enzymes that infrequently cut (such as *Sma*I or *Sac*II) to cleave bacterial DNA at different restriction sites. The use of these infrequently cutting restriction enzymes limits the number of restriction fragments (to between 7 and 20) and ensures that they are relatively large [176]. Generally, the frequency of cutting is inversely proportional to the number of nucleotides in the recognition site [177]. In North America, the isolates are designated with NAP and a number (e.g., NAP1: North America Pulsotype 1) [178]. The technique separates the large fragments of DNA generated based on size using a pulsed-field electrophoresis gel with resulting electrophoresis patterns that are highly discriminatory. However, large amounts of high-molecular-weight DNA have to be read, making the process labour-intensive (Table 6). *C. difficile* typing based on REA is performed using total cellular DNA, which is digested with a frequently cutting restriction enzyme (*Hin*dIII), and the resulting fragments are resolved by classical agarose electrophoresis. This method was shown to be reproducible, highly discriminatory and universally applicable. However, the visual assessment of the large number of fragments in a single gel can be difficult and may be confounded by the presence of extrachromosomal DNA [175] (Table 6).

In Europe, *C. difficile* PCR-ribotyping has been recognised as the dominant typing method. PCR-ribotyping is based on the size variation of the 16S–23S rDNA intergenic spacer regions. A PCR-ribotype is defined as a group of strains that produce an identical band pattern. Therefore, a single band difference warrants a new ribotype [178]. Stubbs et al. (1999) [179] constructed a *C. difficile* PCR-ribotype reference library composed of 2030 isolates, with a total of 116 distinct types identified from environmental, hospital, community practitioner, veterinary and reference sources. The method was performed with agarose gel-based electrophoresis. Bidet et al. (1999) [180] improved the reading of the banding patterns by selecting a partial sequence of the rRNA genes (16S–23S) and the intergenic spacer region with a new set of primers located closer to this intergenic spacer region. The Public Health Laboratory

[2] https://www.bd.com/resource.aspx?IDX=17953.

[3] http://www.cepheid.com/us/cepheid-solutions/clinical-ivd-tests/healthcare-associated-infections/xpert-c-difficile.

[4] http://www.hain-lifescience.de/en/products/microbiology/genotype-cdiff/genotype-cdiff.html.

[5] Pulsed-field Gel Electrophoresis (PFGE) | Pathogens and Protocols | PulseNet | CDC [WWW Document], n.d. URL http://www.cdc.gov/pulsenet/pathogens/pfge.html (accessed 6.18.15).

Table 5
Laboratory test for *C. difficile*.

C. difficile detection	Test	Objective	Characteristics	Confirmation	Advantages	Disadvantages	Study
Toxin/enzyme detection	Cell cytotoxicity assay	Considered the best standard test for identifying toxigenic *C. difficile*	A laboratory cell line (Vero, Hep2, fibroblast, CHO or HeLa cells) is exposed to a filtrate of a stool suspension If *C. difficile* toxins are present, a cytopathic effect is observed after 24–48 h (cell rounding via cytoskeleton disruption) The effect is mainly due to toxin B (which is more cytotoxic than toxin A)	The presence of toxigenic *C. difficile* can be confirmed if a specific antiserum (added later) reverses the effect on the cells	It can detect toxins at picogram levels Most sensitive available test for detection of toxin B	Relatively slows and requires the maintenance of cell lines. If the antiserum does not neutralise the cytopathic effect, which is observed in 21% of cases, the results are inconclusive	[8,165,166]
	Enzyme immunoassay (EIA)	It can detect both toxins A and/or B, and may also detect glutamate dehydrogenase (GDH)[c]	*C. difficile* constitutively produces GDH in easily detectable levels, but only identifies the bacterium and not the presence of toxins (the method is comparable with stool culture)	The method can be combined with cell cytotoxicity assay, EIA for toxins, or culture with further toxin characterisation of the strains If EIA results are negative and culture testing results are positive, it is recommended to isolate the strain from the plate and repeat EIA testing to determine if it is toxigenic	Rapid (15–45 min) Low cost (estimated at 8 USD) Easy to perform and does not require technical training or special equipment Good sensitivity for detection of GDH (96%–100%) Useful in laboratories without culture facilities	Less sensitive than the cell cytotoxicity assay missing 40% of diagnosis Results seem to differ based on the commercial kit used It must be combined with a positive culture)	
Culture	Stool culture	It permits molecular typing of the isolated strains and antibiotic susceptibility determination (essential for epidemiological surveys)	Cycloserine-cefoxitin fructose (CCF) has been the most commonly used medium for *C. difficile* isolation. The medium includes egg-yolk or blood and taurocholate, desoxycholate or cholate, which has been shown to induce germination of spores. Presently, several commercial selective media are available. *The new chromogenic media improve the recovery the bacterium*. Pre-treatment of samples with ethanol shock has been associated with an increase in sensitivity	Molecular typing of the strains	Most sensitive method for detection of *C. difficile* Pre-made agars are used for the clinical recovery of *C. difficile* from faecal samples and not for the semi-quantification of viable spores	Low specificity for CDI[d] Not clinically practical for routine diagnosis: Slow (24–48 h) and its not give information about toxin production	[8,150,166–170]
Molecular methods	Real Time PCR (RT-PCR)	Detection of *C. difficile* toxin genes directly from stool samples[e]	BD GeneOhmTM Cdiff (Becton Dickenson)[a] Xpert® *C. difficile* (Cepheid)[b]	Culture with further characterisation of the strains	Rapid (<4 h for a result), sensitive and specific	Expensive Requires technical training and special equipment	[171–173]

[a] https://www.bd.com/resource.aspx?IDX=17953.
[b] http://www.cepheid.com/us/cepheid-solutions/clinical-ivd-tests/healthcare-associated-infections/xpert-c-difficile.
[c] Specific enzyme of *C. difficile* produced in in both toxigenic and non-toxigenic isolates.
[d] Low specificity for *C. difficile* infection (CDI) because the rate of asymptomatic carriage of *C. difficile* among hospitalised patients is so high.
[e] They have been largely proposed for laboratory diagnosis of CDI but also for quantitative detection of *C. difficile* in hospital environmental samples.

Table 6
C. difficile typing methods.

Method	Characteristics	Advantages	Disadvantages	Reference
Multiplex PCR	The PCR can target: • *tpi* housekeeping gene[a] • an internal fragment of the toxin B (*tcdB* gene) • the 3′ region, deleted or not, of the toxin A (*tcdA* gene) • binary toxin genes (*cdtA* and *cdtB*) • Other deletions in the Paloc genes	Low cost	Process labour-intensive	[140,174]
Pulsed-field gel electrophoresis (PFGE)	The method uses infrequently-cutting restriction enzymes (like *Sma*I or *Sac*II) to cut bacterial DNA at different restriction sites	Highly discriminatory	Process labour-intensive Difficulty of lysing spores Difficulty of interpreting results and the inter-laboratory exchange of the results Equipment and time required	[175,176]
Restriction enzyme analysis (REA)	The method uses frequently-cutting restriction enzymes (*Hind*III) to cut total cellular DNA at different restriction sites	Reproducible and highly discriminatory power Universally applicable	Difficulty of interpreting results and the inter-laboratory exchange of the results	[175]
PCR-ribotyping	A PCR-ribotype is defined as a group of strains that produce an identical band pattern based on the size variation of the 16S–23S rDNA intergenic spacer regions	Dominant typing method Inter-laboratory comparisons easy if standard nomenclature is available or use of Webribo database	Time intensive Equipment and material costs Technical training required Inter-laboratory comparisons difficult if standard nomenclature is not available	[178,182]
QIAxcel® system	For *C. difficile* ribotyping, the detection of *tcdC18* bp deletion, and toxin gene detection Based on an automated electrophoresis platform	Reduce the cost of hands-on time Allows analysis of up to 96 samples per run	Cost of the cartridges and equipment Limited sensitivity and discriminatory power (cannot distinguish between closely related PCR-ribotypes)	[184]
Serotyping - by slide agglutination - by polyacrylamide gel electrophoresis - by ELISA	The method distinguishes variations in *C. difficile* strains based on bacteria surface antigens	Good correlation between methods Reproducible, Rapid and reliable Inter-laboratory comparisons easy	Cross-agglutination caused by flagellar antigens (totally suppressed by ELISA)	[185,186]
Surface-layer protein A gene sequence typing (*slp*AST)	Sequencing of *slpA* gene, which encodes for a surface immuno protein Layer (S-layer)	Good discriminatory power It can be applied to direct typing (without culture) from DNA stool specimens	It has been showed that *C. difficile* genotype is no predictive of antigenic types	[187–189]
Repetitive sequence-based PCR typing (rep-PCR)	Specific repetitive PCR-primers complement the short repetitive sequences dispersed the bacterial genome The amplified DNA fragments provide a genomic fingerprint that can be employed for subspecies discrimination	Automated rep-PCR with a higher discriminatory power than traditional PCR-ribotyping	Requires visual interpretation and technical skills Inter-laboratory reproducibility has not been demonstrated	[190–192]
Random amplified polymorphic DNA (RAPD)	Random amplification of DNA segments by PCR reaction using a single primer of arbitrary nucleotide sequence	Inexpensive Does not require any specific knowledge of the DNA sequence	Must be combined with PCR-ribotyping to obtain higher discriminatory power	[193,194]
Amplified fragment length polymorphism (AFLP)	Genomic DNA is totally digested with two restriction enzymes. This step is followed by ligation of double-stranded oligonucleotide adaptors to the sticky ends of the restriction fragments followed by amplified by PCR	Low cost	Suboptimal reproducibility (variation in the precision of sizing of fragments) Limited application in *C. difficile* typing	[195]
Toxinotyping	Polymerase chain reaction-restriction fragment length polymorphism based method for differentiating strains according to changes in their toxin genes when compared to the reference VPI 10463 strain	Results of toxinotyping and PCR-ribotyping correlated well	Requires technical skills	[175]
Multilocus sequence typing (MLST)	The sequences of internal fragments of housekeeping genes (usually seven) are used to characterise the strains	Inter-laboratory comparisons easy	Time-consuming (several days) Costly and laborious technique Requires specific technical skills	[199,200]
Multilocus variable number tandem repeat analysis (MLVA)	The method utilizes the naturally occurring variation in the number of tandem repeated DNA sequences found in many different loci in the genome. The different lengths of variable number of tandem repeats (VNTR) regions are determined to distinguish among strains	High discriminatory power which allows tracking of outbreaks and determining phylogenetic relationships	Time-consuming Costly and laborious technique Requires specific technical skills Inter-laboratory comparisons difficult	[175,201]
Whole genome sequencing (WGS)	The method reveals the complete DNA of the bacterium at a single time	Provides the most comprehensive collection of an individual's genetic variation Increasingly low cost	Method still under development The large amount of data requires technical skills for further processing and analysis	[203]

[a] Species species-interspecific fragment of the triose phosphate isomerase (*tpi*) housekeeping gene.

Service Anaerobe Reference Unit, Cardiff (UK) has established a ribotyping nomenclature reference database for *C. difficile*. This nomenclature is designated by a three-digit number starting from 001 (ex. PCR-ribotype 027). Currently, the collection of existing PCR-ribotypes and the assignment of new ones is performed by the Health Protection Agency-funded *C. difficile* Ribotype Network (CDRN) in Leeds (UK), which has more than 600 different PCR-ribotypes in the CDRN database [181]. However, in many laboratories, the standard nomenclature is not always available and a local nomenclature is used, making inter-laboratory comparisons difficult [178] (Table 6). Indra et al. (2008) [182] developed a *C. difficile* sequencer-based PCR-ribotyping method based on capillary gel electrophoresis that was proposed in order to solve the problems associated with inter-laboratory comparisons of typing results and to make PCR-ribotyping less time-intensive (Table 6). PCR amplification was performed using a fluorescent label in one of the primers, and the amplicon sizes were determined using an ABI genetic analyser [175]. A database and web-based software programme was created that allows the analysis and comparison of *C. difficile* capillary-sequencer-based PCR-ribotyping data by simply uploading sequencer data files.[6] Janezic et al. (2011) [183] described a modification to PCR-ribotyping that enables the detection of *C. difficile* in stool samples within hours. The designed primers were located partially within the *C. difficile* 16S–23S rRNA intergenic spacer regions and partially within 16S (forward primer) and 23S (reverse primer).

The QIAxcel® system has been proposed as a new method for *C. difficile* ribotyping, the detection of *tcdC18* bp deletion, and toxin gene detection (toxin A, toxin B and binary toxin CDT genes).[7] QIAxcel is based on an automated electrophoresis platform that processes samples in batches of 12 and allows the analysis of up to 96 samples per run. The system does not require the use of fluorescein-labelled primers and displays the data as both a gel-view format and an electropherogram. The system has the potential to reduce the cost of PCR-ribotyping by drastically reducing the hands-on time. The major costs are the purchase of cartridges, the setup of the QIAxcel system hardware, and the BioCalculator analysis. However, the method has limited sensitivity and discriminatory power. It cannot clearly distinguish between closely related ribotypes, such as 027 and 176 [184].

Serotyping distinguishes variations in *C. difficile* strains based on the bacterial surface antigens. Serogrouping by slide agglutination and by polyacrylamide gel electrophoresis have both been traditionally accepted as practical in routine typing [185]. Both methods have shown a good correlation in results and allow the differentiation of 10 major serogroups (A, B, C, D, F, G, H, I, K and X). Specific profiles have been associated with each of the 10 serogroups except for serogroup A. Strains from serogroup A have a common flagellar antigen that is responsible for cross-agglutination. The shearing of the flagella allows the differentiation of 12 different subgroups of serogroup A. Delmée et al. (1993) [186] used an enzyme-linked immunosorbent assay (ELISA) with antisera specific for the 10 *C. difficile* serogroups (A1, B, C, D, F, G, H, I, K, X) and the 12 serogroups within serogroup A (A2 to A12) for the serogrouping of *C. difficile* colonies for 314 positive faecal samples. The authors found that ELISA was a rapid and reliable method for *C. difficile* serotyping and that cross-agglutination caused by flagellar antigens in the slide agglutination method is totally suppressed by ELISA.

Surface-layer protein A gene sequence typing (*slpA*ST) has also been described for *C. difficile* characterisation. *C. difficile* has a surface immuno-protein Layer (S-layer) encoded by the *slpA* gene, and the typing of isolates is performed by the sequencing of this *slpA* gene. It can also be used for direct typing from DNA stool specimens without culture [187]. This method has been reported as a discriminative tool for *C. difficile* characterisation [188]. However, Dingle et al. (2013) [189] showed that the *C. difficile* genotype was not predictive of antigenic types. Therefore, S-layer typing could be useful for explaining the temporal changes and geographic differences in the epidemiology of CDI as well as the way in which isolates (and antigens) are selected for inclusion in *C. difficile* vaccines [189].

Repetitive sequence-based PCR typing (rep-PCR) is another method proposed for the characterisation of *C. difficile* strains [190]. Bacterial genomes contain multiple dispersed short repetitive sequences separating longer single-copy DNA sequences. Specific repetitive PCR primers complement these repetitive sequences, and the amplified DNA fragments provide a genomic fingerprint that can be employed for subspecies discrimination [191]. The DiversiLab system[8] is an automated rep-PCR typing method that has a high discriminatory power when compared to traditional PCR-ribotyping. However, this method requires the visual interpretation of rep-PCR fingerprint patterns and technical skills. Furthermore, interlaboratory reproducibility for this method must be demonstrated prior to its use for *C. difficile* surveillance [192].

Random amplified polymorphic DNA (RAPD) analysis is the 'random amplification' of DNA segments by PCR reaction using a single primer consisting of an arbitrary nucleotide sequence. RAPD is an inexpensive and powerful typing method and does not require any specific knowledge of the DNA sequence of the target microorganism. The amplification of a segment of DNA will be performed depending on positions that are complementary to the primer sequence. Green et al. (2011) [193] used PCR-ribotyping in conjunction with RAPD to further categorise different *C. difficile* types within defined PCR-ribotypes and therefore obtained a higher discriminatory power than either of the methods used alone. Barbut et al. (1993) [194] evaluated genomic fingerprinting of *C. difficile* strains using RAPD and suggested that this method could be an additional valuable tool for epidemiological studies.

In the amplified fragment length polymorphism (AFLP) technique, a small amount of purified genomic DNA is totally digested with two restriction enzymes, one with an average cutting frequency and the other with a higher cutting frequency. This step is followed by the ligation of double-stranded oligonucleotide adaptors to the sticky ends of the restriction fragments, followed by PCR amplification. After final amplification, the selectively amplified fragments are separated by gel electrophoresis and comparison of banding patterns is typically achieved using dedicated fingerprinting analysis software. AFPL has a relatively low cost, but variation in the precision of the sizing of fragments has been observed, leading to suboptimal reproducibility [195]. This method has seen limited application in *C. difficile* typing. Klaassen et al. (2002) [196] reported that AFLP analysis yielded high-resolution and highly reproducible fingerprinting patterns in a short time period (24 h) to evaluate epidemiological relatedness among hospital *C. difficile* isolates. A further study showed that AFLP is better able to discriminate between *C. difficile* reference strains (most of them toxin A+, toxin B+) than PCR-ribotyping. However, for toxin A−, toxin B+ isolates, both methods yielded similar results [138].

Toxinotyping is a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) based method for differentiating *C. difficile* strains according to changes in their toxin genes

[6] https://webribo.ages.at/.

[7] https://www.qiagen.com/be/products/catalog/automated-solutions/detection-and-analysis/qiaxcel-advanced-system/.

[8] http://www.biomerieux-diagnostics.com/diversilab.

when compared to the reference strain VPI 10463. Strains belonging to the same toxinotype have identical changes in the PaLoc region. Toxinotyping is performed by PCR amplification and restriction enzyme digestion of 10 regions of the PaLoc [175]. Currently, 31 different toxinotypes are identified and designated by Roman numerals from I to XXXII. Strains with toxin genes similar to VPI 10463 are classified as toxinotype 0. A total of 12 out of 20 toxinotypes with variant strains produce binary toxin, while most of the toxinotypes not producing binary toxin have toxin genes very similar to the reference strain, VPI 10463[9]. In this context, only one strain resembling VPI 10463 and positive for CDT has been previously described [197]. Toxinotype XXXII has been recently reported and corresponds to a new type of *C. difficile* strain (toxin A−, B+, CDT-) that completely lacks the *tcdA* gene and has an atypical organisation of the PaLoc integration site [198].

Multilocus sequence typing (MLST) analysis uses the sequences of internal fragments of housekeeping genes (usually seven) to characterise the strains. The internal fragments of each gene (450–500 bp) are sequenced on both strands using an automated DNA sequencer. For each housekeeping gene, the different sequences present within a bacterial species are assigned as distinct alleles, and for each isolate, the alleles at each of the seven loci define the allelic profile or sequence type (ST). The data obtained are unambiguous, and the allelic profiles of the isolates can easily be compared to those in a large central database and therefore can be compared between laboratories[10]. There are two MLST databases available for *C. difficile*, each adapted to a different typing scheme: the first is organised according to the scheme described by Griffiths et al. (2010) [199], which is performed with the housekeeping genes *adk*, *atp*, *dxr*, *recA*, *sodA*, *tpi* and *glyA*[11]; and the second is organised according to the scheme described by Lemée et al. (2004) [200], which is performed with the housekeeping genes *aroE*, *dutA*, *gmk*, *groEL*, *recA*, *sodA* and *tpi*.[12] The primary problem with MLST is the time-consuming nature of the method, with the completion of analysis taking several days. In addition, MLST is a relatively costly and laborious technique that requires specific technical skills.

Multilocus variable-number tandem-repeat analysis (MLVA) has been suggested to have higher discriminatory power than other typing methods for investigating the relatedness between *C. difficile* strains [201]. It has been widely used in medical microbiology as an alternative or complement to other typing techniques such as PFGE, rep-PCR or MLST [202]. MLVA utilizes the naturally occurring variation in the number of tandem repeat DNA sequences found in many different loci in the genome. Therefore, the lengths of the variable number of tandem repeats (VNTR) regions are determined to distinguish among the strains. The technique is achieved by a multiplex PCR assay (with primers designed to target different VNTR regions in the genome) and visualised by electrophoresis or automated fragment analysis on a sequencer. The product size is used to calculate the number of repeat units of each locus. The calculated numbers of repeats of the VNTR loci (alleles) are combined, and this provides the MLVA profile. Each unique MLVA profile is given an MLVA type designation. The MLVA profile can be used for the comparison and clustering of the bacterial strains.[13] Compared with traditional PCR ribotyping, MLVA has increased discriminatory power, which allows for the more efficient tracking of outbreaks and has the potential to determine phylogenetic relationships. In addition, MLVA produces digital data with a decreased turnaround time [175].

Whole genome sequencing (WGS) reveals the complete DNA of an organism at a single time and provides the most comprehensive collection of an individual's genetic variation [203]. Sanger sequencing and subsequently Roche 454 and Illumina next-generation sequencing technologies have been applied to study the evolutionary dynamics of *C. difficile* [175] at increasingly low cost [203]. Recent studies have applied WGS to determine the epidemiological relationships between *C. difficile* strains in healthcare settings or in the scientific community employing WGS [204]. Although the method is still under development and the large amount of data obtained requires technical skills for further processing and analysis, it is very probable that in the near future, WGS will replace all other current typing techniques.

Killgore et al. (2007) [205] compared seven different techniques (REA, PGE, PCR-ribotyping, MLST, MLVA, AFLP and *slp*AST) for epidemic *C. difficile* strain typing. They found that all methods appeared to be adequate for detecting an outbreak strain in a particular institution. However, REA or MLVA showed the highest level of discrimination between strains, and they seem to be the most recommended methods to track outbreak strains geographically. However, as neither of the techniques are widely used and little data are available, there is currently no method with proven interlaboratory reproducibility for inter-institutional *C. difficile* tracking.

9. High-throughput sequencing analysis and CDI

16S metagenetics is a culture-independent strategy allowing the identification of bacterial populations present in a large panel of samples. It has been recently introduced to investigate the intestinal microbiota of healthy patients and patients suffering CDI. In the last year, most of the studies reported bacteria at the phylum and class level, but higher taxonomic resolution may reveal more differences in the population structure [206]. Preliminary results have shown that one of the taxa found in high proportions in patients with CDI is *Proteobacteria*, while *Bacteroidetes* proportions are lower in infected patients [207]. In this context, one study has proposed the use of *Bacteroidetes* and *Firmicutes* as probiotics to treat CDI [208]. However, the few available data regarding alterations to the intestinal microbiota of patients with CDI has been obtained in different patient conditions (age, antibiotic treatment, hospitalisation), which explains the high amount of variability between the studies. Despite this limitation, further studies exploring the diversity of the gut microbiota in CDI patients will be critical for further understanding the pathogenesis of *C. difficile* and for developing new approaches for the treatment and prophylaxis of the infection. In addition, recent studies in humans and animals have shown that many of the sequences were not identical to sequence entries present in the available databases [209,210]. Furthermore, among the sequences identical to known entries, the species name was seldom taxonomically defined. As previously reported, these findings underline the complexity of the gut microbiota, stressing the need for further research on taxonomy and functional microbiology [207].

10. Conclusions and perspectives

Eighty years after its discovery, *C. difficile* continues to be the focus of attention in hospitals and an important topic for many research groups worldwide. Recognised as the leading cause of nosocomial antibiotic-associated diarrhoea, the incidence of CDI remains high and in some years has increased, despite the efforts to improve prevention and reduce the spread of the bacterium in

[9] http://www.mf.uni-mb.si/tox/.
[10] http://pubmlst.org/general.shtml.
[11] http://pubmlst.org/cdifficile/.
[12] http://www.pasteur.fr/recherche/genopole/PF8/mlst/Cdifficile2.html.
[13] http://www.mlva.net/.

healthcare settings. Outside of Europe and America, the incidence of CDI infection is also rising. The major research studies of the last decade have been focused on the control of the spread of the bacterium, the rapid diagnosis of CDI, and the efficacy of treatment and recurrence prevention. Different guidelines have been designed to improve the management of the infection. Diagnosis must consider both clinical and laboratory findings. Laboratory tests must be rapid and sensitive; therefore, stool culture is not clinically practical. However, the isolation of the strain is necessary for epidemiological studies. There is a need for highly discriminatory typing methods, and results should be comparable between laboratories. One potential alternative in the near future is whole genome sequencing, now considered as the next-generation typing tool.

The investigation of the gut's microbial communities by new metagenetic analyses will allow researchers to discern whether any alteration of the gut microbiota composition can favour *C. difficile* colonisation, as well as the microbes responsible for rendering individuals less susceptible to the infection. This approach will be critical in the future to further understand the pathogenesis of *C. difficile* and to develop new successful prevention and treatment measures.

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