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By

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Title:

Molecular typing and antibiotic resistance profiles of *Clostridioides difficile* strains isolated in Algeria

Graduated: ……/……/….., jury composed of :

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"Somewhere, something incredible is waiting to be known."

Carl Sagan; Astronomer and scientist.

Abstract

Clostridioides difficile, an anaerobic, Gram-positive, spore-forming bacterial pathogen, is considered as the leading cause of hospital-acquired post-antibiotic diarrhoea. The major virulence factors of *C. difficile* are two toxins, toxin A and toxin B. *C. difficile* can either colonize patients without causing clinical manifestations (healthy carriers), or can cause a spectrum of diseases, ranging from mild diarrhoea to severe forms, such as pseudomembranous colitis, colon perforation, toxic megacolon and sepsis, which can lead to death.

C. difficile infections (CDIs) frequently occur in hospital settings, and are transmitted by the faecal-oral route, following ingestion of *C. difficile* spores, via contaminated hands or environments. Hospitalized patients, those receiving antibiotic therapy, or the elderly are particularly at high risk of developing CDI. The high rate of recurrences and the limited number of effective antimicrobial agents complicate both the diagnosis and management of CDI.

Since 2003, the incidence and the severity of CDI, with high mortality rates, have been steadily increasing, and have become a major public health problem throughout the world. This changes in the epidemiology and clinical presentations of CDI were linked to the emergence and rapid dissemination of a hypervirulent strain called 027.

Between 2016 and 2019, 300 faecal specimens were collected from hospitalized patients with antibiotic-associated diarrhea. *C. difficile* were cultured on ChromID CDIF, and identified by Matrix Assisted Laser Desorption Ionisation-Time Of Flight mass spectrometry (MALDI-TOF MS). Toxin gene profiles were characterized by multiplex PCR. The isolates were typed by PCR ribotyping and Multilocus Sequence Typing (MLST). Antimicrobial susceptibility was tested by the Disc diffusion and E-test method against a panel of 6 antibiotics. The antibiotic resistance genetic determinants for clindamycin, erythromycin and tetracycline were analysed by PCR, and by sequencing for the quinolones.

C. difficile was detected in 18 (6%) of diarrheal patients, and were assigned to 11 different PCR-ribotypes and 12 sequence types: RT085/ST39, FR248/ST259, FR111/ST48, RT017/ST37, RT014/ST2, RT014/ST14, FR247/new ST, RT005/ST6, RT029/ST16, RT039/ST26, RT056/ST34 and RT446/ST58. Three toxin profiles were detected, two toxigenic, A+B+CDT- (33.3%) and A-B+CDT- (11%); and one non-toxigenic, A-B-CDT- (55.5%). The most common ribotypes were the non-toxigenic RT085 (16.7%), followed by the toxigenic RT014 and RT 017 (11.1% each). MLST analysis grouped the isolates into two clades, 1 and 4. Clade 4 was more homogeneous, as it included mainly non-toxigenic isolates. All *C. difficile* isolates were susceptible to metronidazole, vancomycin and moxifloxacin, whereas 72.2% and 16.6% were resistant to clindamycin and tetracycline, respectively.

In conclusion, The prevalence of CDI in this study was comparable to those reported in many studies from Europe, Africa and the middle East. The *C. difficile* strains circulating in our healthcare settings were diverse and include novel RTs. Overall, we believe that our data provide important information regarding the epidemiology of CDI in Algeria, and emphasizes the need for continued surveillance to detect and prevent the spread of *C. difficile*. Further larger studies are needed to assess the true extent of CDI in Algeria.

Keywords: *C. difficile*, nosocomial infections, Epidemiology, antibiotic resistance, ribotyping, MLST.

Résumé

Clostridioides difficile, une bactérie pathogène, anaérobie, Gram positif et sporulante, est considérée comme la principale cause de diarrhées post-antibiotiques, acquises en milieux hospitaliers. Les principaux facteurs de virulence de *C. difficile* sont deux toxines, la toxine A et la toxine B. *C. difficile* peut soit coloniser les patients sans provoquer de manifestations cliniques (porteurs sains), soit provoquer un éventail de maladies, allant de la diarrhée légère à des formes sévères, comme la colite pseudomembraneuse, la perforation du côlon, le mégacôlon toxique et la septicémie, qui sont potentiellement mortelles.

Les infections à *C. difficile* (ICD) surviennent fréquemment en milieux hospitaliers, et sont transmises par la voie féco-orale, suite à l'ingestion de spores de *C. difficile* par l'intermédiaire des mains ou des environnements contaminés. Les patients hospitalisés, ceux qui reçoivent une antibiothérapie ou les personnes âgées sont particulièrement à haut risque de développer une ICD. Le taux élevé de récidives et le nombre limité d'agents antimicrobiens efficaces compliquent à la fois le diagnostic et la prise en charge de l'ICD.

Depuis 2003, l'incidence et la gravité des ICD, associés à des taux de mortalité élevés, n'ont cessé d'augmenter et sont devenues un problème majeur de santé publique dans le monde. Ces changements dans l'épidémiologie et les présentations cliniques des ICD étaient liés à l'émergence et à la diffusion rapide d'une souche hypervirulente appelée 027.

Entre 2016 et 2019, 300 échantillons fécaux ont été prélevés chez des patients hospitalisés souffrant de diarrhée associée aux antibiotiques. *C. difficile* ont été cultivés sur ChromID CDIF et identifiés par spectrométrie de masse à temps de vol avec désorption laser assistée par MALDI-TOF MS (*Matrix Assisted Laser Desorption Ionisation-Time Of Flight Mass Spectrometry*). Les profils de gènes de toxines ont été caractérisés par PCR multiplex. Les isolats ont été typés par PCR-ribotypage et MLST (*Multilocus Sequence Typing*). La sensibilité aux antimicrobiens a été testée par la méthode de diffusion de Disc et le E-test contre un panel de 6 antibiotiques. Les déterminants génétiques de la résistance aux antibiotiques clindamycine, l'érythromycine et la tétracycline ont été analysés par PCR, ou par séquençage pour les quinolones.

C. difficile a été détecté chez 18 (6 %) des patients diarrhéiques et ont été assignés à 11 PCR ribotypes (RT) différents et 12 types de séquence (ST) : RT085/ST39, FR248/ST259, FR111/ST48, RT017/ST37, RT014/ST2, RT014/ST14, FR247/nouveau ST, RT005/ST6, RT029/ST16, RT039/ST26, RT056/ST34 et RT446/ST58. Trois profils de toxines ont été détectés, deux toxigéniques, A+B+CDT- (33,3 %) et A-B+CDT- (11 %) ; et un non-toxinogènique, A-B-CDT- (55,5 %). Les ribotypes les plus communs étaient le non-toxinogène RT085 (16,7 %), suivi des toxinogènes RT014 et RT017 (11,1 % chacun). L'analyse MLST a regroupé les isolats en deux clades, 1 et 4. Le clade 4 était plus homogène, car il comprenait principalement des isolats non toxinogènes. Tous les isolats de *C. difficile* étaient sensibles au métronidazole, la vancomycine et à la moxifloxacine, tandis que 72,2 % et 16,6 % étaient résistants à la clindamycine et à la tétracycline, respectivement.

En conclusion, la prévalence des ICD dans cette étude était comparable à celles rapportées dans de nombreuses études menées en Europe, en Afrique et au Moyen-Orient. Les souches de *C. difficile* circulantes dans nos milieux de soins étaient diverses et comprenaient de nouveaux RT et ST. Dans l'ensemble, nos données fournissent des informations importantes concernant l'épidémiologie des ICD en Algérie et soulignent la nécessité d'une surveillance continue pour détecter et prévenir la propagation de *C. difficile*. D'autres études plus élargies sont nécessaires pour évaluer l'étendue réelle des ICD en Algérie.

Mots clés: *C. difficile*, infections nosocomiales, Epidémiologie, résistances aux antibiotiques, ribotypage, MLST.

ملخص

المطثية العسيرة، أو كلوستريديوديز ديفيسيل هي بكتيريا لا هوائية، إيجابية الغرام، مبوغة، تعتبر السبب الرئيسي للإسهال السريري الناتج عن المضادات الحيوية خاصة عند المرضى المقيمين بالمستشفيات. حيث تتمحور عوامل الخطورة الرئيسية لداء المطثية العسيرة في انتاج نوعين من السموم الخطيرة، وهما السم أ والسم ب. المطثية العسيرة يمكن إما أن تصيب الأشخاص دون التسبب في أعراض مرضية والذين يعتبرون نواقل خاملة، كما يمكن أن تتسبب للأشخاص المصابين بعدة أعراض، تتراوح من الإسهال الخفيف الى الأعراض الأشد فتكا، مثل التهاب القولون، انثقاب القولون، تضخم القولون، والإنتان، كما يمكن في الحالات المتقدمة أن تؤدي للوفاة.

تحدث أغلبية حالات العدوى الناتجة عن الاصابة بداء كلوستريديوديز في المستشفيات، وتنتقل عن طريق الأيدي ومن الأسطح الملوثة نتيجة ابتلاع أبواغ البكتيريا حيث أن أكثر فئة هشة معرضة للخطر هم المرضى في المستشفيات، أو الأشخاص الذين يتلقون العلاج بالمضادات الحيوية، كما أن كبار السن معرضين بشكل خاص للإصابة بهذه العدوى. يؤدي إرتفاع معدل الإصابة المتكررة وتطوير مقاومة للمضادات الحيوية لدى المرضى إلى تعقيد حالات التشخيص والتكفل بالمرضى.

منذ عام 2003، معدل وشدة الإصابة بداء كلوستريديوديز يزداد باستمرار وثبات مع تسجيل إرتفاع محسوس في معدل الوفيات، حيث أن هذا المرض أصبح مشكل رئيسي في مجال الصحة العمومية حول العالم. وقد ارتبطت هذه الزيادات في المعطيات الوبائية بضهور سلالة شرسة متعددة المقاومة و سريعة العدوى تسمى السلالة 027.

بين سنة 2016 و 2019، تم جمع 300 عينة براز من مرضى مصابين بالإسهال المرتبط بالمضادات الحيوية ومقيمين بالمستشفى. تم زرع وعزل بكتيريا كلوستريديوديز ديفيسيل باستخدام أوساط الانبات الخاصة بهذا النوع وتم التعرف عليها باستخدام تقنية مقياس طيف الليزر الكتلي MALDI-TOF MS.

تم الكشف عن البروفيل السمي لهذه البكتيريا باستخدام اختبار تفاعل البوليميراز المتسلسل المتعدد، كما تم التعرف على الانتماء الريبي النوعي والسلالي بالستخدام تقنية PCR Ripotyping و تحليل تحديد تسلسل المواقع المتعددة MSLT.

إختبار الحساسية للمضادة الحيوية تم عن طريق أقراص التوزيع واختبارE ضد قائمة تتكون من 6 مضادات حيوية، كما تم تحليل المحددات الجينية لمقاومة اللكليندامايسين، الإريثروميسين والتتراسيكلين باستعمال تقنية تفاعل البوليميراز المتسلسل للكينولونات.

تم الكشف عن المطثية العسيرة عند 18 مريض (6٪)، وتم تعيينها إلى 11 نوعا مختلفا من أنواع الريبوسومات PCR Ribotypes و 12 نوعا جيني متسلسل مختلف RT085/ST39 :، FR248/ST259، FR111/ST48، RT017/ST37، RT014/ST2، RT014/ST14، RT005/ST6 و RT029/ST16 و RT039/ST26 و RT056/ST34 و RT446/ST58، FR247/new ST.

تم الكشف عن ثلاثة بروفيلات سمية لهذه البكتيريا، إثنين منها سامة (33.3 ٪A+B+CDT- و 11) ٪A-B+CDT-(وواحدة غير سامة(55.5 ٪A-B-CDT-)، حيث كان النوع الريبي الأكثر انتشارا هو RT085 غير السمي 16.7٪، يليه RT014 و 017 RT ب 11% لكل منهما. وقد جمعت دراسة MLST البكيتريا المعزولة في قبيلتين 1 و 4. حيث أن الفرع الحيوي 4 كان أكثر تجانسا، لأنه كان يشمل في الأساس عدد من السلالات الغير سامة. كما أن كل السلالات المعزولة كانت حساسة للميترونيدازول، الفانكومايسين وموكسيفلوكساسين، في حين أن 72.2٪ و 16.6٪ من مجموع السلالات المعزولة كانت مقاومة للكليندامايسين والتتراسيكلين بهذا الترتيب.

في الختام، ان نسبة عدوى كلوستريديوديز ديفيسيل في هذه الدراسة مشابها لتلك الواردة في العديد من الدراسات في أوروبا وأفريقيا والشرق الأوسط، كما أنه لدينا تنوع في سلالات المطثية العسيرة المنتشرة في مختلف المؤسسات الاستشفائية العمومية والتي شملت سلالات جديدة ليست معروفة. وبشكل عام، نعتقد حقيقة أن بياناتنا توفر معلومات وبائية هامة بشأن داء كلوستريديوديز ديفيسيل في الجزائر، وتشدد على أهمية وضرورة المراقبة المستمرة لكشف ومنع انتشار المطثية العسيرة، كما أن المزيد من الدراسات الواسعة ضروري لتقييم المدى الحقيقي لانتشار هذه العدوى في الجزائر.

الكلمات المفتاحية: المطثية العسيرة، عدوى المستشفيات، علم الأوبئة، مقاومة المضادات الحيوية، Riboypage، MLST.

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List of abbreviations

ADP	adenosine diphosphate
bp	base pair
°C	Celsius degree
CCFA	Cycloserine Cefoxitin Fructose agar
CCEY	Cefsulodin-cycloserine-egg yolk agar
CDAD	*Clostridioides difficile* antibiotic-associated diarrhoea
CDI	*Clostridioides difficile* infections (CDI)
C. difficile	*Clostridioides difficile*
CDT	*Clostridioides difficile* binary toxin
CO2	carbon dioxide
CWP	Cell-wall proteins
CWBP	Cell-wall binding proteins
DNA	Deoxyribonucleic acid
dNTP	Deoxy ribo-nucleotide triphosphate
ECDC	European Centre for Disease Prevention and Control
ESCMID	European Society of Clinical Microbiology and Infectious Diseases
g	gram
GDH	glutamate dehydrogenase
H2	Dehydrogen
HCl	hydrogen chloride
HMW-SLP	high molecular weight protein Surface Layer Protein
kDa	Kilo Daltons
kb	kilo base
L	Litre
LCT	Large Clostridial Toxins

LMW-SLP	low molecular weight protein called Surface Layer Protein
m	masse
M	Mole
mg	milligram
MgCl2	magnesium chloride
MLVA	Multiple Locus Variable Number of Tandem Repeats Analysis
MLST	Multilocus Sequence typing
mM	millimole
min	minute
N2	Nitrogen
NAP1	North Americain Pulsed Field 1
ng	nanogram
nm	nanometer
PaLoc	pathogenicity locus
PCM	pseudomembranous colitis
PCR	Polymerase Chain Reaction
PFGE	Pulsed Field Gel Electrophoresis
PH	potential of hydrogen
pm	picomol
PPI	Proton pomp inhibitor
REA	Restriction Endonuclease Analysis
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic acid
rpm	round per minute
RT	Ribotype
s	seconds

SlpA	S layer protein A
SNP	single nucleotide polymorphism
ST	sequence type
TcdA	toxin A
TcdB	toxin B
U	Unite
µg	microgram
µl	microlitre
µm	micrometer
µm	micrometre
µM	micromole
USA	United States of America
UV	ultraviolet
WGS	whole genome sequencing
z	charge

General Introduction

Clostridium difficile, recently renamed as *Clostridioides difficile*, an anaerobic Gram-positive, spore-forming, toxin producing bacteria, is the leading cause of healthcare- associated diarrhoea **(Burke & Lamont, 2014)**. The spectrum of clinical manifestations of *C. difficile* infections (CDI) can range from mild diarrhoea to more severe an life threatening forms such as pseudomembranous colitis, toxic megacolon, colonic perforation and sepsis **(Smits *et al*., 2016)**. The major risk factors for CDI are advanced age (≥65years old), antibiotic exposure, a prolonged hospital stay, gastro-intestinal surgery as well as chronic conditions such as inflammatory bowel diseases **(Smits *et al*., 2016)**. The transmission of *C. difficile* occurs by the faecal-oral route, mainly from hands or environments contaminated by the spores (**Martin *et al*., 2016**).

The problems associated with *C. difficile* are further exacerbated by the increasing rates of multiple **recurrences** (re-apparition of the infections and symptoms within few weeks following successful treatment of an initial episode), as well as the emergence of strains with reduced susceptibility or **resistance** to antimicrobial agents, particularly metronidazole and vancomycin, the first-line antibiotics for the treatment of CDI, limiting further the therapeutic options **(Valiente *et al*., 2014; Peng *et al*., 2017)**. In addition to being clinically problematic, CDI impose an extra burden on the healthcare systems, due to increased healthcare costs, associated with treatments and prolonged hospitalizations **(Kuijper *et al*., 2006; Lessa *et al*., 2015)**.

CDI occur as a result of distribution of the gastrointestinal microbiota, following antibiotic treatment. The main virulence factors of *C. difficile* are the production of two major toxins: toxin A (*tcdA*) and toxin B (*tcdB*), encoded on a 19.6 kb chromosomally-located pathogenicity locus (*PaLoc*), which have cytotoxic and enterotoxic effects, respectively **(Pruitt & Lacy, 2012)**. In addition, certain strains of *C. difficile* produce a third toxin, called binary toxin (CDT), which act as an actin-specific ADP-ribosyltransferase, encoded by the *cdtA* and *cdtB* genes, located outside the PaLoc **(Sun *et al*., 2010)**.

Since the early 2000s, the incidence and the severity of the CDI are growing in various countries around the world, causing high rates of morbidity and mortality particularly among the elderly. This worrying development in the epidemiology *C. difficile* is linked to the emergence and rapid dissemination of hypervirulent strains, such as the epidemic NAP1/BI/027 **(Solanki *et al*., 2021)**.

Although the burden and the epidemiology of CDI in many countries around the world is well documented, CDI is a largely neglected disease in Algeria, and apart from one previous study by **Djebbar *et al*. (2018)**, there is no other information regarding the epidemiology of CDI in Algeria.

General Introduction

Given that CDI are a major source of concern for healthcare systems throughout the world, it has become important to understand the epidemiological situation of CDI in Algeria.

Therefore, the main aim of this study was to gain insight into the molecular epidemiology of *C. difficile* in Algeria.

Specific objectives were:

1. Isolation and identification *C. difficile* isolates from stools of patients admitted to 5 hospitals in the three provinces of Algeria, Chlef, Ain Defla and Batna.

2. Determination of the toxinogenic profiles of the isolates by PCR.

3. Typing of the isolates by using the PCR-ribotyping and MLST techniques.

4. Assessing the antimicrobial resistance profiles of the isolates.

Chapter I: Review of literature

1.1 History

Clostridioides difficile, previously known as *Clostridium difficile*, was first described by **Hall & O'Toole** in 1935 during an investigation of normal intestinal flora of healthy new-born infants and was named *bacillus difficilis*, to reflect its slow growth and its difficult isolation**.** Since then, *C. difficile* was considered as a commensal of human. In 1977, it was classified in the genus *Clostridium*, on the basis of its morphological characters, and was renamed *Clostridium difficile*. The same year, **Larson *et al*, (1977)** determined a link between *C. difficile* and pseudomembranous colitis (PCM), and they also found that *C. difficile* causes antibiotic-induced diarrhea. The following year, **Bartlett *et al*, (1978)** demonstrated that *C. difficile* is the causative agent of PMC.

C. difficile is found in the intestine of humans and animals (**Barbut *et al.*, 2011)** as well as in the environment (soil and water) **(Diaz *et al*., 2018)**, and *C. difficile* infections (CDI) are now recognized as one of the major health care associated infections, responsible for most (15-25%) of *C. difficile* antibiotic-associated diarrhoea (CDAD) and for the majority (95%) of PMC **(Barbut & Petit, 2001)**.

In 2013, *C. difficile* was reclassified in a new genus called *Peptoclostridium* of the family of *Peptostreptococaceae*, based on the sequence of its 16S rDNA **(Yutin & Galperin, 2013)**; and since 2016, it was reclassified in a new genus called *Clostridioides* in the family of *Clostridiaceae* **(Lawson *et al*., 2016)**.

The current *C. difficile* classification is as follows:

Kingdom:	Bacteria
Division:	Firmicutes
Order:	*Clostridiales*
Class:	*Clostridia*
Family:	*Clostridiaceae*
Genus:	*Clostridioides* (formally *Peptoclostridium* and *Clostridium*)
Species:	*Clostridioides difficile*

1.2 Microbiological and morphological characteristics of *C. difficile*

C. difficile is a rod-shaped Gram-positive bacillus of 0.5 to 1.9 µm in diameter by 3 to 16.8 µm in length (**Figure 1a**), strictly anaerobic, spore-forming, generally mobile by peritrichous ciliature. The optimum growth temperature for *C. difficile* is 37°C.

C. difficile forms slightly convex, whitish or greyish colonies with an irregular, lobate or rhizoidal border **(George *et al*., 1979; Hill *et al*., 2013)**, with a broken glass appearance under binocular magnification (**Figure 1b**); and having a characteristic smell of horse manure, due to the production of a metabolite called *p*-cresol **(Carroll, 2011)**.

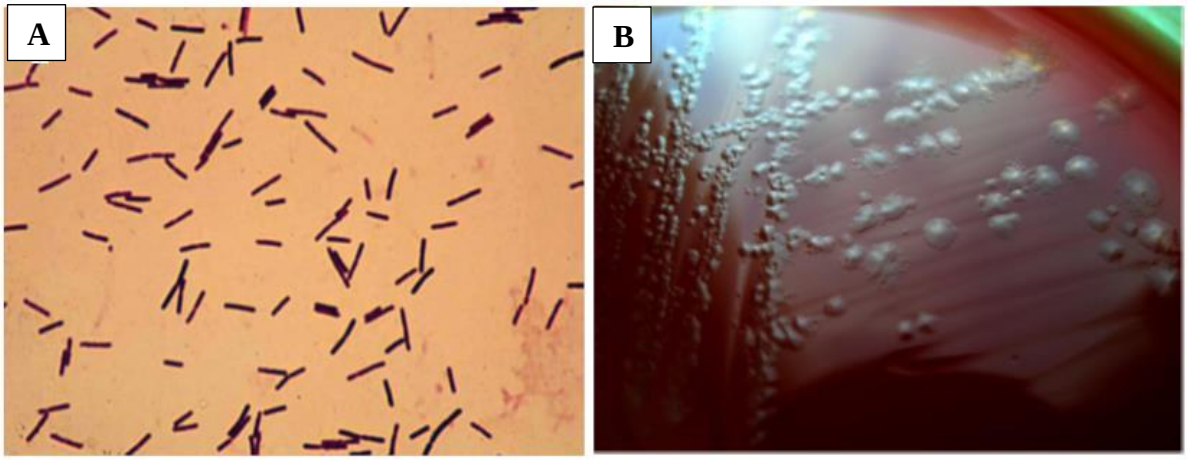

Figure 1. a) Gram staining of *C. difficile* **(Kamiya, 2011); b)** Aspects of *C. difficile* colonies on blood agar supplemented with 5% sheep blood **(Doosti & Mokhtari-Farsani, 2014).**

1.2.1 *C. difficile* spores

C. difficile can occur under a vegetative or a spore form. The spores, which are subterminal to terminal and oval in shape (**Figure 2a**), are highly resistant to disinfectants, heat, desiccation, radiation and hostile conditions. The spores can persist in the environment for a prolonged period of time, and can germinate when the conditions become favourable **(Setlow, 2014)**; and they constitute the principal means of persistence and dissemination of *C. difficile* **(Paredes-Sabja *et al*., 2014)**.

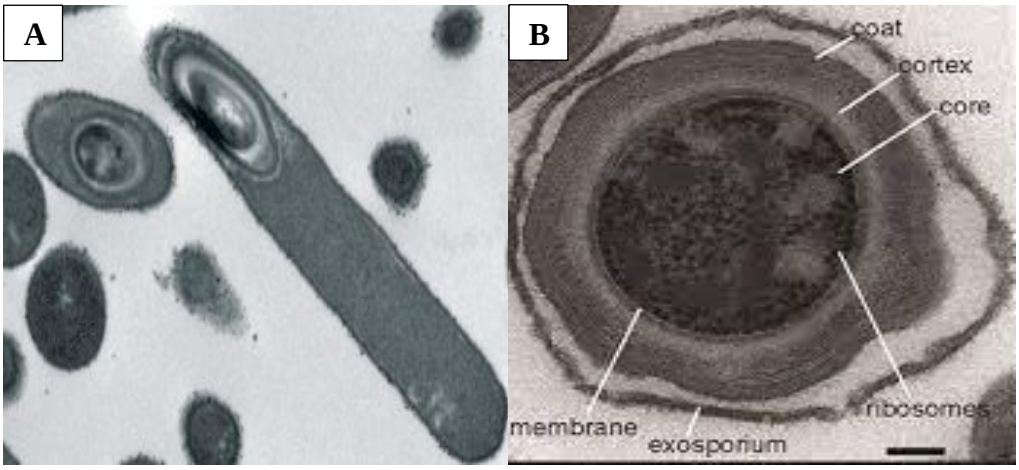

Figure 2. Aspect and structure of *C. difficile* spores. Transmission electron micrograph image of **A**) an endospore **(Ray, 2019)**; and **B**) a sectioned spore **(Awad *et al*., 2014).**

Microscopic examination reveals that the spore of *C. difficile* is composed of a nucleus (containing the chromosome, a complex of ribosomes and a wide variety of nucleoprotein complexes), which is covered by three concentric layers, from the inside out : the cortex (internal layer) formed mainly of peptidoglycan, a dense spore wall (coat), composed mostly of proteins, and an exosporium (outer layer) consisting mainly of glycoproteins (**Figure 2b) (Paredes-Sabja *et al*., 2014)**.

C. difficile sporulation occurs when nutrients become depleted. The exact environmental signals triggering sporulation are not well known **(Abt *et al*., 2016).**

The germination of *C. difficile* spores occurs in the intestinal tract and is induced by bile acids, in particular taurocholate. Binding of bile acids to the germination receptors present on the surface of the spore is necessary to initiate the germination in the colon **(Sorg & Sonenshein, 2008; Sorg & Sonenshein, 2009)**.

It has been documented that infected patients can discharge approximately 10^5 spores per gram of faeces. The spores may last in the environment for a very long time since they are resistant to heat, radiation, chemicals, and antimicrobials agents **(Setlow, 2014)**.

1.3 Clinical manifestations

C. difficile infections (CDI) usually occurs following antibiotic treatment, which disturbs the balance of the intestinal microbiota. The clinical manifestations of CDI vary depending on a wide range of factors, related to the pathogen and to the host; and ranging from asymptomatic carriage, mild diarrhea without colitis, to a serious and life-threatening pathologies,

pseudomembranous colitis (PMC), which can progress into fulminant colitis and toxic megacolon (**Figure 3) (Eckert *et al*., 2013; Seugendo *et al*., 2015)**.

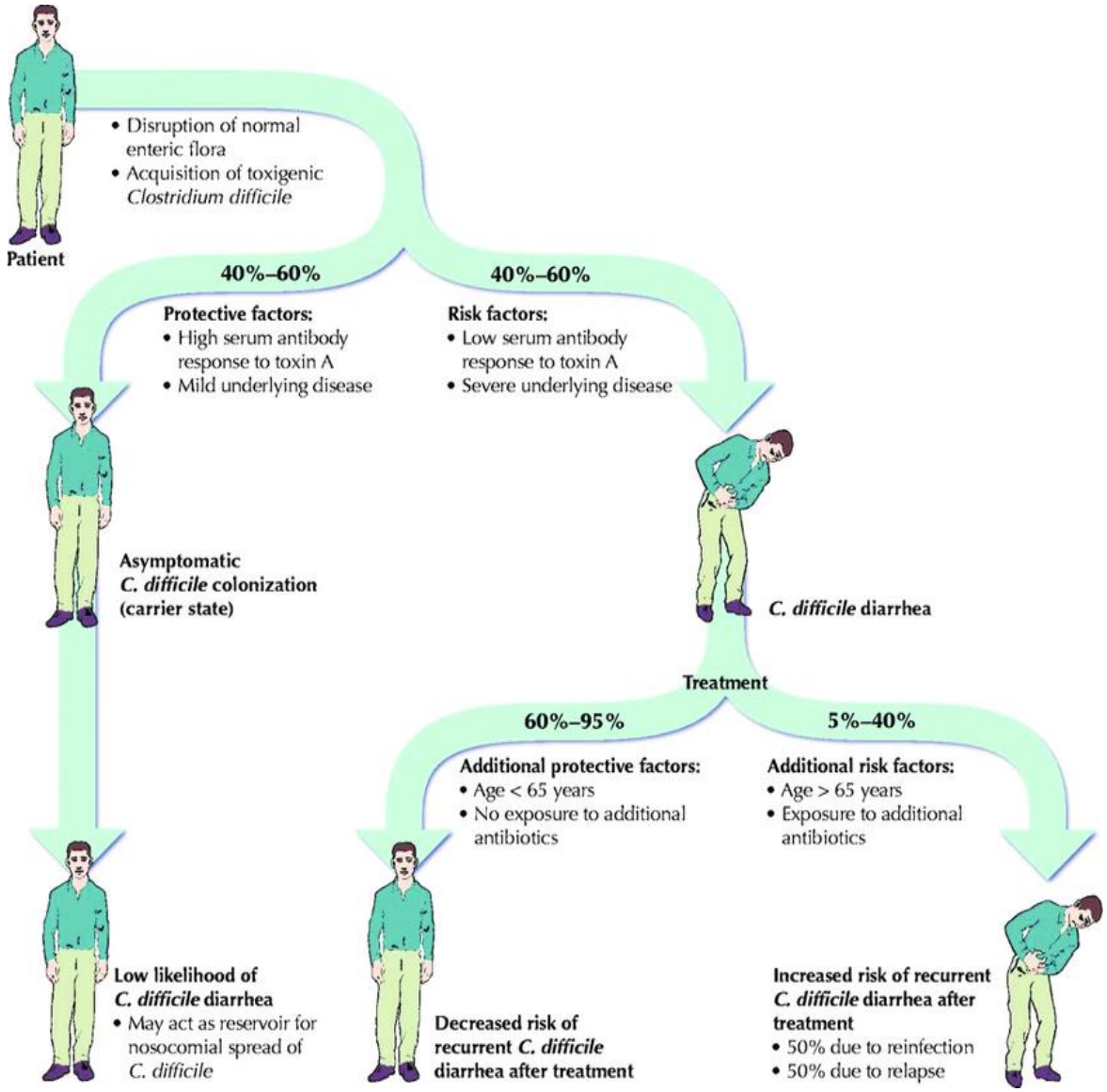

Figure 3. The clinical manifestations of CDI and their associated risk factors **(Khanafer, 2013).**

1.3.1 Asymptomatic carriage

The prevalence of asymptomatic carriage of *C. difficile* in healthy adults, without clinical signs of CDI, was estimated at around 3 %, and can vary between 40 and 60% in hospitalized patients (**Figure 3).** However, it can reach up to 8.3% in children over the age of two, between 3% to 33% in infants and toddlers under the age of two, and between 15% to 63% in newborns **(Bryant & McDonald, 2009; Cerquetti *et al*., 1995)**.

1.3.2 CDAD

CDAD can be defined as three watery or unformed stools per day, without mucus or visible blood, which can be accompanied with moderate fever and mild abdominal pain, most often without marked deterioration in the general condition of the patient. The manifestation of moderate CDAD is often encountered in adults without risk factors for complications (**Figure 3) (Elbeddini & Gerochi, 2021; van Prehn *et al*., 2021)**. Colonic endoscopy in this case would highlight a normal-looking mucosa or at most an erythema, without colitis. In 25% of the cases, the symptoms disappear two to three days after the end of the antibiotic treatment **(Johnson & Gerding, 1998**). The simple diarrhea is the form of CDI most observed in the community, hospitalization is required in the most severe cases **(Al-Eidan *et al*., 2000; Sartelli *et al*., 2019).**

1.3.3 Colitis

The most common complication form of severe CDI is PMC, which is characterized by the presence of yellowish plaques on the mucosa, called pseudomembranes (made up of cellular debris, mucus, fibrin and leukocytes), scattered or confluent depending on the stage of the disease (**Figure 4**). These colitis are associated with a profuse and abundant diarrhea (6 stools/day), most often accompanied with fever, abdominal pain and nausea **(Barbut *et al*., 2004; Bartlett, 1996)**. Other clinical signs of inflammation such as hyperleukocytosis, a high white blood cell and, high creatinine levels and low level of albumin, can also be observed in patients.

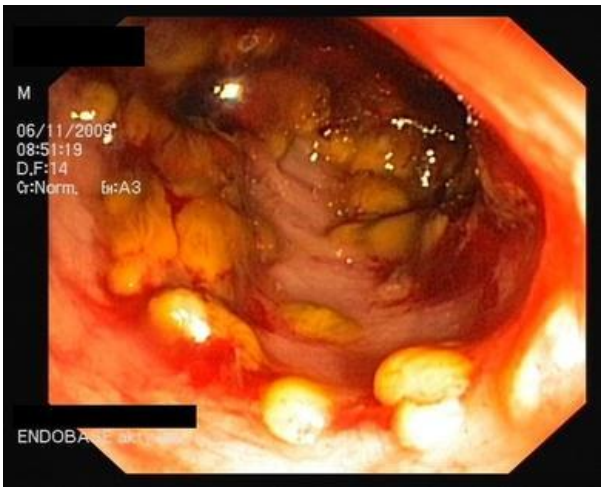

Figure 4. Endoscopic image of pseudomembranous colitis **(Kazanowski *et al*., 2014)**.

PMC may develop into severe complications, fulminant colitis, that may lead to colonic perforations and toxic megacolon, which require urgent surgery, as they are most often associated with multisystem organ failure and sepsis, which could lead to death, particularly among the vulnerable groups of patients, such as the elderly and the immune-compromised **(Farooq *et al*., 2015; Ong *et al*., 2017).**

1.3.4 Recurrences

One of the particularities of CDI is the recurrence of the disease, due to either relapse or reinfection, which is of main concerns for both clinicians and patients. A patient who was effectively treated for a CDI, i.e. disappearance of symptoms, is likely to recur within the two months following the end of the treatment **(Barbut *et al*., 2004)**, the recurrence rate can reach up to 20% after a first infection, and could increase to 50% after a first recurrence **(Valiente *et al*., 2014).** The majority of the recurrent cases of CDI are the result of relapse, caused by infection with the same strain of *C. difficile* that caused the initial episodes of CDI, which persisted in the digestive tract in the form of spores, and the remaining cases of recurrences are due to reinfection with an exogenous strain of *C. difficile*, different from that of the initial episode, most often acquired during hospitalization. The CDI recurrences are more frequent in older patients (above 65), or patients with altered intestinal flora and/or immune-compromised **(Sheitoyan-Pesant *et al*., 2016; Song & Kim, 2019; Tsigrelis, 2020).**

1.4 Burden of CDI

At present, CDIs have become a real challenge for health systems worldwide. From an economic point of view, CDIs increased substantially the cost of patient care, as a result of the increase in the CDI incidence rates, the vulnerable population, the severity of the disease, repeated and longer hospital stays, laboratory diagnostics, surgical complications and medications **(Dubberke & Olsen, 2012; Dubberke & Wertheimer, 2009; McGlone *et al.*, 2012).**

1.5 Pathogenesis of *C. difficile*

The contamination by *C. difficile* occurs by the ingestion of spores through the faecal-oral route, following antibiotic-induced dysbiosis of the gut microbiota. The spores, which resist the stomach acidity, move into the intestine, where they germinate into vegetative cells, to colonize and multiply in the colon **(Figure 5)**. The germination process is induced by the bile acids present in the small intestine **(Denève *et al.*, 2009; Poxton *et al.*, 2001; Sorg & Sonenshein, 2008).**

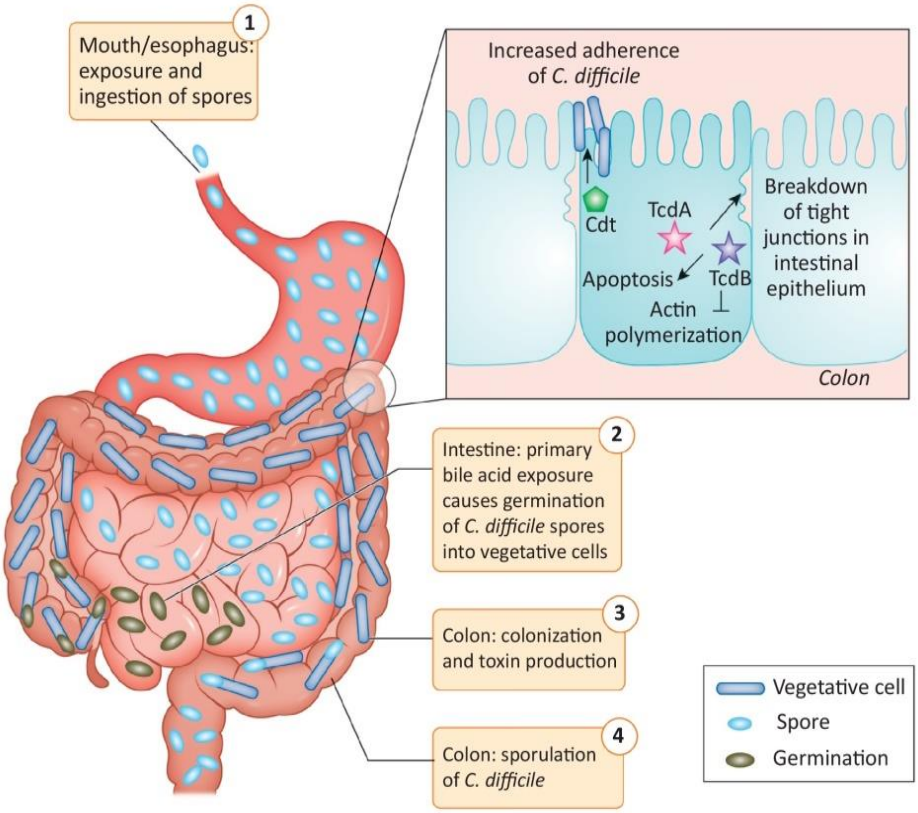

Figure 5. Pathogenesis of *C. difficile* **(Sandhu & McBride, 2018).**

In the second stage of the pathogenesis, the toxigenic strains of *C. difficile* produce two toxins, toxin A (TcdA), an enterotoxin, and toxin B (TcdB) a cytotoxin. These high molecular-weight proteins bind to specific receptors on the cells of the colonic epithelium and penetrate into the cytoplasm, where they modify the cell structure which leads to the breakdown of the epithelium and the increase in the cellular permeability, resulting in diarrhea **(Denève *et al*., 2009; Rupnik *et al*., 2009; Voth & Ballard, 2005)**.

The toxins also induce a host inflammatory response, by the production of various pro-inflammatory cytokines (INFα; IL1; IL6), which can contribute to the development of the colitis forms of the disease **(Czepiel *et al*., 2014; Péchiné & Collignon, 2016; Yu *et al*., 2017)**.

1.6 Virulence factors of *C. difficile*

The pathogenicity of *C. difficile* is a multifactorial process involving several virulence factors which are involved in the different stages of the infection process, attachment, motility, survival, multiplication, colonisation and invasion. Certain virulence factors are associated to the surface of the bacterium and others are extracellular, and certain contribute directly to the pathogenicity of the bacterium while others have complementary roles **(Janoir, 2016)**.

1.6.1 Toxins

1.6.1.1 Toxins A et B

The main virulence factors of *C. difficile* are the two toxins, TcdA and TcdB, which are similar proteins (49% identical and 63% similar) of 308 and 270 kDa, respectively, that belong to the family of Large Clostridial Toxins (LCTs), which also includes the lethal and haemorrhagic toxins from *C. sordellii* (TcsL and TcsH), the cytotoxin from *C. perfringens* (TpeL) and the alpha-toxin from *C. novyi* (Tcn*α*) **(Voth *et al*., 2005)**.

Both toxins are glycosyltransferases that inactivate specifically the families of Rho and Ras GTPases proteins, which are low molecular weight proteins of the GTP-binding family, involved in the regulation of actin cytoskeleton in eukaryotic cells **(Chen *et al*., 2015; Voth & Ballard, 2005)**. This inactivation, which occurs by the addition of a molecule of glucose to a threonine residue on the Rho proteins, causes the disruption of intracellular signalling, which results in the depolymerization of the actin filaments, the disruption of the cytoskeleton organization, the rounding of cells, activation of the apoptosis process and the destruction of

intercellular tight junctions **(Figure 6)**. All these events contribute to an increased fluid permeability of the intestinal mucosa, which is manifested by a profuse diarrhea **(Aktories *et al*., 2017).**

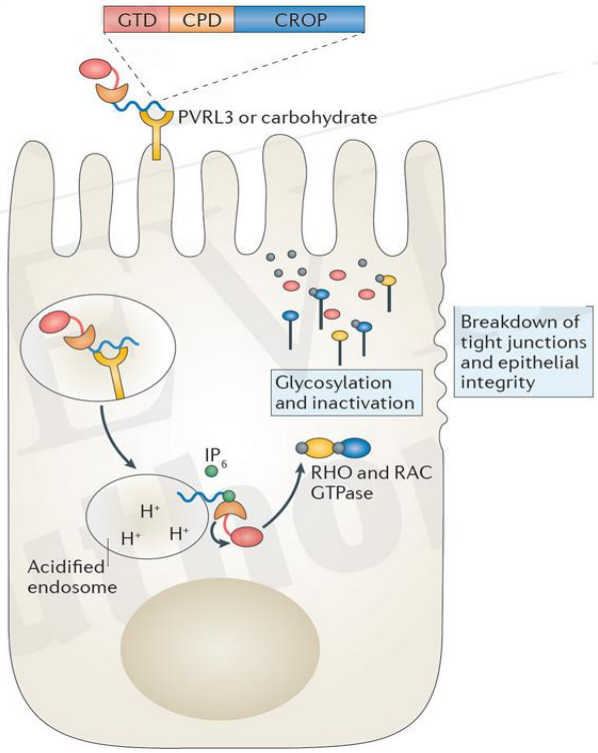

Figure 6. Mechanisms of actions of toxin A and B. The two toxins binds to a specific receptors on the surface of the intestinal epithelial cells. They are then internalized via endocytosis. The amino-terminal fragment, containing the cysteine protease domain (CPD) and the glucosyl transferase domain (GTD), is cleaved in the acidified endosome, and transported into the cytosol, where the GTD is cleaved and released by the CPD. The GTD glycosylates and inactivates the RHO or RAC GTPases which results in the breakdown of tight junctions and epithelial integrity **(Abt *et al*., 2016)**.

In addition, the *C. difficile* toxins A and B trigger a severe inflammatory reaction in the colon, by stimulating the production of high level of cytokines, which can contribute to the development of PMC **(Freeman, 2008)**.

The genes encoding the toxins A (*tcdA*) and B (*tcdB*) are located on a 19.6-kb chromosomal region known as the pathogenicity locus (PaLoc) **(Figure 7) (Barbut *et al*., 2007; Geric *et al*., 2006).** In addition of the genes *tcdA* and *tcdB*, the PaLoc contains three genes *tcdR*, *tcdC* and *tcdE* **(Figure 7)**. The gene *tcdR* encodes a transcriptional regulator, which is a member of the family of alternative extracytoplasmic sigma factor, involved in the regulation of the

expression of *tcdA* and *tcdB* **(Dupuy *et al.*, 2008)**; whereas the gene *tcdC* codes for an anti-sigma factor that inhibits toxin expression **(Matamouros *et al.*, 2007)** and the *tcdE* gene encodes a holine-like protein involved in the secretion of the toxins **(Govind & Dupuy, 2012; Tan *et al.*, 2001).**

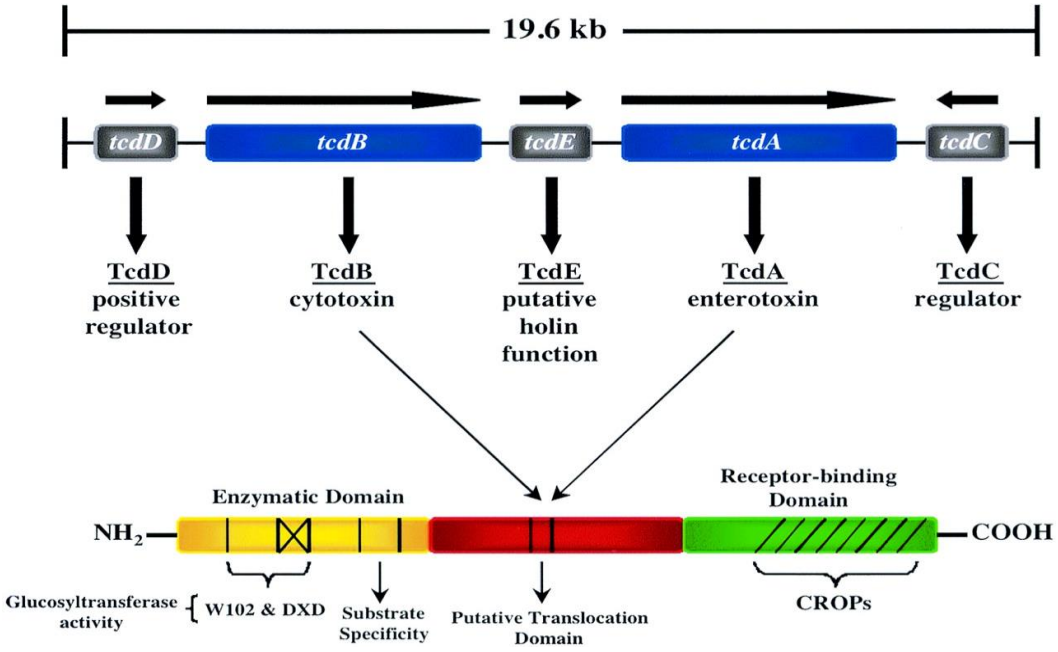

Figure 7. Genetic arrangement of the *C. difficile* pathogenicity locus (PaLoc) and domain structures of the toxins TcdA and TcdB **(Voth & Ballard, 2005)**.

1.6.1.2 Binary toxin

Some strains of *C. difficile* produce a third toxin, known as binary toxin (CDT, *C. difficile* transferase), which is an actin-specific ADP-ribosyltransferase **(Rupnik *et al.*, 2003)**.

The binary toxin, is a 4.3 kDa protein composed of two subunits, CDTa and CDTb. The subunit CDTb binds to specific receptors on the surface of the enterocytes, and allows the internalization of the subunit CDTa by endocytosis into the cytoplasm of the host cell (**Figure 8**). CDTa exerts its ADP-ribosyltransferase activity on actin monomers; and as such, it inhibits the formation of the actin cytoskeleton, causing the ballooning of the cells, which leads to cell death **(Abt *et al.*, 2016; Davies *et al.*, 2011; Sundriyal *et al.*, 2010)**.

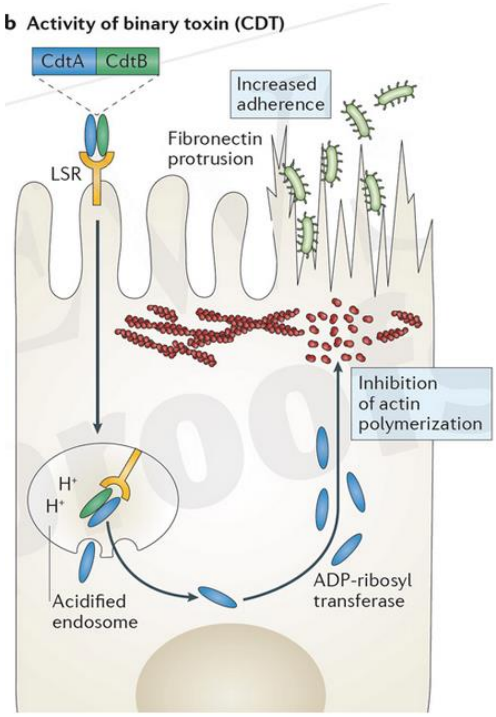

Figure 8. Mechanism of action of the binary toxin. The binary toxin, which consists of a binding subunit (CdtB) and an active subunit (CdtA), binds to a specific receptor on the surface of the intestinal epithelial cells. The binary toxin is internalized via endocytosis. The CdtB subunit creates pores in the acidified endosome to release the CdtA subunit into the cytosol. The ADP-ribosyl transferase activity of CdtA inhibits actin polymerization, causing the ballooning of the cells and leading to cell death **(Abt *et al*., 2016)**.

The contribution of the binary toxin to the virulence is controversial, as certain strains of *C. difficile* that do not produce this toxin remain virulent; however, it was suggested that it increases virulence in the strains that produces it **(Abt *et al*., 2016).**

The genes *cdtA* et *cdtB* coding for the two subunits of the binary toxin are located on the chromosome outside the PaLoc.

In addition to the two main virulence factors, toxin A and B, *C. difficile* produces several other virulence factors that have been shown to play a role in the pathogenicity, including surface-associated factors, which are involved mainly in the adhesion of *C. difficile* to host tissues, and factors that contribute to the colonization and invasion processes **(Janoir, 2016; Vedantam *et al*., 2012).**

1.6.2 Surface-associated factors

For pathogens, adhesion to host cells is an important step during the process of pathogenesis. Several surface proteins and structures involved in adhesion have been identified in *C. difficile*, including the S-layer proteins, fibronectin-binding protein (FbpA), collagen-binding protein (CbpA), flagellar proteins (FliC and FliD), surface polysaccharides, lipoproteins and fimbriae

(Borriello *et al*., 1988; Péchiné *et al*., 2016).

1.6.2.1 S-layer proteins

The Surface Layer (S-layer), is a crystalline outer layer that covers the surface of *C. difficile* cells, which is composed of two superimposed protein layers. The outer layer consists of a low molecular weight protein called Surface Layer Protein (LMW-SLP), a protein of about 36 KDa (P36), and the inner layer consists of a high molecular weight protein Surface Layer Protein (HMW-SLP), with a molecular weight between 42 to 48 KDa (P47) **(Calabi *et al*., 2001; Cerquetti *et al*., 2000; Fagan *et al*., 2009).**

Both the HMW-SLP P47 and the LMW-SLP P36 are encoded by the *slpA* gene and are produced following the post-translational cleavage of the pre-protein SlpA (at serine 321) by a serine protease Cwp84. The N-terminal and the C-terminal portions of the precursor protein SlpA correspond to the LMW-SLP and HMW-SLP, respectively. Following the cleavage of the SlpA, the two subunits, LMW-SLP and HMW-SLP, are then assembled non-covalently at the surface to form an HMW-SLP/LMW-SLP (H/L) complex, the major constituent of the S-layer. The HMW-SLP is able to bind and interact with the extracellular matrix and more particularly type I collagen, thrombospondin and vitronectin **(Calabi *et al*., 2001; Calabi & Fairweather, 2002; Dang *et al*., 2010; Fagan et al., 2009; Janoir et al., 2007; Kirby et al., 2009)**.

There are 29 genes in *C. difficile* genome which code for SlpA-like surface proteins (**Figure 9**), but only a few have been characterized, and their roles in the *C. difficile* colonization was established; of these Cwp84 Cwp66 and CwpV, have adhesin properties, and have been shown to play an important role in the physiopathology of *C. difficile* **(Janoir *et al*., 2007; Waligora *et al*., 2001)**.

All of the proteins in this family have three tandem domains known as CWB2, which allow the binding of these proteins to the cell wall, hence their name CWP for Cell-Wall Proteins

(**Figure 9**). Additionally, the proteins in this family contain other distinct protein domains that confer additional specific functions, such as adhesion to epithelial cells, immune evasion, protection of cell integrity, cell aggregation, resistance to bacteriophages and resistance to antimicrobial compounds (**Figure 9**) **(Bradshaw *et al*., 2018; Fagan & Fairweather, 2014).**

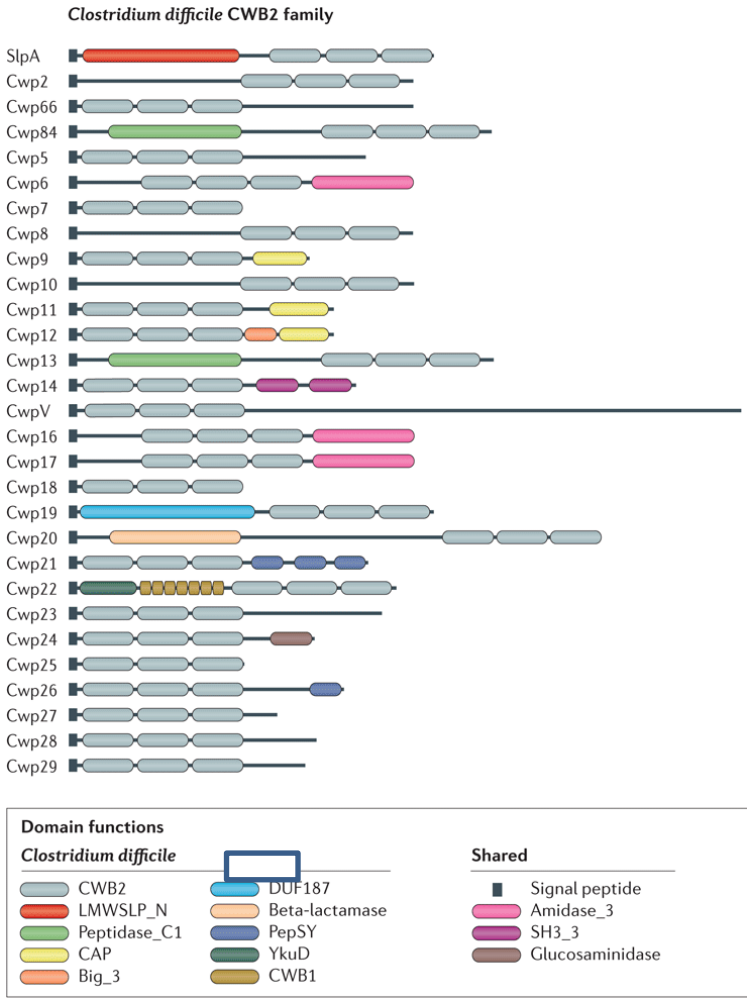

Figure 9. Domain structure of all members of the *Clostridium difficile* cell wall binding (CWB2) protein family. The amino-terminal signal peptide, which is cleaved during translocation by the Sec secretion system, is shown as a black box. The three tandem CWB2 domains are shown in grey. The variable domains that confer different functions are shown in different colours **(Fagan & Fairweather, 2014).**

1.6.2.2 Other surface-associated factors

Other surface proteins of *C. difficile* have been described as having adhesin properties are the fibronectin-binding protein, FbpA **(Barketi-Klai *et al*., 2011; Hennequin *et al*., 2003)**, the

collagen binding protein CbpA **(Tulli *et al*., 2013)** and the heat shock protein GroEL **(Hennequin *et al*., 2001).**

C. difficile also possesses a capsule formed of three types of polysaccharides that covers the bacterium, and protects it against phagocytosis **(Ganeshapillai *et al*., 2008; Jiao *et al*., 2013)**.

In addition to its role in motility and chemotaxis, the flagella, particularly the two major flagellar proteins, the flagellin (FliC) and the cap (FliC), have also been associated with the adhesion and colonization processes of *C. difficile* **(Borriello, 1998; Duan et al., 2013; Lillehoj et al., 2002; Tasteyre et al., 2000).**

C. difficile possesses also lipoproteins **(Bradshaw *et al*., 2019; Kovacs-Simon *et al*., 2014; Simon, 2013)** and fimbriae **(Borriello *et al*., 1988)** on its surface, although their roles in the adhesion is not well understood. However, immunogenic properties of the lipoproteins has been recently demonstrated, making them potential candidates for the development of a vaccine **(Bradshaw *et al*., 2019)**.

1.6.3 Colonization factors

Although the protease Cwp84 is well known to be involved in the cleavage of the pre-protein SlpA, it has also been described to play a role in the proteolytic degradation of fibronectin, vitronectin and laminin **(Janoir *et al*., 2007)**.

In addition, all strains of *C. difficile* produce several hydrolytic enzymes, hyaluronidase, chondrdtin-4-sulphatase, gelatinase and heparinase. The role of these enzymes in the pathophysiology of *C. difficile* is not very well caracterised, but it has been suggested that they could be involved in the destruction of host tissues, which may facilitate colonization and establishment of infection **(Seddon *et al*., 1990)**.

On the other hand, *C. difficile* produces a bacteriostatic phenolic compound, *p*-cresol, which inhibits the growth of certain Gram-negative enteric species such as *Escherichia coli*, *Proteus mirabillis*, and *Klebsiella* spp.; thus, providing *C. difficile* with a competitive colonization advantage over the normal intestinal flora **(Passmore *et al*., 2018; Sebaihia *et al*., 2006; Selmer & Andrei, 2001).**

1.7 Genomics of *C. difficile*

In order to better understand the pathogenicity of *C. difficile*, the sequencing of the first complete genome of strain 630 was performed in 2006 by **Sebaihia *et al***. This virulent and multi-resistant strain was isolated in 1982 from a patient hospitalized with severe PMC in Zurich, Switzerland **(Wüst *et al*., 1982)**. The analysis of this genome revealed the presence of a high percentage (11%) of mobile genetic elements acquired by horizontal transfer, mainly transposons, prophages and genomic islands, that are responsible for the acquisition of a number of virulence and antibiotic resistance genes **(Sebaihia *et al*., 2006)**.

Since then, many genomes of different strains of *C. difficile* have been sequenced, offering the opportunity to compare and understand the genetic diversity between strains, even if they are genetically very close, in order to better to elucidate the molecular mechanisms responsible for the increase in the incidence and severity of CDI, and to understand the evolution (phylogenomics) of this pathogen **(Janezic & Rupnik, 2015; Knight *et al*., 2015; Kociolek *et al*., 2018).**

1.8 Epidemiology of CDI

A large number of studies have been conducted to describe the epidemiology of CDI, and to estimate their prevalence and incidence, particularly in the United States, Canada and Europe. Overall, the data from these studies indicated that the number of cases and the incidence of CDI are increasing around the world, with varying degrees from one country to another.

Since 2003, CDI have evolved in a worrying way, with increasing incidence, severity and lethality. This evolution is linked to the emergence and the rapid dissemination of a hypervirulent clone of *C. difficile* named 027, NAP1 or BI depending on the typing method used **(Dinh *et al*., 2015; Loo *et al*., 2005).**

The hypervirulence of this strain is due to its production of high quantities of the two toxins (16 and 23 times more than the wild type strain, for toxin A and B, respectively), which is caused by base pairs deletion at position 117 in the *tcdC* gene, which encodes the negative regulator of the toxin A and toxin B genes, which leads to the production of an inactive repressor, hence the overproduction of the toxins. This strain also produces the binary toxin and is multi drug-resistant. In addition, the epidemic strain 027 seems to have a greater

capacity for sporulation, which could facilitate its dissemination and survival in the hospital environment **(Fatima & Aziz, 2019; Warny *et al*., 2005)**.

Over the past two decades, this strain has been responsible for many outbreaks worldwide, particularly in North America and Europe, with high morbidity, mortality and recurrence rates. The incidence of CDIs has increased by three folds in Canada and by eight in USA within 10 years **(Denève *et al*., 2009)**.

These infections are mainly nosocomial, and occurring in patients over 65 years of age. Very quickly, the clone 027 became endemic in North America. In 2003, it represented 50% of isolates in the United States and up to 80% in certain hospitals in Quebec. Prior to 1990, it accounted for less than 0.3% of *C. difficile* strains (Clements et al., 2010; Gerding, 2010; Kuijper et al., 2006, 2008; Li et al., 2015; O'Connor et al., 2009).

The clone 027 quickly appeared in Europe, first in the United Kingdom in 2004, where a major epidemic broke out at the Stocke Mandeville Hospital with more than 300 cases and nearly 10% of deaths directly linked to *C. difficile* (**Anon, 2006**).

The clone 027 then affected the Netherlands in July 2005, then Belgium in October 2005 **(Pépin *et al*., 2004)**. The situation in other European countries has also evolved over the same period, where cases have been reported in Sweden, Denmark, Germany, Austria , Poland and Switzerland **(Davies *et al*., 2016).**

In France, *C. difficile* strain 027 emerged in an epidemic form in the Nord-Pas de Calais region at the beginning of 2006. In the years of 2012-2013, an outbreak of CDI due to RT027 was reported in Marseille **(Eckert *et al*., 2013; Lagier *et al*., 2013)**. Compared to the rest of Europe, the incidence of CDI is considerably lower in France and estimated to be 2.28 cases per 10,000 patients per day, 14% of the registered cases are considered to be serious and 4% are fatal, suggesting the effectiveness of the preventive measures in place **(Eckert *et al*., 2013).**

The data from Europe as a whole showed that the incidence rates of CDI varied between 1 and 10 cases per 1,000 admissions **(van Dorp *et al*., 2016)**.

In Asia, the 027 strain has been linked to CDI in several countries, Japan (**Kato *et al*., 2007**), South Korea **(Tae *et al*., 2009)**, Hong Kong **(Cheng *et al*., 2009)**, Singapore **(Lim *et al*.,**

2011), albeit with a much lower overall prevalence (0.3%) **(Borren *et al.*, 2017)**, than in the European countries (19%) **(Davies *et al.*, 2016)**.

Currently this hypervirulent strain RT027 can be found on all continents (except in Africa, where it was not been reported date), however, this clone is not unique, other virulent strains of *C. difficile* were also reported in different countries (**Figure 10**), indicating that the population of circulating *C. difficile* strains are very heterogeneous **(Borren *et al.*, 2017; Collins *et al.*, 2013; Davies *et al.*, 2016)**. This variety of strains indicates also a dynamic evolution of *C. difficile*, with the emergence of new epidemic and hypervirulent strains (RT001, 078, 017, 012, 014/020, 023, 046, ...) around the world **(Arvand *et al.*, 2009; Collins *et al.*, 2013; Couturier *et al.*, 2018; Knight *et al.*, 2017; Krutova *et al.*, 2017; Shaw *et al.*, 2020).**

In Europe, 125 different PCR ribotypes were identified in a multicentric study in 19 different countries, the most frequent were RT027 (19%), RT001/072 (11%) and RT014/020 (10%) **(Davies *et al.*, 2016)**. Nevertheless, the same study revealed substantial variation in the distribution of ribotypes between different countries; for example, in Italy, the most frequent ribotypes were RT018 (22%) and 356 (17%); whereas in the Czech Republic RT176 was the most dominant (38%) **(Davies *et al.*, 2016).**

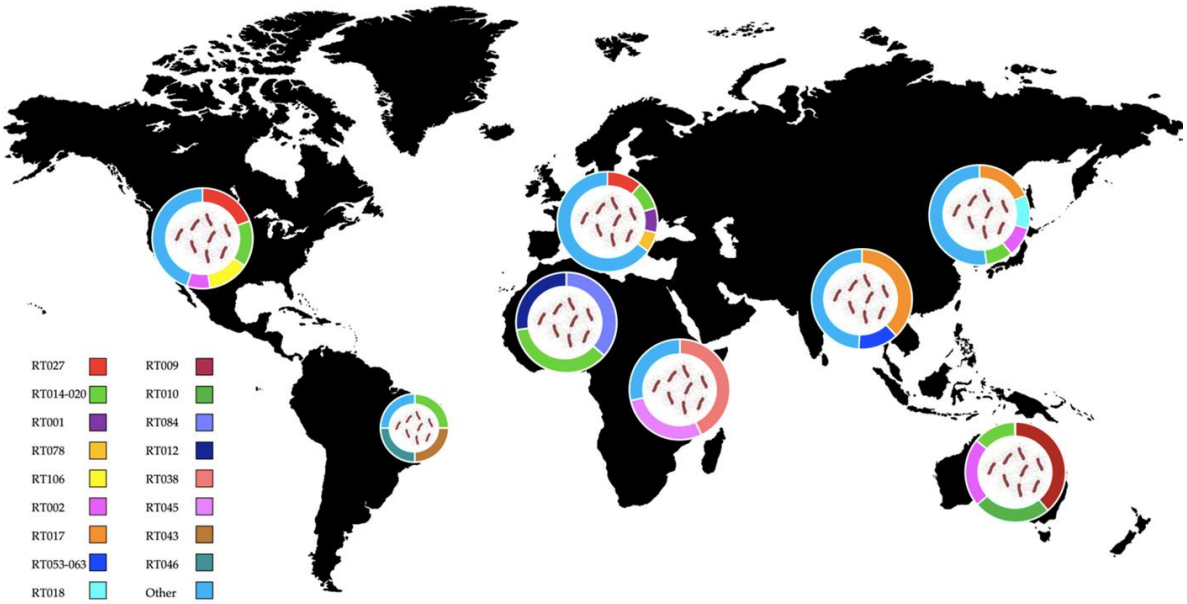

Figure 10. Distribution of the most frequent *C. difficile* ribotypes in the world **(Mengoli *et al.*, 2022).**

In Asia, the most predominant ribotype was RT017 (14%), a toxigenic clone that produces only toxin B, and which caused epidemics in several Asian countries **(Borren *et al.*, 2017; Collins *et al.*, 2013)**. It has been proposed that strains of the RT017 ribotype originated in Asia, then spread to Europe and other continents where they reached epidemic proportions **(Cairns *et al.*, 2017; Imwattana *et al.*, 2019)**.

As in Europe, a variability in the prevalence of specific ribotypes was also observed in other countries. In Japan, the ribotype smz/018 was dominant **(Collins *et al.*, 2013).** The RT001, a hypervirulent clone that causes severe CDI, predominated in the Middle East, mainly in Saudi Arabia (19%) **(Saber *et al.*, 2020)** and Iran (20%) **(Azimirad *et al.*, 2020)**.

Yet another shift in the epidemiology of *C. difficile* was observed following the emergence of community-acquired CDI (CA-CDI), the incidence and severity of which are on the rise since 2000 **(Gupta & Khanna, 2014)**. The affected persons are healthy young adults who had less frequent prior exposure to antibiotics; thus, the risk factors for CA-CDI can be different from those of hospital-acquired CDI (HA-CDI) **(Bloomfield & Riley, 2016)**. It has been suggested that frequent contact with asymptomatic carriers such as children under two years of age or with domestic animals **(Keessen *et al.*, 2013; Knetsch *et al.*, 2014)** are the risk factors for CA-CDI **(Chitnis *et al.*, 2013; Khanna *et al.*, 2012; Ofori *et al.*, 2018; Ogielska *et al.*, 2015)**.

Unlike the hypervirulent strains of the RT027, which are the principal cause of HA-CDI, the virulent strains of the RT078 and RT126 (toxinotype V) are more and more being isolated in CA-CDI in many European countries **(Goorhuis *et al.*, 2008; Hensgens *et al.*, 2012; Janezic *et al.*, 2012; Knetsch *et al.*, 2014; Tkalec *et al.*, 2019; Valerija *et al.*, 2012).**

C. difficile has also been identified as a pathogen in several animal species, such as horses, piglets, cattle and domestic animals **(Weese, 2020)**. The prevalence of CDI in horses varied considerably between studies ranging from 5% to 63%. The mortality rate in horses varied according to the studies, and can reach up to 40% **(Rodriguez *et al.*, 2014)**. the frequency of CDI varied between 23% and 93% in piglets with diarrhea **(Rodriguez *et al.*, 2016)**. The mortality rate in neonatal piglets (1 to 15 days old) was estimated at 50% **(Songer *et al.*, 2000).**

In animals, the clinical signs of CDI vary depending on the species, which make them difficult to diagnose. In pigs, CDI is one of the most important enteric diseases of newborns and is seen mainly in piglets 1-7 days old **(Hopman *et al*., 2011; Keessen *et al*., 2013).**

Although limited epidemiological and genotyping data on *C. difficile* in animals is available, few studies suggested a low genotypic diversity of the *C. difficile* population in animals, compared to that in humans **(Rupnik *et al*., 2009)**. Most of the strains identified in animals are different from those identified in humans; but few were found in both humans and animals. For example, strains of the RT078, which is frequently associated with CA-CDI, are also frequently found in livestock animals and pigs **(Moono *et al*., 2016; Rabold *et al*., 2018)**, which strongly suggest the possibility of transmission of *C. difficile* from animals to humans and reciprocally **(Rabold *et al*., 2018)**. A similar scenario was observed with two other epidemic strains, RT027 and RT017, which were also found in calves in Canada **(Rodriguez-Palacios *et al*., 2006)**. Consequently, *C. difficile* can be considered a zoonotic pathogen and animals could play an important role as reservoirs for this pathogen **(Rabold *et al*., 2018)**.

Furthermore, *C. difficile* has also been frequently found in food destined for human consumption, such as meat, pork, chicken, and with a less frequency in fish, seafood, leafy green vegetables, potatoes and milk **(Pasquale *et al*., 2012; Rodriguez-Palacios *et al*., 2007, 2020)**. Indeed, several ribotypes of *C. difficile* responsible for infections in humans have been identified in food, the most frequent being: RT001, RT002, RT018, RT014, RT015, RT106 and RT 0126 **(de Boer *et al*., 2011; Heise *et al*., 2021; Tkalec *et al*., 2022)**. The RT017, 027 and 078 ribotypes, commonly isolated in Europe in patients with CDI, have also been characterized in food products and in livestock **(Dahms *et al*., 2014; Gould & Limbago, 2010)**. Phylogenetic analysis of whole genomes of *C. difficile* RT078 isolated from humans and animals suggests possible zoonotic transmission of the pathogen **(Knetsch *et al*., 2014; Knight & Riley, 2019)**. No case of *C. difficile* infection directly associated with a food source has been described to date in the literature.

1.8.1 Reservoirs and transmission of *C. difficile*

C. difficile is widely found in different environments, mainly in the digestive tract of humans and many animal species (dogs, horses, cattle, sheep, pigs, fish and poultry), as well as in soil and water and in raw vegetables **(Figure 11)**.

The intestinal tract of humans and many animal species (dogs, horses, cattle, sheep, pigs, fish and poultry) is the main ecological niche for the multiplication of the bacterium and the dissemination of spores in the environment **(Freeman *et al.*, 2010; Rupnik, 2007)**. The asymptomatic carriage of this microbe in human guts was estimated at 3% in adults and between 50 to 70% in children under 2 years old **(Burns & Minton, 2011; Hequette-Ruz *et al.*, 2015)**. *C. difficile* is also found in different environments, such as soil, water, raw vegetables, home and hospital surfaces as well as in the faeces of newborns **(Figure 11) (Gerding *et al.*, 1986).**

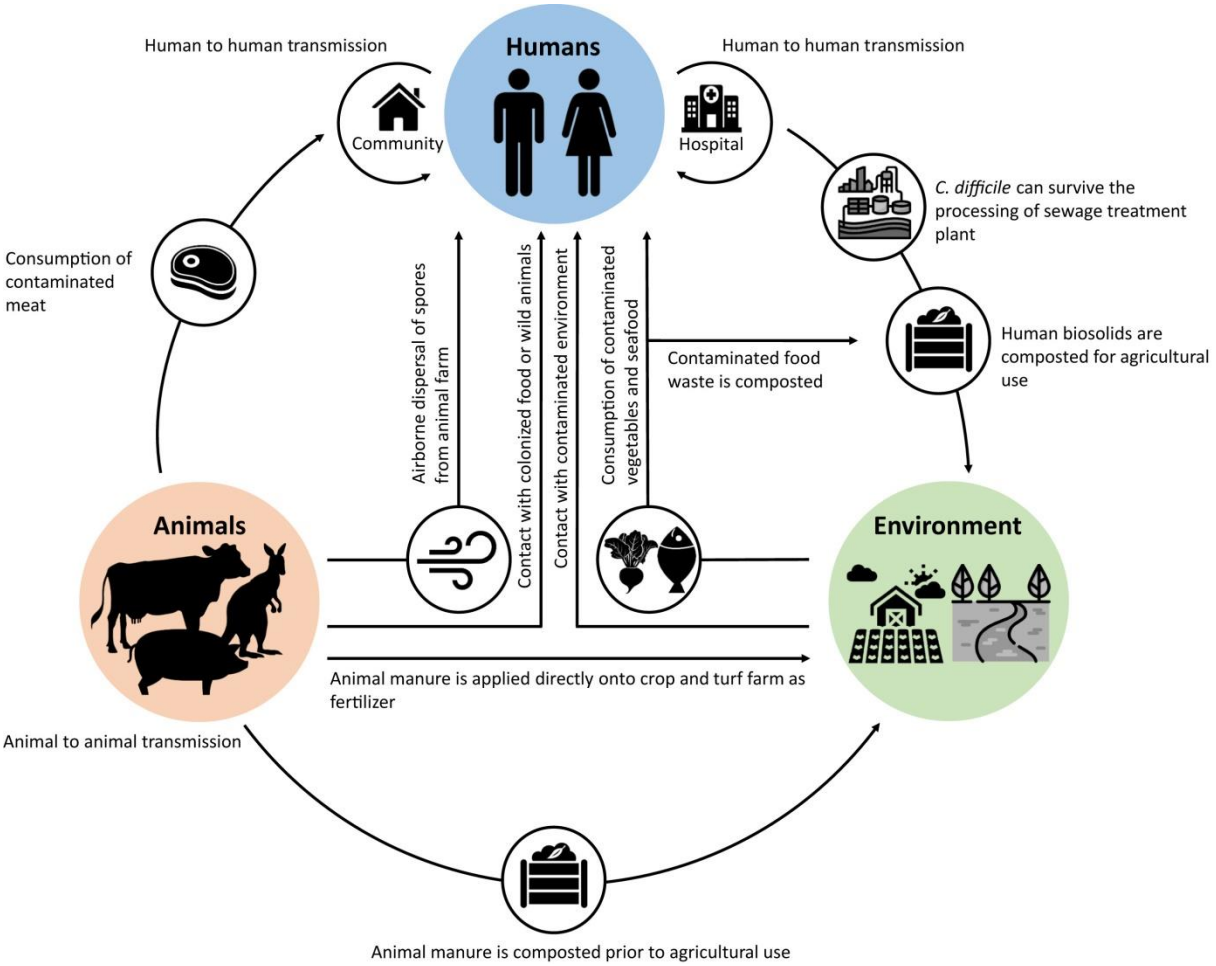

Figure 11. Reservoirs and transmission of *C. difficile* **(Lim *et al.*, 2020)**.

1.8.2 Risk factors

Several risk factors are associated with the development of CDI. The most important are exposure to antibiotics, old age and hospitalization**.**

1.8.3 Antibiotics

Almost all antibiotics have been associated with the development of CDI, including the antibiotics used for the treatment of CDI: metronidazole and vancomycin. However, broad-spectrum penicillins and cephalosporins, clindamycin, and fluoroquinolones have a higher risk of CDI induction than other antibiotics. Antibiotic therapy causes alterations in the intestinal microbial composition, allowing the colonization of *C. difficile* and the development of CDI. In addition, the duration of antibiotic therapy, as well as the combination of different antibiotics, play an important role in CDI **(Buyse *et al*., 2005; Gerding, 2004; Nelson *et al.*, 2017).**

1.8.4 Age

One of the important risk factors for CDI is advanced age, 65 years and over. This can be due to the fact that the old aged tend to have a weakened immune system, underlying diseases, frequent antibiotic treatments, frequent hospitalizations and even changes in the composition of the intestinal microbiota during aging **(Figure 15) (Hookman & Barkin, 2009; McDonald *et al*., 2006; Pépin *et al*., 2004)**.

People under 60 years old have a CDI related mortality risk of 2.5%; in contrast, mortality risk in persons aged between 61 and 70 years old is 4.3%, this rate can increase to 9.4% in those aged between 71 to 80 years, and to 13.5% in 80 years old persons **(Karas *et al*., 2010).**

1.8.5 Hospitalization

The hospital environment is known to play an important role in the transmission of *C. difficile* spores. Thus, prolonged or multiple hospitalizations are factors that have been demonstrated to increase the risk of CDI. This is mainly due to heavy contamination of hospital environments (beds, surfaces and medical devices) with *C. difficile* spores, which can be transferred to patients by the hands of healthcare personnel, or directly by contact with other CDI patients **(Chang & Nelson, 2000).**

1.8.6 Other risk factors

Other important risk factors for the development of CDI are proton pump inhibitors (PPI), acid lowering agents commonly used in the treatment of ulcer, gastroesophageal reflux disease and upper gastrointestinal complications. It has been estimated that the risk of the

development of CDI was 2.5 times higher in patients taking PPI **(Cunningham *et al*., 2003; Khanafer *et al*., 2017; Yearsley *et al*., 2006).**

Other factors such as previous episodes of CDI, gastrointestinal surgery, endoscopic procedures, chronic inflammatory bowel disease, immunosuppression, chemotherapy and agents modifying the intestinal ecosystem (laxatives, acid secretion inhibitors, transit retardants, etc.) are also potential factors for CDI (2012) **(Khanna *et al*., 2012).**

1.9 Diagnostics of CDI

Diagnosis of CDI is based on the presence of typical clinical signs and symptoms, such as diarrhea or PMC (observed by colonoscopy, endoscopy and abdominal imaging); or by several laboratory diagnostic tests which aim at detecting the pathogen and/or its products (such as the toxins or the glutamate dehydrogenase (GDH)) in the stool, by culturing the pathogen, by immune-enzymatic tests or by PCR **(Bartlett & Gerding, 2008; Czepiel *et al*., 2019).**

1.9.1 Endoscopic diagnostic

The diagnosis of a CPM is carried out by an endoscopic examination which reveals the presence of characteristic yellowish aphthoid lesions on the the colon mucosa **(Neumann & Pohl, 2013)**. This procedure is invasive and could not be decisive due the absence of PMC at the beginning of the infection or in the mild cases **(Bartlett & Gerding, 2008; Eckert & Barbut, 2010).**

1.9.2 Laboratory diagnostics

1.9.2.1 Detection of *C. difficile* by culture

A variety of selective culture media are used for the purpose of the laboratory diagnostics of *C. difficile*, the most widely used are CCFA (Cycloserine Cefoxitin Fructose) and CCEY (Cefsulodin-cycloserine-egg yolk agar), supplemented with lysozyme or taurocholate to enhance spore-germinating **(Barbut *et al*., 1995; Bliss *et al*., 1997; Lalande *et al*., 2004).**

Recently, a high-performance chromogenic medium was developed by BioMerieux, ChromID Cdiff (CDIF)®, to improve the isolation and the identification of *C. difficile*. This medium

promotes the germination of spores with high efficiency, allowing a direct detection of *C. difficile* with increased sensitivity **(Carson *et al*., 2013; Perry *et al*., 2010)**.

The culture of toxigenic *C. difficile* from stool samples is considered as the gold standard for the laboratory diagnosis of CDI **(Debast *et al*., 2009)**, with a sensitivity of 90%; however, it is considerably long (72 to 96 hours) and, most importantly, do not distinguish between toxinogenic and non toxinogenic strains. Therefore, complementary tests, such as cytotoxicity or immuno-enzymatic tests or PCR, are needed in order to determine the toxinogenic potential of the strain **(Barbut *et al*., 2003; Delmée *et al*., 2005; Reller *et al*., 2007)**.

1.9.2.2 Cytotoxicity assay

The cytotoxicity assay (CYTA) is considered the reference method for the diagnosis of CDI. This test allows the detection of toxigenic strains of *C. difficile* directly from stools. The test is based on examining the cytopathic effect of the toxin B in stool filtrates on tissue culture (human fibroblasts, Vero, HeLa or Hep-2 cells). The cytopathic effect can be observed by the rounding of the cells **(Carroll & Mizusawa, 2020)**.

Although CYTA is very specific, if positive, it indicates the presence of the disease; however it is less sensitive, laborious, time consuming (24 to 48 hours) and requires an infrastructure adapted for cell culture and microscopy. In addition, the CYTA requires a confirmatory step, involving the neutralization of the activity of the cytotoxin with specific antitoxins from *C. difficile* or *C. sordellii* **(Dupont, 2013; Eastwood *et al*., 2009)**.

1.9.2.3 Toxigenic culture

This test consists of isolating *C. difficile* on selective media followed by the analysis of the strain's toxinogenic potential *in vitro* using the cytotoxicity assay described above, or by other toxin detections tests.

1.9.2.4 Detection of the toxins

The detection of free toxins in the stool can be carried out using several commercially available immuno-enzymatic (IEA) or immuno-chromatographic (ICA) assays which can detect either toxin A or toxin B or both toxins simultaneously. These tests are frequently used in diagnostics laboratories because of their rapidity (results obtained in 30 minutes), simplicity and affordability. Overall, these tests have been described as having good

specificity and low sensitivity **(Carroll & Mizusawa, 2020; Polage *et al*., 2015)**. However, other highly sensitive and specific (99%) enzyme-linked immunosorbent tests (ELISA) have been developed **(Crobach *et al*., 2016).**

1.9.2.5 Detection of glutamate dehydrogenase

The glutamate dehydrogenase (GDH) is an enzyme produced in large amounts by *C. difficile*. The GDH can be detected in the stool either by immuno-enzymatic IE or immuno-chromatographic IC assays. These tests are quite sensitives but not very specifics, as they cannot differentiate between toxigenic and non-toxigenic strains and it has also cross-reactions with other *Clostridium* species producing this enzyme **(Bignardi & Settle, 2010; Carroll & Mizusawa, 2020)**. Thus, a positive result by this test must be followed by another test to confirm the presence of toxins **(Goldenberg *et al*., 2010; Vasoo *et al*., 2014).**

Several commercial diagnostic kits have been developed for the simultaneous detection of *C. difficile* GDH and toxins, such as the VIDAS® C. difficile GDH and toxin A/B (CDAB), the C.DIFF QUIK CHEK® COMPLETE and the RIDASCREEN C. difficile GDH. These tests are rapid, cost-effective, highly sensitive and specific **(Kim *et al*., 2022; Snell *et al*., 2004).**

1.9.2.6 Molecular detection of *C. difficile*

Nucleic acid amplification tests (NAAT), by PCR (Polymerase Chain reaction), conventional or real-time PCR (RT-PCR), using primers targeting the genes coding for the toxin A, B, the binary toxin or the GDH, are rapid, highly sensitive and specific, and are the most commonly used methods for detecting *Clostridium difficile* **(Carroll & Mizusawa, 2020; Jensen *et al*., 2015; van den Berg *et al*., 2013).**

Several rapid NAAT assays that detect the *C. difficile* toxin genes in stool specimens by real-time PCR have been developed, two of such tests are the Cobas® Liat Cdiff test and the Xpert® *C. difficile*/Epi test **(Granato *et al*., 2018)**.

It is worth mentioning that all the CDI diagnostic tests described above have advantages and disadvantages, and differ in term of their specificity, sensitivity, rapidity, affordability and ease of use; and hence no single test alone is ideal **(Huber *et al*., 2013)**. Thus, it is necessary to combine at least two tests in order to optimize the diagnosis of CDI.

Several combinations of tests have been tried **(Goldenberg *et al.*, 2010; Ticehurst *et al.*, 2006; Vasoo *et al.*, 2014)**. However, a multi-step algorithm, using different combinations of GDH, immunological and NAAT tests, has been recently recommended by the Society of Healthcare Epidemiology of America (SHEA) and the Infectious Diseases Society of America (IDSA) (**Figure 12**) **(McDonald *et al.*, 2018)**. This algorithm has been tested and shown that to be the best compromise between the performance of the tests and the clinical reality of the CDI **(McDonald *et al.*, 2018).**

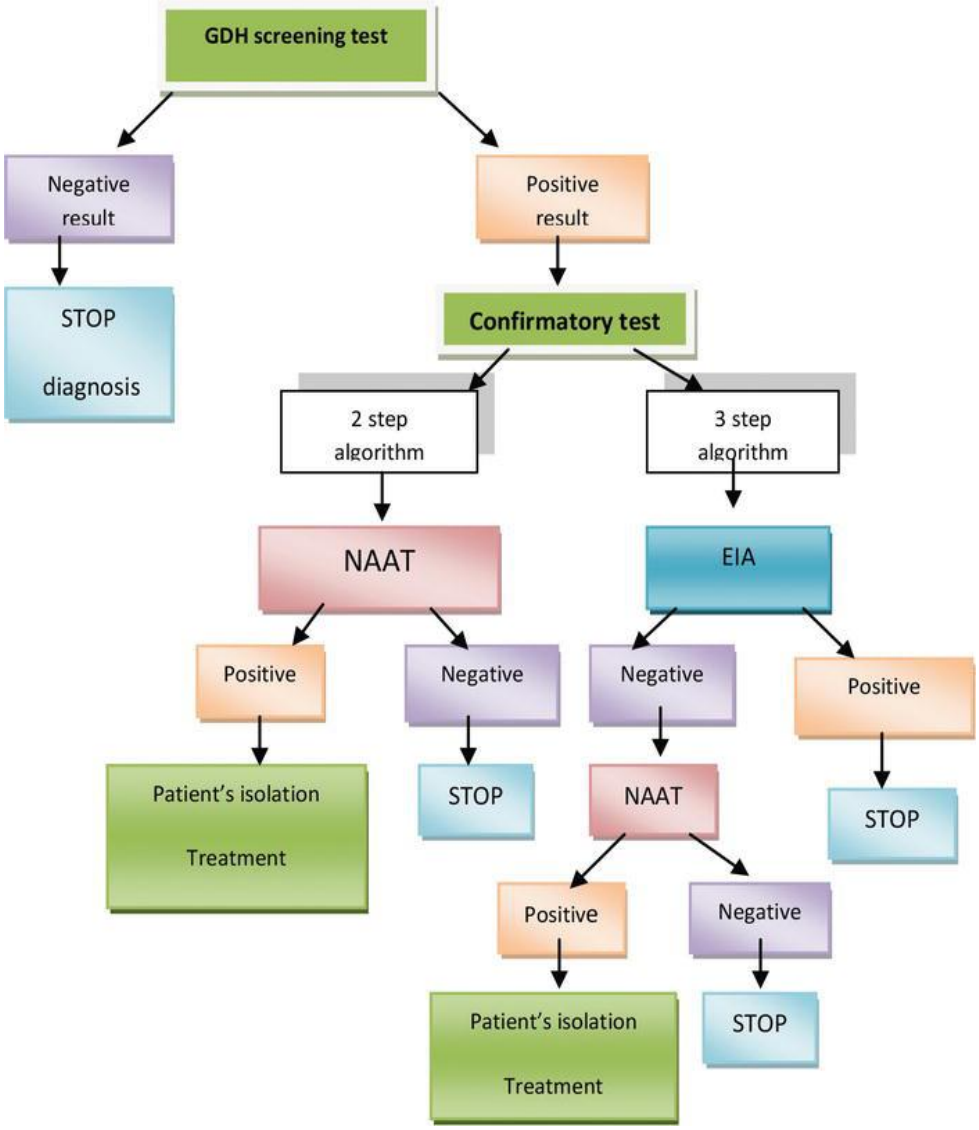

Figure 12. Algorithm steps for *C. difficile* diagnosis (different laboratory approaches) **(Iancu *et al.*, 2017).**

1.10 Molecular typing methods

Several typing methods have been used to characterise the strains of *C. difficile* for surveillance and epidemiological investigation purposes. These methods allow the differentiation between different strains of *C. difficile* in order to understand the epidemiological links between them. All these genotyping methods are used for the detection of DNA polymorphisms in the strains of *C. difficile*, and they are either restriction-based (REA and PFGE), PCR-based (MLVA and PCR-ribotyping), PCR and restriction-based (toxinotyping), PCR and sequencing-based (MLST) or whole genome sequencing-based **(Killgore *et al*., 2008; Knetsch *et al*., 2013)**. The most frequently used of these methods are described below.

1.10.1 Restriction endonuclease analysis

The Restriction Endonuclease Analysis (REA) method is based on the digestion of the total chromosomal DNA of *C. difficile* with a restriction enzyme such as HindIII. The large number of DNA fragments generated are separated by agarose gel electrophoresis, and the profiles of the bands obtained are visualized and compared between different strains. The REA is simple to perform and has a high discriminating power **(Clabots *et al*., 1993; Wren & Tabaqchali, 1987)**.

1.10.2 Pulsed field gel electrophoresis

The Pulsed Field Gel Electrophoresis (PFGE) method involves the use of a restriction enzyme which infrequently cuts the bacterial genome, thereby generating DNA fragments with large sizes. The fragments will then be separated slowly on a polyacrylamide gel subjected to an electric field, in which the voltage is changed in a repetitive manner. This allows large DNA fragments to migrate at varying distances depending on their size. The fragments will then be visualized to reveal any differences between the aspects of the bands **(Alonso *et al*., 2005; Janezic & Rupnik, 2010)**.

1.10.3 Multiple Locus Variable Number of Tandem Repeats Analysis

Multiple Locus Variable Number of Tandem Repeats Analysis (MLVA) is a typing method which target the polymorphism within the regions of the genome called variable copy number of tandem repeat (VNTR). These VNTRs are amplified by multiplex PCR, and the amplicons produced are separated by capillary electrophoresis. Since the number of VNTRs and the

repeats within them vary between different strains, the number and size of the DNA fragments produced by PCR will be different, and as such, different strains will exhibit different banding patterns, called an MLVA profiles. Because of its very high discriminatory power (allowing to distinguish even between closely related bacterial strains), the MLVA is widely used for subtyping of bacterial strains in epidemiological investigations (Marsh et al., 2006; van den Berg et al., 2007)**.**

1.10.4 PCR-ribotyping

This PCR-based method is used to amplify the intergenic regions between the genes encoding 16S and 23S rRNA (interspace regions, IS-regions) in the rRNA operons. The amplified DNA are separated by electrophoresis, and the band profiles of the strains are compared. Since *C. difficile* strains have multiple copies of rRNA operons, which could differ in term of their copy number and of the size of their IS-regions, the profiles of the bands obtained will make it possible to discriminate between the different strains. This highly discriminatory and reproducible typing method is highly recommended for epidemiological investigations, and is most widely used in Europe and Australia **(Bidet *et al*., 1999; Indra *et al*., 2008; O'Neill *et al*., 1996).**

1.10.5 Toxinotyping

Toxinotyping is a typing method that is used to detect the polymorphisms (ponctuel mutations or deletions) within the PaLoc (the chromosomal locus that contains the *tcdA* and *tcdB* toxin genes). This PCR-RFLP-based technique involves the amplification of the PaLoc by PCR, followed by the digestion of the PCR products with restriction enzymes. The DNA fragments obtained are separated by electrophoresis. The number and size of the DNA bands are compared to those of the reference strain VPI 10463 (= toxinotype 0), and *C. difficile* strains are then assigned a toxinotype **(Rupnik, 2010)**. To date, 34 different toxinotypes (I to XXXIV) have been characterized **(Rupnik & Janezic, 2016)**.

1.10.6 Multilocus Sequence Typing

Multilocus Sequence Typing (MLST) is based on PCR amplification followed by sequencing of seven housekeeping genes, which are conserved in bacterial genomes. The sequences of the genes are compared to the sequences of the same genes present in an MLST database. An allele number will be assigned to each sequence. The assigned allele numbers will be

combined to form an MLST type **(Griffiths *et al*., 2010; Lemée & Pons, 2010**). The MLST, with its high discriminating power, allows the grouping of strains into "clades", delineated on the basis of their molecular lineages.

1.10.7 Whole Genome Sequencing Based Typing (phylogenomics)

Although the typing methods for *C. difficile* strains described above are generally effective in differentiating between different isolates, whole genome sequencing (WGS) typing is much more discriminating, as it allows the detection of individual nucleotide changes in the genome, called single nucleotide polymorphism (SNP). The resolution and discrimination offered by WGS are very high, even if the isolates are genetically very close at a very high **(Janezic & Rupnik, 2019; Seth-Smith *et al*., 2021).**

1.11 Treatment

1.11.1 Antibiotherapy

The first step in the treatment of CDI is to stop the antibiotherapy, whenever possible, in order to restore the intestinal microbiota. This simple measure can be sufficient, as it has been shown to improve the clinical symptoms in 15 to 20% of patients within 48 hours, and in most cases no further specific treatment is required **(Gerding *et al*., 1995; Mullane *et al*., 2011; Musher *et al*., 2005)**. Correcting electrolyte and stopping unnecessary medication with proton pump inhibitors should also be envisaged in the treatment of CDI **(Surawicz *et al*., 2013)**.

In case where the interruption of the antibiotherapy is not possible, the option of using other antibiotics with a lower risk of CDI such as aminoglycosides, macrolides or tetracyclines, should be considered **(Musher *et al*., 2005)**.

The choice of treatment against CDI is determined by the severity of the disease and whether it is a first episode or a recurrence **(Bagdasarian *et al*., 2015; Nelson *et al*., 2017)**.

If the discontinuation of the antibiotic therapy is ineffective, or the antibiotics cannot be stopped, the treatment of choice is metronidazole or vancomycin. Both antibiotics have been shown to have the same efficacy in the treatment of the moderate forms of CDI **(Bagdasarian *et al*., 2015; Nelson *et al*., 2017)**. However, metronidazole is favoured for first-line treatment for moderate infections due to its low cost; in contrast, and because of its high cost and the risk of development of resistance in enterococci, vancomycin is recommended as

first-line treatment for severe infections **(Aslam *et al*., 2005)**. The failure and the recurrences rates of vancomycin and metronidazole are similar **(Vardakas *et al*., 2012)**.

A novel antibiotic, fidaxomicin, has been shown recently to be effective in the treatment of CDI and in reducing the recurrences rates **(Bowman & Utter, 2020; Cornely *et al*., 2012; Louie *et al*., 2011)**. Fidaxomicin obtained marketing authorization in 2012, and was included in the updated ESCMID CDI treatment guidance document **(van Prehn et al., 2021)**. Fidaxomicin is a macrocyclic bacteriostatic antibiotic, which acts by inhibiting RNA synthesis in *C. difficile* and reduces the production of toxins and spores. It is orally administered and acts mainly in the intestinal lumen, and is also characterized by its narrow spectrum with, in particular, a lack of activity on Gram-negative bacilli and on Bacteroides; thus, having minimal impact on the intestinal microbiota, compared to other antibiotics **(Louie *et al*., 2012)**.

The antibiotic doses prescribed depend on the severity of the CDI. The following protocols have been proposed by the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) in their treatment guidance documents published in 2014 and updated 2021 **(Figure 13) (Debast *et al*., 2014; van Prehn *et al*., 2021)**.

In case of non-severe forms (moderate diarrhea, leukocytosis < 15,000/µl); the discontinuation of antibiotic therapy is recommended, if possible; if not, fidaxomicin 200 mg twice daily for 10 days is strongly advised for the initial episode of CDI; if access to fidaxomicin is limited, oral vancomycin 125 mg four times daily for 10 days is a suitable alternative. Oral metronidazole 500 mg three times daily for 10 days should_be used only when vancomycin and fidaxomicin are not available **(van Prehn *et al*., 2021)**.

In case of severe forms (Profuse diarrhea, abdominal pain, fever >38.5 C°, leucocyte count ≥ 15,000/µl, rise in serum creatinine >50% above the baseline), a regimen of vancomycin 125 mg four times daily for 10 days or fidaxomicin 200 mg twice daily for 10 days, is recommended.

In severe-complicated CDI or fulminant CD (hypotension, septic shock, elevated serum lactate, ileus, toxic megacolon, bowel perforation or any fulminant course of disease), vancomycin 125 mg four times daily for 10 days or fidaxomicin 200 mg twice daily for 10 days are recommended. When a patient's condition deteriorates or progresses towards severe complicated CDI while on anti-CDI antibiotic therapy, addition of iv tigecycline 50 mg twice

daily (100 mg loading dose) is an option that should be envisaged on a case-by-case basis **(van Prehn *et al*., 2021)**.

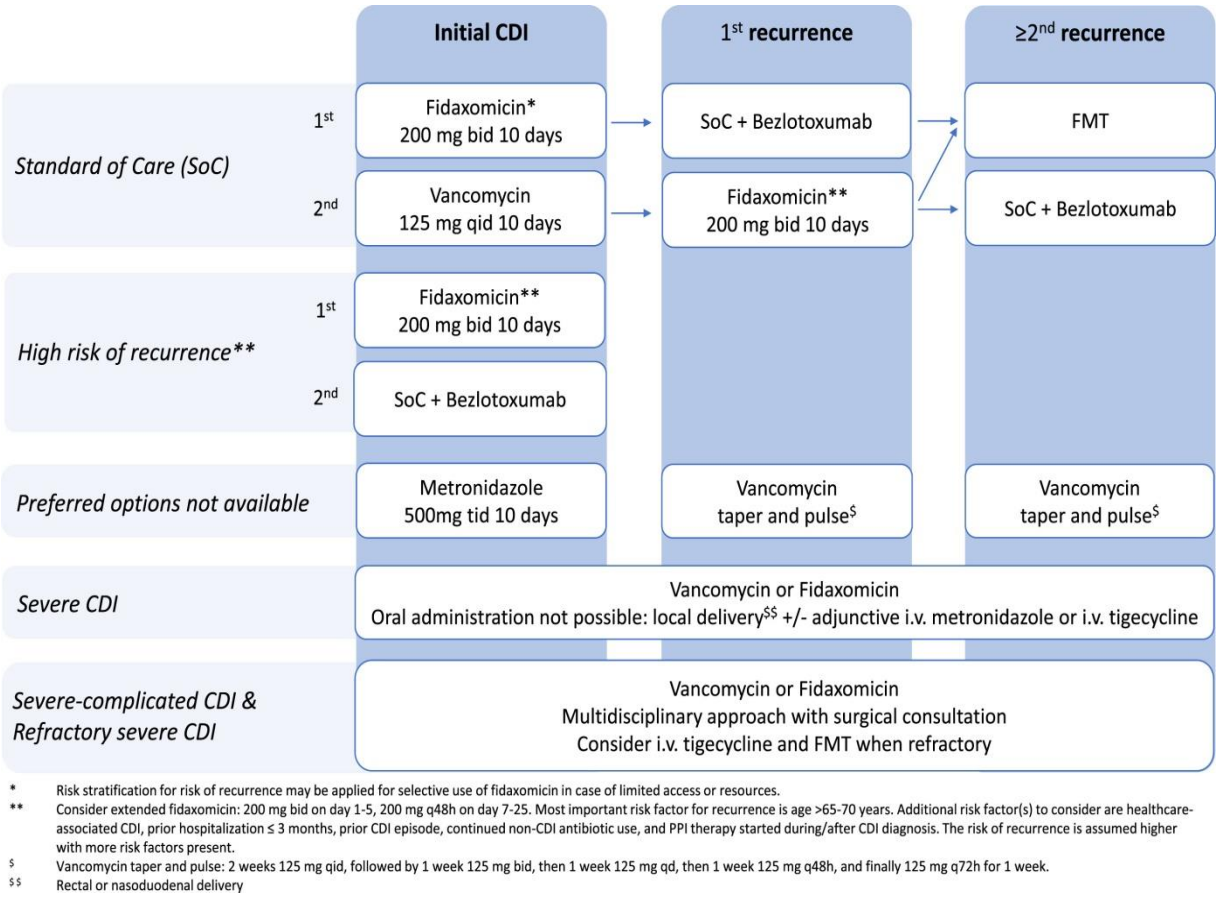

Figure 13. CDI Treatment recommended by the European Society of Clinical Microbiology and Infectious Diseases **(van Prehn *et al*., 2021)**.

1.11.2 Alternative treatments

Considering the fact that disruption of the intestinal microbiota is a prerequisite for the development of CDI, the restoration of the intestinal microbiota and of its barrier effect represents, therefore, a promising alternative strategies to limit the use of antibiotics. Two of these therapeutic strategies, fecal transplantation and probiotics, have been used successfully in the treatment of CDI **(McFarland, 2005)**.

1.11.2.1 Fecal microbiota transplantation

Fecal microbiota transplantation (FMT) consists of introducing a diluted stool preparation from a healthy donor into the patient's digestive tract. This therapeutic approach has been the subject of numerous studies, which showed its effectiveness (a cure of CDI in 92% of cases) in the treatment of severe or recurrent CDI **(Gough *et al.*, 2011; Mattila *et al.*, 2012; van Nood *et al.*, 2013).** However, the use of this type of therapy is still limited due to its high cost, non-acceptability by the patients, as well as concerns about its safety and secondary effects.

1.11.2.2 Probiotics

Probiotics are live non-pathogenic microorganisms which have the potential of restoring the microbial balance in the gastro-intestinal tract.

The potential of probiotics of *Lactobacillus rhamnosus* GG and *Saccharomyces boulardii* in the treatment of different cases of CDI, alone or in association with vancomycin or metronidazole, has been investigated in several studies, and has shown promising results **(Kalakuntla *et al.*, 2019; Pace *et al.*, 2015; Wilkins & Sequoia, 2017).** On the other hand, other studies had concluded that there was insufficient evidence to recommend their systematic use in the treatment of CDI **(Surawicz *et al.*, 2013)**. The diversity of these probiotics agents, and the absence of standardization of the protocols, make it difficult to draw conclusions on their real impact in the treatment of CDI **(Cohen *et al.*, 2010; Surawicz *et al.*, 2013).**

1.11.2.3 Immunotherapy

Passive immunization by the administration of two anti-toxin monoclonal antibodies (actoxumab and bezlotoxumab, anti-toxin A and anti-toxin B, respectively) have been tested in several studies, with bezlotoxumab showing better effectiveness, making it a promising option for the treatment of CDI (Hussack & Tanha, 2016; Johnson & Gerding, 2019; Kufel et al., 2017; Posteraro et al., 2018; Steele et al., 2013; Wilcox et al., 2017; Yang et al., 2015). Indeed, this monoclonal antibody has received the FDA authorization for use in case of recurrent CDI **(Mullard, 2016)**.

1.11.2.4 Vaccination

Vaccination is a promising prophylactic approach which could confer long-term protection against CDI, and could also limit the spread of *C. difficile*. Although there is currently no vaccine against *C. difficile* available on the market, several vaccine candidates are either under clinical trials or under development **(Foglia *et al*., 2012; Leuzzi *et al*., 2014; Pizarro-Guajardo *et al*., 2019; Riley *et al*., 2019; Surawicz *et al*., 2013).**

1.11.2.5 Surgical intervention

Surgical intervention is necessary, or sometimes crucial for the survival, for patients with complicated cases of CDI (fulminant colitis, ileus or toxic megacolon) that has not responded to any of the other treatments **(Butala & Divino, 2010; Hall & Berger, 2008; Lamontagne *et al*., 2007).**

1.12 Prevention and control

The strategies for CDI prevention rely primarily on two mains categories of measures: prevention of the transmission and reducing the risk factors.

The main measures to prevent transmission include isolation of the infected patient, adequate hand washing, wearing of gloves and gowns as well as rigorous disinfection of hospital environments and equipment **(Blanckaert *et al*., 2008; Chang & Nelson, 2000; Oughton *et al*., 2009).** Whereas, the most effective strategies to reduce the risk factors is the rationalization of the use of antibiotics **(Davey *et al*., 2005)**.

Chapter II: Materials and Methods

2.1 Study settings and sample collection

During the period between January 2016 and January 2019, 300 samples of diarrheal stools were collected from patients admitted to five hospitals in three different Wilayas (provinces), University Hospital Benflis Touhami of Batna (East), hospitals Seours Bedj, Merouani Abed and Freres Khelif of Chlef, and Bouabida hospital of Ain-Dafla. The patients were hospitalized mainly in three wards, internal medicine, paediatric and intensive care unit (ICU).

The inclusion criteria for this study were defined by the World Health Organization as patients presenting diarrhoea in the form of 3 or more loose or liquid stools per day, after 3 days of hospital admission with or without antimicrobial therapy (**Debast *et al.*, 2014**). The patients were both males and females of all ages, except children less than 2 years old, due to the high asymptomatic carriage of *C. difficile* in this age group (**Schutze & Willoughby, 2013**).

A single unformed stool sample was collected from each patient using a sterile container and then transferred to the laboratory and conserved in -20°C for further analysis. Each sample was accompanied by a completed questionnaire reporting clinical and socio-demographic information on the patient, such as age, gender, date of admission, previous hospitalization and/or exposure to antibiotics, underlying pathologies, use of PPI, diagnosis, treatment, etc, .. (**Annex I**).

2.2 *C. difficile* culture

The stool samples were treated with 96% ethanol 1:1 (AnalaR NORMAPURE (VWR Chemicals, France) for 30 minutes to eliminate all the bacteria of the intestinal microflora and to select the spores only **(Marler *et al.*, 1992)**. The suspension was then cultured on, ChromID CDIF (Biomérieux, Marcy l'Etoile, France), a chromogenic agar for the selective isolation of *C. difficile*; the compositions of which is given in **Annex II**. Plates were incubated at 37°C for 48 h under anaerobic conditions (10% H2, 5% CO2, 85% N2) using atmosphere generation system AnaeroGen^{Tm} compact 2,5L (Thermo scientific, Japan). Colonies presenting typical morphological characteristics (black and irregular colonies), according to the manufacturers' recommendations, were presumed as *C. difficile* and were

isolated and stored in cryogenic storage vial Microbank (Pro-Lab diagnostics, Canada) at -80°C for further use.

2.3 Confirmation of bacterial identification by MALDI-TOF-MS

MALDI-TOF-MS (Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry) is a rapid and reliable technique for the identification of bacterial species. The technique is based on the analysis of the protein profiles of the bacteria, and allows the differentiation between closely related bacterial species **(Luzzatto-Knaan *et al*., 2015).**

A mass spectrometer consists of three functional units: an ionization chamber, a gas phase; a mass analyzer, and an ion detector (**Figure 14**).

The principle of the analysis by MALDI-TOF consists of bombarding the bacterial peptides (obtained after trypsin digestion) by a laser beam in the ionization chamber. Once the ions are formed, they are transferred into a gas phase and accelerated by a uniform electric field, which directs them to the mass analyzer, where they are separated according to their mass/charge ratio (m/z). The arrival of ions at the end of the flight tube is detected by the ion detector. The time of flight (TOF), which is the time necessary for the ions to reach the detector, is proportional to the square root of m/z. The sum of the ions analyzed forms a spectrum, which will be interpreted by an identification software (**Figure 15**) **(Croxatto *et al*., 2012; Luzzatto-Knaan *et al*., 2015)**.

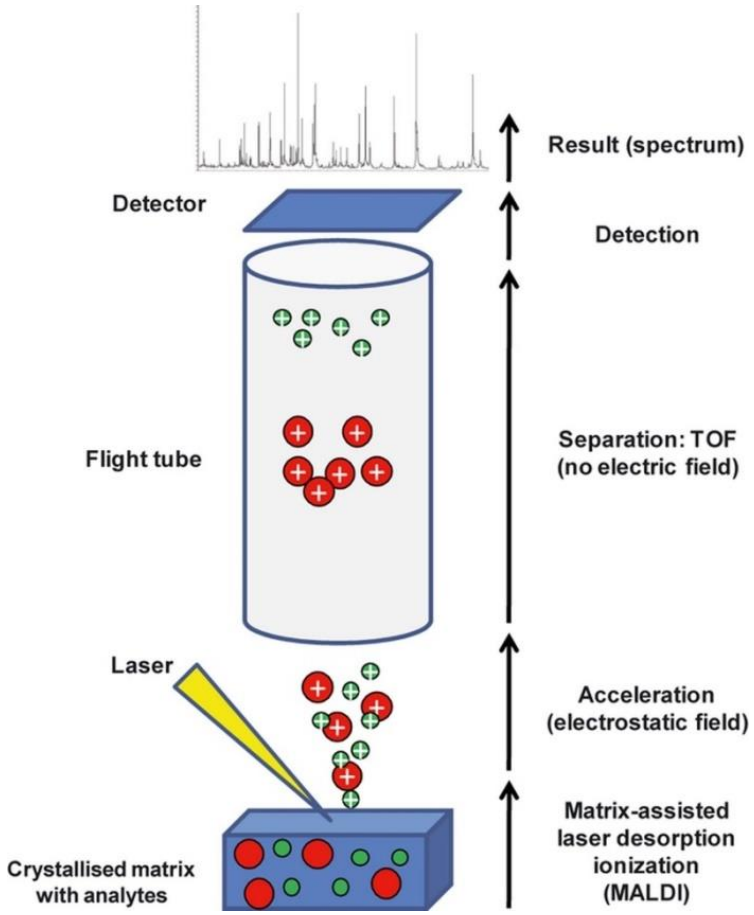

Figure 14. The components and operating principle of a MALDI-TOF-MS **(Croxatto *et al.*, 2012).**

The identification by MALDI-TOF-MS was performed according to the manufacturers' instructions as follows (**Figure 15)**:

- Deposit one colony into one spot of the target plate and leave to dry.

For the positive control, add 1 µl of Bacterial Test Standard (extract of *Escherichia coli* DH5) in a separate well.

- Cover the bacterial spots with 1 µl of matrix HCCA (a-Cyano-4-hydroxycinnamic acid) and leave to dry.

- Place the target plate into the MALDI-TOF-MS and run the analysis.

- After performing the analysis, a mass spectrum is produced and then compared with an internal reference database of mass spectra (**Figure 15)** by the software MBT Compass IVD

software" (Microflex LT BRUKER, USA), and the result of the identification are given with a score between 0 to 3.

An identification is considered highly probable when the score is between 2 and 3, while the identification is considered unreliable when the score is less than 2; between 0 and 1 the identification is not possible. The result can be interpreted only when the positive and negative control are valid.

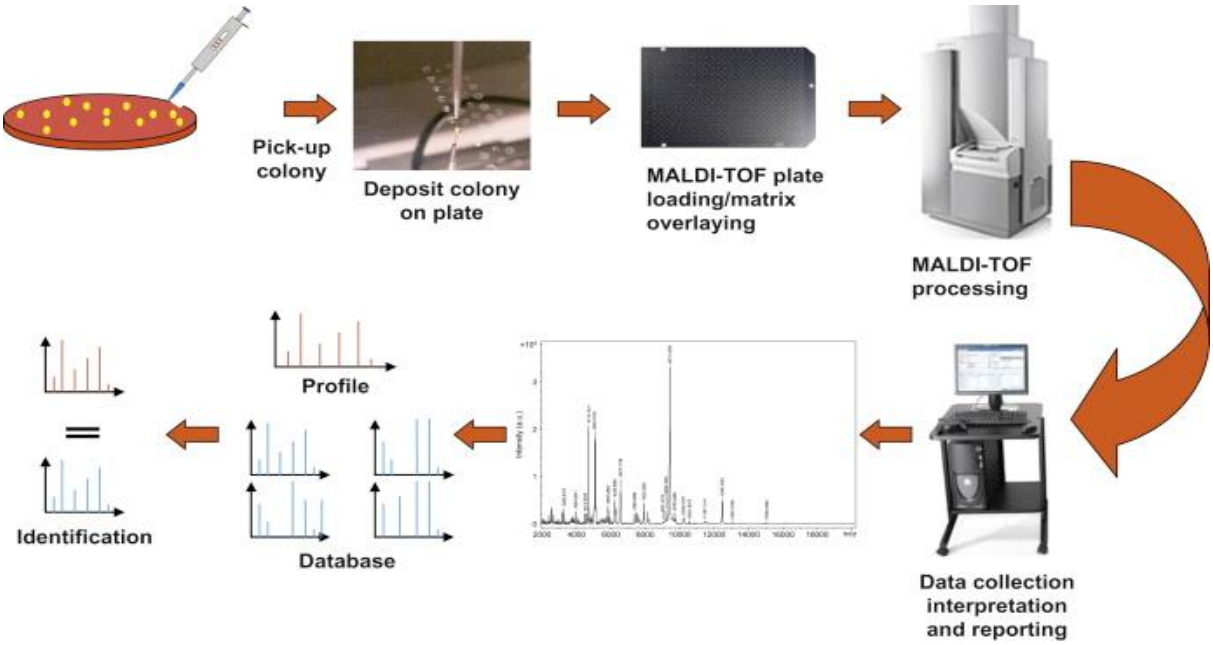

Figure 15. Protocol steps for sample preparation and analysis by MALDI-TOF MS **(Dridi & Drancourt, 2011)**.

2.4 Molecular characterization and toxigenic profiling

2.4.1 DNA Extraction

The extraction of bacterial genomic DNA was carried out using the extraction kit InstaGene Matrix (Bio-Rad, USA), according to the manufacturer's instructions.

- homogenize one bacterial colony in 1 ml of molecular grade H_2O by vortexing for 10 s.

- Centrifuge the mix at 13000 rpm for 5 min, and discard the supernatant.

- Add 200 µl of InstaGene Matrix to the bacterial pellet, vortex for 10s and incubate at 56°C for 30 min.

- Vortex vigorously for 10 seconds and then incubate at 95°C for 8 min and vortex again for 10 s.

- Centrifuge the mix at 13000 rpm for 5 min.

- Transfer the supernatant containing the DNA into another tube and store at -20°C for later use.

The quality and quantity of the extracted DNAs were assessed by measuring the absorbance at 260/280 nm using the NanoDrop spectrophotometer 2000 (Thermo Scientific, USA) **Annex III.**

2.4.2 Toxigenic profiling by multiplex PCR

Different *C. difficile* toxin profiles exist. They are defined by modifications (deletions or mutations) in the genes encoding he toxin A (*tcdA*), toxin B (*tcdB*), the binary toxin (*cdtA*/*cdtB*) or in the PaLoc. The result of these modifications is either an absence, presence or an alteration of the production of the toxins. Therefore, the choice of PCR primers used must take into account all these possible variations in order to determine the toxin profile of the *C. difficile* isolate.

For this purpose, a multiplex PCR assay was carried out using the protocol of The National Reference Centre of *C. difficle* in France. This protocol target seven genes simultaneously, using 7 pairs of primers (**Table 1**) specific to the genes encoding the triose phosphate isomerase (*tpi*), toxin A (*tcdA*), toxin B (*tcdB*), binary toxin (*cdtA*/*cdtB*), deletion in pathogenicity locus (*Lok*), and a possible 18-bp internal in-frame deletion within the gene *tcdC;* to detect the toxigenic/non-toxigenic strains of *C. difficile* and the presumptive identification of the epidemic NAP1/027/BI (**Lemee *et al.*, 2004, Barbut *et al.*, 2011, Person *et al.*, 2011, Braun *et al.*, 1996)**.

The amplification of the *tpi* gene serves as an internal control to confirm the isolates as *C. difficile* (**Lemee *et al.*, 2004**).

The deletion at position 117 in the *tcdC* gene coding for the negative regulator of the toxins A and B, and which is present in all the hypervirulent strains of the PCR ribotype 027 **(Wolff *et al.*, 2009),** was targeted using primers designed by **Persson *et al.* (2011).**

The deletion of the pathogenic locus PaLoc was detected as described by **Braun *et al* (1996)**, using the primers lok 3-F and lok M8-R**.** *C. difficile* RT 027, which carries all the investigated genes, was used as positive control, whereas molecular biology grade water was used as negative control.

Table 1. Primers used in Multiplex PCR.

Target	Primers	Sequence	Amplicon size (pb)	Reference
cdtA	cdtA-F cdtA-R	GGA AAG AAA AGA AGC AGA AAG AAT A TCC CCC TTA CTT ACA TCA TC	607	(Barbut *et al*., 2019)
tcdA	tcdA M8-F tcdA M8-R	AGA TTC CTA TAT TTA CAT GAC AAT AT ATC CCA GGG GCT TTT ACT CC	464	(Lemee *et al*., 2004)
tcdB	tcdB-F tcdB-R	TTA CAA ACA GGT GTA TTT AGT ACA GA TAA ATA CTC CTA TTT GCA TTT CTC C	412	(Barbut *et al*., 2019)
Lok	lok 3-F lok M8-R	TTT ACC AGA AAA AGT AGC TTT AA TTC TGT TGC TTT CCC TAC CCC	620	(Braun *et al*.,1996)
cdtB	cdtB-F (P304) cdtB M8-R	TAA ACA AAG GAG AAT CTG C AGC TTT TTC AAT TGC TTC TCC AA	521	(Barbut *et al*., 2019)
Tpi	tpi M8-F tpi M8-R	AAA GAA GCT ACT AAG GGT ACA AA CAT AAT ATT GGG TCT ATT CCT AC	230	(Lemee *et al*., 2004)
tcdC	tcdC M8-F tcdC M8-R	CATGGTTCAAAATGAAAGACGAC GGTCATAAGTAATACCAGTATCATATCCTTTC	162	(Persson *et al*., 2011)

2.4.3 PCR reaction mix

The amplification was performed in a final volume of 50 µl (**table 2**), including 5µl of DNA, dNTP and Buffer; 0.6 µl for *tcdA*, *cdtA* and *cdtB* primers; and 0.4 µl for *lok*, *tcdB*, *tcdC* and *tpi* primers; 0.5 µl of 5U/µl Taq; and the final volume was completed to 50 µl with H_2O.

Table 2. Multiplex PCR reaction mixes **(Barbut *et al*., 2019).**

Reagent	Initial concentration	Volume (µL)/tube
Primers lok	100 µM	0,4
Primers tcdA	100 µM	0,3
Primers cdtA	100 µM	0,3
Primers cdtB	100 µM	0,3
Primers tcdB	100 µM	0,2
Primers tcdC	100 µM	0,2
Primers tpi	100 µM	0,2
dNTP	2mM	5
Buffer Taq Roche	10X	5
Taq Roche 5U	5U/µL	0,5
H_2O qsp 45µL	/	30,7
DNA	/	5
Final volume		50µL

2.4.4 PCR Amplification

The 96 well PCR plate containing the PCR reaction mixes was placed in ProFlex 96-well PCR System (Applied biosystems, USA) which was programmed as follows:

One initial denaturation step at 94°C for 10 min, followed by 25 cycles of 94°C for 50s (denaturation), 54°C for 40s (annealing) and 72°C for 1min (extension), and a final extension step at 72°C for 10 min (**Barbut *et al.*, 2019**).

2.4.5 Capillary electrophoresis of the PCR products

To verify the presence of amplicons, the PCR products were diluted to 1/25th with H_2O; and 1µl of the diluted DNA was mixed with 10.5 µl HIDI-Formamide (Applied biosystems, USA), and 0.7 µl of internal marker GeneScan600 LIZ (Applied biosystems, USA). The DNA fragments were then separated using high resolution capillary electrophoresis system HITACHI ABI 3500 Genetic Analyzer (Applied biosystems, USA). The results were interpreted using GeneMapper Software version 5.0 (Applied Biosystems, USA) **Annex IV** (**Barbut *et al.*, 2019**).

2.5 Typing of the *C. difficile* isolates

2.5.1 PCR Ribotyping

Analysis of the polymorphisms within the 16S-23S rRNA intergenic regions of the *C. difficile* isolates by PCR Ribotyping was performed according to the European Centre for Disease Prevention and Control (ECDC, https://ecdc.europa.eu/en/home) standard protocol using the primers designed by **Bidet *et al*. (1999)** and shown in **table 3**.

Table 3. PCR Ribotyping primers (**Bidet *et al*., 1999**).

Primer	Sequence
CD1-FAM (Forward)	5´-GTGCGGCTGGATCACCTCCT-3´
CD2 (Reverse)	5´-CCCTGCACCCTTAATAACTTGACC-3´

2.5.1.1 PCR reaction mix

Each PCR reaction was performed in a final volume of 25 µl, containing 12.5 µl HotStartTaq (Qiagen, France) master mix (containing HotStarTaq DNA Polymerase, Buffer, dNTPs, and $MgCl_2$), 2 µl DNA template, 0.25 µl of each primer and 10 µl of water.

2.5.1.2 PCR Amplification

The PCR reaction were carried out in a Veriti 96-Well Fast Thermal Cycler (Applied biosystems, USA) which was programmed as follows:

One initial denaturation step at 95°C for 15 min, followed by 20 cycles of 95°C (denaturation), 57°C (annealing) and 72°C (extension) for 1min each, followed by a final extension step at 72°C for 30 min (**Barbut *et al.*, 2019**).

2.5.1.3 Capillary electrophoresis of the PCR products

The PCR products were mixed with 8.5µl HI-DI Formamide and 0.5µl GenScan 1200 LIZ and then separated by capillary electrophoresis using ABI Hitachi 3500 genetic analyzer (Applied Biosystems, USA) **Annex V.**

Ribotypes were determined using the European database of *C. difficile* Ribotypes WEBRIBO version 2.2 (https://webribo.ages.at/).

2.5.2 Multilocus sequence typing (MLST)

The amplification of the seven loci (*adk*, *atpA*, *dxr*, *glyA*, *recA*, *sodA* and *tpi*) was carried out by the method of **Griffiths *et al*, (2010)** and **Jolley & Maiden, (2010)**, using the primers developed by **(Griffiths *et al*, (2010)**, which are shown in **Table 4**.

Table 4. Primers used in the MLST

Locus	Prime name	Primer sequence	Amplicon size (bp)
adk	adk1F	TTACTTGGACCTCCAGGTGC	635
	adk1R	TTTCCACTTCCTAAGGCTGC	
*atp*A	atpA1F	TGATGATTTAAGTAAACAAGCTG	674
	atpA1R	AATCATGAGTGAAGTCTTCTCC	
Dxr	dxr3F	GCTACTTTCCATTCTATCTG	525
	dxr4R	CCAACTCTTTGTGCTATAAA	
*gly*A	glyA1F	ATAGCTGATGAGGTTGGAGC	625
	glyA1R	TTCTAGCCTTAGATTCTTCATC	
*rec*A	recA2F	CAGTAATGAAATTGGGAGAAGC	705
	recA2R	ATTCAGCTTGCTTAAATGGTG	
*sod*A	sodA5F	CCAGTTGTCAATGTATTCATTTC	585
	sodA6R	ATAACTTCATTTGCTTTTACACC	
tpi	tpi2F	ATGAGAAAACCTATAATTGCAG	640
	tpi2R	TTGAAGGTTTAACACTTCCACC	

bp: base pair.

NB : The size of the amplicon varies with the strain genotype.

2.5.2.1 PCR reaction mix

Each PCR reaction was carried out in a final volume of 50 µl containing 5 µl of 10X PCR buffer (Qiagen, United Kingdom), 1 µl of a 10 µM concentration of each forward and reverse primer, 1 µl of 10 mM dNTP mix (Invitrogen, United Kingdom), 0.25 µl of HotStart *Taq*

DNA polymerase (Qiagen, United Kingdom), and 2 µl of *C. difficile* chromosomal DNA (approximately 10 ng) and 39,75 µl of water.

2.5.2.2 PCR Amplification

The PCR amplification was carried out in Veriti Thermal Cycler (Applied biosystems, USA), with the program: 95°C for 15 min (initial denaturation), followed by 35 cycles of 94°C for 30 s, 50°C for 40 s, and 72°C for 70 s, and a final extension at 72°C for 5 min.

2.5.2.3 Purification of the PCR products

The PCR products were mixed with 20% polyethylene glycol (molecular weight, 8,000) and 2.5 M NaCl and incubated at room temperature for 10 min. The DNA was precipitated by centrifugation at 16000g for 5 mn, and the supernatant was discarded. The pellet was washed by the addition of 125 µl of 70% ethanol and centrifugation as before. The ethanol was removed and the pellet was left to dry, and then resuspended in biology grade water **(Griffiths *et al*., 2010; Schmitz & Riesner, 2006)**.

2.5.2.4 Sequencing of the PCR products

The purified PCR products were sequenced on both strand by the Sanger method using the BigDye Terminator v 3.1 Cycle Sequencing kit (Applied Biosystems, UK). Each sequencing reaction contained 2 µl of purified PCR products, 4 µl of either forward or reverse PCR primer (0.66 M) **(Table 4)**, 0.25 µl of BigDye Terminator v 3.1 Ready Reaction Mix, 1.875 µl of 5X sequencing buffer (1 M MgCl2, 8 ml of 1 M Tris-HCl, pH 9). The final volume was completed to 10 µl with 1.875 µl of H_2O.

The sequencing conditions consisted of 30 cycles of: denaturation at 96°C for 10s, hybridization at 50°C for 5s and elongation at 60°C for 3 min.

The sequencing products were purified with 2 volumes of ethanol and 0.1 volume of 3 M sodium acetate (pH 5.2), followed by centrifugation for 20 min at maximum speed; the pellet was washed with 70% ethanol, and then resuspended in biology grade water.

The sequencing was performed in the sequencer ABI 3730xl DNA analyzer (Applied Biosystems, USA).

2.5.2.5 Sequence analysis

The DNA sequences obtained were assembled and edited using the software STARS (Sequence Typing Analysis Retrieval System, http://pubmlst.org/software/assembly/). Phylogenetic analysis was performed using the program MEGA (Molecular Evolutionary Genetics Analysis) version 4 (http://www.megasoftware.net/). The sequence type (ST) and clade were determined by comparing the sequences of the isolates with the MLST database available at: http://pubmlst.org/cdifficile/) **(Griffiths *et al*., 2010; Jolley *et al*., 2018)**.

2.6 Antibiotic susceptibility tests

The antibiotic susceptibility tests were performed by the Bauer-Kirby disk diffusion method (I2A, France) for Clindamycin (CLD), Erythromycin (ERY), Moxifloxacin (MXF) and Tetracycline (TET); and the Minimum Inhibitory Concentrations (MIC) for Vancomycin (VAN) and Metronidazol (MTZ) were determined using the E-test method (Biomérieux, France).

a bacterial suspension equivalent to 1.5 McFarland was spread on the surface of Brucella blood agar plates supplemented with 0. 5mg/l hemin, 1mg/l Vitamin K1 and 5% sheep blood (Becton Dickinson GmbH, Germany). The plates were left to dry at room temperature, then the antibiotic discs and the E-test strips were deposited on the surface of the agar. The plates were incubated anaerobically at 37°C for 48h in AnaeroPack™ 2.5L Rectangular Jar (Thermo scientific, Japan).

For the disk diffusion method, the reading was carried out by measuring the diameters of the inhibition zones, and the bacteria were classified as susceptible (S) or resistant (R) or intermediate (I), according to the recommendations of the CA-SFM 2019 (Comité de l'Antibiogramme de la Société Française de Microbiologie), (SFM, 2019), who set the breakpoints as follows: TET (30 µg) < 19 mm, MXF (5 µg) < 21 mm, CLD (2 UI) < 15 mm, ERY (15 UI) < 22 mm **(CA-SFM, 2019).**

For the E-test method, the MICs were determined by referring to the breakpoint value of 2 mg/L for VAN and MTZ, set by the European Committee on Antimicrobial Susceptibility Testing **(EUCAST, 2020).**

Multidrug resistance is defined as the concomitant resistance to at least three antibiotics of different classes.

2.7 Detection of antibiotic-resistance genetic determinants

The presence of antibiotic-resistance genetic determinants of clindamycin/macrolides (*ermB*), tetracycline (*tetM*, *tetO*, *tetB[P]*, *tet0/32/0*, *tet40*, *tetA[P]*) and fluoroquinolones (*gyrA*) was investigated by multiplex PCR, using the primers shown in **Table 5 (Dridi *et al.*, 2002; Spigaglia & Mastrantonio, 2004)**.

Table 5. Primers used for the detection of the resistance genetic determinants for clindamycin/macrolides, tetracycline and fluoroquinolones.

Primer	Sequence	Gene target	Amplicon size (bp)	Reference
E5	5′-CTCAAAACTTTTTAACGAGTG-3′	*ermB*	711	(Spigaglia & Mastrantonio, 2004)
E6	5′-CCTCCCGTTAAATAATAGATA-3′			
CL1	5′-ATACAGCATGACCGTTAAAG-3′	*catD*	500	(Spigaglia & Mastrantonio, 2004)
CL2	5′-ATGTGAAATCCGTCACATAC-3′			
gyrA1	5′-AATGAGTGTTATAGCTGGACG-3′	*gyrA*	390	(Dridi *et al.*, 2002)
gyrA2	5′ -TCTTTTAACGACTCATCAAAGT T-3′			
TETMd	5′ -TGGAATTGATTTATCAACGG-3′	*tetM*	1080	(Marchese *et al.*, 1998)
TETMr	5′ -TTCCAACCATACAATCCTTG -3′			

bp: base pair.

2.7.1 PCR reaction mix

PCR amplification reactions were performed in a final volume of 25 µl containing 2 µl of dNTPs (200 µm /µl), 2.5 µl of 10X Taq buffer, 0.5 µl of each primer (25 pm/ µl), 0.75 µl of Taq DNA polymerase (3 U/ µl); 5 µl of *C. difficile* chromosomal DNA (30 ng/ µl), and 13.75 µl of distilled water.

2.7.2 PCR Amplification

The PCR amplification was carried out in Veriti Thermal Cycler (Applied biosystems, USA), with the program: 94°C for 5 min (initial denaturation), followed by 30 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1.5 min.

2.7.3 Gel electrophoresis of the PCR products

To verify the presence of amplicons, 10 µl of PCR products were separated by electrophoresis on an agarose gel at 1.5% (weight/volume) and stained with ethidium bromide. The DNAs were visualized on a Trans illuminator under ultraviolet (UV) and their sizes were determined by comparison with DNA molecular weight markers.

2.7.4 PCR-RFLP of *ermB* genes

Twenty microlitres of PCR products, obtained using the primers E5 and E6 (under the same PCR conditions as those of the multiplex PCR detailed above) were digested with 40 U of the restriction enzyme *Pvu*II. This enzyme has one restriction site in the *C. difficile* 630 *ermB* sequence (SZ-type sequence), but has no restriction site in the *C. perfringens* CP592 *ermB* sequence (SP-type sequence).

Following restriction with *Pvu*II for 60 min at 37°C, the DNA fragments were separated on a 2% agarose gel, stained with ethidium bromide, and visualized on a Trans illuminator under UV light, and their sizes were determined by comparison with DNA molecular weight markers.

If the sequence of the *ermB* gene is similar to the *C. difficile* 630 *ermB*, the restriction with *Pvu*II should produce two DNA fragments of approximately 589 and 122 bp in size, whereas if the sequence of the *ermB* gene is similar to the *C. perfringens* CP592 *ermB* , it will not be cut with *Pvu*II, and only one DNA fragment of 711 bp will be obtained **(Spigaglia & Mastrantonio, 2004)**.

2.7.5 Detection of mutation in *gyrA*

For the detection of the quinolone resistance-determining regions QRDRs, the *gyrA* gene was amplified by PCR using the primers gyrA1 and gyrA2 shown in **table 5**, and the PCR products were sequenced on both strands (with the same primers) by the Sanger method using the

BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, UK) on the DNA sequencer ABI Prism 373A (Applied Biosystems, France) **(Dridi *et al*., 2002)**.

2.8 Statistical analyses

The data were coded using Excel 2013 (Microsoft, Redmond, WA, USA) and analyzed by R software (R Development Core Team, 2016). The distribution of prevalence of *C. difficile* between provinces, ages and sexes of patients was tested by Chi-Square test or Fisher's exact test. A level of P value <0.05 was considered as statistically significant.

Chapter III: Results

3.1 Study population

A total of 300 patients were included during the three year study period, between 2016 and 2019, the majority of which 86% (n=258, 1 hospital) were from the province of Batna, followed by Chlef 10.3% (n=31, 3 hospitals) and Ain Defla 3.7% (n=11, 1 hospital). There were more females (56%, n=168) than males (44%, n=132); the majority of the patients 79% (n=237) were adults (≥19 years old), and only 21% (n=63) were ≤18 years old.

3.2 Identification of *C. difficile*

3.2.1 Identification by culture

From the 300 faecal specimen collected, 53 were presumed as *C. difficile* on the basis of the morphological characteristics of the colonies (black and irregular) on the selective medium ChromID CDIF (Biomérieux, France) (**Figure 16**). The isolates were purified on CLO Agar (Biomérieux, France) before their identification by MALDI-TOF MS.

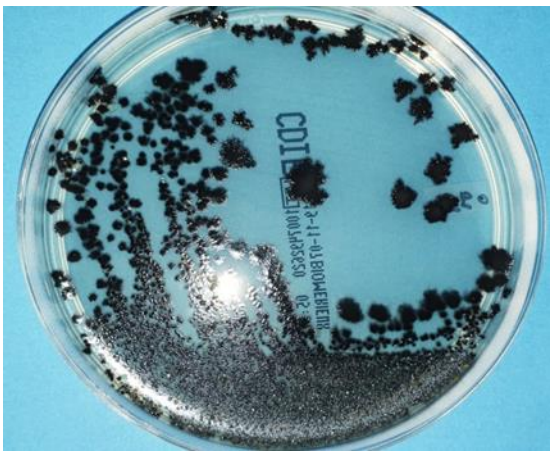

Figure 16. Aspects of *C difficile* colonies on ChromID CDIF agar.

3.2.2 Identification by MALDI-TOF MS

From the 53 suspected isolates, 18 were confirmed to be *C. difficile* by MALDI-TOF MS with score values ≥ 1.71. The results of the identification of the isolates with their scores are shown in **table 6**.

Table 6. Results of the identification of the *C. difficile* isolates by MALDI-TOF MS.

Isolate ID	MALDI-TOF Species Identification	MALDI-TOF score
CD038	*C. difficile*	2,02
CD053	*C. difficile*	2,19
CD093	*C. difficile*	2,13
CD137	*C. difficile*	2,05
CD144	*C. difficile*	1,91
CD147	*C. difficile*	2,16
CD155	*C. difficile*	1,89
CD181	*C. difficile*	2,27
CD190	*C. difficile*	2,05
CD202	*C. difficile*	2,04
CD210	*C. difficile*	2,08
CD213	*C. difficile*	2,26
CDC05	*C. difficile*	2,07
CDC13	*C. difficile*	1,89
CDD04	*C. difficile*	2,15
CDD07	*C. difficile*	1,71
CDF02	*C. difficile*	2,05
CDS0	*C. difficile*	2,08

3.3 Prevalence rates of *C. difficile*

Of the 300 patients, a total of 18 *C. difficile* isolates were recovered, giving an overall prevalence of 6% (CI at 95%: 3.3% - 8.7%).

The highest prevalence was recorded in the province of Ain Defla (18.2%, 2/11), followed by Chlef (9.7%, 3/31) and Batna (5%, 13/258) (**Table 6**). The prevalence was higher (11.1%, 7/63) in patients who were ≤18 years old than those aged ≥19 years old (4.6%, 11/237). The prevalence in females (6.5%, 11/168) was slightly higher than in men (5.3%, 7 /132) (**Table 6**). However, the differences in *C. difficile* prevalence between the three provinces, sexes or age groups were not statistically significant (P value > 0.05).

Table 7. Frequencies of isolation and distribution of *C. difficile* in this study.

	Province			Age		Gender		Ward			
	Ain Defla	Batna	Chlef	≤18 years	≥19 years	M	F	WIM	MIM	PED	ICU
Numbre of samples	11	252	31	63	237	132	168	129	96	63	12
Numbre of *C. difficile* isolates	2	13	3	7	11	7	11	7	3	7	1
Prevalence %	18.2	5	9.7	11.1	4.6	5.3	6.5	5.4	3.1	11.1	8.3

M: Male; F: Female; ICU: intensive care unit; PED: Paediatric; MIM: Men's internal medicine; WIM: Women's internal medicine.

3.4 Detection of the toxin genes

A PCR multiplex assay for the detection of *tcdA*, *tcdB* and *cdtA*/*B* genes, allowed us to distinguish the *C. difficile* isolates, on the basis of the presence/absence of toxin genes, into three toxin genes profiles: 6 (33%) A+B+CDT-, having an intact *tcdA* and *tcdB*, a deleted *cdtA* and the *cdtB* was present as a pseudogene; 2 (11%) A-B+CDT-, had an intact *tcdB*, a deletion within *tcdA*, and *cdtA* and *cdtB* deleted; and 10 (55.5%) A-B-CDT-, that did not carry any of the toxin genes (**Figure 17**). The same analysis revealed that the *tcdC* gene was present (without an internal deletion) in all the toxigenic isolates and absent in all the non-toxigenic isolates.

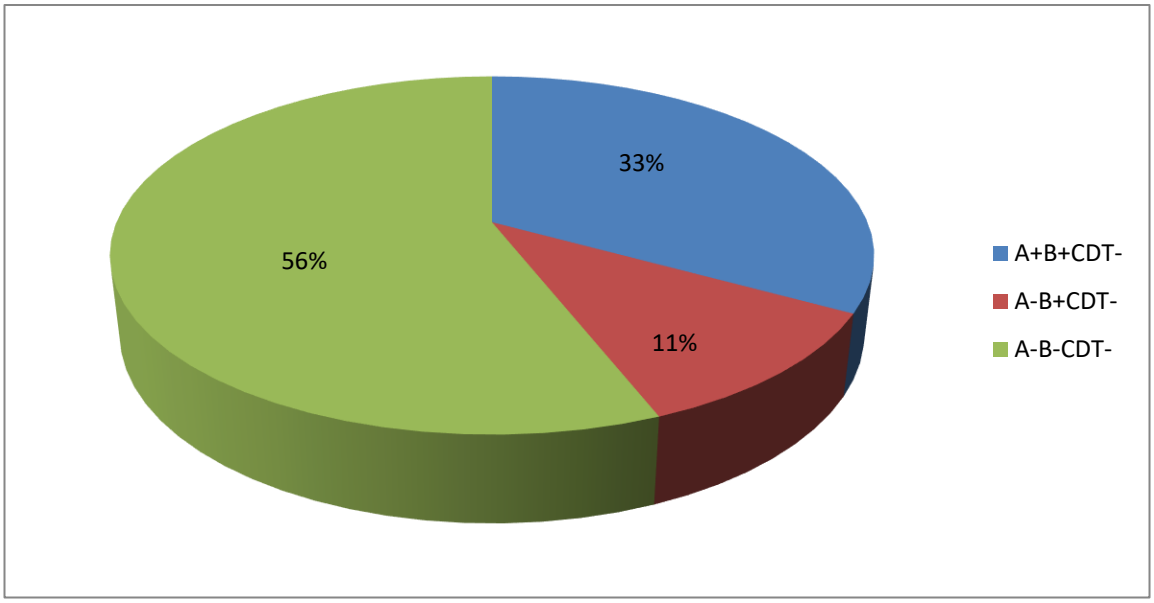

Figure 17. Frequencies of the *C. difficile* toxin gene profiles.

3.5 PCR ribotyping

The PCR ribotyping assigned the 18 *C. difficile* isolates to 11 different ribotypes: RT 085 and FR 248 (16.6 %, n=3 each), FR 111, RT 017 and RT 014 (11.1 %, n=2 each), FR 247, RT 005, RT 029, RT 039, RT 056 and RT 446 (5.5 %, n=1 each) (**Figure 18**). The three unrecognized ribotypes, FR 111, FR 247 and FR 248, detected in this study correspond to ribotypes maintained in the internal database of the French National Reference Laboratory for *C. difficile.*

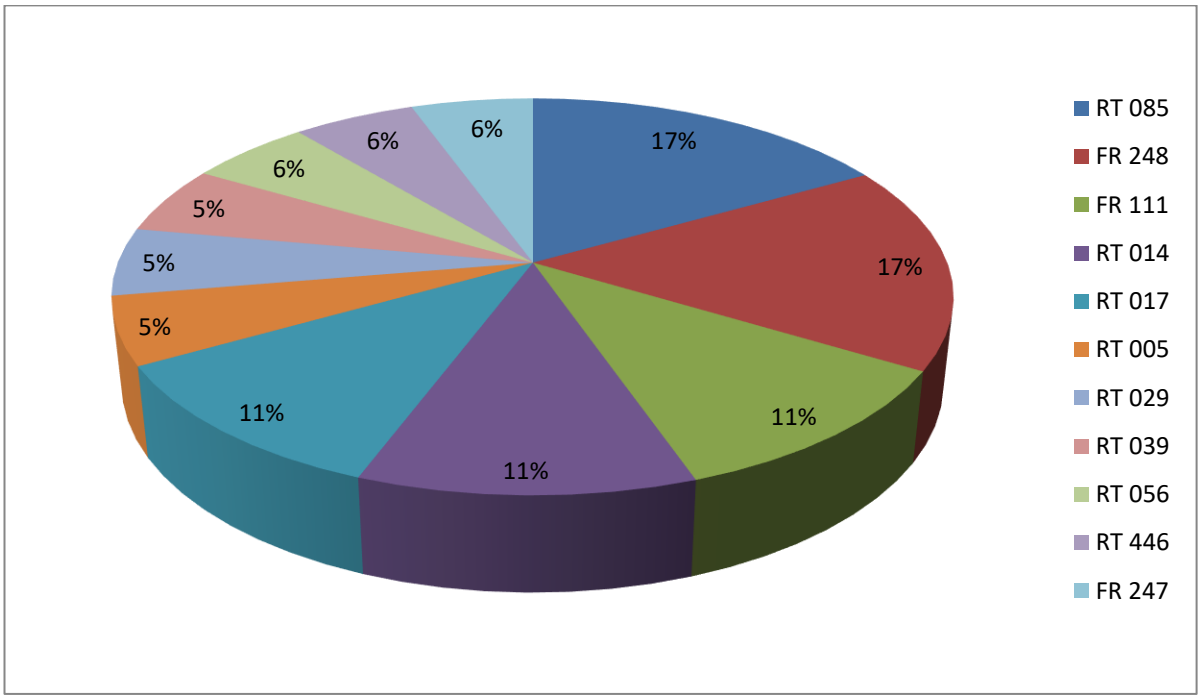

Figure 18. Frequencies of the *C. difficile* PCR ribotypes.

3.6 Multilocus sequence typing

The MLST analysis separated the 18 *C. difficile* isolates into 12 sequence types (ST): ST39 and ST259 (16.7%, n=3 each); ST37 and ST48 (11%, n=2 each); ST2, ST6, ST14, ST16, ST26, ST34, ST58 and one new STs (5.5%, n=1 each) (**Figure 19**).

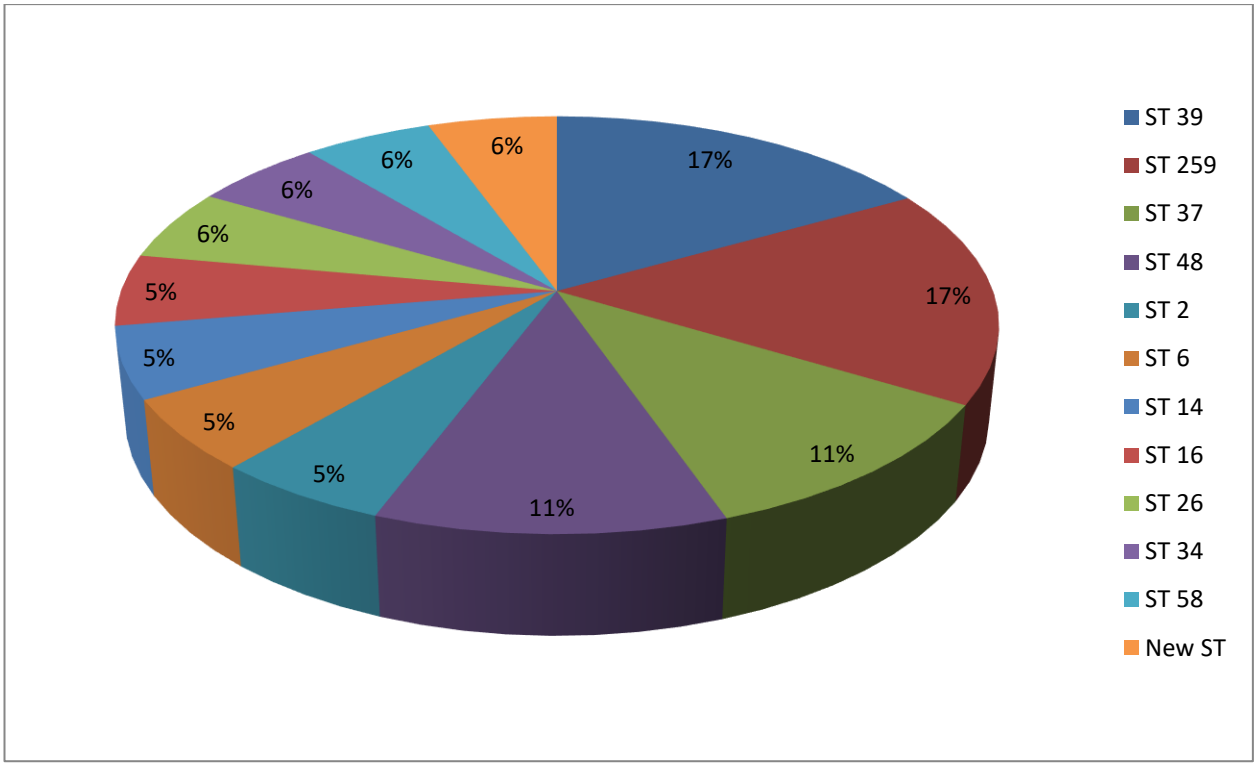

Figure 19. Frequencies of the *C. difficile* Sequence types

In addition, the MLST analysis classified the *C. difficile* isolates into two MLST clades, 1 and 4; Clade 1 was more heterogeneous and consisted of a diverse set of isolates, RT 005/ST6, RT 14/ST2, RT 14/ST14, RT 029/ST16, RT 039/ST26, RT 056/ST34, FR 111/ST48, RT 446/ST58 and FR 247/New ST; whereas clade 4 included RT 85/ST39, RT 17/ST37 and FR 248/ST259. Furthermore, Clade 4, included mostly non-toxigenic isolates (33.33%), with the exception of 2 isolates, which produced toxin B only, and belonged to RT 17/ST37 (**Table 8**).

Table 8. Epidemiological and molecular characteristics of *C. difficile* isolates in the study.

Sample	Province	Hospital	Ward	Year	Gender	Age	Genotyping							
							PaLoc	*tcdA*	*tcdB*	Deletion in *tcdC*	*cdtA*	*cdtB*	Ribotype	Sequence type (Clade)
CD 038	Batna	UH	MIW	2016	F	Ad	+	-	-	-	-	-	RT 085	39 (4)
CD 053	Batna	UH	MIM	2016	M	Ad	+	-	-	-	-	-	RT 039	26 (1)
CD 093	Batna	UH	REA	2016	M	Ad	+	-	-	-	-	-	RT 085	39 (4)
CD 137	Batna	UH	PED	2017	F	Ch	+	-	-	-	-	-	RT 085	39 (4)
CD 144	Batna	UH	PED	2017	M	Ch	+	-	-	-	-	+	FR 111	48 (1)
CD 147	Batna	UH	WIM	2017	F	Ad	+	-	-	-	-	+	FR 111	48 (1)
CD 155	Batna	UH	MIM	2017	M	Ad	-	+	+	+^{NID}	-	+	RT 014	2 (1)
CD 181	Batna	UH	PED	2017	F	Ch	-	+	+	+^{NID}	-	+	RT 014	14 (1)
CD 190	Batna	UH	WIM	2017	F	Ad	-	+	+	+^{NID}	-	+	RT 056	34 (1)
CD 202	Batna	UH	WIM	2017	F	Ad	+	-	-	-	-	-	FR 247	New * (1)
CD 210	Batna	UH	WIM	2017	F	Ad	-	+	+	+^{NID}	-	+	RT 446	58 (1)
CD 213	Batna	UH	MIM	2017	M	Ad	-	+	+	+^{NID}	-	+	RT 005	6 (1)
CD C05	Chlef	Chettia	WIM	2018	F	Ad	-	+	+	+^{NID}	-	+	RT 029	16 (1)
CD C13	Chlef	Chettia	PED	2018	F	Ch	+	-	-	-	-	-	FR 248	259 (4)
CD D04	Ain Defla	S/Bobida	PED	2018	M	Ch	+	-	-	-	-	-	FR 248	259 (4)
CD D07	Ain Defla	S/Bobida	PED	2018	M	Ch	+	-	-	-	-	-	FR 248	259 (4)
CD F02	Chlef	Chorfa	PED	2018	F	Ch	-	+^{ID}	+	+^{NID}	-	-	RT 017	37 (4)
CD S0	Batna	UH	WIM	2018	F	Ad	-	+^{ID}	+	+^{NID}	-	-	RT 017	37 (4)

UH: University hospital, WIM: Women's internal medicine, MIM: Men's internal medicine, PED: Paediatric, F: Female, M: Male, Ad: Adult, Ch: Child, ID: internal deletion, NID: no internal deletion, * The closest match to STs: 69, 104 and 596.

3.7 Phenotypic detection of antibiotic resistance

Antibiotic susceptibility profiles of the *C. difficile* isolates were determined by the disk diffusion method using 4 antibiotic disks, CLD, ERY, MXF and TET (**Figure 20A**); and by the E-test

method against VAN and MTZ (**Figure 20B**); and the results were interpreted according to the recommendations of the CA-SFM 2019. The results showed that all the isolates were susceptible to MTZ and VAN, the first line of antibiotics used for the treatment of CDI, and to MXF, a fourth generation fluoroquinolone (**Figure 21 and 22 and Table 9**).

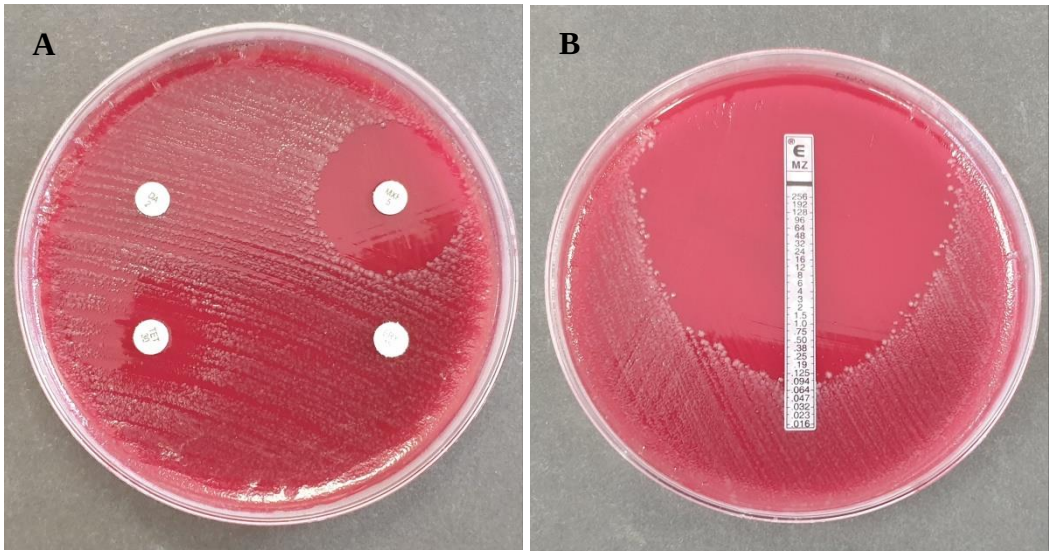

Figure 20. Antibiotic susceptibility tests, A) disk diffusion method for isolate CD S0 against MXF, CLD, ERY and TET; B) E-Test for isolate CD 155 against MTZ strip.
TET: Tetracycline; MXF: Moxifloxacin; ERY: Erythromycin; CLD: Clindamycin; MTZ: Metronidazole.

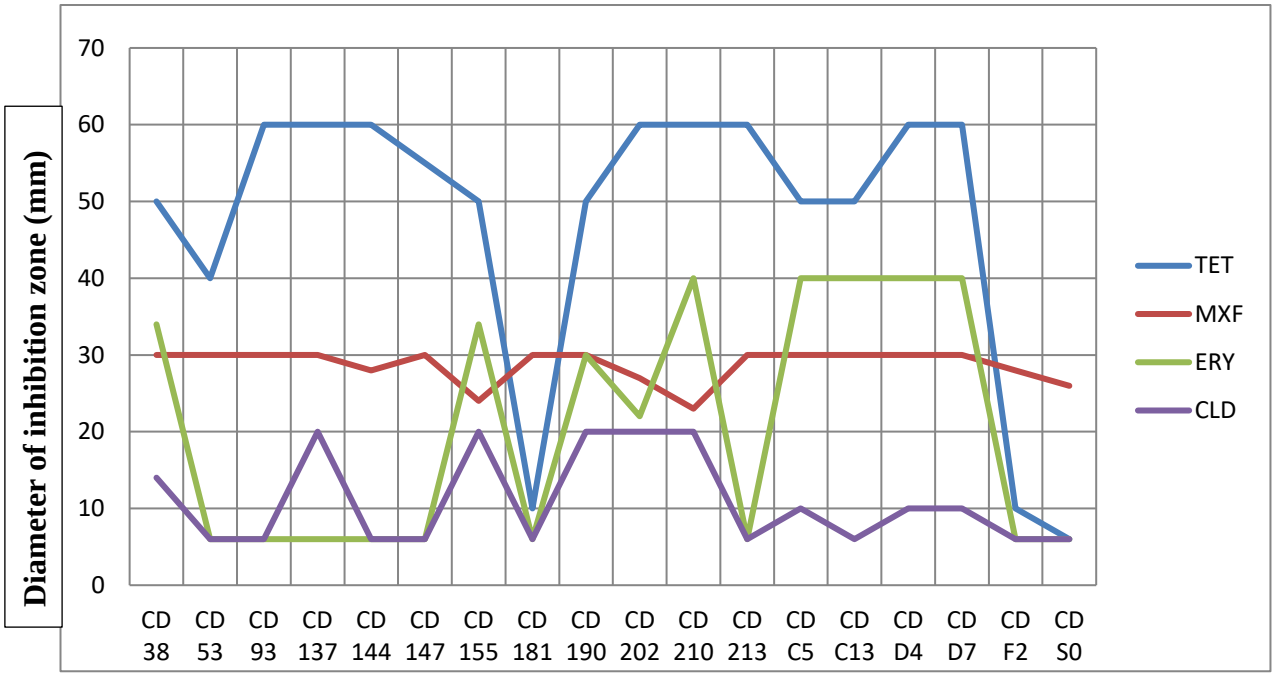

Figure 21. Results of the antimicrobial susceptibility test by the disk diffusion method.
TET: Tetracycline; MXF: Moxifloxacin; ERY: Erythromycin; CLD: Clindamycin.

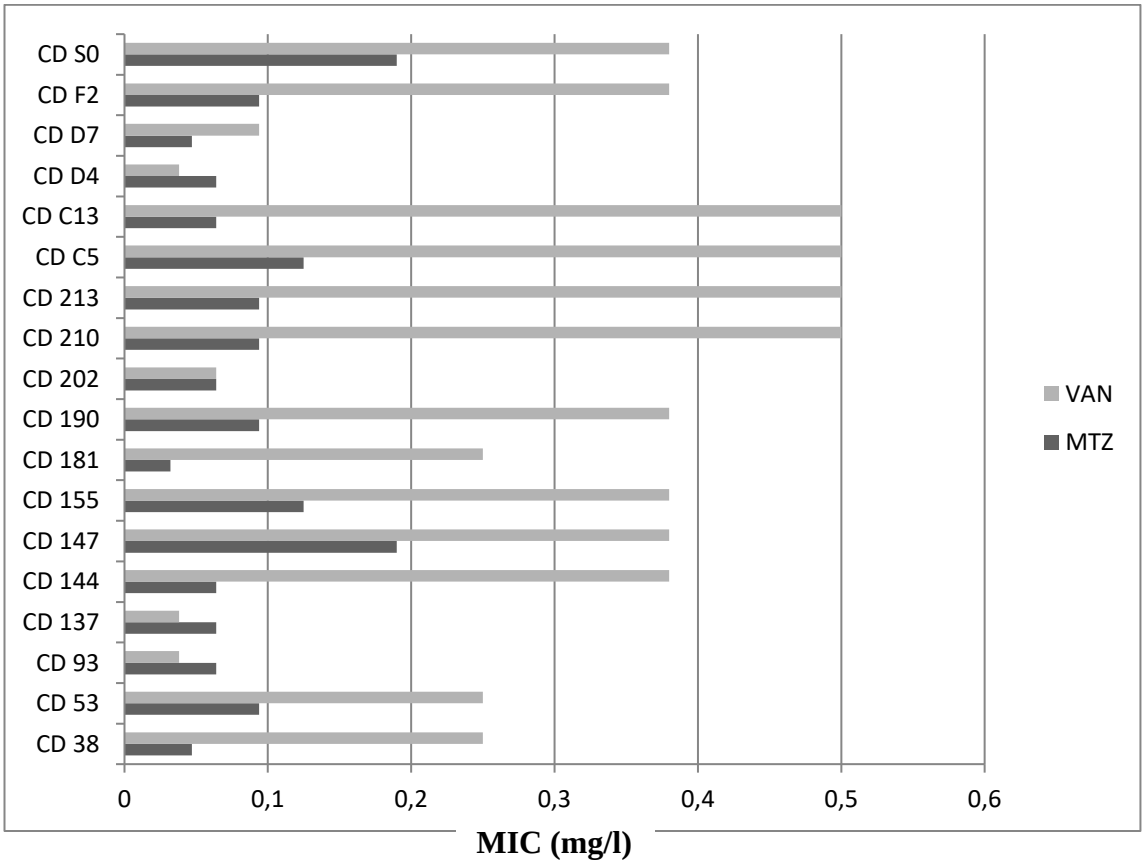

Figure 22. Results of the antimicrobial susceptibility test by the E-test method.
MIC: Minimal inhibitory concentration; VAN: Vancomycin; MTZ: Metronidazole.

Thirteen isolates (72%) were resistant to CLD, 9 (50%) to ERY and 8 (44.4%) isolates were resistant to both CLD and ERY; whereas 3 (17%) isolates were resistant to TET (**Figure 23**).

These 3 TET-resistant isolates were also resistant to CLD and ERY (**Table 9**).

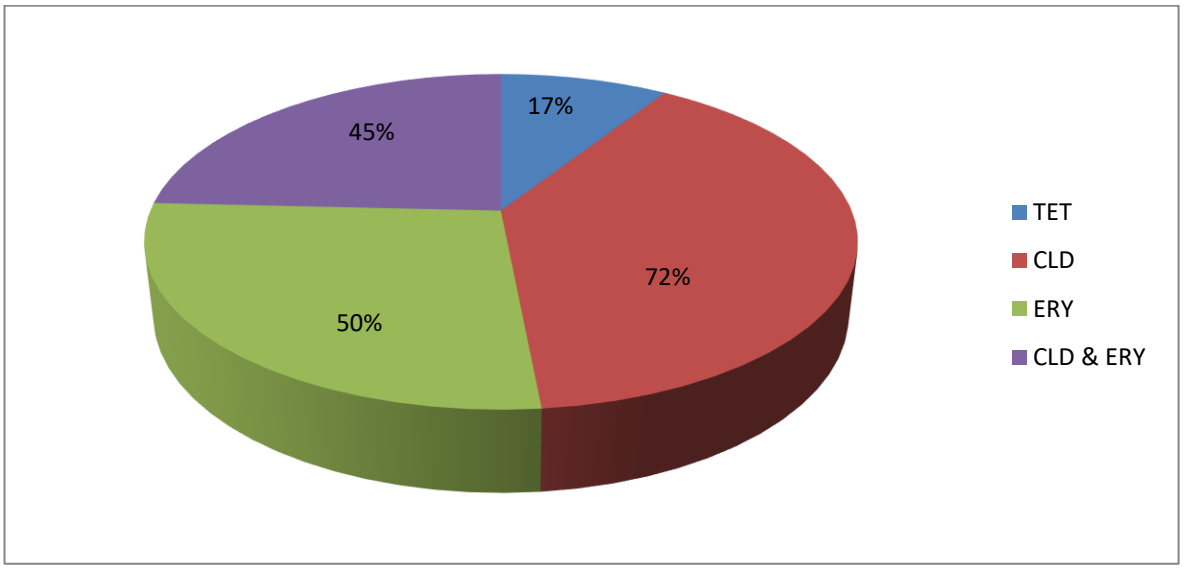

Figure 23. Frequency of antibiotic resistance.
CLD: clindamycin; ERY: erythromycin; TET: tetracycline.

Table 9. Antimicrobial resistance patterns of *C. difficile* isolates.

RT	Isolate	Antibiotic Inhibition zone (mm)						MIC (mg/l)			
		TET		CLD		ERY		MXF		MTZ		VAN	
		S	R *tetM* (+)	S	R *ermB* (+)	S	R	S	R	S	R	S	R
		<15	≥19	<15	≥15	<18	≥21	<4	≥4	<1	≥2		
RT 085	038	S		R +		S		S		S			
	093	S		R +		R +		S		S			
	137	S		S		R +		S		S			
FR 248	C13	S		R		S		S		S			
	D04	S		R		S		S		S			
	D07	S		R		S		S		S			
RT 014	155	S		S		S		S		S			
	181	R +		R +		R +		S		S			
RT 017	F2	R +		R +		R +		S		S			
	S0	R +		R +		R +		S		S			
FR 111	144	S		R +		R +		S		S			
	147	S		R +		R +		S		S			
RT 005	213	S		R +		R +		S		S			
RT 029	C05	S		R		S		S		S			
RT 039	053	S +		R +		R +		S		S			
RT 056	190	S		S		S		S		S			
RT 446	210	S		S		S		S		S			
FR 247	202	S		S		R -		S		S			

3.8 Detection of antibiotic-resistance genes

The presence of the *ermB* gene conferring resistance to the MLSB family of antibiotics, CLD and ERY, and the TET resistance gene, *tetM*, was determined by PCR.

The *ermB* gene was present in 9 (50%) isolates which were resistant to either CLD or ERY, or both. However, one isolate, CD202, which was resistant to ERY did not carry the *ermB* gene.

The *tetM* gene was detected in 3 (16.7%) TET resistant isolates. Interestingly, 1 isolate, CD053, carried the *tetM* gene despite being susceptible to TET.

Sequence analysis of the quinolone resistance-determining regions (QRDR) of *gyrA* did not reveal any substitution in any of our isolates.

Chapter IV: Discussion

C. difficile is the leading cause of hospital-acquired antibiotic-associated diarrhoea **(Burke & Lamont, 2014)**. The CDIs have become a major concern worldwide because of the increase in their frequency and severity. This evolution was due mainly to the emergence and the dissemination of a particularly virulent clones such as the RT 027, and also to the emergence of CDI in the community, in populations previously considered to be at low risk **(Balsells *et al*., 2019).**

Although CDI represent a significant public health concern in many countries, they are, however, largely neglected in Algeria, and epidemiological data on *C. difficile* are scarce, with the exception of two previous studies which were limited to the regions of Algiers or Chlef **(Djebbar *et al*., 2018; Merad & Djellout, 1992)**. Hence, this study was undertaken to investigate the prevalence and the molecular epidemiology of *C. difficile* over a wider geographic region, including five hospitals in three provinces of Algeria, Chlef, Ain Defla and Batna.

The estimated prevalence of *C. difficile* in this study was 6%, which was similar to those found in the two previous Algerian studies (6,9% and 7,6%) **(Djebbar *et al*., 2018; Merad & Djellout, 1992)**, as well as to those reported in several African and Middle-Eastern countries, 4,9% in Ghana **(Janssen *et al*., 2016)**, 6,4% and 7,3% in Tanzania (Seugendo et al., 2015, 2020), 8,6% in Zimbabwe **(Berger *et al*., 2020)**, 9% in Iran (Kouhsari et al., 2019; Kouzegaran et al., 2016), 7,9% in Qatar **(Al-Thani *et al*., 2014)** or 6.8% in Saudi Arabia **(Alalawi *et al*., 2020)**. However, our prevalence is either much lower than those reported in other studies in Lebanon (82,9%) **(Berger *et al*., 2018)** and in South Africa (22%) **(Rajabally *et al*., 2016)**; or higher than those reported in Kuwait (0,5%) **(Jamal *et al*., 2015).**

It is worth mentioning that the prevalence rates vary considerably between different countries and even within the same country. These variations could be due to the heterogeneity between the studies, such as the types and duration of studies, types of populations studied, types of hospital establishments, sampling methods or identification methods used.

Indeed, the prevalence of *C. difficile* in different European countries ranged from 4 to 39% **(Bauer *et al*., 2011)**, and in the USA between 7% and 20% **(Cohen *et al*., 2014)**. As examples of intra-countries variations we can cite the case of South Africa, where the prevalence in 11 studies varied between 1,7% **(September *et al*., 2019)** and 51,8% **(B. R. Kullin *et al*., 2018)**; or Saudi Arabia, between 6.8% and 23.5%, in 5 different studies **(Alalawi *et al*., 2020; Aljafel *et al*., 2020; Althaqafi *et al*., 2022; Hudhaiah & Elhadi, 2019; Saber *et al*., 2020).**

The PCR ribotyping analysis revealed that our isolates belonged to 11 different ribotypes, RT005, RT014, RT017, RT029, RT039, RT056, RT085, RT446 and three unclassified ribotypes, FR 111, FR 247 and FR 248, indicating that our isolates are more genetically diverse compared to those isolated in the previous Algerian study, which reported only four ribotypes **(Djebbar *et al*., 2018)**. Among the 11 ribotypes identified in this study, 10 were new, and only one, RT014, was previously identified in a previous study in Algeria, but in a different hospital **(Djebbar *et al*., 2018).**

Eight (44.4%) *C. difficile* isolates were toxigenic and were shared between six ribotypes, 005, 014, 017, 029, 056 and 446; whereas ten isolates were non toxigenic and belonged to ribotypes RT039, RT085, FR111, FR247 and FR248.

The most prevalent ribotype identified in this study was RT085 (16.7%, n=3), followed by RT014 and RT 017 (11.1%, n=2 each), and one isolate each for the following ribotypes RT 005/RT 029/RT 039/RT 056/RT 446/ (5.6% each).

The dominant RT identified in this study, RT085, is non toxigenic and is rarely reported in the literature; except for one recent study from China, where it was found to be the second most dominant RT (Dai et al., 2020). The three isolates of RT085 were detected only in the eastern province of Algeria (Batna), in three different wards of the same hospital, but were missing in the other two provinces (Ain Defla and Chlef). This finding suggests a possible contamination of the Batna's hospital by *C. difficile* RT085 ribotype, which could facilitate its transmission to patients; as it could also suggest a possible distinct geographical distribution of the *C. difficile* ribotypes circulating in Algeria, with the predominance of RT085 in the region of Batna.

The second most dominant RT, RT014, is toxigenic (A+B+CDT-) and is also among the most prevalent ribotypes in many European countries, where it was reported as epidemic and causing infections in humans, and was also common in several Middle-Eastern countries **(Al-Thani *et al*., 2014; Azimirad *et al*., 2020; Berger *et al*., 2018)**. However, in the African continent, this RT was reported only in Algeria **(Djebbar *et al*., 2018),** Zimbabwe **(Berger *et al*., 2020)** and South Africa **(B. Kullin *et al*., 2017)**. Isolates of RT014 were also found commonly associated with different animals and environments **(Bauer *et al*., 2011; Janezic *et al*., 2014; Koene *et al*., 2012; Moono *et al*., 2017; Tkalec *et al*., 2019)**.

With regard to RT017, a toxigenic RT that produces toxin B only (A-B+CDT-), and, despite lacking the toxin A (due to a deletion within the *tcdA* gene), is considered a major epidemic clone that was responsible for outbreaks of CDIs in several countries around the world

(Cairns *et al*., 2015; Kuijper *et al*., 2001). This RT is particularly predominant in Asia **(Imwattana *et al*., 2019)**, but is less common in the African continent, except for two studies from South Africa that reported the isolation of RT017 at high rates **(B. Kullin *et al*., 2017; Rajabally *et al*., 2016)**; in contrast, it has not been detected so far in any of the Middle-eastern countries.

The toxigenic RT005 (A+B+CDT-) is among the most common ribotypes in patients with CDI in Europe (Freeman et al., 2015, 2020)**,** and it was also isolated, albeit with a low frequency, in a study from Ghana (Seugendo et al., 2018)**,** Lebanon **(Berger *et al*., 2018)** and Zimbabwe **(Berger *et al*., 2020).** RT005 was also isolated from different wild and domestic animals **(Andrés-Lasheras *et al*., 2017; Bandelj *et al*., 2016; Himsworth *et al*., 2014; Williams *et al*., 2018).**

The toxigenic PCR-ribotype RT029 (A+B+CDT-) was previously reported as one of the most frequent RTs among hospitalized patients in Iran (Azimirad et al., 2020; Baghani et al., 2020), and was also isolated from humans in Egypt **(Helmi & Hamdy, 2006),** Lebanon **(Berger *et al*., 2018)**. This RT was also isolated from humans, animals and the environment in Slovenia **(Janezic *et al*., 2012)** and from an agricultural soil fertilized with manure from broiler chickens in a study from Germany **(Frentrup *et al*., 2021)**.

The toxigenic isolate belonging to the PCR-ribotype RT056 (A+B+CDT-) detected in this study, was previously isolated from humans (Baldan et al., 2015; Hong et al., 2020; Tickler et al., 2019, 2020), animals and the environment **(Knight *et al*., 2013; Knight & Riley, 2013; Moono *et al*., 2017)**; and more importantly, it was reported as associated with complicated CDI in hospitalized patients in two studies from Europe **(Bauer *et al*., 2011; Davies *et al*., 2016)**. In addition, a study from England reported that this RT is likely to be associated with CA-CDI **(Fawley *et al*., 2016)**. In Africa, this PCR-ribotype was reported only in Zimbabwe **(Berger *et al*., 2020)**, whereas in the Middle-East it was reported in Qatar **(Al-Thani *et al*., 2014)** and Kuwait **(Jamal *et al*., 2015; Rotimi *et al*., 2003)**.

Concerning the non-toxigenic ribotype RT039, there have been only few reports of it in humans in Australia **(Hong *et al*., 2020; Putsathit *et al*., 2021)**, Mexico **(Aguilar-Zamora *et al*., 2022)**, Kuwait **(Rotimi *et al*., 2003)** and in Iran **(Azimirad *et al*., 2020; Kouhsari *et al*., 2019)**. Surprisingly, several RT039 isolates from Iran were found to carry the toxin genes **(Azimirad *et al*., 2020)**. In addition, isolates of RT039 were also recovered from animals and

foods of animal origin **(Abdel-Glil *et al*., 2018; Janezic *et al*., 2014; Kecerova *et al*., 2019; Koene *et al*., 2012).**

The toxigenic ribotype, RT446 (A+B+CDT-), is catalogued in the WEBRIBO database, but no information is available in the literature concerning its prevalence or its origin.

The remaining three unknown ribotypes, FR111, FR247 and FR248, are all non-toxigenic, and they were assigned to RTs maintained in the internal database of the French National Reference Laboratory for *C. difficile*. It is worth mentioning that the three isolates belonging to the ribotype FR248 were all recovered from children that were admitted to two different hospitals from two provinces (Ain Defla and Chlef), and as such, it is possible that this ribotype could be associated with children.

Interestingly, the hypervirulent ribotypes RT027 or RT078, that were associated with outbreaks of CDI, with greater severity and mortality, in hospitals in North America and Europe were not found in this study nor in the previous Algerian study of **Djebbar *et al*., 2018**. The possible reasons for the absence of the hypervirulent ribotypes RT027 and RT078 in both Algerian studies are the small sample size and the small number of hospitals investigated. Therefore, the results derived from these two studies might not be representative of the whole country. The absence of RT027 and RT078 in Algeria is significant, albeit not totally unexpected, as these two RTs have also not been found so far in any of the African country; in contrast, in the Middle-East, it was detected, albeit with low frequency, in Iran (n=14) **(Jalali *et al*., 2012; Khoshdel *et al*., 2015; Kouzegaran *et al*., 2016)**, Saudi Arabia (n=4) **(Alzahrani & Al Johani, 2013)** and Qatar (n=1) **(Al-Thani *et al*., 2014)**. Similarly, there is very little data on the ribotype RT 078 in Africa and the Middle-East, except for a few reports from Egypt (n=6) and Kuwait (n=9) **(Helmi & Hamdy, 2006; B. Kullin *et al*., 2022; Rotimi *et al*., 2003).**

On the other hand, the isolation of the toxigenic ribotypes RT014, RT017, RT029 and RT 056 in this study is important from an epidemiological point of view, because these ribotypes were responsible for CDIs in humans in several countries around the world (RT017, RT014 and RT056) **(Davies *et al*., 2016)**, or associated with animals (RT014, RT029 and RT056) **(Janezic *et al*., 2012)**, raising concerns about their potential zoonotic transmission.

According to the result of the MLST analysis all the isolates of the same RT belonged to the same ST, with the exception of RT014, which was shared by two different STs, ST2 and ST14 (1 isolate each). This result is on line with previous findings that certain RTs, including

RT014, are associated with more than one ST **(Knetsch *et al*., 2012)**. This finding indicates that MLST has more discriminatory power than PCR ribotyping.

Antibiotics are one of the major risk factors for the development of CDI, given that antibiotic resistance confer a survival advantage for resistant *C. difficile* strains, which results in therapeutic failure and increases the chances of recurrence, and is also a key driver of the evolution and changing epidemiology of *C. difficile* **(Slimings & Riley, 2021).**

Rates of antibiotic resistance of *C. difficile* vary considerably between different studies, due to local antibiotic consumption **(Sholeh *et al*., 2020).**

In the present study, all of the 18 *C. difficile* isolates (100%) were susceptible to vancomycin and metronidazole, the first-line antibiotics for the treatment of CDI **(Igarashi *et al*., 2018; Ofosu, 2016)**, with metronidazole recommended for non-severe CDI (first episode) and vancomycin for severe or recurrent CDI, according to the ESCMID guideline **(Debast *et al*., 2014)**. Thus, the efficacity of this antimicrobial agent against our *C. difficile* isolates is reassuring from a therapeutic point of view. Our finding was similar to that of many studies, which also found 0% resistance rate against these two antibiotics **(Banawas, 2018)**. In addition, a recent systematic review and meta-analysis, which included 111 studies around the world, reported that resistance to these antibiotics was rare, with an estimated pooled antimicrobial resistance rate of 3.2% for metronidazole and 0.6% for vancomycin **(Sholeh *et al*., 2020).**

Similarly, resistance rate to moxifloxacin, a fourth generation fluoroquinolone, was 0% in this study, which is quite different from other studies which reported that resistance to moxifloxacin is common and varied between countries, 8% in France, 12% in Belgium, 20% in Sweden, 43% in Spain, 77% in Hungary and 100% in Poland (Freeman et al., 2015), 33.5% in the United States **(Snydman *et al*., 2015)**, 72.3% in China **(Chen *et al*., 2018)** and 78% in Iran (Baghani et al., 2018). It has been suggested that the excessive and widespread use of the fluoroquinolone antibiotics was responsible for the emergence of the hypervirulent *C. difficile* 027 strains **(Peng *et al*., 2017)**.

Resistance to the two MLSB family of antibiotics, clindamycin and erythromycin, was found in 72% and 55.5% of our *C. difficile* isolates, respectively; which was in line with other studies from different countries around the world, which showed that resistance of clinical isolates of *C. difficile* to these two antibiotics was very common, ranging from 13% to 100% and 8.3% to 100% for erythromycin and clindamycin, respectively **(Peng *et al*., 2017)**. A systematic review and meta-analysis, which included 15 studies from around the world,

estimated a pooled resistance rate of *C. difficile* to clindamycin and erythromycin at 61% for each **(Dilnessa *et al*., 2022).**

The resistance rate to tetracycline in this study (16%) was comparable to those estimated by **(Sholeh *et al*., 2020)** (20%) and **(Banawas, 2022)** (24%) in Saudi Arabia, but lower than that reported by **Dilnessa *et al*., 2022** (35%).

Three isolates belonging to the toxigenic RT 014 (n=1) and RT 017 (n=2), responsible for CDIs in humans around the world, were co-resistant to CLD, ERY and TET. The resistance of RT017 to many antimicrobial agents has been documented in several studies, and was suggested as the driving factor for the success and dissemination of this ribotype throughout the world **(Imwattana *et al*., 2020; Lew *et al*., 2020)**.

The gene *erm*(B), the main genetic determinant for resistance to the MLS_B antibiotics in C. difficile and which is located on a transposon **(Imwattana *et al*., 2021)**, was found in 8 isolates which were resistant to both clindamycin and erythromycin, and in 2 isolates that were resistant to either clindamycin or erythromycin; however, 5 isolates which were resistant to either clindamycin or erythromycin did not harbour the *erm*(B) gene. The absence of the *erm*(B) gene in *C. difficile* strains resistant to clindamycin or erythromycin was previously reported, and it was suggested that the resistance of these strains to the MLS_B antibiotics might be conferred by another unknown mechanism **(Spigaglia & Mastrantonio, 2004; Zhao *et al*., 2020).**

All the *C. difficile* isolates which were resistant to tetracycline carried the *tetM* gene; whereas, none of the other tetracycline resistance genes (*tetO*, *tetB[P]*, *tet0/32/0*, *tet40*, *tetA[P]*) was detected in this study, confirming previous finding that the *tetM* gene, which, like the *erm*(B) gene, is also located on a transposon (Tn*916*, Tn*5397*, Tn*6190* or Tn*6944*) was the most predominant tetracycline resistance gene in *C. difficile* **(Imwattana *et al*., 2021)**. Surprisingly, one isolate was susceptible to tetracycline despite carrying the *tetM*, suggesting that this gene could be inactive due to a mutation.

Isolates which were co-resistant to TET, CLD and ERY harboured both the *tetM* and the *ermB* gene, which suggests that these two genes are located on the same transposon **(Imwattana *et al*., 2021)**.

Genotypic screening for mutations in the quinolone resistance-determining region (QRDR) of the *gyrA* gene did not reveal any substitution, which correlates well with the data from the

phenotypic antimicrobial susceptibility testing (absence of resistance to moxifloxacin, a fourth generation fluoroquinolone).

Conclusion

This study was undertaken to fill the knowledge gap on the burden of the epidemiology of *C. difficile* infections in Algeria, by isolating clinical isolates of *C. difficile* from hospitalized patients, determining their toxinogenic profiles, ribotypes and sequence types, and by assessing their antibiotic resistance patterns.

The estimated prevalence of *C. difficile* in the 5 hospitals investigated (6%), did not change substantially in comparison with the previous Algerian study, but was moderate in comparison with those recorded in many parts of the world.

The results of genotyping demonstrated a great diversity of the *C. difficile* strains circulating in our hospitals, including new ribotypes; as well as a heterogeneous geographical distribution of the ribotypes, with certain ribotypes being presents in certain geographical region and/or hospitals, but absent in others. Almost half of these isolates was toxinogenic.

Although the well-known hypervirulent *C. difficile* strains such as RT027 and RT078 were not detected in this study, other important epidemic clones (RT014 and RT017), which are known to have caused CDI outbreaks in several countries around the world, were present.

Very importantly, metronidazole and vancomycin remain suitable for the treatment CDI in Algeria, as indicated by the full susceptibility of our isolates to these two antibiotics.

The evidence provided by this study will help to expand our understanding of *C. difficile* epidemiology in Algeria, and highlights the importance of active surveillance programs of CDI in Algerian hospitals, continuous monitoring of their antibiotic resistance profiles, as well as the implementation of appropriate prevention and control strategies, in order to reduce the risk of development and transmission of these infections.

There are a number of limitations within this study that needs to be considered; first, and most importantly, the small number of samples (collected from five hospitals in three geographical regions only) and, as such, the findings might not be adequately representative of the entire country; second, our study lacked clinical data on the patients, which limited the correlation between the strain genotypes and the clinical outcomes; third, antibiotic susceptibility testing was not performed against other important antibiotics used for the treatment of CDI such as fidaxomicin and rifaximin. Despite these limitations, our study provides a good cross-sectional view of the under-appreciated epidemiologic situation in Algeria.

However, additional multicenter studies, which should include larger sample size, more hospitals, and cover a wider geographical area, should be conducted, in order to have a more representative picture on the epidemiological situation of CDI in the whole country.

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Annex I

Patient information questionnaire

Fiche de renseignements

Informations générales sur le patient :

Sexe : ○ Homme ○ Femme

Age : ○ Enfant ○ Adulte

Date du prélèvement :

Historique médical du patient :

○ Patient interne* ○ Patient externe

* Si le patient est interne remplir les informations suivantes :

Date d'admission :

Raison(s) d'admission:

Début de la diarrhée :

Antibiotiques pris au cours d'hospitalisation :

Durée de prise d'antibiotiques :

Voie de prise de l'antibiothérapie : ○ Orale ○ IV

Réponse à la thérapie :

Autres pathologie(s) traitée(s) :

Hospitalisation(s) ultérieure(s) : ○ Oui* ○ Non

(*)Raison(s) d'admission :

(*)Fréquence des diarrhées :

Diagnostic histologique (Coloscopie) :

Médecin traitant :

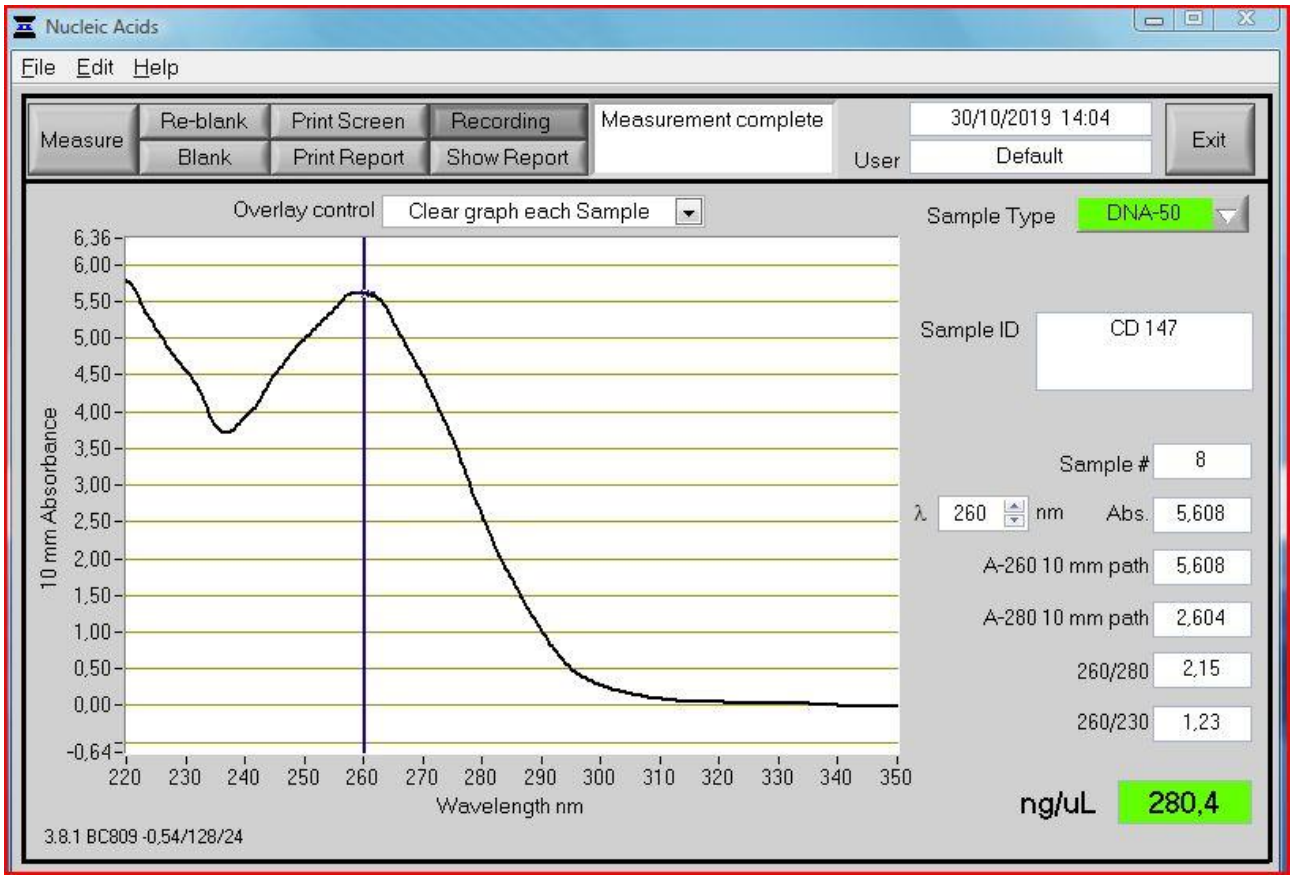
Annex II

Composition of *Clostridium difficile* Agar (CLO)

Peptone de caséine (bovin) 13 g	Peptone de caséine (bovin) 13 g
Peptone de viande (bovin ou porcin) 5 g	Peptone de viande (bovin ou porcin) 5 g
Peptone de coeur (bovine ou porcine) 3 g	Peptone de coeur (bovine ou porcine) 3 g
Amidon de maïs 1 g	Amidon de maïs 1 g
Chlorure de sodium 5 g	Chlorure de sodium 5 g
Agar 13,5 g	Agar 13,5 g
Sang (mouton) 50 mL	Sang (mouton) 50 mL
Cyclosérine 0,100 g	Cyclosérine 0,100 g
Céfoxitine 0,008 g	Céfoxitine 0,008 g
Amphotéricine B 0,002 g	Amphotéricine B 0,002 g
Eau purifiée 1 L	Eau purifiée 1 L
pH: 7.3	

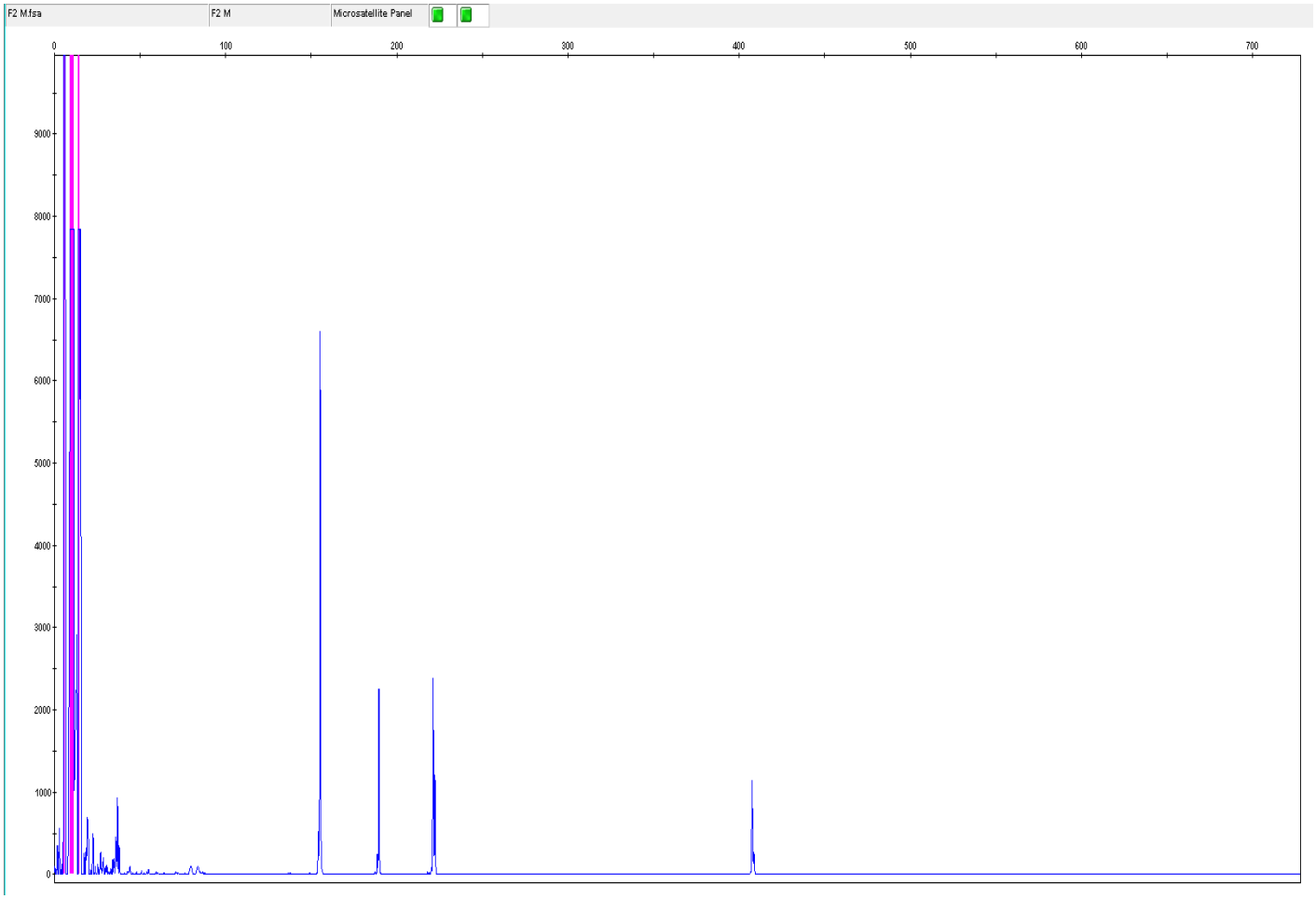
Annex III

DNA quality and quantity assessment by NanoDrop spectrophotometer



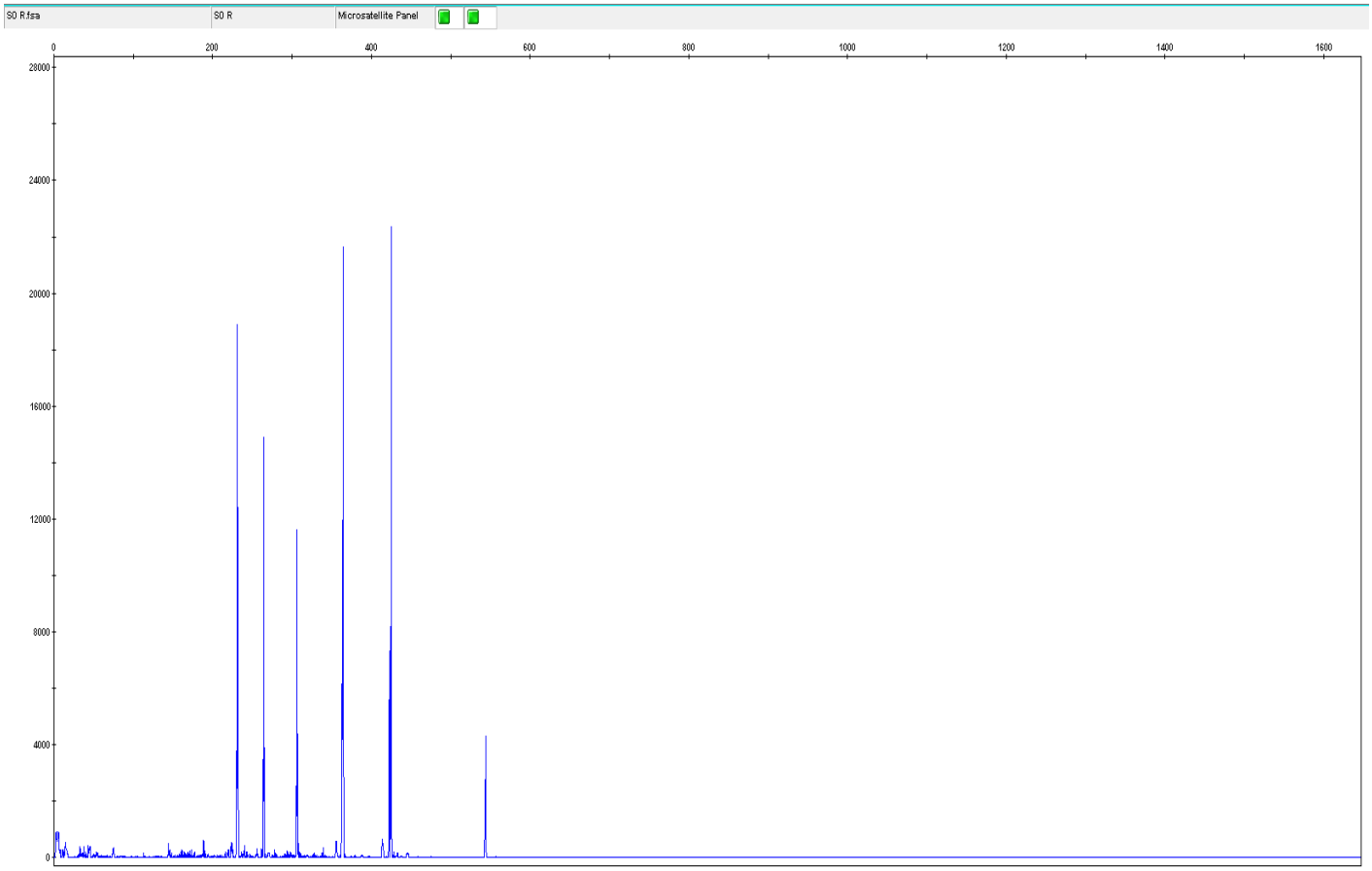
Annex IV

GeneMapper electropherogram for multiplex PCR (strain S0).



Annex V

GeneMapper electropherogram for PCR ribotyping (strain S0).



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Original Article

Molecular epidemiology and antimicrobial resistance patterns of *Clostridioides difficile* isolates in Algerian hospitals

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Abstract

Introduction*: Clostridioides difficile* is a major pathogen responsible for hospital-associated diarrhoea. This study investigated the molecular epidemiology and antibiotic resistance of *C. difficile* isolates in five Algerian hospitals.

Methodology: Between 2016 and 2019, faecal specimens were collected from in-patients and were cultured for *C. difficile*. Isolates were characterised by toxin genes detection, Polymerase Chain Reaction (PCR)-ribotyping, Multilocus Sequence Typing (MLST), antimicrobial susceptibility testing against a panel of antibiotics, and screened for antimicrobial resistance genes.

Results: Out of 300 patient stools tested, 18 (6%) were positive for *C. difficile* by culture, and were found to belong to 11 different ribotypes (RT) and 12 sequence types (ST): RT 085/ST39, FR 248/ST259, FR 111/ST48, RT 017/ST37, RT 014/ST2, RT 014/ST14, FR 247/new ST, RT 005/ST6, RT 029/ST16, RT 039/ST26, RT 056/ST34 and RT 446/ST58. MLST analysis assigned the isolates to two clades, 1 and 4. Clade 4 was more homogeneous, as it mainly included non-toxigenic isolates. Three toxin gene profiles were detected, two toxigenic, A+B+CDT- (33.3%) and A-B+CDT- (11%); and one non-toxigenic, A-B-CDT- (55.5%). All *C. difficile* isolates were susceptible to metronidazole, vancomycin and moxifloxacin.

Conclusions: Overall prevalence of *C. difficile* in our healthcare settings was 6%. Antibiotic resistance rates ranged from 72.2% (clindamycin) to 16.6% (tetracycline). This study highlighted a relatively high genetic diversity in term of ribotypes, sequence types, toxin and antibiotic resistance patterns, in the *C. difficile* isolates. Further larger studies are needed to assess the true extent of *C. difficile* infections in Algeria.

Key words: *Clostridioides difficile*; Algeria; ribotyping; MLST; antibiotic resistance; toxins.

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Introduction

Clostridioides difficile, formerly known as *Clostridium difficile*, a Gram-positive, anaerobic, spore-forming, toxin producing bacteria, is the leading cause of healthcare-associated diarrhoea [1]. The symptoms of *C. difficile* infections (CDI) can range from mild diarrhoea to pseudomembranous colitis or toxic megacolon, a severe form of the disease [2]. The major risk factors for CDI are advanced age (≥ 65 years old), antibiotic exposure, a prolonged hospital stay, gastro-intestinal surgery as well as chronic conditions such as inflammatory bowel diseases [2].

The main virulence factors of *C. difficile* are the production of two major clostridial toxins: toxin A (TcdA) and toxin B (TcdB), encoded on a 19.6 kb chromosomally-located pathogenicity locus (*PaLoc*), which have cytotoxic and enterotoxic effects, respectively [3]. However, certain strains of *C. difficile* produce a third toxin, called binary toxin (CDT), which acts as an actin-specific ADP-ribosyltransferase, encoded by the *cdtA* and *cdtB* genes, located outside the *PaLoc* [4].

The increase in the incidence of CDI reported worldwide over the last two decades was mainly attributed to the emergence of hypervirulent, multidrug-

resistant strains, such as the epidemic NAP1/BI/027 strain [5]. Compared to Europe and North America, CDI is a largely neglected disease in the developing countries and epidemiological data on *C. difficile* are scarce or lacking. In Africa, the highest prevalence of *C. difficile* was reported in Kenya (93.3%) [6] and the lowest in the Ivory-Coast (2%) [7], whereas in the Middle-East, the highest prevalence was recorded in Lebanon (82.9%) [8], and the lowest in Kuwait (0.5%) [9]. In Algeria only one study was conducted reporting a prevalence of 6.9% [10]. The wide variability in the above prevalence estimates is presumably due to a combination of factors such as study designs, type of population studied and *C. difficile* identification methods.

The aim of this study was to investigate the molecular epidemiology and antibiotic resistance of *C. difficile* in five hospitals located in three different provinces of Algeria.

Methodology

Study design and sample collection

Unformed stool samples were collected from patients admitted to five hospitals in three different provinces, Batna, Ain Defla and Chlef; located in the East, Centre and West of Algeria, respectively, between January 2016 and January 2019. All patients included in the study developed diarrhoea, defined as 3 or more loose or liquid stools per day, or more frequently than is normal for the individual (as defined by the World Health Organization, http://www.who.int/topics/diarrhoea) [11], after three days of admission with or without previous antibiotic treatment. Infants under the age of 2 years old are excluded from this study due to the high asymptomatic carriage of *C. difficile* in this group [12].

C. difficile culture and identification

Following alcohol-treatment of the stool samples to eliminate vegetative cells [13], the remaining spores were cultured on a selective medium (*Clostridium difficile* chromogenic agar; ChromID CDIF, Biomérieux, Marcy l'Etoile, France). The plates were incubated at 37 °C for 48 h under anaerobic conditions (10% H, 2.5% CO_2, 85% N_2) using AnaeroGen 2.5L (Thermo scientific, Tokyo, Japan). Black colonies or suspicious non-black colonies (based on the morphological aspect) were identified using Matrix Assisted Laser Desorption Ionisation-Time Of Flight mass spectrometry (MALDI-TOF MS) (Microflex LT BRUKER, Madison, USA).

Molecular identification and toxin genes detection

Genomic DNA was extracted using InstaGene Matrix Kit (Bio-Rad, Hercules, USA), following the manufacturer's instructions. *C. difficile* isolates were stored at -80 °C using Microbank mixed microbial storage vials (Pro-Lab diagnostics, Ontario, Canada).

For molecular characterisation of *C. difficile* isolates and toxin genes, a multiplex PCR assay was carried out according to the protocol of Barbut *et al*., 2019 [14], using seven pairs of primers targeting the following genes: *tpi*, (triose phosphate isomerase), *tcdA* (toxin A), *tcdB* (toxin B) *cdtA* and *cdtB* (binary toxin subunits), the PaLoc and *tcdC* (negative regulator for toxin expression) [15]. *C. difficile* PCR-ribotype (RT) 027, was used as positive control. The amplicons were analysed using a high-resolution capillary electrophoresis detection system (HITACHI ABI 3500 Genetic Analyzer, Applied Biosystems, Massachusetts, USA). The results were visualised using GeneMapper Software version 5.0 (Applied Biosystems, Massachusetts, USA).

Polymerase Chain Reaction (PCR)-ribotyping

PCR-ribotyping was performed according to the protocol recommended by The European Centre for Disease Prevention and Control (ECDC), using primers designed by Bidet *et al* [16]. For the amplification of the 16S-23S rRNA intergenic region, a capillary electrophoresis was performed using a Genetic Analyser (HITACHI ABI 3500, Applied Biosystems, Massachusetts, USA) and electrophoreograms were visualised using GeneMapper Software version 5.0 (Applied Biosystems, Massachusetts, USA). PCR-ribotype (RT) was determined using WEBRIBO database version 2.2 available at: https://webribo.ages.at/. When PCR-ribotyping profiles are unknown, the prefix "FR" was used (French reference laboratory internal nomenclature).

Multilocus sequence typing (MLST)

MLST was performed as described by Griffiths *et al* [17], using PCR primers targeting seven housekeeping genes (*adk*, *atpA*, *dxr*, *glyA*, *recA*, *soda* and *tpi*). The sequence type (ST) and clade were determined by comparing the sequences of strains with the MLST database (http://pubmlst.org/cdifficile).

Antimicrobial susceptibility testing

Antimicrobial susceptibility to clindamycin (CLD), erythromycin (ERY), moxifloxacin (MXF) and tetracycline (TET) was assessed using the disk diffusion method (I2A, France). For metronidazole

(MTZ) and vancomycin (VAN), the minimal inhibitory concentration (MIC) was determined using an E-test (Biomérieux, Marcy l'Etoile, France). Brucella blood agar plates supplemented with 0.5 mg/L hemin, 1mg/L Vitamin K1 and 5% sheep blood (Becton Dickinson GmbH, Heidelberg, Germany) were inoculated with 1.5 MacFarland bacterial suspension. Plates were then incubated for 48 h at 37 °C in an anaerobic atmosphere using AnaeroGen 2.5L (Thermo scientific, Tokyo, Japan). The diameter of inhibition zones was interpreted according to the CA-SFM 2019 recommendations (Antibiotic susceptibility committee of the French society for microbiology) [18]. Breakpoints were set as follows: TET (30 µg) < 19 mm, MXF (5 µg) < 21 mm, CLD (2 UI) < 15 mm, ERY (15 UI) < 22 mm [18]. For MTZ and VAN, MIC breakpoint 2 mg/L was applied as recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [19]. Multidrug-resistance was considered when the strain showed resistance to 3 or more antimicrobial classes.

Detection of antibiotic-resistance genetic determinants

The presence of the resistance genetic determinants for the macrolide-lincosamide-streptogramin B (MLS_B) family of antibiotics (*ermB*), tetracycline (*tetM*, *tetO*, *tetB[P]*, *tet0/32/0*, *tet40*, *tetA[P]*) [20] and fluoroquinolones (*gyrA*) mutations in the quinolone resistance-determining region (QRDR) was investigated as previously described [20,21].

Statistical analyses

The data were coded using Excel 2013 (Microsoft, Redmond, WA, USA) and analyzed by R software (R Development Core Team, 2016). The distribution of prevalence of *C. difficile* between provinces, ages and sexes of patients was tested by Chi-Square test or Fisher's exact test. A level of *p value* < 0.05 was considered as statistically significant.

Results

Prevalence rates of C. difficile

This multi-center, prospective study included a total of 300 patients admitted to five hospitals in three provinces of Algeria over a period of three years. The majority of patients were from the province of Batna (n = 258, 86%, 1 university hospital), followed by Chlef (n = 31, 10.3%, 3 hospitals) and Ain Defla (n = 11, 3.7%, 1 hospital). There were more females (n = 168, 56%) than males (n = 132, 44%); the majority of patients (n = 237, 79%) were adults (≥ 19 years old), and 21% (n = 63) were ≤ 18 years old.

Of the 300 patients, a total of 18 *C. difficile* isolates were cultured, giving an overall prevalence of 6% (CI at 95%: 3.3%-8.7%). The highest prevalence was recorded in the province of Ain Defla (18.2%, 2/11), followed by Chlef (9.7%, 3/31) and Batna (5%, 13/258). The prevalence was higher (11.1%, 7/63) in patients who were ≤ 18 years old than those aged ≥ 19 years old (4.6%, 11/237). The prevalence in females (6.5%, 11/168) was slightly higher than in men (5.3%, 7 /132). However, the differences in *C. difficile*

Table 1. Molecular characterisation of *C. difficile* isolates in the study.

Sample	Province	Hospital	Ward	Year	Gender	Age	Genotyping							
							PaLoc	*tcdA*	*tcdB*	*tcdC*	*cdtA*	*cdtB*	Ribotype	Sequence type (Clade)
CD 038	Batna	UH	MIW	2016	F	Ad	+	-	-	-	-	-	RT 085	39 (4)
CD 053	Batna	UH	MIM	2016	M	Ad	+	-	-	-	-	-	RT 039	26 (1)
CD 093	Batna	UH	REA	2016	M	Ad	+	-	-	-	-	-	RT 085	39 (4)
CD 137	Batna	UH	PED	2017	F	Ch	+	-	-	-	-	-	RT 085	39 (4)
CD 144	Batna	UH	PED	2017	M	Ch	+	-	-	-	-	+	FR 111	48 (1)
CD 147	Batna	UH	MIW	2017	F	Ad	+	-	-	-	-	+	FR 111	48 (1)
CD 155	Batna	UH	MIM	2017	M	Ad	-	+	+	$+^{NID}$	-	+	RT 014	2 (1)
CD 181	Batna	UH	PED	2017	F	Ch	-	+	+	$+^{NID}$	-	+	RT 014	14 (1)
CD 190	Batna	UH	MIW	2017	F	Ad	-	+	+	$+^{NID}$	-	+	RT 056	34 (1)
CD 202	Batna	UH	MIW	2017	F	Ad	+	-	-	-	-	-	FR 247	New * (1)
CD 210	Batna	UH	MIW	2017	F	Ad	-	+	+	$+^{NID}$	-	+	RT 446	58 (1)
CD 213	Batna	UH	MIM	2017	M	Ad	-	+	+	$+^{NID}$	-	+	RT 005	6 (1)
CD C05	Chlef	Chettia	MIW	2018	F	Ad	-	+	+	$+^{NID}$	-	+	RT 029	16 (1)
CD C13	Chlef	Chettia	PED	2018	F	Ch	+	-	-	-	-	-	FR 248	259 (4)
CD D04	Ain Defla	S/Bobida	PED	2018	M	Ch	+	-	-	-	-	-	FR 248	259 (4)
CD D07	Ain Defla	S/Bobida	PED	2018	M	Ch	+	-	-	-	-	-	FR 248	259 (4)
CD F02	Chlef	Chorfa	PED	2018	F	Ch	-	$+^{ID}$	+	$+^{NID}$	-	-	RT 017	37 (4)
CD S0	Batna	UH	MIW	2018	F	Ad	-	$+^{ID}$	+	$+^{NID}$	-	-	RT 017	37 (4)

UH: University hospital; MIW: Women's internal medicine; MIM: Men's internal medicine; PED: Paediatric; F: Female; M: Male; Ad: Adult; Ch: Child; ID: internal deletion; NID: no internal deletion; * The closest match to STs: 69; 104 and 596.

prevalence between the three provinces, genders and age groups were not statistically significant (p value > 0.05).

Detection of tcdA, tcdB, cdtA/B and tcdC genes

A PCR multiplex assay for the detection of *tcdA*, *tcdB* and *cdtA/B* genes, revealed the presence of three toxin genes profiles: six *C. difficile* isolates (33%) with intact *tcdA* and *tcdB,* deleted *cdtA* and the *cdtB* as a pseudogene (A+B+CDT-); two *C. difficile* isolates (11%) revealed a deletion in *tcdA* and intact *tcdB*, deleted *cdtA* and *cdtB* (A-B+CDT-); the remaining ten *C. difficile* isolates (55.5%) did not carry any of the toxin genes (A-B-CDT-) (Table 1). The same analysis revealed that the *tcdC* gene was present (without internal deletion) in all the toxigenic isolates and absent in all the non-toxigenic isolates.

PCR ribotyping and multilocus sequence typing

The 18 *C. difficile* isolates were assigned to 11 different ribotypes and 12 sequence types: RT 085/ST39 (n = 3), FR 248/ST259 (n = 3), FR 111/ST48 (n = 2), RT 017/ST37 (n = 2), RT 014/ST2 (n = 1), RT 014/ST14 (n = 1), FR 247/new ST (n = 1), RT 005/ST6 (n = 1), RT 029/ST16 (n = 1), RT 039/ST26 (n = 1) and RT 056/ST34 (n = 1), RT 446/ST58 (n = 1) (Table 1). The three unrecognized isolates, FR 111, FR 247 and FR 248, detected in this study corresponded to ribotypes maintained in the internal database of the French National Reference Laboratory for *C. difficile*.

Our *C. difficile* isolates were classified into two MLST clades, 1 and 4; (Table 1) clade 1 was more heterogeneous and consisted of a diverse set of isolates, RT 005/ST6, RT 14/ST2, RT 14/ST14, RT 029/ST16, RT 039/ST26, RT 056/ST34, FR 111/ST48, RT 446/ST58 and FR 247/New ST; whereas clade 4 included RT 85/ST39, RT 17/ST37 and FR 248/ST259. In addition, clade 4, include mainly non-toxigenic isolates (33.33%), with the exception of 2 isolates belonging to RT 17/ST37, which produce toxin B only.

Detection of antimicrobial susceptibility and antibiotic-resistance genes

Antibiotic-susceptibility data of the 18 *C. difficile* isolates are presented in Table 2. All isolates were susceptible to MTZ and VAN, the first line of antibiotics used for the treatment of CDI, and to MXF, a fourth-generation fluoroquinolone. Sequence analysis showed no substitution in the QRDR of *gyrA* of all our isolates.

Five isolates (27.7%, 5/18) belonging to RT 005 (n = 1), RT 039 (n = 1), RT 085 (n = 1) and FR 111 (n = 2) were resistant to the MLS_B family antimicrobials CLD and ERY, conferred by the presence of the *ermB* gene.

A similar resistance rate against the macrolide antibiotics CLD was found (27.7%, 5/18) in the isolates

Table 2. Antimicrobial resistance patterns of *C. difficile* isolates.

RT Stains		ATB (mm)						MIC (mg/L)			
		TET		CLD	ERY	MXF		MTZ		VAN	
		S	R	S	R	S	R	S	R	S	R
		tetM (+)		*ermB* (+)							
		< 15	≥ 19	< 15	≥ 15	< 18	≥ 21	< 4	≥ 4	< 1	≥ 2
RT 085	038	S		R +	S	S		S		S	
	93	S		R +	R +	S		S		S	
	137	S		S	R +	S		S		S	
FR 248	C13	S		R	S	S		S		S	
	D04	S		R	S	S		S		S	
	D07	S		R	S	S		S		S	
RT 014	155	S		S	S	S		S		S	
	181	R +		R +	R +	S		S		S	
RT 017	F2	R +		R +	R +	S		S		S	
	S0	R +		R +	R +	S		S		S	
FR 111	144	S		R +	R +	S		S		S	
	147	S		R +	R +	S		S		S	
RT 005	213	S		R +	R +	S		S		S	
RT 029	C05	S		R	S	S		S		S	
RT 039	053	S +		R +	R +	S		S		S	
RT 056	190	S		S	S	S		S		S	
RT 446	210	S		S	S	S		S		S	
FR 247	202	S		S	R -	S		S		S	

RT: ribotype; ATB: antibiotic; R: resistant; S: susceptible; TET: tetracycline; MXF: moxifloxacin; ERY: erythromycin; CLD: clindamycin; MTZ: metronidazole; VAN: vancomycin; MIC: minimal inhibition; concentration; +: presence of the gene; -: absence of the gene.

of the ribotypes RT 29 (n = 1), RT 085 (n = 1) and FR 248 (n = 3); one of which (RT 29) was not found to carry the *ermB* gene.

Two RT 085 (n = 1) and FR 247 (n = 1) isolates were resistant to ERY only. The latter isolate did not carry the *ermB* gene. Three isolates belonging to RT 014 (n = 1) and RT 017 (n = 2), which carried the *ermB* and *tetM* genes, were resistant to CLD, ERY and TET.

The remaining three isolates RT 014 (n = 1), RT 056 (n = 1) and RT 446 (n = 1) were susceptible to CLD, ERY and TET, and were not found to carry the *ermB* and the *tetM* genes. Interestingly, one isolate of the RT 039 harbored the *tetM* gene, but was susceptible to TET.

The two isolates of the RT 014, which belonged to two different STs, ST2 and ST14, also exhibited different antibiotic resistance phenotypes and genotypes; one isolate (RT 014/ST14) was resistant to CLD, ERY and TET, and carried the *ermB* and *tetM* genes; whereas the second (RT 014/ST2) was susceptible to these three antibiotics and did not carry the *ermB* and *tetM* genes. Of note, these two isolates were recovered from two different patients admitted to different wards of the same hospital.

Discussion

C. difficile has been identified as a leading nosocomial pathogen worldwide and the main causative agent of antibiotic-associated diarrhoea in humans [1]. Although *C. difficile* infections were generally regarded as primarily healthcare-associated, and community-acquired, *C. difficile* infections have now emerged as a significant public health concern [2]. Algeria is at the crossroads to Europe, Africa, and the Middle-East; and like in many developing countries, CDI is a largely neglected disease, and epidemiological data on *C. difficile* are scarce. There is, however, one previous study, based in two hospitals in one province [10]. Therefore, this study was conducted to investigate the prevalence and the molecular epidemiology of *C. difficile* over a wider geographic region, in five study sites in three provinces of Algeria. Interestingly, the prevalence of *C. difficile* estimated here (6%) was similar to the previous Algerian study (6.9%) [10].

The prevalence estimated in this study was comparable to those reported in certain African and Middle-Eastern countries such as Ghana (4.9%) [22], Tanzania (6.4% and 7.3%) [23,24], Zimbabwe (8.6%) [25], Iran (9%) [26,27], Qatar (7.9%) [28] and Saudi Arabia (8.4%) [29], and also falls within the range reported in a European multi-country surveillance study, from 4% to 39% [30] and in the United States from 6% to 48% [31].

Compared to the previous Algerian study, which reported the detection of only four ribotypes [10], our study revealed a relatively larger diversity of PCR ribotypes. The most prevalent RT were RT 085 and FR 248 (n = 3, 16.7%, each), followed by RT 014/RT 017/FR 111 (n = 2, 11.1%, each) and RT 005/RT 029/RT 039/RT 056/RT 446/ FR 247 (n = 1, 5.6% each). All isolates of the same RT belonged to the same ST, with the exception of RT 014, which was shared by two different STs, ST2 and ST14 (1 isolate each), which is in agreement with a previous study [32]. Among the above ribotypes, only RT 014 was previously reported in Algeria, but in a different hospital [10].

The ten *C. difficile* isolates were non toxigenic and belonged to ribotypes RT 039, RT 085, FR 111, FR 247 and FR 248; with RT 085 and FR 248 as the most prevalent (n = 3, 16.7%, each). The three isolates of RT 085 were detected in three different wards of the same hospital in the eastern province of Algeria (Batna), but were missing in the two other provinces; suggesting a possible distinct geographic distribution of this ribotype in Algeria. Although, the ribotype RT 085 was reported as more common in China [33], it was rarely reported in other countries.

It is also worth mentioning that all the three isolates belonging to the unclassified ribotype FR 248 were recovered from children, that were admitted to two different hospitals from two provinces (Ain Defla and Chlef), and as such, the possibility of an association of this ribotype with children is plausible.

The remaining non toxigenic ribotype isolated with a lesser frequency (n = 1), RT 039, was previously reported as most common in patients with cystic fibrosis in Western Australia [34], and was also detected in health care settings in Iran [35], and Kuwait [36]. Surprisingly, several isolates of RT 039 from Iran were found to carry the toxin genes [35]. In addition, isolates of RT 039 were also recovered from animal samples in the Netherlands [37] and Egypt [38].

Toxigenic isolates accounted for 44.4% (8/18) of the total and were shared between six ribotypes, 005, 014, 017, 029, 056 and 446; among which, those belonging to RTs 014 and 017 were the most frequent (n = 2, 11.1%, each). Isolates of the RT 014 were the most prevalent ribotype in many European countries, where it was reported as responsible for CDI outbreaks in humans, and also commonly associated with animals and different environments [37,39–42]. The RT 014 was also detected in several countries in the Middles-East, Iran [35], Lebanon [8] and Qatar [28]; whereas in the African continent it was reported only in Algeria [10] and South Africa [43].

The other most prevalent ribotype in this study, RT017, which is characterized by a deletion in the *tcdA* gene and the absence of binary toxin genes and, therefore, A-B+CDT- [44], is the predominant ribotype in Asia, and has also caused major outbreaks of CDIs in several countries around the world [45,46]. To date, the only African country that reported the ribotype RT 017 is South Africa [43,47], but it has not been detected so far in any of the Middle-eastern countries.

The toxigenic PCR-ribotype RT 029 was previously reported as one of the most frequent RTs among hospitalized patients in Iran [35], and was also isolated from humans in Egypt [48] as well as from humans and animals in Costa Rica [49].

The toxigenic isolate belonging to the PCR-ribotype RT 056 detected in this study, was commonly isolated from humans, cattle, vegetables and the environment in Australia [42]. Prior studies reported that RT 056 was frequently associated with complicated CDI in hospitalized patients in Europe [39,50]. The only report to date of this PCR-ribotype in Africa comes from Zimbabwe [25], whereas in the Middle-East it was reported in Qatar [28] and Kuwait [9,36].

Although the toxigenic RT 005 identified in this study is among the most common ribotypes in Europe [51], it was isolated with a low frequency in a study from Ghana [52], and has not been documented so far in the Middle-East.

Importantly, both this and the previous Algerian study failed to detect the hypervirulent ribotypes RT 027 or RT 078. It must be noted that the ribotype RT 027 was not reported in the African continent, whereas, in the Middle-East, it was detected, albeit with low frequency, in Iran (n = 14) [26,53,54], Saudi Arabia (n = 4) [55] and Qatar (n = 1) [28]. Similarly, there is very little data on the ribotype RT 078 in Africa and the Middle-East, except for two reports from Egypt (n = 6) and Kuwait (n = 9) [36,48].

It is worth mentioning that the detection of the toxigenic ribotypes RT014, RT017, RT029 and RT 056 in this study is important and interesting from an epidemiological point of view, given that these ribotypes were reported to be either responsible for CDIs in several countries around the world (RT017, RT 014 and RT 056) [50], or commonly associated with animals (RT 014, RT 029 and RT 056) [56], raising concerns about their potential zoonotic transmission.

Antibiotic susceptibility test results have shown that all 18 *C. difficile* isolates were susceptible to VAN and MTZ, the treatment of choice for CDI [57], as well as to MXF, a fourth generation fluoroquinolone [58], which is in line with the results of several other studies [59].

Thirteen MLS_B-resistant isolates (72.2%, 13/18), carried the *ermB* gene, and were resistant to CLD and/or ERY; whereas two (11.1%, 2/18) MLS_B-resistant isolates were *ermB*-negative but resistant to either CLD or ERY; suggesting that the MLS_B resistance in these isolates might be conferred by other mechanisms; which is in agreement with previous studies [20,60].

We noticed that resistance to TET was always associated with co-resistance to CLD and ERY, as is the case for three isolates belonging to RT 014 (n = 1) and RT 017 (n = 2), which harboured both the *tetM* and the *ermB* gene. The other TET resistance genes investigated in this study (*tetO*, *tetB[P]*, *tet0/32/0*, *tet40*, *tetA[P]*) were not detected in any of our 18 isolates. Surprisingly, one isolate, member of RT 039, was susceptible to TET despite carrying the *tetM* gene. Given that *tetM* is the predominant TET resistance genetic determinant in *C. difficile*, the exact mechanism behind this peculiar phenotype is unclear at this stage; it is possible, however, that the *tetM* gene in this isolate was inactive due to a mutation.

The high rate of resistance of RT 017 to many antimicrobial agents has been largely documented in several studies, and considered as a major contributing factor to the success and dissemination of this ribotype throughout the world [61,62].

There are a number of limitations within this study that needs to be highlighted; first, and most important, our study lacked clinical patient data; second, this is a study based on a small sample size of isolates; third, *C. difficile* isolates were collected in three geographical areas and five hospitals, which may limit the generalization of the *C. difficile* prevalence estimates to the whole country; fourth, lack of antibiotic susceptibility testing against other important antibiotics used for the treatment of CDI such as fidaxomicin and rifaximin. Clearly, larger studies, over wider geographical area and larger number of study sites are merited.

Conclusions

The present study revealed a moderate prevalence of CDI (6%), with a relatively high diversity of *C. difficile* isolates, some of which were toxigenic. All isolates were susceptible to VAN and MTZ; whereas a high proportion of the isolates showed resistance to CLD and/or ERY. Although well-known hypervirulent *C. difficile* strains such as RT 027 and RT 078 were not detected in this study, our findings highlight the significance of this pathogen in a sample of the Algerian

population, and therefore, an active surveillance of CDI is crucial in order to have a more generalized estimation of the burden of this disease in the country.

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Authors' contributions

YB collected the specimens, and isolated, cultured and confirmed the tested isolates. YB, JC, RSZ and FB confirmed the tested isolates with mass spectrometry, carried out the toxin gene profiling and ribotyping; and performed the antibiotic resistance tests. KM performed the MLST and characterized the antibiotic resistance genes. DA helped in the culturing, identification of the isolates and interpretation of the results. SR was involved in the inclusion of one group of patients and provided their specimens. MEB performed the statistical analysis. YB wrote the draft manuscript. FB, KM, DA and MS reviewed the manuscript. MS conceived the study, supervised the research and revised the manuscript.

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