UNIVERSIDAD DE COSTA RICA
SISTEMA DE ESTUDIOS DE POSGRADO

POSIBLES FENOTIPOS ASOCIADOS A LA VIRULENCIA DE LAS CEPAS ST01 Y NO-ST01 NO EPIDÉMICAS DEL CLADO MLST 2 DE *Clostridioides difficile*

Tesis sometida a la consideración de la Comisión del Programa de Estudios de Posgrado en Microbiología, Parasitología, Química Clínica e Inmunología para optar por el grado de Maestría Académica en Microbiología con énfasis en Bacteriología

ADRIANA BADILLA LOBO

Ciudad Universitaria Rodrigo Facio, Costa Rica

2021

Dedicatoria

A mis padres, hermana y amigos.

Agradecimientos

A Luis Enrique Chaves, Gloriana Escalante y Daniela Aguilar, por sus colaboraciones en actividades experimentales.

A Pablo Vargas y Robin Cárdenas, por su indispensable ayuda en el laboratorio.

Al personal del Centro de Investigación en Enfermedades Tropicales (CIET), por siempre brindarme su ayuda en el desarrollo experimental de este proyecto.

A Francisco Vega y Emmanuel Aguilar, por su colaboración en la obtención y preparación de líneas celulares empleadas en esta investigación.

Al Doctor Carlos Chacón por su apoyo y constante motivación.

A los Doctores Carlos Quesada y Esteban Chaves, por su conocimiento, su atención, sus sugerencias y motivación durante esta experiencia académica.

A mi tutor, Dr. César Rodríguez, por su conocimiento, consejos y apoyo. Por siempre motivarme, por su paciencia y compañía, así como por su dedicación durante todo el desarrollo de este proyecto.

"Esta tesis fue aceptada por la Comisión del Programa de Estudios de Posgrado en Microbiología, Parasitología, Química Clínica e Inmunología de la Universidad de Costa Rica, como requisito parcial para optar al grado y título de Maestría Académica en Microbiología con énfasis en Bacteriología"

LAURA MONTURIOL GROSS (FIRMA)
Digitally signed by LAURA MONTURIOL GROSS (FIRMA)
Date: 2021.08.30 08:21:26 -06'00'

Ph. D. Laura Monturiol Gross
Representante del Decano
Sistema de Estudios de Posgrado

CESAR AUGUSTO RODRIGUEZ SANCHEZ (FIRMA)
2021.08.27 10:03:12 -06'00'

Ph.D. César Rodríguez Sánchez
Profesor Guía

ESTEBAN RICARDO CHAVES OLARTE (FIRMA)
PERSONA FISICA, CPF-09-0085-0582.
Fecha declarada: 27/08/2021 02:13:19 PM
Esta representación visual no es fuente de confianza. Valide siempre la firma.

Ph.D. Esteban Chaves Olarte
Lector

Firmado digitalmente por CARLOS ALBERTO QUESADA GOMEZ (FIRMA)
Fecha: 2021.08.27 10:14:41 -06'00'

Ph.D. Carlos Quesada Gómez
Lector

ALBERTO ALAPE GIRON (FIRMA)
Firmado digitalmente por ALBERTO ALAPE GIRON (FIRMA)
Fecha: 2021.08.30 12:12:50 -06'00'

Ph. D. Alberto Alape Girón
Representante del Programa de Posgrado en Microbiología, Parasitología, Química Clínica e Inmunología

ADRIANA BADILLA LOBO (FIRMA)
Firmado digitalmente por ADRIANA BADILLA LOBO (FIRMA)
Fecha: 2021.08.27 00:05:52 -06'00'

Adriana Badilla Lobo
Sustentante

Índice General

Resumen

Clostridioides difficile es una bacteria anaerobia que causa diarrea, principalmente en personas que han recibido terapia con antibióticos. La incidencia y la mortalidad de las infecciones por *C. difficile* (ICD) han aumentado en las últimas décadas, en parte debido a la propagación mundial de cepas epidémicas, como las cepas ST01. Este grupo de aislamientos pertenece al segundo de los ocho clados en los que se clasifica la población global de *C. difficile* conocida hasta la fecha, según la técnica de tipificación de secuencias multilocus (MLST).

El Clado MLST 2 también incluye numerosas cepas distintas de ST01, que a pesar de su estrecha relación filogenética no se han asociado con brotes ni se han estudiado en detalle.

En este trabajo se realizó una revisión narrativa de la literatura, para recopilar información sobre las características microbiológicas, la epidemiología y la presentación clínica de tantos miembros del Clado MLST 2 como fuera posible, con el objetivo de corroborar si el aumento de la virulencia es un rasgo generalizado entre los miembros de este clado. Por otro lado, dado que la capacidad diferencial de las cepas ST01 y no-ST01 para producir brotes y enfermedades graves puede resultar de variaciones en la expresión de fenotipos asociados a la virulencia, una segunda parte de esta tesis se dedicó a comparar experimentalmente siete cepas del Clado 2 de 5 STs, aislados de pacientes con diarrea, con respecto a los fenotipos relacionados con las etapas temprana, intermedia y tardía del desarrollo de la ICD. En detalle, analizamos la adhesión de las esporas a células epiteliales colorrectales (CEC), la inducción de la reacción proinflamatoria mediada por SlpA, la movilidad y la formación de biopelículas. Además, se estudiaron los niveles y la cinética de la síntesis de TcdB, sus efectos citopáticos asociados y afinidad por diferentes dianas y, por último, la susceptibilidad de las suspensiones de esporas al desinfectante dicloroisocianurato de sodio (NaDCC).

Todas las cepas fueron similares en términos de adherencia de las esporas a CEC, movilidad, capacidad de formación de biopelículas a las 24 y 72 h, y susceptibilidad de las esporas al NaDCC. Por otro lado, aunque las cepas difirieron con respecto a su respuesta proinflamatoria inducida por SlpA, capacidad de formación de biopelículas a las 120 h, producción de TcdB y tipos de efectos citopáticos, las diferencias observadas no fueron exclusivas de las cepas ST01.

En conjunto, la revisión y los hallazgos experimentales indican que la mayor virulencia mostrada por las cepas ST01 i*n vivo* es un fenotipo multifactorial y que la combinación de factores implicada sigue siendo esquiva.

Abstract

Clostridioides difficile is an anaerobic bacterium that causes diarrhea, mainly in individuals who have received antibiotic therapy. The incidence and mortality of *C. difficile* infections (CDI) have increased over the last decades, in part due to the global spread of epidemic strains, such as the ST01 strains. This group of isolates belong to the second of the eight clades into which the global *C. difficile* population known to date is classified, according to the multilocus sequence typing technique (MLST).

The MLST Clade 2 also includes numerous non-ST01 strains, which despite their close phylogenetic relationship have not been associated with outbreaks and studied in detail.

In this work, a narrative literature review was performed to gather information on the microbiological features, epidemiology, and clinical presentation of as many MLST Clade 2 members as possible, aiming to corroborate whether increased virulence is indeed a widespread trait among members of this clade. On the other hand, since the differential ability of ST01 and non-ST01 strains to produce severe disease and outbreaks may result from variations in the expression of virulence-associated phenotypes, a second part of this thesis was devoted to experimentally compare seven Clade 2 strains from 5 STs, isolated from patients with diarrhea, regarding phenotypes related to the early, intermediate, and late-stage of CDI development. In detail, we analyzed the adhesion of spores to epithelial colorectal cells (ECC), induction of SlpA-mediated pro-inflammatory reaction, motility, and biofilm formation. In addition, we studied the levels and kinetics of TcdB synthesis, its associated cytopathic effects, target affinities, and lastly, the susceptibility of spore suspensions to the disinfectant sodium dichloroisocyanurate (NaDCC).

All strains were similar in terms of spore adherence to ECC, motility, biofilm formation ability at 24 and 72 h, and NaDCC spore susceptibility. On the other hand, although the strains did differ regarding their SlpA-induced proinflammatory response, biofilm formation capability at 120 h, TcdB production, and types of cytopathic effects, the dissimilarities observed were not exclusive of ST01 strains.

Altogether, the review and the experimental findings indicate that the higher virulence shown by the ST01 strains *in vivo* is a multifactor phenotype and that the implicated combination of factors remains elusive.

Lista de Cuadros

Lista de Figuras

Lista de Abreviaturas

CDI: *Clostridioides difficile* infection.

TcdA: Toxin A.

TcdB: Toxin B.

PaLoc: Locus of pathogenicity.

PFGE: Pulsed-field gel electrophoresis.

MLST: Multilocus sequence typing.

NAP: North-American pulsed-field types.

RT: PCR ribotype.

ST: Sequence type.

HS: Histopathological score.

CA: Community-acquired.

SlpA: Surface layer protein.

Cwp84: Cell-wall protein 84.

GTD: Glycosyltransferase domain.

CPE: Cytopathic effect.

REA: Restriction endonuclease analysis.

MLVA: Multilocus variable-number tandem-repeat analysis.

cgMLST: Core genome MLST.

wgMLST: Whole genome MLST.

GTPases: Guanosine triphosphatases.

FAK: Focal adhesion kinase.

FQ: Fluoroquinolones.

MGEs: Mobile genetic elements.

SNPs: Single nucleotide polymorphisms.

IL1-β: Interleukin 1-β.

TNF-α: Tumoral necrosis factor α.

TcdBv: Variant TcdB.

AST: Aspartate aminotransferase.

CCFA: Cefoxitin-cycloserine-fructose agar.

BHI: Brain heart infusion.

TSA: Trypticase soy agar.

PBS: Phosphate buffer saline.

ECC: Epithelial colorectal cells.

MOI: Multiplicity of infection.

EMEM: Eagle´s minimal essential medium.

CFU: colony forming units.

PVDF: Polyvinylidene difluoride.

MS: Mass spectrometry.

ELISA: Enzyme-linked immunosorbent assay.

CV: Crystal violet.

BHI-CY: BHI broth supplemented with cysteine and yeast extract.

OD: Optical density.

WB: Western blotting.

DMEM: Dulbecco modified Eagle medium.

FBS: Fetal bovine serum.

SDS: Sodium dodecyl sulfate.

ANOVA: Analysis of variance.

HMW: High molecular weight.

LMW: Low molecular weight.

NaDCC: Sodium dichloroisocyanurate.

LRF: Logarithmic reduction factor.

MDDC: Monocyte-derived cells.

Cryo-ET: Cryo-electron tomography.

c-di-GMP: Cyclic diguanosine monophosphate.

***lrp*:** Leucine-responsive regulatory protein.

***flg*:** Flagellar switch.

CROPS: combined repetitive oligopeptides.

RBD: Receptor binding domain.

CDT: binary toxin.

TEM: Transmission electron microscopy.

UNIVERSIDAD DE COSTA RICA

SEP Sistema de Estudios de Posgrado

Autorización para digitalización y comunicación pública de Trabajos Finales de Graduación del Sistema de Estudios de Posgrado en el Repositorio Institucional de la Universidad de Costa Rica.

Yo, Adriana Badilla Lobo, con cédula de identidad 206810479, en mi condición de autor del TFG titulado Posibles fenotipos asociados a la virulencia de las cepas ST01 y no epidémicas no-ST01 del Clado MLST 2 de Clostridioides difficile.

Autorizo a la Universidad de Costa Rica para digitalizar y hacer divulgación pública de forma gratuita de dicho TFG a través del Repositorio Institucional u otro medio electrónico, para ser puesto a disposición del público según lo que establezca el Sistema de Estudios de Posgrado. **SI** [ ] **NO** * [X]

***En caso de la negativa favor indicar el tiempo de restricción:** 1 **año (s).**

Este Trabajo Final de Graduación será publicado en formato PDF, o en el formato que en el momento se establezca, de tal forma que el acceso al mismo sea libre, con el fin de permitir la consulta e impresión, pero no su modificación.

Manifiesto que mi Trabajo Final de Graduación fue debidamente subido al sistema digital Kerwá y su contenido corresponde al documento original que sirvió para la obtención de mi título, y que su información no infringe ni violenta ningún derecho a terceros. El TFG además cuenta con el visto bueno de mi Director (a) de Tesis o Tutor (a) y cumplió con lo establecido en la revisión del Formato por parte del Sistema de Estudios de Posgrado.

FIRMA ESTUDIANTE

Nota: El presente documento constituye una declaración jurada, cuyos alcances aseguran a la Universidad, que su contenido sea tomado como cierto. Su importancia radica en que permite abreviar procedimientos administrativos, y al mismo tiempo genera una responsabilidad legal para que quien declare contrario a la verdad de lo que manifiesta, puede como consecuencia, enfrentar un proceso penal por delito de perjurio, tipificado en el artículo 318 de nuestro Código Penal. Lo anterior implica que el estudiante se vea forzado a realizar su mayor esfuerzo para que no sólo incluya información veraz en la Licencia de Publicación, sino que también realice diligentemente la gestión de subir el documento correcto en la plataforma digital Kerwá.

1. Introduction

1.1 Stages in the pathogenesis of *Clostridioides difficile* infection (CDI)

C. difficile is a Gram-positive, anaerobic, spore-forming bacterium found in intestinal tracts of animals and humans and the environment. This pathogen is a leading cause of antibiotic-associated diarrhea, which ranges from mild to severe and can progress into life-threatening complications such as pseudomembranous colitis, toxic megacolon, or even death [1–3].

C. difficile infection (CDI) initiates with the oral ingestion of spores, which reach the intestinal environment of a host whose microbiota is structurally and functionally altered by the action of antibiotics [4]. In this niche, spores adhere to epithelial cells and sense the presence of primary bile acids and L-glycine, which in concert act as co-germinants and prompt their germination process [5–7].

The flagella [8] and surface layer composed of SlpA [9] of the emerged vegetative cells mediate their migration through the gut [8,10], adhesion to epithelial cells [5,11], and -at least *in vitro*- biofilm formation, which facilitate the colonization process and provide the necessary protection to stress factors [7,12,13].

Once a determined population density has been reached, the main *C. difficile* virulence factors are expressed. Namely, the Rho-glycosylating toxins A (TcdA) and B (TcdB), encoded by *tcdA* and *tcdB* within the so-called locus of pathogenicity (PaLoc) [14,15], and in some cases also CDT, a binary ADP-ribosylating toxin located elsewhere in the chromosome [15]. The biological outcome of intoxication with these effectors in the intermediate CDI stage is the appearance of cellular alterations and a proinflammatory immune response.

Late stages of CDI involve *C. difficile* growth, coverage of other areas of the intestinal tract, and the beginning of a sporulation process [6]. During this stage, a considerable number of genes are sequentially activated to induce the mother cell to generate a forespore that undergoes metabolic and structural transformations resulting in resistance to environmental factors [6,16,17]. Mature spores can adhere to or penetrate intestinal epithelial cells to foster *C. difficile* persistence, or can be released into the environment through the host's stools [6,17,18]. Spores are the vehicle of transmission of this pathogen by the fecal-oral route, are

highly contagious, can persist on diverse surfaces, and resist widely used disinfectants. Consequently, they are problematic in healthcare settings [16].

Though the events associated with each CDI stage may overlap, this segmentation facilitates the understanding of the infection and the study of different virulence factors expressed during CDI, as we do in this work.

1.2 *C. difficile* typing techniques

C. difficile is distinguished by a high level of genotypic and phenotypic diversity. This high diversity has prompted the development of several typing methods [19–22], which have been exploited in concert to explore *C. difficile* phylogeny, population structure, epidemiology, and transmission [22].

In the early 1980s only phenotypic techniques such as serotyping, autoradiography polyacrylamide gel electrophoresis, and immunoblotting were available [19,23]. These methods were replaced by genotype-based methods, as the latter are characterized by better reproducibility, typeability, and discriminatory power [19,23].

The genotypic-based typing methods are classified according to their targets as band- or sequence-based techniques. Among this subgroup, some of the most frequently used techniques are pulsed-field gel electrophoresis (PFGE), PCR ribotyping, and multilocus sequence typing (MLST) [19,20,23]. They classify *C. difficile* isolates in North-American pulsed-field types (NAP), PCR ribotypes (RT), and sequence types (ST), respectively [19,20,23].

Based on MLST, the known population of *C. difficile* is distributed in eight groups: Clades 1 to 5, plus the cryptic clades C-I, C-II, and C-III [3,24]. Most of these clades include isolates implicated in human CDI cases.

Clade 2 is particularly noteworthy as it includes the “hypervirulent” NAP1/RT027/ST01 strain [2,25], which has been implicated in outbreaks worldwide since the early 2000s [26] and has been associated with more severe CDI cases as well as high recurrence and mortality rates. However, this clade also includes other STs of less explored virulence that have been isolated from clinical samples of infected patients, such as ST32, ST41, ST67, ST154, ST638 [2,21,27,28]. The predominance and epidemicity of the NAP1/ST01 strain motivated us to corroborate whether increased virulence is an exclusive feature of this strain or a common

trait within the clade and the contribution of diverse virulence-related phenotypes to this feature.

1.3 Previous evidence of virulence phenotypes in *C. difficile* MLST Clade 2 strains

In a pioneering study aiming to compare of the *in vivo* virulence potential of representatives from ST01, ST41, ST67, and ST252, [29], our research group reported the following results:

Table 1. Biochemical markers and histopathological findings associated with CDI developed in animal models upon infection/intoxication with various *C. difficile* MLST Clade 2 strains.

	Parameter	**LIBA-5758 (ST01)**	**LIBA-5757 (ST67)**	**LIBA-2811 (ST41)**	**LIBA-5809 (ST252)**	**Negative control**
Murine ileal loop model	Weight/length (mg/cm)	69	40	37	33	25
	MPO activity (U/100 mg tissue)	2.6	1.8	0.9	0.8	0.2
	TNF-α (pg/mL)	68	84	56	8	18
	IL-1β (pg/mL)	510	225	270	200	50
	IL-6 (pg/mL)	450	440	470	355	150
	Inflammation (HS)	4	4	1	1	0
	Edema (HS)	3	2	3	2	1
	Epithelial damage (HS)	3	3	0	0	0
Hamster infection model	Survival percentage (%)/day	0/4	30/7	30/7	100/12	100/12
	Neutrophil count (%)	55	45	33	34	35
	Lymphocyte count (%)	40	52	62	60	61
	Monocyte count (%)	3	2	3	2	2
	Creatinine (mg/dL)	0.31	0.21	0.18	0.16	0.22
	Albumin (g/dL)	2,4	3	2.9	2.2	2.9
	Blood urea nitrogen (mg/dL)	27	22	23	21	17
	Alanine amino-transferase (U/L)	120	65	50	62	52
	Aspartate amino-transferase (U/L)	130	210	200	85	95
	Lactate dehydrogenase (U/L)	710	800	620	280	300
	Inflammation (HS)	3	1	1	1	0

	Edema (HS)	4	4	1	1	1
	Epithelial damage (HS)	4	1	0	0	0

HS: Histopathological score scale that ranges from 0 (absence of alterations) to 4 (severe) [29].

Altogether, these results categorically demonstrate that the ST01 strain shows higher virulence in mice and hamsters compared to the non-ST01 strains. However, the factors that differentiate it from the other STs and explain this apparent "hypervirulence" have not been determined. This gap in knowledge is the rationale behind this work.

2. Thesis outline

This thesis is structured in two chapters. Chapter 1 corresponds to a narrative literature review in which STs from the MLST Clade 2 of *C. difficile* are compared regarding their microbiological features, epidemiology, and clinical presentations. Chapter 2, in turn, summarizes the results of *in vitro* experiments representing different stages in the pathogenesis of CDI, such as the determination of spore adherence to epithelial cells (initial stage), characterization of SlpA and the immune response that it induces, quantitation of motility and biofilm formation capacity, toxin production and their associated cellular alterations (middle stages), and the study of the resistance of spores to sodium dichloroisocyanurate (late stages).

2.1 Justification

The *C. difficile* MLST Clade 2 includes the ST01 strain, which has caused outbreaks in different parts of the world, including Costa Rica [30]. These strains are associated with increased infection rates, severity, and mortality [31].

Other STs belonging to the same "hypervirulent clade" have been isolated from samples of patients admitted in Costa Rican hospitals. They have not been implicated in outbreaks but induce CDI symptomatology in infected individuals.

A comparative study of virulence factors expressed by ST01 and non-ST01 strains at various CDI development stages, has the potential to identify key factors involved in the increased virulence that distinguished the former group of isolates. This knowledge has potential diagnostic and therapeutic applications.

2.2 Hypothesis

Compared to non-ST01 strains from the *C. difficile* MLST Clade 2, ST01 isolates produce spores with higher adherence to epithelial colorectal cells, SlpA that induces higher levels of pro-inflammatory cytokines. Besides, they also show increased motility and biofilm formation capacity, hypersecretion of toxin B with high cytopathic potential, and spores distinguished by a higher level of resistance to disinfectants.

2.3 Objectives

2.3.1 Main objective

To determine virulence-associated phenotypes related to the establishment of the CDI, the pathogenic potential, and the dissemination capacity of ST01 and non-ST01 strains of the *C. difficile* MLST Clade 2, to identify factors that explain the increased virulence and epidemic nature of the ST01 strain.

2.3.2 Specific objectives

- To study different strains from the *C. difficile* MLST Clade 2 regarding their spore adherence to epithelial colorectal cells, expression of SlpA, and its effect on the host's immune response. Also, to compare their colonization capacity through assessment of their motility and biofilm formation ability.
- To determine whether the level of TcdB secretion in different strains from the MLST Clade 2, as well as their glycosylation profile, correlate with their *in vivo* virulence potential.
- To estimate the persistence and dissemination capacity of different strains from the MLST Clade 2 by exploring the resistance of their spores to the disinfectant sodium dichloroisocyanurate.

3. Chapter I. Literature review: "Microbiological features, epidemiology, and clinical presentation of *Clostridioides difficile* strains from MLST Clade 2: a narrative review".

Published in Anaerobe, doi: https://doi.org/10.1016/j.anaerobe.2021.102355

3.1 Abstract

Clostridioides difficile is an emerging One Health pathogen and a common etiologic agent of diarrhea, both in healthcare settings and the community. This bacterial species is highly diverse, and its global population has been classified in eight clades by multilocus sequence typing (MLST). The *C. difficile* MLST Clade 2 includes the NAP1/RT027/ST01 strain, which is highly recognized due to its epidemicity and association with severe disease presentation and mortality. By contrast, the remaining 83 sequence types (STs) that compose this clade have received much less attention. In response to this shortcoming, we reviewed articles published in English between 1999 and 2020 and collected information for 27 Clade 2 STs, with an emphasis on STs 01, 67, 41 and 188/231/365. Our analysis provides evidence of large phenotypic differences that preclude support of the rather widespread notion that ST01 and Clade 2 strains are “hypervirulent”. Moreover, it revealed a profound lack of (meta)data for nearly 70% of the Clade 2 STs that have been identified in surveillance efforts. Targeted studies aiming to relate wet-lab and bioinformatics results to patient and clinical parameters should be performed to gain a more in-depth insight into the biology of this intriguing group of *C. difficile* isolates.

3.2 Introduction

Clostridioides difficile is a Gram-positive, strictly anaerobic, spore-forming bacterium, and a leading cause of antibiotic-associated diarrhea worldwide [32,33]. *C. difficile* infection (CDI) is defined by the presence of diarrhea, which varies from mild to severe, and either by detection of *C. difficile* toxins in stool samples or pseudomembranous colitis in a colonoscopy or histopathologic studies [34]. Other potential complications of CDI include toxic megacolon or even death [35].

CDI typically develops among hospital patients with risk factors beyond receiving antibiotic therapy, such as older age, exposure to health care centers, and comorbidities such as

inflammatory bowel disease and immunodeficiency, among others [36]. However, the epidemiology of CDI is changing, and a growing number of community-acquired (CA) cases have been seen among individuals considered to be at low-risk, including young adults and children [37]. Furthermore, many CDI cases now show more severe symptoms and require more prolonged hospitalizations; hence the burden of this disease on health systems continues to increase [36,38].

The major virulence factors of *C. difficile* are toxins TcdA and TcdB, which belong to the large clostridial glycosylating toxin family. Both toxins play a role in symptom development [14,15]. Nonetheless, as demonstrated by assays in animal models, only TcdB seems to be essential for virulence [39]. Other proteins, such as the surface layer protein (SlpA), cell wall protein 84 (Cwp84), flagellar components, and the binary toxin CDT, have also been shown to play roles in CDI pathogenesis [13,40].

Comparative analyses of global collections of *C. difficile* whole genome sequences have demonstrated that TcdB is much more diverse than TcdA (12 vs. 7 protein sequence subtypes), possibly as a result of a higher mutation rate or distinct recombination and lateral gene transfer events in the former protein [41,42]. Both genes appear to be under purifying selection, although *tcdB* shows more positively selected sites [41,42].

TcdB variants differ in their biological features, immunoactivities, and potential pathogenicity, and this heterogeneity correlates with the severity of clinical outcomes during CDI progression. Depending on the sequence of its glycosyltransferase domain (GTD), which influences toxins functionalities and target affinities, TcdB can induce two types of cytopathic effects (CPE): a classical, arborizing, CPE in which cells develop neurite-like protrusions and remain attached to cell culture plates [43], or a TcsL-like effect that is characterized by cell rounding and clumping and surface detachment [43,44].

Together with polymorphisms in toxin alleles, strain differences in virulence have been traced to antimicrobial resistance patterns [45], spore production and germination capabilities [6], and recently to phase-variable signal transduction systems [46], c-di-GMP levels [47], and the production of alarmones [48], among other factors.

The high diversity that distinguishes this species has encouraged the implementation of various typing techniques, some of which have proven useful in epidemiological investigations and the identification of strains with unique biological properties [23,49].

The first *C. difficile* typing techniques were based on phenotypic traits. However, they suffered from low discriminatory power and reproducibility and were replaced by genotypic approaches.

Genotypic typing methods can be classified according to the nature of their targets and as to whether they are band- or sequence-based (Table 2). Whereas pulsed-field gel electrophoresis (PFGE) is frequently used in North America, PCR ribotyping is the most frequently used *C. difficile* genotyping method in Europe. Multilocus sequence typing (MLST) is more suitable for evolutionary studies, outbreak detection, and transmission or population structure studies [20].

Table 2. *C. difficile* genotyping techniques (Modified from Killgore 2008, Knetsch 2013, and Janezic 2019).

Technique		**Target**
Band-based	Restriction endonuclease analysis (REA)	*Hind*III restriction sites
	Pulsed-field gel electrophoresis (PFGE)	*Sma*I restriction sites
	PCR ribotyping	16S-23S intergenic spacer regions
	Multilocus variable-number tandem-repeat analysis (MLVA)	DNA repeat units
Sequence-based	Multilocus sequence typing (MLST)	Intragenic sequences of 7 *adk*, *atpA*, *dxr*, *glyA*, *recA*, *sodA*, and *tpi*
	Core genome MLST (cgMLST)	Non-repetitive genes conserved in all members of a species

	Whole genome MLST (wgMLST)	Accessory and core genes, including repetitive genes and pseudogenes

MLST allows isolate discrimination through sequencing of 405-500 bp DNA fragments of seven or eight housekeeping genes (for *C. difficile*, seven gene fragments, total length 3501 bp). The obtained sequences are compared with reference sequences uploaded to internet-accessible MLST databases (https://pubmlst.org/organisms/clostridioides-difficile/) and used to generate allelic profiles composed of allele numbers assigned to sequence variants of a given locus. Each unique allele profile is assigned a sequence type number that is stored in the database to facilitate interlaboratory comparison [20,49].

As indicated by MLST, the known population of *C. difficile* can be distributed in eight clades (clades 1 to 5, plus clades C-I, C-II, and C-III) [24,49]. Whereas isolates from clades 1-5 are more often associated with humans [21,22], the so-called cryptic clades C-I to C-III mainly include non-toxigenic isolates from the environment [24] and, to a lesser extent, toxigenic strains implicated in CDI [30]. New STs with different host affinities, ecological adaptations, and virulence potentials are continually being identified.

According to a globally-optimized eBURST cluster analysis [50], the *C. difficile* MLST Clade 2 comprises 84 STs distributed in 15 clonal complexes (Figures 1 and 2). It includes the highly recognized NAP1/027/ST01 strain, and for this reason, it has been repeatedly referred to as the "hypervirulent clade" [2,25] although the virulence and pathogenicity potential of most non-NAP1/027/ST01 strains are poorly understood or unknown. To corroborate whether increased virulence is indeed a widespread trait among members of this clade, we compared in this review microbiological, epidemiological and, clinical features of various *C. difficile* Clade 2 sequence types (STs).

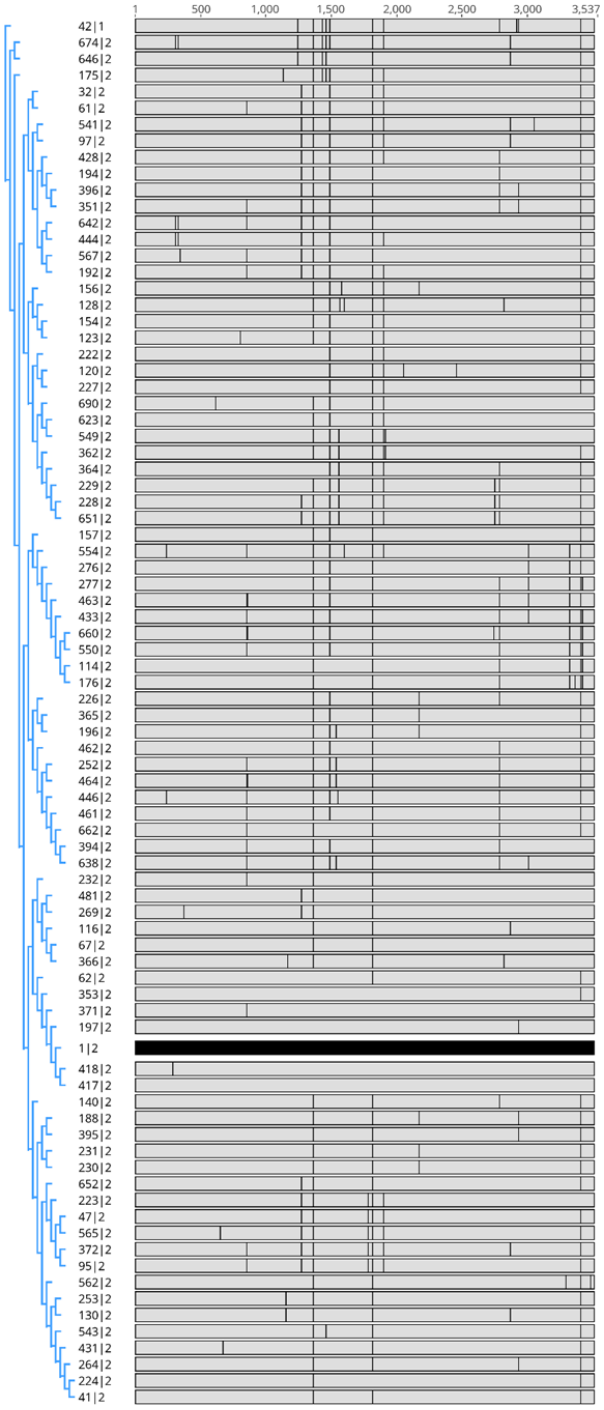
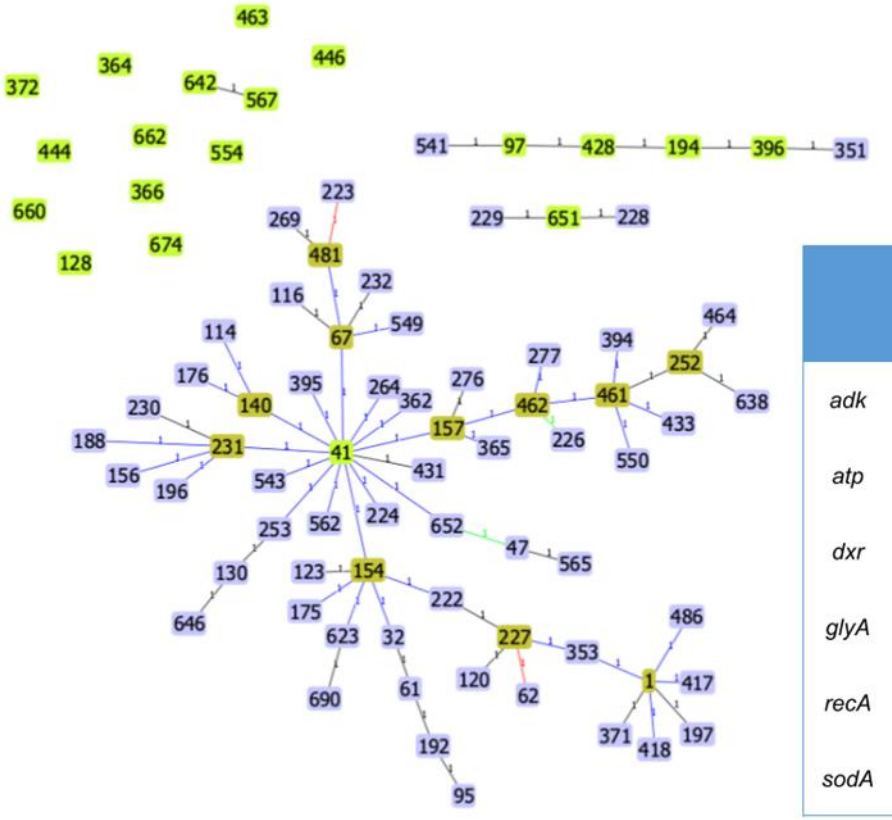


Figure 1. Maximum likelihood phylogenetic tree for 84 sequence types (STs) assigned to the *C. difficile* MLST Clade 2. The tree was generated from an alignment of concatenated DNA allele sequences from the Griffiths scheme that were downloaded from PubMLST. Sequences identifiers include ST and clade. Alignment disagreements to ST01 (black) appear

as vertical black bars. The combined allele sequence of ST42 from Clade 1 was used to root the tree.



	Simpson´s index of diversity	Unique alleles
adk	0.085-0.320	7
atp	0.354-0.574	7
dxr	0.578-0.731	8
glyA	0.792-0.872	8
recA	0.07-0.28	3
sodA	0.552-0.738	9

Figure 2. As indicated by a goeBURST analysis performed with Phyloviz2, the *C. difficile* MLST Clade 2 is composed of 15 clonal complexes. Most STs belong to clonal complex 0 (n=62).

3.3 Search strategy

We collected scientific articles published between 1999 and 2020 in English from the Google Scholar, JSTOR, Scopus, and PubMed databases using keywords such as "*C. difficile* MLST", "*C. difficile* Clade 2", "*C. difficile* ST01", "*C. difficile* ST67", "*C. difficile* ST41", "*C. difficile* typing techniques", "*C. difficile* transmission", "*C. difficile* antibiotic-resistance", "*C. difficile* toxins", "*C. difficile* emergent sequence types". A total of 31 publications were selected to elaborate elementary aspects of *C. difficile*, including its main

virulence factors, CDI pathogenesis, epidemiology, typing techniques and, phylogenetic classification. The number of publications obtained for each ST differed, with a marked predominance of studies on the ST01 strain (n=40). This figure was followed by papers on ST67 and ST41 (ca. 20 publications for each one). Information for STs 188/231/365 was even more scarce (less than 10 publications). A few STs, such as ST62, ST95, ST97, ST114, ST123, ST154, ST192, ST223, ST229, ST264, and ST461-464, have only been analyzed with regards to their virulence factors and origins. No information was found for 55 Clade 2 STs.

3.4 Results

3.4.1 ST01

Microbiological features

ST01 is by far the best-described Clade 2 member. ST01 strains have been reported to overproduce toxins TcdA and TcdB subtype 2 (TcdB2) *in vitro* [41,51,52], arguably as a result of an 18bp deletion and a single-base-pair deletion at position 117 in the gene for the transcriptional regulator TcdC [52,53]. Strains from this ST have been linked to 3- to 23-fold higher titers than those of the main hospital strains, according to results derived from Swedish [31] and Canadian studies [54]. However, other authors have claimed that the toxin levels produced *in vitro* by ST01 isolates and bacteria classified to other STs did not differ [55] or that toxin titers were similar but maintained for a longer period of time in the former group of isolates [56]. Whatever the case might be, these results may not be representative of intestinal conditions [57].

Cell cultures inoculated with TcdA and TcdB from ST01 strains develop a classic arborizing CPE due to glycosylation of the small guanosine triphosphatases (GTPases) RhoA, Rac1, Cdc42, and, to a much lesser extent, of Rap and R-Ras [58]. This morphological change is caused by disaggregation of adaptor proteins, such as vinculin and talin, which trigger the disassembly of stress fibers at focal adhesions [43,59]. TcdA inactivates focal adhesion kinase (FAK) and vinculin by direct contact with Src, inhibiting its kinase activity through a glucosyltransferase activity-independent mechanism [59].

ST01 TcdA and TcdB show increased cytopathic activity. This feature has been attributed to a rapid release of enzymatically active domains into the cell cytosol due to efficient auto-processing [58,60]. Also, ST01 strains synthesize CDT, a member of the Iota family of binary toxins that acts on monomeric globular actin inside epithelial cells, triggering actin cytoskeletal collapse and protrusion of a network of microtubules on the surface of host cells. This structural modification enhances *C. difficile* adherence [61].

An additional factor that presumably contributes to the spread and increased incidence of the ST01 strain is its resistance to fluoroquinolones (FQ) due to a Thre82Ile substitution in the amino acid sequence of the DNA gyrase GyrA [62]. This mutation has been implicated in the transcontinental dissemination of two FQ-resistant ST01 sub-lineages [63]. Other reported mutations related to antibiotic resistance in this ST include a double substitution in the RNA polymerase RpoB (for rifampicin-resistance) and substitutions Glu117Lys and Pro538Leu in the elongation factor G (FusG) for resistance to fusidic acid [63,64]. Some ST01 strains are also resistant to clindamycin, tetracycline, and linezolid due to the acquisition of mobile genetic elements (MGEs) containing *erm*(B), *tetM*, and *cfr*(B) genes, respectively [64,65].

Genomic analyses have revealed that the evolution of ST01 strains is mainly driven by core genome mutations, rather than by homologous recombination [66,67]. Most of these nucleotide substitutions correspond to nonsynonymous single nucleotide polymorphisms (SNPs) in genes for virulence traits or antibiotic resistance [64,67].

Competition studies between ST01, ST02, ST03, and ST08 strains in human fecal bioreactors and a mice model have assigned an increased ecological fitness to ST01 [68]. The exact mechanisms behind this trait are unclear, though it is highly probable that they include an improved ability to compete for limiting nutrients [68]. In this regard, ST01 strains show an SNP in the gene for the trehalose repressor protein TreR (L172I), which confers them the capacity to metabolize low concentrations of trehalose under starvation conditions and increases TcdB production in the presence of this disaccharide [69].

The high sporulation rate of ST01 strains [66] is also a factor to consider, as it likely favors their transmission between patients and from contaminated environments [31,55]. ST01

spores are not only abundant but also highly resistant to stressful environmental conditions and widely used disinfectants, such as quaternary ammonium compounds (benzalkonium chloride) and biguanides (chlorhexidine gluconate), and on a smaller scale to chlorine releasing agents (sodium dichloroisocyanurate) [16].

Epidemiology

ST01 strains gained notoriety in the early 2000s when the number of CDI cases in North America, England, and Europe increased dramatically [51,70,71]. More recently, they have continued causing CDI outbreaks of high severity and mortality in healthcare centers from not only those regions, but also in Latin American countries such as Mexico [72], Honduras [65], Costa Rica [30], Panama [73], and Chile [74].

Besides causing disease in elderly hospitalized inpatient populations with multiple comorbidities, ST01 strains can trigger CDI in children and young adults who have not been received antibiotic therapy [37,75]. However, recent genomic studies have demonstrated that this ST is predominantly nosocomial [76] and that outbreaks associated with it are majorly clonal [51,66]. It should also be noted that patients exposed to FQ are up to 14 times more likely to be affected by ST01 strains than by others [77].

ST01 strains cause many CDI cases and consume more resources because some of them are more likely to give rise to clinical complications [35]. Therefore, as recognized by the diagnostics industry through the development and commercialization of multiple tests, their specific detection improves patient management and CDI control [34,78].

European countries such as the Netherlands have implemented surveillance programs and guidelines that include rapid detection of CDI cases, typing of implicated strains, responsible use of antibiotics, hand hygiene education for hospital staff, disinfection protocols, protective clothing use, and measures related to patient isolation [79]. As a result of these initiatives, the prevalence of ST01 strains in several countries of this region has declined [71,79–81].

Clinical features:

ST01 strains induce strong inflammatory reactions, including neutrophil infiltration and increased myeloperoxidase activity in ileal tissue [77]. They also cause increased secretion of interleukin-1β (IL-1β), IL-6, and tumoral necrosis factor-α (TNF-α), which in concert trigger edema, substantial mucosal disruption, and diarrhea [77,82].

Some studies propose ST01 carriage as a predictor of patient outcome [34,83]. In this regard, ST01 strains are frequently associated with increased disease severity, white blood cells count >15,000 cell/mL, and serum creatinine levels >1.5 mg/dL [84]. Recurrence and mortality rates during the first 30 days of CDI are also more frequent among patients infected with ST01 strains than in those infected with other strains [77,85].

3.4.2 ST67

Microbiological features

Similar to ST01 strains, ST67 isolates i) seem to overexpress *tcdA* and *tcdB in vitro*, ii) give rise to elevated CPE_{50} values, iii) carry CDT genes, and iv) present two types of deletions in *tcdC* [58,86]. On the other hand, members of this ST can harbor either TcdB2 or TcdB4 [41]. Therefore, while some strains induce a classic arborizing CPE, others induce TcsL-like CPEs. The GTD domain of TcdB4 resembles those of variant TcdBs ($TcdB_v$) from $TcdA^-$ strains classified as ST37 [58,87]. This difference holds despite the otherwise high level of identity in the PaLoc sequences of ST67 and ST01 strains, suggesting an ancestral recombination event between ST01 and ST37 isolates [58]. To further argue that the PaLoc of ST67 strains is variable, some authors have reported ST67 isolates with full-length TcdC sequences but with an Asp7Glu substitution [88,89].

As opposed to TcdB2 (classical CPE, ST01 strains), TcdB4 modifies Rac1, Cdc42, Rap, and R-Ras GTPases but does not target RhoA [44,82]. This combined glycosylation profile dismisses vinculin from focal adhesions and disrupts β3-integrin and tyrosine-phosphorylated proteins at cell-to-cell contacts [44]. In addition, R-Ras glycosylation triggers integrin inactivation, leading to loss of adhesiveness to extracellular matrix proteins and ultimately to cell detachment [44].

Whereas some ST67 strains lack antibiotic resistance-associated SNPs in *gyrA* and *gyrB* and display an FQ-susceptible phenotype [58,88], others carry the Thre82Ile substitution in *gyrA* that distinguishes epidemic ST01 strains [62,89]. This heterogeneity is also seen in their antibiotic susceptibility profiles, as some ST67 strains differ concerning their categorization as resistant or susceptible to metronidazole and vancomycin [88–90]. Most strains, however, are susceptible to rifampicin, moxifloxacin, and clindamycin [88].

As with ST01 strains, the trehalose repressor protein TreR of ST67 strains shows an L172I substitution. Therefore, it is anticipated that they would show an advantage over other lineages in competition experiments, as well as increased CDI severity in animal models [31,89].

Epidemiology:

Although ST67 strains have been referred to as "hypervirulent" [86], they are less often recovered than ST01 strains. They have isolated in North America [21,91], Costa Rica [58], Brazil [86], the United Kingdom [66], Finland [92], Thailand [93], and Japan [88,89], yet their dispersion routes have not been explored.

They have been found both in older adults who received antibiotics in hospital settings [88,90], as in individuals ranging from elderly to young from the community, including children who received antibiotic therapy [89].

Clinical features:

In contrast to ST01 strains, a study in animal biomodels demonstrated that ST67 strains prompt a mild or almost undetectable immune cell infiltration in ileal loops. Likewise, cell-free culture supernatants from ST67 isolates induced low MPO activity in this type of tissue and release of low amounts of IL-1β, IL-6, and TNF-α by RAW macrophages [58]. The strains from which these results were derived synthesize high levels of toxins *in vitro*, raising intriguing questions about the pathogenesis of ST67 strains and of CDI in general.

Patients infected with ST67 strains suffer from abdominal pain, repeated vomiting, and mild to severe diarrhea. Moreover, their blood tests show increased leucocyte counts and

creatinine levels [89]. CDI cases attributed to ST67 strains can course with complications such as ascites, ileus, and colonic dilations, pseudomembranous colitis, septic shock, and toxic megacolon [89].

3.4.3 ST41

Microbiological features

As with ST01 strains, ST41 strains synthesize TcdA, TcdB, and CDT [94], and display a truncating mutation in *tcdC* at position 117 [95]. Some isolates, in addition, harbor an 18 bp deletion in positions 316 to 333 of the same gene [96].

The *tcdB* products from ST41 strains can induce either a classical or a TcsL-like CPE [58,96], depending on the presence of TcdB2 or TcdB4, respectively [41]. These opposing features match the substantial difference at a whole-genome level that exists between ST01 and ST41 strains (ca. 10k SNPs) and the early divergence of ST41 from other Clade 2 lineages [87,94].

ST41 strains are susceptible to quinolones, moxifloxacin, ceftriaxone, metronidazole, and vancomycin [87,94]. On the other hand, they show variable susceptibility to clindamycin, as some isolates carry *ermB* [96]. As in ST01 and ST67 strains, an L172I substitution in the trehalose repressor protein TreR have been reported among the ST41 strains [89].

Epidemiology:

ST41 was initially identified as a presumptive ST01 isolate, but further analyses demonstrated that it constituted a separate ST and RT [97]. Though initially isolated in the United States in 2004, ST41 isolates have also been found in South America, Europe, Asia [93], and Australia [94,98], where they participated in a clonal outbreak that lasted from 2010 to 2012 [94]. This ST was also detected in New Zealand during a national survey in 2011[99] and the United Kingdom in early 2012 [100].

Most current knowledge of the epidemiology and ecology of ST41 strains is derived from investigations of isolates recovered in Australia, where it became highly prevalent [100], and

from New Zealand [87,95,99]. In those studies, the majority of ST41 CDI cases were seen in patients <65 years with no recent healthcare exposure [94], common risk factors, or comorbidities, suggesting an unconfirmed, community-based source or reservoir such as root vegetables contaminated with soil or contaminated food and animals [87,101]. One of these strains was transported to the UK by a tourist who visited Australia, highlighting the pathogenic and dissemination potential of ST41 strains [94].

Clinical features:

Patients infected with ST41 are likely to develop severe colitis, fever >38.5^{o}C, white blood cells count >15,000 cell/mL, elevated creatinine levels, and hemodynamic instability, among others. Complications such as pseudomembranous colitis are not rare, as well as abnormalities such as massive intestine distension, colonic wall thickening, and ascites [99]. Also, the mortality rates assigned to CDI cases by ST41 strains can be up to 13 times higher than those recorded for non-ST41 strains [87,95].

3.4.4 STs 188/231/365

Microbiological features:

These STs correspond to RT251. They synthesize TcdA, TcdB2, CDT, and carry a wild type *tcdC* [41,102,103]. All of them induce a classic cytopathic effect [102].

STs 188/231/365 share five/three/four of the seven MLST alleles with ST41 and ST01, respectively, suggesting that they share some degree of evolutionary history [102,103]. Nonetheless, they are not identified in the clinics as ST01 and ST41 strains, possibly due to their lack of polymorphisms in *tcdC*.

Despite their relatedness, STs 188, 231, and 365 differ concerning some phenotypic characteristics. For instance, while ST188 and ST231 strains produce low toxin titers, ST365 toxin levels are comparable to those of ST01 isolates. This difference was explained by the lack of sporulation recorded for ST365 through *in vitro* assays, possibly as a result of a lab setting mutation [102]. Concerning germination and flagellar motility, *in vitro* assays

demonstrated that the germination efficiency of STs 188/231/365 is lower than that of ST01 strains and that ST231 and ST365 isolates reach ST01 motility levels [102].

Isolates from STs 188/231/365 are susceptible to metronidazole, vancomycin, fidaxomicin, rifaximin, amoxicillin-clavulanate, moxifloxacin, meropenem, and tetracycline. On the other hand, ST231 has decreased susceptibility to clindamycin and erythromycin, related to the presence of *ermB* on MGEs [102,103].

Epidemiology:

Although ST188 and ST365 isolates have been seen in North America [102], ST231 strains have been mainly found in New Zealand and Australia, where they have caused severe CDI cases [98,102]. Some ST 188/231/365 strains have been recovered from production animals and meat for human consumption, suggesting that they can disseminate through contaminated food [103,104].

ST231 strains have been found in elderly patients with several comorbidities but also in young, previously healthy individuals with, or without, previous exposure to antibiotic therapy that acquired CA-CDI [103].

Clinical features:

Similar to ST01 strains, ST231 strains caused diarrhea and weight loss, as well as colonic and caecal tissue alterations, such as epithelial damage, crypt hyperplasia, and severe inflammation (neutrophil influx and edema) in a murine model [103].

Human patients infected with ST231 strains suffer from multiple recurrences and also re-infections with different strains. These CDI cases were characterized by the presence of diarrhea and fever in association with abdominal pain and elevated white blood cell counts and C-reactive protein concentrations. Some of them developed pseudomembranous colitis and required colectomy [103].

Table 3 summarizes features that have been reported for STs 01, 67, 41, and 188/231/365.

Table 3. Toxin gene profiles and genotypes implicated in antibiotic resistance reported for ST01, ST67, ST41, and ST 188/231/365 strains (Taken from He 2012, Quesada-Gómez 2016, Hidalgo-Villeda 2018, Saito 2019, Cao 2019, Saldanha 2020, Skinner 2020, Shen 2020, and Guerrero-Araya 2020).

Sequence type(s)	**Toxin profile**					**Genotypes implicated in resistance**	
	tcdA	***tcdB***	***tcdC***	***cdtAB***	**Cytopathic effect**		**Antibiotic**
01	+	TcdB2	18-bp deletion, single bp deletion at position 117	+	Classic[a]	Thre82Ile (*gyrA*)	Fluoroquinolones
						Asp426Asn (*gyrB*)	
						His502Asn, Arg505Lys, Ile548Met (*rpoB*)	Rifampicin
						His455Asn, His455Tyr, Pro538Leu, Glu117Lys (*fusA*)	Fusidic acid
						ermB	Macrolides, lincosamides, streptogramin
						cfr(B), cfr(C)	Linezolid, pleuromutilins, phenicols
						tetM	Tetracycline
67	+	TcdB2 or TcdB4	18-bp deletion, single bp deletion at position 117, Asp7Glu substitution or wild type	+	Classic or TcsL-like	Thre82Ile (*gyrA*)	Fluoroquinolones
						tet genes	Tetracycline
41	+	TcdB2 or TcdB4	18-bp deletion, single bp deletion at	+	Classic or TcsL-like	*ermB*	Macrolides, lincosamides, streptogramin

			position 117				
188/231/365	+	TcdB2 (STs 231 and 365)	Wild type	+	Classic	*ermB* (ST231)	Clindamycin, erythromycin

[a]Classic CPE: arborizing (cells show neurite-like protrusions and remain attached to cell culture plates), TcsL-like CPE: variant (cell rounding and clumping and surface detachment).

3.4.5 Less-studied Clade 2 STs

Though infrequent, strains from ST62, ST95, ST97, ST114, ST123, ST154, ST192, ST223, ST229, ST264, and ST461-464 have been identified in epidemiological surveillance programs in Finland [92], Brazil [105], Japan [106], Costa Rica [28,107], North America, Australia, and Europe [21,25], even as CDI causative agents.

Many of these STs have been misleadingly referred to as ST01-like isolates [92] due to the presence of *tcdA*, *tcdB*, and *cdt* in their genomes [105,106], and also because, with a single exception (ST97), their *tcdC* genes show a deletion at position 117 [92,106]. Some of them are known to carry TcdB2 or TcdB4 (Table 4) and ST154 has toxin plasmids that induce variant CPE [88] (unpublished results). It has been reported that ST95, ST192, ST223, ST229, and ST264 lack mutations in quinolone resistance-determining regions [92]. This bulk of data was for the most part obtained through PCR and Sanger sequencing of discrete genes. Therefore, most genomic and phenotypic features of these STs remain undetermined.

Table 4. Distribution of TcdB subtypes among less frequently isolated *C. difficile* Clade 2 STs (Modified from Shen 2020).

TcdB2		**TcdB4**	
ST32	ST192	ST62	ST196
ST47	ST197	ST114	ST229
ST61	ST362	ST116	ST364
ST95	ST366	ST194	ST396
ST97	ST371		
ST123			

3.4.6 Is the ST01 strain "hypervirulent"?

Numerous studies across the globe have linked increases in the severity, mortality, and recurrence of CDI to the spread of *C. difficile* ST01 strains. For instance, a retrospective study conducted in Québec over 13 years evidenced two-fold or three-fold increments in adjusted odds ratios for mortality and severity, respectively, that coincided with its emergence [108,109]. Similarly, Hubert et al. reported an association between infection with strains that had binary toxin and a partial deletion in *tcdC*, such as ST01, and development of severe diarrhea OR=2.1, (95%CI, 0.98 to 4.6), adjusted for age [110]. In the Netherlands, Goorhuis (2007) detected a clear tendency for more severe diarrhea OR= 1.99 (95% CI, 0.83–4.73), higher attributable mortality OR= 3.30 (95% CI, 0.41–26.4), and more recurrences OR= 1.44 (95% CI, 0.94–2.20) among patients infected with *C. difficile* ST01 than among those infected with other strains [111]. In line with this trend, a multivariate analysis of CDI cases from several US states revealed that the ST01 strain was significantly associated with severe disease, aOR= 1.74, (95% CI 1.36-2.22), severe outcome, aOR= 1.66, (95% CI 1.09–2.54), and death within 14 days after positive stool collection, aOR= 2.12 (95% CI 1.22–3.68) [83]. Another study from this country also found an association between infection with the RT027/ST01 strain and severe CDI and 30-day all-cause mortality, with OR= 1.73 (95% CI, 1.03-2.89) and OR= 2.02 (95% CI, 1.19-3.43), respectively [112].

On the other hand, studies performed in England and the United States have reported opposing findings. For instance, Morgan et al. (2008) could not confirm that patients infected with ST01 strains were more likely to have severe CDI [113]. Cloud et al. (2009) obtained similar findings through the study of 6 different outcomes related to mortality and intensive

care unit admission. None of these outcomes were significantly worse among ST01-infected patients, compared to those infected with other strains [114]. Further studies have also failed to report an association of the ST01 strain with increased disease severity aOR= 0.35; (95% CI, 0.13-0.93), in-hospital mortality aOR= 1.02; (95% CI, 0.53-1.96), or recurrence aOR=1.16; (95% CI; 0.36-3.77), after controlling for potential confounders [115].

Though these contrasting results might be influenced by differences in study design and statistical analysis, sample size, population, severity and mortality definitions, parameters measured, and the bias of observational studies towards abnormal or severe disease presentations, we conclude that robust evidence is still lacking to sustain the claim that the ST01 strain is hypervirulent *in vivo*.

3.4.7 Is "hypervirulence" a frequent trait among Clade 2 STs?

A study done in New Zealand documented more severe disease OR= 9.33 (95% CI, 1.27-82.59) and recurrence OR= 3.78 (95% CI, 0.49-31.85) in patients infected with ST41 strains than in those affected by non-ST41 strains [99]. Likewise, compared to non-ST41 strains, ST41 strains in Australia were 13 times deadlier and more likely to induce severe disease, according to both IDSA/SHEA OR= 4.65 (95% CI, 0.91-23.82) and Zar criteria OR= 8.11 (95% CI, 0.98-67.21) [87]. At first sight these studies support the notion that ST41 strains are hypervirulent. However, these authors compared ST41 with STs from other MLST clades.

The outcomes of infection with a ST231 strain in a murine model were found to be comparable to those elicited by an ST01 strain, including marked weight loss, severe disease within 48 hours of infection, and death [103]. Nonetheless, these similarities have not been confirmed in studies on human CDI.

Some studies have also highlighted ST67 as a hypervirulent causative agent of CDI [86,88,89]. However, others did not find any evidence of severe diarrhea and the development of fulminant colitis in CDI cases caused by isolates from this ST [88].

Although results from animal models do not always reflect what will occur in a human host, it has been shown that the severity of CDI by ST01, ST67, and ST41 strains in a mouse model is influenced by toxin production regulation or the presence of nutritional sources, such as trehalose [69,89]. A recent study compared the pathogenic potential of ST01, ST67, ST41, and ST252 strains using the murine ileal loop model and oral infection of Syrian gold hamsters with spores [29]. In the first bio model, the ST01 isolate tested induced the highest overall level of edema and IL-1β. MPO activity, an indicator of neutrophilic degranulation, and histopathological scores were higher for ST01 and ST67 isolates. In turn, markers of acute inflammation in ileal tissue, such as TNF-α and IL-6, were comparable among ST01, ST67, and ST41 strains. However, this bio model has also delivered contrasting results, for the MPO, IL-1β, and IL-6 levels induced by infection with ST67, ST41, and ST252 isolates were indistinguishable from those measured in non-intoxicated cells [29,58], suggesting marked differences among members of the same ST. In the second bio model, colonization by ST01 isolates occurred sooner, and this ST also caused the highest levels of epithelial damage and lethality measured overall. In this study, ST01 and ST67 induced systemic infection, as indicated by elevated counts of peripheral neutrophils, high levels of alanine aminotransferase (liver malfunctioning) and lactate dehydrogenase (tissue damage), blood urea nitrogen and creatinine (renal function impairment), and changes in serum electrolytes (sodium and chloride), aspartate aminotransferase (AST), and albumin levels. By contrast, ST252 and one ST41 isolate were linked to high survival rates and induced little or no alterations in serum biomarkers [29]. A similar functional comparison of human CDI cases by these STs has not been performed.

This bulk of data, along with our profound lack of information for most Clade 2 members, and the marked phenotypic heterogeneity of isolates from the same ST, lead us to conclude that it is still premature to claim that hypervirulence is a widespread feature among Clade 2 *C. difficile* strains.

3.4.8. Closing remarks and recommendations

The continuous isolation of Clade 2 members as CDI causative agents justify the need for an active epidemiological vigilance, not only focused on ST01 strains [105]. Besides, our findings demonstrate that it is still necessary to expand current knowledge on

the epidemiology and pathogenesis of the Clade 2 STs listed in Table 5 and do not clarify whether the ST01 strain or the whole clade can be regarded as hypervirulent.

Table 5. *C. difficile* Clade 2 STs for which no information was found.

Clonal complex	**ST**
CC0	ST120 ST130 ST140 ST156 ST157 ST175 ST176 ST222 ST223 ST224 ST226 ST227 ST230 ST232 ST253 ST264 ST269 ST276 ST277 ST353 ST394 ST395 ST417 ST418 ST431 ST433 ST461 ST462 ST464 ST481 ST486 ST543 ST549 ST550 ST562 ST565 ST623 ST638 ST646

	ST652 ST690
CC1	ST351 ST428 ST541
CC2	ST228 ST651
CC3	ST128 ST372 ST444 ST446 ST463 ST554 ST567 ST642 ST660 ST662 ST674

To shed light on this matter, it is desirable that future studies include: i) toxin gene profiles, ii) cytotoxicity assays to estimate toxin synthesis levels, designate CPE types, and list TcdB glycosylation targets, iii) experimentation in animal biomodels to measure pro-inflammatory markers, describe histological findings, and determine lethality, iv) antibiotic-susceptibility testing, v) phenotypic and biochemical investigations on the role in host interaction of virulence factors (i.e. SlpA, cell wall proteins, flagella, fimbria, and biofilms), vi) determination of spore formation ability and germination rates, and vii) metabolic studies to estimate adaptation potential to the intestinal environment.

It would also be relevant to relate wet-lab and bioinformatics results to patient metadata and clinical parameters, including, but not limited to, age, exposure to antibiotics, presence of comorbidities, type of CDI onset, clinical progression, recurrence rates, biomarker levels in stools and serum, among others.

4. Chapter II. Manuscript: "Virulence-associated phenotypes of ST01 and non-ST01 strains from the *Clostridioides difficile* MLST Clade 2".

4.1Materials and methods

4.1.1 *C. difficile* strains and cultivation

This study included seven *C. difficile* isolates recovered from CDI patients admitted to Costa Rican national hospitals between 2009 and 2018. These bacteria were obtained from stool samples treated with 96% ethanol which were inoculated onto cefoxitin-cycloserine-fructose agar (CCFA) plates (Oxoid) [77]. Colonies were identified phenotypically with the RapID 32A system (bioMérieux) [77]; and genotypically by an end-point PCR targeting fragments of *tpi*, *tcdA*, *tcdB*, *tcdC*, and *cdtB* [116]. Thereafter, colonies with confirmed identity were typed by PFGE [19,117]. After sequencing by synthesis, it was determined by fastMLST (https://github.com/EnzoAndree/FastMLST) that the seven isolates represent six sequence types (STs) from the *C. difficile* MLST Clade 2 [20]. In this regard, whereas isolates LIBA-5758 and LIBA-5700 were allocated to ST01, LIBA-2811 (ST41), LIBA-7857 (ST47), LIBA-5757 (ST67), LIBA-6656 (ST154), and LIBA-5809 (ST638) were assigned to the STs indicated in parenthesis. The reference strain R20291 (ST01) was included as a positive control because its phenotypes have been widely documented. All phenotypic assays were done with bacteria freshly plated from cryopreservation vials maintained at -80 °C. Unless otherwise indicated, all incubations were done at 37 °C under an atmosphere composed of 90% N_2, 5% H_2, and 5% CO_2 into an anaerobic chamber (Bactron II; Shell Lab).

4.1.2 Spore preparations

Spore suspensions were obtained based on a modification of previously published protocols [5,118]. Briefly, each strain was cultured for 24 h in Brain Heart Infusion (BHI) broth supplemented with 0.1% hemin, 0.1% vitamin K_1, and 0.5% yeast extract. Next, trypticase soy agar (TSA) plates supplemented with 0.5% yeast extract were inoculated with a dilution of the starter culture (0.7 ml in 1000 ml) and incubated for 7 days. Biomass was recovered by flooding the plates with ice-cold sterile distilled water followed by scraping with disposable loops. To separate spores from vegetative cells, the aforementioned suspensions were mixed with non-ionic density gradient medium (Histodenz, Sigma-Aldrich) and

centrifuged the mixtures at 16873 g for 10 min at room temperature. Spore pellets were washed with PBS (0.01 M, pH 7.4) to remove Histodenz traces, resuspended in PBS + BSA 1% w/v, and stored at 4ºC for at least 15 days to allow spore maturation. After this period, spore suspensions were resuspended, diluted, and heated to 55 °C for 20 min to kill remaining vegetative cells. The number of viable and total spores in each suspension was determined by duplicate through cultivation (BHI agar plates supplemented with 0.1% hemin, 0.1% vitamin K_1, 0.01% of sodium taurocholate), at 37 ºC for 48 to 72 h under anaerobiosis and by microscopic counting of Schaeffer and Fulton-stained smears.

4.1.3 Adherence of spores to epithelial colorectal cells (ECC)

The ability of spores to adhere to ECC was assessed using procedures reported in the literature [5]. Briefly, Caco-2 epithelial cell cultures, at a final density of approximately 1.1 x 10^6 cells/well, were inoculated with spores at an MOI (multiplicity of infection) of 4 or 10 in 200 µL of EMEM (Eagle's minimal essential medium) and incubated for 1 h at 37 ºC under aerobiosis. Next, a group of cells was washed three times with PBS to remove unattached spores while a second group of cells was left unwashed to determine the total number of spores that were originally added. Afterwards, both groups of cells were lysed with 0.06% Triton X-100 for 30 min at 37 ºC under aerobic conditions. An aliquot of 100 µL of the resulting cell lysates were spread onto BHI agar plates supplemented with 2% glucose, 0.5% yeast extract, and 0.01% sodium taurocholate which were incubated for 48 h for recording of colony forming units (CFU). Spore adherence was determined with the following formula:

$$[(\text{final CFU/mL}) / (\text{initial CFU/mL})] \times 100$$

To confirm these results, spores adhered to Caco-2 were visualized by indirect immunofluorescence based on a modification of previously published protocols [5,43]. To this end, Caco-2 epithelial cell cultures, grown on 12 mm glass slides at a final density of approximately 1.1 x 10^6 cells / well, were inoculated with spores at an MOI of 4 or 10 in 200 µL of EMEM and incubated for 1 h at 37 ºC under an aerobic atmosphere with 5% CO_2. Thereafter, cells were washed with PBS and fixed with 3.5% paraformaldehyde for 10 min. Fixed cells were blocked for 10 min with 50 mM ammonium chloride and permeabilized with 0.1% Triton X-100 for 10 min. For specific spore detection we exposed the slides for 30 min to a primary rabbit antibody targeting a *C. difficile* spore protein (ab2985, Abcam)

and a secondary antibody for the same time period (Alexa Fluor 546 F(ab) fragment of goat anti-rabbit, Life Technologies). All washes were done with PBS and 0.1% Triton X-100. Slides were mounted with SlowFade® Gold antifade reagent with DAPI (Invitrogen) to protect the cells from bleaching and stain cell nuclei. Images were recorded with a fluorescence microscope (Eclipse 80i, Nikon), a CCD camera (Digital Sight, Nikon), and the NIS-Elements AR 3.0 Software (Nikon). Both assays were performed in triplicate.

4.1.4 SlpA detection and pro-inflammatory response mediation

Preparations enriched in surface layer protein SlpA were obtained from bacteria grown for 18 h in TYT broth (3% bacto tryptose, 2% yeast extract, 0.1% thioglycollate, pH 6.8) as previously described [9,107]. Briefly, bacterial biomass harvested by centrifugation at 18514 g for 15 min at 4 °C (Centrifuge 5810 R, Eppendorf) was washed twice with PBS and resuspended in 0.2 M glycine adjusted at pH 2.2. After 30 min, the supernatants were recovered, neutralized with 2M Tris solution (pH 8.0), filtered through 0.2 µm membranes, and stored in aliquots at -20 °C. Proteins separated by 10% SDS-PAGE were electrotransferred to a polyvinylidene difluoride (PVDF) membrane and probed with a anti-LIBA-5758 (ST01) SlpA mice antisera [107]. For signal detection, membranes were incubated with peroxidase-conjugated anti-mouse antibodies (G21040, Invitrogen) and the immunological complexes were revealed by a chemiluminescence reaction using Lumi-Light Plus Western Blotting substrate (Roche) and a Chemidoc XRS documentation system (Bio-Rad). The identity of the bands was confirmed by mass spectrometry (MS) at the Proteomics Facility of the Clodomiro Picado Institute/University of Costa Rica.

Confluent monolayers of Raw 264.7 macrophages (ATCC TIB-71) were exposed to 20 µg of proteins contained in the SlpA preparations for 12 h under an aerobic atmosphere with 5% CO_2. As positive and negative controls, 5 µg of *Escherichia coli* LPS and a mixture of 0.2 M glycine pH 2.2 with 2 M Tris pH 8.0 were tested, respectively (data not shown). The concentration of TNF-α in supernatants was determined using a commercial mouse TNF-α enzyme-linked immunosorbent assay (ELISA), as instructed by the manufacturer (eBioscience).

4.1.5 Flagellin extraction

To extract flagellin, strains were grown on BHI agar plates supplemented with 0.1% hemin, 0.1% vitamin K_1. After 24 h of incubation, biomass was resuspended in 500 µL of distilled water and vigorously vortexed for 3 min. Supernatants obtained by centrifugation at 16873 g in a benchtop centrifuge (model 5418, Eppendorf) for 5 min at room temperature, were lyophilized and thereafter resuspended in 100 µl of Milli Q water (Merck) [119]. The yield and purity of these preparations were assessed by SDS-PAGE followed by Coomassie staining. Furthermore, the identity of the bands was determined by mass spectrometry at the Proteomics Facility of the Clodomiro Picado Institute/University of Costa Rica.

4.1.6 Motility assays

Motility was first assessed using semisolid agar tubes prepared with BHI broth and 0.175% agar. These media were inoculated through stabbing with a straight platinum needle and incubated for 48 h [120]. Strain M120 (non-motile) was used as a negative control. To evaluate swimming and swarming motility we used bacterial cultures grown in BHI broth for 8 h and pre-reduced plates containing 25 mL of BHI broth and 0.3% or 0.4% agar, respectively [8]. Whereas swimming plates were stab-inoculated with 3 uL of bacterial cultures, swarming plates were spot-inoculated with the same volume. After 48 h of incubation, the diameter of growth was measured with a ruler. Six replicates were obtained for each strain by triplicate [8]. In this series of experiments, strain M120 was again used as a negative control.

4.1.7 Biofilm formation

The capacity of the strains to form biofilms was quantified using crystal violet (CV) staining of growth in plastic microtiter plates [12]. To this end, started cultures were obtained through overnight incubation in BHI broth (Oxoid) supplemented with 0.1% cysteine and 0.5% yeast extract (BHI-CY). Next day, starter cultures were diluted 1:100 with BHI-CY containing 0.1 M glucose and incubated in cell culture 24-well polystyrene plates for 24, 72, and 120 h. A total of three wells were inoculated for each strain (1 mL/well). Wells were washed with PBS and the biomass adhered to their inner surfaces was stained with 1 mL/well of 0.2% CV for 30 min. Subsequently, plates were washed with PBS, and CV was extracted for the same period using 1 mL/well of methanol. The optical density (OD) of the extracts was determined

at 570 nm using a spectrophotometer (Genesys 20, Thermo Scientific). A non-inoculated well was used as a negative control.

4.1.8 Quantification of secreted TcdB

To quantify secreted TcdB, 24 h cultures in TYT broth were centrifuged at 20,000 g for 30 min to remove bacteria, and the supernatants were passed through 0.2 µm filters [77]. Proteins in these cell-free supernatants were concentrated using Strataclean® resin [121] and thereafter separated by duplicate in 7.5% polyacrylamide gels by SDS-PAGE electrophoresis. One gel from each pair was exploited to confirm the presence of TcdB via Western blotting (WB) with an anti-TcdB monoclonal antibody (2CV, tgcBiomics), a goat anti-mouse IgG-horseradish peroxidase conjugate (G-21040 Invitrogen; Life Technologies), a chemiluminescent substrate (Lumi-Light Plus Western blotting substrate, Roche), and a XRS ChemiDoc documentation system (Bio-Rad). The second gel, in turn, was stained with Coomassie blue and analyzed by densitometry using ImageJ (https://imagej.nih.gov/ij/) to approximate the amounts of proteins with TcdB molecular weight and WB signals.

4.1.9 Characterization of toxin-derived cytopathic effects (CPE): titration and kinetics

The biological activity of TcdA/B in the cell-free supernatants described above was studied through intoxication of confluent HeLa cell monolayers grown in 96-well polystyrene plates in Dulbecco modified Eagle medium (DMEM, Gibco) supplemented with 5% fetal bovine serum (FBS, Sigma). Ten-fold dilutions of culture supernatants that originally contained approximately 62 ug of total protein were added to the cell cultures. To verify that the CPE observed was indeed caused by these toxins, the assays were also performed in presence of a single neutralizing antibody raised against TcdA and TcdB (T5000, TechLab, Inc) [77]. The percentage of morphologically altered cells in each well was monitored hourly by phase-contrast microscopy for 24 h using an automated cell imager (Cytation3, BioTek instruments). Cytopathic titers were expressed as the inverse of the dilution of the supernatants that produced 50% cell rounding in the monolayers (CPE_{50}) after 24 h of incubation.

4.1.10 *In vitro* GTPase glycosylation profiles

Glycosylation of monomeric GTPases by cell-free supernatants was examined through WB. Briefly, HeLa cells monolayers grown under the above conditions but in 24-well polystyrene plates, were inoculated with 40 μL of cell-free supernatants that were derived from 24 h cultures in TYT broth ca. 250 ug of total protein). After 24 h of incubation, cells were lysed with 2% sodium dodecyl sulfate (SDS). Lysate proteins were separated in 10% SDS-polyacrilamide gels, electrotransferred to PVDF membranes, and probed with monoclonal anti-Rho A- (ab54835, Abcam) and anti-Rac1- (610651, clone 102, BD Biosciences) antibodies that fail to recognize the glycosylated isoforms of these proteins. B-actin was detected as loading control using an anti-Actin antibody produced in rabbits (A2066, Sigma) [58,82].

4.1.11 Spore response to NaDCC

A dilution-neutralization assay was performed to determine the susceptibility of spore suspensions to NaDCC [17,122]. Briefly, spores adjusted at a concentration of 10^7/mL were mixed with 0.3 g/L bovine albumin and then exposed to 1000 ppm NaDCC for 5 min at room temperature. Aliquots of these suspensions were transferred to tubes containing the neutralizing agent 0.5% sodium thiosulfate (Merck) and allowed to set for 5 min at room temperature. Subsequently, three decimal dilutions were spread onto Brucella agar plates supplemented with 0.01% sodium taurocholate, in duplicate [17,122]. After 48 h, the number of CFU/mL was recorded. In this test, a disinfectant that leads to a reduction in the initial bacterial population of at least 5 logarithms is considered effective [122]. This test was performed three times for each strain.

4.1.12 Statistical analyses

Data are presented as means plus/minus standard deviations. Means were compared by one-way ANOVA tests with Bonferroni correction or Kruskal-Wallis tests followed by Dunn’s multiple comparison tests or Tukey's test, respectively. P values < 0.05 were considered statistically significant, using the statistics software SigmaPlot 14.0.

4.2 Results

4.2.1 Spore adherence to human intestinal epithelial cells

The adherence level of spores from different MLST Clade 2 STs to ECC is comparable

To evaluate the level of adherence of spores to human intestinal cells, we exposed Caco-2 cell cultures to purified spores at an MOI of 4 for 1 hour. A group of cells was washed to remove unattached spores, while a second group of cells was left unwashed to determine the total number of spores added to the cell cultures. Cells were then lysed, and lysates were spread onto agar plates to determine the initial (total) and final (adhered) amounts of spores. All the strains achieved similar adherence percentages, ranging between 83.0 and 86.9% (Fig. 3A). To confirm these results, we attempted to visualize spores adhered to Caco-2 cells through an indirect immunofluorescence microscopy assay (Fig 3B). No interferences due to spore autofluorescence or non-specific interactions involving the secondary antibody were observed (Fig. 3B 1-2) and spores interacting with epithelial cells were detected for all strains (Fig. 3B 3). However, the low intensity of the fluorescent signal emitted by the antibodies that recognize the spores (red), compared to the fluorescence of the eukaryotic cells stained with DAPI (blue), hindered the obtention of confident spore counts.

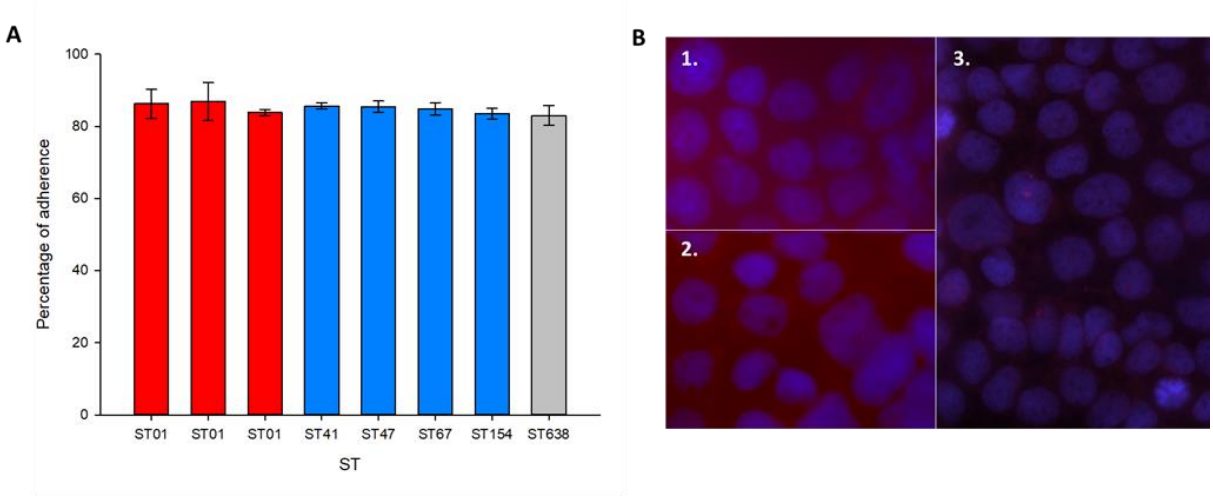


Figure 3. Strains from various MLST Clade 2 STs show a similar percentage of adherence to human intestinal epithelial cells. Spore adherence to Caco-2 cells was determined quantitively by plate counts (A) or qualitatively via epifluorescence microscopy (B). Red bars in A, designated as ST01, correspond to strains R20291, LIBA-5700, and LIBA-5758, from left to right. The results shown in A represent the average of three independent experiments. No statistically significant differences were found at $P < 0.05$ (One-way ANOVA). B) An indirect immunofluorescence assay was performed to confirm the adherence of spores to Caco-2 cells (MOI = 4) grown on 12 mm cover slides and stained with DAPI. Three conditions were imaged: 1) Caco-2 cells exposed to primary and secondary antibodies (no spores, negative control), 2) Caco-2 cells exposed to spores plus secondary antibody only (control for detection of non-specific antibody interactions), and 3) Caco-2 cells exposed to spores plus primary antibody targeting a *C. difficile* spore protein (ab2985, Abcam) and secondary antibody (Alexa Fluor 546, Life Technologies) (test).

4.2.2 SlpA detection and pro-inflammatory response mediation

The MLST Clade 2 strains tested exhibit ST-specific SlpA profiles and a differential pro-inflammatory activity.

SlpA was extracted from cultures of *C. difficile* Clade 2 strains with a low pH glycine buffer, with relatively low levels of contaminating proteins. The identity of the extracted proteins was confirmed based on their anticipated molecular weight and by MS for all strains except for LIBA-7857 (ST47). With a single exception, two bands were seen on SDS-polyacrylamide gels: a high molecular weight (HMW) and a low molecular weight (LMW)

band, migrating around 45-52 kDa and 35-40 kDa, respectively. Different migration patterns were seen among the strains, mainly in their LMW isoforms. A single band of HMW was observed for the ST47 strain tested (Fig. 4A).

Polyclonal mouse antibodies raised against SlpA from strain LIBA-5758 (ST01) were used in a WB analysis of SlpA preparations from the strains under study (Fig. 4B). As expected, this antiserum recognized the HMW and LMW SlpA of the strain used for immunization. Identical recognition patterns were recorded for the other ST01 strains, while the antiserum only recognized the HMW SlpA of the remaining strains, suggesting a rather high level of antigenic similarity in this isoform.

The pro-inflammatory activity triggered by the SlpA preparations was assessed through the measurement of TNF-α released by exposed Raw 264.7 macrophages (Fig. 4C). SlpA from strains LIBA-5700 (ST01), LIBA-5758 (ST01), LIBA-2811 (ST41), and LIBA-6656 (ST154) induced a statistically significant higher secretion of TNF-α (ranging from 1338.13 to 1562.40 pg/mL) than strains R20291 (ST01), LIBA-7857 (ST47), LIBA-5757 (ST67), and LIBA-5809 (ST638) (ranging from 669.43 to 1041.70 pg/mL).

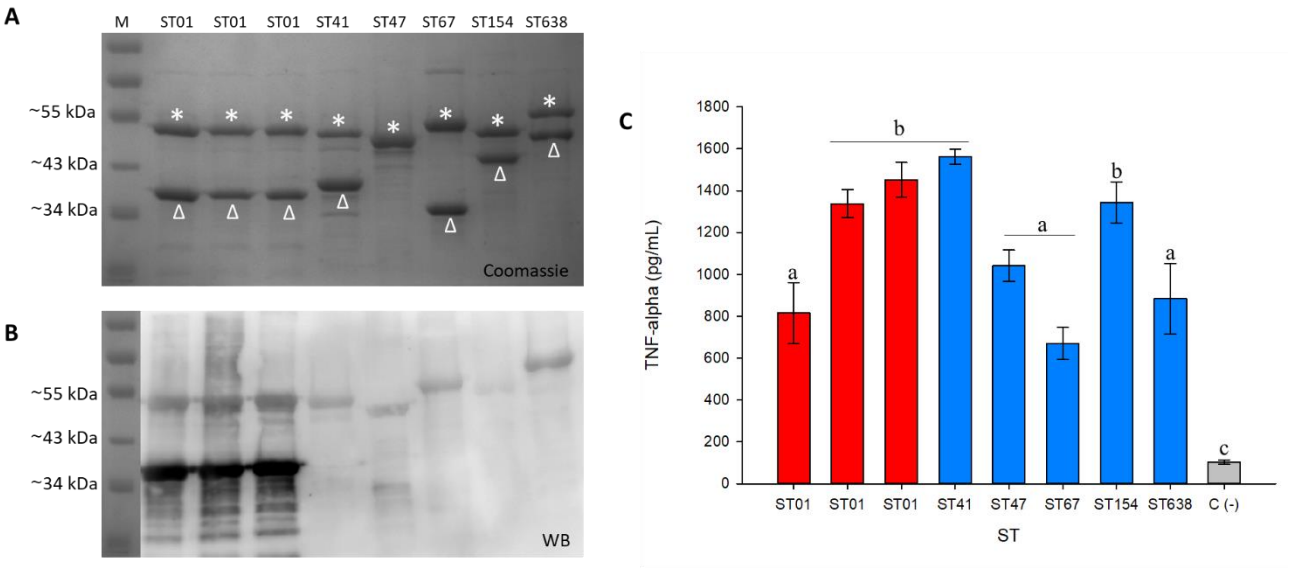


Figure 4. ST-specific SlpA profiles with distinct immunogenic properties and proinflammatory potential. A) Coomassie-stained SDS-PAGE of SlpA preparations obtained for different *C. difficile* MLST Clade 2 strains by low-pH extraction Lane M: PageRuler™ (Thermo Scientific) molecular weight marker. Lanes designated as ST01 correspond to strains R20291, LIBA-5700 and LIBA-5758, respectively. *: HMW SlpA band, Δ: LMW SlpA band. B) Western blotting (WB) of SlpA preparations probed with anti LIBA-5758 (ST01) SlpA mouse antibodies. Immune complexes were revealed by chemiluminescence using Lumi light Plus (Roche). C) 20 µg of SlpA preparations, or glycine with Tris (in a ratio of 10:1) as negative control C (-), were incubated for 12 h with Raw 264.7 macrophages grown on 24-well plates. Thereafter, the amount of TNF-α released to the supernatants was quantified using a mouse TNF-α ELISA kit (eBioscience). Red bars, designated as ST01, represent strains R20291, LIBA-5700 and LIBA-5758, respectively. Non ST01-strains appear in blue. Error bars represent standard deviations from three independent experiments. (One-way ANOVA, with Bonferroni test, $P< 0.05$).

4.2.3 Detection of flagellin and motility assays

All STs tested show comparable swarming and swimming motility.

Flagellin extracted from the cell surface of *C. difficile* strains was separated and visualized by SDS-PAGE (data not shown). In each case, bands of approximately 33 to 36 kDa -the

molecular weight expected for flagellin- were observed in the gel but accompanied by other contaminating proteins. MS of extracted gel bands identified this protein band as the product of *fliC* for strains LIBA-2811 (ST41), LIBA-7857 (ST47), and LIBA-6656 (ST154). For the other strains, however, these bands were identified as SlpA.

All the strains were motile, as revealed by a qualitative screening with soft agar tubes (Fig. 5A). Compared to the negative control, all of the Clade 2 strains showed swarming and swimming motility (Fig. 5B). No differences in swarming motility were seen. By contrast, LIBA-5700 (ST01) showed less swimming motility than the other strains (Fig. 5C).

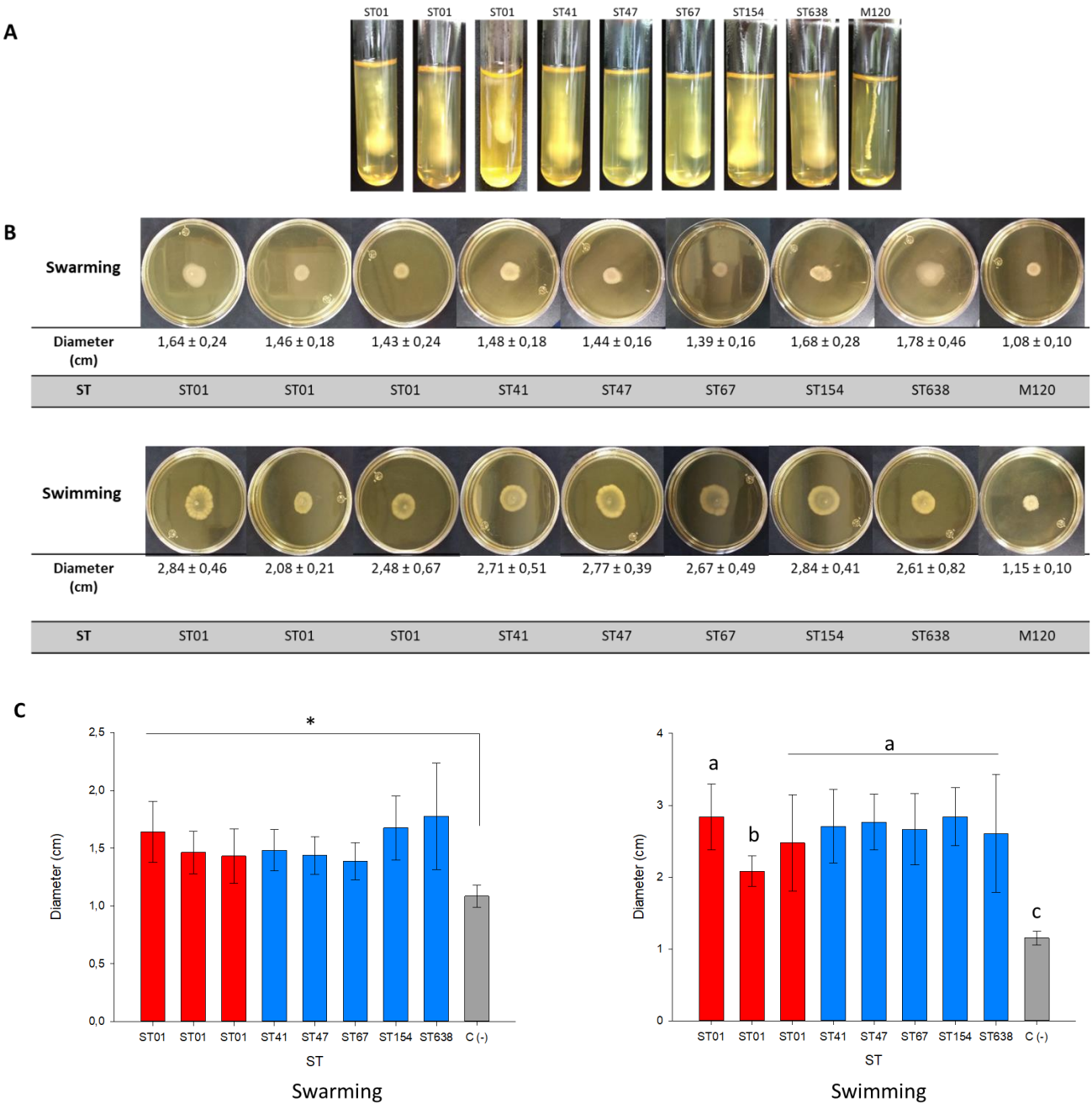


Figure 5. Flagellin extraction and quantitation of swarming and swimming motility.

(A) Soft BHI agar tubes were stab-inoculated with a colony of each strain to screen their motility capacity. Strain M120 is non-motile and was therefore included as a negative control. (B) Soft BHI agar plates were spot-inoculated and stab-inoculated with 4 µL of OD-adjusted bacterial cultures to assay swarming and swimming motility, respectively. Growth diameters in cm (C) were recorded after 48 h. ST01 lanes, images, and bars correspond to strains

R20291, LIBA-5700 and LIBA-5758, from left to right. Red and blue bars in C correspond to ST01 isolates and non-ST01 isolates, respectively. Results were obtained from three independent experiments. (P<0.05 Kruskal-Wallis and Dunn's multiple comparison test).

4.2.4 Biofilm formation

All Clade 2 strains tested show similar biofilm formation ability at 24 and 72 h. A few non conclusive differences emerged at 120 h.

The ability of Clade 2 strains to form biofilms was evaluated by CV staining assay of sessile biomass after 24, 72, and 120 h of growth in microtiter plates (Fig. 6). Maximum figures of biofilm formation were observed at 24 h. Afterwards, biofilm formation decreased gradually to reach the lowest levels at 120 h. All the strains showed significantly higher biofilm formation than the negative control at 24 and 48 h, while at 120 h, the values obtained for strains LIBA-5700 (ST01), LIBA-5758 (ST01), and LIBA-2811 (ST41) were indistinguishable from those observed for the negative control.

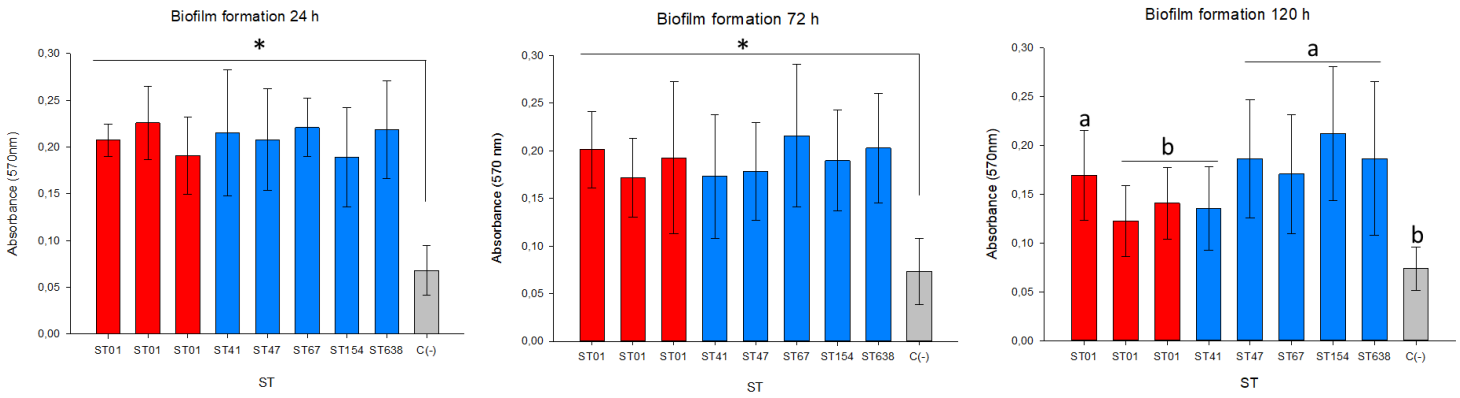


Figure 6. Biofilm formation at 24, 48 and 120 h as determined by CV staining of biomass attached to plastic microtiter plates. Diluted overnight cultures grown in BHIS supplemented with 0.1 M glucose for 3 days were inoculated in triplicate in 24-well plates. Uninoculated culture medium was used as a negative control (C-). The amount of biomass adhered to the plates after the indicated periods of time was estimated by quantitation of extracted crystal violet. Red and blue bars correspond to ST01 isolates and non-ST01 isolates, respectively. Bars designated as ST01 in the three panels correspond to strains R20291, LIBA-5700 and LIBA-5758, from left to right. Each strain was tested in triplicate. Error bars

represent standard deviations. (P<0.05 Kruskal-Wallis with Tukey's tests (24 and 72h), One-way ANOVA, with Bonferroni test (120h).

4.2.5. Quantification of TcdB and effects induced by glycosyltransferase activity.

C. difficile MLST Clade 2 strains produce different amounts of TcdB, which induce distinct cytopathic effects according to their target affinities.

The strains under study were compared regarding their TcdB production after 24 h of growth in TYT broth (Fig. 7A and B). As indicated by densitometric measurements of Coomassie-stained SDS-PAGE bands for which a signal was seen in the WB, strains LIBA-5700 (ST01) (505.9), LIBA-5758 (ST01) (477.6), LIBA-2811 (ST41) (438.2), and LIBA-6656 (ST154) (457.1) produce approximately 1.5 - 4X higher amounts of TcdB than the other STs (with densities ranging from 121.1 to 278.2). The lowest TcdB production was measured for LIBA-5809 (ST638) (Fig. 7B).

The amount of TcdB produced by these strains did not always correlate with the cytopathic potential of cell-free supernatants to HeLa cells (Fig. 7C). In this regard, although the highest titers overall were assigned to the high toxin producer strains LIBA-5700 (ST01) and LIBA-5758 (ST01) (1:10000), strains LIBA-2811 (ST41) and LIBA-6656 (ST154) showed similar titers than strains producing lower amounts of toxin, such as R20291 (ST01), LIBA-7857 (ST47), and LIBA-5757 (ST67) (1:1000), and LIBA-5809 (ST638) (1:100). Our Western Blots revealed an interaction between TcdB of strains LIBA-5700 (ST01), LIBA-5758 (ST01), LIBA-2811 (ST41), LIBA-6656 (ST154), and LIBA-5809 (ST638) and the detection antibody that matched the densitometric measurements. By contrast, strains LIBA-7857 (ST47) and LIBA-5757 (ST67) gave rise to strong chemiluminescent signals, despite having bands of low intensity in the SDS-PAGE gels (Fig. 7C).

To characterize the morphological changes induced by the TcdB toxins, HeLa cells were intoxicated with cell-free supernatants and the cytopathic effects (CPE) were recorded across 24 h. Whereas ST01, ST47, and ST638 strains induced a classical, arborizing CPE, the other strains caused a variant effect characterized by cell rounding, clumping, and surface detachment (Fig. 7C). The time of CPE appearance varied among the strains, with significant differences being evident already after 2 hours of intoxication (Fig 7D). At this time, whereas

cells exposed to two ST01 strains (LIBA-5700 and LIBA-5758) showed the highest percentage of rounded cells, LIBA-5809 (ST638) produced the least amount of morphologically altered cells. An hour later, LIBA-5809 (ST638) maintained the lowest percentage of rounded cells compared to the other strains. At the following time points, although LIBA-2811 (ST41) and LIBA-5809 (ST638) induced a CPE at a slower pace, no significant differences were found. Cells exposed to supernatants from the high toxin producer strains LIBA-5700 (ST01) and LIBA-5758 (ST01) showed the classical CPE already 3 h after intoxication. R20291 (ST01) and LIBA-7857 (ST47), in turn, induced morphological changes after 4 h, followed by LIBA-6656 (ST154), which induced the variant CPE after 5 h. Although LIBA-5757 (ST67) (CPE completed at 6 h) did not produce as much TcdB as LIBA-2811 (ST41), it caused cell alterations 3 h before (variant CPE at 9 h). By contrast, cells intoxicated with LIBA-5809 (ST638) required ca. 13 h to manifest a CPE and preserved their neurite-like protrusions even after 24 h of incubation. The CPE differences observed mirror the glycosylation patterns induced by the strains in HeLa cells, as determined through an *in vitro* assay (Fig. 7E). In this regard, strains that induced a classical CPE glycosylated RhoA and Rac1, while variant CPE-inducing strains, such as ST41, ST67, and ST154, only glycosylated Rac1.

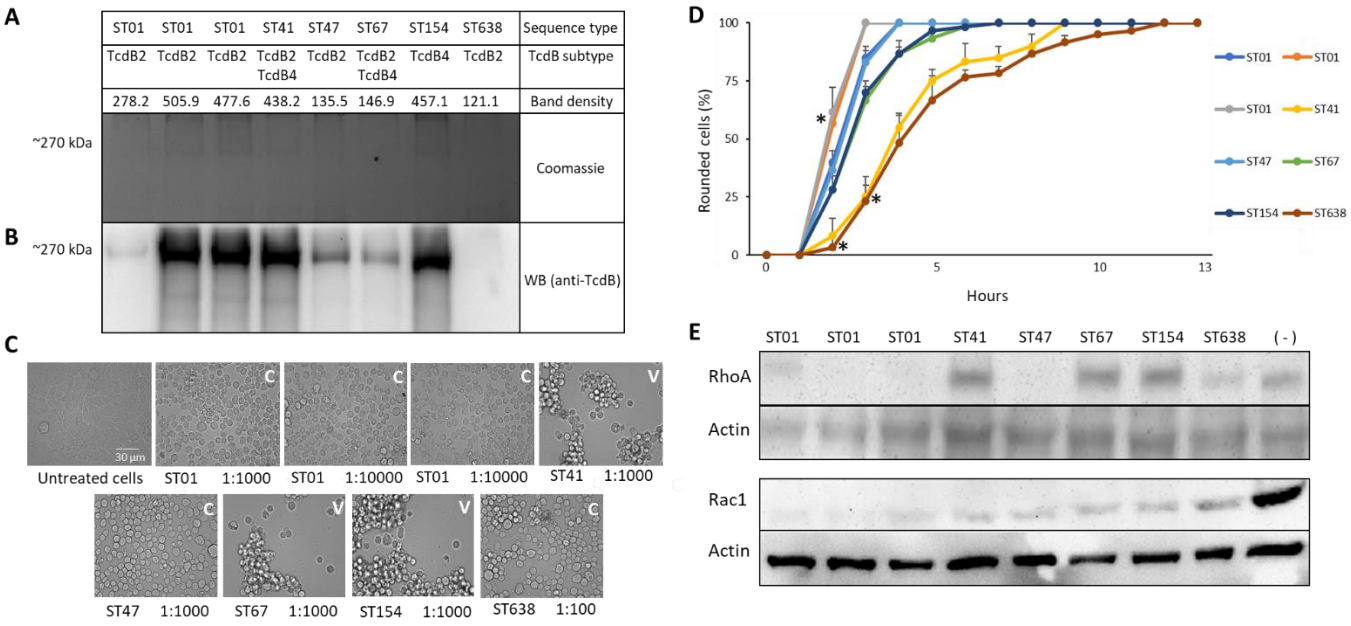


Figure 7. Quantification of TcdB and characterization of cytopathic effects due to target glycosylation. A) Proteins in cell-depleted supernatant were concentrated using StrataClean beads (Agilent Technologies), separated by 7,5% SDS-PAGE, and visualized by Coomassie blue staining. The density of bands of approximately 270 kDa was measured using ImageJ. B) To confirm the identity of the bands seen in the SDS-PAGE a parallel SDS-PAGE gel was transferred to a PVDF membrane, probed with monoclonal antibody to TcdB (tgcBiomics) and the interaction complex was visualized by chemiluminescence using Lumi light Plus (Roche). C) Cytopathic effect (CPE) recorded 24 h after monolayers of HeLa cells were intoxicated with cell-free supernatants of the indicated strains. C: classic CPE, V: variant CPE. The decimal dilution in which CPE_{50} was observed is indicated next to each ST below the pictures. D) Cell rounding was monitored each hour during 24 h to obtain kinetic profiles of CPE appearance. Each strain was tested in triplicate. Error bars represent standard deviations. (*: $P<0.05$ One-way ANOVA, with Bonferroni test). E) Monolayers of HeLa cells were intoxicated with cell-free supernatants for 24 h. Cells were lysed for subsequent evaluation of GTPases glycosylation by WB with antibodies that fail to recognize the glycosylated forms of RhoA and Rac1 (a lack of signal depicts target glycosylation). As a loading control, membranes were revealed with a monoclonal antibody targeting actin. Untreated cells (-) were included as control for unmodified proteins. Lanes or images

designated as ST01 correspond to strains R20291, LIBA-5700, and LIBA-5758, respectively. Images were obtained from three independent experiments.

4.2.6 Spore susceptibility to sodium dichloroisocyanurate (NaDCC)

The susceptibility of spores from MLST Clade 2 strains to NaDCC is similarly high.

To examine the susceptibility of spores from our MLST Clade 2 strains to a clinically relevant disinfectant, we exposed spore suspensions to NaDCC and calculated logarithmic reduction factors (LRF) after 5 minutes of exposure in presence of organic matter (Fig. 8). In this series of assays, spores from the well-studied reference strain CD630 were tested for comparative purposes. The highest LRF were recorded for strains CD630 (LRF: 5.36 ± 0.12) and LIBA-2811 (ST41) (LRF: 5.10 ± 0.05). However, these figures were similar to those determined for strains LIBA-5700 (ST01) (4.82 ± 0.65), R20291 (ST01) (LRF= 4.64 ± 0.58), LIBA-5758 (ST01) (3.82 ± 0.18), LIBA-7857 (ST47) (4.55 ± 0.93), LIBA-5757 (ST67) (3.60 ± 0.64), LIBA-6656 (ST154) (4.86 ± 0.79), and LIBA-5809 (ST638) (4.84 ± 0.47) (One-way ANOVA, $P<0.05$).

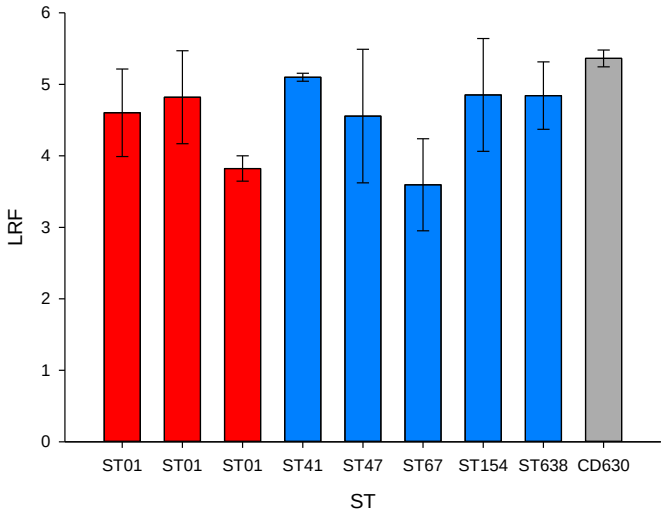


Figure 8. NaDCC showed similar inhibitory sporicidal activity against all MLST Clade 2 *C. difficile* strains tested. The inhibitory activity of NaDCC (1000 ppm) on spores ($1x10^7$ spores/mL) from the *C. difficile* MLST Clade 2 strains under study and a reference laboratory strain (CD630) was tested by triplicate using a dilution-neutralization method. Results obtained were expressed as average log10 reduction factors (LRF), where an LRF = 5 indicates a 99.999% reduction in the original number of endospores. Bars designated as ST01

represent strains R20291, LIBA-5700, and LIBA-5758, respectively. Red and blue bars correspond to ST01 and non-ST01 isolates, respectively. Error bars indicate standard deviations. No statistically significant differences were found at $P<0.05$ (One-way ANOVA).

4.3. Discussion

In this work several virulence-associated phenotypes implicated in different stages of CDI development were analyzed aiming to identify factors that could explain the increased *in vivo* virulence and epidemicity of ST01 isolates, compared to representatives of other ST from the same *C. difficile* MLST clade (Clade 2). The results obtained did not reveal any obvious exclusive patterns or trends, as all strains exhibited similar levels of spore adherence to epithelial cells, motility, biofilm formation at 24 and 72h, and spore resistance to NaDCC. Besides, some ST01 isolates and members of other STs shared phenotypes, including SlpA-mediated secretion of pro-inflammatory cytokines, decreased biofilm formation at 120h, and production of TcdB in high levels. In consequence, the hypothesis raised in this research project is rejected.

4.3.1 Adherence of spores to human epithelial cells

C. difficile is a strictly anaerobic bacterium that forms dormant spores. These structures, which are highly resistant to antibiotics, the host´s immune response, and some disinfectants commonly used in clinical environments [6], is produced via quorum sensing in response to stimuli such as nutrient starvation and other stress factors [6]. They facilitate the persistence of *C. difficile* in the host and the environment, contributing to the recurrence of CDI and the transmission of this pathogen by the fecal-oral route [6,18]. Based on this important role in CDI, we evaluated the level of adherence of spores to human intestinal cells using plate counts and indirect immunofluorescence assays.

As determined by the first technique, all strains assessed achieved similarly high adherence percentages (83.0-86.9%). Firstly, these results confirm that *C. difficile* Clade 2 spores can adhere to enterocyte-like epithelial cells. This attachment, which likely is the initial stage of CDI development, has been claimed to occur through the interaction of spore and host proteins [5]. In this regard, the high levels of adherence of spores from epidemic strains such as R20291 (ST01) to intestinal epithelial cells *in vitro* are mediated by the presence of the

highly hydrophobic exosporium, the outermost layer of spores, with hair-like projections composed by collagen-like BclA3 glycoproteins. These projections interact with collagen domains located on the cellular membrane or the apical microvilli of intestinal cells, and the extracellular matrix proteins fibronectin and vitronectin [5,6,18,123,124]. It is probable that unidentified proteins of 40-45 kDa could directly affect the ultrastructure of the exosporium, since their absence triggers a decrease in spore hydrophobicity and adherence to epithelial cells [5]. However, it also has been reported that mutations in *cdeC*, the coding gene for the cysteine-rich exosporium protein [6,125], causes a deficiently assembled exosporium that lacks the hair-like extensions and has reduced hydrophobicity [124]. Unexpectedly, *cdeC* mutated R20291 spores showed higher adherence than the wild type, suggesting that the correct assembly of exosporium increases the selectivity of the adherence of spores to epithelial cells [124,125]. Furthermore, i*n vitro* assays have revealed that BclA3 is essential for spore entry to T84 and Caco2 cells through BclA3-Fn-$\alpha_5\beta_1$ and Vn-$\alpha_v\beta_1$ integrin internalization pathways [18].

Altogether, our results suggest that the indistinguishable adherence percentages to Caco-2 cells observed for the Clade 2 strains mirror common ultrastructural characteristics among their exosporia and comparable levels of hydrophobicity. These potential similarities, however, seem not to correlate with virulence, as our strains caused different levels of pathology in animal models [29]. Now, considering that the methodology followed in this work does not differentiate adhered- from internalized-spores, and bearing in mind that *C. difficile* spores can enter intestinal epithelial cells, it would be desirable to establish in the future the total amount of spores that interact with cells and the proportion of spores internalized. Moreover, it would be interesting to study new therapeutic strategies for strains with spores with higher internalization rates, including drugs that block the entry of spores into cells, such as nystatin [18], as this could restrict recurrence and thereby, improve the management of CDI patients.

In line with its qualitative nature, the microscopic method allowed us to visually confirm interactions between spores and Caco-2 cells. The primary antibody used in these experiments is directed against the hypothetical protein CD1021, which is homologous to the spore coat assembly protein H from *Bacillus subtilis* encoded by *cotH* [126]. This protein has also been detected in spores from *C. difficile* 630 and R20291 [126]. However, there is

insufficient information about the localization and proportion of this protein in the spores of this bacterium. The low intensity of the fluorescent signal emitted by the spore-recognizing antibodies, compared with that of eukaryotic cells stained with DAPI, hindered a quantitative analysis of the attached spores. It is probable that the target of the primary antibody (protein CD1021) was not extensively distributed on the surface of the spores or that the secondary antibody was not sufficiently sensitive to the presence of primary antibodies coupled with their corresponding antigen. Consequently, future research should deploy antibodies in higher concentrations or directed towards other/additional targets, to generate more intense fluorescent signals. Also, confocal microscopy techniques could be employed to obtain more details of the interaction between spores and epithelial cells.

4.3.2 SlpA detection and pro-inflammatory response mediation

Once spores are established in the intestinal epithelium and exposed to germinants such as bile salts and L-glycine, the germination process is triggered, from which vegetative cells emerge. These developmental forms are responsible for the clinical manifestations of CDI, mainly by the production of toxins, but also through the expression of surface-associated proteins (SAPs) that play roles in adhesion and colonization of the intestinal epithelium, i.e. the surface layer protein A (SlpA), flagellin (FliC), flagellar cap protein (FliD), cell wall protein Cwp66, and cell wall cysteine protease Cwp84 [6,127].

One of the most well-described SAPs is SlpA, the product of *slpA* [128]. SlpA is the primary component of the *C. difficile* S-layer, a two dimensional paracrystalline lattice anchored to the outer surface [11,127,129,130]. In general, studies on other bacteria have shown that this layer is involved in cell shape determination, molecular sieving, transport or storage of nutrients, host cell adhesion and immune system evasion [11], endowing cells with resistance to bactericidal activity of complement (i.e. C3b), immunoglobuline-binding capabilities and resistance to polymorphonuclear leukocytes in the absence of opsonins [131].

SlpA is produced as a single precursor protein, which is secreted and processed by the SecA2 secretion system and Cwp84, to generate 33-34 kDa low molecular weight (LMW) and 41-45 kDa high molecular weight (HMW) subunits [9,130,132]. These proteins induce the release of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6. Furthermore, they

participate in the maturation of monocyte-derived dendritic cells (MDDC) and antibodies production [107,127].

Our results show the presence of two bands in SDS-PAGE gels that, despite showing different molecular weights [9], correspond to LMW and HMW SlpA, as confirmed by MS. These two bands were observed in all the strains evaluated, except LIBA-7857 (ST47), in whose lane no clear evidence of a LMW band was observed. This unusual finding could be due to degradation, variations in the proteolytic processing of the precursor protein or its secretion mechanism, post-translational modifications that affect the migration pattern, and *slpA* sequence variability, among other factors [9,128].

When the proinflammatory response induced by SlpA preparations containing both HMW and LMW and low amounts of other accompanying proteins was measured *in vitro,* all of the Clade 2 strains tested significantly increased the levels of TNF-alpha compared to the negative control, confirming that SlpA induces proinflammatory responses, as seen in previous studies [133]. Strains LIBA-5700 (ST01), LIBA-5758 (ST01), LIBA-2811 (ST41), and LIBA-6656 (ST154) induced the strongest responses, followed by strains R20291 (ST01), LIBA-7857 (ST47), LIBA-5757 (ST67), and LIBA-5809 (ST638). The increased levels of this cytokine, and others, could contribute to the tissular damage that *C.difficile* provokes in animal models and also in CDI patients [107,129].

Though the underlying mechanism that explains differences in immune response induced by the strains has yet to be clarified, it could involve the ability of SlpA to modulate the balance between the Th1/Th2 immune response [129]. In this regard, *C. difficile* SlpA stimulated human monocyte-derived dendritic cells (MDDC) produces high amounts of IL-12p70 and IFN-ϒ pro-inflammatory cytokines associated with the Th1 immune response-, as well as IL-10 and IL-5; which are anti-inflammatory cytokines representative of the Th2 response [129]. Due to its ability to activate the immune system, SlpA should be studied as a potential adjuvant or as part of a multicomponent vaccine against *C. difficile* [129].

Mouse antibodies raised against LIBA-5758 ST01-SlpA not only recognized SlpA from other strains from the same ST, but also, to a lesser extent, HMW SlpA from other STs. By contrast, these antibodies did not react with LMW SlpA from other strains, confirming that HMW SlpA is more conserved than LMW SlpA [9,107,128], which has an extremely high genetic diversity that leads to a lack of immunological cross-reactivity [9,128].

It is also important to consider that the sequence of S-layer cassettes varies because of homologous recombination. These modifications can be seen even among strains of the same ST, as has been reported for ST41 [128]. Variations in SlpA sequence might lead to increased secretion and higher proinflammatory activity, enhancing the virulent phenotype of certain *C. difficile* strains [107]. Consequently, it would be relevant to determine whether the SlpA cassettes of the strains included in this study, in particular that of strain R20291, which induced a lower level of TNF-α compared to other isolates from its ST.

Though SlpA is attached to the cell surface, it is also part of the exoproteome, whereby the amount and type of SlpA secreted might contribute to strain virulence *in vivo* [107,129]. We did not measure this; hence, our assays could be complemented with exoproteome analyses and an evaluation of their biological activity in animal models. It would also be interesting to study potential differences in the levels of anti-SlpA antibodies produced by animals exposed to SlpA extracts of our Clade 2 strains, as well as their neutralizing capacity. These experiments would allow us to clarify whether they inhibit the adhesion of *C. difficile* to epithelial cells or delay the progress of infection in animal models. Besides, it would be valuable to study the anti-SlpA antibody production in patients infected with *C. difficile*, by comparing antibody levels in early and late stages of infection to reveal response trends [133]. In any case, our *in vitro* data indicates that SlpA and its derived pro-inflammatory effect is not useful to differentiate ST01 strains from strains of other STs within the same Clade.

4.3.3 Detection of flagellin and motility assays

FliC and FliD are SAPs involved in flagellar assembly, cellular movement, chemotaxis, adherence, and attachment of *C. difficile* to the intestinal mucus layer, contribute to bacterial survival and host colonization [8,127].

In *C. difficile* 630, *fliC* and *fliD* belong to the F1 regulon, which encodes late-stage genes involved in flagellar filament synthesis. The F2 regulon contains flagellar biosynthetic glycan genes, whereas the early-stage genes required for basal body assembly belong to the F3 regulon. However, this setup can vary among strains [134].

A bacterial flagellin purification protocol and subsequent analysis by SDS-PAGE allowed us obtained bands of approximately 36-39 kDa that fit the flagellin 15-62 kDa molecular weight range expected for this protein in *C. difficile* [119,120]. While bands were identified as

products of the *fliC* gene for strains LIBA-2811 (ST41), LIBA-7857 (ST47), and LIBA-6656 (ST154), in other strains they were identified by MS as SlpA. This likely occurred due to the similar molecular weights of flagellin and LMW SlpA, or possibly because the latter group of strains produce smaller amounts of flagellin or have flagellin proteins of different size or modified post-translationally through glycosylation, as has been shown for strains R20291 (ST01) and 630 [134]. Alternative methodologies could be tested in the future to increase flagellin extraction yields and purity, and they could be complemented with WB using anti-FliC antibodies and MS to confirm the identity of the bands seen in the gels [119, 135].

Although flagellin detection was not confirmed for all strains (data not shown), all of them were confirmed to be motile through assays on motility tubes and soft agar plates. No differences were noted regarding swarming motility, yet strain LIBA-5700 (ST01) showed lower swimming ability than the other strains.

This difference in motility can be the result of divergence or loss of flagellar regulons among strains, together with differences in the number of flagella, as has been reported for clonal isolates of strains such as R20291 from ST01 [134]. For instance, it has been reported that strain R20291 (monotrichous) is less motile than strain *C. difficile* 630 (peritrichous) [8] but also that strain R20291 has multiple flagella and shows increased motility compared to strain 630 [136]. Therefore, it is difficult to determine the genetic variations and mechanisms that influence this phenotype [134]. The presence or absence of flagella, as well as their number and position in the cell, can be confirmed by flagella staining, negative-stained electron micrographs [8], or cryo-electron tomography (cryo-ET) [137].

Flagella may also play a role in establishing associations with human epithelial cells. Indeed, *in vitro* tests have shown that strain R20291with functional flagella displays a greater adherence to Caco-2 cells than mutants with paralyzed flagella or non-flagellated strains [8]. On the other hand, flagellar motility seems not to be essential for colonization and adherence in mice [8]; therefore, these phenotypes need to be studied more extensively to understand the biological role of flagella and motility during CDI development.

From our findings, it is expected that the strains under study can synthesize flagella, establish cell-cell interactions, and produce surfactants [10]. Swarming and swimming motility allows bacteria to move in response to chemical gradients or other signals that directs them to a potential host. Our results indicate that the Clade 2 strains tested can swim -an advantage in

environments with a greater presence of fluids-, and transition to swarming motility -which would allow them to migrate on surfaces such as intestinal epithelial cells [10]-. Hence, these motility mechanisms together could facilitate the adaptation of this pathogen to different sections of the intestinal tract.

The presence of flagella may stimulate the immune system through the toll-like receptor TLR5 [138]. On the other hand, swarm cells may have enhanced resistance to engulfment by macrophages [10] and this could facilitate CDI development and promote persistence in the intestinal environment.

Among other factors, motility depends on virulence regulation factors, such as Cd1, a class I cyclic diguanosine monophosphate (c-di-GMP) sensing riboswitch [138]. The increased levels of c-di-GMP alter flagella assembly or their function, inhibiting cellular motility by truncation of flagellar transcripts, therefore, c-di-GMP negatively regulates flagellar gene expression, and thereby, swimming motility [139]. Motility mechanisms are also influenced by the leucine-responsive regulatory protein (*lrp*). In this respect, a R20291 *lrp*-mutant strain shows defects on swimming motility, possibly due to the decreased transcription of the flagellar regulator SigD, a sigma factor essential for transcription of late-stage flagellar genes [140]. The expression of *sigD* is also affected by the orientation of a genetic ON/OFF switch, termed flagellar switch (*flg* switch), whose inversion is catalyzed by the tyrosine recombinase RecV, and controls phase variable production of flagella and other virulence factors. Bacteria expressing *flg* phase ON can produce flagella. It could improve the bacterial ability to colonize the intestinal mucosa due to increased adhesion and flagella-mediated motility. On the other hand, cells expressing *flg* phase OFF suffer a decrease in the abundance of early-stage flagellar gene transcripts and are, in consequence, non-flagellated. This phenotype could result in evasion of recognition by TLR5, as the absence of flagellin would decrease the activation of the inflammasome and the production of antibodies [138].

Considering the information presented in this subsection, it is unlikely that the strains evaluated differ with regard to the expression of the aforementioned regulatory mechanisms. In our hands, motile behavior did not differentiate some strains ST01 from strains from other STs of the same Clade. Therefore, these factors appear not to be crucial in the establishment of the higher virulent phenotype associated with the ST01 strains.

4.3.4 Biofilm formation

Biofilms are sessile, surface-associated, microbial communities [12] immersed in a matrix generally composed of bacterial proteins, such as SlpA and flagella -in the case of *C. difficile* [8,12,13,119]-, DNA, and surface polysaccharides [12,49]. They protect bacteria from stress, such as exposure to antibiotics and host immune response, and promote a better host colonization [12,13]. For this reason, we evaluated the *in vitro* biofilm formation ability of our *C. difficile* Clade 2 strains at different times.

All strains formed biofilms without differences at 24 and 72 h, with decreases in biofilm formation ability over time. The least abundant biofilms were seen at 120 h, when the results obtained for strains LIBA-5700 (ST01), LIBA-5758 (ST01), and LIBA-2811 (ST41) were indistinguishable from the values obtained for the negative control. These results are consistent with other studies, where the maximum level of biofilm formation showed by strain R20291 (ST01) at 24h gradually dropped until reaching a minimum at 120 h [12].

It has been determined that the early stages of biofilm establishment are more affected by abnormalities on SlpA and the processing of its precursor protein than by flagella, which are more important at later stages [12]. Based on this notion, and on studies dealing with the flagellar switch [138], variations in flagellar expression could have caused the reduced biofilm formation detected for some strains at 120h.

Biofilm formation also depends on c-di-GMP, which inversely modulates motility and cell aggregation [139]. High levels of this second messenger promotes clumping of *C. difficile* cells, and therefore, biofilm formation by increasing adhesins production, as well as extracellular components [139]. Although the levels of c-di-GMP were not measured as part of our assays, it would be interesting to determine whether the concentration of c-di-GMP changes over time between the strains in a distinctive way.

Biofilm formation is also modulated by the expression of CwpV, a surface protein which promotes cell aggregation [141]. This protein, which together with SlpA constitute the S-layer, shows a phase variable expression regulated by the CwpV switch [141,142]. Bacteria on phase ON produce *cwpV* full-length transcripts and consequently express the protein, while transcripts produced in the phase OFF adopt a stem loop structure that induces transcription termination [142]. The inversion of this switch is regulated by RecV, the same regulator of the flagellar switch [138,141]. This coincidence evidences that flagellar

regulatory system is part of a complex system for the control of virulence factors, including toxin production, which is positively regulated by transcription of genes of the F3 regulon, while the expression of F1 genes decrease the transcription and secretion of toxins [143]. These regulatory mechanisms are beyond the scope of this work; however, they should be studied in future research, since they could modulate biofilm formation capacity, *in vitro*, and *in vivo*, between the strains.

Our results showed no differences between the strains in the initial and intermediate stages of biofilm formation, although in the late-stage some ST01 strains and the ST41 strain tested reduced their formation capacity. Nevertheless, biofilm formation capacity does not seem to be a characteristic to which the highly virulent phenotype of the ST01 strains can be attributed.

The human gut is distinguished by a great diversity of microorganisms that establish complex relationships with each other [144]. In this respect, our analysis is limited and partial, since biofilm formation was tested using pure cultures. Furthermore, our assays do not allow us to evaluate interactions with potential receptors on intestinal epithelial cells or the influence of stress factors on biofilm synthesis. These limitations could be partially solved by studying biofilm formation capacity in animal models and through the parallel analysis of mutants for biofilm-related genes [12,141]. Finally, the architecture and composition of these biofilms could be analyzed by confocal microscopy. Similar experiments in the past have proven useful to detect correlations between antibiotic resistance and persistence shown by certain strains [145].

4.3.5 Quantification of toxin B and characterization of effects induced by glycosyltransferase activity

Toxins TcdA and TcdB are the major virulence factors of *C. difficile* and elicit CDI clinical manifestations [1]. Their encoding genes are located in a chromosomal region named the PaLoc, along with genes that encode regulators of toxin expression, TcdR, and TcdC, as well as TcdE, a putative holin associated with toxin secretion [1,60,146].

TcdB is a 270 kDa cytotoxin that harbors a glycosyltransferase domain (GTD) [58] responsible for modifying GTP-binding proteins (GTPases) from the Ras superfamily, such as RhoA and Rac1, among others [15]. These targets are molecular switches involved in several cellular processes, including regulation of the cytoskeleton, migration, intracellular

traffic and other crucial activities [15]. Depending on the affinity of isoforms of TcdB for different substrates, they are classified as classic or variant, as defined by the type of morphological changes that they trigger in eukaryotic cells. [43,58,82].

Based on the essential role of TcdB in *C. difficile* virulence [39], different strains have been compared regarding the amounts of toxin produced, their amino acid sequences, distribution, and biological activity [41]. TcdB is highly diverse, and their natural variants, which have arisen due to recombination and horizontal gene transfer events, are classified into eight subtypes (TcdB1-TcdB8) [41]. Changes in the sequence of TcdB could be selected through an enhancement in virulence and possibly by immune evasion, triggering a different relatedness with animal and human diseases among TcdB variants [41,60].

As indicated in Table 3 from Chapter I, Clade 2 members have either TcdB2 or TcdB4. TcdB2 is the most extensively distributed isoform within this clade, followed by TcdB4, while some rare strains (i.e. from STs ST41 and ST67) can harbor either TcdB2 or TcdB4.

We characterized seven clinical isolates regarding their toxin production levels, as well as their effect at the cellular level *in vitro*, as a consequence of toxin glycosyltransferase activity on two GTPases. We observed that LIBA-5700 (ST01) and LIBA-5758 (ST01), together with ST41 and ST154, secreted the highest amount of TcdB, followed by the R20291 (ST01), ST47, ST67 strains, and lastly, LIBA-5809 (ST638), which produced the least amount of toxin, according with densitometric measurements.

Strains can produce different amounts of toxins because of genetic variations, growth conditions, and the influence of regulatory mechanisms that also coordinate other metabolic processes in response to environmental stimuli [147]. TcdA and TcdB hyperproduction has been mainly attributed to an 18 bp deletion and a frameshift mutation in *tcdC*, the encoding gene for the negative regulator of toxin synthesis [52,53,58,148]. This mutation is present in strain LIBA-5758 (ST01) [52], LIBA-5757 (ST67) [58,86], and LIBA-2811 (ST41) strains [104]. However, this is subject of debate because *tcdC* mutations have not been reported for other strains considered as "hyperproducers" of toxins A and B. On the other hand, strains with truncated *tcdC* alleles do not always hyperproduce toxins, as has been reported for R20291 (ST01) [148–150], and as observed for LIBA-2811 (ST41) here. Besides, restoration of chromosomal mutated *tcdC* to wild type in R20291 (ST01) did not affect toxin synthesis [149], suggesting that TcdC is not a major determinant for *C. difficile* hypervirulence. Several

studies failed to detect an association between toxin production and *tcdC* genotype or disease severity [148–150]. These contrasting results evidence that genomic information needs to be complemented with phenotypic analyzes such as those carried as part of this study, as well as with animal models assays such as those performed by Orozco-Aguilar et al., to determine the *in vivo* biological effects of toxins [29].

C. difficile strains, such as R20291 (ST01), have TcdC-independent regulation of toxin synthesis, that involves proteins codified by quorum signal generation and response pathways genes, such as the Arg quorum-signaling system [151]. It has been reported that inactivation of Arg components decreases toxin production in R20291 (ST01), thus, abnormalities on those proteins could repress expression of toxin genes and lead to colonization defects [147]. Global transcriptional regulators involved in the modulation of several metabolic processes, such as the Lrp, have been recently described to modulate toxin expression among *C.difficile* strains [140]. In this regard, strains with non-functional *lrp* produce significantly higher levels of toxins than those with the wild type allele [140].

Toxin-coding genes, traditionally located on the bacterial chromosome, can also be found in mobile genetic elements (MGEs) such as putative conjugative plasmids [28]. In agreement with our phenotypic findings, the ST154 strain studied here carries an MGE-associated *tcdB* whose product is expected to induce a *Clostridium sordellii*–like cytopathic effect [28]. In addition to its plasmid-borne *tcdB*, ST154 carries a different *tcdB* allele on a chromosomal PaLoc. The contribution of these two *tcdB* alleles to CDI remains unclear [28], however, it could affect toxin expression and be related to the toxin hyperproducer phenotype detected by us. A deeper study of the regulatory mechanisms mentioned above has the potential to clarify the differences in toxin production and cytopathic activity seen among our strains.

Toxin production may be part of adaptive strategies in response to environmental conditions, such as nutritional signals. Glucose and other rapidly metabolized carbon sources, as well as the presence of proline and cysteine, inhibit toxin gene expression through different regulatory mechanisms [147]. Also, the presence of second messengers involved in the regulation of several virulence factors, such as c-di-GMP, decreases the transcription of toxin encoding genes and the positive regulator *tcdR* [147,152]. Toxin synthesis is also influenced by the temperature to which the bacteria are exposed, the phases of the growth curve, as well as by the exposure to sub-inhibitory concentrations of certain antibiotics, among other factors

[146,146]. Therefore, it would be desirable to repeat our toxin related analyses in living systems.

The identity of the bands with the molecular weight expected for TcdB (270 kDa) [1,146] was confirmed through a WB assay in which positive signals were confirmed to be TcdB by MS [58]. The resulting bands speak in favor of immunorecognition variations among the strains, since TcdB produced by ST47 and ST67 strains was more intensively recognized than those of strains R20291 (ST01) and ST638, which exhibited higher or similar densitometric measurements, respectively. These four strains presumably express TcdB2 [41], but there is a chance that their toxin gene sequences vary subtly, at least at the antigenic level. ST67 is predicted to harbor TcdB2 or TcdB4, which could imply an additional source of immunorecognition variability.

To better understand these results, it is necessary to analyze the toxin sequences of these STs. Furthermore, it is desirable to perform quantitative label-free mass spectrometry to accurately quantify the amount of toxin secreted by each strain [153] and to study their effect of other GTPases.

Consistent with preceding studies, we observed that strains causing classical CPE, such as ST01 and ST47, glycosylate RhoA and Rac1, while ST41 and ST67 strains showed a reduced ability to glycosylate RhoA. Additionally, it has been reported that both toxins glycosylate Rap1, Rap2, and R-Ras at low levels. Cdc42 is also modified by classic TcdB, and to a much lesser extent by variant isoforms [41,58].

The analyzed ST41 and ST67 strains elicit a variant CPE, probably associated with TcdB4, even when they could also harbor the TcdB2 subtype [41]. We cannot rule out that the ST154 and ST638 strains tested may have more than one toxin subtype, as ST41 and ST67 do, but it is anticipated that the toxin produced by ST154 is TcdB4, and that the toxigenic phenotype in ST638 is similar to those of strains with TcdB2, based on to the CPE they induce and the GTPase glycosylation profile detected.

Remarkably, RhoA was more evident in cells treated with supernatants containing toxins from LIBA-2811 (ST41), LIBA-5757 (ST67) and LIBA-6656 (ST154), than in non-intoxicated cells, confirming that its overexpression represents a compensatory response mechanism [43].

The observed differences in toxin activity are a consequence of variations at the sequence level between TcdB2 and TcdB4. They share similar CROPs (combined repetitive oligopeptides) and RBD (receptor-binding) domains, but their GTD (glycosyl-transferase) domains differ [58], which is enzymatically responsible for the inactivation of the GTPases that lead to host cellular dysfunction.

Regarding cytopathic potential , although the titers recorded are lower than those reported by our group in previous studies [82], we also observed that the high cytopathic activity induced by ST01 is not distinct from that recorded for ST67 and ST41 strains. These results suggest that this activity and the kinetics of CPE formation do not depend directly on the amount of toxin produced by the strains or the type of CPE that they induce.

It should be noted that toxin A and binary toxin (CDT), whose expression has been reported for strains such as ST01 [61], ST67 [58,86], and ST41 [154], also mediate cytotoxic activity and cytopathic effects. In this respect, though beyond the scope of this thesis, an in-depth functional characterization of TcdA and CDT produced by Clade 2 strains should be included in future studies.

Our results did not allow us to establish a correlation between the amount and type of toxin produced by each strain and its cytopathic potential. Thus, impact of these features on virulence levels associated with Clade 2 strains remain unclear.

4.3.6 Spore response to sodium dichloroisocyanurate (NaDCC)

Some *C. difficile* Clade 2 strains, such as R20291 (ST01) have been associated with increased transmissibility, CDI prevalence, and severity [16]. Spores are at least in part responsible for the persistence of *C. difficile* in patients, and their horizontal transmission [6]. It is estimated that a CDI patient can excrete between $1x10^4$ and up to $1x10^7$ spores per gram of faeces [7,16]. These excreted spores can remain viable in the environment for long periods where they can resist the activity of commonly used disinfection agents [155–158].

Our *in vitro* assessment of NaDCC showed indistinguishable differences in log reductions for all of the strains tested, suggesting that they do not possess factors that confers significant resistance to NaDCC under the studied conditions. Resistance of spores to disinfectants is mediated by variations in the cortex, spore coat, and the exosporium [6,159], which in *C. difficile* include the products of genes such as *spoIVA*, *cotA*, *cotB*, *cotCB*, *cotD*, *cotF*, *cotG*,

sodA, *bclA1*, *bclA2*, *bclA3*, *cdeC*, and *cdeM* [6,160]. Based on the results obtained, no significant differences in the sequences of these genes are expected among the strains studied, or alternatively, if there are some variations, they would not be sufficient to induce a significant difference in resistance to NaDCC in our assay. It would be useful to investigate these gene sequences to have a more complete picture of possible changes distributed between the Clade 2 strains, even when those differences at the sequence level are not directly associated with a significantly increased or decreased resistance to NaDCC. Eventually, if a strain shows important variations in its response to NaDCC, the knowledge acquired at gene sequence level would help to identify the differences most likely involved in the induction of that phenotype. Also, strains could share a very similar mutation profile and display different resistance phenotypes, as well as the contrary.

Besides, environmental and physiological factors, as well as the presence of efflux pumps or ABC transporters and other proteins that interact with external stimuli, play an important role in the spore resistance to biocidal agents [16].

The outermost layers of the spores are related to hydrophobicity and adherence to organic and inorganic surfaces [17]. It has been observed that spores lacking the exosporial layer are less hydrophobic, show reduced adherence to surfaces, and are more susceptible to NaDCC, which evidences the contribution of structural variations in terms of susceptibility to biocidal agents [17]. Furthermore, the spore coat possibly acts as a permeability barrier that hinders the entry of nonspecific molecules to the spore, and in conjunction with the exosporium, protects the enzymes and DNA from harmful molecules and chemicals [17]. Additionally, the cortex probably contributes to the resistance maintaining the dehydration of the spore core. The decreased water level and high viscosity prevent the diffusion of water-soluble antimicrobial agents such as chlorine [159].

Our assay could be complemented with analyzes that study the structure of the spore through transmission electron microscopy (TEM), and also its relative hydrophobicity, which would allow establishing a correlation between the presence of exosporium in the spores and the level of susceptibility to NaDCC shown by them. It could also reveal whether the spores undergo any structural change after exposure to the disinfectant, which affects phenotypes beyond their viability, such as their adherence to surfaces [17,161].

Moreover, NaDCC activity against spores from Clade 2 strains should be assessed on different surfaces, such as contaminated stainless-steel pieces, floors, bed, gowns, curtain rails, soap dispensers, among others, to obtain a more relevant estimation of the *in situ* effectiveness of this agent [157].

5. Closing remarks

This investigation constitutes the most extensive comparison of STs from the MLST Clade 2, including a literature review and *in vitro* experiments for characterizing virulence-associated phenotypes of relevance at different stages of CDI development.

Our narrative review demonstrated:

- A lack of available information for most Clade 2 members.
- That current evidence does not support the widespread view that “hypervirulence” is a common feature within this clade.
- A need for continuous and active surveillance programs to capture more Clade isolates
- The need to combine exhaustive genomic and phenotypic studies with patient data and clinical parameters to deepen our understanding of the epidemiology and pathogenesis of Clade 2 STs.

The experimental part of the thesis, in turn, is summarized in Table 6.

Table 6. Virulence-associated phenotypes of representatives of 6 STs from the *C. difficile* MLST Clade 2.

		ST01			ST41	ST47	ST67	ST154[60]	ST638
Assay[a]		**R20291**	**LIBA-5700**	**LIBA-5758**	**LIBA-2811**	**LIBA-7857**	**LIBA-5757**	**LIBA-6656**	**LIBA-5809**
Spore adherence to ECC (%)		**86.26 ± 3.97**	**86.86 ± 5.25**	**83.81 ± 0.88**	**85.66 ± 0.81**	**85.48 ± 1.53**	**84.89 ± 1.68**	**83.56 ± 1.52**	**82.99 ± 2.75**
SlpA detection and associated antibody response		ST-dependent expression of SlpA isoforms with different apparent molecular weights. ST-dependent immunogenic similarity.							
SlpA and pro-immflamatory response mediation (TNF-α pg/mL)		815.20 ± 146.65	1338.13 ± 66.62	1452.06 ± 82.87	1562.40 ± 36.43	1041.70 ± 74.14	669.43 ± 77.14	1343.96 ± 97.98	883.5 ± 168.52
Motility	Swarming (cm)	**1.64 ± 0.24**	**1.46 ± 0.18**	**1.43 ± 0.24**	**1.48 ± 0.18**	**1.44 ± 0.16**	**1.39 ± 0.16**	**1.68 ± 0.28**	**1.78 ± 0.46**
	Swimming (cm)	**2.84 ± 0.46**	**2.08 ± 0.21**	**2.48 ± 0.67**	**2.71 ± 0.51**	**2.77 ± 0.39**	**2.67 ± 0.49**	**2.84 ± 0.41**	**2.61 ± 0.82**
Biofilm formation (Absorbance	24h	**0.20 ± 0.17**	**0.23 ± 0.03**	**0.19 ± 0.04**	**0.21 ± 0.06**	**0.21 ± 0.05**	**0.22 ± 0.03**	**0.18 ± 0.05**	**0.21 ± 0.05**
	72h	**0.20 ± 0.04**	**0.17 ± 0.04**	**0.19 ± 0.08**	**0.17 ± 0.06**	**0.18 ± 0.05**	**0.21 ± 0.07**	**0.19 ± 0.05**	**0.20 ± 0.05**
	120h	0.17 ± 0.04	0.12 ± 0.03	0.14 ± 0.04	0.13 ± 0.04	0.19 ± 0.06	0.17 ± 0.06	0.21 ± 0.07	0.19 ± 0.07
TcdB subtype		TcdB2	TcdB2	TcdB2	TcdB2 TcdB4	TcdB2	TcdB2 TcdB4	TcdB4	TcdB2

Quantification of secreted TcdB[b]	Non-increased	Increased	Increased	Increased	Non-increased	Non-increased	Increased	Non-increased
CPE	Classical	Classical	Classical	Variant	Classical	Variant	Variant	Classical
Host cell targets of glycosyltransferase activity	RhoA Rac1	RhoA Rac1	RhoA Rac1	Rac1	RhoA Rac1	Rac1	Rac1	RhoA Rac1
Spore susceptibility to NaDCC (logarithmic reduction factor)	**4.60 ± 1.07**	**4.82 ± 0.65**	**3.82 ± 0.52**	**5.10 ± 0.05**	**4.55 ± 0.93**	**3.59 ± 0.64**	**4.85 ± 0.78**	**4.84 ± 0.47**

[a] Results in bold correspond to statistically indistinguishable results

[b]Non-increased: band density below 400, increased: band density above 400.

Altogether, these results demonstrate that the studied STs show great similarity regarding:

i) Spore adherence to intestinal epithelial cell.
ii) Motility capacity.
iii) Initial and intermediate stages of biofilm formation.
iv) Spore susceptibility to NaDCC.

The three former results suggest that the studied strains share characteristics of relevance in the intestinal colonization process. Besides, the comparable susceptibility of their spores to NaDCC is unlikely to influence their spread capability.

- The studied strains express SlpA with different molecular weights, immunorecognition, and pro-inflammatory potential. This response was significantly increased in cells exposed to ST01 proteins, but this was also seen for some non-ST01 strains (i.e., ST41 and ST154).
- Biofilms formed by some ST01 and ST41 strains reduce their stability at 120 h.
- TcdB high level production was shared by some ST01, ST41, and ST154 strains.

- While TcdB produced by strains ST01, ST47, and ST638 induce a classical CPE, strains from ST41, ST67, and ST154 induce the variant CPE, as a result of the modification of different intracellular targets. These observations suggest that toxin overproduction and the type of CPE induced as a result of the glycosylation of different GTPases do not determine the "hypervirulent" phenotype described for ST01 strains.

In summary, we confirmed that ST01 and non-ST01 strains share virulence-associated *in vitro* phenotypes. The factors studied do not explain the increased *in vivo* virulence reported for the ST01 strains compared to the non-ST01 strains by Orozco-Aguilar [29], suggesting that this is a multifactor phenotype.

It is recommended that this work is complemented with experiments such as:

- Structure-visualization techniques, including immunofluorescence microscopy, TEM, or cryo-ET, to analyze in depth the spores, S-layer, flagella, and biofilms of the strains. These experiments would also allow the detection of alterations after exposure to antibiotics or disinfectants.
- Analysis of the presence of SlpA in the exoproteome, as well its biological effects in animal models. In addition to the study of the neutralizing capacity of antibodies against these proteins, obtained by animal immunization.
- Determination of the *in situ* effectiveness of other sporicidal disinfectants.
- Determination of variations in the sequences of genes related to the production of spores and the expression of SlpA, flagella, and toxins, among others, as well as their regulatory mechanisms.
- In-depth comparison of genetic elements related to antibiotic resistance, as an indicator of possible obstacles in the treatment of CDI produced by different strains and their dissemination capacity.

6. References

[1] D.E. Voth, J.D. Ballard, *Clostridium difficile* toxins: mechanism of action and role in disease, Clin Microbiol Rev. 18 (2005) 247–263. https://doi.org/10.1128/CMR.18.2.247-263.2005.

[2] R.A. Stabler, D.N. Gerding, J.G. Songer, D. Drudy, J.S. Brazier, H.T. Trinh, A.A. Witney, J. Hinds, B.W. Wren, Comparative phylogenomics of *Clostridium difficile* reveals clade specificity and microevolution of hypervirulent strains, J Bacteriol. 188 (2006) 7297–7305. https://doi.org/10.1128/JB.00664-06.

[3] B. Elliott, G.O. Androga, D.R. Knight, T.V. Riley, *Clostridium difficile* infection: Evolution, phylogeny and molecular epidemiology, Infect Genet Evol. 49 (2017) 1–11. https://doi.org/10.1016/j.meegid.2016.12.018.

[4] D.A. Burns, D. Heeg, S.T. Cartman, N.P. Minton, Reconsidering the sporulation characteristics of hypervirulent *Clostridium difficile* BI/NAP1/027, PLoS One. 6 (2011) e24894. https://doi.org/10.1371/journal.pone.0024894.

[5] D. Paredes-Sabja, M.R. Sarker, Adherence of *Clostridium difficile* spores to Caco-2 cells in culture, J Med Microbiol. 61 (2012) 1208–1218. https://doi.org/10.1099/jmm.0.043687-0.

[6] D. Paredes-Sabja, A. Shen, J.A. Sorg, *Clostridium difficile* spore biology: sporulation, germination, and spore structural proteins, Trends in Microbiology. 22 (2014) 406–416. https://doi.org/10.1016/j.tim.2014.04.003.

[7] W.K. Smits, D. Lyras, D.B. Lacy, M.H. Wilcox, E.J. Kuijper, *Clostridium difficile* infection, Nat Rev Dis Primers. 2 (2016) 16020. https://doi.org/10.1038/nrdp.2016.20.

[8] S.T. Baban, S.A. Kuehne, A. Barketi-Klai, S.T. Cartman, M.L. Kelly, K.R. Hardie, I. Kansau, A. Collignon, N.P. Minton, The role of flagella in *Clostridium difficile* pathogenesis: Comparison between a Non-Epidemic and an Epidemic strain, PLOS ONE. 8 (2013) e73026. https://doi.org/10.1371/journal.pone.0073026.

[9] E. Calabi, S. Ward, B. Wren, T. Paxton, M. Panico, H. Morris, A. Dell, G. Dougan, N. Fairweather, Molecular characterization of the surface layer proteins from *Clostridium difficile*, Molecular Microbiology. 40 (2001) 1187–1199. https://doi.org/10.1046/j.1365-2958.2001.02461.x.

[10] D.B. Kearns, A field guide to bacterial swarming motility, Nat Rev Microbiol. 8 (2010) 634–644. https://doi.org/10.1038/nrmicro2405.

[11] W.J. Bradshaw, A.K. Roberts, C.C. Shone, K.R. Acharya, The structure of the S-layer of *Clostridium difficile*, J. Cell Commun. Signal. 12 (2018) 319–331. https://doi.org/10.1007/s12079-017-0429-z.

[12] T. Dapa, R. Leuzzi, Y.K. Ng, S. T. Baban, R. Adamo, S. A. Kuehne, M. Scarcelli, N. P. Minton, D. Serruto, M. Unnikrishna, Multiple Factors modulate biofilm formation by the anaerobic pathogen *Clostridium difficile*, Journal of Bacteriology. 3 (2013) 545-555. https://journals.asm.org/doi/abs/10.1128/JB.01980-12.

[13] M.M. Awad, P.A. Johanesen, G.P. Carter, E. Rose, D. Lyras, *Clostridium difficile* virulence factors: Insights into an anaerobic spore-forming pathogen, Gut Microbes. 5 (2014) 579–593. https://doi.org/10.4161/19490976.2014.969632.

[14] G.P. Carter, J.I. Rood, D. Lyras, The role of toxin A and toxin B in the virulence of *Clostridium difficile*, Trends in Microbiology. 20 (2012) 21–29. https://doi.org/10.1016/j.tim.2011.11.003.

[15] K. Aktories, C. Schwan, T. Jank, *Clostridium difficile* Toxin Biology, Annu. Rev. Microbiol. 71 (2017) 281–307. https://doi.org/10.1146/annurev-micro-090816-093458.

[16] L.F. Dawson, E. Valiente, E.H. Donahue, G. Birchenough, B.W. Wren, Hypervirulent *Clostridium difficile* PCR-ribotypes exhibit resistance to widely used disinfectants, PLoS One. 6 (2011). https://doi.org/10.1371/journal.pone.0025754.

[17] L.T. Joshi, A. Welsch, J. Hawkins, L. Baillie, The effect of hospital biocide sodium dichloroisocyanurate on the viability and properties of *Clostridium difficile* spores, Lett Appl Microbiol. 65 (2017) 199–205. https://doi.org/10.1111/lam.12768.

[18] P. Castro-Córdova, P. Mora-Uribe, R. Reyes-Ramírez, G. Cofré-Araneda, J. Orozco-Aguilar, C. Brito-Silva, M.J. Mendoza-León, S.A. Kuehne, N.P. Minton, M. Pizarro-Guajardo, D. Paredes-Sabja, Entry of spores into intestinal epithelial cells contributes to recurrence of *Clostridioides difficile* infection, Nat Commun. 12 (2021) 1140. https://doi.org/10.1038/s41467-021-21355-5.

[19] G. Killgore, A. Thompson, S. Johnson, J. Brazier, E. Kuijper, J. Pepin, E.H. Frost, P. Savelkoul, B. Nicholson, R.J. van den Berg, H. Kato, S.P. Sambol, W. Zukowski, C.

Woods, B. Limbago, D.N. Gerding, L.C. McDonald, Comparison of seven techniques for typing international epidemic strains of *Clostridium difficile*: restriction endonuclease analysis, pulsed-field gel electrophoresis, PCR-ribotyping, multilocus sequence typing, multilocus variable-number tandem-repeat analysis, amplified fragment length polymorphism, and surface layer protein A gene sequence typing, J Clin Microbiol. 46 (2008) 431–437. https://doi.org/10.1128/JCM.01484-07.

[20] D. Griffiths, W. Fawley, M. Kachrimanidou, R. Bowden, D.W. Crook, R. Fung, T. Golubchik, R.M. Harding, K.J.M. Jeffery, K.A. Jolley, R. Kirton, T.E. Peto, G. Rees, N. Stoesser, A. Vaughan, A.S. Walker, B.C. Young, M. Wilcox, K.E. Dingle, Multilocus sequence typing of *Clostridium difficile*, J. Clin. Microbiol. 48 (2010) 770–778. https://doi.org/10.1128/JCM.01796-09.

[21] R.A. Stabler, L.F. Dawson, E. Valiente, M.D. Cairns, M.J. Martin, E.H. Donahue, T.V. Riley, J.G. Songer, E.J. Kuijper, K.E. Dingle, B.W. Wren, Macro and micro diversity of *Clostridium difficile* isolates from diverse sources and geographical locations, PLoS ONE. 7 (2012) e31559. https://doi.org/10.1371/journal.pone.0031559.

[22] S. Janezic, M. Rupnik, Genomic diversity of *Clostridium difficile* strains, Res. Microbiol. 166 (2015) 353–360. https://doi.org/10.1016/j.resmic.2015.02.002.

[23] C.W. Knetsch, T.D. Lawley, M.P. Hensgens, J. Corver, M.W. Wilcox, E.J. Kuijper, Current application and future perspectives of molecular typing methods to study *Clostridium difficile* infections, Euro Surveill. 18 (2013) 20381. https://doi.org/10.2807/ese.18.04.20381-en.

[24] S. Janezic, M. Potocnik, V. Zidaric, M. Rupnik, Highly divergent *Clostridium difficile* strains isolated from the environment, PLOS ONE. 11 (2016) e0167101. https://doi.org/10.1371/journal.pone.0167101.

[25] C.W. Knetsch, E.M. Terveer, C. Lauber, A.E. Gorbalenya, C. Harmanus, E.J. Kuijper, J. Corver, H.C. van Leeuwen, Comparative analysis of an expanded *Clostridium difficile* reference strain collection reveals genetic diversity and evolution through six lineages, Infect. Genet. Evol. 12 (2012) 1577–1585. https://doi.org/10.1016/j.meegid.2012.06.003.

[26] E. Valiente, M.D. Cairns, B.W. Wren, The *Clostridium difficile* PCR ribotype 027 lineage: a pathogen on the move, Clin Microbiol Infect. 20 (2014) 396–404. https://doi.org/10.1111/1469-0691.12619.

[27] C.L. Costa, D. López-Ureña, T. de Oliveira Assis, R.A. Ribeiro, R.O.S. Silva, M. Rupnik, M.H. Wilcox, A.F. de Carvalho, A.O. do Carmo, A.A.M. Dias, C.B.M. de Carvalho, E. Chaves-Olarte, C. Rodríguez, C. Quesada-Gómez, G.A. de Castro Brito, A MLST Clade 2 *Clostridium difficile* strain with a variant TcdB induces severe inflammatory and oxidative response associated with mucosal disruption, Anaerobe. 40 (2016) 76–84. https://doi.org/10.1016/j.anaerobe.2016.06.005.

[28] G. Ramírez-Vargas, C. Rodríguez, Putative Conjugative Plasmids with tcdB and cdtAB Genes in *Clostridioides difficile*, Emerging Infect. Dis. 26 (2020) 2287–2290. https://doi.org/10.3201/eid2609.191447.

[29] J. Orozco-Aguilar, A. Alfaro-Alarcón, L. Acuña-Amador, E. Chaves-Olarte, C. Rodríguez, C. Quesada-Gómez, In vivo animal models confirm an increased virulence potential and pathogenicity of the NAP1/RT027/ST01 genotype within the *Clostridium difficile* MLST Clade 2, Gut Pathog. 12 (2020). https://doi.org/10.1186/s13099-020-00383-4.

[30] C. Quesada-Gómez, C. Rodríguez, M. del M. Gamboa-Coronado, E. Rodríguez-Cavallini, T. Du, M.R. Mulvey, M. Villalobos-Zúñiga, R. Boza-Cordero, Emergence of *Clostridium difficile* NAP1 in Latin America, J. Clin. Microbiol. 48 (2010) 669–670. https://doi.org/10.1128/JCM.02196-09.

[31] T. Akerlund, I. Persson, M. Unemo, T. Norén, B. Svenungsson, M. Wullt, L.G. Burman, Increased sporulation rate of epidemic *Clostridium difficile* Type 027/NAP1, J Clin Microbiol. 46 (2008) 1530–1533. https://doi.org/10.1128/JCM.01964-07.

[32] S. Bletz, S. Janezic, D. Harmsen, M. Rupnik, A. Mellmann, Defining and evaluating a core genome Multilocus Sequence Typing scheme for Genome-Wide Typing of *Clostridium difficile*, J Clin Microbiol. 56 (2018). https://doi.org/10.1128/JCM.01987-17.

[33] M.J.T. Crobach, J.J. Vernon, V.G. Loo, L.Y. Kong, S. Péchiné, M.H. Wilcox, E.J. Kuijper, Understanding *Clostridium difficile* colonization, Clinical Microbiology Reviews. 31 (2018). https://doi.org/10.1128/CMR.00021-17.

[34] S.H. Cohen, D.N. Gerding, S. Johnson, C.P. Kelly, V.G. Loo, L.C. McDonald, J. Pepin, M.H. Wilcox, Clinical Practice Guidelines for *Clostridium difficile* infection in adults: 2010 Update by the Society for Healthcare Epidemiology of America (SHEA) and the Infectious Diseases Society of America (IDSA), Infection Control and Hospital Epidemiology. 31 (2010) 431–455. https://doi.org/10.1086/651706.

[35] S. Tratulyte, J. Miciuleviciene, N. Kuisiene, First genotypic characterization of toxigenic *Clostridioides difficile* in Lithuanian hospitals reveals the prevalence of the hypervirulent ribotype 027/ST1, Eur. J. Clin. Microbiol. Infect. Dis. 38 (2019) 1953–1959. https://doi.org/10.1007/s10096-019-03633-9.

[36] S.M. Vindigni, C.M. Surawicz, *C. difficile* infection: Changing epidemiology and management paradigms, Clin Transl Gastroenterol. 6 (2015) e99. https://doi.org/10.1038/ctg.2015.24.

[37] S. Khanna, D.S. Pardi, S.L. Aronson, P.P. Kammer, R. Orenstein, J.L. St Sauver, W.S. Harmsen, A.R. Zinsmeister, The epidemiology of community-acquired *Clostridium difficile* infection: a population-based study, Am. J. Gastroenterol. 107 (2012) 89–95. https://doi.org/10.1038/ajg.2011.398.

[38] E. Balsells, T. Shi, C. Leese, I. Lyell, J. Burrows, C. Wiuff, H. Campbell, M.H. Kyaw, H. Nair, Global burden of *Clostridium difficile* infections: a systematic review and meta-analysis, J Glob Health. 9 (2019) 010407. https://doi.org/10.7189/jogh.09.010407.

[39] D. Lyras, J.R. O'Connor, P.M. Howarth, S.P. Sambol, G.P. Carter, T. Phumoonna, R. Poon, V. Adams, G. Vedantam, S. Johnson, D.N. Gerding, J.I. Rood, Toxin B is essential for virulence of *Clostridium difficile*, Nature. 458 (2009) 1176–1179. https://doi.org/10.1038/nature07822.

[40] A.M. Hoegh, J.B. Nielsen, A. Lester, A. Friis-Møller, K. Schønning, A multiplex, internally controlled real-time PCR assay for detection of toxigenic *Clostridium difficile* and identification of hypervirulent strain 027/ST-1, Eur. J. Clin. Microbiol. Infect. Dis. 31 (2012) 1073–1079. https://doi.org/10.1007/s10096-011-1409-5.

[41] E. Shen, K. Zhu, D. Li, Z. Pan, Y. Luo, Q. Bian, L. He, X. Song, Y. Zhen, D. Jin, L. Tao, Subtyping analysis reveals new variants and accelerated evolution of

Clostridioides difficile toxin B, Communications Biology. 3 (2020) 1–8. https://doi.org/10.1038/s42003-020-1078-y.

[42] M.J. Mansfield, B.J.-M. Tremblay, J. Zeng, X. Wei, H. Hodgins, J. Worley, L. Bry, M. Dong, A.C. Doxey, Phylogenomics of 8,839 *Clostridioides difficile* genomes reveals recombination-driven evolution and diversification of toxin A and B, BioRxiv. (2020) 2020.07.09.194449. https://doi.org/10.1101/2020.07.09.194449.

[43] E. Chaves-Olarte, E. Freer, A. Parra, C. Guzmán-Verri, E. Moreno, M. Thelestam, R-Ras glucosylation and transient RhoA activation determine the cytopathic effect produced by toxin B variants from toxin A-negative strains of *Clostridium difficile*, J. Biol. Chem. 278 (2003) 7956–7963. https://doi.org/10.1074/jbc.M209244200.

[44] E. Chaves-Olarte, P. Löw, E. Freer, T. Norlin, M. Weidmann, C. von Eichel-Streiber, M. Thelestam, A novel cytotoxin from *Clostridium difficile* serogroup F is a functional hybrid between two other large clostridial cytotoxins, J. Biol. Chem. 274 (1999) 11046–11052. https://doi.org/10.1074/jbc.274.16.11046.

[45] Z. Peng, A. Addisu, S. Alrabaa, X. Sun, Antibiotic resistance and toxin production of *Clostridium difficile* isolates from the hospitalized patients in a large hospital in Florida, Front Microbiol. 8 (2017) 2584. https://doi.org/10.3389/fmicb.2017.02584.

[46] E.M. Garrett, O. Sekulovic, D. Wetzel, J.B. Jones, A.N. Edwards, G. Vargas-Cuebas, S.M. McBride, R. Tamayo, Phase variation of a signal transduction system controls *Clostridioides difficile* colony morphology, motility, and virulence, PLOS Biology. 17 (2019) e3000379. https://doi.org/10.1371/journal.pbio.3000379.

[47] E.B. Purcell, R. Tamayo, Cyclic diguanylate signaling in Gram-positive bacteria, FEMS Microbiol Rev. 40 (2016) 753–773. https://doi.org/10.1093/femsre/fuw013.

[48] C. Wolz, T. Geiger, C. Goerke, The synthesis and function of the alarmone (p)ppGpp in firmicutes, International Journal of Medical Microbiology. 300 (2010) 142–147. https://doi.org/10.1016/j.ijmm.2009.08.017.

[49] A. Martínez-Meléndez, R. Morfin-Otero, L. Villarreal-Treviño, S.D. Baines, A. Camacho-Ortíz, E. Garza-González, Molecular epidemiology of predominant and emerging *Clostridioides difficile* ribotypes, Journal of Microbiological Methods. 175 (2020) 105974. https://doi.org/10.1016/j.mimet.2020.105974.

[50] A.P. Francisco, M. Bugalho, M. Ramirez, J.A. Carriço, Global optimal eBURST analysis of multilocus typing data using a graphic matroid approach, BMC Bioinformatics. 10 (2009) 152. https://doi.org/10.1186/1471-2105-10-152.

[51] V.G. Loo, L. Poirier, M.A. Miller, M. Oughton, M.D. Libman, S. Michaud, A.-M. Bourgault, T. Nguyen, C. Frenette, M. Kelly, A. Vibien, P. Brassard, S. Fenn, K. Dewar, T.J. Hudson, R. Horn, P. René, Y. Monczak, A. Dascal, A predominantly clonal multi-institutional outbreak of *Clostridium difficile*-associated diarrhea with high morbidity and mortality, N. Engl. J. Med. 353 (2005) 2442–2449. https://doi.org/10.1056/NEJMoa051639.

[52] H. Jia, P. Du, H. Yang, Y. Zhang, J. Wang, W. Zhang, G. Han, N. Han, Z. Yao, H. Wang, J. Zhang, Z. Wang, Q. Ding, Y. Qiang, F. Barbut, G.F. Gao, Y. Cao, Y. Cheng, C. Chen, Nosocomial transmission of *Clostridium difficile* ribotype 027 in a Chinese hospital, 2012–2014, traced by whole genome sequencing, BMC Genomics. 17 (2016). https://doi.org/10.1186/s12864-016-2708-0.

[53] D. Bakker, W.K. Smits, E.J. Kuijper, J. Corver, TcdC does not significantly repress toxin expression in *Clostridium difficile* 630ΔErm, PLoS One. 7 (2012). https://doi.org/10.1371/journal.pone.0043247.

[54] M. Warny, J. Pepin, A. Fang, G. Killgore, A. Thompson, J. Brazier, E. Frost, L.C. McDonald, Toxin production by an emerging strain of *Clostridium difficile* associated with outbreaks of severe disease in North America and Europe, Lancet. 366 (2005) 1079–1084. https://doi.org/10.1016/S0140-6736(05)67420-X.

[55] M. Merrigan, A. Venugopal, M. Mallozzi, B. Roxas, V.K. Viswanathan, S. Johnson, D.N. Gerding, G. Vedantam, Human hypervirulent *Clostridium difficile* strains exhibit increased sporulation as well as robust toxin production, J. Bacteriol. 192 (2010) 4904–4911. https://doi.org/10.1128/JB.00445-10.

[56] J. Freeman, S.D. Baines, K. Saxton, M.H. Wilcox, Effect of metronidazole on growth and toxin production by epidemic *Clostridium difficile* PCR ribotypes 001 and 027 in a human gut model, J Antimicrob Chemother. 60 (2007) 83–91. https://doi.org/10.1093/jac/dkm113.

[57] D.J. Fairley, J.P. McKenna, M. Stevenson, J. Weaver, C. Gilliland, A. Watt, P.V. Coyle, Association of *Clostridium difficile* ribotype 078 with detectable toxin in

human stool specimens, J Med Microbiol. 64 (2015) 1341–1345. https://doi.org/10.1099/jmm.0.000165.

[58] C. Quesada-Gómez, D. López-Ureña, N. Chumbler, H.K. Kroh, C. Castro-Peña, C. Rodríguez, J. Orozco-Aguilar, S. González-Camacho, A. Rucavado, C. Guzmán-Verri, T.D. Lawley, D.B. Lacy, E. Chaves-Olarte, Analysis of TcdB Proteins within the hypervirulent clade 2 reveals an impact of RhoA glucosylation on *Clostridium difficile* proinflammatory activities, Infect Immun. 84 (2016) 856–865. https://doi.org/10.1128/IAI.01291-15.

[59] H. Kim, S.H. Rhee, C. Pothoulakis, J.T. LaMont, *Clostridium difficile* toxin A binds colonocyte Src causing dephosphorylation of focal adhesion kinase and paxillin, Exp Cell Res. 315 (2009) 3336–3344. https://doi.org/10.1016/j.yexcr.2009.05.020.

[60] J.M. Lanis, S. Barua, J.D. Ballard, Variations in TcdB activity and the hypervirulence of emerging strains of *Clostridium difficile*, PLOS Pathogens. 6 (2010) e1001061. https://doi.org/10.1371/journal.ppat.1001061.

[61] D.M. Anderson, M.J. Sheedlo, J.L. Jensen, D.B. Lacy, Structural insights into the transition of *Clostridioides difficile* binary toxin from prepore to pore, Nature Microbiology. 5 (2020) 102–107. https://doi.org/10.1038/s41564-019-0601-8.

[62] M. Mac Aogáin, S. Kilkenny, C. Walsh, S. Lindsay, G. Moloney, T. Morris, S. Jones, T.R. Rogers, Identification of a novel mutation at the primary dimer interface of GyrA conferring fluoroquinolone resistance in *Clostridium difficile*, Journal of Global Antimicrobial Resistance. 3 (2015) 295–299. https://doi.org/10.1016/j.jgar.2015.09.007.

[63] M. He, F. Miyajima, P. Roberts, L. Ellison, D.J. Pickard, M.J. Martin, T.R. Connor, S.R. Harris, D. Fairley, K.B. Bamford, S. D'Arc, J. Brazier, D. Brown, J.E. Coia, G. Douce, D. Gerding, H.J. Kim, T.H. Koh, H. Kato, M. Senoh, T. Louie, S. Michell, E. Butt, S.J. Peacock, N.M. Brown, T. Riley, G. Songer, M. Wilcox, M. Pirmohamed, E. Kuijper, P. Hawkey, B.W. Wren, G. Dougan, J. Parkhill, T.D. Lawley, Emergence and global spread of epidemic healthcare-associated *Clostridium difficile*, Nat Genet. 45 (2013) 109–113. https://doi.org/10.1038/ng.2478.

[64] E. Guerrero-Araya, C. Meneses, E. Castro-Nallar, A.M. Guzmán D, M. Álvarez-Lobos, C. Quesada-Gómez, D. Paredes-Sabja, C. Rodríguez, Origin, genomic

diversity and microevolution of the *Clostridium difficile* B1/NAP1/RT027/ST01 strain in Costa Rica, Chile, Honduras and Mexico, Microb Genom. 6 (2020). https://doi.org/10.1099/mgen.0.000355.

[65] F. Hidalgo-Villeda, E. Tzoc, L. Torres, E. Bu, C. Rodríguez, C. Quesada-Gómez, Diversity of multidrug-resistant epidemic *Clostridium difficile* NAP1/RT027/ST01 strains in tertiary hospitals from Honduras, Anaerobe. 52 (2018) 75–78. https://doi.org/10.1016/j.anaerobe.2018.06.001.

[66] K.E. Dingle, D. Griffiths, X. Didelot, J. Evans, A. Vaughan, M. Kachrimanidou, N. Stoesser, K.A. Jolley, T. Golubchik, R.M. Harding, T.E. Peto, W. Fawley, A.S. Walker, M. Wilcox, D.W. Crook, Clinical *Clostridium difficile*: clonality and pathogenicity locus diversity, PLoS ONE. 6 (2011) e19993. https://doi.org/10.1371/journal.pone.0019993.

[67] T. Murillo, G. Ramírez-Vargas, T. Riedel, J. Overmann, J.M. Andersen, C. Guzmán-Verri, E. Chaves-Olarte, C. Rodríguez, Two groups of cocirculating, epidemic Clostridiodes difficile strains microdiversify through different mechanisms, Genome Biol Evol. 10 (2018) 982–998. https://doi.org/10.1093/gbe/evy059.

[68] C.D. Robinson, J.M. Auchtung, J. Collins, R.A. Britton, Epidemic *Clostridium difficile* strains demonstrate increased competitive fitness compared to nonepidemic isolates, Infect Immun. 82 (2014) 2815–2825. https://doi.org/10.1128/IAI.01524-14.

[69] J. Collins, C. Robinson, H. Danhof, C.W. Knetsch, H.C. van Leeuwen, T.D. Lawley, J.M. Auchtung, R.A. Britton, Dietary trehalose enhances virulence of epidemic *Clostridium difficile*, Nature. 553 (2018) 291–294. https://doi.org/10.1038/nature25178.

[70] L.C. McDonald, G.E. Killgore, A. Thompson, R.C. Owens, S.V. Kazakova, S.P. Sambol, S. Johnson, D.N. Gerding, An epidemic, toxin gene–variant strain of *Clostridium difficile*, New England Journal of Medicine. 353 (2005) 2433–2441. https://doi.org/10.1056/NEJMoa051590.

[71] A. Cheknis, S. Johnson, L. Chesnel, L. Petrella, S. Sambol, S.E. Dale, J. Nary, P. Sears, D.M. Citron, E.J.C. Goldstein, D.N. Gerding, Molecular epidemiology of *Clostridioides* (*Clostridium*) *difficile* strains recovered from clinical trials in the US,

Canada and Europe from 2006-2009 to 2012-2015, Anaerobe. 53 (2018) 38–42. https://doi.org/10.1016/j.anaerobe.2018.05.009.

[72] A. Camacho-Ortiz, D. López-Barrera, R. Hernández-García, A.M. Galván-De Los Santos, S.M. Flores-Treviño, J.M. Llaca-Díaz, H.J. Maldonado-Garza, H.J.M. Garza, F.J. Bosques-Padilla, E. Garza-González, First report of *Clostridium difficile* NAP1/027 in a Mexican hospital, PLoS ONE. 10 (2015) e0122627. https://doi.org/10.1371/journal.pone.0122627.

[73] D. López-Ureña, C. Quesada-Gómez, E. Miranda, M. Fonseca, E. Rodríguez-Cavallini, Spread of epidemic *Clostridium difficile* NAP1/027 in Latin America: case reports in Panama, J. Med. Microbiol. 63 (2014) 322–324. https://doi.org/10.1099/jmm.0.066399-0.

[74] C. Hernández-Rocha, J. Barra-Carrasco, M. Pizarro-Guajardo, P. Ibáñez, S.M. Bueno, M.R. Sarker, A.M. Guzman, M. Álvarez-Lobos, D. Paredes-Sabja, Epidemic *Clostridium difficile* ribotype 027 in Chile, Emerging Infectious Diseases. 18 (2012) 1370–1372. https://doi.org/10.3201/eid1808.120211.

[75] K. Dulęba, M. Pawłowska, M. Wietlicka-Piszcz, *Clostridium difficile* infection in children hospitalized due to diarrhea, Eur J Clin Microbiol Infect Dis. 33 (2014) 201–209. https://doi.org/10.1007/s10096-013-1946-1.

[76] X. Didelot, D.W. Eyre, M. Cule, C.L. Ip, M.A. Ansari, D. Griffiths, A. Vaughan, L. O'Connor, T. Golubchik, E.M. Batty, P. Piazza, D.J. Wilson, R. Bowden, P.J. Donnelly, K.E. Dingle, M. Wilcox, A.S. Walker, D.W. Crook, T.E. A Peto, R.M. Harding, Microevolutionary analysis of *Clostridium difficile* genomes to investigate transmission, Genome Biology. 13 (2012) R118. https://doi.org/10.1186/gb-2012-13-12-r118.

[77] C. Quesada-Gómez, D. López-Ureña, L. Acuña-Amador, M. Villalobos-Zúñiga, T. Du, R. Freire, C. Guzmán-Verri, M. del M. Gamboa-Coronado, T.D. Lawley, E. Moreno, M.R. Mulvey, G.A. de C. Brito, E. Rodríguez-Cavallini, C. Rodríguez, E. Chaves-Olarte, Emergence of an outbreak-associated *Clostridium difficile* variant with increased virulence, J Clin Microbiol. 53 (2015) 1216–1226. https://doi.org/10.1128/JCM.03058-14.

[78] M.J.T. Crobach, T. Planche, C. Eckert, F. Barbut, E.M. Terveer, O.M. Dekkers, M.H. Wilcox, E.J. Kuijper, European Society of Clinical Microbiology and Infectious Diseases: update of the diagnostic guidance document for *Clostridium difficile* infection, Clin. Microbiol. Infect. 22 Suppl 4 (2016) S63-81. https://doi.org/10.1016/j.cmi.2016.03.010.

[79] M.P. Hensgens, A. Goorhuis, D.W. Notermans, B.H. van Benthem, E.J. Kuijper, Decrease of hypervirulent *Clostridium difficile* PCR ribotype 027 in the Netherlands, Eurosurveillance. 14 (2009) 19402. https://doi.org/10.2807/ese.14.45.19402-en.

[80] D. Ashiru-Oredope, M. Sharland, E. Charani, C. McNulty, J. Cooke, ARHAI Antimicrobial Stewardship Group, Improving the quality of antibiotic prescribing in the NHS by developing a new Antimicrobial Stewardship Programme: Start Smart--Then Focus, J. Antimicrob. Chemother. 67 Suppl 1 (2012) i51-63. https://doi.org/10.1093/jac/dks202.

[81] R. Herbert, J. Hatcher, E. Jauneikaite, M. Gharbi, S. d'Arc, N. Obaray, T. Rickards, M. Rebec, O. Blandy, R. Hope, A. Thomas, K. Bamford, A. Jepson, S. Sriskandan, Two-year analysis of *Clostridium difficile* ribotypes associated with increased severity, J Hosp Infect. 103 (2019) 388–394. https://doi.org/10.1016/j.jhin.2019.06.003.

[82] D. López-Ureña, J. Orozco-Aguilar, Y. Chaves-Madrigal, A. Ramírez-Mata, A. Villalobos-Jimenez, S. Ost, C. Quesada-Gómez, C. Rodríguez, P. Papatheodorou, E. Chaves-Olarte, Toxin B Variants from *Clostridium difficile* strains VPI 10463 and NAP1/027 share similar substrate profile and cellular intoxication kinetics but use different host cell entry factors, Toxins (Basel). 11 (2019). https://doi.org/10.3390/toxins11060348.

[83] I. See, Y. Mu, J. Cohen, Z.G. Beldavs, L.G. Winston, G. Dumyati, S. Holzbauer, J. Dunn, M.M. Farley, C. Lyons, H. Johnston, E. Phipps, R. Perlmutter, L. Anderson, D.N. Gerding, F.C. Lessa, NAP1 strain type predicts outcomes from *Clostridium difficile* infection, Clin. Infect. Dis. 58 (2014) 1394–1400. https://doi.org/10.1093/cid/ciu125.

[84] L.C. McDonald, D.N. Gerding, S. Johnson, J.S. Bakken, K.C. Carroll, S.E. Coffin, E.R. Dubberke, K.W. Garey, C.V. Gould, C. Kelly, V. Loo, J. Shaklee Sammons, T.J.

Sandora, M.H. Wilcox, Clinical practice guidelines for *Clostridium difficile* infection in adults and children: 2017 Update by the Infectious Diseases Society of America (IDSA) and Society for Healthcare Epidemiology of America (SHEA), Clin. Infect. Dis. 66 (2018) e1–e48. https://doi.org/10.1093/cid/cix1085.

[85] K.M. Tamez-Torres, P. Torres-González, F. Leal-Vega, A. García-Alderete, N.I. López García, R. Mendoza-Aguilar, A. Galindo-Fraga, M. Bobadilla-del Valle, A. Ponce de León, J. Sifuentes-Osornio, Impact of *Clostridium difficile* infection caused by the NAP1/RT027 strain on severity and recurrence during an outbreak and transition to endemicity in a Mexican tertiary care center, International Journal of Infectious Diseases. 65 (2017) 44–49. https://doi.org/10.1016/j.ijid.2017.09.022.

[86] R.N. Pires, A.A. Monteiro, G.Z. Saldanha, D.R. Falci, C.F.B. Caurio, T.C.T. Sukiennik, F.C. Adam, A.C. Pasqualotto, A.F. Martins, Hypervirulent *Clostridium difficile* strain has arrived in Brazil, Infect Control Hosp Epidemiol. 39 (2018) 371–373. https://doi.org/10.1017/ice.2017.280.

[87] S.K. Lim, R.L. Stuart, K.E. Mackin, G.P. Carter, D. Kotsanas, M.J. Francis, M. Easton, K. Dimovski, B. Elliott, T.V. Riley, G. Hogg, E. Paul, T.M. Korman, T. Seemann, T.P. Stinear, D. Lyras, G.A. Jenkin, Emergence of a ribotype 244 strain of *Clostridium difficile* associated with severe disease and related to the epidemic ribotype 027 strain, Clin. Infect. Dis. 58 (2014) 1723–1730. https://doi.org/10.1093/cid/ciu203.

[88] R. Saito, Y. Usui, A. Ayibieke, J. Nakajima, I. Prah, K. Sonobe, Y. Aiso, S. Ito, Y. Itsui, Y. Hadano, Y. Nukui, R. Koike, S. Tohda, Hypervirulent clade 2, ribotype 019/sequence type 67 *Clostridioides difficile* strain from Japan, Gut Pathogens. 11 (2019) 54. https://doi.org/10.1186/s13099-019-0336-3.

[89] H. Cao, S.C.-Y. Wong, W.-C. Yam, M.C.-J. Liu, K.-H. Chow, A.K.-L. Wu, P.-L. Ho, Genomic investigation of a sequence type 67 *Clostridium difficile* causing community-acquired fulminant colitis in Hong Kong, Int. J. Med. Microbiol. 309 (2019) 270–273. https://doi.org/10.1016/j.ijmm.2019.05.003.

[90] G.Z. Saldanha, R.N. Pires, A.P. Rauber, D. de Lima-Morales, D.R. Falci, J. Caierão, A.C. Pasqualotto, A.F. Martins, Genetic relatedness, Virulence factors and Antimicrobial resistance of *C. difficile* strains from hospitalized patients in a

multicentric study in Brazil, J Glob Antimicrob Resist. 22 (2020) 117–121. https://doi.org/10.1016/j.jgar.2020.01.007.

[91] Y. Luo, E. Cheong, Q. Bian, D.A. Collins, J. Ye, J.H. Shin, W.C. Yam, T. Takata, X. Song, X. Wang, M. Kamboj, T. Gottlieb, J. Jiang, T.V. Riley, Y.-W. Tang, D. Jin, Different molecular characteristics and antimicrobial resistance profiles of *Clostridium difficile* in the Asia-Pacific region, Emerg Microbes Infect. 8 (2019) 1553–1562. https://doi.org/10.1080/22221751.2019.1682472.

[92] M. Krutova, O. Nyc, J. Matejkova, E.J. Kuijper, J. Jalava, S. Mentula, The recognition and characterisation of Finnish *Clostridium difficile* isolates resembling PCR-ribotype 027, Journal of Microbiology, Immunology and Infection. 51 (2018) 344–351. https://doi.org/10.1016/j.jmii.2017.02.002.

[93] P. Ngamskulrungroj, S. Sanmee, P. Pusathit, P. Piewngam, B. Elliott, T.V. Riley, P. Kiratisin, Molecular epidemiology of *Clostridium difficile* infection in a large teaching hospital in Thailand, PLOS ONE. 10 (2015) e0127026. https://doi.org/10.1371/journal.pone.0127026.

[94] D.W. Eyre, L. Tracey, B. Elliott, C. Slimings, P.G. Huntington, R.L. Stuart, T.M. Korman, G. Kotsiou, R. McCann, D. Griffiths, W.N. Fawley, P. Armstrong, K.E. Dingle, A.S. Walker, T.E. Peto, D.W. Crook, M.H. Wilcox, T.V. Riley, Emergence and spread of predominantly community-onset *Clostridium difficile* PCR ribotype 244 infection in Australia, 2010 to 2012, Euro Surveill. 20 (2015) 21059. https://doi.org/10.2807/1560-7917.es2015.20.10.21059.

[95] D.A. Collins, L.A. Selvey, A. Celenza, T.V. Riley, Community-associated *Clostridium difficile* infection in emergency department patients in Western Australia, Anaerobe. 48 (2017) 121–125. https://doi.org/10.1016/j.anaerobe.2017.08.008.

[96] A.M. Skinner, L. Petrella, F. Siddiqui, S.P. Sambol, C.A. Gulvik, D.N. Gerding, C.J. Donskey, S. Johnson, Unique clindamycin-resistant Clostridioides difficile strain related to fluoroquinolone-resistant epidemic BI/RT027 strain, Emerg. Infect. Dis. 26 (2020) 247-254. https://doi.org/10.3201/eid2602.181965.

[97] E. Valiente, L.F. Dawson, M.D. Cairns, R.A. Stabler, B.W. Wren, Emergence of new PCR ribotypes from the hypervirulent *Clostridium difficile* 027 lineage, J Med Microbiol. 61 (2012) 49–56. https://doi.org/10.1099/jmm.0.036194-0.

[98] A.C. Cheng, D.A. Collins, B. Elliott, J.K. Ferguson, D.L. Paterson, S. Thean, T.V. Riley, Laboratory-based surveillance of *Clostridium difficile* circulating in Australia, September - November 2010, Pathology. 48 (2016) 257–260. https://doi.org/10.1016/j.pathol.2016.02.005.

[99] M.N. De Almeida, H. Heffernan, A. Dervan, S. Bakker, J.T. Freeman, H. Bhally, S.L. Taylor, T.V. Riley, S.A. Roberts, Severe *Clostridium difficile* infection in New Zealand associated with an emerging strain, PCR-ribotype 244, N. Z. Med. J. 126 (2013) 9–14.

[100] C.A. Huber, L. Hall, N.F. Foster, M. Gray, M. Allen, L.J. Richardson, J. Robson, R. Vohra, S. Schlebusch, N. George, G.R. Nimmo, T.V. Riley, D.L. Paterson, Surveillance snapshot of *Clostridium difficile* infection in hospitals across Queensland detects binary toxin producing ribotype UK 244, Commun Dis Intell Q Rep. 38 (2014) E279-284.

[101] V. Tkalec, S. Janezic, B. Skok, T. Simonic, S. Mesaric, T. Vrabic, M. Rupnik, High *Clostridium difficile* contamination rates of domestic and imported potatoes compared to some other vegetables in Slovenia, Food Microbiol. 78 (2019) 194–200. https://doi.org/10.1016/j.fm.2018.10.017.

[102] S. Hong, D.R. Knight, B. Chang, R.J. Carman, T.V. Riley, Phenotypic characterisation of *Clostridium difficile* PCR ribotype 251, an emerging multi-locus sequence type clade 2 strain in Australia, Anaerobe. 60 (2019) 102066. https://doi.org/10.1016/j.anaerobe.2019.06.019.

[103] M.C. Wehrhahn, C. Keighley, J. Kurtovic, D.R. Knight, S. Hong, M.L. Hutton, D. Lyras, Q. Wang, R. Leong, T. Borody, M. Edye, T.V. Riley, A series of three cases of severe *Clostridium difficile* infection in Australia associated with a binary toxin producing clade 2 ribotype 251 strain, Anaerobe. 55 (2019) 117–123. https://doi.org/10.1016/j.anaerobe.2018.11.009.

[104] D.A. Collins, P. Putsathit, B. Elliott, T.V. Riley, Laboratory-based surveillance of *Clostridium difficile* strains circulating in the Australian healthcare setting in 2012, Pathology. 49 (2017) 309–313. https://doi.org/10.1016/j.pathol.2016.10.013.

[105] A.N. Diniz, C.A. de Oliveira Júnior, E.G. Vilela, H.C.P. Figueiredo, M. Rupnik, M.H. Wilcox, W.N. Fawley, D.S. Blanc, F.C. Faria Lobato, R.O.S. Silva, Molecular

epidemiology of *Clostridioides* (previously *Clostridium*) *difficile* isolates from a university hospital in Minas Gerais, Brazil, Anaerobe. 56 (2019) 34–39. https://doi.org/10.1016/j.anaerobe.2019.01.010.

[106] Y. Usui, Y. Nukui, R. Koike, S. Tohda, R. Saito, Draft genome sequence of a *Clostridioides difficile* sequence type 97 strain belonging to hypervirulent Clade 2, Microbiol Resour Announc. 9 (2020). https://doi.org/10.1128/MRA.00245-20.

[107] C. Quesada-Gómez, T. Murillo, G. Arce, A. Badilla-Lobo, C. Castro-Peña, J. Molina, D. López-Ureña, S. González-Camacho, B. Lomonte, C. Chacón-Díaz, C. Rodríguez, E. Chaves-Olarte, Proteogenomic analysis of the *Clostridium difficile* exoproteome reveals a correlation between phylogenetic distribution and virulence potential, Anaerobe. 62 (2020) 102151. https://doi.org/10.1016/j.anaerobe.2020.102151.

[108] J. Pépin, L. Valiquette, M.-E. Alary, P. Villemure, A. Pelletier, K. Forget, K. Pépin, D. Chouinard, *Clostridium difficile*-associated diarrhea in a region of Quebec from 1991 to 2003: a changing pattern of disease severity, CMAJ. 171 (2004) 466–472. https://doi.org/10.1503/cmaj.1041104.

[109] J. Pépin, L. Valiquette, B. Cossette, Mortality attributable to nosocomial *Clostridium difficile*–associated disease during an epidemic caused by a hypervirulent strain in Quebec, CMAJ. 173 (2005) 1037–1042. https://doi.org/10.1503/cmaj.050978.

[110] B. Hubert, V.G. Loo, A.-M. Bourgault, L. Poirier, A. Dascal, É. Fortin, M. Dionne, M. Lorange, A portrait of the geographic dissemination of the *Clostridium difficile* North American Pulsed-Field Type 1 strain and the epidemiology of *C. difficile*-Associated Disease in Québec, Clin Infect Dis. 44 (2007) 238–244. https://doi.org/10.1086/510391.

[111] A. Goorhuis, T. Van der Kooi, N. Vaessen, F.W. Dekker, R. Van den Berg, C. Harmanus, S. van den Hof, D.W. Notermans, E.J. Kuijper, Spread and epidemiology of *Clostridium difficile* Polymerase Chain Reaction Ribotype 027/Toxinotype III in The Netherlands, Clin Infect Dis. 45 (2007) 695–703. https://doi.org/10.1086/520984.

[112] K. Rao, D. Micic, M. Natarajan, S. Winters, M.J. Kiel, S.T. Walk, K. Santhosh, J.A. Mogle, A.T. Galecki, W. LeBar, P.D.R. Higgins, V.B. Young, D.M. Aronoff, *Clostridium difficile* ribotype 027: relationship to age, detectability of toxins A or B in

stool with rapid testing, severe infection, and mortality, Clin Infect Dis. 61 (2015) 233–241. https://doi.org/10.1093/cid/civ254.

[113] O.W. Morgan, B. Rodrigues, T. Elston, N.Q. Verlander, D.F.J. Brown, J. Brazier, M. Reacher, Clinical severity of *Clostridium difficile* PCR ribotype 027: a case-case study, PLoS One. 3 (2008) e1812. https://doi.org/10.1371/journal.pone.0001812.

[114] J. Cloud, L. Noddin, A. Pressman, M. Hu, C. Kelly, *Clostridium difficile* strain NAP-1 is not associated with severe disease in a nonepidemic setting, Clin Gastroenterol Hepatol. 7 (2009) 868-873.e2. https://doi.org/10.1016/j.cgh.2009.05.018.

[115] K.A. Bauer, J.E.W. Johnston, E. Wenzler, D.A. Goff, C.H. Cook, J.-M. Balada-Llasat, P. Pancholi, J.E. Mangino, Impact of the NAP-1 strain on disease severity, mortality, and recurrence of healthcare-associated *Clostridium difficile* infection, Anaerobe. 48 (2017) 1–6. https://doi.org/10.1016/j.anaerobe.2017.06.009.

[116] G. Ramírez-Vargas, D. López-Ureña, A. Badilla, J. Orozco-Aguilar, T. Murillo, P. Rojas, T. Riedel, J. Overmann, G. González, E. Chaves-Olarte, C. Quesada-Gómez, C. Rodríguez, Novel Clade C-I *Clostridium difficile* strains escape diagnostic tests, differ in pathogenicity potential and carry toxins on extrachromosomal elements, Scientific Reports. 8 (2018) 13951. https://doi.org/10.1038/s41598-018-32390-6.

[117] J.A. Karlowsky, G.G. Zhanel, G.W. Hammond, E. Rubinstein, J. Wylie, T. Du, M.R. Mulvey, M.J. Alfa, Multidrug-resistant North American pulsotype 2 *Clostridium difficile* was the predominant toxigenic hospital-acquired strain in the province of Manitoba, Canada, in 2006-2007, J Med Microbiol. 61 (2012) 693–700. https://doi.org/10.1099/jmm.0.041053-0.

[118] D. Paredes-Sabja, C. Bond, R.J. Carman, P. Setlow, M.R. Sarker, Germination of spores of *Clostridium difficile* strains, including isolates from a hospital outbreak of Clostridium difficile-associated disease (CDAD), Microbiology (Reading). 154 (2008) 2241–2250. https://doi.org/10.1099/mic.0.2008/016592-0.

[119] S. M. Twine, C. W. Reid, A. Aubry, D. R. McMullin, K. M. Fulton, J. Austin, S. S. Logan, Motility and flagellar glycosylation in *Clostridium difficile*, Journal of Bacteriology. 191 (2009) 7050-7062. https://journals.asm.org/doi/abs/10.1128/JB.00861-09.

[120] A. Tasteyre, M.-C. Barc, T. Karjalainen, P. Dodson, S. Hyde, P. Bourlioux, P. Borriello, A *Clostridium difficile* gene encoding flagellin, Microbiology (Reading). 146 (Pt 4) (2000) 957–966. https://doi.org/10.1099/00221287-146-4-957.

[121] A. Otto, S. Maaß, F. Bonn, K. Büttner, D. Becher, An easy and fast protocol for affinity bead-based protein enrichment and storage of proteome samples, Methods Enzymol. 585 (2017) 1–13. https://doi.org/10.1016/bs.mie.2016.09.012.

[122] A.P. Fraise, M. a. C. Wilkinson, C.R. Bradley, S. Paton, J. Walker, J.-Y. Maillard, R.L. Wesgate, P. Hoffman, J. Coia, C. Woodall, C. Fry, M. Wilcox, Development of a sporicidal test method for *Clostridium difficile*, J Hosp Infect. 89 (2015) 2–15. https://doi.org/10.1016/j.jhin.2014.09.014.

[123] L. T. Joshi, D. S. Phillips, C. F. Williams, A. Alyousef, L. Baillie, Contribution of spores to the ability of *Clostridium difficile* to adhere to surfaces, Applied and Environmental Microbiology. 78 (2012) 7671-7679. https://journals.asm.org/doi/abs/10.1128/AEM.01862-12.

[124] P. Mora-Uribe, C. Miranda-Cárdenas, P. Castro-Córdova, F. Gil, I. Calderón, J.A. Fuentes, P.I. Rodas, S. Banawas, M.R. Sarker, D. Paredes-Sabja, Characterization of the adherence of *Clostridium difficile* spores: The integrity of the outermost layer affects adherence properties of spores of the epidemic strain R20291 to components of the intestinal mucosa, Front. Cell. Infect. Microbiol. 6 (2016). https://doi.org/10.3389/fcimb.2016.00099.

[125] J. Barra-Carrasco, V. Olguín-Araneda, A. Plaza-Garrido, C. Miranda-Cárdenas, G. Cofré-Araneda, M. Pizarro-Guajardo, M.R. Sarker, D. Paredes-Sabja, The *Clostridium difficile* exosporium cysteine (CdeC)-rich protein is required for exosporium morphogenesis and coat assembly, J Bacteriol. 195 (2013) 3863–3875. https://doi.org/10.1128/JB.00369-13.

[126] R. Rajagopal, AP. Wei, Antibodies to *Clostridium difficile* spores and uses thereof, Patent Application Publication (2011) Patent No.: US 8.697.374 B2.

[127] A. Aliramezani, M. Talebi, M. Douraghi, Polymorphisms in the genes encoding surface associated proteins of *Clostridioides difficile* isolates, Infect Genet Evol. 86 (2020) 104598. https://doi.org/10.1016/j.meegid.2020.104598.

[128] K.E. Dingle, X. Didelot, M.A. Ansari, D.W. Eyre, A. Vaughan, D. Griffiths, C.L.C. Ip, E.M. Batty, T. Golubchik, R. Bowden, K.A. Jolley, D.W. Hood, W.N. Fawley, A.S. Walker, T.E. Peto, M.H. Wilcox, D.W. Crook, Recombinational switching of the *Clostridium difficile* S-layer and a novel glycosylation gene cluster revealed by large-scale whole-genome sequencing, J Infect Dis. 207 (2013) 675–686. https://doi.org/10.1093/infdis/jis734.

[129] C.M. Ausiello, M. Cerquetti, G. Fedele, F. Spensieri, R. Palazzo, M. Nasso, S. Frezza, P. Mastrantonio, Surface layer proteins from *Clostridium difficile* induce inflammatory and regulatory cytokines in human monocytes and dendritic cells, Microbes Infect. 8 (2006) 2640–2646. https://doi.org/10.1016/j.micinf.2006.07.009.

[130] M.M. Merrigan, A. Venugopal, J.L. Roxas, F. Anwar, M.J. Mallozzi, B.A.P. Roxas, D.N. Gerding, V.K. Viswanathan, G. Vedantam, Surface-layer protein A (SlpA) is a major contributor to host-cell adherence of *Clostridium difficile*, PLoS One. 8 (2013) e78404. https://doi.org/10.1371/journal.pone.0078404.

[131] M. Sára, U.B. Sleytr, S-Layer proteins, J Bacteriol. 182 (2000) 859–868. https://doi.org/10.1128/JB.182.4.859-868.2000.

[132] P. Oatley, J.A. Kirk, S. Ma, S. Jones, R.P. Fagan, Spatial organization of *Clostridium difficile* S-layer biogenesis, Sci Rep. 10 (2020) 14089. https://doi.org/10.1038/s41598-020-71059-x.

[133] A. Mizrahi, J.-F. Bruxelle, S. Péchiné, A. Le Monnier, Prospective evaluation of the adaptive immune response to SlpA in *Clostridium difficile* infection, Anaerobe. 54 (2018) 164–168. https://doi.org/10.1016/j.anaerobe.2018.09.008.

[134] E. Stevenson, N.P. Minton, S.A. Kuehne, The role of flagella in *Clostridium difficile* pathogenicity, Trends Microbiol. 23 (2015) 275–282. https://doi.org/10.1016/j.tim.2015.01.004.

[135] E. Valiente, L. Bouché, P. Hitchen, A. Faulds-Pain, M. Songane, L.F. Dawson, E. Donahue, R.A. Stabler, M. Panico, H.R. Morris, M. Bajaj-Elliott, S.M. Logan, A. Dell, B.W. Wren, Role of glycosyltransferases modifying type B flagellin of emerging hypervirulent *Clostridium difficile* lineages and their impact on motility and biofilm formation, Journal of Biological Chemistry. 291 (2016) 25450–25461. https://doi.org/10.1074/jbc.M116.749523.

[136] R.A. Stabler, M. He, L. Dawson, M. Martin, E. Valiente, C. Corton, T.D. Lawley, M. Sebaihia, M.A. Quail, G. Rose, D.N. Gerding, M. Gibert, M.R. Popoff, J. Parkhill, G. Dougan, B.W. Wren, Comparative genome and phenotypic analysis of *Clostridium difficile* 027 strains provides insight into the evolution of a hypervirulent bacterium, Genome Biol. 10 (2009) R102. https://doi.org/10.1186/gb-2009-10-9-r102.

[137] J. Chu, J. Liu, T.R. Hoover, Phylogenetic distribution, ultrastructure, and function of bacterial flagellar sheaths, Biomolecules. 10 (2020). https://doi.org/10.3390/biom10030363.

[138] B.R. Anjuwon-Foster, R. Tamayo, A genetic switch controls the production of flagella and toxins in *Clostridium difficile*, PLoS Genet. 13 (2017) e1006701. https://doi.org/10.1371/journal.pgen.1006701.

[139] E.B. Purcell, R.W. McKee, S.M. McBride, C.M. Waters, R. Tamayo, Cyclic diguanylate inversely regulates motility and aggregation in *Clostridium difficile*, J Bacteriol. 194 (2012) 3307–3316. https://doi.org/10.1128/JB.00100-12.

[140] K.-Y. Chen, J. Rathod, Y.-C. Chiu, J.-W. Chen, P.-J. Tsai, I.-H. Huang, The transcriptional regulator Lrp contributes to toxin expression, sporulation, and swimming motility in *Clostridium difficile*, Front. Cell. Infect. Microbiol. 9 (2019). https://doi.org/10.3389/fcimb.2019.00356.

[141] C.B. Reynolds, J.E. Emerson, L. de la Riva, R.P. Fagan, N.F. Fairweather, The *Clostridium difficile* cell wall protein CwpV is antigenically variable between strains, but exhibits conserved aggregation-promoting function, PLOS Pathogens. 7 (2011) e1002024. https://doi.org/10.1371/journal.ppat.1002024.

[142] J.E. Emerson, C.B. Reynolds, R.P. Fagan, H.A. Shaw, D. Goulding, N.F. Fairweather, A novel genetic switch controls phase variable expression of CwpV, a *Clostridium difficile* cell wall protein, Molecular Microbiology. 74 (2009) 541–556. https://doi.org/10.1111/j.1365-2958.2009.06812.x.

[143] A. Aubry, G. Hussack, W. Chen, R. KuoLee, S.M. Twine, K.M. Fulton, S. Foote, C.D. Carrillo, J. Tanha, S.M. Logan, Modulation of toxin production by the flagellar regulon in *Clostridium difficile*, Infect Immun. 80 (2012) 3521–3532. https://doi.org/10.1128/IAI.00224-12.

[144] A. Martínez-Meléndez, R. Morfin-Otero, L. Villarreal-Treviño, S.D. Baines, A. Camacho-Ortíz, E. Garza-González, Analysis of biofilm production and expression of adhesion structures of circulating *Clostridioides difficile* strains from Mexico, Enfermedades Infecciosas y Microbiología Clínica. (2021). https://doi.org/10.1016/j.eimc.2021.01.017.

[145] L. F. Dawson, J. Peltier, C. L. Hall, M. A. Harrison, M. Derakhshan, H. A. Shawn, N. F. Fairweather, B. W. Wren, Extracellular DNA, cell surface proteins and c-di-GMP promote biofilm formation in *Clostridioides difficile*, Scientific Reports, 11 (2021). https://www.nature.com/articles/s41598-020-78437-5.

[146] R. Chandrasekaran, D.B. Lacy, The role of toxins in *Clostridium difficile* infection, FEMS Microbiol Rev. 41 (2017) 723–750. https://doi.org/10.1093/femsre/fux048.

[147] I. Martin-Verstraete, J. Peltier, B. Dupuy, The Regulatory Networks That Control *Clostridium difficile* toxin synthesis, Toxins (Basel). 8 (2016). https://doi.org/10.3390/toxins8050153.

[148] S.D. Goldenberg, G.L. French, Lack of association of tcdC type and binary toxin status with disease severity and outcome in toxigenic *Clostridium difficile*, J Infect. 62 (2011) 355–362. https://doi.org/10.1016/j.jinf.2011.03.001.

[149] S.T. Cartman, M.L. Kelly, D. Heeg, J.T. Heap, N.P. Minton, Precise manipulation of the *Clostridium difficile* chromosome reveals a lack of association between the tcdC genotype and toxin production, Appl Environ Microbiol. 78 (2012) 4683–4690. https://doi.org/10.1128/AEM.00249-12.

[150] A.M.O. Paiva, L. de Jong, A.H. Friggen, W.K. Smits, J. Corver, The C-terminal domain of *Clostridioides difficile* TcdC is exposed on the bacterial cell surface, BioRxiv. (2019) 872283. https://doi.org/10.1101/872283.

[151] C. Darkoh, H. L. DuPont, S. J. Norris, H. B. Kaplan, Toxin synthesis by *Clostridium difficile* is regulated through Quorum Signaling, mBio, 6 (2015). https://journals.asm.org/doi/full/10.1128/mBio.02569-14.

[152] E.B. Purcell, R.W. McKee, D.S. Courson, E.M. Garrett, S.M. McBride, R.E. Cheney, R. Tamayo, A nutrient-regulated cyclic diguanylate phosphodiesterase controls *Clostridium difficile* biofilm and toxin production during stationary phase, Infect Immun. 85 (2017). https://doi.org/10.1128/IAI.00347-17.

[153] H. Moura, R.R. Terilli, A. Woolfitt, Y.M. Williamson, G. Wagner, T.A. Blake, M. Solano, J. Barr, Proteomic analysis and label-free quantification of the large *Clostridium difficile* toxins, International Journal of Proteomics. (2013). https://doi.org/10.1155/2013/293782.

[154] D.W. Eyre, T.E.A. Peto, D.W. Crook, A.S. Walker, M.H. Wilcox, Hash-based Core Genome Multilocus Sequence Typing for *Clostridium difficile*, J Clin Microbiol. 58 (2019). https://doi.org/10.1128/JCM.01037-19.

[155] A.D. Russell, Bacterial resistance to disinfectants: present knowledge and future problems, J Hosp Infect. 43 Suppl (1999) S57-68. https://doi.org/10.1016/s0195-6701(99)90066-x.

[156] P. Vohra, I. Poxton, Efficacy of decontaminants and disinfectants against *Clostridium difficile*, Journal of Medical Microbiology. 60 (2011) 1218–24. https://doi.org/10.1099/jmm.0.030288-0.

[157] L. Doan, H. Forrest, A. Fakis, J. Craig, L. Claxton, M. Khare, Clinical and cost effectiveness of eight disinfection methods for terminal disinfection of hospital isolation rooms contaminated with *Clostridium difficile* 027, J Hosp Infect. 82 (2012) 114–121. https://doi.org/10.1016/j.jhin.2012.06.014.

[158] A. Medaglia-Mata, R. Starbird-Pérez, E. Sánchez-Chacón, C. Rodríguez, Disinfection of surfaces contaminated with *Clostridioides difficile* endospores using NaCl-derived electrochemically activated solution, Journal of Hospital Infection. 105 (2020) 670–677. https://doi.org/10.1016/j.jhin.2020.04.023.

[159] S. F. Bloomfield, M. Arthur, Effect of chlorine-releasing agents on *Bacillus subtilis* vegetative cells and spores, Letters in Applied Microbiology. 8 (1989) 101-104. https://sfamjournals.onlinelibrary.wiley.com/doi/10.1111/j.1472-765X.1989.tb00233.x

[160] P. Calderón-Romero, P. Castro-Córdova, R. Reyes-Ramírez, M. Milano-Céspedes, E. Guerrero-Araya, M. Pizarro-Guajardo, V. Olguín-Araneda, F. Gil, D. Paredes-Sabja, *Clostridium difficile* exosporium cysteine-rich proteins are essential for the morphogenesis of the exosporium layer, spore resistance, and affect *C. difficile* pathogenesis, PLoS Pathog. 14 (2018) e1007199. https://doi.org/10.1371/journal.ppat.1007199.

[161] C. Dyer, L.P. Hutt, R. Burky, L.T. Joshi, Biocide Resistance and Transmission of *Clostridium difficile* spores spiked onto clinical surfaces from an American health care Facility, Appl Environ Microbiol. 85 (2019). https://doi.org/10.1128/AEM.01090-19.